Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

difenoconazole (ISO); 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether

EC Number: -
CAS Number: 119446-68-3

CLH-O-0000007004-85-01/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

10 June 2021

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification: Difenoconazole (ISO); 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-Chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether

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Index Number: 613-RST-VW-Y

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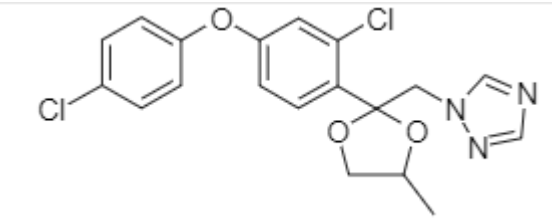
Version number: 2 **Date: 10 March 2020**

CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether
Other names (usual name, trade name, abbreviation)	-
ISO common name (if available and appropriate)	Difenoconazole
EC number (if available and appropriate)	-
EC name (if available and appropriate)	
CAS number (if available)	119446-68-3
Other identity code (if available)	687
Molecular formula	$C_{19}H_{17}Cl_2N_3O_3$
Structural formula	
SMILES notation (if available)	O1C[CH](C)O[C]1(Cn1ncnc1)c1c(Cl)cc(Oc2ccc(Cl)cc2)cc1
Molecular weight or molecular weight range	406.3 g/mol
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	Difenoconazole has two diastereomeric (cis and trans) pairs of enantiomers; these are known as (2R, 4R), (2S, 4S), (2S, 4R) and (2R, 4S). The cis and trans diastereoisomers are present in the ratio range 0.8 to 1.6 respectively and the enantiomers are present as racemic mixtures *i.e.* 1:1 ratio. The manufacturing process of difenoconazole is not stereoselective.
Description of the manufacturing process and identity of the source (for UVCB substances only)	Not applicable
Degree of purity (%) (if relevant for the entry in Annex VI)	Minimum purity ≥ 940 g/kg

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)
Difenoconazole CAS No. 119446-68-3	Min. 94%	Not listed	**Acute Tox 4; H302** **Eye Irrit. 2; H319**

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
Toluene CAS No. 108-88-3	Maximum content of toluene in the technical material is 5 g/kg (0.5%)	**Flam. Liq. 2; H225** **Repr. 2; H361d** **Asp. Tox. 1; H304** **STOT RE 2 (*); H373**** **Skin Irrit. 2; H315** **STOT SE 3; H336**	-	-

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
None					

Table 5: Test substances (non-confidential information) (this table is optional)

Identification of test substance	Purity	Impurities and additives (identity, %, classification if available)	Other information	The study(ies) in which the test substance is used

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 6:

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No entry on Annex VI										
Dossier submitters proposal	-	Difenoconazole; 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether;	-	119446-68-3	Acute Tox. 4 Eye Irrit. 2 Aquatic Acute 1 Aquatic Chronic 1	H302 H319 H400 H410	GHS07 GHS09 Wng	H302 H319 H410		Oral: ATE = 1453 mg/kg bw M = 10 M = 10	
Resulting Annex VI entry if agreed by RAC and COM	613-RST-VW-Y -	Difenoconazole (ISO); 1-({2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl}methyl)-1H-1,2,4-triazole; 3-Chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether	-	119446-68-3	Acute Tox. 4 Eye Irrit. 2 Aquatic Acute 1 Aquatic Chronic 1	H302 H319 H400 H410	GHS07 GHS09 Wng	H302 H319 H410		Oral: ATE = 1453 mg/kg bw M = 10 M = 10	

Table 7: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	Data lacking	Yes
Flammable gases (including chemically unstable gases)	Hazard class not applicable	No
Oxidising gases	Hazard class not applicable	No
Gases under pressure	Hazard class not applicable	No
Flammable liquids	Hazard class not applicable	No
Flammable solids	Data conclusive but not sufficient for classification	Yes
Self-reactive substances	Data lacking	Yes
Pyrophoric liquids	Hazard class not applicable	No
Pyrophoric solids	Data conclusive but not sufficient for classification	Yes
Self-heating substances	Data conclusive but not sufficient for classification	Yes
Substances which in contact with water emit flammable gases	Data conclusive but not sufficient for classification	Yes
Oxidising liquids	Hazard class not applicable	No
Oxidising solids	Data conclusive but not sufficient for classification	Yes
Organic peroxides	Hazard class not applicable	No
Corrosive to metals	Data conclusive but not sufficient for classification	Yes
Acute toxicity via oral route	**Harmonised classification proposed**	**Yes**
Acute toxicity via dermal route	Data conclusive but not sufficient for classification	Yes
Acute toxicity via inhalation route	Data conclusive but not sufficient for classification	Yes
Skin corrosion/irritation	Data conclusive but not sufficient for classification	Yes
Serious eye damage/eye irritation	**Harmonised classification proposed**	**Yes**
Respiratory sensitisation	Data lacking	No
Skin sensitisation	Data conclusive but not sufficient for classification	Yes
Germ cell mutagenicity	Data conclusive but not sufficient for classification	Yes
Carcinogenicity	Data conclusive but not sufficient for classification	Yes
Reproductive toxicity	Data conclusive but not sufficient for classification	Yes
Specific target organ toxicity-single exposure	Data conclusive but not sufficient for classification	Yes
Specific target organ toxicity-repeated exposure	Data conclusive but not sufficient for classification	Yes
Aspiration hazard	Hazard class not applicable	No

Hazard class	Reason for no classification	Within the scope of public consultation
Hazardous to the aquatic environment	**Aquatic Acute 1; H400; M = 10** **Aquatic Chronic 1; H410; M = 10**	Yes
Hazardous to the ozone layer	Data lacking	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Difenoconazole is not currently listed in Annex VI of Regulation (EC) 1272/2008.

Difenoconazole is a fungicide used as an active substance in plant protection products (PPP). Difenoconazole was included in Annex I to Directive 91/414/EEC by Commission Directive 2008/69/EC and has been deemed to be approved under Commission Implementing Regulation (EU) No 540/2011 in accordance with Regulation (EC) No 1107/2009, which was amended in accordance with Commission Implementing Regulation 1100/2011.

The EFSA peer review (EFSA Journal 2011;9(1):1967) of the pesticide risk assessment of difenoconazole proposed the following classification for difenoconazole based on the available data at the time: Xn, R22 'Harmful if swallowed'; R53.

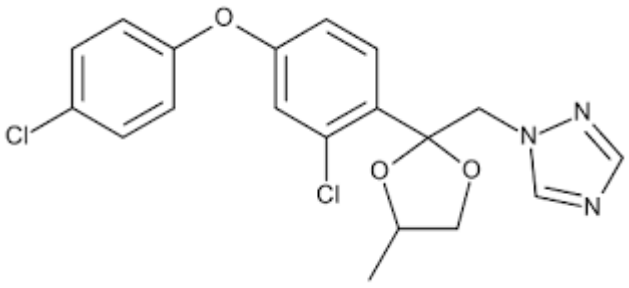
Regarding the renewal of difenoconazole as an active substance in the context of PPP Regulation, a Renewal Assessment Report (RAR, 2019) in accordance with Commission Regulation (EC) No. 686/2012 has been developed by the Spanish CA. This CHL report is based on all relevant information from the RAR that has been adequately evaluated for hazard identification purposes in accordance with the CLP criteria. Since the draft RAR is not publicly available yet, a sanitized version of it has been included as Annex I to this CLH report.

At the time of submission of this CLH report, difenoconazole is not registered under REACH (Regulation (EC) 1907/2006).

RAC general comment

Difenoconazole is an active substance of a plant protection products used as a fungicide. It is not currently listed in Annex VI of Regulation (EC) 1272/2008, although in the past an EFSA peer review of the pesticide risk assessment proposed a classification of difenoconazole as Xn, R22; R53.

The chemical structure is shown below:



The CLH report was prepared by the Dossier Submitter (DS) mainly based on the available data from the Renewal Assessment Report developed in accordance with the Commission

Regulation (EC) No. 844/2012. At the time of submission of the CLH report, difenoconazole had not been registered under REACH.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

In accordance with article 36(2) of Regulation (EC) 1272/2008 on classification, labelling and packaging of substances and mixtures, being difenoconazole an active substance in the meaning of Plant Protection Product (PPP) Regulation, it should now be considered for harmonised classification and labelling for all physico-chemical, human health and environmental endpoints. This Annex VI dossier presents a classification and labelling proposal based on the information provided for the assessment of difenoconazole under Regulation (EC) 1107/2009.

5 IDENTIFIED USES

Difenoconazole is an active substance of a plant protection product (PPP) and it is used as a fungicide.

6 DATA SOURCES

This CLH Report is mainly based on the available data from the Renewal Assessment Report (RAR, 2019) developed in accordance with Commission Regulation (EC) No. 844/2012 by the Spanish CA. Information on data sources used in this CLH Report are included in section 14 (References) and 15 (Annexes).

7 PHYSICOCHEMICAL PROPERTIES

Table 8: Summary of physicochemical properties

Property	Value	Comment (e.g. measured or estimated)	Reference
Physical state at 20°C and 101.3 kPa	Difenoconazole (Batch No. AMS 255/3). Purity 99.3%, (cis/trans ratio: 0.7) Odorless white fine crystalline powder (at 25ºC)	Visual and organoleptic assessment	**Das, R., 1999b** **CA B.2.3/01**
	Difenoconazole technical. (Batch No. V7). Purity not stated Difenoconazole technical is an off-white powder with a slightly sweet odour.	Visual and organoleptic assessment	**Das, R., 1993** **CA B.2.3/01**
Melting/freezing point	Difenoconazole (Batch No. AMS 255/3). Purity 99.3% (cis/trans ratio: 0.7) Melting point: 82.0-83.0 ºC with an estimated accuracy of ± 0.4ºC	Method: EEC A.1 OECD 102 OPPTS 830.7200 GLP: Yes	**Das, R., 1999a** **CA B.2.1/01**
Boiling point	Difenoconazole (Batch No. AMS 255/3). Purity 99.3% (cis/trans ratio: 0.7) Difenoconazole decomposes before boiling at atmospheric pressure. Decomposition begins at about 337 ºC. From the vapour pressure study, difenoconazole would boil at 100.8ºC at the reduced pressure of 3.7 mPa.	Method: EEC A.2 OECD 103 OPPTS 830.7220 GLP: Yes	**Das, R., 1997** **CA B.2.1/02**
Relative density	Difenoconazole (Batch AMS 255/3). Purity 99.3%. 1.39 x 10^3 kg/m^3 at 22ºC, corresponding to a relative density of 1.39 .	Method: OECD 109 GLP: Yes	**Füldner, H. H., 1999** **DAR (2006) B.2.1.2**
Vapour pressure	Difenoconazole (Batch No. 255/102). Purity 99.0%. 3.32 x 10^{-8} Pa at 25ºC.	Method: EEC A.4 OECD 104 GLP: No	**Rordorf, B., 1988** **CA B.2.2/01**
Surface tension	Difenoconazole (Batch No. 255/4). Purity 99.1%. 63.2 mN/m at 22.5º C ± 0.5ºC (90% of saturation concentration).	Method: EEC A.5 OECD 115 GLP: Yes	**O'Connor, B., 2015** **CA B.2.12/01**
Water solubility	Difenoconazole (Batch No. AMS 255/102). Purity 99.0%. At pH 7.2 and 25ºC ± 0.1ºC, solubility = 15 mg/L ± 1.3 mg/L. The test substance does not dissociate at environmentally relevant pH and the solubility in water should therefore not be affected by changes of pH in the pH interval 4-10. Both isomers (cis/trans) had about the same solubility in water.	Method: EEC A.6 OECD 105 GLP: Yes	**Stulz, J., 1994** **CA B.2.5/01**
Partition coefficient n-octanol/water	Difenoconazole (Batch No. AMS 255/3). Purity 99.3% (cis/trans ratio 0.7). Log Pow = 4.36 ± 0.02 at 25º and a pH of approx. 8.	Method: EEC A.8 OECD 107 OPPTS 830.7550 GLP: Yes	**Kettner, R., 1999b** **CA B.2.7/01**
Flash point	Not applicable since the melting point is > 40ºC.	-	-
Flammability	Difenoconazole (Batch No. SMO4H493). Purity: 97.4%. Not flammable.	Method: EEC A.10 GLP: Yes	**Jackson, W., 2012** **CA B.2.9/01**
Explosive properties	Difenoconazole (Batch No. SMO4H493). Purity: 97.4%. Not explosive.	Method: EEC A14 GLP: Yes	**Jackson, W., 2012** **CA B.2.11/01**
Self-ignition temperature	Difenoconazole (Batch No. SMO4H493). Purity: 97.4%. No auto-ignition below the melting point.	Method: EEC A.16 GLP: Yes	**Jackson, W., 2012** **CA B.2.9/02**

Property	Value	Comment (e.g. measured or estimated)	Reference
Oxidising properties	Difenoconazole (Batch No. SMO4H493). Purity: 97.4%. Difenoconazole is not an oxidizing substance.	Method: EEC A.17 GLP: Yes	**Jackson, W., 2012** **CA B.2.13/01**
Solubility in organic solvents	Difenoconazole (Batch No. WM 806228). Purity 94.6% (cis/trans ratio 1.30) At 25ºC: Acetone: >500 g/L Dichloromethane: >500 g/L Ethyl acetate: >500 g/L Hexane: 3.0 g/L Methanol: >500 g/L Octanol: 110 g/L Toluene: >500 g/L	Method: CIPAC MT 157.3 GLP: Yes	**Kettner, R., 1999a** **CA B.2.6/01**
Dissociation constant	Difenoconazole (Batch No. AMS 255/3) Purity 99.3% ± 0.3% (cis/trans ratio 0.7) pKa 1.07 ± 0.18 (deprtonotaion of the triazole)	Method: OECD 112 (titration methods) GLP: Yes	**Hörmann, A., 1999** **CA B.2.8/01**
Viscosity	N/A – solid	-	-

8 EVALUATION OF PHYSICAL HAZARDS

8.1 Explosives

Table 9: Summary table of studies on explosive properties

Method	Results	Remarks	Reference
EEC A.14 GLP: Yes	Not explosive. Purity: 97.4% (Batch No. SMO4H493)		**Jackson, W., 2012 B.2.11/01 (AS)**

8.1.1 Short summary and overall relevance of the information provided on explosive properties

Difenoconazole was tested for explosive properties using the EC Method A.14 and was found not to be explosive. However, this test method is not comparable to the test procedures for classification of explosive properties that are described in Part I of the UN Recommendations on the Transport of Dangerous Goods (RTDG), Manual of Tests and Criteria (7th Revision) according to CLP criteria.

8.1.2 Comparison with the CLP criteria

Difenoconazole contains a triazole substituent, i.e. contiguous nitrogen atoms, which is associated with explosive properties, as reflected in Table A6.1 described in Appendix 6 of the UN RTDG, Manual of Tests and Criteria (7th Revision). In order to fulfill the criteria for non-classification stated in the Regulation EC 1272/2008 data established in Annex I 2.1.4.3 (c) must be provided (exothermic decomposition energy). This information is not available and therefore, a data gap remains although, based on the outcome of the test study method EC A.14 and handling experience the explosive properties of difenoconazole are considered of low concern.

8.1.3 Conclusion on classification and labelling for explosive properties

Not classified –data lacking.

8.2 Flammable gases (including chemically unstable gases)

Hazard not applicable (solid).

8.3 Oxidising gases

Hazard not applicable (solid).

8.4 Gases under pressure

Hazard not applicable (solid).

8.5 Flammable liquids

Hazard not applicable (solid).

8.6 Flammable solids

Table 10: Summary table of studies on flammable solids

Method	Results	Remarks	Reference
EEC A.10 GLP: Yes	Not flammable. Purity: 97.4% (Batch No. SMO4H493)		**Jackson, W., 2012 B.2.9/01 (AS)**

8.6.1 Short summary and overall relevance of the provided information on flammable solids

In the Test Method EC A.10 study, the substance did not ignite on contact with the ignition source (not flammable).

8.6.2 Comparison with the CLP criteria

The method used for classification purposes according to CLP criteria is the UN Test N.1 described in the UN RTDG, Manual of Tests and Criteria (7th revision). However, as reflected in the CLP Guidance and ECHA Guidance on Information Requirements and Chemical Safety Asssessment (R.7.1.10.3), if the result of an A.10 method indicates that a classification as a flammable solid does not apply (result: not highly flammable), no more testing is necessary.

Difenoconazole was classified as not flammable in the EC Method A.10, hence no classification is required.

8.6.3 Conclusion on classification and labelling for flammable solids

Not classified – conclusive but not sufficient for classification.

8.7 Self-reactive substances

8.7.1 Short summary and overall relevance of the provided information on self-reactive substances

No data provided.

8.7.2 Comparison with the CLP criteria

A self-reactive substance corresponds to a thermally unstable solid liable to undergo a strongly exothermic decomposition even without participation of oxygen (air). The classification procedures for self-reactive substances need not be applied if a) there are no chemical groups in the molecule associated with explosive or self-reactive properties or b) for a single organic substance, the estimated self-accelerating decomposition temperature (SADT) for a 50 kg package is greater than 75º C or the exothermic decomposition energy is less than 300 J/g.

Difenoconazole contains a triazole substituent, i.e. contiguous nitrogen atoms, which is associated with explosive properties, as reflected in Table A6.1 described in Appendix 6 of the UN Manual of Tests and Criteria (7th Revision). No data on thermal stability are available according to the method described in Part II section 20.3.3.3 of the UN RTDG, Manual of Test and Criteria (7^{th} revision).

8.7.3 Conclusion on classification and labelling for self-reactive substances

Not classified – data lacking.

8.8 Pyrophoric liquids

Hazard not applicable (solid).

8.9 Pyrophoric solids

8.9.1 Short summary and overall relevance of the provided information on pyrophoric solids

No studies are available. However, difenoconazole does not ignite spontaneously in contact with air based on experience of handling and use.

8.9.2 Comparison with the CLP criteria

According to Section 2.10.4.1 of Annex 1 of CLP, the classification procedure for pyrophoric solids need not be applied when experience in manufacture and handling shows that the substance does not spontaneously ignite upon coming into contact with air at normal temperatures. There are no reports in the available studies of difenoconazole spontaneously igniting when in contact with air.

Therefore, difenoconazole does not meet the criteria for classification as a pyrophoric solid.

8.9.3 Conclusion on classification and labelling for pyrophoric solids

Not classified – conclusive but not sufficient for classification.

8.10 Self-heating substances

Table 11: Summary table of studies on self-heating substances

Method	Results	Remarks	Reference
EEC A.16 GLP: Yes	Not auto-ignition below the melting point. Purity: 97.4% (Batch No. SMO4H493)		**Jackson, W., 2012 B.2.9/02 (AS)**

8.10.1 Short summary and overall relevance of the provided information on self-heating substances

Difenoconazole is not an auto-inflammable substance when tested for auto-flammability using the method EC A.16.

8.10.2 Comparison with the CLP criteria

According to the ECHA Guidance on the Application of the CLP Criteria (version 5.0 July 2017), the test method A.16 is not deemed appropriate to evaluate the self-heating property of solids towards a CLP classification. However, substances with a low melting point (< 160ºC) should not be considered for classification in this hazard class. Difenoconazole has a measured melting point of 82-83ºC and it is expected completely molten up to the cut-off temperature of 160ºC. Therefore, difenoconazole does not meet the criteria for classification as a self-heating substance.

8.10.3 Conclusion on classification and labelling for self-heating substances

Not classified – conclusive but not sufficient for classification.

8.11 Substances which in contact with water emit flammable gases

8.11.1 Short summary and overall relevance of the provided information on substances which in contact with water emit flammable gases

No data provided.

8.11.2 Comparison with the CLP criteria

According to Section 2.12.4.1 of Annex I of CLP, the classification procedure for this hazard class need not be applied if the chemical structure of the substance or mixture does not contain metals or metalloids, or experience in production or handling shows that the substance does not react with water or the substance is known to be soluble in water to form a stable mixture. According to the mentioned criteria, classification for this hazard class is not needed for difenoconazole.

8.11.3 Conclusion on classification and labelling for substances which in contact with water emit flammable gases

Not classified – conclusive but not sufficient for classification.

8.12 Oxidising liquids

Hazard not applicable (solid).

8.13 Oxidising solids

Table 12: Summary table of studies on oxidising solids

Method	Results	Remarks	Reference
EEC A.17 GLP: Yes	Difenoconazole is not an oxidizing substance. Purity: 97.4% (Batch No. SMO4H493)		**Jackson, W., 2012 B.2.13/01 (AS)**

8.13.1 Short summary and overall relevance of the provided information on oxidising solids

Difenoconazole was tested for its oxidizing properties according to the method EEC A.17 and the result shows it is not an oxidizing solid. However, this test method is not comparable to the test procedure for classification of oxidising solids according to CLP criteria, which is test O.1, described in Part III subsection 34.4.1 of the UN RTDG, Manual of Tests and Criteria (7th revision).

8.13.2 Comparison with the CLP criteria

According to Section 2.14.4.1 point b) of Annex I of CLP, for organic substances the classification procedure for this hazard class shall not apply if the substance or mixture contains oxygen, fluorine or chlorine and these elements are chemically bonded only to carbon or hydrogen. Difenoconazole does not contain such chemical groups and it was not an oxidizing substance according to test method EC A.17. Therefore, classification for this class is not applicable to difenoconazole.

8.13.3 Conclusion on classification and labelling for oxidising solids

Not classified – conclusive but not sufficient for classification.

8.14 Organic peroxides

Difenoconazole is not an organic peroxide. It does not contain the bivalent O-O- structure and it is not thermally unstable.

8.15 Corrosive to metals

8.15.1 Short summary and overall relevance of the provided information on the hazard class corrosive to metals

No data derived in accordance with the recommended test method in CLP (test in Part III, sub-section 37.4 of the UNRTDG Manual of Tests and Criteria) have been provided.

8.15.2 Comparison with the CLP criteria

According to the ECHA Guidance on the Application of the CLP Criteria (version 5.0 July 2017), the UN Test C.1 excludes solids while it considers ‘solids that may become liquid upon transportation’. Difenoconazole is supplied as a dry solid and its measured melting point is > 55ºC, which is the test temperature required in the UN Test C.1 test. Furthermore, evidence from manufacture and handling

shows that difenoconazole is not corrosive to metals. Therefore, difenoconazole does not meet the criteria for classification as corrosive to metals.

8.15.3 Conclusion on classification and labelling for corrosive to metals

Not classified – conclusive but not sufficient for classification.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Based on the available information, the DS proposed no classification of difenoconazole for the following hazards: flammable solid, phyrophoric solid, self-heating substance, substance that in contact with water emit flammable gases, oxidising solid and corrosive to metals. DS proposed no classification of difenoconazole for explosivity and self-reactive substance due to data lacking.

Comments received during consultation

The applicant acknowledged the data gaps identified for some physical hazards (explosivity and self-reactive substances) and confirmed that the missing studies according to the CLP Regulation (UN RTDG methods) would be conducted to fulfil the data requirement by October 2020. RAC noted that these studies were not finally submitted.

Assessment and comparison with the classification criteria

Difenoconazole contains a triazole substituent, i.e. contiguous nitrogen atoms, which structure is associated with explosive properties. In addition the exothermic decomposition energy is not available and therefore it is not possible to know whether the criteria for classification is fulfilled. However, RAC notes that, based on the negative result of one study with method EC A.14 and the handling experience, the concern for the explosive properties of difenoconazole is low. Overall, **based on lack of data, RAC supports the DS proposal for no classification of difenoconazole for explosive properties.**

An EC A.10 study showed as difenoconazole did not ignite on contact with the ignition source. However, as reflected in the CLP Guidance and ECHA Guidance on Information Requirements and Chemical Safety Assessment (R.7.1.10.3), if the result of an A.10 method indicates that a substance is not flammable, no more testing is necessary. Overall, **RAC supports the DS's proposal for no classification of difenoconazole as flammable solid.**

As commented above, difenoconazole contains a chemical structure structurally associated with explosive properties. However, no data on thermal stability are available. Thus, **based on lack of data, RAC supports the DS's proposal for no classification of difenoconazole as a self-reactive substance.**

There are no reports demonstrating that difenoconazole spontaneously ignites upon coming into contact with air at normal temperatures. In addition, RAC notes that if a substance does

not ignite upon contact with a very hot flame (as in an EU A.10 test) or upon heating, it will not ignite spontaneously at room temperature. Thus, **RAC supports the DS's proposal for no classification of difenoconazole as a pyrophoric solid.**

According to CLP criteria substances with melting point lower than 160 °C should not be considered for classification for self-heating substances. Difenoconazole has a melting point of 82-83 °C. Moreover, an EC A.16 test showed no auto-ignition below the melting point. Overall, **RAC supports the DS's proposal for no classification of difenoconazole for self-heating substances.**

Difenoconazole does not contain metals or metalloids. Moreover, the experience in production and handling shows that the substance does not react with water and is soluble in water forming a stable solution. Thus, **RAC supports the DS's proposal for no classification of difenoconazole for substances in contact with water emit flammable gases.**

Difenoconazole is not an oxidizing substance in an EC A.17 test. However, this method is not considered in the CLP criteria for the classification of oxidising solids. According to the CLP screening procedure, if the substance contains oxygen, fluorine or chlorine and these elements are chemically bonded only to carbon or hydrogen the classification is not warranted. Thus, **RAC supports the DS's proposal for no classification of difenoconazole for oxidising solids.**

There is no data derived in accordance with recommended methods for setting classification for substances corrosive to metals. However, only solids with a melting point below 55°C need to be tested, see CLP guidance 2.16.4.1., in addition, RAC notes that based on handling experience difenoconazole is not corrosive to metals. Overall, **RAC support the DS's proposal for no classification of difenoconazole as corrosive to metals.**

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 13: Summary table of toxicokinetic studies

	Type of study/guideline, deviation if any/species/route/dosage/test substance	Results/Remarks	Reference
In vivo single and repeated dose study	**E, D, M** Comparable to OECD TG 417 (1984) GLP: Yes, except in one study (Anonymous, 1988) Sprague Dawley Crl:CD®BR rats (♂, ♀) Oral dosage (by gavage): - Single dosing: [^{14}C] 0.5 mg/kg bw (group 1) and [^{14}C] 300 mg/kg bw (group 2) - Repeated dosing: Unlabelled 0.5 mg/kg bw for 14 d + single [^{14}C] 0.5 mg/kg bw (group 3) Unlabelled difenoconazole. Purity: 94.5% [^{14}C-pheny]-difenoconazole. Radiopurity: 98.6% [^{14}C-triazole]-difenoconazole. Radiopurity: 98% Vehicle: - Group 1 (labelled): Hi Sil 233-ethanol solution + 1% CMC - Group 2 (labelled): Acetone + Hi Sil 233-ethanol solution + 1% CMC - Group 3 (unlabelled/labelled): Hi Sil 233-ethanol solution + 1% CMC **Study acceptable as relevant**.	This summary is based on several study reports. The biological part is included in Anonymous study reports (1987a, 1987b), excretion and distribution in Anonymous study report (1988) and metabolism in Anonymous study report (1990) and Anonymous supplementary report (1993). Excretion: > 98% of the radioactivity administered recovered in all groups, the majority (>78%) in faeces, regardless of ^{14}C-label, dose level, dose regime or sex; most of the remainder of the excreted radioactivity was detected in urine. T½ of excretion: ≈ 20 h (low dose), ≈ 22 h (multiple low doses) and 33-48 h (high dose). Tissue residues: ≤ 1% of the dose, with highest levels in plama and fat in all dose groups (phenyl label), and only quantifiable residues in liver of rats given the high dose (triazole label). More radioactivity in tissues of rats dosed with [^{14}C-phenyl]-difenoconazole than in those dosed with [^{14}C-triazole]-difenoconazole. For both labelled forms, residues in ♀ tissues tended to be slightly lower than those in ♂. Faecal metabolites: Based on metabolite profiles, there were three major metabolites that were later identified. Metabolite A: Hydroxy-CGA 205375 (two isomers) → 53.4% of dose Metabolite B: Hydroxy-difenoconazole (two diastereomers) → 12.3% of dose Metabolite C: CGA 205375 (only detected at the high dose) → 12.6% of dose Urinary metabolites: The urinary metabolite profiles were more complex than of faeces with greater variability between label, dose and sex groups. Phenyl labelled urines showed a trend towards less polar metabolites and greater complexity in distribution than triazole labelled ones. Identified metabolites (triazole label): CGA 71019 (1,2,4-triazole) as one major metabolite. Identified metabolites (phenyl label): CGA 205375 (0.2% of dose) and its sulphate conjugate (2.8% of dose), hydroxy-CGA 205375 (1.7% of dose) and its sulphate conjugate (2.0% of dose), and hydroxy acetic acid (1.8% of dose); no single unknown metabolite accounts > 1.1% of dose. Tissue metabolites: CGA 189138 (chlorophenoxy-chlorobenzoic acid), identified in phenyl labelled liver samples. Metabolism: The major steps involve hydrolysis of the ketal resulting in CGA 205375 (metabolite C) with the ketone CGA 205374 as a postulated but not identified intermediate and hydroxylation on the outer phenyl ring of the parent (metabolite B) and in CGA 205375 (metabolite A). As a minor process cleavage of the alkyl chain between the triazole and the inner phenyl ring occur, resulting in a hydroxy acetic acid or an acetic acid moiety and free triazole. Sulfate conjugates were identified for CGA 205375 and hydroxy-CGA 205375.	**Anonymous 1 (1987a) B.6.1.1-03 (AS)** **Anonymous 2 (1987b) B.6.1.1-04 (AS)** **Anonymous 3 (1988) B.6.1.1-05 (AS)** **Anonymous 4 (1990, 1993) B.6.1.1-06 (AS)**

	Type of study/guideline, deviation if any/species/route/dosage/test substance	Results/Remarks	Reference
In vivo single dose study	**A, D, E, K** Comparable to OECD TG 417 (1984) GLP: No Sprague Dawley (SPF) rats (♂, ♀) Oral dosage (by gavage): 0.5 and 300 mg/kg bw. Unlabelled difenoconazole. Purity: not stated [^{14}C-pheny]-difenoconazole. Radiopurity: >97% Vehicle: - Low dose: Hi Sil 233-ethanol solution (labelled) + 1% CMC. - High dose: 1) Acetone (unlabelled) + Hi Sil 233-ethanol solution (labelled) + 1% CMC; 2) Toluene (labelled and unlabelled) + 1% CMC (only used for determining blood kinetics in ♀). **Study acceptable as relevant**	Absorption: 80-90% (low dose); 40-60% (high dose) 48 h post-dosing Bile elimination: 73-76% (low dose); 39-56% (high dose) Urinary excretion in bile duct cannulated rats: 9-14 % (low dose); 1% (high dose) Faecal excretion in bile duct cannulated rats: 2-4% (low dose); 17-22% (high dose). Enterohepatic circulation demonstrated: Bile from low dose treated male rats injected intraduodenally to other bile duct cannulated rats was re-absorbed by 48 h and 80% re-excreted in bile, 4% in urine and 14% in faeces. Blood kinetics: - AUC: 3-6 µg equiv.h/mL (low dose); 1710-2460 µg equiv.h/mL (high dose) - Cmax: 0.17-0.33 µg/g (low dose); 30- 48 µg/g (high dose) - Tmax: 0.5 -2 h (low dose); 4 h (high dose) - T½ : 3-4 d (both dose groups) No difference in blood kinetics when vehicle containing Hi Sil 233 or not (high dose) Tissue residues: The highest radioactivity was found in the liver, kidneys and adrenal glands (♂, ♀), and in the Harderian glands and adipose tissue (♀) 2 h after the low dose, and in fat, liver, Harderian glands, adrenal glands, kidneys and pancreas (♂, ♀) 4 h after the high dose. Residues in ♀ tissues tended to be lower than in ♂ tissues at 24-48 h and thereafter. At 7 d, only fat had comparable levels (low dose) or higher (high dose) than those present in plasma.	**Anonymous 5 (1992) B.6.1.1-01 (AS)**
In vivo reneated dose study	**A, D, BA, E, K, M** OECD TG 417 (1984) GLP: Yes HanBrl: WIST (SPF) rats (♂) Oral dosage (by gavage): 0.5 mg/kg bw for 14 d. 4 animals assigned to subgroups which were sacrificed at different time points after 1st dosing, i.e. day 1 (T1), day 7 (T2), day 14 (T3) and day 20 (T4). Unlabelled difenoconazole. Purity: 99.3% (only used for analytical purposes) [^{14}C-pheny]-difenoconazole. Radiopurity: 98.5% Vehicle: Polyethylene gyclol 200/ethanol/water 1/1/3 (v/v) **Study acceptable as relevant**.	Absorption: Difenoconazole was rapid and almost completely absorbed. >98% of dose was excreted within 7 d after the last dose (86% in faeces and 12% in urine). Excretion: A steady state was reached 3 d after 1st dosing. At plateau, the daily dose excreted was ≈ 85% in faeces and ≈ 12% in urine. After the dosing period the daily excretion decreased rapidly. Blood kinetics: A plateau is reached after 11 d after start of dosing. After cessation of dosing, the blood concentration was half the maximum concentration within 4 d – a moderately fast decline. Tissue residues: Most of the selected tissues reached a plateau 7 d after start of dosing, except liver, kidneys, fat and pancreas which reached the maximum residue at the end of the dosing period. The highest residues levels were found in liver and kidney. The depletion of test substance for most tissues was moderately fast with T½ of 4-6 d, more rapid in liver, kidneys and pancreas (T½ of 1-3 d) and slower in fat (T½ of 9 d). At the end of the experimental period < 0.5% of the administered dose remained in tissues. Metabolite profiles: The metabolite pattern of urine and faeces was essentially the same for all analysed time intervals (day 0-1, day 6-7, and day 13-14) although there were some slight quantitative differences between the first time interval and the last. At least 9 metabolite fractions in urine and at least 5 in faeces were revealed. Less than 2% of the dose was excreted in faeces as unchanged parent (Fr. 5) and 4-6% corresponded to CGA 205375 (Fr 3).	**Anonymous 6 (2003a) B.6.1.1-02 (AS)**

	Type of study/guideline, deviation if any/species/route/dosage/test substance	Results/Remarks	Reference
In vitro study	**M** *In vitro* comparative metabolism (HLM and RMN) There are no specific testing regulations/guidelines GLP: Yes Dosage: 10 µM Unlabelled difenoconazole. Purity: 88.2% [^{14}C-pheny]-difenoconazole. Radiopurity: 99.8% [^{14}C-triazole]-difenoconazole. Radiopurity: 99.2%	Difenoconazole was metabolised in HLM and RLM, being the metabolism NADPH-dependent and comparable for the two radiolabels, suggesting that no cleavage of difenoconazole occurred. The metabolic pattern of difenoconazole in RLM was qualitatively similar to HLM. All the human metabolites formed were detected in rat.	**Thibaut, R., 2017 B.6.1.1-07 (AS)**

A – Absorption; D – Distribution; BA – Bioaccumulation; M – Metabolism; E – Excretion; K– Kinetics; HLM - Human liver microsomes; RLM - Rat liver microsomes

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

Following oral administration of [^{14}C-phenyl]- or [^{14}C-triazole]-difenoconazole as a single dose of 0.5 or 300 mg/kg bw, or multiple doses of 0.5 mg/kg bw (labelled on the last day and unlabelled the previous 14 days) to rats, > 98% of the radioactivity administered was recovered in faeces (78-95%) and urine (8-22%), 7 days after dosing. T½ of excretion was ≈ 20 h (low dose), ≈ 22 h (multiple low doses) and 33-48 h (high dose). Most of the radioactivity was excreted in the faeces (<78%), mainly in samples collected in the 12-24 h and 24-48 h time period, regardless of ^{14}C-label, dose level, dose regime or sex. Rats given [^{14}C-triazole] label excreted the radioactivity faster than rats given [^{14}C-phenyl] label (multiple and high dose groups), and the female metabolism of the ^{14}C-phenyl label was slower than the corresponding metabolism in males (high dose group). Tissue residues were low at sacrifice (7 days post-dosing) and represented ≤ 1% of dose. There was more radioactivity in tissues of phenyl label dosed rats than in those of triazole label dosed rats indicating that the bridge between the phenyl and triazole rings must sometimes be susceptible to metabolic cleavage. For phenyl label, the highest levels of radioactivity were found in plasma and fat (fat level was higher than plasma level for low and high dose groups). For triazole label, the only quantifiable residues were found in liver of rats treated with the high dose. For both labelled forms, residues in female tissues tended to be slightly lower than those in males.

Following a single oral dose of 0.5 mg [^{14}C-phenyl]-difenoconazole/kg bw administered to rats, maximum blood concentrations were reached at 2 h (♂) and 0.5 h (♀), followed by a rapid decline with T½ of 4-6 h up to 12 h and T½ of 3-4 d from 24-168 h. After a single oral dose of 300 mg [^{14}C-phenyl]-difenoconazole/kg bw, peak blood concentrations were reached after ≈ 4 h, with T½ of 22-24 h up to 72 h and 3-4 days from 96 to 168 h. In females, Cmax and AUC reached only about 50% of the respective values in males (low dose) and were 63% and 70% of the respective values in males (high dose). Blood levels were proportional to the dose. When rats were bile duct cannulated, the systemic dose was eliminated predominantly via bile, accounting for 73-76% (low dose) and 39-56% (high dose), urinary excretion accounted 9-14% (low dose) and 1% (high dose) and faecal excretion accounted 2-4% (low dose) and 17-22% (high dose), which confirm an absorption of 80-90% (low dose) and 40-60% (high dose) within 48 h after dosing. When bile from males dosed with difenoconazole at 0.5 mg/kg bw was administered intraduodenally to other bile duct cannulated rats, 80% of the dose was re-eliminated via bile and just 4% in urine, thereby demonstrating entero-hepatic recirculation. The highest tissue radioactivity was found in the liver, kidneys and adrenal glands (both sexes), and in the Harderian glands and adipose tissue (♀) 2 h after the low dose, and in fat, liver, Harderian glands, adrenal glands, kidneys and pancreas (both sexes) 4 h after the high dose. Residues in female tissues tended to be lower than in male tissues at 24-48 h and thereafter. At 7 days only fat had comparable levels (low dose) or higher (high dose) than those present in plasma.

Following 14 daily doses of 0.5 mg [^{14}C-phenyl]-difenoconazole/kg bw administered to male rats, >98% of dose was excreted within 7 d after the last dose (86% in faeces and 12% in urine). Since it is assumed that the majority of absorbed test substance re-enters the intestinal tract by biliary excretion and is finally excreted with faeces, difenoconazole absorption was considered to be rapid and almost complete. A steady state in terms of excretion was reached 3 days after the 1st dosing; at plateau the daily dose excreted accounted for ≈ 85% in faeces and ≈12% in urine; after the last dosing the daily excretion decreased rapidly. The blood kinetics showed increasing concentrations with ongoing administrations and a plateau was reached 11 days after the start of dosing; after cessation of dosing, the blood concentration was half the maximum concentration within 4 days – a moderately fast decline. The residues in most of the selected tissues reached a plateau 7 days after start of dosing, except liver, kidneys, fat and pancreas which reached the maximum residue at the end of the dosing period; however, based on the figure of residues *vs* time, it is assumed that for these tissues the plateau levels will be reached within 2 or 3 weeks of multiple dosing. The highest residues levels were found in liver and kidney, followed by plasma and lungs. Tissue depletion was moderately fast in most tissues (T½ of 4-6 days), more rapid in liver, kidneys and pancreas (T½ of 1-3 days) and slower in fat (T½ of 9 days). 7 days after the last dosing < 0.5% of dose remained in tissues.

From all of above it follows that difenoconazole showed no potential for accumulation in body tissues.

Metabolites profiles were obtained using day 2 or day 3 individual urine and faeces samples from one male and one female of each of following dosing groups: 1) a single oral dose of 0.5 mg [^{14}C-phenyl] or [^{14}C-triazole]-difenoconazole/kg bw; 2) a single oral dose of 300 mg [^{14}C-phenyl] or [^{14}C-triazole]-difenoconazole/kg bw; 3) 14 daily oral doses of 0.5 mg unlabelled difenoconazole/kg bw followed by a single oral dose of 0.5 mg [^{14}C-phenyl]- or [^{14}C-triazole]-difenoconazole/kg. Some sex differences in the relative abundance of the faecal metabolites were observed, but in most cases the abundances are very similar when like samples of the different labels are compared, indicating that in the major faecal metabolites both ring systems are intact. The three major faecal metabolites (A, B and C) were isolated from combined samples of four phenyl labelled high dose females. Metabolite A was identified as hydroxy-CGA 205375 (53.4% of dose), metabolite B as hydroxy-difenoconazole (12.3% of dose) and metabolite C as CGA-205375 (12.6% of dose). Metabolites A and B each separated into two isomers. In terms of the NIH shift mechanism, the metabolites A1 and A2 would result from chloride retention and chloride shift, respectively. For metabolite B, only diastereomers of the chloride shift substitution are observed. Metabolite profiles for urine were complex, with a greater variability between label, dose and sex groups than was observed for the faeces. The triazole labelled urines contained a major polar metabolite, not observed in the phenyl labelled urines. The phenyl labelled urines showed in general a trend toward less polar metabolites and greater complexity in distribution than triazole labelled ones. From pooled day-2 or 3-urine samples of four triazole labelled high dose males, one major metabolite (≥ 10%) was isolated, and identified as CGA-71019 (1, 2, 4-triazole). From pooled day-1-urine samples of three phenyl labelled high dose females, metabolites were isolated and identified as CGA-205375 (0.2%) and its sulphate conjugate (2.8%), hydroxy-CGA 205375 (1.7%) and its sulphate conjugate (2%), and hydroxy acetic acid (1.8%), and no single unknown urine metabolite accounts for more than 1.1% of dose. Due to the low levels of tissue deposition in the rat, liver was chosen as a representative tissue because, it exhibited comparable phenyl label distribution to other tissues, and was the only organ showing detectable triazole label residues. One metabolite was isolated from the combined liver samples of phenyl labelled high dose males and identified as CGA 189138 (chlorophenoxy-chlorobenzoic acid).

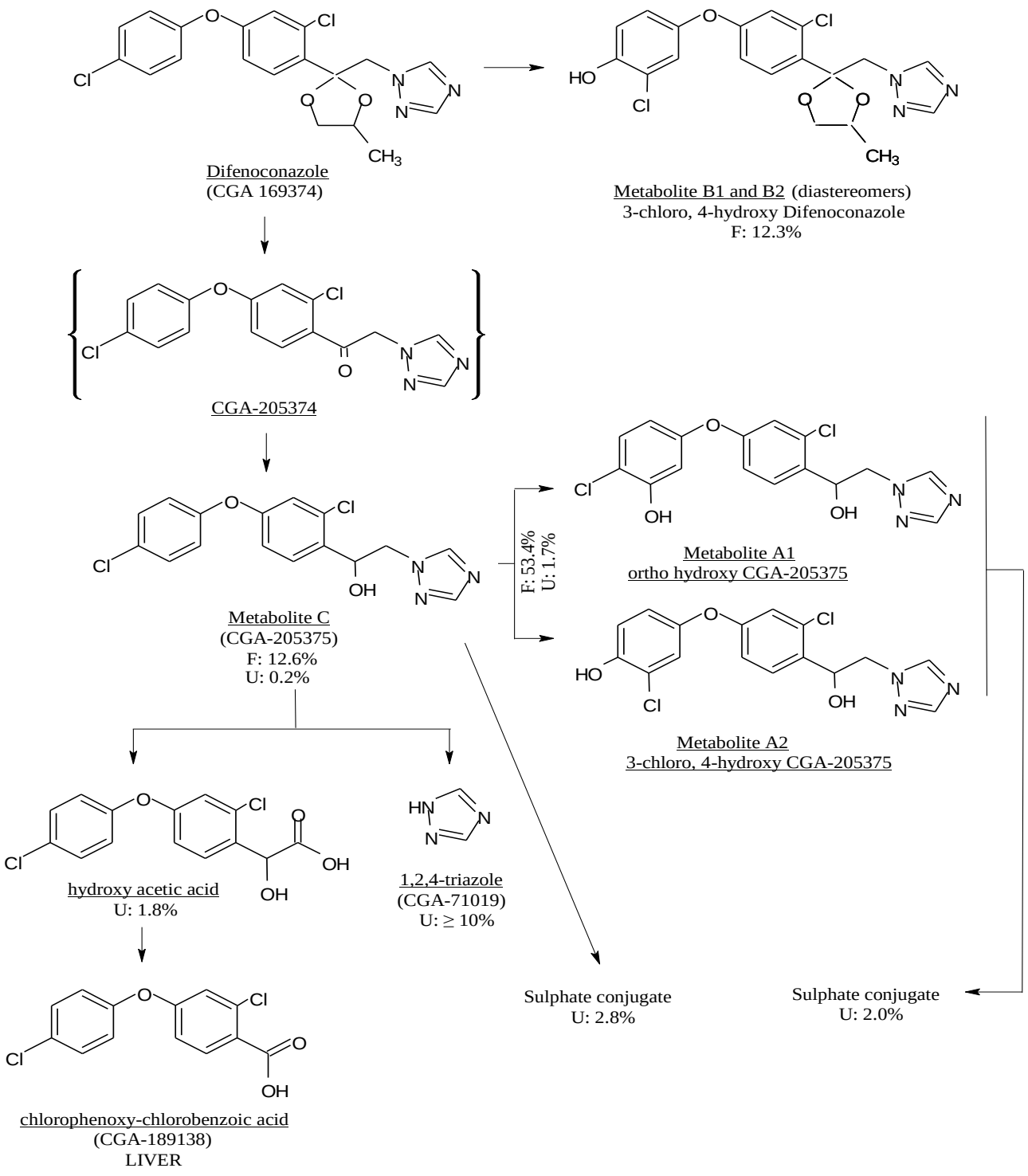
The metabolite profiles in urine and faeces were not essentially influenced by multiple dosing. Following 14 daily doses of 0.5 mg [^{14}C-phenyl]-difenoconazole/kg bw administered to male rats, the metabolite pattern of urine and faeces was essentially the same for all analysed time intervals (day 0-1, day 6-7, and day 13-14) although there were some slight quantitative differences between the first time interval and the last. At least 9 metabolite fractions in urine and at least 5 in faeces were revealed. In faeces, < 2% of the daily dose was excreted as difenoconazole (Fr. 5) and 4-6% as CGA 205375 (Fr 3).

It can be said that the difenoconazole molecule was extensively metabolised. The presence of 1, 2, 4 triazole and hydroxyacetic acid in urine and that of chlorophenoxy-chlorobenzoic acid in liver provides evidence that some bridge cleavage is occurring. It appears likely, that the triazole and the hydroxyacetic acid are excreted in urine because of their polar nature while the free phenyl acid is absorbed by the tissues due to its lipophilic character. The extensive biliary elimination was consistent with the relatively high molecular weights of the major metabolites detected in faeces.

The proposed metabolic pathway is shown in Figure 1. The major steps of the metabolism of difenoconazole in the rat involve hydrolysis of the ketal resulting in CGA 205375 (metabolite C) with the ketone CGA 205374 as a postulated but not identified intermediate and hydroxylation on the outer phenyl ring of the parent (metabolite B) and in CGA 205375 (metabolite A). As a minor process cleavage of the alkyl chain between the triazole and the inner phenyl ring occur, resulting in a hydroxy acetic acid or an acetic acid moiety and free triazole. Sulphate conjugates were identified for CGA 205375 and for hydroxy-CGA 205375.

Finally, results of the *in vitro* comparative metabolism study showed that difenoconazole was metabolised by both human and rat liver microsomes (HLM and RLM), being the metabolism NADPH-dependent and comparable for [^{14}C-phenyl]- and [^{14}C-triazole]- labels, suggesting that no cleavage of difenoconazole occurred. The metabolic pattern of difenoconazole in RLM was qualitatively similar to HLM. All the human metabolites formed were detected in rat. Therefore, it can be said that all potential human metabolites of difenoconazole have been tested in the pivotal toxicology species, thus demonstrating its relevance to derive human toxicological reference values.

Figure 1: Metabolic Pathway of Difenoconazole in Animals



U: urine; F: faeces; figures refer to % of dose in faeces or urine

10 EVALUATION OF HEALTH HAZARDS

10.1 Acute toxicity - oral route

Table 14: Summary table of animal studies on acute oral toxicity

<table>
<tr><th>Method, guideline, deviations if any</th><th>Species, strain, sex, no/group, test substance, dose levels, duration of exposure</th><th>Value
LD_{50}</th><th>Reference</th></tr>
<tr>
<td>Acute oral toxicity study in rats
OECD TG 401 (1981)
GLP: Yes
Study acceptable</td>
<td>Species: Rat
Strain: Sprague-Dawley
Oral (gavage)
Single dose
Purity: Not specified
Vehicle: 3% corn starch with 1% polysorbate 80
5 Rats/sex/group
Doses: 0, 1000, 2000, 3000 mg/kg bw
14-day observation period</td>
<td>Mortality:
<table><tr><th>Dose mg/kg bw</th><th>Males Mortality</th><th>Females Mortality</th></tr><tr><td>0</td><td>0/5</td><td>0/5</td></tr><tr><td>1000</td><td>2/5</td><td>2/5</td></tr><tr><td>2000</td><td>2/5</td><td>2/5</td></tr><tr><td>3000</td><td>5/5</td><td>5/5</td></tr></table>Clinical signs: hypoactivity, stains around the mouth, perineal staining, ataxia, lacrimation, soft faeces, hypothermia, salivation, spasms, postration, chromodacryorrhea, and postration in both males and females.
Necropsy: solid red clot and/or red stomach in males and females of the 2000 mg/kg bw dose group.
Body weight: slight decrease in body weight in both males and females in the 2000 mg/kg bw dose group.
LD_{50} = 1453 mg/kg bw for both sexes</td>
<td>Anonymous 7 (1987) B.6.2.1-01 (AS)</td>
</tr>
<tr>
<td>Acute oral toxicity study in mice
OECD TG 401 (1987)
GLP: Yes
Study acceptable</td>
<td>Species: Mouse
Strain: Tif:MAG f (SPF)
Oral (gavage)
Single dose
Purity: Not specified
Vehicle: Arachis oil
5 Mice/sex/group
Doses: 1000, 2000 mg/kg/bw
14-day observation period</td>
<td>Mortality:
<table><tr><th>Dose mg/kg bw</th><th>Males Mortality</th><th>Females Mortality</th></tr><tr><td>1000</td><td>0/5</td><td>1/5</td></tr><tr><td>2000</td><td>1/5</td><td>2/5</td></tr></table>Clinical signs: piloerection, abnormal body positions, dyspnea, reduced locomotor activity and ataxia.
Necropsy: no treatment-related effects.
Body weight: no treatment-related effects.
LD_{50} >2000 mg/kg bw for both sexes</td>
<td>Anonymous 8 (1990) B.6.2.1-02 (AS)</td>
</tr>
</table>

10.1.1 Short summary and overall relevance of the provided information on acute oral toxicity

Two acute oral toxicity studies were evaluated.

The acute oral toxicity study in rats (B.6.2.1-01) reported an estimated LD_{50} value (both sexes) equal to 1453 mg/kg bw/day.

The acute oral toxicity study in mice (B.6.2.1-02) reported an observed LD_{50} value (both sexes) greater than 2000 mg/kg bw/day.

10.1.2 Comparison with the CLP criteria

The LD_{50} values obtained in rats and mice were 1453 mg/kg bw/day and greater than 2000 mg/kg bw/day, respectively. Based on the classification criteria under Regulation (EC) No. 1272/2008 the LD_{50} in rats is below the threshold value of 2000 mg/kg, which grants the classification for acute oral toxicity.

10.1.3 Conclusion on classification and labelling for acute oral toxicity

In rats, ATE was 1453 mg/kg bw. Therefore, according to the criteria under Regulation (EC) No. 1272/2008 difenoconazole is classified for acute oral toxicity, Acute Tox. 4 (H302).

10.2 Acute toxicity - dermal route

Table 15: Summary table of animal studies on acute dermal toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group, test substance, dose levels, duration of exposure	Value LD_{50}	Reference
Acute dermal toxicity study in rabbits OECD TG 402 (1981) GLP: Yes **Study acceptable**	Species: Rabbit Strain: New Zealand White Dermal route (occlusive dressing) Purity: Not specified Vehicle: Ethanol 5 Rabbits/sex/dose Dose: 2010 mg/kg bw (limit test) Single dose 24h exposure 14-day observation period	**Mortality:** none **Clinical signs**: mild cutaneous effects including erythema (2 males and 1 female) and desquamation of the skin in all animals on day 7 and all males and 2 females on day 14. **Necropsy**: no treatment-related effects **Body weight**: no treatment-related effects. **LD_{50} > 2010** mg/kg bw for both sexes	**Anonymous 9 (1987a) B.6.2.2 (AS)**

10.2.1 Short summary and overall relevance of the provided information on acute dermal toxicity

The acute dermal toxicity study in rabbits (B.6.2.2) reported an observed LD_{50} value (both sexes) greater than 2010 mg/kg bw/day.

10.2.2 Comparison with the CLP criteria

LD_{50} greater than 2010 mg/kg bw is above the threshold value of 2000 mg/kg bw for triggering acute dermal toxicity classification.

10.2.3 Conclusion on classification and labelling for acute dermal toxicity

Data available indicates that difenoconazole does not require classification for acute dermal toxicity.

10.3 Acute toxicity - inhalation route

Table 16: Summary table of animal studies on acute inhalation toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group, duration of exposure	Test substance, dose levels, form and particle size (MMAD)	Value LC_{50}	Reference
Acute inhalation toxicity study in rats OECD 403 (1981) GLP: Yes **Study acceptable**	Species: Rat Strain: Tif: RAI f (SPF) Nose-only exposure for 4h followed by a 14-day observation period Single dose 5 Rats/sex/dose	Purity: 96.2% Suspension of difenoconazole with 5% Sipernat 50 S (inert silica). Maximum achievable concentration. Parameter / Value: Nominal concentration (mg/m^3): 3967 Gravimetric concentration (mg/m^3): 3458 ± 137 Mean exposure concentration corrected for 5% Sipernat 50 S (mg/m^3): 3285 Particle size MMAD (µm): 1.1 - 1.5 GSD: 2.8 – 3.8 Particles < 3 µm: 73-81%	**Mortality:** none **Clinical signs**: piloerection, hunched posture, dyspnea and reduced locomotor activity. **Necropsy**: no treatment-related effects. **Body weight**: lower body weight gain in males on the first week after exposure. **LC_{50} > 3.3** mg/L (both sexes)	**Anonymous 10 (1991) B.6.2.3 (AS)**

10.3.1 Short summary and overall relevance of the provided information on acute inhalation toxicity

The acute inhalation toxicity study in rats (B.6.2.3) reported an observed LC_{50} value (both sexes) greater than 3.3 mg/L (maximum attainable concentration).

10.3.2 Comparison with the CLP criteria

The four-hour inhalation study in rats reported an $LC_{50} \geq 3.3$ mg/L (maximum attainable concentration). According to the classification criteria under Regulation (EC) No. 1272/2008 the threshold for no classification for acute inhalation toxicity is an $LC_{50} > 5$ mg/L for dusts or mists.

However, considering that the maximum attainable concentration did not produce any mortality, no classification for acute inhalation toxicity is therefore proposed.

10.3.3 Conclusion on classification and labelling for acute inhalation toxicity

Data available indicates that difenoconazole does not require classification for acute inhalation toxicity.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The CLH report contains two acute oral toxicity studies in rats and mice reporting LD_{50} of 1453 mg/kg bw and greater than 2000 mg/kg bw; respectively. The CLH report also contains one acute dermal toxicity study in rabbits with an LD_{50} greater than 2010 mg/kg bw. The only acute inhalation toxicity study yielded a LC_{50} greater than 3.3 mg/L. Based on the result of these studies, the DS proposed no classification of difenoconazole for acute dermal and inhalation toxicity and classification for acute oral toxicity category 4 (H302) with and ATE of 1453 mg/kg bw.

Comments received during consultation

One Member State Competent Authority (MSCA) supported the classification of difenoconazole as Acute Tox. 4; H302 and the oral ATE of 1453 mg/kg bw.

Assessment and comparison with the classification criteria

The table below summarises all the available studies for assessment of acute toxicity of difenoconazole.

Table: Summary of animal studies on acute toxicity with difenoconazole.

Study	Dose level	Results	Reference
Acute oral toxicity study in rats OECD TG 401 GLP: Yes Sprague-Dawley rats Oral (gavage) 5 rats/sex/group 14-day observation period	Vehicle: 3% corn starch with 1% polysorbate 80 Doses: 0, 1000, 2000, 3000 mg/kg bw Purity: Not specified	<u>Clinical signs:</u> hypoactivity, stains around the mouth, perineal staining, ataxia, lacrimation, soft faeces, hypothermia, salivation, spasms, prostration, chromodacryorrhea, and prostration in both males and females. <u>Necropsy:</u> solid red clot and/or red stomach in males and females of the 2000 mg/kg bw dose group. <u>Body weight:</u> slight decrease in body weight in both males and females in the 2000 mg/kg bw dose group. DOSE (mg/kg bw) / MORTALITY Males / Females 0 — 0/5 / 0/5 1000 — 2/5 / 2/5 2000 — 2/5 / 2/5	Anonymous 7, 1987 B.6.2.1-01 (AS)

		3000 5/5 5/5 **LD_{50} = 1453 mg/kg bw for both sexes**	
Acute oral toxicity study in mice OECD TG 401 (1987) GLP: Yes Tif: MAG f (SPF) mouse Oral (gavage) 5 mice/sex/group 14-day observation period	Vehicle: Arachis oil Purity: Not specified Doses: 1000, 2000 mg/kg bw	Clinical signs: piloerection, abnormal body positions, dyspnoea, reduced locomotor activity and ataxia. Necropsy: no treatment-related effects. Body weight: no treatment-related effects. DOSE (mg/kg bw) / MORTALITY Males / Females 1000 0/5 1/5 2000 1/5 2/5 **LD_{50} >2000 mg/kg bw for both sexes**	Anonymous 8, 1990 B.6.2.1-02 (AS)
Acute dermal toxicity Study OECD TG 402 (1981) GLP: Yes New Zealand White rabbits Occlusive dressing 5 rabbits/sex/dose 14-day observation period	Vehicle: Ethanol Purity: Not specified Dose: 2010 mg/kg bw (limit test) 24h exposure	Clinical signs: mild cutaneous effects including erythema (2 males and 1 female) and desquamation of the skin in all animals on day 7 and all males and 2 females on day 14. Necropsy: no treatment-related effects Body weight: no treatment-related effects. Mortality: none **LD_{50} > 2010 mg/kg bw for both sexes**	Anonymous 9, 1987a B.6.2.2 (AS)
Acute inhalation toxicity study OECD 403 (1981) GLP: Yes Tif: RAI f (SPF) rats 5 rats/sex/dose Nose-only 14-day observation period	Exposure for 4 h Purity: 96.2% Suspension of difenoconazole with 5% Sipernat 50 S (inert silica) (maximum achievable concentration) Nominal concentration: 3967 mg/m^3 Gravimetric concentration: 3458 ± 137 mg/m^3	Clinical signs: piloerection, hunched posture, dyspnoea and reduced locomotor activity. Necropsy: no treatment-related effects. Body weight: lower body weight gain in males on the first week after exposure. Mortality: none **LC_{50} > 3.3 mg/L**	Anonymous 10, 1991 B.6.2.3 (AS)

	Particle size MMAD: 1.1 - 1.5 µm Particles < 3 µm: 73-81%

Comparison with the criteria

Two acute oral toxicity studies performed following OECD Guidelines and GLP compliant yielded LD_{50} of 1453 mg/kg bw (rats) and greater than 2000 mg/kg bw (mice). The LD_{50} estimated for rats fulfils the criteria for classification within category 4 (LD_{50} greater than 300 mg/kg bw and lower than 2000 mg/kg bw). Thus, classification of difenoconazole for acute oral toxicity is warranted. **RAC supports the DS's proposal for classification of difenoconazole as Acute Tox. 4; H302** via the oral route with the ATE derived from the LD_{50} in rats rounded to 1450 mg/kg bw.

One acute dermal toxicity study performed following OECD Guideline and GLP compliant yielded a LD_{50} greater than 2000 mg/kg bw. This LD_{50} is above the threshold value of 2000 mg/kg bw considered for triggering classification. Thus, **RAC supports the DS's proposal for no classification of difenoconazole for acute dermal toxicity.**

One acute inhalation toxicity study performed following OECD Guideline and GLP compliant yielded a LC_{50} greater than the maximum attainable concentration of 3.3 mg/L. The threshold for triggering classification for acute inhalation is 5.0 mg/L for dust and mist. However, considering that 3.3 mg/L caused no mortalities, RAC notes that it is very unlikely that the LC_{50} could be below 5.0 mg/L. Overall, **RAC supports the DS's proposal for no classification of difenoconazole for acute inhalation toxicity.**

10.4 Skin corrosion/irritation

Table 17: Summary table of animal studies on skin corrosion/irritation

Method, guideline, deviations if any	Species, strain, sex, no/group, test substance, dose levels, duration of exposure	Results -Observations and time point of onset -Mean scores/animal -Reversibility	Reference
Primary dermal irritation study in rabbits Method comparable to OECD 404 (2002) GLP: Yes Deviations: Skin reactions were scored at 30min instead of 60min after patch removal. Both the temperature (21-25°C) and humidity (66-77%) of the animal room deviated	Species: Rabbit Strain: Hra: (New Zealand White) SPF 3 Rabbits/sex/dose Purity: 91.5% Vehicle Dose: 0.5 g moistened with 0.9% saline. Test item applied under semi-occluded conditions. Exposure: 4 hours	No mortality occurred. One female rabbit was found with a grade 1 erythema at 30 min post patch removal. This effect was totally reversible at 24h. The average irritation scores observed at 24h, 48h and 72h for both erythema and edema were **0**. Conclusion: **Non-irritant**	**Anonymous 11 (1991a) Anonymous 12 (1992) (Supplemental information irritation study) B.6.2.4. (AS)**

Method, guideline, deviations if any	Species, strain, sex, no/group, test substance, dose levels, duration of exposure	Results -Observations and time point of onset -Mean scores/animal -Reversibility	Reference
from the TG 404 recommendations of 20±3°C and 50-60%, respectively **Study acceptable**			

10.4.1 Short summary and overall relevance of the provided information on skin corrosion/irritation

The primary dermal irritation study in rabbits (B.6.2.4) reported a mild erythema in one female 30min after patch removal, which was totally reversible at 24 hours. The average irritation scores at 24h, 48h and 72h of 0 for both erythema and edema. Despite the slight deviation from the method, i.e. 30min rather than 60min post-patch removal, the observed irritation on the skin was reversible at 24 hours.

10.4.2 Comparison with the CLP criteria

The average irritation scores observed at 24h, 48h and 72h were 0 for both erythema and edema, hence below the threshold for classification as skin irritant.

10.4.3 Conclusion on classification and labelling for skin corrosion/irritation

Data available indicates that difenoconazole does not require classification for skin irritation.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole for skin irritation/corrosion based on the result of an irritation study in rabbits showing no erythema and no oedema.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The table below summarises the available study for assessment of dermal irritation/corrosion of difenoconazole.

Table: Summary of the animal study on skin corrosion/irritation with difenoconazole.

Study	Dose level	Results	Reference
Primary dermal irritation study in rabbits Method comparable to OECD TG 404 (2002) GLP: Yes Deviations: Skin reactions were scored at 30 min instead of 60 min after patch removal Hra: (New Zealand White) SPF rabbits 3 rabbits/sex/dose	Purity: 91.5% 0.5 g moistened with 0.9% saline Test item applied under semi-occluded conditions Exposure: 4 hours	One female rabbit was found with a grade 1 erythema at 30 min post patch removal This effect was totally reversible at 24 h The average irritation scores observed at 24h, 48h and 72h for both erythema and oedema were 0	Anonymous 11, 1991a Anonymous 12, 1992 Supplemental information irritation study B.6.2.4. (AS)

RAC supports the DS's proposal for no classification of difenoconazole for dermal irritation or corrosion since no erythema or oedema were observed.

10.5 Serious eye damage/eye irritation

Table 18: Summary table of animal studies on serious eye damage/eye irritation

<table>
<tr><th>Method, guideline, deviations if any</th><th>Species, strain, sex, no/group, test substance, dose levels, duration of exposure</th><th>Results
- Observations and time point of onset
- Mean scores/animal
- Reversibility</th><th>Reference</th></tr>
<tr>
<td>Primary eye irritation study in rabbits

Method comparable to OECD 405 (2002)

GLP: Yes

Deviations: The temperature and humidity of the animal room deviated from guideline recommendation

Study acceptable</td>
<td>Species: Rabbit

Strain: Hra: (New Zealand White) SPF

Purity: 91.5%

3 Rabbits/sex/unwashed group

2 male and 1 female rabbit/washout group (test item was washed off 30 seconds after instillation)

Dose: 0.05 g test item (0.1 ml weight equivalent) undiluted was instilled into the right eye. The left eye served as control.

Observations after 1h, 24h, 48h, 72h and 96h (end of study).</td>
<td>No mortality occurred. No systemic signs of toxicity were noted during the study.

Signs of irritation were observed in the cornea, iris and conjunctiva of unwashed eyes, which were reversible by day 4 of the study. Reactions in the eyes of the washout group were milder and shorter in duration.

The mean scores calculated at 24h, 48h and 72h are shown in the following table for both washed and unwashed groups:
<table>
<tr><th>Treatment</th><th>Cornea</th><th>Iris</th><th colspan="2">Conjunctiva</th></tr>
<tr><th></th><th></th><th></th><th>Redness</th><th>Chemosis</th></tr>
<tr><td rowspan="5">Unwashed group</td><td>0.6</td><td>0.6</td><td>2</td><td>1</td></tr>
<tr><td>0.3</td><td>0.6</td><td>2.6</td><td>0.6</td></tr>
<tr><td>0.3</td><td>0.3</td><td>2</td><td>0.6</td></tr>
<tr><td>1.3</td><td>0.6</td><td>2.6</td><td>1.6</td></tr>
<tr><td>0</td><td>0.3</td><td>1</td><td>0.3</td></tr>
</table>
</td>
<td>Anonymous 13 (1991b) B.6.2.5. (AS)</td>
</tr>
</table>

			0	0	1.6	0.6
		Washout group	0	0.3	1	0.6
			0	0	1.3	0.3
			0	0	1.3	0

Results: 4/6 animals in the washout group showed redness scores ≥2 observed at 24h, 48h and 72h. The effects were reversible at 96h.

Conclusion: **Eye irritant**

10.5.1 Short summary and overall relevance of the provided information on serious eye damage/eye irritation

The primary eye irritation study in rabbits (B.6.2.5) reported conjunctival erythema in four out of six rabbits in the unwashed test group with scores ≥2 (mean score calculated at 24, 48 and 72h). One animal form this test group also showed corneal lesion of 1.3 score. These effects were reversible by day 4.

10.5.2 Comparison with the CLP criteria

According to the ECHA Guidance on the application of the CLP criteria (July 2017) when 6 rabbits are used in the eye irritation study the test material is considered irritant to the eye when conjunctival erythema is ≥ 2 in at least 4/6 animals. The irritation scores reported in the study are therefore within the threshold criteria under Regulation (EC) No. 1272/2008 required for classification of the test item as an eye irritant.

10.5.3 Conclusion on classification and labelling for serious eye damage/eye irritation

Based on the data available and according to the criteria under Regulation (EC) No. 1272/2008 difenoconazole is classified as eye irritant category 2, Eye Irrit. 2 (H319).

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The DS proposed the classification of difenoconazole as Eye Irrit. 2; H319 based on the results of one assay comparable to OECD TG 405 showing conjunctival erythema in four out of six rabbits with scores ≥ 2 (mean score calculated at 24, 48 and 72h).

Comments received during consultation

One MSCA supported the classification of difenoconazole as Eye Irrit. 2; H319.

Assessment and comparison with the classification criteria

The table below summarises the available study for assessment of serious eye damage/eye irritation of difenoconazole.

Table: Summary of the animal study on eye irritation/corrosion with difenoconazole.

Study	Dose level	Results	Reference
Primary eye irritation study in rabbits Method comparable to OECD TG 405 (2002) GLP: Yes Hra: (New Zealand White) SPF rabbits 3 rabbits/sex/unwashed group 2 male and 1 female rabbit/washout group	Purity: 91.5% Washout group: test item was washed off 30 seconds after instillation Dose: 0.05 g test item (0.1 mL weight equivalent) undiluted was instilled into the right eye. Observations after 1h, 24h, 48h, 72h and 96h	Mean scores calculated at 24h, 48h and 72h:	Anonymous 13, 1991b B.6.2.5. (AS)

			Conjunctiva	
	Cornea	Iris	Redness	Chemosis
Unwashed	0.6	0.6	2	1
	0.3	0.6	2.6	0.6
	0.3	0.3	2	0.6
	1.3	0.6	2.6	1.6
	0	0.3	1	0.3
	0	0	1.6	0.6
Washed	0	0.3	1	0.6
	0	0	1.3	0.3
	0	0	1.3	0

Signs of irritation were reversible by day 4

Comparison with the criteria

According to the classification criteria, a reversible conjunctival redness ≥ 2 in 2 of 3 animals warrants classification. The table above shows as four of six rabbits showed a reversible redness score ranging between 2 and 2.6. Thus, the criteria are met and **RAC supports the DS's proposal for classification of difenoconazole as Eye Irrit. 2; H319 (causes serious eye irritation).**

10.6 Respiratory sensitisation

10.6.1 Short summary and overall relevance of the provided information on respiratory sensitisation

No data available.

10.6.2 Comparison with the CLP criteria

No data available.

10.6.3 Conclusion on classification and labelling for respiratory sensitisation

In the absence of any data, no classification can be drawn.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole for respiratory sensitisation based on lack of data.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC notes that: i) there are no data indicating evidence of respiratory tract irritation with difenoconazole; ii) the acute inhalation study showed no evidence of respiratory system impairment; and iii) rabbit dermal and eye irritation studies indicated lack of irritant potential on skin and mucosal membranes. Overall, RAC agrees the DS's assessment that no classification is warranted for difenoconazole for respiratory sensitisation based on lack of data. However, RAC notes that due to an omission, this hazard class was not open for consultation, although the DS proposal was made available.

10.7 Skin sensitisation

Table 19: Summary table of animal studies on skin sensitisation

<table>
<tr><th>Method, guideline, deviations if any</th><th>Test substance, species, strain, sex, no/group</th><th>Dose levels, duration of exposure and results</th><th>Reference</th></tr>
<tr>
<td>Modified Buehler Test in guinea pigs
Guideline: OECD 406 (1981)
Deviations: The number of animals used in the study is less than the recommended, i.e. 10 vs 20 recommended by the guideline. Also, information on the size of the pads is not provided.
GLP: Yes
Study acceptable</td>
<td>Species: Guinea Pig
Strain: Hartley
Sex: Females
Purity: Not specified
4 animals in preliminary test
10 animals in main test/group</td>
<td><u>Preliminary test</u>: The irritation potential of the test item was determined in 4 animals at a dose of 0.5 g neat. No information is given on the outcome of this test.

<u>Main test</u>: the test procedure was a modification of the Buehler method. Group designations and concentrations of test chemical and positive control (DNCB) used during each phase of the test procedure are summarised as follows:
<table>
<tr><th>Group</th><th>No. of animals</th><th>Topical induction</th><th>Topical challenge</th></tr>
<tr><td>1. non-sensitised</td><td>10</td><td>not performed</td><td>Difenoconazole (0.5 g neat)</td></tr>
<tr><td>2. sensitised</td><td>10</td><td>Difenoconazole (0.5 g neat)</td><td>Difenoconazole (0.5 g neat)</td></tr>
<tr><td>3. non-sensitised</td><td>10</td><td>not performed</td><td>positive control 0.05%</td></tr>
<tr><td>4. sensitised</td><td>10</td><td>positive control 0.05%</td><td>positive control 0.05%</td></tr>
</table>
<u>Topical induction phase– days</u>: 1-22</td>
<td>Anonymous 14 (1987b) B.6.2.6 (AS)</td>
</tr>
</table>

Method, guideline, deviations if any	Test substance, species, strain, sex, no/group	Dose levels, duration of exposure and results	Reference
		An unknown area on one flank was shaved prior to dosing. Test chemical or positive control was applied to a gauze patch of unknown size and placed on the flanks of group-2 and 4 animals (the sensitized groups), with an occlusive tape and left in place for 6 h. The induction process was repeated on days 1, 3, 6, 8, 10, 13, 15, 17, 20 and 22 giving a total of ten 6-hour exposures. At exposure termination, patches were removed and the test site was wiped with 95% ethanol and water. Dermal observations were made 24 h after each induction dose. Animals of groups 1 and 3 were left untreated during the induction phase. Topical challenge phase– day: 36 The test chemical was applied, in a similar manner to the induction treatments, to a previously untreated skin site of all animals, including non-sensitised group-1-and 3-animals. Skin sites were examined 24 and 48 hours after the challenge dose. Results: No erythema was observed at induction in the difenoconazole-treated group 1 (0/10). No positive reactions observed at challenge of both sensitised and non-sensitised groups treated (groups 1 and 3). Conclusion: **Non-sensitiser**	

10.7.1 Short summary and overall relevance of the provided information on skin sensitisation

A modified Buehler test (B.6.2.6) was carried out in female guinea pigs. A positive control (*i.e.* DNCB) was also tested using the same procedure.

A preliminary test was performed with four animals to assess the irritancy of the test item. The main test was perfomed using two groups of 10 animals each per test item (difenoconazole and positive control):

- Non-sensitising group: No topical induction. A single dose of test item (0.5g difenoconazole or 0.05% DNCB) was applied undiluted to the right flank area of each animal on test day 36 for approximately 6 hours. Skin sites were examined 24 and 48 hours after the challenge dose.
- Sensitised group: Topical induction with the test item (0.5g difenoconazole or 0.05% DNCB) was performed on test days 1, 3, 6, 8, 10, 13, 15, 17, 20 and 22. The test item was applied to a gauze patch and placed on the left flank of each animal. A challenge dose was applied on day 36 of the study on the right flank for 6 hours. Skin sites were examined 24 and 48 hours after the challenge dose.

No response was observed in any of the animals from both non-sensitised and sensitised groups treated with difenoconazole. Based on the results of the study, difenoconazole did not show skin sensitising properties.

10.7.2 Comparison with the CLP criteria

No response was observed in any of the animals from both non-sensitised and sensitised groups treated with difenoconazole. Based on the degree of the skin reactions (sensitisation rate <15%) and according to the the CLP criteria, difenoconazole does not meet the threshold for classification as skin sensitiser.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole for skin sensitisation based on negative results of a modified Buehler test in guinea pigs.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The table below summarises the available study for assessment of skin sensitisation of difenoconazole.

Table: Summary of the animal study on skin sensitisation with difenoconazole.

Study	Dose level	Results	Reference
Modified Buehler Test in guinea pigs Guideline: OECD TG 406 (1981) Deviations: 1) The number of animals used in the study (10) is less than the recommended (20); 2) Information on the size of the pads is not provided. GLP: Yes Hartley guinea pig Females	Purity: Not specified Topical induction: 6h occlusive exposures to 0.5 g difenoconazole or 0.05% positive control on days 1, 3, 6, 8, 10, 13, 15, 17, 20 and 22. Topical challenge: 6h occlusive exposures to 0.5 g difenoconazole or 0.05% positive control on days 36.	No erythema was observed at induction in the difenoconazole-treated group 1 (0/10). No positive reactions were observed after challenge with difenoconazole (0/10).	Anonymous 14, 1987b B.6.2.6 (AS)

No response was observed in the sensitised animals. However, RAC notes that in the modified Buhler test no erythema was observed at induction, and according to the CLP Guidance section 3.4.2.2.4, the Buhler test should be conducted at the highest induction dose causing mild skin irritation. Moreover, since difenoconazole is a solid it should have been applied in a vehicle rather than directly in solid state. Thus, due to these uncertainties, **RAC support's no classification of difenoconazole in this case, due to inconclusive data.**

10.8 Germ cell mutagenicity

Table 20: Summary table of mutagenicity/genotoxicity tests *in vitro*

Method, guideline, deviations if any	Test system	Test substance and dosage	Results	Remarks	Reference
Bacterial gene mutation (Ames test) Comparable to OECD TG 471 (1983) and OECD TG 472 (1983). Deviations from the current OECD TG 471(1997): None GLP: Yes **Study acceptable as relevant**	*S. typhimurium:* TA1535, TA1537, TA98, TA100 *E. coli*: WP2*uvrA* S9 from livers of rats induced with Aroclor 1254.	Difenoconazole Purity: 91.8% Solvent: DMSO Exp 1 and 2 340, 681, 1362, 2723, 5447 µg/plate (±S9) with all strains Exp. 3 85, 170, 340, 681, 1362 µg/plate (±S9) with TA1537 &TA98	Negative	Due to a strong growth-inhibiting effect in TA1537 & TA98 at the higher dose levels in Exp 1 and 2, a 3rd exp with these strains was carried out, using a lower concentration range.	**Ogorek, B., 1990** **B.6.4.1.1 (AS)**
Mammalian cell gene mutation test Comparable to OECD TG 476 (1984) Deviations from the current OECD TG 490 (2016): For TK mutants, the selective agent 5-bromodeoxyuridine (BUdR) was used instead of trifluorothymidine (TFT). GLP: Yes **Study acceptable as relevant**	Mouse lymphoma L5178Y $TK^{+/-}$ cells S9 from livers of rats induced with Aroclor 1254	Difenoconazole Purity: 94.5% Solvent: DMSO Exp. 1 (4h) 8, 16, 32, 48, 64, 72, 80 µg/mL (-S9) 5, 10, 20, 30, 40, 45, 50 µg/mL (+S9) Exp. 2 (4 h) 15, 30, 60, 90, 120, 135, 150 µg/mL (-S9) 3, 6, 12, 18, 24, 27, 30 µg/mL (+S9) Exp. 3 (4 h) 12, 24, 48, 72, 96, 108, 120 µg/mL (-S9)	Negative	Exp. 1: No toxicity (-S9) and toxicity above 20 µg/mL (+S9). Exp. 2: Toxicity from 120 µg/mL (-S9) and at 30 µg/mL (+S9). Exp. 3: Toxicity from 72 µg/mL (-S9)	**Dollenmeier, P., 1986a** **B.6.4.1.2 (AS)**
Mammalian cell chromosome aberration test OECD TG 473 (1997) Deviations from the current OECD TG 473 (2016): No long term treatment (-S9); mitotic index (MI) instead of relative population doubling (RPD) or relative increase in cell count (RICC) as toxicity measure; 200 instead of 300 metaphases per concentration analysed. GLP: Yes **Study acceptable as support information**, since no long term treatment (-S9) and equivocal results (+S9).	Chinese hamster ovary (CHO) cells S9 from livers of rats induced with Aroclor1254	Difenoconazole Purity: 94.3% Solvent: DMSO Exp. 1 (3 h) 21.99, 27.49, 34.36 µg/mL (-S9) 34.36, 53.69, 67.11 µg/mL (+S9) Exp. 2. (3 h) 21.99, 27.49, 34.36 µg/mL (-S9) 34.36, 53.69, 67.11, 83.89 µg/mL (+S9)	Negative (-S9) Equivocal (+S9)	The frequency of chromosomal aberrations exceeds the historical negative control range at 67.11 µg/mL, in Exp. 1 (+S9), might be due to cytotoxicity (59% reduction in MI), but this effect was not repeated at 67.11 µg/mL (45% reduction in MI) in Exp. 2 (+S9	**Lloyd, M., 2001** **B.6.4.1.3.1.1 (AS)**
Mammalian cell chromosome aberration test OECD TG 473 (1997) Deviations from the current OECD TG 473 (2016): In Exp. 2, two instead of three concentrations analysed; MI as toxicity measure instead of	Chinese hamster ovary (CHO) cells S9 from livers of rats induced with Aroclor 1254.	Difenoconazole Purity: 94.3% Solvent: DMSO Exp. 1 (3-h, -S9) 26.3, 39.5, 59.3 µg/mL Exp. 2 (3 h, +S9) 11.7, 17.6 µg/mL Exp. 3 (21 h, -S9)	Negative (-S9) Equivocal (+S9)	In exp. 4 (+S9), at 17.6 µg/mL, the frequency of chromosomal aberrations exceeds the historical negative control range but this	**Ogorek, B., 2001** **B.6.4.1.3.1.2 (AS)**

Method, guideline, deviations if any	Test system	Test substance and dosage	Results	Remarks	Reference
RPD or RICC; 200 instead of 300 metaphases/concentration analysed. GLP: Yes **Study acceptable as support information**, since equivocal results (+S9).		2.3, 5.2, 11.7 µg/mL Exp. 4 (3 h, +S9) 7.8, 11.7, 17.6 µg/mL		effect was not repeated in exp. 2 (+S9), and there was no sign of cytotoxicity at this concentration.	
Mammalian cell chromosome aberration test Comparable to OECD TG 473 (1983) Deviations from the current OECD TG 473 (2016): 100 instead of 300 metaphases per concentration analysed; no long term treatment (-S9). GLP: Yes **Study acceptable as support information**, due to no long term treatment (-S9), and a low number of metaphases per concentration analysed.	Human lymphocytes S9 from livers of rats induced with Aroclor 1254	Difenoconazole Purity: 94.5% Solvent: DMSO Exp.1 (3 h, -S9) 2.5, 5, 10, 20, 40 µg/mL) Exp. 2 (3 h, +S9) 2.5, 5, 10, 20, 40 µg/mL	Negative	Cytotoxicity at the two highest dose levels in both experiments (±S9)	**Strasser, F., 1985 B.6.4.1.3.1.3 (AS)**
Mammalian cell chromosome aberration test OECD TG 473 (1997) Deviations from the current OECD TG 473 (2016): 200 instead of 300 metaphases per concentration analysed. GLP: Yes **Study acceptable as relevant**	Human lymphocytes S9 from livers of rats induced with phenobarbital and β-naphthoflavone	Difenoconazole Purity: 94.3% Solvent: DMSO Exp. 1 (3 h) 5, 30, 75 µg/mL (-S9) 5, 30, 62 µg/mL (+S9) Exp. 2 1, 5, 10 µg/mL (20 h, -S9) 5, 30, 50 µg/mL (3-h, +S9	Negative	Cytotoxicity at the highest dose levels in both experiments (±S9)	**Fox, V., 2001 B. 6.4.1.3.1.4 (AS)**
DNA damage (UDS test) OECD TG 482 (1987), which was deleted on 2nd April 2014 Deviations from OECD TG 482 (1987): None GLP: Yes **Study acceptable as supplementary information** since it is not required.	Primary hepatocytes from male TIF: RAIf (SPF) rats	Difenoconazole Purity: 91.8% Solvent: DMSO Exp 1 and 2 0.46, 1.39, 4.17, 12.5, 25, 50 µg/mL	Negative		**Hertner, T., 1992 B.6.4.1.4 (AS)**
Photomutagenicity				Waiver	

Table 21: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells *in vivo*

Method, guideline, deviations if any	Test system	Test substance and dosage	Results	Remarks	Reference
Mammalian chromosome aberration in somatic cells (Micronucleus test) OECD TG 474 (1983) Deviations from the current OECD TG 474 (2016): Animals acclimatised for 1	Tif: MAGf (SPF) mice	Difenoconazole Purity: 91.8% Vehicle: Arachis oil Dosing by oral gavage 1st part 1600 mg/kg bw with	Negative	At 1600, 800 and 400 mg/kg bw, mice showed clinical symptoms of piloerection laterocumbency and ataxia, but no	**Anonymous 15 (1991) B.6.4.2.1 (AS)**

Method, guideline, deviations if any	Test system	Test substance and dosage	Results	Remarks	Reference
day instead of for 5 days; 1000 instead of 2000 polychromatic erythrocytes for each animal analysed per dose level; not justified bone marrow sampling at 16 h post dosing; no proof of exposure of the bone marrow. GLP: Yes **Study acceptable as relevant** since, based on the weight of evidence, bone marrow has been adequately exposed.		sampling times at 16, 24 and 48 h after treatment 2nd part 400, 800, 1600 mg/kg bw with a single sampling time at 24 h after treatment		mortality. No cytotoxicity on blood forming cells.	

10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

- The potential genotoxicity of difenoconazole has been investigated in an appropriate battery of *in vitro* and *in vivo* genotoxicity assays. *In vitro* tests for gene mutation (Ames test, mouse lymphoma cell assay), chromosomal aberration (cytogenetic assay in CHO cells and human lymphocytes) and DNA damage (unscheduled DNA synthesis) and *in vivo* tests for chromosomal aberration (mouse micronucleus test) were conducted.
- *In vitro*, difenoconazole was negative in both bacterial and mammalian cell assays for gene mutation, negative for chromosomal aberration in both cytogenetic assays using isolated human lymphocytes and negative for DNA damage in the unscheduled DNA synthesis (UDS) assay. Increases in chromosomal aberrations were reported in both cytogenetic assays using CHO cells, but only at high concentrations inducing cytotoxicity (or not) and they were not clearly reproducible either between repeat examinations of the same slides, between experiments or across studies. Therefore, these observations are not considered of significance in light of the negative results of other genotoxicity assays, including those of other *in vitro* and *in vivo* cytogenetic assays.
- *In vivo*, difenoconazole was negative in the micronucleus (MN) test. Deviations from the current OCDE TG 474 (2016) regarding to requirements for the acclimatisation period, the number of polychromatic erythrocytes scored for micronuclei and the proof of exposure of the bone marrow were noted. As the bone marrow is a well-perfused tissue, systemic bioavailability of a test substance can be considered as a line of evidence of bone marrow exposure. Lines of evidence based on systemic bioavailability of the test substance should be assessed with a weight of evidence (WoE) approach. There are the following lines of evidence: a) In the MN test, clinical signs (ataxia, laterocumbency, piloerection were considered to indicate systemic toxicity, b) In a 3-month feeding study in mouse, a diffuse/centrilobular hepatocyte enlargement was histopathologically detected, evaluated as being related to the test substance and considered as a line of evidence of systemic bioavailability. Therefore, it is demonstrated that the bone marrow has been adequately exposed to difenoconazole in the MN test and that deficiencies noted in the study design do not invalidate the negative result obtained.
- No photomutagenicity study was provided. Whilst photomutagenicity testing is potentially triggered, the *in vitro* 3T3 NRU phototoxicity assay returned a negative result and thus no photomutagenicity testing is considered to be necessary. However, difenoconazole is an UVB

absorber and the irradiation wavelength used in the phototoxicity assay (>330 nm) is not appropriate for UVB absorbers. Since there is not a validated test method for testing phototoxicity at UVB wavelength, it cannot be concluded on the phototoxicity of difenoconazole.

In conclusion, based on the weight of evidence difenoconazole is considered to be non-genotoxic.

10.8.2 Comparison with the CLP criteria

Difenoconazole was not mutagenic in a valid *in vivo* somatic cell mutagenicity test and so according to the guidance on the application of the CLP criteria no classification is warranted. The overall body of toxicological data from a number of *in vitro* and *in vivo* assays indicates that difenoconazole is of no genotoxic concern. Therefore no classification for mutagenicity under the CLP regulation is required.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Not classified (conclusive but not sufficient for classification).

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

DS proposed no classification of difenoconazole for germ cell mutagenicity based on negative *in vitro* results in the following tests: *in vitro* tests for gene mutation (Ames test, mouse lymphoma cell assay), chromosomal aberration (cytogenetic assay in CHO cells and human lymphocytes) and DNA damage (unscheduled DNA synthesis); and also based on negative *in vivo* tests for chromosomal aberration (mouse micronucleus test).

Comments received during consultation

One company-manufacturer supported the proposal for no classification of difenoconazole for germ cell mutagenicity.

Assessment and comparison with the classification criteria

The tables below summarise the *in vitro* and *in vivo* studies found in the CLH report for assessing the difenoconazole potential for inducing germ cell mutagenicity.

Table: Summary of mutagenicity/genotoxicity *in vitro* studies with difenoconazole.

Method	Tested concentrations	Results	Reference
Bacterial gene mutation (Ames test) Comparable to OECD TG 471 (1983) and OECD TG 472 (1983) GLP: Yes	Purity: 91.8% Solvent: DMSO Exp. 1 and 2: 340, 681, 1362, 2723, 5447 µg/plate (±S9) with all strains	**Negative**	Ogorek, 1990 B.6.4.1.1 (AS)

S. typhimurium: TA1535, TA1537, TA98, TA100 *E. coli:* WP2uvrA S9 from livers of rats induced with Aroclor 1254	Exp. 3: 85, 170, 340, 681, 1362 µg/plate (±S9) with TA1537 & TA98		
Mammalian cell gene mutation test Comparable to OECD TG 476 (1984) GLP: Yes Mouse lymphoma L5178Y TK+/- cells S9 from livers of rats induced with Aroclor 1254	Purity: 94.5% Solvent: DMSO Exp. 1 (4h): 8, 16, 32, 48, 64, 72, 80 µg/mL (-S9); 5, 10, 20, 30, 40, 45, 50 µg/mL (+S9) Exp. 2 (4h): 15, 30, 60, 90, 120, 135, 150 µg/mL (-S9); 3, 6, 12, 18, 24, 27, 30 µg/mL (+S9) Exp. 3 (4h): 12, 24, 48, 72, 96, 108, 120 µg/mL (-S9)	Exp. 1: No toxicity (-S9) and toxicity above 20 µg/mL (+S9). Exp. 2: Toxicity from 120 µg/mL (-S9) and at 30 µg/mL (+S9). Exp. 3: Toxicity from 72 µg/mL (-S9) **Negative**	Dollenmeier, 1986a B.6.4.1.2 (AS)
Mammalian cell chromosome aberration test OECD TG 473 (1997) GLP: Yes Chinese hamster ovary (CHO) cells S9 from livers of rats induced with Aroclor1254	Purity: 94.3% Solvent: DMSO Exp. 1 (3h): 21.99, 27.49, 34.36 µg/mL (-S9); 34.36, 53.69, 67.11 µg/mL (+S9) Exp. 2. (3h): 21.99, 27.49, 34.36 µg/mL (-S9); 34.36, 53.69, 67.11, 83.89 µg/mL (+S9)	The frequency of chromosomal aberrations exceeds the historical negative control range at 67.11 µg/mL, in Exp. 1 (+S9), might be due to cytotoxicity (59% reduction in MI), but this effect was not repeated at 67.11 µg/mL (45% reduction in MI) in Exp. 2 (+S9) **Negative (-S9)** **Equivocal (+S9)**	Lloyd, 2001 B.6.4.1.3.1.1 (AS)
Mammalian cell chromosome aberration test OECD TG 473 (1997) GLP: Yes Chinese hamster ovary (CHO) cells S9 from livers of rats induced with Aroclor 1254	Purity: 94.3% Solvent: DMSO Exp. 1 (3h): 26.3, 39.5, 59.3 µg/mL (-S9) Exp. 2 (3h): 11.7, 17.6 µg/mL (+S9) Exp. 3 (21h): 2.3, 5.2, 11.7 µg/mL (-S9) Exp. 4 (3h): 7.8, 11.7, 17.6 µg/mL (+S9)	In exp. 4 (+S9), at 17.6 µg/mL, the frequency of chromosomal aberrations exceeds the historical negative control range, but this effect was not repeated in exp. 2 (+S9), and there was no sign of cytotoxicity at this concentration. **Negative (-S9)** **Equivocal (+S9)**	Ogorek, 2001 B.6.4.1.3.1.2 (AS)

Mammalian cell chromosome aberration test Comparable to OECD TG 473 (1983) GLP: Yes Human lymphocytes S9 from livers of rats induced with Aroclor 1254	Purity: 94.5% Solvent: DMSO Exp.1 (3h): 2.5, 5, 10, 20, 40 µg/mL (-S9) Exp. 2 (3h): 2.5, 5, 10, 20, 40 µg/mL (+S9)	Cytotoxicity at the two highest dose levels in both experiments (±S9) **Negative**	Strasser, 1985 B.6.4.1.3.1.3 (AS)
Mammalian cell chromosome aberration test OECD TG 473 (1997) GLP: Yes Human lymphocytes S9 from livers of rats induced with phenobarbital and β-naphthoflavone	Purity: 94.3% Solvent: DMSO Exp. 1 (3h): 5, 30, 75 µg/mL (-S9); 5, 30, 62 µg/mL (+S9) Exp. 2: 1, 5, 10 µg/mL (20h, -S9); 5, 30, 50 µg/mL (3h, +S9)	Cytotoxicity at the highest dose levels in both experiments (±S9) **Negative**	Fox, 2001 B. 6.4.1.3.1.4 (AS)
DNA damage (UDS test) OECD TG 482 (1987) GLP: Yes Primary hepatocytes from male TIF: RAIf (SPF) rats	Purity: 91.8% Solvent: DMSO Exp 1 and 2: 0.46, 1.39, 4.17, 12.5, 25, 50 µg/mL	**Negative**	Hertner, 1992 B.6.4.1.4 (AS)

Difenoconazole was negative in both bacterial and mammalian cell assays for gene mutation, negative for chromosomal aberration in both cytogenetic assays using isolated human lymphocytes and negative for DNA damage in the unscheduled DNA synthesis assay. Increases in chromosomal aberrations were reported in both cytogenetic assays using CHO cells, but only at high concentrations, sometimes concurrently with cytotoxicity. These chromosomal aberrations were not clearly reproducible between experiments or across studies. Therefore, these observations are not considered of significance by RAC, especially considering the negative results of other *in vitro* genotoxicity assays.

Table: Summary of mutagenicity/genotoxicity *in vitro* studies with difenoconazole.

Method	Tested concentrations	Results	Reference
Micronucleus test OECD TG 474 (1983) GLP: Yes	Purity: 91.8% Vehicle: Arachis oil Dosing by oral gavage	At 1600, 800 and 400 mg/kg bw, mice showed clinical symptoms of piloerection, laterocumbency and ataxia, but no mortality	Anonymous 15, 1991 B.6.4.2.1 (AS)

Tif: MAGf (SPF) mice	1st part: 1600 mg/kg bw with sampling times at 16, 24 and 48h after treatment	No cytotoxicity on blood forming cells
	2nd part: 400, 800, 1600 mg/kg bw with a single sampling time at 24h after treatment	**Negative**

Difenoconazole was negative in the *in vivo* micronucleus test. Deviations from the current OECD TG 474 (2016) requirements were noted on the acclimatisation period, the number of polychromatic erythrocytes scored for micronuclei and the proof of exposure of the bone marrow. The toxicokinetic studies do not provide evidence that difenoconazole was able to reach bone marrow, although the clinical signs reported in the micronucleus test and the systemic toxicity reported in the 3-month feeding study in mouse, suggest systemic bioavailability.

In addition to the micronucleus test summarised in the table above, an additional *in vivo* micronucleus test without the deficiencies stated above (see below the section Supplemental information - In depth analyses by RAC) was provided. This study demonstrated that, up to the maximum tolerable dose of 320 mg/kg bw/day in male mice, difenoconazole is not able to induce clastogenicity or aneugenicity.

Overall, RAC does not consider difenoconazole a substance of genotoxic concern and supports the DS's proposal for **no classification of difenoconazole for germ cell mutagenicity.**

Supplemental information - In depth analyses by RAC

In a supplemental study provided difenoconazole was tested up to the maximum tolerated dose level (MTD) to evaluate its potential to cause damage to chromosomes or cell division apparatus, or to cause interference with the cell cycle, leading to the formation of micronuclei in developing reticulocytes in the bone marrow of young adult mice.

In the range-finding phase, groups of 3 male and/or 3 female mice were given difenoconazole, at 320 mg/kg bw/day or 500 mg/kg bw/day. The maximum tolerable dose was confirmed as 320 mg/kg bw/day in male and female mice. As there were no inter-sex differences in toxicity, the main study was conducted in males only, with the high dose selected as 320 mg/kg bw/day. Proof of exposure was conducted as part of the range-finding study to demonstrate that the bone marrow was exposed to the test item, via LC-MS/MS analysis of test item in the whole blood and plasma of animals given 320 mg/kg bw/day difenoconazole. The presence of difenoconazole, was confirmed by analysis of the study samples using a validated method.

For the main study phase, 4 groups, each of 6 male mice were dosed with vehicle alone (negative controls given peanut oil) or 80, 160 or 320 mg/kg bw/day difenoconazole, on 2 successive days, approximately 24 hours apart. A positive control group, also of 6 male mice, was given a single 1 mg/kg bw intraperitoneal injection of mitomycin C. Blood samples were taken from all main study animals approximately 48 hours after the final dose administration. A minimum of 4000 and a maximum of approximately 20000 reticulocytes were scored for the presence of micronuclei for each animal and the frequency of micronucleated reticulocytes was statistically analysed.

There were no statistically significant increases in frequency of micronucleated reticulocytes in male mice given any dose level of difenoconazole, compared with the negative control

group. There was no evidence of a statistically significant reduction in the percentage of reticulocytes given difenoconazole, and, since proof of exposure to the bone marrow was demonstrated in the range finding phase of the study, this indicated a lack of toxicity of difenoconazole, to the bone marrow. The animals dosed with mitomycin C had statistically significant increases in the number of micronucleated reticulocytes compared with the concurrent control group; which demonstrated that the test system was capable of detecting a known clastogen. There was no statistically significant decrease in the percentage of reticulocytes in the positive control group, indicating a lack of toxicity to the bone marrow.

In conclusion, there was no evidence of clastogenicity or aneugenicity following oral (gavage) administration of difenoconazole, up to the maximum tolerable dose of 320 mg/kg bw/day in male mice and therefore, difenoconazole is considered to be neither clastogenic nor aneugenic in the mouse micronucleus test.

10.9 Carcinogenicity

Table 22: Summary table of animal studies on carcinogenicity

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
[Effects statistically significant and dose-related unless stated otherwise as not
significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>Long-term toxicity and carcinogenicity study in rats (2-years)
<u>Lab:</u> Hazleton Laboratories America.
<u>Guideline</u>: OECD TG 453 (1981)
<u>GLP</u>: Yes
<u>Rat strain:</u> Sprague-Dawley ♂ and ♀
<u>No. animals</u>: 80/sex/dose group Recovery animals: 10/sex/ supplementary group (control or high dose groups)
Study acceptable</td><td>Difenoconazole
<u>Purity</u>: 94.5% weeks 1-20; and 95% weeks 21-104
Oral (diet)
<u>Doses</u>: 0, 10, 20, 500 and 2500 ppm, equivalent to:
Males: 0, 0.5, 1, 24.1 and 124 mg/kg bw/d
Females: 0, 0.6, 1.3, 32.8 and 170 mg/kg bw/d
104-weeks feed exposure
Recovery animals: 52-weeks fed exposure + 4 weeks control diet</td><td><u>Mortality</u>: no evidence of any treatment-related effect
<table>
<tr><th rowspan="2">Parameter at week 104</th><th colspan="5">Males (ppm)</th><th colspan="5">Females (ppm)</th></tr>
<tr><th>0</th><th>10</th><th>20</th><th>500</th><th>2500</th><th>0</th><th>10</th><th>20</th><th>500</th><th>2500</th></tr>
<tr><td>% survival [a]</td><td>61</td><td>50</td><td>57</td><td>61</td><td>64</td><td>48</td><td>50</td><td>44</td><td>61</td><td>58</td></tr>
<tr><td>% mortality</td><td>39</td><td>50</td><td>43</td><td>39</td><td>36</td><td>52</td><td>50</td><td>56</td><td>39</td><td>42</td></tr>
</table>
[a]Percent survival adjusted for accidental deaths, recovery animals and scheduled sacrifices

<u>Clinical signs</u>: no treatment related clinical signs.

<u>Neoplastic changes</u>: Neoplastic changes were considered to be incidental due to the lack of a dose response and/or no increases of the incidence of neoplasia in treated animals.
<table>
<tr><th rowspan="2">Neoplastic findings</th><th colspan="5">Males (ppm)</th><th colspan="5">Females (ppm)</th></tr>
<tr><th>0</th><th>10</th><th>20</th><th>500</th><th>2500</th><th>0</th><th>10</th><th>20</th><th>500</th><th>2500</th></tr>
<tr><td>Hepatocellular adenoma[B]</td><td>4</td><td>1</td><td>0</td><td>0</td><td>0</td><td>3</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Hepatocellular carcinoma[M]</td><td>1</td><td>3</td><td>1</td><td>1</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Histiocytic sarcoma[X]</td><td>1</td><td>1</td><td>0</td><td>0</td><td>3</td><td>1</td><td>0</td><td>0</td><td>1</td><td>1</td></tr>
<tr><td>Leukaemia granulocytic[X]</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>Malignant lymphoma, histiocytic[X]</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Malignant lymphoma, lymphocytic[X]</td><td>1</td><td>0</td><td>1</td><td>0</td><td>3</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Mesothelioma[X]</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>Paraganglioma[M]</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Number of animals examined</td><td>90</td><td>80</td><td>80</td><td>80</td><td>90</td><td>90</td><td>80</td><td>80</td><td>80</td><td>90</td></tr>
</table>
[B]Primary, benign neoplasm; [M]: Primary, malignant neoplasm; [X]: Other neoplasm</td><td>Anonymous 16 (1989a)
Anonymous 17 (1992) (HCD)
B.6.5.1 (AS)</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
		2500 ppm (124♂/170♀ mg/kg bw/day) **Bodyweight and bodyweight gain:** ▪ (↓) bw in ♂/♀ on weeks 52 (11%/23%), 76 (8/23%) and 104 (only significant in ♀: 22%) ▪ (↓) bw gain in ♂/♀ from week 13 until the end of the study (11-22% lower than the control in ♂and 32-40% in ♀). **Food consumption:** ▪ (↓) food consumption in ♂/♀ on weeks 52, 76 and 104 (<8% in ♂, and <15% in ♀), corresponding to reductions on bw and bw gain. **Haematology:** ▪ (↓) RBC in ♀ on week 28 (10%) ▪ (↓) Hb in ♀ on week 28 (8%) and week 53 (7%) ▪ (↓) Hct in ♂/♀ on week 53(10% ndr/13%) and, in ♀, also on week 28 (13% ncdr) ▪ (↓) MCV in ♂/♀ on week 53 (3%/4%) and, in ♀, also on week 28 and 79 (4% and 3%, ncdr) ▪ (↑) MCH in ♂ on week 28 and 53 (4% and 5% ndr) ▪ (↑) MCHC in ♂/♀ on weeks 28 (3%/5%), 53 (8%/8%) and 79 (10%/7%) ▪ (↓) platelets in ♂ on weeks 28(17%), 53 (24%), 79 (22%) and 104 (19% ndr) ▪ (↓) total WBC in ♂/♀ on week 104 (30%/36% ncdr) ▪ (↓) total no. segmented neutrophiles in ♂/♀ on week 104 (28%/49%) ▪ (↓) total no. lymphocytes in ♂ on week 104 (31%) **Clinical chemistry:** ▪ (↑) albumin in ♂/♀ on week 28 (5%/7%) and, in ♂, also on weeks 53 (5%) and 104 (21% ndr) ▪ (↓) globulin in ♂ on weeks 53 (10%) and 104 (17% ncdr) ▪ (↑) albumin/globulin (A/G) ratio in ♂ on weeks 53 (18% ndr) and 104 (48% ndr) ▪ (↑) alanine aminotransferase (ALAT) in ♀ on weeks 28 (59% ncdr) and 53 (32%) ▪ (↓) alanine aminotransferase (ALAT) in ♂ on week 53 (115%) ▪ (↑) glucose in ♂/♀ on week 28 (12%/8%) ▪ (↑) cholesterol in ♂/♀ on week 28 (23%/28% ncdr) and, in ♂, also on week 104 (48% ndr) ▪ (↓) total bilirubin in ♂/♀ on week 28 (44%/67%) and, in ♀, also on weeks 53 (73%) and 79 (69%) **Organ weights:** ▪ Carcass: (↓) abs wt in ♂/♀ on week 53 (11% ncdr/21%) and, in ♀, also on week 104 (22% ncdr) ▪ Liver: (↑) rel wt in ♂/♀ on week 53 (14% ncdr/48% ncdr) and, in ♀, also on week 104 (43% ncdr) ▪ Adrenals: (↓) abs wt in ♂ on week 53 (29%) ▪ Spleen: (↓) abs wt in ♀ on recovery sacrifice week 57 (18%) ▪ Ovaries: (↑) abs and rel wt on week 104 (90% and 132% ncdr, respectively) **Histopathology:** *Non-neoplastic changes:* ▪ (↑) incidence of hepatocellular hypertrophy in ♂ at terminal sacrifice (89% compared to 17.5% in control group) and ♀ (84% compared to 12.5% in control group) **500 ppm** (24.1 ♂/32.8 ♀ mg/kg bw/day) **Bodyweight and bodyweight gain:** ▪ (↓) bw in ♀ on week 52 (6% ncdr) ▪ (↓) bw gain in ♂/♀ on weeks 13, 24 and 52 (6-7% lower than the control in ♂and 10-11% in ♀).	

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
[Effects statistically significant and dose-related unless stated otherwise as not
significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td></td><td></td><td>
<u>Haematology:</u>
▪ (↓) Hb in ♀ on week 28 (5%)
▪ (↓) MCV in ♂ on week 79 (5%)
▪ (↓) platelets in ♂ on weeks 28 (9%) and 53 (11%)
<u>Clinical chemistry:</u>
▪ (↑) alanine aminotransferase (ALAT) in ♀ on week 28 (41% ncdr)
▪ (↓) alanine aminotransferase (ALAT) in ♂ on week 53 (42%)
<u>Organ weights:</u>
▪ Ovaries: (↑) rel wt (109% ncdr)
<u>Histopathology:</u>
Non-neoplastic changes:
▪ (↑) incidence of hepatocellular hypertrophy in ♂ at terminal sacrifice (65% compared to 17.5% in control group) and ♀ (34% compared to 12.5% in control group)
20 ppm (1 ♂/1.3 ♀ mg/kg bw/day)
<u>Bodyweight gain:</u>
▪ (↓) bw gain in ♂ on weeks 13 (5% lower than the control).
<u>Haematology:</u>
▪ (↓) Hb in ♀ on week 28 (4%)
▪ (↑) Hb in ♂ on week 28 (8% ndr)
▪ (↑) Hct in ♂ on week 28 (7% ndr)
10 ppm (0.5 ♂/0.6 ♀ mg/kg bw/day)
<u>Haematology:</u>
▪ (↑) MCV in ♂ on week 79 (4% ndr)
<u>Organ weights:</u>
▪ Ovaries: (↑) rel wt (41% ncdr)
NOAEL$_{toxicity}$: 20 ppm corresponding to 1/1.3 mg/kg bw/day for ♂ and ♀ respectively.
NOAEL$_{carcinogenicity}$: >2500 ppm corresponding to 124/170 mg/kg bw/day for ♂ and ♀ respectively.
</td><td></td></tr>
<tr><td>Carcinogenicity study in mouse (78-weeks)
<u>Lab:</u> Hazleton Laboratories America.
<u>Guideline</u>: OECD TG 451 (1981)
<u>GLP:</u> Yes
<u>Mouse strain</u>:
CD-1® (ICR) ♂ and ♀
<u>No. animals:</u> 60/sex/dose group Recovery animals: 10/sex/ supplementary group (control, 2500 and 4000 ppm groups)</td><td>Difenoconazole
<u>Purity</u>: 94.5% weeks 1-20; and 95% weeks 21-80
Oral (diet)
<u>Doses</u>: 0, 10, 30, 300, 3000-2500* and 4500 ppm, equivalent to:
Males: 0, 1.5, 4.7, 46.3, 507.6-423 and 819 mg/kg bw/d
Females: 0, 1.9, 5.6, 57.8, 615.6-513 and 983 mg/kg bw/d
78-weeks feed exposure</td><td>
<u>Mortality</u>: All (70) the ♀ in the 4500 ppm dose group died or were humanely sacrificed during the first 2 weeks. Survival for the 4500 ppm ♂group was significantly lower than control.
At 3000 ppm, 15 ♀ died/were sacrified on week 1, which led to a reduction in dose to 2500 ppm for ♂ and ♀ during week 2. An additional ♀ died during week 2. On week 3, 10 control ♀ were moved to 2500 ♀ group to maintain the size; 3 of these ♀ were humanely sacrified during their first week of treatment. After the initial mortality in ♀ of this group, there was no remarkable treatment-effect on survival.
<table>
<tr><th rowspan="2">Number of animals</th><th colspan="6">Males (ppm)</th></tr>
<tr><th>0</th><th>10</th><th>30</th><th>300</th><th>3000-2500[a]</th><th>4500</th></tr>
<tr><td>Initiation</td><td>70</td><td>60</td><td>60</td><td>60</td><td>70</td><td>70</td></tr>
<tr><td>Died/human sacrifice weeks 1-3</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>11</td></tr>
<tr><td>Interim sacrifice week 53</td><td>10</td><td>10</td><td>10</td><td>10</td><td>10</td><td>10</td></tr>
<tr><td>Post-recovery sacrifice week 57</td><td>9[b]</td><td>0</td><td>0</td><td>0</td><td>10</td><td>10</td></tr>
<tr><td>% Mortality at termination[c]</td><td>38</td><td>36</td><td>46</td><td>51</td><td>32</td><td>63</td></tr>
<tr><td>% Survival to termination[c]</td><td>62</td><td>64</td><td>54</td><td>49</td><td>68</td><td>37**</td></tr>
<tr><td></td><td colspan="6">Females (ppm)</td></tr>
<tr><td>Initiation</td><td>70</td><td>60</td><td>60</td><td>60</td><td>70</td><td>70</td></tr>
<tr><td>Re-assignment Day 10[d]</td><td>-10</td><td></td><td></td><td></td><td>+10</td><td></td></tr>
<tr><td>Died/human sacrifice weeks 1-3</td><td>0</td><td>0</td><td>0</td><td>0</td><td>19</td><td>70</td></tr>
<tr><td>Interim sacrifice week 53</td><td>10</td><td>10</td><td>10</td><td>10</td><td>10</td><td>0</td></tr>
<tr><td>Post-recovery sacrifice week 57</td><td>0[e]</td><td>0</td><td>0</td><td>0</td><td>10</td><td>0</td></tr>
<tr><td>% Mortality at termination[c]</td><td>51</td><td>29</td><td>40</td><td>30</td><td>35</td><td>-</td></tr>
<tr><td>% Survival to termination[c]</td><td>49</td><td>71</td><td>60</td><td>70</td><td>65</td><td>-</td></tr>
</table>
</td><td>Anonymous 18 (1989b)
B.6.5.2 (AS)</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
[Effects statistically significant and dose-related unless stated otherwise as not
significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>Study acceptable</td><td>Recovery animals:
52-weeks fed exposure +
4 weeks control diet

*The original dose was 3000 ppm but due to early mortality the dose was reduced to 2500 ppm at the beginning of week 2</td><td>
[a]Dose level 3000 ppm through week 1, [b]one recovery-group animal died during recovery, [c]excluding interim and post-recovery sacrifices, [d]at the beginning of 3rd week, 10 ♀s from the control group were moved to 2500 ppm group to maintain an adequate sample size in this last group for the duration of the study, [e]no control females were sacrificed as recovery animals

<u>Clinical signs</u>: Thinness, hunched appearance and rough haircoat were noted more frequently in 2500 ppm ♀ group and in 4500 ppm ♂ group when compared to controls. In 4500 ppm ♂ also (↑) in the incidence of reduced motor activity was observed.

<u>Neoplastic changes</u>: Statistical analysis of liver adenomas and carcinomas revealed significant increases for males of 2500 and 4500 ppm groups and for females of 2500 ppm group. The incidence of adenomas and carcinomas was already elevated in the 4500 ppm males at the interim and recovery sacrifices.
<table>
<tr><th colspan="7">Males (ppm)</th></tr>
<tr><th>Mice</th><th>0</th><th>10</th><th>30</th><th>300</th><th>3000-2500[a]</th><th>4500</th></tr>
<tr><td colspan="7">Hepatocellular adenoma</td></tr>
<tr><td>U</td><td>0/20</td><td>3/17</td><td>1/23</td><td>2/26</td><td>9/16</td><td>6/34</td></tr>
<tr><td>I</td><td>0/10</td><td>1/10</td><td>2/10</td><td>0/10</td><td>1/10</td><td>2/10</td></tr>
<tr><td>R</td><td>0/9</td><td>-</td><td>-</td><td>-</td><td>0/10</td><td>3/10</td></tr>
<tr><td>T</td><td>4/31</td><td>6/32</td><td>5/27</td><td>7/24</td><td>3/34</td><td>9/16</td></tr>
<tr><td>Total
%</td><td>4/70
65</td><td>10/59
17%</td><td>8/60
13%</td><td>9/60
15%</td><td>13/70*
19%</td><td>20/70**
29%</td></tr>
<tr><td colspan="7">Hepatocellular carcinoma</td></tr>
<tr><td>U</td><td>0/20</td><td>0/17</td><td>1/23</td><td>0/26</td><td>1/16</td><td>4/34</td></tr>
<tr><td>I</td><td>0/10</td><td>0/10</td><td>0/10</td><td>0/10</td><td>0/10</td><td>2/10</td></tr>
<tr><td>R</td><td>0/9</td><td>-</td><td>-</td><td>-</td><td>1/10</td><td>1/10</td></tr>
<tr><td>T</td><td>1/31</td><td>0/32</td><td>0/27</td><td>0/24</td><td>3/34</td><td>6/16</td></tr>
<tr><td>Total
%</td><td>1/70
1%</td><td>0/59
0%</td><td>1/60
2%</td><td>9/60
0%</td><td>5/70
7%</td><td>13/70**
19%</td></tr>
<tr><th colspan="7">Females (ppm)</th></tr>
<tr><th>Mice</th><th>0</th><th>10</th><th>30</th><th>300</th><th>2500[a]</th><th></th></tr>
<tr><td colspan="6">Hepatocellular adenoma</td><td></td></tr>
<tr><td>U</td><td>0/26</td><td>0/14</td><td>0/21</td><td>0/15</td><td>5/21</td><td></td></tr>
<tr><td>I</td><td>0/10</td><td>0/10</td><td>0/10</td><td>0/10</td><td>1/10</td><td></td></tr>
<tr><td>R</td><td>-</td><td>-</td><td>-</td><td>-</td><td>0/10</td><td></td></tr>
<tr><td>T</td><td>0/24</td><td>0/35</td><td>0/29</td><td>1/35</td><td>10/29</td><td></td></tr>
<tr><td>Total
%</td><td>0/60
0%</td><td>0/59
0%</td><td>0/60
0%</td><td>1/60
2%</td><td>16/70**
23%</td><td></td></tr>
<tr><td colspan="6">Hepatocellular carcinoma</td><td></td></tr>
<tr><td>U</td><td>0/26</td><td>0/14</td><td>0/21</td><td>0/15</td><td>2/21</td><td></td></tr>
<tr><td>I</td><td>0/10</td><td>0/10</td><td>0/10</td><td>0/10</td><td>0/10</td><td></td></tr>
<tr><td>R</td><td>-</td><td>-</td><td>-</td><td>-</td><td>0/10</td><td></td></tr>
<tr><td>T</td><td>0/24</td><td>0/35</td><td>1/29</td><td>0/35</td><td>2/29</td><td></td></tr>
<tr><td>Total
%</td><td>0/60
0%</td><td>0/59
0%</td><td>1/60
2%</td><td>0/60
0%</td><td>4/70
6%</td><td></td></tr>
</table>
Data shown:number of animals with finding/number of animals examined. U: unscheduled deaths, I: interim sacrifice week 53, R: recovery sacrifice week 57, T: terminal sacrifice week 79-80.
[a]Dose level 3000 ppm until day 21.
Bonferroni adjustment. * p ≤0.05 ** p ≤ 0.01

4500 ppm ♂ (819 mg/kg bw/day; no ♀survived: 983 mg/kg bw/day)
<u>Bodyweight and bodyweight gain:</u>
(↓) bw on week 56 (7%).
(↓) bw gain through weeks 0-76 (34%)
</td><td></td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
		Clinical chemistry: (↑) alanine aminotransferase (ALAT) on weeks 53 (280% ncdr) and 79 (311% ncdr) (↑) alkaline phosphatase (ALP) on week 79 (444% ncdr) (↑) Sorbitol dehydrogenase (SDH) on weeks 53 (378%) and 79 (353% ncdr). However, it was n.s. after 4-week recovery period (week 57) **Gross pathology (liver):** *Unscheduled deaths*: (↑) incidence of liver enlargement (41% ncdr, compared with an incidence of 10% in control group), pale areas (38%, compared with an incidence of 0% in control group) and masses (29% ncdr, compared with an incidence of 0% in control group). *Terminal sacrifice* (week 79): (↑) incidence of liver enlargement (50% ncdr, compared with an incidence of 16% in control group), pale areas (56% ncdr, compared with an incidence of 3% in control group) and masses (44% ncdr, compared with an incidence of 10% in control group). **Organ weights:** Carcass: (↓) abs wt on recovery week 57 (14%) Liver/gall bladder: (↑) abs and rel wt on weeks 53 (63%/77%, respectively) and 79 (112% ncdr/121% ncdr, respectively) Brain: (↓) rel wt on week 53 (6% ncdr) **Histopathology:** *Non-neoplastic changes (liver):* (↑) individual cell necrosis (76%, compared with a 7% in control group) (↑) focal/multifocal necrosis (23%, compared with a 6% in control group) (↑) hepatocyte hypertrophy (81% ncdr, compared with a 24% in control group) (↑) liver fatty change (44%, compared with a 1% in control group) (↑) liver bile stasis (71% ncdr, compared with a 1% in control group) *Neoplastic changes (liver):* (↑) hepatocellular adenoma (29% ncdr, compared with a 6% in control group) (↑) hepatocellular carcinoma (19%, compared with a 1% in control group) **3000-2500 ppm** (507.6-423 ♂/615.6-513 ♀ mg/kg bw/day) **Bodyweight and bodyweight gain:** (↓) bw ♂/♀ on week 52 (6%/6% ncdr), and (↓) bw in ♀ also on weeks 60 (7%), 72 (8%) and 76 (8%) (↓) bw gain in ♀ through weeks 0-76 (22%) (↓) bw gain in ♂ through weeks 0-52 (21%), although it was n.s. on week 76 **Haematology:** (↑)segmented neutrophils in ♀ (19%) (↓)lymphocytes in ♀ (38%) **Clinical chemistry:** (↑) alanine aminotransferase (ALAT) on week 53 in ♂ (247% ncdr), and on week 79 in ♀ (528% ncdr) (↑) Sorbitol dehydrogenase (SDH) on week 53 in ♂ (298%) and on week 79 in ♂/♀ (125% ncdr/160% ncdr). **Gross pathology (liver):** *Unscheduled deaths*: (↑)incidence of liver enlargement in ♂ (25% ncdr, compared with an incidence of 10% in control group), pale areas in ♀ (29%, compared with an incidence of 3% in control group) and masses (44% ncdr/19% ncdr, compared with an incidence of 0%/0% in control groups). *Terminal sacrifice* (week 79): (↑) incidence of liver enlargement ♂/♀ (24%/45% ncdr, compared with an incidence of 16%/0% in control groups), pale areas in ♂/♀ (35% ncdr/41% ncdr, compared with an incidence of 3%/0% in control groups) and masses (15% ncdr/28%, compared with an incidence of 10%/0% in control groups). **Organ weights:** Carcass: (↓) abs wt in ♂, on recovery week 57 (10%). Also (↓) abs wt in ♀, on week 79 (8%)	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
		Liver/gall bladder: (↑) abs and rel wt in ♂ on weeks 53 (34%/38%, respectively) and (44% ncdr/39% ncdr, respectively). Also (↑) abs and rel wt in ♀ on weeks 53 (41%/46%, respectively) and 79 (82%/99%, respectively) **Histopathology:** *Non-neoplastic changes (liver):* (↑) individual cell necrosis in ♂/♀ (74%/39%, compared with a 7%/5% in control groups) (↑) focal/multifocal necrosis in ♂ (16%, compared with a 6% in control group) (↑) hepatocyte hypertrophy in ♂/♀ (87% ncdr/76% ncdr, compared with a 24%/3% in control groups) (↑) liver fatty change in ♂/♀ (19%/11%, compared with a 1%/0% in control groups) (↑) liver bile stasis in ♂/♀ (80% ncdr/71%, compared with a 1%/0% in control groups) *Neoplastic changes (liver):* (↑) hepatocellular adenoma in ♂/♀ (19% ncdr/23%, compared with a 6%/0% in control groups) (↑) hepatocellular carcinoma in ♂/♀ (7%/6%, compared with a 1%/0% in control groups) **300 ppm** (46.3 ♂/57.8 ♀ mg/kg bw/day) **Bodyweight and bodyweight gain:** (↓) bw gain in ♀ through weeks 0-13 (16%) (↓) bw gain in ♂ through weeks 0-52 (15%), although it was n.s. on week 76 **Clinical chemistry:** (↑) Sorbitol dehydrogenase (SDH) on week 53 in ♂ (98%) **Organ weights:** Liver/gall bladder: (↑) abs and rel wt in ♀ on week 53 (20%/17%, respectively) **Histopathology:** *Non-neoplastic changes (liver):* (↑) individual cell necrosis in ♂ (22%, compared with a 7% in control group) (↑) hepatocyte hypertrophy in ♂ (43% ncdr, compared with a 24% in control group) $NOAEL_{toxicity}$: **30 ppm** corresponding to **4.7/5.6 mg/kg bw/day** for ♂ and ♀ respectively. $NOAEL_{carcinogenicity}$: **300 ppm** corresponding to **46.3/57.8 mg/kg bw/day** for ♂ and ♀ respectively.	

Table 23: Summary table of other (mode of action) studies relevant for carcinogenicity

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results **[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]**	Reference
Oral study of 14-days in mice. Lab: Ciba-Geigy Limited Guideline: No test method available GLP: No	**Mortality:** 6 animals died: 5 animals due to malapplication: 1 control, 2 control-recovery, 1 at DFZ 400 mg/kg bw/day, 1 at DFZ recovery 400 mg/kg bw/day. 1 additional control animal died spontaneously on the day of sacrifice. **Clinical signs:** No signs of toxicity were observed throughout the treatment period. **400 mg/kg bw/day**	**Anonymous 19 (1992)** **B.6.8.2.2.1 (AS)**

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

Test substance: Difenoconazol, (purity: 91.8%; batch: P.807002)

Reference substances:

Phenobarbitone (PB) 3-methylcholan-threne (3-MC) Nafenopin (NAF)

Route administration: Oral gavage (DFZ) Intraperitoneally (PB, 3-MC and NAF)

Mice strain (male): CIBA-GEIGY breeding station Tif:MAGf (SPF)

Doses:
DFZ: 0, 1, 10, 100, 400 mg/kg bw/day for 14 days. Recovery animals: 0 and 400 mg/kg bw/day for 14 + 28 days for recovery. 9 animals per group.
PB: 40 mg/kg bw/day for 4 days. 6 animals
3-MC: 80 mg/kg bw/day for 2 days. 6 animals
NAF: 100 mg/kg bw/day for 4 days (6 animals) and 50 mg/kg bw/day for 6 days (3 animals)
Study acceptable

Bodyweight:

Unaffected

Organs weight (liver): (↑) abs (79%)

Treatment	Dose (mg/kg bw/day)	Bodyweight (g)	Liver weight (g)
Control	0	29.7	1.17
DFZ	400	28.2	**2.09*** **
Control rec.	0	34.8	1.41
DFZ rec.	400	34.5	1.40

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;rec: recovery group*

Protein content:

(↑) Cytochrome P-450 (323%)
(↑) CYP1A (35%)
(↓) CYP2B (43%)
(↑) CYP3A (316%)
(↓) CYP4A (17%)

Treatment	Dose (mg/kg bw/day)	Cytochrome P450 [a]	CYP1A (RAU)	CYP2B (RAU)	CYP3A (RAU)	CYP4A (RAU)
Control	0	18.5	100	100	100	100
DFZ	400	**78.3*** **	135	57	416	83
Control rec.	0	15.1	100	100	100	100
DFZ rec.	400	15.3	118	97	91	78

The data show the immunochemically detectable relative protein content of liver microsomal cytochromes P-450 crossreactive with monoclonal antibodies: d15 (CYP1A), be4 (CYP2B), p6 (CYP3A) and clo4 (CYP4A). RAU: Relative Area Units, from densitometric scans of single western blots (samples pooled). rec: recovery group.
*[a]: *= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test*

Enzyme activities:

(↑) MEH (245%)
(↑) Microsomal morphine UDP-GT (59%) n.s.
(↑) Microsomal 1-naphtol UDP-GT (20) n.s.
(↑) EROD (231%)
(↑) PROD (3246%)
(↑) Lauric acid 11-hydroxylase (130%)
(↓) Lauric acid 12-hydroxylase (17%, ndr, n.s.)

Treatment	Dose (mg/kg bw/day)	MEH	Microso-mal morphine UDP-GT	Microso-mal 1-naphtol UDP-GT	EROD	PROD
Control	0	61.8	684	815	2.27	0.89
DFZ	400	**213.7*** **	1090	983	**7.52*** **	**29.78*** **
Control rec.	0	68.3	669	914	4.17	1.07
DFZ rec.	400	60	715	911	2.59	1.05

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are nmol/min/g liver

Treatment	Dose (mg/kg bw/day)	Lauric acid 11-hydroxylase	Lauric acid 12-hydroxylase
Control	0	24.1	45.8
DFZ	400	**55.4*** **	37.8
Control rec.	0	20.6	19.3
DFZ rec.	400	18.4	15.6

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are nmol/min/g liver

100 mg/kg bw/day

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

Bodyweight:

(↓) bw (11%, ndr)

Organs weight (liver):

Unaffected

Treatment	Dose (mg/kg bw/day)	Bodyweight (g)	Liver weight (g)
Control	0	29.7	1.17
DFZ	100	**26.3***	1.32

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;rec: recovery group*

Protein content:

(↑) Cytochrome P-450 (161%)
(↑) CYP1A (23%)
(↓) CYP2B (49%)
(↑) CYP3A (115%)
(↓) CYP4A (53%)

Treatment	Dose (mg/kg bw/day)	Cytochrome P450 [a]	CYP1A (RAU)	CYP2B (RAU)	CYP3A (RAU)	CYP4A (RAU)
Control	0	18.5	100	100	100	100
DFZ	100	**48.2*** **	123	51	215	47

The data show the immunochemically detectable relative protein content of liver microsomal cytochromes P-450 crossreactive with monoclonal antibodies: d15 (CYP1A,be4 (CYP2B), p6 (CYP3A) and clo4 (CYP4A). RAU: Relative Area Units, from densitometric scans of single western blots (samples pooled). rec: recovery group.
*[a]: *= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test*

Enzyme activities:

(↑) MEH (50%) n.s.
(↑) Microsomal morphine UDP-GT (28%) n.s.
(↑) Microsomal 1-naphtol UDP-GT (24%) n.s.
(↑) EROD (285%)
(↑) PROD (1839%)
(↑) Lauric acid 11-hydroxylase (78%)
(↓) Lauric acid 12-hydroxylase (38%, ndr, n.s.)

Treatment	Dose (mg/kg bw/day)	MEH	Microsomal morphine UDP-GT	Microsomal 1-naphtol UDP-GT	EROD	PROD
Control	0	61.8	684	815	2.27	0.89
DFZ	100	92.8	882	1013	**8.76*** **	**17.26*** **

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are mol/min/g liver

Treatment	Dose (mg/kg bw/day)	Lauric acid 11-hydroxylase	Lauric acid 12-hydroxylase
Control	0	24.1	45.8
DFZ	100	**43*** **	28.4

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are mol/min/g liver

10 mg/kg bw/day

Bodyweight:

Unaffected

Organs weight (liver):

Unaffected

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

Treatment	Dose (mg/kg bw/day)	Bodyweight (g)	Liver weight (g)
Control	0	29.7	1.17
DFZ	10	29.2	1.34

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;rec: recovery group*

Protein content:

(↓) Cytochrome P-450 (8%) n.s.
(↑) CYP1A (9%)
(↓) CYP2B (38%)
(↓) CYP3A (37%, ndr)
(↓) CYP4A (51%)

Treatment	Dose (mg/kg bw/day)	Cytochrome P450 [a]	CYP1A (RAU)	CYP2B (RAU)	CYP3A (RAU)	CYP4A (RAU)
Control	0	18.5	100	100	100	100
DFZ	10	16.9	109	62	63	49

The data show the immunochemically detectable relative protein content of liver microsomal cytochromes P-450 crossreactive with monoclonal antibodies: d15 (CYP1A), be4 (CYP2B), p6 (CYP3A) and clo4 (CYP4A). RAU: Relative Area Units, from densitometric scans of single western blots (samples pooled). rec: recovery group.
*[a]: *= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test*

Enzyme activities:

(↓) MEH (5%) n.s.
(↑) Microsomal morphine UDP-GT (5%) n.s.
(↑) Microsomal 1-naphtol UDP-GT (19%) n.s.
(↑) EROD (56%) n.s.
(↑) PROD (140%)
(↑) Lauric acid 11-hydroxylase (8%) n.s.
(↓) Lauric acid 12-hydroxylase (67%) ndr

Treatment	Dose (mg/kg bw/day)	MEH	Microsomal morphine UDP-GT	Microsomal 1-naphtol UDP-GT	EROD	PROD
Control	0	61.8	684	815	2.27	0.89
DFZ	10	58.6	724	972	3.56	**2.14****

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are mol/min/g liver

Treatment	Dose (mg/kg bw/day)	Lauric acid 11-hydroxylase	Lauric acid 12-hydroxylase
Control	0	24.1	45.8
DFZ	10	26.2	**14.9*****

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are mol/min/g liver

1 mg/kg bw/day

Bodyweight:

Unaffected

Organs weight (liver):

Unaffected

Treatment	Dose (mg/kg bw/day)	Bodyweight (g)	Liver weight (g)
Control	0	29.7	1.17
DFZ	1	30.6	1.25

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;rec: recovery group*

Protein content:

Cytochrome P450 (5%) n.s.
(↓) CYP1A (5%)
(↓) CYP2B (12%)

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

(↓) CYP3A (20%, ndr)
(↓) CYP4A (37%)

Treatment	Dose (mg/kg bw/day)	Cytochrome P450 [a]	CYP1A (RAU)	CYP2B (RAU)	CYP3A (RAU)	CYP4A (RAU)
Control	0	18.5	100	100	100	100
DFZ	1	17.5	95	88	80	63

*The data show the immunochemically detectable relative protein content of liver microsomal cytochromes P450 crossreactive with monoclonal antibodies: d15 (CYP1A), be4 (CYP2B), p6 (CYP3A) and clo4 (CYP4A). RAU: Relative Area Units, from densitometric scans of single western blots (samples pooled). rec: recovery group.[a]: *= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test*

Enzyme activities:

(↑) MEH (19%) n.s.
(↑) Microsomal morphine UDP-GT (16%) n.s.
(↑) Microsomal 1-naphtol UDP-GT (10%) n.s.
(↑) EROD (31%) n.s.
(↑) PROD (124%)
(↑) Lauric acid 11-hydroxylase (7%) n.s.
(↓) Lauric acid 12-hydroxylase (48%) ndr

Treatment	Dose (mg/kg bw/day)	MEH	Microsomal morphine UDP-GT	Microsomal 1-naphtol UDP-GT	EROD	PROD
Control	0	61.8	684	815	2.27	0.89
DFZ	1	73.7	797	897	2.98	**1.99****

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are nmol/min/g liver

Treatment	Dose (mg/kg bw/day)	Lauric acid 11-hydroxylase	Lauric acid 12-hydroxylase
Control	0	24.1	45.8
DFZ	1	25.9	**23.5****

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are nmol/min/g liver

Reference substances (PB, 3-MC and DFZ) treatment:

Body weight:

- PB: unaffected
- 3-MC: unaffected
- NAF: (↑ 17%)

Organ weight (liver):

- PB: unaffected
- 3-MC: (↑) abs (28%)
- NAF: (↑) abs (88%)

Treatment	Dose (mg/kg bw/day)	Bodyweight (g)	Liver weight (g)
Control	0	29.7	1.17
PB	40	29.5	1.33
3-MC	80	28.3	**1.50*****
NAF	50	**35.0***	**2.20*****

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test*

Protein content:

- PB:

(↑) Cytochrome P-450 (80%)
(↑) CYP1A (43%)
(↓) CYP2B (5%)
(↑) CYP3A (287%)
(↓) CYP4A (33%)

- 3-MC:

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

(↑) Cytochrome P-450 (147%)
(↑) CYP1A (4432%)
(↓) CYP2B (34%)
(↓) CYP3A (33%)
(↓) CYP4A (50%)

- NAF:

(↑) Cytochrome P-450 (40%) n.s.
(↓) CYP1A (39%)
(↓) CYP2B (28%)
(↑) CYP3A (227%)
(↑) CYP4A (810%)

Treatment	Dose (mg/kg bw/day)	Cytochrome P450 [a]	CYP1A (RAU)	CYP2B (RAU)	CYP3A (RAU)	CYP4A (RAU)
Control	0	18.5	100	100	100	100
PB	40	**33.3*****	143	95	387	67
3-MC	80	**45.8*****	4532	66	67	50
NAF	50	26.0	61	72	327	910

The data show the immunochemically detectable relative protein content of liver microsomal cytochromes P-450 crossreactive with monoclonal antibodies: d15 (CYP1A), be4 (CYP2B), p6 (CYP3A) and clo4 (CYP4A). RAU: Relative Area Units, from densitometric scans of single western blots (samples pooled). rec: recovery group.
*[a]: *= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test*

Enzyme activities:

Treatment	Dose (mg/kg bw/day)	MEH	Microsomal morphine UDP-GT	Microsomal 1-naphtol UDP-GT	EROD	PROD
Control	0	61.8	684	815	2.27	0.89
PB	40	**132.9*****	1021	**1478*****	**7.67*****	**14.44*****
3-MC	80	71.5	972	**1245****	**105*****	**2.34*****
NAF	50	**205.9*****	994	**1791*****	3.55	1.47

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are nmol/min/g liver

Treatment	Dose (mg/kg bw/day)	Lauric acid 11-hydroxylase	Lauric acid 12-hydroxylase
Control	0	24.1	45.8
PB	40	**48.5*****	43.9
3-MC	80	28.1	**23.4****
NAF	50	**83.7*****	**391.1*****

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are nmol/min/g liver

- PB:

(↑) MEH (115%)
(↑) Microsomal morphine UDP-GT (49%) n.s.
(↑) Microsomal 1-naphtol UDP-GT (81%)
(↑) EROD (237%)
(↑) PROD (1522%)
(↑) Lauric acid 11-hydroxylase (101%)
(↓) Lauric acid 12-hydroxylase (4%) n.s.

- 3-MC:

(↑) MEH (15%) n.s.
(↑) Microsomal morphine UDP-GT (42%) n.s.
(↑) Microsomal 1-naphtol UDP-GT (52%)
(↑) EROD (4525%)
(↑) PROD (162%)

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

(↑) Lauric acid 11-hydroxylase (16%) n. s.
(↓) Lauric acid 12-hydroxylase (49%)

- NAF:

(↑) MEH (233%)
(↑) Microsomal morphine UDP-GT (45%) n.s.
(↑) Microsomal 1-naphtol UDP-GT (119%)
(↑) EROD (56%) n.s.
(↑) PROD (65%) n. s.
(↑) Lauric acid 11-hydroxylase (247%)
(↑) Lauric acid 12-hydroxylase (753%)

Testosterone hydroxylation in mice treated with all doses of DFZ, PB, 3-MC and NAF

Treatment	Dose (mg/kg bw/day)	Total activity	2β-H-T	6β-H-T	15β-H-T	16β-H-T	2α-H-T
Control	0	81.8	10	22.6	2.4	3.9	1.4
DFZ	1	113.5	6.2	**43.2****	**4.6****	1.7	2.5
	10	**117.3***	**5.3***	**37.6***	**4.1***	3	1.7
	100	**407.3*****	**20.4****	**194.2*****	**14.7*****	6.4	4.8
	400	**544*****	**32.6*****	**287.6*****	**22.6*****	**8.9***	**5.8****
Control rec.	0	107.1	9.5	37.9	3.6	3.3	3.3
DFZ rec.	400	97.3	**4.7****	32.5	3.99	1.7	1.8
PB	40	**281.4*****	14.5	**149.5*****	**11.4*****	4	2
3-MC	80	**124****	7	32.4	**4***	3.9	1
NAF	50	**370.3*****	21.9	**220.4*****	**14.8*****	3.9	1.7

Treatment	Dose (mg/kg bw/day)	6α-H-T	7α-H-T	16α-H-T	Androste-nedione	Unidentified test metabolite
Control	0	3.3	9.6	6.3	17.1	5.2
DFZ	1	**5.9*****	10	7.7	**23.9***	7.7
	10	**7.6*****	11.3	7.7	**31.9*****	7
	100	**26.1*****	16.6	**15.7*****	**90.8*****	**17.5*****
	400	**29*****	15.4	**18.8*****	**104.7*****	**18.6*****
Control rec.	0	4.07	9.6	6.4	21.2	8.3
DFZ rec.	400	**6.2***	8.8	6.7	22.5	8.5
PB	40	**15.9*****	16.9	**13.3*****	**40.9*****	**13*****
3-MC	80	**7.3*****	9.1	**13.6****	**36.6*****	**9****
NAF	50	**6.8*****	**19.8***	**23.8*****	**40.6*****	**16.6*****

**= p<0.05, ** = p<0.01, *** = p<0.001, Dunnett's test;* rec: recovery group; Units are nmol/min/g liver

- The level of total testosterone hydroxylation was induced 6-fold in a dose-related manner, in mice at 400 mg/kg bw/day DFZ. Except for 7α-hydroxy-testosterone, all testosterone metabolites were increased between 3 and 12.5 fold in mice at 400 mg/kg bw/day DFZ.
- Increases of 6β- , 15β-, 6α- and 16α- hydroxy-testosterone also appear in the treatment with PB.

Peroxisomal fatty acid β-oxidation and GST activity with all doses of DFZ, PB, 3-MC and NAF

- A slight, dose-dependent and not statistically significant decrease of peroxisomal fatty acid β-oxidation was observed in mice treated with DFZ, that was reversible after 28 days of recovery. PB and 3-MC did not affect the peroxisomal fatty acid β-oxidation whereas the treatment with NAF produced an increase of 4.4 fold in this process.

<table>
<tr><th>Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td></td><td>▪ The activity of cytosolic glutathione S-transferase (GST) in mice treated with 400 mg/kg bw/day DFZ was increased 1.4 fold. A similar level of induction was seen with PB, and to a lesser extent with NAF. 3-MC reduced this enzyme activity to 1.3 fold. All these changes in cytosolic GST activity were not statistically significant.
CONCLUSION
The treatment with DFZ at 400 mg/kg bw/day in mice produced changes that were similar to those with PB, as increases of microsomal proteins (microsomal morphine UDPGT and microsomal 1-naphtol UDPGT) activities, increases of cytochrome P450 content, changes in CYP isoenzymes levels, increases of liver enzymes (MEH, EROD, PROD) activities and increases of testosterone hydroxylation.</td><td></td></tr>
<tr><td>In vitro study with CD-1 mice hepatocytes.

Lab: CXR Biosciences Ltd

Guideline: No test method available

GLP: No

Test substance: Difenoconazole (batch No. SMO3E4125; purity 93.9%)

Dose levels: Primary monolayer cultures of hepatocytes were cultured in Leibowitz CL15 medium for 4 hours to allow adherence. The medium was changed and the hepatocytes exposed to PB (at 10, 100 and 1000 µM), to EGF[a] (25ng/ml), to difenoconazole (at 0.5, 1, 2, 4, 8 and 12.5 µM) or to 0.5% DMSO (vehicle) for 96 h.
[a] Epidermal Growth factor (positive control for replicative DNA synthesis).

Study acceptable</td><td>Cytotoxicity:
No cytotoxicity at the tested doses (>97% ATP depletion at 25 µM during the pre-test).
Hepatocellular proliferation:
<table>
<tr><th rowspan="2">Treatment</th><th rowspan="2">Concentration</th><th colspan="2">S-phase labelling index</th></tr>
<tr><th>Mean</th><th>%</th></tr>
<tr><td>Negative control</td><td>0.5% [v/v] DMSO</td><td>1.79</td><td>100</td></tr>
<tr><td rowspan="6">DFZ</td><td>0.5 µM</td><td>2.16</td><td>120.7</td></tr>
<tr><td>1 µM</td><td>2.47*</td><td>137.9</td></tr>
<tr><td>2 µM</td><td>2.59**</td><td>144.7</td></tr>
<tr><td>4 µM</td><td>2.75**</td><td>153.7</td></tr>
<tr><td>8 µM</td><td>2.83**</td><td>158.5</td></tr>
<tr><td>12.5 µM</td><td>3.04**</td><td>170.1</td></tr>
<tr><td rowspan="3">PB</td><td>10 µM</td><td>2.08</td><td>116.4</td></tr>
<tr><td>100 µM</td><td>2.39*</td><td>133.7</td></tr>
<tr><td>1000 µM</td><td>2.29</td><td>128.2</td></tr>
<tr><td>EGF</td><td>25 ng/mL</td><td>13.89**</td><td>776.6</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett
Cytochrome P450 (CYP) enzymes mRNA assessment:
<table>
<tr><th>Treatment</th><th>Concentration</th><th>Cyp2b10 mRNA</th><th>Cyp3a11 mRNA</th></tr>
<tr><td>Negative control</td><td>0.5% [v/v] DMSO</td><td>1.00</td><td>1.00</td></tr>
<tr><td rowspan="6">DFZ</td><td>0.5 µM</td><td>0.99</td><td>1.25</td></tr>
<tr><td>1 µM</td><td>1.08</td><td>1.14</td></tr>
<tr><td>2 µM</td><td>1.11</td><td>1.24</td></tr>
<tr><td>4 µM</td><td>1.27</td><td>1.33</td></tr>
<tr><td>8 µM</td><td>0.62*</td><td>1.00</td></tr>
<tr><td>12.5 µM</td><td>0.43**</td><td>0.68</td></tr>
<tr><td rowspan="3">PB</td><td>10 µM</td><td>0.90</td><td>1.01</td></tr>
<tr><td>100 µM</td><td>1.67**</td><td>1.84**</td></tr>
<tr><td>1000 µM</td><td>3.32**</td><td>6.51**</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett; Relative to control normalised to 1.
Enzyme activities:
<table>
<tr><th rowspan="2">Enzyme activities: Treatment</th><th rowspan="2">Concentration</th><th colspan="2">PROD</th><th colspan="2">BROD</th><th colspan="2">BQ</th></tr>
<tr><th>Mean</th><th>%</th><th>Mean</th><th>%</th><th>Mean</th><th>%</th></tr>
<tr><td>Negative control</td><td>0.5% DMSO</td><td>29.4</td><td>100</td><td>121.7</td><td>100</td><td>0.23</td><td>100</td></tr>
<tr><td rowspan="6">DFZ</td><td>0.5 µM</td><td>30.4</td><td>103.6</td><td>145.6</td><td>119.6</td><td>0.2</td><td>87</td></tr>
<tr><td>1 µM</td><td>30.4</td><td>103.3</td><td>122.3</td><td>100.5</td><td>0.27</td><td>116.2</td></tr>
<tr><td>2 µM</td><td>24.5</td><td>83.3</td><td>106.4</td><td>87.5</td><td>0.23</td><td>96.6</td></tr>
<tr><td>4 µM</td><td>29.3</td><td>99.6</td><td>129.4</td><td>106.4</td><td>0.38</td><td>160.8</td></tr>
<tr><td>8 µM</td><td>30.2</td><td>102.7</td><td>132.1</td><td>108.6</td><td>0.45**</td><td>193.1</td></tr>
<tr><td>12.5 µM</td><td>27</td><td>91.8</td><td>126</td><td>103.5</td><td>0.54**</td><td>228.5</td></tr>
<tr><td rowspan="2">PB</td><td>10 µM</td><td>31.1</td><td>106.0</td><td>132.3</td><td>108.8</td><td>0.22</td><td>93</td></tr>
<tr><td>100 µM</td><td>43</td><td>146.2</td><td>155.9</td><td>128.2</td><td>0.26</td><td>111.1</td></tr>
</table></td><td>Vardy A. (2016a)
B.6.8.2.2.2-01
(AS)</td></tr>
</table>

<table>
<tr><th>Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td></td><td>
<table>
<tr><td></td><td>1000 µM</td><td>109.7**</td><td>373.4</td><td>560.8**</td><td>461</td><td>0.85**</td><td>360.9</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett.
CONCLUSION
Treatment of isolated male CD-1 mouse hepatocyte cultures with difenoconazole at concentrations up to 12.5 µM resulted in increases in replicative DNA-synthesis as determined by the S-phase labelling index.
However, difenoconazole did not increase either Cyp2b10 or Cyp3a11 mRNA levels. PROD and BROD activities were also unchanged, but difenoconazole did increase BQ activity (indicative of CYP3A activity) in concentration-dependent manner.
Treatment with the positive controls PB and EGF gave the expected set of responses, indicating the suitability of the system.
</td><td></td></tr>
<tr><td>In vitro study with human hepatocytes
Lab: CXR Biosciences Ltd
Guideline: No test method available
GLP: No
Test substance: Difenoconazole (batch No. SMO3E4125; purity 93.9%)
Dose levels: Primary male human hepatocytes in Cryopreserved Hepatocytes Plating Medium for up to 6 hours to allow adherence and, then the medium was changed to Leibowitz HCL15 medium and the hepatocytes exposed to PB (at 10, 100 and 1000 µM), to Difenoconazole (at 0.5, 1, 2, 4, 6 and 8 µM), to EGF[a] (25ng/ml) or to 0.5% DMSO (vehicle) for 96 h.

[a] Epidermal Growth factor (positive control for replicative DNA synthesis).
Study acceptable</td><td>
<u>Cytotoxicity:</u>
Treatment with 6 µM and 8 µM DFZ resulted in hepatocellular cytotoxicity with ATP levels being reduced to 75% and 49% of control, respectively. Treatment with PB did not cause a statistically significant decrease in ATP levels.
<table>
<tr><th rowspan="2">Treatment</th><th rowspan="2">Concentration</th><th colspan="2">ATP content (luminescence units)</th></tr>
<tr><th>Mean</th><th>%</th></tr>
<tr><td>Negative control</td><td>0.5% [v/v] DMSO</td><td>241442</td><td>100</td></tr>
<tr><td rowspan="7">DFZ</td><td>0.5 µM</td><td>267111</td><td>110.6</td></tr>
<tr><td>1 µM</td><td>269272</td><td>111.5</td></tr>
<tr><td>2 µM</td><td>246431</td><td>102.1</td></tr>
<tr><td>4 µM</td><td>222670</td><td>92.2</td></tr>
<tr><td>6 µM</td><td>181000**</td><td>75.0</td></tr>
<tr><td>8 µM</td><td>118468**</td><td>49.1</td></tr>
<tr><td rowspan="3">PB</td><td>10 µM</td><td>251895</td><td>104.3</td></tr>
<tr><td>100 µM</td><td>258924</td><td>107.2</td></tr>
<tr><td>1000 µM</td><td>228311</td><td>94.6</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett
<u>Hepatocellular proliferation:</u>
<table>
<tr><th rowspan="2">Treatment</th><th rowspan="2">Concentration</th><th colspan="2">S-phase labelling index</th></tr>
<tr><th>Mean</th><th>%</th></tr>
<tr><td>Negative control</td><td>0.5% [v/v] DMSO</td><td>0.29</td><td>100</td></tr>
<tr><td rowspan="7">DFZ</td><td>0.5 µM</td><td>0.27</td><td>93.5</td></tr>
<tr><td>1 µM</td><td>0.28</td><td>96.8</td></tr>
<tr><td>2 µM</td><td>0.34</td><td>116.6</td></tr>
<tr><td>4 µM</td><td>0.31</td><td>105.2</td></tr>
<tr><td>6 µM</td><td>0.25</td><td>85</td></tr>
<tr><td>8 µM</td><td>0.16*</td><td>53.4</td></tr>
<tr><td rowspan="3">PB</td><td>10 µM</td><td>0.30</td><td>101.1</td></tr>
<tr><td>100 µM</td><td>0.28</td><td>95.8</td></tr>
<tr><td>1000 µM</td><td>0.33</td><td>114.3</td></tr>
<tr><td>EGF</td><td>25 ng/mL</td><td>1.89**</td><td>647.2</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett
<u>Enzyme activities</u>:
<table>
<tr><th rowspan="2">Treatment</th><th rowspan="2">Concentration</th><th colspan="2">PROD[a]</th><th colspan="2">BROD[a]</th><th colspan="2">BQ[b]</th></tr>
<tr><th>Mean</th><th>%</th><th>Mean</th><th>%</th><th>Mean</th><th>%</th></tr>
<tr><td>Negative control</td><td>0.5% DMSO</td><td>0.15</td><td>100</td><td>1.28</td><td>100</td><td>0.21</td><td>100</td></tr>
<tr><td rowspan="4">DFZ</td><td>0.5 µM</td><td>0.32**</td><td>210.7</td><td>2.24</td><td>175.4</td><td>0.29</td><td>138.1</td></tr>
<tr><td>1 µM</td><td>0.25</td><td>162.9</td><td>2.02</td><td>158</td><td>0.28</td><td>132</td></tr>
<tr><td>2 µM</td><td>0.27*</td><td>177.7</td><td>2.78*</td><td>217.1</td><td>0.27</td><td>127.1</td></tr>
<tr><td>4 µM</td><td>0.47**</td><td>306.1</td><td>4.49**</td><td>351.5</td><td>0.19</td><td>88</td></tr>
</table>
</td><td>Vardy A. (2016b)
B.6.8.2.2.2-02
(AS)</td></tr>
</table>

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

	6 µM	**0.45****	292.2	**2.99****	233.9	**0.07****	33.3
	8 µM	**0.32****	207.7	**3.31****	259.3	**0.04****	19.8
PB	10 µM	0.15	99	1.54	120.6	0.26	123.9
	100 µM	0.19	125.7	**2.65***	207.8	**0.40****	187.3
	1000 µM	**0.33****	216.8	**5.40****	423.1	**0.52****	248.6

**= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett*

CONCLUSION

Treatment of isolated male human hepatocyte cultures with difenoconazole at concentrations up to 8 µM had no effect on replicative DNA-synthesis, as determined by the S-phase labelling index.
However, difenoconazole led to PROD and BROD activities induction, which are mainly representative of CYP2B and CYP2B/3A, respectively. In contrast, BQ activity was unaffected by treatment with difenoconazole at the lower concentrations, and then reduced at the higher concentrations assessed in the presence of cytotoxicity.

***In vitro* study with mice hepatocytes.**

Lab: CXR Biosciences Ltd
Guideline: No test method available
GLP: No
Test substance: Difenoconazole (batch No. SMO3E4125; purity 93.9%)
Dose levels: Hepatocytes prepared from livers of male CD-1 mice (treated with 500 ppm PB during 14 days) were incubated with Difenoconazole at 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µM, along with a vehicle control (dimethylformamide).

Study acceptable

Enzyme activities:

DFZ concentration (µM)	PROD (CYP2B) activity		BQ (CYP3A) activity	
	pmols resorufin formed/min/mg protein	% control	nmols 7-OH formed/min/mg protein	% control
0	28.38	100	1.93	100
0.01	29.826	105.1	1.566	81.3
0.03	21.172	74.6	1.163[a]	60.4[a]
0.1	21.481	75.7	1.762	91.5
0.3	13.067	46.0	1.874	97.3
1	13.676	48.2	1.535	79.7
3	5.95	21.0	1.181	61.3
10	4.056	14.3	0.806	41.9
30	3.331	11.7	0.467	24.2
100	0.81	2.9	0.315	16.3
300	-	-	0.094	4.9

[a] Value removed from the data analysis

Inhibition of PROD Activity by difenoconazole: IC_{50} = 0.371 µM

Inhibition of BQ Activity by difenoconazole: IC_{50} = 6.911 µM

CONCLUSION

Difenoconazole inhibited both CYP2B and CYP3A induction in an *in vitro* assay when the enzymatic activities were assessed using liver microsomes prepared from PB-induced male CD-1 mice.
The IC50 values revealed difenoconazole to be a more potent inhibitor of the PROD activity compared to the BQ activity.

Vardy A. (2016c)
B.6.8.2.2.2-03
(AS)

CAR transactivation study
Lab: Department of Veterinary and Biomedical Sciences. Penn

Viability:

Treatment	Conc.	% viability				
		Untransfected	Empty vector	*hCAR3*	*mCAR3*	*rCAR3*

Omiecinski C. (2016)
B.6.8.2.2.2-04
(AS)

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

State University.
Guideline: No test method available
GLP: Yes, with exceptions.
Test substance: Difenoconazole (batch No. SMO3E4125; purity 93.9%)
Dose levels: Expression vectors for CAR3 variants of mouse, rat and human with a CYP2B6 response element-luciferase reporter were transfected into COS-1 cells[a] along with necessary cofactors. After a expression time (16-18h) cells were incubated during 24 h with DFZ (at 1, 3, 10 and 30 µM) and with the following CAR ligands (positive controls):
- CITCO (substrate for human CAR3) at 5 µM.
- TBPOBOP (substrate for mouse CAR3) at 0.5 µM.
- Clotrinazole (CLOT, substrate for rat CAR3) at 10 µM.
In all treatments, DMSO levels never exceeded 0.1% (v/v).

[a] Primate derived.

Study acceptable

DMSO	**0.1% v/v**	100	100	100	100	100
CITCO	**5 µM**	94.3	100.4	93.1	96.1	90.4
TCPOBOP	**0.5 µM**	109.3	91.8	112.9	110.6	94.6
CLOT	**10 µM**	101.2	103.7	105.1	97.4	93.0
DFZ	**1 µM**	96.8	97.5	114.6	117.9	85.9
	3 µM	96.4	100.6	99.1	101.4	84.5
	10 µM	96.0	95.2	94.6	97.2	**78.3****
	30 µM	89.8	94.9	84.4	87.2	**73.1****

***= p<0.01, ANOVA + Dunnett; Conc.: concentration*

CAR3 reporter assay:

Treatment	Concentration	Luciferase activity			
		Empty vector		*hCAR3*	
		Norm. value	Fold change	Norm. value	Fold change
DMSO	**0.1% v/v**	0.00221	1.0	0.00269	1.0
CITCO	**5 µM**	0.00222	1.0	0.05128	**19.0****
TCPOBOP	**0.5 µM**	0.00258	1.17	0.00233	0.86
CLOT	**10 µM**	0.00235	1.05	0.01026	**3.81****
DFZ	**1 µM**	0.00243	1.1	0.00279	1.04
	3 µM	0.00226	1.02	0.00286	1.05
	10 µM	0.00217	0.98	0.00268	0.98
	30 µM	0.00174	0.79	0.00215	0.8

***= p<0.01, ANOVA + Dunnett, Norm.value: normalized value*

Treatment	Concentration	*mCAR3*		*rCAR3*	
		Norm. value	Fold change	Norm. value	Fold change
DMSO	**0.1% v/v**	0.0034	1.0	0.0027	1.0
CITCO	**5 µM**	0.01666	4.9	0.00292	1.08
TCPOBOP	**0.5 µM**	0.13209	**38.81****	0.00297	1.1
CLOT	**10 µM**	0.07451	**21.9****	0.22661	**83.83****
DFZ	**1 µM**	0.00493	1.44	0.00298	1.1
	3 µM	0.01144	3.35	0.00295	1.08
	10 µM	0.03499	**10.27****	0.00349	1.28
	30 µM	0.05875	**17.27****	0.00447	1.66

***= p<0.01, ANOVA + Dunnett; Norm.value: normalized value*

CONCLUSION

Under conditions of this assay, difenoconazole was a direct activator of mouse CAR and not an activator of human CAR. A small increase in activation of rat CAR (1.6-fold) was observed at 30 µM difenoconazole, but this difference was not statistically significant, indicating that difenoconazole was at most a low potency activator of rat CAR.

PXR transactivation study Lab: Indigo	**Viability:** **Treatment** / **Concentration** / **% viability**	**Korrapati M. & Sherf B. (2016)** **B.6.8.2.2.2-05**

<table>
<tr><th>Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>Biosciences, Inc.
Guideline: No test method available
GLP: No
Test substance: Difenoconazole (batch No. SMO3E4125; purity 93.9%)
Dose levels: Expression vectors were constructed with the ligand binding domains of PXR variants of mouse, rat and human fused to the DNA binding domain of the transcription factor Gal4 and with a Gal4 response luciferase reporter. These vectors were transfected into HEK cells (human embryonic kidney). After 16-18 h of expression, cells were incubated during 24h with DFZ at 13.7, 41.2, 123, 370, 1111, 3333, 10000, 30000 nM and with pregnenolone-16α-carbonitrile and TO901317 at appropriate ranges of concentrations. In all treatments, DMSO levels never exceeded 0.1% (v/v).
Study acceptable</td><td>
<table>
<tr><th></th><th></th><th>human</th><th>rat</th><th>mouse</th></tr>
<tr><td>DMSO</td><td>0.1% v/v</td><td>100</td><td>100</td><td>100</td></tr>
<tr><td rowspan="9">DFZ</td><td>13.7 Nm</td><td>97</td><td>100</td><td>97</td></tr>
<tr><td>41.2 nM</td><td>98</td><td>100</td><td>99</td></tr>
<tr><td>123 nM</td><td>99</td><td>101</td><td>97</td></tr>
<tr><td>370 nM</td><td>99</td><td>101</td><td>99</td></tr>
<tr><td>1111 nM</td><td>100</td><td>98</td><td>101</td></tr>
<tr><td>3333 nM</td><td>101</td><td>101</td><td>101</td></tr>
<tr><td>10000 nM</td><td>92</td><td>95</td><td>98</td></tr>
<tr><td>30000 nM</td><td>89</td><td>88</td><td>97</td></tr>
</table>
<u>PXR assay:</u>
<table>
<tr><th rowspan="2">Treatment</th><th rowspan="2">Concentration</th><th colspan="3">Luciferase activity fold-change</th></tr>
<tr><th>human</th><th>rat</th><th>mouse</th></tr>
<tr><td>DMSO</td><td>0.1% v/v</td><td>1.0</td><td>1.0</td><td>1.0</td></tr>
<tr><td rowspan="8">DFZ</td><td>13.7 nM</td><td>1.1</td><td>1.1</td><td>1.2</td></tr>
<tr><td>41.2 nM</td><td>0.96</td><td>0.93</td><td>1.2</td></tr>
<tr><td>123 nM</td><td>0.77</td><td>1.1</td><td>1.3</td></tr>
<tr><td>370 nM</td><td>1.1</td><td>1.0</td><td>1.5</td></tr>
<tr><td>1111 nM</td><td>1.1</td><td>1.0</td><td>1.8**</td></tr>
<tr><td>3333 nM</td><td>1.4</td><td>1.0</td><td>1.4</td></tr>
<tr><td>10000 nM</td><td>1.3</td><td>0.42**</td><td>1.0</td></tr>
<tr><td>30000 nM</td><td>0.56</td><td>0.20**</td><td>0.61</td></tr>
<tr><td rowspan="8">TO901317</td><td>4.57</td><td>5.3</td><td>-</td><td></td></tr>
<tr><td>13.7</td><td>14</td><td>0.79</td><td>1.2</td></tr>
<tr><td>41.1</td><td>23**</td><td>1.1</td><td>1.3</td></tr>
<tr><td>123</td><td>40**</td><td>1.3</td><td>1.4</td></tr>
<tr><td>370</td><td>54**</td><td>1.3</td><td>1.2</td></tr>
<tr><td>1111</td><td>71**</td><td>3.4**</td><td>1.1</td></tr>
<tr><td>3333</td><td>59**</td><td>12**</td><td>2.0**</td></tr>
<tr><td>10000</td><td>-</td><td>38**</td><td>3.5**</td></tr>
<tr><td rowspan="8">Pregnenolone-16α-carbonitrile</td><td>4.88</td><td>0.74</td><td>0.92</td><td>1.2</td></tr>
<tr><td>19.5</td><td>1.0</td><td>1.1</td><td>1.3</td></tr>
<tr><td>78.1</td><td>0.86</td><td>1.2</td><td>1.8</td></tr>
<tr><td>313</td><td>1.1</td><td>1.8</td><td>1.7</td></tr>
<tr><td>1250</td><td>1.3</td><td>18**</td><td>2.5**</td></tr>
<tr><td>5000</td><td>1.0</td><td>74**</td><td>3.3**</td></tr>
<tr><td>20000</td><td>1.3</td><td>108**</td><td>4.0**</td></tr>
</table>
***= p<0.01, ANOVA + Dunnet; In triplicate.*
CONCLUSION
Based on the results of these luciferase reporter assays, difenoconazole is not an activator of human, rat, or mouse PXR. Positive controls gave the expected results.
</td><td>(AS)</td></tr>
<tr><td>Acute toxicity and toxicokinetics study (1- and 7-day) in CD-1 and C57BL/6J mice
Lab: CXR</td><td><u>Bodyweights:</u>
<u>1 or 7-Day Oral gavage treatment (Part 1 and 2):</u>
No significant effect on terminal bodyweights in any treatment group in either strain.
<u>Single intravenous injection (Part 3):</u>
↓ bodyweight in CD1 and C57BL/6J mice, but attributable to the intravenous dosing and serial</td><td>Anonymous 20 (2017a)
B.6.8.2.2.3-01
(AS)</td></tr>
</table>

<table>
<tr><th>Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>Biosciences Ltd
Guideline: No test method available.
GLP: No
Test substance: Difenoconazole (purity: ≥ 93.9%; batch: SM03E4125)
Route administration: Oral gavage and intravenous injection
Mice strain (male): Charles River CD-1 and Envigo C57BL/6J.
Dose levels/No animals:
Part 1: 1-Day Oral Gavage.
✓ Dose:
CD-1 mice: 0, 15, 45, 150, 400 mg/kg bw/day.
C57BL/6J mice: 0, 15, 150 mg/kg bw/day
✓ No animals: 5/group/strain
Part 2: 7-Day Oral Gavage.
✓ Dose:
CD-1 mice: 0, 15, 45, 150, 400 mg/kg bw/day.
C57BL/6J mice: 0, 15, 150 mg/kg bw/day
✓ No animals: 5/group/strain
Part 3: 1-Day Intravenous.
✓ Dose:
CD-1 mice: 1 mg/kg
C57BL/6J mice: 1 mg/kg
✓ No animals: 5/group/strain</td><td>blood sampling procedures.
<u>Clinical chemistry (ALT, AST and ALP):</u>
<u>7-Day Oral gavage treatment (Part 2):</u>
CD-1: No consistent dose-related changes
C57BL/6J: No consistent dose-related changes
<u>Toxicokinetic Analysis</u>
<u>1 or 7-Day Oral gavage treatment (Part 1 and 2):</u>
CD-1: Metabolism of DFZ induced with oral doses above 45 mg/kg
C57BL/6J: Metabolism of DFZ induced with oral doses at 150 mg/kg/day
The results in both strains of mice indicate that the increase in exposure with respect to dose is non-linear over the dose range tested in these repeated dose studies.
<table><tr><th rowspan="2">DFZ dose [a]</th><th rowspan="2">Day</th><th colspan="2">CD-1 mice</th><th colspan="2">C57BL/6J mice</th></tr><tr><th>AUC(0-tau)</th><th>Rac</th><th>AUC(0-tau)</th><th>Rac</th></tr><tr><td rowspan="2">15</td><td>1</td><td>2550</td><td rowspan="2">0.9</td><td>2334</td><td rowspan="2">0.9</td></tr><tr><td>7</td><td>2166</td><td>2005</td></tr><tr><td rowspan="2">45</td><td>1</td><td>5550</td><td rowspan="2">0.5</td><td colspan="2" rowspan="2">No data</td></tr><tr><td>7</td><td>2693</td></tr><tr><td rowspan="2">150</td><td>1</td><td>82241</td><td rowspan="2">0.1</td><td>34991</td><td rowspan="2">0.2</td></tr><tr><td>7</td><td>6432</td><td>7559</td></tr><tr><td rowspan="2">400</td><td>1</td><td>111858</td><td rowspan="2">0.1</td><td colspan="2" rowspan="2">No data</td></tr><tr><td>7</td><td>13041</td></tr></table>[a]: mg/kg bw/day; AUC(0-tau): the area under the blood concentration versus time curve from time zero over the dosing interval, in ng.h/mL. Rac: Accumulation ratio = AUC(0-tau) following repeat dosing (Day 7) / AUC(0-tau) after a single dose (Day 1)
<u>Single intravenous injection (Part 3):</u>
Difenoconazole was eliminated within 4-8 hours in both CD-1 and C57BL/6J mice.
<u>Organ weight (liver):</u>
<u>7-Day Oral gavage treatment (Part 2):</u>
CD-1:
▪ ↑ Absolute liver weight (1.5-fold) at 400 mg/kg bw/day
▪ ↑ Relative liver weight (1.5-fold) at 400 mg/kg bw/day
▪ ↑ Relative liver weight (1.2-fold) at 150 mg/kg bw/day
C57BL/6J:
▪ ↑ Absolute liver weight (1.3-fold) at 150 mg/kg bw/day
▪ ↑ Relative liver weight (1.2-fold) at 150 mg/kg bw/day
<table><tr><th rowspan="2">7-Day Oral Gavage DFZ</th><th colspan="3">C57BL/6J mice</th><th colspan="5">CD-1 mice</th></tr><tr><th>C</th><th colspan="2">DFZ</th><th>C</th><th colspan="4">DFZ</th></tr><tr><th>Dose [a]</th><th>0</th><th>15</th><th>150</th><th>0</th><th>15</th><th>45</th><th>150</th><th>400</th></tr><tr><td>Terminal Bodyweight (g)</td><td>22.2</td><td>22.66</td><td>23.96</td><td>35.42</td><td>34.26</td><td>32.42</td><td>33.78</td><td>34.96</td></tr><tr><td>Absolute liver weight (g)</td><td>1.17</td><td>1.23</td><td>1.49**</td><td>1.89</td><td>1.96</td><td>1.85</td><td>2.19</td><td>2.77**</td></tr><tr><td>Relative liver weight (%)</td><td>5.26</td><td>5.44</td><td>6.20**</td><td>5.31</td><td>5.72</td><td>5.71</td><td>6.5**</td><td>7.93**</td></tr></table>[a]: mg/kg bw/day; *= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett
<u>Liver histopathology:</u></td><td></td></tr>
</table>

<table>
<tr><th>Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>Liver enzymes:
- PROD: marker for CYP2B
- BROD: marker for CYP2B/3A
-BQ: marker for CYP3A
Study acceptable</td><td>7-Day Oral gavage treatment (Part 2):
CD-1:
▪ ↑ centrilobular hepatocellular hypertrophy at dose ≥ 150 mg/kg bw/day
▪ ↓ glycogen at dose ≥ 150 mg/kg bw/day
▪ ↑ incidence of cytoplasmic vacuolation at 400 mg/kg bw/day (4/5 animals)
▪ No treatment-related ↑ in inflammatory cell focus
C57BL/6J:
▪ ↑ centrilobular hepatocellular hypertrophy at dose 150 mg/kg bw/day
▪ ↓ glycogen at 150 mg/kg bw/day
▪ No incidence of cytoplasmic vacuolation at any dose level.
▪ No treatment-related ↑ in inflammatory cell focus

Enzymatic activities:
7-Day Oral gavage treatment (Part 2):
CD-1:
↑ total P450 content at 150 (↑ 3.7-fold) and 400 mg/kg bw/day (↑ 4.6-fold)
↑ PROD activities at 45, 150 and 400 mg/kg bw/day (↑ 13.5, 28.3 and 41.7-fold, respectively)
↑ BROD activities at 45, 150 and 400 mg/kg bw/day (↑ 25.6, 67 and 107-fold, respectively)
↑ BQ activities at 45, 150 and 400 mg/kg bw/day (↑ 1.4, 2.3 and 2.4-fold, respectively)
C57BL/6J:
↑ total P450 content at 150 mg/kg bw/day (↑ 3.7-fold)
↑ PROD activities at 150 mg/kg bw/day (↑ 54.4-fold)
↑ BROD activities at 150 mg/kg bw/day (↑ 110.7-fold)
↑BQ activities at 150 mg/kg bw/day (↑ 1.8-fold)

<table>
<tr><th>Strain</th><th>Dose (mg/kg bw/day)</th><th>Total P450 (nmol/mg)</th><th>PROD [a]</th><th>BROD [a]</th><th>BQ [b]</th></tr>
<tr><td rowspan="5">CD-1</td><td>0</td><td>0.48</td><td>4.82</td><td>21.35</td><td>1.99</td></tr>
<tr><td>15</td><td>0.82</td><td>8.12</td><td>64.01</td><td>2.48</td></tr>
<tr><td>45</td><td>0.86</td><td>64.99**</td><td>545.76*</td><td>2.81*</td></tr>
<tr><td>150</td><td>1.78**</td><td>136.34**</td><td>1429.55**</td><td>4.59**</td></tr>
<tr><td>400</td><td>2.18**</td><td>200.98**</td><td>2287.18**</td><td>4.85**</td></tr>
<tr><td rowspan="3">C57BL/6J</td><td>0</td><td>0.40</td><td>4.21</td><td>17.37</td><td>2.41</td></tr>
<tr><td>15</td><td>0.54</td><td>16.22</td><td>152.60</td><td>2.95</td></tr>
<tr><td>150</td><td>1.48**</td><td>228.81**</td><td>1923.47**</td><td>4.44**</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett. [a]: pmol resorufin/min/mg; [b]: nmol 7-OH/min/mg

Toxicogenomic analysis:
7-Day Oral gavage treatment (Part 2):
CD-1:
↑ Cyp2b10 mRNA at 45, 150 and 400 mg/kg bw/day (↑ 11, 76 and 248 -fold, respectively)
↑ Cyp3a11 mRNA at 45, 150 and 400 mg/kg bw/day (↑ 1.8, 3.5 and 4.3 -fold, respectively)
↑ Gadd45β mRNA at 400 mg/kg bw/day n.s (↑ 2.7- fold)
C57BL/6J:
↑ Cyp2b10 mRNA at 15 and 150 mg/kg bw/day (↑ 52 and 438 -fold, respectively)
↑ Cyp3a11 mRNA at 15 and 150 mg/kg bw/day (↑ 1.4 and 2.3 -fold, respectively)
↓ Gadd45β mRNA at 15 mg/kg bw/day (↓ 0.3 - fold) and ↑ Gadd45β mRNA at 150 mg/kg bw/day n.s. (3.2 –fold).</td><td></td></tr>
</table>

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

Strain	Dose (mg/kg bw/day)	Liver *Cyp2b10* mRNA	Liver *Cyp3a11* mRNA	Liver *Gadd45β* mRNA
CD-1	0 (control)	1.00	1.00	1.00
	15	**11.21***	1.26	0.22
	45	**76.09****	**1.77***	0.38
	150	**187.13****	**3.49*****	0.26
	400	**247.84*****	**4.28*****	2.73
C57BL/6J	0 (control)	1.00	1.00	1.00
	15	**52.2***	**1.36***	**0.26***
	150	**437.57*****	**2.31*****	3.24

**= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett. Results are expressed as fold change relative to control, where control values were normalized to 1.00*

CONCLUSION

Difenoconazole treatment produced increased liver weights accompanied by centrilobular hypertrophy and decreased glycogen content in the liver at doses ≥150 mg/kg bw/day in both strains of mouse and an increased incidence of cytoplasmic vacuolation at 400 mg/kg bw/day in CD-1 mice. Furthermore, there were dose-related increases in hepatic total cytochrome P450 content and on PROD, BROD and BQ activities (markers for CYP2B, CYP2B/3A and CYP3A, respectively) along with increases of *Cyp2b10* and *Cyp3a11* mRNAs levels in both strains of mouse. The toxicokinetic data indicated that metabolism of DFZ was induced and that the increase in exposure with respect to dose was non-proportional over the dose range tested in the repeated dose studies in both strains of mouse.

Overall, difenoconazole administration resulted in similar effects in male CD-1 and C57BL/6 mice at dose levels tested.

<table>
<tr><th>Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>Acute toxicity and toxicokinetics study (1- and 7-day) in Car/Pxr double KO and hCAR/hPXR mice
Lab: CXR Biosciences Ltd
Guideline: No test method available.
GLP: No
Test substance: Difenoconazole (purity: 93.9%; batch: SM03E4125)
Route administration: Oral gavage and intravenous injection
Mice strain (male): Taconic Biosciences Inc. Car/Pxr double KO, hCAR/hPXR and C57BL/6NTAc (WT)
Dose levels/No animals:
Part 1: 1-Day Oral Gavage.
✓ Dose: Car/Pxr double KO mice: 0, 15, 150 mg/kg bw/day.
hCAR/hPXR mice: 0, 15, 150 mg/kg bw/day
✓ No animals: 5/group/strain
Part 2: 7-Day Oral Gavage.
✓ Dose: Car/Pxr double KO mice: 0, 15, 150 mg/kg bw/day.
hCAR/hPXR mice:</td><td><u>Bodyweights:</u>
<u>1 or 7-Day Oral gavage treatment (Part 1 and 2):</u>
▪ No significant effect on terminal bodyweights in any treatment group.
▪ ↓ bodyweight change in Car/Pxr double KO mice at 150 mg/kg for 7 days.
<table>
<tr><th rowspan="2">7 Day Oral Gavage DFZ</th><th colspan="3">Car/Pxr double KO mice</th><th colspan="3">hCAR/hPXR mice</th></tr>
<tr><th>C</th><th colspan="2">DFZ</th><th>C</th><th colspan="2">DFZ</th></tr>
<tr><th>Dose (mg/kg/d)</th><th>0</th><th>15</th><th>150</th><th>0</th><th>15</th><th>150</th></tr>
<tr><td>Day 1 Bodyweight (g)</td><td>21.66</td><td>24.74</td><td>23.12</td><td>22.66</td><td>23.32</td><td>24.20</td></tr>
<tr><td>Terminal Bodyweight (g)</td><td>21.76</td><td>24.16</td><td>22.10</td><td>22.04</td><td>23.50</td><td>24.08</td></tr>
<tr><td>Body weight change (g)</td><td>0.10</td><td>-0.58</td><td>-1.02*</td><td>-0.62</td><td>0.18</td><td>-0.12</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett.
<u>Single intravenous injection (Part 3):</u> Unchanged
<u>Clinical chemistry (ALT, AST and ALP)</u>
<u>7-Day Oral gavage treatment (Part 2):</u>
Car/Pxr double KO: No consistent dose-related changes
hCAR/hPXR: No consistent dose-related changes
<u>Toxicokinetic Analysis</u>
<u>1 or 7-Day Oral gavage treatment (Part 1 and 2):</u>
Car/Pxr double KO:
▪ ↑ difenoconazole clearance after 7-day treatment at 150 mg/kg bw/day (Rac=0.5)
▪ ↑ difenoconazole clearance after 7-day treatment at 15 mg/kg bw/day (Rac=0.6)
hCAR/hPXR:
▪ ↑ difenoconazole clearance after 7-day treatment at 150 mg/kg bw/day (Rac=0.4)
▪ No ↑ difenoconazole clearance after 7-day treatment at 15 mg/kg bw/day (Rac=0.8)
The $AUC_{(0\text{-}tau)}$ increased non-proportionally with respect to dose in both the Car/Pxr double KO mice and hCAR/hPXR mice.
<table>
<tr><th rowspan="2">DFZ (mg/kg bw/day)</th><th rowspan="2">Day</th><th colspan="2">Car/Pxr double KO mice</th><th colspan="2">hCAR/hPXR mice</th></tr>
<tr><th>$AUC_{(0\text{-}tau)}$</th><th>Rac</th><th>$AUC_{(0\text{-}tau)}$</th><th>Rac</th></tr>
<tr><td>15</td><td>1</td><td>396</td><td rowspan="2">0.6</td><td>366</td><td rowspan="2">0.8</td></tr>
<tr><td>15</td><td>7</td><td>254</td><td>293</td></tr>
<tr><td>150</td><td>1</td><td>23762</td><td rowspan="2">0.5</td><td>15375</td><td rowspan="2">0.4</td></tr>
<tr><td>150</td><td>7</td><td>11349</td><td>6138</td></tr>
</table>
$AUC_{(0\text{-}tau)}$: the area under the blood concentration versus time curve from time zero over the dosing interval, in ng.h/mL. Rac: Accumulation ratio = $AUC_{(0\text{-}tau)}$ following repeat dosing (Day 7)/ $AUC_{(0\text{-}tau)}$ after a single dose (Day 1).
<u>Single intravenous injection (Part 3):</u>
Difenoconazole was eliminated within 4-8 hours in Car/Pxr double KO, hCAR/hPXR and C57BL/6NTAc (WT) mice.
<u>Organ weight (liver):</u>
<u>7-Day Oral gavage treatment (Part 2):</u>
Car/Pxr double KO:
▪ No effect on absolute liver weight
▪ No effect on relative liver weight
hCAR/hPXR:
▪ ↑ Absolute liver weight (1.28 -fold) at 150 mg/kg bw/day</td><td>Anonymous 21 (2017b)
B.6.8.2.2.3-02
(AS)</td></tr>
</table>

<table>
<tr><th>Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>0, 15, 150 mg/kg bw/day
✓ <u>No animals</u>: 5 animals/group/strain
Part 3: 1-Day Intravenous.
✓ <u>Dose:</u>
Car/Pxr double KO mice: 1 mg/kg
hCAR/hPXR mice: 1 mg/kg
C57BL/6NTAc (WT): 1 mg/kg
✓ <u>No animals</u>: 5/group/strain
<u>Liver enzymes:</u>
- PROD: marker for CYP2B
- BROD: marker for CYP2B/3A
-BQ: marker for CYP3A
Study acceptable</td><td>
↑ Relative liver weight (1.18 -fold) at 150 mg/kg bw/day
<table>
<tr><th rowspan="2">7 Day Oral Gavage DFZ</th><th colspan="3">Car/Pxr double KO mice</th><th colspan="3">hCAR/hPXR mice</th></tr>
<tr><th>C</th><th colspan="2">DFZ</th><th>C</th><th colspan="2">DFZ</th></tr>
<tr><th>Dose (mg/kg/d)</th><th>0</th><th>15</th><th>150</th><th>0</th><th>15</th><th>150</th></tr>
<tr><td>Terminal Bodyweight (g)</td><td>21.76</td><td>24.16</td><td>22.10</td><td>22.04</td><td>23.50</td><td>24.08</td></tr>
<tr><td>Absolute liver Weight (g)</td><td>1.20</td><td>1.26</td><td>1.14</td><td>1.19</td><td>1.29</td><td>1.52**</td></tr>
<tr><td>Relative liver weight (%)</td><td>5.56</td><td>5.21</td><td>5.18</td><td>5.38</td><td>5.50</td><td>6.34**</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett.
<u>Liver histopathology</u>
<u>7-Day Oral gavage treatment (Part 2):</u>
Car/Pxr double KO:
No treatment-related changes.
No treatment-related ↑ in inflammatory cell focus
hCAR/hPXR:
↑ mild centrilobular hepatocellular hypertrophy at 150 mg/kg bw/day (4/5)
Fat vacuolation and ↓ glycogen in the absence of hypertrophy (1/5)
No treatment-related ↑ in inflammatory cell focus
<u>Enzymatic activities</u>
<u>7-Day Oral gavage treatment (Part 2):</u>
Car/Pxr double KO:
No consistent dose-related changes in the total P450
No ↑ PROD, BROD and BQ activities
hCAR/hPXR:
No consistent dose-related changes in the total P450
↑ PROD activities n. s. at 150 mg/kg bw/day (↑ 1.9 -fold)
↑ BROD activities at 150 mg/kg bw/day (↑ 2.3 -fold)
↑ BQ activities at 150 mg/kg bw/day (↑ 1.5 -fold)
<table>
<tr><th>Strain</th><th>Dose (mg/kg bw/day)</th><th>Total P450 (nmol/mg)</th><th>PROD[a]</th><th>BROD[a]</th><th>BQ[b]</th></tr>
<tr><td rowspan="3">Car/Pxr double KO mice</td><td>0 (control)</td><td>0.49</td><td>2.30</td><td>13.13</td><td>4.59</td></tr>
<tr><td>15</td><td>0.42</td><td>2.70</td><td>16.21</td><td>4.06</td></tr>
<tr><td>150</td><td>0.58</td><td>3.12</td><td>18.06</td><td>4.49</td></tr>
<tr><td rowspan="3">hCAR/hPXR mice</td><td>0 (control)</td><td>0.76</td><td>23.41</td><td>115.93</td><td>6.52</td></tr>
<tr><td>15</td><td>0.82</td><td>14.36</td><td>79.79</td><td>6.06</td></tr>
<tr><td>150</td><td>0.88</td><td>43.53</td><td>268.46**</td><td>10.09**</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001, ANOVA + Dunnett. [a]: pmol resorufin/min/mg; [b]: nmol 7-OH/min/mg
<u>Toxicogenomic analysis</u>
<u>7-Day Oral gavage treatment (Part 2):</u>
Car/Pxr double KO:
No consistent dose-related changes in Cyp2b10, Cyp3a11 and Gadd45β mRNA levels at 15 and 150 mg/kg bw/day
hCAR/hPXR:
↑ Cyp2b10 mRNA n.s. at 150 mg/kg bw/day (↑ 12.9 -fold)
↑ Cyp3a11 mRNA at 150 mg/kg bw/day (↑ 2.7 -fold)
Gadd45β mRNA unaffected
</td><td></td></tr>
</table>

<table>
<tr><th>Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td></td><td>
<table>
<tr><th>Strain</th><th>DFZ (mg/kg bw/day)</th><th>Liver Cyp2b10 mRNA</th><th>Liver Cyp3a11 mRNA</th><th>Liver Gadd45β mRNA</th></tr>
<tr><td rowspan="3">Car/Pxr double KO mice</td><td>0 (control)</td><td>1.00</td><td>1.00</td><td>1.00</td></tr>
<tr><td>15</td><td>0.50</td><td>1.26</td><td>0.90</td></tr>
<tr><td>150</td><td>0.25</td><td>1.20</td><td>0.83</td></tr>
<tr><td rowspan="3">hCAR/hPXR mice</td><td>0 (control)</td><td>1.00</td><td>1.00</td><td>1.00</td></tr>
<tr><td>15</td><td>1.46</td><td>3.19</td><td>2.39</td></tr>
<tr><td>150</td><td>12.92</td><td>2.69**</td><td>2.51</td></tr>
</table>
*= p<0.05, ** = p<0.01, *** = p<0.001; ANOVA + Dunnett. Results are expressed as fold change relative to control, where control values were normalized to 1.00

CONCLUSION
The main differences between both strains of mice treated with difenoconazole are:
- Absolute and relative liver weight were unaffected in Car/Pxr double KO but both weights significantly increased in hCAR/hPXR male mice.
- Histopathological analysis showed a dose-related mild centrilobular hypertrophy only in hCAR/hPXR mice livers, whereas Car/Pxr double KO mice livers showed no treatment-related changes.
- The analysis of liver enzyme activities and of corresponding mRNA expression levels in the liver showed no response at any dose level in male Car/Pxr double KO. However, in hCAR/hPXR mice at 150 mg/kg bw/day, BROD and BQ enzyme activities (markers for CYP2B/3A and CYP3A, respectively) showed statistically significant increases and Cyp2b10 and Cyp3a11 mRNA levels were higher than controls.
- The toxicokinetic data indicated that metabolism of DFZ was induced and that the increase in exposure with respect to dose was non-proportional over the dose range tested in the repeated dose studies in both strains of mouse.

Taken together, these data suggest that the hepatic effects of difenoconazole are dependent on the presence of a functional CAR and/or PXR.
</td><td></td></tr>
<tr><td>Hepatocellular proliferation and liver enzymatic induction study (7-day) in Car/Pxr double KO, C57BL/6NTAc WT and CD-1 WT mice
Lab: CXR Biosciences Ltd
Guideline: No test method available.</td><td>
<u>Clinical observations</u>
A difenoconazole-treated C57BL/6NTAc WT mouse appeared subdued at day 7 after the last dosage.
Upon gross dissection, the small intestine contained blood.

<u>Bodyweights</u>
▪ No significant effect on terminal bodyweights in any mouse strain treated with DFZ.
▪ No significant effect on terminal bodyweights in any mouse strain treated with PB.
▪ ↓ bodyweight change in CD-1 WT and Car/Pxr double KO treated with PB.

<table>
<tr><th>Parameters</th><th>Control</th><th>DFZ [a]</th><th>Control (Saline)</th><th>PB [b]</th></tr>
</table>
</td><td>Anonymous 22 (2017)
B.6.8.2.2.3-03 (AS)</td></tr>
</table>

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

GLP: No

Test substance: Difenoconazole (purity: 93.9%; batch: SM03E4125)

Reference substance:

Phenobarbital sodium salt (purity: ≥ 99.9%; batch: SLBJ3684V)

Route administration: Oral gavage.

Animals were also implanted subcutaneously with Alzet osmotic pumps containing BrdU to analyse the hepatocellular proliferation.

Vehicle:

Carboxymethyl-cellulose (CMC) to difenoconazole.

Normal saline to phenobarbital.

Mice strain (male): Charles River UK CD-1 WT, Taconic Biosciences Inc. C57BL/6NTAc WT and *Car/Pxr* double KO

Dose levels:

Difenoconazole (DFZ): 0, 150 mg/kg bw/day for 7 days.

Phenobarbital (PB): 0, 80 mg/kg bw/day for 7 days.

No animals:

12/group/strain

Liver enzymes:

- EROD: marker for CYP1A

- PROD: marker for CYP2B

CD-1 WT mice				
Day 1 Bodyweight (g)	37.18	37.13	37.63	37.12
Terminal Bodyweight (g)	37.42	37.27	38.53	36.08
Body weight change (g)	0.24	0.14	0.91	**-1.03****
C57BL/6NTAc WT mice				
Day 1 Bodyweight (g)	26.25	26.16	26.34	26.33
Terminal Bodyweight (g)	26.98	26.93	26.75	26.42
Body weight change (g)	0.73	0.76	0.41	0.09
***Car/Pxr* double KO mice**				
Day 1 Bodyweight (g)	25.17	24.83	25.33	25.64
Terminal Bodyweight (g)	25.89	24.47	25.73	25.28
Body weight change (g)	0.73	0.36	0.39	**-0.37***

**= p<0.05, ** = p<0.01, *** = p<0.001, two-tailed Student's t-Test.[a]: 150 mg/kg bw/day; [b]: 80 mg/kg bw/day*

Clinical chemistry (ALT, AST and ALP)

No consistent treatment-related changes in CD-1 WT, C57BL/6NTAc WT and *Car/Pxr* double KO. The isolated statistically significant differences in CD-1 WT and C57BL/6NTAc WT mice were not considered an effect of treatment.

Parameter	Strain	Control	DFZ [a]	Control	PB [b]
ALP (U/L)	**CD-1 WT**	73.20	**90.43****	76.55	80.56
	C57BL/6NTAc WT	96.30	94.58	98.58	105.5
	***Car/Pxr* double KO**	104.09	113.39	104.32	105.35
ALT (U/L)	**CD-1 WT**	45.26	**63.32****	51.43	**82.35***
	C57BL/6NTAc WT	50.41	53.53	40.36	51.95
	***Car/Pxr* double KO**	39.30	50.77	35.99	41.95
AST (U/L)	**CD-1 WT**	96.54	92.28	112.57	159.06
	C57BL/6NTAc WT	153.29	139.35	107.84	**155.98***
	***Car/Pxr* double KO**	155.71	177.55	151.36	157.83

**= p<0.05, ** = p<0.01, *** = p<0.001, two-tailed Student's t-Test.[a]: 150 mg/kg bw/day; [b]: 80 mg/kg bw/day*

Organ weight (liver)

CD-1 WT (DFZ):
- ↑ Absolute liver weight (↑ 1.3 fold)
- ↑ Relative liver weight (↑ 1.2 fold)

CD-1 WT (PB):
- ↑ Relative liver weight (↑ 1.1 fold)

C57BL/6NTAc WT (DFZ):
- ↑ Absolute liver weight (↑ 1.2 fold)
- ↑ Relative liver weight (↑ 1.2 fold)

C57BL/6NTAc WT (PB):
- ↑ Absolute liver weight (↑ 1.2 fold) in mice treated with PB
- ↑ Relative liver weight (↑ 1.2 fold) in mice treated with PB

Car/Pxr double KO (DFZ):
- No effect on absolute and relative liver weight

Car/Pxr double KO (PB):
- No effect on absolute and relative liver weight

Parameters	Control	DFZ [a]	Control	PB [b]

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

- BROD: marker for CYP2B/3A

-BQ: marker for CYP3A

-LAH (12-OH lauric acid): marker for CYP4A

Study acceptable

CD-1 WT mice				
Terminal Bodyweight (g)	37.42	37.27	38.53	36.08
Absolute liver weight (g)	2.06	**2.6****	2.2	2.39
Relative liver weight (%)	5.5	**7.0****	5.8	**6.5****
C57BL/6NTAc WT mice				
Terminal Bodyweight (g)	26.98	26.93	26.75	26.42
Absolute liver Weight (g)	1.53	**1.77****	1.51	**1.8****
Relative liver weight (%)	5.65	**6.64****	5.64	**6.78****
***Car/Pxr* double KO mice**				
Terminal Bodyweight (g)	25.89	24.47	25.73	25.28
Absolute liver weight (g)	1.39	1.38	1.29	1.35
Relative liver weight (%)	5.4	5.6	5.0	5.4

**= p<0.05, ** = p<0.01, *** = p<0.001, two-tailed Student's t-Test.*[a]*: 150 mg/kg bw/day;*
[b]*: 80 mg/kg bw/day*

Liver histopathology:

CD-1 WT (DFZ and PB):
- ↑ centrilobular hepatocellular hypertrophy
- No treatment-related ↑ in inflammatory cell focus

C57BL/6NTAc WT (DFZ and PB):
- ↑ centrilobular hepatocellular hypertrophy
- ↑ in inflammatory cell focus (3/12 control vs. 6/12 DFZ; 5/12 control vs. 6/12 PB) [a]

[a]: All animals with minimal grade finding (severity grade 1) except 2 animals with mild grade finding present (severity grade 2) in the PB treated group.

Car/Pxr double KO (DFZ and PB):
- No treatment-related changes present in the liver
- No treatment-related ↑ in inflammatory cell focus

Enzymatic activities:

CD-1 WT (DFZ):
- ↑ EROD, PROD, BROD, BQ and LAH activities

CD-1 WT (PB):
- ↑ EROD, PROD, BROD, BQ and LAH activities

C57BL/6NTAc WT (DFZ):
- ↑ EROD, PROD, BROD, BQ and LAH activities

C57BL/6NTAc WT (PB):
- ↑ EROD, PROD, BROD and BQ activities
- No changes in LAH activities

Car/Pxr double KO (DFZ):
- No changes in EROD, PROD and BQ activities
- ↓ BROD activities
- ↑ LAH

Car/Pxr double KO (PB):
- ↓ EROD and BROD activities
- No changes in PROD, BQ and LAH activities

Strain	Group	EROD[a]	PROD[a]	BROD[a]	BQ[b]	LAH[c]
CD-1 WT	**Control**	36.12	1.41	5.62	4.15	4.82

Type of study, laboratory, guideline, GLP, test substance(purity), route administration, strain, dose levels, no animals/group, acceptability	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference

	DFZ [d]	**81.18****	**42.60****	**358.69****	**9.53****	**6.94****
Fold induction		**↑ 2.2**	**↑ 30.1**	**↑ 63.8**	**↑ 2.3**	**↑ 1.4**
C57BL/ 6NTAc WT	**Control**	38.14	2.36	9.32	4.11	5.70
	DFZ [d]	**100.79****	**68.64****	**473.06****	**10.42****	**11.40****
Fold induction		**↑ 2.6**	**↑ 29.1**	**↑ 50.8**	**↑ 2.5**	**↑ 2**
***Car/Pxr* double KO**	**Control**	50.32	1.99	12.46	4.42	2.64
	DFZ [d]	50.32	1.32	**6.27***	3.71	**5.87****
Fold induction		1	↓ 0.6	**↓ 0.5**	↓ 0.8	**↑ 2.2**

**= p<0.05, ** = p<0.01, *** = p<0.001, two-tailed Student's t-Test. [a]: pmol resorufin/min/mg; [b]: nmol 7-OH/min/mg; [c]: nmol 12-OH/10min/mg protein; [d]: 150 mg/kg bw/day*

Strain	Dose	EROD[a]	PROD[a]	BROD[a]	BQ[b]	LAH[c]
CD-1 WT	**Control**	35.37	1.15	4.73	3.20	4.06
	PB [d]	**141.37****	**170.17****	**630.47****	**12.52****	**5.86****
Fold induction		**↑ 4**	**↑ 147.9**	**↑ 133.2**	**↑ 3.9**	**↑ 1.4**
C57BL/ 6NTAc WT	**Control**	37.06	2.19	7.94	4.21	4.72
	PB [d]	**174.01****	**220.98****	**619.49****	**11.13****	4.79
Fold induction		**↑ 4.7**	**↑ 100.9**	**↑ 78**	**↑ 2.6**	1.01
***Car/Pxr* double KO**	**Control**	53.02	1.42	6.53	4.35	2.11
	PB [d]	**41.43****	1.40	**5.42***	4.79	2.69
Fold induction		**↓ 0.78**	0.9	**↓ 0.83**	1.1	1.3

**= p<0.05, ** = p<0.01, *** = p<0.001, two-tailed Student's t-Test. [a]: pmol resorufin/min/mg; [b]: nmol 7-OH/min/mg; [c]: nmol 12-OH/10min/mg protein; [d]:* 80 mg/kg bw/day

Hepatocellular proliferation (measured as the BrdU labelling index)

CD-1 WT (DFZ and PB): ↑ BrdU labelling index
C57BL/6NTAc WT (DFZ and PB): ↑ BrdU labelling index
Car/Pxr double KO (DFZ): ↑ BrdU labelling index
Car/Pxr double KO (PB): No effect on BrdU labelling index

BrdU Labelling Index	Control	DFZ [a]	Control	PB [b]
CD-1 WT	0.81	**5.58****	0.59	**7.26****
C57BL/6NTAc WT	1.03	**6.96****	1.18	**11.59****
***Car/Pxr* double KO**	0.45	**1.31****	1.08	1.33

**= p<0.05, ** = p<0.01, *** = p<0.001, two-tailed Student's t-Test.; [a]: 150 mg/kg bw/day; [b]: 80 mg/kg bw/day*

CONCLUSION

In CD-1 WT and C57BL/6NTAc WT mice, DFZ caused increases in absolute and relative liver weight, hepatocellular proliferation and centrilobular hypertrophy, accompanied by liver enzymes (EROD, PROD, BROD and BQ) induction, suggesting activation of CAR and possibly PXR nuclear hormone receptors.
In the *Car/Pxr* double KO mice, there was not increase in absolute and relative liver weight, nor in centrilobular hypertrophy neither in liver enzymes induction, suggesting these were all CAR-mediated events. However, there was hepatocellular proliferation (albeit much reduced),
that may be a consequence of the low BrdU labelling index for control (CMC) mice. Additionally, the formation of 12-OH lauric acid was also higher than the control response in both the wild-type and *Car/Pxr* double KO mice treated with DFZ.
PB caused the expected results in all three mouse strains evaluated, except in CD-1 WT mice by an slight increase in LAH.

10.9.1 Short summary and overall relevance of the provided information on carcinogenicity

Two long-term toxicity/oncogenicity studies were conducted with difenoconazole, one in rats and one in mice (*Anonymous 16, 1989a; Anonymous 18, 1989b*).

In a 2-year long-term toxicity and carcinogenicity study in rats, difenoconazole was tested at dose levels of 0, 10, 20, 500 and 2500 ppm, equivalent to 0, 0.5, 1, 24.1 and 124 mg/kg bw/day for males and 0, 0.6, 1.3, 32.8 and 170 mg/kg bw/day for females (rationale for selection of doses was not provided in the report).

No mortality or clinical signs were associated to treatment.

Bodyweights of the 2500 ppm animals tended to be lower than those of control animals throughout the study and significant differences were recorded at weeks 52, 76 and 104, with decreases of 8-23%. In the 2500-ppm group of animals significantly lower values for body weight gain were noted from week 13 until study termination. The bodyweight gains of males and females of 2500 ppm group at week 104 were 11% and 37% lower than the control males and females, respectively. There were also a statistically reduction in bodyweight gain at 500 ppm at weeks 13, 24 and 52 in both sexes although it did not persist at termination (week 104). The significant reduction in weight gain in males of 20 ppm at week 13 was considered negligible.

The mean food consumption values for the animals of 2500 ppm group were significantly lower than for the control values at weeks 52, 76 and 104. These significant reductions were < 8% in males and < 15% in females, corresponding to the observations of reduced bodyweight and lower bodyweight gain. There were no records over the water intakes.

Ophthalmological examinations of the control and 2500 ppm animals revealed no treatment-related findings.

Haematologycal analysis showed a decrease in red cell mass (RBC count, haemoglobin, haematocrit) in females of the 2500 ppm group especially early in the study. There were significant changes in mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentrations (MCHC) but in most cases the red cell parameters were not significantly affected at study termination. Platelet counts were significantly lower than control values for males in the 500 ppm and the 2500 ppm groups. Leukocyte counts (WBC) were depressed for 2500 ppm males and females at week 104 (reduction of 30% and 36% in males and females, respectively), resulting from lower absolute segmented neutrophil and lymphocyte counts.

Blood chemistry revealed an increased albumin and decreased globulin levels in males of 2500 ppm group throughout the study, resulting in increased A/G ratios. In 2500 ppm females, only albumin levels were elevated, and only on week 28.

Alanine aminotransferase (ALAT) levels were increased in 500 and 2500 ppm males on week 53 (↑ 42% and 115%, respectively). However, ALAT levels were decreased in 500 and 2500 ppm females on week 28 (↓41% and 59%, respectively) and also in 2500 ppm females on week 53 (↓32%).

There was a transient decrease in glucose in males and females (↓12% and 8%, respectively) at week 28 and an increase in total cholesterol at weeks 28 and 104 (↑23 and 48% respectively) in males and on week 28 (↑28%) in females. There were decreases in total bilirubin in 2500 ppm males at week 28 (↓44%) and 2500 ppm females on weeks 28, 53 and 79 (↓67, 73 and 69% respectively) that could reflect the reductions in haemoglobin values.

Nevertheless, these differences were considered not relevant due to the low magnitude of the change, inconsistency across study intervals and/or the lack of a dose response.

Urinalysis revealed an increase in urine ketone bodies and a decrease in pH in 2500 ppm males at week 28, which would be consistent with the diminished nutritional status of these animals.

Macroscopic examinations did not reveal any treatment-related findings.

The terminal carcass weights of the 2500 ppm animals were lower than controls at weeks 53 in both sexes and 104 in females. There was no difference in carcass weight between the groups after the 4-week recovery period.

Absolute liver weights were unaffected among the groups during the study. However, the relative liver weights for the 2500 ppm animals were higher than control values at weeks 53 (↑14% in males and 48% in females) and 104 (↑18% no significant in males and ↑ 44% in females), but were similar to control values following the 4- weeks recovery period. The time points and dose groups of the reductions in carcass weights and increase in relative liver weights correspond.

The other statistically significant differences, that were considered not to be toxicologically relevant, were decreased absolute adrenal weights at week 53 in 2500 ppm males, decreased spleen weights at week 57 in 2500 ppm females, and increased ovary weights at week 104 in the 2500 ppm group, attributable to ovarian cysts with similar prevalence among treated and untreated groups of survivors and non-survivors.

Non-neoplastic changes revealed by histopathological examinations consisted in an increased incidence and severity of hepatocellular hypertrophy in 500 and 2500 ppm animals at study termination. For males, the incidence was 65 and 89% in the 500 and 2500 ppm dose groups, respectively, compared to 17.5% in control group. Corresponding values for females were 34 and 84%, compared to 12.5% in control group. These changes were not evident at the interim sacrifice at week 53.

No other treatment-related histomorphologic alterations were noted. A similar frequency and severity of commonly seen spontaneous disease lesions and incidental findings were observed in control and experimental rats of both sexes.

No neoplastic changes were considered relevant, due to the lack of a dose response and/or the low incidence and there were no increases in neoplasia in treated animals.

Since no evidence of carcinogenicity was observed at tested dose levels, the **NOAEL for carcinogenicity** was considered to be greater than 2500 ppm, **equivalent to > 124 and 170 mg/kg bw/day** for males and females, respectively. NOAEL for toxicity was **20 ppm** corresponding to **1 and 1.3 mg/kg bw/day** for males and females, respectively.

In a 78-week carcinogenicity study in mice, difenoconazole was tested at dose levels of 0, 10, 30, 300, 3000-2500 and 4500 ppm, equivalent to 0, 1.5, 4.7, 46.3, 507.6-423 and 819 mg/kg bw/day for males and 0, 1.9, 5.6, 57.8, 615.6-513 and 983 mg/kg bw/day for females. In the fifth group, the original dose of 3000 ppm was reduced to 2500 ppm at the beginning of week 2, due to early mortality.

The rationale for selection of dose levels was not provided in the report.

All (70) females in the 4500 ppm dose group died or were sacrificed in a moribund condition during the first 2 weeks. Eleven males (out of 70) in the 4500 ppm dose group died or were sacrificed for the same reason during the first 3 weeks of the study. At the next lower dose, 3000 ppm, 15 (out of 70) females died or were sacrificed during the first week, which led to a reduction to 2500 ppm for both sexes of this dose group, beginning at week 2 of the study. After the lowering of dose, one additional female died during the week 2 of experiment. At the beginning of week 3, 10 females of the control group were moved to 2500 ppm group to maintain an adequate sample size of this last

group for the duration of the study (replacement animals); 3 of these 10 females were sacrificed due to moribund during their first week of exposure to 2500 ppm. After the initial mortality in females of 2500 ppm group during the first 3 weeks of the study, there was no remarkable effect on survival.

Since no control females were sacrificed as recovery animals (week 57), the statistical analysis of the findings observed on the 2500 ppm recovery (female) group was impossible.

In males, survival for the 4500 ppm group was significantly less than control which contributed to a statistically significant negative overall trend in survival.

Clinical signs observed in this study included higher incidence of thinness, hunched appearance and rough haircoat in females of 2500 ppm group and in males of 4500 ppm group, compared to controls. The incidence of reduced motor activity was increased for the 4500 ppm males when compared with control.

There was a dose-dependent reduction in body weight of treated animals. Males of the two highest dose groups (2500 and 4500 ppm) had significantly lower body weights (↓6% and 7%, respectively) than controls from week 1 throughout to week 56. Females of the 2500 ppm group had significantly lower body weight (<10%) throughout the study period.

Dose-related significantly lower cumulative body weight gain was noted. Mean body weight gains were significantly decreased through 76 weeks for the groups of 4500 ppm in males (↓ 34%) and 2500 ppm in females (↓ 22%), through weeks 52 and 13 for males of 300 ppm, 2500 ppm and 4500 ppm groups (↓15%, 21% and 32% in week 52; ↓ 16%, 19% and 64% in week 13, respectively) and through week 52 in females of 2500 ppm group (↓ 23%) and week 13 in females of 300 ppm and 2500 ppm groups (↓16% and 33%, respectively).

There were no statistically significant differences in weekly food consumption among the groups for weeks 1 to 76. Body weight and food consumption values for recovery animals at week 56 were similar to the pre-recovery weights at week 52.

Ophthalmological examinations revealed no ocular finding attributable to treatment.

Haematologycal analysis showed an increase of the percent of segmented neutrophil count (↑ 19%) in females of the 2500 ppm group at week 79; the percent of lymphocytes was decreased (↓ 38%) compared to controls, but the biological significance of this finding is unclear. The absolute leukocyte differential count was not recorded.

Clinical chemistry revealed statistically significant increases in liver enzyme values in the highest dose groups at weeks 53 and 79. Mean ALAT values were elevated for groups of 2500 ppm and 4500 ppm males at week 53 and for 4500 ppm males and 2500 ppm females at week 79. Mean ALP values were increased for males of 4500 ppm group at week 79. Mean SDH values were increased for males of 2500 ppm and 4500 ppm groups at weeks 53 and 79, males of 300 ppm group at week 53 and females of 2500 ppm group at week 79.

After a 4-week recovery period the values approximated control values indicating that the changes are at least in part reversible.

Macroscopic examinations noted for unscheduled deaths included an increased overall incidence of liver findings (enlargement, pale areas and masses) in males and females of 2500 ppm group and in males of 4500 ppm group.

There were no remarkable gross observations at the week 53 interim sacrifice and the week 57 recovery sacrifice.

Remarkable observations recorded for the liver at terminal sacrifice included enlargement, pale areas and masses. At terminal sacrifice in males at 4500 ppm the incidences of liver enlargement, pale areas and masses were 50%, 56% and 44%, respectively, whereas in females at 2500 ppm were 45%, 41% and 28%, respectively.

The body (carcass) weight for females of the highest dose-group was lower than control at termination of the study.

Mean absolute and relative liver weight values were significantly higher than control values for males of 2500 ppm and 4500 ppm groups and females of 2500 ppm group at weeks 53 and 79, and for females of 300 ppm at week 53. In males at 300 ppm at week 53 the relative liver weight was also increased although without statistical significance.

Liver weights in males of recovery group (week 57) were lower than the weights for the animals at week 53 (interim sacrifice), indicating reversibility. The fact that no control females were sacrificed as recovery animals, made the statistical analysis impossible.

The other statistically significant differences were not considered toxicologically relevant due to inconsistency or no dose-response pattern.

Non-neoplastic changes found at study termination were observed in liver. The following hepatocelullar findings were significantly increased in males of 2500 ppm and 4500 ppm groups and in females of 2500 ppm group, focal/multifocal necrosis (males only), individual cell necrosis, fatty change, hepatocyte hypertrophy and bile stasis. A statistically significantly increased incidence was also noted for individual cell necrosis and hypertrophy in males of 300 ppm group.

The incidences of individual cell necrosis, hepatocyte hypertrophy, fatty change and bile stasis in the liver of males of 4500 ppm group were lower after the 4-week recovery period than that observed after 53 weeks of treatment, indicative of partial recovery.

Also neoplastic changes were detected in mice. Statistical analysis of liver adenomas and carcinomas revealed significant increases for males of 2500 and 4500 ppm groups, and for females of 2500 ppm group. The incidence of adenomas and/or carcinomas was already elevated in the 4500 ppm males at the interim and recovery sacrifices.

No other microscopic lesions observed in other organ tissues were considered attributable to treatment with difenoconazole.

Hepatocellular adenomas and carcinomas were observed in liver in male mice at 4500 ppm (819 mg/kg bw/day) and male and female mice at 2500 ppm (423/513 mg/kg bw/day for males/females, respectively). The **NOAEL for carcinogenicity** was considered to be 300 ppm in both sexes, **equivalent to 46.3 and 57.8 mg/kg bw/day** for males and females, respectively.

The **NOAEL for toxicity** was considered to be 30 ppm, **equivalent to 4.7 and 5.6 mg/kg bw/day** for males and females, respectively.

Liver tumours mode of action

In the carcinogenicity study in mice (*Anonymous 18, 1989b*) following dietary administration for up 78 weeks, hepatocellular adenomas and carcinomas were observed in liver in male mice at 4500 ppm (819 mg/kg bw/day) and male and female mice at 2500 ppm (423/513 mg/kg bw/day for males/females, respectively). The incidence was statistically significant for males of 2500 and 4500 ppm groups, ($p<0.05$ and $p<0.01$, respectively), and for females of 2500 ppm group ($p<0.01$). In addition, the incidence of adenomas and/or carcinomas was already elevated in the 4500 ppm males at the interim and recovery sacrifices. These results are showed in the following table:

% Total hepatocellular tumours[b]	Males (ppm)						Females (ppm)				
	0	10	30	300	2500[a]	4500	0	10	30	300	2500[a]
	Males (mg/kg bw/day)						Females (mg/kg bw/day)				
	0	1.5	4.7	46.3	423	819	0	1.9	5.6	57.8	513
Hepatocellular adenoma (%)	6	17	13	15	19	29	0	0	0	2	23
Hepatocellular carcinoma (%)	1	0	2	0	7	19	0	0	2	0	6
Hepatocellular adenoma and carcinoma (%)	7	17	15	15	**26***	**48****	0	0	2	2	**29****

[a]: Dose level 3000 ppm through day 21; * p<0.05, ** p<0.01, *** p<0.001, Bonferroni; [b]: Includes tumours observed on unscheduled deaths, interim sacrifice at week 53, recovery sacrifice at week 57 and terminal sacrifice animals at week 79-80.

The mode of action studies were performed to analyse the mechanism by which difenoconazole induced liver adenomas/carcinomas at high doses in mice in the long-term toxicity study and their relevance to humans.

The treatment related findings seen in the long-term studies reflected the same effects in the short-term studies, with the liver as a principle target of difenoconazole toxicity. Effects on the liver were noted in both rats and mice, as an evident increase in liver weight, hypertrophy as well as hepatocellular enlargement, single cell necrosis, hepatocellular vacuolation and fatty change in both sexes. This was accompanied by hepatocellular adenomas and carcinomas in both sexes of mice.

The results of both apical and mechanistic studies with difenoconazole provide some evidence which supports that the increase in incidence of liver tumours in mice might be mediated via a well characterized, non-genotoxic, rodent-specific phenobarbital (liver CYP2B inducer/CAR activator)-like MoA.

It is postulated by the applicant that difenoconazole induces liver tumours by a mechanism initiated by activation of the constitutive androstane receptor (CAR) which results in altered expression of CAR-responsive genes, including induction of pro-proliferative/anti-apoptotic genes (*Bhandal et al., 2017*). CAR-mediated stimulation of cell proliferation (and associated replicative DNA synthesis) promotes an environment permissive for increased cell replication. Suppression of apoptosis promotes an environment that would allow a spontaneously mutated cell to clonally expand without it being removed by apoptotic processes. Over time, under the promoting effect of this pro-proliferative and anti-apoptotic milieu, spontaneously initiated cells progress to pre-neoplastic foci, and after clonal expansion eventually ultimately result in hepatocellular adenoma and carcinoma. In addition to the induction of pro-proliferative and anti-apoptotic genes, CAR activation in male mice also results in the induction of a number of other genes, including some coding for members of specific cytochrome P450 families of isozymes, particularly those of CYP2b and, to a lesser extent, CYP3a. The activation of CAR and induction of pro-proliferative/anti-apoptotic genes are considered to be causal key effects, being necessary and directly resulting in the induction of liver tumours, whereas the effects on cytochrome P450s are considered to be associative key effects in that while they are a characteristic hallmark of CAR activation, they are not central to the induction of liver tumours. A further associative key event is liver hypertrophy, which is caused by proliferation of the smooth endoplasmic reticulum as a consequence of cytochrome P450 induction. This hypertrophy, in combination with the increased proliferation, in turn results in an increase in liver weight. When the CAR-dependent liver weight increase is sufficiently large and prolonged, it results in fatty change/bile stasis, and ultimately a late-onset necrosis of hepatocytes. This effect illustrates the excessive nature of these doses, but serves as a late acting, modulatory factor for the CAR-dependent process that produces a pro-proliferative milieu that is the initial and main driver for the carcinogenic process.

The CAR activation MoA mimics that of phenobarbital (PB), a mode of action which is accepted to be of no relevance for humans and to be rodent-specific *(Elcombe et al., 2014; Holsapple, 2006).*

Some of the effects of CAR activators observed in rodent liver can also be demonstrated in human liver. For example, PB and other CAR activators can induce CYP enzymes in both rodent liver and in human liver *(Elcombe et al., 2014).* Treatment with PB has been shown to increase liver size in humans, which is due to hepatocyte hypertrophy and proliferation of the smooth endoplasmic reticulum *(Aiges et al., 1980; Pirttiaho et al., 1978, 1982).* However, in terms of the human relevance, the key species difference is that while CAR activators are mitogenic agents in rodent hepatocytes, they do not appear to stimulate replicative DNA synthesis in human hepatocytes.

For phenobarbital it has been shown *in vitro* that there is a difference in ability between rodent and human hepatocytes in producing cell proliferation through CAR activation. Studies with cultured hepatocytes have demonstrated that phenobarbital was able to induce CYP2b forms in both rat and human hepatocytes, but cell proliferation only in rat hepatocytes *(Hirose et al., 2009; Parzefall et al., 1991).* Apparently a similar result has been observed for mouse versus human hepatocytes, given the results reported for phenobarbital *(Elcombe et al., 2014).*

Next to indications that human hepatocytes are refractory to the hyperplastic effects of PB, no convincing evidence of a specific and relevant role of phenobarbital in human liver cancer risk can be extracted from the epidemiological data (PB has been used as a sedative, hypnotic and antiepileptic drugs for many years at doses comparable to those in rodent bioassays) *(Monro, 1993, La Vecchia & Negri, 2014).*

The key role of increased cell proliferation in a CAR activator MOA for mice liver tumour formation has been demonstrated in studies performed in mice lacking *Car*. In such *Car* knockout mice, PB does not stimulate replicative DNA synthesis in hepatocytes and does not promote liver tumours *(Huang et al., 2005; Wei et al., 2000; Yamamoto et al., 2004).* Besides, phenobarbital caused no effects on cell proliferation in *in vivo* studies in humanized liver chimeric mice (*Yamada et al, 2014*) although in contrast cell proliferation was observed in hCAR/hPXR mice exposed to phenobarbital (*Braeuning et al., 2014*).

It is broadly recognized that the PB-like MOA for induction of rodent liver tumor is qualitatively not plausible for humans due to differences in rodent and human responses to CAR activation. Thus, compounds that cause rat or mouse liver tumors through this CAR-mediated MOA, similar to PB, would not be expected to increase the risk of liver tumor development in humans.

Assessment and evidences of the postulated MoA for liver tumours induced by difenoconazole using the framework developed by IPCS and ILSI/HESI or the OECD guidance for Adverse Outcome Pathway development. Modified Bradford Hill Considerations.

The guidance provided by ECHA recommends following the IPCS framework (IPCS, 2007) when evaluating the MoA data to explain carcinogenicity findings in animals and their relevance to humans.

This postulated mode of action and the human relevance for the difenoconazole-induced liver tumours is assessed by applying the MoA/Human Relevance Framework (HRF) developed by the International Programme on Chemical Safety (IPCS) of the World Health Organization (WHO) *(Sonich-Mullin et al., 2001; Boobis et al., 2006)* and the International Life Science Institute (ILSI/HESI) *(Meek, M.E. et al, 2003; Meek, M.E. et al, 2014, Holsapple et al., 2006).* This framework considers systematically data on apical and mode of action for effects regarding this mechanism of hepatic tumour formation and their relevance to humans and use a weight-of–evidence approach based on the Bradford Hill criteria.

THE IPCS CONCEPTUAL MOA FRAMEWORK FOR EVALUATING ANIMAL CARCINOGENESIS:

1. Postulated MoA (theory of the case)
2. Key events
3. Concordance of dose-response and Temporal Association
4. Strength, consistency and specificity of association of tumour response with key events.
5. Biological plausibility and coherence
6. Other modes of action
7. Uncertainties, Inconsitencies, and Data Gaps assessment of postulated mode of action.
8. Assessment of postulated mode of action

This approach is also consistent with the Adverse Outcome Pathway (AOP) process developed by the OECD, for which a CAR Liver Tumor AOP has been described *(Peffer et al., 2017)* (http://aopwiki.org/aops/107). The OECD encourages scientists to capture these AOPs in an online tool known as AOP wiki as part of the AOP process *(Kleinstreuer et al., 2016; OECD, 2013; OECD, 2016).* It should be noted that in the MoA proposed in the current document, the initial step KE1 (CAR activation) can also be described by the equivalent term "Molecular Initiating Event" (MIE) in the recommended nomenclature of an AOP *(OECD, 2016; Peffer et al., 2017*). Similarly, the final step of KE5 (increase in hepatocellular adenomas/ carcinomas) can also be described as an "Adverse Outcome" (AO) in the AOP wiki nomenclature.

A MoA consists of a series of key event (KEs), which are integral to tumor formation, providing the dose is sufficiently high and the duration of exposure is sufficiently long. A MoA can also include associative events (AEs), which are not required for tumor development, but can be used as markers for certain required KEs. In addition, modulating factors (ModFs) may be identified that are not necessary for tumor development, but can modulate the severity or dose response kinetics of KEs leading to tumor development.

1. Postulated MoA for the induction of hepatocellular tumours in mice

The proposed mode of action for difenoconazole liver tumours consists on the activation of the Constitutive Androstane Receptor (CAR) in the liver. CAR activation conduces to increased expression of pro-proliferative and anti-apoptotic genes in the liver and an early, transient, increase in hepatocellular proliferation. Over time, the increased hepatocellular foci as a result of clonal expansion of spontaneously mutated cells in the mouse results in slight increases in liver tumour incidence compared to concurrent controls.

2. Listing of key events identified in experimental animals

In a review article *(Elcombe et al., 2014)* it is analysed the evidence that mouse or rat liver tumors that occur via a CAR MoA are not relevant to humans based on qualitative differences between the species. This review paper is used as a basis for defining the key events and associative events that are part of this MoA. The CAR activation is the molecular initiating event for the cellular pathway ultimately leading to the apical adverse outcome of liver tumours in PB treated rodents. In this evaluation to analyse the human relevance of PB-induced rodent liver tumour MoA (non-genotoxic) mediated through CAR activation the following key events were identified: KE1: CAR activation, KE2: altered gene expression specific to CAR activation, KE3: increased cell proliferation, KE4: clonal expansion leading to altered foci and KE5: liver adenomas/carcinomas. It has to be mentioned that, in a more recent publication *(Peffer et al., 2018)*, the author notes that altered foci at tumorigenic

doses are not observed with all CAR activators, so this author considers that demonstration of this key event is not critical. In addition to these key events in the pathogenesis of hepatocellular tumors in rodents, reversibility of hepatic effects upon discontinuance of treatment is considered as a necessary data to support this MoA.

Associative events for this MoA, although do not constitute direct evidence of causality of CAR-mediated MoA, they provide associative support of a CAR-mediated MoA and are commonly seen following exposure to PB-like xenobiotic compounds. Altered gene expression leads to several associative events, out of which the following ones have been considered as the most feasible to demonstrate as part of a regulatory dataset *(Peffer et al., 2018):* AE1: Increased CYP2B, CYP3A enzyme activity and/or protein, AE2: Hepatocellular hypertrophy in the centrilobular region of the liver, AE3: Increased liver weight. Additional associative events are: decreased apoptosis and altered epigenetic changes specific to CAR activation and with inhibition of gap junctional intercellular communication and oxidative stress being modulating factors *(Elcombe et al., 2014).*

PB treatment first led to early observable key and associative events (e.g CAR activation, altered gene expression, cell proliferation, enzymatic activation, apoptosis suppression, hypertrophy, and liver weight increase). While effects on some key and associative events including CAR activation, altered gene expression, CYP induction and hypertrophy are observed from early time points throughout the period of PB treatment, the stimulation of cell proliferation in normal hepatocytes is only observed at early time points. For most CAR activators, the stimulation of cell proliferation, assessed as the labelling index (i.e. the percentage of hepatocyte nuclei undergoing replicative DNA synthesis), in rat and mouse liver is transient and not sustained, primarily observed in the first 1-3 weeks after treatment begins, and then returns to a similar rate as in control animals *(Kolaja et al., 1996a; Orton et al., 1996; Philips et al., 1997; Whysner et al., 1996).* However, while hepatocyte labelling index returns to control levels with sustained treatment, overall cell proliferation is still enhanced due to the increase in the total number of hepatocytes per animal. Increased cell proliferation is also important in the growth of altered hepatic foci. At longer treatment times, rates of cell proliferation are enhanced in altered hepatic foci, which typically develop relatively late in long-term studies. Short-term mechanistic studies in mice with PB or CAR-associated compounds typically do not develop hepatocellular foci for months *(Goldsworthy and Fransson-Steen 2002).* In promotion studies where altered hepatic foci were produced by initiation with diethylnitrosamine (DEN), PB was found to increase replicative DNA synthesis within the foci *(Kolaja et al.; 1996b,c; Elcombe et al., 2014).* Clonal expansion leading to altered foci and liver adenomas/carcinomas are only observed after chronic treatment with PB.

In the Table 24 are showed the key and associative events of the CAR activation MOA *(Elcombe et al., 2014; Peffer et al., 2018).*

Table 24: Key events and associative events in the MoA

Key events	Associative events
Key event 1: CAR nuclear receptor activation	
Key event 2: Altered gene expression specific to CAR activation	Enzyme induction (CYP2B and CYP3A) Hepatocellular hypertrophy Liver weight increase Inhibition of apoptosis Epigenetic changes
Key event 3: Increased cell proliferation	
Key event 4: Clonal expansion leading to foci/areas of altered hepatocytes (eosinophilic)	

Key event 5: Liver adenomas/carcinomas	
Modulating factor	
Gap junctional intercellular communication	
Oxidative stress	

In evaluating the difenoconazole data set, the profile of effects was examined for the strength of association, consistency and specificity to determine whether key events occurred consistently across difenoconazole studies, whether these key events were linked in a biologically plausible manner, and whether these key events exhibited the expected concordance across dose-response and temporal relationship. Thus, repeat dose guideline studies, which include subcronic, chronic toxicity/oncogenicity studies and studies to the reproduction as well as specific MoA studies, were examined for evidence to support the CAR-mediated MoA for difenoconazole.

The tables below show the experimental evidences for the key and associative events of a CAR-mediated induction of liver tumours in rats, mice, dogs and humans studies with difenoconazole.

Table 25a: Evidences for the key events in rats, mice, dogs and humans

Key events	Rats	Mice	Dogs	Humans
CAR activation	YES: Difenoconazole produced a slight trend no significant toward direct activation of rat CAR in the transactivation study. *Omiecinski C, 2016*	YES: Difenoconazole was a direct activator of mouse CAR in the transactivation study. Increases in absolute and relative liver weight, hepatocellular proliferation, centrilobular hypertrophy and hepatic enzymes induction are not observed in *Car/Pxr* double KO mice, therefore they are CAR-dependent effects. *Omiecinski C, 2016* *Anonymous 21, 2017b* *Anonymous 22, 2017*	Not determined	NO: Difenoconazole was not a direct activator of human CAR in the transactivation study. In *hCAR/hPXR* mice, there were CAR-dependent effects as increases in absolute and relative liver weight, centrilobular hypertrophy and hepatic enzymes induction. *Omiecinski C, 2016* *Anonymous 21, 2017b*
Altered gene expression specific to CAR activation	Not determined	YES: Increases in *Cyp2b10* and *Cyp3a11* mRNAs levels. The liver mRNA expression levels showed no response at any dose in male *Car/Pxr* double KO. However, in *hCAR/hPXR* mice, increases in *Cyp2b10 and Cyp3a11* mRNA levels were observed. *Anonymous 20, 2017a* *Anonymous 21, 2017b*	Not determined	Not determined
Increased cell proliferation	*In vitro*: Not determined *In vivo*: Not observed/ reported	*In vitro*: YES *In vivo*: YES *Vardy A., 2016a* *Anonymous 22, 2017*	Not determined	*In vitro*: NO *Vardy A., 2016b*

Key events	Rats	Mice	Dogs	Humans
Clonal expansion leading to altered foci	Not observed/ reported	YES: Increase in inflammatory cell foci in C57BL/6NTAc WT mice. *Anonymous 22, 2017*	Not observed/ reported	Not determined
Liver adenomas/ carcinomas	Not observed/ reported	YES: Hepatocellular adenomas and carcinomas were observed in liver in male mice at 4500 ppm (819 mg/kg bw/day) ($p<0.01$) and male and female mice at 2500 ppm (♂ $p<0.05$ and ♀<0.01) (423/513 mg/kg bw/day for males/females, respectively). *Anonymous 18, 1989b*	Not observed/ reported	Not determined

Table 25b: Evidences for the associative events in rats, mice, dogs and humans

Associative events	Rats	Mice	Dogs	Humans
Enzyme induction (CYP2B and CYP3A)	Not determined	*In vitro*: YES Increases in hepatic enzymes levels, especially CYP3A *In vivo*: YES Increases in hepatic enzymes levels, especially CYP1A, CYP2B*, CYP3A and UDPGT. *Vardy A., 2016a* *Anonymous 19, 1992* (*In this study CYP2B levels decrease) *Anonymous 20, 2017a* *Anonymous 21, 2017b* *Anonymous 22, 2017*	Not determined	*In vitro*: YES Increases in hepatic enzymes levels, especially, CYP2B/3A. *In vivo*: Not determined *Vardy A., 2016b*
Hepatocellular hypertrophy	YES *Anonymous 36, 2000* *Anonymous 16, 1989a and 17, 1992*	YES *Anonymous 18, 1989b* *Anonymous 20, 2017a* *Anonymous 21, 2017b* *Anonymous 22, 2017*	NO *Anonymous 34, 1987* *Anonymous 35, 1988*	Not determined
Increased liver weight	YES *Anonymous 29, 1986a* *Anonymous 30, 1986b* *Anonymous 31, 1987a* *Anonymous 31, 1987a* *Anonymous 36, 2000* *Anonymous 16, 1989a and 17, 1992*	YES *Anonymous 33, 1987b* *Anonymous 18, 1989b* *Anonymous 20, 2017a* *Anonymous 21, 2017b* *Anonymous 22, 2017*	YES *Anonymous 34, 1987*	Not determined
Inhibition of apoptosis	Not determined	Not determined	Not determined	Not determined
Epigenetic changes	Not determined	Not determined	Not determined	Not determined

Mice apical and mode of action studies show key events of this MoA as CAR activation, altered gene expression, increased cell proliferation, clonal expansion leading to altered foci and liver tumours,

and also associative events as enzyme induction, hepatocellular hypertrophy and increased liver weight.

Increased liver weight and centrilobular hepatocellular hypertrophy were also seen in apical studies in rat. In addition, a non- significant trend toward direct activation of rat CAR was observed in rats. Regarding the induction of hepatic enzymes typical of PB-like MoA, this associative event was only experimentally evaluated in male mice mechanistic studies with difenoconazole, where activation of CYP 1A, CYP 2B, CYP 3A and UDPGT were observed. At this regard, it is well known that for compounds acting through CAR activation, a similar MoA operates for both mice and rats and therefore, the activation of hepatic enzymes typical of PB-like MoA could be plausible in rats.

3. Concordance of dose-response and temporal association

The dose response and temporal relationships for the Key and Associative Events measured in the *in vivo* studies in mice and rats are presented below (Table 26 and Table 27).

Quantification (degree of change) is not shown for each dose in order to keep the tables clearer, but the positive or negative effect of each strain is included (+ or – in the table). The responses for the key and associative events are shown as positive or negative referred to whether they support or not the mode of action (green or red color respectively in the table). Key event 4 (foci) and key event 5 (Formation of liver tumours) are generally not applicable to subchronic studies and therefore are labelled as "not applicable (NA)" in the tables, although the histopathological outcome was measured.

The *in vitro* studies results are not shown in these tables in order to make the comparations between doses easier.

Table 26: Concordance of dose-response and temporal relationships in studies in mice

Reference	Dose (mg/kg bw/day) ♂/♀	**Key event 2:** Altered gene expression specific to CAR activation	**Associative event[1]** Enzyme induction	**Associative event[1]** Hepatocellular hypertrophy	**Associative event[1]** Increased liver weight	**Key event 3:** Increased cell proliferation	**Key event 4:** Clonal expansion leading to altered foci	**Key event 5:** Liver adenomas/ carcinomas
	Ordered from low to high dosage	**Key and associative events are shown in order from earliest event to later (left to right). Results show the time that the event was observed. Quantitative changes in severity are not shown.**						
MICE								
Anonymous 19, 1992	**1 (♂)**	ND	+ 2 weeks	ND	- 2 weeks	ND	NA	NA
Anonymous 18, 1989b	**1.5/1.9**	ND	ND	- 79 weeks	- 79 weeks	ND	- 79 weeks	- 79 weeks
Anonymous 33, 1987b	**3.3/4.6**	ND	ND	- 13 weeks	- 13 weeks	ND	NA	NA
Anonymous 18, 1989b	**4.7/5.6**	ND	ND	- 79 weeks	- 79 weeks	ND	- 79 weeks	- 79 weeks
Anonymous 19, 1992	**10 (♂)**	ND	+ 2 weeks	ND	- 2 weeks	ND	NA	NA
Anonymous 20, 2017a	**15 (♂)**	+ 1 week	- 1 week	- 1 week	- 1 week	ND	- 1 week	NA
Anonymous 21, 2017b	**15 (♂)**	- 1 week	- 1 week	- 1 week	- 1 week	ND	- 1 week	NA
Anonymous 33, 1987b	**34.2/45.2**	ND	ND	+ 13 weeks [a] (♂)	+ 13 weeks (rel in ♂)	ND	NA	NA
Anonymous 20, 2017a	**45 (♂)**	+ 1 week	+ 1 week	- 1 week	- 1 week	ND	- 1 week	NA
Anonymous 18, 1989b	**46.3/57.8**	ND	ND	+ 79 weeks (♂)	- 79 weeks	ND	- 79 weeks	- 79 weeks
Anonymous 19, 1992	**100 (♂)**	ND	+ 2 weeks	ND	- 2 weeks	ND	NA	NA

Reference	Dose (mg/kg bw/day) ♂/♀	**Key event 2:** Altered gene expression specific to CAR activation	**Associative event[1]** Enzyme induction	**Associative event[1]** Hepatocellular hypertrophy	**Associative event[1]** Increased liver weight	**Key event 3:** Increased cell proliferation	**Key event 4:** Clonal expansion leading to altered foci	**Key event 5:** Liver adenomas/ carcinomas
	Ordered from low to high dosage	**Key and associative events are shown in order from earliest event to later (left to right). Results show the time that the event was observed. Quantitative changes in severity are not shown.**						
Anonymous 20, 2017a	**150 (♂)**	+ 1 week	+ 1 week	+ 1 week	+ 1 week (abs and rel in C57BL/6J; rel in CD-1)	ND	- 1 week	NA
Anonymous 21, 2017b	**150 (♂)**	+ 1 week in *hCAR/hPXR* - 1 week in *Car/Pxr* double KO	+ 1 week in *hCAR/hPXR* - 1 week in *Car/Pxr* double KO[2]	+ 1 week in *hCAR/hPXR* - 1 week in *Car/Pxr* double KO[2]	+ 1 week (abs and rel in *hCAR/ hPXR)* - 1 week in *Car/Pxr* double KO[2]	ND	- 1 week	NA
Anonymous 22, 2017	**150 (♂)**	ND	+ 1 week	+ 1 week in CD-1 WT and C57BL/6NTAc WT - 1 week in *Car/Pxr* double KO[2]	+ 1 week (abs and rel in CD-1 WT and C57BL/6NTAc WT) - 1 week in *Car/Pxr* double KO[2]	+ 1 week in CD-1 WT, C57BL/6NTAc WT and *Car/Pxr* double KO[3]	- 1 week in CD-1 WT and *Car/Pxr* double KO + 1 week in C57BL/6NTAc WT [4]	NA
Anonymous 20, 2017a	**400 (♂)**	+ 1 week	+ 1 week	+ 1 week	+ 1 week (abs and rel)	ND	- 1 week	NA
Anonymous 19, 1992	**400 (♂)**	ND	+ 2 weeks	ND	+ 2 weeks (abs) Reversible after a 28-day recovery period	ND	NA	NA
Anonymous 33, 1987b	**440/639**	ND	ND	- 13 weeks	+ 13 weeks (abs and rel in ♂/♀)	ND	NA	NA
Anonymous 18, 1989b	**507.6/615.6 -423/513** (due to early mortality the dose was at the beginning of week 2)	ND	ND	+ 79 weeks (♂/♀)	+ 53 weeks (rel in ♂/♀) Reversible after recovery (week 57) + 79 weeks (abs and rel in ♂/♀)	ND	- 79 weeks	+ 79 weeks (♂/♀)
Anonymous 18, 1989b	**819/983** (Females at this dose group died during the first 2 weeks)	ND	ND	+ 79 weeks (♂)	+ 53 weeks (abs and rel in ♂) Reversible after recovery (week 57) + 79 weeks (abs and rel in ♂)	ND	- 79 weeks	+ 79 weeks (♂)

Positive response in a key event (dark green); positive response in an associative event (pale green); negative response in a key event (dark red); negative response in an associative event (pale red); ND: Not determinated (white boxes); NA: Not applicable (white boxes).
- : negative effect, + : positive effect, [a]: Observed as centrilobular hepatocellular enlargement;
[1]Associative events are referred to key event 2 (Altered gene expression specific to CAR activation)
[2]The negative effect in Car/Pxr double KO supports the mode of action.
[3]The positive effect in Car/Pxr double KO is against the mode of action.
[4]The effect in C57BL/6NTAc WT *and Car/Pxr double KO (from* C57BL/6NTAc background*) supports the mode of action. Nevertheless, in the additional WT strain (CD-1) it was not supported.*

Table 27: Concordance of dose-response and temporal relationships in studies in rats

Reference	Dose (mg/kg bw/day) ♂/♀	Key event 2: Altered gene expression specific to CAR activation	Associative event[1] Enzyme induction	Associative event[1] Hepatocellular hypertrophy	Associative event[1] Increased liver weight	Key event 3: Increased cell proliferation	Key event 4: Clonal expansion leading to altered foci	Key event 5: Liver adenomas/ carcinomas
	Ordered from low to high dosage	**Key and associative events are shown in order from earliest event to later (left to right). Results show the time that the event was observed. Quantitative changes in severity are not shown.**						
RATS								
Anonymous 16, 1989a and 17, 1992	**0.5/0.6**	ND	ND	- 104 weeks	- 104 weeks	ND	- 104 weeks	- 104 weeks
Anonymous 16, 1989a and 17, 1992	**1/1.3**	ND	ND	- 104 weeks	- 104 weeks	ND	- 104 weeks	- 104 weeks
Anonymous 31, 1987a	**1.3/1.7**	ND	ND	- 13 weeks	- 13 weeks	ND	NA	NA
Anonymous 32, 2006a	**2.8/3.2**	ND	ND	ND	ND	ND	NA	NA
Anonymous 30, 1986b	**3.3/3.5**	ND	ND	- 13 weeks	- 13 weeks	ND	NA	NA
Anonymous 36, 2000	**10/10**	ND	ND	- 4 weeks	- 4 weeks	ND	NA	NA
Anonymous 31, 1987a	**13/17**	ND	ND	- 13 weeks	+ 13 weeks (rel in ♀)	ND	NA	NA
Anonymous 32, 2006a	**17.3/19.5**	ND	ND	ND	ND	ND	NA	NA
Anonymous 30, 1986b	**20/21**	ND	ND	- 13 weeks	+ 13 weeks (rel in ♂/♀)	ND	NA	NA
Anonymous 16, 1989a and 17, 1992	**24.1/32.8**	ND	ND	+ 104 weeks (♂/♀)	- 104 weeks	ND	- 104 weeks	- 104 weeks
Anonymous 29, 1986a	**27/27**	ND	ND	- 4 weeks	- 4 weeks	ND	NA	NA
Anonymous 31, 1987a	**51/66**	ND	ND	- 13 weeks	+ 13 weeks (abs and rel in ♂/♀)	ND	NA	NA
Anonymous 36, 2000	**100/100**	ND	ND	- 4 weeks	- 4 weeks	ND	NA	NA
Anonymous 31, 1987a	**105/131**	ND	ND	+ 13 weeks [a] (♂/♀)	+ 13 weeks (abs and rel in ♂/♀)	ND	NA	NA
Anonymous 32, 2006a	**107/120**	ND	ND	ND	+ 13 weeks (abs and rel in ♂/♀)	ND	NA	NA
Anonymous 30, 1986b	**121/129**	ND	ND	- 13 weeks	+ 13 weeks (abs and rel in ♂/♀) Reversible after recovery (week 17) in ♂/♀	ND	NA	NA
Anonymous 16, 1989a and 17, 1992	**124/170**	ND	ND	+ 104 weeks (♂/♀)	+ 53 weeks (rel in ♂) Reversible after recovery (week 57) + 104 weeks (rel in ♀)	ND	- 104 weeks	- 104 weeks
Anonymous 29, 1986a	**156/166**	ND	ND	- 4 weeks	+ 4 weeks (abs and rel in ♂; rel in ♀)	ND	NA	NA

Reference	Dose (mg/kg bw/day) ♂/♀	Key event 2: Altered gene expression specific to CAR activation	Associative event[1] Enzyme induction	Associative event[1] Hepatocellular hypertrophy	Associative event[1] Increased liver weight	Key event 3: Increased cell proliferation	Key event 4: Clonal expansion leading to altered foci	Key event 5: Liver adenomas/ carcinomas
	Ordered from low to high dosage	**Key and associative events are shown in order from earliest event to later (left to right). Results show the time that the event was observed. Quantitative changes in severity are not shown.**						
Anonymous 31, 1987a	**214/275**	ND	ND	+ 13 weeks [a] (♂/♀)	+ 13 weeks (abs and rel in ♂/♀)	ND	NA	NA
Anonymous 29, 1986a	**914/841**	ND	ND	- 4 weeks	+ 4 weeks (abs and rel in ♂; rel in ♀)	ND	NA	NA
Anonymous 36, 2000	**1000/1000**	ND	ND	+ 4 weeks (♂/♀)	+ 4 weeks (abs and rel in ♂, rel in ♀)	ND	NA	NA

Positive response in a key event (dark green); positive response in an associative event (pale green); negative response in a key event (dark red); negative response in an associative event (pale red); ND: Not determinated (white boxes); NA: Not applicable (white boxes).
- : negative effect, + : positive effect, [a]: Observed as diffuse hepatocellular enlargement
[1]Associative events are referred to key event 2 (Altered gene expression specific to CAR activation)

There are two relationships of interest in the MoA evaluation. First, whether the Key Events show a sequential (temporal) relationship such that Key Events 1 and 2 precede Key Events 3 and 4, which occur before Key Events 5 and the second relationship examines dose-response, and whether Key Events show an incidence and severity consistent with doses.

Temporal Association

The data are available for the effect of treatment with difenoconazole in male and female at various time points, ranging from 1 to 79 weeks in mice (Table) and from 4 to 104 weeks in rats (Table 27). The temporality of the different events of the MoA proceeds in the expected order. If a key event (or events) is essential element for carcinogenesis, it must precede the appearance of tumours. Difenoconazole treatment first led to early events as altered gene expression specific to CAR activation, enzymatic activation, hypertrophy and liver weight increase. The final adverse outcome effect of formation of hepatocellular tumours (Key Event 5) only occurs in mice and it is a late event, only observed at 79 weeks.
The clonal expansion leading to altered foci (key event 4) has only been observed in a 7-day mode of action study (C57BL/6NTAc WT mice) (*Anonymous 22, 2017*) because there were an increase in inflammatory cell foci. However in the carcinogenicity study in mice (*Anonymous 18, 1989b*) in which hepatocellular adenomas and carcinomas were observed (key event 5), the clonal expansion leading to altered foci (key event 4) was not observed.

Concordance of dose-response

Effects of difenoconazole on a number of the key and associative events showed similar dose-dependency with the incidence of tumours only observed at the high doses in male and female mice. Liver tumor formation is a progression from hepatocellular hypertrophy to increased cell proliferation to tumors; liver histopathologic changes were increased in incidence and severity with a higher dose of treatment with difenoconazole, contributing to the biological plausibility of this MoA.
In the carcinogenicity study in mice (*Anonymous 18, 1989b*), hepatocellular adenomas and carcinomas were observed in liver in male mice at 4500 ppm (819 mg/kg bw/day) ($p<0.01$) and male and female mice at 2500 ppm (423/513 mg/kg bw/day for males/females, respectively) ($p<0.05$ and $p<0.01$, respectively). At 2500 ppm (423/513 mg/kg bw/day for males/females, respectively) there were also hepatocellular hypertrophy and increased liver weight in male and female mice. The same occurred at 4500 ppm (819 mg/kg bw/day) in male mice, since females at this dose died during the

first 2 weeks. In this study the cell proliferation was not determined and the clonal expansion leading to altered foci was not observed.
Although in some cases, ordered key events are not observed in the same study, overall, the key and associative events observed in mice and rats receiving difenoconazole occurred in a logical temporal sequence and in a dose-dependent manner.

4. Strength, consistency and specificity

The weight of evidence linking the key and associative events with the toxicological response is consistent with the hepatic effects observed in many apical and mechanistic studies in **mice**. Although some of the studies to demonstrate the key events presented uncertainties, there is information for all of them which contributes to the consistency of this MoA.

In mice carcinogenicity study, liver adenomas and carcinomas were reported to occur after difenoconazole treatment with 423 and 513 mg/kg bw/day in males and females respectively. Besides, liver weight and hepatocyte hypertrophy frequency increased from 46.3 and 57.8 mg/kg bw/day in males and females respectively and this effect was reversible. According to the results from the CAR transactivation assay (*Omiecinski C., 2016*) difenoconazole is a direct activator of mouse CAR and therefore a PB-like MoA might be a plausible explanation for the carcinogenic findings. Reversibility of the non-neoplasic cellular changes is also in accord with the proposed CAR MoA.

Consistent with this CAR activation, after a 7-days treatment difenoconazole produced increases in *Cyp2b10* and *Cyp3a11* mRNAs levels evident from 15 mg/kg bw/day, dependent on CAR and/or on PXR as they were not observed in *Car/Pxr* double KO mice. Moreover, CAR activation was indirectly evident as an increment in the Cytochrome P450 proteins levels after exposure to difenoconazole in several studies (*Anonymous 22, 2017; Anonymous 19, 1992*).

It was also reported an increment in the PROD, BROD and BQ activities (indicative of CYP2B, CYP2B or CYP3A and CYP3A activation, respectively) from 45 mg/kg bw/day after a 7-days treatment (*Anonymous 20, 2017a*), as well as higher activity of BROD after a 14-days exposure *(Anonymous 19, 1992)* and increased BQ activity in an *in vitro* study (*Vardy A., 2016a*). The difenoconazole-dependent liver weight increase accompanied by centrilobular hypertrophy can be considered as an event associated to the altered expression of these proteins. Both liver enzyme inductions and liver weight variations provoked by difenoconazole were not observed in *Car/Pxr* double KO mice (i.e. they were mediated by CAR and/or by PXR). Based on the results of the luciferase reporter assay, difenoconazole does not seem to be an activator of human, rat or mouse PXR (*Korrapati M. & Sherf B., 2016*) and therefore it could be expected a similar pattern from the single *Car* $^{-/-}$ knockout mice.

Another measured key event consistent with the proposed MoA was the hepatocellular proliferation. A difenoconazole-dependent increment in the replicative DNA synthesis was observed in mouse hepatocyte cultures treated *in vitro (Vardy A., 2016a).* This effect was also observed *in vivo* although, in contrast to the PB effect, it was only partially dependent on *Car/Pxr* as there was still hepatocellular proliferation (albeit much reduced) in *Car/Pxr* knockout mice (*Anonymous 22, 2017).*

Foci of altered hepatocytes (as increase in inflammatory cell foci) were found in the C57BL/6NTAc mice and not in the *Car/Pxr* double KO mice, but it should be highlighted that this was only reported in a 7-days study *(Anonymous 22, 2017).*

Taken together, the incidence of hepatocellular tumours in mice is lower than the incidence of earlier effects, which contributes to the strength of the proposed MoA in this species.

A small and not significant increase in **rat** CAR activation was observed in the transactivation assay (*Omiecinski C., 2016*), indicating that difenoconazole was at most a low potency activator of CAR in

this system. Coherent with this finding, in the carcinogenicity study in rats *(Anonymous 16, 1989a and 17, 1992),* tumours were not observed, but an increment in the incidence of hepatocellular hypertrophy was reported at terminal sacrifice from 24.1 and 32.8 mg/kg bw/day. These observations suggest that under these conditions of dosage and exposure a low activation of CAR by difenoconazole might be able to the increase hepatocytes hypertrophy but not enough to provoke tumors.

The available data in this analysis indicate that the MoA cannot be exactly applicable to **humans**. According to the transactivation assay difenoconazole was not an activator of human CAR (*Omiecinski C., 2016*). However, *Cyp2b10* and *Cyp3a11* mRNA levels and enzyme activities were increased in *hCAR/hPXR* mice *(Anonymous 21, 2017b)* and a similar effect with increased enzyme activities was observed in the *in vitro* assay with human hepatocytes *(Vardy A., 2016b),* suggesting difenoconazole-dependent CAR activation in humans as it occurs after phenobarbital treatment (*Elcombe et al., 2014).*

Nevertheless, despite this possible CAR activation, no significant increment in human hepatocytes proliferation was detected *(Vardy A., 2016b),* as expected for a PB-like activator of CAR.

5. Biological plausibility and coherence

The CAR activation could be a plausible MoA for liver tumour formation in rodents caused by difenoconazole. The succession of the most of key and associative events are consistent with the PB-like mechanism. The observed reversibility of early key events upon cessation of treatment with difenoconazole is also consistent with the proposed MoA, i.e. the non-neoplastic cellular changes were reset by the normal feedback-control systems and reversed.

The liver is the most common target tissue affected in carcinogenicity studies in rats and mice (*Gold et al., 2001*). This may be due to the fact that the liver is the major site of metabolic processing of xenobiotics and one of the first organs exposed following absorption from the gastrointestinal tract if administered orally, as in the case of the carcinogenicity studies with difenoconazole.

The induction of liver tumours in male mice subsequent to the activation of CAR is a comprehensively studied and characterised MOA for a number of compounds, including the archetypal CAR activator phenobarbital (*Whysner et al., 1996; Meek et al., 2003; Holsapple et al., 2006*), the potent mouse CAR activator TCPOBOP (*Huang et al., 2005*) and the insecticide sulfoxaflor (*LeBaron et al., 2013*). In addition, the stated MoA and the clear dependence on CAR activation are consistent with the data for cyproconazole, another triazole fungicide that caused liver tumours in mice but not in rats (as for difenoconazole) in a CAR-dependent manner (*Peffer et al., 2007*). Also, the 7-days study using *Car/Pxr* double KO mice has shown that when CAR is knocked out, some of the key events and associative events in the postulated MoA do not occur (*Anonymous 22, 2017).*

However, there are a number of uncertainties and another potential alternative mode of action that could derive in liver tumours. All of them have to be taken into account in a weigh-of-evidence assessment.

Other modes of action

To define a MoA in liver, it is critical to ensure that other MoAs do not contribute significantly to hepatocarcinogenesis. In addition to CAR activation, other mechanisms may be involved in difenoconazole-induced tumorigenesis in mice liver.

Several MoAs have been identified for liver carcinogenesis and those applicable to the rodent model are listed in publications by *Cohen (2010)* and *Klaunig et al. (2012).* These include mechanisms in which a chemical can increase the risk of cancer by damaging DNA directly (DNA reactive) or indirectly by increasing the number of DNA replications (non–DNA reactive). Several nongenotoxic mechanisms for hepatocarcinogenesis in rodents include sustained cytotoxicity, oxidative stress/damage, inflammation, infection, iron (copper) overload, increased apoptosis, immunosuppression, porphyria or receptor-mediated. MoAs for hepatocellular carcinogens that cause receptor mediated hepatocellular proliferation include: aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPAR- α) activation, estrogens and statins (*Boobis et al., 2009; Holsapple et al., 2006; Klaunig et al., 2003; Meek et al., 2003; Williams, 1997a*).

The alternative MoAs for difenoconazole-induced hepatocellular carcinogenesis are presented below (Table 28).

Table 28: Alternative MoAs resulting in liver tumors

MoA	Data relating to difenoconazole	Conclusion
DNA reactivity and mutagenicity	DNA reactivity can be excluded since the genotoxicity testing *in vivo* and *in vitro* of difenoconazole gave no evidence of a genotoxic potential.	Unlikely
Cytotoxicity and regenerative hyperplasia	In the oral 90-day study in mice (*Anonymous 33, 1987b*) at 7500 ppm (1320/1917 mg/kg bw/day in males/females, respectively) and 15000 ppm (2640/3834 mg/kg bw/day in males/females, respectively) hepatotoxicity was observed by hepatocellular enlargement and necrosis of individual hepatocytes for both sexes. Furthermore, in the carcinogenicity study in mice (*Anonymous 18, 1989b*), necrosis of individual hepatocytes, focal/multifocal necrosis of hepatocytes, bile stasis and fatty change were observed for both sexes at 79 weeks. Since these findings occurred accompanied of increases in liver weights, they are considered a secondary effect to excessively large increases in liver weight and liver size, since literature has shown late-onset necrosis will occur as a secondary effect to very large increases in liver weight and liver size (*Maronpot et al., 2010; Hall et al., 2012*). A small increase in the incidence of mild to moderate single-cell necrosis can sometimes occur, particularly after longer-term treatment of mice with CAR activators. However, more severe/diffuse necrosis in the liver suggests that an alternative MoA via cytotoxicity might be operative (*Hall et al., 2012*). The limited amount of hepatic necrosis (single cell or focal) observed in the *in vivo* mouse treated with difenoconazole studies is in contrast with the pattern of effects seen with classic cytotoxic carcinogens that cause a diffuse necrosis (widespread multifocal hepatocyte death) in the liver that progressed to a sustained regenerative hyperplasia, as is the case of chloroform and carbon tetrachloride.In the mice study in which hepatic tumours were observed only localized areas of hepatic necrosis were seen (in both sexes). Therefore, it is not considered that cytotoxicity is an additional MoA involved in the hepatocellular tumour formation.	Unlikely
Estrogenic activity	Difenoconazole does not present structural similarity with estrogens. The *in silico* mechanistic data indicated that the probability of binding of difenoconazole on estrogen receptors is low and ToxCast ER model data showed negative results for estrogens (B.6.8.3).	Unlikely

Statin-like activity	It has not been experimentally shown that difenoconazole has not activity as an HMG-CoA reductase inhibitor.	Plausible
Aryl Hydrocarbon Receptor (AhR) activation	Difenoconazole produces increases in EROD activity and increases in CYP1A protein levels in liver microsomes of treated mice and these markers are greatly increased by AhR activators. However, these increases are clearly lower than the increases produced in these markers by the reference substance 3-methylcholanthrene (3-MC) that is an AhR agonist (*Anonymous 19, 1992*).	Unlikely
Peroxisome proliferator-activated receptor alpha (PPARα) activation	Difenoconazole produced a slight decrease in lauric acid 12-hydroxylase (LAH) activity and in CYP4A levels of protein in liver fractions of treated mice and did not increase peroxisomal lipid beta-oxidation (*Anonymous 19, 1992).* Each of these markers are greatly increased by peroxisome proliferators. However, in the *Anonymous 22, 2017* study an increase in LAH has been observed in different strains of mice treated with difenoconazole. No reference substance (like the PPARα agonist nafenopin, NAF) was tested in the same study and therefore the relevancy of such increase is unclear. The plausibility of this MoA cannot be rejected, but the low repetitiveness of the result suggests it is not probable.	Plausible but not probable
Immunosuppresion	No changes in the immune system or immune cells were detected in difenoconazole studies.	Unlikely

6. Uncertainties, inconsistencies and data gaps

The following uncertainties were found:

- Firstly, the transactivation assay (*Omiecinski C., 2016*) was only carried out with modified versions of mouse, rat and human CAR genes. Although it was explained that the APYLT insertion only affects the constitutive activity of CAR, its potential interference to the ligand binding cannot be discarded without further justification.

- There is no explanation about why double *Car/Pxr* knockout mice are analysed instead of single *Car* knockout animals. Furthermore, in the PXR transactivation assay *(Korrapati M. & Sherf B., 2016)*, a non-dose response increment of PXR activation was observed after difenoconazole treatment in mouse. It is also noticed that PXR activation was tested with the PXR ligand binding domain (of each species) fused to the Gal4 protein and not with the actual receptor. The presence of positive controls contributes to reduce the level of uncertainty from this experiment.

- Taking into account that in humans there are several CAR proteins derived from alternative splicing (e. g. *hCAR1*,*hCAR2*, *hCAR3*), no details were given about which *hCAR* version was inserted in the mice employed in the 1 and 7 day investigative *in vivo* assay *(Anonymous 21, 2017b).*

- It is unclear if the lack of information about the presence of altered hepatocytes foci in the carcinogenicity study in mice is because this parameter was not considered in the experimental design or if foci were investigated but they were not found in the liver (during the assessment this second option was assumed). These foci were observed only in C57BL/6NTAc mice in the 7 days study *(Anonymous 22, 2017),* although according to this mode of action this should not be an early stage event. Nevertheless, it is noticed that, according to AOP number 107 from AOPwiki, no data for the key event of increased altered foci are available in CD-1 mice treated with phenobarbital whereas studies in male C57BL/10J mice also treated with PB show a clear increase in altered foci.

- No tumours were observed in the rat carcinogenesis study (*Anonymous 16, 1989a and 17, 1992),* although the results from the *in vitro* assay (*Omiecinski C., 2016)* suggested that CAR was also activated by difenoconazole in this species. However this could be explained as a delay in the response of this test system.

The following points were considered as inconsistencies:

- The reason for the lack of increase of *Cyp2b10* and *Cyp3a11* mRNA levels with difenoconazole treatment in mouse primary hepatocytes *in vitro (Vardy A., 2016c)* is unknown. If CAR activation is proposed as the mode of action for the tumourigenic effect of difenoconazole in mice, an increment of the transcription levels in these two genes should occur. Moreover, it would have been desirable that the same information could be available for the expression of the orthologous genes in human hepatocytes.

- After *in vitro* difenoconazole treatment of mouse cells *(Vardy A., 2016a)* only BQ activity increased in concentration-dependent manner but unexpectedly neither PROD nor BROD activities changed, while in human cells the three activities were incremented *(Vardy A., 2016b).* Accordingly, in the oral 14-days study *(Anonymous 19, 1992),* CYP2B protein level (whose markers are PROD and BROD) did not increase as expected from the proposed MoA. Unexpectly, in the same study PROD enzyme activity was increased and this pattern was similar with phenobarbital tested in this study. This CYP2B-independent activation of PROD after difenoconazole treatment was not justified. Moreover, using liver microsomes prepared from PB-induced male CD-1 mice, difenoconazole inhibited both CYP2B and CYP3A induction in an *in vitro* assay when the enzymatic activities were assessed *(Vardy A., 2016c).* A similar *in vitro* PB pre-treatment was not tested in human cells. This unexpected inhibition was not justified and the potential consequences for the proposed mode of action were not addressed.

- Contrary to the proposed mode of action, an increase in hepatocellular proliferation was also reported in the *Car/Pxr* double KO mice treated with difenoconazole, although it was much reduced than the proliferation calculated in *wild-type* mice (CD-1 and C57BL/6NTAc) with the same treatment. In contrast to this, in the case of the *Car/Pxr* double KO mice treated with phenobarbital there was not an increase in hepatocellular proliferation, as it was expected (*Anonymous 22, 2017).*

- The formation of 12-OH lauric acid was higher in both *wild-type* and *Car/Pxr* double KO mice treated with difenoconazole than in untreated controls (*Anonymous 22, 2017).* In this study it was suggested that these results might be indicative of a small PPARα nuclear hormone receptor induction caused by difenoconazole, and that this induction could explain the residual hepatocellular proliferation in the *Car/Pxr* KO mice through a CAR-dependent block of PPARα transcription. However, the presence of 12-OH lauric acid also in wild-type mice indicates that this alternative explanation should be dismissed, since if an active CAR suppresses the PPARα transcription, and PPARα transcription was observed in the wild-type where CAR activation was observed, it could mean that DFZ can also activate PPARα to such an extend to overcome the transcription block from CAR.

The following data gap was detected:

- The lack of a HMG-CoA reductase activity study to rule out that difenoconazole could be related to an statin-like mode of action.

7. Assessment of postulated mode of action

When proposing a CAR MoA for liver tumours induced by a test compound, there are critical parameters to be included in the final mechanistic data package, which should contain (at a minimum) demonstration of the molecular initiating event (CAR activation, KE1) and the obligatory key event of increased cell proliferation (KE3) *(Peffer et al., 2018).*

In the analysis of postulated MoA for hepatocellular tumour caused by difenoconazole, there are experimental evidences for these crucial key events as CAR activation (KE1) and increased cell proliferation (KE3) in mice. Furthermore, all key events have been observed as well as the associative key events enzyme induction, hepatocellular hypertrophy and increased liver weight in mice.

Therefore, there is a clear evidence that CAR receptor activation is involved in tumorogenic action of difenoconazole in the liver of mice.

Conclusion on liver tumours

Difenoconazole caused an increased incidence of hepatocellular tumours (combined adenomas and carcinomas) in a carcinogenicity study in mice. However, this increase has low relevance to humans due to the following reasons:

1. Difenoconazole has been investigated for genotoxicity, and tested negative in a battery of standard *in vitro* and *in vivo* studies so, difenoconazole is considered a non-genotoxic agent. The mechanism behind tumour formation in the mice as genotoxic can be excluded.
2. The liver tumours appeared only in one species (mouse), but not in rat.
3. A long time of latency: the highest incidence of hepatocellular adenomas and carcinomas was only observed at the end of study (79 weeks).
4. There are experimental evidences supporting a PB-like MoA for the induction of liver tumours in mice. There are findings consistent with a PB-like response, as the induction of CYP450 of the 2B/3A families, observations of increased liver weight, centrilobular hepatocellular hypertrophy and hepatocellular proliferation. Furthermore, like with PB, the appearance of adenomas occurred only after chronic administration of difenoconazole.
5. Similarly to phenobarbital (known CAR inducer), difenoconazole did not induce hepatocellular proliferation (prerequisite for tumour formation) in human hepatocytes, in contrast to mice.
6. Low plausibility for the most of other potential alternative MoAs for liver carcinogenesis induced by difenoconazole.

The results of the studies indicate that difenoconazole and phenobarbital share some common mechanisms and there is similarity of the mode of action between the two substances.

Although data for concordance analysis with PB are limited, since it has only been tested along with difenoconazole in two *in vitro* assays (*Vardy A., 2016a* and *Vardy A., 2016b*) and in two *in vivo* studies (*Anonymous 19, 1992* and *Anonymous 22, 2017),* and there are some uncertainties, inconsistencies and data gaps, in the overall assessment it can be considered that difenoconazole presents a PB-like MoA.

Epidemiologic data on PB with human exposures similar to those that are carcinogenic to rodents, show that it is not a human liver carcinogen and that the MoA for PB-induced rodent liver tumours is not relevant to humans (*IARC, 2001; Olsen et al., 1989, Whysner et al., 1996, Friedman et al. 2009, La Vecchia and Negri, 2014*).

Furthermore, the absence of a cell proliferation response in human tissues appears to be a common feature of a number of CAR activators and this correlates with a lack of hepatocarcinogenesis in humans (*Elcombe et al., 2014*).

10.9.2 Comparison with the CLP criteria

Comparison with criteria for Category 1A classification: In accordance with the criteria in the CLP regulation, classification for carcinogenicity Category 1A is reserved for substances known to have carcinogenic potential in humans. In the absence of human data, category 1A is not triggered.

Comparison with criteria for Category 1B classification: In accordance with the criteria in the CLP regulation, classification for carcinogenicity Category 1B is reserved for substances that are presumed to be carcinogenic in humans, and is largely based on data from animal studies where there is sufficient evidence to demonstrate animal carcinogenicity (presumed human carcinogen).

There are liver tumors in a single species (mice). In order to assess the strength of evidence and to conclude whether difenoconazole triggers Cat.1B, Cat.2 or no classification, the Guidance on the Application of the CLP Criteria (version 5.0, July 2017) in section 3.6.2.2.2. establishes certain important factors which may be taken into consideration when assessing the overall level of concern. These factors are displayed in the Table 29 below.

Table 29: Factors to be taken into consideration in assessing the overall level of concern of the difenoconazole-induced liver tumours

	Liver tumours
Genotoxicity	Difenoconazole is not genotoxic
Tumour type and background incidence	Mouse: Hepatocellular tumours (adenomas and carcinomas) in male and female. Rat: There were not liver tumours.
Multi-site responses	No. The tumours only appeared in liver.
Progression of lesions to malignancy	Yes, because both adenomas and carcinomas have been observed.
Reduced tumour latency	No, since the majority of liver tumours in mice occurred at a later stage of the study (79 weeks).
Whether response single or several species	Liver tumours only in a single species: mice.
Whether response is in single or both sexes	Liver tumours appeared in both sexes: males and females.
Structural similarity to a substance(s) for which there is good evidence of carcinogenicity	Not noted
Routes of exposure	Only experimental studies by oral route are available. Dietary oral (relevant for humans)
Comparison of absorption, distribution, metabolism and excretion between test animals and humans	No human data available.
Possibility of a confounding effect of excessive toxicity at test doses	No. All female mice (70) at 819 mg/kg bw/day died or were sacrificed in a moribund condition during the first 2 weeks in the carcinogenicity study. Eleven males (out of 70) in the 983 mg/kg bw/day dose group died or were sacrificed for the same reason during the first 3 weeks of the study. At the next lower dose, 615.6 mg/kg bw/day, 15 (out of 70) females died or were sacrificed during the first week,

	Liver tumours
	which led to a reduction in dose to 423/513 mg/kg bw/day for males/females, respectively, of this dose group, beginning at week 2 of the study. These deaths are attributed to the excessive toxicity of treatment. However, the mechanism of cytotoxicity and regenerative hyperplasia is unlikely to be the cause of the liver tumours after difenoconazol exposure because of the limited amount of hepatic necrosis (single cell or focal) observed in the *in vivo* mice studies.
Mode of action and its relevance for humans	Mechanistic studies provide experimental evidence supporting a PB-like CAR activation dependent MoA for liver tumours in mice. The evaluation of data indicates that alternative MoAs are not probable in mice (immunosuppression, AhR activation, estrogen, cytotoxicity and regenerative hyperplasia, DNA reactivity and mutagenicity). However there are not experimental data to rule out other alternative MoAs as PPARα activation and statins-like activity. Difenoconazole activates CAR in mice and might activate it also in humans (as PB does). As expected for a PB-like activator of CAR, an increment in cell proliferation was detected in mice hepatocytes but not in human hepatocytes. Taking all together, the proposed mode of action is rodent-specific and not regarded as relevant to humans.

Comparison with criteria for Category 2 classification: In accordance with the criteria in the CLP regulation, classification for carcinogenicity Category 2 is reserved for substances where there is evidence obtained from human and/or animal studies but which is not sufficiently convincing to place the substance in Category 1.

Liver tumours in mice: mechanistic data suggest that these tumours are not relevant to humans and therefore not considered for classification. MoA supported: CAR activation, altered gene expression specific to CAR activation, increased cell proliferation, clonal expansion leading to foci/areas of altered hepatocytes and liver adenomas/carcinomas. This mode of action closely mimics that of phenobarbital, is rodent-specific and non-relevant for humans.

PB has been shown not to increase cell proliferation in cultured human hepatocytes and the development of altered hepatic foci in humans has not been reported in the literature (*Elcombe et al., 2014*). Similarly, difenoconazole has been shown not to increase cell proliferation in cultured human hepatocytes (*Vardy A., 2016b*). It is, therefore, not likely that liver tumours would occur through this MoA as a consequence of difenoconazole exposure in humans.

Moreover, the data from a number of epidemiological studies in patients after extended treatment with PB, report no evidence of increased liver tumor risk (*Friedman et al., 2009; IARC, 2001; La Vecchia & Negri, 2014; Olsen et al., 1989, 1995; Whysner et al., 1996*).

On overall, the finding of liver tumours in mice after difenoconazole exposure is not considered to be of relevance to humans.

10.9.3 Conclusion on classification and labelling for carcinogenicity

Not classified (conclusive but not sufficient for classification).

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole based on: i) a 2-year long-term toxicity and carcinogenicity study in rats showing no relevant neoplastic changes; ii) a 78-week carcinogenicity study in mice showing adenomas and carcinomas in liver: iii) a number of mechanistic studies suggesting that the liver adenomas and carcinomas in mice are induced through a phenobarbital-like mode of action (MoA) based on constitutive androstane receptor (CAR) activation.

Comments received during consultation

One MSCA questioned that difenoconazole was able to activate the rat CAR. The DS replied recognizing the uncertainties as regard the CAR activation in rats and noting that this issue has no final impact in the proposal of classification since liver tumours were not observed in rats.

One company-manufacturer supported the proposal for no classification of difenoconazole for carcinogenicity.

Assessment and comparison with the classification criteria

2-year long-term toxicity and carcinogenicity study in rats

Difenoconazole was tested at dose levels of 0, 10, 20, 500 and 2500 ppm, equivalent to 0, 0.5, 1, 24 and 124 mg/kg bw/day for males and 0, 0.6, 1.3, 33 and 170 mg/kg bw/day for females. The table above (summary of repeated dose toxicity studies in rats with difenoconazole) shows the main non-neoplastic findings of this study.

No mortality or clinical signs were associated to the treatment. In general, bodyweights at the top dose tended to be lower than those of control animals. Minor alterations in clinical chemistry and haematology were also noted. Nevertheless, these differences were considered not relevant due to the low magnitude of the change, inconsistency across study intervals and/or the lack of a dose response. Macroscopic examinations did not reveal any treatment-related findings. Absolute liver weights were unaffected among the groups during the study. However, the relative liver weights for the 2500 ppm animals were higher than control values at weeks 53 (↑14% in males and 48% in females) and 104 (↑18% no significant in males and ↑ 44% in females) but were similar to control values following the 4 weeks recovery period. Other alterations in other organ weights (adrenals, ovaries, spleen) were not considered as toxicologically relevant.

Non-neoplastic changes revealed by histopathological examinations consisted in an increased incidence and severity of hepatocellular hypertrophy in 500 and 2500 ppm animals at study termination. For males, the incidence was 65 and 89% in the 500 and 2500 ppm dose groups,

respectively, compared to 17.5% in the control group. Corresponding values for females were 34 and 84%, compared to 12.5% in the control group.

No neoplastic changes were considered relevant, due to the lack of a dose response and/or the low incidence and there were no increases in neoplasia in treated animals.

78-week carcinogenicity study in mice

Difenoconazole was tested at dose levels of 0, 10, 30, 300, 3000-2500 and 4500 ppm, equivalent to 0, 1.5, 4.7, 46, 508-423 and 819 mg/kg bw/day for males and 0, 1.9, 5.6, 58, 616-513 and 983 mg/kg bw/day for females. In the fifth group, the original dose of 3000 ppm was reduced to 2500 ppm at the beginning of week 2, due to early mortality. Table summary of repeated dose toxicity studies in rats with difenoconazole shows the main non-neoplastic findings of this study.

All (70) females in the 4500 ppm dose group died or were sacrificed in a moribund condition during the first 2 weeks. Eleven males (out of 70) in the 4500 ppm dose group died or were sacrificed for the same reason during the first 3 weeks of the study. At the next lower dose, 3000 ppm, 15 (out of 70) females died or were sacrificed during the first week, which led to a reduction to 2500 ppm for both sexes of this dose group, beginning at week 2 of the study. After the lowering of dose, one additional female died during the week 2 of experiment. At the beginning of week 3, ten females of the control group were moved to 2500 ppm group to maintain an adequate sample size of this last group for the duration of the study (replacement animals); 3 of these 10 females were sacrificed due to moribund condition during their first week of exposure to 2500 ppm. After the initial mortality in females of 2500 ppm group during the first 3 weeks of the study, there was no remarkable effect on survival.

Clinical signs observed in this study included higher incidence of thinness, hunched appearance and rough haircoat in females of 2500 ppm group and in males of 4500 ppm group, compared to controls. The incidence of reduced motor activity was increased for the 4500 ppm males when compared with control.

There was a dose-dependent reduction in body weight of treated animals. Males of the two highest dose groups (2500 and 4500 ppm) had significantly lower body weights (↓6% and 7%, respectively) than controls from week 1 throughout to week 56. Females of the 2500 ppm group had significantly lower body weight (< 10%) throughout the study period. Mean body weight gains were significantly decreased through 76 weeks for the groups of 4500 ppm in males and 2500 ppm in females.

Haematological analysis showed some alterations of unclear biological significance. Clinical chemistry revealed statistically significant increases in liver enzyme values. However, these changes were at least in part reversible.

Macroscopic examinations noted for unscheduled deaths included an increased overall incidence of liver findings (enlargement, pale areas and masses) in males and females of 2500 ppm group and in males of 4500 ppm group. Remarkable observations recorded for the liver at terminal sacrifice included enlargement, pale areas and masses. At terminal sacrifice in males at 4500 ppm the incidences of liver enlargement, pale areas and masses were 50%, 56% and 44%, respectively, whereas in females at 2500 ppm were 45%, 41% and 28%, respectively. Mean absolute and relative liver weight values were significantly higher than control values for males of 2500 ppm and 4500 ppm groups and females of 2500 ppm group.

Liver weights in males of recovery group (week 57) were lower than the weights for the animals at week 53 (interim sacrifice), indicating reversibility.

Non-neoplastic changes found at study termination were observed in liver. The following hepatocellular findings were significantly increased in males of 2500 ppm and 4500 ppm groups and in females of 2500 ppm group, focal/multifocal necrosis (males only), individual cell necrosis, fatty change, hepatocyte hypertrophy and bile stasis. A statistically significantly increased incidence was also noted for individual cell necrosis and hypertrophy in males of 300 ppm group. The incidences of individual cell necrosis, hepatocyte hypertrophy, fatty change and bile stasis in the liver of males of 4500 ppm group were lower after the 4-week recovery period than that observed after 53 weeks of treatment, indicative of partial recovery.

Neoplastic changes detected in mice are summarised in the tables below for males and females, respectively. Statistical analysis of liver adenomas and carcinomas revealed significant increases for males of 2500 and 4500 ppm groups, and for females of 2500 ppm group. The incidence of adenomas and/or carcinomas was already elevated in the 4500 ppm males at the interim and recovery sacrifices.

Table: Incidences of hepatocellular adenoma and carcinoma in male mice. * = Statistically significant for p ≤ 0.05 ** = Statistically significant for p ≤ 0.01.

ppm	**0**	**10**	**30**	**300**	**3000-2500**	**4500**
mg/kg bw/day	**0**	**1.5**	**4.7**	**46**	**508-423**	**819**
Adenoma						
Unscheduled deaths	0/20	3/17	1/23	2/26	9/16	6/34
Interim sacrifice	0/10	1/10	2/10	0/10	1/10	2/10
Recovery sacrifice week 57	0/9	-	-	-	0/10	3/10
Terminal	4/31	6/32	5/27	7/24	3/34	9/16
Total	4/70 (6%)	10/59 (17%)	8/60 (13%)	9/60 (15%)	**13/70*** (19%)	**20/70**** (29%)
Carcinoma						
Unscheduled deaths	0/20	0/17	1/23	0/26	1/16	4/34
Interim sacrifice	0/10	0/10	0/10	0/10	0/10	2/10
Recovery sacrifice week 57	0/9	-	-	-	1/10	1/10
Terminal	1/31	0/32	0/27	0/24	3/34	6/16
Total	1/70 (1%)	0/59 (0%)	1/60 (2%)	9/60 (0%)	5/70 (7%)	**13/70**** (19%)

Table: Incidences of hepatocellular adenoma and carcinoma in female mice. * = Statistically significant for p ≤ 0.05 ** = Statistically significant for p ≤ 0.01.

ppm	**0**	**10**	**30**	**300**	**3000-2500**
mg/kg bw/day	**0**	**1.9**	**5.6**	**58**	**616-513**
Adenoma					
Unscheduled deaths	0/26	0/14	0/21	0/15	5/21
Interim sacrifice	0/10	0/10	0/10	0/10	1/10
Recovery sacrifice week 57	-	-	-	-	0/10
Terminal	0/24	0/35	0/29	1/35	10/29
Total	0/60 (0%)	0/59 (0%)	0/60 (0%)	1/60 (2%)	**16/70**** (23%)
Carcinoma					
Unscheduled deaths	0/26	0/14	0/21	0/15	2/21
Interim sacrifice	0/10	0/10	0/10	0/10	0/10
Recovery sacrifice week 57	-	-	-	-	0/10
Terminal	0/24	0/35	1/29	0/35	2/29
Total	0/60 (0%)	0/59 (0%)	1/60 (2%)	0/60 (0%)	4/70 (6%)

Mechanistic study: Oral study of 14-days in mice

Mice were orally dosed with difenoconazole (purity 91.8%) at 0, 1, 10, 100 and 400 mg/kg bw/day for 14 days. The number of animals per each experimental group was nine. Forty mg/kg bw/day of phenobarbital (PB) were intraperitoneally administered to six mice for 4 days. Eighty mg/kg bw/day of 3-methylcholanthrene (3-MC) were intraperitoneally administered to six mice for two days. One hundred mg/kg bw/day of nafenopin (NAF) were intraperitoneally administered to three mice for six days. The mice strain was Tif:MAGf (SPF). The effects of each treatment on bodyweight, liver weight protein content of different P450 isoforms, P450 enzymatic activities, testosterone hydroxylation, peroxisomal fatty acid oxidation and glutathione S-transferase were determined. The results are summarised in the table below.

Table: Effect of difenoconazole and other reference substances on hepatic function of mice. ns= Non-significant.

	Difenoconazole				PB	3-MC	NAF
mg/kg bw/day	**1**	**10**	**100**	**400**	**40**	**80**	**100**
Bodyweight	ns	ns	↓11%	ns	ns	ns	↑17%
Liver weight	ns	ns	ns	↑79%	ns	↑28%	↑88%
Cytochrome P-450	↑5% ns	↑8% ns	↑161%	↑323%	↑80%	↑147%	↑40% ns
CYP1A	↑5%	↑9%	↑23%	↑35%	↑43%	↑4432%	↓39%
CYP2B	↑12%	↑38%	↑49%	↑43%	↑5%	↓34%	↑28%
CYP3A	↑20%	↑37%	↑115%	↑316%	↑287%	↓33%	↑227%
CYP4A	↑37%	↑51%	↑53%	↑17%	↑33%	↓50%	↑810%
MEH	↑19% ns	↑5% ns	↑245%	↑245%	↑115%	↑15% ns	↑233%
Microsomal morphine UDPGT	↑16% ns	↑5% ns	↑50% ns	↑59% ns	↑49% ns	↑42% ns	↑45% ns
Microsomal 1-naphtol UDPGT	↑10% ns	↑19% ns	↑28% ns	↑20% ns	↑81% ns	↑52%	↑119%
EROD	↑31% ns	↑56% ns	↑285%	↑231%	↑237%	↑4525%	↑56% ns
PROD	↑124%	↑140%	↑1839%	↑3246%	↑1522%	↑162%	↑65% ns
Lauric acid 11-hydroxylase	↑7% ns	↑8% ns	↑78%	↑130%	↑101%	↑16% ns	↑247%
Lauric acid 12-hydroxylase	↓48%	↓67%	↓38% ns	↓17% ns	↓4% ns	↓49%	↑753%

MEH: microsomal poxide hydrolase UDPGT uridine 5'-diphospho-glucuronosyltransferase: EROD: ethoxyresorufin-*O*-deethylase PROD: pentoxyreso-rufin-*O*-dependentylase

The level of total testosterone hydroxylation was induced 6-fold in a dose-related manner, in mice at 400 mg/kg bw/day difenoconazole. Except for 7α-hydroxy-testosterone, all testosterone metabolites were increased between 3- and 12.5-fold in mice at 400 mg/kg bw/day of difenoconazole. PB caused increases of 6β- , 15β-, 6α- and 16α-hydroxy-testosterone.

A slight, dose-dependent and not statistically significant decrease of peroxisomal fatty acid β-oxidation was observed in mice treated with difenoconazole. PB and 3-MC did not affect the peroxisomal fatty acid β-oxidation whereas the treatment with NAF produced an increase of 4.4-fold in this process.

The activity of cytosolic glutathione S-transferase in mice treated with 400 mg/kg bw/day difenoconazole was increased 1.4-fold. A similar level of induction was seen with PB, and to a

lesser extent with NAF. 3-MC reduced this enzyme activity to 1.3-fold. All these changes in cytosolic GST activity were not statistically significant.

In conclusion, the treatment with difenoconazole at 400 mg/kg bw/day in mice produced changes that were similar to those with PB, as increases of microsomal proteins (microsomal morphine UDPGT and microsomal 1-naphtol UDPGT) activities, increases of cytochrome P450 content, changes in CYP isoenzymes levels, increases of liver enzymes (MEH, EROD, PROD) activities and increases of testosterone hydroxylation.

Mechanistic study: In vitro study with CD-1 mice hepatocytes

Primary monolayer cultures of hepatocytes were cultured in Leibowitz CL15 medium for 4 hours to allow adherence. The medium was changed and the hepatocytes exposed to PB at 10, 100 and 1000 µM, to Epidermal Growth Factor (EGF, positive control for replicative DNA synthesis) at 25 ng/mL, or to difenoconazole (purity 93.9%) at 0.5, 1, 2, 4, 8 and 12.5 µM or to 0.5% DMSO (vehicle) for 96h.

There were no cytotoxicity at the tested doses since 25 µM difenoconazole caused around 3% ATP depletion. Treatment of isolated male CD-1 mouse hepatocyte cultures with difenoconazole at concentrations up to 12.5 µM resulted in increases in replicative DNA-synthesis as determined by the S-phase labelling index. However, difenoconazole did not increase either Cyp2b10 or Cyp3a11 mRNA levels. PROD and BROD (benzyloxyresorufin-*O*-debenzyl-ase) activities were also unchanged, but difenoconazole did increase BQ activity (benzyloxyquinoline-*O*-debenzylation; indicative of CYP3A activity) in concentration-dependent manner. Treatment with the positive controls PB and EGF gave the expected set of responses, indicating the suitability of the system.

Mechanistic study: In vitro study with human hepatocytes

Primary male human hepatocytes in Cryopreserved Hepatocytes Plating Medium were cultured for up to 6 hours to allow adherence. Then the medium was changed to Leibowitz HCL15 medium and the hepatocytes exposed to PB at 10, 100 and 1000 µM, to difenoconazole at 0.5, 1, 2, 4, 6 and 8 µM, to EGF at 25 ng/mL or to 0.5% DMSO (vehicle) for 96h.

Treatment with 6 µM and 8 µM difenoconazole resulted in hepatocellular cytotoxicity with ATP levels being reduced to 75% and 49% of control, respectively. Treatment with PB did not cause a statistically significant decrease in ATP levels. Treatment of isolated male human hepatocyte cultures with difenoconazole at concentrations up to 8 µM had no effect on replicative DNA-synthesis, as determined by the S-phase labelling index. However, difenoconazole led to PROD and BROD activities induction, which are mainly representative of CYP2B and CYP2B/3A, respectively. In contrast, BQ activity was unaffected by treatment with difenoconazole at the lower concentrations, and then reduced at the higher concentrations assessed in the presence of cytotoxicity.

Mechanistic study: CAR transactivation study

Expression vectors for CAR variants of mouse, rat and human with a CYP2B6 response element-luciferase reporter were transfected into COS-1 cells along with necessary cofactors. After an expression time (16-18h) cells were incubated during 24h with difenoconazole (purity 93.9%) at 1, 3, 10 and 30 µM and with the following CAR ligands (positive controls): CITCO

(substrate for human CAR) at 5 µM; TBPOBOP (substrate for mouse CAR) at 0.5 µM; and clotrinazole (substrate for rat CAR) at 10 µM.

Under conditions of this assay, difenoconazole was a direct activator of mouse CAR and not an activator of human CAR. A small increase in activation of rat CAR (1.6-fold) was observed at 30 µM difenoconazole, but this difference was not statistically significant, indicating that difenoconazole was at most a low potency activator of rat CAR.

Mechanistic study: PXR transactivation study

Expression vectors were constructed with the ligand binding domains of pregnane X receptor (PXR) variants of mouse, rat and human fused to the DNA binding domain of the transcription factor Gal4 and with a Gal4 response luciferase reporter. These vectors were transfected into HEK cells (human embryonic kidney). After 16-18h of expression, cells were incubated during 24h with difenoconazole (purity 93.9%) at 13.7, 41.2, 123, 370, 1111, 3333, 10000, 30000 nM and with pregnenolone-16α-carbonitrile and TO901317 at appropriate ranges of concentrations.

Based on the results of these luciferase reporter assays, difenoconazole is not an activator of human, rat or mouse PXR. Positive controls gave the expected results.

Mechanistic study: Acute toxicity and toxicokinetic study (1- and 7-day) in CD-1 and C57BL/6J mice

Male Charles River CD-1 and Envigo C57BL/6J mice were treated in following three different conditions as follows. Condition 1 (five animals/group/strain): Treatment for 1 day by oral gavage at 0, 15, 45, 150 and 400 mg/kg bw/day CD-1 mice or 0, 15 and 150 mg/kg bw/day C57BL/6J mice. Condition 2 (five animals/group/strain): Treatment for 5 days by oral gavage at 0, 15, 45, 150 and 400 mg/kg bw/day CD-1 mice or 0, 15 and 150 mg/kg bw/day C57BL/6J mice. Condition 3 (five animals/group/strain): Intravenous treatment for 1 day at 1 mg/kg bw/day both strains.

Difenoconazole treatment produced increased liver weights accompanied by centrilobular hypertrophy and decreased glycogen content in the liver at doses ≥ 150 mg/kg bw/day in both strains of mouse and an increased incidence of cytoplasmic vacuolation at 400 mg/kg bw/day in CD-1 mice. Furthermore, there were dose-related increases in hepatic total cytochrome P450 content and on PROD, BROD and BQ activities (markers for CYP2B, CYP2B/3A and CYP3A, respectively) along with increases of Cyp2b10 and Cyp3a11 mRNAs levels in both strains of mouse. Overall, difenoconazole administration resulted in similar effects in male CD-1 and C57BL/6 mice at dose levels tested.

Mechanistic study: Acute toxicity and toxicokinetic study (1- and 7-day) in CAR/PXR double KO and hCAR/hPXR mice

Taconic Biosciences Inc. CAR/PXR double KO, hCAR/hPXR and C57BL/6NTAc (WT) mice were treated in following three different conditions as follows. Condition 1 (five animals/group/strain): Treatment for 1 day by oral gavage at 0, 15 and 150 mg/kg bw/day (all strains). Condition 2 (five animals/group/strain): Treatment for 5 days by oral gavage at 0, 15 and 150 mg/kg bw/day (all strains). Condition 3 (five animals/group/strain): Intravenous treatment for 1 day at 1 mg/kg bw/day (all stains).

Absolute and relative liver weight were unaffected in CAR/PXR double KO but both weights were significantly increased in hCAR/hPXR mice. Histopathological analysis showed a dose-related mild centrilobular hypertrophy only in hCAR/hPXR mice livers, whereas CAR/PXR double KO mice livers showed no treatment related changes. The analysis of liver enzyme activities and of corresponding mRNA expression levels in the liver showed no response at any dose level in male CAR/PXR double KO mice. However, in hCAR/hPXR mice at 150 mg/kg bw/day, BROD and BQ enzyme activities (markers for CYP2B/3A and CYP3A, respectively) showed statistically significant increases and Cyp2b10 and Cyp3a11 mRNA levels were higher than controls. In conclusion, these data suggest that the hepatic effects of difenoconazole are dependent on the presence of a functional CAR and/or PXR.

Mechanistic study: Hepatocellular proliferation and liver enzymatic induction study (7-day) in CAR/PXR double KO, C57BL/6NTAc WT and CD-1 WT mice

Charles River UK CD-1 WT, Taconic Biosciences Inc. C57BL/6NTAc WT, CAR/PXR double KO and CD-1 WT mice were treated (12 animals/group/strain) by oral gavage for 7 days with either 150 mg/kg bw/day difenoconazole or 80 mg/kg bw/day PB.

In CD-1 WT and C57BL/6NTAc WT mice, difenoconazole caused increases in absolute and relative liver weight, hepatocellular proliferation and centrilobular hypertrophy, accompanied by liver enzymes (EROD, PROD, BROD and BQ) induction, suggesting activation of CAR and possibly PXR nuclear hormone receptors. In the CAR/PXR double KO mice, there was not increase in absolute and relative liver weight, nor in centrilobular hypertrophy in liver enzymes induction, suggesting these were all CAR-mediated events. However, there was hepatocellular proliferation (albeit much reduced), that may be a consequence of the low BrdU labelling index for control (CMC) mice. Additionally, the formation of 12-OH lauric acid was also higher than the control response in both the wild type and CAR/PXR double KO mice treated with difenoconazole. PB caused the expected results in all three-mouse strains evaluated, except in CD-1 WT mice by a slight increase in lauric acid 12-hydroxylase (LAH).

Proposed mode of action

The applicant proposes a MoA consistent with the Adverse Outcome Pathway 107 developed by the OECD (AOPwiki, http://aopwiki.org/aops/107) entitled "*Constitutive androstane receptor activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat*".

The proposed MoA for difenoconazole liver tumours consists of the activation of the CAR in the liver. CAR activation conduces to increased expression of pro-proliferative and anti-apoptotic genes in the liver and an early, transient, increase in hepatocellular proliferation. Over time, the increased hepatocellular foci because of clonal expansion of spontaneously mutated cells in the mouse results in slight increases in liver tumour incidence compared to concurrent controls. In a review article (Elcombe *et al.*, 2014), it is analysed the evidence that mouse or rat liver tumours that occur via a CAR MoA are not relevant to humans based on qualitative differences between the species. The table below shows the key and associative events of the CAR activation MoA.

Table: Key events and associative events in the mode of action based on AOP number 107.

Key events	Key events Associative events
Key event 1: CAR nuclear receptor activation	
Key event 2: Altered gene expression specific to CAR activation	Enzyme induction (CYP2B and CYP3A) Hepatocellular hypertrophy Liver weight increase
Key event 3: Increased cell proliferation	
Key event 4: Clonal expansion leading to foci/areas of altered hepatocytes (eosinophilic)	
Key event 5: Liver adenomas/carcinomas	

The tables below show the experimental evidence for the key and associative events of a CAR mediated induction of liver tumours in rats, mice, dogs and humans studies with difenoconazole.

Table: Evidence for the key events in rats, mice, dogs and humans.

Key events	Rats	Mice	Dogs	Humans
CAR activation	YES: Difenoconazole produced a slight trend no significant toward direct activation of rat CAR in the transactivation study Omiecinski, 2016	YES: Difenoconazole was a direct activator of mouse CAR in the transactivation study. Increases in absolute and relative liver weight, hepatocellular proliferation, centrilobular hypertrophy and hepatic enzymes induction are not observed in CAR/PXR double KO mice, therefore they are CAR-dependent effects. Omiecinski, 2016 Anonymous 21, 2017b Anonymous 22, 2017	Not determined	NO: Difenoconazole was not a direct activator of human CAR in the transactivation study. In hCAR/hPXR mice there were CAR-dependent effects as increases in absolute and relative liver weight, centrilobular hypertrophy and hepatic enzymes induction. Omiecinski, 2016 Anonymous 21, 2017b
Altered gene expression specific to CAR activation	Not determined	YES: Increases in Cyp2b10 and Cyp3a11 mRNAs levels. The liver mRNA expression levels showed no response at any dose in male CAR/PXR double KO. However, in hCAR/hPXR mice, increases in Cyp2b10 and Cyp3a11 mRNA levels were observed. Anonymous 20, 2017a Anonymous 21, 2017b	Not determined	Not determined
Increased cell proliferation	*In vitro:* Not determined	*In vitro:* YES *In vivo:* YES Vardy, 2016a	Not determined	*In vitro*: NO Vardy, 2016b

	In vivo: Not observed/ reported	Anonymous 22, 2017		
Clonal expansion leading to altered foci	Not observed/ reported	YES: Increase in inflammatory cell foci in C57BL/6NTAc WT mice. Anonymous 22, 2017	Not observed/ reported	Not determined
Liver adenomas/ carcinomas	Not observed/ reported	YES: Hepatocellular adenomas and carcinomas were observed in liver in male mice at 4500 ppm (819 mg/kg bw/day) ($p < 0.01$) and male and female mice at 2500 ppm (♂ $p < 0.05$ and ♀ < 0.01) (423/513 mg/kg bw/day for males/females, respectively). Anonymous 18, 1989b	Not observed/ reported	Not determined

Table: Evidence for the associative events in rats, mice, dogs and humans.

Associative events	**Rats**	**Mice**	**Dogs**	**Humans**
Enzyme induction (CYP2B and CYP3A)	Not determined	*In vitro:* YES Increases in hepatic enzymes levels, especially CYP3A. *In vivo:* YES Increases in hepatic enzymes levels, especially CYP1A, CYP2B*, CYP3A and UDPGT. Vardy, 2016a Anonymous 19, 1992 (*In this study CYP2B levels decrease) Anonymous 20, 2017a Anonymous 21, 2017b Anonymous 22, 2017	Not determined	*In vitro:* YES Increases in hepatic enzymes levels, especially, CYP2B/3A. *In vivo:* Not determined Vardy, 2016b
Hepatocellular hypertrophy	YES Anonymous 36, 2000 Anonymous 16, 1989a and 17, 1992	YES Anonymous 18, 1989b Anonymous 20, 2017a Anonymous 21, 2017b	NO Anonymous 34, 1987 Anonymous 35, 1988	Not determined

		Anonymous 22, 2017		
Increased liver weight	YES Anonymous 29, 1986a Anonymous 30, 1986b Anonymous 31, 1987a Anonymous 36, 2000 Anonymous 16, 1989a and 17, 1992	YES Anonymous 33, 1987b Anonymous 18, 1989b Anonymous 20, 2017a Anonymous 21, 2017b Anonymous 22, 2017	YES Anonymous 34, 1987	Not determined

The whole database, including the toxicity and mechanistic studies, meets the key events of this CAR activation mechanism, including altered gene expression, increased cell proliferation, clonal expansion leading to altered foci and liver tumours. This same database also provides evidence for the associative events such as enzyme induction, hepatocellular hypertrophy and increased liver weight.

Uncertainties, inconsistencies and data gaps in the proposed mode of action

The following uncertainties were found:

- The applicant explained that double CAR/PXR knockout mice were utilised instead of single CAR knockout animals, as it is almost impossible to split the two nuclear receptors because of shared ligands, co-activators and response elements. Furthermore, in the PXR transactivation assay (Korrapati & Sherf, 2016), a non-dose related increment of PXR activation was observed after difenoconazole treatment in mouse. It is also noted that PXR activation was tested with the PXR ligand-binding domain (of each species) fused to the Gal4 protein and not with the actual receptor. The presence of positive controls reduces the level of uncertainty from this experiment.
- In humans, there are several CAR proteins derived from alternative splicing (e.g. hCAR1,hCAR2, hCAR3), no details were given about which hCAR version was inserted in the mice employed in the 1 and 7-day investigative *in vivo* assay (Anonymous 21, 2017b).
- It is unclear if the lack of information about the presence of altered hepatocytes foci in the carcinogenicity study in mice is because this parameter was not considered in the experimental design or if foci were investigated but they were not found in the liver. These foci were observed only in C57BL/6NTAc mice in the 7 days study (Anonymous 22, 2017), although according to this MoA this should not be an early stage event. Nevertheless, it is noticed that, according to AOP number 107 from AOPwiki, no data for the key event of increased altered foci are available in CD-1 mice treated with PB whereas studies in male C57BL/10J mice also treated with PB show a clear increase in altered foci.
- No tumours were observed in the rat carcinogenesis study (Anonymous 16, 1989a and 17, 1992), although the results from the *in vitro* assay (Omiecinski, 2016) suggested that CAR was also activated by difenoconazole in this species. However, this could be explained as a delay in the response of this test system.

The following points were considered as inconsistencies:

- The reason for the lack of increase of Cyp2b10 and Cyp3a11 mRNA levels with difenoconazole treatment in mouse primary hepatocytes *in vitro* (Vardy, 2016c) is unknown. If CAR activation is proposed as the MoA for the tumourigenic effect of difenoconazole in mice, an increment of the transcription levels in these two genes should occur. Moreover, it would have been desirable that the same information could be available for the expression of the orthologous genes in human hepatocytes.
- After *in vitro* difenoconazole treatment of mouse cells (Vardy, 2016a) only BQ activity increased in concentration-dependent manner but unexpectedly neither PROD nor BROD activities changed, while in human cells the three activities were incremented (Vardy, 2016b). Accordingly, in the oral 14-day study (Anonymous 19, 1992), CYP2B protein level (whose markers are PROD and BROD) did not increase as expected from the proposed MoA. Unexpectedly, in the same study PROD enzyme activity was increased and this pattern was similar with PB tested in this study. This CYP2B-independent activation of PROD after difenoconazole treatment was not justified. Moreover, using liver microsomes prepared from PB-induced male CD-1 mice, difenoconazole inhibited both CYP2B and CYP3A induction in an in vitro assay when the enzymatic activities were assessed (Vardy, 2016c). A similar *in vitro* PB pre-treatment was not tested in human cells. This unexpected inhibition was not justified and the potential consequences for the proposed MoA were not addressed.
- Contrary to the proposed MoA, an increase in hepatocellular proliferation was also reported in the CAR/PXR double KO mice treated with difenoconazole, although it was much reduced compared to the proliferation observed in wild-type mice (CD-1 and C57BL/6NTAc) with the same treatment. In contrast to this, in the case of the CAR/PXR double KO mice treated with PB there was not an increase in hepatocellular proliferation, as would be expected (Anonymous 22, 2017).
- The formation of 12-OH lauric acid was higher in both wild-type and CAR/PXR double KO mice treated with difenoconazole than in untreated controls (Anonymous 22, 2017). In this study it was suggested that these results might be indicative of a small PPARα nuclear hormone receptor induction caused by difenoconazole, and that this induction could explain the residual hepatocellular proliferation in the CAR/PXR KO mice through a CAR-dependent block of PPARα transcription. However, the presence of 12-OH lauric acid also in wild-type mice indicates that this alternative explanation should be dismissed, since if an active CAR suppresses the PPARα transcription, and PPARα transcription was observed in the wild-type where CAR activation was observed, it could mean that difenoconazole can also activate PPARα to such an extend to overcome the transcription block from CAR.

The following data gap was detected:

- The lack of an HMG-CoA reductase activity study to rule out that difenoconazole could be related to a statin-like MoA.

Other potential modes of action

To define a MoA in liver, it is critical to ensure that other modes of action do not contribute significantly to hepatocarcinogenesis. In addition to CAR activation, other mechanisms may be involved in difenoconazole-induced tumorigenesis in mice liver. The plausibility of other possible modes of action is discussed in the table below.

Table: Assessment of alternative MoA for difenoconazole induced liver tumours.

Mode of Action	Data relating to difenoconazole	Conclusion
Mutagenicity	DNA reactivity and mutagenicity can be excluded since the genotoxicity testing *in vivo* and *in vitro* of difenoconazole gave no evidence of a genotoxic potential.	Unlikely
Cytotoxicity and regenerative hyperplasia	In the oral 90-day study in mice at 7500 ppm and 15000 ppm, hepatotoxicity was observed by hepatocellular enlargement and necrosis of individual hepatocytes (table 'summary of repeated dose toxicity studies in rats with difenoconazole'). Furthermore, in the carcinogenicity study in mice necrosis of individual hepatocytes, focal/multifocal necrosis of hepatocytes, bile stasis and fatty change were observed (table 'summary of repeated dose toxicity studies in rats with difenoconazole'). Since these findings occurred accompanied of increases in liver weights, they are considered a secondary effect to excessively large increases in liver weight and liver size, since literature has shown late-onset necrosis will occur as a secondary effect to very large increases in liver weight and liver size (Maronpot *et al.*, 2010; Hall *et al.*, 2012). A small increase in the incidence of mild to moderate single-cell necrosis can sometimes occur, particularly after long-term treatment of mice with CAR activators. However, more severe/diffuse necrosis in the liver suggests that an alternative MoA via cytotoxicity might be operative (Hall *et al.*, 2012). The limited amount of hepatic necrosis (single cell or focal) observed in the *in vivo* mouse treated with difenoconazole studies is in contrast with the pattern of effects seen with classic cytotoxic carcinogens that cause a diffuse necrosis (widespread multifocal hepatocyte death) in the liver that progressed to a sustained regenerative hyperplasia, as is the case of chloroform and carbon tetrachloride. In the mice study in which hepatic tumours were observed only localized areas of hepatic necrosis were seen in both sexes. Therefore, it is not considered that cytotoxicity is an additional MoA involved in the hepatocellular tumour formation.	Unlikely
Estrogenic activity	Difenoconazole does not present structural similarity with oestrogens. The *in silico* mechanistic data indicated that the probability of binding of difenoconazole on oestrogen receptors is low and ToxCast ER model data showed negative results for oestrogens (B.6.8.3).	Unlikely
Statin-like activity	It has not been experimentally shown that difenoconazole has not activity as an HMG-CoA reductase inhibitor.	Plausible
Aryl Hydrocarbon Receptor (AhR) activation	Difenoconazole produces increases in EROD activity and increases in CYP1A protein levels in liver microsomes of treated mice and these markers are greatly increased by AhR activators. However, these increases are clearly lower than the increases produced in these markers by the reference substance 3-MC that is an AhR agonist (Anonymous 19, 1992).	Unlikely
Peroxisome proliferator activated receptor alpha (PPARα) activation	Difenoconazole produced a slight decrease in LAH activity and in CYP4A levels of protein in liver fractions of treated mice and did not increase peroxisomal lipid beta-oxidation (Anonymous 19, 1992). Each of these markers are greatly increased by peroxisome proliferators. However, in the Anonymous 22, 2017 study an increase in LAH has been observed in different strains of mice treated with difenoconazole. No reference substance (like the PPARα agonist nafenopin, NAF) was tested in the same study and therefore the relevancy of such increase is unclear. The plausibility	Plausible, but not probable

	of this MoA cannot be rejected, but the low repetitiveness of the result suggests it is not probable.	
Immunosuppression	No changes in the immune system or immune cells were detected in difenoconazole studies.	Unlikely

Comparison with criteria

Category 2 is reserved for substances with evidence of carcinogenicity not sufficiently convincing to place the substance in Category 1A or 1B and can be set if the evidence of carcinogenicity is restricted to a single experiment, as is the case of difenoconazole. However, a full range of investigative studies was performed to determine the MoA of difenoconazole in the mouse. These experiments provide experimental evidence suggesting that liver carcinogenicity is induced through a PB-like MoA (CAR activation → increase of replicative DNA synthesis → hypertrophy → carcinogenesis); which can be considered as not relevant for humans. However, RAC notes that some uncertainties and inconsistences remain (see the assessment above) and this, considering that alternative modes of action have not been fully ruled-out, is insufficient evidence to support the non-relevance of the observed liver tumours for humans. Therefore, RAC considers that classification of **difenoconazole as Carc. 2; H351 (Suspected of causing cancer)** is warranted.

10.10 Reproductive toxicity

A two-generation study in rats is available to investigate the effects of difenoconazole on sexual function and fertility. One developmental toxicity study in rats and one in rabbits (oral) are also available.

10.10.1 Adverse effects on sexual function and fertility

Table 30: Summary table of animal studies on adverse effects on sexual function and fertility

For more detailed information see dRAR B.6. chapter 6.6.1.1.

Method, guideline, GLP, species, strain, sex, no./group deviations, acceptability	Test substance, route administration, dose levels, duration of exposure, parameters observed	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
Multigeneration reproductive toxicity study in rat Method/ Guideline: OECD 416 (1981). GLP: compliant Rat strain: Sprague Dawley CRCD VAF/PLUS. Sex: ♂ and ♀ No. animals (groups): F_0 and F_1: 30	Test substance: Difenoconazole (CGA 169374), (Purity: 97.4 %). Route administration: Oral (diet). Doses: 0, 25, 250 or 2500 ppm equivalent to: F_0 and F_1 (mean) ♂: 0, 1.68, 16.8, or 171 mg/kg bw/day ♀: 0, 1.88, 18.8, 189	**PARENTAL TOXICITY** **2500 ppm (171/189 mg/kg bw/day)** **F_0 adults** *Body weight* ▪ ↓ During premating and mating period in ♂/♀ (4-8% ♂ and 4-15% ♀) ▪ ↓ Throughout gestation and lactation in ♀ (13%). *Body weight gain* ▪ ↓ During premating period in ♂/♀ (0 to 77 days ↓12% ♂ and ↓30%♀) ▪ ↓ Throughout gestation in ♀ (0-7 days ↓34% and 0-20 days ↓10%). ▪ ↓ Throughout lactation in ♀ (0-7 days ↓52% and 0-20 days ↓26%).	**Anonymous 23, (1988)** **B.6.6.1.1 (AS)**

Method, guideline, GLP, species, strain, sex, no./group deviations, acceptability	Test substance, route administration, dose levels, duration of exposure, parameters observed	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
rats/sex/dose. Deviations from OECD TG 416 (2001): No oestrus cyclicity, landmarks of sexual development, no sperm analysis and ovarian follicle counts. The histopathology undertaken is limited in adults and offspring and, in particular, the target organ (liver) toxicity has not been evaluated. **Study acceptable**	mg/kg bw/day. Exposure: Pre-mating treatment: F_0 (77 days) F_1 (98 days) Treatment continued in F_0 and F_1 throughout gestation and lactation. Parameters observed F_0 and F_1 parental: Mortality, clinical signs, bw and bw gain, food consumption, gross pathology, histopathology (performed on sex organs and the pituitary at 0 and 2500 ppm), organ wts (testes and ovaries) and ophthalmoscopic examinations *F_0 , F_1 and F_2 offspring* Mean litter size, number of live pups at birth, pup sex ratios, number of viable and stillborn pups, clinical signs, external malformations (hematoma, tail-agenesis), pup wts, gross pathology, Ophthalmoscopic examinations. *Reproductive:* Mating, fertility, pregnancy and gestation indices	*Food consumption* ▪ ↓ Overall during premating period in ♂/♀ (♂ 9% and ♀11%) ▪ ↓ Throughout gestation (13%) in ♀. *Organ weights* ↑ Relative testes wt (9%). No histopathology associated. **F_1 adults** *Body weight* ▪ ↓ During premating and mating period in ♂/♀ (29-16% ♂ and 22-19% ♀) ▪ ↓ Throughout gestation (22-19%) and lactation (22-18%) in ♀ *Body weight gain* ▪ ↓ During premating period (0 to 98 days) in ♂/♀ (↓10% ♂ and ↓22%♀) ▪ ↓ Throughout gestation in ♀ (0-7 days ↓30% and 0-20 days ↓7%). *Food consumption* ▪ ↓ Overall during premating period in ♂/♀ (♂ 11% and ♀17%) ▪ ↓ Throughout gestation (16-22%) in ♀. *Organ weights* ▪ ↑ Relative testes wt (14%) and ovaries wt (33%) due to the reduction of terminal body weight (16% and 22% respectively). No histopathology associated. **250 ppm (16.8/18.8 mg/kg bw/day)** **F_0 adults:** *Body weight gain* ▪ ↓ in ♀ during the 1st week of gestation (17%). This reduction was not associated with alterations in food consumption and therefore, was not related to treatment. **F_1 adults** *Body weight* ▪ ↓ During premating period, days: 0-7 (8%) in ♂. This reduction was not associated with alterations in food consumption and therefore, was not related to treatment. *Food consumption* ▪ ↓ During the 2nd week of gestation period, days 7-14 (10%) in ♀, but this difference was considered to be incidental. **25 ppm (1.68/1.88 mg/kg bw/day)** There were no treatment-related effects. **NOAEL** parental toxicity: **250 ppm** (equivalent to approx. **16.8 mg/kg bw/day**) based on decreased body weight, body weight gain and food consumption of successive generations (F_0 and F_1) at 2500 ppm. **REPRODUCTIVE PARAMETERS** There were no treatment-related effects. **NOAEL** reproductive toxicity > **2500 ppm (>189 mg/kg bw/day)** based on no effects observed at the top dose tested in F_0 and F_1 generations. **OFFSPRING TOXICITY** **2500 ppm (171/189 mg/kg bw/day)**	

Method, guideline, GLP, species, strain, sex, no./group deviations, acceptability	Test substance, route administration, dose levels, duration of exposure, parameters observed	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
		F1 offspring: *Live birth index* ▪ ↓ in the percentage survival of ♂ pups from days 0 to 4 pre-cull [95.2% *vs* 98.7% control (ndr)]. *Pup weight* ▪ ↓ ♂/♀ at birth (6%/-), day 4 precull (13%/11%), day 4 postcull (14%/11%), day 7 (23%/20%), day 14 (27%/26%) and day 21 (30%/29%). **F2 offspring:** *Pup weight* ▪ ↓ ♂/♀ at birth (8.2%/7.4%), day 4 precull (14.1%/13.1%), day 4 postcull (14.5%/13.9%), day 7 (20.5%/19.9%), day 14 (26.2%/25.6%) and day 21 (32.9%/31.8%). **250 ppm (16.8/18.8 mg/kg bw/day)** **F1 offspring:** *Pup weight* ▪ ↓ ♂ on day 21 (7%). As this reduction was slight and not associated with any other reductions in body weight during the period at this dose, it was considered to be incidental **F2 offspring:** There were no treatment-related effects. **25 ppm (1.68/1.88 mg/kg bw/day)** There were no treatment-related effects in F1 and F2 offspring generation. **NOAEL offspringl toxicity: 250 ppm** (equivalent to approx. **16.8 mg/kg bw/day**) based on decreased pups weight in males and females F1 and F2 pups through lactation period at 2500 ppm.	

Table 31: Summary table of human data on adverse effects on sexual function and fertility

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No evidence of adverse health effects in humans on sexual function and fertility				

Table 32: Summary table of other studies relevant for toxicity on sexual function and fertility

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

The potential effects of difenoconazole on fertility and reproductive performance have been investigated in a standard 2-generation study in rat (B.6.6.1.1) at doses up to 2500 ppm (approximately equivalent to 171/189 mg/kg bw/day for males and females respectively).

This study is previous to the current test guideline (OECD 416, 2001) and it is therefore deficient in some endpoints including oestrus cyclicity, landmarks of sexual development, sperm analysis and

ovarian follicle counts. The histopathology undertaken is limited in adults and offspring and, in particular, the target organ (liver) toxicity has not been evaluated. However, the conclusions reached are robust and the omissions/deviations are considered unlikely to alter these conclusions.

Toxicity in the parental animals (F_0 and F_1) were observed at 2500 ppm. It included reduced body weight, body weight gain and food consumption.

In F_0 parents during the pre-mating period the reduction of body weight was 8% for males and 15% for females, the reduction of body weight gain was 12 to 14% for males and 30% for females and during mating the reduction of body weight was approximately 9% for males, and persisted into gestation (body weight was 12% lower and body weight gain was 34%) and lactation (body weight was 13% lower and body weight gain was 52% lower) for females. Treatment-related reductions in food consumption were also noted in both sexes of F_0 during pre-mating (9% for males and 11% for females) and in females during gestation (13%).

F_1 animals also showed reductions in mean body weight and weight gain during the pre-mating period (body weight was more than 15% lower for males and females; body weight gain was 10% lower for males and 22% for females), this reduction persisted in females into gestation and lactation (body weight was more than 20% lower and body weight gain was 30% lower during the first week of gestation). Treatment-related reductions in food consumption were also noted in both sexes of F_1 during pre-mating (11% for males and 17% for females) and in females during gestation (17%) at this dose level.

No adverse effect of difenoconazole on sexual function or the fertility of the rat was identified at dose levels which induced some parental toxicity. Furthermore, there were no effects of difenoconazole on the development of the offspring other than lower body weights at birth. In F1 offspring generation there was a slight decrease, in percentage survival of male pups from days 0 to 4 pre-cull at 2500 ppm (95.2% vs 98.7% control). Although this decrease was statistically significant, it was slight and was not dose dependent, so it was considered incidental.

Treatment-related reductions in pup weights of F1 and F2 offspring generations were observed at 2500 ppm for both sexes through lactation period [lactation days 0, 4 (pre-and post-cull), 7, 14 and 21]. These reductions were statistically significant on all assessment occasions except for females on lactation day 0 of F1 offspring generation. These reductions were associated with reductions in body weight and body weight gain in F0 and F1 female parents during this period at this dose. There are no data about the feed consumption in the female parents during that period.

10.10.3 Comparison with the CLP criteria

Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A, known human reproductive toxicant) or from animal data (Category 1B, presumed human reproductive toxicant).

According to the CLP criteria a classification of a substance in category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on reproduction in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects.

Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

No human information is available on the effects of difenoconazole on the reproductive system. Information from a reliable 2-generation study in rats showed that difenoconazol has no effects on fertility and reproductive performance. Consequently, classification is not warranted.

10.10.4 Adverse effects on development

Table 33: Summary table of animal studies on adverse effects on development

Method, guideline, GLP, species, strain, sex, no./group deviations, acceptability	**Test substance, route administration, dose levels, duration of exposure, parameters observed**	**Results** [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	**Reference**
Rat developmental toxicity study Method: US EPA 83-3 comparable to OECD 414 (1981). GLP: Yes. Rat strain: Crl:COBS®CD®(SD)BR rats. Sex: 25 mated females/group. *23 females instead of 25 in the 100 mg/kg bw/day treatment group.* Deviations from OECD 414: The exposure period was from day 6 to 15 of gestation instead of the recommended period from implantation (e.g. day 5 post mating) to the day prior to scheduled caesarean section (day 20). Furthermore, it is recommended to include the	Test substance: Difenoconazole (CGA 169374), (Batch FL-851406, Purity: 95.7 %). Route administration: Oral (gavage). Doses: 0, 2, 15.6, 100 and 200 mg/kg bw/day Exposure: days 6-15 (gestation period) Parameters observed: *Maternal data:* mortality, clinical signs, bw, bw gain, food consumption. *Reproductive data:* no. of pregnancies, no. of corpora lutea, implantation sites, no. of early and late resorptions and live litter size. *Foetal data:* Foetus wt, foetus sex and foetus alterations (external, visceral and skeletal)	***Maternal toxicity*** **200 mg/kg bw/day** *Clinical signs* ▪ ↑ Excess salivation in 19/25 dams *vs* 0/25 controls (76%). ▪ ↑ Red vaginal exudate in 3/25 dams *vs* 0/25 controls (12%). *Body weight* ▪ ↓ During treatment period, on day 8 (14%) on days 10, 15 and 19 (4-7%). *Body weight gain* ▪ ↓ During treatment [days: 6-15 (56%), and 0-20 (12%)]. *Food consumption* ▪ ↓During the treatment period 6-16 (10-44%). After treatment (days 17-20), ↑ food consumption (12%) **100 mg/kg bw/day** *Clinical signs* ▪ ↑ Excess salivation in 14/23 dams *vs* 0/25 controls (61%). ▪ ↑ Red vaginal exudate in 3/25 dams *vs* 0/25 controls (13%). *Body weight gain* ▪ ↓ During treatment period, days 6-15 (23%). *Food consumption* ▪ ↓ During treatment, days 6-12 (11-17%). **15.6 and 2 mg/kg bw/day** No treatment related effects. **$NOAEL_{maternal}$ 15.6 mg/kg/day** based on decreased body weight gain and food consumption at 100 mg/kg bw/day. ***Developmental toxicity*** ***Reproductive data*** **200 mg/kg bw/day** ▪ 1/25 dams with total implant loss ▪ ↑ No. of early resorptions per litter (1.7 *vs* 0.7 control. HCD:	**Anonymous 24, (1987)** **Anonymous 25, (1992)** **(Suplemental information Teratology study)** **B.6.6.2.1 (AS)**

<table>
<tr><th>Method, guideline, GLP, species, strain, sex, no./group deviations, acceptability</th><th>Test substance, route administration, dose levels, duration of exposure, parameters observed</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>following measurements: wt of the gravid uteri including cervix, anogenital distance and thyroid hormones which were not performed in this study.

Study acceptable.</td><td></td><td>
0.3-1.4) (ndr, ns).
▪ ↑ No. of late resorptions per litter (2 vs 0 control. HCD: 0-0.1) (ndr, ns).
▪ ↓ Live litter size (12.2 vs 14.1 control. HCD: 12.3-15.8) (ns).
▪ ↑ No. post implantation loss (9.8 vs 4.8 control. HCD: 2.1-9.4) (ndr) (ns).
<u>100, 15.6 and 2 mg/kg bw/day</u>
No treatment related effects.
Foetal data
<u>Foetal alterations</u>
<u>200 mg/kg bw/day</u>
Foetal external alterations
▪ 1 Foetus was found with an umbilical hernia (ndr) (n.s).
Foetal skeletal alterations
Vertebral thoracic:
▪ ↑ Incidence foetal (3.1%) and litter (16.7%; n.s.) of thoracic central bifid.
▪ ↑ Incidence foetal (1.9%) and litter (4.2%; n.s.) of uni-laterally ossified.
<table>
<tr><th>Skeletal alteration</th><th>Incidence</th><th>Control</th><th>200 mg/kg bw/day</th><th>(1) HCD</th></tr>
<tr><td colspan="2">No. of fetuses examined</td><td>182</td><td>160</td><td>3417</td></tr>
<tr><td colspan="2">No. of litters examined</td><td>25</td><td>24</td><td>413</td></tr>
<tr><td rowspan="2">Thoracic central bifid</td><td>Foetus</td><td>0/182</td><td>5/160 (3.1%)**</td><td>31/3417 (0.91%)</td></tr>
<tr><td>Litter</td><td>0/25</td><td>4/24 (16.7%)</td><td>30/413 (7.26%)</td></tr>
<tr><td rowspan="2">Thoracic central unilateral ossification</td><td>Foetus</td><td>0/182</td><td>3/160 (1.9%)**</td><td>2/3417 (0.06%)</td></tr>
<tr><td>Litter</td><td>0/25</td><td>1/24 (4.2%)</td><td>2/413 (0.48%)</td></tr>
</table>
** = p ≤ 0.01. (1) HCD: Historical Control Data for Crl:COBS®CD®(SD)BR. Argus Research Laboratories, INC.(Data for 1985 to 1986).
Ossification sites
▪ ↑ No. of hyoid (0.95 vs 0.72 in controls), no. of thoracic vertebrae (13.24 vs 13 controls) and no. of ribs (13.21 vs 13 controls).
▪ ↓ No. of lumbar vertebrae (5.75 vs 6 in controls) and sternal sternum (3.4 vs 3.73 in controls).
<table>
<tr><th>Ossification sites/litter</th><th>Incidence</th><th>Control</th><th>200 mg/kg bw/day</th></tr>
<tr><td colspan="2">No. of fetuses examined</td><td>182</td><td>160</td></tr>
<tr><td colspan="2">No. of litters examined</td><td>25</td><td>24</td></tr>
<tr><td>No. of hyoid ossification sites</td><td>foetus/litter</td><td>0.72</td><td>0.95*</td></tr>
<tr><td>No. of thoracic ossification sites</td><td>foetus/litter</td><td>13.00</td><td>13.24**</td></tr>
<tr><td>No. of lumbar ossification sites</td><td>foetus/litter</td><td>6.00</td><td>5.75**</td></tr>
<tr><td>No. of ribs</td><td>foetus/litter</td><td>13.00</td><td>13.21**</td></tr>
<tr><td>No. of sternal ossification sites</td><td>foetus/litter</td><td>3.73</td><td>3.40*</td></tr>
</table>
*p≤0.05; **p≤0.01
<u>100 mg/kg bw/day</u>
Foetal external alterations
▪ 1 foetus with depressed left eye bulge (ndr) (n.s).
▪ 1 foetus with agenesis of the diaphragmatic lobe (ndr) (n.s).
In absence of dose-dependency, these irreversible changes were considered incidental
</td><td></td></tr>
</table>

<table>
<tr><th>Method, guideline, GLP, species, strain, sex, no./group deviations, acceptability</th><th>Test substance, route administration, dose levels, duration of exposure, parameters observed</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td></td><td></td><td>
Foetal skeletal alterations
Vertebral thoracic:
▪ ↑ Foetuses (1.2%) and litter (8.7%) incidence of thoracic central bifid (n.s.).
<table>
<tr><th>Skeletal alteration</th><th>Incidence</th><th>Control</th><th>100 mg/kg bw/day</th><th>HCD</th></tr>
<tr><td colspan="2">No. of fetuses examined</td><td>182</td><td>160</td><td>3417</td></tr>
<tr><td colspan="2">No. of litters examined</td><td>25</td><td>24</td><td>413</td></tr>
<tr><td rowspan="2">Thoracic central bifid</td><td>Foetus</td><td>0/182</td><td>2/168 (1.2%)</td><td>31/3417 (0.91%)</td></tr>
<tr><td>Litter</td><td>0/25</td><td>2.23 (8.7%)</td><td>30/413 (7.26%)</td></tr>
</table>
No significant
<u>15.6 and 2 mg/kg bw/day</u>
No treatment related effects.
NOAEL developmental: 15.6 mg/kg bw/day based on increased incidences of some skeletal alterations in foetuses at 100 mg/kg bw/day.
</td><td></td></tr>
<tr><td>Rabbit developmental toxicity study.
<u>Method</u>: US EPA FIFRA 83-3 comparable to OECD 414 (1981).
<u>GLP</u>: Yes.
<u>Rabbit strain</u>: New Zealand White.
<u>Sex and no. animals</u>: 20 mated females/group
<u>Deviations from OECD 414</u>: The exposure period was during period of organogenesis (approximately days 7 to 19) instead of the recommended period from implantation (e.g. day 5 post mating) to the day prior to scheduled caesarean section (day 28).
Historical control data only pertains to fetal wt. Data were not presented for the other parameters evaluated in this investigation.</td><td><u>Test substance</u>: Difenoconazole technical (CGA 169374), batch FL-851406, purity 95.7%.
<u>Dose levels</u>: 0, 1, 25 and 75 mg/kg bw/day.
<u>Exposure</u>: from day 7 to 19 of presumed gestation.
<u>Parameters observed:</u>
Maternal data:
Clinical signs, mortality, body wts measurements, food consumption, ophtalmoscopic examinations
Reproductive data:
No. of corpora lutea, uterus and ovaries wt, no. of pregnancies dams, no. aborted, no. of implantation sites, number of resorptions, number of live and dead foetuses.
Foetal data:
Foetus wt, foetus sex</td><td>
<u>Maternal toxicity</u>
<u>75 mg/kg bw/day</u>
Mortality
▪ 1/19 animal died on gestational day 18 following a period of apparent treatment-related anorexia.
▪ In addition, 2/19 abortions on gestational days 18 and 24, respectively.
These abortions and the death observed were attributed to treatment.
Clinical signs
▪ ↑ stool variations 12/19 vs 2/19 controls (secondary to variation in food consumption) included the two dams with abortions.
Body weight gain
▪ ↓ 34% days 0-29 and initial weight loss (days: 7-10 (1,200%), days 10-14 (83%).
Food consumption
▪ ↓ Days 9-10 (49%), days 13-14 (48%), days 17-18 (35%), and 18-19 (26%).
<u>25 mg/kg bw/day</u>
No effects related with treatment
Clinical signs
▪ ↑ stool variations 7/19 vs 2/19 controls (secondary to variation in food consumption).
Body weight gain
▪ ↓ between days 0-29 full study (34%) and 0-29 U[1] full study (110%, ns).
[1] Corrected bw 0 terminal bw minus the wt of the uterus, ovaries, placetas and fetuses.
This reduction was seen to be a result of initial variations and the cumulative effects of slight reductions than a toxic response per se.
Food consumption
▪ ↓ Days: 23-24 (30%) and 26-27 (36%). Ndr, at 75 mg/kg bw day there is an increase.
<u>1 mg/kg bw/day</u>
No effects related with treatment
</td><td>Anonymous 26, (1987).
Anonymous 27, (1992)
(Report addendum)
B.6.6.2.2 (AS)</td></tr>
</table>

Method, guideline, GLP, species, strain, sex, no./group deviations, acceptability	Test substance, route administration, dose levels, duration of exposure, parameters observed	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
Study acceptable.	and foetus alterations (external, visceral and skeletal)	*Mortality* One animal died from dosing accidents on gestation day 16. *Food consumption* ▪ ↓ Days: 23-24 (31.5%) and 26-27 (34.4%). Ndr, at 75 mg/kg bw day there is an increase. **NOAEL** maternal **25 mg/kg bw/day** based on decreased body weight gain and food consumption, abortion and death at 75 mg/kg bw/day. ***Developmental toxicity*** ***Reproductive data*** **75 mg/kg bw/day** ▪ ↑ No. of early resorption (0.6 *vs* 0.3 control) (n.s). Total resorptions (0.9 *vs* 0.5 control) (n.s). ▪ ↑ No. of post implantation losses (0.92 *vs* 0.47 controls) (n.s). The mean % of implants per dam was 12.9% vs 7.4% control. ▪ ↑ Prenatal deaths (0.13 vs. 0.07 control) (n.s). **25 and 1 mg/kg bw/day** No treatment related effects. ***Foetal data*** *Foetal alterations* **75 mg/kg bw/day** *Visceral malformations* ▪ 1 horseshoe kidney and one partial cryptophthalmos (ndr) (n.s). These malformations were considered to be spontaneous and not related to treatment. **25 mg/kg bw/day** *External alterations* ▪ 1 foetus with raised, discoloured area on the ventral thoracic region (ndr) (n.s), which may be have resulted from technical manipulation and was considered to be incidental. *Visceral malformations* ▪ 1 foetus with microcephaly (ndr) (n.s). This malformation were considered to be spontaneous and not related to treatment. **1 mg/kg bw/day** No treatment related effects. **NOAEL** developmental **25 mg/kg bw/day** based on increased in the number and percent of resorptions (mainly early), increased in post implantation loss and prenatal death at 75 mg/kg bw/day.	

Table 34: Summary table of human data on adverse effects on development

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No evidence of adverse health effects in humans				

Table 35: Summary table of other studies relevant for developmental toxicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

Short summary and overall relevance of the provided information on adverse effects on development

The developmental toxicity of difenoconazole (CGA169374 technical) was investigated in two prenatal developmental toxicity studies, one in rat (B.6.6.2.1) and one in rabbit (B.6.6.2.2). Both studies predate the current OECD Test Guideline Number 414 (2001) and do not include the recommended extended dosing period (i.e. from implantation to one day prior to the day of scheduled kill). However, both studies are considered adequate and relevant for evaluation of the potential of difenoconazole to induce developmental effects. No evidence of teratogenicity was observed in either species.

In the **rat study** (B.6.6.2.1), at the highest dose tested of 200 mg/kg bw/day the onset of dosing was associated with a 14% loss of body weight (days 6-8). For the dosing period (days 6-15) the body weight gain was reduced by approximately 56% and the overall reduction (GD 0-20) was 12% lower than controls. Furthermore, a decrease of the food consumption was observed during days 6-16 though the most remarkable decrease was seen on days 8-9 (44%). The incidence of excess salivation was significantly increased in 19 out of 25 (76%) dams. There was one female at this dose level with severe weight loss that totally resorbed its litter.

In the reproductive effects, there was an increase in the number of early and late resorptions, increase in post implantation loss and decrease in litter size. Although these differences were not statistically significant or dose-dependent were outside historical controls.

In reference to foetal alterations, there were statistically significant alterations in the foetal ossification sites (an increase in the average number of ossified hyoid, number of thoracic vertebrae and mean number of ribs and a decrease in the average number of lumbar vertebrae and sternal sternum). In addition, there was an increased incidence of some skeletal alterations in litters (thoracic central bifid). Although these differences were not statistically significant, they were dose-dependent and out of the historical controls (16.7 *vs* 7.26 HCD).

At the intermediate dose, 100 mg/kg bw/day, the onset of dosing was associated with an overall statistically significant reduction in body weight gain of 23% (days 6-15), compared with controls and reduction in food consumption of approximately 14% (day 6-12). The incidence of excess salivation was significantly increased in 14 out of 23 (61%) dams. Full recovery was made in the post-dosing period and hence there was no effect on foetal body weight or ossification.

There was an increased incidence of some skeletal alterations in litters (thoracic central bifid). Although these differences were not statistically significant, they were dose-dependent (at this dose level and higher doses) and out of the historical controls (8.7 *vs* 7.26 CH).

No effects were seen in dams treated with 2 or 15.6 mg/kg bw/day.

In the **rabbit study** (B.6.6.2.2), at the highest dose tested of 75 mg/kg bw/day the onset of dosing was associated with loss body weight gain (days 7-10; 10-14 and 0-29) and loss of food consumption (days 9-19), abortion in two rabbits and death following anorexia in another rabbit.

There were no significant differences in pregnancy or litter parameters among the groups. There was an increase in the number and percent of resorptions (mainly early, 0.6 *vs* 0.3 control), increase in

post implantation loss (12.9%), and prenatal death (0.13 vs 0.07 control). Although these differences were not statistically significant the increase was noteworthy and, given the absence of historical controls, it cannot be ruled out that they are related to the treatment. No treatment-related external, visceral or skeletal abnormalities were seen.

No effects were seen in dams or in foetuses from dams treated with 1 or 25 mg/kg bw/day.

The effects observed in the offspring in a **2-generation study in rat** (B.6.6.1.1) at doses up to 2500 ppm (approximately equivalent to 171/189 mg/kg bw/day for males and females respectively) were:

In F_1 offspring generation there was a slight decrease, in percentage survival of male pups from days 0 to 4 pre-cull at 2500 ppm (95.2% *vs* 98.7% control). Although this decrease was statistically significant, it was slight and was not dose dependent, so it was considered incidental.

Treatment-related reductions in pup weights of F_1 and F_2 offspring generations were observed at 2500 ppm for both sexes through lactation period [lactation days 0, 4 (pre-and post-cull), 7, 14 and 21]. These reductions (6-30%) were statistically significant on all assessment occasions except for females on lactation day 0 of F_1 offspring generation. These reductions were associated with reductions in body weight and body weight gain in F_0 and F_1 dams during this period at this dose.

10.10.5 Comparison with the CLP criteria

Substances are classified in Category 1 for developmental toxicity when they are known to have produced an adverse effect on development in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with development in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A, known human reproductive toxicant) or from animal data (Category 1B, presumed human reproductive toxicant).

According to the CLP criteria a classification of a substance in category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on development is considered not to be a secondary non-specific consequence of other toxic effects.

Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on development is considered not to be a secondary non-specific consequence of the other toxic effects.

No human information is available on the effects of difenoconazole on development, but there is information from 2 reliable developmental studies in rat and rabbit and 2-generation study in rat.

In rat prenatal developmental toxicity study of difenoconazole, the effects observed were an increase in the number of early and late resorptions, increase in post implantation loss and decrease in litter size and skeletal alterations.

The effects observed in the offspring in a 2-generation study in rat were reductions in pup weights of F_1 and F_2 offspring generations.

In rabbit prenatal developmental toxicity study of difenoconazole, the effects observed were increase in the number of resorptions (mainly early), post implantation losses, and prenatal deaths.

According to *Annex I, section 3.7.2.4.2 of the Guidance on the Application of the CLP Criteria Version 5.0 – July 2017*, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species. However, the limited number of studies which have investigated the relationship between developmental effects and general maternal toxicity have failed to demonstrate a consistent, reproducible relationship across species.

According to the results of submitted studies, no irreversible effects such as structural malformations, foetal embryo/lethality, and significant postnatal functional deficiencies were observed. The effects observed were minor developmental changes and were not statistically significant or dose dependent and they could be associated with maternal toxicity. Consequently, classification is not warranted.

10.10.6 Adverse effects on or via lactation

The classification is intended to indicate when a substance may cause harm due to its effects on or via lactation and is independent of consideration of the reproductive or developmental toxicity of the substance. This can be due to the substance being absorbed by women and adversely affecting milk production or quality, or due to the substance (or its metabolites) being present in breast milk in amounts sufficient to cause concern for the health of a breastfed child.

The reproductive study available, does not provide evidence of adverse effects in the offspring due to transfer in the milk or adverse effect on the quality of the milk. Toxicokinetics studies do not indicate the likelihood that the substance can be potentially present in breast milk.

Table 36: Summary table of human data on effects on or via lactation

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No evidence of adverse health effects in humans				

Table 37: Summary table of other studies relevant for effects on or via lactation

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.10.7 Comparison with the CLP criteria

The classification is intended to indicate when a substance may cause harm due to its effects on or via lactation and is independent of consideration of the reproductive or developmental toxicity of the substance. There were no effects to warrant classification of difenoconazole, for effects on or via lactation.

10.10.8 Conclusion on classification and labelling for reproductive toxicity

Not classified (conclusive but not sufficient for classification).

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The DS proposed no classification of difenoconazole for sexual function and fertility based on a 2-generation reproductive toxicity study showing no alterations in reproductive parameters and no significant offspring toxicity.

The DS proposed no classification of difenoconazole for developmental toxicity based on one teratogenicity study in rats and one teratogenicity study in rabbits showing minor developmental changes that were not statistically significant or dose-dependent and that could be associated to maternal toxicity.

The DS proposed no classification of difenoconazole for adverse effects on or via lactation since the reproductive study does not provide evidence of adverse effects in the offspring due to transfer in the milk or adverse effect on the quality of the milk.

Comments received during consultation

One company-manufacturer supported the proposal for no classification of difenoconazole for reproductive toxicity.

Assessment and comparison with the classification criteria

2-generation reproductive toxicity study

The table below summarises the 2-generation reproductive toxicity study with difenoconazole. This study is deficient in some endpoints including oestrus cyclicity, landmarks of sexual development, sperm analysis and ovarian follicle counts. The histopathology undertaken is limited in adults and offspring and, in particular, the target organ (liver) toxicity has not been evaluated.

Toxicity in the parental animals (F0 and F1) were observed at 2500 ppm. It included reduced body weight, body weight gain and food consumption. In F0 parents during the pre-mating period the reduction of body weight as regard control group was 8% for males and 15% for females, the reduction of body weight gain was 12 to 14% for males and 30% for females and during mating the reduction of body weight as regard the control was approximately 9% for males and persisted into gestation (body weight was 12% lower and body weight gain was 34%) and lactation (body weight was 13% lower and body weight gain was 52% lower) for females. F1 animals also showed reductions in mean body weight and weight gain during the pre-mating period (body weight was more than 15% lower for males and females; body weight gain was 10% lower for males and 22% for females), this reduction persisted in females into gestation and lactation (body weight was more than 20% lower and body weight gain was 30% lower during the first week of gestation).

No adverse effect of difenoconazole on sexual function or the fertility of the rat was identified at dose levels which induced some parental toxicity. Furthermore, there were no effects of

difenoconazole on the development of the offspring other than lower body weights at birth. In F1 offspring generation there was a slight decrease, in percentage survival of male pups from days 0 to 4 at 2500 ppm.

Treatment-related reductions in pup weights of F1 and F2 offspring generations were observed at 2500 ppm for both sexes through lactation period. These reductions were statistically significant on all assessment occasions except for females on lactation day 0 of F1 offspring generation.

Table: Summary of animal studies on adverse effects on sexual function and fertility with difenoconazole.

Method	Results	Reference
2-generation reproductive toxicity study OECD TG 416 (1981) GLP: compliant Sprague Dawley rats F0 and F1: 30 rats/sex/dose Deviations: No oestrus cyclicity, landmarks of sexual development, no sperm analysis and ovarian follicle counts Purity: 97.4 % Oral (diet) 0, 25, 250 or 2500 ppm F0 and F1 (mean): males: 0, 1.7, 17, or 171 mg/kg bw/day females: 0, 1.9, 19, 189 mg/kg bw/day Pre-mating treatment: F0 (77 days); F1 (98 days); Treatment continued in F0 and F1 throughout gestation and lactation	**PARENTAL TOXICITY** See table summary of repeated dose toxicity studies in rats with difenoconazole **REPRODUCTIVE TOXICITY** There were no treatment-related effects **OFFSPRING TOXICITY** <u>2500 ppm (171/189 mg/kg bw/day)</u>	Anonymous 23, 1988 B.6.6.1.1 (AS)

	F1 offspring		F2 offspring	
	males	females	males	females
Survival (days 0 to 4 pre-cull)	95.2% vs 98.7%	-	-	-
Pup weight (birth)	↓6%	-	↓8.2%	↓7.4%
Pup weight (day 4 pre-cull)	↓13%	↓11%	↓14%	↓13%
Pup weight (day 4 post-cull)	↓14%	↓11%	↓15%	↓14%
Pup weight (day 7)	↓23%	↓20%	↓21%	↓20%
Pup weight (day 14)	↓27%	↓26%	↓26%	↓26%
Pup weight (day 21)	↓30%	↓29%	↓33%	↓32%

<u>250 ppm (17/19 mg/kg bw/day)</u>

	F1 offspring		F2 offspring	
	males	females	males	females
Pup weight (birth)	-	-	-	-
Pup weight (day 4 pre-cull)	-	-	-	-
Pup weight (day 4 post-cull)	-	-	-	-
Pup weight (day 7)	-	-	-	-
Pup weight (day 14)	-	-	-	-
Pup weight (day 21)	↓7%	-	-	-

Developmental toxicity study in rats

The study was performed following the OECD TG 414 guideline in compliance with GLP. Crl rats were dosed by gavage with 0, 2, 16, 100 and 200 mg/kg bw/day of difenoconazole (purity 95.7%) during gestation days 6-15. Table summary of repeated dose toxicity studies in rats with difenoconazole summarises the maternal toxicity. At the highest dose of 200 mg/kg bw/day the onset of dosing was associated with a 14% loss of body weight (days 6-8) as regard the control group. For the dosing period (days 6-15) the body weight gain was reduced by approximately 56% and the overall reduction (GD 0-20) was 12% lower as regard controls. Excess salivation was significantly increased in 19 out of 25 (76%) dams. There was one female at this dose level with severe weight loss that totally resorbed its litter.

At the intermediate dose, 100 mg/kg bw/day, the onset of dosing was associated with an overall statistically significant reduction in body weight gain of 23% (days 6-15) compared to control. The incidence of excess salivation was significantly increased in 14 out of 23 (61%) dams. Full recovery was made in the post-dosing period and hence there was no effect on foetal body weight or ossification.

The table below summarises the foetal alterations. At the top dose, there were statistically significant alterations in the foetal ossification sites (an increase in the average number of ossified hyoid, number of thoracic vertebrae and mean number of ribs and a decrease in the average number of lumbar vertebrae and sternal sternum). In addition, there was an increased incidence of some skeletal alterations in litters (thoracic central bifid). At 100 mg/kg bw/day there was an increased incidence of some skeletal alterations in litters (thoracic central bifid). Although these differences were not statistically significant, they were dose-dependent (at this dose level and higher doses) and out of the historical control data (8.7 vs 7.26 HCD). No treatment-related effects on foetus were noted at 16 and 2 mg/kg bw/day.

Table: Foetal alterations detected in the teratogenicity study in rats with difenoconazole. * = Statistically different form control for p ≤ 0.05 * = Statistically different form control for p ≤ 0.01.

		Control	**200 mg/kg bw/day**	**100 mg/kg bw/day**	**HCD**
Examined foetuses		**182**	**160**	**160**	**3417**
Examined litters		**25**	**24**	**24**	**413**
Thoracic central bifid	foetus	0/182	5/160 (3.1%)**	2/168 (1.2%)	31/3417 (0.91%)
Thoracic central bifid	litter	0/25	4/24 (17%)	2/23 (8.7%)	30/413 (7.3%)
Thoracic central unilateral ossification	foetus	0/182	3/160 (1.9%)**	-	2/3417 (0.06%)
Thoracic central unilateral ossification	litter	0/25	1/24 (4.2%)	-	2/413 (0.48%)
No. of hyoid ossification sites	foetus/litter	0.72	0.95*	-	-
No. of thoracic ossification sites	foetus/litter	13.0	13.2**	-	-
No. of lumbar ossification sites	foetus/litter	6.0	5.8*	-	-
No. of ribs	foetus/litter	13.0	13.2**	-	-
No. of sternal ossification sites	foetus/litter	3.7	3.4*	-	-

Developmental toxicity study in rabbits

The study was performed following OECD TG 414 guideline and observing GLP procedures. New Zealand White rabbits were dosed by gavage with 0, 1, 25 and 75 mg/kg bw/day of

difenoconazole (purity 95.7%) during gestation days 7-19. Table summary of repeated dose toxicity studies in rats with difenoconazole summarises the maternal toxicity. At the highest dose the onset of dosing was associated with loss body weight gain (days 7-10; 10-14 and 0-29), abortion in two rabbits and death following anorexia in another rabbit.

There were no significant differences in pregnancy or litter parameters among the groups. There was an increase in the number and percent of resorptions (mainly early, 0.6 vs 0.3 control), increase in post implantation loss (12.9%), and prenatal death (0.13 vs 0.07 control). Although these differences were not statistically significant the increase was noteworthy and, given the absence of HCD, it cannot be ruled out that they are related to the treatment. No treatment-related external, visceral or skeletal abnormalities were seen. No effects were seen in dams or in foetuses from dams treated with 1 or 25 mg/kg bw/day.

Comparison with criteria

Difenoconazole caused no adverse effects on sexual function or fertility at dose levels causing parental toxicity. Overall, RAC supports the DS's proposal for **no classification of difenoconazole for sexual function and fertility.**

According to the results of submitted studies, no irreversible effects such as structural malformations, foetal embryo/lethality, and significant postnatal functional deficiencies were observed. The effects observed were minor developmental changes that could be associated with maternal toxicity (excess salivation in 19/25 dams, ↓56% body weight gain days 6-15 and ↓12% body weight gain on days 0-20). A slight decrease in the percentage survival of male pups from days 0 to 4 pre-cull at 2500 ppm was noted. However, it was slight and not sufficient for supporting a classification. Treatment-related reductions in pup weights of F1 and F2 offspring generations in both sexes were noted at the top dose through lactation period. However, these reductions could be associated with reductions in body weight and body weight gain in F0 and F1 female parents during this period at this dose. Overall, RAC supports the DS's proposal for **no classification of difenoconazole for developmental toxicity.**

The classification is intended to indicate when a substance may cause harm due to its effects on or via lactation and is independent of consideration of the reproductive or developmental toxicity of the substance. This can be due to the substance being absorbed by women and adversely affecting milk production or quality, or due to the substance (or its metabolites) being present in breast milk in amounts sufficient to cause concern for the health of a breastfed child.

The available reproductive study does not provide evidence of adverse effects in the offspring due to transfer in the milk or adverse effect on the quality of the milk. Toxicokinetic studies do not indicate the likelihood that the substance can be potentially present in breast milk. Thus, there were no effects to warrant classification of difenoconazole for effects on or via lactation. RAC supports the DS's proposal for **no classification of difenoconazole for adverse effects on or via lactation.**

10.11 Specific target organ toxicity-single exposure

10.11.1 Short summary and overall relevance of the provided information on specific target organ toxicity – single exposure

Specific target organ toxicity (single exposure) is defined as specific, non-lethal target organ toxicity arising from a single exposure to a substance or mixture. Relevant information for STOT SE is covered by acute toxicity studies in form of clinical observations, and macroscopic and microscopic pathological examination that can reveal hazards that may not be life-threatening but could indicate functional impairment. Acute toxicity studies are included in section 10.1.

STOT SE 3

STOT SE3 includes narcotic effects and respiratory tract irritation. These are target organ effects for which a substance does not meet the criteria to be classified in Categories 1 or 2.

According to the results of the acute inhalation study (see Table 16), respiratory tract irritation was not observed upon administration of difenoconazole.

Narcotic effects were not observed in acute toxicity studies.

STOT SE 1 and 2

STOT-SE Category 1 and 2 is assigned on the basis of findings of 'significant' or 'severe' toxicity. In this context, 'significant' means changes which clearly indicate functional disturbance or morphological changes which are toxicologically relevant. 'Severe' effects are generally more profound or seriouis than 'significant' effects and are of considerably adverse nature with significant impact on health. Both factors have to be evaluated by weight of evidence and expert judgement.

Table 38: Summary table of animal studies on STOT SE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Acute oral toxicity study in rats OECD TG 401 (1981) Rat, Sprague Dawley 5 Rats/sex/group **Study acceptable** *Guideline value for classification:* *STOT SE 1≤ 300 mg/kg bw/day* *STOT SE 2≤ 2000 mg/kg bw/day*	Purity: Not specified Oral (gavage) Doses: 0, 1000, 2000, 3000 mg/kg bw	**Clinical signs** Hypoactivity, stains around the mouth, perineal staining, ataxia, lacrimation, soft faeces, hypothermia, prostration, chromodacryorrhea, chromorhinorrhea, spasms, salivation, unkept appearance, rhinorrhoea, hypopnoea, ptosis. **Body weight** Slight decrease in mean male and female body weight gain at the 2000 mg/kg bw group. **Necropsy** Pronounced stomach lesions in deceased animals during the study.	**Anonymous 7 (1987) B.6.2.1.-01 (AS)**
Acute oral toxicity study in the mouse OECD TG 401 (1981) Mouse, Tif: MAG f (SPG) mice	Purity: Not specified Oral (gavage) Doses: 1000, 2000 mg/kg bw	**Clinical signs** Piloerection, abnormal body positions, dyspnea, reduced locomotor activity and ataxia. Animals in the 2000 mg/kg bw group also showed tonic spasms. **Necropsy**	**Anonymous 8 (1990) B.6.2.1.-02 (AS)**

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, route of exposure, dose levels, duration of exposure**	**Results**	**Reference**
5 animals/sex/group **Study acceptable** *Guideline value for classification:* *STOT SE 1≤ 300 mg/kg bw/day* *STOT SE 2≤ 2000 mg/kg bw/day*		No relevant findings.	
Acute neurotoxicity study in rats OECD 424 (1997) EEC B.43 GLP: Yes Rat, Alpk:APfSD (Wistar-derived) 10 rats/sex/dose **Study acceptable** *Guideline value for classification:* *STOT SE 2 ≤ 2000 mg/kg bw* *STOT SE 1 ≤ 300 mg/kg bw*	Purity: 94.3% Oral (gavage) Doses of 0, 25, 200 and 2000 mg/kg bw Parameters observed: Mortality, clinical signs, body weight and food consumption, functional observational battery (FOB), motor activity, brain weights and neuropathology.	No mortalities occurred during the study. **2000 mg/kg bw** Clinical signs *Significance of statistical tests not available on clinical signs* ▪ Reduced splay reflex [day 1 in ♂ (1/10) and ♀ (1/10); day 7 in ♀ (2/10)] ▪ Upward curvature of spine [day 1 in ♂ (8/10) and ♀ (9/10)] ▪ Decreased activity [day 1 in ♂ (6/10) and ♀ (7/10)] ▪ Piloerection [day 1 in ♂ (3/10) and ♀ (5/10)] ▪ Sides pinched [day 1 in ♂ (3/10) and ♀ (7/10)] ▪ Anormal (tio-toe) gait [day 1 in ♂ (3/10) and ♀ (8/10)] Body weight and food consumption ▪ (↓) bw in ♂ [day 1 (7%), day 8 (5%) and day 15 (4%)] and ♀ [day 1 (7%)] ▪ (↓) Food consumption in ♂ [week 1 (19%)] FOB ▪ (↑) Time to tail flick in ♀ [day 1 (5.6 *vs.* 4.1 of controls)] ▪ (↓) Fore-limb grip strength in ♂ [day 1 (26%)] ▪ (↑) Fore-limb grip strength in ♀ [day 15 (22%)] ▪ (↑) Hind limb grip strength in ♀ [day 8 (17%)] Motor activity ▪ (↑) in ♂ [day 1 (55%)] ▪ (↓) in ♀ [day 1 (37%); day 8 (31%)] ▪ **200 mg/kg bw** Body weight ▪ (↓) Bw in ♂/♀ [day 1 (2%/ 2%)] FOB ▪ (↓) Fore-limb grip strength in ♂ [day 1 (23%)] ▪ (↓) Hind-limb grip strength in ♂ [day 15 (21%)] Motor activity ▪ (↑) in ♂ [day 1 (50%)] **NOAEL general toxicity:** 200 ppm mg/kg bw/day **NOAEL neurotoxicity:** > 2000 ppm mg/kg bw/day	**Anonymous 28 (2006)** **B.6.7.1.1. (AS)**

10.11.2 Comparison with the CLP criteria

Effects observed in the range of STOT SE 1 (guidance value for classification: ≤ 300 mg/kg bw) observed in male rats in the acute neurotoxicity study (B.6.7.1.1) included increased motor activity on day 1, decreased fore-limb grip strength on day 1 and decrease in hind-limb grip strength on day 15. The effecs observed on day 1 reversed at the end of the study and therefore, they are not considered adverse. The statistically-significant difference from control in hind-limb grip is not considered to be related to treatment as this effect was not seen in the high or low dose groups. Overall, these effects are not indicative of 'significant' or 'sever' changes and therefore, they are not regarded for STOT SE 1.

The only effects observed in the range for STOT SE 2 after oral administration included in Table 33 are not relevant for classification (guidance value for classification: ≤ 2000 mg/kg bw and >300 mg/kg bw):

- All treatment-related motor activity and clinical signs (reduced splay reflex, upward curvature of spine, decreased activity, sides pinched, anormal (tio-toe) gait) observed on day 1 showed complete recovery by day 5 (males) and day 7 (females).
- Increased fore-limb grip strength in females on day 15 was statistically-different to control animals. This difference was only seen in females and at one time-point and therefore, this observation is considered to be incidental to treatment with difenoconazole.

No signs were observed to be regarded for classification for STOT SE 3 according to CLP Regulation (respiratory tract irritation and narcotic effects).

10.11.3 Conclusion on classification and labelling for STOT SE

Difenoconazole does not require classification for STOT SE according to CLP Regulation.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

DS proposed no classification of difenoconazole for STOT SE based on the lack of non-lethal target organ toxicity, narcotic effects or respiratory tract irritation found in the acute toxicity studies and in the acute neurotoxicity study in rats.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The CLH report contains information about four different acute toxicity studies; which are summarised in the table above and the acute neurotoxicity study summarised in the table below.

Table: Summary of acute neurotoxicity study in rat with difenoconazole.

Study	Dose level	Results			Reference
Acute neurotoxicity study OECD 424 (1997) EEC B.43 GLP: Yes Alpk:APfSD rats 10 rats/sex/dose Oral (gavage)	Purity: 94.3% Doses of 0, 25, 200 and 2000 mg/kg bw	No mortalities occurred during the study 2000 mg/kg bw			Anonymous 28, 2006 B.6.7.1.1. (AS)
			males	females	
		Clinical signs (statistical significance not available)			
		Reduced splay reflex (day 1)	1/10	1/10	
		Reduced splay reflex (day 7)	0/10	2/10	
		Upward curvature of spine (day 1)	8/10	9/10	
		Decreased activity (day 1)	6/10	7/10	
		Piloerection (day 1)	3/10	5/10	
		Sides pinched (day 1)	3/10	7/10	
		Abnormal gait (day 1)	3/10	8/10	
		Body weight and food consumption			
		Body weight (day 1)	↓7%	↓7%	
		Body weight (day 8)	↓5%	-	
		Body weight (day 15)	↓4%	-	
		Food consumption (week 1)	↓19%	-	
		Functional Observational Battery			
		Time to tail flick (day 1)	-	5.6 vs 4.1 controls	
		Fore-limb grip strength (day 1)	↓26%	-	
		Fore-limb grip strength (day 15)	-	↑22%	
		Hind limb grip strength (day 8)	-	↑17%	
		Motor activity			
		Day 1	↑55%	↓37%	
		Day 8	-	↓31%	
		200 mg/kg bw			
			males	females	
		Body weight (day 1)	↓2%	↓2%	
		Fore-limb grip strength (day 1)	↓23%	-	
		Hind limb grip strength (day 15)	↓23%	-	
		Motor activity			
		Day 1	↑50%	-	

The effects noted in the range warranting classification as STOT SE 1 (≤ 300 mg/kg bw) were observed in the acute neurotoxicity study at 200 mg/kg bw and included increased motor activity on day 1, decreased fore-limb strength on day 1 and decrease in hind-limb strength on day 15 (table above). Both effects observed on day 1 were reverted at the end of the study.

The reduction in hind limb grip strength reported on day 15 was not noted at 2000 mg/kg bw; which suggests that this effect could be incidental.

The effects reported in the acute oral and inhalation toxicity studies (table summary of animal studies on acute toxicity with difenoconazole) could be considered for classification as STOT SE 2. However, the effects in oral toxicity were noted at doses causing mortalities and therefore should not be used for setting STOT SE classification in order to avoid double classification. The effects noted in the acute inhalation toxicity study seems to be unspecific rather than organ specific and thus could not be used for warranting STOT SE classification (table summary of animal studies on acute toxicity with difenoconazole).

The top dose (2000 mg/kg bw) in the acute neurotoxicity study is borderline for warranting classification as STOT SE 2. At this dose level, the effects observed were treatment-related motor activity and clinical signs observed on day 1 (table summary of acute neurotoxicity study in rat with difenoconazole) and that were fully reverted by day 5 (males) and on day 7 (females). These effects are not considered by RAC severe enough for supporting a classification. Moreover, at this borderline dose also an increment in fore-limb grip strength in females on day 15 was noted. However, this difference was only seen in females and at one time-point and therefore, this observation is considered by RAC as incidental to treatment with difenoconazole.

No narcotic effects or respiratory tract irritation were observed in the available studies (tables above). Thus, classification as STOT SE 3 is not warranted.

Overall, **RAC supports the DS's proposal for no classification of difenoconazole for STOT SE.**

10.12 Specific target organ toxicity-repeated exposure

Table 39: Summary table of animal studies on STOT RE

For more detailed information please refer to RAR B.6 (AS) Chapter 6.3

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
ORAL ROUTE			

28-day cumulative oral toxicity (feeding) study in the rat OECD 407 (1981) GLP: No Rats, SPF-bred Wistar (initial age of 4 weeks) 10 rats/sex/dose Deviations: Necropsy done on day 33; dose intervals are higher than recommended (6 to 7 vs 2 to 4) No full histopathological examination was carried out, as required by TG, *i.e.* only the livers and gross lesions were histopathologically examined. HC: Historical Control; Research Consulting Company AG, Switzerland, Feb 81-Oct 84 (Wistar/Han male and females rats, age from 7 to 9 weeks) **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 300 mg/kg bw/day* *STOT RE 1 ≤ 30 mg/kg bw/day* *(28-day oral study)*	Purity: 95% Oral (diet) Doses of 0, 250, 1500 or 10000 ppm equivalent to 0, 27, 156/166 and 914/841 mg/kg bw/day (♂/♀) Parameters observed: Mortality, clinical signs, body weight and food consumption, biochemistry, haematology, urinalysis, organ weights, gross pathology and histopathology on liver and gross lessions. No statistical tests were performed on food consumption	No mortalities occurred during the study **10000 ppm** (914♂/841♀ mg/kg bw/day) <u>Body weight and food consumption</u> *Significance of statistical tests not available on food consumption* ▪ (↓) bw in ♂/♀ [week 1 (30%/29%), week 2 (39%/36%), week 3 (40%/36%) and week 4 (42%/36%)] ▪ (↓) Food consumption in ♂/♀ [week 1 (75%/71%), week 2 (41%/44%), week 3 (33%/41%) and week 4 (36% /44%)] <u>Heaematology</u> ▪ (↓) Hb in ♂/♀ [(6%/9%] within HC ▪ (↓) Haematocrit in ♂/♀ (4%/9%) within HC ▪ (↓) MCV in ♂/♀ (6%/8%) within HC ▪ (↓) MCH in ♂/♀ (7%/9%) within HC ▪ (↓) Thromboplastin time in ♂/♀ (8%/9%) out of HC in ♂ ▪ (↓) Platelets in ♀ (12%) within HC ▪ (↑) Reticulocyte count in ♀ (68%) within HC <u>Clinical chemistry</u> ▪ (↑) Cholesterol in ♂/♀ (230%/231%) out of HC ▪ (↓) Sodium in ♂/♀ (1% ncdr/1% ncdr) within HC ▪ (↑) Inorganic phosphorus in ♀ (15% ncdr) within HC ▪ (↑) γ-Glutamyl transferase in ♂/♀ (75%/73%) no HC provided. ▪ (↑) AP in ♂/♀ (37%/68%) out of HC ▪ (↑) AST in ♂ (11%) within HC ▪ (↑) Albumin abs levels in ♂/♀ (15%/9%) out of HC and (↑) rel levels in ♂/♀ (20%/5% ndr) out of HC ▪ (↓) α1-Globulin abs levels in ♂/♀ (29%/10%) out of HC in ♂ and rel levels in ♂/♀ (26%/13%) out of HC in ♂ ▪ (↓) β-globulins abs levels in ♂ (29%) and rel levels in ♂ (27%) out of HC ▪ (↑) α2-globulin abs levels in ♀ (21%) and rel levels in ♀ (21%) within HC ▪ (↓) ɤ-Globulin rel levels in ♀ (29%) within HC ▪ (↑) A/G ratio in ♂/♀ (58%/13% ncdr) out of HC <u>Urinalysis</u> ▪ (↑) Ketones in ♂/♀ <u>Organ weights</u> ▪ (↓) Terminal bw (carcass) in ♂/♀ (44 % ncdr/40%) ▪ Liver: (↓) abs wt in ♂ (14% ndr) and (↑) rel wt in ♂/♀ (54%/74%) ▪ Kidneys: (↓) abs wt in ♂/♀ (37%/28%) and (↑) rel wt in ♂/♀ (13%/20%) ▪ Brain: (↓) abs wt in ♂/♀ (14%/11%) and (↑) rel wt in ♂/♀ (55% 48%) ▪ Heart: (↓) abs wt in ♂/♀ (39%/35%) ▪ Spleen: (↓) abs wt in ♂/♀ (38% ndr/42%) ▪ Thymus: (↓) abs wt in ♂/♀ (49% ndr/47% ndr) ▪ Testes: (↓) abs wt (18%) and (↑) rel wt (49%) ▪ Ovaries: (↓) abs wt (56%) and (↓) rel wt (40%) ▪ Adrenals: (↓) abs wt in ♀ (29%) and (↑) rel wt in ♂ (50%) ▪ Thyroid: (↓) abs and rel wt in ♂ and (↑) rel wt in ♀. No data on the magnitude of variations available. **1500 ppm** (156♂/166♀ mg/kg bw/day) <u>Heaematology</u> ▪ (↓) Thromboplastin time in ♂/♀ (6%/4%) out of HC in males. <u>Clinical chemistry</u> ▪ (↑) Albumin abs levels in ♂ (8%) and rel levels in ♂ out of HC (10%). ▪ (↓) α1-Globulin abs levels in ♂ (9%) and rel levels in ♂ (8%) within HC. ▪ (↑) α1-Globulin abs levels in ♀ (10%) within HC. ▪ (↓) β-globulins abs levels in ♂ (22%) and rel levels in ♂ out of HC (21%)	**Anonymous 29 (1986a) B.6.3.1.1 (AS)**

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
		▪ (↑) A/G ratio in ♂ (26%) out of HC Urinalysis ▪ (↑) Ketones in ♂ Organ weights ▪ (↓) Terminal bw in ♀ (carcass) (6%) ▪ Liver: (↑) abs wt in ♂ (17% ndr) and (↑) rel wt in ♂/♀ (22%/15%) ▪ Kidneys: (↓) abs wt in ♀ (8%) and (↑) rel wt in ♂ (10%) **250 ppm** (27♂/27♀ mg/kg bw/day) Haematology ▪ (↓) Thromboplastin time in ♀ (4%) out of HC Clinical chemistry ▪ (↑) Albumin rel levels in ♂ (8%) within HC ▪ (↓) α1-Globulin abs levels in ♂ (14%) and rel levels in ♂ (12%) within HC ▪ (↓) β-Globulins abs levels in ♂ (13%) and rel levels in ♂ (12%) within HC (↑) A/G ratio in ♂ (18%) within HC Urinalysis ▪ (↑) Ketones in ♂ Organ weight ▪ Terminal bw (carcass) (↓ ncdr) in ♂ (14%) ▪ Liver: (↓) abs wt in ♂ (20% ndr) ▪ Spleen: (↓) abs wt in ♂ (25% ndr) **NOAEL**: 1500 ppm (156 ♂ and 166 ♀mg/kg bw/day) **LOAEL**: 10000 ppm (914♂ and 841 ♀mg/kg bw/day)	
13-week oral toxicity (feeding) study in the rat OECD 408 (1981) GLP: No (quality control by the laboratory itself) Rats, SPF Wistar (initial age of 4 weeks) 10 rats/sex/dose. Additional satellite group of 10 animals/sex/dose at 0 and 1500 ppm continued on diet during a 4-week recovery period Deviations: Dose intervals are higher than recommended (6 fold vs 2 to 4). Wet weights of the epididymidis, uterus and spleen were not recorded; histopathology of mammary gland,	Purity: 94.5% Oral (diet) Doses of 0, 40, 250 or 1500 ppm equivalent to 0, 3.3/3.5, 20/21 and 121/129 mg/kg bw/day (♂/♀) Parameters observed: Mortality, clinical signs, body weight and food and water consumption, biochemistry, haematology, urinalysis, organ weights and histopathology of selected tissues. Hearing tests and ophthalmoscopy examinations was carried out during pre-tests	No mortalities occurred during the study. No treatment-related clinical symptoms or signs of toxicity were evident. **1500 ppm** (121♂/129♀ mg/kg bw/day) Body weight and food consumption *Significance of statistical tests not available on food and water consumption (not presented due to low ns (2-4 cages)) and body weight gain (not performed).* ▪ (↓) Bw in ♂/♀ [week 9 (12% /9%), week 13 (13% /10%)]. At week 17 (recovery period) 9%/4% respectively n.s. ▪ (↓) Food consumption in ♂/♀ [week 9 (11%/13%) and week 13 (11%/11%)]. ▪ (↓) Water consumption in ♂/♀ [week 4 (10%/14%), week 9 (17%/25% ndr), week 13 (16%/23% ncdr)]. Heaematology ▪ (↓) RBC in ♂ [week 13 (3%) and week 17 (3%)] within HC ▪ (↓) WBC in ♂ [week 13 (16%)] within HC ▪ (↓) Platelet count in ♂ [week 13 (12% ncdr)] within HC Clinical chemistry ▪ (↑) Phosphorus in ♀ [week 13 (17% ncdr)] and in ♂/♀ [week 17 (8%/17%)] within HC ▪ (↑) ALP in ♂/♀ [week 13 (38%/48%)] and in ♂ [week 17 (13%)] within HC ▪ (↑) Urea in ♂ [week 13 (10% ncdr) and week 17 (11%)] within HC ▪ (↑) Chloride in ♂ [week 13 (2% ndr)] within HC ▪ (↑) GGT in ♂ [week 13 (34%)] within HC ▪ (↑) LDH in ♂ [week 17 (14%)] within HC ▪ (↓) Creatinine in ♀ [week 13 (17%)] within HC ▪ (↓) Cholesterol in ♀ [week 17 (15%)] within HC ▪ (↓) Sodium in ♀ [week 13 (1%)] within HC	**Anonymous 30 (1986b) B.6.3.2.1.1 (AS)**

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
skin and salivary glands was not performed. Sensory reactivity to external smituly was not examined. HC: Historical Control; Research Consulting Company AG, Switzerland, Jun 81-Oct 84 (Wistar/Han male and females rats, age from 19 to 36 weeks) **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 100 mg/kg bw/day* *STOT RE 1 ≤ 10 mg/kg bw/day* *(90-day oral study)*	and at the end of treatment.	▪ (↑) Potassium in ♀ [week 17 (10%)] within HC ▪ (↓) Protein in ♂ [week 13 (4%)] and in ♀ [week 17 (4%) within HC ▪ (↑) Relative albumin in ♂ [week 13 (10%)] within HC ▪ (↓) Relative α1-globulin in ♂/♀ [week 13 (21% ndr / 5% ndr)] within HC ▪ (↑) Relative α1-globulin in ♀ [week 17 (9%)] within HC ▪ (↓) Relative β-globulins in ♂ [week 13 (9% ncdr)] within HC ▪ (↑) A/G ratio [week 13 (22%)] within HC <u>Urinalysis</u> ▪ (↓) pH in ♂ [week 13 (6 *vs.* 7 in controls)] within HC ▪ (↑) Ketones in ♂ [week 13 (score of 2 *vs.* 1 in controls)] ▪ (↑) Urobilinogen [week 13 (score of 1 *vs.* 0 in controls)] ▪ (↓) Specific gravity in ♀ [(1.02 *vs.* 1.03 in controls)] within HC ▪ (↓) Blood in ♀ (score of 0 *vs.* 1 in controls) <u>Organ weights</u> ▪ Terminal bw (↓) in ♂/♀ (14%/13%) ▪ Liver: (↑) abs wt in ♂/♀ [week 13 (18%/22%] and (↑) rel wt in ♂/♀ [week 13 (33%/39%)] ▪ Brain: (↑) rel wt in ♂/♀ [week 13 (20%/25%) and week 17 (20%/13%)] ▪ Testes: (↑) rel wt [week 13 (11%)] ▪ Ovaries: (↑) rel wt [week 13 (25%)] ▪ Heart: (↓) abs wt [week 13 (11%) and (↓) rel wt [week 13 (magnitude n.a.)] **250 ppm** (20♂/21♀ mg/kg bw/day) <u>Clinical chemistry</u> ▪ (↑) Chloride in ♂ [week 13 (ncdr)] within HC ▪ (↑) Relative albumin in ♂ [week 13 (5%)] within HC ▪ (↓) Relative β-globulins in ♂ [week 13 (13% ncdr)] within HC <u>Urinalysis</u> ▪ (↑) Urobilinogen [week 13 (score of 1 *vs.* 0 in controls)] ▪ (↓) Blood in ♀ (score of 0 *vs.* 1 in controls) <u>Organ weights</u> ▪ Liver: (↑) rel wt in ♂/♀ [week 13 (11% / 19%)] **40 ppm** (3.3♂/3.5♀ mg/kg bw/day) <u>Clinical chemistry</u> ▪ (↑) Chloride in ♂ [week 13 (ncdr)] within HC ▪ (↓) Relative β-globulins in ♂ [(7% ncdr)] within HC **NOAEL**: 250 ppm (20♂ and 21♀ mg/kg bw/day) **LOAEL**: 1500 ppm (121 ♂ and 129 ♀ mg/kg bw/day)	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
13-Week oral toxicity (feeding) study in the rat OECD 408 (1981) GLP: Yes Rats, SPF Wistar 15 rats/sex/dose 10 rats/sex as control group No historical control data provided Deviations: Wet weights of uterus and thymus were not recorded; histology of a mammary gland was not performed. **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 100 mg/kg bw/day* *STOT RE 1 ≤ 10 mg/kg bw/day* *(90-day oral study)*	Purity: 94.5% Oral (diet) Doses of 0, 20, 200, 750, 1500 or 3000 ppm equivalent to 0, 1.3/1.7, 13/17, 51/66, 105/131 and 214/275 mg/kg bw/day (♂/♀) Parameters observed: Mortality, clinical signs, body weight and food and water consumption, biochemistry, haematology, urinalysis, organ weights and histopathology. Ophthalmoscopy examinations was carried out during pre-tests and at the end of treatment.	Mortality: 1♀ at 1500 ppm considered incidental and 1♂ at 200 ppm regarded accidental. 7♀ after blood sampling on day 90: 1 at 0 ppm, 1 at 200 ppm, 2 at 750 ppm, 1 at 1500 ppm and 2 at 3000 ppm). Clinical signs: Discomfort and hunched appearance, alopecia, lacrimation, swollen or enlarged ears, thinnes, scores, chromodacryorrhea, soft faeces and exophthalmos. No dose pattern observed with these effects. **3000 ppm** (121♂/129♀ mg/kg bw/day) Bodyweight *(no statistics performed on this parameter)* ▪ (↓) Bw in ♂ [week 1 (12% ndr), week 13 (10%)] and in ♀ [week 4 (15%), week 8 (18%) and week 13 (20%)] Bodyweight gain ▪ (↓) Bw gain in ♂/♀ [weeks 0-13 (50%/57%)] Heaematology ▪ (↓) RBC in ♂/♀ [week 13 (7% ndr/11% ncdr)] ▪ (↓) Ht in ♂/♀ [week 13 (8% ndr/14%)] ▪ (↓) Hb in ♀ [week 13 (8%)] Clinical chemistry ▪ (↑) BUN in ♂ [week 13 (38%)] ▪ (↓) Total bilirubin in ♂/♀ (for both sexes 0.0 in treated group *vs.* 0.1 in controls) Urinalysis *(no statistical analysis performed)* ▪ (↑) Ketones in ♂ (1.5 *vs.* 0.5 of controls) Organ weight ▪ (↓) Terminal bw in ♀ (17% ncdr). ▪ Liver: (↑) abs wt in ♂/♀ (22%/32%) and liver rel wt (40%/67%) ▪ Adrenals: (↓) abs wt in ♂ (17% ncdr) ▪ Brain: (↑) rel wt in ♀ (16%) ▪ Heart: (↑) rel wt in ♂ not pairwise significant but with significative positive-trend along dose levels. ▪ Kidneys: (↑) rel wt in ♀ not pairwise significant but with significative positive-trend along dose levels. Histopathology ▪ Diffuse hepatocyte enlargement ♂ (10/10 *vs.* 1/10 of controls) and ♀ (10/10 *vs.* 0/10 of controls) **1500 ppm** (105♂/131♀ mg/kg bw/day) Bodyweight ▪ (↓) Bw in ♀ [week 4 (10%), week 8 (11%) and week 13 (11%)] Bodyweight gain ▪ (↓) Bw gain in ♀ [weeks 0-13 (34%)] Heaematology ▪ (↓) RBC in ♂/♀ [week 13 (10% ndr/9% ncdr)] ▪ (↓) Ht in ♂/♀ [week 13 (11% ndr/12%)] ▪ (↓) Hb in ♀ [week 13 (5%)] Clinical chemistry ▪ (↑) BUN in ♂ [week 13 (23%)] ▪ (↓) Total bilirubin in ♀ (0.0 in treated group *vs.* 0.1 in controls) Organ weight ▪ Terminal bw (↓) in ♀ (6% ncdr) ▪ Liver: (↑) abs wt in ♂/♀ (31%/27%) and (↑) rel wt (32%/35%)	**Anonymous 31 (1987a) B.6.3.2.1.2 (AS)**

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
		Histopathology ▪ Diffuse hepatocyte enlargement: ♂ (10/10 *vs.* 1/10 of controls) and ♀ (4/10 *vs.* 0/10 of controls) **750 ppm** (51♂/66♀ mg/kg bw/day) Bodyweight ▪ (↓) Bw in ♀ [week 13 (7%)] Bodyweight gain ▪ (↓) Bw gain in ♀ [weeks 0-13 (18%)] Heaematology ▪ (↓) RBC in ♂ [week 13 (8% ndr)] ▪ (↓) Ht in ♂ [week 13 (9% ndr)] Organ weight ▪ Liver: (↑) abs wt in ♂/♀ (26%/24%), (↑) rel wt in ♂/♀ (21%/28%) **200 ppm** (13♂/17♀ mg/kg bw/day) Bodyweight gain ▪ (↓) Bw gain in ♀ [weeks 0-13 (16%)] Organ weight ▪ Liver: (↑) rel wt in ♀ (21%) **NOAEL**: 750 ppm (51 ♂ and 66 ♀ mg/kg bw/day) **LOAEL**: 1500 ppm (105 ♂ and 131 ♀ mg/kg bw/day	
Oral 90-day neurotoxicity study in rats (subchronic) OECD 424 (1997) EEC B.43 GLP: Yes Rat, Alpk:APfSD 10 rats/sex/dose **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 100 mg/kg bw/day* *STOT RE 1 ≤ 10 mg/kg bw/day* *(90-day oral study)*	Purity: 94.3% Oral (diet) Doses of 0, 40, 250 and 1500 ppm equivalent to 0, 2.8/3.2, 17.3/19.5 and 107/120.2 mg/kg bw/day (♂/♀) Parameters observed: Mortality, clinical signs, body weight and food consumption, functional observational battery (FOB), motor activity, macroscopic pathology, neurohistopathology, brain and liver weights	No mortalities occurred during the study. No treatment-related clinical symptoms or signs of toxicity were evident. **1500 ppm** (107♂/120.2♀ mg/kg bw/day) Bodyweight and food consumption ▪ (↓) Bw in ♂ [week 2 (4%), week 6 (7%), week 8 (7%), week 12 (9%), week 13 (9%)] and (↓) bw in ♀ [weeks 6 (5%), week 8 (4%), week 12 (4%), week 13 (6%)] ▪ (↓) Food consumption in ♀ [week 13 (7% ndr)] FOB ▪ (↑) Time to tail flick in ♀ on week 5 (6.7 *vs.* 4.8 of controls) ▪ (↓) Fore-limb grip strength in ♀ [week 9 (24%)] ▪ (↓) Hind limb grip strength in ♂ [week 2 (23%), week 9 (18%), week 14 (27%)] Organ weight ▪ Liver: (↑) abs wt in ♂/♀ (28%/36%) and liver wt adjusted for bw in ♂/♀ (38%/45%). **250 ppm** (17.3♂/19.5♀ mg/kg bw/day) Food consumption ▪ (↓) Food consumption in ♀ [week 13 (7% ndr)] FOB ▪ (↓) Hind limb grip strength in ♂ [week 14 (20%)] **NOAEL general toxicity:** 250 ppm (17.3 ♂ and 19.5 ♀ mg/kg bw/day) **NOAEL neurotoxicity:** > 1500 ppm (107 ♂ and 120.2 ♀ mg/kg bw/day)	**Anonymous 32 (2006a) B.6.7.1.2. (AS)**

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
13-Week oral toxicity (feeding) study in the mouse OECD 408 (1981) GLP: Yes Mouse, CD-1 (ICR) strain 15 mice/sex/dose 20 mice/sex as control group No historical control data provided Deviations: Blood samples and terminal pathology examinations at week 13 were done only in 10 animals per sex and group. No Biochemical determinations not performed. The wet weights of the uterus and thymus not recorded. The female mammary gland not preserved or examined histologically. **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 100 mg/kg bw/day* *STOT RE 1 ≤ 10 mg/kg bw/day* *(90-day oral study)*	Purity: 94.5% Oral (diet) Doses of 0, 20, 200, 2500, 7500 and 15000 ppm equivalent to 0, 3.3/4.6, 34.2/45.2, 440/639, 1320/1917 and 2640/3834 mg/kg bw/day (♂/♀) Parameters observed: Mortality, clinical signs, body weight and food and water consumption, haematology, eye examination, gross pathology, organ weights and histopathology examinations.	Mortality: There was 100% mortality within the first 3 weeks of the study in the 7500 and 15000 ppm dose groups. Additionally: 0 (1♂), 20 (1♂), 200 (2♀ one of them incidental) and 2500 (1♀). Deaths below 7500 ppm can be regarded as incidental. Clinical signs: Thinness, hunched posture, languor and tremor observed for early deaths. Clinical signs in the remaining 4 groups did not show a dose pattern. Females in the 2500 ppm dose group showed polypnea during the first week of the study. Other observations in more than one animal included alopecia, thinness, lacrimation, opaque, small or ulcerated eye and swollen abdomen. **2500 ppm** (440♂/639♀ mg/kg bw/day) Body weight ▪ (↓) Bw gain in ♀ [weeks 0-13 (21%)] Organ weight ▪ Terminal bw (↓) in ♀ (15% ncdr) ▪ Liver: (↑) abs wt in ♂/♀ (82%/70%) and rel wt in ♂/♀ (94%/86%) ▪ Heart: (↓) abs wt in ♀ (8%) ▪ Ovaries: (↓) abs wt (25%) Macropathology ▪ Liver enlargement ♂ (6/10 *vs.* 0/9) and ♀ (7/9 *vs.* 0/10) ▪ Liver pale area ♂ (1/10 *vs.* 0/9) and ♀ (1/9 *vs.* 0/10) ▪ Liver prominent reticular pattern ♂ (4/10 *vs.* 0/9) Histopathology ▪ Diffuse hepatocyte enlargement ♂ (10/10 *vs.* 0/9) and ♀ (9/9 *vs.* 0/10) ▪ Hepatic vacuolization ♂ (7/10 *vs.* 1/9) and ♀ (7/9 *vs.* 1/9) ▪ Sinusoidal cell pigmentation ♂ (3/10 *vs.* 0/9) ▪ Coagulative necrosis ♀ (4/9 *vs.* 0/10) **200 ppm** (34.2♂/ 45.2♀ mg/kg bw/day) Organ weight ▪ Liver: (↑) rel wt in ♂ (15%) Histopathology ▪ Diffuse hepatocyte enlargement ♂ (1/10 *vs.* 0/9) and ♀ (2/8 *vs.* 0/10) ▪ Centrilobular hepatocellular enlargement ♂ (9/10 *vs.* 2/9) **NOAEL**: 20 ppm (3.3♂ and 4.6 ♀ mg/kg bw/day) **LOAEL**: 200 ppm (34.2 ♂ and 45.2 ♀ mg/kg bw/day)	**Anonymous 33 (1987b) B.6.3.2.2.1 (AS)**
28-Week oral toxicity (feeding) study in dogs OECD 452 (1981) GLP: Yes Dog, Beagle 3 animals/sex/dose No historical control data provided Deviations: Duration of the study should be 12 months.	Purity: 96.1% Oral (diet) Doses of 0, 100, 1000, 3000 and 6000 ppm equivalent to 0, 6/3.4, 31.3/34.8, 96.6/110.6 and 157.8/203.7 mg/kg bw/day (♂/♀) Parameters observed: Mortality, clinical signs,	No mortalities occurred during the study. Clinical signs: Lenticular opacity in one female at 3000 ppm and all animals at 6000 ppm during weeks 20-29. **6000 ppm** (157.8♂/203.7♀ mg/kg bw/day) Body weight and food consumption ▪ (↓) Bw in ♂/♀ [week 28 (30%/32%)] ▪ (↓) Bw gain in ♂/♀ throughout the study ▪ (↓) Food consumption in ♂ [weeks 1 to 28 (35%-87%)] ▪ (↓) Food consumption in ♀ [weeks 1 to 4 (40%-78%)] Ophtalmological findings ▪ (↑) Bilateral subcapsular, equatorial, anterior cortical and posterior cortical lenticular aberrations (cataracts) in all dogs. Subsequent examinations revealed slight to marked progression of the lenticular aberration	**Anonymous 34 (1987) B.6.3.2.3.1 (AS)**

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
Number of animals should be 4 per sex in each dose group. **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 50 mg/kg bw/day* *STOT RE 1 ≤ 5 mg/kg bw/day* *(6-Month oral study)*	individual body weight, food consumption, haematology, eye examination, gross pathology, organ weights and histopathology examinations on selected tissues. Physical examinations on weeks 13 and 28.	<u>Heaematology</u> ▪ (↑) Platelet count in ♂ [week 14 (60%) and week 28 (121%)] <u>Clinical chemistry</u> ▪ (↓) Total protein in ♀ [week 28 (15%) (ncdr)] ▪ (↓) Calcium in ♀ [week 28 (14%)] ▪ (↑) ALP in ♂ [week 17 (136%) and week 28 (78% n.s.)] and ♀ [week 17 (48% n.s.) and week 28 (287% ndr)] <u>Organ weight</u> ▪ Terminal body wt (↓) in ♂/♀ (31%/31%) ▪ Brain: rel wt in ♂/♀ (34%/33%) ▪ Heart: (↓) abs wt in ♂ (30%) ▪ Prostate: (↓) abs/rel wt (61%/45%) ▪ Salivary gland: (↓) abs wt in ♂ (28%) ▪ Uterus: (↓) abs/rel wt (83% n.s./78% n.s.), ▪ Ovaries: (↓) abs/rel wt (48% n.s./27% n.s.) ▪ Kidneys: (↑) rel wt in ♀ (50%) ▪ Liver: (↑) abs wt in ♂/♀ (44% n.s./11% n.s.) and rel wt in ♂/♀(44% n.s./65%). <u>Macropathology</u> ▪ Bilateral ocular opacity (1♂ and 1♀) <u>Histopathology</u> ▪ Eyeball-ciliary body: minimal acute purulent inflammation ♂ (1/3) and minimal cysts ♂ (1/3) ▪ Moderate cataract left eye ♂ (2/3), ♀ (1/3) ▪ Minimal cataract right eye ♂ (1/3), ♀ (1/3) ▪ Severe cataract right eye ♀ (2/3) **3000 ppm** (96.6♂/110.6♀ mg/kg bw/day) <u>Food consumption</u> ▪ (↓) Food consumption in ♂ [week 1(50%), week 2 (23%) and week 4 (16%)] <u>Clinical chemistry</u> ▪ (↑) ALP in ♀ [week 17 and 28 (243% ndr/455% ndr)] <u>Organ weight</u> ▪ Brain: (↑) rel wt in ♀ (14%) ▪ Liver: (↑) abs/rel wt in ♀ (27% ndr/41%) <u>Ophtalmological findings</u> ▪ (↑) Bilateral subcapsular, equatorial, anterior cortical and posterior cortical lenticular aberrations (cataracts) in 1♂ and 1♀. Subsequent examinations revealed slight to marked progression of the lenticular aberration <u>Macropathology</u> ▪ Bilateral ocular opacity (1♀) <u>Histopathology</u> ▪ Minimal cataract left eye ♀ (1/3) ▪ Moderate cataract left eye ♂ (1/3) and ♀ (1/3) ▪ Minimal cataract right eye ♂ (1/3) ▪ Severe cataract right eye ♀ (1/3) **1000 ppm** (31.3♂/34.8♀ mg/kg bw/day) <u>Food consumption</u> ▪ (↓) Food consumption in ♂ [week 2 (28%), week 3 (28%) and week 4 (26%)] **NOAEL**: 1000 ppm (31 ♂ and 35 ♀ mg/kg bw/day)	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
		LOAEL: 3000 ppm (96.6 ♂ and 110.6 ♀ mg/kg bw/day)	
Oral 1-year dietary study in dogs OECD 452 (1981) ~87/302/EEC B.30 GLP: Yes Dog Beagle 4 dogs/sex/dose No historical control data available **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 25 mg/kg bw/day* *STOT RE 1 ≤ 2.5 mg/kg bw/day* *(1-year oral study)*	Purity: 96.1% Oral (diet) Doses of 0, 20, 100, 500 and 1500 ppm equivalent to 0, 0.7/0.6, 3.4/3.7, 16.4/19.4 and 51.2/44.3 mg/kg bw/day (♂/♀) Parameters observed: Mortality, clinical signs, individual body weight, food consumption, haematology, gross pathology, organ weights and histopathology examinations on selected tissues. Physical examinations on weeks 14, 25, 39 and 52. Eye examinations on weeks 11, 27, 39 and 51.	No mortalities occurred during the study. No treatment-related clinical symptoms or signs of toxicity were evident. Clinical signs: Minor faecal changes and emesis observed in one or two animals in each group and swollen appendage in one female at 20 ppm (considered not treatment-related). Corneal opacity was seen in one female of the control group. **1500 ppm** (51.2♂/44.3♀ mg/kg bw/day) Body weight and food consumption ▪ (↓) Bw gain in ♀ [week 1 (5%)] ▪ (↓) Food consumption in ♀ [week 1 (23%), week 5 (19%), week 10 (24%) and week 51 (29%)] Heaematology ▪ (↓) Reticulocyte count in ♀ [week 52 (63%)] Clinical chemistry ▪ (↓) BUN in ♂ [week 52 (27% ndr)] ▪ (↑) ALP in ♂ [week 13 (42%), week 26 (98%) and week 52 (143%)] ▪ (↑) Sodium in ♀ [week 52 (5%)] **500 ppm** (16.4♂/19.4♀ mg/kg bw/day) Clinical chemistry ▪ (↑) AP in ♂ [week 52 (55%)] Organ weight ▪ Adrenals: (↓) rel wt in ♂ (23% ndr) **100 ppm** (3.4♂/3.7♀ mg/kg bw/day) Clinical chemistry ▪ (↓) BUN in ♂ [week 52 (29% ndr)] **NOAEL**: ≥ 1500 ppm (≥ 51.2 ♂ and 44.3 ♀mg/kg bw/day) **LOAEL**: not achieved	**Anonymous 35 (1988) B.6.3.3.1.1 (AS)**
DERMAL ROUTE			
28-Day repeated dose dermal toxicity study in rats. OECD 410 (1981) ~92/69/EEC B.9 GLP: Yes Rat, HanIbm: Wist (SPF) 10 rats/sex/dose No historical control data provided Deviations: Purity of test material does not meet guidance specifications. Clinical observations should be made at least	Purity: 91.8% Dermal suspended in 1% carboxymethylcellulose in 0.1% Tween 80 and distilled water. Doses of 0, 10, 100 and 1000 mg/kg bw/day (♂/♀) Application for 6h/day for 5 days/week for the first 3 weeks and everyday thereafter. Parameters observed: Mortality twice	No mortalities occurred during the study. No treatment-related clinical symptoms or signs of toxicity were evident. No signs of skin irritation in the skin application site were noted. **1000 mg/kg bw/day** Food consumption ▪ (↓) Food consumption in ♂ [week 1 (10%)] Clinical chemistry ▪ (↓) Total bilirubin in ♂ (37%) ▪ (↓) Globulin in ♂ (8%) ▪ (↓) Calcium in ♂ (4%) ▪ (↑) Albumin in ♂ (11%) Organ weight ▪ Liver: (↑) abs wt in ♂ (12%) and (↑) rel wt in ♂/♀ (16%/11%) Histopathology ▪ Skin application site - Hyperkeratosis ♂ (6/10 *vs.* 2/10 of controls) and ♂ (10/10 *vs.* 4/10 of controls) ▪ Minimal hepatocellular hypertrophy ♂ (7/10 *vs.* 2/10 of controls), ♀ (7/10 *vs.* 1/10 of controls) ▪ Thyroid - hypetrophy of follicular epithelium ♂ (8/10 with severity of 2.0 *vs.* 8/10 of controls with severity of 1.56) and ♀ (9/10 with severity	**Anonymous 36 (2000) B.6.3.4.2.1 (AS)**

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results [Effects statistically significant and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
once daily instead of once weekly. **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 600 mg/kg bw/day* *STOT RE 1 ≤ 60 mg/kg bw/day* *(28-day dermal study)*	daily, eye examinations, clinical signs, individual body weight, food consumption, haematology, gross pathology and organ weights. Histopathology examinations on selected tissues.	of 1.7 *vs.* 7/10 of controls with severity of 1.3) **NOAEL**: 100 mg/kg bw/day ♂ and ♀ **LOAEL**: 1000 mg/kg bw/day ♂ and ♀	

Other studies relevant for STOT RE

Other long-term exposure studies, *i.e*. carcinogenicity and reproductive toxicity studies, can also provide evidence of specific target organ toxicity that could be used in the assessment of classification.

Chapter 10.9: 2-year long term toxicity study in rats (Anonymous 16, 1989) and 18-month long-term toxicity study in mice (Anonymous 18, 1989b) and 14-days study in rat (Anonymous 19, 1992).

Chapter 10.10: multigeneration study in rats (Anonymous 23, 1988), developmental study in rats (Anonymous 24, 1987 and Anonymous 25, 1992) and developmental study in rabbits (Anonymous 26, 1987 and Anonymous 27, 1992).

These studies are properly summarised in the corresponding chapters.

Furthermore, two additional studies to assess the cataractogenic potential of difenoconazole are summarised in the table below.

Table 40: Summary table of other studies relevant for STOT RE

Type of study/data Test substance	Relevant information about the study (as applicable)	Observations	Reference
56 Day feeding cataractogenicity in young chicken No test method available GLP: No Chicken, Hisex 5 chickens/sex in treated groups 3 chickens/sex in negative and positive control groups **Study acceptable** *Guideline value for classification:* *STOT RE 2 ≤ 161 mg/kg bw/day* *STOT RE 1 ≤ 16 mg/kg bw/day* *(56-day study)*	Purity not stated Oral (diet) Dose 0, 5000 ppm equivalent to 317.6 mg/kg bw/day (♂/♀) Vehicle: Ol. Arachidis PH. H. VI (10%) Positive control: 2,4-dinitrophenol at 2500 ppm (158.9 mg/kg bw/day) Parameters observed: Mortality, clinical signs, individual body weight, food consumption. Eye examinations and histopathological examinations carried out on day 57.	**5000 ppm** (♂/♀ 317.6 mg/kg bw/day) Mortality 5000 ppm (1♀) on day 36. Clinical signs: Ruffled feathers in all animals from day 7 until termination. Slight reduced locomotor activity on days 9 and 10 and between day 13 and 23. Body weight and food consumption ▪ (↓) Bw in ♂/♀ [day 28 and day 56] ▪ (↓) Food consumption in ♂/♀ throughout the study. Eye examinations *Treated group:* Lens alterations observed throughout the study in ♂ (5/5) and ♀ (2/5) of which ♂ (4/5) and ♀ (1/5) were irreversible by day 56. *Positive control group*: marked lens opacities on days 3 and 7 and became slight alterations until study termination (except for one female which had no findings after day 38). *Negative control group*: no lesions Histopathology	**Anonymous 37 (1987) B.6.8.2.1-01 (AS)**

Type of study/data Test substance	Relevant information about the study (as applicable)	Observations	Reference
		Treated group: Initial changes in the lens, indicative of cataract, in 3/5 males and 1/5 females. The lesions comprised slight swelling of the epithelial cells either at the equator or anteriorly, and/or necrosis of the lens fibres posteriorly, under the capsule or in the outer cortex. *Positive control group*: 2/3 males developed changes indicative of cataract and one female showed a slight swelling of the lens epithelium at the equator. *Negative control group*: no lesions. **Conclusion**: Difenoconazole has the potential to be cataractogenic in young chicken when administered orally in food over a period of 56 days.	
18-Week oral toxicity (feeding) study in dogs; assessment of cataractogenic potential No test method available GLP: Yes Dog, beagle Group 1 (G1): 1 dog/sex Group 2 (G2): 2 dogs/sex **Study acceptable as supporting information.** *Guideline value for classification:* *STOT RE 2 ≤ 72 mg/kg bw/day* *STOT RE 1 ≤ 7.2 mg/kg bw/day* *(18-week study → G1)* *STOT RE 2 ≤ 428 mg/kg bw/day* *STOT RE 1 ≤ 42.8 mg/kg bw/day* *(3-week study → G2)* HCD only available for haematology and clinical biochemistry	Purity: 96.1% Oral (diet) G1 doses (18-week treatment): 6000 ppm (days 1-8), 3000 ppm (days 9-63), 4000 ppm (days 64-127) equivalent to 213.8, 106.8 and 142.5 mg/kg bw/day, respectively. G2 doses (3-week of treatment and 15 week of recovery): 6000 ppm (days 1-8), 3000 ppm (days 9-21) and 0 ppm (days 22-127) equivalent to 213.8 and 106.8 mg/kg bw/day, respectively. Parameters observed: Mortality, clinical signs, individual body weight, food consumption, ophthalmoscopic examinaitons, blood samples (pre-test, week 3, 13 and 19), clinical biochemistry and gross pathology. Eyes and tissues with macroscopic changes submitted for histopathological examinations.	No mortalities occurred during the study. Clinical signs: Vomiting observed in 1♂ (G1) and 1♀ (G2). Faecal changes (mucus and worms/red areas) in 1♀ (G1) during weeks 6-9 and 1♂ (G2) during week 10. Diarrhoea in 1♀ (G2) during week 14. Eye examinations No signs of cataractogenic potential of the test and no histological alterations observed in the eye. Histopathology ▪ Lungs: slight interstitial pneumonia 1/1♂ (G1) and 1/2♂ (G2) and 1/1♀ (G1) and 1/2♀ (G2) and moderate bronchopneumonia 1/2♀ (G2.) ▪ Small intestinte follicular hyperplasia: severe 1/1♂ of both sexes (G1) and 1/2♂ (G2) and 2/2♀ (G2) and moderate in 1/2♂ (G2) ▪ Large intestine follicular hyperplasia: moderate 1/1 of both sexes (G1) and severe 2/2 of both sexes (G2). ▪ Spleen: congestion severe in all animals of G1 and G2. ▪ Cervical lymph node (erythrohagocytosis): slight in 1/2♂ (G2) and moderate in 1/1♂ (G1). ▪ Cervical lymph node (sinus oedema): moderate 1/1♂ (G1) and 1/2♂ (G2) and 1/2♀ (G2) and severe 1/2♂ (G2). ▪ Cervical lymphoid (hyperplasia): slight 1/2 ♂ (G2) and moderate 1/2♀ (G2). ▪ Ovaries cyst 1/1♀ (G1) and 1/2♀ (G2). **Conclusion**: Treatment of dogs with difenoconazole at doses of 3000 and 6000 ppm (equivalent to 106.8 and 213.8 mg/kg bw/day) for 18 weeks did not result in formation of cataracts.	**Anonymous 38 (1989) B.6.8.2.1-02 (AS)**

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

Oral route

Studies in rats:

In a 28-day dietary study in rats (B.6.3.1.1), the liver was identified as the target organ with adverse effects at the highest tested dose level of 10000 ppm (914/841 mg/kg bw/day).

Increases in relative liver weights were observed in both sexes from 1500 ppm (156/166 mg/kg bw/day) but not accompanied by macroscopic or microscopic findings. However, at 10000 ppm liver effects are deemed adverse since cholesterol levels and hepatic enzymes (AST, CGT and AP) were increased with values out of the historical control data provided.

The relative kidney weight in males was increased (10%) in a dose-dependent manner from 1500 ppm. The adversity of these effect at 1500 ppm is doubtful regarding the low magnitude of the increase (10%) and the absence of macroscopic findings. It has to be noted that histopatholgy in this organ was not performed. Additionally, increase in ketones in males at doses ≥ 200 ppm was attributed to reduced food consumption and/or prolonged fasting.

Liver effects at 10000 ppm (914/841 mg/kg bw/day) were above the threshold extrapolated value for STOT RE 2 from a 90-day study (≤ 300 mg/kg bw/day) and therefore, classification does not apply.

In a 90-day dietary study in rats (B.6.3.2.1.1), the target organ was the liver, with effects observed from 250 ppm (20/21 mg/kg bw/day) but considered adverse only at 1500 ppm (121/129 mg/kg bw/day).

Increases in both absolute and relative liver weights were observed in both sexes in the 1500 ppm dose group with significant increases in ALP levels in both sexes and a slight decrease in total protein concentration in males though this biochemical parameters were reversed by the end of the recovery period. Histopatholgy revealed no effects in liver. Significant increases in relative liver weights at 250 ppm occurred not showing induction of liver enzymes or histopathological lesions, hence these are considered as an adaptive response.

The dose at which effects were observed was 1500 ppm (121/129 mg/kg bw/day) is above the cut-off value for STOT RE 2 for a 90-day repeat dose study (≤ 100 mg/kg bw/day) and therefore, STOT RE is not required.

A second 90-day dietary study in rats (B.6.3.2.1.2), the target organ was the liver with adverse effects from 1500 ppm (105/131 mg/kg bw/day) in both sexes.

Adverse effects in liver were seen in both sexes from 1500 ppm with increases in the absolute and relative liver weights accompanied by increases in the incidence and severity of diffuse hepatocellular enlargement. The absolute and relative liver weights were increased in all animals from 750 ppm and also the liver relative weight in females at 200 ppm. However, these effects observed at 200 and 750 ppm in liver occurred in absence of histopathological findings and they are considered a normal adaptive response to increased work load and below the threshold of liver toxicity in SD rats.

The liver effects observed at 1500 ppm (105/131 mg/kg bw/day) are above the cut-off value for STOT RE 2 for a 90-day repeat dose study (≤ 100 mg/kg bw/day), hence no classification is regarded for STOT RE.

In a 13-week oral neurotoxicity study in rats (B.6.7.1.2) there were no effects on nervous system. Effects on liver weight in both sexes were observed at 1500 ppm (107/120.2 mg/kg bw/day) but no

clinical chemistry or histopathology of this organ was performed. In any case, liver effects are above the cut-off value for a 90-day repeated dose study STOT RE 2 (≤ 100 mg/kg bw/day).

In a 2-year long-term toxicity and carcinogenicity study in rats (B.6.5.1) (refer to section 10.9) the target organ was the liver from 500 ppm (24.1/32.8 mg/kg bw/day).

There was an increase incidence of hepatocellular hypertrophy in both male and female rats at terminal sacrifice observed from 500 ppm (24.1/32.8 mg/kg bw/day). There were also increases in relative liver weight observed in both sexes at 2500 ppm (124/170 mg/kg bw/day) on week 53 though they were similar to controls after recovery on week 57.

The liver effects observed at 500 ppm (24.1/32.8 mg/kg bw/day) are above the extrapolated cut-off value for STOT RE 2 for a 2-year study (≤ 12.5 mg/kg bw/day), hence no classification is regarded for STOT RE.

In a multigeneration study in rats (B.6.6.1.1) there were no effects that showed target organ toxicity up to the highest tested dose of 2500 ppm (172/192 mg/kg bw/day). Toxicity in the parental animals of the F0 and F1 animals included reductions in mean body weight and body weight gain at 2500 ppm during pre-mating period in both sexes and persisted during gestation and lactation for females. Neonatal toxicity was also observed at the highest tested dose. Reduced body weight of the F1 and F2 pups was observed throughout the lactation period.

The highest tested dose of 2500 ppm (172/192 mg/kg bw/day) is above the extrapolated cut-off value for STOT RE 2 for a multigeneration study considering a time of dosing in the interval of 70-120 days (75-130 mg/kg bw/day), hence no classification is regarded for STOT RE 2.

In a teratology study in rats (B.6.6.2.1) there were no effects that showed target organ toxicity up to the highest tested dose of 200 mg/kg bw/day. Maternal toxicity effects included reduced body weight gain and food consumption. The highest tested dose (200 mg/kg bw/day) did not show relevant effects for STOT RE 2 classification.

Studies in mice:

In a 90-day dietary study in mice (B.6.3.2.2.1) the target organ was the liver from 200 ppm (34/45 mg/kg bw/day).

Increases in absolute and relative weights were seen in both sexes at 2500 ppm (440/639 mg/kg bw/day) accompanied by increased incidence of difusse hepatocyte enlargement and hepatic vacuolisation in both sexes and coagulative necrosis in females. Biochemical determinations were not performed. The increased liver weight at 200 ppm (34.2/45.2 mg/kg bw/day) in males was statistically significant and dose-dependent and it was accompanied by centrilobular hepatocellular enlargement (9/10♂ *vs*. 2/9 control♂). This finding was classified as minimum (8) or slight (1), restricted to males of this dose group and not observed at 2500 ppm. However, liver hypertrophy starts in the centrilobular hepatocytes, spreading to the intermediate zone as it progresses, and finally observed as diffuse hypertrophy all around the lobule of the liver. This would explain the absence of centrilobular hepatocyte enlargement at 2500 ppm since 10/10 animals at this dose level had diffuse hepatocellular enlargement. Since clinical chemistry is not available, the adversity of this finding cannot be disregarded.

The liver effects observed at 200 ppm (34/45 mg/kg bw/day) are within the interval for STOT RE 2 for a 90-day repeat dose study (≤ 100 mg/kg bw/day and ≥ 10 mg/kg bw/day).

In a 78-week carcinogenicity study in mice (B.6.5.2) the target organ was the liver with carcinogenic response. Non-neoplastic changes in the liver were observed at 300 ppm (equivalent to 46.3/57.8 mg/kg bw/day) and above (3000-2500 ppm and 4500 ppm) and these included increase in liver

absolute and relative weight values. Histopathological findings in the liver included individual cell necrosis, fatty changes, hepatocyte hypertrophy and bile stasis observed in males at 2500 ppm and 4500 ppm and females at 2500 ppm. Increase in individual cell necrosis and hepatocyte hypertrophy were also observed in males at 300 ppm.

The liver effects observed at 300 ppm (46.3/57.8 mg/kg bw/day) are above the extrapolated cut-off value for STOT RE 2 for a 18-week study (≤ 16.7 mg/kg bw/day), hence no classification is regarded for STOT RE.

In a supplementary MOA study in mice (14 days oral) (B.6.8.2.2.1) a wide range of liver enzyme activities and CYP protein determinations were evaluated, along with liver weights and liver histopathology. Increased liver weight was observed in animals treated with 400 mg/kg bw/day (↑79%). All changes were reversible after the 28-day recovery period. Microsomal cytochrome P-450 contents were significantly elevated in mice treated with 100 and 400 mg/kg bw/day difenoconazole although the levels returned to control values during the recovery period. Difenoconazole caused changes in markers of CYP2B (decrease in protein content) and/or CYP3A enzyme induction. No significant changes in CYP1A or CYP4A protein content. Activities of 7-ethoxyresorufin O-de-ethylase (EROD) and 7-pentoxaresorufin O-depentylase (PROD) were increased approximately 3- and 30- fold in mice treated with 400 mg/kg bw/day difenoconazole, respectively. Induction of lauric acid 11-hydrolase activity was observed at 100 and 400 mg/kg bw/day. On the contrary, a slight inhibition of lauric acid 12-hydroxylase activity was observed at all dose levels.

Since there are effects in the liver from the dose of 100 mg/kg bw/day, applying the Haber´s rule, for effects ≤ 600 mg/kg bw/day the substance could be classified as STOT RE Category 2. Nevertheless, the reversibility of the effects, the absence of histopathological findings and the availability of other longer studies suggest that no classification is regarded for STOT RE.

Studies in dogs:

In a 6-month dietary study in dogs (B.6.3.2.3.1) the target organs were the eye and the liver.

With respect to the effects on eye, clinical signs included lenticular opacity in one female at 3000 ppm and all animals at 6000 ppm during weeks 20 to 29. The ophthalmoscopic examinations revealed cataracts in all dogs from week 11 at 6000 ppm and one male and all females at 3000 ppm. Macroscopic examinations revealed treatment-related bilateral ocular opacity in one female at 3000 ppm and one male and one female at 6000 ppm. Histopatholgy revealed at 6000 ppm, moderate to severe cataracts in the left lens of all animals and minimal to severe cataracts in the right lens. At 3000 ppm, cataracts were noted for one male (bilateral) and two females (both unilateral), which were not seen in the ophthalmoscopic examinations. Effects in eyes at both 3000 and 6000 ppm are regarded adverse. The cataractogenic effects observed at 3000 ppm (96.6/110.6 mg/kg bw/day) are above the extrapolated cut-off value for STOT RE 2 for a 6-month study (≤ 50 mg/kg bw/day), hence no classification is regarded for STOT RE.

Absolute and relative liver weights were increased in females from 3000 ppm and in males at 6000 ppm (not significant) with increased ALP on week 28 from 3000 ppm in females though not clearly dose-related. The significance of this liver effect is doubtful since histopatholgy did not revealed any finding on this organ. In any case, 3000 ppm (96.6/110.6 mg/kg bw/day) is above the extrapolated cut-off value for STOT RE 2 for a 6-month study (≤ 50 mg/kg bw/day)

In a 1-year dietary study in dogs (B.6.3.3.1.1) there were no effects that showed target organ toxicity up to the highest tested dose, *i.e*. 1500 ppm (51.2/44.3 mg/kg bw/day). No cataractogenicity was observed in this study in contrast to the 6-month repeat dose study. Increased levels of ALP were observed in males at 1500 ppm during the course of the study whereas only at week 52 in male dogs

at 500 ppm and had no correlates of macro- or microscopic findings. Consequently, no effects for STOT RE were found in this study.

In an 18-week dietary study in dogs for the assessment of caractogenic potential (B.6.8.2.1-02), considered only as supporting information, the experiment consisted of two treated groups: group 1 with treatment during 18 weeks for 1 dog/sex at doses of 106.8-213.8 mg/kg bw/day and group 2 with 3 weeks of treatment at doses of 106.8-213.8 mg/kg bw/day and 15 weeks of recovery.

No signs of cataractogenic potential nor histological alterations in the eye were observed in any animals. Histopatholgy findings in other organs revealed effects in lungs, small and large intestine, spleen, cervical lymph node and ovaries in both groups. These findings were clearly above the extrapolated value for STOT RE 2 for a 18-week study (≤ 72 mg/kg bw/day) (group 1) but in the range for the extrapolated value for STOT RE 2 for a 3-week study (42.8-428 mg/kg bw/day) (group 2). However, the MSCA regards difficult to evaluate the relevance of these findings considering that the study was performed not following a guideline with a low number of animals and absence of control animals or historical control data for histopathology.

Studies in rabbits:

In a teratology study in rabbits (B.6.6.2.2) there were no effects that showed target organ toxicity up to the highest tested dose, *i.e*. 75 mg/kg bw/day. Maternal toxicity observed at the highest tested dose included body weight loss and food consumption. No relevant effects are considered for STOT RE 2 at the highest tested dose level.

Studies in chickens:

In a 56-day feeding caractogenicity study in chickens (B.6.8.2.1-01) the target organ was the eye at the only tested dose level with difenoconazole at 5000 ppm (317.6 mg/kg bw/day).

Effects in the eyes included lens alterations observed throughout the study in 5/5 males and 2/5 females of which ♂ (4/5) and ♀ (1/5) were irreversible by day 56. Histopathology revealed changes indicative of cataracts in 3/5 males and 1/5 females.

The effects in the eye observed at 5000 ppm (317.6 mg/kg bw/day) are above the extrapolated cut-off value for STOT RE 2 for a 56-day study (161 mg/kg bw/day), hence no classification is assigned for STOT RE 2.

Dermal route

In a 28-day dermal study in rats (B.6.3.4.2.1), the target organs were the liver, skin and thyroid, with adverse effects observed at 1000 mg/kg bw/day.

Histopathological examination showed increase in the absolute liver weight in males and relative liver weight in both sexes accompanied by increased incidence of minimal centrilobular hepatocellular hypertrophy in both sexes at 1000 mg/kg bw/day.

Thyroid effects were observed in both sexes with increase in the grade of severity of the hypertrophy of the thyroid follicular epithelium in males (2.0 *vs.* 1.6 control) and females (1.7 *vs.* 1.3) with increased incidence in females (9/10 *vs.* 7/10) at 1000 mg/kg bw.

Hyperkeratosis in both sexes (6/10 *vs.* 2/10 control males and 10/10 *vs.* 4/10 control females) was also observed at 1000 mg/kg bw.

Liver, skin and thyroid effects were observed at 1000 mg/kg bw/day, which is clearly above the threshold value for STOT RE 2 (≤ 600 mg/kg bw/day) and therefore, these effects are not regarded for STOT RE classification.

10.12.2 Comparison with the CLP criteria

Classification for repeated dose toxicity depends on the type of effects and the dose at which the effects are observed. The CLP criteria state that STOT RE is assigned on the basis of findings of 'significant' or 'severe' toxicity. In this context, 'significant' means changes that clearly indicate functional disturbance or morphological changes that are toxicologically relevant. 'Severe' effects are generally more profound or serious than 'significant' effects and are of a considerably adverse nature that significantly impact on health.

Table 41: Summary table of relevant effect for STOT RE classification

Dose levels and duration of exposure	Effest relevant for STOT RE [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr)/ncdr (not clearly dose-related)]	Reference
LIVER		
28-Day Cumulative oral toxicity (feeding) study in the rat Doses of 0, 250, 1500 or 10000 ppm equivalent to 0, 27, 156/166 and 914/841 mg/kg bw/day (♂/♀)	**STOT RE 2 (≤ 300 mg/kg bw/day)** 1500 ppm (156♂/166♀ mg/kg bw/day) Organ weights: ▪ Liver: (↑) abs wt in ♂ (17% ndr) and (↑) rel wt in ♂/♀ (22%/15%) **MSCA opinion:** Increases in liver absolute and relative weights were not accompanied by histopathological findings or clinical chemistry. These effects were regarded as an adaptive response.	**Anonymous 28 (1986a) B.6.3.1.1 (AS)**
13-Week oral toxicity (feeding) study in the rat Doses of 0, 40, 250 or 1500 ppm equivalent to 0, 3.3/3.5, 20/21 and 121/129 mg/kg bw/day (♂/♀)	**STOT RE 2 (≤ 100 mg/kg bw/day):** 250 ppm (20♂/21♀ mg/kg bw/day) Organ weights: ▪ Liver: (↑) rel wt in ♂/♀ [week 13 (11%/19%)] **MSCA opinion**: Increases in liver relative weights were not accompanied by histopathological findings or clinical chemistry. This effect is considered an adaptative response not toxicologically relevant.	**Anonymous 29 (1986b) B.6.3.2.1.1 (AS)**
13-Week oral toxicity (feeding) study in the rat Doses of 0, 20, 200, 750, 1500 or 3000 ppm equivalent to 0, 1.3/1.7, 13/17, 51/66, 105/131 and 214/275 mg/kg bw/day (♂/♀)	**STOT RE 2 (≤ 100 mg/kg bw/day)** 750 ppm (51♂/66♀ mg/kg bw/day) Organ weight ▪ Liver: (↑) abs wt in ♂/♀ (26%/24%) and (↑) rel wt in ♂/♀ (21%/28%) 200 ppm (13♂/17♀ mg/kg bw/day) Organ weight ▪ Liver: (↑) rel wt in ♀ (21%) **MSCA opinion:** Increases in liver absolute and relative weights were not accompanied by histopathological findings or clinical chemistry. These effects were considered a normal adaptive response.	**Anonymous 30 (1987a) B.6.3.2.1.2 (AS)**

Dose levels and duration of exposure	Effest relevant for STOT RE [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr)/ncdr (not clearly dose-related)]	Reference
13-Week oral toxicity (feeding) study in the mouse Doses of 0, 20, 200, 2500, 7500 and 15000 ppm equivalent to 0, 3.3/4.6, 34.2/45.2, 440/639, 1320/1917 and 2640/3834 mg/kg bw/day (♂/♀).	**STOT RE 2 (≤ 100 mg/kg bw/day)** 200 ppm (34.2♂/ 45.2♀ mg/kg bw/day) Organ weight ▪ Liver: (↑) rel wt in ♂ (15%) Histopathology ▪ Diffuse hepatocyte enlargement ♂ (1/10) and ♀ (2/8) ▪ Centrilobular hepatocellular enlargement ♂ (9/10) **MSCA opinion:** Effects relevant for STOT RE classification. The increase in liver relative weight was statistically significant and dose-dependent. The histopathological finding was increased incidence of centrilobular hepatocyte enlargement (9/10 ♂ vs 2/9 control ♂). This finding was classified as minimum (8) or slight (1), and it was restricted to males of this group. However, liver hypertrophy starts in the centrilobular hepatocytes, spreading to the intermediate zone as it progresses, and finally observed as diffuse hypertrophy all around the lobule of the liver. This would explain the absence of centrilobular hepatocyte enlargement at 2500 ppm since 10/10 animals at this dose level had diffuse hepatocellular enlargement. Since clinical chemistry is not available the adversity of this finding cannot be disregarded.	**Anonymous 32 (1987b)** **B.6.3.2.2.1 (AS)**

10.12.3 Conclusion on classification and labelling for STOT RE

Liver

The main target organ was the liver according to the results of the available studies. The only effects deemed relevant were found in the oral 90-day dietary study in the mouse with centrilobular hepatocyte enlargement in 9/10 males (8 minimal and 1 slight) along with increased relative liver weight in males. These effects were observed at a dose level (200 ppm, i.e. 34.2♂/ 45.2♀ mg/kg bw/day) below guidance value for STOT RE 2 (100 mg/kg bw/day) classification and not observed at the immediate highest dose level (2500 ppm, *i.e.* 440♂/639♀ mg/kg bw/day) since the lesion evolved to diffuse hepatocellular enlargement, observed in all males at 2500 ppm. It has to be noted that liver hypertrophy starts in the centrilobular hepatocytes, spreading to the intermediate zone as it progresses, and finally observed as diffuse hypertrophy all around the lobule of the liver. Since clinical chemistry is not available the adversity of this finding cannot be disregarded.

The effects in liver below extrapolated value for STOT RE 2 were only observed in one specie (mice) one sex (male) and the grade of severity of centrilobular hepatocyte enlargement. Besides, 78-week treatment with the same strain of mice did not show liver effect below STOT RE 2 cut-off values. Considering the whole available data from all studies in other species, it can be concluded that difenoconazole does not cause liver toxicity at dose levels below guidance values for STOT RE classification.

Eye

With regards to the effects observed in the eye following a 6-month oral repeat dose study in dogs, two supplementary studies have been provided to assess the cataractogenic potential of difenoconazole: a 56-day caractogenicity study in chicken and a 18-week feeding study in dogs. Despite the changes in the eyes indicative of cataracts observed in chickens, no eye damage was induced in the dog following an 18-week oral exposure. Furthermore, the formation of cataracts was not detected in dogs following a longer exposure in a 1-year repeat dose oral study. In the absence of ocular findings at shorter and longer exposures, the cataractogenic effects detected in dogs at 6-months are not regarded toxicologically significant.

The dose at which cataractogenic effects were observed in chickens (5000 ppm equivalent to 317.6 mg/kg bw/day) is clearly above the guidance value for STOT RE 2 classification. Therefore, STOT RE classification is not proposed.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The DS identified liver and eye as targets of repeated dose toxicity studies. However, according to DS, liver effects are an adaptive response rather than a toxic effect or do not appear at dose levels below the guidance values for STOT RE classification. On the other hand, DS considered that the capability of difenoconazole to induce cataracts are not toxicologically relevant or are above the guidance values for STOT RE classification. Overall, DS proposed no classification of difenoconazole for STOT RE.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The table contained in Annex III summarises the repeated dose toxicity studies with difenoconazole.

Comparison with the criteria

The battery of repeated dose toxicity studies showed the following effects: i) alterations in body weight and body weight gain; ii) alterations in blood and clinical chemistry; iii) alterations in organ weights; iv) clinical signs; v) mortalities; vi) eye alterations; and, vii) liver alterations.

The Guidance on the Application of the CLP Criteria establishes that clinical observations or small changes in bodyweight gain, food consumption as well as small changes in clinical biochemistry or haematology, when such changes or effects are of doubtful or minimal toxicological importance as shown above cannot be used for supporting a classification as STOT RE. RAC also notes that the alterations in organs weight other than liver were not accompanied with histopathological alterations or organ dysfunction and therefore these effects were neither considered for classification.

Clinical signs and mortalities noted in the table above were considered a consequence of general toxicity, without a specific target organ and therefore these clinical effects do not warrant classification, especially considering that in all cases these clinical signs appeared at dose levels above the guideline values for supporting classification.

The 28 weeks repeated dose toxicity study in dogs reported lenticular aberrations (cataracts), although at dose levels above the limit for warranting classification. These ocular alterations were also confirmed, also at doses above the guideline limit value, in a 56-day cataractogenicity study in chicken. However, an 18-week toxicity study in dogs for assessing the cataractogenic potential of difenoconazole failed in the detection of these effects in the

treated animals. Moreover, it is also noted that the formation of cataracts in the 1-year repeated dose toxicity study in dogs was not observed. Overall, RAC does not consider that the eye alterations reported above were enough for supporting a STOT RE classification.

The table above shows that liver is the target organ of difenoconazole. Difenoconazole caused liver alterations (mainly increases in absolute and relative weights with histopathological alterations as individual cell necrosis, focal/multifocal necrosis, hepatocyte hypertrophy, liver fatty change and bile stasis) in major or minor extension in the following studies: 28-days dietary study in rats; two 90-days dietary studies in rats; 13-weeks oral neurotoxicity study in rats; 2-year long-term toxicity and carcinogenicity study in rats; 90-days dietary study in mice; 78-week carcinogenicity study in mice; and, 28 weeks dietary study in dogs.

The assessment of the hepatic toxicity presented above shows that the relevant effects are below the guideline values for supporting classification:

- Increases in absolute and relative liver weights between 15-22% at 156-166 mg/kg bw/day in the 28-day oral toxicity study in rat (STOT RE 2 ≤ 300 mg/kg bw/day)
- Increases in relative liver weights between 11-19% at 20-21 mg/kg bw/day in the 13-week oral toxicity study in rat (STOT RE 2 ≤ 100 mg/kg bw/day)
- Increases in absolute and relative liver weights between 21-28% at 51-66 mg/kg bw/day in the 13-week oral toxicity study in rat (STOT RE 2 ≤ 100 mg/kg bw/day)
- Increases in relative liver weights of 16-21% at 13-17 mg/kg bw/day in the 13-week oral toxicity study in rat (STOT RE 2 ≤ 100 mg/kg bw/day)
- Increases in relative liver weights of 15% together with diffuse hepatocyte enlargement (1/10 males and 2/8 females) and centrilobular hepatocellular enlargement in 9/10 males at 34-45 mg/kg bw/day in the 13-week oral toxicity study in mouse (STOT RE 2 ≤ 100 mg/kg bw/day)

In most of the cases reported above, increases in absolute and relative liver weight were not accompanied by histopathological findings or clinical chemistry and, at these concentrations, are considered by RAC as adaptive rather than adverse effects and therefore do not warrant classification. Among all the hepatotoxicity effects observed below the guideline levels for warranting classification only one case was accompanied by histopathological alterations (8 cases of minimum and 1 case of slight centrilobular hepatocellular enlargement). This is not considered by RAC robust enough for supporting classification. Overall, **RAC supports the DS's proposal for no classification of difenoconazole for STOT RE.**

RAC evaluation of aspiration toxicity

Summary of the Dossier Submitter's proposal

DS considered that this hazard is not applicable for difenoconazole.

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC notes that the hazard aspiration toxicity is not relevant for fine crystalline powders and therefore concurs with the DS assessment. However, RAC does not propose a conclusion on this hazard class as it was not open for consultation and thus is outside of RAC's mandate.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Difenoconazole is a fungicide active substance considered under Directive 91/414/EEC (subsequently Regulation 1107/2009) for representative use as a foliar spray. Available environmental fate and ecotoxicology studies have been considered and summarised in the original Draft Renewal Assessment Report, 2019 (RAR, Volume 3, Annex B8 and Annex B9) and the renewal of approval dossier.

The key information pertinent to determining the environmental hazard classification for Difenoconazole is presented below. Unless otherwise stated, these studies were conducted in accordance with GLP and the validity criteria of the representative test guideline, if applicable. Full robust summaries of these studies are presented in Annex 1 to this dossier.

11.1 Rapid degradability of organic substances

Difenoconazole is considered not readily biodegradable. It is hydrolytically stable at pH4 to 9 and it is also stable under direct photolytic conditions with half-lifes between 11.8 and more than 1000 years.

Table 42: Summary of relevant information on rapid degradability

Method	Results	Remarks	Reference
Ready biodegradability. 92/69/EEC (corresponding to OECD Test Guideline No. 301B)	0% biodegradation in 29 days. Results indicate Difenoconazole is "not readily biodegradable"	The study is considered acceptable	Baumann W. (1993)
Hydrolysis of ^{14}C-Difenoconazole at pH 5, 7 and 9. EPA, 540/9-82-021, October 1982	After 30 days of incubation at 25ºC, 95.2%, 100.5% and 101.9% of added radiocarbon remained as difenoconazole. Difenoconazole is hydrolytically stable in solutions at pH 5 to 9 at 25ºC over a period of 30 days	The study is considered acceptable	Atkins R.H.; (1991)
Aerobic mineralisation. OECD guideline 309	<u>Difenoconazole:</u> DT_{50}: 104.7 and 146.7 days (at concentrations 10µg/L and	The study is accepted but further information related to metabolite "B" is	Gartner, C.; Herrechen, 2016

Method	Results	Remarks	Reference
(November 2004)	95µg/L respectively) Max. 16.3% mineralisation after 61 days. Metabolites: Difenoconazole alcohol (CGA 205375) Triazole acetic acid (CGA 142856) CGA 199312 Metabolite "B"	necessary.	
Aerobic aquatic metabolism in water/sediment systems. BBA Guideline Part IV; 5 – 1 (1990); Dutch Registration Guideline, Section G.2 (1987); US EPA 540/9-82-021.	DT50/DT90 (days): Water phase: 2.16/7.16 (pond system) 5.52/18.3 (river system) Whole system: 318/>1000 (pond system) 300/>998 (river system) Metabolites: Difenoconazole alcohol (CGA 205375)	The study is acceptable. Kinetic evaluation of the raw data by Terry (2015c).	González Valero, J. 1993. Terry A; 2015c
Aerobic aquatic metabolism in water/sediment systems. OECD Draft Guideline: Aerobic and anaerobic transformation in water/sediment systems (August 2000)	Difenoconazole rapidly absorbed on to the sediment. It is very persistent in sediments at low temperature. Max. 2.9% mineralisation after 181 days. Metabolite: Difenoconazole alcohol (CGA 205375)	It is considered only acceptable for the route of degradation of Difenoconazole	Ulbrich, R. 1997
Aerobic aquatic metabolism in water/sediment systems. EPA Guideline 162 – 4; OECD 308.	DT50/DT90 (days): Water phase: 3.2/10.6 (river system) Whole system: 1113/2300 (river system) Sediment phase: 690/>1000 (river system) Metabolites: <5% at the end of the study.	The study is acceptable. Kinetic evaluation of the raw by Terry (2015c).	Lin, Y. 2006 Terry, A. 2015c
Aerobic aquatic metabolism in water/sediment systems. OECD 308; EPA Guideline 712-C-018 and EPA 712-C-08-019;.OPPTS 835.4300	DT50/DT90 (days): 14C-Triaxolyl label: Water phase: 2.53/8.41 (Swiss Lake) 2.47/8.19 (Calwich Abbey) Whole system:	The study is acceptable.	Yeomans, P.; Mould, R. 2018

Method	Results	Remarks	Reference
and OPPTS 835.4400	167/533 (Swiss Lake) 703/>1000 (Calwich Abbey) 14C-2-Chlorophenoxy label: Water phase: 2.13/7.08 (Swiss Lake) 2.46/8.16 (Calwich Abbey) Whole system: 164/546 (Swiss Lake) 470/>1000 (Calwich Abbey) Metabolites: Difenoconazole alcohol (CGA 205375) CGA 199312		
Aqueous Photolysis of Difenoconazole [^{14}C-Triazole] under laboratory conditions. Guidelines: SETAC (1995); OECD/GD(97)21; EPA OPPTS 835.2210.	Difenoconazole is stable to direct photolysis in aqueous systems at pH 7 at 25ºC over a period of 15 days.	The study is considered acceptable	Gaauw, van der A. (2002a)
Quantum Yield of the photochemical degradation of Difenoconazole in aqueous solution. Guidelines: SETAC (1995); OECD/GD(97)21; EPA OPPTS 835.2210	The quantum yield of difenoconazole was determined to be 0.0155. Calculated environmental half-lives were between 11.8 and >10000 years.	The study is considered acceptable	Hennecke D. (2002a)

11.1.1 Ready biodegradability

Author(s): Baumann W. 1993
Title: Report on the test for ready biodegradability of Difenoconazole in the carbon dioxide evolution test.
Guidelines: 92/69/EEC (corresponding to OECD Test Guideline No. 301B)
GLP: Yes

This study determinated the biodegradability of difenoconazole in the carbon dioxide evolution test. Activated sludge collected from a sewage treatment plant prepared in accordance with the guideline was dosed with difenoconazole and incubated for 29 days at 22ºC in duplicate and test concentrations of 26.0 and 26.7 mg/L. Evolved carbon dioxide was trapped in sodium hydroxide. The CO_2 traps were sampled on days 0, 3, 6, 8, 10, 15, 20, 24, 28 and 29 and the carbon content determined in a

carbon analyser. Percentage biodegradation was calculated from content of inorganic carbon in absorption flask (corrected for blank) over the calculated theoretical organic carbon content.

Findings:
No biodegradation of difenoconazole was found after 29 days (0% biodegradation). Biodegradation of reference compound was >70% after 6 days.

Conclusion:
The study is considered aceptable and Difenoconazole is not readily biodegradable under the conditions of this test.

11.1.2 BOD_5/COD

No data available.

11.1.3 Hydrolysis

A study to address the data requirement of hydrolytic degradation (Atkins, R. H. 1991b) was included in the submission for Annex I inclusion under Directive 91/414/EEC and was deemed acceptable following evaluation and peer review at EU level (2006).

Author(s): Atkins R.H.; 1991b
Title: Hydrolysis of ^{14}C-Difenoconazole at pH 5, 7 and 9
Guidelines: EPA, 540/9-82-021, October 1982
GLP: Yes

The kinetics of hydrolysis of 14C-triazole labelled difenoconazole was studied in aqueous solution under acid, neutral and basic conditions.
^{14}C-difenoconazole solution was prepared at a concentration of 2 mg/l in acetonitrile and aliquots mixed with sterile buffers which were incubated for 30 days in the dark in triplicate at 25ºC and under 3 pH conditions 5-7-9. Samples were taken at 0, 2, 7, 12, 16, 19, 23, 27 and 30 days of incubation. Recoveries were determined by LSC and a quantitative analysis was done by HPLC and confirmatory GC-MS to analyse day 30 samples.

Findings:

Total radioactive recoveries were between 101 and 103% applied radiocarbon (AR). The high recoveries indicated that no volatile products could have been produced.

Little or no degradation of difenoconazole occurred, the parent accounted for between 95.2%, 100.5% and 102% (AR) in the solutions after 30 days. The calculated half-lives for Difenoconazole were significantly greater tan 30 days (>1000 days). Only two unknown degradates were detected throughout the study in quantities <1.2% of applied radiocarbon

Conclusion:
The study is considered aceptable and Difenoconazole is hydrolytically stable in solutions at pH 5 to 9 at 25ºC over a period of 30 days.

11.1.4 Other convincing scientific evidence

No data available

11.1.4.1 Field investigations and monitoring data (if relevant for C&L)

No data available

11.1.4.2 Inherent and enhanced ready biodegradability tests

No data available

11.1.4.3 Water, water-sediment and soil degradation data (including simulation studies)

Aerobic mineralisation.

A new study (Gartner, C.; Herrechen (2016)) was submitted for the EU review on the aerobic mineralisation of Difenoconazle in surface water. The study followed OCED guideline 309 (November 2004) and was conducted to GLP. A summary is provided below, with a robust summary provided in Annex I of this dossier.

Author(s): Gartner C., Herrechen; 2016
Title: Difenoconazole - Aerobic Mineralisation of [^{14}C]-Difenoconazole in Surface Water
Guidelines: OECD Guideline 309
GLP: Yes

The extent of mineralisation and the rate and route of degradation of difenoconazole was investigated in Calwich Abbey natural lake water. Difenoconazole [triazolyl-U-^{14}C] and [chlorophenoxy-U-^{14}C] labels were separately applied to the water at nominal concentrations of 10 and 95 µg/L (low and high, respectively).

The systems were incubated under aerobic conditions in the dark at 20ºC, pH 8.9, and DO 8.4 mg/L for up to 61 days and samples were analyzed on 0, 7, 14, 21, 28, 47 and 61 in duplicate samples.

At both rates, the [chlorophenoxy-U-^{14}C] label was also applied to sterilised test systems. At each sampling time, the quantity of radioactivity in the water was determined by liquid scintillation counting (LSC). Any volatile radioactivity was continuously flushed from the vessels, collected in traps and analysed. A mass balance was determined for each sample.

Separate reference samples (treated with ^{14}C-sodium benzoate at 10 µg/L) of natural water were prepared to determine whether a viable microbial population was present in the test system but were only analysed at the end of the test.

The radioactive residue in the water samples was characterized and quantified by HPLC analysis, and confirmatory analysis were performed using TLC and Mass Spectrometry.

Findings:

The mean mass balance in all samples was 96.3 % AR (range 89.9 to 102.8 % AR).

For the non-sterilised, viable test systems Difenoconazole decreased to a mean of 61.9 %- 71.1% AR (10 µg/L) and 71.6 % AR - 78.9 % AR (95 µg/L) by 61 DAT.

The resulting degradation rates (DT50) of difenoconazole were estimated by fitting single first-order kinetics (SFO) to the data:

Test concentration (µg/L)	**DegT_{50} (days)**
10	104.7
95	146.7

It can be seen from the data that there is a dose dependence. The lower degradation of Difenoconazole was found in high test concentrations.

For the sterilised samples, difenoconazole was found to be stable, with 92% AR remaining at 61 DAT which demonstrates that the degradation of difenoconazole in natural water is microbially mediated.

Depending on the radiolabel, mineralisation to CO_2 was different:

	10 µg/L	**95 µg/L**
[triazolyl-U-14C]-Difenoconazole	0.6%	0.4%
[chlorophenoxy-U-14C]-Difenoconazole	16.3%	11.4%

In the 10 µg/L treatment of [triazolyl-U-^{14}C]-Difenoconazole, CGA205375 was observed > 5% AR in two consecutive measurements and it accounted for up to 10.3% AR at the end of the study (61 d).

CGA142856 was also a relevant metabolite, reaching a 7.6 %AR at the end of the study (61d) in this treatment.

In addition, up to eight discrete unknown components were also observed. Two of them exceeded 5 % of applied activity: unknown A and unknown B.

- At the high concentration of triazole label, the highest levels of unknown A were 9.8 % AR and it reached 5.9% AR at the end of the study (61 DAT).This metabolite was successfully identified by HR LC-MS as CGA199312. RMS considers that according to these results, CGA199312 should be included in the definition of residue for surface water risk assessment.

- The unknown degradate B was further split into several peaks based on additional TLC analysis. Only in one sample (10 µg/L, 47 DAT) did one component exceed 5% AR (5.4 % AR) and at only

one time point. At the next sampling interval at 61days of incubation no component is exceeding 5% AR. However, these results were obtained from the replicate where the lowest amount of radioactivity was detected after 61 d. Therefore, it cannot be ensure that the largest individual component did not exceed 5% AR at the end of the study or 10% AR at 47days. Additionally, the TLC examples included in the report were not enough to demonstrate that the unknown B is a multicomponent neither the %AR described above. Consequently, further information is required to confirm that any individual component of unknown B accounted for more than 10 % of the amount of active substance added, 5% in at least two sequential measurements, or >5 % at the end of the study.

The main degradate of [chlorophenoxy-U-^{14}C]-Difenoconazole was CGA205375, which reached a maximum of 5.9 % (10 µg/L, 61 DAT) and 2.3 % AR (95 µg/L, 47 DAT). CGA189138 was found at 1.7 %AR (high concentration only, 47 DAT). No unknown degradates were observed.

The study is accepted but further information is necessary related to metabolite "B".

Water/sediment studies.

Regarding water/sediment system, two studies (Gonzalez-Valero, 1993, and Ulbrich R.; 1997) were included in the submission for Annex I inclusion under Directive 91/414/EEC and were deemed acceptable following evaluation and peer review at EU level (2006).

In addition, two new studies (Lin, 2006, and Yeomans and Mould, 2018) were submitted for the EU review on the degradation of Difenoconazole in water/sediment systems. The studies followed the OECD guideline 308 (April 2002) and were conducted to GLP.

A kinetics assessment (Terry, 2015c) was performed in accordance with FOCUS degradation kinetics guidance (2006, 2011, 2014) on the raw data generated from Gonzalez-Valero (1993) and Lin (2006) studies.

Summaries of these studies are presented below, with robust summaries presented in Annex 1 of this dossier.

Author(s): González Valero, J.; 1993
Title: Metabolism of Difenoconazole under aerobic conditions in aquatic systems.
Guidelines: BBA Guideline Part IV; 5-1 (1990); Dutch Registration Guideline, Section G.2: Behaviour in Water, Ministry of Social Affairs, January 1987; US EPA 540/9-82-021.
GLP: Yes

Route and rate of degradation of [^{14}C-chlorophenyl]-difenoconazole in pond and river systems were investigated. The test substance was applied to the water phase at concentrations of 0.17 mg/L (corresponding to direct over spray of 100 g a.s./ha, , 0.06 m deep water) or related to sediment: 1.5 mg/kg dw (pond system), 0.47 mg/kg dw (river system). The systems were incubated for 183 days, with the water phases maintained under aerobic conditions at 20.0 ºC in the dark. Water and sediment samples were taken at 0, 1, 3, 7, 14, 22, 32, 59, 90, 127 and 183 days after treatment

Radioactivity in the water phases was quantified by LSC .The composition of the radioactivity in the water and sediment phases was determined by HPLC and TLC.

Findings:

The distribution and characterisation of radioactivity was expressed in terms of combined water and sediment phases.

Total radioactive recoveries were between 90 and 110% of AR.

Percentage of the applied radioactivity recovered in the water phase of each systems were from 87.7% (0d) to 2.1% (183d) in pond system and from 79.8% (0d) to 2.9% (183d) in river system and dissipation rates of radioactivity from the water phase were to 1-2 days.

Difenoconazole decreased from 89-96% of the applied radioactivity day 0 to 61-71% at study termination. Besides $^{14}CO_2$ no other volatile compounds were measured. Up to 3.9% of the applied radioactivity was evolved as $^{14}CO_2$. Bound residues increased to maximum 13.9% at study termination in the pond system.

CGA205375 was the only metabolite identified as >10% of the applied radioactivity (11.6% of the applied radioactivity on day 90 in the river system). An unknown aquatic metabolite M3 was observed > 5% AR in consecutive measurements and under the new data requirements needs to be addressed in the risk assessment (5.7% at 127d and 7.8% at 183d).

Since the identification of metabolites in this study for first EU approval was insufficient, a new water/sediment study was submitted for the purpose of renewal (see below Lin, 2006). According to Lin (2006) no metabolite was formed over 4% AR. Therefore, RMS considers that M3 should be not considered further in the environmental risk assessment.

The raw data of this study was used for recalculation of $DegT_{50}$ of Difenoconazole. The kinetics of this study has been re-assessed accoording to FOCUS degradation kinetics guidance (2006; 2011; 2014) and normalized to reference conditions in a separate report (see below Terry, 2015c).

Author(s): Ulbrich R.; 1997
Title: Metabolism of ^{14}C labelled Difenoconazole in aquatic systems under aerobic conditions at 8ºC.
Guidelines: OECD Draft Guideline: Aerobic and anaerobic transformation in water/sediment systems. August 2000.
GLP: Yes

The objective of this study was to investigate the dissipation and degradation of ^{14}C-Difenoconazole in pond and river systems at rates of 0.1 mg/L (corresponding to direct over spray of 100 g a.s./ha, 0.1 m deep water), or as related to sediment; 0.49 mg/kg dw (pond) and 0.34 mg/kg dw (river system). The systems were incubated for 181 days (river system) and 183 days (pond system), with the water phases maintained under aerobic conditions, at 8±1ºC, in the dark. Sampling time points were taken on days 0*, 1, 3, 7*, 14*, 28, 42*, 91, 120*/122* and 181*/183 (* in duplicate).

Findings:

Percentage of the applied radioactivity recovered in the water phase of each system were from 90.2% (0d) to 1.1% (183d) in pond system and from 95.2% (0d) to 1.8% (181d) in river system. The radioactivity rapidly disappeared from the water column. Characterisation of radioactivity in the two systems showed that Difenoconazole rapidly adsorbed on to the sediment.

Low amounts of $^{14}CO_2$ evolved, with maximum amounts of 1.9 and 2.9% in pond and river systems respectively, at the end of incubation. No other volatile products were detected. Unextracted radioactivity increased over the study, to maximum 11.4 and 9.8% at study termination (days 181/183).

No metabolites were identified as >10% of the applied radioactivity, but CGA205375 accounted two sequential measurements for more than 5%AR.

It is clear that difenoconazole is very persistent in sediments at low temperature. Since difenoconazole degraded less than 20%AR at the end of the study and it is an 8ºC study, no kinetics are derived from this study. It is considered only acceptable for the route of degradation of difenoconazole in water/sediment system.

Author(s): Lin Y; 2006
Title: Difenoconazole – Aerobic aquatic metabolism of [triazolyl-3,5]^{14}C-Difenoconazole.
Guidelines: EPA Guideline Number 162-4. OECD 308.
GLP: Yes

The degradation of Difenoconazole was investigated in a sandy loam sediment flooded with river water treated at maximum field of 125 g a.i. /ha (0.16 ppm dry sediment equivalent and 0.032 ppm total water/sediment system) with [Triazolyl-3,5]^{14}C-difenoconazole and was aerobically incubated at 25ºC in darkness. Non-sterile sediment/water (50 g/200 mL) systems were utilised. The sediment/water test systems were classified as kinetic systems in this report. During the course of the incubation, samples (total of 11 sampling points from day 0 to day 112) were harvested for extraction and quantitative/qualitative analysis. Samples dosed at an elevated concentration of 1.5 ppm (dry sediment equivalent) were incubated as bulk samples and harvested as bio-synthesizers to provide sufficient quantity of degradates for identification of the radioactive components.

Findings:

The recoveries in the mass balance ranged from 92.05% to 102.81% AR.

Radioactivity rapidly decreased in aqueous fraction from 87.86%AR (85.57% Difenoconazole) at day 0 to less than 5% (2.98% Difenoconazole) at day 28, but radioactivity found in sediment increased from 7.18% at day 0 to 85.79% (81.53% Difenoconazole) at day 112 and a maximum of 91.8% Difenoconazole at day 28.

In total system, AR decreased from 95.04% at day 0 (85.57% Difenoconazole) to 88.74% at day 112 (81.53% Difenoconazole). Non extractable radioactivity from sediment increased from 0.13% at day 0 to 8.87% at day 112 and volatiles accounted for less than 0.6%.

Difenoconazole degraded less than 20%AR at the end of the study. Thus, only five metabolites were detected in the system with %AR below 5% at the end of the study.

The raw data of this study was used for recalculation of $DegT_{50}$ of Difenoconazole. The kinetics of this study have been re-assessed according to FOCUS degradation kinetics guidance (2006; 2011; 2014) and normalized to reference conditions in a separate report (see below Terry, 2015c).

Author(s): Terry A; 2015c

Title: Difenoconazole – Calculation of persistence and modelling endpoints from water/sediment study data.
Guidelines: FOCUS 2006.
GLP: No

This report presents the calculations of $DegT_{50}$, $DegT_{90}$, DT_{50} and DT_{90} values for difenoconazole, for both persistence and modelling endpoints in water/sediment systems.

The degradation of difenoconazole has been studied in three equilibrated water/sediment systems in two studies (Gonzalez-Valero, 1993 and Lin, 2006). The original data from these studies were used to calculate the rate of degradation of difenoconazole in water/sediment systems, and the rate of dissipation of difenoconazole from the water compartment, following the guidance in FOCUS Kinetics (2006).

The RMS accept the trigger and modelling values purposed by the applicant. Temperature values of 25ºC were normalized to 20ºC using Q10 of 2.58:

		DT50/DT90 (Days)		
		Water Phase	**Whole System**	**Sediment**
Gonzalez-Valero (1993)	Pond system	2.16/7.16	318/>1000	-
	River system	5.52/18.3	300/>998	-
Lin (2006)	River system	3.20/10.6	1113/2300	690/>1000

Author(s): Yeomans, P, Mould, R.; 2018
Title: Difenoconazole – Aerobic Aquatic-Sediment Metabolism of ^{14}C-Difenoconazole.
Guidelines: OECD Guideline No. 308; EPA 712-C-08-018 and EPA 712-C-08-019, OPPTS 835.4300 and OPPTS 835.4400; SETAC-EUROPE Procedures for Assessing Environmental Fate and Ecotoxicity of Pesticides: Section 8.2 (Aerobic Aquatic Degradation).
GLP: Yes

The rate and route of degradation of ^{14}C-triazolyl ring labelled Difenoconazole and ^{14}C-2-chlorophenoxy labelled Difenoconazole was investigated in two different water sediment systems: Calwich Abbey (silt loam) and Swiss Lake (sand). ^{14}C-labelled Difenoconazole was applied to the water at a nominal amount of 30 µg/L (equivalent to a single maximum application rate of 125 g a.i/ha). The actual application rates achieved were 123 and 125 g ai/ha, based on actual application concentrations of 41.7 and 42.4 µg/L for triazolyl ring labelled and 2-chlorophenoxy labelled Difenoconazole, respectively.

The systems were incubated in the laboratory under aerobic conditions and maintained in the dark at 20 ± 2ºC for up to 181 days. For each system, duplicate samples were taken for analysis from 0 DAT through 181 DAT. At each sampling time, the water phase was separated from the sediment phase by decanting. Extractable ^{14}C-residues were characterized by HPLC and its quantitation confirmed by TLC. Any volatile radioactivity was continuously flushed from the vessels, collected in traps and analysed via Liquid Scintillation Counting (LSC). A mass balance is determined for each sample.

Findings:

The recoveries in overall mass balance for the four systems were between 93%-95%AR.

Radioactivity (% Applied Radioactivity, AR) rapidly decreased in aqueous fraction (mean total water residues) in every system:

	^{14}C-Triazolyl		**^{14}C-2-Chlorophenoxy**	
	0 DAT	**181 DAT**	**0 DAT**	**181 DAT**
Calwich Abbey	81.2%	2.4%	78.3%	0.8%
Swiss Lake	85.1%	12.3%	74.5%	10.0%

Radioactivity (% AR) found in sediment (mean total sediment residues) increased in every system.

	^{14}C-Triazolyl		**^{14}C-2-Chlorophenoxy**	
	0 DAT	**181 DAT**	**0 DAT**	**181 DAT**
Calwich Abbey	6.5%	83.6%	7.0%	83.3%
Swiss Lake	2.8%	61.1%	11.2%	60.0%

And radioactivity (% AR) found in total system (mean total water and sediment extractable residues) decreased in every system.

	^{14}C-Triazolyl		**^{14}C-2-Chlorophenoxy**	
	0 DAT	**181 DAT**	**0 DAT**	**181 DAT**
Calwich Abbey	87.5%	85.9%	85.4%	84.1%
Swiss Lake	87.8%	73.4%	85.7%	69.9%

Non extractable radioactivity from sediment increased from not detected (<0.01%AR) at 0DAT in every system to 7.9%AR, 9.5%AR, 23.8%AR and 22.7%AR for triazolyl and clorophenoxy labels in Calwich Abbey and Swiss Lake, respectively. Volatiles accounted for less than 2.7%AR.

For triazolyl label, difenoconazol degraded between 9.3%AR to 13.2%AR in Calwich Abbey and Swiss Lake, respectively. For chlorophenoxy label, difenoconazol degraded between 46.6%AR to 41.6%AR in Calwich Abbey and Swiss Lake, respectively. Thus, only two metabolites detected were accounted for >10%AR (max. CGA199312: 24.0%AR, triazolyl label Swiss Lake 181DAT; max. CGA205375: 11.4%AR, chlorophenoxy label Calwich Abbey 181DAT).

The half-lives (DT50) of ^{14}C- Difenoconazole in the water and in the total water-sediment system (from the HPLC analysis), were determined using a Single First Order (SFO) kinetic model (KinGUIv2.1) for each incubation condition. The kinetics of this study have been assessed according to FOCUS degradation kinetics guidance (2006, 2011, 2014):

		DT50/DT90 (Days)	
		Water Phase	**Whole System**
Swiss Lake	^{14}C-Triaxolyl	2.53/8.41	167/533
	^{14}C-2-Chlorophenoxy	2.13/7.08	164/546

Calwich Abbey	^{14}C-Triaxolyl	2.47/8.19	703/>1000
	^{14}C-2-Chlorophenoxy	2.46/8.16	470/>1000

The water/sediment studies suggest that Difenoconazole mainly disappears from aquatic systems by physical-chemical processes. Partitioning to sediment is the main route of dissipation of Difenoconazole in water sediment systems primarily binding to sediment.

Difenoconazole can be considered as not rapidly degradable in the aquatic environment from the water/sediment system studies carried out. Although short DT50 and DT90 values were registered for the water phases (DT50 between 2.13 and 5.52 days and DT90 between 7.08 and 18.3 days), Difenoconazole disappears by dissipation process, binding to sediment. And at the end of the above studies, the maximum carbon dioxide increased to 3.9% AR indicating minimal mineralization.

11.1.4.4 Photochemical degradation

There is a study on photochemical degradation under laboratory conditions (Gaauw, van der A. 2002a).

Authors: Gaauw, van der A. 2002a
Title: Aqueous Photolysis of Difenoconazole [^{14}C-Triazole] under laboratory conditions.
Guidelines: SETAC (1995); OECD/GD(97)21; EPA OPPTS 835.2210.
GLP: Yes

Solutions of radiolabelled difenoconazole were irradiated with artificial sunlight (xenon arc light) at 25 °C and pH 7. The irradiation was carried out for a continuous period of 15 days. Corresponding control samples were maintained under the same conditions but in the dark. Samples were taken for analysis at a range of time intervals up to 15 days (0, 3, 6, 8, 10 and 15 days) with radiochemical quantification by LSC and chromatographic analysis by HPLC and TLC.

Findings:

After 15 days of continuous irradiation, difenoconazole represented 91% of the applied radioactivity. Besides difenoconazole, three other radioactive fractions were detected, however, none exceeded 6.3% of the applied radioactivity. Co-chromatography using HPLC showed that none of the fractions corresponded to the available reference items.

For the dark control, individual recoveries ranged between 95% and 101% AR.

Conclusions:

Difenoconazole is stable to direct photolysis in aqueous systems.

Author(s): Hennecke D.; 2002a
Title: Quantum Yield of the photochemical degradation of Difenoconazole in aqueous solution.
Guidelines: SETAC (1995); OECD/GD(97)21; EPA OPPTS 835.2210
GLP: Yes

For determination of the quantum yield, difenoconazole was dissolved in purified, de-ionised water containing 10% of acetonitrile as inert co-solvent. Irradiated samples and corresponding dark control samples were incubated at 25±1ºC (irradiated) and 22ºC (dark controls) and analysed by HPLC.

Based on the assumption that the quantum yield is independent of the wavelength for a discrete absorption band, the irradiation experiments were performed at 290 ± 4 nm with the intention to measure higher degradation rates because absorption decreases rapidly at higher wavelengths. Samples were irradiated for 0.5, 1, 2, 3, 4 and 6 hours.

Initial test substance concentrations for the quantum yield determination were 10.1 mg/L, 20.0 mg/L and 41.1 mg/L and were irradiated for 0.5, 1, 2, 3, 4 and 6 hours. After 6 hours irradiation the observed degradation was approximately 37% on all tested concentrations. Therefore these samples were not used for determination of quantum yield (0-30% transformation was used as criteria since otherwise degradation products may disturb the correct determination). Control samples were prepared and stored in the dark.

Findings:

The maximum absorption was at approximately 272.5 nm showing an absorption band which tails into the spectrum of sunlight (wavelengths > 290 nm), but no separate absorption band above 290 nm was observed. The molar absorption coefficient at 290 nm ε_{290} was 552.1 $L*mol^{-1}*cm^{-1}$ and at 295 nm ε_{295} was 139.3 $L*mol^{-1}*cm^{-1}$.

The quantum yield was determined to be 0.0155. Environmental half-lives were predicted using a computer program based on a model developed by Frank and Klöpffer (1989)[1]. The determined UV/Vis absorption coefficients and the calculated quantum yield were used as input and no dissipation processes other than photolysis were considered. The calculation was performed for pure water. The calculated half lives of difenoconazole at 52° North were between 11.8 years and >10000 years, depending on the season.

Conclusions:
Direct photolysis is assessed to be an insignificant process for degradation of Difenoconazole in surface water.

Overall conclusions on degradation.

Difenoconazole is considered not readily biodegradable according to the result of the biodegradation test presented (0 %), following OECD 301 B guideline. The ready biodegradability criterion stated in this guideline considers substances readily biodegradable when 70% biotic degradation takes place in the 10 days window within the 28 days long duration test.

[1] Frank K and Klöpffer W (1989) A Convenient Model and Program for the Assessment of Abiotic Degradation of Chemicals in Natural Waters. Ecotox. Environ. Safety, 17; 323-332.

Difenoconazole shows hydrolytic stability at pH values of 5, 7 and 9 at 25ºC under sterile conditions in the dark for 30 days and it is considered hydrolytically stable at environmentally relevant temperatures and pH values.

In an aerobic mineralization study Difenoconazole degraded with DT_{50} values of 104.7 and 146.7, depending on test concentration, to the following metabolites: CGA205375 and CGA142856.

The water/sediment study suggests that Difenoconazole mainly disappears from aquatic systems by physical-chemical processes and not by microbial degradation. Partitioning to sediment is the main route of dissipation of Difenoconazole in water sediment systems primarily binding to sediment. Although short DT50 and DT90 values were registered for the water phases (DT50 between 2.13 and 5.52 days and DT90 between 7.08 and 18.3 days), Difenoconazole disappears by dissipation process, binding to sediment. And at the end of the above studies, the maximum carbon dioxide increased to 3.9% AR indicating minimal mineralization.

Photodegradation of Difenoconazole was measured being insignificant in water

Due to the results summarized above, Difenoconazole can be considered as a not rapidly degradable substance in the environment, according to the CLP criteria.

11.2 Environmental transformation of metals or inorganic metals compounds

Not applicable.

11.2.1 Summary of data/information on environmental transformation

Not applicable.

11.3 Environmental fate and other relevant information

Soil adsorption

In the RAR, three studies on adsorption and desorption in soils were considered valid for Difenoconazole (Adam, 2006a, Atkins, 1191a and Spare, 1988). Difenoconazole is considered being immobile for adsorption and desorption in soil based on KfOC values (6139 mg/L, 5966 mg/L and 3206 mg/L). The results indicate Difenoconazole would be moderately to strongly adsorbed to soil or sediment. Water/sediment studies confirm this with high levels of partitioning into sediment.

There are also three studies (Adam, 2006b, Walsh, 2008 and Völkel, 2002) on adsorption and desorption in soils for GCA205375 metabolite. This metabolite is also considered being immobile for adsorption and desorption in soil based on KfOC values (5180 mg/L, 2079 mg/L and 2660 mg/L).

On the other hand, the study on adsorption and desorption in soils for GCA142856 metabolite (Scachci et al. 2002) indicates that it is mobile in the tested soils (KfOC value 9.12 mg/L).

These results do not impact the conclusions regarding degradation (not rapidly) according to CLP criteria and there are not summarised below although their robust summaries are presented in Annex 1 of this dossier.

Volatilisation.

Based on the very low vapour pressure (3.32×10^{-8} Pa, 25 °C) and low Henry´s law constant (9.0×10^{-7} Pa m3 mol-1 at 25ºC), Difenoconazole is virtually non-volatile, therefore, significant exposure to air is not to be expected. However, there are also two volatilization studies on Difenoconazole which were provided in the RAR and in which the results show that Difenoconazole is not expected to volatilize significantly from soil. These results do not impact the degradation classification and there are not summarised below although their robust summaries are presented in Annex 1 of this dossier.

11.4 Bioaccumulation

Table 43: Summary of relevant information on bioaccumulation

Method	Results	Remarks	Reference
Partition coefficient n-octanol/water EEC A.8, OECD 107, OPPTS 830.7550: shake flask method	log P_{ow} = 4.36 ± 0.02 at 25 °C and a pH of approximately 8 (unbuffered distilled water). The determination was only performed at one pH since Difenoconazole does not dissociate at environmentally relevant pH.	This study is accepted.	Kettner, R. (1999b) CA 2.7/01
Bioconcentration test in bluegill sunfish. US EPA FIFRA 72-6	Difenoconazole whole fish BCF: 320.	- No attempt was made to characterise the radioactivity, hence the BCF is based on total residues - Only one concentration was tested, while the guidelines require at least two exposure levels	Anonymous (1987)
Bioconcentration test in bluegill sunfish. US EPA FIFRA 72-6	Difenoconazole whole fish BCF: 330.	- Only one concentration was tested, while the guidelines require at least two exposure levels	Anonymous (1992).

11.4.1 Estimated bioaccumulation

As experimental data are available, estimations of bioaccumulation potential are not required.

11.4.2 Measured partition coefficient and bioaccumulation test data

Author(s): Kettner, R. (1999b).
Title: Octanol/water partition coefficient of Difenoconazole.
Guidelines: OECD 107; OPPTS 830.7550
GLP: Yes

This study aimed to determine the partition coefficient n-octanol/water of Difenoconazole at 25ºC.

Findings:

The following result was obtained for Difenoconazole:
logPow = 4.36 ± 0.02 at 25ºC and a pH of approximately 8 (unbuffered distilled water).
The determination was only performed at one pH since Difenoconazole does not dissociate at environmental relevant pH.

With regard to bioaccumulation of Difenoconazole, two 28 days dynamic studies on bioconcentration by bluegill sunfish (*Lepomis macrochirus*) were carried out. They were generally conducted in accordance with the referred guidelines, excep that only one concentration was tested in each study, while the guidelines require at least two exposure levels.
However, the two available studies together are considered to fulfil the requirement of more than one exposure concentration.

Author(s): Anonymous (1987).
Title: Uptake, depuration and bioconcentration of ^{14}C-Difenoconazole by bluegill sunfish (*Lepomis macrochirus*).
Guidelines: US EPA FIFRA 72-6
GLP: Yes

This study aimed to determine the uptake rate, depuration rate and bioconcentration of [14C]-Difenoconazole in bluegill sunfish (*Lepomis macrochirus*).

Bluegill sunfish were exposed in a flow through test system to a nominal concentration of 0.02 mg/L of [^{14}C]-Difenoconazole for a period of 28 days followed by a 14 day period of depuration in fresh water. Mortality and abnormal behaviour were recorded.

Findings:

The mean water concentration of Difenoconazole over the 28-day exposure period was 0.018 mg/L, i.e. 90% of nominal. However, due to malfunction of the diluter apparatus on day 27, the measured concentration on day 28 was 0.031 mg/L, i.e. 155% of nominal. Therefore day 28 data was not considered in the calculation of uptake rate, depuration rate or bioconcentration factor.

With the exception of two fish that died between days 0 and 3 of the uptake phase, all fish remained healthy throughout the study. The mean ^{14}C residue concentrations for fillet, viscera and whole fish were 5.9, 8.0 and 4.7 ppm respectively, after 28 days of continuous exposure. Analysis of fish samples taken during the depuration phase, indicated that 50% of accumulated residues were eliminated after 1.1 days in fresh water and that fish had eliminated 99, 100 and 100% of residues from fillet, viscera and whole body, respectively, by day 14.

When exposed continuously to 0.018 mg/L Difenoconazole, a steady state concentration in fish tissues was reached after 3.7 days of exposure and complete depuration occurred within 14 days of transfer to clean water.

Uptake rate constants, depuration rate constants and bioconcentration factors for ^{14}C difenoconazole in bluegill sunfish are presented below:

Uptake rate constant (whole fish)	200 (±14)
Depuration rate constant	0.62 (±0.044)
Depuration half-life (days)	1.1 (±0.079)
Bioconcentration factor (mg/kg whole fish mg/L water)	320 (±32)
Time to reach 90% of steady state (days)	3.7 (±0.26)

Conclusions:
The samples from day 28 could not be used for the calculations of uptake rate and bioconcentration factors, but since steady state was reached after less than four days, this would not have a significant impact on the results. No attempt was made to characterise the radioactivity, hence the BCF is based on total residues.
Only one concentration was tested, while the guidelines require at least two exposure levels. Otherwise, the study was generally conducted in accordance with the referred guidelines. Since a second study is available (Fackler, 1992) there is sufficient information from two different test concentrations.

Author(s): Anonymous (1992).
Title: Bioconcentration and elimination of ^{14}C-residues by bluegill (*Lepomis macrochirus*) exposed to Difenoconazole
Guidelines: USEPA FIFRA 72-6
GLP: Yes

This study aimed to determine the uptake rate, depuration rate and bioconcentration of [14C]-Difenoconazole in bluegill sunfish (*Lepomis macrochirus*).

Bluegill sunfish were exposed in a flow through test system to a nominal concentration of 1.0 µg/L of [^{14}C]-Difenoconazole for a period of 28 days followed by a 14 day period of depuration in fresh water. After an equilibration period, fish were monitored daily for mortality and abnormal behavior. After 28 days, remaining fish were transferred to fresh water for a further 14 days for metabolite analyses. Further, hexane/methanol extractions of muscle tissue were made to determine the relative distribution of nonpolar and polar radioactivity on day 28 of exposure.

Findings:

With the exception of 8 fish that died in the difenoconazole-treated tank, all fish were healthy and exhibited normal behaviour throughout the study. The mean Difenoconazole water concentration was 1.1 µg/L, i.e.110% of nominal, over the 28-day exposure period, and remained below 0.26 µg/L during the depuration phase.

The mean ^{14}C residue concentrations for fillet, viscera and whole fish were 180, 610 and 340 µg/kg, respectively, after 28 days continuous exposure. Analysis of fish samples taken during the depuration phase, indicated that 50% of accumulated residues were eliminated by day 3 and that fish had eliminated 96, 98 and 97% of residues from fillet, viscera and whole body, respectively, by day 14.
When exposed continuously to 1.0 mg/L Difenoconazole, a steady state concentration in fish tissues was reached after 3 days of exposure and 97% depuration occurred within 14 days of transfer to clean water.

Estimated uptake rate constants, depuration rate constants and bioconcentration factors are presented below.

	Fillet	Viscera	Whole fish
Uptake rate constant	140	870	270
Depuration rate constant	0.86	1.5	0.84
Bioconcentration factor	170	570	330
Time to reach steady state (estimated by RMS)	ca 3 days	ca 3 days	ca 3 days

Analyses of the methanol (polar) and hexane (non-polar) solvent extractions of edible tissues on day 28 revealed that 35% of the residues were extractable with methanol, 19% with hexane and 48% were not extractable with either solvent.

Conclusions:
The radioactivity in tissue samples from edible parts on day 1 was more than 3 times higher than the steady state level that was reached from day 3, and no explanation was given in the study report. A whole fish BCF calculated based on day 1 concentrations would be 645. However, in samples from day 3 onwards, the concentrations remained stable throughout the exposure phase, and therefore the proposed BCF values based on the steady state concentrations are considered reasonable.
Further, the steady state BCF values are consistent with the previously referred study (Forbis, 1987). Also in this study, only one concentration level was tested.

To conclude, on the basis of the available information, whether the substance has the potential to bioconcentrate in aquatic organisms or not, the two BCF values of 320 and 330 (in whole fish) from the dynamic studies should be compared to the CLP criteria. Thus, Difenoconazole does not meet the criterion established by CLP (as the experimentally determined BCF value is <500), so no bioconcentration in fish is expected. The experimental log Kow value of 4.36 is greater than the trigger value of 4 in the CLP Regulation and so indicates a potential for bioaccumulation. Nevertheless as experimental derived BCF values are more preferred than log Kow values for classification purposes, the above mentioned BCF <500 would already determine that bioconcentration is no expected for this active substance.

11.5 Acute aquatic hazard

A brief summary of the aquatic toxicity studies evaluated during Annex I inclusion of Difenoconazole and submitted for the purposes of EU renewal is reported below. From all available ecotoxicity tests on this substance only information considered adequate, reliable and relevant for the classification proposal has been included.

The available acute toxicity data for relevant metabolites of Difenoconazole (CGA 71019, CGA 142586) revealed toxicity values > 1 mg/L. Therefore, the studies with these metabolites are not described here in detail. On the other hand, the toxicity data of metabolite CGA205375 revealed toxicity values to fish < 1 mg/L and the study with this metabolite is described below.

Table 44: Summary of relevant information on acute aquatic toxicity

Method	Species	Test material	Results[1]	Remarks	Reference
Acute toxicity to fish.	Rainbow trout (*Salmo gairdneri)*	Difenoconazole technical (96.1% purity)	96h-LC50 = 1.1 mg/L, based on mean	Accepted	Anonymous 1990a

US EPA FIFRA 72-1			measured concentrations		
Acute toxicity to fish. US EPA FIFRA 72-1	Bluegill sunfish (*Lepomis macrochirus)*	Difenoconazole technical (96.1% purity)	96h-LC50 = 1.21 mg/L based on mean measured concentrations	Accepted	Anonymous 1988.
Acute toxicity to fish. US EPA FIFRA 72-3	Sheepshead minnow (*Cyprinodon variegates)*	Difenoconazole technical (96% purity)	96h-LC50 = 1.16 mg/L based on mean measured concentrations	Accepted	Anonymous 1993.
Acute toxicity to fish. OPPTS Draft Guideline 850.1075 and OECD Guideline 203.	Fathead minnow (*Pimephales promelas)*	Difenoconazole technical (97.3% purity)	96h-LC50 = 1.9 mg/L based on mean measured concentrations	Accepted	Anonymous 2011
Acute toxicity to fish. OECD Guideline 203.	Rainbow trout (*Salmo gairdneri)*	CGA 205375 (triazolylalcohol) (99% purity)	96h-LC50 = 0.66 mg/L, based on mean measured concentration	Accepted	Anonymous (2001a)
Acute toxicity to aquatic invertebrates. US EPA FIFRA 72-2	Water flea (*Daphnia magna)*	Difenoconazole technical (96.1% purity)	48h-LC50 = 0.77 mg/L based on mean measured concentrations	Accepted	Forbis, A.D. 1988a.
Acute toxicity to aquatic invertebrates. US EPA FIFRA 72-3	Mysid shrimp (*Mysidopsis bahia)*	Difenoconazole technical (95% purity)	48h-LC50 = 0.15 mg/L based on mean measured concentrations	Accepted	Surprenant, D. C. 1990c.
Acute toxicity to aquatic invertebrates. US EPA FIFRA 72-3	Eastern oysters (*Crassostrea virginica)*	Difenoconazole technical (95% purity)	48h-LC50 > 0.3 mg/L based on mean measured concentrations	The power of the statistical evaluation is probably low, since there was a large variation in shell deposition rate among both control and treated shells so this value would be considerd as	Surprenant, D. C. 1990d.

				supplementary information. Accepted	
Acute toxicity to algae or other aquatic plants. OECD Guideline 201	Green algae (*Scenedesmus subspicatus*)	Difenoconazole technical (91.8% purity)	72h-E_bC50 = 0.032 mg/L based on mean measured concentrations	Accepted	Grade, R. (1993b)
Acute toxicity to algae or other aquatic plants. Statistical Re-analysis	Green algae (*Scenedesmus subspicatus*)	Difenoconazole technical (91.8% purity)	**72h-E_rC50 = 0.0876 mg/L** based on mean measured concentrations	Statistical Re-analysis of data from the previous study (Grade, 1993b). Accepted	Taylor, S. & Pickering, F. (2016b).
Acute toxicity to algae or other aquatic plants. OECD Guideline 201	Freshwater green alga (*Pseudokirchneriella subcapitata)*	Difenoconazole technical (94.4% purity)	72h-ErC50 = 1.2 mg/L based on mean measured concentrations	Accepted	Hoberg, J. R. (2006).
Acute toxicity to algae or other aquatic plants. US EPA FIFRA 122-2	Duckweed (*Lemna gibba)*	Difenoconazole technical (96.1% purity)	14d-EC50 = 18.5 mg/L (frond number) 14d-EC50 = 9.9 mg/L (dry weight), based on nominal concentrations	Results should be treated with caution due to no analytical measurements were made to verify the test concentrations. Supplementary information	Drottar, K. (1986)
Acute toxicity to algae or other aquatic plants. OECD 221 (2006); US EPA, OPPTS 850.4400 (1996).	Duckweed (Lemna gibba)	Difenoconazole technical (94.4% purity)	For the frond number: 7d-EbC50 = 1.8 mg/L 7d-ErC50 >6.5 mg/L based on mean measure concentrations	Accepted.	Hoberg, J. R. (2006d).

[1] Indicate if the results are based on the measured or on the nominal concentration

11.5.1 Acute (short-term) toxicity to fish

With regard to acute (short-term) toxicity to fish of Difenoconazole, four studies were carried out. Three of these studies (Surprenant, 1990a; Bowman, 1988; Machado, 1993) were already evaluated during Annex I inclusion of Difenoconazole and they were accepted. The last one (Fournier, 2011) was submitted for the purpose of EU renewal.

Author(s): Anonymous (1990a).
Title: Acute toxicity of Difenoconazole to rainbow trout (*Salmo gairdneri*) under flow-through

conditions
Guidelines: US EPA FIFRA 72-1
GLP: Yes

The 96-hour LC_{50} of Difenoconazole to rainbow trout (*Salmo gairdneri*) was assessed under continuous flow-through conditions. Two replicates each were exposed to nominal concentrations of 0.45, 0.69, 1.1, 1.6 and 2.5 mg/L of Difenoconazole. Mortality and abnormal behaviour were recorded at 24, 48, 72 and 96 hours. Temperature, pH and dissolved oxygen concentrations were also recorded at 24 hour intervals and water samples collected at test initiation and test termination were analysed for test substance concentration by HPLC.

Findings:

Mean measured difenoconazole concentrations corresponded to 84-129% of nominal concentrations. The 96-hour LC50 for Difenoconazole was calculated to be 1.1 mg/L, based on mean measured concentrations. It was noted that no NOEC value could be determined from this study, since sublethal effects were observed at all test concentrations.

Author(s): Anonymous (1988).
Title: Acute toxicity of Difenoconazole technical to bluegill sunfish (*Lepomis macrochirus*).
Guidelines: US EPA FIFRA 72-1
GLP: Yes

The 96-hour LC_{50} of Difenoconazole to bluegill sunfish (*Lepomis macrochirus*) was assessed under static conditions. Exposure nominal concentrations were 0.32, 0.56, 1.0, 1.8 and 3.2 mg/L. Mortality and abnormal behaviour were monitored at 24, 48, 72 and 96 hours. Temperature, pH and dissolved oxygen concentrations were also recorded at 24 hour intervals and water samples collected at test initiation and test termination were analysed for test substance concentration by HPLC.

Findings:

The study was conducted in accordance with the referred guidelines, although more than one replicate would have been preferred, although not strictly required.
Measured concentrations at termination of the test were lower than 80 % (70 – 78%) so the LC_{50} should be calculated based on mean measured concentrations. And according to OECD Guidelines 203 if the data obtained are not suitable for standard methods of calculation of the LC_{50} (for example cases like this, where the dose response curve goes from 0% to 100% mortality between two subsequent concentrations), and if the concentration interval is less than a factor of 2, then the geometric mean of the highest concentration causing no immobility (nominal concentration of 1.0 mg as/L) and the lowest causing 100% immobility (nominal concentration of 1.8 mg as/L) can be used as an approximate LC_{50}. Hence the LC_{50} from this study can be considered to be 1.21 mg/L, considering the geometric mean measured concentration of tested levels.

Author(s): Anonymous (1993).
Title: Difenoconazole - Acute toxicity to sheepshead minnow (*Cyprinodon variegates*) under flow-through conditions.

Guidelines: US EPA FIFRA 72-3.
GLP: Yes

The 96-hour LC_{50} of Difenoconazole to sheepshead minnow (*Cyprinodon variegates*) was assessed under flow-through conditions. Exposure nominal concentrations were 0.32, 0.54, 0.9, 1.5 and 2.5 mg/L. Mortality and abnormal behaviour were monitored at 24, 48, 72 and 96 hours. Temperature, pH and dissolved oxygen concentrations were also recorded daily and water samples collected, at test initiation and test termination, were analysed for test substance concentration by HPLC.

Findings:

The study was conducted in accordance with the referred guidelines. At the 96-h two lowest test levels, the measured concentrations were significantly higher than the initial values, due to a malfunction of the diluter system. Therefore, only the initial measured values were used for these levels when the LC_{50} was calculated.
Mean measured difenoconazole concentrations corresponded to 74 – 100% of nominal concentrations.
According to OECD Guidelines 203 if the data obtained are not suitable for standard methods of calculation of the LC_{50} (for example cases like this, where the dose response curve goes from 0% to 100% mortality between two subsequent concentrations), and if the concentration interval is less than a factor of 2, then the geometric mean of the highest concentration causing no immobility and the lowest causing 100% immobility can be used as an approximate LC_{50}. Hence the LC_{50} from this study can be considered to be 1.16 mg/L, considering the geometric mean measured concentration of tested levels.

Author(s): Anonymous (2011).
Title: Difenoconazole - Acute toxicity to Fathead minnow (*Pimephales promelas*) under static-renewal conditions.
Guidelines: OPPTS Draft Guideline 850.1075 and OECD Guideline 203.
GLP: Yes

The acute toxicity of Difenoconazole to fathead minnow (*Pimephales promelas*) was determined under static-renewal conditions. Exposure nominal concentrations were 0.25, 0.50, 1.0, 2.0, 4.0 and 8.0 mg/L (0.25, 0.33, 0.66, 1.4, 2.6 and 5.1 mg/L mean measured). Mortality and symtoms of toxicity were made at 0, 6, 24, 48, 72 and 96 hours. Temperature, pH and dissolved oxygen concentrations were also recorded daily. The test concentrations were verified by chemical analysis of Difenoconazole at 0, 48 and 96 hours using an HPLC/UV method.

Findings:

Mean measured difenoconazole concentrations ranged from 64 to 99% of nominal values. Analysis of quality control samples resulted in measured concentrations in the range of 92 to 106% of the nominal fortified values confirming the appropriate precision and quality control was maintained.
The 96h-LC50 was estimated by binomial probability (CETIS^{TM} Version 1.8.0 (Ives, 2009)). Based on mean measured concentrations, the 96h-LC50 was estimated to be 1.9 mg/L. The 96h-NOEC, based on mortality and sub-lethal effects was determined to be 0.66 mg/L.

METABOLITES

Author(s): Anonymous (2001a)
Title: CGA 205375 - Acute toxicity to rainbow trout (*Salmo gairdneri*).
Guidelines: OECD 203
GLP: Yes

The 96-hour LC_{50} of CGA205375 to rainbow trout (*Salmo gairdneri*) was assessed under static conditions. Exposure nominal concentrations were 0.12, 0.25, 0.5, 1.0 and 2.0 mg/L of Difenoconazole. Mortality and symptoms of toxicity were monitored at 24, 48, 72 and 96 hours. Temperature, pH and dissolved oxygen concentrations were also recorded at 24 hour intervals and water samples collected at test initiation and test termination were analysed for test substance concentration by HPLC.

Findings:

Mean measured difenoconazole concentrations corresponded to 44-61% of nominal concentrations. The 96-hour LC50 for CGA 205375 was calculated to be 0.66 mg/L, based on mean measured concentrations.

11.5.2 Acute (short-term) toxicity to aquatic invertebrates

With regard to acute (short-term) toxicity to aquatic invertebrates of Difenoconazole, three studies were carried out (Forbis, 1988a; Surprenant, 1990c; Surprenant, 1990d). All of them were already evaluated during Annex I inclusion of Difenoconazole and they were accepted.

Author(s): Forbis, A. D. (1988a).
Title: Acute toxicity of Difenoconazole to *Daphnia magna*.
Guidelines: USEPA FIFRA 72 - 2.
GLP: Yes

The acute toxicity of Difenoconazole to *Daphnia magna* was determined under static conditions. Exposure nominal concentrations were 0.56, 1.0, 1.8, 3.2 and 5.6 mg/L. The daphnids were monitored at 24 and 48 hours for mortality. Temperature, pH and dissolved oxygen concentrations were recorded at 0 and 48 hours.Water samples were collected at 0 and 48 hours for analysis of test substance concentration by HPLC.

Findings:

Overall mean measured difenoconazole concentrations corresponded to 90–100% of nominal concentrations.
Based on mean measured concentrations, the 48h-LC50 for Difenoconazole in *Daphnia magna* was estimated to be 0.77 mg/L.

Author(s): Surprenant, D. C. (1990c).
Title: Difenoconazole: Acute toxicity to mysid shrimp (*Mysidopsis bahia*) under flow-through conditions.
Guidelines: USEPA FIFRA 72 - 3.
GLP: Yes

The acute toxicity of Difenoconazole to *Mysidopsis bahia* was determined under flow-through conditions. Two replicates each were exposed to nominal concentrations of 0.036, 0.055, 0.085, 0.13 and 0.2 mg/L. Mortality was monitored daily. Temperature, pH and dissolved oxygen were recorded daily and water samples were collected on day 0 and 4 for analysis of test substance concentration by HPLC.

Findings:

Mean measured difenoconazole concentrations corresponded to 81-95% of nominal concentrations. Based on mean measured concentrations, the 48h-LC50 for Difenoconazole in *Mysidopsis bahia* was estimated to be 0.15 mg/L

Author(s): Surprenant, D. C. (1990d).
Title: Difenoconazole: Acute toxicity to eastern oysters (*Crassostrea virginica*) under flow-through conditions.
Guidelines: USEPA FIFRA 72 - 3.
GLP: Yes

The acute toxicity of Difenoconazole to *Crassostrea virginica* was determined under flow-through conditions. Two replicates each were exposed to nominal concentrations of 0.044, 0.088, 0.18, 0.35, and 0.7 mg/L. The oysters were monitored daily for abnormalities and mortality. After 96 hours oysters were removed for measurement of shell growth. Temperature, pH and dissolved oxygen were recorded daily and water samples were collected on day 0 and 4 for analysis of test substance concentration by HPLC.

Findings:

Mean measured difenoconazole concentrations corresponded to 43-145% of nominal concentrations. Throughout the exposure period, oysters did not exhibit any abnormalities and suffer any mortality at any exposure concentration. Shell growth data indicates that shell deposition was 35% lower in oysters exposed to 0.3 mg/L difenoconazole, than in untreated oysters.

Based on mean measured concentrations, the 96h-LC50 > 0.3 mg/L. The power of the statistical evaluation is probably low, since there was a large variation in shell deposition rate among both control and treated shells so this value would be considerd as supplementary information.

11.5.3 Acute (short-term) toxicity to algae or other aquatic plants

With regard to acute (short-term) toxicity to algae of Difenoconazole, two studies were carried out.

(Grade, 1993b and Hoberg, 2006). These studies were already evaluated during Annex I inclusion of Difenoconazole and they were accepted. For the purpose of EU renewal and according to Commission Regulation (EU) No 283/2013, a satatistical re-analysis of data from Grade (1993b) was presented (Taylor & Pickering, 2016b) and it is also sumarised below.

Author(s): Grade, R. (1993b).
Title: Report on the growth inhibition test of Difenoconazole tech. to green algae (*Scenedesmus subspicatus*).
Guidelines: OECD Guideline 201.
GLP: Yes

The potential toxicity of Difenoconazole to green algae (*Scenedesmus subspicatus*) was investigated under static conditions for 72 hours. Exposure nominal concentrations were 0.0123, 0.037, 0.11, 0.33 and 1.0 mg/L. Cultures were maintained for 3 days under a constant temperature and continuous light. Cell density was assessed faily using a cell counter. Samples of culture solutions were taken immediately prior to exposure and after 72 hours for analysis of test substance concentrations by GLC.

Findings:

GLC analysis showed that mean initial difenoconazole concentrations for nominal concentrations of 0.0123 and 0.037 mg/L were below the limits of detection (<0.04 mg/L) while mean initial concentrations for nominal concentrations of 0.11, 0.33 and 1.0 mg/L were 77, 65 and 70% of nominal.
Based on mean measured values for those concentrations above the limit of detection and assuming the actual concentration of those doses below the limit of detection were 70% of nominal, 72 hour E_bC_{50} and NOEC parameters were estimated to be 0.032 and 0.0086 mg/L, respectively.

Author(s): Taylor, S. & Pickering, F. (2016b).
Title: Difenoconazole - Report on the growth inhibition test of Defenoconazole to green algae (*Scenedesmus subspicatus*). Statistical Re-analysis.
Guidelines:
GLP:

This study is based on the previous data repoted by Grade (1993b) submitted and evaluated in the previous EU review of difenoconazole (DAR, May 2006). In that study the effect of five active substance concentrations (0.0123, 0.037, 0.11, 0.33, 1.0 mg/L nominal) towards *Scenedesmus subspicatus* was investigated. However, the mean measured concentrations of the test item at the highest three nominal concentrations were 0.085, 0.215 and 0.70 mg/L, whereas it was not possible to determine the mean measured concentrations at the nominal concentrations of 0.0123 and 0.037 mg/L.

Findings:

On the basis that the mean recoveries for the highest three treatments were 71%, the proposed assumption that the real concentrations of the nominal values 0.0123 and 0.037 mg/L were 0.0086 and 0.026 mg/L respectively, was considered reasonable.

The EC50 value reported was 0.032 mg/L which was below the limit of detection of the active substance (0.04 mg/L). In addition, that value was based on biomass only (area under the growth curve).

In this study, the aforementioned data were re-analysed to provide the EC10, EC20 and EC50 for the response variables yield and growth rate:

Yield	Growth rate
72h-EyC10 = 0.0114 mg a.s./L	72h-ErC10 = 0.0150 mg a.s./L
72h-EyC20 = 0.0156 mg a.s./L	72h-ErC20 = 0.0274 mg a.s./L
72h-EyC50 = 0.0282 mg a.s./L	72h-ErC50 = 0.0876 mg a.s./L

Author(s): Hoberg, J. R. (2006).
Title: Difenoconazole: Toxicity to Freshwater green alga (*Pseudokirchneriella subcapitata*).
Guidelines: OECD Guideline 201.
GLP: Yes

The toxicity of Difenoconazole to green algae (*Pseudokirchneriella subcapitata*) was determined under static conditions for 96 hours. Exposure nominal concentrations were 0.072, 0.18, 0.45, 1.1, 2.8 and 7.0 mg/L. Cultures were maintained under a constant temperature and continuous light. Cell density was assessed daily. The test concentrations were verified by chemical analysis at 0 and 96 hours, using HPLC.

Findings:

Chemical analysis of the test solutions showed that measured concentrations ranged from 41 to 83% of the nominal values, and mean measured concentrations were used (0.059, 0.15, 0.36, 0.89, 2.3 and 4.5 mg a.s./L).
Statical analysis (t-test) determined no significant differences between control and solvent control in cell density, biomass and growth rate, and thus data from both controls were pooled.
The NOEC for total biomass was 0.059 mg/L. Although the statistical method of fit the data is not specified, the 72h- EbC50 was reporetd to be 0.56 mg/L. A significant reduction in growth rate at concentration of 0.89 mg as/L is found compared to pooled controls, and the 72-h NOEC was 0.36 mg a.s./L. The 72h-ErC50 was reporetd to be 1.2 mg/L.

In addition to the above studies just presented, another three studies (Hoberg, 2006a; Hoberg, 2006b and Hoberg, 2006c), and their stastical re-analysis (Taylor & Allen, 2016d; Taylor & Allen, 2016e; Taylor & Allen, 2016f, respectively), conducted with additional algal species have been submitted in order to evaluate toxicity of Difenoconazol in other algae species. Despite they are not required by the Regulation 283/2013, these studies do not met the validity criteria of OECD 201 Guideline needed

for approval of active substances in the EU. Additionally, data needed for fulfilling validity criteria of the tests are not provided. Therefore, these studies and their re-analysis are not considered valid and they are not summarized below though their full robust summaries are presented in Annex 1 to this dossier.

Effects on aquatic macrophytes.

Author(s): Drottar, K. R. (1986).
Title: Acute toxicity of Difenoconazole to duckweed (*Lemna gibba G3*).
Guidelines: US EPA FIFRA 122-2.
GLP: Yes

The toxicity of Difenoconazole to duckweed (*Lemna gibba G3*).) was determined under static conditions for 14 days. Exposure nominal concentrations were 1.25, 2.5, 5, 10, 20 and 40 mg/L. Frond number and dry weight biomass was assessed after 14 days.

Findings:

Based on nominal concentrations, the 14-day EC50 for frond number and dry weight were 18.5 and 9.9 mg/L, respectively.

According to the previous evaluation carried out by the RMS (Sweden), results should be treated with caution due to no analytical measurements were made to verify the test concentrations. However, due to this data will not be used in the risk assessment and difenoconazole is not an herbicide or growth regulator, the test in higher plants is not required by Regulation 283/2013.

This study is considered as supplementary information.

Author(s): Hoberg, J. R. (2006d).
Title: Difenoconazole: 7-day toxicity test with duckweed (*Lemna gibba*).
Guidelines: OECD Guideline-method 221: Lemna sp. Growth inhibition test (2006); US EPA Ecological Effects test guidelines, OPPTS 850.4400 (1996).
GLP: Yes

The toxicity of Difenoconazole to the aquatic plant *Lemna gibba* was determined in a 7-day static test. Exposure nominal concentrations were 0.15, 0.38, 0.96, 2.4, 6.0 and 15 mg/L. Assessment of frond number were made on days 0, 3, 5 and 7. Fronds were harvested for measurement of dry weight after 7 days. Temperature was measured continuously, light intensity was recorded once at test start and pH was recorded on days 0, 3, 5 and 7 days.

The test concentrations were verified by chemical analysis of Difenoconazole at days 0 and 7, using HPLC with ultra violet-visible detection

Findings:

At the start of the test, the concentrations of the test item were found to be in the range 40 to 92% of the nominal values and at the end of the test were in the range 43 to 85%. Mean measured concentrations were used for the calculation and reporting of results.

For frond number, the 7-d EC_{50} for biomass (E_bC_{50}) and growth rate (E_rC_{50}) for Difenoconazole to *Lemna gibba* were 1.8 and >6.5 mg a.s./L respectively, based on mean measured concentrations.

The 7-d NOEC was determined to be 0.11 mg a.s./L and the 7-day LOEC was determined to be 0.30 mg a.s./L.
The study is accepted.

11.5.4 Acute (short-term) toxicity to other aquatic organisms

No data are available.

11.6 Long-term aquatic hazard

A brief summary of the aquatic toxicity studies evaluated during Annex I inclusion of Difenoconazole and submitted for the purposes of EU renewal is reported below. From all available ecotoxicity tests on this substance only information considered adequate, reliable and relevant for the classification proposal has been included.

Related to metabolites of Difenoconazole, only metabolite CGA 205375 toxicity data to sediment dwelling organisms are presented. The study with this metabolite is described below.

Table 45: Summary of relevant information on chronic aquatic toxicity

Method	Species	Test material	Results[1]	Remarks	Reference
Fish early life stage. US EPA FIFRA 72-4	Fathead minnow (*Pimephales promelas*)	Difenoconazole technical (96.1% purity)	32-NOEC = 0.0076 mg/L, based on mean measured concentrations.	Accepted	Anonymous 1987b
Fish early life stage. US EPA FIFRA 72-4	Fathead minnow (*Pimephales promelas*)	Difenoconazole technical (96.1% purity)	34-NOEC = 0.0076 mg/L, 34d-EC_{10} = 0.0129 mg/L, based on mean measured concentrations.	Statistical Re-analysis of data from the previous study (Surprenant, 1987b). Accepted	Anonymous 2016a
Fish early life stage. US EPA FIFRA 72-4	Fathead minnow (*Pimephales promelas*)	Difenoconazole technical (95% purity)	30d-(post hatch)NOEC = 0.0087 mg/L, based on mean measured concentrations.	Accepted	Anonymous 1990b
Fish full life-cycle. OPPTS Draft Guideline 850.1500.	Fathead minnow (*Pimephales promelas*)	Difenoconazole technical (97.4% purity)	NOEC (90d post hatch) = 0.0036 mg/L, EC_{10} (90d post hatch) = 0.02151 mg/L based on mean measured concentrations.	Accepted	Anonymous (2009) Anonymous (2016)
Long term and chronic toxicity to aquatic invertebrates. US EPA FIFRA 72-4	*Daphnia magna*	Difenoconazole technical (96.1% purity)	21-day NOEC = 0.0056 mg/L, based on mean measured concentrations	Accepted	Forbis, A. D. (1988b)

Long term and chronic toxicity to aquatic invertebrates.	*Daphnia magna*	Difenoconazole technical (96.1% purity)	21-day NOEC = 0.0056 mg/L, 21d-EC_{10} = 0.0078 mg/L, based on mean measured concentrations	Statistical Re-analysis of data from the previous study (Forbis, 1988b). Accepted	Taylor, S. & Pickering, F. (2016a)
Long term and chronic toxicity to aquatic invertebrates. OPPTS Guideline 850.1350 and FIFRA Guideline 72-4.	Mysids *(Americamysis bahia)*	Difenoconazole technical (94.4% purity)	28-day NOEC = 0.0046 mg/L, based on mean measured concentrations	Accepted	Lee, M. R. (2009)
Long term and chronic toxicity to aquatic invertebrates.	Mysids *(Americamysis bahia)*	Difenoconazole technical (94.4% purity)	28-day NOEC = 0.0046 mg/L, based on mean measured concentrations	Statistical Re-analysis of data from the previous study (Lee, 2009). Accepted	Taylor, S. & Allen, M. (2016b)
Long term and chronic toxicity to aquatic invertebrates. OPPTS Guideline 850.1350 and ASTME Guideline 1191-03a (2008).	Mysids *(Americamysis bahia)*	Difenoconazole technical (94.4% purity)	**28-day NOEC = 0.0023 mg/L**, based on mean measured concentrations	Accepted	Sayers, L. E. (2014)
Long term and chronic toxicity to aquatic invertebrates.	Mysids *(Americamysis bahia)*	Difenoconazole technical (94.4% purity)	28-day NOEC = 0.0023 mg/L, based on mean measured concentrations	Statistical Re-analysis of data from the previous study (Sayers, 2014). Accepted	Taylor, S. & Allen, M. (2016c)
Acute toxicity to algae or other aquatic plants. OECD Guideline 201	Green algae (*Scenedesmus subspicatus*)	Difenoconazole technical (91.8% purity)	72h-NOEC = 0.0086 mg/L, based on mean measured concentrations	Accepted	Grade, R. (1993b)
Acute toxicity to algae or other aquatic plants. OECD Guideline 201	Freshwater green alga (*Pseudokirchneriella subcapitata)*	Difenoconazole technical (94.4% purity)	72h-NOEC = 0.36 mg/L, based on mean measured concentrations	Accepted	Hoberg, J. R. (2006).
Chronic toxicity to sediment dwelling organisms ASTM E1706 (1995)	Midge larvae (*Chironomus riparius*)	Difenoconazole technical (91% purity)	Water phase: 28-day NOEC = 0.015 mg/L, based on mean measured concentrations.	Accepted	Van der Kolk, J. (1999)

			Sediment phase: 28d- NOEC = 0.00525 mg/Kg, based on mean measured concentration. This value is estimated. EFSA recalculated the sediment concentration in the test system.		
Chronic toxicity dwelling organisms OECD 218 (2004)	Midge larvae (*Chironomus riparius*)	Difenoconazole technical (96.6% purity)	28d-NOECemergence = 14 mg/Kg dry sediment (corresponding to 0.038 mg/L) 28d-NOECdevelopmental = 8.2 mg/Kg dry sediment (corresponding to 0.018 mg/L). Mean measured concentrations.	Accepted	Eckenstein, H. (2014)
Chronic toxicity to sediment dwelling organisms OECD proposed guideline for toxicity test with Chiromonidae, May 1998; BBA Guideline proposal 1995	Midge larvae (*Chironomus riparius*)	CGA 205375 (synonomous with CGA 2113910) (99%purity)	26-dy NOEC = 0.4 mg/L (water column) 28-day NOEC = 10 mg/Kg (sediment)	Accepted	Grade (2001)

[1] Indicate if the results are based on the measured or on the nominal concentration

11.6.1 Chronic toxicity to fish

Fish early life stage toxicity tests.

Author(s): Anonymous (1987b)
Title: The toxicity of Difenoconazole to fathead minnow (*Pimephales promelas*) embryo and larva.
Guidelines: US EPA FIFRA 72-4
GLP: Yes

The 32-day NOEC of Difenoconazole to fathead minnow (*Pimephales promelas*) was assessed under flow-through conditions. Exposure nominal concentrations were 0.0062, 0.012, 0.025, 0.05 and 0.1 mg/L of Difenoconazole. Larvae were monitored daily for behavioural abnormalities and survival

was estimated twice weekly. Larval weight and length was recorded 34 days after test initiation. Temperature, pH, dissolved oxygen and total water hardness were recorded daily and water samples were collected on days 0, 1, 4 and weekly thereafter until test termination for analysis of test substance concentration by HPLC.

Findings:

Overall mean measured Difenoconazole concentrations corresponded to 98-123% of nominal concentrations.
Difenoconazole concentrations up to 0.1 mg/L had no significant effect on embryo survival but significantly reduced larval survial to 49%. Exposure to concentrations of 0.014, 0.029, 0.049 and 0.1 mg/L, also caused significant reductions in the length and/or wet weight of larvae after 30 days.
Due to reductions in larval weight seen following exposure to 0.014 mg/L, the NOEC for Difenoconazole in fathead minnows was estimated to be 0.0076 mg/L.

This fish early life stage toxicity (ELS) study was conducted in 1987, prior to the existence of OECD Guideline 210. Therefore, compliance of the study with the validity criteria of the actual version of OECD 210 (adopted 2013) and the calculation of the E(L)C10 and E(L)C20 values have been conducted by applicant as required by Commission Regulation 283/2013.
For details of this re-evaluations, see the study Taylor & Allen (2016) below.

Author(s): Anonymous (2016a)
Title: The toxicity of Difenoconazole to fathead minnow (*Pimephales promelas*) embryo and larva. Statistical Re-analysis.
Guidelines:
GLP:

This study is based on the previous data repoted by Anonymous (1987) submitted and evaluated in the previous EU review of difenoconazole (DAR, May 2006). That study did not provide estimates of the EC_{10} and EC_{20} for the response variables evaluated. Consequently, the data generated have been re-analysed in an attempt to provide these values.

Probit analysis with linear maximum likelihood regression was used to determine the concentration response function. Chi^2 was used as a goodness of fit measure. The results of the Probit analysis and their 95% and 99% confidence limits (Fiellers theorem, 1954) were estimated.

Findings:

The validity criteria of OECD 210 (2013) were fulfilled. The endpoints were considered reliable for larval length and weight.

The most sensitive endpoints were based on fish weight.

32-days NOEC = 0.0076 mg a.s./L,

32-days EC10 = 0.01298 mg a.s./L

32-days EC20 = 0.0196 µg a.s./L

Based on mean measured concentrations.

Author(s): Anonymous (1990b)
Title: Difenoconazole technical: Toxicity to fathead minnow (*Pimephales promelas*) embryo and larva.
Guidelines: US EPA FIFRA 72-4
GLP: Yes

The 32-day NOEC of Difenoconazole to to fathead minnow (*Pimephales promelas*) was assessed under flow-through conditions. Exposure nominal concentrations were 0.0013, 0.0025, 0.005, 0.01 and 0.02 mg/L of Difenoconazole.

Hatching was recorded daily until day 4 when 25 live larvae were selected from those surviving in each incubation cup and transferred to one of two larval growth cylinders in each aquaria for 60 days post-hatch exposure. Larval behaviour and mortality were monitored daily. Larval length was determined on post-hatch days 30 and 60 while wet weight was recorded on day 60. Temperature, pH and dissolved oxygen were recorded daily while total hardness was measured on day 0 and weekly thereafter. Water samples were collected on days 0 and 4 and weekly thereafter, for analysis of test substance concentration by HPLC

Findings:

Overall mean measured Difenoconazole concentrations corresponded to 90-160% of nominal concentrations.
Difenoconazole concentrations up to 0.019 mg/L had no significant effect on embryo survival or larvae survival measured on days 30 and 60 post-hatch. Concentrations up to 0.0087 mg/L did not significantly affect larvae length on day 30, exposure to 0.019 mg/L difenoconazole significantly reduced length from 24 mm in controls to 23 mm. At measurement made on day 60 post-hatch, larvae length and wet weight was not significantly affected by concentrations up to 0.019 mg/L.

Based on larval length measured 30 days post-hatch, the NOEC for difenoconazole in fathead minnow was 0.0087 mg/L (measured concentration).

<u>Fish full life cycle test.</u>
The next study was already evaluated following the Evaluation of Confirmatory data after Annex I inclusion of Difenoconazole and it was included in the Addendum to the DAR of Difenoconazole (September 2014).

Author(s): Anonymous (2009)
Title: Difenoconazole: Fish full life-cycle test with fathead minnow (*Pimephales promelas*).
Guidelines: Adapted from OPPTS Draft Guideline 850.1500 to include endocrine endpoints.
GLP: Yes

The toxicity of difenoconazole on the life-cycle of the fathead minnow *(Pimephales promelas)* was investigated. Fish were exposed to the following range of nominal concentrations of 1, 2, 4, 8 and 16 µg/L, and a dilution water control.

The biological endpoints evaluated were first generation (F0) hatching success, survival, growth (total length and wet weight) and reproduction (spawning frequency and fecundity), histological sex ratio as well as plasma vitellogenin concentration (VTG) and gonad histopathology; second generation (F1) hatching success, survival, growth, histological sex ratio, plasma vitellogenin concentration (VTG) and gonad histopathology.
Temperature, pH and dissolved oxygen were measured daily and total hardness, total alkalinity and specific conductance was measured weekly.

Findings:

Overall mean measured Difenoconazole concentrations corresponded to 88-110% of nominal concentrations.

The study was well performed and reported. Since the statistically significant effects on growth at 7.8 µg/L was slight (4.1%), limited to males in the first generation out of two, and there was no corresponding significant effect on wet weight of the same group, it is agreed that the NOAEC of 7.8 µg/L would be relevant for growth related parameters. It is also agreed that the proposed NOAEC of 7.8 µg/L would cover possible effects on hatching success and survival of the off-spring. During peer review, the applicant added that "the effect on male length at 95-dph is also not consistent with other available chronic fish studies for difenoconazole."

A NOEC = 3.6 µg/L (measured concentration) will be used in the risk assessment as a conservative approach (as agreed during the peer review of the Confirmatory data; EFSA supporting publication 2014:EN-680).

Author(s): Anonymous, (2016)
Title: Difenoconazole – Life Cycle Test with the Fathead Minnow (*Pimephales promelas*). Statistical Re-analysis.
Guidelines:
GLP:

This study is based on the previous data repoted by Anonymous (2009) submitted and evaluated in the previous EU review of difenoconazole (DAR, May 2006). That study did not provide estimates of the EC_{10}, EC_{20} and EC_{50} for the response variables evaluated as part of the original study. Consequently, the data generated have been re-analysed in an attempt to provide these values.

Probit analysis with linear maximum likelihood regression was used to determine the concentration response function. Chi^2 was used as a goodness of fit measure. The results of the Probit analysis and their 95% and 99% confidence limits (Fiellers theorem, 1954) were estimated.

Findings:

EC_{10} (90d post-hatch) = 0.02151 mg/L (male wet weight)

NOEC (90d post-hatch) = 0.0036 mg/L (length, measured concentration)

11.6.2 Chronic toxicity to aquatic invertebrates

Author(s): Forbis, A. D. (1988b).
Title: Chronic toxicity of Difenoconazole to *Daphnia magna* under flow-through test conditions.
Guidelines: USEPA FIFRA 72 - 4.
GLP: Yes

The chronic toxicity of Difenoconazole to *Daphnia magna* was determined under flow-through conditions. Four replicate cultures each were exposured to nominal concentrations of 0.0036, 0.006, 0.012, 0.022 and 0.05 mg/L. The daphnids were monitored daily for mortality and reproductive success. Temperature, pH and dissolved oxygen concentrations were recorded on days 0, 4, 7 and 21. Water samples were collected on the same days for analysis of test substance concentration by HPLC.

Findings:

Mean measured Difenoconazole concentrations corresponded to 93 – 108% of nominal concentrations.

The NOEC was based on the number of young per adult and based on mean measured concentrations, the 21-day NOEC for Difenoconazole in *Daphnia magna* was estimated to be 0.0056 mg/L (reproduction). The data of this study were re-analysed (Taylor, S. & Pickering, F. (2016a) below) and the 21-day NOEC of 0.0056 mg/L was confirmed.

Author(s): Taylor, S. & Pickering, F. (2016a).
Title: Difenoconazole – Chronic Toxicity to Daphnia magna under flow-through conditions. Statistical re-analysis.
Guidelines:

The previous study (Forbis, A. D., 1988b) did not provide estimates of the EC10 and EC20 for the response variables evaluated as part of the original study. Additionally, an EC50 value was not presented for some of the variables. Consequently, the data generated in this study have been re-analysed and the 21-day NOEC of 0.0056 mg/L (reproduction) was confirmed.

Author(s): Lee, M. R. (2009)
Title: Difenoconazole – Life cycle toxicity test with mysids (*Americamysis bahia*).
Guidelines: Draft OPPTS Guideline 850.1350 and FIFRA Guideline 72 – 4.
GLP: Yes

The effect of difenoconazole on the survival and reproduction of the mysid *Americamysis bahia* was determined over 28 days under flow-through conditions. The study was run with nominal concentrations of 0.38, 0.76, 1.5, 3.0, 6.1 and 12 µg a.s./L. The test incorporated two replicate cultures for each concentration and one control treatment. Adult mysids (F_0) were monitored daily for mortality and reproductive success. The pairing chambers with F_1 mysids were established to monitor mysid survival 96 hours post release. Temperature, pH, dissolved oxygen and salinity were recorded

daily. Water samples were collected prior to test initiation and on days 0, 7, 14, 21 and 28 for analysis by LC/MS/MS.

Findings:
Mean measured Difenoconazole concentrations corresponded to 75 – 80% of nominal concentrations and variability was less than 20% across all treatments.

Based on measured concentrations, the 28-day NOEC (based on reproduction) for Difenoconazole in *Americamysis bahia* was determined to be 4.6 µg/L. The Lowest Observed Effect Concentration (LOEC) was determined to be 9.3 µg a.s./L.
The data of this study were re-analysed (Taylor, S. & Allen M., (2016b) below) and the 28-day NOEC of 4.6 µg/L was confirmed.

Author(s): Taylor, S. & Allen, M. (2016b).
Title: Difenoconazole – Life cycle toxicity test with mysids (*Americamysis bahia*) following Draft OPPTS Guideline 850.1350 and FIFRA Guideline 72 – 4. Statistical re-analysis.
Guidelines:

The previous study (Lee, M. R. 2009) did not provide estimates of the EC_{10} and EC_{20} for the response variables evaluated as part of the original study. Additionally, some of the variables did not provide estimates of the EC_{50} value. Consequently, the data generated in this study have been re-analysed and the 28-day NOEC of 4.6 µg/L (reproduction) was confirmed

The endpoints evaluated were mortality F_0 and F_1 and also reproduction, body weight and body length in F_0. Adverse effects were only observed in reproduction and no clear dose-response relationship could be established. In fact, the relationship was not significant, ($p(F) = 0.882$ and $p(F) = 0.891$) as a result no reliable ECx values could be calculated.

However, the mean value of offspring was lower in all tested concentration than in control although statistical significant differences were observed only at highest tested concentration of 9.3 µg/L.

Anyway, this concerns is not enough to exclude this study from the risk assessment. And the study is accepted. 28-day NOEC = 4.6 µg/L (reproduction).

Author(s): Sayers, L. E. (2014)
Title: Difenoconazole – Life cycle toxicity test with mysids (*Americamysis bahia*).
Guidelines: OCSPP Guideline 850.1350 (1996); ASTM E Guideline 1191 – 03a (2008).
GLP: Yes

This study was performed to determine the chronic (full life-cycle) toxicity of Difenoconazole to the mysid, *Americamysis bahia*, under flow-through conditions. 28-day survival, male and female survival, reproduction (based on mean young produced per female per reproductive day), male and female growth (total body length and dry weight) and survival of F1 mysid were calculated as test endpoints. The study was run with nominal concentrations of 0.75, 1.5, 3.0, 6.0 and 12 µg a.s./L. The test incorporated four replicate cultures for each concentration and eight were maintained for the

control. Adult mysids (F0) were monitored daily for mortality and reproductive success. The pairing chambers with F1 mysids were established to monitor mysid survival 96 hours post release and observations of stress, abnormal behavior and survival were made. Temperature, pH, dissolved oxygen and salinity were measured in each replicate on day 0, and alternated between replicates daily thereafter throughout the exposure period. Water samples were collected prior to test initiation and on days 0, 7, 14, 21 and 28 for analysis by LC/MS/MS.

Findings:
Mean measured Difenoconazole concentrations corresponded to 83 – 120% of nominal concentrations and variability was less than 20% across all treatments.

The most sensitive indicator of toxicity for Difenoconazole and *A. bahia* was 28-day survival. Based on this endpoint and the mean measured concentrations of Difenoconazole, the No-Observed-Effect Concentration (NOEC) was determined to be 2.3 µg/L. The Lowest-Observed-Effect Concentration (LOEC) for mysids was determined to be 4.8 µg/L. Since no concentration tested resulted in ≥50% mortality, the 7, 14, 21 and 28-day LC50 values were empirically estimated to be >10 µg/L, the highest mean measured difenoconazole concentration tested.

The data of this study were re-analysed (Taylor, S. & Allen M., (2016c) below) and the 28-day NOEC of 2.3 µg/L was confirmed.

Author(s): Taylor, S. & Allen, M. (2016c).
Title: Difenoconazole – Life cycle toxicity test with mysids (*Americamysis bahia*) following. Statistical re-analysis.
Guidelines:

The previous study (Sayers, 2014) did not provide estimates of the EC_{10} and EC_{20} for the response variables evaluated as part of the original study. Additionally, some of the variables did not provide estimates of the EC_{50} value. Consequently, the data generated in this study have been re-analysed.

The endpoints evaluated were mortality F_0 and F_1 and also reproduction, body weight and body length in F_0.

The most sensitive endpoint was the survival at 28 days considering male and female jointly obtaining a NOEC = 2.3 µg as/L.

However, statistical differences were observed at 1.2 µg as/L tested concentration compared to the control for survival (only considering males) and at 2.3 µg as/L tested concentration for dry body weight of males. In both cases, effects were detected only on males and not in female. The effects observed seems to have random behaviour as no clear dose-response relationship can be established. Thus, the biological meaningful is questionable and NOEC survival = 2.3 µg as/L is consired acceptable.

For reproduction, statistical effect were observed at 10 µg as/L for the mean number of offspring per female which results on a NOEC = 4.8 µg as/L.

The results of this study showed no clear dose –response relationship for all endpoints measured during the experiment. Consequently, ECx values cannot be considered reliable.

11.6.3 Chronic toxicity to algae or other aquatic plants

Please refer to previous point 11.5.3 where the toxicity tests with the parent on algae and Lemna are included.

11.6.4 Chronic toxicity to other aquatic organisms

Sediment dwelling organisms

Author(s): van der Kolk, J. (1999)
Title: Difenoconazole: Chronic effects on midge larvae (*Chironomus riparius*) in a water/sediment system.
Guidelines: ASTM E1706 (1995)
GLP: Yes

The 28-day NOEC of Difenoconazole to midge larvae (*Chironomus riparius*) was assessed under static conditions. Exposure nominal concentrations were 0.05, 0.5, 5 and 50 mg/kg dry sediment of Difenoconazole. Cultures were monitored daily for numbers of emerged midge. Water samples were collected on day 21 for analysis of test substance concentration by HPLC.

Findings:

Due to the lack of analytical measurements in the sediment phase, the NOEC based on sediment concentration should also be treated with caution. However, since no effects were observed in the study, and since other aquatic invertebrates are indicated to be much more sensitive to difenoconazole, this study is considered to be of sufficient quality for the assessment of the risk for sediment-dwelling organisms. NOEC based on the measured concentration in the water phase on day 21 was 0.015 mg/L.

As no measurements of sediment concentrations were conducted, EFSA recalculates the sediment concentrations in the test system from the measured water concentration. 28-day NOEC = 0.00525 mg/kg.

Author(s): Eckenstein, H. (2014).
Title: Difenoconazole – Effects on the development of sediment dwelling larvae of *Chironomus riparius* in a water/sediment system with spiked sediment.
Guidelines: OECD 2018 (2004)
GLP: Yes

The effects of difenoconazole on the development of *Chironomus riparius* were determined under static conditions. Organisms were exposed to nominal concentrations of 5.0, 10, 20, 40 and 80 mg difenoconazole/kg dry sediment. The Difenoconazole concentration in water and sediment of test vessels was determined by HPLC-MS/MS on days 0, 7 and 28.

Findings:

The mean overall emergence ratio in the control and solvent control ranged from 74 to 83%. In the sediment, the concentrations of Difenoconazole remained rather stable during the study period with recoveries from 70 to 97% of nominal concentration. The total amount of test item determined per test vessel as percentage of the nominal amount ranged from 71% to 82% on day 0, from 74% to 97% on day 7 and from 70% to 85% on day 28. Thus, the amount of test item determined in the supernatant water is negligible.

This kind of study (spiked-sediment) is designed to assess the effects of prolonged exposure of chemicals to the sediment-dwelling larvae of Chironomus and it is recommended for substances that persist in sediment during long time periods. However, the exposure route of difenoconazole through water column would be also relevant. Thus, the key endpoint would be reported considering also the water concentration and the results obtained for test samples in water from original study in order to present the endpoint in terms of mg as/L water.

Based on initial measured concentration:

28-day NOEC emergence = 14 mg/kg dry sediment (corresponding to 0.038 mg as/L)

28-day NOEC developmental = 8.2 mg/kg dry sediment (corresponding to 0.018 mg as/L)

28-day EC10 emergence = 12 mg/kg dry sediment

METABOLITES

Author(s): Grade (2001)
Title: Toxicity tes of CGA 211391 (metabolite of CGA 169374) on sediment dwelling *Chironomus riparius* under static conditions.
Guidelines: OECD proposed guideline for toxicity test with Chiromonidae, May 1998; BBA Guideline proposal 1995
GLP: Yes

The 28-day NOEC of CGA 2113915 (synonymous with CGA205375) to *Chironomus riparius* was assessed under static conditions. Exposure nominal concentrations were 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 mg/L or mixed with sediment: 2.5, 5, 10, 20, 40 and 80 mg/Kg dw sediment. Cultures were monitored daily for numbers of emerged midge. Temperature, pH and dissolved oxygen concentration were recorded once a week. Water samples were collected on days 0, 2, 7, 14 and 26/28 for analysis of test substance concentration by HPLC. Sediment concentrations were determined in samples collected on days 0, 7 and 26/28.

Findings:

The study was well performed and reported, although the extrapolated EC_{50} values should be treated with caution. No degradation of the test substance took place during the study. The NOEC values are considered valid for the risk assessment

Based on effects on emergence or developmental rate in *Chironomus riparius* the 26-day NOEC for exposure via the water column was 0.4 mg/L and the 28-day NOEC for exposure via sediment was 10 mg/kg.

11.7 Comparison with the CLP criteria

Endpoint	CLP classification criteria	Difenoconazole data	Conclusions
Rapid degradability	Demostrated rapid/not rapid degradation	Not readily biodegradable and not rapidly degradable	Not rapidly degradable
Short-term toxicity	LC_{50}/EC_{50} value	Adequate data for fish, aquatic invertebrates, algae and aquatic plants. 72h-E_rC50 = 0.0876 mg/L Green algae (*Scenedesmus subspicatus*) (Taylor, S. & Pickering, F., 2016b).	Aquatic Acute 1
Long-term toxicity	NOEC value	Adequate data for fish, aquatic invertebrates, algae and aquatic plants. 28-day NOEC = 0.0023 mg/L Mysids (*Americamysis bahia*) (Sayer, L.E., 2014)	Aquatic Chronic 1
Bioaccumulation potential	BCF ≥ 500, or if absent, log Kow ≥ 4	Two experimental BCF values considered valid: 320 and 330; and Log Kow = 4.36	Bioconcentration is not expected

11.7.1 Acute aquatic hazard

Full acute data set was available for Difenoconazole as there were acute studies on fish, aquatic invertebrates, algae and aquatic plants, covering the three trophic levels. Also studies with metabolites (CGA 71019, CGA 205375 and CGA 142586) were available for all trophic levels although only CGA205375 presented a toxicity value < 1 mg/L on fish. However, classification proposal is based on studies conducted with Difenoconazole as the lowest and the most reliable endpoint values.

Based on the available data, the lowest acute toxicity endpoint is *Scenedesmus subspicatus* **ErC50(72h)= 0.0876 mg/l**. This endpoint will establish the M factor needed for the CLP environmental classification.

It is concluded that Difenoconazole does fulfil the criteria for classification and it should be classified according to Regulation (EC) No. 1272/2008 as:

Aquatic Acute 1 with M factor of 10.

CLP criteria:

- for EC50 acute toxicity values below or equal to 1 mg/l [ErC50(72h) = 0.0876 mg/l ≤ 1 mg/l] and

- for M factor, acute toxicity value in the range 0.01 < L(E)C50 ≤ 0.1 mg/L.

11.7.2 Long-term aquatic hazard (including bioaccumulation potential and degradation)

Bioaccumulation

The log Kow values for Difenoconazole is 4.36 which is greater than the CLP log Kow trigger value of > 4 intended to identify substances with a potential to bioaccumulate under CLP criteria. Two studies are available to establish measured BCF estimates. According to CLP guidance, measured estimates should be used in preference when available to conclude on the bioaccumulation potential of a substance (BCF ≥ 500 indicates bioaccumulation potential). Therefore, these data have been used to conclude on the potential for bioaccumulation of Difenoconazole. The BCF estimates are 320 and 330. Both BCF estimates are lower than the CLP trigger value of 500 and, therefore, Difenoconazole is considered to have low potential to bioaccumulate.

Degradation

A ready biodegradability test (OECD test guideline 301B) shows that Difenoconazole being not readily biodegradable for purposes of classification as the pass level criteria of ready biogradation test (70 % of DOC removal or 60 % of theoretical oxygen demand) within 28 days was not reached (0% biodegradation in 29 days).

According to hydrolysis tests (OECD test guideline 111 “Hydrolysis as a function of pH”), Difenoconazole is hydrolytically stable in solutions at pH 4 to 9. According to the criteria in CLP guidance, the substance might be considered as rapidly degradable for classification purposes only when the longest half-life determined within the pH range of 4-9 is shorter than 16 days and the hydrolysis products formed do not fulfil the classification criteria as hazardous for aquatic environment. As Difenoconazole is hydrolytically stable and no degradation products have been detected or were detected in quantities < 1.2% of applied radioactivity, Difenoconazole does not fulfil the CLP criteria of being rapidly degradable.

In an aerobic mineralization study Difenoconazole degraded with DT_{50} values of 104.7 and 146.7, depending on test concentration, to the following metabolites: CGA205375 and CGA142856.

The water/sediment studies suggest that Difenoconazole mainly disappears from aquatic systems by physical-chemical processes. Partitioning to sediment is the main route of dissipation of Difenoconazole in water sediment systems primarily binding to sediment.

Difenoconazole can be considered as not rapidly degradable in the aquatic environment from the water/sediment system studies carried out. Although short DT50 and DT90 values were registered for the water phases (DT50 between 2.13 and 5.52 days and DT90 between 7.08 and 18.3 days), Difenoconazole disappears by dissipation process, binding to sediment. And at the end of the above studies, the maximum carbon dioxide increased to 3.9% AR indicating minimal mineralization.

Photodegradation of Difenoconazole was measured being insignificant in water.

Overall, degradation information does not provide sufficient data to show that Difenoconazole is ultimately degraded to > 70% within 28 days (equivalent to a half-life of less than 16 days) or being transformed to non-classifiable products. Therefore, Difenoconazole is considered being **not rapidly degradable** according to the CLP criteria.

Toxicity

Long-term aquatic toxicity data regarding technical Difenoconazole are available for fish, aquatic invertebrates including sediment dwelling organisms, algae and other aquatic plants (i.e. there is appropriate data for all three trophic levels that need to be assessed for CLP classification). Classification proposal is based on studies conducted with Difenoconazole although there were acute and chronic studies available for metabolites.

The lowest NOErC value is the measured **28d-NOEC of 0.0023 mg a.s./L** for mysids *(Americamysis bahia)* (derived from Sayers, 2014). This is > 0.001 mg/L but ≤ 0.01 mg/L, and since Difenoconazole is considered to be 'not rapidly degradable' as well as not potentially bioaccumulative, it should be classified according to Regulation (EC) No. 1272/2008 as:

Aquatic Chronic 1 with a chronic M-factor of 10.

CLP Criteria:

- Aquatic long-term toxicity reflected by a valid endpoint for invertebrates reproduction NOEC (28d)=0.0023 mg/L, and
- For the M factor, Difenoconazole is considered not rapidly degradable substance and its long-term toxicity value is in the range of 0.001 to 0.01 (NOEC = 0.0023 mg/L).

11.8 CONCLUSION ON CLASSIFICATION AND LABELLING FOR ENVIRONMENTAL HAZARDS

Taking into account all the information and the assessment summarized in the previous sections 11.7.1 and 11.7.2, the following classification class and category can be concluded for this active substance:

According to Table 4.1.0 (a) and (b)(i), Difenoconazole meets the CLP Regulation criteria for being classified as **Aquatic Acute 1 with M factor of 10** and **Aquatic Chronic 1 with M factor of 10.**

Therefore, the proposal for classificacion for Difenoconazole is:

Aquatic Acute 1; H400: Very toxic to aquatic life. M-factor 10

Aquatic Chronic 1; H410: Very toxic to aquatic life with long lasting effects. M-factor 10

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Regarding the renewal of difenoconazole as an active substance in the context of the PPP regulation, a Renewal Assessment Report has been developed and the CLH report also relied on data submitted in the context of the application for approval as an active substance under Regulation (EC) No 1107/2009.

Overall, the DS concluded that difenoconazole is not rapidly degradable, has a low potential for bioaccumulation and proposed classification based on aquatic acute toxicity to algae and aquatic chronic toxicity to invertebrates:

Aquatic Acute 1 with an M-factor of 10, based on the lowest measured 72h E_rC_{50} value of 0.0876 mg/L for *Scenedesmus subspicatus* and Aquatic Chronic 1 with an M-factor of 10, based on the lowest measured 28-d NOEC of 0.0023 mg/L for *Americamysis bahia*.

Degradation

A ready biodegradability test (OECD TG 301B) showed that 0% biodegradation of difenoconazole was observed after 29 days (Baumann, 1993). Therefore, difenoconazole was considered as not readily biodegradable.

The results of a hydrolysis study (OECD TG 111, GLP) showed that difenoconazole is hydrolytically stable in solutions at pH 4 to 9 at 25ºC over a period of 30 days (Atkins, 1991).

Difenoconazole is stable to direct photolysis in aqueous systems at pH 7 at 25ºC over a period of 15 days (Gaauw, 2002a). Direct photolysis is assessed to be an insignificant process for degradation of difenoconazole in surface waters (Hennecke, 2002a).

In an aerobic mineralisation study (OECD TG 309, GLP), difenoconazole degraded with DT_{50} values of 104.7 and 146.7 days depending on test concentration (10 µg/L and 95 µg/L respectively) to the major metabolites CGA205375 and CGA142856 (Gartner and Herrechen, 2016).

Regarding the water/sediment system, four studies (Gonzalez-Valero, 1993; Ulbrich, 1997; Lin, 2006; Yeomans and Mould, 2018) were submitted. A kinetics assessment (Terry, 2015c) was performed in accordance with FOCUS degradation kinetics guidance. Although short DT_{50} and DT_{90} values were registered for the water phases (DT_{50} between 2.13 and 5.52 days and DT_{90} between 7.08 and 18.3 days), difenoconazole disappears via dissipation, binding to sediment. At the end of the above studies, the maximum carbon dioxide increased to 3.9%

AR, indicating minimal mineralization. Thus, difenoconazole can be considered as not rapidly degradable in the aquatic environment from the water/sediment system studies carried out.

Overall, due to the results summarised above, the DS concluded that degradation information does not provide sufficient data to show that difenoconazole is ultimately degraded to a level equal to or greater than 70% within 28 days (equivalent to a half-life of less than 16 days) or is degraded to non-classifiable products. Therefore, difenoconazole was considered by the DS as being not rapidly degradable, according to the CLP criteria.

Aquatic Bioaccumulation

Two 28 days studies (US EPA FIFRA 72-6) on bioconcentration by bluegill sunfish (*Lepomis macrochirus*) were carried out. Only one concentration was tested in each study, while the test guidelines require at least two exposure levels. The two available studies examined together were considered to fulfil the requirement of more than one exposure concentration.

The studies were conducted in a flow-through system to nominal concentrations of 0.02 mg/L (Anonymous, 1987) and 1.0 µg/L (Anonymous, 1992) for a period of 28 days followed by a 14 days period of depuration in fresh water.

For the whole fish, the steady-state bioconcentration factor (BCF) was calculated to be 320 L/kg and 330 L/kg for the treatment levels of 0.02 mg/L and 1.0 µg/L, respectively.

The determined log K_{OW} of 4.36 ± 0.02 at pH 8 and 25^{0}C (OECD TG 107) meets the CLP trigger value of ≥ 4 indicating a potential for bioaccumulation. The log K_{OW} was not pH dependent as difenoconazole did not dissociate at environmentally relevant pHs (Kettner, 1999b).

Consequently, as preference in CLP is given to experimentally derived BCF values (in this case equal to 330 L/kg, which is below the CLP criterion of ≥ 500), the DS concluded that difenoconazole can be considered as having low potential for bioaccumulation.

Aquatic Toxicity

The aquatic toxicity test results from available acute and chronic studies for all trophic levels of difenoconazole are summarised in the following table and sections. Acute and chronic aquatic toxicity data on difenoconazole are available for fish, invertebrates, algae and aquatic plants. Algae is the most acutely sensitive trophic group. Invertebrates are the most chronically sensitive trophic group. All provided studies were considered as acceptable and reliable by the DS.

Table: Acute Aquatic toxicity

Test organism	Guideline, test method	Short-term result (endpoint)	Reference / Test item
Fish			
Salmo gairdneri	US EPA FIFRA 72-1 / GLP	96h LC_{50} = 1.1 mg/L (mm)	Anonymous, 1990a / Difenoconazole (96.1%)
Lepomis macrochirus	US EPA FIFRA 72-1 / GLP	96h LC_{50} = 1.21 mg/L (mm)	Anonymous, 1988 / Difenoconazole (96.1%)
Cyprinodon variegates	US EPA FIFRA 72-3 / GLP	96h LC_{50} = 1.16 mg/L (mm)	Anonymous, 1993 / Difenoconazole (96%)
Pimephales promelas	OECD TG 203; OPPTS Draft Guideline 850.1075 / GLP	96h LC_{50} = 1.9 mg/L (mm)	Anonymous, 2011 / Difenoconazole (97.3%)

Salmo gairdneri	OECD TG 203 / GLP	96h LC_{50} = 0.66 mg/L (mm)	Anonymous, 2001a / CGA205375 (triazolylalcohol 99%)
Aquatic invertebrates			
Daphnia magna	US EPA FIFRA 72-2 / GLP	48h LC_{50} = 0.77 mg/L (mm)	Forbis, 1988a / Difenoconazole (96.1%)
Mysidopsis bahia	US EPA FIFRA 72-3 / GLP	48h LC_{50} = 0.15 mg/L (mm)	Surprenant, 1990c / Difenoconazole (95%)
Crassostrea virginica	US EPA FIFRA 72-3 / GLP	48h LC_{50} > 0.3 mg/L (mm)	Surprenant, 1990d. / Difenoconazole (95%)
Algae / other aquatic plants			
Scenedesmus subspicatus	OECD TG 201 / GLP	72h E_bC_{50} = 0.032 mg/L (mm)	Grade, 1993b / Difenoconazole (91.8%)
Scenedesmus subspicatus	Statistical re-analysis of data from the previous study (Grade, 1993b)	**72h E_rC_{50} = 0.0876 mg/L (mm)**	Taylor and Pickering / 2016b / Difenoconazole (91.8%)
Pseudokirchneriella subcapitata	OECD TG 201 / GLP	72h E_rC_{50} = 1.2 mg/L (mm)	Hoberg, 2006 / Difenoconazole (94.4%)
Lemna gibba	US EPA FIFRA 122-2 / GLP	14d EC_{50} = 18.5 mg/L (frond number) (nom) 14d EC_{50} = 9.9 mg/L (dry weight) (nom)	Drottar, 1986 / Difenoconazole (96.1%)
Lemna gibba	OECD TG 221; US EPA, OPPTS 850.4400 / GLP	7d E_bC_{50} = 1.8 mg/L 7d E_rC_{50} > 6.5 mg/L (frond number) (mm)	Hoberg, 2006d / Difenoconazole (94.4%)

mm: mean measured concentration, nom: nominal concentration

Four studies have been submitted on the acute toxicity of difenoconazole to fish. The reported 96h LC_{50} values of difenoconazole in all studies with fish were above 1 mg/L based on mean measured concentration. In addition, toxicity data of metabolite CGA205375 have been submitted as well. The reported 96h LC_{50} values of metabolite CGA 205375 (triazolylalcohol) was 0.66 mg/L based on mean measured concentration.

Three studies have been submitted on the acute toxicity of difenoconazole to aquatic invertebrates. The reported 48h LC_{50} values of difenoconazole in all studies with invertebrates varied between 0.15 – 0.77 mg/L, based on mean measured concentration.

Two original studies have been submitted on the acute toxicity of difenoconazole to algae. The indicated acute toxicity value were 72h E_bC_{50} = 0.032 mg/L with *Scenedesmus subspicatus* (Grade, 1993b) and 72h E_rC_{50} = 1.2 mg/L with *Pseudokirchneriella subcapitata* (Hoberg, 2006), based on mean measured concentration. However, the reported value of 0.032 mg/L from the study with *Scenedesmus subspicatus* (Grade, 1993b) was below the limit of detection of the active substance (0.04 mg/L). Therefore, statistical re-analysis (Taylor and Pickering, 2016b) of data from this study (Grade, 1993b) was presented as well. The re-calculated acute toxicity value 72h E_rC_{50} = 0.0876 mg/L was derived.

Two studies have been submitted on the acute toxicity of difenoconazole to aquatic macrophytes. The reported 14 and 7- day EC_{50} values of difenoconazole in both studies were above 1 mg/L.

Overall, the DS proposed to classify difenoconazole as Aquatic Acute 1 based on the 72h E_rC_{50} for *Scenedesmus subspicatus* of 0.0876 mg/L mean measured concentration based on the

provided statistical re-analysis. As this acute toxicity value falls within the 0.01 < L(E)C_{50} ≤ 0.1 mg/L range, the acute M-factor proposed by the DS was 10.

Table: Aquatic Chronic toxicity

Test organism	Guideline, test method	Long-term result (endpoint)	Reference / Test item
Fish			
Fathead minnow *(Pimephales promelas)*	Fish early life stage US EPA FIFRA 72-4 / GLP	32d NOEC = 0.0076 mg/L (mm)	Anonymous, 1987b / Difenoconazole (96.1%)
Fathead minnow *(Pimephales promelas)*	Statistical re-analysis of data from the previous study (Anonymous, 1987b)	34d NOEC = 0.0076 mg/L 34d EC_{10} = 0.0129 mg/L (mm)	Anonymous, 2016a / Difenoconazole (96.1%)
Fathead minnow *(Pimephales promelas)*	Fish early life stage US EPA FIFRA 72-4 / GLP	30d NOEC $_{(post\ hatch)}$ = 0.0087 mg/L (mm)	Anonymous, 1990b / Difenoconazole (95%)
Fathead minnow *(Pimephales promelas)*	Fish full life-cycle OPPTS Draft Guideline 850.1500 / GLP	90d $NOEC_{(post\ hatch)}$ = 0.0036 mg/L	Anonymous, 2009 / Difenoconazole (97.4%)
Fathead minnow *(Pimephales promelas)*	Statistical re-analysis of data from the previous study (Anonymous, 2009)	90d $NOEC_{(post\ hatch)}$ = 0.0036 mg/L 90d $EC_{10\ (90d\ post\ hatch)}$ = 0.02151 mg/L (mm)	Anonymous, 2016 / Difenoconazole (97.4%)
Aquatic invertebrates			
Water flea *(Daphnia magna)*	US EPA FIFRA 72-4 / GLP	21d NOEC 0.0056 mg/L (mm)	Forbis 1988b / Difenoconazole (96.1%)
Water flea *(Daphnia magna)*	Statistical re-analysis of data from the previous study (Forbis, 1988b)	21d NOEC 0.0056 mg/L (mm) 21d EC_{10} = 0.0046 mg/L (mm)	Taylor and Pickering, 2016a / Difenoconazole (96.1%)
Mysids *(Americamysis bahia)*	OPPTS Guideline 850.1350 and FIFRA Guideline 72-4 / GLP	28d NOEC 0.0046 mg/L (mm)	Lee, 2009 / Difenoconazole (94.4%)
Mysids *(Americamysis bahia)*	Statistical re-analysis of data from the previous study (Lee, 2009)	28d NOEC 0.0046 mg/L (mm)	Taylor and Allen, 2016b / Difenoconazole (94.4%)
Mysids *(Americamysis bahia)*	OPPTS Guideline 850.1350 and ASTME Guideline 1191-03a / GLP	**28d NOEC = 0.0023 mg/L (mm)**	Sayers, 2014 / Difenoconazole (94.4%)
Mysids *(Americamysis bahia)*	Statistical Re-analysis of data from the previous study (Sayers, 2014)	28d NOEC = 0.0023 mg/L (mm)	Taylor and Allen, 2016c / Difenoconazole (94.4%)
Algae /other aquatic plants			
Green algae *(Scenedesmus subspicatus)*	OECD TG 201 / GLP	72h NOEC = 0.0086 mg/L (mm)	Grade, 1993b / Difenoconazole (91.8%)
Green algae *(Scenedesmus subspicatus)*	Statistical re-analysis of data from the previous study (Grade, 1993b)	72h NOEC = 0.0086 mg/L (mm) 72h E_rC_{10} = 0.015 mg/L	Taylor and Pickering, 2016b / Difenoconazole (91.8%)
Freshwater green algae *(Pseudokirchneriella subcapitata)*	OECD TG 201 / GLP	72h NOEC = 0.36 mg/L (mm)	Hoberg, 2006 / Difenoconazole (94.4%)

Duckweed *(Lemna gibba)*	OECD TG 221; US EPA, OPPTS 850.4400 / GLP	7d NOEC = 0.11 mg/L (mm)	Hoberg, 2006d / Difenoconazole (94.4%)
Sediment dwelling organisms			
Midge larvae *(Chironomus riparius)*	ASTM E1706 / GLP	Water phase: 28d NOEC = 0.015 mg/L (mm) Sediment phase: 28d NOEC = 0.00525 mg/kg (mm)	Van der Kolk, 1999 / Difenoconazole (91%)
Midge larvae *(Chironomus riparius)*	OECD TG 218 / GLP	28d $NOEC_{emergence}$ = 14 mg/kg dry sediment (corresponding to 0.038 mg/L) 28d $NOEC_{developmental}$ = 8.2 mg/kg dry sediment (corresponding to 0.018 mg/L) (mm)	Eckenstein, 2014 / Difenoconazole (96.6%)
Midge larvae *(Chironomus riparius)*	OECD proposed guideline for toxicity test with Chironomidae; BBA Guideline proposal	26d NOEC = 0.4 mg/L (water column) 28d NOEC = 10 mg/kg (sediment)	Grade, 2001 / CGA205375 (triazolylalcohol 99%)

mm: mean measured concentration, nom: nominal concentration

Three original studies and a re-assessment of two studies have been submitted on the chronic toxicity of difenoconazole to fish. The original and re-assessed NOEC values of difenoconazole fall in the range above 0.001 to below 0.01 mg/L, based on mean measured concentration. The Probit analysis with linear maximum likelihood regression was used to determine the concentration response function in the re-assessment. Although EC_{10} values of difenoconazole originally not were estimated they have been estimated during re-analysis.

Three original studies have been submitted on the chronic toxicity of difenoconazole to invertebrates. All of them have been re-assessed by the Dossier Submitter and the originally obtained chronic toxicity values were confirmed. The reported chronic toxicity values of difenoconazole on invertebrates slightly differ but were within the same range of above 0.001 to below 0.01 mg/L. The lowest chronic toxicity value was 28-day NOEC = 0.0023 mg/L for *Americamysis bahia*, based on mean measured concentration.

Three original studies and the re-assessment of one study have been submitted on the chronic toxicity of difenoconazole to algae / other aquatic plants. The statistical re-analyses confirmed the obtained NOEC and provided estimated EC_{10} values. In addition, two studies on the chronic toxicity of difenoconazole and one study on the chronic toxicity of metabolite (CGA205375) with non-biting midge (*Chironomus riparius*) in a water/sediment system have been submitted.

Overall, the DS proposed to classify difenoconazole as Aquatic Chronic 1 based on the 28-day NOEC for *Americamysis bahia* of 0.0023 mg/L, based on mean measured concentration. As the substance was considered not rapidly degradable and chronic toxicity falls within the $0.001 < NOEC \leq 0.01$ mg/L range, the chronic M-factor proposed by the DS was 10.

Comments received during consultation

Two MSCAs submitted comments on the environmental part of the DS's CLH proposal. Both of them agreed with the proposed classification by the DS without further comments.

Assessment and comparison with the classification criteria

Degradation

A ready biodegradation study (OECD TG 301B) with difenoconazole indicated 0% degradation after 29 days, indicating that difenoconazole is not readily biodegradable.

No hydrolysis of difenoconazole was observed and substance was stable in solutions at pH 4 to 9 at 25°C over a period of 30 days (EPA, 540/9-82-021). Calculated half-lives for difenoconazole were above 1000 days.

Difenoconazole was stable to direct photolysis in aqueous systems at pH 7 at 25°C over a period of 15 days (EPA, OPPTS 835.2210). After 15 days of continuous irradiation, difenoconazole represented 91% of the AR. Detected radioactive fractions do not exceeded 6.3% AR. The calculated half-lives of difenoconazole at 52°N were between 11.8 years and above 10000 years, depending on the season. Thus, difenoconazole is stable to direct photolysis in aqueous systems and direct photolysis is an insignificant process for degradation of difenoconazole in surface water.

In an aerobic mineralisation study (OECD TG 309), max mineralisation after 61 days was 16.3% AR. Difenoconazole degraded with DT_{50} values of 104.7 and 146.7 depending on the test concentration (10 µg/L and 95 µg/L respectively) to the major metabolites CGA205375 and CGA142856. For the non-sterilised, viable test systems Difenoconazole decreased to a mean of 61.9 - 71.1% AR (10 µg/L) and 71.6 - 78.9% AR (95 µg/L) after 61 days. For the sterilised samples, difenoconazole was found to be stable, with 92% AR remaining at 61 day. Thus, degradation of difenoconazole in natural water is microbially mediated.

Water/sediment studies suggest that difenoconazole mainly dissipates from aquatic systems by physical-chemical processes. Partitioning to sediment is the main route of dissipation of difenoconazole in water sediment systems primarily via binding to sediment. For the water phase, DT_{50} values were 2.16 days (pond system) and 5.52 days (river system). DT_{90}s were 7.16 days (pond system) and 18.3 days (river system). The whole system DT_{50} values were 318 days (pond system), 300 days (river system) and DT_{90} > 1000 days (pond system), > 998 days (river system). Based on the other study results, the DT_{50} of water phase was 3.2 days and DT_{90} – 10.6 days. However, for whole system the DT_{50} was 1113 days and the DT_{90} – 2300 days. At the end of the above studies, the maximum carbon dioxide increased to 3.9% AR indicating minimal mineralization.

Overall, due to the results summarised above, RAC agrees with the assessment of the DS that difenoconazole is not ultimately degraded to ≥ 70% within 28 days (equivalent to a half-life < 16 days), or rapidly transformed to non-classifiable products. Consequently, RAC agrees that difenoconazole should be considered as **not rapidly degradable**.

Aquatic Bioaccumulation

In the two available experimental studies to determine the bioconcentration potential, the determined whole fish BCF values of 320 and 330 L/kg for difenoconazole is below the CLP trigger value of ≥ 500. Although only one concentration was tested in each study while the test guidelines require at least two exposure levels, RAC agrees with the DS that two available studies together could be considered as fulfilling the requirement of more than one exposure concentration. Therefore, the whole fish calculated steady-state bioconcentration factor (BCF) is 320 L/kg and 330 L/kg for the treatment levels of 0.02 mg/L and 1.0 µg/L, respectively.

The derived Log K_{OW} value of 4.36 (pH 8 at 25°C) meets the CLP trigger value for indication of bioaccumulation (Log K_{OW} ≥ 4). However, following the CLP regulation (section 4.1.2.8.1), the available, reliable experimental BCF determined in fish is taken in preference to the Log K_{OW}. Therefore, based on the BCF_{fish} below 500, RAC agrees with the DS that difenoconazole is **not bioaccumulative** according to the CLP criteria.

Aquatic Toxicity

RAC notes that there are reliable acute and chronic aquatic toxicity data for all trophic levels. RAC agrees that the provided studies are acceptable and reliable. The most acutely sensitive trophic group is algae and most chronically sensitive trophic group is aquatic invertebrates. Chronic toxicity values for aquatic invertebrates slightly varied, but within the same order of magnitude. In addition, most of the chronic toxicity values for fish and algae were also within the same order of magnitude as chronic toxicity values for invertebrates.

Consequently, RAC agrees that the lowest acute toxicity endpoint for aquatic acute classification is the 72h E_rC_{50} value for *Scenedesmus subspicatus* of 0.0876 mg/L, based on re-assessment of the study results. The lowest chronic endpoint for aquatic chronic classification is the 28d NOEC value for *Americamysis bahia* of 0.0023 mg/L, based on mean measured concentration and confirmed by the re-assessment of the study.

Conclusion on classification

Difenoconazole is considered as not rapidly degradable and does not fulfil the criteria for bioaccumulation. Based on the available and reliable information, RAC agrees with the DS that difenoconazole warrants classification as:

Aquatic Acute 1 based on E_rC_{50} = 0.0876 mg/L for *Scenedesmus subspicatus.* As this acute toxicity value falls within the 0.01 < L(E)C_{50} ≤ 0.1 mg/L range, the **acute M-factor is 10**.

Aquatic Chronic 1 based on NOEC = 0.0023 mg/L for *Americamysis bahia*. As this chronic toxicity value falls within the 0.001 < NOEC ≤ 0.01 mg/L range, the **chronic M-factor is 10**.

12 EVALUATION OF ADDITIONAL HAZARDS

12.1 Hazardous to the ozone layer

12.1.1 Short summary and overall relevance of the provided information on ozone layer hazard

Global effects such as contributions to global warming potential (GWP), ozone depleting potential (OPD) and photochemical ozone creation potential (POCP) are considered if there is a high probability for evaporation and persistence in the gas phase, which can be expressed by the volatility in terms of the vapour pressure and the Henry constant.

There are no data provided regarding the hazard of difenoconazole to the ozone layer, the Ozone Depleting Potential (ODP) of difenoconazole has not been measured because Difenoconazole residues are unlikely to occur and persist in the atmosphere, due to the low volatility (vapor pressure: 3.32×10^{-8} Pa at 25ºC) and the rapid photochemical degradation in air of the active substance. Any accumulation of difenoconazole in the troposphere is therefore unlikely to occur.

12.1.2 Comparison with the CLP criteria

A substance is considered hazardous to the ozone layer if the available evidence concerning its properties and its predicted or observed environmental fate and behaviour indicate that it may present a danger to the structure and/or the functioning of the stratospheric ozone layer.

Any substances having an ODP of greater than or equal to the lowest ODP (i.e. 0.005) of the substances currently listed in Annex I to Regulation EC No 1005/2009 should be classified as hazardous to the ozone layer (category 1).

Although no specific data have been provided for this hazard, considering the chemical structure and other available information on the physcio-chemcial properties, Difenoconazole is not expected to be hazardous to stratospheric ozone.

12.1.3 Conclusion on classification and labelling for hazardous to the ozone layer

Not classified, data lacking.

13 ADDITIONAL LABELLING

No additional labelling is proposed.

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15 ANNEXES

A sanitized version of the Renewal Assessment Report (RAR, 2019) of the active substance difenoconazole has been included as Annex I.