Advice on toxicological evaluation of 1,2,4-triazole

isclaimer: due to confidentiality certain parts of the advice have been deleted for the purpose of publication

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1	2015/04/28	
2	2015/05/24	Feedback after reach committee
3	2015/06/21	ED part elaborated
4	2015/10/10	Follow-up WCSR 3: typing error: OECD TG 408 instead of 409; references to original studies instead of to secondary sources
5	2015/10/10	Addition of conclusions of a follow-up advice
6	2015/10/27	Correction of references to the only reproduction study

Contents

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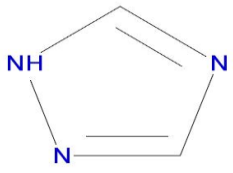
Structural formula:



Conclusions of the evaluation	Tick relevant box(es)
Concern not clarified; Need to request further information from the Registrant(s)	X
Concern clarified; No need of further risk management measures	
Concern clarified; Need for risk management measures;	

1 Deliverable 0: Stability of 1,2,4-triazole and its metabolites

In available animal studies, 1,2,4-triazole is rapidly and nearly completely absorbed, widely distributed in tissues, with no evidence of significant accumulation and excreted mainly in the urine at 80-100% of the administered dose in the first 24h. No significant metabolism occurs in animals and the sole toxicologically significant compound found is the 1,2,4-triazole itself.

2 Deliverable 1: Reprotoxicity

Conclusions:

Development: Developmental toxicity has been clearly demonstrated for 1,2,4-Triazole in rats and rabbits. Foetotoxicity, reflected by skeletal deviations, a diminution of foetal weight and an increase in the number of runts was already observed in rats at doses that were not toxic for the dams. In addition to this foetotoxicity, malformations have been observed in rats and rabbits at maternal toxic doses. In rats, some malformations (microphtalmia) were not related to the test substance but other malformations occurred at a rate above historical values (hydronephrosis) or at a rate for which historical values were not given (cleft palate, undescended testicle). However, cleft palate was observed in 3 different litters when this malformation is known to occur rarely spontaneously in rats. Moreover, the high rate of resorptions (53%) may have masked the number of malformations. In

rabbits, foetotoxicity and urinary tract malformations occurred concurrently to severe maternal toxicity. The historical values of these malformations are unknown.

Cleft palate and hydronephrosis are malformations commonly observed with some triazole-derivatives.

It was therefore important to analyze the toxicological profile of the different triazole-derivatives to better assess the observations made with 1,2,4-triazole.

Triazole-derivatives are antimycotic compounds used as fungicides in agriculture but also as antimycotic in human and veterinary medicine. The common target for all triazoles in fungi is the enzyme CYP51, involved in the steroid biosynthesis and therefore in the formation of the fungal walls.

In mammals, different Cytochrome P450 enzymes are also potential targets of the triazole-derivatives. Depending of the azoles and the tissue, different specific inhibition or induction have been described.

1,2,4-Triazole itself is a metabolite of different triazole-derivatives but the proportion of this metabolite may considerably differ from one compound to another. Amounts varying from 1% to 65 % have been found in the urine of rats exposed to different azoles. No correlation between the proportion of cranio-facial malformations and the amount of 1,2,4-triazole was noted.

Cleft palate is a specific malformation implying a disturbance in the process of cranial morphogenesis and the most validated hypothesis to explain this disturbance is the alteration in the endogenous level of Retinoic Acid. Excess in Retinoic Acid is responsible of a series of malformations and resulted also in increased rates of fetal resorption and stillbirths. This excess of Retinoic Acid caused by at least some triazole-derivatives would be the consequence of the inhibition of the CYP26 family. These enzymes are expressed differently during embryonic development, depending of time and tissue.

To compare the teratogenic potential of different triazole-derivatives, different alternative in vitro models have been used.

In 2011, de Jong et al compared some of these models, the mouse embryonic stem cell test (STEM), the rat whole embryo culture (WEC) and the zebrafish embryotoxicity test (ZET) to assess the potency of six 1,2,4 triazoles according to the developmental toxicity. The approach used for the comparison with the in vivo potency was a BMD approach. BMD10 skeletal variations in vivo was compared to BMC05 total morphological score for WEC, BMC05 general morphological score for ZET and BMC50 of beating embryoid bodies for STEM. Unfortunately, the parent compound, 1,2,4-triazole was not included in the study. Nonetheless, they concluded that using the approach explained, the ZET gave the best correlation with the in vivo observations, followed by the STEM.

Menegola et al have used the rat whole embryo model to investigate the teratogenic activity of different azoles. In one of the studies, 1,2,4-triazole was compared to two well-known teratogenic triazoles, flusilazole and fluconazole. These two triazole-derivatives compounds showed similar teratogenic effects as concentrations as low as 3.125 to 250 µM for flusilazole and 62.5 to 500 µM for fluconazole but no teratogenic activity was detected with 1,2,4-Triazole. Concentrations as high as 5000 µM induced only slight developmental retardation and blood discoloration.

Taking into account all this information in a Weight of Evidence approach, a more severe classification for 1,2,4-triazole could be discussed.

Even if no correlation exist between the proportion of cranial malformations and amount of 1,2,4-triazole in the urine of rats exposed to different triazole-derivatives and if no teratogenic activity has been shown in an in vitro model, it is not excluded that 1,2,4-triazole is teratogenic at high doses in vivo. This is suggested by the typical malformations, characteristic from the triazole-derivatives, observed in rats and rabbits exposed during gestation. The historical values of these malformations

were not given but it is well known that some of them, like cleft palates, are very rare. In the developmental study in rats, this malformation has been observed in 3/25 litters at the high dose. Moreover, the high level of resorptions at this dose (53%) may have masked these malformations. Finally, the high rate of resorptions in itself, if not directly linked to maternal toxicity, would justify classification. It would be useful to know if this effect, which is also one of the effects caused by excess of retinoic acid in the embryo, would occur in the absence of maternal toxicity.

A new developmental study in rats with intermediate doses between 100mg/kg bw and 200 mg/kg bw could help to detect any dose-related effect, mainly concerning the rate of resorptions. Malformations could also be easier detected with a lower rate of resorptions.

Fertility: 1,2,4-Triazole had a marked effect on index fertility and on the number of implantations at a dose causing degenerative effects in the cerebellum of the parents. The body weight was also reduced at this high dose but no clinical symptoms were observed. At the lower doses, no effect was observed on fertility, except a decrease in the number of corpora lutea at the intermediate dose in P0 and in F1 and a lower testicular sperm count in P0, that was statistically significant at the lowest dose. This effect on sperm count was not dose-related.

The clear effect on fertility being observed in the presence of other toxic effects, the question is to know if this fertility effect can be considered as a secondary non-specific consequence of the systemic toxicity. In this case, it is not possible to decide on this point as no clinical signs and only a moderate decrease in body weight gain was observed in the parents at the highest dose and no direct relation between the degenerative lesions in the cerebellum and the effect on fertility can be established. Even if a decrease in sperm counts was observed in the males, this effect could not have caused the drastic decrease in the fertility index. Moreover, at the lower doses, the same reduction in testicular sperm counts had no effect on fertility.

Based on these data, and due to the above-mentioned uncertainties, a category 2 for fertility is warranted for 1,2,4-triazole.

An extended-one generation study with a better choice of doses could be proposed to decide on a final classification.

4. Advice:

Historical values for some malformations (undescended testicles and cleft palate) observed in the developmental rat study because they are missing and could help in the decision for classification. Before recommending new studies, a discussion would be useful. Hence, the doses used in the developmental study and in the fertility study do not allow a final decision on classification, as effects at the intermediary doses are unknown.

5. References:

(1) Menegola E, Broccia ML, Di Renzo F, Giavini E. Antifungal triazoles induce malformations in vitro. Reprod Toxicol. 2001 Jul-Aug;15(4):421-7.

(2) De Jong E, Barenys M, Hermsen SA, Verhoef A, Ossendorp BC, Bessems JG, Piersma AH. Comparison of the mouse Embryonic Stem cell Test, the rat Whole Embryo Culture and the Zebrafish Embryotoxicity Test as alternative methods for developmental toxicity testing of six 1,2,4-triazoles. Toxicol Appl Pharmacol. 2011 Jun 1;253(2):103-11.

3 Deliverable 2: Neurotoxicity

3.1 Conclusions:

Chronic dietary administration of 1,2,4 triazole seems to cause neurotoxic effects in rat and mice. In a combined toxicity/neurotoxicity study in rats effects reflecting potential neurotoxicity were observed from 183 mg/kg and higher. Observations of the functional behaviour revealed effects in both sexes including ungroomed appearance, muscle fasciculations, tremor, gait in-coordination, decreased activity in the open field, and decreased rearing. There was a slight decrease in both sexes in absolute brain weight and microscopic findings were observed in both sexes in the brain and nerve tissue. No neurotoxic effects were seen at 33 mg/kg/d. Also in two-generation studies with rats and mice, and in developmental toxicity study in rabbits neurotoxic effects were observed. However potential neurotoxic effects were seen at concentrations where other effects (e.g. effects on reproduction, body weight) were also observed. The NOAEL values used for setting an ADI (0-0,2 mg/kg bw/d, EFSA 2009) and for risk assessment (Reference Dose of 0,03 mg/kg bw/d, US EPA 2006b) are based on effects on development/fertility/reproduction and are considered to be protective enough for neurotoxic effects.

A remaining point of concern may be the potential developmental neurotoxicity that was seen in the F1- and F2-generation in one developmental rat study, at concentrations lower than those causing neurotoxic effects in the parents. However in another 2-generation rat study, no developmental neurotoxicity was observed in the F1- or F2-generation at concentrations lower than those causing neurotoxic effects in the P-generation(US EPA, July 2009 HPV Challenge Program. Test plan submission 1,2,4-triazole)

3.2 Advice:

The concern is clarified.

3.3 References

EFSA, 2009. Scientific Opinion on Risk Assessment for a Selected Group of Pesticides from the Triazole Group to Test Possible Methodologies to Assess Cumulative Effects from Exposure through Food from these Pesticides on Human Health. EFSA Panel on Plant Protection Products and their Residues (PPR Panel). European Food Safety Authority, Parma, Italy

US-EPA, 2006b. Memorandum: Subject: Waiver request for Triazole acute Neurotoxicity Study. Scientific data reviews EPA Series 361. December 14, 2006

US EPA, July 2009. HPV Challenge Program. Test plan submission 1,2,4-triazole

4 Deliverable 3a: Genotoxicity

4.1 Concern:

In the registration dossier of 1,2,4-triazole, 5 in vitro and 1 in vivo genotoxicity tests are negative. No carcinogenicity test is available.

However, based on data from aminotriazole (which belongs to the same family) carcinogenicity and endocrine disruption cannot be excluded.

Indeed, 3-amino-triazole induced inconsistent genotoxic effects in vitro, but no genotoxic effects in vivo. Thyroid tumors have been observed in rats and mice. Mechanism of these tumours are not genotoxic. An endocrine mode of action is presumed. Even if the relevance for the humans of this tumour induced mechanism is still under debate (INRS, toxicological fiche of Aminotriazole, edition 2008).

Changes of thyroid hormones levels have also been observed in fish following 3-amino-1,2,4-triazole exposure (Changes of thyroid hormone levels and related gene expression in Chinese rare minnow (Gobiocypris rarus) during 3-amino-1,2,4-triazole exposure and recovery, Li et al., 2009).

4.2 Open literature

No additional information on other genotoxicity studies with 1,2,4-triazole is found in the open literature.

1,2,4-triazole was reviewed by JMPR in 2008. The meeting concluded that 1,2,4-triazole is unlikely to be genotoxic, based on the results of in vitro tests: 2 Ames tests, the forward mutation and test for chromosomal aberrations (same in vitro studies as discussed in this report).

1,2,4-triazole was also found not mutagenic (same in vitro studies as discussed in the JMPR 2008 report and as discussed in this report) as discussed by the US EPA HPV Challenge Program, 2009.

EFSA (2009) concluded that a number of adverse effects common to several triazoles has been observed in laboratory animals, such as developmental effects, effects on reproduction, hepatotoxicicty, hepatocarcinogenicity in mice and the production of other types of tumours (thyroid, testis), via non-genotoxic mechanisms.

The Californian EPA's Office of Environmental Health Hazard Assessement (OEHHA) presented a table with genotoxicity finding for various triazole antifungal agents during a public meeting of the proposition 65 Carcinogen identification Committee in 2001:

Table 3. Genotoxicity findings for various triazole antifungal agents

Chemical	Gene mutation		Chromosomal effects		UDS
	Salmonella	Other	Micronucleus	Other	
Cyproconazole (U.S. EPA, 1991; 1992; 2008)	-	-	-	+ (CHO cells)	NA
Difenoconazole (U.S. EPA, 1994)	-	-	-	NA	-
Etaconazole (U.S. EPA, 1998; 2000)	-	-	NA	NA	NA
Fenbuconazole (U.S. EPA, 2001a)	-	-	NA	-	-
Fluconazole (Fucic *et al.*, 2008)	NA	NA	+ (*in vivo*: mouse)	NA	NA
Flusilazole (IPCS, 1995)	-	-	-	-	-
Hexaconazole (U.S. EPA, 2000)	-				-
Myclobutanil (Ross *et al.*, 2009)	-	-	NA	-	-
Propioconazole (Ross *et al.*, 2009)	-	+ (*in vivo:* mouse)	-	-	-
Tebuconazole (U.S. EPA, 2010; CDPR, 2003)	-	-	-	-	-
Triadimefon (Ross *et al.*, 2009)	-	+ (*in vivo:* mouse)	+ (*in vivo*: rat)	+ (*in vivo*: rat)	-
Triadimenol (U.S. EPA, 1998; CDPR, 2000)	-	-	-	-	-
Uniconazole (U.S. EPA, 1998; 2000)	-	-	+ (*in vivo*: mouse)	+ (CHO cells)	-

UDS = Unscheduled DNA synthesis
NA = Not available

Table 4. Genotoxicity findings for triazole antifungal agent listed as a carcinogen under Proposition 65

Chemical	Gene mutation		Chromosomal effects		DNA effects	
	Salmonella	Other	Micronucleus	Other	UDS	Other
Epoxiconazole (U.S. EPA, 2001b)	-	-	-	-	-	-

The prediction of mutagenicity, carcinogenicity, development toxicity, and skin sensitization with the CEASAR programs was performed for a set of 27 conazoles by Bolčič-Tavčar and Vračko (2012). The CAESAR programs were developed to support the European Community Regulation on chemicals and their safe use (REACH) and follow the OECD principles for (Q)SAR models used for regulatory purposes. The CAESAR mutagenicity model is built on a large data set of 4204 compounds with their Ames test results. For all structures the descriptor pool was calculated using MDL software. BestFirst algorithm from the Waikato Environment for Knowledge Analysis software was applied to select the 27 relevant descriptors. Modelling combines support to vector algorithm and a rule-based system checking for structural alerts. The predictions were compared to the currently valid classification of the 27 substances in the EU or on the classification proposed at expert meetings in the Pesticide Risk Assessment and Peer Review (PRAPeR) group. The predicted classification for mutagenicity was in good agreement with regulatory classification. 1,2,4-triazole was predicted as non-mutagen.

4.3 Conclusions

In vitro, 1,2,4-triazole tested negative in the Ames tests, the mouse lymphoma assay and the chromosomal aberration test. In addition, 1,2,4-triazole tested also negative in the in vivo micronucleus test. By going through the open literature, no further concern or reasoning for additional genotoxicity testing was detected. 1,2,4-triazole is unlikely to have a genotoxic potential. No further testing is needed.

If data from other studies (repeated-dose toxicity studies, reprotoxicity studies) indicate that 1,2,4-triazole is possibly a carcinogen, than further research should be done on the mechanism to induce tumor formation by 1,2,4-triazole, which is more than likely not genotoxic.

4.4 References

(1) INRS, toxicological fiche of Aminotriazole, edition 2008.

(2) Li W, Zha J, Spear PA, Li Z, Yang L, Wang Z. Changes of thyroid hormone levels and related gene expression in Chinese rare minnow (Gobiocypris rarus) during 3-amino-1,2,4-triazole exposure and recovery. Aquat Toxicol. 2009 Apr 2;92(1):50-7.

(3) Bolčič-Tavčar M, Vračko M. Prediction of mutagenicity, carcinogenicity, developmental toxicity, and skin sensitization with CEASAR program for a set of conazoles. Arc Hig Rada Toksikol. 2012;63:283-292.

(4) US EPA HPV Challenge Program. Test Plan Submission 1H-1,2,4-Triazole. July 2009

(5) EFSA panel on Plant Protection Products and their Residues, Scientific Opinion on Risk Assessment for a Selected Group of Pesticides from the Triazole Group to Test Possible Methodologies to Assess Cumulative Effects from Exposure through Food from these Pesticides on Human Health. EFSA Journal 2009; 7 (9); 1167[187 pp.]

(6) JMPR, 2008. Triazole fungicide metabolites. JMPR 2008:437-490

(7) OEHHA, 2011. Chemicals for CIC Consultation: Triazole Antifungal Agents. www.oehha.org/prop65/public_meetings/CIC101211/101211Triazole_cic.pdf

(8) US EPA HPV Challenge Program, 2009. Test Plan Submission 1H-1,2,4-triazole.

5 Deliverable 3b: Carcinogenicity

5.1 Genotoxicity and carcinogenicity of 1,2,4-Triazole

Conclusions:

A large number of parent triazole-derivative pesticides have been classified as carcinogens (most also non-mutagenic), but the relevance of that finding to expected effects of free triazole may be limited. The types of tumours associated with exposure to the parent chemicals are most commonly hepatocellular adenomas/carcinomas in mice. Other tumour types vary considerably (including liver tumours, thyroid tumours, ovarian tumours, testicular tumours, and bladder tumours). None of the tumour types are clearly associated with the proportion of free triazole formed in available rat metabolism studies.

Regarding the substance (1,2,4-triazole) itself: it was found negative in several genotoxicity test conducted following OECD guidelines (several Ames test in vitro, an assay for forward mutation, and a test for chromosomal aberration) and therefore is unlikely to be genotoxic. As for non-genotoxic carcinogenic actions, the comparative studies involving triazoles pesticides shows that 1,2,4-triazole does not show any of the activities of the parent substances (parent in the context of 1,2,4-triazole being a metabolite of the studied pesticide).

Advice:

1,2,4-triazole was found negative in several genotoxicity test conducted following OECD guidelines and therefore is unlikely to be genotoxic. As for non-genotoxic carcinogenic actions, the studies involving triazoles pesticides shows that 1,2,4-triazole does not show any of the activities of the parent substances. The weak point being that there are not much data specific to 1,2,4-triazole, in this context, its properties are compared to the carcinogenic properties of the parent substances, there are no specific chronic cancer study on 1,2,4-triazole. But if we stay in the context of REACH, the substance falling in the 1-10 tonnes/per annum group, regarding the genotoxicity, the information requirement stops at the Ames test.

6 Deliverable 5: Endocrine Disruption

6.1 Concern:

Based on data from aminotriazole (which belongs to the same family as 1,2,4-triazole) endocrine disruption cannot be excluded. Indeed, 3-amino-triazole induced inconsistent genotoxic effects in vitro, but no genotoxic effects in vivo. Thyroid tumours have been observed in rats and mice. Mechanism of these tumours are not genotoxic. An endocrine mode of action is presumed. Even if the relevance for the humans of this tumour induced mechanism is still under debate (INRS, toxicological fiche of Aminotriazole, edition 2008).
Changes of thyroid hormones levels have also been observed in fish following 3-amino-1,2,4-triazole exposure (Changes of thyroid hormone levels and related gene expression in Chinese rare minnow (Gobiocypris rarus) during 3-amino-1,2,4-triazole exposure and recovery, Li et al., 2009).

6.2 Conclusions:

No indication of an endocrine disruption properties of 1,2,4-triazole is found.

6.3 Advice:

No need to request further information.

7 Deliverable 6: Follow-up advice

Concern

On the basis of the rapporteurs' findings and advices given to the BE CA, the registrants of 1,2,4-triazole provided some additional information on the remaining areas of concern, namely reprotoxicity and neurotoxicity. The follow-up advice was therefore requested from the WCSR in order to analyse the new information and to list arguments to support the demand of extended one generation reprotoxicity study (including investigation of DNT, DIT, thyroid ED mode of action).

7.2.**Advice:**

The rapporteur considered that the 2-generation study does not answer to several questions because of the bad choice of doses. Due to the fact that the space between the NOAEL and the LOAEL is too large, we don't know what can happen between these doses, we don't know if the effect on fertility is linked or not to the neurotoxic effects. The neurodevelopmental study would be the first priority but other questions would not be answered with this study and it is why an EOGRTS would be indicated to answer to the question on the effect on fertility. If an EOGRTS is conducted, the endocrine-related endpoints could be better investigated even if the evidence for a potent endocrine disruptor is not there. In a first time, we would recommend to conduct a steroidogenesis assay to see if 1,2,4-triazole has an effect at this level.