Committee for Risk Assessment

RAC

Annex 2

Response to comments document (RCOM)

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

4-nitrosomorpholine

EC Number: -
CAS Number: 59-89-2

CLH-O-0000007006-81-01/F

Adopted

10 June 2021

COMMENTS AND RESPONSE TO COMMENTS ON CLH: PROPOSAL AND JUSTIFICATION

Comments provided during consultation are made available in the table below as submitted through the web form. Any attachments received are referred to in this table and listed underneath, or have been copied directly into the table.

All comments and attachments including confidential information received during the consultation have been provided in full to the dossier submitter (Member State Competent Authority), the Committees and to the European Commission. Non-confidential attachments that have not been copied into the table directly are published after the consultation and are also published together with the opinion (after adoption) on ECHA's website. Dossier submitters who are manufacturers, importers or downstream users, will only receive the comments and non-confidential attachments, and not the confidential information received from other parties. Journal articles are not confidential; however they are not published on the website due to Intellectual Property Rights.

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Substance name: 4-Nitrosomorpholine
EC number: -
CAS number: 627-564-6
Dossier submitter: Germany

CARCINOGENICITY

Date	Country	Organisation	Type of Organisation	Comment number
20.08.2020	Sweden		MemberState	1
Comment received				
The Swedish CA supports classification of 4-nitrosomorpholine (CAS No. 59-89-2) as specified in the proposal. SE agrees with the proposal to set the SCL to 0.001% based on the high potency of the substance.				
Dossier Submitter's Response				
The comment by the Swedish CA is acknowledged.				
RAC's response				
Thank you for your comment.				

MUTAGENICITY

Date	Country	Organisation	Type of Organisation	Comment number
21.08.2020	France		MemberState	2
Comment received				
There are appropriate in vitro and *in vivo* studies available, which, in a weight of evidence, clearly show that 4-nitrosomorpholine is a mutagen in liver. 4-Nitrosomorpholine is known to be a hepatotoxicant and has liver as a target organ. The substance is clastogen in the presence of S9 in vitro, the liver should be a target for *in vivo* follow up. Liver clinical effects were reported in only one genotoxicity/repeated dose toxicity study (Hayashi et al, 2015). Both *in vivo* micronucleus tests available using hepatocytes (Ashby and Lefevre, 1989, Hayashi et al., 2015) were positive. Even if there is no validated OECD TG available for liver as target tissue, there is ongoing work to develop an OECD guideline for liver MN. Thus, test results can be regarded as relevant for classification. Hayashi et al. (2015) and				

Ashby and Lefevre (1989) did not report positive controls or historical controls for the published tests, however the studies gave positive results. In Hayashi et al. (2015), toxic effects in liver (single cell necrosis) related to treatment have been detected already at the lowest dose tested (5 mg/kg bw) of-N-nitrosomorpholine in 80% of rats orally treated for 14 days. We agree with DS that the influence of high liver toxicity on the test outcome (MNHEPs) in liver cells remains still unclear in terms of dose. The liver micronucleus trial results indicate a high sensitivity for the repeat dose liver micronucleus assay in detecting hepatocarcinogen (Sui et al, 2015). In order to reach a conclusion, hepatocarcinogenicity of 4 Nitrosomorpholine needs to be discussed in order to evaluate the liver toxicity data from Hayashi et al. (2015) for reliability in terms of a possible classification.

Despite negative results in in vivo heritable germ cell mutagenicity test, namely a dominant lethal test, an in vivo experimental for 4 NMOR in which some aspects of toxicokinetic like distribution have been examined separately shows the presence of 4 NMOR in testis (28 dpm/mg wet tissue of radioactivity in testis).

The classification of mutagens in Category 2 is based on positive evidence obtained from
(i) in vivo mammalian somatic cell mutagenicity tests or
(ii) other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.

Classification based on weight of evidence:
There is an in vivo database for 4-nitrosomorpholine that might be worth discussing. All of the available in vivo genotoxicity studies are publications and despite limitations in test design and reporting, those studies could be considered reliable. There is one available in vivo genotoxicity study which is positive and which can be identified as key study and as reliable even if the positive controls are missing. We agree that in all studies with positive results higher dose levels from 100 mg/kg bw and above were applied. Dose levels above MTD could interfere with the validity of the results of a genotoxicity study and could lead to false (positive) results. For 4-nitrosomorpholine a LOAEL (14 days) of 5 mg/kg bw/d was derived for oral administration in rats. The positive MN liver test by Hayashi et al. (2015) is to be explored as reported/measured toxicity and clinical effects were observed and liver is the target organ. There is ongoing work on OECD TG on liver MN. There is, in our opinion, no reason to disregard the studies. It is possible to assess, based on all reported positive and negative in vivo genotoxicity studies, if the positive effect following oral administration was robust and valid. Most of the positive studies were performed using intraperitoneal administration.

Contrary to DS, we disagree to say that the entire database is contradictory. The available key study like the MN test (Hayashi et al., 2015) is to be considered by RAC. In summary, a robust classification in Category 2 based on weight of evidence can be warranted if the data of the MN test is considered despite limitations as well as the whole results of available in vivo genotoxicity studies.

Conclusions on classification and labelling
There are mutagenicity assays with positive evidences for 4-nitrosomorpholine and the current data of the MN test could be sufficient to fulfil the classification criteria for mutagenicity in Category 2. Hence, at present, a classification and labelling of 4-nitrosomorpholine as mutagenic is to be discussed.

Dossier Submitter's Response

The German CA acknowledges the comment of the French CA.

However, a weight-of-evidence approach as proposed by the French CA is viewed critically by DECA for classification of 4-nitrosomorpholine for germ cell mutagenicity because of the lack of *in vivo* data with adequate quality. DECA interpretes the Guidance on the Application of CLP Criteria as such, that "at least one positive valid *in vivo* mammalian somatic cell mutagenicity test" (…) or "at least one positive valid *in vivo* mammalian somatic cell genotoxicity test, supported by positive *in vitro* mutagenicity results" is necessary to warrant classification in Cat. 2" (compare section 'classification as a Category 2 mutagen' on page 366 in the guidance). A weight-of-evidence approach using expert judgement becomes necessary in the case that there are also (valid) negative or equivocal data available (related to section 3.5.2.4 on 366 of the guidance) but not in the case if only data exist that cannot be considered robust enough for classification. None of the available *in vivo* genotoxicity studies for 4-nitrosomorpholine is identified as key study and as sufficiently reliable. There is no *in vivo* genotoxicity study available which was performed according to an international accepted guideline (e.g. OECD). All of the available *in vivo* genotoxicity studies have major limitations mainly related to missing information on toxicity, i.e. clinical effects and cytotoxicity. Therefore, the relevance of the positive results observed cannot be assessed (i.e. if being false-positive responses).

In addition, the positive MN test using hepatocytes in rat (Hayashi et al., 2015) is considered as not reliable despite the fact that toxicological information was available for this test. OECD TG 474 is validated for bone marrow as target tissue only. There is currently no validated OECD TG available for liver as target tissue (e.g. regarding upper limits of toxicity, age of animals, correct sampling times etc.), noting that there is ongoing work to develop an OECD guideline for liver MN. As long as there is no validated OECD TG available, test results cannot be regarded as robust enough for classification. Moreover, Hayashi et al., 2015 did not report positive controls or historical controls for the published test. Toxic effects in liver (single cell necrosis) have been detected already at the lowest dose tested (5 mg/kg bw). In the opinion of the DECA, the influence of high liver toxicity on the test outcome (MNHEPs) in liver cells still remains unclear. DECA acknowledges that if a validated assay becomes available, the data from Hayashi et al., 2015 could be reevaluated and assessed for reliability in terms of a possible classification at a later time. However, it is strongly discouraged by DECA for the time being to base a classification on a single *in vivo* test for which no validated OECD guideline is available and for which positive controls and historical controls have not been reported.

Overall, DECA interpretes the *in vivo* database to be contradictory, because positive and negative results are found for the same genotoxic endpoint in the same test system (chromosomal aberrations, MN tests). From 16 *in vivo* genotoxicity studies using a validated test system (MN assay based on bone marrow exposure, comet assay, UDS test, dominant lethal assay and chromosomal aberration test), six studies were negative (4 MN studies, 1 CA test, 1 dominant lethal assay), two studies yielded ambiguous results (1 CA, 1 MN test) and eight of the studies were positive (5 MN, 1 Comet, 2 UDS).

In all relevant studies yielding negative results lower dose levels up to 125 mg/kg bw 4-nitrosomorpholine were applied. In all studies with positive results, higher dose levels of 100 mg/kg bw and above were applied. The applied dose levels are critical for the tests as the MTD should be the highest dose administered and dose levels used should preferably cover a range from the MTD to a dose producing little or no toxicity (compare Section 33 of OECD TG 474). Dose levels above MTD could interfere with the validity of the results of a genotoxicity study and could lead to false (positive) results. For 4-nitrosomorpholine, a LOAEL of (14 days) of 5 mg/kg bw/d was derived for oral substance administration in rats (see section 4.8.1). Moreover, an oral LD50 of 282 mg/kg in rats was reported and Hayashi

et al., 2015 described deteriorated conditions in all animals after oral administration of 100 mg/kg for 1 week in rats. Besides the MN test by Hayashi et al. (2015), in none of the available positive *in vivo* genotoxicity studies toxicity and clinical effects were reported/measured. It cannot be deduced from the available genotoxicity studies whether doses applied lead to elicited severe systemic effects. Thus, it is not possible to conclude if the observed effects are due to real genotoxicity or are secondary effects due to cytotoxicity. The fact that most of the positive studies were performed using intraperitoneal substance administration, in which a higher bioavailability is assumed, underpins the uncertainty of the (toxic) effect of the dose levels applied and potentially false-positive results.

Negative results were reported in six available *in vivo* genotoxicity studies with intraperitoneal and oral substance administration of lower doses of 4-nitrosomorpholine. However, all these studies are also considered not to be reliable and sufficiently robust to conclude a negative outcome because they are not performed according/similar to a validated guideline.

All in all, the entire *in vivo* database is considered inconclusive, because negative and positive results for the same genotoxic endpoint in the same test system (chromosome aberrations, MN tests) are described. At the same time, a reliable *in vivo* key study is not available.

DECA strongly discourages to justify a classification on a weight of evidence approach based on data considered not reliable or on a single *in vivo* test (Hayashi et al., 2015) for which no validated OECD guideline is available to date and in which positive controls and historical controls have not been reported.

DECA acknowledges that in a weight-of-evidence approach there is a concern for genotoxicity for the substance, however, the database is considered not robust enough for classification.

RAC's response

Thank you for your comment and response. RAC acknowledeges the limitations in the database but considered after a weigh-of-evidence assessment of the *in vivo* studies that classification germ cell mutagenicity was warranted.

Date	Country	Organisation	Type of Organisation	Comment number
20.08.2020	Sweden		MemberState	3

Comment received

We propose to consider whether a more holistic weight of evidence assessment could be useful in concluding on the mutagenicity classification. This would include the results of all the mutagenicity studies, in combination with carcinogenic studies indicating that a mutagenic mechanism may be involved. Although the mutagenicity studies are judged to be of low reliability, there are several positive studies as well as tumor data that point to a mutagenic property of this substance that may warrant classification as Muta 2.

Dossier Submitter's Response

The German CA supports that the positive results observed in the *in vitro* and *in vivo* genotoxicity studies could hint to a genotoxic mode of action for carcinogenicity.

However, a classification as Muta 2 is not warranted because of the lack of *in vivo* data with adequate quality and the high uncertainty due to contradictory results from the *in vivo* studies (related to the same genotoxicity endpoint in the same test system). The German CA strongly discourages to justify a Muta 2 classification on a weight of evidence approach based on *in vivo* studies considered not reliable and robust.
RAC's response
Thank you for your comment. See response to comment 2.