Committee for Risk Assessment

RAC

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

10 June 2021

CLH-O-0000007006-81-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **4-nitrosomorpholine**

EC Number: **-**

CAS Number: **59-89-2**

The proposal was submitted by **Germany** and received by RAC on 14 May 2020.

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Germany has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on 22 June 2020. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by 21 August 2020.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Nathalie Printemps**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **10 June 2021** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	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 current Annex VI entry										
Dossier submitters proposal	613-RST-VW-Y	4-nitrosomorpholine	-	59-89-2	Carc. 1B STOT RE 1	H350 H372 (liver)	GHS08 Dgr	H350 H372 (liver)		Carc. 1B; H350: C≥ 0.001%	
RAC opinion	613-RST-VW-Y	4-nitrosomorpholine	-	59-89-2	Carc. 1B Muta 2 STOT RE 1	H350 H341 H372 (liver)	GHS08 Dgr	H350 H341 H372 (liver)		Carc. 1B; H350: C≥ 0.001%	
Resulting Annex VI entry if agreed by COM	613-RST-VW-Y	4-nitrosomorpholine	-	59-89-2	Carc. 1B Muta 2 STOT RE 1	H350 H341 H372 (liver)	GHS08 Dgr	H350 H341 H372 (liver)		Carc. 1B; H350: C≥ 0.001%	

GROUNDS FOR ADOPTION OF THE OPINION

HUMAN HEALTH HAZARD EVALUATION

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

Summary of the Dossier Submitter's proposal

The liver was identified as the main target organ in rats. The evaluation of STOT RE was based on five oral (drinking water) rat repeated-dose toxicity studies. Two were sub-acute studies and three were long-term studies related to carcinogenicity. These studies were neither GLP nor OECD TG compliant. Most of the studies investigated a limited number of parameters (e.g. liver). Nevertheless, the dossier submitter (DS) considered that the studies provided relevant information for this endpoint.

In these studies, the following adverse liver toxic effects were noted at doses relevant for classification as STOT RE 1 (≤ 10 mg/kg bw/d):

- Single cell necrosis in centribular hepatocytes,
- Diffuse inflammatory cell infiltration,
- Acinocentral loss of glycogen,
- Scarring,
- Fibrosis,
- Postnecrotic cirrhosis,
- Decreased absolute and relative liver weight.

The DS pointed out that in the studies which also investigated the carcinogenic potential of the substance, these liver findings occurred concurrently with preneoplastic and neoplastic effects.

Based on a weight-of-evidence assessment, the DS concluded that the liver effects observed after oral treatment of rats fulfilled the criteria for classification of 4-nitrosomorpholine as STOT RE 1, H371 (liver).

Comments received during consultation

No comments were received.

Assessment and comparison with the classification criteria

The DS presented five repeated-dose toxicity studies in rats (Hayahi *et al*., 2015; Weber and Bonnasch, 1994; Lijinsky *et al*., 1975 and 1976, Moore *et al*., 1989). A summary of study results is provided under "Supplemental information - In depth analyses by RAC" in the background document.

In Hayashi *et al*. (2015), groups of male rats (n=5 per groups) were treated for 14 days with 0, 5, 10 or 30 mg/kg bw/d 4-nitrosomorpholine *via* drinking water. Although the study was not performed according to the relevant OECD TG, it is well described and is considered acceptable for classification purposes. Liver weight was statistically significantly decreased at 30 mg/kg bw/d (20% compare to control). A dose-related increase in the severity and incidence of liver findings were noted in rats. Single cell necrosis was observed in all rats at 30 mg/kg bw/d and was associated with anisokaryosis, proliferation of perilobular oval cells and diffuse inflammatory cell

infiltration. Histopathological findings found in the liver are reported in the table below. No excessive general toxicity was noted in the study.

Dose (mg/kg)	Control	5	10	30
Hypertrophy of centrilobular hepatocytes				
None	5			
Minimal		5		
Mild			5	
moderate				5
Single cell necrosis in centrilobular hepatocytes				
None	5	1		
Minimal		3		
Mild		1	5	5
Anisokaryosis in hepatocytes				
None	5	5	5	1
Minimal				4
Proliferation of perilobular oval cells				
None	5	5	5	
Minimal				1
Mild				3
moderate				1
Diffuse inflammatory cell infiltration				
None	5	5	5	1
Minimal				4

The liver effects can be considered adverse and irreversible where necrosis occurred. In this study single cell necrosis was associated with other liver findings such as proliferation of perilobular oval cells, increasing the concern. After correction for exposure duration (14 days), a minimum effective dose of 0.8 mg/kg bw/d can be calculated for cell necrosis (observed at 5 mg/kg) for a 90 day study using Haber's rule. Mild to moderate liver findings were observed at the top dose of 30 mg/kg bw/d (corresponding to an effective dose of 4.7 mg/kg bw/d after correction for exposure duration) in this study. These values are below the upper guidance value for classification as STOT RE 1.

A dose and time-related increase in severity of liver damage was also reported by Weber and Bonnasch (1994). Findings indicative of hepatocyte degeneration and necrosis were already noted after a 7-week exposure at 24 mg/kg bw/d 4-nitrosomorpholine. After correction for exposure duration, an effective dose slightly above the upper guidance value for classification in category 1 is obtained (13 mg/kg). However, it is noted that although single cell necrosis was observed at lower doses in the study, the time at which the effects appeared is not known.

The studies of Lijinsky *et al*. (1975 and 1976) are considered of lower weight as no controls were used and as information on actual doses were not provided. In Lijinsky *et al*. (1976), extensive but focal postnecrotic cirrhosis was noted in most livers of rats exposed to 4-nitrosomorpholine for 30-weeks at 0.3 and 1.5 mg/kg. In Lijinsky *et al*. (1975), liver necrosis, massive scaring, biliary hyperplasia and telangiectasis was noted in rats exposed for 30 weeks to 4-nitrosomorpholine at 1.4 mg/kg bw/d in drinking water. These two studies support the conclusion that 4-nitrosomorpholine is a severe hepatotoxicant at low dose levels, which are relevant for classification as STOT RE 1.

The sub-acute toxicity study published by Moore *et al*. (1989) investigated the adrenals only. The reported effects were not sufficient to support classification STOT RE.

No data are available on other species.

On the basis of the observed dose-related increase in severe liver findings (e.g. necrosis) in four studies, RAC agrees with the DS's proposal to **classify 4-nitrosomorpholine as STOT RE 1; H372 (liver)**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS concluded that based on *in vitro* data, 4-nitrosomorpholine caused gene mutation in bacterial and mammalian cells after metabolic activation. No reliable *in vitro* cytogenicity studies were identified by the DS.

The DS considered the entire *in vivo* database to be inconclusive. The DS pointed out that both positive and negative results were obtained for the same endpoint and that no key studies could be identified. In addition, according to the DS, positive results were mainly obtained at high dose levels, in the absence of information on cytotoxicity. Therefore, the DS considered the database not robust enough for classification.

As the classification criteria are mainly based on *in vivo* results, no classification was proposed.

Comments received during consultation

Two member states commented that a classification as Muta. 2 may be warranted based on a weight-of-evidence assessment of the studies.

Assessment and comparison with the classification criteria

In vitro data

Gene mutation in bacteria

Ten Ames assays were reported by the DS. All recommended strains were tested. Dose levels up to 10000 µg/plate were used. The studies used either preincubation methods or plate incorporation. S9 from mice, rats or hamsters for metabolic activation were included. Only four studies were considered reliable with restrictions by the DS (Klimisch 2). RAC agrees that the Rowland *et al*. (1981) study should be disregarded due to lack of reporting. Positive results were obtained in the other nine studies in *S. thyphimurium* TA 100, TA 1535 or *E. coli* WP2. When reported, no cytotoxicity was noted. With the exception of one study (rated unreliable, no information on dose levels), metabolic activation was required to induce a positive result. The substance was negative in TA 98 and TA 1537.

RAC concludes that 4-nitrosomorpholine is mutagenic in bacteria in the presence of metabolic activation.

Summary of reverse mutation assays in bacteria cells

Test system	Without metabolic Activation	With metabolic activation	Lowest effective dose (ED)*	Reliability (DS)	Reference
S. typhimurium TA 100, TA 98	-	+ (TA 100) - (TA 98)	33 µg/plate (Hamster S9) 333 µg/plate (rat S9) No cytotoxicity	2	Zeiger *et al*., 1992
E.coli WP2 uvrA	-	+	No information on ED or cytotoxicity	2	Matsushima *et al*., 1981
S. typhimurium TA 1537, TA 100, TA 98	-	+ (TA100) - (TA98, TA 1537)		2	MacDonald, 1981
S. typhimurium TA 1537, TA 100, TA 98	-	+ (TA 100) - (TA 98, TA 1537)		2	Nagao *et al*., 1981
S. typhimurium TA100, TA 98	+	+	No information on dose levels	3	Ichinotsubo, 1981
S. typhimurium TA100, TA 1530	-	+	25 mM No cytotoxicity	3	Khudoley *et al*., 1981
S. typhimurium TA 1535	-	+	1000 µg/plate No cytotoxicity	3	Andrews *et al,* 1980
S. typhimurium TA 1535	-	+	0.01 µmol/plate No cytotoxicity	3	Zeiger *et al*, 1978
S. typhimurium TA 1535, TA 1536, TA 1538	-	+ (TA1535) - (TA1537, TA1536 and TA1538)	1080 µM No cytotoxicity	3	Gomez RF, 1974

***** information retrieved by RAC from the original study reports when available.

Mammalian cell results

Three micronucleus studies were performed on various cell lines (Human foetal cells, rat digestive tract cells, rat primary hepatocytes). Although no metabolic activation was used, the cells were considered metabolically active. Positive results were observed in all three studies. When cytotoxicity was analysed, the positive results were not secondary to cytotoxicity (e.g. Mueller-Tegethoff, 1995).
Two studies investigated chromosomal aberrations in various cell types (Human HepG2, primary rat hepatocytes, V79, human VH10 fibroblasts). Although no metabolic activation was used, the primary hepatocytes were considered to be metabolically active. Positive results were obtained in all cell types except in human VH10 fibroblasts. No data was available on cytotoxicity.

The DS disregarded these five studies, because the cell cultures were mostly non-standard and a positive control was not always included. RAC acknowledges the limitations but considers that the database strongly indicate that the substance induces chromosomal mutations or formation of micronuclei *in vitro*.

Regarding *in vitro* gene mutation in mammalian cells, positive results were observed in the two available studies (Jotz *et al*., 1981 and Rochinova *et al*., 2004). In the most reliable study (Jotz *et al*., 1981), the results were positive only in presence of metabolic activation. The positive results were reported in presence of slight cytotoxicity (not further specified in the CLH report). These studies indicate that 4-nitrosomorpholine induces gene mutations in mammalian cells *in vitro*.

Test system	Endpoint	Test condition	Without met. Act.	With met. Act.	Lowest effective dose (ED)	Reference
Klimisch score 2 (DS's assessment)						
Mouse lymphoma cells	Gene mutation (TK locus)	similar to standard guideline	-	+	330 µg/ml Cytotoxicity within acceptability criteria	Jotz *et al*., 1981
Klimisch score 3 (DS's assessment)						
V79 cells	Gene mutation (6-TG)	30 min exposure	+	+	15 mmol/L with met. Act., 20 mmol/L without met., act., Slight cytotoxicity	Robichova *et al*., 2004a
Human fetuses cells (HuFoe-15), rat digestive tract cells (IEC-17, IEC-18), hamster V79 cells	Micronucleus	24h treatment	- (V79) + (HuFoe-15, IEC-18, IEC-17)	ND	ED not provided (0.1 to 100µg/mL were tested) Cytotoxicity not determined	Glatt *et al*., 1990
Primary rat hepatocytes	Micronucleus	4h treatment	+	ND	10-6 M Mitotic Index: 50% at 10-4	Mueller-Tegethoff *et al*., 1995
Primary rat hepatocytes	Micronucleus	Exposure duration not stated	+	ND	0.116 mg/mL No data on cytotoxicity	Slamenova *et al*., 2002
Primary rat hepatocytes	Chromosomal aberrations	3h exposure time	+	ND	0.116 mg/mL No data on cytotoxicity	Slamenova *et al*., 2002
human fibroblasts (VH10 cells), hamster lung fibroblasts (V79), Human HepG2 hepatoma cells	Chromosomal aberrations	0,5 or 43h exposure (HepG2), 23h (V79) and 41h (VH10)	- (VH10), + (V79), + HepG2	ND	0.25 mmol/L (V79 and HepG2 after 43h exposure) and 10 mmol/L (HepG2 after 0.5h exposure) Cytotoxicity not determined	Robichova *et al*., 2004b

ND: no data; met. act.: metabolic activation

Overall, RAC agrees with the DS that, based on *in vitro* genotoxicity data, 4-nitrosomorpholine causes gene mutations in bacterial and mammalian cells after metabolic activation. In addition, RAC considers that they provide a strong indication that 4-nitrosomorpholine also causes chromosomal mutations *in vitro* in mammalian cells.

In vivo data

The DS disregarded all the available *in vivo* studies. RAC agrees that the study of Roehrborn *et al*. (1973) provides slight evindece, as only a short meeting abstract is available. Regarding other studies, an in-depth analysis of the limitations of the *in vivo* studies has been performed by RAC (see below). Based on this analysis, RAC agrees that four additional studies should be considered to have a similarly low weight due to major deficiencies (Salamone *et al*., 1981, Tsuchimoto *et al*., 1981, Korr *et al*., 2001, Ramaya *et al*., 1980). Regarding other studies, although RAC

acknowledges the limits of the studies, they are considered acceptable for classification purposes in a weight of evidence assessment.

Four bone marrow micronucleus assays were available in rats or mice, following single or two intraperitoneal (ip) administrations (24h apart). Three of these studies were positive at dose levels ≥ 100 mg/kg bw/d (Nerseyan *et al*., 2002, Wakata *et al*., 1998 and Morita *et al*., 1997). Negative results were obtained at lower dose levels (Kirkhart *et al*., 1981). No details on toxicity was provided in these studies. Nevertheless, in Kirkhart *et al*. (1981) the highest dose of 32 mg/kg bw/d in mice was considered as 50% of the LD_{50} in ICR mice (ip), suggesting that doses ≥100 mg/kg bw/d may produce toxicity in mice. Nevertheless, in Tsuda *et al*. (2000), no excessive toxicity was noted in mice following single ip administration of 250 mg/kg. Therefore, there are uncertainties concerning the toxicity observed in animals in these studies.

Only one bone marrow micronucleus study is available following oral administration (Hayashi *et al*., 2015). Negative results were obtained at dose levels up to 30 mg/kg bw/d in the presence of slight bone marrow toxicity. In contrast, in this study, a dose-related increase in liver micronuclei was observed at ≥ 10 mg/kg. At this dose, mild centrilobular hypertrophy and minimal single cell necrosis were already noted in rats. Cytotoxicity is one of the issues encountered in the liver micronucleus assay. The relationship between liver micronucleus formation and cytotoxicity is unclear (Uno *et al*., 2015). Therefore, it is difficult to interpret the results of this liver micronucleus study. Strengths and weaknesses of the liver micronucleus assay were discussed in the 6th international workshop on genotoxicity testing (Uno *et al*., 2015). Regarding the usefulness of the assay, the liver micronucleus assay is expected to detect genotoxicants that require metabolic activation. It is stated that substances, such as 4-nitrosomorpholine, that form unstable reactive liver metabolites, would be expected to be more active at this site than in bone marrow. In the same line, Hayashi *et al*. (2015) pointed out that the active genotoxic metabolite of 4-nitrosomorpholine might not reach the bone marrow, which may explain the differences in results between the liver and the bone marrow micronucleus assay.

In Ashby *et al*. (1989), positive results were obtained in an unscheduled DNA synthesis assay in liver after a single oral gavage dose at 100 mg/kg.

A dominant lethal assay was negative in the mouse.

One *in vivo* comet assay is available in mice and was performed on stomach, colon, liver, kidney, bladder, lung, brain and bone marrow. 4-nitrosomorpholine was administered once intraperitoneally at 250 mg/kg. No death, morbidity or distinctive clinical signs were noted. DNA damage was statistically significantly increased in all organs ($p<0.001$) except in brain and bone marrow. At necropsy, although macroscopic findings were noted in the liver, no necrosis was observed. No other histopathological findings were reported. The authors of the study suggested that the absence of positive results in bone marrow may reflect the low genotoxic activity at this site compared to other organs (e.g. the liver). In the study, similar results were obtained with the other tested dialkyl N-nitrosamines.

Method	Test system	Test condition	Results	Effective dose level	Reference
Studies considered acceptable for classification purposes based on WOE assessment (DS's score: Klimisch 3)					
DNA damage in stomach, colon, liver, kidney, bladder, lung, brain, bone marrow (similar to OECD TG)	Mouse (male)	Comet assay, ip (single dose) Sampling time: 3, 9 and 24h after treatment	+ (stomach, colon, liver, kidney, bladder, lung) - (brain, bone marrow)	250 mg/kg Liver macroscopic findings but no necrosis observed	Tsuda *et al*., 2000
Unscheduled DNA synthesis in liver (similar to OECD TG)	Rat (male)	Oral: gavage (single dose), 2.5 and 12h exposure time	+	10 mg/kg bw/d in preliminary study and 100 mg/kg bw/d in main study No data on clinical findings	Ashby *et al*., 1989
Dominant lethal (similar to OECD TG)	Mouse (male and female)	ip	-	35 mg/kg bw/d as a top dose (reduced mating at 50 and 100 mg/kg)	Parkin *et al*., 1973
Micronucleus formation (similar to OECD TG)	Rat (male)	Oral, gavage, 14-day treatment, sampling 24h after treatment	-	Bone marrow toxicity at 30 mg/kg bw/d (top dose)	Hayashi *et al*., 2015
Micronucleus formation in liver			+	10 mg/kg Hepatic lesions observed in all dose groups, decreased liver weight at 30 mg/kg	
Micronucleus (similar to OECD TG)	Rat (male)	Two ip administrations	+	100 mg/kg Evidence of myelotoxicity	Neresyan *et al*., 2002
Micronucleus (similar to OECD TG)	Rat (male)	Two ip administrations Harvest 24h after treatment	+	180 mg/kg No data on toxicity	Wakata *et al*., 1998
Micronucleus (similar to OECD TG)	Mouse (male and females)	Single ip dose Sampling 18h after treatment	+	250 mg/kg bw/d No data on toxicity	Morita *et al*., 1997
		Single ip dose: Sampling 24h after treatment	+	500 mg/kg bw/d No data on toxicity	
Micronucleus (Similar to OECD TG)	Mouse (male)	Two ip administrations Sampling 6 or 24h after treatment	-	32 mg/kg bw/d was the maximum dose tested	Kirkhart, 1981
Studies considered of lower weight (DS's score: Klimisch 3)					
Chromosomal aberration	Mouse (F1, male)	ip (no further information)	-	50 mg/kg bw/d as top dose	Ramaya *et al*., 1980

Method	Test system	Test condition	Results	Effective dose level	Reference
Micronucleus (similar to OECD TG)	Mouse (male)	Two ip administrations, Sampling 6h after treatment	-	32 mg/kg bw/d was the maximum dose tested	Tsuchimoto *et al*., 1981
Micronucleus (similar to OECD TG)	Mouse (sex not specified)	One or two ip injections Sampling: 48, 72, 96h after final second treatment or 30, 48h and 72h after single injection	- (single injection) + (Two injections)	40% and 80% of LD_{50} (no exact data and no effective dose provided) No data on cytotoxicity	Salamone, 1981
Unscheduled DNA synthesis in liver	Rat (male)	Oral: gavage (single dose), direct injection of 3H-thymidine after treatment	+	200 mg/kg bw/d	Korr *et al*., 2001

Ip: intraperitoneal

Mechanism of action and structural similarity

4-nitrosomorpholine belongs to the chemical groups of N-nitrosamines and is extensively metabolised in mammals. According to Koissi and Fishbein (2014), alpha-hydrocylation of 4-nitrosomorpholine leads to an intermediate which is assumed to form reactive electrophilic alkyldiazonium ions.

QSAR data also support the hypothesis. The QSAR toolbox revealed an alert for an Aryl N-nitroso group. As stated by the DS in the CLH report, the formation of reactive electrophilic alkyldiazonium ions is generally considered relevant for alkylnitrosamides.

The DS noted that they did not find alkylating agents from the same class that have a harmonised classification for germ cell mutagenicity. Nevertheless, there is no information on whether this endpoint was assessed for these compounds.

In the carcinogenicity database for 4-nitrosomorpholine, there are indications that the substance is a genotoxic carcinogen. Indeed, tumours were observed at multiple sites, without a threshold and after a short latency period.

Comparison with classification criteria

Classification in category 2 may be based on positive results from at least one valid *in vivo* mammalian somatic cell mutagenicity or genotoxicity test, supported by positive *in vitro* mutagenicity data after metabolic activation.

RAC agrees with the DS that valid positive *in vitro* mutagenicity data were available.

RAC acknowledge that all the *in vivo* studies had limitations and that no key studies, fully compliant with OECD TG, could be identified. Nevertheless, some of the studies were considered comparable to the relevant OECD TGs. Positive results were consistently obtained using intraperitoneal administration in micronucleus assays at doses ≥ 100 mg/kg, indicating intrinsic genotoxic properties. The negative results observed at lower dose levels in ip studies and the negative oral micronucleus assay in the bone marrow support weak activity of the substance in this organ. Nevertheless, positive results in the comet assay, UDS and micronucleus assay were obtained in the rat liver, which is the target organ of the substance for carcinogenicity. Although there are uncertainties on the genotoxicity in animals from the UDS and in the liver micronucleus assay, no excessive toxicity was reported in the comet assay. These positive results are

supported by positive results obtained with 4-nitrosomorpholine *in vitro* in several endpoints after metabolic activation. Based on a weight-of-evidence evaluation of the database, 4-nitrosomorpholine warrant classification at least for somatic cell mutagenicity.

There are no data available in the dossier on the potential of 4-nitrosomorpholine to reach the germ cells. Therefore, the substance does not fulfil the criteria for classification in category 1B.

Overall, RAC concludes that **classification of 4-nitrosomorpholine as Muta. 2, H341 is warranted**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

Twenty-eight studies with a Klimisch reliability of 2 were assessed by the DS. None of the studies were fully compliant with standard test guidelines. Only four of these studies (2 in rats and 2 in hamsters) were considered of sufficient quality to allow an assessment of the carcinogenic potential of the substance (Lijinsky *et al*., 1988; Weber and Bannasch, 1994, Cardesa *et al*., 1990; Ketkar *et al*., 1983). Other studies had shortcomings (e.g. absence of controls) but were considered as supporting.

In Lijinsky *et al*. (1988), a clear dose-related increase in liver tumours (hepatocellular adenoma and carcinoma, hemangioma) were noted in female rats (males not investigated) treated with 4-nitrosomorpholine in drinking water, following 50 or 100-week exposure. In addition, although not clearly dose-dependent, at higher doses, an increased incidence of thyroid, oesophagus and tongue tumours were noted. The incidences of these tumours were outside the historical control range values published by NTP in 2010.

In weber and Bannasch (1994), male rats were treated with 4-nitrosomorpholine at 6 to 24 mg/kg bw/d for 7 to 80 weeks in drinking water. A dose and time-related increase in liver tumours was noted. Increasing dose levels shortened the time to liver tumour occurrence. In this study, only the liver was examined.

The DS stated that all the other carcinogenicity studies in rats supported the liver carcinogenic potential of 4-nitrosomorpholine, independently of rat strain, sex and route of exposure. The DS noted that liver preneoplastic lesions were already noted following a single high oral dose (320 mg/kg). Oesophageal and thyroid tumours were also reported in several other studies. Nasal tumours were only increased in one study (Garcia and Lijinsky, 1972) but was considered of unclear relevance as the nasal cavity was analysed only in a few animals.

In Ketkar *et al*. (1983) and Cardesa *et al*. (1990), a dose-related increase in tumour incidence of the respiratory and digestive tracts were noted in male and female hamsters. No effects on survival was noted in these studies, in contrast to rats. In supporting studies, the carcinogenic potential of 4-nitrosomorpholine for the respiratory tract was also seen using other routes of administration (intratracheal administration, subcutaneous, oral inhalation) and independently of strain and sexes. An increase in respiratory tract tumours in hamsters was already noted after a single high subcutaneous dose(Althoff *et al*., 1974). The DS highlighted that the liver was not the main target organ for carcinogenicity in hamsters.

Based on tumours observed in rats and hamsters, by any route of exposure, the DS considered that there was sufficient evidence of carcinogenicity. The most reliable studies were carried out at different time and laboratories (Lijinsky *et al*., 1988; Weber and Bannasch, 1994, Cardesa *et al*., 1990; Ketkar *et al*., 1983). In addition, the DS pointed out that in the studies, malignant

tumours occurred to an unusual degree of incidences and that there were multiple tumour sites. Several supporting studies also provided evidence of reduced tumour latency for the liver findings.

The DS suggested that the severe decrease in survival noted in the rat studies may be due to the carcinogenic potential of the substance.

The DS further noted that 4-nitrosomorpholine belongs to the group of substances known as nitrosamines. Three N-nitrosamines are classified Carc. 1B in the CLP regulation (dimethyl nitrosamine, 2,2-(nitrosoimino)bisethanol and nitrosodi-n-proplamin).

N-nitrosamines are assumed to have a mutagenic mode of action. The DS concluded that the observed liver rat tumours at low dose levels, after a short latency period in multiple organs hint towards a genotoxic mechanism of action. Moreover, the need of metabolic activation to obtain a positive result in the Ames test further supports this hypothesis.

Overall, the DS proposed to classify 4-nitrosomorpholine as Carc. 1B, H350.

Specific concentration limit (SCL)

The DS proposed a specific concentration limit of 0.001% for 4-nitrosomorpholine. The DS used the most sensitive species and tumour type in lifespan studies for the derivation of T25. Using the highest net liver tumour incidence of 15% observed in the oral rat study (Lijinsky *et al*., 1988) at 0.02 mg/kg bw/d (converted from mg/L by the DS), considering all liver tumour types, a T25 of 0.032 mg/kg bw/d was obtained. Since the T25 was well below the limit of 1 mg/kg bw/d for high potency carcinogens, an **SCL of 0.001%** was proposed by the DS instead of 0.01% generally recommended for high potency carcinogens.

Comments received during public consultation

One Member State agreed with the proposal to classify N-nitrosomorpholine as Carc. Cat. 1B and to set a SCL at 0.001%.

Assessment and comparison with the classification criteria

In the dossier, twenty-eight published carcinogenicity studies with 4-nitrosomorpholine of reliability 2 (Klimisch) were presented (some studies were reported as separate studies by the DS when different exposure conditions were performed in the same study).

Fourteen carcinogenicity drinking water studies (11 in rats, 2 in hamsters and 1 in mice) were available, the study duration varied between 8 weeks and whole lifetime exposure and doses varied between 0.003 mg/kg bw/d and 24 mg/kg bw/d. Three oral gavage studies were presented (two in rats a one in hamsters). Study duration varied from single exposure (320 mg/kg) to 30 weeks. One 6-week inhalation study was available in SD female rats at 0.5 mg/kg. In addition, four subcutaneous, one intratracheal and one intravesicular (via the bladder) studies were available.

Six strains of rat (Sprague-Dawley, Fisher F344, albino random bred, WS/Shi, SD/gShi, MRC), one strain of mouse (A/J) and three strains of hamster (Chinese, European and Syrian) were tested. Only eight studies investigated both sexes.

In agreement with the DS, RAC also considered 4 of the published carcinogenicity studies to be key studies (Lijinsky *et al*., 1988 (50 and 100-week exposure), Ketkar *et al*., 1983, Weber and Bannasch, 1994). Although not fully compliant with OECD TG 451, RAC agrees with the DS that the published results of these studies are sufficiently reliable and relevant to assess the carcinogenic potential of 4-nitrosomorpholine.

Rats

In Lijinsky *et al*. (1988), female F344 rats were treated with 4-nitrosomorpholine at different dose levels for 50 or 100 weeks in drinking water. In addition, two higher doses were tested for 25 and 40 weeks. A dose-related and statistically significant (trend test) increase in liver tumours (hepatocellular adenoma, carcinoma and hemangiosarcoma) was noted after both 50 and 100 weeks of exposure. A time-related increase in incidence was also noted. At the two highest doses (40 and 100 mg/L), 96% and 100% of animals had benign or malignant liver tumours after 40 or 25 weeks, respectively. Body weight changes or non-neoplastic findings were not reported. A dose-related decrease in survival was noted in the study. Historical control data published by NTP in 2010 are not considered relevant as they were from a different laboratory and a different period of time compared to the Lijinsky *et al*. (1988) study.

Increases in tongue and thyroid malignant tumours were also noted but without a clear dose-reponse relationship and in a lower number of animals as compare to liver. In addition, an increase in esophagus tumours were seen at ≥ 2.6 mg/L but without a clear dose-response relationship(not seen in controls or in treated groups up to this dose level).

Results after 50 or 100-week exposure are provided in the table below (as published in Lijinsky *et al*., 1988).

mg/L	0	0.07	0.18	0.45	1.1	2.6	6.4	16
mg/kg*	**0**	**0.0035**	**0.009**	**0.023**	**0.055**	**0.13**	**0.32**	**0.8**
100-week exposure								
Hepatocellular carcinoma	0/80	1/100	0/99	0/47	1/48	7/48	16/24	Na
Haemangio-sarcoma	0/80	0/100	0/99	0/47	0/48	5/48	13/24	na
Begnin or malignant	1/80 (1%)	6/100 (6%)	5/99 (5%)	7/47 (15%)	9/48 (19%)	22/48 (46%)	23/24 (96%)	Na
50-week exposure								
Hepatocellular carcinoma	0/80	na	na	0/48	1/48	5/48	7/24	15/23
Haemangio-sarcoma	0/80	na	na	0/48	0/48	1/48	0/24	8/23
Benign or malignant	1/80 (1%)	na	na	6/48 (13%)	7/48 (15%)	15/48 (31%)	14/24 (58%)	22/23 (96%)

* conversion performed assuming consumption of 20mL drinking water per rat per day (Lijinsky *et al*., 1988) and 0.4kg bw (weight of older rats in table 3.18 of the CLP guidance document (v.5.0)); The conversion value and incidences slightly differ from table 14.4 of the CLH dossier.
na: not available.

Consistent to the result of this study, Weber and Bannasch (1994), reported a dose-related increase in pre-neoplastic lesions and liver tumours in male rats, demonstrating that the effect was not sex or strain-specific. Time-dependency was also noted in the study as the first tumours were observed after 27 weeks at 6 mg/kg bw/d and 15 weeks at 24 mg/kg. The large reduction of survival noted in the study was considered to be related to the carcinogenic effect of the substance.

In all the other presented supporting studies in rats, increases in liver tumour incidences were noted, independently of strain, sex and route of exposure (drinking water, inhalation, gavage). The relevance of other tumour-types identified in other supporting studies (kidney, esophagus, thyroid, nasal cavity) in rats are difficult to interpret due to missing controls, missing historical

control data, single dose levels and as only selected number of organs were analysed in the studies. Nevertheless, the studies support the results of Lijinsky (1988) and indicate that 4-nitrosomorpholine is a multi-site carcinogen.

Hamsters

In Ketkar *et al*. (1983), male and female Syrian gold hamsters were orally given in drinking water containing 0.010%, 0.005% and 0.001% 4-nitrosomorpholine. The doses were stated to correspond to 1/20, 1/40 and 1/150 of the LD_{50}. Dose-related increases in respiratory tract and digestive tract tumours were observed. In males, body weights were decreased but the decrease was not statistically significant. No effect on survival was noted in either sex. The authors reported that in the respiratory tract, the main target organs were the larynx and trachea (papillary polyps, papillomas and epidermoid carcinomas). The authors reported that most tumours found in the liver were hepatocellular adenomas and carcinomas but that cholangiocellular and endothelial tumours were also observed. For liver tumours no incidences were provided. Tumour latency decreased with increasing dose of 4-nitrosomorpholine.

Results published in Ketkar *et al*. (1983) are reported in the table below.

Dose [mg/kg bw/d]*	**Total number of tumour bearing animals**	**Respiratory tract tumours (incidence)**	**Digestive tract tumours (incidence)**
Males			
0	8/50 (16 %)	0/50 (0 %)	0/50 (0 %)
0.9	12/29 (41.4 %)	8/29 (27.6 %)	4/29 (13.79%)
3.4	14/29 (48.3)	13/29 (44.8 %)	9/29 (31.3 %)
6.1	26/30 (86.7 %)	21/30 (70 %)	18/30 (60 %)
Females			
0	3/50 (6%)	0/50 (0%)	0/50 (0%)
1	14/28 (50%)	14/28 (50%)	0/28 (0%)
3.9	17/30 (56.7%)	16/30 (53.3%)	2/30 (6.67%)
8.3	23/30 (76.7%)	22/30 (73.3%)	6/30 (20%)

*conversion based on mean weekly intake as provided in the published study.

Consistent with these findings, Cardesa *et al*. (1990) also found a dose-related increase in laryngotracheal tumours in Syrian male and female hamsters following life-time treatment with 4-nitrosomorpholine in drinking water. Examination was restricted to the respiratory tract. Decreased survival was only noted in females. No data on body weight, clinical or non-neoplastic findings were available.

Other supporting studies in hamsters either *via* oral or other routes of exposure supported the conclusion that the respiratory tract is a target organ of 4-nitrosomorpholine carcinogenicity in hamsters. In contrast to rats, liver tumours were not consistently reported in the studies.

Mice

Only one 10-week oral drinking water study was available in mice, which was of low reliability. Investigations were restricted to lung adenomas. An increased incidence in lung adenoma was noted after 10 weeks of exposure at the single dose tested (3.6 mg/kg). Nevertheless, as the DS noted, a very high background incidence of this tumour type was found in controls. Therefore, although indicative of potential carcinogenicity in mice, the results should be considered with care.

Mode of action

RAC agrees with the DS that the occurrence of tumours at low dose levels, in multiple organs and after short latency period indicate a non-threshold genotoxic mode of action. Metabolism and hepatotoxicity seems to play a role in the carcinogenic potential of the substance.

Classification of other N-nitrosamines as Carc. 1B further support classification of the substance for carcinogenicity.

Overall evaluation and comparison with the criteria

According to the CLP criteria, category 1B is indicated when relevant malignant neoplasms were observed in at least two species. In the case of 4-nitrosomorpholine, increased incidences of liver, digestive or respiratory tracts tumours were observed in both rats and hamster in both sexes after exposure via the oral route. These findings provide sufficient evidence of carcinogenicity. Carcinogenicity was noted following exposure via all tested routes of administration.

Therefore, **Carc. cat. 1B (H350) is warranted** for 4-nitrosomorpholine**.**

Specific concentration limit

In line with the EC (1999) guidance, RAC agrees with the DS to calculate T25 values based on liver tumours in female rats observed following life-time dietary exposure (Lijinsky *et al*., 1988). Treatment started at 8 weeks of age and the duration of the study was 100 weeks. Although animals were treated for 5 days out of 7 each week, RAC agrees with the DS that no correction should be done, as it was already included in the conversion from mg/L to mg/kg bw/d. The lowest effective dose in female rats for liver carcinoma was 0.13 mg/kg bw/d. At this dose, 7/48 female rats showed liver tumours (14.6%). No background correction is needed as no tumours were seen in controls. The T25 is equal to 0.21 mg/kg bw/d (T25 = 100/104 x 25/14.6x 0.13 mg 4-nitrosomorpholine/kg bw/d). Considering (consistent wit the approach of the DS) all liver tumours types (benign and malignant), a T25 of 0.037 mg/kg bw/d is obtained (100/104 x 25/15 x 0.023). This value differs slightly from the T25 calculated by the DS. This may be due to differences in the underlying assumption used to convert dose levels from mg/L to mg/kg bw/d in the study.

Considering respiratory tract tumours in hamsters after whole life time exposure (Ketkar *et al*., 1983), a higher T25 of 0.5 mg/kg bw/d is obtained (T25= 25/50 * 1 mg 4-nitrosomorpholine/kg bw per day).

According to the document EC (1999), a T25 < 1 mg/kg bw/d is the starting point for considering a substance as a high potency carcinogen and an SCL of 0.01% could be assigned according to the CLP guidance.

Nevertheless, other considerations should be considered for assigning a potency class:

- *Dose-response relationship*
  here is no data indicating a supralinear dose-response.

- *Site/species/strain/gender activity*
  4-nitrosomorpholine is a multi-site carcinogen in both sexes and in three species. This provides support for a high potency carcinogen.

- *Mechanism including genotoxicity*
  4-nitrosomorpholine was found to be a genotoxicant and a non-threshold carcinogen. In addition, the carcinogenic mode of action may be relevant to humans.

- T*oxicokinetics*
  there is no data suggesting that the toxicokinetic behavior would be different in animals and humans.

- *Other elements*
  the very short latency period observed in the studies increase the concern. Indeed, tumours were already noted after single administration. 4-nitrosomorpholine reduced tumour latency in several published studies.

Overall, RAC considers that based on these other considerations **an SCL of 0.001%**, as proposed by the DS, for 4-nitrosomorpholine is appropriate.

Additional references

Uno *et al*. (2015). Recommended protocols for the liver micronucleus test: Report of the IWGT working group. Mutation research. 783; 13-18

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).