CLH report

Proposal for Harmonised Classification and Labelling

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

Substance Name:

4-nitrosomorpholine

EC Number: -

CAS Number: 59-89-2

Index Number: -

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CONTENTS

Part A.

Part B.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1 Substance identity

Substance name:	***4-nitrosomorpholine***
EC number:	**-**
CAS number:	***59-89-2***
Annex VI Index number:	**-**
Degree of purity:	***≥ 80 % w/w***

1.2 Harmonised classification and labelling proposal

Table 2 The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	none
Current proposal for consideration by RAC	Carc. 1B, H350, SCL = 0.001 % STOT RE 1, H372
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Carc. 1B, H350, SCL = 0.001 % STOT RE 1, H372

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3 Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	Not assessed in this dossier.			
2.2.	Flammable gases				
2.3.	Flammable aerosols				
2.4.	Oxidising gases				
2.5.	Gases under pressure				
2.6.	Flammable liquids				
2.7.	Flammable solids				
2.8.	Self-reactive substances and mixtures				
2.9.	Pyrophoric liquids				
2.10.	Pyrophoric solids				
2.11.	Self-heating substances and mixtures				
2.12.	Substances and mixtures which in contact with water emit flammable gases				
2.13.	Oxidising liquids				
2.14.	Oxidising solids				
2.15.	Organic peroxides				
2.16.	Substance and mixtures corrosive to metals				
3.1.	Acute toxicity - oral				
	Acute toxicity - dermal				
	Acute toxicity - inhalation				
3.2.	Skin corrosion / irritation				
3.3.	Serious eye damage / eye irritation				
3.4.	Respiratory sensitisation				
3.4.	Skin sensitisation				
3.5.	Germ cell mutagenicity	**None**	--	**None**	**Data inconclusive**
3.6.	Carcinogenicity	**Carc. 1B, H350**	**SCL = 0.001 %**	**None**	--
3.7.	Reproductive toxicity	Not assessed in this dossier.			
3.8.	Specific target organ toxicity – single exposure				
3.9.	Specific target organ toxicity – repeated exposure	**STOT RE1, H372**	--	**None**	--
3.10.	Aspiration hazard	Not assessed in this dossier.			
4.1.	Hazardous to the aquatic environment				
5.1.	Hazardous to the ozone layer				

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Hazard pictograms:

GHS08: Health hazard

Signal word:

Dgr: Danger

Hazard statements:

H350: May cause cancer
H372: Causes damage to organs (liver) through prolonged or repeated exposure

Proposed notes assigned to an entry:

-

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

4-nitrosomorpholine has not previously been assessed for harmonised classification by RAC.

2.2 Short summary of the scientific justification for the CLH proposal

Based on an assessment of available animal carcinogenicity studies it can be concluded that a classification as Carc. 1B (H350) is warranted for 4-nitrosomorpholine. The results of numerous reliable and supporting studies indicate a high carcinogenic potential of 4-nitrosomorpholine and show that 4-nitrosomorpholine induces tumours in different species (rat, hamster, mice), different organs and independent from the administration route applied (oral, inhalation, intratracheal, subcutaneous). A number of similarities of tumour organs and tumour types were observed across studies, species and routes. The findings for 4-nitrosomorpholine are in line with numerous other N-nitrosamines known to be potent carcinogens. There is no registration of 4-nitrosomorpholine up to date.

Moreover, there are appropriate animal studies available for 4-nitrosomorpholine which, in a weight of evidence, clearly show that 4-nitrosomorpholine is a hepatotoxicant after oral treatment of rats. Toxic effects to the liver included single cell necrosis in centribular hepatocytes, diffuse inflammatory cell infiltration, an acinocentral loss of glycogen, scarring, fibrosis, significant reduced mean absolute and relative liver weights and postnecrotic cirrhosis. These effects are considered to be relevant for human health, are in line with effects described in Section 3.9.2.7.3 d, e and f (CLP Regulation) and were observed at low doses (compared to equivalent

guidance values) warranting classification as STOT RE 1 H372 (Causes damage to organs (liver) through prolonged or repeated exposure).

2.3 Current harmonised classification and labelling

4-nitrosomorpholine has currently no harmonised classification (Annex VI, CLP Regulation).

2.4 Current self-classification and labelling

The self-classification as available from the C&L Inventory Database (May 2020) includes self-classification of a total of 48 notifiers.

Self-classification for carcinogenicity (Carc. 2, H351) was done by 43 notifiers. 44 notifiers classified for acute toxicity (Acute Tox. 3, H301), one for mutagenicity (Muta. 2 H341) and one for reproductive toxicity (Repr. 2, H361).

4 out of 48 notifiers did not consider any self-classification of 4-nitrosomorpholine for human health hazards.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

According to article 36(1) of the CLP Regulation substances that fulfil the criteria for classification for carcinogenicity (category 1A, 1B or 2) shall normally be subject to harmonised classification and labelling. Based on an assessment of the numerous available carcinogenicity studies for 4-nitrosomorpholine it can be concluded that a classification as Carc. 1B (H350) is warranted for 4-nitrosomorpholine. Hence, action is needed at community level as currently there exists no harmonised classification for 4-nitrosomorpholine as Carc. 1B (H350).

At present, there is no registration of 4-nitrosomorpholine. But 4-nitrosomorpholine has been detected as impurity in higher amounts in consumer products (e.g. snow sprays). Without harmonised classification (Carc. 1B), restrictions laid down in Annex XVII No. 28-30 (REACH Regulation) cannot be applied to protect the general public from 4-nitrosomorpholine containing consumer products. Hence, a harmonised classification for 4-nitrosomorpholine would enable implementation of appropriate REACH Regulation processes related to a carcinogenic substance.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

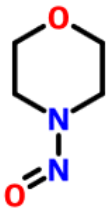
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 4 Substance identity

EC number:	-
EC name:	-
CAS number (EC inventory):	-
CAS number:	59-89-2
CAS name:	Morpholine, 4-nitroso-
IUPAC name:	4-nitrosomorpholine
CLP Annex VI Index number:	-
Molecular formula:	$C_4H_8N_2O_2$
Molecular weight range:	116.12 g/mol

Structural formula:



1.2 Composition of the substance

Table 5 Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
4-nitrosomorpholine		80 – 100 %w/w	

Table 6 Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
-			

Table 7 Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
-				

1.2.1 Composition of test material

1.3 Physico-chemical properties

Table 8 Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Yellow crystals	O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Whitehouse Station, NJ: Merck and Co., Inc., 2006., p. 1147	handbook data
	Yellow crystals. Golden liquid with many crystals at 68°F.	National Toxicology Program, Institute of Environmental Health Sciences, National Institutes of Health (NTP). 1992. National Toxicology Program Chemical Repository Database. Research Triangle Park, North Carolina: NTP.	secondary source
Melting/freezing point	29 °C	O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Whitehouse Station, NJ: Merck and Co., Inc., 2006., p. 1147	handbook data
Boiling point	224-224.5°C at 747 mmHg	O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Whitehouse Station, NJ: Merck and Co., Inc., 2006., p. 1147	handbook data
Density	1.32 ± 0.1 g/cm^3 (Temp: 20 °C; Press: 760 Torr)	Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2015 ACD/Labs)	calculated
Vapour pressure	0.036 mm Hg at 20 deg C; 0.19 mm Hg at 40 deg C (est)	Klein RG; Toxicol 23: 135-47 (1982)	handbook data
Surface tension	50.3 ± 7.0 dyne/cm	Calculated using ACD/I-Lab Software (v12.1.0.50375)	calculated
Water solubility	Miscible in water in all proportions.	IARC. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Geneva: World Health Organization, International Agency for Research on Cancer, 1972-PRESENT. (Multivolume work). Available at: http://monographs.iarc.fr/index.php , p. V17: 263 (1978)	handbook data
Partition coefficient n-octanol/water	-0.594±0.273 (T = 25°C)	Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2015 ACD/Labs)	calculated
Granulometry	none		
Solubility in organic solvents	Soluble in organic solvents.	IARC. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Geneva: World Health Organization, International Agency for Research on Cancer, 1972-PRESENT. (Multivolume work). Available at: http://monographs.iarc.fr/ENG/Classification/index.php p. V17: 263 (1978)	handbook data

2 MANUFACTURE AND USES

No registered manufacture/use.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not evaluated in the present dossier.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

Table 9 Studies related to experimental non-human toxicokinetic information for 4-nitrosomorpholine

Method	Results	Remarks	Reference
***In vivo* distribution and excretion of [3,5-^{14}C] radiolabelled 4-nitrosomorpholine and metabolites**, no guideline followed intravenous and subcutaneous rat (Sprague-Dawley) male dose: 2.5 mg/kg bw (single dose) **exposure regime**:intravenous: sacrifice 1 min, 15 min, 1 h, 4 h and 24 h after injection; subcutaneous: sacrifice 4 and 24 h after injection **Parameters investigated:** whole body autoradiography	- 1 min after sacrifice with low temperature autoradiography (study of non-metabolised 4-nitrosomorpholine): homogeneous labelling of most tissues → *indication that non-metabolized 4-nitrosomorpholine passes cellular membranes freely* - at all measured time points after injection high levels of non-volatile metabolites in liver and nasal mucosa and some labelling of oesophageal mucosa and intestinal content → *indication that liver, nasal mucosa and oesophageal mucosa are target organs and probably main organs for metabolism* - homogeneous background radioactivity in most other tissues → *probably due to incorporation of labelled one- and/or two-carbon fragments via normal metabolic pathways (acc. to authors)*	**supporting study** (2 reliable with restrictions) Restrictions: only few results reported, number of animals not given, radioactivity not reported at all measured time points, results are not presented separately for intravenous and subcutaneous injection **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Loefberg B, Tjaelve H (1985)
***In vivo* distribution and excretion of [3,5-^{14}C] radiolabelled 4-nitrosomorpholine and metabolites**, no guideline followed intravenous single dosing observation: 8h after injection rat (Sprague-Dawley) male dose: 2.5 mg/kg bw (single dose) **Parameters investigated:** radioactivity determined in various tissues, faeces and urine, analysis of [**^{14}C]**-CO_2 exhalation, (rats individually in metabolism cages)	**Distribution:** levels of radioactivity in various tissues [dpm/mg wet tissue]: - liver: 260 - nasal olfactory mucosa: 121 - kidney: 77 - lung: 62 - oesophagus: 57.4 - pancreas: 50 - small intestine: 46 - submaxillary salivary gland: 46 - tongue: 37 - forestomach: 33 - heart: 29 - testis: 28 - brain: 21.4 **Excretion**: - within 1 h about 2.4 % of administered dose exhaled as [**^{14}C]**-CO_2 - within 8 h 4.7 % of administered dose exhaled as CO_2 - urine: 63.3 % of dose within 8 h - faeces: 2.6 % of dose within 8 h	**supporting study** (2 reliable with restrictions) Restrictions**:** no analytical identification of metabolites in urine or faeces, only one time point measured **Test material: 4-itrosomorpholine** **Analytical purity:** no data, commercial substance source	Loefberg B, Tjaelve H (1985)
***In vivo* distribution of [3,5-^{14}C] radiolabelled 4-nitrosomorpholine and metabolites in posterior nasal**	- most marked labelling over subepithelial glands (Bowman	**supporting study** (2 reliable with	Loefberg B, Tjaelve H (1985)

region, no guideline followed intravenous rat (Sprague-Dawley) male dose: **2.6 mg/kg bw** (single dose) Exposure regime: sacrifice 4 h after injection **Parameters investigated:** radioactivity distribution in posterior nasal region	glands) in the lamina propria mucosae	restrictions) Restrictions: only one rat, only one dose level, only one time point **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	
***In vitro* metabolism of [3,5-^{14}C] radiolabeled 4-nitrosomorpholine in rat tissue**, no guideline followed Test procedure: - Pieces of nasal olfactory mucosa, liver, oesophagus, kidney cortex, lung and trachea excised from non-treated rats and incubated in Krebs-Ringer phosphate buffer containing 1.03 µCi (0.07 mM) 4-nitrosomorpholine - incubation for 60 min at 37°C under oxygen atmosphere - radioactivity from formed labelled CO_2 detected - adding metyrapone and using Carbon monoxide atmosphere to study if metabolism is cytochrome P-450 dependent	- production of [^{14}**C]**-CO_2 significantly increased compared to control (boiled liver) in nasal olfactory mucosa, liver and oesophagus - [^{14}**C]**-CO_2 yields lower if metyrapone added or using carbon monoxide atmosphere → *results indicate that metabolism is cytochrome P-450 dependent (acc. to authors)*	**supporting study** **(2 reliable with restrictions**) **Rationale:** no standardised guideline available, specifity of metyrapone and carbon monoxide atmosphere for *cytochrome P-450not discussed* **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Loefberg B, Tjaelve H (1985)
***In vitro* metabolism of [^{3}H] radiolabeled 4-nitrosomorpholine in isolated rat liver microsomes,** no guideline followed - rat liver microsomes prepared and incubated with labelled 4-nitrosomorpholine (no concentration and exposure time given) - investigation of (ethyl acetate) microsome extracts with thin-layer chromatography (TLC) after 4-nitrosomorpholine exposure)(due to small amounts mass spectra were not obtained)	**Metabolites identified:** - N-nitroso 2-hydroxymorpholine identified as one 4-nitrosomorpholine metabolite in microsome extract - some other possible metabolites were present in immobile phase and could not be identified	**supporting study** **2 (reliable with restrictions)** Rationale: no details on 4-nitrosomorpholine source and purity, identification method was TLC, no details on methods (such as 4-nitrosomorpholine concentration), no detailed documentation of results **Test material: 4-nitrosomorpholine** **Analytical purity: and source** no data	Manson D, Cox PJ, Jarman M (1978)
***In vivo* determination of urinary metabolites of [^{3}H] radiolabeled 4-nitrosomorpholine,** no guideline followed route of administration: no data	**Excretion:** - dichloromethan extracts of the urine at pH 7.0 contained mainly 4-nitrosomorpholine (non-metabolised) (TLC, silicat gel)	**supporting study** **2 (reliable with restrictions)** Rationale: no administration route given, no details on dosing, no details on	Manson D, Cox PJ, Jarman M (1978)

rat number of animals: 2 **dose:** total dose 40 mg/rat controls: no **additional information on method:** urine of two rats collected over 24 h, urine extracts with different solvents (e.g. dichloromethane and ethyl acetate), analysed using TLC and mass spectrometry	**Urinary metabolites:** - ethyl acetate extracts contained a component which was identified as N-nitrosodiethanolamine (MS) - N-nitroso 2-hydroxymorpholine not detected	rat strain, main identification method was TLC, no details on method, no detailed documentation of results, no quantification data, no details on 4-nitrosomorpholine source and purity **Test material:** 4-n**itrosomorpholine** **Analytical purity:** no data	
***In vivo* determination of blood elimination of 4-nitrosomorpholine,** no guideline followed intravenous exposure: single dose observation: 0.5, 1, 2, 4 and 8 h after injection rat (Wistar) male 5 animals **dose: 6.3 mg/kg** (single dose) **Additional information on method:** 0.1 mL blood collected from plexus orbitalis, quantification of 4-nitrosomorpholine using GC with a chemoluminiscence detector	**Blood concentration of 4-nitrosomorpholine:** - 30 min after injection blood concentration about 3.8 µg/mL blood - 8 h after injection blood concentration about 1 µg/mL blood - linear decrease of 4-nitrosomorpholine concentration during 8h after injection	**supporting study** 2 (reliable with restrictions) **Test material: 4-nitrosomorpholine** **Analytical purity:** 99 %	Maduagwu EN, Frei E, Frank N, Spiegelhalder B, Preussmann R (1983)
***In vivo* determination of urinary metabolites of 4-nitrosomorpholine,** no guideline followed intraperitoneal single dosing rat (Fischer 344) male 2 animals **dose: 125 or 150 mg/kg bw** (single dose) **Additional information on method:** urine collected over 48 h, metabolites determined via GLC-MS, 4-nitrosomorpholine determined using GLC	**Metabolites identified in urine:** - 16 % of dose (2-hydroxyethoxy)acetic acid - 33 % of dose nitroso(2 -hydroxyethyl)glycin - 12 % of dose nitrosodiethanolamine **Unchanged 4-nitrosomorpholine in urine:** - 1.5 % of dose	supporting study **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	Hecht SS, Young R (1981)
***In vitro* metabolism of [^{3}H] radiolabeled 4-nitrosomorpholine in isolated rat liver microsomes,** no	**Metabolites identified:** - (2hydroxy-ethoxy)acetaldehyde	supporting study **Test material: 4-**	Hecht SS, Young R (1981)

guideline followed - liver microsomes obtained from male F344 rats which had been given Aroclor 1254 - incubation with 23.2 mg 4-nitrosomorpholine for 20 min at 37°C - mixture analysed by HPLC and MS Control: heat-inactivation of microsomes	detected - this metabolite not detected in heat-deactivated microsomes	**nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	
***In vivo* dermal absorption of 4-nitrosomorpholine,** no guideline followed dermal: clipped area of the upper dorsal skin single dose observation: 24 h rat (F344) male 3 -7 animals (three independent experiments) Dose: **5 mg/rat** (no data on animal weight and age) Vehicle: water or ethyl acetate Controls: yes (no data) Additional information on method: analysis of 4- nitrosomorpholine using gas chromatography, at several sampling points (0, 1, 2, 4, 6, 8, 24 h) 0.1 mL blood samples taken from tail vein	**Blood concentrations of 4-nitrosomorpholine:** - 4-nitrosomorpholine concentration between 2 and 12 µg/mL at all sampling points using ethyl acetate as vehicle and 0.3 to 5 µg/mL using water as vehicle - it is reported that 4-nitrosomorpholine was not detected 24 h after treatment (no data are shown)	supporting study **2 (reliable with restrictions)** Rationale: no data on controls, no data on values 0 h and 24 h after treatment, high variability between animals, results not related to weight or age or of animals, high variability between similar experiments in urine concentration, only one dose tested **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 %, non-commercial substance source	Lijinsky W, Losikoff AM, Sansone EB (1981)
***In vivo* oral absorption of 4-nitrosomorpholine,** no guideline followed oral: gavage single dose observation: 24 h rat (F344) male 4 animals Dose: **5 mg/rat** (no data on animal weight and age) Vehicle: water	**Blood concentrations of 4-nitrosomorpholine** - at all measured time points 4 to 11 µg/mL 4- nitrosomorpholine found in blood samples (data not related to weight or age of animals)	supporting study **2 (reliable with restrictions)** Rationale: no data on controls, no data on values 0 h and 24 h after treatment, high variability between animals, results not related to weight or age or of animals, only one dose tested **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 %, non-commercial substance source	Lijinsky W, Losikoff AM, Sansone EB (1981)

Controls: yes (no data) Additional information on method: analysis of 4- nitrosomorpholine using gas chromatography, at several sampling points (0, 1, 2, 4, 6, 8, 24 h) 0.1 mL blood samples taken from tail vein			
***In vivo* distribution, elimination and metabolism of [^{14}C] radiolabeled 4-nitrosomorpholine,** no guideline followed intraperitoneal single dose observation: 24 h (distribution study) and 30 h (elimination and metabolism study) rat male 4 animals (distribution study) or 2 animals (elimination and metabolism study) Dose: **400 mg/kg bw** Vehicle: no data Controls: no Additional information on method: - distribution study: 1, 3, 8, 18 and 24 h after injection 4-nitrosomorpholine concentrations were determined in blood, liver, kidney, spleen, lung, small intestine, large intestine and brain using polaropgraph - elimination and metabolism study: urine and faeces and exhaled radiolabeled CO_2 collected for 30 h after injection	**Distribution study:** - following injection 4-nitrosomorpholine rapidly distributed throughout animal - within 24 h after injection no accumulation in certain tissue observed - concentration in all tissues decreased over 24 h - concentrations in tissues reduced to less than 10 % of the initial value within 18 h after injection **Elimination and Metabolism study:** - within 30 h after injection only 3.3 % of the radioactivity injected exhaled as **[^{14}C]-**CO_2 - within 30 h after injection 81 % excreted in the urine - three radiolabeled compounds found in urine: two of these were supposed to be identified as 4-nitrosomorpholine and nitrosodiethanolamine using paper chromatography	Supporting study **2 (reliable with restrictions)** Rationale: considered as only partly reliable as a very high dose in the acute level was applied to the rats, chemical identification was based on an outdated method which is not considered to be unambiguous **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 %, non-commercial substance source	Stewart BW, Swann PF, Holsman JW, Magee PN (1974)

4.1.2 Human information

There is currently no information available.

4.1.3 Summary and discussion on toxicokinetics

Toxicokinetics studies fully compliant with a standardised guideline such as OECD Test Guideline (TG) 417, in which all aspects of toxicokinetics (absorption, distribution, elimination and metabolism) are examined, were not available for 4-nitrosomorpholine. However, there exist several experimental *in vivo* and *in vitro* studies for 4-nitrosomorpholine in which some aspects of toxicokinetics have been examined separately. These studies are documented in Table 9 and in the technical dossier. The results of these studies are discussed below.

Absorption

There are two *in vivo* studies available related to absorption of 4-nitrosomorpholine by the oral and dermal administration routes (Lijinsky et al., 1981). Male F344 rats were treated dermally and orally with a single dose of 4-nitrosomorpholine (5 mg) and observed for 24 h. 4-nitrosomorpholine concentrations in blood samples from the tail vein, taken at different time points after treatment, were examined using gas chromatography. After dermal treatment 4-nitrosomorpholine concentrations between 2 and 12 µg/mL were found at all sampling points using ethyl acetate as vehicle and 0.3 to 5 µg/mL using water as vehicle. It was reported that 4-nitrosomorpholine was not detected 24 h after treatment. After oral treatment 4-nitrosomorpholine concentrations between 4 to 11 µg/mL were found in blood samples at all measured time points. The data indicate similar absorption rates after oral and dermal treatment. However, the studies are considered as not reliable due to several reasons including no data on 4-nitrosomorpholine concentrations in blood from controls and at the beginning of treatment, no data on age and weight of animals, high biological variability of data and only one tested dose level. Thus, the results of these studies are not further discussed here.

Distribution

Four studies related to *in vivo* distribution, three performed by Loefberg and Tjaelve, 1985 and one by Stewart et al., 1974, are available for 4-nitrosomorpholine.

The three collectively reported distribution studies in rats by Loefberg and Tjaelve, 1985 with intravenous substance administration of a single dose (about 2.5 mg/kg bw) of radiolabeled [3,4 - ^{14}C] 4-nitrosomorpholine comprise a qualitative whole body autoradiography study, a quantitative distribution study and a distribution study specifically related to the nasal region. In the whole body autoradiography study it was shown that non-metabolised 4-nitrosomorpholine was rapidly distributed homogeneously in most rat tissues 1 min after injection. The authors concluded that 4-nitrosomorpholine pass freely through cellular membranes. At all later analysis time points (15 min to 24 h after treatment) tissue bond radioactivity concentrated in liver and nasal mucosa and to a lower extent also in the oesophageal mucosa. This indicates that these organs are target organs for 4-nitrosomorpholine in rats which is in line with the observed carcinogenicity in these organs in rats (see section 4.10). In addition, homogeneous background radioactivity was detected in most other tissues. According to the authors, this is probably due to incorporation of labelled one- and/or two-carbon fragments via normal metabolic pathways. Concordant to the qualitative results highest levels of radioactivity were found in the liver and nasal olfactory mucosa in the quantitative distribution study. In the distribution study specifically related to the rat posterior nasal region the most marked labelling was detected over subepithelial glands (Bowman glands) in the lamina propria mucosae.

Stewart et al, 1974, who treated rats intraperitoneally with a single high dose of 400 mg/kg bw of radiolabeled 4-nitrosomorpholine, also found a rapid distribution of 4-nitrosomorpholine throughout all tissues after injection. However, in contradiction to the results of Loefberg and Tjaelve, 1985, they did not observe an accumulation of radioactivity in any of the tissues tested.This could be due to the comparable high dose (400 mg/kg bw versus 2.5 mg/kg bw) used by the authors which might lead to observation of unspecific diffusion rather than specific distribution, metabolism and elimination processes.

Elimination

Maduagwu et al., 1983 investigated the *in vivo* elimination of 4-nitrosomorpholine from the blood in rats after a single intravenous dose of 6.3 mg/kg bw over 8 h after treatment. 30 min after

injection the blood concentration was about 3.8 µg/mL blood. 8 h after injection the 4-nitrosomorpholine concentration in blood was reduced to one fourth to about 1 µg/mL blood. Within the observed 8 h after treatment a linear decrease of 4-nitrosomorpholine concentration was observed.

Loefberg and Tjaelve, 1985 examined urine and faeces of rats for radioactivity after treatment with a single intravenous dose (2.5 mg/kg bw) of radiolabeled 4-nitrosomorpholine. It was found that 8 h after injection 63.3 % of the 4-nitrosomorpholine dose was excreted with the urine and 2.6 % with the faeces. These results are in line with the findings by Maduagwu et al. 1983 and indicate that 4-nitrosomorpholine is metabolised. However, it was not distinguished in the study if radioactivity was originating from metabolites or non-metabolised 4-nitrosomorpholine. The authors further investigated the exhaled radiolabeled CO_2 over 8 h after single dosing with radiolabeled 4-nitrosomorpholine. 4.7 % of the administered dose was exhaled as CO_2 within 8 h after injection which also indicates metabolism of 4-nitrosomorpholine. Interestingly, about half of this amount was already exhaled during the first hour. Altogether, 8 hours after injection of a single intravenous dose of 4-nitrosomorpholine in rats about 70 % of the dose was eliminated with the urine, faeces and the exhaled CO_2.

The fast elimination of a single dose of 4-nitrosomorpholine was also observed by Stewart et al., 1974 after injection of a high single dose of 400 mg/kg bw radiolabeled 4-nitrosomorpholine in rats. Within 30 h after injection 81 % of the dose was excreted in the urine. They also found a quite low rate of elimination via the exhaled CO_2. Within 30 h after injection only 3.3 % of the radioactivity injected was exhaled as CO_2.

Hecht and Young, 1981, who determined urinary metabolites in 4-nitrosomorpholine treated rats found that only 1.5 % of the 4-nitrosomorpholine dose was excreted with the urine as non-metabolised 4-nitrosomorpholine via GLC-MS analysis (Hecht and Young, 1981). This is in contrast to Manson et al., 1978 and Stewart et al., 1974 who detected higher levels of non-metabolised 4-nitrosomorpholine in the urine of treated rats. But the results of Hecht and Young, 1981 using GLC-MS are considered to be more unambiguous compared to Manson et al., 1978 and Stewart et al., 1974 using TLC as chemical analysis techniques.

Altogether, data on elimination of 4-nitrosomorpholine can be taken as indication that 4-nitrosomorpholine is metabolised to a high extent. Suggested metabolism pathways are discussed in the following.

Metabolism

Three metabolites of 4-nitrosomorpholine namely nitroso(2-hydroxyethyl)glycin (33 % of dose), (2-hydroxyethoxy)acetic acid (16 % of dose) and nitrosodiethanolamine (12 % of dose) (Figure 1 and 2) were identified by GLC-MS *in vivo* in the urine of male rats which were treated with an intraperitoneal single dose of 4-nitrosomorpholine (Hecht and Young, 1981). The supposed metabolism pathways to obtain these three metabolites are shown in the following figures (Figure 1 and 2).

Nitroso(2-hydroxyethyl)glycine is supposed to originate from *β-hydroxylation* of 4-nitrosomorpholine via formation of nitroso-2-hydroxymorpholine as an intermediate (Figure 1) (Hecht and Young, 1981, Loeppky et al., 2005). The intermediate nitroso-2-hydroxymorpholine was found *in vitro* in metabolism studies using isolated rat liver microsomes treated with radiolabeled 4-nitrosomorpholine (Manson et al., 1978, Jarman and Manson, 1986).

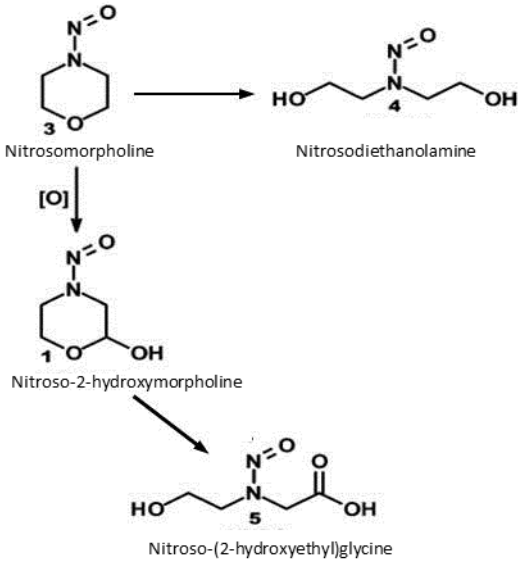


Figure 1: Figure modified from Loeppky et al., 2005 (Scheme 1): Supposed metabolism pathways of 4-nitrosomorpholine to either nitrosodiethanolamine or nitroso-2-hydroxyethylglycine by *β-hydroxylation* of 4-nitrosomorpholine via nitroso-2-hydroxymorpholine as intermediate.

(2-Hydroxyethoxy)acetic acid is supposed to originate from *α-hydroxylation* of 4-nitrosomorpholine via 3-hydroxy-N-nitrosomorpholine and (2-hydroxethoxy)acetaldehyde as intermediates (Hecht and Young, 1981, Koissi et al, 2012, Koissi and Fishbein, 2013, Kim and Fishbein, 2003) (Figure 2). Whereas the (2-hydroxethoxy)acetaldehyde was detected in an *in vitro* metabolism study using isolated rat liver microsomes treated with radiolabeled 4-nitrosomorpholine (Hecht and Young, 1981), the presumed intermediate α-hydroxynitrosamine (3-hydroxy-N-nitrosomorpholine) is instable and supposed to rapidly decompose to a highly reactive diazonium ion intermediate (Koissi and Fishbein, 2013, Koissi et al., 2012). This is suggested to be capable of alkylating DNA (Koissi and Fishbein, 2013).

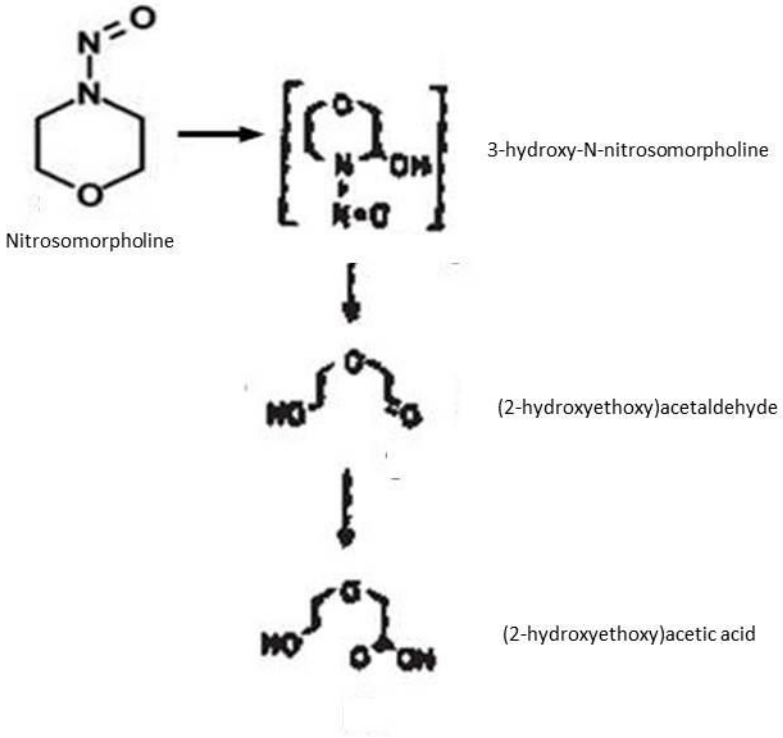


Figure 2: Figure modified from Hecht and Young et al., 1981 (Chart 1): Supposed metabolism of 4-nitrosomorpholine by *α-hydroxylation* to (2-hydroxyethoxy)acetic acid via the unstable 3-hydroxy-N-nitrosomorpholine and (2-hydroxethoxy)acetaldehyde as intermediates.

In a study by Löfberg and Tjälve, 1985 metabolism rates of various rat tissues *in vitro* for 4-nitrosomorpholine were studied by investigation of the formation rate of radiolabeled CO_2 by each tissue treated with [3,4 -^{14}C]- radiolabeled 4-nitrosomorpholine. Pieces of nasal olfactory mucosa, liver, oesophagus, kidney cortex, lung and trachea were excised from non-treated rats and incubated with 4-nitrosomorpholine. Statistically significant increased production of radiolabeled CO_2 compared to the control (boiled liver) was found for the nasal olfactory mucosa, liver and the oesophagus. The highest metabolism rate was detected for the nasal olfactory mucosa. The results indicate that the nasal olfactory mucosa, the liver and the oesophagus are the major organs for 4-nitrosomorpholine metabolism and are in line with the high radioactivity detected in these organs after treatment of rats *in vivo* with radiolabelled 4-nitrosomorpholine (Löfberg and Tjälve, 1985). Production of radiolabelled CO_2 *in vitro* in liver tissue was reduced if metyrapone was added and highly reduced under carbon monoxide atmosphere. According to the authors, it can be suggested that 4-nitrosomorpholine metabolism might be cytochrome P450 dependent.

4.2 Acute toxicity

Not evaluated in the present dossier.

4.3 Specific target organ toxicity – single exposure (STOT SE)

Not evaluated in the present dossier.

4.4 Irritation

Not evaluated in the present dossier.

4.5 Corrosivity

Not evaluated in the present dossier.

4.6 Sensitisation

Not evaluated in the present dossier.

4.7 Repeated dose toxicity

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

Table 10 Summary table of relevant oral repeated dose toxicity studies

Method	Results	Remarks	Reference
Repeated dose toxicity study, 14 d, sub-acute, (no guideline followed) oral (gavage) Exposure: **14 days** (daily) rats (Crl:CD(SD)) male 5 animals per group **5, 10 and 30 mg/kg bw/d** (nominal gavage) Vehicle: water Controls: untreated animals **Parameters investigated:** clinical effects and mortality, body weights, gross pathology (liver), liver weight, histopathology of the liver	**Clinical effects and mortality:** *30 mg/kg bw/d:* - in 2/ 5 animals the stool volume was decreased - 1/5 animals showed emaciation **Body weights:** *30 mg/kg bw/d*: - significant decrease in mean body weights at day 4, 8 and 11 of about 30 % compared to controls **Gross pathology, weight and histopathology of liver:** *5 mg/kg bw/d:* - 5/5 animals minimal hypertrophy of centribular hepatocytes (controls 0/5) - in 3/5 animals minimal and 1/5 mild single cell necrosis in centrilobular hepatocytes (controls 0/5) *10 mg/kg bw/d:* - in 5/5 animals mild hypertrophy of centribular hepatocytes observed - in all 5/5 animals mild single cell necrosis in centrilobular hepatocytes *30 mg/kg bw/d*: - discoloration of liver in 4/5 animals - significant reduced absolute and relative mean liver weights compared to controls (mean absolute liver weights: controls: 11.86 g, 30 mg/kg bw/d: 6.91 g; mean relative liver weight: controls: 3.68 g per 100 g, 30 mg/kg bw/d: 2.96 g per 100 g) - in 5/5 animals mild hypertrophy of	**key study** **2 (reliable with restrictions)** **Rationale**: well documented study, Restrictions: no guideline followed, only male animals tested, exposure for only 14 days, data only on specific clinical parameters experimental result **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 %, commercial substance source	Hayashi A, Kosaka M, Kimura A, Wako Y, Kawasako K, Hamada S (2015)

	centribular hepatocytes observed - in 4/5 animals minimal anisokaryosis in hepatocytes - in 1/5 animal minimal, in 3/5 mild and 1/5 animal moderate proliferation of centrilobular oval cells - in 5/5 animals mild single cell necrosis in centrilobular hepatocytes - in 4/5 animals minimal diffuse inflammatory cell infiltration **LOAEL (14 days):** 5 mg/kg bw/d (nominal) (male) based on single cell necrosis in hepatocytes in 4/5 animals		
Repeated dose toxicity study, 20 to 50 weeks, sub-chronic to chronic, (**no guideline followed**) oral (drinking water) Exposure: **daily**, **50 weeks** (6 mg/kg bw/d, 30 animals); **37 weeks** (12 mg/kg bw/d, 25 animals); **20 weeks** (24 mg/kg bw/d, 11 animals) rat (Sprague-Dawley) male **6, 12, 24 mg/kg bw/d** (nominal in water) Vehicle: water **Parameters investigated:** Body weights, gross pathology (liver), histopathology of the liver, (no other clinical parameters or organs investigated)	**Body weights:** *6 mg/kg bw/d (50 weeks)* - 13 % lower mean body weight compared to controls *12 mg/kg bw/d (37 weeks)* - 19 % lower mean body weight compared to controls *24 mg/kg bw/d (20 weeks)* - 32 % lower mean body weight compared to controls **Gross pathology and histopathology of liver:** *6 mg/kg bw/d (50 weeks)* - 67 % of animals showed hepatocellular adenomas and 57 % hepatocellular carcinomas *12 mg/kg bw/d (37 weeks)* - 76 % of animals showed hepatocellular adenomas and 56 % hepatocellular carcinomas *24 mg/kg bw/d (20 weeks)* - numerous single cell necrosis - acinocentral loss of glycogen - occurrence of megalocytes - bile ductular proliferations - fibrosis - at weeks 15 and 20 severe cirrhosis - cholangiofibrosis, cholangiomas and multiple hepatocyte nodules - after 20 weeks: 64 % of animals showed hepatocellular carcinoma **LOAEL: (50 weeks)** ≤ 6 mg/kg bw/d	**supporting study** **2 (reliable with restrictions)** **Rationale:** no guideline followed, no data on substance purity, non-commercial substance source, analysis of only very specific clinical effects experimental result **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	Weber E, Bannasch P (1994a)
Repeated dose toxicity study, 30 weeks, sub-chronic (no guideline followed)	**Survival:** 0.3 mg/kg bw/d - 100 % at about 70 weeks - 50 % at about 100 weeks	**supporting study** **2 (reliable with restrictions)** Rationale: no control	Lijinsky W, Taylor HW, Keefer LK (1976)

oral: drinking water Exposure: 5 days a week for **30 weeks** Observation: whole life span rat (Sprague-Dawley) male 30 animals/ dose group (3/cage) **0.3 and 1.5 mg/kg bw/d** (nominal in water) Vehicle: water **Parameters investigated:** Survival, gross pathology, histopathology of major organs (no other effect investigated)	- 10 % at about 110 weeks 1.5 mg/kg bw/d - 100 % at about 10 weeks - 50 % at about 80 weeks - 10 % at about 100 weeks **Gross pathology:** *0.3 and 1.5mg/kg bw/d:* - in all livers white foci (1mm-1cm size) scattered throughout parenchyma in all lobes and replaced 50-90 % of normal liver - occasional small biliary-retention cysts and telangiectasia - discrete areas of scarring and fibrosis - biliary hyperplasia with ductal hyperplasia **Histopathology of liver (non-neoplastic effects)** *0.3 and 1.5 mg/kg bw/d:* - extensive but focal postnecrotic cirrhosis (most livers of both groups) - cysts - telangiectatic sinuses - vascular channels occasionally filled with thrombi and leukocytic debris	animals included, only two dose levels tested, result documentation restricted to liver, results reported only cumulatively for both dose levels (no documentation of results separately for the two dose groups) **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	
Repeated dose toxicity study, 30 weeks, sub-chronic (no guideline followed) oral: drinking water Exposure: 5 days a week for **30 weeks** Observation: whole life span rat (Sprague-Dawley) male 30 animals **1.4 mg/kg bw/d** (nominal in water) Vehicle: water **Parameters investigated:** Survival, gross pathology, histopathology of major organs (results reported for liver only), no other effect parameters investigated)	**Survival:** - 100 % at week 10 - 87.7 % at week 50 - 46.7 % at week 80 - 6.6 % at week 100 **Gross pathology and histopathology of liver** (non-neoplastic effects): - necrosis - massive scarring - biliary hyperplasia - telangiectasis	**supporting study** **2 (reliable with restrictions)** **Rationale:** no control animals, only one dose level tested, only male animals, restriction of non-neoplastic result description to liver, investigation of very few effect parameters **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	Lijinsky W, Taylor HW (1975)
Repeated dose toxicity study, 7 weeks, sub-acute (no guideline followed)	**Histopathology of adrenal cortex:** - focal lesions (eosinophilic cell foci and pale cell foci) in zona reticularis/fasciculata or the zona	**Supporting study** **2 (reliable with restrictions)** Rationale: no	Moore MA; Weber E; Mayer D; Bannasch P (1989)

oral: drinking water Exposure: **7 weeks** (daily) Observation time: 4, 20, 44 weeks rat (Sprague-Dawley) male 6.0* mg/kg bw/d (120 mg/L, nominal in water) Vehicle: water **Parameters investigated:** histopathology of adrenal cortex no other effect parameters investigated *dose was estimated with assumption of 20 mL/d/rat water consumption	glomerulosa developed earlier and at significantly higher levels compared to controls	standardised guideline followed, only one effect parameter investigated (histopathology of adrenal cortex), no survival and no body weights reported, no data on water consumption per day, effect analysis only after cessation of treatment (earliest 4 weeks), only one dose level, no data on purity of 4-nitrosomorpholine **Test material: 4-nitrosomorpholine** **Analytical purity**: no data, non-commercial source	

4.7.1.2 Repeated dose toxicity: inhalation

4.7.1.3 Repeated dose toxicity: dermal

4.7.1.4 Repeated dose toxicity: other routes

4.7.1.5 Human information

There is currently no information available.

4.7.1.6 Other relevant information

4.7.1.7 Summary and discussion of repeated dose toxicity

There are no oral, inhalation or dermal-repeated dose toxicity studies available for 4-nitrosomorpholine which were performed according or equivalent/similar to a standardised guideline. Most of the available studies for 4-nitrosomorpholine with repeated dose administration were performed to investigate carcinogenic or genotoxic effects (see section 4.9 and 4.10). However, in none of these studies a comprehensive investigation of clinical effects, gross pathology or histopathology (non-neoplastic effects) was performed and in most cases effect analysis was restricted to the liver.

Hayashi et al., 2015 investigated the sub-acute toxicity of 4-nitrosomorpholine in a 14-d drinking water study in male Sprague-Dawley rats. The study was intended as a dose-range finding study for a micronucleus test and next to the body weights only gross pathological and histopathological effects of the liver were investigated. Nevertheless, the results of the study are considered reliable with restrictions. Three dose levels (5, 10 and 30 mg/kg bw/d) of 4-nitrosomorpholine and 5 rats per dose group were tested. At 5 and 10 mg/kg bw/d all animals showed minimal hypertrophy of the centribular hepatocytes. Moreover minimal and mild single cell necrosis in the centribular

hepatocytes were observed in most animals. These effects are considered to be treatment related as they became more prominent in animals treated with 30 mg/kg bw/d and were observed in a dose-dependent manner. At 30 mg/kg bw/d, also several adverse clinical and pathological effects were found. These included significant decreased body weights of about 30 % compared to controls, with one animal suffering from emaciation, and reduced absolute and relative liver weights. Moreover, for 5/5 animals minimal to moderate proliferation of the centrilobular oval cells and minimal diffuse inflammatory were observed in the liver at this dose level. The observed hepatic lesions indicate that 4-nitrosomorpholine is a hepatotoxicant. From the results a LOAEL of 5 mg/kg bw/d was derived. Lower doses have not been tested in the study.

Three studies related to carcinogenicity in rats (Weber and Bannasch, 1994, Lijinsky et al., 1976 and Lijinsky and Taylor, 1975) contain some hints on repeated dose toxicity (non-neoplastic effects) of 4-nitrosomorpholine. From the studies it can be concluded that 4-nitrosomorpholine, in addition to the observed neoplastic/tumorigenic effects as described in section 4.10, causes liver toxicity. Severe and extensive cirrhosis of the liver was found after daily oral treatment of rats with 24 mg/kg bw/d 4-nitrosomorpholine for 20 weeks (Weber and Bannasch, 1994) and already at a dose of 0.3 mg/kg bw/d after 30 weeks of oral treatment of rats (5 days a week) (Lijinsky et al., 1976). Lijinsky and Taylor, 1975 further reported necrosis and massive scarring in the liver after oral treatment of rats for 30 weeks (5 days a week) with a dose of 1.4 mg/kg bw/d 4-nitrosomorpholine. In the studies by Lijinsky et al., 1976 and Lijinsky and Taylor, 1975 a reduced survival rate of the rats after repeated oral dosing for 30 weeks was found. Reduced survival after repeated 4-nitrosomorpholine administration is also discussed in section 4.10.

In a sub-acute study specifically related to effects to the adrenal cortex Moore et al., 1989 found focal lesions in the zona reticularis/fasciculata and zona glomerulosa at significantly higher levels compared to controls after oral treatment of rats for 7 weeks with a daily dose of 6 mg/kg bw/d 4-nitrosomorpholine.

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

4.8.1 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

Even though there are no repeated toxicity studies available for 4-nitrosomorpholine which were entirely performed according or equivalent/similar to a standardised guideline, in some available repeated dose toxicity studies the effects of 4-nitrosomorpholine to the rat liver have intensively been investigated (Hayashi et al., 2015; Weber and Bannasch, 1994; Lijinsky et al., 1976; Lijinsky and Taylor, 1975). Treatment of rats orally with low doses of about 0.3 and 1.5 mg/kg bw/d, respectively for 30 weeks resulted in adverse liver lesions such as an extensive postnecrotic cirrhosis, scarring, cysts and telangiectasis next to prominent neoplastic and preneoplastic effects (Lijinsky et al., 1976, Lijinsky and Taylor, 1975). Treatment related single cell necrosis in centribular hepatocytes were detected in 80 % of rats orally treated for only 14 days with a 4-nitrosomorpholine dose of 5 mg/kg bw/d (Hayashi et al., 2015). The 14-d treatment of rats with 30 mg/kg bw/d lead to cell necrosis in hepatocytes in all treated animals, to diffuse inflammatory cell infiltration in 80 % of the treated animals and also to significant reduced mean absolute and relative liver weights compared to controls (Hayashi et al., 2015). Based on the findings in this study a LOAEL (14 days) of 5 mg/kg bw/d (nominal) (male) for liver toxicity could be derived. At higher doses (up to 24 mg/kg bw/d) and after a longer treatment time (20 weeks) Weber and Bannasch, 1994 observed an acinocentral loss of glycogen, the occurrence of megalocytes, fibrosis and severe cirrhosis in the liver in orally treated rats. The liver has been identified as target organ for carcinogenicity in 4-nitrosomorpholine treated rats as described in section 4.10. In the sub-chronic to chronic studies described above, observed non-neoplastic effects in the liver occurred in parallel to preneoplastic and neoplastic effects (Weber and Bannasch, 1994; Lijinsky et al., 1976; Lijinsky and Taylor, 1975). Based on the weight of evidence from several although not fully guideline-compliant studies it can be concluded that 4-nitrosomorpholine is hepatotoxic already at low doses and after short treatment times in rats.

4.8.2 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

According to the CLP Regulation substances are classified as specific target organ toxicants (STOT) following repeated exposure by the use of expert judgement, on the basis of weight of all evidence available, including the use of recommended guidance values.

In the Guidance on the Application of the CLP Criteria (section 3.9.2) it is recommended that the most appropriate animal data on repeated dose toxicity for use in hazard characterisation are primarily obtained from studies conforming to internationally agreed test guidelines. However, studies not conforming to conventionally agreed test guidelines are considered also to provide relevant information for this endpoint and, if evaluated on a case by case basis by expert judgement, could be used in the context of a total weight of evidence assessment for STOT RE classification. For 4-nitrosomorpholine there are no repeated toxicity studies available which were fully compliant with a standardised guideline. Nevertheless, the repeated dose toxicity studies published by Hayashi et al., 2015; Weber and Bannasch, 1994; Lijinsky et al., 1976; Lijinsky and Taylor, 1975 provide useful information on liver toxicity of 4-nitrosomorpholine and are considered to be useful in a weight of evidence assessment for STOT RE classification of 4-nitrosomorpholine.

Classification Criteria for specific target organ toxicity- repeated exposure are as follows:

‘Category 1: Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of:

- Reliable and good quality evidence from human cases or epidemiological studies; or
- Observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations.’

There exists no reliable and good quality evidence from human cases or epidemiological studies for 4-nitrosomorpholine.

But there are appropriate animal studies available which, in a weight of evidence, clearly show that 4-nitrosomorpholine is a hepatotoxicant after oral treatment of rats. Toxic effects to the liver included single cell necrosis in centribular hepatocytes, diffuse inflammatory cell infiltration, an acinocentral loss of glycogen, occurrence of megalocytes, cysts, telangiectasis, scarring, fibrosis, significant reduced mean absolute and relative liver weights and postnecrotic cirrhosis. These effects are considered to be relevant for human health and are in line with effects described in Section 3.9.2.7.3 d, e and f (CLP Regulation) supporting classification.

Guidance values to assist in Category 1 classification are summarised in Table 3.9.2 (CLP Regulation). Effects observed at a dose level of ≤ 10 mg/kg bw/d after oral treatment in a 90-day repeated-dose study normally justify classification in Category 1. Equivalent guidance values other than that for 90-day studies can be established by expert judgement and by application of Haber's rule according to section 3.9.2.9.5. of the CLP Regulation.

The very severe toxic liver effect of postnecrotic cirrhosis was observed in orally treated rats at a dose level of 0.3 mg/kg bw/d after 30 weeks (approximately 210 days) of 4-nitrosomorpholine treatment (Lijinsky et al., 1976). Using the Haber`s rule for a 210-day study a general equivalent guidance value of about 4.3 mg/kg bw/d can be established from Table 3.9.2 (CLP Regulation) warranting Category 1 classification. The observed liver effects at a dose level of 0.3 mg/kg bw/d occurred one magnitude lower than this established guidance value justifying a Category 1 classification. This is supported by the LOAEL of 5 mg/kg bw/d (nominal) (male) for liver toxicity derived from the oral 14-day repeated dose study (Hayashi et al. 2015). Using the Haber`s rule for an oral 14-day study a general equivalent guidance value of about 60 mg/kg bw/d can be established warranting Category 1 classification. The established LOAEL of 5 mg/kg bw/d is 12-times lower than this guidance value.

From the results of the studies by Lijinsky et al., 1976 and Hayashi et al., 2015 it can be concluded that effects to the liver after oral treatment were produced at low exposure concentrations relevant for Category 1 classification. Hence, for 4-nitrosomorpholine the classification as STOT RE 1 H372 (Causes damage to organs (liver) through prolonged or repeated exposure) is justified.

There are no dermal or inhalation repeated-dose toxicity studies available for 4-nitrosomorpholine. Hence, not all relevant routes of exposure by which 4-nitrosomorpholine is hepatotoxic can be identified. It cannot be proven that no other routes than oral cause the hazard. Information on exposure route is not included in this STOT RE classification.

4.8.3 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

Based on the comparison of the available repeated-dose toxicity data for 4-nitrosomorpholine with the criteria laid down in the CLP Regulation it is justified to classify 4-nitrosomorpholine as **STOT RE 1 H372 (Causes damage to organs (liver) through prolonged or repeated exposure)**.

4.9 Germ cell mutagenicity (Mutagenicity)

4.9.1 Non-human information

4.9.1.1 *In vitro* data

The results of the relevant *in vitro* genotoxicity studies are summarised in Table 11.

Table 11 Summary table of *in vitro* mutagenicity studies

Method	Results	Remarks	Reference
***in vitro* mammalian cell micronucleus test** (aneugenic and clastogenic effects) **(no guideline followed)** HuFoe-15 cells (rat liver and human fetus) (met. act.: without) IEC-18 cells (rat, digestive tract) (met. act.: without) IEC-17 cells (rat, digestive tract) (met. act.: without) V79 (Chinese hamster lung fibroblasts) (met. act.: without) **0.1, 0.3, 1, 3, 10, 30 and 100 µg/mL** Positive control substance: benzo(a)pyrene Vehicle/Negative controls: yes Vehicle: DMSO or acetone Number of cells scored: 2000 Cytotoxicity measured: no **Additional information on method:** 4-nitrosomorpholine added to the cells after 18 h of incubation, termination after 24 h, harvesting of cells: 24 h for V79 cells, 48h for IEC-17 and IEC-18 and 60h for HuFoe-15 cells after termination of exposure	**Evaluation of results:** **positive** in HuFoe-15, IEC-18, IEC-17 cells (without met. act.) **negative** in V79 cells without met. act. **Test results:** **4-nitrosomorpholine:** - Positive for HuFoe-15 cells without met. act : up to 4 fold increase of micronucleated cells compared to controls; cytotoxicity: not determined ; vehicle controls valid; positive controls valid - Positive for IEC-18 cells without met. act.: up to 2 fold increase of micronucleated cells compared to controls; cytotoxicity: not determined; vehicle controls valid; positive controls valid - Positive for IEC-17 cells without met. act.: up to about 2.5 fold increase of micronucleated cells compared to controls; cytotoxicity: not determined; vehicle controls valid; positive controls valid) -for all positive results significant positive trends in concentration-dependence were obtained - Negative for Chinese hamster lung fibroblasts (V79) without met. act.; cytotoxicity: not determined; vehicle controls valid; positive controls valid	**disregarded study** **3 (not reliable)** **Rationale:** performance of test in non-standard cell cultures (except for V79), tests performed without metabolic activation system, no use of cytoB: no data on cytotoxicity (such as RPD or RICC), no data on historical controls, no data on source and analytical purity of 4-nitrosomorpholine, no adequate positive control **Test material: N-4-nitrosomorpholine** **Analytical purity and source:** no data	Glatt H, Gemperlein I, Setiabudi F, Platt KL, Oesch F (1990)
***in vitro* mammalian cell micronucleus test** (aneugenic and clastogenic effects) **(no guideline followed)** primary hepatocytes: rat (met. act.: without) **6 concentrations between 10E-6 and 10E-4 M** Positive control substance(s): benzo(a)pyrene; cyclophosphamide Vehicle/Negative controls: included Vehicle: DMSO (only for positive controls) Number of cells scored: 8000 from	Evaluation of results: **positive (**without met. act.) (cells supposed to be metabolically competent) Test results (data presented in figure only): **positive** without met. act.; - Mitotic index: about 50 % of controls at highest concentration (10E-4M) - concentration-dependent increase of micronucleated cells, up to 3 fold compared to controls -cytotoxicity: yes; vehicle controls valid; positive controls: not valid	**disregarded study** **3 (not reliable)** **Rationale:** non-standard cell culture, invalid results for positive control, tests performed without metabolic activation system, no data on historical controls **Test material: 4-nitrosomorpholine** **Analytical purity**: no data, commercial substance source	Mueller-Tegethoff K, Kasper P, Mueller L (1995)

two replicates Cytotoxicity measured: yes (mitotic index) **Additional information on method:** cells treated for 4 hours and harvested after a 72 h incubation period.			
***in vitro* mammalian cell micronucleus test** (aneugenic and clastogenic effects) **(no guideline followed)** hepatocytes: primary, rat (met. act.: without) **0.116 mg/mL** Positive control substance(s): benzo(a)pyrene (5 µg/mL) Negative controls: included Vehicle: DMSO (only for positive controls) Number of cells scored: 1000 from two replicates Cytotoxicity measured: no **Additional information on method:** expression time was 48 h, 3 h before end of cultivation colchicine added, 3 independent experiments	**Evaluation of results:** **positive** (without met. act.) (cells might be metabolically competent) **Test results:** - positive without metabolic activation: significant increase of micronuclei of about 2.5 fold compared to controls in all three independent experiments - cytotoxicity: not determined; negative controls valid; positive control: not adequate (needs metabolic activation)	**disregarded study** **3 (not reliable)** **Rationale**: Non-standard cell culture, no adequate positive control, only one dose level tested, test performed without metabolic activation system, cytotoxicity not investigated, only 1000 cells scored, no data on historical controls **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Slamenová D, Chalupa I, Robichová S, Gábelov A, Farkašová T, Hrušovská (2002)
***in vitro* mammalian chromosome aberration (no guideline followed)** hepatocytes: primary, rat (met. act.: without) **4-nitrosomorpholine: 0.116 mg/mL** Positive control substance(s): benzo(a)pyrene (5 µg/mL) Negative controls: included Vehicle: MEM diluted with PBS Number of metaphases scored: 200 Cytotoxicity measured: no **Additional information on method:** 3 h exposure time, harvesting after 48 h, 3 h before harvesting colchicine added, metaphases analysed for chromatid gaps and breaks, isochromatid gaps and breaks and exchanges, 3 independent experiments performed	**Evaluation of results:** **positive** (without met. act.) (cells might be metabolically competent) **Test results:** - positive without met act.: significant increase of chromosomal aberrations compared to controls (3 to 5 fold) in three independent experiments; cytotoxicity: not determined; negative controls valid; positive controls not valid (not adequate (needs met. act.)) (Positive control: Benzo(a)pyrene: significant increase (2-fold) only in one out of three independent experiments: result considered as ambiguous) .	**disregarded study** **3 (not reliable)** **Rationale:** Non-standard cell culture, no adequate positive control, only one dose level, without metabolic activation system, cytotoxicity not investigated, only 200 metaphases scored **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Slamenová D, Chalupa I, Robichová S, Gábelov A, Farkašová T, Hrušovská (2002)

***in vitro* mammalian chromosome aberration test (no guideline followed)** primary human VH10 cells fibroblasts(met. act.: without) Chinese hamster lung fibroblasts (V79) (met. act.: without) human HepG2 hepatoma cells(met. act.: without) **0.125, 0.25, 0.5, 1 and 2 mmol/L** (for HepG2 cells (exposure for 0.5 h)**:** 0.5, 1, 2, 5, 10, 20, 26 mmol/L)) Positive control substance(s): no Negative /vehicle controls: yes Vehicle: PBS buffer Number of metaphases scored: 100 Cytotoxicity measured: no **Additional information on method:** preincubation period 26 h, exposure: 43 h and 0.5 h for HepG2, 23 h for V79 cells and 41 h forVH10 cells (in second experiment with HepG2 cells 0.5 h exposure), cells harvested 3 h after adding of colchicine	Evaluation of results: **Positive** in standard cell line and human non-standard hepatoma cells (without met. act.) **Negative** in primary human cell culture (without met. act.) Test results: **VH10 cells: negative** without met. act.; cytotoxicity: not determined; vehicle controls valid; no positive control **V79 cells: positive** without met. act.: clear concentration-dependent increase of chromosomal aberrations up to about 7-fold (highest concentration) compared to controls, increase was significant compared to controls at 0.25, 0.5 and 1 mmol/L; cytotoxicity: not determined ; vehicle controls valid; no positive control **HepG2 cells:** **43 h exposure: positive** without met. act.: clear concentration dependent increase of chromosomal aberrations up to about 6-fold compared to controls (except at highest concentrations, here only 13 metaphases scored) , increase was significant increased compared to controls at 0.25, 0.5, 1 and 2 mmol/L; cytotoxicity: not determined; vehicle controls valid; no positive controls **0.5 h exposure**: positive without met. act.: clear concentration dependent increase of chromosomal aberrations up to about 30-fold compared to controls, increase was significant increased compared to controls at 10, 20 and 26 mmol/L; cytotoxicity: not determined; vehicle controls valid; no positive control)	**disregarded study** **3 (not reliable)** Rationale: no positive control, no data on substance purity, substance source non-commercial, only 100 metaphases scored, no metabolic activation system, no data on cytotoxicity, HepG2 and VH10 cells are not considered as standard cell cultures **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial source	Robichova S, Slamenova D, Chalupa I, Sebov L (2004)
bacterial reverse mutation assay, Ames test) similar to OECD TG 471 S. typhimurium TA 1535 (met. act.: with and without) Test concentrations: 10 concentrations: 5, 10, 25, 50, 100, 250, 500, 1000 µg Positive control substance(s): no Negative/vehicle control: yes Vehicle: DMSO	**Evaluation of results:** **positive (**with met. act.) **negative (**without met. act.) **Test results:** positive with met. act.: concentration-dependent increase in revertants; cytotoxicity: not determined; vehicle controls valid; no positive controls negative without met. act.**;**	**disregarded study** **3 (not reliable)** Rationale: no positive controls, only one strain tested **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 % (non-commercial)	Andrews AW, Lijinsky W (1980)

Cytotoxicity measured: no	cytotoxicity: not determined; vehicle controls valid; no positive controls		
bacterial reverse mutation assay (Ames test) (no guideline followed) S.typhimurium TA1535, TA1536, TA1537, TA1538 (met. act.: with and without) Test concentrations: **Experiment 1** (TA1535, TA1536, TA1537, TA1538): 1000 µmol/ plate **Experiment 2** (TA1535): 7 concentrations between 1 and 1000 µmol/plate Positive control substance(s): no Negative control: yes Vehicle: no data	**Evaluation of results:** **Positive** (with met. act.) **Test results:** **Experiment 1:** **positive** for TA 1535 with met. act.; cytotoxicity: no; negative controls valid; no positive control (base-pair substitution principle) **negative** for TA 1535; without met. act.; cytotoxicity: no; negative controls valid; no positive control (base-pair substitution principle) **negative** for TA1536, TA1537, TA1538 with and without met. act.; cytotoxicity: no; negative controls valid; no positive control (frameshift mutation principle) **Experiment 2:** **positive** for TA 1535 with met. act**.,** concentration dependent linear increase in revertants	**disregarded study** **3 (not reliable)** **Rationale:** no positive controls, TA 1536 and TA 1538 non-standards strains, S9 system obtained from untreated rats, no detailed data on experimental conditions, concentration - dependence studied only for one experimental condition (strain TA1535 with metabolic activation), no vehicle controls, no data on source or analytical purity of 4-nitrosomorpholine **Test material: 4-nitrosomorpholine** **Analytical purity and source**: no data	Gomez RF, Johnston M, Sinskey AJ (1974)
bacterial reverse mutation assay (Ames test) similar to OECD TG 471 S. typhimurium TA 1535 (met. act.: with and without) with metabolic activation: **0.01, 0.05, 0.1, 0.5, 1.0 µmol/plate** without metabolic activation: **1.0 µmol/plate** Positive control substance(s): no Negative/vehicle control: yes Vehicle: phosphate buffer Cytotoxicity measured: no	**Evaluation of results:** **Positive** (with met. act.) Test results: **positive** with met. act.: concentration-dependent increase of revertants up to 26 fold compared to control; cytotoxicity: not determined; negative controls valid; no positive controls **negative** TA 1535 without met. act.; cytotoxicity: not determined; negative controls valid, no positive controls	**disregarded study** **3 (not reliable)** **Rationale:** no positive controls, only one strain of S. typhimurium used (TA-1535), S9 from untreated rats, only one concentration tested without metabolic activation **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Zeiger E, Sheldon AT (1978)

bacterial reverse mutation assay (Ames test) similar to OECD TG 471 S. typhimurium TA 100 (met. act.: with and without) S. typhimurium TA 98 (met. act.: with and without) TA100: **33, 100, 333, 1000, 1666, 3333, 6666 µg/plate** TA98: **100, 333, 1000, 3333, 6666 and 10000** µg/plate Positive control substance(s): Congo red Negative/ vehicle control: yes Vehicle: water	**Evaluation of results:** **Positive** (with met. act.) **Negative** (without met. act.) **Test results:** **positive** for TA 100 with met. act.: concentration-dependent increase of revertants obtained (see table 1), number of revertants in the highest tested concentration more than 8 fold compared to vehicle/negative control and more than double compared to positive control; vehicle controls valid; positive controls valid **negative** for TA 100 without met. act.; vehicle controls valid; positive controls valid **negative** for TA 98 with and without met. act., vehicle controls valid; positive controls valid	**key study** **2 (reliable with restrictions)** **Rationale:** Study equivalent to standardised guideline, restrictions: not according to GLP, only two Salmonella strains tested **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Zeiger E, Anderson B, Haworth S, Lawlor T, Mortelmans K (1992)
bacterial reverse mutation assay (Ames test) similar to OECD TG 471 S. typhimurium, other: TA 1530 (met. act.: with) S. typhimurium TA 100 (met. act.: with) **1, 2.5, 12.5 and 25 mM** Positive control substance(s): no Negative control: yes Vehicle: DMSO	**Evaluation of results:** **Positive (**with met. act.) **Test results:** **positive** for TA1530 with met. act.; cytotoxicity: no; vehicle controls valid: no data; no positive controls **positive** for TA100 with met. act., cytotoxicity: no; vehicle controls valid: no data; no positive controls - for both strains a concentration-dependent increase of revertants, at 25 mM about 1750 to 2000 revertants per plate	**disregarded study** **3 (not reliable)** **Rationale:** no positive controls, only two strains tested, TA1530 not considered a standard strain, no data on negative controls shown, testing only with using of metabolic activation, only four concentrations **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Khudoley V, Malaveille C, Bartsch H (1981)
***in vitro* gene mutation study in mammalian cells** (HPRT test**) similar to OECD TG 476** (*In vitro* Mammalian Cell Gene Mutation Test) Chinese hamster lung fibroblasts (V79) (met. act.: with and without) **10, 15 and 20 mmol/L** Positive control substance(s): N-ethyl-N-nitro-N-nitrosoguanidine; aflatoxin Negative control: yes (but no data) Vehicle: PBS buffer	**Evaluation of results:** **Positive** (with and without met. act.) **Test results:** **positive** with met. act. in two highest test concentrations, significant increase of 6-TG resistant mutations, cytotoxicity: yes (slight cytotoxic effects); negative controls valid; positive controls valid **positive** without met. act. in highest concentration; cytotoxicity: yes (slight cytotoxic effects); negative	**disregarded study** **3 (not reliable)** **Rationale:** 4-nitrosomorpholine source non-commercial and no analytical purity given **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	Robichova S; Slamenova D; Gabelova A; Sedlak J; Jakubikova J (2004)

Cytotoxicity measured: yes **Additional information on method:** exposure only 30 min; expression time 7 and 9 days	controls valid; positive controls valid		
bacterial reverse mutation assay (Ames test) similar to OECD TG 471 E. coli WP2 uvr A pKM 101 (met. act.: with and without) E. coli WP2 uvr A (met. act.: with and without) **8 concentrations between 10 and 10000 µg/plate** (results presented in figure only) Positive control substance(s): benzo(a)pyrene Vehicle control: yes Preincubation method	**Evaluation of results:** **positive** **(**with met. act.) **Test results:** - *positive* for E. coli WP2 uvr A pKM 101 with met. act.; vehicle controls valid; positive control valid - *negative* for E. coli WP2 uvr A pKM 101 without met. act., vehicle control: no data; positive control valid - *positive* for E. coli WP2 uvr A with met. act., vehicle controls valid; positive control valid - *negative* for E. coli WP2 uvr A without met. act; vehicle control: no data; positive controls valid	**key study** **2 (reliable with restrictions)** **Rationale:** similar to standardised guideline, not according to GLP only two standard strains tested **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, but collaborative study	Matsushima T, Takamoto Y, Shirai A, Sawamura M, Sugimura T (1981)
bacterial reverse mutation assay (Ames test) similar to OECD TG 471 S. typhimurium TA 1537 (met. act.: with and without) S. typhimurium TA 98 (met. act.: with and without) S. typhimurium TA 100 (met. act.: with and without) **0, 2000, 5000, 10000 µg/plate** Positive control substance(s): benzo(a)pyrene; 9,10 - dimethylanthracene; cyclophosphamide Negative controls: yes (vehicle) Vehicle: DMSO	**Evaluation of results:** **Positive (**with met. act.) **Test results:** - *positive* for TA 100 with met. act.; concentration dependent increase in revertants, more than 2-fold compared to controls at highest conc., vehicle controls valid; positive controls valid - *negative* for TA 100 without met. act.; vehicle controls valid, positive controls valid - *negative* for TA 98 with met. act., vehicle controls valid, positive controls valid for benz(a)pyrene - negative for TA 1537 with met. act.; vehicle controls valid; no data on positive controls - no data for results on TA98 and TA1537 without met. act.; no data for positive controls	**key study** **2 (reliable with restrictions)** **Rationale:** study similar to standardised guideline, restrictions: only three strains tested, positive control data not shown for the test strain TA1537 **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, but collaborative study	MacDonald DJ (1981)
bacterial reverse mutation assay (Ames test) similar to OECD TG 471 S. typhimurium TA 100 (met. act.: with and without) S. typhimurium TA 98 (met. act.: with	**Evaluation of results:** **Positive** (with and without met. act.) **Test results:** - positive for TA 100 with and without met. act.; vehicle controls:	**disregarded study** **3 (not reliable)** **Rationale:** data for positive controls not valid for TA98, only two strains tested, no detailed data on results	Ichinotsubo D, Mower H, Mandel M (1981)

and without) Test concentrations: no detailed data Positive control substance(s): benzo(a)pyrene; cyclophosphamide; 9,10-dimethylbenzanthracene Negative controls: no Vehicle: DMSO	no data; positive controls valid - positive for TA 98 with and without met. act.; vehicle controls: no data; positive controls not valid	are presented, data on negative controls not shown, no data on tested concentrations **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, but collaborative study	
bacterial reverse mutation assay (Ames test) similar to OECD TG 471 S. typhimurium TA 100 (met. act.: with and without) S. typhimurium TA 98 (met. act.: with and without) S. typhimurium TA 1535 (met. act.: with and without) S. typhimurium TA 1537 (met. act.: with and without)		**disregarded study** **4 (not assignable)** **Rationale:** detailed result data are missing (for 4-nitrosomorpholine results are presented only for TA1535, no negative controls are reported, positive control data only shown for TA100 and TA98) reliability assessment of the study not possible **Test material: 4-nitrosomorpholine** **Analytical purity:** no data	Rowland I, Severn B (1981)
bacterial reverse mutation assay (Ames test) similar to OECD TG 471 S. typhimurium, other: TA 1537, TA98, TA100 (met. act.: with and without) Test concentrations: four between 0 and 2000 µg/plate (no exact data as results shown in figure only) Positive control substance(s): benzo(a)pyrene;cyclophosphamide;9,10-dimethylanthracene Negative controls: yes Vehicle: DMSO preincubation method	**Evaluation of results:** **positive** (with met. act.) **Test results:** - *positive* for TA 100 with met. act., concentration dependent increase in revertants up to 3-fold compared to control, vehicle controls valid; positive controls valid - *negative* for TA 100 without met. act.; vehicle controls valid; positive controls valid - *negative* for TA 98 with and without met. act.; vehicle controls valid; positive controls valid - *negative* for TA 1537 with and without met. act.; vehicle controls valid; positive controls valid	**key study** **2 (reliable with restrictions)** **Rationale:** study similar to standardised guideline, negative controls valid, positive controls valid, restrictions: only three Salmonella strains tested, only four 4-nitrosomorpholine concentrations tested, no data on cytotoxicity, not according to GLP **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, but collaborative study	Nagao M,Takahashi Y (1981)
***in vitro* gene mutation study in mammalian cells**, Mouse lymphoma assay (MLA) using the Thymidine Kinase Gene, **similar to OECD TG 490**	**Evaluation of results:** **positive** (with met. act.) **negative**	**key study** **2 (reliable with restrictions)** **Rationale:** study	Jotz MM, Mitchell AD (1981)

mouse lymphoma L5178Y cells (met. act.: with and without) Test concentrations: **214.2, 329.6, 507, 780, 1200 µg/mL** Positive control substance(s): 3-methylcholanthrene Negative controls: yes Vehicle: 1 % DMSO	(without met. act.) **Test results:** **positive** with met. act.: clear concentration-dependent increase in the mutation frequency (MF) of L5179Y cells up to 2.5 fold compared to control at the highest concentration and above the Global Evaluation Factor (GEF) at the four highest concentrations; cytotoxicity: within acceptability criteria; solvent control: negative (MF within acceptability criteria); positive control: positive (MF within acceptability criteria) **negative** without met. act.; cytotoxicity: within acceptability criteria; positive control: positive (MF within acceptability criteria); solvent control: negative (MF within acceptability criteria)	similar to standardised guideline, standardised cell culture, valid positive and negative controls, cytotoxicity reported and > 10 %, 5 doses tested, concentration dependent increase in mutation frequency, restriction: study not according to GLP **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, substance source within collaborative study	

4.9.1.2 *In vivo* data

Table 12 Summary table of relevant *in vivo* mutagenicity studies

Method	Results	Remarks	Reference
Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test) intraperitoneal rat (albino random bred) male 10 animals **100 mg/kg bw** (nominal injected), two administrations (24 h interval) Positive control substance(s): 30 mg/kg cyclophosphamide Negative controls = Vehicle controls: yes Vehicle: distilled water	**Evaluation of results:** **positive** **Test results:** - genotoxicity: positive (0.77 % micronuclei) - toxicity: not examined - vehicle controls valid (0.16 % micronuclei, but no comparison to historical controls) - positive controls valid (2.2 % micronuclei)	**disregarded study** **3 (not reliable)** **Rationale:** only one dose level tested, no data on toxicity, no data on ratio of immature to total erythrocytes, only 2000 erythrocytes screened per sample, no data on substance purity **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Neresyan AK, Muradyan RE (2002)
Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test) intraperitoneal rat (Fischer 344) male at least 4 animals **180 mg/kg bw** (nominal injected) two administrations (24 h interval) Positive control substance(s): yes, cyclophosphamide (20 mg/kg, single oral administration via gavage, 56 rats) Vehicle controls: yes Vehicle: distilled water **Additional information on method:** bone marrow micronucleated polychromatic immature erythrocytes and micronucleated reticulocytes from peripheral blood examined after treatment, harvesting 24 h after final treatment	**Evaluation of results:** **positive** **Test results:** *bone marrow micronucleated polychromatic erythrocytes:* - 4-nitrosomorpholine: 0.63 % - negative control valid (0.14 %) - positive control valid (1.8 %) *micronucleated reticulocytes from peripheral blood:* - 4-nitrosomorpholine: 0.31 % - negative control valid (0.07 %) - positive control valid (0.8 %, mean from 78 rats)	**disregarded study** **3 (reliable with restrictions)** **Rationale:** one dose level tested only, no data on clinical effects and toxicity to bone marrow, only 2000 cells screened, only at least 4 animals per group (no detailed information on exact number) **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 % (commercial substance source)	Wakata A, Miyamae Y, Sato S, Suzuki T, Morita T, Asano N, Awogi T (1998)

Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test) intraperitoneal mouse (ddY) male 5 animals **125, 250, 500, 1000, 2000 mg/kg** bw (nominal injected) single administration Positive control substance(s): yes, 0.5 mg/kg mitomycin (no results are shown) Vehicle controls: yes Vehicle: saline **Additional information on method:** sampling already 18 h after treatment, screening of only 1000 bone marrow micronucleated polychromatic erythrocytes	**Evaluation of results:** **positive** **Test results:** - Percent of micronucleated polychromatic erythrocytes: dose [mg/kg] % significance 0: 0.06 125: 0.16 no 250: 0.36 yes 500: 0.94 yes 1000: 0.94 yes 2000: 0.54 yes trend analysis: 0.000 (highly significant) - significant dose-dependent increase of micronucleated polychromatic erythrocytes - vehicle controls valid (no data on historical controls) - data on positive control are not shown	**disregarded study** **3 (not reliable)** **Rationale:** no data on positive controls, no data on historical controls, only 1000 bone marrow micronucleated polychromatic erythrocytes scored per animal, sampling after single substance administration already 18 h after treatment, no data on toxic effects in bone marrow or clinical effects (test concentration very high) **Test material): 4-4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Morita T, Asano N, Awogi T, Sasaki YF, Sato S, Shimada H, Sutou S (1997)
Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test) intraperitoneal mouse (ddY) male 3 animals **250, 375, 500 mg/kg** bw (nominal injected) single administration Positive control substance(s): yes, 0.5 mg/kg mitomycin (no results are shown) Vehicle controls: yes Vehicle: saline **Additional information on method:** sampling 24 h after treatment, screening of 1000 bone marrow micronucleated polychromatic erythrocytes	**Evaluation of results:** **positive** **Test results:** - percent of micronucleated polychromatic erythrocytes: dose (mg/kg) % significance 0: 0.20 250: 0.33 no 375: 0.40 no 500: 1.13 yes - trend analysis: 0.000 (highly significant) - significant increase of micronuclei compared to controls at highest dose - vehicle controls valid (no data on historical controls) - data on positive control are not shown	**disregarded study** **3 (not reliable)** **Rationale:** no results for positive controls shown, only 1000 bone marrow micronucleated polychromatic erythrocytes scored per animal, no data on clinical effects and toxic effects in bone marrow, only three animals tested **Test material): 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Morita T, Asano N, Awogi T, Sasaki YF, Sato S, Shimada H, Sutou S (1997)

Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test) intraperitoneal mouse (ddY) male 3 animals **125, 250 mg/kg bw** (nominal injected) two administrations (24h interval) Positive control substance(s): yes, 0.5 mg/kg mitomycin (no results are shown) Negative controls: no, Vehicle controls: yes Vehicle: saline **Additional information on method:** sampling 18 h after last treatment, screening of 1000 bone marrow	**Evaluation of results:** **positive** **Test results:** - percent of micronucleated polychromatic erythrocytes: dose (mg/kg) % 0: 0.20 125: 0.53 250: 0.65 (no data on statistical significance) - significant increase of micronuclei compared to controls in highest test concentration - vehicle controls valid (no data on historical control) - data on positive control are not shown	**disregarded study** **3 (not reliable)** **Rationale:** no results for positive controls shown, only 1000 bone marrow micronucleated polychromatic erythrocytes scored per animal, no data on toxic effects in bone marrow, only three animals tested, no data on clinical effects, no data on historical controls **Test material): 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Morita T, Asano N, Awogi T, Sasaki YF, Sato S, Shimada H, Sutou S (1997)
Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test) intraperitoneal mouse (ddY) male 5 animals **31, 63, 125 mg/kg** bw (nominal injected) two administrations (24 h interval) Positive control substance(s): yes, 0.5 mg/kg mitomycin (no results are shown) Vehicle controls: yes Vehicle: saline **Additional information on method:** sampling 24 h after last treatment, screening of 1000 bone marrow micronucleated polychromatic erythrocytes	**Evaluation of results:** **negative** **Test results:** - percent of micronucleated polychromatic erythrocytes: dose (mg/kg) % significance 0: 0.24 31: 0.24 no 63: 0.30 no 125: 0.26 no - trend analysis: 0.3352 (not significant) - no increase in micronuclei compared to control - vehicle controls valid (but no data on historical controls) - data on positive control are not shown	**disregarded study** **3 (not reliable)** **Rationale:** no results for positive controls shown, only 1000 bone marrow micronucleated polychromatic erythrocytes scored per animal, no data on toxic effects in bone marrow, no data on clinical effects, no data on historical controls **Test material): 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Morita T, Asano N, Awogi T, Sasaki YF, Sato S, Shimada H, Sutou S (1997)
Micronucleus assay similar to OECD TG **474** (Mammalian Erythrocyte Micronucleus Test) intraperitoneal	Evaluation of results: **negative** Test results: - at all three dose levels in males	**disregarded study** **3 (not reliable)** **Rationale:** no positive control, sampling 6 h after final treatment,	Tsuchimoto T, Matter BE (1981)

mouse (CD-1) male/female **0, 8, 16, 32 mg/kg bw (**nominal injected) 2 animals per dose and sex Number of treatments: 2 (24 h apart) Time of sampling: 6 h after final treatment Negative control : yes Positive control substance(s): no **Number of immature erythrocytes scored per animal: 1500**	and females no increase of micronuclei in immature erythrocytes compared to controls - *Toxicity:* no ratio of total immature to total erythrocytes given (highest dose tested is 50 % of LD50) - vehicle control: valid - positive control: not examined	scoring of only 1500 immature erythrocytes per animal, only animals per group, no data on bone marrow toxicity, no data on clinical effects **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	
Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test) intraperitoneal mouse (ICR) male 4 animals per dose **0,8,16, 32 mg/kg bw** (nominal injected) Number of treatments: 2 (at 0 and 24 h) Time of sampling: after 6 and 24 h after final treatment Vehicle control: yes Positive control substance(s): yes, trimethylphosphate (TMP) **Number of immature erythrocytes scored per animal: 1000**	Evaluation of results: **negative** Test results: - at all three dose levels no increase of micronuclei in immature erythrocytes compared to controls - *Toxicity:* no ratio of total immature to total erythrocytes given (highest dose tested is 50 % of LD50) - vehicle control: valid - positive control: valid	**disregarded study** **3 (not reliable)** **Rationale:** only 1000 immature erythrocytes scored per animal, only 4 animals per group, no data on clinical effects and effects on bone marrow, no criteria given for dose selection **Test material (common name): 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Kirkhart B (1981)
Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test), many deviations intraperitoneal mouse (B6C3F1) 5 animals per group **80 % of LD50 and 40 % of LD50 (no exact data)** Number of treatments: experiment 1: 2 (24 h apart), experiment 2: single treatment	Evaluation of results: **ambiguous** Test results: *Genotoxicity:* negative in experiment 1 and positive and negative in experiment 2 *Toxicity:* no ratio of total to immature to total erythrocytes given (highest concentration is 80 % of LD50) vehicle control: no	**disregarded study** **3 (not reliable)** **Rationale:** no direct positive control (data on cyclophosphamide, included as one of 41 test substances: not valid), no negative/vehicle controls, only two dose levels tested, one dose above MTD, only 500 immature erythrocytes scored, sampling after two treatments not between 18 and 24h,	Salamone MF, Heddle JA, Katz M (1981)

Time of sampling: 48, 72, 96 h after final treatment (experiment 1) and 30, 48 and 72 h after single treatment vehicle control : no Positive control substance(s): no (cyclophosphamide was included as one of 41 test substances but with invalid results) **Number of immature erythrocytes scored per animal: 500**	positive control: no (data on cyclophosphamide not valid)	toxicity on bone marrow not reported **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	

Micronucleus assay similar to OECD TG 474 (Mammalian Erythrocyte Micronucleus Test) oral (gavage) rat (Crl:CD(SD)) male 5 animals per group **5, 10, 30 mg/kg bw** (nominal conc.) (dose selection related to observed clinical effects) 14 days (daily) Positive control substance(s): no Vehicle controls: yes Vehicle: water **Additional information on method:** sampling 24 h after treatment, screening of 2000 bone marrow	**Evaluation of results:** **negative** **Test results:** - no (significant) difference in number of micronucleated immature erythrocytes compared to control animals at all tested dose - statistically significant decrease in the proportion of immature erythrocytes at 30 mg/kg compared with controls of about 7 % **clinical effects:** - no animal died - body weights: significant decrease compared to control at 30 mg/kg - at 30 mg/kg significant decrease of absolute liver weight compared to controls - at 30 mg/kg decreased stool volume in 2 of 5 animals and one animal with emaciation - no abnormal signs in other dose groups - hepatic lesions observed in all dose groups	**disregarded study** **3 (not reliable)** **Rationale:** no positive control, no data on historical controls **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 %, commercial substance source	Hayashi A, Kosaka M, Kimura A, Wako Y, Kawasako K, Hamada S (2015)
***In vivo* micronucleus test using hepatocytes in rat** oral (gavage) rat Test concentrations: 5, 10 and 30 mg/kg bw	**Evaluation of results:** **positive** **Test results:** micronucleated hepatocytes in the LMN (liver micronucleus assay) assay after treatment stastically significant and dose-dependent	**disregarded study** **3 (not reliable)** **Rationale:** No standardised guideline available for *in vivo* micronucleus using hepatocytes **Test material: 4-nitrosomorpholine**	Hayashi A, Kosaka M, Kimura A, Wako Y, Kawasako K, Hamada S (2015)

***In vivo* micronucleus test using hepatocytes in rat** oral (gavage) rat Test concentrations: 10 and 100 mg/kg bw	**Evaluation of results:** **positive** **Test results:** High incidences of micronucleated hepatocytes in the LMN (liver micronucleus assay) assay after treatment	**disregarded study** **3 (not reliable)** **Rationale:** No standardised guideline available for *in vivo* micronucleus using hepatocytes, test results **Test material: 4-nitrosomorpholine**	Ashby and Lefevre (1989)
Chromosome aberration assay in bone marrow cells, no guideline followed intraperitoneal mouse (F1 of CBAxC57BL) male (no data on animal number) **50 mg/kg** (nominal conc.) **Positive control:** Cyclophosphamide Vehicle controls: yes	**Evaluation of results:** **negative** **Test results:** - number of chromosomal aberrations in treated animals was similar to controls - vehicle controls valid - positive controls valid	**disregarded study** **3 (not reliable)** **Rationale:** No data on substance purity, only one dose level tested, no data on clinical effects **Test material: 4-nitrosomorpholine** **Analytical purity:** no data	Ramaya LK, Pomerantzeva MD, Vilkina GA (1980)
Mammalian bone marrow chromosomal aberration test, no guideline followed Subcutaneous Rat (SD) no information on number of experimental animals Chromosome analysis after 5th and 15th treatment in bone marrow cells	**Ambiguous** After 5th treatment significant increase of chromosomal aberrations After 15th treatment decrease in chromosomal aberrations	**disregarded study** **4 (not assignable)** **Rationale:** Meeting Abstract only **Test material: 4-nitrosomorpholine** **Analytical purity:** no data	Roehrborn G and Neher J (1973)
Dominant lethal assay similar to OECD TG 478 (Genetic Toxicology: Rodent Dominant Lethal Test) intraperitoneal mouse (C57BL (male), BALB/C (female) F1 hybrid mice) male 7 males at each dose level **35, 50, 100 mg/kg** (nominal conc.) Positive control substance(s): methylmethanesulfonate (50 mg/kg bw) Vehicle controls: yes Vehicle: water	**Evaluation of results:** **negative** **Test results:** - 50 & 100 mg/kg: testing not possible as reduced incidence of mating - 35 mg/kg bw: no significant difference in number and percent of dead implants between treatment and control group - positive control valid	**disregarded study** **3 (not reliable)** **Rationale:** no information on source and purity of 4-nitrosomorpholine, only about 200 implants investigated **Test material: 4-nitrosomorpholine** **Analytical purity:** no data; no data on substance source	Parkin R; Waynforth HB; Magee PN (1973)

Additional information on method: about 200 implants were screened			
Alkaline single cell electrophoresis assay similar to OECD TG 489 intraperitoneal mouse (ddY) male 4 animals **250 mg/kg bw** one administration sampling time: 3, 8 and 24 h after treatment Positive control: no Negative control: untreated animals Vehicle: saline	**Evaluation of results:** **positive** **Test results:** - positive in cells of stomach, colon, liver, kidney, bladder, lung at all sampling times - negative in cells of brain and bone marrow toxicity: no information negative control: valid positive control: not available	**disregarded study** **3 (not reliable)** **Rationale:** only one dose level tested, no data on historical controls, no data on toxicity **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Tsuda S, Matsusaka N, Madarame H, Miyamae Y, Ishida K, Satoh M, Sekihashi K, Sasaki YF (2000)
Unscheduled DNA synthesis test, no guideline followed oral: gavage rat (Wistar) male 4 animals per group **200 mg/kg** (nominal conc.) Vehicle: distilled water Vehicle control: distilled water Positive control substance(s): no Additional information on method: rats directly injected with 3H-thymidine after treatment, autoradiographs from liver, kidney, urethra, prostate etc. prepared	Evaluation of results: **positive** Test results: - test results positive - vehicle controls valid (but no data on historical controls) - positive controls: not examined	**disregarded study** **3 (not reliable)** **Rationale:** No standardised guideline followed, labelled thymidine was injected in rats directly (*in vivo* effect on DNA repair/UDS), no positive control substance included, only one dose level tested **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	Korr H, Botzem B, Schmitz C, Enzmann H (2001)
Unscheduled DNA synthesis test similar to OECD TG 486 (UDS Test with Mammalian Liver Cells *in vivo*) oral: gavage rat (Alderley Park) male preliminary experiment: 1 animal/group	**Evaluation of results:** **positive** **Test results**: *Preliminary experiment:* - a increase in NG was observed in preliminary study at all dose levels *Main experiment:* - 2.5 h exposure time: 2/3 animals	**disregarded study** **3 (not reliable)** **Rationale:** only one dose level tested in main experiment, in preliminary experiment only one animal per dose level , no data on clinical effects at all tested concentrations, no	Ashby J, Lefevre PA (1989)

main experiment: 3 animals/group preliminary experiment**:10, 50, 100, 200 mg/kg** (nominal conc.) main experiment: **100 mg/kg** (nominal conc.) Single doses Exposure times: preliminary experiment: 2.5 h, main experiment: 2.5 and 12 h Positive control substance(s): N- Nitrosodimethylamine for 2.5 h exposure, 6-dimethylaminophenylazobenzthiazole (6BT) for 12 h exposure Vehicle controls: yes Cells screened:150 cells from three slides examined per animal	increase in NG compared to controls; vehicle controls valid; positive control valid - 12 h exposure time: 3/3 animals with increase in NG compared to controls; vehicle controls valid; positive control valid	explanation on dose selection for main experiment **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, commercial substance source	

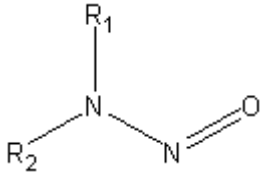
4.9.2 Human information

No data were available.

4.9.3 Other relevant information

An inquiry using the QSAR Toolbox (QSAR Toolbox version 4.2) using the profiling tool revealed the aryl N-nitroso group in 4-nitrosomorpholine as a structural alert for *in vitro* and *in vivo* mutagenicity (Figure 3).

Alkyl and aryl N-nitroso groups



R1= Aliphatic or aromatic carbon
R2= Any atom/group

Figure 3: 4-nitrosomorpholine was analysed with the QSAR Toolbox (version 4.2) and `aryl N-nitroso groups' were identified with the profiling tool as alerts for *in vivo* and *in vitro* mutagenicity.

4.9.4 Summary and discussion of mutagenicity

In order to evaluate the available data for 4-nitrosomorpholine, a literature search with defined keywords was performed in various databases (RTECS, Toxcenter, Toxnet, REAXIS, Chemlist, ISI Web of Knowlege).

In vitro studies:

There are four bacterial reverse mutation assays (Ames-Tests) available for 4-nitrosomorpholine which were considered to be reliable with restrictions (Zeiger et al., 1992, Matsushima et al., 1981, MacDonald, 1981 and Nagao and Takahashi, 1981). All four studies were performed similar to the OECD TG 471. They indicate a positive mutagenic potential of 4-nitrosomorpholine when using a metabolic activation system in the *S. typhimurium* strains TA100 and TA1535 and the E.coli strains WP2 uvrA and WP2 uvrA (pKM101). Reliability restrictions of these four studies were related to the number of tested strains ($\leq$ 3) within one test and that the studies have not been performed according to GLP.

Other available bacterial reverse mutation assays studies for 4-nitrosomorpholine (Andrews and Lijinsky, 1980, Gomez et al., 1974, Zeiger and Sheldon, 1978, Khudoley et al., 1981, Ichinotsubo et al., 1981, Rowland and Severn, 1981) were considered not to be reliable mainly due to missing positive controls. Moreover, in the report 'Evaluation of short-term tests for carcinogens: Report of the international collaborative program - Progress in mutation research' (de Serres and Ashby, 1981) eight more bacterial reverse mutation assays have been published for 4-nitrosomorpholine with positive results in at least one tested strain mainly TA100 (referring to studies of Brooks and Dean, 1981, Richold and Jones, 1981, Martire et al., 1981, Simon and Shepherd, 198, Trueman, 1981, Baker and Bonin, 1981, Venitt and Crofton-Sleigh, 1981, Garner et al., 1981). As reliable and unambiguous bacterial reverse mutation assays have already been identified and included in the present CLH-report and technical dossier it is supposed that no more information can be gathered

from the eight additional studies. Hence, these were not assessed for reliability and not included in the CLH-report.

There is one *in vitro* gene mutation study in mammalian cells, a Mouse Lymphoma Assay (MLA) using the thymidine kinase gene, available for 4-nitrosomorpholine (Jotz and Mitchell, 1981). This study was performed similar to the OECD TG 490 and was considered to be reliable with restrictions. The MLA indicates a positive mutagenic potential of 4-nitrosomorpholine using a metabolic activation system (negative without). This is supported by another available *in vitro* mammalian cell gene mutation test using the Hprt gene (Robichova et al., 2004) in which also positive results were found for 4-nitrosomorpholine. However, this study was considered not to be reliable mainly due to missing positive controls.

Reliable *in vitro* cytogenicity studies in mammalian cells are not available for 4-nitrosomorpholine. Two available *in vitro* mammalian chromosomal aberration tests (Slamenova et al., 2002 and Robichova et al., 2004) and three *in vitro* mammalian cell micronucleus tests (Slamenova et al., 2002, Mueller-Tegethoff et al., 1995 and Glatt et al., 1990), which all gave positive results for 4-nitrosomorpholine, were considered not to be reliable due to several reasons including either missing positive controls or usage of non-standard cell cultures. The reasons are specified in Table 11 and in the technical dossier.

Overall, from the *in vitro* genotoxicity data of the available reliable assays, it can be concluded that 4-nitrosomorpholine causes gene mutations in bacterial and mammalian cells after metabolic activation.

There is evidence for metabolisation of 4-nitrosomorpholine in mammals from *in vivo* studies in rats (Hecht and Young, 1981). The supposed two main metabolisation pathways via *α-hydroxylation* and *β-hydroxylation* for 4-nitrosomorpholine are presented in section 4.1 (toxicokinetics). *α-Hydroxylation* of 4-nitrosomorpholine leads to the intermediate 3-hydroxy-N-nitrosomorpholine which is assumed to be rapidly decomposed to a diazonium ion capable of alkylating DNA (Koissi and Fishbein, 2013, Figure 4). This could be the underlying mode of action of the observed positive mutagenicity results. The formation of reactive electrophilic alkyldiazonium ions have been generally discussed for alkylnitrosamides (Miller and Miller, 1981).

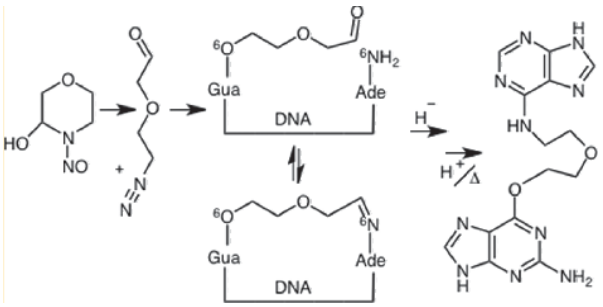


Figure 4: Figure taken from Koissi and Fishbein, 2013: Assumed decomposition of 3-hydroxy-N-nitrosomorpholine to a highly reactive diazonium ion capable of alkylating DNA

The positive *in vitro* mutagenicity findings for 4-nitrosomorpholine are qualitatively supported by results from some of the mutagenicity-related profilers in the QSAR Toolbox (version 4.2), cf. Table 13.

Table 13 Profiling of 4-nitrosomorpholine with respect to mutagenicity using relevant profilers from the OECD QSAR Toolbox (v. 4.2)[$]

Profiler type	Profiler name and description (excerpt from QSAR Toolbox profiler scheme description)	Output
General Mechanistic	**DNA binding by OASIS:** The profiler is based on the Ames Mutagenicity model part of the OASIS TIMES system. The profiler consists of 85 structural alerts responsible for the interaction with DNA analysed in the Ames Mutagenicity model. The scope of the profiler is to investigate the presence of alerts within target molecules which may interact with DNA. The list of 85 structural alerts has been separated into eight mechanistic domains. Each of the mechanistic domains is separated into mechanistic alerts. The profiling result assigns a target to the corresponding structural alert, mechanistic alerts and domain.	SN1: Nucleophilic attack after carbenium or nitrosonium ion formation: N-nitroso compounds
	DNA binding by OECD: A profiler compiling mechanistic organic chemistry fragments (in the form of structural alerts) for the binding of organic compounds to DNA.The profiler was created following the mapping of existing structural alerts for mutagenicity and carcinogenicity. The mapping was performed to achieve maximum overlap and usability whilst restricting redundancy in the alerts, and to ensure that the alerts related to the molecular initiating event of covalent DNA binding by OECD. A total of 60 alerts have been created; of these all but two are supported by mechanistic information and meta data. The alerts cross six broad organic chemistry mechanisms.	SN1: Carbenium ion formation: N-nitroso (alkylation) SN2: Nitrosation-SN2: Nitroso-SN2
Endpoint Specific	**DNA alerts for AMES by OASIS:** The profiler is based on the Ames Mutagenicity model part of the OASIS TIMES system. The profiler is based on the 85 structural alerts responsible for the interaction of chemicals with DNA extracted from the Ames Mutagenicity model. The scope of this profiler is to investigate the presence of alerts within the target molecules responsible for interaction with DNA related to Ames mutagenicity. This profiler accounts for incapability of some chemicals having an alert to interact with DNA due to electronic and steric factors. This is explicitly defined by inhibition masks associated with some alerts. The list of 85 structural alerts has been separated into eight mechanistic domains. Each of the mechanistic domains has been separated into mechanistic alerts. 31 of the alerts have been updated. The profiling result outcome assigns a target to the corresponding structural alert, mechanistic alerts and domain.	No alert found[§]
	DNA alerts for CA and MNT by OASIS: The profiler is based on the 85 structural alerts responsible for interaction of chemicals with DNA extracted from the Chromosomal aberrations model. There is a slight difference between DNA alerts in the in vitro Ames and CA models justified by the different local training set chemicals in both models. The scope of this profiler is to investigate the presence of alerts within the target molecules responsible for the interaction with DNA related to Chromosomal aberration and Micronucleus tests. This profiler accounts for incapability of some chemicals having an alert to interact with DNA due to electronic and steric factors. This is explicitly defined by inhibition masks associated with some alerts. The list of 85 structural alerts has been separated into eight mechanistic domains. Each of the mechanistic domains has been separated into mechanistic alerts. The profiling result outcome assigns a target to the corresponding structural alert, mechanistic alerts and domain.	
	Protein binding alerts for Chromosomal aberration by OASIS: The profiler is based on 33 structural alerts accounting for interactions of chemicals with specific proteins, such as topoisomerases, cellular protein adducts, etc. Associated with clear mechanistic justification, these alerts are included as a second reactivity component (complementing DNA reactivity) in the in vitro Chromosomal aberrations OASIS TIMES mutagenicity model. The scope of this profiler is to investigate the ability of target molecules to elicit clastogenicity. Functionalities which bring about steric (or electronic) hindrance in molecules and thus impede interactions with proteins are explicitly defined and associated with some of the alerts as “inhibition” masks.	

Profiler type	Profiler name and description (excerpt from QSAR Toolbox profiler scheme description)	Output
	***In vitro* mutagenicity (Ames test) alerts by ISS:** This profiler is based on the Mutagenicity/Carcinogenicity module of the software Toxtree. It works as a decision tree for estimating *in vitro* (Ames test) mutagenicity, based on a list of 30 structural alerts (SAs). The SAs for mutagenicity are molecular functional groups or substructures known to be linked to the mutagenic activity of chemicals. As one or more SAs embedded in a molecular structure are recognised, the system flags the potential mutagenicity of the chemical. The present list of SAs is a subset of the original Toxtree list, obtained by eliminating the SAs for nongenotoxic carcinogenicity.	Alkyl and aryl N-nitroso groups
	***In vivo* mutagenicity (Micronucleus) alerts by ISS:** This profiler is based on the ToxMic rulebase of the software Toxtree. This rulebase provides a list of 35 structural alerts (SAs) for a preliminary screening of potentially *in vivo* mutagens. These SAs are molecular functional groups or substructures that are known to be linked to the induction of effects in the *in vivo* micronucleus assay. The compilation of SAs for the *in vivo* micronucleus assay in rodents provided here is based on both the existing knowledge on the mechanisms of toxic action and on a structural analysis of the chemicals tested in the assay.	

[$] A detailed documentation of these profilers is available within the Toolbox software, which can be downloaded from https://www.qsartoolbox.org/de/download; [§] Note that these alert compilations are not (and do not claim to be) exhaustive, therefore absence of an alert cannot be interpreted as absence of effect.

In vivo studies

Reliable *in vivo* genotoxicity tests (heritable germ or somatic cell mutagenicity tests and other cell genotoxicity assays) in mammals are not available for 4-nitrosomorpholine. None of the available *in vivo* studies are ‘study reports` and none are performed according to an international by accepted guideline. All available studies are publications and relevant limitations considering test design and reporting are found. The limitations are considered to be major for each individual study and, hence, all available studies were considered invalid. Not a single key study is identified. The limitations mainly are related to missing information on toxicity, i.e. clinical effects and cytotoxicity. Clinical effects were reported in only one study (Hayashi et al, 2015). In addition, in many cases only one dose level was included in the study or positive controls were missing.

There exist several *in vivo* Mammalian Erythrocyte Micronucleus Tests in rats or mice similar to OECD TG 474 (Neresyan and Muradyan, 2002, Wakata et al., 1998, Morita et al., 1997, Hayashi et al., 2015, Kirkhart, 1981, Tsuchimoto and Matter, 1981 and Salamone et al., 1981). However, all tests were considered not to be reliable as in none of these tests criteria for dose selection and selection of the highest tested dose e.g. by determining clinical effects or the MTD in the animals were given. Moreover, in most of these tests no positive controls were included. Interestingly, obtained results for 4-nitrosomorpholine were ambiguous. At high doses (of about 100 to 2000 mg/kg bw, i.p.), supposed to be of higher systemic toxicity (see section 4.7), positive results were found. At low doses (of about 5 to 32 mg/kg, oral and i.p.) negative results were obtained. The rationales for the limited reliability for each of these studies are specified in Table 12 and the technical dossier.

Moreover, there are two *in vivo* micronucleus tests available using hepatocytes (Ashby and Lefevre, 1989, Hayashi et al., 2015). Both tests were positive. However, the results of the tests are not considered relevant for classification for the time being and the studies are disregarded from assessment. 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.) even if 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 relevant for classification. Moreover, Hayashi et al., 2015 and Ashby and Lefevre, 1989 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 DS, the influence of high liver toxicity on the test outcome (MNHEPs) in liver cells remains still unclear. When 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.

An available negative *in vivo* chromosomal aberration assay in mouse bone marrow cells (Ramaya et al., 1980), a negative rodent dominant lethal test (Parkin et al., 1973), two positive *in vivo* UDS tests in rats (Ashby and Lefevre, 1989, Korr et al., 2001) and a positive comet assay (Tsuda et al., 2000), with 4-nitrosomorpholine were also considered not to be reliable. The rationales for the assessed reliability for each of these studies are specified in Table 12 and in the technical dossier.The overall consistency of the positive results in the MN assays (bone marrow), comet assay, and UDS tests in a weight of evidence approach regarding a possible classification is discussed in section 4.9.5.

Further available genotoxicity tests for 4-nitrosomorpholine which were performed using outdated test systems for which either OECD test guidelines have been deleted or standardised test guidelines do not exist[1] were not considered to be relevant and to contribute to a classification decision in line with the criteria of the CLP Regulation. This include the *in vitro* alkaline elution test (Martelli et al., 1988), *in vivo* alkaline elution test (Brambilla et al., 1987), *in vitro* unscheduled DNA synthesis test (Martelli et al., 1988, Martin and McDermid, 1981), *in vitro* comet assay (Lazarova et al., 2006, Robichova and Slamenova, 2001, Slamenova et al., 2002), *in vivo* sister-chromatid exchange test (Kligerman et al., 1985), *in vitro* sister-chromatid exchange test (Evans and Mitchell, 1981), Gene mutation assay in *Saccharomyces cerevisiae* (Metha and vonBorstel, 1981, Sharp and Parry, 1981, Zimmermann and Scheel, 1981), mitotic recombination assay in *Saccharomyces cerevisiae* (Parry and Sharp, 1981), wing spot test in *Drosophila melanogaster* (Negishi et al., 1991), Drosophila mosaic test (Surjan et al., 1985), *in vivo* mammalian lymphocyte chromosome aberration test (Newton et al., 1981) and the host mediated assay for *Salmonella typhimurium* (Braun and Schoeneich, 1975, Zeiger, 1971, Zeiger, 1973). These studies gave ambiguous results and are shortly summarised in the table shown in Annex I.

4.9.5 Comparison with criteria

According to the CLP Regulation mutagens may be classified in hazard categories 1A, 1B or 2.

The classification of mutagens in Category 1A is based on positive evidence from human epidemiological studies. For 4-nitrosomorpholine there are no data available on mutagenicity from human epidemiological studies. Hence, classification in Category 1A is not warranted.

The classification of mutagens in Category 1B is based on:

(i) positive results from *in vivo* heritable germ cells mutagenicity tests in mammals or

(ii) positive results from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells or

(iii) positive results from tests showing mutagenic effects in germ cells of humans.

For 4-nitrosomorpholine *in vivo* heritable germ cells mutagenicity tests in mammals, reliable *in vivo* somatic cell mutagenicity tests in mammals in combination with some evidence that the substance

[1] Concerning OECD Test Guidelines for the Testing of Chemicals and test methods described in the Regulation (EG) No. 440/2008 (11.12.2015)

has potential to cause mutations in germ cells and tests showing mutagenic effects in germ cells of humans are not available.

The only available *in vivo* heritable germ cell mutagenicity test, namely a dominant lethal test (Parkin et al., 1973) yielded negative results. Due to limitations the test was considered not reliable.

Hence, the limited database does not allow a decision finding and classification in Category 1B.

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.

Even though there are *in vitro* tests with positive results available for 4-nitrosomorpholine there exist no reliable *in vivo* mammalian somatic cell mutagenicity tests or other reliable *in vivo* somatic cell genotoxicity tests for 4-nitrosomorpholine. Hence, classification in Category 2 based on these criteria is not warranted.

Classification based on weight of evidence:

Generally, a weight of evidence approach is regarded critically with respect to the weak *in vivo* database for 4-nitrosomorpholine. None of the available *in vivo* genotoxicity studies is identified as key study and as reliable. There is no *in vivo* genotoxicity study available, which was performed according to an international accepted guideline (study report). All of the available *in vivo* genotoxicity studies are publications and due to major limitations in test design and reporting, all those studies are considered not reliable.

Nevertheless, in the following the available data are discussed in terms of consistency of the results. For 4-nitrosomorpholine 16 *in vivo* genotoxicity studies have been identified which were performed using a relevant test system (MN assay (bone marrow), comet assay, UDS test, dominant lethal assay, chromosomal aberration test). Eight of the studies were positive, two studies yielded ambiguous results and six studies were negative.

Interestingly, 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 from 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 found 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, non of the available *in vivo* studies reported/measured toxicity and clinical effects. Thus, from the available studies it cannot be derived if the applied dose was the MTD or above. It is not possible to decide, based on all reported positive and negative *in vivo* genotoxicity studies, if the positive effect was robust and valid. The fact that most of the positive studies were performed using intraperitoneal substance administration, where a higher bioavailability is assumed, underpins the uncertainty of the (toxic) effect of the dose levels applied.

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 valid and sufficiently robust to conclude on a negative outcome.

All in all, the entire database is contradictory. A valid key study is not available.In summary, a robust classification in Category 2 based on weight of evidence due to major limitations and contradictory results of all available *in vivo* genotoxicity studies is not warranted.

Classification based on chemical structure activity relationship:

According to criteria laid down in the CLP Regulation, substances which are positive in *in vitro* mammalian mutagenicity assays shall also be considered for classification as Category 2 mutagens if they show a chemical structure activity relationship to known germ cell mutagens. As there exists one reliable positive *in vitro* mammalian mutagenicity assay for 4-nitrosomorpholine (Jotz and Mitchell, 1981) it was assessed if there are chemical structure activity relationships to known germ cell mutagens. Known germ cell mutagens are listed in Annex VI of the CLP regulation (Muta. 1A/B mutagens). However, none of the listed chemicals classified as Muta. 1 A/B was found to belong to the chemical group of N-nitrosamines or N-nitrosamides (possessing alkyl and aryl N-nitroso groups). Vice versa, none of the identified structure analogues to 4-nitrosomorpholine (using the profiling tool of the QSAR Toolbox and searching for alkyl and aryl N-nitroso groups) has been listed in Annex VI as Muta. 1 A/B. So far, a harmonised classification was available only for three N–nitrosamines, namely dimethylnitrosamine (CAS 62-75-9) and 2,2-(nitrosoimino)bisethanol (CAS 1116-54-7) and nitrosodi-n-propylamin (CAS 621-64-7). These three substances are classified as Carc.1B but not as Muta.1 or 2. It is concluded that presently there exist no germ cell mutagens with structure activity relationship to 4-nitrosomorpholine for which a classification as germ cell mutagen has been agreed. Hence, a classification in Category 2 based on structural similarities cannot be proposed.

4.9.6 Conclusions on classification and labelling

Even though there are mutagenicity assays with positive evidences for 4-nitrosomorpholine the current data are not sufficient to fulfil the classification criteria for mutagenicity in Categories 1 or 2. Hence, at present, a classification and labelling of 4-nitrosomorpholine as mutagenic is not justified.

4.10 Carcinogenicity

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

The results of oral carcinogenicity studies are summarised in Table 14. For some of these studies detailed results are shown in separate tables 14.1 to14.8.

Table 14 Relevant oral carcinogenicity studies

Method	Results	Remarks	Reference
Carcinogenicity study, 50 weeks exposure, life-time observation (no guideline followed) oral: drinking water Exposure: **50 weeks** (5 days a week) Observation: whole life span rat (MRC rats) male/female 15 animals/group (5/cage)* **3.6 mg/kg bw/d** (nominal in water) Vehicle: water No control group Statistics: no data (not applied) **Experimental design:** Animals treated with 100 mg/L 4-nitrosomorpholine solution in drinking water over night for 5 days a week (5 animals/cage 100 mL solution); during the day rats received tap water **Parameters investigated:** Survival, gross pathology (no results reported), histopathology: neoplastic effects (number of animals with tumours in various organs, no detailed data on organs examined) *Females: only 14 animals autopsied	**Survival:** *Females* - 100 % at week 20 - 0 % at week 50 *Males* - 100 % at week 30 - 7 % at week 50 **Neoplastic effects:** *Females* 13/14 (93 %) animals with tumours: - 13/14 (93 %) liver - 1/14 (7 %) esophagus - 5/14(35 %) nasal cavity - 1/14 (7 %) mammary gland *Males* 15/15 (100 %) animals with tumours : - 1/15 (7 %) larynx - 13/15 (87 %) liver - 2/15 (13 %) esophagus - 9/15 (60 %) nasal cavity - 1/15 (7 %) stomach	**Supporting study** **2 (reliable with restrictions):** **Rationale:** No guideline followed, no controls included, 15 animals/group only, only one dose level included, no detailed clinical investigation, no histopathology of non-neoplastic effects **Test material: 4-nitrosomorpholine** **Analytical purity**: > 99 %	Garcia H, Lijinsky W (1972)

Carcinogenicity study, 30 weeks exposure, life-time observation (no guideline followed) oral: drinking water Exposure: **30 weeks** (5 days a week) Observation: whole life span rat (Sprague-Dawley) male 30 animals/group **1.4 mg/kg bw/d** (nominal in water) Vehicle: water No control group Statistics: no data (not applied) **Experimental design:** 60 mL 4-nitrosomorpholine solution (0.34 mM) solution provided to three rats per cage 5 days a week, on the two remaining days rats received tap water **Parameters investigated:** survival, gross pathology, histopathology (neoplastic and non-neoplastic effects, ≥ 20 organs examined)	**Survival:** - 100 % at week 10 - 87.7 % at week 50 - 46.7 % at week 80 - 6.6 % at week 100 **Gross pathology and histopathology of liver (non-neoplastic effects):** - necrosis - massive scarring - biliary hyperplasia - telangiectasis **Neoplastic effects:** 16/30 (53 %) male animals with liver tumours: - tumours mostly of hepatocellular origin and two Kupffer cell sarcoma - tumours appeared benign and malignant	**Supporting study** **2 (reliable with restrictions) Rationale:** No standardised guideline followed, no controls included, 30 animals/group only, only one dose level tested, only male animals, no detailed clinical investigation **Test material: 4-nitrosomorpholine** **Analytical purity:** no exact data, non-commercial substance source, no detectable impurities (MS)	Lijinsky W, Taylor HW (1975)

Carcinogenicity study, 30 weeks exposure, life-time observation (no guideline followed) oral: drinking water Exposure: **30 weeks** (5 days a week) Observation: whole life span (max. for 126 weeks) rat (Sprague-Dawley) male 30 animals/group **0.3 mg/kg bw/d** (nominal in water), **1.5 mg/kg bw/d** (nominal in water) Vehicle: water Controls: 9 males, 9 females (documented in Taylor and Lijinsky, 1975, Cancer Res, 35, 812-815) Statistics: no data related to comparison of treatment groups and controls **Experimental design:** 3 animals per cage received 60 mL 4-nitrosomorpholine solution (0.35 mM (40.6 mg/L) and 0.07 mM(8 mg/L)) for five days a week, solution administered per cage was consumed completely each day, on the two remaining days rats received tap water **Parameters investigated:** survival, gross pathology and histopathology (neoplastic and non-neoplastic effects) of major organs (no detailed data on organs examined)	**Survival:** *0.3 mg/kg/d* - 100 % at about 70 weeks - 50 % at about 100 weeks - 10 % at about 110 weeks *1.5 mg/kg/d* 100 % at about 10 weeks 50 % at about 80 weeks 10 % at about 100 weeks **Gross pathology:** *0.3 and 1.5 mg/kg/bw/d* - all livers white foci (1mm-1cm size) scattered throughout parenchyma in all lobes and replaced 50-90 % of normal liver - occasional small biliary-retention cysts and telangiectasia **Histopathology of liver (non-neoplastic effects)** *0.3 and 1.5 mg/kg/bw/d* - extensive focal postnecrotic cirrhosis - biliary hyperplasia with ductal hyperplasia - cysts - telangiectatic sinuses - vascular channels occasionally filled with thrombi and leukocytic debris **Neoplastic effects:** *Controls:* various benign endocrine tumours (no detailed data) *0.3 mg/kg bw/d* 12/30 (40 %) animals tumour bearing: - 11/30 (37 %) hepatocellular tumours - 1/30 (3 %) hemangioendothelial tumour (liver) *1.5 mg/kg bw/d* 18/30 (60 %) animals tumour bearing: - 16/30 (53 %) hepatocellular tumours - 2/30 (7 %) hemangioendothelial tumours (liver) → concentration dependent increase in liver tumours	**Supporting study** **2 (reliable with restrictions) Rationale**: No standardised guideline followed, 30 animals/group only, only males tested, only two dose levels tested, no detailed clinical investigation, no body weights measured (before, during and after exposure) **experimental result** **Test material: 4-nitrosomorpholine** **Analytical purity:** no exact data, non-commercial substance source, no detectable impurities (MS)	Lijinsky W, Taylor HW, Keefer LK (1976)

Carcinogenicity study, 50 weeks exposure, life-time observation (no guideline followed) oral: drinking water Exposure: **50 weeks** (5 days a week) Observation: whole life span (max. for 126 weeks) rat (Fischer 344) male/female 20 males and 20 females per group Males: **0.6 and 1.4 mg/kg bw/d** (nominal in water);(16 mg/L and 40 mg/L) Females **1.4 mg/kg bw/d** (nominal in water); (40 mg/L) Vehicle: water Controls: no controls included Statistics: no data (not applied) **Experimental design**: 80 mL of 4-nitrosomorpholine solutions were provided to 4 animals per cage 5 days a week, at the two remaining days animals received tap water, solution administered per cage almost consumed completely each day **Parameters investigated**: survival, gross pathology and histopathology (non-neoplastic and neoplastic) of major organs (no detailed data on organs examined)	**Survival:** Males: *0.6 mg/kg bw/d:* - 100 % at 30 weeks, 50 % at 80 weeks, 0 % at 110 weeks *1.4 mg/kg bw/d:* -100 % at 40 weeks, 30 % at 60 weeks 0 % at 80 weeks Females: *1.4 mg/kg/d:* - 100 % at 40 weeks, 7 % at about 60 weeks, 0 % at 70 weeks **Neoplastic effects:** Males: *0.6 mg/kg bw/d* 20/20 (100 %) animals tumour bearing: - 18/20 (90 %) Liver Carcinoma - 4/20 (20 %) Liver Sarcoma - 2/20 (10 %) Esophagus Papilloma - 1/20 (5 %) Forestomach Papilloma - 1/20 (5 %) Tongue Papilloma - 5/20 (25 %) Leukemia - 1/20 (5 %) tumours in pituitary *1.4 mg/kg bw /d* 20/20 (100 %) animals tumour bearing: - 19/20 (95 %) Liver Carcinoma - 15/20 (75 %) Liver Sarcoma - 7/20 (20 %) Esophagus Papilloma - 6/20 (20 %) Leukemia Females: *1.4 mg/kg bw/d* - 20/20 (100 %) animals tumour bearing - 17/20 (89 %) Liver Carcinoma - 16/20 (80 %) Liver Sarcoma - 3/20 (15 %) Esophagus Carcinoma - 9/20 (45 %) Esophagus Papilloma - 9/20 (45 %) Leukemia - 1/20 (5 %) tumours in pituitary General findings (females and males): - the liver tumours were mainly hepatocellular carcinomas, hemangioendothelial sarcomas and a few cholangiocarcinomas - occasional the following tumours were observed: nasal carcinoma, lung adenocarcinoma, spleen hemangiosarcoma, neurosarcoma, ear carcinoma, head carcinoma, mesothelioma, thyroid carcinomas, testis carcinomas, adrenal pheochromocytoma (numbers of all these tumours not different compared to historical controls of the laboratory of authors)	**Supporting study** **2 (reliable with restrictions) Rationale**: No standardised guideline followed, no controls included, 20 animals/group only, two dose levels tested only, no detailed clinical investigation, no body weights measured (before, during and after exposure), no data on results of histopathological (non-neoplastic) investigations, no data on purity of 4-nitrosomorpholine and non-commercial substance source **Test material: 4-nitrosomorpholine** **Analytical purity**: no data given, non-commercial substance source	Lijinsky W; Reuber MD (1982)

Carcinogenicity study, whole life span (100 weeks), similar to OECD TG 451 oral: drinking water Exposure: 5 days a week, **100 weeks** observation: whole life span rat (Fischer 344) female 100 to 24 animals (see Table 14.2) per dose group **0,0.003, 0.007, 0.02, 0.04; 0.09, 0.23 mg/kg bw/d** (nominal in water) Vehicle: water (with max. 0.2 % ethanol) Controls: 80 untreated animals Statistics: Cox exact test (trend test) **Experimental design:** 80 mL of 4-nitrosomorpholine solutions provided to 4 animals per cage for 5 days a week (0.07 mg/L - 100 mg/L), at two remaining days animals received tap water, solution administered almost consumed completely each day, due to high mortality in the three highest dose groups, animals were treated less than 100 weeks **Parameters investigated**: survival, gross pathology and histopathology (non-neoplastic and neoplastic effects) of ≥ 20 organs	**Survival:** - significant decrease compared to controls at 0.09 and 0.23 mg/kg bw/d **Neoplastic effects:** *controls:* - high rates of spontaneous tumours of: Adrenal medulla: 8/80 (10 %) Leukemia. 31/80 (38.8 %) Mammary: 25/80 (31.3 %) Pituitary: 42/80 (52.5 %) Uterus: 9/80 (11.3 %) - low liver tumour incidence: 1/80 (1.25 %) *Treated animals:* - highly significant dose- dependent increase of liver tumours after treatment (e.g. hepatocellular carcinoma, hemangiosarcoma, hepatocellular adenoma) (see Table 14.2); Cox exact trend test: P < 0.0001 for hepatocellular carcinoma, hemangiosarcoma and any benign or malignant tumours - at the highest dose level liver tumours (any benign or malignant) in 96 % of the animals - increase of tumour rates at higher dose levels of tumours of thyroid and tongue (see Table 14.2) (12.5 % at highest dose)	**key study** **2 (reliable with restrictions)** **Rationale:** similar to standardised guideline, controls included; *Restrictions*: no males tested, no daily dosing, no detailed clinical investigation performed, no body weights measured (before, during and after exposure), no data on histopathology (non-neoplastic effects), no vehicle controls (water with max. 0.2 % ethanol) **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 %	Lijinsky W, Kovatch RM, Riggs CW, Walters PT (1988)

Carcinogenicity study, exposure 50 weeks, observation whole life span, similar to OECD TG 451 oral: drinking water Exposure**: 5 days a week**, **50 weeks** (for doses up to 0.83 mg/kg bw/d) (2.07 mg/kg bw/d: 40 weeks exposure, 5.2 mg/kg bw/d: 25 weeks exposure) Observation: whole life span rat (Fischer 344) female 24 to 48 animals per dose group (see Table 14.4) **0, 0.02, 0.04, 0.09, 0.23, 0.58, 1.43, 3.58 mg/kg bw/d** (nominal in water) Vehicle: water (with max. 0.2 % ethanol) Controls: 80 untreated animals Statistics: Cox exact test (trend test) **Experimental design:** 80 mL of 4-nitrosomorpholine solutions provided to 4 animals per cage for 5 days a week (0.45 to 100 mg/L), at two remaining days animals received tap water, solution administered almost consumed completely each day, due to high mortality in the two highest dose groups, animals were treated less than 50 weeks **Parameters investigated**: survival, gross pathology and histopathology (non-neoplastic and neoplastic effects) of ≥ 20 organs	**Survival:** - significant decrease at 0.04, 0.09, 0.23, 0.58, 1.43, 3.58 mg/kg bw/d compared to controls - survival of animals treated with the three highest doses was highly reduced (see Table 14.3) **Neoplastic effects:** *controls:* - spontaneous tumours of: Adrenal medulla: 8/80 (10 %) Leukemia: 31/80 (38.8 %) Mammary: 25/80 (31.3 %) Pituitary: 42/80 (52.5 %) Uterus: 9/80 (11.3 %) - Low liver tumour incidence: 1/80 (1.25 %) *treated animals:* - highly significant dose-dependent increase of liver tumours after treatment with 4-nitrosomorpholine (e.g. hepatocellular carcinoma, hemangiosarcoma, hepatocellular adenoma); Cox exact trend test: P < 0.0001 for hepatocellular carcinoma, hemangiosarcoma and any benign or malignant tumours - at the highest dose level liver tumours in 100 % of the animals - increase of tumour rates in higher dose levels of tumours of esophagus, thyroid and tongue (see Table 14.4) (note: high mortality rates and liver tumour rates at these dose groups)	**key study** **2 (reliable with restrictions)** **Rationale**: similar to standardised guideline, controls included; *Restrictions:* treatment time 50 weeks only, no males tested, no detailed clinical investigation performed, no body weights measured (before, during and after exposure), no data on histopathology (non-neoplastic effects), no vehicle controls (water with 0.2 % ethanol) **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 %	Lijinsky W, Kovatch RM, Riggs CW, Walters PT (1988)

Carcinogenicity study, exposure 50 weeks, observation whole life span, (no guideline followed) oral: drinking water Exposure: **50 weeks (5 days a week)** Observation: whole life span (max. for 124 weeks) rat (Fischer 344) female 20 animals per dose group **0.95 mg/kg bw/d** (nominal in water) Vehicle: water Untreated control group included Statistics: no data (not applied) **Experimental design:** 80 mL (26.5 mg/L) 4-nitrosomorpholine solutions provided to 4 animals per cage for 5 days a week**,** on the two remaining days animals received tap water, solution administered per cage almost consumed completely each day **Investigated parameters:** survival, gross pathology and histopathology (non-neoplastic and neoplastic effects) of major organs (no detailed data on organs examined)	**Survival:** 100 % 50 weeks (controls: 60 weeks) 50 % 64 weeks (controls: 110 weeks) 0 % 80 weeks (controls: > 124 weeks) **Neoplastic effects:** *controls*: 0/20 (0 %) tumour bearing *treated animals*: - Liver Hepatocellular tumour: 19/20 (95 %) - Liver hemangiosarcoma: 10/20 (50 %) - Lung tumours: 1/20 (5 %) - Thyroid follicular cell: 2/20 (10 %) - Kidney adenoma 1/20 (5 %) - Adrenal cortex 1/20 (5 %) - Brain astrocytoma 1/20 (5 %)	**supporting study** **2 (reliable with restrictions) Rationale:** No standardised guideline followed, 20 animals/group only, one dose level tested only, no daily dosing, no detailed clinical investigation, no body weights measured (before, during and after exposure), no data on histopathology (non-neoplastic effects), no data on substance purity, non- commercial substance source experimental result **Test material: 4-nitrosomorpholine** **Analytical purity:** no data given, non-commercial substance source	Hecht SS, Lijinsky W, Kovatch RM, Chung FL, Saavedra JE (1989)

Carcinogenicity study, exposure 8 weeks, observation 12 weeks, (no guideline followed) oral: drinking water Exposure: **daily for 8 weeks** Observation: 12 weeks rat (WS/Shi) male 16 animals per dose group (effective no. of animals for analysis: 15) **17 mg/kg bw/d** (0.02 % solution) (nominal in water) Vehicle: water No controls included Statistics: not relevant as no controls included **Parameters investigated:** Survival, bw, relative liver weight, gross pathology of liver and lungs, histopathology (neoplastic and non-neoplastic effects) of liver and lungs	**Survival:** - after 12 weeks observation: 4/16 (25 %) survived **Body weight:** - increase of 61 % within treatment and observation period (initial 161 g, final 318 g) **Relative liver weight:** - final 16.6 g/100g **Neoplastic effects:** - hepatocellular carcinoma: 15/15 (100 %); induction time 117 days (no exact data on determination, presumably death time)	**supporting study** **2 (reliable with restrictions) Rationale**: No standardised guideline followed, no controls included, short treatment time (8 weeks), one dose level tested only, 16 animals/group only, males only, investigation of hepatocellular carcinoma and lung metastasis only, no detailed clinical investigation, no data on histopathology (non-neoplastic effects) **Test material: 4-nitrosomorpholine** **Analytical purity:** no data given, commercial substance source	Murai T, Mori S, Hosono M, Iwakura Y, Takashima A, Oohara T, Makino (2000)
Carcinogenicity study, exposure 10 weeks, observation one year, (no guideline followed) oral: drinking water Exposure: 5 days a week, 10 weeks Observation: one year after begin of treatment rat (albino random-bred rats) male treatment group: 31 animals, control group: 19 animals **8.9 mg/kg bw/d** (nominal in water) Vehicle: water Untreated controls included Statistics: no data related to comparison of treatment groups and controls **Parameters investigated:** gross pathology, histopathology (non-neoplastic and neoplastic effects) of major organs (no detailed data on organs examined)	**Neoplastic effects:** *control group:* -1/19 (5.3 %) rats with tumour (extrahepatic, testicular Leydig cell tumour) *treatment group:* 23/31 (74 %) rats with tumours: - Hepatocellular adenomas: 10/31 (32 %) - Hepatocellular carcinoma: 9/31 (29 %) - Other intrahepatic neoplasms: 3/31 (9.6 %) - Renal cell carcinoma: 1/31 (3 %)	**supporting study** **2 (reliable with restrictions) Rationale:** No standardised guideline followed, short treatment time (10 weeks), 19 to 31 animals/group, males only, one dose level tested only, no daily dosing, no detailed clinical investigation, no bw, no data on histopathology (non-neoplastic effects), no data on substance purity or source **Test material: 4-nitrosomorpholine** **Analytical purity:** no data	Nersesyan AK, Muradyan RE (2002)

Carcinogenicity study, exposure 8 weeks, observation 12 weeks, (no guideline followed) oral: drinking water Exposure: daily for **8 weeks** Observation: 12 weeks rat (SD/gShi) male 16 animals per dose group **14 mg/kg bw/d** (nominal in water) Vehicle: water No controls included Statistics: not relevant as no controls included **Parameters investigated:** Survival, bw, relative liver weight, gross pathology of liver and lungs, histopathology (neoplastic and non-neoplastic effects) of liver and lungs	**Survival:** - after 12 weeks observation 14/16 (88 %) survived **Body weight:** - Final body weight was 426 ± 37 g (initial 174 g); (increase of 144 %) **Relative liver weight:** - final 4.1 g/100g **Neoplastic effects:** - hepatocellular carcinoma: 1/16 (6 %); induction time 135 days (no exact data on determination, presumably death time)	**supporting study** **2 (reliable with restrictions) Rationale**: No standardised guideline followed, no controls included, short treatment time (8 weeks), one dose level tested only, 16 animals/group only, males only, investigation of hepatocellular carcinoma and lung metastasis only, no detailed clinical investigation, no data on histopathology (non-neoplastic) **Test material: 4-nitrosomorpholine** **Analytical purity:** no data given, commercial substance source	Murai T, Mori S, Hosono M, Iwakura Y, Takashima A, Oohara T, Makino (2000)
Carcinogenicity study, exposure 8 weeks, observation 12 weeks, (no guideline followed) oral: drinking water Exposure: **daily for 8** weeks Observation: 12 weeks rat (F344/DuCrj) male 16 animals per dose group **17 mg/kg bw/d** (nominal in water) Vehicle: water No controls included Statistics: not relevant as no controls included **Parameters investigated:** Survival, bw, relative liver weight, gross pathology of liver and lungs, histopathology (neoplastic and non-neoplastic effects) of liver and lungs	**Survival:** - After 20 week's observation time: 11/16 survived (69 %) **Body weight:** - Final body weight was 296 ± 14 g (initial 123 g) (increase of 143 %) **Relative liver weight:** - final 8.8 g/100g **Neoplastic effects:** - hepatocellular carcinoma: 13/15 (87 %); induction time 131 days (no exact data on determination, presumably death time)	**supporting study** **2 (reliable with restrictions) Rationale**: No standardised guideline followed, no controls included, short treatment time (8 weeks), one dose level tested only, 16 animals/group only, males only, investigation of hepatocellular carcinoma and lung metastasis only, no detailed clinical investigation, no data on histopathology (non-neoplastic) **Test material: 4-nitrosomorpholine** **Analytical purity:** no data given, commercial substance source	Murai T, Mori S, Hosono M, Iwakura Y, Takashima A, Oohara T, Makino (2000)

Carcinogenicity study, up to 80 weeks (no guideline followed) oral: drinking water Exposure: daily for **7,11,15,20,27, 37,50,65 or 80 weeks** (stop experiment) rat (Sprague-Dawley) male 5 to 30 animals per treatment group (s. Table 14.5) **0, 6, 12, 24 mg/kg bw/d** (nominal in water) Vehicle: water Untreated controls included Statistics: no data (not applied) **Parameters investigated**: bw, gross pathology and histopathology (non-neoplastic and neoplastic effects) of the liver	**Body weights:** - exposure to 24,12, and 6 mg/kg bw/d resulted in mean body weight reduction of 32, 19, and 13 % in comparison to controls from week 11 on **Non-neoplastic:** - after treatment with 24 mg/kg bw/d for 7 weeks numerous single cell necroses, an acinocentral loss of glycogen, occurrence of megalocytes, bile ductular proliferations and fibrosis - after 11 weeks of treatment with 24 mg/kg bw/d cirrhosis, cholangiofibrosis, cholangiomas and multiple hepatocyte nodules - after week 15 and 20 severe cirrhosis after treatment with 24 mg/kg bw/d **Neoplastic effects:** - clear dose- and time-dependent increase in incidence of hepatocellular adenomas and carcinomas (see Table 14.5) in treated animals - dose- and time-dependent increase of preneoplastic lesions in treated animals - 24 mg/kg bw/d: first tumours after 15 weeks of treatment (time of sacrifice) - 12 mg/kg bw/d: first tumours after 20 weeks of treatment (time of sacrifice) - 6 mg/kg bw/d: first tumours after 27 weeks of treatment (time of sacrifice) - controls: first tumours after 80 weeks (Table 14.5) (time of sacrifice)	**key study** **2 (reliable with restrictions)** **Rationale for restrictions:** no guideline followed, 5 to 30 animals/group only, only male animals, no detailed clinical investigation, gross pathology and histopathology restricted to liver experimental result **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	Weber E, Bannasch P (1994a)

Carcinogenicity study, 30 weeks (no guideline followed) oral: gavage Exposure: **twice weekly 30 weeks** (3-day interval) Observation time: until animals were moribund or died naturally rat (Fischer 344) female 12 animals per group **3.6 mg/kg bw/day (10 mg/rat/week)** (actual ingested) Vehicle: corn oil/ethyl acetate Controls: vehicle controls Statistics: no data (not applied) **Parameters investigated:** survival, gross pathology**,** histopathology (non-neoplastic and neoplastic effects) of major organs (no detailed data on organs examined)	**Survival:** *controls:* 100 %: about 70 weeks 6 %: about 110 weeks *treatment group:* 100 %: about 20 weeks 0 %: at 30 weeks Median week of death: 26 **Neoplastic effects:** *controls:* - No tumours were observed 0/12 (0 %) *treatment group:* - Tumours of liver: 11/12 (91.7 %) - Tumours of esophagus: 8/12 (66.7 %) - Tumours of thyroid: 2/12 (16.7 %)	**supporting study** **2 (reliable with restrictions)** **Rationale**: no guideline followed, one dose level tested only, 12 animals/group only, females only, no daily dosing, no detailed clinical investigation, no body weights measured, no results of histopathology (non-neoplastic effects) **Test material: 4-nitrosomorpholine** **Analytical purity:** no data given, non-commercial substance source	Lijinsky W, Saavedra JE, Kovatch RM (1991a)
Carcinogenicity study, 10 weeks (no guideline followed) oral: drinking water Exposure: daily for 10 weeks Observation time: 20 weeks mouse (A/J) female 40 animals per treatment group **3.6 mg/kg bw/d** (0.2 µmol/mL) (nominal in water) Vehicle: water Untreated controls included Statistics: Student´s *t*-test, χ^2 test **Parameters investigated:** lung tumour incidence (adenomas)	**Neoplastic effects:** - For treatment group a significantly ($P < 0.01$) higher lung tumour incidence (100 % of treated mice) was observed compared to controls (40 % of mice).	**supporting study** **2 (reliable with restrictions) Rationale**: No standardised guideline followed, one dose tested only, short treatment time, no data on survival, no body weights measured, no detailed clinical investigation, investigation restricted to lung adenomas **Test material: 4-nitrosomorpholine** **Analytical purity:** no detailed data given ("pure according to TLC and NMR analysis"), non-commercial substance source	Hecht SS, Lijinsky W, Kovatch RM, Chung FL, Saavedra JE (1989)

Carcinogenicity study, 26 weeks, observation 50 weeks (no guideline followed) oral: gavage Exposure: once weekly for **26 weeks** Observation time: 50 weeks hamster, Syrian male 20 animals per treatment group **6.7 mg/kg bw/d** (nominal in water) (0.2 mL of 26 mg/mL solution) Vehicle: water Untreated controls included Statistics: no data (not applied) **Parameters investigated:** survival, gross pathology, histopathology (non-neoplastic and neoplastic effects) of major organs (no detailed data on organs examined)	**Survival:** *controls:* 100 % about 40 weeks 50 % > 80 to < 90 weeks 0 % > 90 weeks *treated animals:* 100 % about 20 weeks 50 % > 20 to < 30 weeks 0 % 50 weeks **Neoplastic effects:** *controls:* - 3/20 (15 %): Forestomach papilloma *treated animals:* - Liver hemangiosarcoma: 1/20 (5 %) - Nasal carcinomas: 15/20 (75 %) - Lung adenomas: 1/20 (5 %) - Trachea adenomas: 6/20 (30 %)	**supporting study** **2 (reliable with restrictions) Rationale:** Not according to standardised guideline, only one dose level tested, 20 animals/group only, no daily dosing, short treatment time, only male animals, no detailed clinical investigation, no body weights measured, no data on histopathology results (non-neoplastic), no data on purity of substance, non-commercial substance source **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non- commercial source	Lijinsky W, Kovatch RM, Knutsen GL (1984)

Carcinogenicity study, life-time, similar to OECD TG 451 oral: drinking water Exposure: daily for **whole life span** hamster, Syrian male/female 30 animals per treatment group, 50 animals per control group females: **1.0, 3.9, 8.3 mg/kg bw/d** (nominal in water) males: **0.9, 3.4, 6.1 mg/kg bw/d** (nominal in water) Vehicle: water Untreated controls included Statistics: no data (not applied) **Parameters investigated:** survival, body weights, clinical examination, gross pathology, histopathology (neoplastic effects and non-neoplastic effecs) of major organs (no detailed data on organs examined)	**Survival:** - no significant differences between controls and treatment groups (males about 62 weeks, females about 48 weeks) **Body weight:** - no significant differences between controls and treatment groups **Neoplastic effects:** *controls (males and females):* - some spontaneous tumours (see Tables 14.6 and 14.7) - no tumours in either the respiratory or digestive tract *treatment groups (males and females)* - highly significant dose-dependent increase in incidence of tumours of the respiratory (larynx and trachea) and digestive tract compared to controls (see Tables 14.6 and 14.7) - all observed tumours in other organs in the treatment groups did not show dose-dependence and are considered as tumours occurring spontaneously - tumour latency decreased with increasing 4-nitrosomorpholine doses (see Tables 14.6 and 14.7) - increased tumour incidences of liver described in text but no detailed data shown	**supporting study** **2 (reliable with restrictions)** **Rationale:** Similar to guideline; *Restrictions:* 30 animals/ treatment group only, no results on clinical investigations reported, no data on histopathology (non-neoplastic effects) **Test material: 4-nitrosomorpholine** **Analytical purity:** 99.5 %	Ketkar MB, Holste J, Preussmann R, Althoff J (1983)

Carcinogenicity study, life-time, similar to OECD TG 451 oral: drinking water Exposure: **daily whole life span** hamster, Syrian male/female 30 animals per treatment group, 50 animals per control group females: **1.0, 3.9, 8.3 mg/kg bw/d** (nominal in water) males: **0.9, 3.4, 6.1 mg/kg bw/d** (nominal in water) (concentrations were calculated related to Ketkar et al. 1983, due to the same treatment procedure in the same laboratory) Vehicle: water Untreated controls included Statistics: no data (not applied) **Parameters investigated:** survival, clinical examination, body weights, gross pathology of major organs (no details on organs examined), histopathology (non-neoplastic and neoplastic effects) of laryngo-tracheal tract	**Survival** *treated males:* - no effects in survival compared to controls *treated females:* - decrease in survival in higher concentrations: *controls:* 67.5 weeks (50 animals), 1.05 mg/kg bw/d: 60 weeks , 3.89 mg/kg bw/d: 47 weeks, 8.2 mg/kg bw/d: 41 weeks) **Neoplastic effects:** - data are only reported for laryngo-tracheal tumours *controls:* males: 0/50 (0 %) females: 0/50 (0 %) *treated males*: - dose-dependent increase in laryngo-tracheal tumours: 0.87 mg/kg bw/d: 6/29 (20.7 %) 3.4 mg/kg bw/d: 13/29 (44.8 %) 6.1 mg/kg bw/d: 24/30 (80 %) *treated females:* - dose-dependent increase in laryngo-tracheal tumours: 1.05 mg/kg bw/d: 12/28 (42.9 %) 3.89 mg/kg bw/d: 14/30 (46 %) 8.2 mg/kg bw/d: 20/30 (66 %)	**key study** **2 (reliable with restrictions)** **Rationale**: similar to guideline; *Restrictions*: 30 animals/ treatment groups only, no data on clinical effects, no data on body weights, neoplastic data restricted to respiratory tract **Test material: 4-nitrosomorpholine** **Analytical purity:** 99.5 %	Cardesa A, Garcia-Bragado F, Ram¡rez J, Ernst H (1990)

Carcinogenicity study, single dosing, observation up to 93 weeks (no guideline followed) oral: gavage Exposure: **single dosing** Observation: animals sacrificed after different time points: 0-3 weeks,4 weeks, 9-22 weeks, 55-93 weeks rat (Sprague-Dawley) male 0-3 weeks: 29 animals, 4 weeks: 6 animals, 9-22 weeks: 6 animals, 27-40 weeks: 8 animals, 55-93 weeks: 13 animals **320 mg/kg bw (nominal in water)** Vehicle: tap water Untreated controls included Statistics: no data **Parameters investigated:** survival, gross pathology and histopathology (non-neoplastic and neoplastic effects) of liver, kidney, spleen, heart, lung	**Mortality:** 17/62 animals died in first three weeks due to acute effect of 4-nitrosomorpholine **Histopathology** *preneoplastic effects:* - some preneoplastic effects in bile (no data on controls) - clear cell tubules (6/8 animals after 27 weeks observation and 7/11 animals after 55 weeks observation) and acidophilic epitheliomes (3/11 animals after 55 weeks observation) in kidney (none in controls) - increase of oncocytic tubules (2/8 animals after 27 weeks observation and 8/11 animals after 55 weeks observation) in kidney compared to controls - increase in clear cell, acidophilic and basophilic foci in liver compared to controls - slightly increase of oncocytic tubules, oncocytic epitheliomes , chromophobe tubules and cysts in liver compared to control group *neoplastic effects:* - hepatocellular carcinoma in 1/11 (9 %) treated animals (after 55 weeks) - cholangiofibroma (bile) in 2/13 (15 %) treated animals (after 55 weeks) - basophilic epithelioma (kidney) in 17/19(89 %) treated animals	**supporting study** **2 (reliable with restrictions) Rationale**: no guideline followed, high single dosing in acute level, 6-29 animals/group only, no detailed clinical analysis of the animals (very detailed examination and documentation of neoplastic and pre-neoplastic effects), results of non-neoplastic histopathology not reported, no data on analytical purity and substance source **Test material: 4-nitrosomorpholine** **Analytical purity and substance source:** no data	Bannasch P, Mayer D, Krech R (1979)

Carcinogenicity study, 20 to 65 weeks (no guideline followed) oral: drinking water Exposure: continuously for **20, 25, 30, 35, 40, 45, 50, 55, 60 and 65 weeks** Observation time: no rat (Sprague-Dawley) male 4 to 12 rats per group **0.5 mg/kg bw/d** (nominal in water, 1mg/100mL) Vehicle: water Untreated controls included (but no results reported) Statistics: no data (not applied) **Parameters investigated:** gross pathology and histopathology (neoplastic effects) of liver	**Neoplastic effects:** 20 - 25 weeks of exposure: 0/8 (0 %) animals with liver tumours 30- 35 weeks of exposure: 0/8 (0 %) animals with liver tumours 40-45 weeks of exposure: 7/16 (44 %) animals with liver tumours (2 adenomas and 5 carcinomas) 50-55 weeks of exposure: 13/14 (93 %) animals with liver tumours (4 adenomas and 10 carcinomas) 60-65 weeks of exposure: 24/24 (100 %) animals with liver tumours (5 adenomas and 40 carcinomas)	**supporting study** **2 (reliable with restrictions) Rationale:** no guideline followed, no data on environmental conditions of animals, 4-12 animals/group only, no data on controls, no mortality, no detailed clinical investigation, examination of liver only, no data on 4-nitrosomorpholine purity and source **Test material: 4-nitrosomorpholine** **Analytical purity and substance source:** no data	Cortinovis C, Klimek F, Nogueira E (1991)

Carcinogenicity study, 2 years (no guideline followed) oral: drinking water Exposure: 5 days/week for 2 years rat (MRC-Wistar) male 48 animals per group **6.4 mg/kg bw/d** (nominal in water) Vehicle: distilled water Untreated controls included (50 animals) Statistics: no data (not applied) **Parameters investigated:** survival, water and food consumption was examined, gross pathology and histopathology (non-neoplastic and neoplastic effects) of major organs, no details given on organs examined	**Survival** *controls:* 96 % (48/50): 60 weeks 78 % (39/50): 80 weeks 24 % (12/50): 100 weeks 4 % (2/50): 120 weeks *treatment group:* 85 % (41/48): 18 weeks 29 % (14/48): 30 weeks 2 % (1/48): 40 weeks 0 % (0/48): 50 weeks **Food consumption** controls: data not shown treatment group: 22±6 g/rat/day **Water consumption:** controls: data not shown treatment group: 24±5 mL/rat/day **Neoplastic effects:** *treatment group:* - details are presented in Table 14.8 - high liver tumour incidences (liver cell carcinoma, liver kupffer cell sarcoma, liver cholangiocarcinoma) compared to controls - induction of liver tumour metastases in lung - no increase in tumour incidence in other organs besides the liver - decrease of latency of spontaneous tumours (brain and forestomach) compared to latencies observed for the controls	**supporting study** **2 (reliable with restrictions) Rationale:** no guideline followed, only one dose tested, no daily substance administration, no detailed clinical analysis, no data on bw, no data on histopathology (non-neoplastic effects), no data on purity and source of substance **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	Mirvish SS, Pelfrene AF, Garcia H, Shubik P (1976)

Table 14. 1 Lijinsky et al., 1988 (2 years): Survival in controls and at the highest dose

Survival	0 mg/kg bw/d	0.23 mg/kg bw/d
100 %	ca. 75 to 90 weeks	ca. 30 to 80 weeks
50 %	ca. 110 to 120 weeks	ca. 100 weeks
0 %	ca. 125 weeks	105 weeks

Table 14. 2 Lijinsky et al., 1988 (2 years): Organs with dose-dependent increase of tumour rate after 4-nitrosomorpholine treatment of rats for up to 100 weeks

Dose (mg/kg bw/day)	Time of treatment (weeks)	Liver[a]				Thyroid	Tongue
		Any tumour (benign or malignant)	Hepatocellular carcinoma	Hemangiosarcoma	Hepatocellular adenoma	C-cell carcinoma	Squamous cell papilloma or carcinoma
0	100	1/80 (1 %)	0/80 (0 %)	0/80 (0 %)	1/80 (1 %)	2/80 (2.5 %)	2/80 (2.5 %)
0.003	100	6/100 (6 %)	1/100 (1 %)	0/100 (0 %)	5/100 (5 %)	0/100 (0 %)	1/100 (1 %)
0.007	100	5/99 (5 %)	0/99 (0 %)	0/99 (0 %)	5/99 (5 %)	0/100 (0 %)	3/100 (3 %)
0.02	100	7/47 (15 %)	0/47 (0 %)	0/47 (0 %)	6/47 (13 %)	1/48 (2 %)	0/48 0 %)
0.04	100	9/48 (19 %)	1/48 (2 %)	0/48 (0 %)	8/48 (16 %)	5/48 (10 %)	1/48 (2 %)
0.09	100	22/48 (46 %)	7/48 (15 %)	5/48 (10 %)	15/48 (31 %)	4/48 (8 %)	1/48 (2 %)
0.23	100	23/24 (96 %)	16/24 (67 %)	13/24 (54 %)	15/24 (62.5 %)	3/24 (12.5 %)	3/24 (12.5 %)
Cox exact test (trend test)		P < 0.0001	P < 0.0001	P < 0.0001	No data	No data	No data

[a]Cholangioma were observed in 1/48 animals at 0.023 mg/kg bw/d and 1/24 animals at 0.33 mg/kg bw/d

Table 14. 3 Lijinsky et al., 1988 (1 year): Survival in controls and at the four highest tested doses

Survival	0 mg/kg bw/d	0.23 mg/kg bw/d	0.58 mg/kg bw/d	1.43 mg/kg bw/d	3.58 mg/kg bw/d
100 %	ca. 75 to 90 weeks	ca. 30 to 80 weeks	ca. 30 to 75 weeks	ca. 30 weeks	ca. 25 weeks
50 %	ca. 110 to 120 weeks	ca. 105 weeks	ca. 80 weeks	ca. 55 weeks	ca. 30 weeks
0 %	ca. 125 weeks	ca. 125 weeks	ca. 100 weeks	ca. 60 weeks	ca. 40 weeks

Table 14. 4 Lijinsky et al., 1988(1 year): Organs with concentration-dependent increase of tumour rate after 4-nitrosomorpholine treatment of rats for up to 50 weeks

Dose (mg/kg bw/day)	Time of treat-ment (weeks)	Liver[a]				Esophagus	Thyroid	Tongue
		Any tumour (benign or malignant)	Hepato-cellular carcinoma	Hemangio-sarcoma	Hepato-cellular adenoma	Squamous cell papilloma or carcinoma	C-cell carcinoma	Squamous cell papilloma or carcinoma
0	100	1/80 (1 %)	0/80 (0 %)	0/80 (0 %)	1/80 (1 %)	0/80 (0 %)	2/80 (2.5 %)	2/80 (2.5 %)
0.02	50	6/48 (12.5 %)	0/48 (0 %)	0/48 (0 %)	6/48 (12.5 %)	0/48 (0 %)	1/48 (2 %)	2/48 (4 %)
0.04	50	7/48 (14.6 %)	1/48 (2 %)	0/48 (0 %)	6/48 (12.5 %)	0/48 (0 %)	4/48 (8 %)	0/48 (0 %)
0.09	50	15/48 (31 %)	5/48 (10 %)	1/48 (2 %)	11/48(22.9 %)	0/48 (0 %)	8/48 (16.7 %)	0/48 (0 %)
0.23	50	14/24 (58 %)	7/24 (29 %)	0/24 (0 %)	9/24 (37.5 %)	0/24 (0 %)	5/24 (20 %)	1/24 (4.2 %)
0.58	50	22/23 (96 %)	15/23 (65 %)	8/23 (35 %)	15/23 (65 %)	3/24 (12.5 %)	2/24 (8 %)	2/24 (8 %)
Cox exact test (trend test)*		**P < 0.0001**	**P < 0.0001**	**P < 0.0001**	**No data**	**No data**	**No data**	**No data**
1.43	40	23/24 (96 %)	16/24 (67 %)	23/24 (96 %)	11/24(45.8 %)	13/24(54.2 %)	2/24 (8 %)	4/24 (17 %)
3.58	25	24/24 (100 %)	15/24 (63 %)	24/24 (100 %)	20/24 (83 %)	5/24 (20 %)	0/24 (0 %)	0/24 (0 %)

*Cox exact test performed only for doses of 0.02 to 0.58 mg/kg bw/d, [a] cholangioma: 0; hepatocholangioadenoma 2/24 at 0.83 mg/kg bw/d

Table 14. 5 Weber and Bannasch, 1994a Incidence and number of hepatocellular tumours

Time of treatment (weeks)	control		6 mg/kg bw/d		12 mg/kg bw/d		24 mg/kg bw/d	
	Hepato-cellular adenomas	Hepato-cellular carcinomas	Hepato-cellular adenomas	Hepato-cellular carcinomas	Hepato-cellular adenomas	Hepato-cellular carcinomas	Hepato-cellular adenomas	Hepato-cellular carcinomas
7	0/10 (0 %)	0/10 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	a	0/5 (0 %)
11	0/10 (0 %)	0/10 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	a	0/18 (0 %)
15	0/10 (0 %)	0/10 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	a	4/15 (27 %)
20	0/8 (0 %)	0/8 (0 %)	0/6 (0 %)	0/6 (0 %)	2/6 (33 %)	0/5 (0 %)	a	7/11 (64 %)
27	0/10 (0 %)	0/10 (0 %)	2/6 (33 %)	0/6 (0 %)	5/6 (83 %)	1/6 (17 %)	-	
37	0/10 (0 %)	0/10 (0 %)	2/8 (25 %)	3/8 (38 %)	19/25 (76 %)	14/25 (56 %)	-	
50	0/10 (0 %)	0/10 (0 %)	20/30 (67 %)	17/30 (57 %)	-	-	-	
65	0/10 (0 %)	0/10 (0 %)	-	-	-	-	-	
80	2/5 (40 %)	0/5 (0 %)	-	-			-	

a: large number of nodules observed which could not clearly be distinguished from true adenomas

Table 14. 6 Ketkar et al. 1983: Incidence and latency of tumours observed in 4-nitrosomorpholine treated male hamsters

A, harderian gland adenoma; B, salivary duct adenoma; C, thyroid adenoma; D, spleen haemangioendothelioma; E, papillary polypintestine;

Dose [mg/kg bw/d]	sex	Total number of tumour bearing animals	Respiratory tract		Digestive tract		Other organs (not dose dependent)
			Tumour Incidence	Tumour Latency	Tumour Incidence	Tumour Latency	
0	males	8/50 (16 %)	0/50 (0 %)	-	0/5 (0 %)	-	1 x B,P, O,J 2 x C, 4 x I
0.9	males	12/29 (41.4 %)	8/29 (27.6 %)	82.88 ± 11.33	4/29 (13.79)	-	1 x H,I, K,Q 2 x C
3.4	males	14/29 (48.3)	13/29 (44.8 %)	82.54 ± 15.13	9/29 (31.3 %)	81.22 ± 20.38	1 x F,D,G,R 2 x B,C
6.1	males	26/30 (86.7 %)	21/30 (70 %)	70.05 ± 12.82	18/30 (60 %)	69.39 ± 14.63	1x A,G,I,J,K

F, forestomach papilloma; G, colon adenocarcinoma; H, thyroid adenocarcinoma; I, adrenal cortical adenoma; J, adrenal haemangioma; K, cortical carcinoma; L, uterine leiomyoma; M, uterine adenocarcinoma; N, uterine adenoma; 0, testicular leydig cell tumour; P, malignant schwannoma; Q, malignant lymphoma; R, intestine leiomyosarcoma.

Table 14. 7 Ketkar et al., 1983: Incidence and latency of tumours observed in 4-nitrosomorpholine orally-treated female hamsters

Dose [mg/kg bw/d]	sex	Total number of tumour bearing animals	Respiratory tract		Digestive tract		Other organs (not dose dependent)
			Tumour Incidence	Tumour Latency	Tumour Incidence	Tumour Latency	
0	females	3/50 (6 %)	0/50 (0 %)	-	0/5 (0 %)	-	2 x C 1 x L
1.0	females	14/28 (50 %)	14/28 (50 %)	65.14 ± 8.64	0/5 (0 %)	84.5 ± 11.33	1 x Q
3.9	females	17/30 (56.7 %)	16/30 (53.3 %)	56.75 ± 11.59	2/30 (6.67 %)	78.00 ± 11.33	1 x B,C,G,M,N
8.3	females	23/30 (76.7 %)	22/30 (73.3 %)	45.73 ± 11.61	6/30 (20 %)	52.17 ± 7.08	1 x B,D, E, Q, I 2x C

A, harderian gland adenoma; B, salivary duct adenoma; C, thyroid adenoma; D, spleen haemangioendothelioma; E, papillary polypintestine;
F, forestomach papilloma; G, colon adenocarcinoma; H, thyroid adenocarcinoma; I, adrenal cortical adenoma; J, adrenal haemangioma; K, cortical carcinoma; L, uterine leiomyoma; M, uterine adenocarcinoma; N, uterine adenoma; 0, testicular leydig cell tumour; P, malignant schwannoma; Q, malignant lymphoma; R, intestine leiomyosarcoma.

Table 14. 8 Mirvish et al., 1976: tumour incidence in 4-nitrosomorpholine treated MRC rats

Tumour type	Controls		4-nitrosomorpholine treated rats (6.4 mg/kg bw/d)	
	Incidence	Latency (weeks)	Incidence	Latency (weeks)
Forestomach tumours: Squamous cell papilloma	2/48 (4 %)	77 ± 23	1/41 (2.4 %)	32
Forestomach tumours: Squamous cell carcinoma	0/48 (0 %)	-	0/41 (0 %)	-
Liver cell carcinoma	0/48 (0 %)	-	17/41 (41.5 %)	30 ± 5
Liver Kupffer cell sarcoma	0/48 (0 %)	-	28/41 (68.3 %)	28 ± 4
Liver Cholangiocarcinoma	0/48 (0 %)	-	1/41 (2.4 %)	34
Liver tumours (other types)	0/48 (0 %)	-	0/41 (0 %)	-
Liver tumours metastases in lung	0/48 (0 %)	-	24/41 (58.5 %)	28 ± 4
Brain	2/48 (4 %)	100 ± 8	2/41 (4.8 %)	36 ± 6
Testis	8/48 (16.7 %)	100 ± 13	0/41 (0 %)	-
Adrenal	3/48 (6.25 %)	99 ± 6	0/41 (0 %)	-
Pituitary adenoma	1/48 (2 %)	77	0/41 (0 %)	-

4.10.1.2 Carcinogenicity: inhalation

The results of carcinogenicity studies by exposure via inhalation are summarised in Table 15.

Table 15 Relevant inhalation carcinogenicity studies

Method	Results	Remarks	Reference
Carcinogenicity study, 6 weeks (no guideline followed) inhalation: vapour (whole body) Exposure**: 6 weeks** (4h/day, 4-5 days a week, in total 29 administrations) Observation: no data (presumably whole life span) rat (Sprague-Dawley) female 24 animals per treatment group, 17 animals per control group **0.5 mg/kg bw/d** (nominal inhaled) = **0.0077 mg/L** (in the breathing air) Vehicle: unchanged (no vehicle) Untreated controls included Statistics: no data (not applied) **Parameters investigated:** body weights, histopathology (neoplastic effects) of major organs (no detailed data)	**Body weights:** - no differences between controls and treatment group **Neoplastic effects:** *controls:* - adenomas of mammary gland: 2/17 (11.8 %) - adenocarcinomas of mammary gland: 2/17 (11.8 %) - pheochromocytomas of suprarenal glands: 3/17 (17.6 %) - adenomas of pituitary gland: 2/17 (11.8 %) *treatment group:* - hepatocellular carcinomas: 4/24 (16.7 %) - liver neoplastic nodules: 5/24 (20.8 %) - nasal region: mucoepidermoidal carcinoma: 1/24 (4.2 %) - brain neuroblastoma:1/24 (4.2 %) - follicular carcinoma of thyroid gland: 1/24 (4.2 %)	**supporting study** **2 (reliable with restrictions)** **Rationale**: no guideline followed, only one dose tested, 24 animals/group only, treatment for only 6 weeks, no males tested, no data on survival, clinical investigations, histopathology (non-neoplastic effects) **Test material: 4-nitrosomorpholine** **Form:** vapour **Analytical purity and substance source:** no data	Klein RG, Spiegelhalder B, Preussmann R (1990)

Carcinogenicity study, 5 weeks (no guideline followed) inhalation: vapour (whole body) Exposure: **5 weeks** (4h/day, 4-5 days a week, in total 21 administrations Observation: no data (presumably the whole life span) Hamster (Syrian) males 32 animals in treatment group, 31 animals in control group **1.8 mg/kg bw/d** (nominal inhaled) = **0.014 mg/L** (in the breathing air) Vehicle: unchanged (no vehicle) Untreated controls included Statistics: no data (not applied) **Parameters investigated:** body weights, histopathology (neoplastic effects) of major organs (no detailed data)	**Body weights:** - no differences between controls and treatment group observed **Neoplastic effects:** *controls:* - cholangiomas of liver: 4/31 (12.9 %): - pheochromocytomas of suprarenal glands: 5/31 (16.1 %) - leukemia: 1/31 (3.2 %) *treatment group:* - hepatocellular carcinomas: 2/32 (6.2 %) - liver hemangioendothelioma: 1/32 (3.1 %) - neurogenic sarcoma: 2/32 (20.8 %) - adenocarcinoma of the spleen: 1/32 (3.1 %) - adenocarcinoma of the stomach: 1/32 (3.1 %) - papilloma of the forestomach: 4/32 (12.5 %) - papilloma of the trachea: 5/32 (15.6 %)	**supporting study** **2 (reliable with restrictions)** **Rationale:** no guideline followed, only one dose tested, 24 animals/group only, treatment for only 6 weeks, no females tested, no data on survival, clinical investigations, histopathology (non-neoplastic effects) **Test material: 4-nitrosomorpholine** **Form:** vapour **Analytical purity and substance source:** no data	Klein RG, Spiegelhalder B, Preussmann R (1990)

4.10.1.3 Carcinogenicity: dermal

There are no dermal carcinogenicity studies available for 4-nitrosomorpholine.

4.10.1.4 Carcinogenicity: other routes of administration

Table 16 Relevant carcinogenicity studies with other administration routes than oral, dermal, inhalation

Method	Results	Remarks	Reference
Carcinogenicity study, 30 weeks (no guideline followed) intravesicular Exposure: twice per week for **30 weeks** Observation time: until animals were moribund or died naturally rat (Fischer 344) female 12 animals per group **3.6 mg/kg bw/day** (nominal injected) Vehicle: 25 % ethanol solution in water controls (vehicle) included Statistics: no data (not applied) **Parameters investigated:** survival, gross pathology and histopathology (non-neoplastic and neoplastic effects) of ≥ 20 organs	**Survival:** controls: 102 weeks (median) treatment group: 35 weeks (median) **Neoplastic effects:** *controls:* - no tumours observed (0/12 (0 %)) *treatment group:* - liver tumours: 58 % of animals - nasal tumours : 100 % of animals - esophagus tumours: 17 % of animals	**supporting study** **2 (reliable with restrictions) Rationale:** no guideline followed, non-standard substance administration via intravesicular injection, 12 animals/group only, one dose level only, females only, high ethanol concentrations in vehicle (25 %), dose resulted in low survival of the treated animals, no data on clinical examination, no results of histopathology (non- neoplastic effects) **Test material: 4-nitrosomorpholine** **Analytical purity:** > 98 %	Lijinsky W, Thomas BJ, Kovatch RM (1991b)

Carcinogenicity study, life time (no guideline followed) subcutaneous Exposure: once weekly for the **whole life** hamster, Syrian male/female 20 animals per group females: **4.0** (1/20 LD50), **8.0** (1/10 LD50), **16.1** (1/5 LD50) **mg/kg bw/d** (nominal injected) males: **3.5** (1/20 LD50), **7.0** (1/10 LD50), **14.1** (1/5 LD50) **mg/kg bw/d** (nominal injected) Vehicle: (controls received saline) Controls (treated with saline were included) Statistics: no data (not applied) **Parameters investigated:** survival, gross pathology and histopathology (non-neoplastic and neoplastic effects) of major organs (no detailed data)	**Survival:** *males:* 3.5 mg/kg bw/d: 35 weeks 7.0 mg/kg bw/d: 32 weeks 14.1 mg/kg bw/d: 25 weeks *females:* 4.0 mg/kg bw/d: 31 weeks 8.0 mg/kg bw/d: 28 weeks 16.1 mg/kg bw/d: 24 weeks **Neoplastic effects:** - dose-dependent increase in trachea tumour incidence in male and females (see Table 16.1) - the highest dose level resulted in a 100 % trachea tumour incidence in males and 84 % in females (see Table 16.1) - high but no dose-dependent incidence of tumours of the nasal cavity (see Table 16.1) - very few tumours in larynx and lungs (as no data of controls are given, these could be spontaneous tumours) - data of controls are not shown	**supporting study** **2 (reliable with restrictions) Rationale:** no guideline followed, no daily dosing, 20 animals/group only, no standard administration route (subcutan), no detailed clinical investigation, no control data (results) reported, no body weights, no data on histopathology (non-neoplastic effects), no data on 4-nitrosomorpholine source and purity **Test material: 4-nitrosomorpholine** **Analytical purity and source:** no data	Haas H, Mohr U, Krueger FW (1973)

Carcinogenicity study, life time (no guideline followed) subcutaneous Exposure: once weekly for **whole life** hamster, European male/female 10 animals per treatment group females: **3.5** (0.05 LD50), **7.0** (0.1 LD50), **14.1** (0.2 LD50) **mg/kg bw/d** (nominal injected) males: **3.1** (0.05 LD50), **6.1** (0.1 LD50), **12.3** (0.2 LD50) **mg/kg bw/d** (nominal injected) untreated controls included (20 animals in control groups) Statistics: no data (not applied) **Parameters investigated:** survival, gross pathology and histopathology (non-neoplastic and neoplastic effects) of major organs (no detailed data)	**Survival:** *males:* controls: no data 3.1 mg/kg bw/d: 23.7 weeks 6.1 mg/kg bw/d: 23.4 weeks 12.3 mg/kg bw/d: 17.6 weeks *females:* controls: no data 3.5 mg/kg bw/d: 29.3 weeks 7.0 mg/kg bw/d: 23.5 weeks 14.1 mg/kg bw/d: 20.5 weeks **Body weights:** - controls (males and females): steady increase until week 40 - treatment group: increase at the beginning (approx. 15 weeks), after that highly decrease *Males* (bw at about 25 weeks): controls: about 440 g 3.1 mg/kg bw/d: about 330 g 6.1 mg/kg bw/d: about 300 g 12.3 mg/kg bw/d: about 230 g *Females* (bw at about 25 weeks): controls: about 350 g 3.5 mg/kg bw/d: about 270 g 7.0 mg/kg bw/d: about 130 g 14.1 mg/kg bw/d: about 100 **g** **Neoplastic effects:** - controls: no neoplasms - 100 % tumour incidence at almost all dose levels in females and males of nasal cavity tumours (see Table 16.2) - dose-dependent increase of tumours of trachea in females and males (see Table 16.2) - increased tumour incidence in forestomach in females and males - dose-dependent increase in tumour incidence of esophagus/mouth tumours in females and males (see Table 16.2) - (tumours reported for other organs seem to be spontaneous: low tumour incidence, no dose-dependence) (see Table 16.2)	**supporting study** **2 (reliable with restrictions) Rationale:** no guideline followed, 10 animals/group only, no daily dosing, no vehicle described, no relevant administration route (subcutan), no data on 4-nitrosomorpholine source and purity, no examination of clinical effects, no data on histopathology (non-neoplastic effects) experimental result **Test material: 4-nitrosomorpholine** **Analytical purity and source:** no data	Mohr U, Reznik G, Reznik-Schueller H (1974)

Carcinogenicity study, life time (no guideline followed) subcutaneous Exposure: once weekly for **whole life** hamster, Chinese (Cricetulus griseus) male/female 20 animals per group females: **1.2** (1/20 LD50), **2.3** (1/10 LD50), **4.7** (1/5 LD 50) **mg/kg bw/d** (nominal injected) males: **1.2** (1/20 LD50), **2.3** (1/10 LD50), **4.7** (1/5 LD 50) **mg/kg bw/d** (nominal injected) Controls treated with vehicle included Statistics: no data (not applied) **Parameters investigated:** survival, gross pathology and histopathology (non-neoplastic and neoplastic effects) of major organs (no detailed data)	**Survival:** *males:* controls: 84.35 weeks 1.2 mg/kg bw/d: 49.65 weeks 2.3 mg/kg bw/d: 36.20 weeks 4.7 mg/kg bw/d: 24.25 weeks *females:* controls: 88.00 weeks 1.2 mg/kg bw/d: 37.85 weeks 2.3 mg/kg bw/d: 32.40 weeks 4.7 mg/kg bw/d: 21.74 weeks **Neoplastic effects:** - in controls no neoplastic effects (see Table16.3) - overall a 100 % tumour incidence in treated female and male hamsters (see Table 16.3) - highly increased tumour incidences in nasal cavity (males and females), tongue palate, pharynx, esophagus and forestomach in males and females (see Table 16.3) - the highest incidence (about 90 % of the animals) of tumours in esophagus and forestomach (see Table 16.3) - some increase (<35 %) in tumour incidences in larynx and lungs (see Table 16.3) - increase clearly dose-dependent for nasal tumours (females), tumours of the tongues palate (males and females), the esophagus and forestomach (males and females) (see Table 16.3) - for the organs brain, cheek pouch and the liver the observed few tumours in females and males are considered to be spontaneous (see Table 16.3)	**supporting study** **2 (reliable with restrictions) Rationale**: no guideline followed, 20 animals/group only, no daily dosing, no relevant administration route (subcutan), no data on 4-nitrosomorpholine source and purity, no examination of clinical effects, no data on histopathology (non-neoplastic effects) **Test material: 4-nitrosomorpholine** **Analytical purity and source:** no data	Reznik G, Mohr U, Kmoch N (1976)

Carcinogenicity study, 15 weeks exposure, whole life time observation (no guideline followed) intratracheal Exposure: once weekly for **15 weeks** Observation: for whole life hamster, Syrian male 30 animals per treatment group (21 were examined) **0.13 mg/kg bw/d (0.1 mg/week/animal)** Vehicle: 0.025 M phosphate buffer solution Controls treated with vehicle included (39 animals per control group; 27 were examined) Statistics: log-rank test **Parameters investigated:** survival, gross pathology and histopathology (neoplastic and non-neoplastic effects) of main visceral organs and organs with tumours (no detailed data which organs examined)	**Survival:** *survival after 15 instillations:* controls: 29/39 survived (74 %) treatment group: 22/30 survived (73 %) - no effect in survival after 15 instillations compared to controls - high rate of dead animals due to intratracheal instillations *overall survival* 50 % survival: controls: about 400 weeks treatment group: about 250 weeks 0 % survival: controls: 850 weeks treatment group: about 700 weeks **Neoplastic effects: (related to examined animals)** *controls:* - lung tumours: 1/27 (3.7 %) (benign) - no other tumours reported *treatment group:* - tumours of trachea: 9/21 (43 %); significantly different from the control ($P < 0.001$) - no other tumours reported	**supporting study** **2 (reliable with restrictions) Rationale:** no guideline followed, 30 animals/group only, one dose tested only, high rate of dead animals after intratracheal instillations no detailed clinical investigation, no data on bw, no data on histopathology of non-neoplastic effects **Test material: 4-nitrosomorpholine** **Analytical purity:** > 99 %, commercial substance source	Ishinishi N, Tanaka A, Hisanaga A, Inamasu T, Hirata M (1988)

Carcinogenicity study, single dosing observation (no guideline followed) subcutaneous Exposure: **single dosing** Observation: for whole life hamster, Syrian male/female 5 animals per group **50, 100, 200, 400 mg/kg bw** (nominal conc.) Vehicle: physiological saline Controls: untreated (20 females and 20 males) **Parameters investigated:** gross pathology and histopathology (neoplastic effects and non-neoplastic effects) of major organs (no detailed data)	**Neoplastic effects:** *controls:* - no tumours in the respiratory system observed - some tumours observed in Harderian gland, parathyroid gland, adrenal gland and forestomach (no detailed data) *treatment group:* - dose-dependent increase of tumours in the respiratory system - total number of tumour bearing hamsters with tumours in respiratory system: 25 mg/kg: 2/10 (20 %) 50 mg/kg: 3/10 (30 %) 100 mg/kg: 7/10 (70 %) 200 mg/kg: 3/10 (30 %) - tumours mostly in trachea, but also in nasal cavity, larynx, bronchi - first tumours developed between 42 and 51 weeks (presumably time of death) - some not dose-dependent tumours also observed in other organs including Harderian gland, thyroid gland , parathyroid gland, forestomach one hepatocellular adenoma (no detailed data)	**supporting study** **2 (reliable with restrictions) Rationale:** no guideline followed, single dosing only, 5 animals/group only, no data on survival, no data on body weights, no detailed clinical investigation, no data on histopathology (non-neoplastic effects) **Test material: 4-nitrosomorpholine** **Analytical purity:** no data, non-commercial substance source	Althoff J, Wilson R, Cardesa A, Pour P (1974)

Table 16. 1 Results of Haas et al. 1973: Tumour incidences in hamsters treated subcutaneously with 4-nitrosomorpholine

Dose [mg/kg bw/d	Nasal tumours	Larynx tumours	Trachea tumours	Lung tumours
males				
3.5	7/19 (36.8 %)	0/19 (0 %)	16/19 (84 %)	0/19 (0 %)
7	10/18 (55.6 %)	0/18 (0 %)	17/18 (94 %)	1/18 (5.6 %)
14	7/18 (38.9 %)	1/18 (5.6 %)	18/18 (100 %)	1/18 (5.6 %)
females				
4	3/16 (18.8 %)	0/16 (0 %)	11/16 (68.8 %)	0/16 (0 %)
8	7/17 (41.2 %)	0/17 (0 %)	10/17 (58.8 %)	1/17 (5.9 %)
16	6/19 (31.6 %)	0/19 (0 %)	16/19 (84.2 %)	1/19 (5.3 %)

Table 16. 2 Results of Mohr et al., 1974: Tumour incidences in hamsters (European) treated subcutaneously with 4-nitrosomorpholine

Dose [mg/kg bw/d	Nasal cavity	Nasopharyngealduct	Larynx	Trachea	Lungs	Forestomach	Palate	Mouth cheek pouch, esophagus
males								
3.1	10/10 (100 %)	1/10 (10 %)	2/10 (20 %)	2/10 (20 %)	2/10 (20 %)	2/10 (20 %)	1/10 (10 %)	3/10 (30 %)
6.1	9/10 (90 %)	2/10 (20 %)	3/10 (30 %)	5/10 (50 %)	1/10 (10 %)	2/10 (20 %)	2/10 (20 %)	3/10 (30 %)
12.3	10/10 (100 %)	1/10 (10 %)	2/10 (20 %)	4/10 (40 %)	3/10 (30 %)	5/10 (50 %)	2/10 (20 %)	4/10 (40 %)
females								
3.5	9/10 (90 %)	2/10 (20 %)	0/10 (0 %)	1/10 (10 %)	2/10 (20 %)	4/10 (40 %)	0/10 (0 %)	1/10 (10 %)
7.0	10/10 (100 %)	0/10 (0 %)	4/10 (40 %)	7/10 (70 %)	2/10 (20 %)	4/10 (40 %)	2/10 (20 %)	6/10 (60 %)
14.1	10/10 (100 %)	1/10 (10 %)	0/10 (0 %)	5/10 (50 %)	1/10 (10 %)	6/10 (60 %)	1/10 (10 %)	4/10 (40 %)

Table 16. 3 Results of Reznik et al., 1976: Tumour incidences in Chines hamsters treated subcutaneously with 4-nitrosomorpholine

Dose [mg/kg bw/d	Total tumour incidence in %	Nasal cavity	Brain	Larynx	Lungs	Cheek pouch	Tongue palate	Pharynx	Esophagus	Forestomach	Liver
males											
controls	0	0/19 (0 %)	0/19 (0 %)	0/19 (0 %)	0/19 (0 %)	0/19 (0 %)	0/19 (0 %)	0/19 (0 %)	0/19 (0 %)	0/19 (0 %)	0/19 (0 %)
1.2	100	2/15 (13 %)	1/15 (7 %)	1/15 (7 %)	5/15 (33 %)	0/15 (0 %)	0/15 (0 %)	3/15 (20 %)	7/15 (47 %)	12/15 (80 %)	0/15 (0 %)
2.3	100	0/20 (0 %)	0/20 (0 %)	7/20 (35 %)	1/20 (5 %)	0/20 (0 %)	7/20 (35 %)	6/20 (30 %)	14/20 (70 %)	16/20 (80 %)	1/20 (5 %)
4.7	100	6/19 (32 %)	0/19 (0 %)	1/19 (5 %)	0/19 (0 %)	1/19 (5 %)	11/19 (58 %)	5/19 (26 %)	17/19 (90 %)	16/19 (84 %)	0/19 (0 %)
females											
controls	0	0/20 (0 %)	0/20 (0 %)	0/20 (0 %)	0/20 (0 %)	0/20 (0 %)	0/20 (0 %)	0/20 (0 %)	0/20 (0 %)	0/20 (0 %)	0/20 (0 %)
1.2	100	8/17 (47 %)	1/17 (6 %)	3/17 (18 %)	1/17 (6 %)	0/17 (0 %)	7/17 (41 %)	4/17 (24 %)	14/17 (82 %)	12/17 (71 %)	0/17 (0 %)
2.3	73.7	8/19 (42 %)	1/19 (5 %)	6/19 (32 %)	1/19 (5 %)	0/19 (0 %)	7/19 (37 %)	4/19 (21 %)	13/19 (68 %)	9/19 (47 %)	0/19 (0 %)
4.7	90	10/20 (59 %)	0/20 (0 %)	3/20 (15 %)	3/20 (15 %)	1/20 (5 %)	12/20 (60 %)	8/20 (40 %)	18/20 (90 %)	18/20 (90 %)	0/20 (0 %)

4.10.2 Human information

No human data (case reports or epidemiological studies) that identified the relationship between cancer and exposure were available specifically for 4-nitrosomorpholine.

4.10.3 Other relevant information

In 1978, the IARC assessed 4-nitrosomorpholine for its carcinogenic potential. The IARC concluded that there is sufficient evidence for a carcinogenic effect of 4-nitrosomorpholine and the substance was classified as Group 2B (possibly carcinogenic to humans) due to the lack of human data (IARC, 1978).

4-nitrosomorpholine is listed in the 13th Report on Carcinogens of the National Toxicology Program (NTP, 2014). It was already first listed in the Second Annual Report on Carcinogens in 1981. In the report it is concluded that 4-nitrosomorpholine is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals. The report refers to the IARC report (IARC, 1978).

In their Opinion on Nitrosamines and Secondary Amines in Cosmetic Products (SCCS 2012) the SCCS determined a T25 value for 4-nitrosomorpholine based on experimental carcinogenicity data (Lijinsky et al., 1988, Lijinsky and Reuber, 1982 (2 substudies) and Hecht et al., 1989). The estimated mean T25 value based on liver carcinomas in four rat cancer studies was 0.094 mg/kg bw/d for 4-nitrosomorpholine (details in Annex I of SCCS, 2012). It was concluded that, based on the estimated T25, 4-nitrosomorpholine belongs to the most potent carcinogenic nitrosamines comparable to nitrosodimethylamine (T25 0.058 mg/kg bw/d) and nitrosodiethylamine (T25 0.085 mg/kg bw/d).

Further, 4-nitrosomorpholine has been classified as carcinogen of category 2 by the German Committee on Hazardous Substances (AGS) (AGS 2007). 12 N-nitrosamines including 4-nitrosomorpholine were classified as carcinogens by MAK (MAK 2012).

4.10.4 Summary and discussion of carcinogenicity

4-nitrosomorpholine belongs to the chemical group of nitrosamines. Many nitrosamines are known to be potent carcinogens in animals (rodents). Hence, there are many studies available in which the carcinogenic potential of 4-nitrosomorpholine was investigated and 4-nitrosomorpholine has often been applied as model substance in carcinogenesis research (e.g. Bannasch et al., 1978a, Bannasch et al., 1978b, Bannasch et al. 1972, Bannasch et al., 1980, Reznik-Schller, 1977, Romen et al., 1972, Volm et al., 1990).

From all available studies dealing with the carcinogenicity of 4-nitrosomorpholine, 28 studies were identified to be relevant for the assessment of the carcinogenic potential. All identified relevant carcinogenicity studies are summarised in the tables 14 to 16. Most of these studies were performed in rats by oral substance administration (14 drinking water studies and two studies by gavage). Three of the studies are oral studies in hamsters (two by drinking water and one by gavage) and one is a drinking water study in mice. There further exist two inhalation carcinogenicity studies with 4-nitrosomorpholine, one in hamsters and one in rats. In addition, five studies using non-standard substance administration routes are available: three subcutaneous studies in hamsters, one intratracheal study in hamsters and one study in rats using intravesicular substance administration. No dermal carcinogenicity study is available for 4-nitrosomorpholine.

All of the identified relevant carcinogenicity studies with 4-nitrosomorpholine are journal articles and none of these were fully compliant to the standardised test guidelines such as OECD TG 451 or 452. In none of the available studies a detailed clinical examination, the investigation of haematological parameters and clinical chemistry of the treated animals were performed. In many cases, the analytical purity of the used 4-nitrosomorpholine was not given. Nevertheless, five of the relevant studies (three oral studies in rats and two oral studies in hamsters) were considered to be of

sufficient quality (similar to OECD test guidelines, reliable with restrictions) to allow a substantiated assessment of the carcinogenicity potential of 4-nitrosomorpholine as they were performed using more than one dose level, a sufficient number of animals (mostly > 20 animals per group), control animals and a relevant route of substance administration. All other studies had shortcomings mainly including i.e. the lack of controls, the testing of one dose level only, using of a low number of animals or short treatment times. These studies were considered as supporting studies. Shortcomings and the assessed reliability status of each study are summarised in Tables 14 to 16 and in the technical dossier.

If doses were not given as mg/kg bw/d in the respective studies, which was the case for many of the available studies, the doses were calculated as mg/kg bw/d to enable a comparative assessment. Calculated doses for all studies are documented in Tables 14 to 16 and the technical dossier. Especially for the drinking water studies the dose levels could be given as estimates only as an exact calculation as mg/kg bw/d was not possible because the exact drinking water consumption per rat was not measured and in many studies body weights of the animals were not given. In such cases, estimates for body weights for rats and mice were used as described in the 'Guidance on the application of the CLP Criteria' (Table 3.9.2-c, Version 4.0, 2013) and for hamsters in the Guidance on IR & CSA-Chapter R.8 (Table R.8-3, Version 2.1, 2012).

Overall, from all reliable and also supporting studies it can be summarised that 4-nitrosomorpholine highly increases the tumour incidences in multiple organs in female and male rats, female and male hamsters and female mice (no mice study with male animals available) after oral, inhalation, intratracheal or subcutaneous treatment at low doses. The results of the relevant studies are discussed and summarised in detail below.

Oral studies in rats

There are three oral carcinogenicity studies available in rats considered to be of sufficient quality (reliable with restriction) to enable a substantiated assessment of the carcinogenic potential of 4-nitrosomorpholine (Lijinsky et al., 1988, 50 weeks and 100 weeks exposure time; Weber and Bannasch, 1994). All other available oral studies with 4-nitrosomorpholine in rats are considered to be supporting studies and will be discussed in relation to Lijinsky et al., 1988 and Weber and Banasch, 1994.

In the study by Linjinsky et al., 1988 who treated female Fischer 344 rats with six dose levels (0.003, 0.007, 0.02, 0.04, 0.09 and 0.23 mg/kg bw/d) of 4-nitrosomorpholine for 100 weeks with the drinking water, a highly significant ($P < 0.0001$) dose-dependent increase in liver tumours was observed . Most of the tumours were hepatocellular carcinoma, hemangiosarcoma and hepatocellular adenoma. At the highest dose tested (0.23 mg/kg bw/d) the tumour incidence in the liver was 96 % (any benign or malignant liver tumours). Even for the lowest tested dose of 3 µg/kg bw/d a tumour rate of 6 % (benign or malignant tumours in liver) was found. Observed liver tumour incidence (any benign or malignant neoplasms) in the internal controls was in maximum 1 % which is also well in accordance with historical controls (see Table 17). The high liver tumour incidences at the two highest doses correlated with a significant but moderate reduction of survival. Up to and including a dose of 0.04 mg/kg bw/d no significant differences in survival were observed compared to controls. No other clinical effects besides neoplastic effects were measured or reported in the study and no information on the cause of death is available from the study publication. The DS (Dossier Submitter) interpretation is that a life shortening effect of the liver tumours may be assumed. Besides to the liver, 4-nitrosomorpholine treatment of rats also resulted in slightly increased tumour rates (up to 12.5 % at 0.23 mg/kg bw/d) in the thyroid and tongue (Lijinsky et al., 1988) compared to internal and historical controls (see Table 17). For both organs the highest tumour incidences were found for the highest tested dose.

In the study by Linjinsky et al., 1988 a second experiment is reported using the same experimental conditions but a shorter treatment time (50 weeks). Seven different dose levels, administered with the drinking water (0.02, 0.04, 0.09, 0.23, 0.58, 1.43 and 3.58 mg/kg bw/d), were examined. This study is also considered as reliable with restrictions. Due to high mortality in the two highest doses, exposure time was reduced to 40 and 25 weeks, respectively. Overall, a highly significant ($P < 0.0001$) dose-dependent increase in the total liver tumour incidence was observed after 50 weeks of treatment. Consistent to the results after 100 weeks of treatment, most of the liver tumours were hepatocellular carcinoma, hemangiosarcoma and hepatocellular adenoma. A 12.5 % rate of any benign or malignant liver tumours was already detectable at the lowest tested dose of 0.02 mg/kg bw/day. The highest tested dose lead to a 100 % liver tumour incidence in the rats. As expected, liver tumour incidences were lower compared to 100 weeks of treatment at all tested dose levels, e.g. at 0.23 mg/kg bw/d a lower liver tumour incidence was observed 50 weeks after treatment (58 % benign or malignant neoplasms, 29 % hepatocellular carcinoma and 0 % hemangiosarcoma) than 100 weeks after treatment (96 % benign or malignant neoplasms, 67 % hepatocellular carcinoma and 54 % hemangiosarcoma). Also in this study, the high liver tumour rate in the three highest test doses correlated with a high mortality. All animals treated with 3.58 mg/kg bw/d had died after 40 weeks. Controls animals of the same experiment survived 125 weeks. This is in line with the DS interpretation that a life shortening effect of the liver tumours may be assumed.

50 weeks after treatment rats also developed tumours of the thyroid (≥ 0.04 mg/kg bw/d: 8 %) and the tongue (≥ 0.023 mg/kg bw/d: 4.2 %). For both organs tumour incidences in internal and historical controls are low (≤ 2.5 %) (Table 17). Moreover, at doses ≥ 0.58 mg/kg bw/d increased tumours incidences in the esophagus (up to 54.2 %) were observed. For the historical and also internal controls of female F344 rats a 0 % tumour incidence in the esophagus is reported. Incidences for tumours of the thyroid, the tongue and esophagus did not show a clear dose-dependence for the three highest doses tested. For the thyroids (C-cell carcinoma), the highest tumour incidence (12.5 %) was found at 0.23 mg/kg bw/d and for the tongue (17 %) at the second highest dose level of 1.43 mg/kg bw/d, but a 0 % tumour incidence was detected for both organs at the highest tested dose level (3.58 mg/kg bw/d). Similarly, for the esophagus, the highest found tumour incidence in treated animals was about 54 % at 1.43 mg/kg bw/d but only 20 % at the highest dose (3.58 mg/kg bw/d). A possible reason could be the high mortality observed in the high dose groups.

The internal controls in the study by Lijinsky et al., 1988 showed moderate to high numbers of spontaneous leukemia, adenoma and carcinoma of the adrenal cortex, pheochromocytoma of the adrenal medulla, fibroadenoma in the mammary, carcinoma and adenoma in the pituitary and tumours in the uterus (Table 17). These spontaneous tumour incidences are well comparable with the incidences reported for the historical controls of female F344 rats (Table 17). Observed tumours in organs of 4-nitrosomorpholine treated animals which occurred at about the same levels as in internal control animals and which showed high variations in incidence at different dose levels were considered to be spontaneous and not treatment related. Treatment related tumours in other organs than liver, esophagus, thyroids and tongue were not detected for 4-nitrosomorpholine in these studies.

Table 17 Comparison of overall tumour incidences from historical controls of the NTP Historical Controls Report (NTP 2010) (female F344/N rats, about 700 day's exposure, water) with internal controls in the study of Lijinsky et al., 1988 for selected organs.

	NTP Historical Controls Report (NTP 2010) (female F344 rats, about 700 days exposure, water)	**Lijinsky et al., 1988 (female F344 rats, about 700 days exposure, water)**
Liver (any tumour)	0.67 % (hemangiosarcoma 0 %, hepatocellular carcinoma 0 %)	1.25 %
Esophagus (any tumour)	0 %	0 %
Thyroids C-cell adenoma: C-cell carcinoma: F-cell adenoma: F-cell carcinoma:	 13.79 % 2.07 % 0.69 % 0 %	 5 % 2.5 % 0 % 0 %
Leukemia (lymphocytic, monocytic, mononuclear or undifferentiated)	24.7 %	38.75 %
Tongue (squamous cell carcinoma or papilloma)	1.33 %	2.5 %
Adrenal cortex (adenoma and carcinoma)	0.67 %	2.5 %
Adrenal medulla (Pheochromocytoma)	5.44 %	10 %
Mammary (fibroadenoma)	74 %	31.25 %
Pituitary (carcinoma or adenoma)	57.05 %	53.75 %
Uterus (stromal polyp or stromal sarcoma)	21.33 %	11.25 %

Overall, results obtained from a 50 and 100 week 4-nitrosomorpholine treatment of Fischer 344 rats (Lijinsky et al., 1988) are consistent and show that 4-nitrosomorpholine induces tumours in different organs (liver, thyroid, tongue and esophagus) in rats at low doses.

In the study by Weber and Bannasch, 1994, considered to be of sufficient quality (reliable with restrictions), in which male Sprague-Dawley rats were orally treated with three different dose levels (6 to 24 mg/kg bw/d) of 4-nitrosomorpholine with the drinking water for different treatment times (7 to 80 weeks), both, a clear dose- and time-dependent increase in hepatocellular adenomas and carcinomas were observed. The higher the dose level and the longer the treatment time the more preneoplastic and neoplastic lesions in the liver developed. At 6 mg/kg bw/d first tumours were observed after 27 weeks and at 24 mg/kg bw/d first tumours were observed already after 15 weeks. Thus, it can be concluded that, in addition to the observed increase in liver tumour incidence, 4-nitrosomorpholine also shortens the time to liver tumour occurrence at increasing dose levels. Again it was observed that 4-nitrosomorpholine treatment caused highly reduced survival. This is interpreted to be due to its carcinogenic action as high mortality was correlated to high liver tumour incidence (about 56 % to 64 % hepatocellular carcinomas). No other organs than the liver were examined in the study by Weber and Bannasch, 1994.

The findings by Lijinsky et al, 1988 and Weber and Bannasch, 1994 further indicate that 4-nitrosomorpholine increases the liver tumour incidence in different rat strains and in both male and female rats. Hereby, from the data it could be suggested that female Fisher F344 rats are gradually more susceptible to 4-nitrosomorpholine carcinogenicity than male Sprague-Dawley rats as lower incidence of hepatocellular carcinoma and adenoma was reported for a 50-week treatment of male Sprague-Dawley rats with 6 mg/kg bw/d (57 % and 67 %) compared with the 25-week treatment of female Fischer 344 rats with a lower dose of 3.58 mg/kg bw/d (63 % and 83 %). This is supported by the survival data which was observed to be longer for male Sprague-Dawley rats than for female Fischer 344 rats (Lijinsky et al., 1988).

The high liver tumour incidence in orally 4-nitrosomorpholine treated rats found by Lijinsky et al., 1988 and Weber and Bannasch, 1994 was also observed in all other available oral studies in rats at different 4-nitrosomorpholine dose levels, different treatment times and in different rat strains (Garcia and Lijinsky, 1972; Lijinsky and Taylor, 1975; Lijinsky et al., 1976; Lijinsky and Reuber, 1982; Hecht et al., 1982; Murai et al., 2000; Nersesyan and Muradyan 2002; Lijinsky et al., 1991 and Cortinovis et al., 1991, Mirvish et al., 1976). As these studies are considered to be of limited quality (for example due to a short exposure time, low number of exposed animals, no data on controls, testing of single doses, testing of only one dose level etc.) these are not discussed here in detail. Some interesting overall findings will be summarised below. All these studies are regarded as supporting studies.

In the solely available study (Linjinsky et al., 1991a) using repeated gavage substance administration (all other oral rat studies are drinking water studies) of a dose of 3.6 mg/kg bw/d 4-nitrosomorpholine twice weekly for 30 weeks in Fischer 344 rats a similar liver tumour incidence (91 %, 11/12 animals) was found compared to Lijinsky et al., 1988. Consistently to Lijinsky et al., 1988, always a dose-dependence of the liver tumour incidence was found if more than one dose level was tested (Lijinsky and Reuber, 1982; Lijinsky et al., 1976). Moreover, the results of the studies confirm the observed time-dependency of the 4-nitrosomorpholine induced carcinogenicity (Weber and Bannasch, 1994) as similar liver tumour incidences are observed after higher doses combined with shorter treatment times and lower doses combined with longer treatment times (e.g. Murai et al., 2000: at 17 mg/kg bw/d 100 % hepatocellular carcinomas are observed after 8 weeks of treatment and in Cortinovis at al., 1991: at 0.5 mg/kg bw/d 100 % animals with liver tumours observed after 60 to 65 weeks of treatment). Mirvish et al., 1976 who treated MRC Wistar rats with 6.4 mg/kg bw/d 4-nitrosomorpholine for 2 years with the drinking water generally found a short latency for liver tumours between 28 and 34 weeks after 4-nitrosomorpholine treatment which underlines the results obtained by Lijinsky et al., 1988.

The results of the supporting studies further confirm that 4-nitrosomorpholine induces liver tumours independent of the rat strain as liver tumours were observed in F344 rats (Murai et al., 2000; Hecht et al., 1982, Lijinsky and Reuber, 1982, Lijinsky et al., 1991), MRC rats (Garcia and Lijinsky, 1972; Mirvish et al., 1976), Sprague-Dawley rats (Lijinsky et al., 1976, Lijinsky and Taylor, 1975, Cortinovis et al., 1991), WS/Shi rats (Murai et al., 2000), albino random-bred rats (Nersesyan and Muradyan, 2002) and SD/gShi rats (Murai et al., 2000) after oral treatment with 4-nitrosomorpholine. The data by Murai et al., 2000, Lijinsky et al., 1988 and Weber and Bannasch (1994) indicate different susceptibilities of different rat strains towards 4-nitrosomorpholine induced liver carcinogenicity, however there are not enough studies available with different rat strains using the same experimental conditions (such as similar dose, sex, treatment time, observation duration and route) to allow a sound conclusion on that point.

Results from the supporting studies also confirm that 4-nitrosomorpholine treatment results in liver tumours independent of the rat sex. However, no general conclusion on sex susceptibility for 4-

nitrosomorpholine induced carcinogenicity can be drawn as there exist no studies where male and female animals were tested using the same experimental conditions and dose levels.

It is further remarkable that some time-dependent preneoplastic effects (clear cell, acidophilic and basophilic foci) and some neoplastic nodules in the liver were already detectable after treatment of rats with a single (acute) oral dose (320 mg/kg bw/d) of 4-nitrosomorpholine (Bannasch et al., 1979).

Consistently to Lijinsky et al., 1988, an increase in tumour incidences in organs besides the liver has also been observed in other studies after oral treatment with 4-nitrosomorpholine. Increased tumour rates in the esophagus to a high extent (up to 66.7 %) after oral treatment of rats were found by Lijinsky et al., 1991, Garcia and Lijinsky, 1972 and Lijinsky and Reuber, 1982 in female and male rats. Hereby, a dose-dependence for esophagus tumour numbers was observed in male rats (Lijinsky and Reuber, 1982). These findings are in line with the study by Lijinsky et al., 1988 and support the finding that 4-nitrosomorpholine next to the liver induces tumours in the esophagus. However, there are also studies in which no tumours of the esophagus were reported (Lijinsky and Taylor, 1975, Nersesyan and Muradyan, 2002, Hecht et al., 1982 and Lijinsky et al., 1976). This could be due to several reasons, namely that the esophagus was not examined (examined organs are not reported in detail in many studies) or the different susceptibility of different rat strains. The observed increased rate of thyroid tumours by Lijinsky et al., 1988, was confirmed by findings of Lijinsky et al., 1991 and Hecht et al., 1982. Moreover, in the study by Garcia and Lijinsky, 1972 tumours in the nasal cavity were found to a high extent (up to 60 %) in MRC rats after 4-nitrosomorpholine treatment (3.6 mg/kg bw/d). As these were observed only for MRC rats and were not found in any of the other studies including Lijinsky et al., 1988, it remains unclear to the DS whether this was a strain-specific effect or whether this resulted from the fact that the nasal cavity was not examined in other studies. All other tumours reported in the available supporting oral rat studies in other than the above described organs (liver, esophagus, thyroid) (Lijinsky and Reuber, 1982; Nersesyan and Muradyan, 2002; Garcia and Lijinsky, 1972; Hecht et al., 1982) were considered to be spontaneous. Their observed rates were comparable to the incidence observed either the internal controls or the historical controls for Fischer F344 rats (NTP 2010).

Inhalation studies in rats

There is one inhalation study available for 4-nitrosomorpholine in rats with some shortcomings in the testing procedure and which was considered as supporting study (Klein et al. 1990). Sprague-Dawley rats were exposed to a low dose (whole body) (0.0077 mg/L) 4-nitrosomorpholine vapour for only 6 weeks (4h/day, 4-5d/week). Neoplastic nodules in the liver were observed in 20.8 % (5/24) of the treated animals and hepatocellular carcinomas were found for 16.7 % (4/24) of the treated animals. The results of the study indicate that 4-nitrosomorpholine generates liver tumours also via the inhalation substance administration route. For the other reported tumours (nasal region, brain neuroblastoma, carcinoma of the thyroid gland), which were detected at very low incidences in only one animal each, it is not possible to distinguish if these tumours occur treatment related or are spontaneous.

Studies in rats - other administration routes than oral, dermal and via inhalation

There is one study available for 4-nitrosomorpholine in rats using other substance administration routes than oral, dermal or by inhalation (Lijinsky et al. 1991b). Lijinsky et al., 1991b performed a study using intravesicular substance administration twice weekly for 30 weeks in female Fischer 344 rats. The study has some shortcomings e.g. as performed in 12 animals only, using one dose level and a vehicle with a high ethanol content (25 %) and is considered as supporting study (not reliable). High tumour incidences were observed for the liver (58 %). In addition, a 100 % tumour

incidence was found for nasal tumours and 17 % of the treated animals developed tumours of the esophagus. No tumours were found in the vehicle controls. The results of the study, despite the shortcomings of the study, underline the observed carcinogenic potential of 4-nitrosomorpholine.

Oral studies in hamsters

Two of the available oral studies in hamsters (Ketkar et al., 1983 and Cardesa et al., 1990) are considered to be of sufficient quality (reliable with restrictions) as they were performed using simultaneously more than one dose level, a sufficient number of animals (mostly ≥ 30 animals per group) and control animals. Both studies were conducted orally in female and male Syrian hamsters with treatment for the whole life span. Ketkar et al., 1983 observed a dose-dependent increase in tumour incidence in the respiratory and digestive tract in female and male hamsters. The tumour rate in the respiratory tract increased to 70 % for the males and 73.3 % for the females at the highest tested dose levels (6.1 and 8.3 mg/kg bw/d, respectively). Tumours in the digestive tract occurred at low - moderate incidences (compared to the respiratory tract) in males and females. Hereby, the incidence in females was much lower compared to males. This could hint to a lower susceptibility of 4-nitrosomorpholine induced digestive tract carcinogenicity of female hamsters compared to males. Interestingly, the liver seems not to be the main target organ for 4-nitrosomorpholine in hamsters as observed for the rats. The occurrence of liver tumours after 4-nitrosomorpholine treatment in hamsters has in fact been described in the study by Ketkar et al., 1983 but no detailed data on the incidences and tumour types have been reported. Data by Ketkar et al., 1983 further support the time-dependency of 4-nitrosomorpholine induced carcinogenicity as decreased tumour latency was observed with increasing 4-nitrosomorpholine doses. No tumours were observed in the internal controls. The findings by Cardesa et al., 1990 are in line with study by Ketkar et al., 1990 as also dose-dependent increases in the rates of laryngo-tracheal tumours were detected in male and in female hamsters. Highest incidence was 80 % for males and 66 % for females at the highest tested dose levels (6.1 and 8.3 mg/kg bw/d, respectively). In that study no other organs beside the respiratory tract were examined. No tumours were observed in the internal controls. In contrast to rats, in both studies at doses up to 8.3 mg/kg bw/d no differences in survival compared to controls have been observed in male hamsters. The treatment-relationship of the observed decrease in survival of female hamsters in Cardesa et al., 1990 could not be assessed due to inconsistent control survival data in Ketkar et al., 1983 and Cardesa et al., 1990.

In the study by Lijinsky et al., 1984, who treated Syrian male hamsters at one dose level (6.7 mg/kg bw/d) orally via gavage once weekly for 26 weeks, also increased incidences of tumours in the respiratory tract (75 % nasal carcinomas and 30 % trachea adenomas) were found. Tumours of the lung and the liver only occurred in one out of 20 animals. Even if the study was considered as not reliable, the results again indicate that the respiratory tract is the target organ of 4-nitrosomorpholine in hamsters. The nasal cavity was a target site following oral treatment.

Inhalation studies in hamsters

There is one inhalation study available for hamsters (Klein et al. 1990). This study has some shortcomings in the experimental setup and was considered as not reliable. Hamsters were treated with only one dose (1.8 mg/kg bw/d) of vapour of 4-nitrosomorpholine for a very short exposure period (5 weeks) for 4h/day. The treated animals showed an increased rate of tumours in the trachea (15.6 %). This finding is consistent to the results observed after oral exposure. In the treated animals also slightly increased incidences of liver tumours, neurogenic sarcoma, adenocarcinoma of the spleen and stomach and papilloma of the forestomach were detected. However, from the shown data it cannot be concluded if these tumours occurred spontaneously or treatment-related as only one dose was tested and for the control animals a quite high rate of spontaneous tumours (e.g. liver cholangiomas (13 %), pheochromocytoma of suprarenal glands (16 %) and leukemia) was found.

The study using intratracheal substance administration (Ishinishi et al., 1988) in hamsters, although not guideline-compliant, confirmed the respiratory tract as target organ by treatment via the inhalation route. After intratracheal treatment of hamsters with a dose of 0.13 mg/kg bw/d 4-nitrosomorpholine once weekly for 15 weeks tumours in the trachea occurred in 43 % (9/21) of the treated animals. For the controls no tumours of the respiratory tract were found and for the treated animals no other tumours were observed.

Studies in hamsters- other administration routes than oral, dermal and via inhalation

There are three studies available with repeated subcutaneous substance administration in hamsters (Haas et al., 1973, Mohr et al., 1974, Reznik et al., 1976). All three studies were performed with similar experimental setup; but three different hamster strains namely Chinese, European and Syrian hamsters were used. In each experiment female and male animals were examined. Hamsters were treated in three dose groups related to the respective LD50 value (1/5, 1/10 and 1/20 LD50) subcutaneously once weekly for their whole life. Due to the reasons specified in Table 16 and the technical dossiers all three studies were considered to be of limited reliability. In all three studies 4-nitrosomorpholine treated hamsters showed increased mostly dose-dependent incidences of tumours of the respiratory tract. Hereby, the highest incidences were found for tumours of the nasal cavity (up to 55.6 % in male and 41.2 % in female Syrian hamsters, up to 50 % in male and 70 % in female European hamsters and up to 32 % in male and 59 % in female Chinese hamsters) and the trachea (up to 100 % in male and 84.2 % in female Syrian hamsters, up to 50 % in male and 70 % in female European hamsters). Only very few tumours were detected in the larynx and the lung. The data support that organs of the respiratory tract (mainly nasal cavity and trachea) are the target organs of the 4-nitrosomorpholine treatment in hamsters as respiratory tract tumours occurred independently from the administration route (subcutaneous, oral and by inhalation). Tumours of the liver, as observed for rats, were not reported in the tested hamster strains. In contrast to Syrian hamsters, in Chinese and European hamsters increased dose-dependent tumour incidences in organs of the digestive tract have also been observed. This included tumours of the forestomach, mouth, esophagus, tongue and pharynx. The highest tumour rates were observed for the esophagus and forestomach (up to 90 % of the male and female animals) in Chinese hamsters. The results hint to a different strain susceptibility of hamsters towards 4-nitrosomorpholine after subcutaneous substance administration. Interestingly, tumours of the digestive tract were detected in Syrian hamsters after oral administration. Further, in contrast to data obtained for oral substance administration, the results of the subcutaneous studies in hamsters do not hint to different sex susceptibilities. Tumours were found in similar organs and at similar rates in both sexes.

Althoff et al., 1974 investigated tumour incidences in hamsters after single subcutaneous dosing. Animals were observed for their whole life span after treatment. Single dosing already resulted in a highly increased incidence of tumours of the respiratory tract namely in the trachea, nasal cavity, larynx and bronchi at about 42 to 51 weeks after treatment. This is in line with the results of Bannasch et al., 1979 who observed neoplastic effects after single dosing with 4-nitrosomorpholine in rats.

Studies in mice

There is one study available in mice (A/J) with 4-nitrosomorpholine treatment (Hecht et al., 1989). Mice were treated orally by the drinking water with one dose of 3.6 mg/kg bw/d 4-nitrosomorpholine daily for ten weeks. The study has many shortcomings as specified in Table 14 and was considered of limited reliability. Investigation was restricted to lung adenomas. For the treatment group a significantly higher lung tumour incidence (100 %, $P < 0.01$) was observed. However, the lung tumour rate of controls was also quite high (40 %) which is considered critical as for B6C3F1 mice a 0 % tumour incidence of lung adenomas is reported in the historical controls

(NTP 2010, mice). Nevertheless, the results confirm the high carcinogenic potential of 4-nitrosomorpholine and show that 4-nitrosomorpholine induces tumours in different species.

Summarising, the results of all available 4-nitrosomorpholine studies considered as reliable clearly show that 4-nitrosomorpholine highly increases the tumour incidences in female and male rats and hamsters at low doses after oral treatment. In rats mainly tumours of the liver (benign or malignant neoplasms, hepatocellular carcinoma and adenoma, hemangiosarcoma) but also of the digestive tract (esophagus, tongue), the thyroid gland and the nasal cavity were observed. In hamsters 4-nitrosomorpholine treatment resulted in tumours of the respiratory and digestive tract. Tumour incidences showed a dose- and time-dependency. The results of the numerous supporting studies confirm the high carcinogenic potential of 4-nitrosomorpholine. It can be concluded that 4-nitrosomorpholine induces tumours in different species (rat, hamster, mice) and independent from the administration route applied (oral, inhalation, intratracheal, subcutaneous). Moreover, a number of similarities of tumour organs and tumour types were observed across studies, species and routes. In addition, it was found that increased rates of neoplastic effects were already observed after single dosing (acute doses) in hamsters and rats.

4.10.5 Comparison with criteria

According to the CLP Regulation carcinogens may be classified in hazard categories 1A, 1B or 2.

The CLP criteria for classification in category 1A (known or presumed human carcinogens) are as follows (Table 3.6.1):

"A substance is classified in category 1 for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as: Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence."

There are no studies available in which the epidemiological evidence regarding the carcinogenicity of 4-nitrosomorpholine to humans was investigated. Hence, classification in category 1A is not appropriate.

According to Table 3.6.1 (CLP Regulation) substances are classified into category 1B if there are animal experiments for which there is sufficient evidence to demonstrate animal carcinogenicity. In the following it is assessed if a sufficient evidence to demonstrate animal carcinogenicity from all available animal carcinogenicity studies can be derived.

According to section 3.6.2.2.1 a classification is made on the basis of evidence from reliable and acceptable studies and on all existing data. A systematic literature enquiry was performed for 4-nitrosomorpholine and the assessment was based on all available relevant carcinogenicity studies. The first search was on 15/04/2013 in databases Scopus, Sience Direct, ISI Web of Knowledge, Toxline (incl. PubMed), DIMDI with a search strategy using substance name, CAS, CMR, toxicity, human data, human health; 168 references were retrieved. A second search was on 01/07/2015 in databases (Toxnet, RTECS, Toxcenter, REAXIS, Chemlist, ISI Web of Knowledge, DIMDI, Scopus, Science direct) using the search strategy (substance name, CAS, toxicokinetic, CMR endpoints (incl. several subterms), repeated dose (subchronic, longterm) toxicity); > 1400 references were retrieved. All identified relevant carcinogenicity studies with 4-nitrosomorpholine were published as journal articles. None of these were fully compliant with standardised guidelines such as OECD TG 451 or 452. Nevertheless, some of the available studies were considered to be of sufficient quality to allow a substantiated assessment of the carcinogenicity of 4-nitrosomorpholine.

From the results of the studies considered to be reliable (reliable with restrictions) it can be concluded that for 4-nitrosomorpholine several criteria for a sufficient evidence of carcinogenicity are fulfilled:

(a) From the results of the studies a causal relationship could be established between the agent and an increased incidence of an appropriate combination of benign and malignant neoplasms in two species of animals (rats and hamsters).

(b) Moreover, this relationship could be established in more than two independent studies for rats and hamsters carried out at different times and in different laboratories (Lijinsky et al., 1988, Weber and Bannasch, 1994, Cardesa et al., 1990 and Ketkar et al., 1983).

(c) Further, in each of these studies malignant neoplasms occurred to an unusual degree with regard to incidence and site and there were strong findings of tumours at multiple sites.

Additional studies for 4-nitrosomorpholine, which were considered to have shortcomings in the experimental setup or documentation, were included in the assessment in a weight of evidence approach and their consistency to the studies considered reliable (with restrictions) was examined. All of these studies confirmed the carcinogenic potential of 4-nitrosomorpholine.

In the following a number of other factors as described in section 3.6.2.2.6 of the CLP Regulation are considered to enable conclusions on the overall likelihood if 4-nitrosomorpholine poses a carcinogenic hazard in humans.

a) tumour type and background incidence

The incidence of observed tumours in rats and mice were compared to background incidences found in both, the internal controls (if included in the study) and the historical controls as reported in NTP 2010 for rats and mice. The rates of tumours observed in hamsters were compared to the background incidence in the internal controls.

The highest tumour rates (100 %) in 4-nitrosomorpholine-treated rats were observed in the liver. Increased tumour rates were also found for the esophagus (up to 54 %), the thyroid (up to 12.5 %, C-cell carcinoma) and the tongue (up to 17 %). Historical control and internal control tumour incidences in these organs in F344 rats are below 2.5 %. In 4-nitrosomorpholine treated hamsters increased incidences (up to 73.3 %) of tumours of the respiratory tract and the digestive tract were observed. In all studies no tumours in the respiratory or digestive tract were reported for the internal controls. Hence, in rat and hamsters strong findings of tumour incidences highly above the background incidences reported for historical or internal controls were found after 4-nitrosomorpholine treatment.

If two or more dose levels of 4-nitrosomorpholine were tested in the studies, for most of the tumours showing an increased incidence compared to controls a dose-dependency was observed, which is generally taken as positive evidence of carcinogenic activity.

Considering the type of tumours, all organs for which increased tumour incidences have been observed after 4-nitrosomorpholine treatment have equivalents in humans (e.g. liver, thyroid gland, esophagus, tongue, nasal cavity, trachea, and pharynx). The DS concludes that the observed carcinogenic potential of 4-nitrosomorpholine is relevant for humans.

b) multi-site responses

In the reliable studies in both, rats and hamsters, increased tumour incidences were found in two or more organs. In rats mainly tumours of the liver (benign or malignant neoplasms, hepatocellular

carcinoma and adenoma, hemangiosarcoma) but also of the digestive tract (esophagus, tongue), the thyroid gland and the nasal cavity were observed. In hamsters 4-nitrosomorpholine treatment resulted in tumours of the respiratory (nasal cavity, trachea, pharynx) and digestive tract. Hence, it can be concluded that multi-site responses occur after treatment with 4-nitrosomorpholine which can be taken as strong evidence of carcinogenicity. Types of tumours were independently from the used substance administration routes.

c) Progression of lesions to malignancy

4-nitrosomorpholine treatment resulted in increased incidences of both benign and malignant tumours. In repeated dose toxicity studies with 4-nitrosomorpholine in rats (Hayashi et al., 2015, Weber and Bannasch, 1994, Lijinsky et al., 1976, Lijinsky and Taylor, 1975) numerous liver lesions (e.g. white foci scattered throughout the liver parenchyma) were observed at low doses. These are considered to be pre-stages for benign and malignant tumours (Lijinsky et al., 1976) hinting to progression of lesions to malignancy.

d) Reduced tumour latency

There are few reliable studies with 4-nitrosomorpholine available that have investigated time-dependency of 4-nitrosomorpholine treatment related neoplastic effects (e.g. Weber and Bannasch, 1994) or measured the time until tumour development (Mirvish et al., 1976). Mirvish et al., 1976 generally found a short latency for liver tumours between 28 and 34 weeks after 4-nitrosomorpholine treatment. Weber and Bannasch, 1994 found that at increasing 4-nitrosomorpholine dose levels tumours time-dependently developed after shorter exposure times. The data indicate that latency of 4-nitrosomorpholine caused tumours is rather short and decreases with increasing 4-nitrosomorpholine doses.

e) Whether responses are in single or both sexes

Increased tumour incidences compared to controls were found in female and male hamsters and rats.

f) Whether responses are in a single species or several species

Increased tumour incidences were observed in several species (namely rats, hamsters and mice) and strains.

g) Structural similarity to a substances for which there is good evidence of carcinogenicity

4-nitrosomorpholine belongs to the chemical group of nitrosamines. So far, three N–nitrosamines, namely dimethylnitrosamine (CAS 62-75-9) and 2,2-(nitrosoimino)bisethanol (CAS 1116-54-7) and nitrosodi-n-propylamin (CAS 621-64-7), were found to possess a harmonised classification (Annex VI of the CLP Regulation) as Carc.1B. Thus, a carcinogenic potential based on structural similarity can well be expected for 4-nitrosomorpholine.

h) Routes of exposure

Increased tumour incidences in rats and hamsters were observed in studies using the oral and inhalation substance administration route. This supporting, tumours were found after intravesicular substance administration of 4-nitrosomorpholine in rats and after intratracheal and subcutaneous substance administration in hamsters. No dermal studies were available for 4-nitrosomorpholine. Genotoxic carcinogens are generally suspected to be carcinogenic by any route. Hence, this findings would support a genotoxic mode of action for 4-nitrosomorpholine.

i) Comparison of absorption, distribution, metabolism and excretion between test animals and humans

No data on absorption, distribution, metabolism and excretion of 4-nitrosomorpholine are available for humans.

j) The possibility of a confounding effect of excessive toxicity at test doses

None of the available studies was performed compliant with a standardised guideline and generally no detailed clinical examination of the treated animals was performed. In the most studies no other toxic effects besides neoplastic effects, body weight gain or mortality were reported. In most cases 4-nitrosomorpholine treatment resulted in a decreased body weight or high decrease in survival at the doses tested. The observed decrease of survival was dose-dependent and correlated with the tumour incidence. Thus, it can be suggested that the observed carcinogenicity of 4-nitrosomorpholine is responsible for the decrease in survival at higher dose levels. In the study by Lijinsky et al., 1988 increased liver tumour incidences (benign and malignant) were already found at very low dose levels which did not affect body weight or survival. It can be concluded that described toxic effects are not confounding with regard to the carcinogenic potential of 4-nitrosomorpholine.

k) Mode of action and its relevance for humans

4-nitrosomorpholine belongs to the chemical group of N-nitrosamides. N-nitroso compounds represent a well-established class of chemical carcinogens with an anticipated mutagenic mode of action (Woo and Lai, 2005). Genetic events play central roles in the overall process of cancer development. The formation of highly reactive alkyldiazonium ions within the metabolic activation pathway is discussed for N-nitroso compounds which are known to react with nucleophiles in cellular macromolecules such as proteins and nucleic acids. Whereas metabolisation products have been identified for 4-nitrosomorpholine confirming this theory (see section 4.1) the available mutagenicity data presently do not justify the classification of the chemical as Muta. 1 or 2. However, the positive mutagenic results mainly found with the Ames test (with metabolic activation) support that the mutagenic action of 4-nitrosomorpholine after metabolism might play a key role in the observed carcinogenicity. Moreover, the carcinogenic effects observed at very low dose levels, after short latency periods and in multiple organs could indicate a non-threshold (genotoxic) mode of action. In repeated dose toxicity studies with 4-nitrosomorpholine in rats (Hayashi et al., 2015, Weber and Bannasch, 1994, Lijinsky et al., 1976, Lijinsky and Taylor, 1975) numerous single cell necroses and severe cirrhosis in the liver were observed hinting to hepatotoxicity of 4-nitrosomorpholine. As in rats mainly tumours of the liver were found, the observed hepatotoxicity seems to be linked to the observed carcinogenicity. The observed hepatotoxic action could be well in line with the anticipated mutagenic action of 4-nitrosomorpholine. Moreover, it can be concluded that these findings support the theory that metabolism seems to play a key role in the observed 4-nitrosomorpholine carcinogenesis processes in rats as the liver is the main organ for metabolism. The three anticipated coupled processes of metabolism, genotoxicity and hepatotoxicity as basis for the observed carcinogenicity action of 4-nitrosomorpholine in rats is of high relevance in humans.

Altogether it can be summarised that the available data for 4-nitrosomorpholine are sufficient to allow a substantiated evaluation of the carcinogenic potential of that substance. All criteria mentioned in section 3.6.2. (CLP Regulation) are matched to conclude a clear **sufficient evidence** of carcinogenicity for 4-nitrosomorpholine.

4.10.6 Conclusions on classification and labelling

Based on the comparison of the available carcinogenicity data for 4-nitrosomorpholine with the criteria laid down in the CLP Regulation it is justified to classify 4-nitrosomorpholine as **Carc. 1B (H350: May cause cancer).**

Specific concentration limits for Category 1 carcinogens:

For 4-nitrosomorpholine a specific concentration limit is proposed. The specific concentration limit has been determined as recommended in the "Guidelines for setting specific concentration limits for carcinogens" (EC 1999).

For the purpose of setting specific concentration limits a T25 value should be calculated. Below, the T25 value for 4-nitrosomorpholine is determined as described in the guideline mentioned above in section 3.1 'determination of the T25 value'.

The data for determination of the T25 value should preferentially be from oral lifetime studies in mammals. Four lifetime oral carcinogenicity studies in mammals have been identified in the total data set of 4-nitrosomorpholine, namely two lifetime studies in rats (Lijinsky et al., 1988 and Mirvish et al., 1976) and two lifetime studies in hamsters (Cardesa et al., 1990 and Ketkar et al., 1983). The T25 value shall be chosen based on the most sensitive species. Hence, as the hamster is less sensitive compared to the rat in both studies by Cardesa et al., 1990 and Ketkar et al., 1983, the studies in rats are considered more suitable for T25 determination. Hereby, the study by Lijinsky et al., 1988 is chosen for the calculation of T25 value as in the study by Mirvish et al., 1976 only one (higher) dose level was tested.

The study by Lijinsky et al., 1988 was not performed according to a guideline but for this study all criteria are met which are proposed by the "Guideline for setting specific concentration limits for carcinogens" (EC 1999): a) Animals of the test were mammals (rats), b) administration was begun early in life (8 weeks old rats), c) route of administration was via drinking water, d) the substance was bioavailable for systemic absorption, e) test agent was administered alone, f) exposure was chronic (5 days a week), g) duration was lifetime, i) research design included a control group, k) pathology data were reported for the number of animals with tumours rather than total numer of tumours, and l) results reported were original data. Thus, the study is considered as suitable for derivation of the T25 value.

Calculation of T25 value based on the study by Lijinsky et al., 1988:

As the T25 value was not incidentally obtained from the experimental results of Lijinsky et al., 1988 it is calculated from other tumour incidences using linear intrapolation as proposed by the guideline (EC1999). Thus, calculation is based on an observed net 15% incidence of liver tumours. Liver tumours were the most sensitive type of tumours observed for 4-nitrosomorpholine in rats. 15 % tumour incidence was observed at a dose level of 0.02 mg/kg bw/d if any liver tumour is included (hepatocellular carcinoma, hemangiosarcoma and hepatocellular adenoma) and at 0.09 mg/kg bw/d if only hepatocellular carcinoma are included. Accordingly, the respective 'preT25' values are 0.033 mg/kg bw/d based on all observed liver tumours and 0.15 mg/kg bw/d based on only hepatocellular carcinoma. These 'preT25' values are not corrected for dosing of 5 instead of 7 days per week, as this has already been considered while calculation of dose levels (from mg/L in the drinking water study to 'mg/kg bw/d'). However, as dosing was terminated after 100 weeks and not after the standard lifespan of 104 weeks, 'preT25' values are corrected by the factor 100/104. Resulting T25 values are 0.032 mg/kg bw/d and 0.144, respectively. As in the case of 4-nitrosomorpholine all observed types of liver tumours are considered relevant; relevant for humans

and for classification, the more sensitive 'preT25'values **of 0.032 mg/kg bw/d is chosen as the true T25 value.**

It is noted that this value (0.032 mg/kg bw/d) is slightly lower compared to the T25 value published by SCCS (0.094 ± 0.036 mg/kg bw/d). In the SCCS document no details for calculation are given. Calculation by SCCS is based on four studies with lower then lifetime exposure (Lijinsky et al., 1988, Lijinsky and Reuber, 1982 and Hecht et al., 1989). In the SCCS document it is not highlighted that in the study by Lijinsky et al, 1988 two exposure times (50 weeks and 100 weeks) have been applied. Moreover, it is not mentioned on what type of tumours and tissues the calculcation was based on. The recalculation of dose levels in mg/kg bw/d (which is not given as mg/kg bw/d in all the studies) is also not documented. These points might be responsible for the different T25 values obtained.

Determination of the specific concentration limit based on T25 of 0.032 mg/kg bw/d:

With the calculated T25 value of ≤ 1 mg/kg bw/d, 4-nitrosomorpholine belongs to the carcinogens of high potency according to the "Guidelines for setting specific concentration limits for carcinogens" (EC 1999) and the CLP Guidance (3.6.2.5). Category 1 carcinogens showing high potency will normally be given a specific concentration limit of 0.01 %, an order of magnitude lower than the general limit of 0.1 % (see EC 1999).

However, the guidance document EC (1999) indicates that lower SCL values than 0.01% for high potency category 1 carcinogens can be assigned on a case-by-case basis. the estimated T25 value for 4-nitrosomorpholine is more than 10-fold lower than the limit for 'high potency' a 10-fold lower SCL is also considered suitable. Therefore, a **SCL for 4-nitrosopmorpholine of 0.001 %** is proposed.

4.11 Toxicity for reproduction

Not evaluated in the present dossier.

4.12 Other effects

Not evaluated in the present dossier.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Not evaluated in the present dossier.

6 OTHER INFORMATION

Not evaluated in the present dossier.

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8 ANNEXES

Table A- 1 Short summaries of genotoxicity tests for 4-nitrosomorpholine which were performed using outdated test systems for which either OECD test guidelines have been deleted or standardised test guidelines do not exist.

Method	Results	Remarks	Reference
In vitro host-mediated assay with *S. typhimurium*	n.a.	No standardised guideline available for the host-mediated assay with *S. typhimurium*	Baun R, Schoeneich J (1975)
In vitro host-mediated assay with *S. typhimurium* Test concentrations: 0 – 500 mg/kg bw	Positive	No standardised guideline available for the host-mediated assay with *S. typhimurium*	Zeiger E, Legator MS (1971)
Mitotic gene conversion in Saccharomyces cerevisiae Test concentration: no data With and without using S9 mix	Negative But considered as "false negative" as significant induced levels of convertants at concentrations of 150 µg/mL and above	Guideline for Gene Mutation Assay in Saccharomyces cerevisiaea (OECD TG 480) deleted in 2014	Sharp DC, Parry JM (1981)
Mitotic gene conversion in *Saccharomyces cerevisiae* Test concentration: 2 µg/mL With using S9 mix	Negative	Guideline for Gene Mutation Assay in Saccharomyces cerevisiaea (OECD TG 480) deleted in 2014	Zimmermann FK, Scheel I (1981)
Mitotic recombination assay in *Saccharomyces cerevisiae* Test concentrations: no data With and without S9 mix	Positive	Guideline for Mitotic recombination assay in *Saccharomyces cerevisiae* (OECD TG 481) was deleted in 2014	Parry JM, Sharp DC (1981)
Haploid yeast reversion assay with *Saccharomyces cerevisiae* (XV185-14C) Test concentrations: 88.9 and 889 µg/mL with and without S9 microsomal fraction	Positive/negative 4-nitrosomorpholine positive without S9 and negative with S9	Guideline for Gene Mutation Assay in *Saccharomyces cerevisiae* (OECD TG 480) deleted in 2014	Metha RD and vonBorstel RC (1981)
In vitro comet assay in primary rat lymphocytes, testicular cells, type II pneumocytes and hepatocytes Test concentrations: 1.7, 3.4 and 5.1 mM	Positive 4-nitrosomorpholine induced moderate but significant increase of DNA strand breaks in pneumocytes and hepatocytes (1.7 – 5.1 mM)	No standardised guideline available for in vitro alkaline comet assay	Lazarova M, Labaj J, Eckl P, Slamenova D (2006)

In vitro Comet Assay in hepatocytes Test concentrations: 0.116 mg/mL	Positive Significant increase of " % of tail DNA" compared to control	No standardised guideline available for in vitro Comet assay	Slamenová D, Chalupa I, Robichová S, Gábelov A, Farkašová T, Hrušovská L, Bacová G, Šebová L, Eckl P, Bresgen N, Zeitheim P, Schneider P, Wsólová L (2002)
In vitro comet assay in mammalian cells (in human colon carcinoma Caco-2 cells) Test concentrations: 0.93, 1.7, 3.4, 5.1 mmol/L	Positive Concentration-dependent increase of DNA breakage, significant difference compared to control at all tested dose levels	No standardised guideline available for in vitro alkaline comet assay	Robichova S, Slamenova D (2001)
In vitro alkaline elution test (DNA fragmentation) and in vitro UDS test using primary hepatocytes Test concentrations: 1.0, 1.8, 3.2 mM	Positive positive dose related responses for 4-nitrosomorpholine (1-3.2 mM)	In vitro UDS test guideline (OECD TG 482) deleted in 2014, No standardised guideline available for in vitro alkaline elution test	Martelli A, Robbiano L, Gazzaniga GM, Brambilla G (1988)
In vitro UDS test using HeLa Cells test concentrations: 0.1 to 100 µg/mL with and without liver metabolizing system	Negative 4-nitrosomorpholine was inactive with and without liver metabolizing system	In vitro UDS test guideline (OECD TG 482) deleted in 2014	Martin CN, McDermid AC (1981)
In vitro sister-chromatid exchange in Chinese hamster cells	n.a.	(OECD TG 479 test: in vitro sister-chromatid exchange assay in mammalian cells was deleted in 2014)	Evans E, Mitchell AD (1981)
In vivo mammalian lymphocytes chromosome aberration test in rats Test concentration: 200, 250, 300 mg/kg bw (lymphocytes collected from blood taken from abdominal aorta)	Positive Significant increase in number of chromosomal aberrations at 250 and 300 mg/kg bw	No guideline available for mammalian chromosome aberration test in lymphocytes	Newton MF, Bahner B, Lilly LJ (1977)
Wing spot test in *Drosophila melanogaster* Test concentrations: 5, 25, 50 µmol/vial	Positive 4-nitrosomorpholine with clearly positive activities in the test	No standardised guideline available for the wing spot test in *Drosophila melanogaster*	Negishi T, Shiotani T, Fujikawa K, Hayatsu H (1991)

Drosophila mosaic test Test concentrations: 0.07, 0.21, 0.64, 1.92 mmol/kg bw	Positive Significant increased frequency of mosaicism compared to controls observed at 0.21, 0.64 and 1.92 mmol/kg, positive concentration dependence	No standardised guideline available for the Drosophila mosaic test	Surjan A, Kocsis Z, Csik M, Pinter A, Török G, Börzsönyi M, Szabad J (1985)
In vivo alkaline elution test (DNA fragmentation) in rat Oral (gavage) Test concentrations:0.4 mg/kg (measurement of viscometric parameters of DNA in liver cell nuclei obtained by liver perfusion)	Positive Statistically significant changes of DNA viscometric parameters in liver cell nuclei after treatment	No standardised guideline available for an in vivo alkaline elution test (DNA fragmentation)	Brambilla G, Carlo P, Finollo R, Sciaba L (1987)
In vivo sister-chromatid exchange (SCE) test in mouse (i.p.) Test concentrations: 37.5, 75, 150, 300 mg/kg bw	Positive 4-nitrosomorpholine induced significant dose-related increases in SCE frequency	No standardised guideline available for in vivo sister-chromatid exchange (SCE) test (OECD TG 479 test: in vitro sister-chromatid exchange assay in mammalian cells was deleted in 2014)	Kligerman AD, Erexson GL, Wilmer JL (1985)