Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

1,4-dichloro-2-nitrobenzene

EC Number: 201-923-3
CAS Number: 89-61-2

CLH-O-0000007202-85-01/F

Adopted

1 December 2022

1 December 2022

CLH-O-0000007202-85-01/F

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

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

Chemical name: **1,4-dichloro-2-nitrobenzene**

EC Number: **201-923-3**

CAS Number: **89-61-2**

The proposal was submitted by **Netherlands** and received by RAC on **2 November 2021.**

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

PROCESS FOR ADOPTION OF THE OPINION

Netherlands has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **13 December 2021**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **11 February 2022**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Annemarie Losert**

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

The RAC opinion on the proposed harmonised classification and labelling was adopted on **1 December 2022** by **consensus**.

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

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	1,4-dichloro-2-nitrobenzene	201-923-3	89-61-2	Carc. 1B	H350	GHS08 Dgr	H350			
RAC opinion	TBD	1,4-dichloro-2-nitrobenzene	201-923-3	89-61-2	Carc. 1B	H350	GHS08 Dgr	H350			
Resulting Annex VI entry if agreed by COM	TBD	1,4-dichloro-2-nitrobenzene	201-923-3	89-61-2	Carc. 1B	H350	GHS08 Dgr	H350			

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GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

In this RAC opinion 1,4-dichloro-2-nitrobenzene is abbreviated as DCNB.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The dossier submitter (DS) presented the available data consisting of five different bacterial reverse mutation assays, one bacterial SOS response assay, and three in vitro mammalian genotoxicity assays – two chromosome aberration assays and one gene mutation assay.

The DS further reported that there were no in vivo data (neither in somatic nor in germ cells) or human data available.

The studies are presented in table 1 (See Appendix 1).

The DS concluded that four of the five available bacterial reverse mutation assays had major methodological deficiencies and therefore considered only the study by MHWJ (1994a) as reliable. The procedure in this study was comparable to OECD TG 471 and it was conducted according to GLP. It tested five concentrations of DCNB ranging from 39.06 to 5000 µg/plate in the presence/absence of metabolic activation. The DS noted that various expert panels had differentially assessed the mutagenicity of DCNB in the respective *Salmonella typhimurium* strains and that IARC in its 2020 evaluation of DCNB (IARC, 2020) only cited from OECD SIDS, as the original documents were not accessible. The DS stated that the evaluation criteria applied by the Ministry of health and Welfare Japan (1994a) were not clear as the study was only available in Japanese. Therefore, the DS applied the criteria established by Zeiger et al. (1992) and Kier et al. (1986), summarised in Mortlmans & Zeiger (2000), which are in accordance with OECD TG 471 and are shortly summarised in the CLH report. Applying these criteria the DS concluded that *Salmonella typhimurium* strain TA100 gave clearly positive results, with dose-dependence, in the presence and absence of metabolic activation with rat liver S9-mix, the results for *Salmonella typhimurium* strain TA98 were inconclusive without metabolic activation (only a marginally positive result was observed in one test but not confirmed in the second one) and with S9-mix the result was negative. The other tested strains namely *Salmonella typh.* strain TA1535, TA1537, and *E. coli* WP2uvrA were negative with and without metabolic activation. Details on concentration-response relationship and strain-specific responses are documented in Annex I of the CLH report.

The study by Shimizu et al. (1983) only included tests without metabolic activation. Positive results were obtained for TA100 & TA98, whereas TA1535 & 1537 gave negative results. The study did not follow OECD TG 471 and was not conducted according to GLP. Deviations from the guideline were that no metabolic activation was applied, no confirmatory tests were conducted, not all five required tester strains were included and the selection of the positive control was unusual (OECD TG 471 recommends the applied positive control substance, N-ethyl-*N'*-nitro-*N*-nitrosoguanidine, for *E. coli* strains, but not for *Salmonella* strains). Details on concentration-response relationship and strain-specific responses are documented in Annex I of the CLH report.

Black et al. (1985) gave negative results, but as it only tested a single, very low concentration (1 µg/plate) in only one strain, it cannot be regarded as a true negative.

The studies by Kawai et al. (1985) and Jin & Qian (1991) again gave positive results in some strains, including for TA 100 with and without metabolic activation as well as for a modified TA1535 strain (with plasmid pSK1002) which is in line with the results for TA 100 from MHJW (1994a) and Shimizu et al. (1983), but negative results for TA98, which is in contrast to the observations made by MHJW (1994a) and Shimizu et al. (1983). These two studies are, however, poorly reported and have deviations from OECD TG 471 (see table 1).

The DS reported that Jin & Qian (1991) investigated DCNB without metabolic activation in a bacterial SOS response assay, which is approved for water and wastewater and detects primary DNA damage caused by genotoxic substances (Ode, 2016). The study concluded that DCNB was an SOS mutagen (for more details see table 1), however, the DS considered the result as of limited reliability as the study had methodological and reporting deficiencies.

The DS further mentions three in vitro mammalian genotoxicity assays, one gene mutation assay (HPRT test) and two chromosome aberrations assays. The information on the in vitro HPRT test (BUA, 1991), which was negative and the chromosome aberration assay in Chinese hamster V79-cells (BUA, 1991), which did not clearly indicate clastogenic properties of DCNB, was only available from second source and the reliability was not assignable. In a third mammalian in vitro assay DCNB's ability to induce chromosome aberration was assessed according to OECD TG 473. The DS stated that the study was conducted according to GLP, but had some inconsistencies in the report mainly in respect of observed cytotoxicity (Klimisch 2). Cytotoxicity was only investigated in a preliminary test in which 50% growth inhibition was seen at 0.12mg/mL and 0% cell growth at 0.24mg/mL. No doses in between were tested, but at higher doses, less cell growth inhibition was seen (at 0.95mg/mL and 1.9mg/mL growth inhibition was reported to be only 9 and 23% respectively). The effects (statistically significant increase in in incidence of structural aberrations, statistically significant increase in number of polyploid cells), which were only seen at the top dose (0.15mg/mL) after 48h continuous incubation without metabolic activation, are therefore considered to have occurred in the presence of considerable cytotoxicity >55% (but value unclear and from preliminary test). The DS concluded that a clear distinction between true genotoxicity and cytotoxicity was not possible. No effects were seen at the lower doses or upon shorter exposure duration (6h with and without metabolic activation and 24h without metabolic activation). The DS concluded that the observed increase in chromosomal aberrations and polyploidy was equivocal. Further information on the test protocol and the numerical values can be found in table 1 and in Annex I of the CLH report.

The DS concluded that there are some indications for a mutagenic potential of DCNB in in vitro bacterial reverse mutation assays, i.e. in the only reliable study by NHJW (1994a) positive results were obtained for strains TA100 (with and without metabolic activation) and TA98 (without metabolic activation, though only in one of two tests). Positive results for these two strains, as well as for other strains were seen in the remaining fours studies which had deviations from the test guideline.

The only in vitro mutagenicity test in mammalian cells considered to be of sufficient reliability, i.e. the chromosomal aberration test by NHJW (1994b), still had some deficiencies and gave an equivocal result and there are no in vivo studies available (neither in somatic cells nor in germ cells).

The DS compared the available data with the CLP criteria for classification as germ cell mutagen as follows:

There are no epidemiological data available that could support classification of 1,4-dichloro-2-nitrobenzene in Category 1A.

There are neither experimental data from *in vivo* heritable germ cell mutagenicity tests in mammals nor from *in vivo* somatic cell mutagenicity tests in mammals with 1,4-dichloro-2-nitrobenzene available and thus classification in Category 1B is not supported.

Classification in Category 2 CLP requires positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from either somatic cell mutagenicity tests, in mammals. Or other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.

These criteria are also not met because no *in vivo* somatic cell genotoxicity tests in mammals exist for 1,4-dichloro-2-nitrobenzene.

However, CLP also includes a note, which says that substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2.

An in vitro mammalian mutagenicity assay exists for DCNB, i.e. an in vitro chromosome aberration study similar to OECD TG 473 (MHWJ, 1994b). This study has however some deficiencies and no clear indication for genotoxicity was observed (details are outlined in table 1 and in the text from the previous section).

The DS concluded that the existing data from in vitro non-mammalian or mammalian mutagenicity assays are not sufficient to assign a classification as a Category 2 mutagen.

In conclusion the DS proposed no classification for germ cell mutagenicity based on inconclusive data. In addition, he stated that the available data also do not allow to exclude a genotoxic potential of 1,4-dichloro-2-nitrobenzene.

Comments received during consultation

Two MSCA commented and supported the proposal and, like the DS, also pointed out that a mutagenic potential cannot be excluded based on the available data, with inconsistencies in the in vitro data and the lack of an in vivo follow-up study.

One of the MS CAs also referred to additional in silico analyses (using QSAR TB 4.5, Derek Nexus 6.1.1, Sarah Nexus 3.1.1, Leadscope Model Applier LSMA 3.1.0-40) giving several alerts for DCNB's potential to induce (in vitro) genotoxicity.

The DS concluded that these data would support the current proposal.

RAC agrees that these in silico results, support the concern raised by some of the in vitro data, and no final conclusion on DCNB's mutagenic properties is possible.

Assessment and comparison with the classification criteria

RAC concurs with the DS's presentation and analysis of the available data. In line with the DS RAC concludes that the available studies, which consist of in vitro tests only, are inconclusive and RAC supports **no classification for germ cell mutagenicity, based on inconclusive data**.

RAC supports the DS's statement that the available data also do not allow a genotoxic potential of DCNB to be excluded.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS presented the relevant data consisting of two chronic toxicity/carcinogenicity studies one in rats and one mice (Yamazaki et al., 2006), as well as two sub-chronic toxicity studies one in rats and one in mice (Yamazaki et al. 2005). The studies complied with GLP and were conducted similar to guideline OECD TG 453 and OECD TG 408, respectively.

None of these studies are reported in the registration dossier publicly available on the ECHA dissemination website (ECHA Dissemination, 02/09/2022).

The studies are presented in in detail the CLH report and its Annex I; however, the most relevant information is presented in table 2 & 3 below and summarised in the text. The most relevant findings for the assessment of the carcinogenic potential of DCNB are summarised in tables 4 – 7.

***Table 2**: Summary table of animal studies on carcinogenicity – modified table 10 from the CLH report (Findings are always stated for the respective doses in this sequence: 0, 320, 800 and 2000 ppm. Statistically significant results are indicated in **bold** text/numbers as significant in trend test (**trend**) or by pairwise comparison.)*

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Chronic toxicity and carcinogenicity study, similar to OECD TG 453 Deviations: limited reporting Reliability: 2 GLP: yes F344/DuCrj (SPF) male and female rats, 50 per sex and group	1,4-dichloro-2-nitrobenzene purity: > 98.8% 0, 320, 800 or 2000 ppm (w/w) in diet; equivalent to 0, 10, 25 and 63 mg/kg bw/d for males and 0, 17, 44 and 109 mg/kg bw/d for females using bw 475 g for males and 275 g for females and food intake 15 g/d cited by HCN (2018) doses were based on results from the sub-chronic study in rats (Yamazaki et al., 2005, see table 3**Error! Reference**	Survival (until termination) was 40/50, 44/50, 41/50, and 39/50 in males and 38/50, 35/50, 39/50, and 34/50 in females. No significant difference in survival rate analysis was observed between any treated groups and controls for both sexes. At 2000 ppm terminal body weights in males and females were decreased by 15% and 20% compared to their respective controls. Relative liver weight was statistically significantly (P≤0.01) increased in all treated animals of both sexes compared to controls. Relative kidney weight was statistically significantly (P≤0.01) increased in all treated males and in females at 800 and 2000 ppm compared to controls. <u>Non-neoplastic lesions:</u> Hematopoesis in bone marrow 5/50, 9/50, 9/50, **14/50#** in females <u>Neoplastic lesions:</u> **Females:** No increased tumour incidences were observed. **Males:** Tumours and related non-	Yamazaki et al. (2006) Cited also by HCN (2018) IARC (2020)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
	source not found.) daily oral exposure via diet for 2 years (104 weeks)	neoplastic & pre-neoplastic findings are summarised in table 4	
Chronic toxicity and carcinogenicity study, similar to OECD TG 453 Deviations: limited reporting Reliability: 2 GLP: yes Crj:BDF1 (SPF) male and female mice, 50 per sex and group	1,4-dichloro-2-nitrobenzene purity: > 98.8% 0, 320, 800 or 2,000 ppm (w/w) in diet; equivalent to 32, 80 and 200 mg/kg bw/d for males and 41, 103, and 257 mg/kg bw/d for females using bw 45 g for males, 35 g for females and food intake 4.5 g/d, cited by HCN (2018) doses were based on results from a sub-chronic study in mice (Yamazaki et al., 2005, see table 3) daily oral exposure via diet for 2 years (104 weeks)	No significant difference in the survival rate between groups of treated mice and controls was observed in survival analysis according to Kaplan-Meier (data not shown in the publication). Survival (until termination) was 27/49, 35/50, 26/50, and 18/50 in males and 30/50, 27/50, 28/50, and 23/50 in females. After the 65th week of administration a lower survival rate was observed in mice of both sexes at 2,000 ppm. At 2000 ppm an increased number of deaths before the administration period ended was observed for mice of both sexes due to liver tumours; deaths were 7, 8, 11, and 23 for males and 0, 3, 4, and 6 for females. <u>Non-neoplastic lesions:</u> Hemosiderin deposition in kidney 1/49, 6/50, 6/50, **25/50##** in males Erythropoiesis in bone marrow 7/49, 4/50, 14/50, **23/50##** in males <u>Neoplastic lesions:</u> Tumours and related non-neoplastic & pre-neoplastic findings seen in males and females are summarised in table 5	Yamazaki et al. (2006) Cited also by HCN (2018) IARC (2020)

and ## … significantly different at P ≤ 0.05 and P ≤ 0.01 by Chi-square test
* and ** … significantly different at P ≤ 0.05 and P ≤ 0.01 by Fisher's exact test
Trend test performed by Peto test
a: one male mouse died accidentally during administration

***Table 3**: Summary table of other animal studies relevant for carcinogenicity (Table 11 from the CLH report, slightly adapted/corrected → additions are marked grey)*

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
Subchronic toxicity study, similar to OECD TG 408 Reliability: 2 GLP: yes F344/Du Crj (SPF) male and female rats, 10 per sex and group	1,4-dichloro-2-nitrobenzene purity: > 99.9%	0, 1481, 2222, 3333, 5000 or 7500 ppm (w/w) in diet, equivalent to: 0, 93, 135, 207, 316 & 474 mg/kg bw/day in males and 0, 106, 162, 238, 342 & 4548 mg/kg bw/day in females doses were based on results from a sub-acute study in rats daily oral exposure via diet for 90 days	Incidences are always stated for doses at 0, 1481, 2222, 3333, 5000 or 7500 ppm (if not indicated otherwise). Statistically significant results are indicated in **bold**. All rats of treated and control groups survived the administration period and no significant difference in survival rate analysis was observed. Feed intake was statistically significantly lower at 2222 ppm and above in males and 3333 ppm in females. A statistically significant reduced terminal body weight in males and females was observed at 2222 ppm and above, respectively. Only doses 2222 ppm or less did not lead to a body weight decrement above 10%, except in females dosed at 2222 ppm (13%). A dose-dependent retardation of growth was seen in treated animals of both sexes, being more notable in treated males. Yellow coloured urine was observed in treated rats of both sexes. Increase in absolute and relative liver weights was seen in all treated rats of both sexes, except for absolute live weights of high-dosed male rats. In all treated rats of both sexes the relative kidney weights was significantly increased. Absolute and relative testes weights were decreased in rats at 2222 ppm and above, but ovary weight was not affected. In male rats at 2222 ppm and above and in female rats at 5000 and above relative spleen weights were significantly increased. At necropsy, three males at 5000 ppm and all males of the high dose group had accentuated lobular structures of the liver. Increased methaemoglobin levels were observed in 5000 ppm-dosed females and high-dosed males. Haematological parameters were changed at different doses in rats. In males, significant increases gamma-	Yamazaki et al. (2005)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			GTP and alanine aminotransferase levels were seen at 5000 ppm and above and at 7500 ppm, respectively. In female mice, gamma-GTP was increased at 3333 ppm and above. Total cholesterol, phospholipid, total protein, and albumin were significantly increased in all treated rats, except total protein in high dosed rats and albumin in high-dosed male rats. In male rats dosed up to 3333 ppm blood urea nitrogen was increased and in female rats at 5000 ppm and above. <u>Incidences for histopathological lesions in males:</u> Centrilobular hypertrophy hepatocytes in liver: 0/10, **6/10*, 10/10**, 10/10**, 9/10**, 8/10**** Centrilobular vacuolar changes in liver: 0/10, 0/10, 0/10, **6/10*, 10/10**, 10/10**** Total hyaline droplets in kidney: 10/10 (consisiting of 8 animals with moderate and 2 with marked dorplets), **10/10** (all marked) ,10/10** (all marked), 10/10** (all marked), 10/10** (all marked), 9/10* (5 slight, 4 moderate)** Granular casts in kidney: 0/10, **10/10**, 10/10**, 10/10**,** 0/10, 0/10 Cytoplasmic basophilia in kidney: 0/10, **10/10**, 10/10**, 10/10****, 1/10, 0/10 Eosinophilic droplet: proximal tubule in kidney: 0/10, 0/10, 0/10, 0/10, 0/10, 0/10 Deposition of hemosiderin in spleen: 0/10, 1/10, **10/10**, 10/10**, 10/10**, 10/10**** Increased extramedullary haematopoiesis in spleen: 0/10, 0/10, 1/10, 4/10, **9/10**, 10/10**** Germ cell necrosis in testis: 0/10, 0/10, **6/10*, 10/10**, 10/10**,**	

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			10/10** Debris of spermatic elements in epididymis: 0/10, 0/10, **6/10*, 10/10**, 10/10**, 10/10**** Disappearance of sperm in epididymis: 0/10, 0/10, 0/10, **10/10**, 10/10**, 10/10**** <u>Incidences for histopathological lesions in females:</u> Centrilobular hypertrophy hepatocytes in liver: 0/10, **10/10**, 10/10**, 10/10**, 10/10**, 10/10**** Centrilobular vacuolar changes in liver: 0/10, 0/10, 0/10, 0/10, 0/10, **8/10**** Total hyaline droplets in kidney: 0/10, 0/10, 0/10, 0/10, 0/10, 0/10 Eosinophilic droplet: proximal tubule in kidney: 0/10, **8/10**, 10/10**, 10/10**, 10/10****, 4/10 Deposition of hemosiderin in spleen: 0/10, **8/10**, 10/10**, 10/10**, 10/10**, 9/10**** Increased extramedullary haematopoiesis in spleen: 0/10, 0/10, 0/10, 2/10, **8/10**, 9/10****	
Subchronic toxicity study, similar to OECD TG 408 Reliability: 2 GLP: yes Crj:BDF1 (SPF) male and	1,4-dichloro-2-nitrobenzene purity: > 99.9%	0, 1481, 2222, 3333, 5000 or 7500 ppm (w/w) in diet, equivalent to: 0, 245, 374, 530, 775 & 1647 mg/kg bw/day in males and 0, 284, 428, 613, 936 & 1601 mg/kg bw/day in females doses were based on results from a sub acute study in mice	Incidences are always stated for doses at 0, 1481, 2222, 3333, 5000 or 7500 ppm (if not indicated otherwise). Statistically significant results are indicated in bold. During the administration period, at 5000 ppm one male mice and at 7500 ppm 4 male and 4 female mice died (causes could not be confirmed). One accidental death of a male mice at 1481 ppm occurred. Feed intake was statistically significantly lower at 7500 in mice of both sexes. A statistically significantly reduced terminal body weight in males and females was observed at 7500 ppm. Yellow coloured urine was observed in treated mice of both	Yamazaki et al. (2005)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
female mice, 10 per sex and group		daily oral exposure via diet for 90 days	sexes. Increase in absolute and relative liver weights was seen in all treated mice of both sexes, except for absolute liver weights of 1481 ppm dosed female mice. In males at 3333 ppm and above and in females at 5000 ppm and above, the relative kidney weights were significantly increased. Absolute and relative testes weights were decreased in mice at 7500 ppm, but ovary weight was not affected. In mice of both sexes at 2222 ppm and above relative spleen weights were significantly increased. Animals found dead or in moribund state had thymus atrophy. Increased methaemoglobin levels were observed in 7500 ppm-dosed mice of both sexes. Haematological parameters were changed at different doses in mice. Significant increased alanine aminotransferase and aspartate aminotransferase levels were seen at 2222 ppm and above and at 3333 ppm and above in female and male mice, respectively. Total cholesterol, phospholipid, total protein, and albumin were also increased in treated mice but to a lesser extent compared to rats, except total protein in high dosed rats and albumin in high-dosed male rats. Blood urea nitrogen was increased in female mice at 5000 ppm and above. <u>Incidences for histopathological lesions in males:</u> Focal necrosis in liver: 1/10, 1/9, 2/10, 3/10, 5/10, 1/10 Single cell necrosis in liver: 1/10, 1/9, 3/10, **8/10**, 10/10**, 10/10**** Deposit of needle-like body in liver: 0/10, 0/9, **5/10*, 9/10**, 10/10**, 9/10**** Centrilobular hypertrophy hepatocytes in liver: 0/10, **9/9**, 10/10**,**	

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			9/10, 10/10**, 10/10**** Deposition of hemosiderin in spleen: 2/10, 6/9, **10/10**, 9/10**, 10/10**,** 7/10 Increased extramedullary haematopoiesis in spleen: 0/10, 1/9, **5/10*, 9/10**, 10/10**, 6/10*** Germ cell necrosis in testis: 0/10, 0/9, 0/10, 0/10, 0/10, **10/10**** Debris of spermatic elements in epididymis: 0/10, 0/9, 0/10, 0/10, 0/10, **10/10**** Disappearance of sperm in epididymis: 0/10, 0/9, 0/10, 0/10, 1/10, **6/10*** <u>Incidences for histopathological lesions in females:</u> Focal necrosis in liver: 0/10, 0/10, 0/10, 0/10, 0/10, 0/10 Single cell necrosis in liver: 0/10, 2/10, 4/10, **10/10**, 10/10**, 6/10*** Deposit of needle-like body in liver: 0/10, 0/10, **10/10**, 10/10**, 10/10**, 9/10**** Centrilobular hypertrophy hepatocytes in liver: 0/10, **10/10**, 10/10**, 10/10**, 10/10**, 10/10**** Deposition of hemosiderin in spleen: 0/10, **10/10**, 7/10**, 10/10**, 10/10**, 8/10**** Increased extramedullary haematopoiesis in spleen: 0/10, 2/10, 4/10, **10/10**, 10/10**, 5/10***	

* and ** … significantly different at P ≤ 0.05 and P ≤ 0.01 by Chi-square test

In line with the assessment by HCN (2018) the DS judged the dietary carcinogenicity study in rats and mice by Yamazaki et al. (2006) as Klimisch 2, due to deficiencies in reporting. The DS considered the number of animals per treatment groups sufficiently large and the observations and examinations made as suitable. RAC agrees with this assessment.

In the rat study there was no increase in mortality at any dose level, in mice a lower survival rate was observed in top-dose mice of both sexes and an increased number of animals dying due to liver tumours before end of administration period was seen.

In both rats and mice, general toxicity can be summarised as a decrease in terminal body weight in males and females of the top dose/mid and top dose, respectively, a dose dependent suppression in growth rate and some effects on organ weights and blood and blood-biochemical parameters as well as yellow stained urine at higher doses (details are listed in Table 2 and Annex I of the CLH report). Food consumption was not affected in mice nor rats.

Carcinogenic effects observed included:

- Liver adenoma and carcinoma in male rats
- Renal cell adenoma and carcinoma in male rats
- Zymbal gland adenoma in male rats
- Liver adenoma in female mice as well as liver carcinoma and hepatoblastoma in male and female mice

In female rats no neoplastic lesions were observed.

The DS discussed the relevance of these tumours seen in in experimental animals for humans for each tumour type separately, considering the scarcely available information on Modes of Action (MoAs).

Liver tumours in male rats and male and female mice

***Table 4**: Relevant findings to assess the observed liver tumours in male rats (Yamazaki et al., 2006)*

Tumour type	**0 ppm**	**320 ppm**	**800 ppm**	**2000 ppm**	**HCD Mean of 1249 male rats(maximum incidence in any study)**
Females					
Body weight (g)	248 ± 36	238 ± 23	234 ± 32	199 ± 26	
Liver weight (g)	6.317 ± 0.921	6.790 ± 0.930	7.267 ± 0.924§	7.086 ± 0.923§	
Liver weight (%)	2.583 ± 0.413	2.864 ± 0.415§	3.152 ± 0.502§	3.572 ± 0.217§	
Males					
Body weight (g)	384 ± 28	360 ± 48$	353 ± 22§	328 ± 25§	
Liver weight (g)	10.394 ± 1.540	11.508 ± 2.020§	11.946 ± 1.759§	12.361 ± 1.199§	
Liver weight (%)	27.16 ± 0.449	3.268 ± 0.848§	3.397 ± 0.588§	3.778 ± 0.588§	
Basophilic hepatocellular foci	21/50	22/50	**32/50 ##**	**40/50 ##**	
Hepatocellular adenoma	0/50	1/50	0/50	**6/50*** (trend)	
Hepatocellular carcinoma	0/50	0/50	1/50 (2%)	2/50 (4%)	0.2% (2%)
Hepatocellular adenoma & carcinoma combined	0/50	1/50	1/50	**8/50*** (trend)	

and ## … significantly different at P ≤ 0.05 and P ≤ 0.01 by Chi-square test
* and ** … significantly different at P ≤ 0.05 and P ≤ 0.01 by Fisher's exact test
$ and § … significantly different at P ≤ 0.05 and P ≤ 0.01 by Dunnet exact test
Trend test performed by Peto test

The observations can be summarised as a dose related increase in pre-neoplastic and neoplastic lesions, exceeding the upper range of HCD for hepatocellular carcinoma (no other HCDs were presented). No metastasis of the liver tumours to other organs was reported.

***Table 5**: Relevant findings to assess the observed liver tumours in male and female mice (Yamazaki et al., 2006)*

Tumour type	**0 ppm**	**320 ppm**	**800 ppm**	**2000 ppm**	**HCD**
Males					
Body weight (g)	48.8 ± 6.1	46.8 ± 8.4	41.4 ± 8.4§	32.0 ± 3.0§	
Liver weight (g)	2.168 ± 1.533	2.520 ± 1.014	3.467 ± 1.436§	5.722 ± 1.957§	
Liver weight (%)	4.713 ± 4.288	5.465 ± 3.197	8.976 ± 4.789§	17.918 ± 5.911§	
Centrilobular hypertrophy with nuclear atypia of hepatocytes	0/49[a]	**38/50 ##**	**39/50 ##**	**40/50**	
Acidophilic hepatocellular foci	0/49	2/50	**7/50 #**	**11/50 ##**	
Hepatocellular adenoma	17/49	21/50	20/50	16/50	
Hepatocellular carcinoma	15/49	15/50	23/50	**31/50 **** (trend)	
Hepatoblastoma	1/49	**10/50 ****	**12/50 ****	**25/50 **** (trend)	5/1047 (21 studies)+
Hepatocellular adenoma & carcinoma and Hepatoblastoma combined	6/50	8/50	**29/50***	**39/50*** (trend)	
Animals dying after wk 65 (before end of study period) – the increase in the top dose was stated to be related to animals dying from liver tumour.	7	8	11	23	
Females					
Body weight (g)	34.5 ± 7.2	34.7 ± 5.6	33.8 ± 5.1	28.6 ± 2.9§	
Liver weight (g)	1.625 ± 0.820	1.511 ± 0.356	2.028 ± 0.518§	4.251 ± 1.538§	
Liver weight (%)	4.801 ± 2.414	4.437 ± 1.130	6.152 ± 1.882§	15.195 ± 6.151§	

Centrilobular hypertrophy with nuclear atypia of hepatocytes	0/50	**15/50 ##**	**29/50 ##**	**35/50 ##**	
Hepatocellular adenoma	5/50	5/50	**17/50***	**16/50*** (trend)	
Hepatocellular carcinoma	1/50	3/50	**15/50***	**31/50*** (trend)	
Hepatoblastoma	0/50	0/50	0/50	2/50	0/1047 (21 studies)+
Hepatocellular adenoma & carcinoma and Hepatoblastoma combined	6/50	8/50	**29/50***	**39/50*** (trend)	
Animals dying after wk 65 (before end of study period) – the increase in the top dose was stated to be related to animals dying from liver tumour.	0	3	4	6	

and ## … significantly different at P ≤ 0.05 and P ≤ 0.01 by Chi-square test
* and ** … significantly different at P ≤ 0.05 and P ≤ 0.01 by Fisher's exact test
§ … significantly different at P ≤ 0.01 by Dunnet exact test
Trend test performed by Peto test
+ … JBRC historical control data (Yamazaki et al., 2006)

The observations can be summarised as a dose related increase in non-neoplastic (males and females) and pre-neoplastic (males) and neoplastic lesions (males and females), clearly exceeding the available HCDs for hepatoblastoma in males and females (no other HCDs were presented). The DS pointed out that hepatoblastoma is a rather rare tumour type in mice. In this respect RAC refers to Turusov et al (2002), a review of the biology of hepatoblastoma, which supports the DS's statement. Metastasis of DCNB-induced hepatocellular carcinoma of male and female mice and hepatoblastoma of male mice to the lungs was reported.

The DS pointed out that also in the sub-chronic studies in rat and mouse (Yamazaki et al., 2005; see table 3) the liver was identified as one of the target organs and summarised the relevant effects as increased liver weight, centrilobular hypertrophy of hepatocytes in both species, elevated serum activities of liver related transaminases in males and females as well as single cell necrosis in mice.

RAC notes that also in the carcinogenicity studies liver toxicity associated blood biochemical parameters were affected in males and females of both species in mid and top dose, in some instances already at the low dose (details can be found in Annex I of the CLH report).

The DS noted that from the literature it is known that hepatocellular proliferation can be a driver for the development of substance-induced hepatocarcinogenesis and that for certain chemicals a progressive lesion development from first appearing chemical induced hepatocellular foci to adenoma and subsequently carcinoma can be regarded as biological and morphological continuum (e.g. Takahashi et al., 2002). RAC notes that some steps of this sequence of events was seen in the livers of mice and rats exposed to DCNB, but this information does not help to clarify the exact underlying MoA.

Yamazaki et al. (2006) suggested *"A genotoxic mode of action is thought to operate in the DCNB-induced hepatocarcinogenicity"*. In this respect the DS referred to the section on germ cell mutagenicity and stated that there is evidence from an in vitro bacterial genotoxicity study that DCNB can act as mutagen, but that due to the lack of in vivo heritable germ cell or somatic cell mutagenicity tests in mammals, there is insufficient data to support a genotoxic MoA.

The DS also referred to the IARC evaluation (IARC, 2020) assessing the carcinogenic potential of various nitrobenzenes, which reported the following ToxCast and/or Tox21 high-throughput screening assay results for DCNB:

DCNB was active in 3 out of 54 assay endpoints – the three induced reporter transcripts were *"human aryl hydrocarbon receptor response element (AhRE, responsive to AhR), human nuclear receptor subfamily 1, group I, member 2 response element (PXRE, responsive to NR1I2); and human peroxisome proliferator-activated receptor gamma (PPARγ) transcription factors in the HepG2 human liver cell line"*, with the latter being regarded as the most sensitive one (IARC, 2020). IARC (2020) also concluded that there is weak evidence that DCNB is gentotoxic.

In addition to the MoAs listed by the DS as potential causes for the observed liver tumours, RAC also refers to the observed liver toxicity seen in rats and mice, after 90 days and after 2 years exposure to DCNB. Liver toxicity was indicated by increased liver weight, centrilobular hepatocellular hypertrophy and changes in liver related blood biochemical parameters in rats and mice, and in mice additionally single cell necrosis was seen at the higher doses which were only tested in the 90 day study. The liver related blood biochemical changes were seen at the same doses or even at lower doses at which liver tumours were seen in rats and mice. A contribution of recurrent inflammation and regenerative growth to the formation of liver tumours cannot be excluded.

The DS concluded that the information from carcinogenicity studies in rats and mice clearly indicates that exposure to DCNB induced tumours in the liver. Liver tumours occur in humans and no data exist that could link these observations to a MoA which is not relevant for humans (like e.g. via PPARα activation). RAC notes that in contrast there are indications that MoAs with relevance for humans are active, including some indications for genotoxicity, AhR activation or cytotoxicity. The potential contribution of these MoAs to the formation of the observed lvier tumours is however not sufficiently investigated. The DS concluded that the liver tumours seen in male rats and male and female mice should be considered relevant for humans.

Renal tumours in male rats

***Table 6**: Relevant findings to assess the observed renal tumours in male in rats (Yamazaki et al., 2006)*

Tumour type	**0 ppm**	**320 ppm**	**800 ppm**	**2000 ppm**	**HCD Mean of 1249 male rats (maximum incidence in any study)**
Females					
Body weight (g)	248 ± 36	238 ± 23	234 ± 32	199 ± 26	
Kidney weight (g)	1.713 ± 0.138	1.738 ± 0.133	1.766 ± 0.123	1.670 ± 0.113	
Kidney weight (%)	0.703 ± 0.086	0.733 ± 0.053	0.769 ± 0.118§	0.849 ± 0.085§	
Chronic progressive nephropathy (CPN, total)	24/50	23/50	32/50	28/50	
Urothelial hyperplasia in pelvis	10/50	5/50	15/50	6/50	
Mineralisation of papilla	9/50	9/50	9/50	17/50	
Males					
Body weight (g)	384 ± 28	360 ± 48$	353 ± 22§	328 ± 25§	
Kidney weight (g)	2.634 ± 0.221	2.802 ± 0.399	2,757 ± 0.299	2.853 ± 0.300§	
Kidney weight (%)	0.690 ± 0.086	0.799 ± 0.202§	0.785 ± 0.120§	0.873 ± 0.106§	
Chronic progressive nephropathy (CPN, total)	46/50	**49/50 ##**	**50/50 ##**	**49/50 ##**	
Urothelial hyperplasia in pelvis	1/50	**8/50 #**	**36/50 ##**	**39/50 ##**	
Mineralisation of papilla	0/50	**2/50**	**47/50 ##**	**48/50 ##**	
Renal cell adenoma	0/50	0/50	0/50	2/50 (4%)	0.16% (2%)
Renal cell carcinoma	0/50	1/50 (2%)	0/50	1/50 (2%)	0.16% (2%)
Renal cell adenoma and carcinoma combined	0/50	1/50	0/50	3/50 (trend)	

and ## … significantly different at P ≤ 0.05 and P ≤ 0.01 by Chi-square test
* and ** … significantly different at P ≤ 0.05 and P ≤ 0.01 by Fisher's exact test
$ and § … significantly different at P ≤ 0.05 and P ≤ 0.01 by Dunnet exact test
Trend test performed by Peto test

The DS summarised the observations as a borderline increase in incidences of renal cell adenoma (4% compared to 0.16% in the HCDs) in male rats of the highest dose group and an increase in combined incidences of renal cell adenoma and carcinoma in male rats supported by a positive trend test. In one high dose male metastisation of renal cell carcinoma to the lung was noted. No increase in pre-neoplastic lesions was seen in any dose group, but non-neoplastic lesions like

urothelial hyperplasia in the renal pelvis and renal papilla mineralisation were statistically significantly increased with dose. Additionally, chronic progressive nephropathy, which is attributed to advanced age of rats, was statistically significantly increased in all exposed males and an increase in severity was also seen with dose (see table 6 and Annex I of the CLH report).

In the sub-chronic toxicity study by Yamazaki et al. (2005) male rats had increased incidences of hyaline droplets and granular casts at the renal proximal tubules (for details see table 3), both positive for staining with α2u-globulin antibody.

The DS concluded that the observations made in male rats (i.e. α2u-globulin positive hyaline droplets and granular casts, increased mineralisation of the papilla, urothelial hyperplasia in the renal pelvis, increase in chronic progressive nephropathy and increase in adenomas and carcinomas) were indicative for a MoA specific to male rats. This MoA involves α2u-globulin-induced nephropathy which is characterised by α2u-globulin accumulation as hyaline droplets, epithelial degeneration and necrosis, which leads to cell proliferation, chronic progressive nephropathy (often in older rats), atypical hyperplasia within the proximal tubules, and progression to renal tumours (Capen et al., 1999, Swenberg & Lehman-McKeeman, 1999). According to the CLP guidance kidney tumours in male rats which are associated with substances causing α2u-globulin nephropathy are not considered relevant for humans.

The DS listed the criteria established by IARC which all must be met in order to identify a substance as causing renal tumours in male rats solely via α2u-globulin formation, i.e. lack of genotoxicity, male rat specificity for nephropathy and renal tumorigenicity, indication of the characteristic sequence of histopathological changes, of which protein droplet accumulation is obligatory, identification of the protein accumulating in the tubule cells as α2u-globulin, reversible binding of the chemical or metabolite(s) to α2u-globulin, induction of sustained increased cell proliferation in the renal cortex and similarities in dose response relationship of the tumour outcome with the histopathological endpoints (protein droplets, α2u-globulin accumulation, cell proliferation) (Capen et al., 1999).

The DS further reported that IARC (2020) considered the criteria as not fulfilled for DCNB, as they considered the identification of the chemical accumulating in the tubule cells as α2u-globulin was not given and binding of DCNB or a metabolite to α2u-globulin was not demonstrated. The DS further reported that Yamazaki et al. (2006) concluded that the genotoxic potential of DCNB could not be clearly ruled out (as discussed in detail in the section on germ cell mutagenicity). In addition Yamazaki et al. (2006) also described a possible analogy to reactive metabolites of glutathione and cysteine conjugates of tetrachloroethylene, which were biotransformed from the corresponding hepatic glutathione S-conjugate by γ-glutamyltranspeptidase and cysteine conjugate ß-lyase in the kidney and were shown to be mutagenic in the Ames test. Further details to support the relevance of this metabolic pathway for tetrachloroethylene are cited by Yamazaki et al. (2006), i.e. Elfarra et al. (1986) & Dekant et al. (1986) and described in the CLH report. Yamazaki et al. (2006) concluded that as DCNB, like tetrachloroethylene, is excreted as cysteine conjugate (*N*-acetyl-*S*-(4-chloro-3-nitrophenyl)-*L*-cysteine) in the urine (see information on toxicokinetics presented in the CLH report) it can be assumed that it is metabolised to the glutathione conjugate in the liver before, followed by reaction to *S*-(4-chloro-3-nitrophenyl) –*L*-cysteine in the kidney. Yamazaki et al. (2006) concluded that the formation of reactive and possibly mutagenic metabolites in the kidney, catalysed by ß-lyase, could contribute to the development of DCNB-induced chronic nephrotoxicity and nephrocarcinogenicity, but stated that the contribution of α2u-globulin formation could not be ruled out.

The DS concluded that there is clear evidence for renal tumours in male rats but, as the α2u-globulin-induced nephropathy might also be involved, the relevance of these tumours to humans was unclear.

In line with the DS, RAC concludes that the kidney was a clear target organ of DCNB-induced toxicity.

Kidney toxicity was seen in male and female rats, both in the carcinogenicity study (Yamazaki et al., 2006) and in the sub-chronic study (Yamazaki et al., 2005). There was a dose dependent increase in relative kidney weight in both sexes, which was statistically significant for all dosed groups in males, as well as in mid- and top-dose females. Absolute kidney weight was statistically significantly increased only in top-dose males. In addition a dose dependent increase in blood urea nitrogen (BUN) was seen in male and female rats, which was statistically significant in mid and top-dose males, and in females of all dose groups. Kidney toxicity was also seen in the sub-chronic and in the 2-year studies in mice, as relative kidney weight was increased in male and female mice at higher doses and BUN was increased in females of the top doses of both studies.

RAC notes that next to increases in BUN also histopathological examination revealed some increase in chronic progressive nephropathy, also in female rats, but the increase was not statistically significant, there was no dose dependence and no increase in severity was observed. In top-dose females the incidence of mineralised papilla was increased, but not statistically significant.

The table below directly compares the results for DCNB with the criteria developed by IARC.

***Table 7**: Comparison of the observations made upon DCNB exposure with the IARC criteria*.

Lack of genotoxicity	**Not adequately assessed, some evidence for genotoxicity in bacteria**
Male rat specificity for nephropathy and renal tumorigenicity	Tumours only seen in male rats; Nephropathy in males: *histopathology* - clear increase in incidence and severity in nephropathy in males; *blood biochemistry* – dose dependent increase in BUN, statistically significant in mid and top dose; Nephropathy in females: *histopathology* - some increase in incidence, no clear dose dependence, not statistically significant, no increase in severity; *blood biochemistry* – dose dependent increase in BUN, statistically significant in all dose groups
Indication of the characteristic sequence of histopathological changes, of which protein droplet accumulation is obligatory	Male rats: evidence for α2u-globulin positive hyaline droplets and granular casts, urothelial hyperplasia in the renal pelvis, increase in chronic progressive nephropathy and increase in adenomas and carcinomas
Identification of the protein accumulating in the tubule cells as α2u-globulin	Yes, in males. Eosinophilic droplets in females - α2u-globulin negative (see table 3) ➔ indicates that nephrotoxicity is not only caused by α2u-globulin pathway.
Reversible binding of the chemical or metabolite(s) to α2u-globulin	Not investigated

Induction of sustained increased cell proliferation in the renal cortex	Not investigated
Similarities in dose response relationship of the tumour outcome with the histopathological endpoints (protein droplets, α2u-globulin accumulation, cell proliferation)	<u>Male rats – 90 day study (the low dose in this study was comparable to the top dose of the 2 year study, where kidney adenoma and carcinoma were seen):</u> *Hyaline droplets:* in all animals including controls – in controls 8/10 moderate, 2/10 marked, in the dosed groups mainly marked severity was observed; *Granular casts:* not seen in animals of the control and the two top-dose groups, but in 10/10 animals in the low and the next two higher dose groups; <u>Male rats – 2 year study:</u> *Chronic progressive nephropathy:* in all dose groups, some increase with dose, increase in severity with dose ➔ Only slight increase in renal adenoma and carcinoma in the top dose (Except a single incidence of carcinoma in the low dose) ➔ no clear dose concordance *[Urothelial hyperplasia (pelvis):* steep increase with dose, to comparable levels in mid and top dose. → Not considered a pre-neoplastic lesion, as renal cell adenoma/carcinoma are located in the renal cortex. *Mineralisation of papilla:* steep increase with dose, to comparable levels in mid and top dose. → Not considered a pre-neoplastic lesion, as renal cell adenoma/carcinoma are located in the renal cortex.]

Overall RAC agrees with the DS that it cannot be excluded that next to the α2u-globulin pathway, also another MoA / other MoAs could contribute to the observed tumour formation. In conclusion, human relevance cannot be excluded for the observed renal tumours.

<u>Zymbal gland tumours in male rats</u>

Table 8*: Incidence of Zymbal gland adenomas in male rats (Yamazaki et al., 2006)*

Control	**320 ppm**	**800 ppm**	**2000 ppm**	**HCD Mean of 1249 male rats (maximum incidence in any study)**
0/50	0/50	0/50	4/50 (8%) (trend)	0.2% (2%)

Yamazaki et al. (2006) identified a positive trend for Zymbal gland adenomas in male rats with an incidence in the top dose clearly exceeding the upper range of the historical control data. They further concluded that the increase was marginal but substance-related.

The DS referred to the CLP guidance, which lists Zymbal gland tumours among those occurring in tissues with no human equivalent. The guidance states that although there is no human equivalent, human relevance cannot be fully excluded as such tumours indicate that the test material has the ability to induce tumours in the tested species. The DS also referred to a publication by Pohl & Fouts (1983) who detected cytochrome P450 activity in Zymbal glands of rats and mice, indicating that reactive metabolites of carcinogens may be formed, which could lead to tumour formation. The DS further stated that the tumours were of benign nature and as no MoA was identified, the relevance for humans was unclear. Overall, the DS concluded that these tumours would only give supportive evidence for carcinogenicity, but that the evidence was not sufficient on its own.

Comparison with the CLP classification criteria

The DS compared the available evidence with the CLP criteria for classification as carcinogen. The DS excluded Category 1A as there was no evidence from humans available.

The DS further stated that the data by Yamazaki et al. (2006) provided sufficient evidence that there is a causal relationship between the occurrence of tumours in two animal species and assessed further aspects necessary to decide on whether a classification in Category 1B was justified. The DS's assessment of these relevant aspects are summarised in the table below. Some additional aspects are included by RAC.

Table 9*: Weight of evidence assessment of the available information for the tumours seen in mouse and rat upon treatment with DCNB*

Factor	Evidence with DCNB	Conclusion
Tumour type Considering background incidence and HCD	Hepatocellular adenoma and carcinoma in male rats: Hepatocellular adenoma: statistically significantly increased in the top dose, trend Hepatocellular carcinoma: increased in mid and top dose, exceeding historical controls in the top dose Hepatocellular adenoma and carcinoma combined: statistically significantly increased in the top dose, trend	Supportive for classification
	Hepatocellular adenoma in female mice: statistically significant increase in mid and top dose, trend observed Hepatocellular carcinoma in male and female mice: Dose dependent increase, statistically significantly increased in top-dose males and mid and top-dose females, trend observed	Supportive for classification
	Hepatoblastoma in male mice: Dose dependent increase. Statistically significant increase in all dose groups. Exceeding historical controls in all dose groups. Rare tumour.	Supportive for classification

	Hepatoblastoma in female mice: Two incidences in the top dose (2/50) – exceeded historical controls.	
	Renal cell adenoma and carcinoma in male rats: Renal cell adenoma – increased above historical controls in the top dose. Renal cell adenoma – one single incidence in both the low and top dose group (upper range of historical controls) Renal cell adenoma and carcinoma combined - trend	Supportive for classification
	Zymbal gland adenoma in male rats: Increase in the top dose, clearly exceeding historical controls Only benign, no histological equivalent in humans	Supportive for classification
Multi-site responses	Yes	Increased concern
Progression of lesions to malignancy	Yes, for liver (carcinoma and hepatoblastoma) and kidney (carcinoma). Hepatoblastoma – highly malignant tumour. No progression to malignancy for Zymbal gland adenoma in male rats	Increased concern
Metastisation	Metastasis of hepatocellular carcinoma of male and female mice and hepatoblastoma of male mice to the lungs was observed. In one high dose male metastisation of renal cell carcinoma to the lung was noted.	Increased concern
Tumour – cause of death	Number of mice that died due to liver tumours was increased in top dose males and females.	Increased concern
Reduced tumour latency	Not indicated – data not available	-
Whether responses are in single sex or both	Both sexes in mice had malignant tumours, in rats only tumours in males	Increased concern
Whether responses are in a single species or several	Tumour formation occurred in rats and mice.	Increased concern
Structural similarity to a substance(s) for which there is good evidence of carcinogenicity	No	-
Routes of exposure	Oral	-
Comparison of ADME between test animals and humans	No species-specific differences identified in the available toxicokinetic studies.	-
The possibility of a confounding effect of excessive toxicity at test doses	In the rat study there was no increase in mortality at any dose level, in mice a lower survival rate was observed in top dose mice of both sexes and an increased number of animals dying due to liver tumours before end of administration period was seen. In both rats and mice, general toxicity can be summarised as a decrease in terminal body weight in males and females of the top dose/mid and top dose, a dose dependent suppression in growth rate and some effects on organ weights	-

	and blood and blood-biochemical parameters as well as yellow stained urine at higher doses (details are listed in Table 2 and Annex I of the CLH report). Food consumption was not affected in mice nor rats.	
Mode of action and its relevance for humans	a) Genotoxic MoA - potentially relevant for all observed tumour types: There is some support for genotoxic activity of DCNB – positive results in the only reliable bacterial gene mutation assay, some support from 4 further bacterial gene mutation assays, equivocal result from a reliable in vitro mammalian chromosome aberration test; but no in vivo follow up study available (neither in somatic nor in germ cells) – inconclusive data (see section germ cell mutagenicity). b) Cytotoxicity – liver tumours: There is evidence for cytotoxicity in the liver of rats and mice. Inflammation and recurrent growth could be a relevant MoA. Contribution of this MoA cannot be excluded. c) Liver tumours: Some information from ToxCast and Tox 21 high-throughput screening assays – active in 3 of 54 assays related to AhR, PXRE and PPARγ d) Renal tumours in male rats: Support for α2u-globulin induced renal cell tumours in male rats: details can be found in table 7. A contribution of this MoA is plausible, but contribution of other MoAs cannot be excluded. Other potential MoAs include: genotoxicity (inconclusive), cytotoxicity induced by other mechanisms than α2u-globulin induced nephropathy	a) inconclusive b) MoA plausible –relevant to humans. c) Information insufficient to draw any conclusion d) Contribution of α2u-globulin MoA plausible, but other MoAs cannot be completely excluded.

Three different malignant tumour types were observed in two different species (hepatocellular carcinoma in male rats and male and female mice, hepatoblastoma in male and female mice and renal cell carcinoma in male rats).

No underlying mode of action could be identified for the observed liver tumours (hepatocellular adenoma / carcinoma as well as hepatoblastoma). These tumours are considered relevant for humans.

The available MoA data for the observed renal tumours in male rats indicate, that the formation and deposition of α2u-globulin in renal cells could contribute to tumour formation, however, other MoAs could not be sufficiently ruled out.

Considerable increase in malignant tumours was detected in two species (hepatocellular carcinoma) and two sexes (hepatocellular carcinoma, hepatoblastoma) and at multiple site in rats (kidney and liver). These tumours are considered relevant for humans as the underlying modes of action where either not sufficiently investigated or MoAs that are relevant for humans could not be excluded.

The DS concluded that there is clear evidence from animal studies of carcinogenicity and that the available evidence is sufficiently convincing to place the substance in Category 1B.

This proposal is largely in agreement with the assessments of DCNB by IARC (2020) and HCN (2018).

Potency and concentration limits for the classification of mixtures

CLP recommends the derivation of specific concentration limits for substances with high or low carcinogenic potency, while carcinogens of medium potency have generic concentration limits only.

In order to decide on a substance's potency, the CLP guidance recommends to derive T25 values established by Dybing et al. (1997) and refers to the guidance document EC (1999). In line with EC (1999) the DS selected the endpoints with the highest relevance for humans and highest incidence, i.e. the statistically significant increase in incidence of hepatoblastoma in male mice at 320 ppm (approx. 32 mg/kg bw/day) of 10/50 compared to 1/49 in the control and hepatocellular carcinoma in female mice at 800 ppm (approx. 103 mg/kg bw/day) of 15/50 compared to 1/50 in the control. The resulting T25 values were 44.5 mg/kg bw/day for hepatoblastoma in male mice and 92.0 mg/kg bw/day for hepatocellular carcinoma in female mice. Both T25 values lie between 1 mg/kg bw/day and 100 mg/kg bw/day, which is the dose range assigned to medium potency carcinogens (EC, 1999). The DS therefore concluded that DCNB is a medium potency carcinogen and the generic concentration limit of 0.1% shall be applied.

Comments received during consultation

Two MSCA submitted comments and supported the proposal.

Assessment and comparison with the classification criteria

RAC concurs with the presentation and analysis of the available data and supports the proposal of the DS that **classification of DCNB as Carc 1B is warranted**.

RAC also agrees with the selection of the increased incidence in hepatoblastoma in male mice at 320 ppm and the increase in hepatocellular carcinoma in female mice at 800 ppm to calculate T25 values in order to assess the potency of DCNB's carcinogenic potential. As the resulting T25 values fall within the medium potency group, RAC agrees with the DS's conclusion to apply generic concentration limits.

Additional references

Turusov *et al*. (2002): Hepatoblastomas in Mice in the US National Toxicology Program (NTP) Studies. Toxicologic Pathology, 30(5), 580-591

ANNEXES:

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

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

Appendix

Table 1*: Summary table of mutagenicity/genotoxicity tests in vitro (Table 9 from the CLH report, slightly adapted/corrected → additions are marked as grey highlight)*

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
Non-mammalian experimental systems				
Bacterial Reverse Mutation Assay According to Japanese Guideline for Screening Mutagenicity testing of chemicals; similar to OECD TG 471 Deviations: selection of positive controls, applied evaluation criteria (biological relevance poorly considered) GLP: yes Reliability: 2 (study report only available in Japanese with Tables in English; selection of positive control)	1,4-dichloro-2-nitrobenzene Purity: >99.5% Impurities: <0.5% an isomer of 1,4-dichloro-2-nitrobenzene (no further information provided) Solvent: DMSO	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2uvrA Plate incorporation method - preliminary cytotoxicity test (all strains) 0, 50, 150, 500, 1500, and 5000 µg/plate with or without S9-mix Plate incorporation method - <u>first test:</u> TA100: 0, 78.13, 156.3, 312.5, 625, 1250, and 2500 µg/plate with or without S9-mix TA1535 and TA1537: 0, 39.06, 78.13, 156.3, 312.5, 625, and 1250 µg/plate without S9-mix; 0, 78.13, 156.3, 312.5, 625, 1250, and 2500 µg/plate with S9-mix WP2uvrA: 0, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate with or without S9-mix TA98: 0, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate without S9-mix; 0, 78.13, 156.3, 312.5, 625, 1250, and 2500	In the first test a precipitate of the test substance was observed on the surface of the agar plates at 1250, 2500, and 5000 µg/plate. <u>TA100:</u> positive (+/-S9); cytotoxic at ≥1250 µg/plate in first test and at 2500 µg/plate in second test <u>TA98:</u> Inconclusive: first test negative (+/-S9); cytotoxic at ≥2500 µg/plate (-S9) and ≥1250 µg/plate (+S9); second test positive (-S9); negative (+S9); cytotoxic at 5000 µg/plate (-S9) and at ≥1250 µg/plate (+S9)	Ministry of Health and Welfare Japan (MHWJ) (1994a), (Japanese, Tables in English) HCN (2018) IARC (2020) OECD (1996)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
		µg/plate with S9-mix Plate incorporation method - second test: TA100, TA1535, and TA1537: 0, 78.13, 156.3, 312.5, 625, 1250, and 2500 µg/plate with or without S9-mix WP2uvrA: 0, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate with or without S9-mix TA98: 0, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate without S9-mix; 0, 78.13, 156.3, 312.5, 625, 1250, and 2500 µg/plate with S9-mix +/- liver S9-mix from phenobarbital and 5,6-benzoflavone pre-treated male SD rats Media: histidine selective Plates: 3 No. replicates: 2 Positive controls: yes	TA1535: negative (+/- S9); cytotoxic at ≥625 µg/plate in first test and ≥1250 µg/plate in second test TA1537: negative (+/- S9); cytotoxic at ≥625 µg/plate (-S9) and ≥1250 µg/plate (+S9) in first test and at ≥1250 µg/plate (+/- S9) in second test WP2uvrA: negative (+/- S9); cytotoxic at 5000 µg/plate (-S9) and ≥2500 µg/plate (+S9) in first test and at ≥2500 µg/plate (+S9) in second test	
Bacterial Reverse Mutation Assay Ames test No explicit mentioning of	1,4-dichloro-2-nitrobenzene Purity: >99.6% Impurities: not provided Solvent: DMSO	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100 & TA1538 (strain TA1538 was not listed in table 9 of the CLH report) → did not test all	positive for TA98 and TA100 (-S9); cytotoxic at 6553.6 µg/plate positive for TA1538 (-S9); cytotoxic at	Shimizu et al. (1983) ECHA Dissemination (2021)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
OECD TG. Deviations: yes from OECD TG 471, not all required strains were tested, no confirmatory test performed, not tested with S9-mix, selection of positive control GLP: no Reliability: 3		required 5 strains (did not test E. coli WP2uvrA) Pre-incubation method Test concentrations: 0, 51.2, 102.4, 204.8, 409.6, 819.2, 1638.4, 3276.8, and 6553.6 µg/plate without S9-mix No confirmatory test performed. Tests with S9-mix were only carried out if tests without S9-mix were negative. ➔ RAC notes that no test with S9-mix was conducted for the strains TA 1535 & TA 1537, despite the negative results without S9-mix. Evaluation criteria: Positive, if number of revertant colonies is more than twice than the colonies on the control plate. Media: histidine selective Plates: 3 No. replicates: 2 Positive controls: yes	≥3276.8 µg/plate negative for TA1535 and TA1537 (-S9); no clear dose-response observed; cytotoxic at 6553.6 µg/plate	HCN (2018) BUA (1991)
Bacterial Reverse Mutation	1,4-dichloro-2-nitrobenzene (named 2,5-	*Salmonella typhimurium* TA100	Test 1: negative TA100 (-S9);	Black et al. (1985)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Assay Ames test No explicit mentioning of OECD TG or GLP. Deviations: yes from OECD TG 471, only one strain used, not tested with S9-mix, no data on cytotoxicity, limited information on study design and results; only one very low dose tested → cannot be considered a "true negative" * Reliability: 3	dichloronitrobenzene) purity: commercially available, no further information (purity given in the disseminated database is for dinitrochlorobenzene) Impurities: not provided Solvent: DMSO	Plate incorporation method Test 1: test concentration of 1 µg/plate without S9-mix Media: histidine selective Plates: 4 No. replicates: 1 [Test 2: test substance named as dinitrochlorobenzene, thus the test was not considered in this evaluation] Positive control: yes	Number of revertants per plate (Mean ± SEM) were 231 ± 8 for 1,4-dichloro-2-nitrobenzene and 242 ± 10 for control	ECHA Dissemination (2021) HCN (2018)
Bacterial Reverse Mutation Assay Ames test No explicit mentioning of OECD TG or GLP. Deviations: yes from OECD TG 471, only TA98 and TA100 strains tested, no	1,4-dichloro-2-nitrobenzene (named 2,5-dichloronitrobenzene) purity: not provided, used purest available commercial product Impurities: not provided Solvent: DMSO	*Salmonella typhimurium* TA98 and TA100 Pre-incubation method Test concentrations: 0, 250, and 500 µg/plate with and without S9-mix Replicates: 2 Positive controls: yes	negative TA98 (+/- S9); no increase in number of revertants observed positive TA100 (+/- S9); number of revertants increased to twice that of control	Kawai et al. (1987), (Japanese, Tables in English) BUA (1991) ECHA Dissemination (2021)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
data on cytotoxicity, limited information on study design and results as study is in Japanese Reliability: 3		Evaluation criteria: Positive, if the increase in number of revertant colonies is more than twice the control. → this is in agreement with the requirements of OECD TG 471		
Bacterial Reverse Mutation Assay No explicit mentioning of OECD TG or GLP. Deviations: yes from OECD TG 471, only tested one test strain (modified TA 1535), not tested with S9-mix, very limited documentation Reliability: 3	1,4-dichloro-2-nitrobenzene (named 2,5-dichloronitrobenzol) Purity: not provided Impurities: not provided Solvent: DMSO	*Salmonella typhimurium* strain TA1535/pSK1002 Test concentrations: 0, 50, 500, 1000, 1250, 2500, 5000, and 12250 µg/mL without S9-mix Tested until limit concentration. No confirmatory test performed. Media: histidine selective Plates: 3 Number of replicates: 2 Positive control: yes	positive (-S9)	Jin and Qian (1991) IARC (2020)
SOS response assay No explicit mentioning of OECD TG or GLP. Deviations:	1,4-dichloro-2-nitrobenzene (named 2,5-dichloronitrobenzol) Purity: not provided Impurities: not provided	*Salmonella typhimurium* strain TA1535/pSK1002 (*umuC*'-'*lacZ*) Test concentrations: 0, 10, 100, 500, and 1000 µg/mL without S9-mix	positive (-S9) Further information from the publication: The effect was dose dependent for colony formation and	Jin and Qian (1991) HCN (2018) IARC (2020)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
yes, limited data Reliability: 3	Solvent: DMSO	No. replicates: 2 2-fold increase in colonies per plate and β-galactosidase activity above the control levels was defined as positive	ß-galactosidase activity and the study authors concluded that DCNB is an SOS mutagen.	
Mammalian Cells				
Chromosomal aberration According to Japanese Guideline for Screening Mutagenicity testing of chemicals; similar to OECD TG 473 Deviations: cytotoxicity not determined for test concentrations in main test GLP: yes Reliability: 2	1,4-dichloro-2-nitrobenzene Purity: 99.5% Impurities: <0.5% isomer of dichloronitrobenzene (no further information provided) Solvent: DMSO	Chinese hamster lung cells (CHL) Test 1 without S9-mix continuous treatment for 24 or 48 hours, test concentrations: 0, 0.04, 0.08, and 0.15 mg/mL Test 2 without S9-mix treatment for 6 h, test concentrations: 0, 0.024, 0.047, and 0.094 mg/mL Test 3 with S9-mix treatment for 6 h, test concentrations: 0, 0.024, 0.047, and 0.094 mg/mL S-9 fraction from the liver of Phenobarbital and 5,6-Benzoflavone induced male SD derived rats with NADPH-generating system	CLH report submitter: Test 1: No statistically significant increase in structural aberrations or number of polyploid cells observed after 24 h treatment without S9-mix. Cytotoxic at the highest test concentration (0.15 mg/mL) A statistically significant increase in structural aberrations and number of polyploid cells was observed after 48 h treatment without S9-mix in the highest concentration (0.15 mg/mL), which was cytotoxic (only 104 cells analysed instead of 200	Ministry of Health and Welfare Japan (1994b), (Japanese, Tables in English) mentioned in Kusakabe et al. (2002); Morita et al. (2012) IARC (2020) OECD (1996) HCN (2018)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
		Media: RPMI 1640 medium plus 10% foetal calf serum plus phytohaemagglutinin Plates/test: 2 No. replicates: 1 Positive control: yes	cells). → equivocal Test 2 and 3: negative and number of polyploid cells not affected	
Chromosome aberration study in mammalian cells No explicit mentioning of OECD TG or GLP. Deviations: no information Reliability: 4 (secondary source)	1,4-dichloro-2-nitrobenzene Purity: not provided Impurities: not provided Solvent: not provided	Chinese hamster V79-cells Test 1 without S9-mix treatment for 4 h, test concentrations: 0, 10, 50, and 100 µg/mL Test 2 with S9-mix treatment for 6, test concentrations: 0, 20, 100, and 200 µg/mL	Original study not available to the dossier submitter BUA: 18 h after 4 h treatment: Test 1 and Test 2 were negative 28 h after 4 h treatment: Test 1 showed a tendency to an increase in chromosome aberrations at 100 µg/mL (-S9); Test 2 a statistically significantly increase in chromosome aberrations was observed at cytotoxic concentrations without a clear concentration-response →negative without S9-mix →inconclusive with S9-mix	BUA (1991)

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
HPRT assay No explicit mentioning of OECD TG or GLP. Deviations: yes, limited data Reliability: 4 (secondary source)	1,4-dichloro-2-nitrobenzene Purity: not provided Impurities: not provided Solvent: not provided	Chinese hamster V79-cells Test concentrations: 25 - 250 µg/mL with or without S9-mix Test 2 with S9-mix treatment for 6, test concentrations: 0, 20, 100, and 200 µg/mL	CLH report submitter: Original study not available thus an own assessment could not be performed BUA: negative (+/- S9)	BUA (1991)

* … The study was intended to investigate whether certain impurities could be responsible for mutagenic effects of 1-chloro-2,4-dinitrobenzene (CAS No. 97-00-7), explaining why this low dose was tested.