Committee for Risk Assessment

RAC

Annex 1
Background document
to the 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

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

1 December 2022

CLH report

Proposal for Harmonised Classification and Labelling

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

Chemical name:

1,4-Dichloro-2-nitrobenzene

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

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

Index Number: **N/A**

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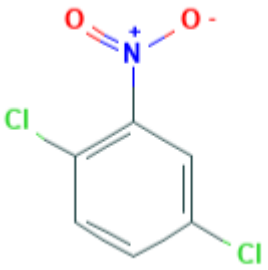
CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	1,4-dichloro-2-nitrobenzene
Other names (usual name, trade name, abbreviation)	Benzene, 1,4-dichloro-2-nitro- 2,5-Dichloronitrobenzene 2,5-Dichloro-1-nitrobenzene Benzene, 2,5-dichloronitro- Nitro-p-dichlorobenzene
ISO common name (if available and appropriate)	Not applicable
EC number (if available and appropriate)	201-923-3
EC name (if available and appropriate)	1,4-dichloro-2-nitrobenzene
CAS number (if available)	89-61-2
Other identity code (if available)	ICSC Number: 1618 RTECS Number: CZ5260000 UN number: 1578 PubChemCID: 6977
Molecular formula	$C_6H_3Cl_2NO_2$
Structural formula	
SMILES notation (if available)	C1=CC(=C(C=C1Cl)[N+](=O)[O-])Cl
Molecular weight or molecular weight range	192 g/mol
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	-
Description of the manufacturing process and identity of the source (for UVCB substances only)	Not relevant
Degree of purity (%) (if relevant for the entry in Annex VI)	≥ 80 wt %

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP) by registrants*
1,4-dichloro-2-nitrobenzene, CAS number: 89-61-2 EC number: 201-923-3	Confidential information, see confidential annex	No harmonised classification available	Acute Tox. 4 (H302) Skin Irrit. 2 (H315) Skin Sens. 1 (H317) Eye Irrit. 2 (H319) Muta. 2 (H341) Aquatic Chronic 3 (H412)

* ECHA C&L Inventory, 2021, Information on Chemicals - Classification & Labelling Inventory, European Chemicals Agency. Online: http://echa.europa.eu/information-on-chemicals/cl-inventory

1,4-Dichloro-2-nitrobenzene is a mono-constituent substance (CAS number: 89-61-2). The current self-classification by the registrants is given in Table 2. The frequency of hazard classifications among all notifications was retrieved from PubChem on 02/02/2021 and is given below. In total, 89 companies provided notifications with hazard classifications (10 aggregated notifications).

One company reported 1,4-dichloro-2-nitrobenzene as not meeting GHS hazard criteria.

Hazard classifications occurring in at least 10% of notifications:

Hazard code	Hazard statement	% of notifications
H302	Harmful if swallowed	62.5
H319	Causes serious eye irritation	57.95
H411	Toxic to aquatic life with long lasting effects	93.18

Except for the self-classification by the registrant, no CMR properties were notified.

The test substance is 1,4-dichloro-2-nitrobenzene in all studies where the test substance was explicitly stated. The purity is given in the study records below if available.

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
No information on impurities available	Confidential information			

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling

Additive (Name and numerical identifier)	**Function**	**Concentration range (% w/w minimum and maximum)**	**Current CLH in Annex VI Table 3 (CLP)**	**Current self-classification and labelling (CLP)**	**The additive contributes to the classification and labelling**
No information on additives available					

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 5: For substance with no current entry in Annex VI of CLP

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATEs	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 submitter's proposal	TBD	1,4-dichloro-2-nitrobenzene	201-923-3	89-61-2	Carc. 1B	H350	GHS08 Dgr	H350		-	

Table 6: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)	hazard class not assessed in this dossier	No
Oxidising gases	hazard class not assessed in this dossier	No
Gases under pressure	hazard class not assessed in this dossier	No
Flammable liquids	hazard class not assessed in this dossier	No
Flammable solids	hazard class not assessed in this dossier	No
Self-reactive substances	hazard class not assessed in this dossier	No
Pyrophoric liquids	hazard class not assessed in this dossier	No
Pyrophoric solids	hazard class not assessed in this dossier	No
Self-heating substances	hazard class not assessed in this dossier	No
Substances which in contact with water emit flammable gases	hazard class not assessed in this dossier	No
Oxidising liquids	hazard class not assessed in this dossier	No
Oxidising solids	hazard class not assessed in this dossier	No
Organic peroxides	hazard class not assessed in this dossier	No
Corrosive to metals	hazard class not assessed in this dossier	No
Acute toxicity via oral route	hazard class not assessed in this dossier	No
Acute toxicity via dermal route	hazard class not assessed in this dossier	No
Acute toxicity via inhalation route	hazard class not assessed in this dossier	No
Skin corrosion/irritation	hazard class not assessed in this dossier	No
Serious eye damage/eye irritation	hazard class not assessed in this dossier	No
Respiratory sensitisation	hazard class not assessed in this dossier	No
Skin sensitisation	hazard class not assessed in this dossier	No
Germ cell mutagenicity	data inconclusive	Yes
Carcinogenicity	harmonised classification proposed: Carc 1B H350	Yes
Reproductive toxicity	hazard class not assessed in this dossier	No
Specific target organ toxicity-single exposure	hazard class not assessed in this dossier	No
Specific target organ toxicity-repeated exposure	hazard class not assessed in this dossier	No
Aspiration hazard	hazard class not assessed in this dossier	No
Hazardous to the aquatic environment	hazard class not assessed in this dossier	No
Hazardous to the ozone layer	hazard class not assessed in this dossier	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

There is no harmonised classification and labelling available for 1,4-dichloro-2-nitrobenzene. The substance has not been included in former activities on harmonised classification.

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

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There is no requirement for justification that action is needed at community level.

The substance has CMR properties (germ cell mutagenicity and carcinogenicity). Harmonised classification and labelling for CMR is a community-wide action under article 36 of the CLP regulation.

5 IDENTIFIED USES

1,4-Dichloro-2-nitrobenzene is used as an intermediate in the production of fine chemicals, pharmaceuticals, pigments, pesticides, and ultraviolet absorbers and as a laboratory agent (ECHA Dissemination, 2021; HCN, 2018; IARC, 2020; OECD, 1996). In the European Union, it is registered for use at industrial sites including the production of pulp, paper, and paper products and textiles, leather, and fur as well as the inclusion into/onto articles like fabrics, textiles, and apparel (ECHA Dissemination, 2021). According to ECHA disseminated database, the source of exposure of professional workers and consumers to 1,4-dichloro-2-nitrobenzene are cleaning and maintenance products (e.g., washing and cleaning products) (ECHA Dissemination, 2021).

6 DATA SOURCES

Systematic searches for publications and other relevant data were performed based on the following databases:

- U.S. National Library of Medicine, Pubmed.gov
- TOXNET, ChemIDplus, IPCS, eChemPortal
- Medline, SciSearch, Biosis, PQscitech, Chemical Abstracts (HCA), Embase (at host STN International)

The REACH registration dossier for 1,4-dichloro-2-nitrobenzene (last modified: 1 September 2020) publicly available from ECHA's disseminated database (ECHA Dissemination, 2021) has been analysed for study references, which then have been considered as data sources for this CLH report.

Relevant reviews and monographs with toxicological risk assessments on 1,4-dichloro-2-nitrobenzene were analysed for study references. Used reviews are from the International Agency for Research on Cancer (IARC) (2020), Health Council of the Netherlands (HCN) (2018), Organisation for Economic Co-operation and Development Screening Information Data Sets (OECD SIDS) (1996), and GDCh-Advisory Committee on Existing Chemicals of Environmental Relevance (BUA report BUA (1991). The respective primary sources within the reviews were retrieved when possible.

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7 PHYSICOCHEMICAL PROPERTIES

Table 7: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Solid, crystalline	ECHA Dissemination (2021)	visual observation
Melting/freezing point	53.1 °C	ECHA Dissemination (2021)	measured, at 97.21 kPa
Boiling point	241.3 °C	ECHA Dissemination (2021)	measured, at 97.21 kPa
Relative density	1.262 g/cm³	ECHA Dissemination (2021)	density measured, at 20 °C
Vapour pressure	0.673 Pa	ECHA Dissemination (2021)	reported from handbook, measured
Surface tension		ECHA Dissemination (2021)	waived
Water solubility	84.095 mg/l	ECHA Dissemination (2021)	measured, at 25 °C
Partition coefficient n-octanol/water	2.87	ECHA Dissemination (2021)	measured, at 25 °C and pH 6.5
Flash point	114 °C	ECHA Dissemination (2021)	measured, at 97.13 kPa
Flammability	No data	ECHA Dissemination (2021)	Information requirement waived
Explosive properties	No data	ECHA Dissemination (2021)	Information requirement waived
Self-ignition temperature	465 °C	ECHA Dissemination (2021)	reported from secondary source (peer-reviewed data base), measured
Oxidising properties	No data	ECHA Dissemination (2021)	Information requirement waived
Granulometry	D50: > 53 ≤ 150 µm	ECHA Dissemination (2021)	measured
Stability in organic solvents and identity of relevant degradation products	No data	ECHA Dissemination (2021)	
Dissociation constant	No data	ECHA Dissemination (2021)	
Viscosity	No data	ECHA Dissemination (2021)	

8 EVALUATION OF PHYSICAL HAZARDS

Not performed for this substance.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 8: Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
Repeated dermal toxicity study/absorption; Rabbits were dermally exposed to 100, 200, 400 mg /kg bw 1,4-dichloro-2-nitrobenzene (purity unknown) once daily for up to 15 days. Non guideline, non-GLP.	1,4-dichloro-2-nitrobenzene is absorbed after repeated dermal exposure; signs of systemic toxicity were present (reduction of erythrocytes and haemoglobin level, hyperaemia, erythropoiesis, and iron pigmentation in spleen) and mortalities occurred.	Original study report not available, only secondary source	(BUA, 1991)
Metabolism study; Groups of female rabbits (6-10 animals per group) were given 0.4 g/kg bw 1,4-dichloro-2-nitrobenzene (purity unknown) once orally via gavage administration as aqueous suspension. Urine samples were collected for 72 hours and analysed by paper chromatography and absorption spectra. Non guideline, non-GLP.	Main urinary metabolites were determined to be excreted as mercapturic acid (9-33%), glucuronide (8-56%) and sulphate (3-21%). Dichloroaniline was excreted to a lesser degree (free: 10-19%, combined: 1%). Of the mercapturic acid metabolites, the isolated metabolites could be assigned to 2,5-dichloroaniline (13%), N-acetyl-S-(4-chloro-2-nitrophenyl)-l-cysteine (2%), and 4-amino-2,5-dichlorophenol (1%). Recovery in urine: 92% of administered dose		Bray et al. (1957) BUA (1991)
Metabolism study; A group of 3 male F344/DuCrj rats was exposed for 2 days to 1,4-dichloro-2-nitrobenzene (purity unknown) orally via diet containing 1% 1,4-dichloro-2-nitrobenzene. Urine samples of all three animals were collected for 24 hours, pooled as one sample, and analysed by UV, nuclear overhauser effect (NOE) method of ^{1}H-NMR, and liquid chromatography (LC)-MS/MS. Non guideline, non-GLP.	Clear yellow coloured urine observed in treated rats versus clear colourless urine in controls Main urinary metabolite was determined to be an N-acetylcysteine conjugate, namely N-acetyl-S-(4-chloro-3-nitrophenyl)-l-cysteine		Ohnishi et al. (2004) ECHA Dissemination (2021)
In vitro studies investigated the conjugation of glutathione to 1,4-dichloro-2-nitrobenzene by microsomal glutathione S-transferase purified from rat and human liver. Morgenstern et al. (1988) reported that 1,4-dichloro-2-nitrobenzene with a purity of 99% was used. Keen et al. (1976) performed a	1,4-Dichloro-2-nitrobenzene is a substrate of glutathione S-transferase but it can also react to thioether in the absence of the enzyme.		BUA (1991) Morgenstern et al. (1988) Keen et al. (1976)

Method	Results	Remarks	Reference
purification of 1,4-dichloro-2-nitrobenzene by distillation under pressure, however the purity is not provided. Activities were determined in an enzyme assay in potassium phosphate at pH 6.5-7.0.			

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

There is no information for 1,4-dichloro-2-nitrobenzene from humans or guideline toxicokinetic studies available. Only limited data on absorption, metabolism, and excretion was identified from animal studies conducted in rabbits and rats.

Absorption of 1,4-dichloro-2-nitrobenzene after single oral or repeated dermal exposure was observed in rabbits (Bray et al., 1957; BUA, 1991; Ohnishi et al., 2004). After oral administration of 0.4 g 1,4-dichloro-2-nitrobenzene/kg body weight (bw) by gavage to female rabbits 92% of the administered dose was recovered as metabolites in the urine (Bray et al., 1957; BUA, 1991). The main urinary metabolites detected were: mercapturic acid (9-33% of applied dose), glucuronide (8-56% of applied dose) and sulphate (3-21% of applied dose). The isolated metabolites of mercapturic acid metabolites were investigated further and determined to be 2,5-dichloroaniline (13% of applied dose), *N*-acetyl-*S*-(4-chloro-2-nitrophenyl)-*L*-cysteine (2% of applied dose), and 4-amino-2,5-dichlorophenol (1% of applied dose) (Bray et al., 1957).

Ohnishi et al. (2004) orally exposed three male rats to 1% 1,4-dichloro-2-nitrobenzene in diet for 2 days and collected urine samples. A clear yellow coloured urine of treated rats was observed and an *N*-acetylcysteine conjugate, namely *N*-acetyl-*S*-(4-chloro-3-nitrophenyl)-*L*-cysteine was identified as the main urinary metabolite (ECHA Dissemination, 2021; Ohnishi et al., 2004).

In vitro studies by Keen et al. (1976) and Morgenstern et al. (1988) demonstrated that 1,4-dichloro-2-nitrobenzene can react with glutathione to thioether either in the presence and absence of glutathione S-transferase (BUA, 1991).

Based on the findings of Bray et al. (1957), IARC reported a scheme as shown in Figure 1 demonstrating the metabolism of absorbed 1,4-dichloro-2-nitrobenzene to predominantly an aniline metabolite and to a phenol metabolite. Afterwards secondary conjugation of both metabolites by mercapturic acid, glucuronide, sulphate or *N*-acetylcysteine will take place (IARC, 2020).

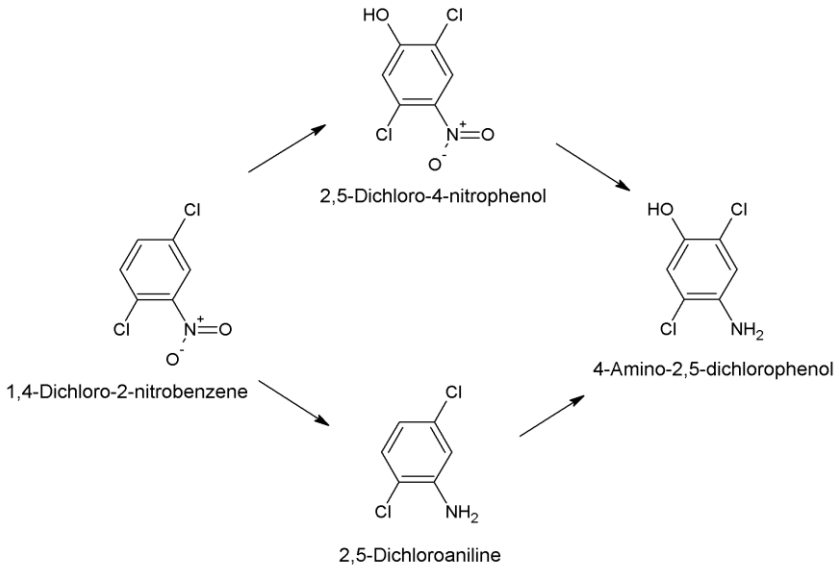

Figure 1: Identified urinary metabolites of 1,4-dichloro-2-nitrobenzene after oral exposure to rabbits, according to IARC (2020); original data by Bray et al. (1957).

To conclude, 1,4-dichloro-2-nitrobenzene is highly absorbed after oral as well as dermal exposure as indicated by the high fraction recovered from the urine. Absorbed 1,4-dichloro-2-nitrobenzene is intensively metabolised and mainly excreted via urine.

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

Evaluation not performed for this substance.

10.2 Acute toxicity - dermal route

Evaluation not performed for this substance.

10.3 Acute toxicity - inhalation route

Evaluation not performed for this substance.

10.4 Skin corrosion/irritation

Evaluation not performed for this substance.

10.5 Serious eye damage/eye irritation

Evaluation not performed for this substance.

10.6 Respiratory sensitisation

Evaluation not performed for this substance.

10.7 Skin sensitisation

Evaluation not performed for this substance.

10.8 Germ cell mutagenicity

Table 9: Summary table of mutagenicity/genotoxicity tests in vitro

Method, guideline, deviations if any	Test substance,	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference

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% isomer of dichloronitrobenzene (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 - first test: 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 µg/plate with S9-mix Plate incorporation method - second test TA100, TA1535, and TA1537: 0, 78.13,	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) <u>TA1535:</u> negative (+/-S9); cytotoxic at ≥625 µg/plate in first test and ≥1250 µg/plate in second test <u>TA1537:</u> 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 <u>WP2uvrA:</u> negative (+/-S9); cytotoxic at 5000 µg/plate (-S9) and ≥2500 µg/plate (+S9) in first test and at ≥2500 µg/plate (+S9) in second	Ministry of Health and Welfare Japan (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**
		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	test	
Bacterial Reverse Mutation Assay Ames test No explicit mentioning of 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	1,4-dichloro-2-nitrobenzene Purity: >99.6% Impurities: not provided Solvent: DMSO	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100 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 test without S9-mix	positive for TA98 and TA100 (-S9); cytotoxic at 6553.6 µg/plate positive for TA1538 (-S9); cytotoxic at ≥3276.8 µg/plate negative for TA1535 and TA1537 (-S9); no clear dose-response observed; cytotoxic at 6553.6 µg/plate	Shimizu et al. (1983) ECHA Dissemination (2021) HCN (2018) BUA (1991)

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
Reliability: 3		were negative. 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		
Bacterial Reverse Mutation 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, only one low concentration tested, limited information on study design and results Reliability: 3	1,4-dichloro-2-nitrobenzene (named 2,5-dichloronitrobenzene) purity: commercially available, no further information (purity given in the disseminated database is for dinitrochlorobenzene) Impurities: not provided Solvent: DMSO	*Salmonella typhimurium* TA100 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	Test 1: negative TA100 (-S9); Number of revertants per plate (Mean ± SEM) were 231 ± 8 for 1,4-dichloro-2-nitrobenzene and 242 ± 10 for control Only one low concentration tested; therefore, no conclusion can be drawn	Black et al. (1985) ECHA Dissemination (2021) HCN (2018)
Bacterial Reverse Mutation Assay Ames test No explicit mentioning of OECD TG or GLP.	1,4-dichloro-2-nitrobenzene (named 2,5-dichloronitrobenzene) purity: not provided, used purest available commercial product Impurities: not	*Salmonella typhimurium* TA98 and TA100 Pre-incubation method Test concentrations: 0, 250, and 500 µg/plate with and	negative TA98 (+/- S9); no increase in number of revertants observed positive TA100 (+/- S9); number of revertants increased twice that of control	Kawai et al. (1987), (Japanese, Tables in English) BUA (1991)

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Observations**	**Reference**
Deviations: yes from OECD TG 471, only TA98 and TA100 strains tested, no data on cytotoxicity, limited information on study design and results as study is in Japanese Reliability: 3	provided Solvent: DMSO	without S9-mix Replicates: 2 Positive controls: yes Evaluation criteria: Positive, if the increase in number of revertant colonies is more than twice than the control.		ECHA Dissemination (2021)
Bacterial Reverse Mutation Assay No explicit mentioning of OECD TG or GLP. Deviations: yes from OECD TG 471, only test strain TA tested, 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 No. 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: yes, limited data	1,4-dichloro-2-nitrobenzene (named 2,5-dichloronitrobenzol) Purity: not provided Impurities: not provided Solvent: DMSO	*Salmonella typhimurium* strain TA1535/pSK1002 (*umu*C'-'*lac*Z) Test concentrations: 0, 10, 100, 500, and 1000 µg/mL without S9-mix	positive (-S9)	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**
Reliability: 3		No. replicates: 2 2-fold increase in colonies per plate and β-galactosidase activity above the control levels was defined as positive		
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 Media: RPMI 1640 medium plus 10% foetal calf serum plus phytohaemagglutinin Plates/test: 2 No. replicates: 1	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 cells). → equivocal Test 2 and 3: negative and number of polyploid cells not affected	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**
		Positive control: yes		
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)
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)

Neither mutagenicity/genotoxicity tests in mammalian cells *in vivo* nor human data are available.

10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

There are only *in vitro* data available for the assessment of germ cell mutagenicity of 1,4-dichloro-2-nitrobenze. No *in vivo* data and no human data were identified.

In total 1,4-dichloro-2-nitrobenzene has been investigated in nine *in vitro* assays, which are summarised in Table 9. *In vitro* data are available from five Bacterial Reverse Mutation Assays (one Ames Test similar to OECD TG 471), a SOS response assay, two Mammalian Chromosome Aberration Assays in Chinese Hamster Lung cells (one similar to OECD TG 473) and a Mammalian Cell Forward Gene Mutation (CHL V-79/HPRT) Assay.

Due to methodological deficiencies of four Bacterial Reverse Mutation Assays reported in Table 9 only the assay reported by Ministry of Health and Welfare Japan (1994a) is regarded as reliable. In this study, conducted similar to OECD 471 and according to GLP, 1,4-dichloro-2-nitrobenzene was tested at five concentrations ranging from 39.06 to 5000 µg/plate in the presence and absence of metabolic activation. Various expert panels have differentially assessed the mutagenicity of 1,4-dichloro-2-nitrobenzene in the respective *Salmonella typh.* strains. It has to be mentioned that in its evaluation from 2020, IARC cited the results from OECD SIDS as the original documents were not accessible (IARC, 2020). Unfortunately, the applied evaluation criteria from Ministry of Health and Welfare Japan (1994a) are not clear as the study is only available in Japanese. Thus, for evaluating the results of *Salmonella typh.* strains, the dossier submitter followed the procedure established by Zeiger et al (1992) and Kier et al. (1986) and summarised in Mortelmans and Zeiger (2000), which are in accordance with the OECD TG 471. A positive result is given if *"a reproducible, dose-related increase in the number of revertant colonies in one or more strains. […]"* are observed (Mortelmans and Zeiger, 2000). *"[…] f no dose-related increase in the number of revertant colonies is observed in at least two independent experiments […]"* then the substance is assessed as non-mutagenic (Mortelmans and Zeiger, 2000). If neither a clear positive nor negative result can be given, it is inconclusive (Mortelmans and Zeiger, 2000). Biological relevance is decisive and thus a two-fold increase in the number of revertants compared to controls is regarded as relevant in tester strains TA98, TA100, and *E. coli* WP2 uvrA. Due to a higher reversion frequency the two-fold value is too insensitive for tester strains TA1535 and TA1537 for which a three-fold rule is applied (Kier et al., 1986).

A clearly positive result, which was dose-dependent, was observed in the presence and absence of the metabolic activating rat liver S9-mix in the *Salmonella typh.* strain TA100. For the *Salmonella typh.* strain TA98 an inconclusive result was obtained without metabolic activation, as only a marginal positive result was observed in one test but not confirmed in the second one. In the presence of metabolic activation *Salmonella typh.* strain TA98 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 the Annex 1.

Other tests conducted with *Salmonella typh.* that are considered less reliable (reduced number of strains tested, documentation deficiencies) provide suggestive evidence as they show positive responses in some of the tested strains. However, both positive and negative results were observed for the same strain in different studies underlining that the results of these studies are ambiguous and not suitable to derive a conclusion.

The SOS response test is a bacterial genotoxicity assay approved for water and wastewater examination, which identifies primary DNA-damage caused by genotoxic substances (Oda, 2016). The induced DNA-damage cannot be repaired and leads to the expression of an enzyme which activity is determined colorimetrically. In the SOS response test conducted in *Salmonella typh.* strain 1535/pSK1002 1,4-dichloro-2-nitrobenzene is considered to be an SOS mutagen in the absence of a metabolic activating system. Due to poor data reporting and methodological deficiencies, the study was regarded to have limited reliability by the dossier submitter.

Of the three *in vitro* genotoxicity assays conducted in mammalian cells, 1,4-dichloro-2-nitrobenzene was reported to be negative in a HPRT test and not a clear clastogene in a chromosome aberration assay conducted in Chinese hamster V79-cells. Both assays are only available from a secondary source and the reliability was therefore not assignable (Reliability 4).

In a second chromosome aberration assay similar to OECD TG 473 and according to GLP (reliability 2, although some inconsistencies exist in the report), Chinese hamster lung cells were exposed to 1,4-dichloro-2-nitrobenzene either continuously for 24 or 48 hours without metabolic activation or for 6 hours with or without metabolic activation. After 6-hour treatment with or without metabolic activation 1,4-dichloro-2-nitrobenzene did not induce structural chromosome aberrations at any of the tested concentrations. After 48 hours in the continuous treatment without metabolic activation, a statistically significant increased incidence of structural aberrations was observed at the highest tested concentration. Additionally, the number of polyploid cells was statistically significantly increased. Both effects were not observed until after the 24 h-treatment at the same concentration level, which was evaluated by the authors of the study to be a cytotoxic concentration (Ministry of Health and Welfare Japan, 1994b). No data on the cytotoxicity of this test series (respective concentrations and exposure durations) was provided. Cytotoxicity was only investigated in a

preliminary test, in which a cell growth of 55% was observed at 0.12 mg/mL and 0% at 0.24 mg/mL without any further concentrations tested in between (growth inhibition was less at even higher concentrations, 0.95 and 1.9 mg/mL, with growth rates between 9 and 23%, which raises doubt regarding consistency of the reported effects). Therefore, it is assumed that a treatment for 48 hours with a concentration of 0.15 mg/mL resulted in cytotoxicity that is in the borderline range at which a reliable evaluation can no longer be done, since a distinction between true genotoxicity or a genotoxic effect due to cytotoxicity is no longer possible. It also has to be noted that only 104 cells instead of planned 200 cells could be analysed at 0.15 mg/mL. Thus, the observed increased incidence of structural chromosome aberrations at the highest tested concentration after 48 hour-treatment is considered to be equivocal. Details on the performed test method and numerical values are documented in the Annex 1.

In conclusion, there are no data on germ cell mutagenicity of 1,4-dichloro-2-nitrobenzene available. For bacteria, genotoxicity assays suggest some mutagenic potential of 1,4-dichloro-2-nitrobenzene. A chromosome aberration study conducted in mammalian cells gave an equivocal result.

10.8.2 Comparison with the CLP criteria

For potential classification on germ cell mutagenicity, criteria from the CLP Regulation (EC, 2008)[1] were applied:

a) Comparison with Category 1 criteria

- *The classification in Category 1A is based on positive evidence from human epidemiological studies (EC, 2008)*

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

- *The classification in Category 1B is based on positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals (EC, 2008)*
- *Classification in Category 1B can also be based on "positive result(s) 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. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells". (EC, 2008)*

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.

b) Comparison with Category 2 criteria

- *Classification in category 2 is based on:*

  — positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:

  — somatic cell mutagenicity tests in vivo, in mammals; or

  — other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays. (EC, 2008)

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

However, in EC (2008) a note is also stated, which needs to be considered:

Note: 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 mutagens. (EC, 2008)

[1] REGULATION (EC) No 1272/2008 considering all ATPs published until January 2021

An *in vitro* mammalian mutagenicity assay exists for 1,4-dichloro-2-nitrobenzene. As already stated in Table 9, an *in vitro* chromosome aberration study similar to OECD TG 473 conducted in CHL cells gave a negative result 24 h after continuous exposure to 1,4-dichloro-2-nitrobenzene without metabolic activation. After 48 hours without metabolic activation, an equivocal result was observed at a cytotoxic concentration. Therefore, no clear positive genotoxicity was observed in *in vitro* mammalian mutagenicity assays. The existing data from *in vitro* non-mammalian or mammalian mutagenicity assays is not regarded as sufficient to assign a classification as a category 2 mutagen.

Overall, no epidemiological data and no *in vivo* heritable germ cell / somatic cell mutagenicity tests in mammals are available. In addition, the *in vitro* bacterial assays suggest there may be some mutagenic potential, but the classification criteria are not met in the absence of a clear positive response in mammalian cells.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Classification as a germ cell mutagen is not warranted because of insufficient data.

The data available also does not allow to exclude a genotoxic potential of 1,4-dichloro-2-nitrobenzene.

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.

10.9 Carcinogenicity

Yamazaki et al. (2006) conducted chronic toxicity/carcinogenicity studies in rats and mice. The studies complied with GLP and were conducted similar to OECD TG 453, with various doses, sufficient large number of animals per treatment group, and suitable observations and examinations. Both studies were not reported in the registration dossier publicly available on the disseminated database of ECHA (ECHA Dissemination, 2021). In its evaluation from 2018, HCN assigned a reliability according to the Klimisch score of 2 (HCN, 2018). The dossier submitter agrees with this score. In the following table the results relevant for the endpoint carcinogenicity are reported. Both studies are reported in detail in Annex I.

Table 10: Summary table of animal studies on carcinogenicity

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	1,4-dichloro-2-nitro-benzene purity: > 98.8% 0, 320, 800 or 2000 ppm (w/w) in diet; equivalent to	Incidences are always stated for doses at 0, 320, 800 and 2000 ppm (if not indicated otherwise). Statistically significant results are indicated in **bold** text/numbers as significant in trend test (**trend**) or by pairwise comparison. 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	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**
Reliability: 2 GLP: yes F344/DuCrj (SPF) male and female rats, 50 per sex and group	10, 25 and 63 mg/kg bw/d for males and 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 a subchronic study in rats (see Table 11) daily oral exposure via diet for 2 years (104 weeks)	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. The relative testis weight was statistically significantly (P≤0.01) increased, but the absolute testis weight was not significantly increased in males at 2000 ppm compared to controls. <u>non-neoplastic lesions:</u> Chronic progressive nephropathy (CPN, total) 46/50**, 49/50$^{\#\#}$, 50/50$^{\#\#}$, 49/50$^{\#\#}$** in males and 24/50, 23/50, 32/50, 28/50 in females Urothelial hyperplasia in pelvis 1/50, **8/50$^{\#}$, 36/59$^{\#\#}$, 39/50$^{\#\#}$** in males Mineralisation of papilla 0/50, 2/50, **47/50$^{\#\#}$, 48/50$^{\#\#}$** in males Hematopoesis in bone marrow 5/50, 9/50, 9/50, **14/50$^{\#}$** in females No increased tumour incidences were observed in females. Males: <u>Pre-neoplastic lesions:</u> Basophilic hepatocellular foci: 21/50, 22/50, **32/50$^{\#\#}$**, **40/50$^{\#\#}$** (n.a.) <u>Neoplastic lesions:</u> Liver: Hepatocellular adenoma: 0/50, 1/50, 0/50, **6/50*** (**trend**) Hepatocellular carcinoma: 0/50, 0/50, 1/50 (2%), 2/50 (4%) Hepatocellular adenoma and carcinoma (combined): 0/50, 1/50, 1/50, **8/50*** (**trend**) Kidney: Renal cell adenoma: 0/50, 0/50, 0/50, 2/50 (4%) Renal cell carcinoma: 0/50, 1/50 (2%), 0/50, 1/50 (2%) Renal cell adenoma and renal cell carcinoma (combined): 0/50, 1/50, 0/50, 3/50 (**trend**) Zymbal gland: Zymbal gland adenoma 0/50, 0/50, 0/50, 4/50 (8%) (**trend**) Historical control incidence in 1249 male rats (maximum incidence in any study): hepatocellular carcinoma, 0.2% (2%); renal cell adenoma, 0.16%	

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		(2%); renal cell carcinoma, 0.16% (2%); Zymbal gland adenoma, 0.2% (2%).	
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-nitro-benzene 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 subchronic study in mice daily oral exposure via diet for 2 years (104 weeks)	Incidences are always stated for doses at 0, 320, 800 and 2000 ppm (if not indicated otherwise). Statistically significant results are indicated in **bold** text/numbers as significant in trend test (**trend**) or by pairwise comparison: 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> Centrilobular hypertrophy with nuclear atypia of hepatocytes 0/49[a], **38/50[##], 39/50[##], 40/50[##]** in males and 0/50, **15/50[##], 29/50[##], 35/50[##]** in females Hemosiderin deposition in kidney 1/49, 6/50, 6/50, **25/50[##]** in males Erythropoiesis in bone marrow 7/49, 7/50, 14/50, **23/50[##]** in males <u>Pre-neoplastic lesions:</u> Acidophilic cell foci: 0/49, 2/50, **7/50[#], 11/50[##]** in males <u>Neoplastic lesions:</u> Males: Hepatocellullar 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**), historical control data: 5/1,047 in 21 studies Hepatocellular adenoma, hepatoblastoma and carcinoma (combined): 26/49, 34/50, **41/50**, 45/50**** (**trend**) Hepatocellular carcinoma and hepatoblastoma metastasized to lungs. Females: Hepatocellular adenoma: 5/50, 5/50, **17/50*, 16/50*** (**trend**)	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
		Hepatocellular carcinoma: 1/50, 3/50, **15/50*, 31/50*** (**trend**) Hepatoblastoma: 0/50, 0/50, 0/50, 2/50, historical control data: 0/1,047 in 21 studies Hepatocellular adenoma, hepatoblastoma and carcinoma (combined): 6/50, 8/50, **29/50*, 39/50*** (**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

Trend test performed by Peto test.

a: one male control mouse died accidentally during administration

Table 11: Summary table of other studies relevant for carcinogenicity

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/DuCrj (SPF) male and female rats, 10 per sex and group	1,4-dichloro-2-nitro-benzene purity: > 99.9%	0, 1481, 2222, 3333, 5000 or 7500 ppm (w/w) in diet (0, 93, 135, 207, 316 or 474 mg/kg bw/d for males and 0, 106, 162, 238, 342 or 458 mg/kg bw/d for females) doses were based on results from a subacute 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	Yamazaki et al. (2005)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			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-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 (consisting of 8 animals with moderate and 2 with marked droplets), **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**, 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**,**	

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			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 female mice, 10 per sex and group	1,4-dichloro-2-nitro-benzene purity: > 99.9%	0, 1481, 2222, 3333, 5000 or 7500 ppm (w/w) in diet (0, 245, 374, 530, 775 or 1647 mg/kg bw/d for males and 0, 284, 428, 613, 936 or 1601 mg/kg bw/d for females); doses were based on results from a subacute study in mice 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. 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 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	Yamazaki et al. (2005)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			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**, 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

10.9.1 Short summary and overall relevance of the provided information on carcinogenicity

There are no human data available for assessing the carcinogenic potential of 1,4-dichloro-2-nitrobenzene. Inhalation carcinogenicity studies were not identified for 1,4-dichloro-2-nitrobenzene.

Reliable oral chronic toxicity/carcinogenicity studies conducted in rats and mice, which were complying to GLP and similar to OECD TG 453 were available. Carcinogenic effects were observed in these studies (see Table 10) including:

- 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.

Regarding classification and labelling for carcinogenicity it is necessary to assess if the observed neoplastic lesions in experimental animals are relevant for humans. This will be discussed for each observed tumour type separately, taking into account the sparsely available information on the Mode of Action (MoA).

10.9.1.1 Liver tumours in male rats and male and female mice

In a chronic toxicity/carcinogenicity study, Yamazaki et al. (2006) reported a statistically significant increase in incidences of hepatocellular adenoma as well as hepatocellular adenoma and carcinoma (combined) in male rats exposed to 1,4-dichloro-2-nitrobenzene. In the highest exposure group (2000 ppm) the incidence of hepatocellular carcinoma was higher than the upper range of the historical control data. Basophilic cell foci, a pre-neoplastic lesion, was also statistically significantly and dose-related increased. Metastisation of liver tumours to other organs or tissues was not observed.

In this study, general toxicity after chronic exposure was characterised by decrease in terminal body weight, changes in blood, and blood biochemistry parameters as well as absolute and relative organ weights (for details see Table 10 and Annex I). Hepatotoxicity was observed as increased relative liver weights in rats of both sexes and centrilobular hypertrophy of hepatocytes in mice of both sexes. Although these effects were also noted in low dose exposure groups their occurrence and severity is limited and thus not regarded as excessive toxicity.

In mice hepatocarcinogenicity of 1,4-dichloro-2-nitrobenzene was evident as significantly increased incidences of liver adenoma in female mice, liver carcinoma in male and female mice, and liver adenoma, carcinoma, and hepatoblastomas (combined) in male and female mice were observed (Yamazaki et al., 2006). An increase in mice dying due to liver tumours before the end of the treatment period was observed (7, 8, 11, and 23 males and 0, 3, 4, and 6 females for 0, 320, 800, and 2000 ppm). Statistically significantly dose-relatedly increased incidences of acidophilic cell foci, a pre-neoplastic and proliferative lesion, was observed in male mice.

Hepatoblastoma is a tumour type with a low background rate in mice. Incidences of hepatoblastomas were statistically significantly increased in all exposed male animals and increased in female mice of the highest exposure group compared to controls (1 and 0 affected animals for male and female controls, resp.) and also compared to historical control data (5 cases out of 1047 males; no case out of 1047 females). Hepatocellular carcinoma of male and female mice as well as hepatoblastomas of male mice metastasised into the lung.

Data from subchronic oral toxicity studies with 1,4-dichloro-2-nitrobenzene conducted in rats and mice identified the liver and kidney as the target organs (for details see Table 11) (Yamazaki et al., 2005). Toxic effects observed in the liver included increased liver weights and centrilobular hypertrophy of hepatocytes in both species as well as single cell hepatic necrosis and elevated serum activity levels of liver-associated transaminases in mice. Clear histopathological changes in hepatocytes were only observed in mice with markedly enlarged cytoplasm and various sizes and shapes of nuclei.

From the literature it is known that continuous hepatocellular proliferation can be a driver for the development of substance-induced hepatocarcinogenicity. Regarding neoplastic findings in murine liver, Takahashi et al (2002) reported progressive lesion development from first appearing of vinyl carbamate-induced hepatocellular foci to adenoma and subsequently carcinoma, which can be regarded as a biological and morphological continuum.

Yamazaki et al. (2006) suggested for 1,4-dichloro-2-nitrobenzene that *"A genotoxic mode of action is thought to operate in the DCNB-induced hepatocarcinogenicity"*. As mentioned in section 10.8.1, there is evidence from an *in vitro* bacterial genotoxicity study that 1,4-dichloro-2-nitrobenzene can act as a mutagen. However, due to lack of data on *in vivo* heritable germ cell or somatic cell mutagenicity tests in mammals, there is insufficient data to support a genotoxic MoA.

In its evaluation from 2020, IARC assessed the carcinogenic potential of various nitrobenzenes by comparing their activity in standardised biochemical and cell-based assays across a few endpoints that are linked to common characteristics of carcinogens. In case of 1,4-dichloro-2-nitrobenzene, ToxCast and/or Tox21 high-throughput screening assays found to be active in 3 out of 54 assay endpoints investigated concerning *"Modulates receptor-mediated effects"*. 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"*, whereas the latter was regarded as the most sensitive one (IARC, 2020). By comparing typical key characteristics of carcinogens with 1,4-dichloro-2-nitrobenzene IARC resumed that there is *"weak evidence that 1,4-dichloro-2-nitrobenzene is genotoxic"* (IARC, 2020).

Although the exact MoA is not known, data from carcinogenicity studies in rats and mice indicate clearly that exposure to 1,4-dichloro-2-nitrobenzene induced tumours in the liver. As liver tumours also occur in humans and no data exist that link 1,4-dichloro-2-nitrobenzene to a MoA which is not relevant for humans (e.g., via PPARα), 1,4-dichloro-2-nitronbenzene's induction of liver tumours in male rats and mice of both sexes is considered as relevant for humans.

10.9.1.2 Renal tumours in male rats

After chronic exposure to 1,4-dichloro-2-nitrobenzene in rats, Yamazaki et al. (2006) reported a positive trend test for the increase in combined incidences of renal cell adenoma and carcinoma in male rats. Compared to the historical control data, a borderline increase in incidences of renal cell adenoma (4% compared to 0.16% in historical controls) in male rats of the highest exposure group was observed. In one case of a high exposed male, metastisation of the renal cell carcinoma to lung was noted. An increase in pre-neoplastic lesion was not seen in any dose group. But non-neoplastic lesions like urothelial hyperplasia in renal pelvis and renal papilla mineralisation were statistically significantly increased in a dose-dependent manner. Additionally, chronic nephropathy, which is attributed to advanced age of rats, was statistically significantly increased in all exposed dose groups.

In the subchronic toxicity study by Yamazaki et al. (2005) male rats had increased incidences of hyaline droplets and granular casts at the renal proximal tubules after subchronic oral exposure to 1,4-dichloro-2-nitrobenzene (for details see Table 11). Both observed alterations were positive for staining with α_{2u}-globulin antibody. These findings are suggesting a rat-specific α_{2u}-globulin-induced nephropathy whose MoA is characterised by α_{2u}-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 and Lehman-McKeeman, 1999). Kidney tumours in male rats associated with substances causing α_{2u}-globulin nephropathy are not considered relevant for humans (ECHA, 2017). In the literature a vast number of chemicals, e.g., hydrocarbons, 1,4-dichlorobenzene are known to cause α_{2u}-globulin-induced nephropathy. In females an increase in blood urea nitrogen level and of eosinophilic droplets in cytoplasm of proximal tubular epithelial cells, which were negative for staining with α_{2u}-globulin antibody, were observed. This indicates that the test substance causes nephrotoxicity not solely via α_{2u}-globulin pathway (Takahashi et al., 2002).

In order to identify if a substance's MoA of causing renal tumours is solely driven by α_{2u}-globulin IARC established criteria, which all must be met, and are the following:

- Lack of genotoxic activity,
- 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 α_{2u}-globulin,
- Reversible binding of the chemical or metabolite to α_{2u}- globulin,
- Induction of sustained increased cell proliferation in the renal cortex,
- Similarities in dose-response relationship of the tumour outcome with the histopathological endpoints (protein droplets, α_{2u}-globulin accumulation, cell proliferation) (Capen et al., 1999).

IARC (2020) concluded that 1,4-dichloro-2-nitrobenzene is not fulfilling all criteria as *"criteria not met included chemical identification of the protein accumulating in tubule cells as α_{2u}-globulin, and reversible binding of 1,4-dichloro-2-nitrobenzene or a metabolite to α_{2u}-globulin"*.

Yamazaki et al. (2006) indicate that *"renal tumors in male rats that are produced by exposure to substances that produce α_{2u}-globulin nephropathy need not be considered in assessing potential neoplastic health risks to humans, when attributed exclusively to α_{2u}-globulin accumulation and non-mutagenic substances"*. As mentioned previously in section 10.8.1, 1,4-dichloro-2-nitrobenzene was mutagenic in a bacterial reverse mutation test and the result of an *in vitro* chromosome aberration assay was equivocal, thus the genotoxic MoA of 1,4-dichloro-2-nitrobenzene is unclear. However, Yamazaki et al. (2006) also refer that *"reactive metabolites of glutathione and cysteine conjugates of trichloroethylene, S-(1,2-dichlorovinyl)glutathione and S- (1,2-dichlorovinyl)-L-cysteine, which were biotransformed from the corresponding hepatic glutathione S-conjugate by γ-glutamyltranspeptidase and cysteine conjugate β-lyase in the kidney"* and were shown to be mutagenic in an Ames test. As support Yamazaki et al (2006) made reference to a study from Elfarra et al. (1986), in which male rats were exposed to S-(1,2-dichlorovinyl)glutathione and S- (1,2-dichlorovinyl)-L-cysteine intraperitoneally, *"indicating a crucial role of the renal γ-glutamyltranspeptidase and cysteine conjugate β-lyase in the trichloroethylene-induced nephropathy"* and studies from Dekant et al. (1986) which showed that *"N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine was identified as a urinary metabolite of trichloroethylene, and that thioacylating metabolites of S-(1,2- dichlorovinyl)-L-cysteine, which were formed by renal cysteine conjugate β-lyase, might contribute to the nephrotoxic and mutagenic effects".*

1,4-Dichloro-2-nitrobenzene is excreted in urine as *N*-acetyl-S-(4-chloro-3-nitrophenyl)-*L*-cysteine (for details see section 9.1) and considering the findings of Elfarra et al. (1986) and Dekant et al. (1986) it can be assumed that it was metabolized to the glutathione conjugate in the liver, followed by reaction to *S*-(4-chloro-3-nitrophenyl)-*L*-cysteine in the kidney. Thus, Yamazaki et al. (2006) summarised *"it can be rational to infer that reactive and possibly mutagenic metabolites, which are presumably produced from S-(4-chloro-3-nitrophenyl)-L-cysteine by cysteine conjugate β-lyase in the kidneys contribute to the development of DCNB-induced nephrocarcinogenicity and chronic nephrotoxicity, although the α_{2u}-globulin-induced nephropathy can not be totally ruled out as a causative factor of nephrocarcinogenicity and nephrotoxicity"*.

Taking all into account, there is evidence for 1,4-dichloro-2-nitrobenzene-induced renal tumours. However, α_{2u}-globulin-induced nephropathy might also be involved and thus the relevance of these tumours to humans is unclear.

10.9.1.3 Zymbal gland adenoma in male rats

In male rats a positive trend in increase in incidences of Zymbal gland adenoma was observed. Compared to the historical controls, the increase clearly exceeded the upper range for the high-dosed males (Yamazaki et al., 2006). Yamazaki et al. (2006) assessed the occurrence of these tumours as substance-related but only marginal due to the increase in tumour incidence.

The Guidance on the Application of the CLP criteria (ECHA, 2017) lists tumours in the Zymbal gland in context that "*some of the commonly used animal species have some tissues with no equivalent in humans"* and further describes *"Zymbal's glands are located beneath squamous epithelium at the anterior and posterior aspect of the ear canal. The external portion of the gland in rats is 3 to 5 millimetres in diameter."*. Although Zymbal glands are not occurring in humans and thus its relevance in humans seems to be low, it cannot be excluded completely. Pohl and Fouts (1983) detected cytochrome P450 activity in Zymbal glands

of rats and mice, indicating that reactive metabolites of carcinogens may be formed, which can lead to tumour formation. Tumours occurring in the Zymbal gland after chronic exposure to 1,4-dichloro-2-nitrobenzene were of benign nature only. As no information on the mode of action is available for these tumours, their human relevance remains unclear.

In conclusion, the relevance of Zymbal gland tumours in male rats for the classification of 1,4-dichlor-2-nitrobenzene as carcinogenic is unclear. Overall, this evidence is therefore considered merely supportive for carcinogenicity, rather than clear evidence.

Table 12: Compilation of factors to be taken into consideration in the hazard assessment

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
F344/DuCrj (SPF) rats	**Liver** hepatocellular adenoma: Control: none (0/50) Historical control data: not reported hepatocellular carcinoma: Control: none (0/50) Historical control data: only reported for carcinoma; 3 cases (0.2%) in 1249 male rats in 25 studies with maximum incidence of 2% hepatocellular adenoma and carcinoma (combined): Control: none (0/50) Historical control data: not reported	Yes	Yes, adenoma transformed to carcinoma, see Table 10	Not applicable	Single, only observed in male rats	Not assumed to be due to excessive toxicity	Oral by diet	Relevant; discussed in detail in section 10.9.1.1
	Kidney Renal cell adenoma: Control: none (0/50) Historical control data: 2 cases (0.16%) in 1249 male rats in 25 studies with maximum incidence of 2%	Yes, one renal cell carcinoma of a high-dosed male rat metastasised to lung	Yes, adenoma transformed to carcinoma, see Table 10	Not applicable	Single, only observed in male rats	Not assumed to be due to excessive toxicity	Oral by diet	Relevance uncertain; discussed in detail in section 10.9.1.2

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
	Renal cell carcinoma: Control: none (0/50) Historical control data: 2 cases (0.16%) in 1249 male rats in 25 studies with maximum incidence of 2% Renal cell adenoma and carcinoma (combined): Control: none (0/50) Historical control data: not reported							
	Zymbal gland Zymbal gland adenoma: Control: none (0/50) Historical control data: 3 cases (0.2%) in 1249 male rats in 25 studies with maximum incidence of 2%	Yes	only (non-malignant) adenoma observed	Not applicable	Single, only observed in male rats	Not assumed to be due to excessive toxicity	Oral by diet	Relevance uncertain; discussed in detail in section 10.9.1.3
Crj:BDF1 (SPF) mice	**Liver** hepatocellular adenoma Control: 17/49; 5/50 in females hepatocellular carcinoma: Control: 15/49 in males; 1/50 in females Historical control data: not reported	Yes, hepatocellular carcinoma of male and female mice as well as hepatoblastoma of male mice metastasised to lung	Yes, adenoma transformed to carcinoma, see Table 10	Not determined	Both sexes	Not assumed to be due to excessive toxicity	Oral by diet	Relevant; discussed in detail in section 10.9.1.1

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
	<u>hepatoblastoma:</u> Control: 1/49 in males; 0/50 in females Historical control data: 5 cases in 1047 males and 0 cases in 1047 females <u>hepatoblastoma, adenoma, and carcinoma (combined):</u> Control: 26/49 in males; 6/50 in females Historical control data: not reported							

10.9.2 Comparison with the CLP criteria

IARC (2020) classified 1,4-dichloro-2-nitrobenzene: "*possibly* carcinogenic to humans (Group 2B)", based on *sufficient* evidence in animals and *inadequate* evidence in humans, according to the IARC criteria.

For potential classification of carcinogenicity, the criteria from the CLP Regulation (EC, 2008) supported by explanations from the Guidance on the Application of the CLP criteria (ECHA, 2017) were applied. The incidences of adenoma and carcinoma were considered. For potential classification of 1,4-dichloro-2-nitrobenzene, classification criteria were analysed accordingly.

- *The classification in Category 1A and 1B is based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived from:*

  — *human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or*

  — *animal experiments for which there is sufficient evidence to demonstrate animal carcinogenicity (presumed human carcinogen).*

  In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals. (EC, 2008)

The classification in Category 1A as a known human carcinogen applies for:

- *A substance may be further distinguished as Category 1A [if] classification is largely based on human evidence […].(EC, 2008)*

No epidemiological data for 1,4-dichloro-2-nitrobenzene is available, thus Category 1A is not warranted.

The classification in Category 1B as a presumed human carcinogen applies for:

- *A substance may be further distinguished […] as Category 1B [if] classification is largely based on animal evidence. (EC, 2008)*
- *Further EC regards as sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence [..](EC, 2008)*

Based on the data provided by Yamazaki et al. (2006) a causal relationship between the substance and an increased incidence of tumours in male and female mice as well as in male rats was observed. However, the criterion above is closely linked to the relevance for humans and additional considerations as discussed below.

The following additional considerations from CLP section 3.6.2.2.6 are relevant for concluding on a classification for carcinogenicity:

- (a) tumour type and background incidence;

  The tumour types observed in rodents after chronic oral exposure to 1,4-dichloro-2-nitrobenzene included hepatoadenoma and -carcinoma (male rats, and male and female mice), hepatoblastoma (male and female mice), renal cell adenoma and carcinoma (combined) (male rats), and Zymbal gland tumours (male rats). All tumour types listed above were elevated to a relevant degree compared to background incidence (or historical control data in the case of hepatoblastoma in female mice) in the individual studies.

- (b) multi-site responses;

  In rats, multi-site responses after chronic exposure to 1,4-dichloro-2-nitrobenzene were observed. Organs affected were liver, kidney, and Zymbal gland, although not all with a clear human relevance. Mice repeatedly exposed to 1,4-dichloro-2-nitrobenzene had different types of tumours in the liver.

  Metastisation of tumours to lung was observed in rodents. In mice, hepatocellular carcinoma of male and female mice as well as hepatoblastoma of male mice metastasised to lung. For renal cell carcinoma, it was observed that one case of a high-dosed male rat metastasised to lung.

- (c) progression of lesions to malignancy;

  In case of hepatocellular adenoma and renal cell adenoma the potential progress to malignancy was identified.

- (d) reduced tumour latency;

  This criterion was not addressed as the studies of Yamazaki et al. (2006) did not provide information on reduced tumour latency.

- (e) whether responses are in single or both sexes;

  Regarding the liver tumours a significant increase in hepatocellular adenoma was identified in male rats and female mice. Hepatocellular carcinoma were significantly increased in male and female mice and not statistically significantly increased in male rats. The combined incidence of hepatocellular adenoma and carcinoma was significantly increased in male rats as well as in male and female mice. Hepatoblastoma of the liver were seen in mice of both sexes. Renal cell adenoma and carcinoma (combined) as well as Zymbal gland adenoma were described in male rats only. In female rats, no tumour formation was observed.

- (f) whether responses are in a single species or several species;

  Tumour formation in the liver occurred in both rats and mice. Renal cell carcinoma and Zymbal gland adenoma were only identified in male rats.

- (g) structural similarity to a substance(s) for which there is good evidence of carcinogenicity;

  In its monography from 2020, IARC evaluated together with 1,4-dichloro-2-nitrobenezene the following chloronitrobenzenes: 2,4-dichloro-2-nitrobenzene (CAS No. 611-06-3), 2-chloronitrobenzene (CAS No. 88-73-3), and 4- chloronitrobenzene (CAS No.: 100-00-5) (IARC, 2020). For these chloronitrobenzenes IARC investigated their potential for carcinogenicity and concluded that they are “possibly carcinogenic to humans” (Group 2B) based on sufficient evidence in experimental animals (IARC, 2020). A harmonised classification is solely available for 4-chloronitrobenzene, which is classified for the CMR properties as a mutagen category 2 (Muta. 2) and carcinogen category 2 (Carc. 2) (ECHA C&L Inventory, 2021).

  However, the evidence for their carcinogenicity does not help to eliminate uncertainties on the mode of action and human relevance of the tumours observed in the studies conducted with 1,4-dichloro-2-nitrobenzene.

- (h) routes of exposure;

  In all available studies 1,4-dichloro-2-nitrobenzene was applied orally via diet.

- (i) comparison of absorption, distribution, metabolism and excretion between test animals and humans;

  There are no indications of significant differences in the toxicokinetic data for humans and animals. However, data for humans and animals is too limited to draw a final conclusion.

- (j) the possibility of a confounding effect of excessive toxicity at test doses;

  The available critical studies gave no concern for a confounding effect by excessive toxicity at test doses. General toxicity after chronic exposure to 1,4-dichloro-2-nitrobenzene was noted by decreased terminal body weight, changes in blood and clinical biochemistry parameters, and organ weights. Adverse effects in kidney and liver were also observed in the low dose exposure group. However, these effects are not regarded as excessive toxicity caused at the tested doses.

- (k) mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity.

  These aspects are discussed in detail in section 10.9.1.

Overall, there is sufficient evidence in animals for carcinogenicity with regard to tumours in the liver (multispecies and sex). A classification in Category 1B is therefore justified. The carcinogenic potential of 1,4-dichloro-2-nitrobenzene is further supported by tumour findings in other organs in the rat although their human relevance is unclear.

The classification of a substance in Category 2 as a suspected human carcinogen is based on the following, according to CLP Regulation (EC, 2008):

The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited (15) evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

The evidence observed in animal studies as described above is not considered limited but clear evidence for carcinogenicity. The evidence is sufficiently convincing to place the substance in Category 1B. Therefore, classification in Cat 2 is not warranted.

10.9.3 Conclusion on classification and labelling for carcinogenicity

Classification in category

Carc. 1B, 'H350: May cause cancer',

is warranted.

This conclusion is largely in accordance with the classification,

- by IARC (2020), who classify 1,4-dichloro-2-nitrobenzene as "possibly carcinogenic to humans" (Group 2B) based on sufficient evidence in experimental animals,
- by Health Council of the Netherlands (HCN, 2018), who assign the category 1B "presumed to be carcinogenic to humans" to 1,4-dichloro-2-nitrobenzene

To account for the contribution of a hazardous substance to the classification of mixtures based on the potency of the hazardous substance the concept of specific concentration limit (SCL) was established.

"Specific concentration limits and generic concentration limits are limits assigned to a substance indicating a threshold at or above which the presence of that substance in another substance or in a mixture as an identified impurity, additive or individual constituent leads to the classification of the substance or mixture as hazardous". (EC, 2008)

In order to derive SCLs for carcinogens, the Guidance on the Application of the CLP criteria (ECHA, 2017) refers to the T25 concept established by Dybing et al. (1997) and a European guideline document (EC, 1999). For the calculation of T25 the endpoints with highest relevance for humans and highest incidence have to be considered. From the carcinogenicity studies performed in rodents (for details see Table 10) the statistically significantly increase in incidence of hepatoblastoma in male mice at 320 ppm (approx. 32 mg/kg bw/d) of 10/50 compared to 1/49 in the control and hepatocellular carcinoma in female mice at 800 ppm (approx. 103 mg/kg bw/d) of 15/50 compared to 1/50 in the control were used. The calculated T25 values were 44.5 mg/kg bw/d for hepatoblastoma in male mice and 92.0 mg/kg bw/d for hepatocellular carcinoma in female mice, respectively. In both cases, the T25 value is in the range of 1 mg/kg bw/d ≤ 100 mg/kg bw/d, which assigns 1,4-dichloro-2-nitrobenzene as a carcinogen of medium potency (EC, 1999). Thus, the generic concentration limit of 0.1% can be applied.

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 3Table 11) daily oral exposure via diet for 2 years (104 weeks)	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-neoplastic & pre-neoplastic findings are summarised in table 4	Yamazaki et al. (2006) Cited also by HCN (2018) IARC (2020)
Chronic toxicity and carcinogenicity study, similar to OECD TG 453 Deviations: limited reporting Reliability: 2 GLP: yes	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	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	Yamazaki et al. (2006) Cited also by HCN (2018) IARC (2020)

Crj:BDF1 (SPF) male and female mice, 50 per sex and group	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)	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	

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	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	Yamazaki et al. (2005)

group			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-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**, 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 female mice, 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, 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 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. 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 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.	Yamazaki et al. (2005)

			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**, 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	0/50	**15/50**	**29/50**	**35/50**	

hypertrophy with nuclear atypia of hepatocytes		**##**	**##**	**##**	
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 ±	238 ±	234 ±	199 ±	

	36	23	32	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.

Zymbal gland tumours in male rats

***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. Hepatoblastoma in female mice: Two incidences in the top dose (2/50) – exceeded historical controls.	Supportive for classification
	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	a) inconclusive b) MoA plausible – relevant to humans. c) Information insufficient to draw

	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	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).

<u>Potency and concentration limits for the classification of mixtures</u>

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.

10.10 Reproductive toxicity

Evaluation not performed for this substance.

10.11 Specific target organ toxicity-single exposure

Evaluation not performed for this substance.

10.12 Specific target organ toxicity-repeated exposure

Evaluation not performed for this substance. However, evaluation of 90-day repeated dose toxicity studies in rat and mice is included in the overall assessment of the endpoint carcinogenicity in section 10.9.

10.13 Aspiration hazard

Evaluation not performed for this substance.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Evaluation not performed for this substance.

12 EVALUATION OF ADDITIONAL HAZARDS

Evaluation not performed for this substance.

13 ADDITIONAL LABELLING

Not applicable.

14 ANNEXES

Please see separate document for Annex I and confidential Annex I.

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Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

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

International Chemical Identification:

1,4-Dichloro-2-nitrobenzene

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

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

Index Number: **N/A**

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Version number: 03 **Date: October 2021**

ANNEX TO ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 1,4-DICHLORO-2-NITROBENZENE

CONTENTS

1 PHYSICAL HAZARDS

Evaluation not performed for this substance.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Evaluation not performed for this substance.

3 HEALTH HAZARDS

Acute toxicity

3.1 Acute toxicity - oral route

Evaluation not performed for this substance.

3.2 Acute toxicity - dermal route

Evaluation not performed for this substance.

3.3 Acute toxicity - inhalation route

Evaluation not performed for this substance.

3.4 Skin corrosion/irritation

Evaluation not performed for this substance.

3.5 Serious eye damage/eye irritation

Evaluation not performed for this substance.

3.6 Respiratory sensitisation

Evaluation not performed for this substance.

3.7 Skin sensitisation

Evaluation not performed for this substance.

3.8 Germ cell mutagenicity

3.8.1 In vitro data

3.8.1.1 Study 1

Study reference:

Anonymous, Bacterial Reverse Mutation Test CAS 89-61-2, Ministry of Health and Welfare Japan, 1994 (Ministry of Health and Welfare Japan, 1994a).

Detailed study summary and results:

Test type

An *in vitro* bacterial reverse mutation assay according to Japanese Guideline for Screening Mutagenicity testing of chemicals; similar to OECD TG 471, was conducted with 1,4-dichloro-2-nitrobenzene.

Main deviations from the OECD TG were the use of a positive control not recommended in the OECD TG and biological relevance was poorly considered by the applied evaluation criteria. GLP compliance is given.

The study report is in Japanese with results tables in English, thus it was not always possible to provide all information.

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 1,4-dichloro-2-nitrobenzene
- Degree of purity: > 99.5%
- Impurities: < 0.5% isomer of dichloronitrobenzene (exact isomer not given)
- Batch number: not provided

Administration/exposure

- Strains: *Salmonella typhimurium* TA 98, TA 100, TA 1535, and TA 1537, and *E. coli* WP2 uvr A
- Target gene: Histidine
- Type and composition of metabolic activation system:
  - species and cell type: rat, liver microsomal enzymes from liver homogenate (S9-mix)
  - quantity: not provided
  - induced or not induced: induced
  - chemicals used for induction: phenobarbital and 5,6-benzoflavone
  - co-factors used: not provided
- Test concentrations:
  - preliminary cytotoxicity test (all strains) 0, 50, 150, 500, 1500, and 5000 µg/plate with or without S9-mix
  - Mutation assay (plate incorporation method):

  - Experiment 1:
    - 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
    - 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
  - Experiment 2:
    - 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
    - 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
  - Number of plates: 3
  - Number of replicates: 2
- Vehicle: DMSO
- Statistical methods: A statistical evaluation according to hypothesis testing was not performed.

Results and discussion

- Tested dose levels based on preliminary cytotoxicity test (0, 50, 150, 500, 1500, and 5000 µg/plate), tested up to maximum concentration according to guideline
- Cytotoxic concentrations with and without metabolic activation:
  - reported at 1250 µg/plate with or without metabolic activation
- Genotoxic effects with and without metabolic activation:
  results according to study authors, derivation of CLH dossier submitter given in brackets:
  - test strain TA100: positive with and without metabolic activation
  - test strain TA1535: negative without metabolic activation, positive with metabolic activation (CLH dossier submitter regards its evaluation criteria of a three-fold higher increase in the number of revertants compared to controls as not reached for TA1535, thus TA1535 is considered to be negative)
  - test strains TA98, TA1537, and WP2uvrA: negative with and without metabolic activation (in case of TA98 the CLH dossier submitter regards its evaluation criteria of a two-fold

higher increase in the number of revertants compared to controls as reached in one of the two tests, thus TA98 is considered to be inconclusive)

- Concurrent negative (solvent/vehicle) and positive control data:
  - solvent control: yes, valid
  - positive control: yes (sodium azide (SA, without S9-mix, strain TA1535), 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF2, without S9-mix, strains TA98, TA100, and strain WP2uvrA), 9-aminoacridine (9-AA, without S9-mix, TA1537), and 2-aminoanthracene (2-AA, with S9-mix, all strains), valid
- Test-specific confounding factors:
  - Effects of pH: not provided
  - Effects of osmolality: not provided
  - Water solubility: not provided
  - Precipitation: precipitation of test substance observed at 1250, 2500, and 5000 µg/plate without metabolic activation in experiment 1
- Statistical results:
  - no statistical evaluation of results available,
- Information needed to adequately assess data for reliability:
  - mean number of revertant colonies per plate and standard deviation: numerical values are provided in Table 1 and Table 2
  - evaluation criteria applied by the study authors:
    - positive: if the increase in the number of mutant colonies compared to controls is more than doubled and the increase is reproducible or dose-dependent

Table 1: Results of bacterial reverse mutation test (experiment 1) of 1,4-dichloro-2-nitrobenzene

With or without S9 mix	Concentration of test substance (µg/plate)	Number of revertants (number of colonies/plate, Mean ± S.D.)				
		TA100	TA1535	WP2uvrA	TA98	TA1537
Without S9-mix	0	114 106 116 (112 ± 5.3)	7 8 8 (8± 0.6)	29 24 18 (24 ± 5.5)	38 34 18 (30 ± 10.6)	3 10 7 (7 ± 3.5)
	39.06	ND	18 9 11 (13 ± 4.7)	ND	ND	11 8 5 (8 ± 3.0)
	78.13	240 254 232 (242 ± 11.1)	10 16 19 (15± 4.6)	ND	ND	9 5 4 (6 ± 2.6)
	156.3	292 372 381 (348 ± 49.0)	11 11 11 (11 ± 0.0)	18 22 18 (19 ± 2.3)	27 21 26 (25 ± 3.2)	9 8 8 (8 ± 0.6)
	312.5	573 555 545 (558 ± 14.2)	22 18 18 (19 ± 2.3)	20 19 17 (19 ± 1.5)	30 32 27 (30 ± 2.5)	9 4 8 (7 ± 2.6)
	625	664 720 700 (695 ± 28.4)	21* 17* 17* (18 ± 2.3)	12 21 14 (16 ± 4.7)	19 22 32 (24 ± 6.8)	7* 10* 6* (8 ± 2.1)
	1250[#]	395* 539* 662*	16* 30* 15*	11 21 15	30 18 31	1* 0* 0*

With or without S9 mix	Concentration of test substance (µg/plate)	Number of revertants (number of colonies/plate, Mean ± S.D.)				
		TA100	**TA1535**	**WP2uvrA**	**TA98**	**TA1537**
		(532 ± 133.6)	(20 ± 8.4)	(16 ± 5.0)	(26 ± 7.2)	(0 ± 0.6)
	2500#	591* 354* 527* (491 ± 122.6)	ND	11 7 14 (11 ± 3.5)	32* 30* 50* (37 ± 11.0)	ND
	5000#	ND	ND	14* 11* 12* (12 ± 1.5)	28* 28* 26* (27 ± 1.2)	ND
With S9-mix	0	113 124 95 (111 ± 14.6)	8 8 7 (8 ± 0.6)	19 25 26 (23 ± 3.8)	28 31 30 (30 ± 1.5)	8 11 9 (9 ± 1.5)
	78.13	208 231 186 (208 ± 22.5)	6 10 9 (8 ± 2.1)	ND	48 33 34 (38 ± 8.4)	12 12 9 (11 ± 1.7)
	156.3	240 254 240 (245 ± 8.1)	13 14 7 (11 ± 3.8)	18 21 10 (16 ± 5.7)	28 34 27 (30 ± 3.8)	13 9 12 (11 ± 2.1)
	312.5	329 380 286 (332 ± 47.1)	9 11 11 (10 ± 1.2)	14 18 15 (16 ± 2.1)	27 43 35 (35 ± 8.0)	6 6 11 (3 ± 2.9)
	625	438 415 460 (438 ± 22.5)	6 6 14 (9 ± 4.6)	14 11 25 (17± 7.4)	37 37 39 (38± 1.2)	9 15 12 (12 ± 3.0)
	1250	623* 636* 575* (611 ± 32.1)	0* 0* 0* (0 ± 0.0)	15 27 14 (19 ± 7.2)	34* 24* 30* (29 ± 5.0)	6* 11* 8* (8± 2.5)
	2500	0* 0* 0* (0 ± 0.0)	0* 0* 0* (0 ± 0.0)	10* 10* 13* (11± 1.7)	7* 6* 2* (5 ± 2.6)	0* 0* 0* (0 ± 0.0)
	5000	ND	ND	9* 14* 4* (9 ± 5.0)	ND	ND
Without S9-mix	AF2 (0.01 µg)	419 433 451 (434 ± 16.0)		118 133 101 (117 ± 16.0)		
	AF2 (0.1 µg)				734 663 717 (705 ± 37.1)	
	SA (0.5 µg)		371 358 316 (348 ± 28.7)			
	9-AA (80 µg)					2417 2199 2136 (2251 ± 147.5)
With S9-mix	2-AA (0.5 µg)				248 272 185 (235 ± 44.9)	
	2-AA (1 µg)	925 804 994 (908 ± 96.2)				
	2-AA (2 µg)		303 289 279 (290 ± 12.1)			201 222 215 (213 ± 10.7)
	2-AA (10 µg)			1298 1222 1416 (1312 ± 97.8)		

Positive controls AF2: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, SA: sodium azide, 9-AA: 9-aminoacridine and 2-AA: 2-aminoanthracene

#: Precipitant was observed on surface of agar plates

*: Inhibition of bacteria growth was observed

ND: not determined

Table 2: Results of bacterial reverse mutation test (experiment 2) of 1,4-dichloro-2-nitrobenzene

With or without S9 mix	Concentration of test substance (µg/plate)	Number of revertants (number of colonies/plate, Mean ± S.D.)

		TA100	TA1535	WP2uvrA	TA98	TA1537
Without S9 mix	0	108 92 101 (100 ± 8.0)	9 7 9 (8 ± 1.2)	14 18 17 (16 ± 2.1)	17 19 19 (18 ± 1.2)	7 6 6 (6 ± 0.6)
	78.13	245 235 197 (226 ± 25.3)	18 9 6 (11 ± 6.2)	ND	ND	6 5 10 (7 ± 2.6)
	156.3	346 324 336 (335± 11.0)	14 9 15 (13± 3.2)	18 18 18 (18 ± 0.0)	22 33 26 (27 ± 5.6)	4 4 12 (7 ± 2.6)
	312.5	635 531 536 (567 ± 58.7)	12 10 12 (11 ± 1.2)	21 21 18 (20 ± 1.7)	43 32 59 (45 ± 13.6)	8 4 10 (7 ± 3.1)
	625	761 709 719 (730 ± 27.6)	13 18 12 (14 ± 3.2)	15 12 20 (16 ± 4.0)	47 34 48 (43 ± 7.8)	7 8 8 (8 ± 0.6)
	1250	720 816 800 (779 ± 51.4)	11* 13* 19* (14± 4.2)	13 11 16 (13 ± 2.5)	37 44 38 (40± 3.8)	3* 7* 8* (6 ± 2.6)
	2500	659* 686* 737* (694 ± 39.6)	14* 14* 19* (16 ± 2.9)	17 18 12 (16 ± 3.2)	48 35 42 (42 ± 6.5)	8* 6* 6* (7 ± 1.2)
	5000	ND	ND	19 22 16 (19 ± 3.0)	45* 48* 33* (42 ± 7.9)	ND
With S9 mix	0	140 109 113 (121 ± 16.9)	9 11 18 (13 ± 4.7)	12 17 17 (153 ± 2.9)	20 29 26 (25 ± 4.6)	9 6 7 (7 ± 1.5)
	78.13	229 212 203 (215 ± 13.2)	11 9 11 (14 ± 4.6)	ND	43 34 33 (37 ± 5.5)	13 16 14 (14 ± 1.5)
	156.3	270 267 301 (279 ± 18.8)	8 19 16 (14 ± 5.7)	26 17 28 (24 ± 5.9)	32 26 29 (29 ± 3.0)	15 10 12 (12 ± 2.5)
	312.5	396 342 413 (384 ± 37.1)	16 9 11 (12 ± 3.6)	25 21 19 (22 ± 3.1)	34 26 39 (33 ± 6.6)	8 8 13 (10 ± 2.9)
	625	536 546 554 (545 ± 9.0)	15 12 19 (15 ± 3.5)	21 16 20 (19 ± 2.6)	23 33 26 (27 ± 5.1)	15 8 11 (11 ± 3.5)
	1250	697 686 611 (665 ± 46.8)	12* 13* 12* (12 ± 0.6)	14 18 15 (16 ± 2.1)	21* 19* 42* (27 ± 12.7)	5* 7* 11* (8 ± 3.1)
	2500	35* 49* 89* (58 ± 28.0)	0* 0* 0* (0 ± 0.0)	8* 13* 15* (12 ± 3.6)	9* 3* 6* (6 ± 3.0)	0* 0* 0* (0 ± 0.0)
	5000	ND	ND	3* 5* 5* (4 ± 1.2)	ND	ND
Without S9-mix	AF2 (0.01 µg)	357 322 366 (348 ± 23.2)		115 140 149 (135 ± 17.6)		
	AF2 (0.1 µg)				593 624 696 (638 ± 52.8)	
	SA (0.5 µg)		212 225 228 (222 ± 8.5)			
	9-AA (80 µg)					1103 1060 993 (1052 ± 55.4)
With S9-mix	2-AA (0.5 µg)				371 384 400 (385 ± 14.5)	
	2-AA (1 µg)	898 784 873 (852 ± 59.9)				
	2-AA (2 µg)		343 337 291 (324 ± 28.4)			226 254 253 (244 ± 15.9)
	2-AA (10 µg)			1136 1229 1281 (1215 ± 73.5)		

Positive controls AF2: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, SA: sodium azide, 9-AA: 9-aminoacridine and 2-AA: 2-aminoanthracene

#: Precipitant was observed on surface of agar plates

*: Inhibition of bacteria growth was observed

ND: not determined

3.8.1.2 Study 2

Study reference:

Anonymous, *In vitro* mammalian chromosome aberration test CAS 89-61-2, Ministry of Health and Welfare Japan, 1994 (Ministry of Health and Welfare Japan, 1994b).

Detailed study summary and results:

Test type

An *in vitro* mammalian chromosome aberration test according to Japanese Guideline for Screening Mutagenicity testing of chemicals; similar to OECD TG 473, was conducted with 1,4-dichloro-2-nitrobenzene. Main deviation from the OECD TG was cytotoxicity not determined for test concentrations in the main test. GLP compliance is given.

The study report is in Japanese with results tables in English, thus it was not always possible to provide all information.

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 1,4-dichloro-2-nitrobenzene
- Degree of purity: > 99.5%
- Impurities: < 0.5% isomer of dichloronitrobenzene (exact isomer not given)
- Batch number: not provided

Administration/exposure

- Strain or cell type or cell line, target gene: Chinese hamster lung cells (CHL)
- Type and composition of metabolic activation system:
  - species and cell type: rat liver, S9 mix
  - quantity: not provided
  - induced or not induced: induced
  - chemicals used for induction: phenobarbital and 5,6-benzoflavone
- co-factors used: not provided
- Test concentrations, and reasoning for selection of doses:
  - prior to cytogenetic assays a growth inhibition test (6 hours without S9 mix) was conducted
  - Assay 1 without S9-mix continuous treatment for 24 or 48 hours, test concentrations: 0, 0.04, 0.08, and 0.15 mg/mL
  - Assay 2 without S9-mix treatment for 6 h, test concentrations: 0, 0.024, 0.047, and 0.094 mg/mL

  - Assay 3 with S9-mix treatment for 6 h, test concentrations: 0, 0.024, 0.047, and 0.094 mg/mL
- Vehicle: RPMI 1640 medium plus 10% foetal calf serum plus phytohaemagglutinin, test substance is diluted in DMSO
- Method of application: in medium
- Number of plates: 2
- Number of replicates: 1
- Statistical methods:
  - Statistical significance was determined by Fisher's Exact probability test method for the frequency of appearance of cells with chromosomal abnormalities

Results and discussion

- Justification for choice of tested dose levels (e.g. dose-finding studies): Tested concentrations were based on a dose-range finding test
- Cytotoxic concentrations with and without metabolic activation: cytotoxicity was only tested in the preliminary growth inhibition test, for details see Table 3 and Table 4
- Genotoxic effects (e.g., positive, negative, unconfirmed, dose-response, equivocal) with and without metabolic activation
  - Assay 1, 24-hour exposure without metabolic activation:
    - negative, no statistically significant increase in structural aberrations or number of polyploid cells observed, cytotoxic at the highest test concentration (0.15 mg/mL)
  - Assay 1, 48-hour exposure without metabolic activation:
    - equivocal, statistically significant increase in structural aberrations and number of polyploid cells was observed in the highest concentration (0.15 mg/mL), which was cytotoxic (only 104 cells analysed instead of 200 cells)
  - Assay 2, 6-hour exposure without metabolic activation:
    - negative and number of polyploid cells not affected
  - Assay 3, 6-hour exposure with metabolic activation:
    - negative and number of polyploid cells not affected
- Concurrent negative (solvent/vehicle) and positive control data:
  - negative control: yes, valid
  - solvent control: yes, valid
  - positive control: yes (cyclophosphamide (with metabolic activation) and mitomycin C (without metabolic activation)), valid
- Test-specific confounding factors:
  - Effects of pH: not provided

  - Effects of osmolality: not provided
  - Water solubility: not provided
  - Precipitation: no precipitation reported
- Statistical results: after test item treatment, a statistically significant increase was observed in Assay 1
- Information needed to adequately assess data for reliability:
  - numerical values presented in Table 5 and Table 6
  - evaluation criteria: in Japanese, according to Ishidate et al. 1987 (no further details, as citation in Japanese)
- Observations regarding reported cytotoxicity:
  - Although the study in general seems to be well performed, some discrepancies were observed regarding the reported cytotoxicity data: cytotoxicity was lower at the highest doses compared to 0.12 – 0.24 mg/mL and at 0.15 mg/mL was lower after 48h compared to 24h (Table 5)

Table 3: Growth inhibition of CHL cells continuously treated with 1,4-dichloro- 2-nitrobenzene for 48 hours without S9 mix from two measurements

Concentration in mg/mL	Cell growth (% of control)		
	Measurement 1	Measurement 2	Average
0	100	100	100.0
0.06	89	76	82.5
0.12	53	58	55.5
0.24	0	0	0.0
0.48	1	3	2.0
0.95	19	21	20.0
1.90	9	21	15.0

Table 4: Growth inhibition of CHL cells treated with 1,4-dichloro-2-nitrobenzene for 6 hours with and without S9 mix from two measurements

Concentration in mg/mL	S9 mix with (+) or without (-)	Cell growth (% of control)		
		Measurement 1	Measurement 2	Average
0	-S9	100	100	100.0
0.06	-S9	93	78	85.5
0.12	-S9	15	0	7.5
0.24	-S9	0	0	0.0
0.48	-S9	6	5	5.5
0.95	-S9	9	6	7.5
1.90	-S9	9	11	10.0
0	+S9	100	100	100.0
0.06	+S9	92	79	85.5
0.12	+S9	2	2	2.0
0.24	+S9	6	2	4.0
0.48	+S9	14	9	11.5

Concentration in mg/mL	S9 mix with (+) or without (-)	Cell growth (% of control)		
		Measurement 1	Measurement 2	Average
0.95	+S9	19	23	21.0
1.90	+S9	19	17	18.0

Table 5: Chromosome analysis of Chinese hamster cells (CHL) continuously treated with 1,4-dichloro-2-nitrobenzene (DCN) without S9 mix

Group	Concen-tration (mg/mL)	Expo-sure (h)	No. of cells analysed	No. of cells with structural aberrations									No. of cell with aberrations		Poly-ploid[4] (%)	Judgement[5]	
				gap	ctb	cte	csb	cse	f	mul[2]	total	Others[3]	TAG (%)	TA (%)		SA	NA
Control			200	1	0	0	0	0	0	0	1	0	1 (0.5)	0 (0.0)	0.13		
Solvent[1]	0	24	200	1	1	0	0	0	0	0	2	0	2 (1.0)	1 (0.5)	0.63		
DCN	0.04	24	200	1	2	0	0	0	0	0	3	0	3 (1.5)	2 (1.0)	0.75	-	-
DCN	0.08	24	200	1	1	0	0	0	0	0	2	0	2 (1.0)	1 (0.5)	1.25	-	-
DCN	0.15	24	6	0	0	1	0	0	0	0	1	0	1 (16.7)	1 (16.7)	0.00[6]	Tox	Tox
MC	0.00005	24	200	9	34	98	5	1	4	10	161	1	97* (48.5)	95* (47.5)	0.38	+	-
Solvent[1]	0	48	200	6	0	0	0	0	0	0	6	0	5 (2.5)	0 (0.0)	0.13		
DCN	0.04	48	200	3	1	1	0	0	1	0	6	0	5 (2.5)	2 (1.0)	0.38	-	-
DCN	0.08	48	200	1	0	0	0	0	0	0	1	0	1 (0.5)	0 (0.0)	0.50	-	-
DCN	0.15	48	104	4	7	8	1	1	1	0	22	0	11* (10.6)	8* (7.7)	4.70*[7]	+	Tox
MC	0.00005	48	200	18	44	106	2	4	6	50	230	0	93* (46.5)	87* (43.5)	0.38	+	-

Abbreviations: gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring etc.), f: acentric fragment (chromatid type), mul: multiple aberrations, TAG: total no. of cells with aberrations, TA: total no. of cells with aberrations except gap, SA: structural aberration, NA: numerical aberration, MC: mitomycin C, Tox: toxic.

1) Dimethyl sulfoxide was used as solvent. 2) More than ten aberrations in a cell were scored as 10.

3) Others, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations.

4) Eight hundred cells were analysed in each group. 5) Judgement was done on the basis of the criteria of Ishidate et al. (1987); no further details as citation in Japanese.

6) Fifteen cells were analysed. 7) One hundred and forty-nine cells were analysed. *: Significantly different from solvent control at $p<0.05$.

Table 6: Chromosome analysis of Chinese hamster cells (CHL) continuously treated with 1,4-dichloro-2-nitrobenzene (DCN) without S9 mix

Group	Concen-tration (mg/mL)	-/+ S9 mix; expo-sure (h)	No. of cells analysed	No. of cells with structural aberrations									No. of cell with aberrations		Poly-ploid[4] (%)	Judgement[5]	
				gap	ctb	cte	csb	cse	f	mul[2]	total	Others[3]	TAG (%)	TA (%)		SA	NA
Control			200	1	0	0	0	0	0	0	1	0	1 (0.5)	0 (0.0)	0.50		
Solvent[1]	0	-S9; 6	200	2	0	0	0	0	0	0	2	0	2 (1.0)	0 (0.0)	0.25		
DCN	0.024	-S9; 6	200	2	0	0	0	0	0	0	2	0	2 (1.0)	0 (0.0)	0.25	-	-
DCN	0.047	-S9; 6	200	3	1	0	0	0	0	0	4	1	4 (2.0)	1 (0.5)	0.25	-	-
DCN	0.094	-S9; 6	200	4	0	1	0	0	0	0	5	0	5 (2.5)	1 (0.5)	1.00	-	-
CPA	0.005	-S9; 6	200	1	1	0	0	0	0	0	2	0	1 (0.5)	1 (0.5)	0.50	-	-
Solvent[1]	0	+S9; 6	200	1	1	0	0	0	0	0	2	0	2 (1.0)	1 (0.5)	0.25		
DCN	0.024	+S9; 6	200	3	1	0	0	0	0	0	4	2	4 (2.0)	1 (0.5)	0.50	-	-
DCN	0.047	+S9; 6	200	3	3	0	0	0	1	0	7	0	4 (2.0)	4 (2.0)	0.50	-	-
DCN	0.094	+S9; 6	200	4	2	4	0	0	0	0	10	1	7 (3.5)	4 (2.0)	1.00	-	-
CPA	0.005	+S9; 6	200	15	96	327	0	0	11	190	639	0	170* (85.0)	168* (84.0)	0.13	+	-

Abbreviations: gap: chromatid gap and chromosome gap, ctb: chromatid break, cte: chromatid exchange, csb: chromosome break, cse: chromosome exchange (dicentric and ring etc.), f: acentric fragment (chromatid type), mul: multiple aberrations, TAG: total no. of cells with aberrations, TA: total no. of cells with aberrations except gap, SA: structural aberration, NA: numerical aberration, CPA: cyclophosphamide.

1) Dimethyl sulfoxide was used as solvent. 2) More than ten aberrations in a cell were scored as 10.

3) Others, such as attenuation and premature chromosome condensation, were excluded from the no. of structural aberrations.

4) Eight hundred cells were analysed in each group. 5) Judgement was done on the basis of the criteria of Ishidate et al. (1987); no further details as citation in Japanese.

*: Significantly different from solvent control at $p<0.05$.

3.8.1.3 Study 3

Study reference:

Shimizu, M., Yasui, Y., Matsumoto, N., Structural specificity of aromatic compounds with special reference to mutagenic activity in Salmonella typhimurium - a series of chloro- or fluoro-nitrobenzene derivatives, Mutation Research, 116 (3-4), 1983, 217-238 (Shimizu et al., 1983).

Detailed study summary and results:

Test type

The mutagenic activity of 1,4-dichloro-2-nitrobenzene was assessed in an *in vitro* bacterial reverse mutation assay by the pre-incubation method with *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 exposed to 0 to 6553.6 µg/plate in the absence of metabolic activation. An assay with the same test setting in the presence of metabolic activation was solely performed, if the previous test was negative. The assay was neither conducted according to OECD TG nor GLP compliance. Deviations from OECD TG 471 were the lack of all required five tester strains, no testing in the presence of a metabolic activation system, performing no confirmatory tests, and the selection of positive control (*N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) used in tester strains TA100 and TA1535 instead of in tester strains *E. coli* WP2, WP2 uvrA).

Test substance

- The test material used in the study is equivalent to the substance identified in the CLH dossier: 1,4-dichloro-2-nitrobenzene
- Degree of purity: > 99.6%
- Impurities: not provided
- Batch number: not provided

Administration/exposure

- Strains: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538
    - Strains detecting frameshift mutations: TA98, TA1537, and TA1538
    - Strains detecting base-pair substitutions: TA100 and TA1535
- Target gene: Histidine
- Type and composition of metabolic activation system:
    - species and cell type: male Sprague-Dawley rats, liver microsomal enzymes from liver homogenate (S9-mix)
    - quantity: 0.5 mL of S9-mix
    - induced or not induced: induced

  - chemicals used for induction: polychlorinated biphenyls (PCB), 500 mg/kg bw
  - co-factors used: not provided
- Test concentrations:
  - preliminary cytotoxicity test (all strains) 10 concentrations tested from 0.1 or 0.01 µg/plate to the toxic dose level (no further information)
  - Mutation assay (pre-incubation method):
    - Experiment 1:
      - For all strains: 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
  - Number of plates: 3
  - Number of replicates: 2
- Vehicle: DMSO
- Statistical methods: A statistical evaluation according to hypothesis testing was not performed.

Results and discussion

- Tested dose levels based on preliminary cytotoxicity test (0, 51.2, 102.4, 204.8, 409.6, 819.2, 1638.4, 3276.8, and 6553.6 µg/plate), tested higher than the maximum concentration according to OECD TG 471 (5000 µg/plate)
- Cytotoxic concentrations with and without metabolic activation:
  - reported at 3276.8 µg/plate for TA1538 and at 6553.6 µg/plate for all other test strains without metabolic activation
- Genotoxic effects with and without metabolic activation:
  - authors of the study regarded 1,4-dichloro-2-nitrobenzene as mutagenic for both types of strain causing frameshift mutations or base-pair substitutions
- Concurrent negative (solvent/vehicle) and positive control data:
  - negative control: no
  - solvent control: yes, valid
  - positive control: yes (*N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG without S9-mix, strain TA100 and TA1535), 2-nitrofluorene (2-NF, without S9-mix, strains TA98 and TA1538), 9-aminoacridine (9-AA, without S9-mix, TA1537), valid
- Test-specific confounding factors:
  - Effects of pH: not provided
  - Effects of osmolality: not provided
  - Water solubility: not provided
  - Precipitation: not reported

- Statistical results:
  - no statistical evaluation of results available,
- Information needed to adequately assess data for reliability:
  - mean number of revertant colonies per plate and standard deviation: numerical values are provided in Table 7
  - evaluation criteria applied by the study authors:
    - positive: if the increase in the number of mutant colonies is more than twice compared to controls

Table 7: Results of bacterial reverse mutation test of 1,4-dichloro-2-nitrobenzene in test strains TA98, T100, TA1535, TA1537, and TA1538 without metabolic activation

Concentration of test substance (µg/plate)	Number of revertants (Mean ± S.D.)				
	TA98	TA1538	TA1537	TA100	TA1535
DMSO (control, 0.05 mL)	28 ± 6	22 ± 7	8 ± 3	181 ± 23	32 ± 8
51.2	39 ± 5	31 ± 4	8 ± 2	170 ± 16	40 ± 7
102.4	56 ± 9	36 ± 6	12 ± 3	355 ± 23	54 ± 6
204.8	71 ± 11	58 ± 7	10 ± 3	499 ± 68	50 ± 8
409.6	105 ± 32	80 ± 11	6 ± 1	742 ± 83	55 ± 8
819.2	130 ± 38	92 ± 14	9 ± 2	852 ± 87	84 ± 13
1638.4	124 ± 45	88 ± 19	10 ± 2	938 ± 119	78 ± 19
3276.8	101 ± 39	13 ± 6*	11 ± 3	1252 ± 236	28 ± 7
6553.6	0*	0*	0*	90 ± 18*	0*
ENNG (2 µg)	ND	ND	ND	1994 ± 377	ND
ENNG (10 µg)	ND	ND	ND	ND	2489 ± 287
2-NF (2 µg)	1798 ± 258	ND	ND	ND	ND
2-NF (5 µg)	ND	1659 ± 228	ND	ND	ND
9-AA (100 µg)	ND	ND	1288 ± 198	ND	ND

Positive controls ENNG: N-ethyl-N'-nitro-N-nitrosoguanidine, 2-NF: 2-nitrofluorene, 9-AA: 9-aminoacridine

*: Inhibition of bacteria growth was observed

ND: not determined

3.8.2 Animal data

No studies available.

3.8.3 Human data

No studies available.

3.8.4 Other data

No studies available.

3.9 Carcinogenicity

3.9.1 Animal data

3.9.1.1 Study 1

Study reference:

Yamazaki, K.; Aiso, S.; Matsumoto, M.; Kano, H.; Arito, H.; Nagano, K.; Yamamoto, S.; Matsushima, T., Carcinogenicity and chronic toxicity of 1,4-dichloro-2-nitrobenzene in rats and mice by two years feeding Industrial Health, 44 (2), 230-243, 2006 (Yamazaki et al., 2006).

Detailed study summary and results:

Test type

In a chronic toxicity and carcinogenicity study (similar to OECD TG 453) male and female rats were exposed to concentrations of the test substance in diet (0, 320, 800 or 2000 ppm) for 2 years and signs of chronic toxicity and carcinogenic activity as well as incidences of tumours were evaluated. GLP compliance is given (according to OECD Principle of Good Laboratory Practice).

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 1,4-dichloro-2-nitrobenzene (DCNB)
- Degree of purity: > 98.8%
- Impurities: determined to be free from impurities and degradation products after analyses by gas chromatography and infrared spectrometry
- Batch number: no information available

Test animals

- Species/strain/sex: rat / F344/DuCrj (SPF) / male and female
- No. of animals per sex per dose: 50 per sex and dose
- Age and weight at the study initiation: 6-weeks old, no information available on weight at study initiation

Administration/exposure

- Route of administration – oral (feed)
- duration of test/exposure period: 2 years (104 weeks)
- doses/concentration levels, rationale for dose level selection: The selected concentrations were 0, 320, 800 or 2000 ppm DCNB (w/w) in diet, based on data on body weight gain and toxicity from a

subchronic toxicity study in rats (13 weeks, via diet) the highest administered concentration level of 2000 ppm was not exceeding the maximum tolerated dose (MTD) determined according to the guidelines of the National Cancer Institute (NCI) and International Agency for Research on Cancer (IARC)

- frequency of treatment: daily, continuous
- control group and treatment: yes, concurrent treatment
- historical control data: yes, from the Japan Bioassay Research Center (JBRC)
- post exposure observation period: no
- diet preparation: a spiral mixer was used for mixing DCNB and γ-irradiation-sterilized CRF-1 powdered diet (Oriental Yeast Co., Tokyo, Japan) for 20 minutes afterwards the diet preparation was stored at 4 °C until further use; diet preparation was performed every 2 weeks during the study duration of 2 years; feeder in cages were filled once every week with the respective (control) diet
    - achieved concentration: analytical verification by gas chromatography found that DCNB concentrations in the powdered diet were 89.7 to 109.1% of target concentrations at the time of preparation
    - stability and homogeneity of the preparation: if time of preparation was set at 100% a decrease of initial concentration to 87.8 to 92.5% on 15th day after preparation was observed, analytical verification was conducted by gas chromatography and infrared spectrometry
- actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test
    - calculation of actual doses according to the Health Council of the Netherlands (2018): equivalent to 10, 25, and 63 mg/kg bw/d for males and 17, 44, and 109 mg/kg bw/d for females using a body weight of 475 g for males and 275 g for females and a food intake of 15 g/d
- Statistical methods:
    - Dunnet's test was performed for analysing body weight, food consumption, and haematological as well as blood biochemical parameters
    - Kaplan-Meier method and log-rank test were performed for determining statistical significance of survival rates
    - Chi-square test was used for analysing urinary data and incidences of non-neoplastic lesions
    - Fisher's exact test was performed for the statistical analysis of incidences of neoplastic lesions and the Peto test was used for determining a positive trend of dose-response relationship for neoplastic incidences

Results and discussion

- mortality and time to death (indicate number died per sex per dose and time to death):

  - survival (until termination) in groups 0, 320, 800, and 2000 ppm were 40/50, 44/50, 41/50, and 39/50 in males and 38/50, 35/50, 39/50, and 34/50 in females
  - neither an exact number nor time to death is given (no further details were provided)
  - survival rate analysis did not show a significant difference between any DCNB-treated groups and controls for both sexes (data not shown in publication)
- clinical signs:
  - at 320, 800 and 2000 ppm: yellow coloured urine throughout study period was observed in both sexes
- body weight gain (for details see Table 8):
  - terminal body weight was statistically significant ($P \leq 0.01$, exception at 320 ppm in males: ($P \leq 0.05$) decreased in all treated males and females at 2000 ppm in comparison to controls
  - terminal body weights in males were decreased by approx. 6% at 320 ppm and 8% at 800 ppm compared to control
  - at 2000 ppm terminal body weights in males and females were decreased by 15% and 20% compared to their respective controls
- growth rate:
  - dose-related suppression in growth rates was observed in DCNB-exposed rats of both sexes (see Figure 1A)
- food consumption: no effects (data not shown in publication)
- ophthalmoscopic examination: no information available
- clinical chemistry (for details see Table 9):
  - γ-glutamyltransferase (GTP) was statistically significant ($P \leq 0.01$, exception in males at 320 ppm: $P \leq 0.05$) increased in all DCNB-exposed males and females compared to controls
  - total cholesterol, phospholipids, and blood urea nitrogen (BUN) were statistically significant ($P \leq 0.01$) increased in males at 800 and 2000 ppm and in all DCNB-exposed females compared to controls
  - triglyceride was statistically significant ($P \leq 0.01$, exception at 800 ppm: $P \leq 0.05$) increased in DCNB-exposed males at 800 and 2000 ppm compared to control
  - total protein and albumin were statistically significant ($P \leq 0.01$, exception at 800 ppm albumin: $P \leq 0.05$) increased in females at 800 and 2000 ppm and glucose was only statistically significant ($P \leq 0.01$) increased in females at 2000 ppm compared to controls
  - liver enzymes (alanine aminotransferase (ALT) and aspartate transaminase (AST)) were not increased in any of the DCNB-exposed groups of both sexes compared to controls
- haematology (for details see Table 10):
  - haematocrit was statistically significant ($P \leq 0.01$) decreased in 2000 ppm females compared to control

- haemoglobin concentration was statistically significant (P≤0.01, exception at 800 ppm: P≤0.05) decreased in females at 800 and 2000 ppm compared to control
- no significant differences in haematological parameters between DCNB-exposed males and controls were observed

- urinalysis:
  - protein was present in urine of males at 2000 ppm compared to control
  - lowered pH was measured in urine of males at 800 and 2000 ppm compared to control (data not shown in publication)
- organ weights (for details see Table 8):
  - relative liver weight was statistically significant (P≤0.01) increased in all DCNB-exposed animals of both sexes compared to controls
  - relative kidney weight was statistically significant (P≤0.01) increased in all DCNB-exposed males and in females at 800 and 2000 ppm compared to controls
  - relative testis weight was statistically significant (P≤0.01) increased and absolute testis weight was not significantly increased in males at 2000 ppm compared to controls
- necropsy findings:
  - incidence of chronic progressive nephropathy (CPN) characterised by slightly tanned and granular surface in the kidney was observed in DCNB-exposed males, which was dose-dependently increased (7/50, 10/50, 27/50, and 32/50 at 0, 320, 800, and 2000 ppm)
  - increased incidence of liver nodules in males at 2000 ppm (0/50, 2/50, 2/50, and 8/50 at 0, 320, 800, and 2000 ppm)
- histopathological findings:

  <u>non-neoplastic lesions (see Table 11):</u>
  - chronic progressive nephropathy (CPN) was observed in all dose groups of both sexes, but the total number of CPN was statistically significant (P≤0.01) increased only in all DCNB-exposed males compared to controls
  - incidences of marked and severe CPN were (not statistically significant) increased compared to control in all DCNB-exposed males in a dose-related manner
  - incidences of urothelial hyperplasia in pelvis were statistically significant (P≤0.01, exception at 320 ppm: P≤0.05) increased in all DCNB-exposed males compared to controls
  - mineralisation of papilla was statistically significant (P≤0.01) increased in males at 800 and 2000 ppm compared to controls
  - haematopoiesis in bone marrow was statistically significant (P≤0.05) increased in females at 2000 ppm compared to controls

  <u>pre-neoplastic lesions (see Table 12):</u>
  - incidence of basophilic cell foci was statistically significant (P≤0.01) dose-dependently increased in DCNB-exposed males at 800 and 2000 ppm compared to controls

- no dose-related incidences of acidophilic cell foci were observed in DCNB-exposed males and females compared to controls
- no increased incidences of atypical tubule hyperplasia and proliferative lesion in proximal tubule epithelium were observed in any DCNB-exposed males and females compared to controls

neoplastic lesions (see Table 12):

- hepatocellular tumours lacked normal lobular architecture, which compressed adjacent hepatic parenchyma
- hepatocellular carcinoma were characterised by cells with irregular-shaped nuclei with a pseudo-glandular arrangement indicative of pronounced structural atypia
- renal cell adenoma had a size of less than 5 mm, no anaplastic feature or prominent cellular pleomorphism and did not metastasised to other organs

- tumour incidence data by sex, dose and tumour type:
  - increased tumour incidences were only found in DCNB-exposed males
  - incidences of hepatocellular adenoma (0/50, 0/50, 1/50, and 6/50*, P≤0.05) and hepatocellular adenoma or carcinoma (0/50, 1/50, 1/50, and 8/50*, P≤0.05) were statistically significantly increased in males at 2000 ppm compared to the control group; also a significant positive trend of the dose-tumour incidence relationship was indicated by Peto test (P≤0.01)
  - hepatocellular carcinoma (0/50, 0/50, 1/50, and 2/50) were observed in males at 800 and 2000 ppm; at 2000 ppm, the incidence was 4% (2/50) at 2000 ppm without statistical significance, but exceeded the upper bound of the range of historical control data (3/1249 (0.2%) in 25 studies, with a maximum incidence of 2%)
  - incidences of renal adenoma (0/50, 0/50, 0/50, and 2/50) and renal carcinoma (0/50, 1/50, 0/50, and 1/50) occurred in males without a statistical significance compared to controls
  - incidences of renal adenoma (4%) and renal carcinoma (2%) were observed in male rats at 2000 ppm, but only the incidence of renal adenoma exceeded the upper bound of the range of historical control data (2/1249 (0.16%) in 25 studies, with a maximum incidence of 2% for renal adenoma and carcinoma, respectively)
  - for combined incidences of renal adenoma and carcinoma a significant dose-related incidence relationship was shown by Peto test (P≤0.05)
  - incidence of Zymbal gland adenoma in male rats was 0/50, 0/50, 0/50, and 4/50; at 2000 ppm no statistical significance compared to the control group was observed; however a significant positive trend of the dose-tumour incidence relationship was indicated by Peto test (P≤0.01)

- at 2000 ppm the incidence of Zymbal gland adenoma was 8% in male rats, which exceeded the upper bound of the range of historical control data (3/1249 (0.2%) in 25 studies, with a maximum incidence of 2%)
- incidences of interstitial tumours were high in DCNB-exposed rats of both sexes as well as in controls, a dose-related relationship was not observed

- local or multi-site responses:
  - tumours were observed in liver, kidney and the Zymbal gland
  - malignant liver tumours did not metastasised to any other organs
  - in a male rat at 2000 ppm one malignant renal cell carcinoma metastasised into the lung
- progression of lesions to malignancy:
  - basophilic cell foci, which are a pre-neoplastic lesion, adenoma and carcinoma were observed in the liver of male rats
- gender and/or species-specific responses:
  - in male rats an α_{2u}-globulin-induced nephropathy cannot be excluded
  - species-specificity of DCNB-induced Zymbal gland tumours in male rats is unsolved
- mode of action (genotoxic, non-genotoxic):
  - Yamazaki et al. (2006) suggest that a genotoxic mode of action might take place in DCNB-induced hepatocarcinogenicity as shown by *in vitro* genotoxicity studies and further supported by data obtained in a chronic toxicity/carcinogenicity study in mice (see section 3.9.1.2)
- toxic response data by sex and dose:
  - chronic exposure to DCNB caused toxic effects in liver, kidney and blood which were reported in detail in this section, see above
- tumour latency: no information available

- Effect levels (given in Yamazaki et al. (2006)):
  - LOEL: 14 mg/kg bw/d based on hepato- and nephrotoxicity

Table 8: Absolute and relative organ weights of rats at termination after exposure to DCNB orally via diet for 2 years

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Number of animals	40	44	41	39	38	35	39	34
Body weight (g)	384 ± 28	360 ± 48*	353 ± 22**	328 ± 25**	248 ± 36	238 ± 23	234 ± 32	199 ± 26**
Liver (g)	10.394 ± 1.540	11.508 ± 2.020**	11.946 ± 1.759**	12.361 ± 1.199**	6.317 ± 0.921	6.790 ± 0.930	7.267 ± 0.924**	7.086 ± 0.923**
Liver (%)	2.716 ± 0.449	3.268 ± 0.848**	3.397 ± 0.588**	3.778 ± 0.405**	2.583 ± 0.413	2.864 ± 0.415**	3.152 ± 0.502**	3.572 ± 0.217**
Kidneys (g)	2.634 ±	2.802 ±	2.757 ±	2.853 ±	1.713 ±	1.738 ±	1.766 ±	1.670 ±

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
	0.221	0.399	0.299	0.300**	0.138	0.133	0.123	0.113
Kidneys (%)	0.690 ± 0.086	0.799 ± 0.202**	0.785 ± 0.120**	0.873 ± 0.106**	0.703 ± 0.086	0.733 ± 0.053	0.769 ± 0.118**	0.849 ± 0.085**
Testes (g)	2.769 ± 1.171	2.957 ± 1.333	2.659 ± 1.118	3.137 ± 1.158	-	-	-	-
Testes (%)	0.720 ± 0.301	0.822 ± 0.360	0.755 ± 0.319	0.954 ± 0.349**	-	-	-	-

Given as Mean ± S.D.

* and ** significantly different at p ≦0.05 and p ≦0.01 by Dunnett's test, respectively

Table 9: Blood biochemical parameters of rats at termination after exposure to DCNB orally via diet for 2 years

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Number of animals	40	41	41	39	38	34	38	34
Total protein (g/dL)	6.5 ± 0.3	6.6 ± 0.4	6.6 ± 0.3	6.5 ± 0.3	6.6 ± 0.5	6.7 ± 0.3	6.9 ± 0.4**	7.0 ± 0.4**
Albumin (g/dL)	3.3 ± 0.2	3.3 ± 0.3	3.3 ± 0.2	3.3 ± 0.2	3.8 ± 0.3	3.9 ± 0.2	4.0 ± 0.3*	4.1 ± 0.2**
A/G ratio	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Glucose (mg/dL)	149 ± 17	143 ± 21	148 ± 13	149 ± 23	145 ± 18	153 ± 15	153 ± 15	158 ± 11**
Total Cholesterol (mg/dL)	175 ± 50	187 ± 45	219 ± 45**	217 ± 51**	126 ± 21	149 ± 25**	165 ± 25**	175 ± 24**
Triglyceride (mg/dL)	80 ± 61	103 ± 79	125 ± 70*	146 ± 79**	44 ± 31	53 ± 47	55 ± 53	60 ± 113
Phospholipid (mg/dL)	245 ± 76	267 ± 61	299 ± 55**	312 ± 63**	222 ± 45	251 ± 43**	275 ± 40**	292 ± 33**
AST (IU/L)	85 ± 43	243 ± 774	92 ± 50	113 ± 123	141 ± 194	116 ± 86	114 ± 61	108 ± 44
ALT (IU/L)	38 ± 17	70 ± 154	40 ± 15	49 ± 49	59 ± 66	54 ± 29	54 ± 28	55 ± 24
γ-GTP (IU/L)	12 ± 8	25 ± 33*	31 ± 24**	38 ± 21**	5 ± 5	7 ± 4**	8 ± 3**	10 ± 5**
BUN (mg/dL)	17.2 ± 3.4	20.7 ± 10.9	23.8 ± 5.1**	30.7 ± 9.6**	16.3 ± 3.6	17.4 ± 2.0*	17.8 ± 2.1**	19.4 ± 2.2**

Given as Mean ± S.D.

* and ** significantly different at p ≦0.05 and p ≦0.01 by Dunnett's test, respectively
AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, γ-GTP: γ-Glutamyl transpeptidase, BUN: Blood urea nitrogen

Table 10: Haematological parameters of rats at termination after exposure to DCNB orally via diet for 2 years

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Number of animals	40	41	41	39	38	34	38	34
RBC (10^6/μL)	8.21 ± 1.25	8.20 ± 1.73	8.62 ± 1.52	8.86 ± 1.69	8.09 ± 1.30	8.21 ± 1.10	8.03 ± 0.99	7.99 ± 1.51
Hb (g/dL)	13.6 ± 2.4	13.4 ± 2.7	14.1 ± 2.3	14.1 ± 2.7	14.6 ± 2.2	14.5 ± 1.6	14.3 ± 1.6*	13.6 ± 2.7**
Hematocrit (%)	41.4 ± 6.2	40.9 ± 7.2	42.8 ± 6.0	42.7 ± 7.7	43.2 ± 5.8	43.0 ± 3.9	42.3 ± 4.5	40.9 ± 7.1**

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Methaemoglobin level (%)	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.4	0.4 ± 0.3	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.3
WBC (10^3/µL)	7.37 ± 5.53	10.56 ± 14.39	8.32 ± 10.62	7.08 ± 2.25	7.22 ± 28.68	2.82 ± 2.59	3.65 ± 8.71	2.42 ± 1.85

Given as Mean ± S.D.

* and ** significantly different at p ≦0.05 and p ≦0.01 by Dunnett's test, respectively

RBC: Red blood cell counts, Hb: Hemoglobin concentration, WBC: White blood cell counts

Table 11: Incidences of non-neoplastic lesions in rats exposed to DCNB orally via diet for 2 years

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Number of animals	50	50	50	50	50	50	50	50
Kidney								
Chronic progressive nephropathy								
Total	46	49**	50**	49**	24	23	32	28
Slight	26	6	2	1	22	21	28	26
Moderate	15	27	10	5	2	0	2	2
Marked	4	14	34	32	0	2	1	0
Severe	1	2	4	11	0	0	1	0
Mineralization: papilla	0	2	47**	48**	9	9	9	17
Urothelial hyperplasia: pelvis	1	8*	36**	39**	10	5	15	6
Bone marrow								
Haematopoiesis: increased	8	10	13	10	5	9	9	14*

* and **: Significantly different at p ≤ 0.05 and p ≤ 0.01 by Chi-square test, respectively.

Table 12: Incidences of neoplastic and pre-neoplastic lesions in rats exposed to DCNB orally via diet for 2 years

	Male					Female				
Dose in ppm	**0**	**320**	**800**	**2000**	**Peto test**	**0**	**320**	**800**	**2000**	**Peto test**
Number of animals	50	50	50	50		50	50	50	50	
Liver										
Hepatocellular adenoma	0	1	0	6*	↑↑	0	0	0	0	
Hepatocellular carcinoma	0	0	1	2		0	0	0	0	
Hepatocellular adenoma and carcinoma (combined)	0	1	1	8*	↑↑	0	0	0	0	
Basophilic cell foci	21	22	32##	40##		26	20	18	25	
Acidophilic cell foci	14	11	6	18		2	1	1	8	
Kidney										
Renal cell adenoma	0	0	0	2		0	0	0	0	
Renal cell carcinoma	0	1	0	1		0	0	0	0	
Renal cell adenoma and carcinoma (combined)	0	1	0	3	↑	0	0	0	0	
Atypical tubule hyperplasia	0	0	2	2		1	0	0	0	
Zymbal gland										

	Male					Female				
Dose in ppm	**0**	**320**	**800**	**2000**	**Peto test**	**0**	**320**	**800**	**2000**	**Peto test**
Adenoma	0	0	0	4	↑↑	0	0	0	0	

* and **: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Fisher's exact test, respectively.

↑ and ↑↑: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Peto test, respectively.

and ##: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Chi-square test, respectively.

3.9.1.2 Study 2

Study reference:

Yamazaki, K.; Aiso, S.; Matsumoto, M.; Kano, H.; Arito, H.; Nagano, K.; Yamamoto, S.; Matsushima, T., Carcinogenicity and chronic toxicity of 1,4-dichloro-2-nitrobenzene in rats and mice by two years feeding Industrial Health, 44 (2), 230-243, 2006 (Yamazaki et al., 2006).

Detailed study summary and results:

Test type

In a chronic toxicity and carcinogenicity study (similar to OECD TG 453) male and female mice were exposed to concentrations of the test substance in diet (0, 320, 800 or 2000 ppm) for 2 years and signs of chronic toxicity and carcinogenic activity as well as incidences of tumours were evaluated. GLP compliance is given (according to OECD Principle of Good Laboratory Practice).

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier: 1,4-dichloro-2-nitrobenzene (DCNB)
- Degree of purity: > 98.8%
- Impurities: determined to be free from impurities and degradation products after analyses by gas chromatography and infrared spectrometry
- Batch number: no information available

Test animals

- Species/strain/sex: mice / Crj:BDF_1 (SPF) / male and female
- No. of animals per sex per dose: 50 per sex and dose
- Age and weight at the study initiation: 6-weeks old, no information available on weight at study initiation

Administration/exposure

- Route of administration – oral (feed)
- duration of test/exposure period: 2 years (104 weeks)

- doses/concentration levels, rationale for dose level selection: The selected concentrations were 0, 320, 800 or 2000 ppm DCNB (w/w) in diet, based on data on body weight gain and toxicity from a subchronic toxicity study in rats (13 weeks, via diet) the highest administered concentration level of 2000 ppm was not exceeding the maximum tolerated dose (MTD) determined according to the guidelines of the National Cancer Institute (NCI) and International Agency for Research on Cancer (IARC)
- frequency of treatment: daily, continuous
- control group and treatment: yes, concurrent treatment
- historical control data: yes, from the Japan Bioassay Research Center (JBRC)
- post exposure observation period: no
- diet preparation: a spiral mixer was used for mixing DCNB and γ-irradiation-sterilized CRF-1 powdered diet (Oriental Yeast Co., Tokyo, Japan) for 20 minutes afterwards the diet preparation was stored at 4 °C until further use; diet preparation was performed every 2 weeks during the study duration of 2 years; feeder in cages were filled once every week with the respective (control) diet
    - achieved concentration: analytical verification by gas chromatography found that DCNB concentrations in the powdered diet were 89.7 to 109.1% of target concentrations at the time of preparation
    - stability and homogeneity of the preparation: if time of preparation was set at 100% a decrease of initial concentration to 87.8 to 92.5% on 15th day after preparation was observed, analytical verification was conducted by gas chromatography and infrared spectrometry
- actual doses (mg/kg bw/day) and conversion factor from diet/drinking water test
    - calculation of actual doses according to the Health Council of the Netherlands (2018): equivalent to 32, 80, and 200 mg/kg bw/d for males and 41, 103, and 257 mg/kg bw/d for females using a body weight of 45 g for males and 35 g for females and a food intake of 4.5 g/d
- Statistical methods:
    - Dunnet's test was performed for analysing body weight, food consumption, and haematological as well as blood biochemical parameters
    - Kaplan-Meier method and log-rank test were performed for determining statistical significance of survival rates
    - Chi-square test was used for analysing urinary data and incidences of non-neoplastic lesions
    - Fisher's exact test was performed for the statistical analysis of incidences of neoplastic lesions and the Peto test was used for determining a positive trend of dose-response relationship for neoplastic incidences

Results and discussion

- mortality and time to death (indicate number died per sex per dose and time to death):
    - survival (until termination) in groups 0, 320, 800, and 2000 ppm were 27/49[1], 35/50, 26/50, and 18/50 in males and 30/50, 27/50, 28/50, and 23/50 in females
    - neither an exact number nor time to death is given (no further details were provided)
    - survival rate analysis did not show a significant difference between any DCNB-treated groups and controls for both sexes (data was not shown in publication)
    - after the 65th week of administration a lower survival rate was observed in mice of both sexes at 2000 ppm
    - at 2000 ppm an increased number of deaths before administration ended was observed for mice of both sexes dying due to liver tumours; deaths were 7, 8, 11, and 23 for males and 0, 3, 4, and 6 for females
- clinical signs:
    - yellow coloured urine throughout the study period was observed in all DCNB-exposed mice of both sexes
- body weight gain (for details see Table 13):
    - terminal body weight was statistically significant decreased in treated males at 800 and 2000 ppm and females at 2000 ppm in comparison to controls
    - at 2000 ppm terminal body weights in males and females were decreased by 34% and 17% compared to their respective controls
- growth rate: a dose-related reduction was observed in treated animals of both sexes, predominantly in high dosed males and females during the last 30 weeks (see Figure 1B)

[1] One male control mouse died accidentally during the administration period

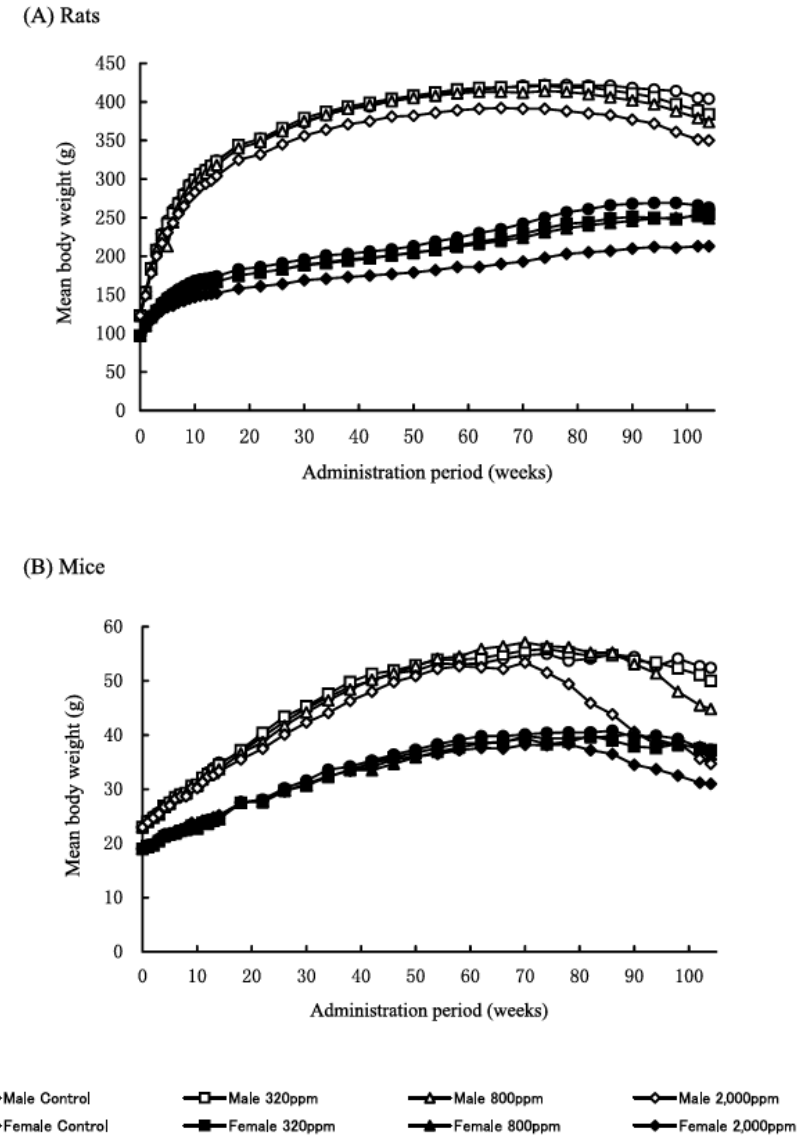

Figure 1: Graphical presentation of growth rate curves of rats (A) and mice (B) after exposure to control and 3 different DCNB-containing diets for 2 years by Yamazaki et al. (2006)

- food consumption: no effects (data not shown in publication)
- ophthalmoscopic examination: no information available
- clinical chemistry (for details see Table 14):
    - γ-glutamyltransferase (GTP) was statistically significant (P≤0.01) increased in all DCNB-exposed males and females compared to controls
    - alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and phospholipid were statistically significant (P≤0.01, exception LDH at 800 ppm: P≤0.05) increased in DCNB-exposed males at 800 and 2000 ppm compared to controls

  - total cholesterol was statistically significant (P≤0.01, exception at 320 ppm: P≤0.05) increased in all DCNB-exposed males compared to controls
  - glucose and triglyceride were statistically significant (P≤0.01) decreased in DCNB-exposed males at 2000 ppm compared to control
  - alanine aminotransferase (ALT) was statistically significant (P≤0.01, exception at 320 ppm: P≤0.05) increased in all DCNB-exposed females compared to controls
  - aspartate transaminase (AST) and total cholesterol were statistically significant (P≤0.01, exception at 800 ppm: P≤0.05) increased in DCNB-exposed females at 800 and 2000 ppm compared to control
  - alkaline phosphatase (ALP) and phospholipid were statistically significant (P≤0.01) increased in DCNB-exposed females at 800 and 2000 ppm compared to controls
  - lactate dehydrogenase (LDH) and blood urea nitrogen (BUN) were statistically significant (P≤0.01) increased in DCNB-exposed females at 2000 ppm compared to controls
  - glucose was statistically significant (P≤0.05) decreased in DCNB-exposed females at 2000 ppm compared to control
- haematology (for details see Table 15):
  - red blood cell count, haematocrit, and haemoglobin concentration were statistically significant (P≤0.01) increased in 2000 ppm females compared to control
  - no significant differences in haematological parameters between DCNB-exposed males and controls were observed
- urinalysis: no effects (data not shown in publication)
- organ weights (for details see Table 13):
  - relative and absolute liver weight were statistically significant (P≤0.01) increased in DCNB-exposed mice of both sexes at 800 and 2000 ppm compared to controls
  - relative kidney weight was statistically significant (P≤0.01) increased in males at 800 and 2000 ppm and in females 2000 ppm compared to controls
  - relative testis weight was statistically significant increased (P≤0.01) and absolute testis weight was not significantly increased in males at 2000 ppm compared to controls
- necropsy findings:
  - dose-dependent increased incidences of liver nodules in DCNB-exposed mice of both sexes (24/49, 30/50, 42/50, and 46/50 for males and 30/50, 5/50, 9/50, and 27/50 for females at 0, 320, 800, and 2000 ppm)
- histopathological findings:

  <u>non-neoplastic lesions (see Table 16):</u>
  - incidences of centrilobular hypertrophy with nuclear atypia of hepatocytes were statistically significant (P≤0.01) increased in all DCNB-exposed males and females in a dose-related manner compared to control

- hemosiderin deposition in kidney and erythropoiesis in bone marrow were statistically significant (P≤0.01) increased in males at 2000 ppm compared to controls

preneoplastic lesions (see Table 17):

- a dose-related increased incidence of acidophilic cell foci was observed in DCNB-exposed males at 800 and 2000 ppm compared to controls statistical significance was P≤0.01, exception at 800 ppm: P≤0.05

neoplastic lesions (see Table 17):

- hepatocellular carcinoma were characterised by marked cellular pleomorphism, including the advent of large and irregular-shaped tumour cells, and structure atypia including pseudo-glandular and papillary-like structures associated with sinusoidal dilatation
- hepatoblastoma had compared to normal hepatocytes smaller, more markedly basophilic, denser in cellularity and more elongated-shaped cells

- tumour incidence data by sex, dose and tumour type:
  - incidences of hepatocellular adenoma (5/50, 5/50, 17/50**, and 16/50**, P≤0.01) and hepatocellular carcinoma (1/50, 3/50, 15/50**, and 31/50**, P≤0.01) were statistically significantly increased in females at 800 and 2000 ppm compared to the control group; also a significant positive trend of the dose-tumour incidence relationship was indicated by Peto test (P≤0.01)
  - incidence of hepatocellular carcinoma (15/49, 15/50, 23/50, and 31/50**, P≤0.01) were statistically significantly increased in males at 2000 ppm; also a significant positive trend of the dose-tumour incidence relationship was indicated by Peto test (P≤0.01)
  - incidence of hepatoblastoma was statistically significantly increased in all DCNB-exposed males (1/49, 10/50**, 12/50**, and 25/50**, P≤0.01) compared to controls (historical control data: 5/1047 in 21 studies)
  - incidence of hepatoblastoma in females was 4% (2/50) at 2000 ppm without statistical significance, but exceeded the upper bound of the range of historical control data (0/1047 in 21 studies)
  - for combined incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma a statistically significant increase in mice of both sexes was observed at 800 and 2000 ppm (26/49, 34/50, 41/50**, and 45/50** for males and 6/50, 8/50, 29/50**, and 39/50** for females, P≤0.01) and a significant dose-related incidence relationship was shown by Peto test (P≤0.01)
- local or multi-site responses:
  - only tumours of the liver were observed, however, of different cellular origin (hepatocarcinoma and hepatoblastoma)
  - in liver tumour masses were multifocally in DCNB-exposed mice compared to single occurrence in controls

  - 18 of 69 hepatocellular carcinoma and 17 of 47 hepatoblastoma in DCNB-exposed males and also 4 of 49 hepatocellular carcinoma in DCNB-exposed females metastasised into the lung

- progression of lesions to malignancy:
  - acidophilic cell foci, which are a pre-neoplastic and proliferative lesion, adenoma and carcinoma were observed in the liver of male and female mice
  - in female mice hepatocellular
- gender and/or species-specific responses: malignant tumours (carcinoma and hepatoblastoma) were observed in both male and female mice; carcinogenicity in rats was shown in a separate study (see section 3.9.1.1)
- mode of action (genotoxic, non-genotoxic):
  - Yamazaki et al. (2006) suggest that a genotoxic mode of action might take place in DCNB-induced hepatocarcinogenicity as shown by *in vitro* genotoxicity studies and further supported by data obtained in a chronic toxicity/carcinogenicity study in rats (see section 3.9.1.1)
- toxic response data by sex and dose:
  - survival rates in mice at 2000 ppm were decreased due to increased number of tumour deaths before the 2-year administration had ended
- tumour latency: no information available

Table 13: Absolute and relative organ weights of mice at termination after exposure to DCNB orally via diet for 2 years

	Male				**Female**			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Number of animals	27	35	26	18	30	27	28	23
Body weight (g)	48.8 ± 6.1	46.8 ± 8.4	41.4 ± 8.4**	32.0 ± 3.0**	34.5 ± 7.2	34.7 ± 5.6	33.8 ± 5.1	28.6 ± 2.9**
Liver (g)	2.168 ± 1.533	2.420 ± 1.014	3.467 ± 1.436**	5.722 ± 1.957**	1.625 ± 0.820	1.511 ± 0.356	2.028 ± 0.518**	4.251 ± 1.538**
Liver (%)	4.713 ± 4.288	5.465 ± 3.197	8.976 ± 4.789**	17.918 ± 5.911**	4.801 ± 2.414	4.437 ± 1.130	6.152 ± 1.882**	15.195 ± 6.151**
Kidneys (g)	0.612 ± 0.049	0.667 ± 0.230	0.649 ± 0.076	0.675 ± 0.150	0.479 ± 0.169	0.484 ± 0.167	0.459 ± 0.057	0.488 ± 0.103
Kidneys (%)	1.274 ± 0.191	1.498 ± 0.837	1.602 ± 0.230**	2.152 ± 0.667**	1.433 ± 0.522	1.430 ± 0.533	1.377 ± 0.179	1.712 ± 0.352**
Testes (g)	0.225 ± 0.039	0.215 ± 0.046	0.215 ± 0.038	0.205 ± 0.028	-	-	-	-
Testes (%)	0.470 ± 0.102	0.469 ± 0.116	0.533 ± 0.109	0.644 ± 0.075**	-	-	-	-

Given as Mean ± S.D.

* and ** significantly different at p ≦0.05 and p ≦0.01 by Dunnett's test, respectively

Table 14: Blood biochemical parameters of mice at termination after exposure to DCNB orally via diet for 2 years

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Number of animals	24	32	24	16	23	26	27	22
Glucose (mg/dL)	196 ± 34	200 ± 48	183 ± 58	152 ± 26**	161 ± 52	145 ± 59	162 ± 30	140 ± 32*
Total Cholesterol (mg/dL)	117 ± 34	153 ± 50*	202 ± 78**	219 ± 51**	85 ± 72	88 ± 23	96 ± 24*	194 ± 75**
Triglyceride (mg/dL)	44 ± 22	52 ± 22	41 ± 21	25 ± 11**	32 ± 22	42 ± 33	37 ± 17	27 ± 12
Phospholipid (mg/dL)	213 ± 54	270 ± 84	363 ± 139**	380 ± 95**	146 ± 77	157 ± 38	191 ± 42**	365 ± 138**
AST (IU/L)	159 ± 201	197 ± 509	287 ± 349**	990 ± 2046**	96 ± 55	1,244 ± 5,547	149 ± 83*	347 ± 242**
ALT (IU/L)	112 ± 149	225 ± 490	353 ± 387**	1241 ± 2112**	40 ± 21	595 ± 2558*	116 ± 49**	528 ± 439**
LDH (IU/L)	1194 ± 3133	940 ± 2008	2214 ± 4539*	9267 ± 19122**	366 ± 184	4,245 ± 18,679	515 ± 419	1796 ± 1592**
ALP (IU/L)	117 ± 27	253 ± 356	766 ± 900**	891 ± 598**	152 ± 63	218 ± 171	305 ± 203**	1164 ± 979**
γ-GTP (IU/L)	3 ± 3	6 ± 10	7 ± 8	22 ± 14**	3 ± 2	4 ± 5	4 ± 4	27 ± 15**
BUN (mg/dL)	20.1 ± 2.7	23.9 ± 15.4	22.9 ± 5.6	21.7 ± 3.3	18.2 ± 9.5	18.1 ± 7.2	16.0 ± 2.4	20.8 ± 4.2**

Given as Mean ± S.D.

* and ** significantly different at p ≦0.05 and p ≦0.01 by Dunnett's test, respectively
AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, γ-GTP: γ-Glutamyl transpeptidase, LDH: Lactate dehydrogenase, ALP: Alkaline phosphatase, BUN: Blood urea nitrogen.

Table 15: Haematological parameters of mice at termination after exposure to DCNB orally via diet for 2 years

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Number of animals	23	32	24	16	23	26	27	22
RBC (10^6/μL)	9.63 ± 1.48	9.30 ± 1.67	9.48 ± 1.60	9.09 ± 1.64	9.12 ± 1.77	9.39 ± 1.86	9.39 ± 0.97	10.44 ± 1.03**
Hb (g/dL)	13.2 ± 1.8	13.0 ± 2.2	13.2 ± 2.1	12.6 ± 2.1	13.1 ± 2.0	13.8 ± 2.3	13.6 ± 1.5	14.8 ± 1.3**
Hematocrit (%)	43.1 ± 5.0	42.5 ± 6.8	43.6 ± 6.7	42.2 ± 6.1	42.8 ± 5.6	45.2 ± 6.6	44.1 ± 4.2	48.8 ± 4.3**
WBC (10^3/μL)	3.20 ± 1.78	2.87 ± 1.21	3.40 ± 1.52	2.95 ± 1.38	3.67 ± 3.61	3.05 ± 3.24	4.40 ± 6.24	3.56 ± 3.36

Given as Mean ± S.D.

* and ** significantly different at p ≦0.05 and p ≦0.01 by Dunnett's test, respectively

RBC: Red blood cell counts, Hb: Hemoglobin concentration, WBC: White blood cell counts

Table 16: Incidences of non-neoplastic lesions in mice exposed to DCNB orally via diet for 2 years

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Number of animals	49[a]	50	50	50	50	50	50	50

	Male				Female			
Dose in ppm	**0**	**320**	**800**	**2000**	**0**	**320**	**800**	**2000**
Kidney								
Hepatocellular hypertrophy with nuclear atypia: centrilobular	0	38**	39**	40**	0	15**	29**	35**
Kidney								
Deposition of hemosiderin	1	6	6	25**	1	0	0	2
Bone marrow								
Erythropoiesis: increased	7	7	14	23**	2	2	0	4

* and **: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Chi-square test, respectively.

[a]: One male control mouse died accidentally during the administration period.

Table 17: Incidences of neoplastic and pre-neoplastic lesions in mice exposed to DCNB orally via diet for 2 years

	Male					Female				
Dose in ppm	**0**	**320**	**800**	**2000**	**Peto test**	**0**	**320**	**800**	**2000**	**Peto test**
Number of animals	49[a]	50	50	50		50	50	50	50	
Liver										
Hepatocellular adenoma	17	21	20	16		5	5	17**	16**	↑↑
Hepatocellular carcinoma	15	15	23	31**	↑↑	1	3	15**	31**	↑↑
Hepatoblastoma	1	10**	12**	25**	↑↑	0	0	0	2	
Hepatocellular adenoma, hepatoblastoma and carcinoma (combined)	26	34	41**	45**	↑↑	6	8	29**	39**	↑↑
Acidophilic cell foci	0	2	7[#]	11[##]		1	7	3	3	

* and **: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Fisher's exact test, respectively.

↑ and ↑↑: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Peto test, respectively.

[#] and [##]: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Chi-square test, respectively.

[a]: One male control mouse died accidentally during the administration period.

3.9.2 Human data

No studies available.

3.9.3 *In vitro* data (e.g. in vitro germ cell and somatic cell mutagenicity studies, cell transformation assays, gap junction intercellular communication tests)

No studies available.

3.9.4 Other data (e.g. studies on mechanism of action)

No studies available.

3.10 Reproductive toxicity

Evaluation not performed for this substance.

3.11 Specific target organ toxicity – single exposure

Evaluation not performed for this substance.

3.12 Specific target organ toxicity – repeated exposure

Evaluation not performed for this substance.

3.13 Aspiration hazard

Evaluation not performed for this substance.

4 ENVIRONMENTAL HAZARDS

Evaluation not performed for this substance.

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