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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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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Evaluation not performed for this substance.

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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≤0.01, exception at 320 ppm in males: (P≤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≤0.01, exception in males at 320 ppm: P≤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≤0.01) increased in males at 800 and 2000 ppm and in all DCNB-exposed females compared to controls
  - triglyceride was statistically significant (P≤0.01, exception at 800 ppm: P≤0.05) increased in DCNB-exposed males at 800 and 2000 ppm compared to control
  - total protein and albumin were statistically significant (P≤0.01, exception at 800 ppm albumin: P≤0.05) increased in females at 800 and 2000 ppm and glucose was only statistically significant (P≤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≤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 ($10^3/\mu L$)	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

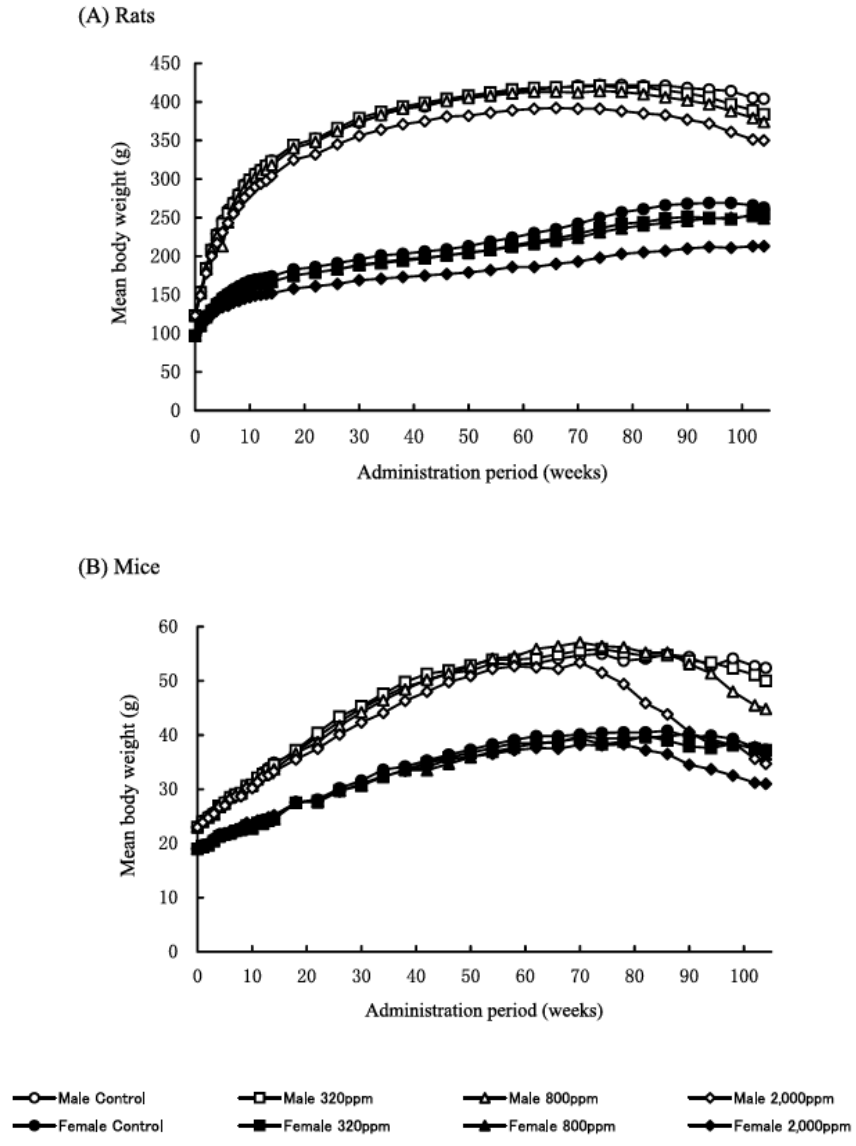

Figure 1: Graphical presentation of growth rate curves of rats (A) and mice (B) after exposure to control and 3 different DCNB-containing diets for 2 years by Yamazaki et al. (2006)

- 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.

5 REFERENCES

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