CLH report

Proposal for Harmonised Classification and Labelling

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

Chemical name:

4-Methylimidazole

EC Number: 212-497-3

CAS Number: 822-36-6

Index Number:

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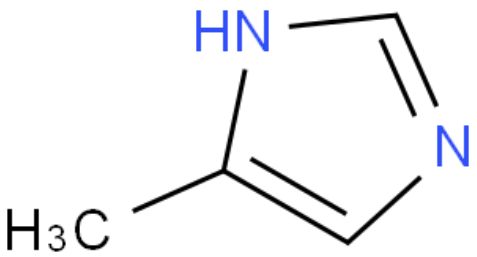
CONTENTS

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)	4-methyl-1H-imidazole
Other names (usual name, trade name, abbreviation)	
ISO common name (if available and appropriate)	
EC number (if available and appropriate)	212-497-3
EC name (if available and appropriate)	4-methylimidazole
CAS number (if available)	822-36-6
Other identity code (if available)	
Molecular formula	C4 H6 N2
Structural formula	
SMILES notation (if available)	C1(C)=CN=CN1
Molecular weight or molecular weight range	82.11
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)	-
Degree of purity (%) (if relevant for the entry in Annex VI)	-

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)
4-methylimidazole	**>99%**	-	286 notifiers in totally 14

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)
			aggregated notifications, and 1 joint entry: Acute Tox. 4 (oral, dermal) Acute Tox. 3 (dermal, inhal.) Skin Irrit. 2 Eye Irrit. 2 STOT SE 3 (upper tract, CNS, resp. tract irrit., Skin Corr. 1, 1A, 1B, 1C Skin Sens. 1, 1B Eye Dam. 1 Carc. 2 7 notifiers notified no classification (C&L Inventory, ECHA, 30 Nov 2020)

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
Not relevant				

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
Not relevant					

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	4-methylimidazole	212-497-3	822-36-6	Carc. 1B Repr. 1B	H350 H360Fd	GHS08 Dgr	H350 H360Fd			

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 conclusive but not sufficient for classification**	Yes
Carcinogenicity	**Harmonised classification proposed**	Yes
Reproductive toxicity	**Harmonised classification proposed**	Yes
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

4-methylimidazole has no harmonised classification and labelling according to CLP.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

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

5 IDENTIFIED USES

Used as intermediate for chemical reactions in manufacture of chemicals and chemical products. Also, 4-methylimidazole occurs in food and beverages as it is formed in the Maillard reaction process.

6 DATA SOURCES

In addition to ECHA and REACH registration, search were made with Google, Pubmed, Web of science and Toxline. Central studies come from National Toxicology Program (NTP) (2004; 2007), reviewed in a monograph by IARC in 2013 (IARC, 2013). In addition, during the DS compilation of the draft CLH report, an NTP reproductive and developmental toxicity study in rats following a continuous breeding protocol was made available (NTP 2019) and the results published by Behl *et al*., 2020).

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 (light yellow powder)		
Melting/freezing point	56 deg C	Pubchem	Freezing point not given
Boiling point	263 deg C	Danish QSAR database online	
Relative density			
Vapour pressure	0.00703 mm Hg 0.9373 Pa	Danish QSAR database online	
Surface tension			
Water solubility	82460 mg/L	Danish QSAR database online	
Partition coefficient n-octanol/water	log Kow 0.61 log Kow exp 0.23	Danish QSAR database online	
Flash point			
Flammability			
Explosive properties			
Self-ignition temperature			
Oxidising properties			
Granulometry			
Stability in organic solvents and identity of relevant degradation products			
Dissociation constant			

Property	Value	Reference	Comment (e.g. measured or estimated)
Viscosity			

8 EVALUATION OF PHYSICAL HAZARDS

Hazard class not assessed in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 8. Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
Single dose toxicokinetic study in male and female F-344 rats and B6C3F1 mice administered 50 and 150 mg/kg [^{14}C] 4-methylimidazole by gavage (non-guideline study). Metabolism of 4-methylimidazole in rat and mouse lung and liver microsomes and S-9 fractions, and *in vivo* in rats and mice.	Of the orally administered (gavage) 4-methylimidazole dose, 41–70% and 79–89% of the radioactivity were excreted in the urine of mice and rats, respectively after 48 h. The majority of the radioactivity eliminated in the urine was in the form of unchanged 4-methylimidazole. In rat urine, this accounted for 73–78% of the urinary radioactivity. In mice, this accounted for 60–77%. Renal clearance was the major excretion pathway in both species. The minor degree of metabolism of 4-methylimidazole was similar between rats and mice. No metabolites were detected after incubation with rat or mouse lung and liver microsomes, or lung S-9 fractions (n=3). Tissue recovery of ^{14}C-radiolabeled 4-methylimidazole in mice was 0.06-0.14% in liver, 0.011-0.027% in kidney, 0.003-0.008% in lung, and 1.32-2.62% in the carcass following oral exposure to 50 and 150 mg/kg bw. In mice, between 92 and 96% of the administered radioactivity was recovered from the excreta, tissues and carcass and cage rinse. In rats, tissue recoveries were 0.05-0.09% in liver, 0.007-0.010% in the kidney, 0.003-0.005% in the lung, and 1.50-2.02% in the carcass following oral exposure to 50 and 150 mg/kg bw. In rats, between 95 and 98% of the administered radioactivity was recovered from the excreta, tissues and carcass	Test material 4-methylimidazole (99.8% purity). Male and female F-344 rats and B6C3F1 mice were obtained from Charles River (number of animals per in vivo test group unclear).	Fennell *et al*., 2019.

Method	Results	Remarks	Reference
	and cage rinse. Overall, 4-methylimidazole was readily absorbed and distributed systemically, and was excreted largely unchanged without significant bioaccumulation.		
Single-dose toxicokinetic studies in male and female F344/N rats and B6C3F1 mice. Dose 10, 50, 100 mg/kg bw, oral administration by gavage. Similar to OECD TG 417.	4-Methylimidazole was rapidly absorbed when administered by gavage to male and female F344/N rats and B6C3F1 mice. Plasma concentration vs. time described by a one-compartment model with first-order absorption and elimination. Elimination half-life values ranged from 1-8 hours in rats and from 21 to 87 minutes in mice and increased with dose in both sexes of both species.	Test material 4-methylimidazole (99% purity). Details of NTP (2007) Study: Conventional F344/N Rats and B6C3F1 male mice. Blood samples were collected using the retroorbital puncture method for rats and cardiac puncture for mice (three rats and three mice were bled at each time point, deviating from recommandations of 4 animals of one sex in OECD TG 417).	NTP, 2007
Single-dose toxicokinetic studies in F344/N rats and B6C3F1 mice. Dose 10 mg/kg bw intravenous administration. Method similar to OECD TG 417.	The plasma concentration versus time data following intravenous administration in rats and mice was described as a one-compartment model with first-order elimination.	Route of administration: single dose by intravenous injection.	Same study as above, NTP, 2007.
Single-dose toxicokinetic studies in F344/N rats. Method similar to OECD TG 417.	Following gavage administration of 5, 50, or 150 mg/kg 4-methylimidazole to F344/N rats, peak plasma concentration was reached between 0.5, 1.0, and 3.0 hours, respectively. At 150 mg/kg bw, the plasma concentration of [14C]-4-methylimidazole was almost constant during the first 5 hours after gavage; at lower doses, the decline was more rapid. The estimated terminal half-life was dose dependent. The results suggest that the elimination of parent 4-methylimidazole was saturable. Using the total urinary recovery of parent 4-methylimidazole, the estimated bioavailability was approximately 60% to 70%. Little or no metabolism of 4-methylimidazole was found. Fecal, biliary, or respirated elimination of radioactivity was	Test material: [14C]-4-methylimidazole. Route of administration: single gavage dose of 5, 50 and 150 mg/kg bw in rats and mice. Single intravenous dose dose of 5 mg/kg bw.	Yuan and Burka, 1995.

Method	Results	Remarks	Reference
	negligible. Elimination after an intravenous dose of 5 mg/kg bw can be described by a two-compartment process with an estimated half-life of 1.8 h and an estimated apparent volume of distribution of 2.3 litre/kg. Metabolism and renal clearance were saturated by a 50 mg/kg bw oral dose.		
Single-dose toxicokinetic studies in F344/N rats and B6C3F1 mice. Method similar to OECD TG 417.	In rats, the uptake at 5 minutes after a single 216 mg/kg bw intraperitoneal injection was highest in the intestines, followed by blood, liver, stomach, and kidney. The compound was excreted unchanged in urine, beginning approximately 30 minutes after injection, and reached approximately 90% within 8 hours.	Route of administration: single dose by intraperitoneal injection.	Hidaka, 1976.
Inhibition of p-nitrophenol hydroxylase in rat liver microsomes.	Hargreaves et al. (1994) reported that 4-methylimidazole was a strong inhibitor of p-nitrophenol hydroxylase in rat liver. p-Nitrophenol is a cytochrome P450 2E1 substrate.	Liver microsomes prepared from male Sprague-Dawley rats. 4-Methylimidazole was incubated with liver microsomes obtained from acetone-pretreated rats, and effects on metabolism investigated.	Hargreaves *et al*., 1994.
Single-dose toxicokinetic studies in ewes. Method similar to OECD TG 417.	In ewes, one half of an oral dose (20 mg/kg bw) was absorbed in about 27 minutes, and the maximum plasma level was reached 5 hours after oral administration. The bioavailability calculated using plasma data from three ewes was 69%, and the biological half-life was 9.03 hours. Only 0.07 mg/kg bw of the oral dose was recovered in urine unchanged. Metabolites were not detected. 4-Methylimidazole forms complexes with heme-containing enzymes such as cytochrome P450 and results in inhibition of mixed function oxidase activity (Karangwa et	Route of administration: single oral dose. Deviating from recommandations of use of 4 rats (recommended species) of one sex in OECD TG 417.	Karangwa *et al*., 1990.

Method	Results	Remarks	Reference
	al., 1990).		
Single-dose toxicokinetic studies in goats and heifers. Method similar to OECD TG 417.	In goats and heifers (i.e. a young cow before it having the first calf), the mean residence time of 4-methylimidazole when administered orally or intravenously was about 5 hours, and the volume of distribution was 0.9 L/kg bw in both goats and heifers. 4-Methylimidazole and its metabolites were excreted mainly in urine, but also in milk and feces. Goats metabolised 4-methylimidazole to a higher extent than heifers which excreted the major part as the unchanged compound. Metabolites identified included 5-methyl hydantoin and 2-methylhydantoic acid, an unidentified metabolite, and urea. The administered 4-methylimidazole was distributed mainly to the liver, kidney, and lung.	Route of administration: single oral or intravenous dose. Deviating from recommandations of use of 4 rats (recommended species) of one sex in OECD TG 417.	Nielsen *et al*., 1993.
Toxicokinetic studies in pregnant and postpartum cows, and in mice fed with cow´s milk.	In pregnant and postpartum cows (1-2 cows) and in mice, 4-methylimidazole was found in cow milk following oral administration. Toxic, and even lethal, doses in cows did not produce signs of toxicity in the calves. No effects were seen in mice receiving the cow´s milk.	Route of administration: oral dose,	Morgan and Edwards, 1986,

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

4-Methylimidazole was rapidly absorbed when administered by gavage to male and female F344/N rats and B6C3F1 mice. Post-dose plasma samples were analyzed for 4-methylimidazole, to calculate absorption and elimination half-life. Elimination half-life values ranged from 1 – 8 hours in rats and from 21 to 87 minutes in mice and increased with dose in both sexes of both species. The plasma concentration versus time data following gavage and intravenous administration in rats and mice was described as a one-compartment model with first-order elimination. No metabolites were described in the report (NTP, 2007).

Following gavage administration to rats with 4-methylimidazole in doses of 5, 50, and 150 mg/kg bw, peak plasma concentration was reached between 0.5, 1.0, and 3.0 hours, respectively, and estimated terminal half-life was 1.1, 4.3 and 7.4 hours (Yuan and Burka, 1995 – study conducted to facilitate the interpretation of the chronic NTP studies). After an intravenous injection of 5 mg/kg bw, the elimination half-life was 1.8 hours. "Little or no metabolism of 4-methylimidazole was found by gavage and intraperitoneal administration"

(citation from the paper). A minor urinary metabolite was found in a saturable process (neither C_{max} or AUC[1] of the metabolite increased with dose), suggested by the authors to be a sulphate-conjugate of 4-methylimidazole and thus a product of detoxification. No other metabolites were described. Fecal, biliary, or respirated elimination of radioactivity was negligible.

In rats, the uptake at 5 minutes after a single 216 mg/kg bw intraperitoneal injection was highest in the intestines, followed by blood, liver, stomach, and kidney (Hidaka, 1976). The compound was excreted unchanged in urine, beginning approximately 30 minutes after injection, and reached approximately 90% within 8 hours.

Only one study identified metabolites of 4-methylimidazole. The study was done in two goats and two heifers, and the major metabolites identified included 5-methyl hydantoin and 2-methylhydantoic acid and an unidentified metabolite. Although mainly excreted in urine, 4-methylimidazole was also found in milk following oral or intravenous administration in several species, indicating transfer to the offspring via lactation (Nielsen et al., 1993; Morgan and Edwards, 1986). However, DS question the validity of these pilot studies.

In rats and mice, orally administered 4-methylimidazole was readily absorbed and distributed systemically, and was excreted largely unchanged in urine without significant bioaccumulation. The minor degree of metabolism of 4-methylimidazole was similar between rats and mice *in vivo*. Identified metabolites in both species were 4-hydroxymethylimidazole, its glucuronide, and other oxidized products, including methylhydantoin. No metabolites were detected after incubation with rat or mouse lung and liver microsomes, or lung S-9 fractions (Fennell *et al*., 2019).

4-Methylimidazole forms complexes with heme-containing enzymes such as cytochrome P450 and results in inhibition of mixed function oxidase activity (Karangwa et al., 1990; Hargreaves et al., 1994). Binding by heme may therefore prolong its half-life. The elimination half-life of 4-methylimidazole is regarded as long enough to allow the manifestation of 4-methylimidazole toxicity.

In conclusion, 4-methylimidazole is rapidly absorbed, widely distributed, metabolised to a low degree in the liver, and rapidly eliminated in mammals without significant bioaccumulation after oral gavage and intravenous injection. The DS assumes that toxicity of 4-methylimidazole stems from the parent chemical itself, and not from a metabolite since metabolism is almost absent in the available studies.

10 EVALUATION OF HEALTH HAZARDS

10.1 Acute toxicity - oral route

Hazard class not assessed in this dossier.

10.2 Acute toxicity - dermal route

Hazard class not assessed in this dossier.

10.3 Acute toxicity - inhalation route

Hazard class not assessed in this dossier.

10.4 Skin corrosion/irritation

Hazard class not assessed in this dossier.

[1] Cmax is the maximum plasma concentration observed after an extravascular (e.g. oral) dose. AUC is the area under the concentration-time-curve, i.e. a measure of the total systemic exposure of a substance.

10.5 Serious eye damage/eye irritation

Hazard class not assessed in this dossier.

10.6 Respiratory sensitisation

Hazard class not assessed in this dossier.

10.7 Skin sensitisation

Hazard class not assessed in this dossier.

10.8 Germ cell mutagenicity

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

Method, guideline, deviations if any, reliability	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
Bacterial Reverse Mutation Assay. Similar to OECD TG 471 Coded aliquots sent to two laboratories a) performed at SRI International, b) at Environmental Health Research and Testing, Inc., (EHRTI). In a), the high dose used, i.e. 10,000 µg /plate, exceeds the current OECD 471, Bacterial Reverse Mutation Test, recommendations of 5,000 μ g/plate (OECD, 1997), however, four lower doses were evaluated, down to 100 μ g/plate. Deviation from OECD TG 471 by:	Test material 4-methylimidazole (>99% purity). Test concentrations in study a): 0, 100, 333, 1000, 3333 and 10000 µg/plate, Test concentrations in study b): 1, 3.3, 10, 20, 33 µg/plate. Both studies with and without hamster or rat liver S9. Highest test concentration limited by experimental design to 10000 µg/plate in study a) and by toxicity to 33 µg/plate in study b)	Main test: Salmonella typhimurium strains TA97, TA98, TA100, and TA1535. Metabolic activation: with and without 10% or 30% hamster or rat liver S9 activation enzymes. In study b) there is no explanation for cytotoxicity observed at concentrations above 33 µg/plate given in NTP, 2007.	4-Methylimidazole (up to 10000 µg/plate) was not mutagenic in Salmonella typhimurium strains TA97, TA98, TA100, or TA1535, when tested with and without 10% or 30% hamster or rat liver S9 activation enzymes.	NTP, 2007.

Method, guideline, deviations if any, reliability	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
The study did not include strain TA 102 or *E. coli* WP2*uvrA* since protocols used were developed and in place prior to the 1997 OECD guideline 471. Reliability 1				
Bacterial Reverse Mutation Assay OECD TG 471-compliant. Reliability 1	Test material 4-methylimidazole (99% purity). Concentrations (plate incubation methodology): 0, 5, 15.81, 50, 158.1, 500, 1581, and 5000 10000 µg/plate. 0, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate in the pre-incubtion test, both methodologies with and without rat and mouse liver S9, in addition to rat and mouse lung S9 activation enzymes.	Salmonella typhimurium strains TA98, TA1535, TA1537, TA100 and TA102 Metabolic activation: with and without 10% mouse and rat liver S9 in addition to mouse and rat lung S9 activation enzymes.	4-Methylimidazole (up to 5000 µg/plate) was not mutagenic in Salmonella typhimurium strains TA98, TA1535, TA1537, TA100 and TA102. Consistent negative results were obtained both in the absence and presence of exogenous metabolism, regardless of whether metabolic activity was provided by S9 from induced rat liver or lung, or mouse liver or lung. No cytotoxicity was observed.	Beevers and Adamson, 2016.
Sister chromatid exchange (SCE), chromosome aberration (CA) and micronucleus (MN) tests in human peripheral lymphocytes (non-guideline study) from 4 blood donors. CA test	Test material 4-methylimidazole (98 % purity). Concentrations: 300, 450, 600 µg/ml for 24 h and 48 h periods.	Whole blood cells (lymphocytes) from four healthy donors (two males and two females) were used for preparation of cells for the SCE, CA and the MN in vitro test. To score SCE, a total of 100 second metaphases per concentration (25 cells per donor) were	In 48 h treatment period 450, 600 µg/ml of 4-methylimidazole induced SCE. 4-Methylimidazole induced CA in the cells at all concentrations both for 24 h and 48 h treatment groups, and led to chromatid and chromosome breakage and formation of fragments. 4-Methylimidazole induced formation of MN at 600 and 750 µg/ml in 24 h and 48 h treatment	Celik and Topaktas, 2018.

Method, guideline, deviations if any, reliability	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
comparable to OECD TG 473. MN test comparable to OECD TG 487. TG 479 (in vitro sister chromatid exchange test in mammalian cells) is not a valid guidance because of a lack of understanding of the mechanism(s) of action of the effect detected by the test. Deviations (methodological) vs TG 473 and TG487: -Test concentrations selected should cover a range from that producing cytotoxicity and including concentrations at which there is moderate and little or no cytotoxicity; -Cells should be exposed to the test chemical with metabolic activation for 3-6 hours, and sampled at a time equivalent to about 1.5 normal cell cycle lengths after the beginning of treatment; - Recommendation of counting at least 2000		analyzed. CAs were evaluated in 100 well-spread metaphases per donor. For the MN test, only 1000 binucleate cells were scored per concentration and not 2000 as recommended in OECD TG 487.	groups. 4-Methylimidazole negatively affected the mitosis in 24 h treatment group at 600 µg/ml, while the same effect was seen at all concentrations in 48 h treatment. 4-Methylimidazole decreased the proliferation index at all concentrations in 24 h treatment group and at 600 and 750 µg/ml in 48 h treatment period. 4-Methylimidazole significantly decreased the nuclear division index at all concentrations in 24 h and 48 h treatment periods. A genotoxic chromosomal effect was observed, concurrently with cytotoxicity. Potentially the observed increased genotoxicity could be an indirect consequence of high cytotoxicity.	

Method, guideline, deviations if any, reliability	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations	Reference
binucleated cells for MN assessment; - Slides not blinded before scoring; - Negative control groups were not included for the 48h treatments, where the most significant positive responses were observed; -At least 300 well-spread metaphases should be scored per concentration and control. Reliability 3				

Table 10. Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells in vivo

Method, guideline, deviations if any, reliability	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Micronucleated erythrocytes in rat and mouse bone marrow. Similar to OECD TG 475. Reliability 1	Test material 4-methylimidazole (99.5% purity). Doses: 0, 25, 50, 100 mg/kg bw.	F344/N male rats. B6C3F1 male mice. Intraperitoneal administration, three times at 24-hour intervals on three consecutive days in both species (n=5).	No effects (no increases in the frequencies of micronucleated erythrocytes were seen in bone marrow of male rats or male mice). No significant alterations in percent micronucleated polychromatic erythrocytes (PCEs), a rough indicator of bone marrow toxicity, in the mouse bone marrow test. In bone marrow of male rats, percent PCEs declined with increasing dose and were significantly depressed at the highest dose.	NTP, 2007. Protocol according to Shelby *et al*., 1993.
Mouse peripheral blood micronucleus test. Food and Drug	Test material 4-methylimidazole (99% purity).	14-week toxicity study (where peripheral blood for the micronucleus test were obtained in week	No effects (no increases in 14-week peripheral blood micronucleus tests in male and female mice).	NTP, 2007. MN peripheral blood assays

Method, guideline, deviations if any, reliability	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Administration Good Laboratory Practices Regulations (21 CFR, Part 58) Reliability 1	Doses: 100, 240, 440, 915 or 1840 mg/kg bw/day to male mice and 110, 240, 540, 1130 or 3180 mg/kg bw/day to female mice.	14). B6C3F1 mice. Exposure 7 days/week by feed, available ad libitum. The number of male and female mice with erythrocytes scored were 5 for all exposure groups except for female mice exposed to 3180 mg/kg bw/day where the erythrocytes scored derived from only 3 animals.	No bone marrow toxicity observed.	were performed according to MacGregor *et al.*, 1990
Chromosomal aberration in the bone marrow cells of Swiss Albino mice (non-guideline study). Comparable to OECD TG 475. Deviations (methodological) vs TG 475: - No formal quality control (QC) and quality assurance (QA) procedures were reported; - Minimum of 5 animals/sex/group (instead of 3 animals/sex/group as used in this study); - 200 metaphase cells per animal should be examined for abberations; - Non-recommended (i.p.) route of administration without justification;	Test material 4-methylimidazole (98 % purity).	Male and female adult Swiss Albino mice. Body weight 33-40 g (unclear whether this was at arrival or at start of dosing). 4-Methylimidazole was dissolved in double distilled water and administered as single dose of 0.5 mL per mouse by intraperitoneal administration. Single intraperitoneal injection with 100, 130 and 160 mg/kg bw (LD_{50}) to males and females (three females and three males instead of 5 were allocated per dosing group, not randomized). CA and mitotic index (MI) of the mouse bone marrow cells were analyzed 12 h and 24 h after treating the animals with 4-methylimidazole. 100 metaphase cells per mouse were investigated for aberrations, instead of 200 as recommended in OECD TG 475. Cytotoxicity as reduced mitotic index was reported at 160 mg/kg bw after 12 h and at all	4-Methylimidazole increased the percentage of chromosomal aberrations at all concentrations after 12 h and at highest concentration after 24 h. The mitotic index decreased in comparison with control at highest concentration for 12 h and at all concentrations for 24 h.	Norizadeh Tazehkand *et al.*, 2016

Method, guideline, deviations if any, reliability	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
- 24 h harvest time is earlier than the recommended 36-42 h second harvest time; -The dose levels used should preferably cover a range from the maximum to a dose producing little or no toxicity. When target tissue (bone marrow) toxicity is observed at all dose levels tested, further study at non-toxic doses is advisable; -Identification of the maximum tolerated dose (i.e. without evidence of study-limiting toxicity, e.g. body weight depression or hematopoietic system cytotoxicity (here, LD50 was regarded as MTD; 160 mg/kg); -Inclusion of a negative control group (only included at one sample time point) and a minimum of three dose levels generally separated by a factor of 2 (here, dose levels were 100, 130 and 160 mg/kg bw); -No historical data available for comparison with negative and positive controls;		concentrations after 24 h,		

Method, guideline, deviations if any, reliability	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
- No observations (clinical signs) reported for treated animals; - Uncoded slides, potentially introducing bias; -Individual animal data should have been included in tabular form Reliability 3				

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

4-methylimidazole was not mutagenic in *Salmonella typhimurium* with or without metabolic activation from hamster, mouse or rat liver S9, or mouse and rat lung S9 (NTP, 2007; Beevers and Adamson, 2016). No cytotoxicity was observed in the *Salmonella* strains by Beevers and Adamson (2016) or by NTP (2007) in the study conducted at SRI International in concentrations up to 10000 µg/plate.
4-Methylimidazole did not induce micronuclei in mouse peripheral blood erythrocytes sampled at the end of the 14 weeks study after receiving 4-methylimidazole continously in the feed in concentrations up to 10000 ppm (1840 and 3180 mg/kg bw/day in male and female mice, respectively). Nor did 4-methylimidazole induce micronuclei in rat or mouse bone marrow cells from animals injected intraperitoneally three times with 24-hour intervals with up to 100 mg/kg bw (NTP, 2007). No bone marrow toxicity (cytotoxicity) was observed in any of these micronucleus studies, except for in rats in the intraperitoneal study where the percent of PCEs declined with increasing dose of 4-methylimidazole and was significantly depressed at the highest dose.

Additionally, structure activity relationship (SAR) analysis revealed no genotoxic potential (i.e. no structural alerts of 4-methylimidazole associated with mutagenicity *in vitro* or *in vivo*) using the softwares Osiris, Case Ultra, ToxTree and DEREK (Krishna *et al*., 2014; Howard and Choksi ,2020).

In contrast to this, Celik and Topaktas (2018) report that 4-methylimidazole has a cytotoxic and genotoxic effect (CA, SCE and MN) *in vitro* in human peripheral blood lymphocytes from 4 donors. Cytotoxicity was observed at all concentration levels where chromosomal genotoxicity was reported, i.e. at 600 and 750 µg/ml for MN and CA, and 450, 600 and 750 µg/ml for SCE (doses were 300, 450, 600 and 750 µg/ml for 24 h and 48 h periods). This academic study had major deviations compared to OECD TG 473 (e.g. indirect effects of cytotoxicity may affect the reported genotoxicity), this study has low reliability.

Norizadeh Tazehkand *et al*. (2016) report that 4-methylimidazole have genotoxic and cytotoxic effect in mouse, shown as increased percentage of chromosomal abberations and decreased mitotic index. Cytotoxicity as reduced mitotic index was reported at 160 mg/kg bw after 12 h and at all concentrations after 24 h. This academic study had major deviations compared to OECD test guidelines and also low reliability. The work by Norizadeh Tazehkand et al. was performed by the same research group as Celik and Topaktas (2018). These genotoxicity findings are not verified by other researchers.

No germ cell mutagenicity studies are retrieved.

10.8.2 Comparison with the CLP criteria

Classification criteria

CATEGORY 1: "Substances known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cells of humans. Substances known to induce heritable mutations in the germ cells of humans."

Category 1A: "The classification in Category 1A is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans."

Category 1B: According to CLP to classify a compound as Cat 1B the following criteria must be fulfilled: "The classification in Category 1B is based on: – positive result(s) from *in vivo* heritable germ cell mutagenicity tests in mammals; or – 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.."

Category 2: Classification criteria for category 2, from CLP: "Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans.

DS assessment and conclusion:

No epidemiological studies are available so Cat 1A is not justified. No germ cell mutagenicity studies are available.

In vivo: 4-methylimidazole did not induce micronuclei in erythrocytes from bone marrow in male rats or mice treated intraperitoneally three times at 24-hour intervals, or in peripheral blood samples from male and female mice receiving up to 10000 ppm in feed for 14 weeks (equivalent to average daily doses of approximately 40, 80, and 170 mg/kg bw). Cat 1B is not justified based on these results.

In vitro: 4-methylimidazole in concentrations up to 10000 µg/plate with and without metabolic activation with hamster and rat liver/lung S9 did not induce cytotoxicity or mutations in *Salmonella typhimurium* strains TA97, TA98, TA100, and TA1535. In one study the maximum concentration was limited to 33 µg/plate due to cytotoxicity. No mutagenicity was seen. Genotoxicity manifested as chromosomal effects (CA, SCE and MN formation) was observed in human lymphocytes treated with 4-methylimidazole *in vitro* for 24 and 48 hours, however it occured concurrently with cytotoxicity and was based on only four blood donors. Cat 2 is not justified based on these results.

An overall assessment of *in vitro* and *in vivo* genotoxicity studies on 4-methylimidazole show that no classification or labelling according to CLP criteria are justified.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

No germ cell mutagenicity studies are retrieved. An overall assessment of *in vitro* and *in vivo* genotoxicity studies on 4-methylimidazole indicates that no classification or labelling according to CLP criteria is justified.

10.9 Carcinogenicity

Carcinogenicity of 4-methylimidazole has been investigated in two NTP 2-year studies; one in rats and one in mice. The tumour types of which the incidence was increased in the respective studies are considered in detail below.

The administered doses were based on findings in the 14 weeks study reported previously by NTP (NTP, 2004). In the two year-studies, the top doses were equal to doses that slightly reduced body weights in the 14 weeks studies to 95, 94, 93 and 88% of the body weight of the control animals in male (2500 ppm in feed) and female rats (5000 ppm in feed), and male and female mice (both 1250 ppm), respectively. In male rats in the 14 weeks study, slight changes in hematology and clinical chemistry parameters were seen in the 2500 ppm group, as well as increased liver weight and vacuolization in hepatocytes, but the effects were not considered detrimental for a 2-year study. In female rats, slight changes in hematology and clinical chemistry parameters were observed, and absolute and relative spleen weights were reduced compared to controls in the 14 weeks study. In mice in the 14 weeks-study, the top dose of 1250 ppm did not induce changes in hematology, clinical chemistry, organ weights, or histopathology.

The historical database for the 2-year bioassays include studies using the same route of administration, and also receiving the same NTP-2000 diet. In general, the concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in the NTP bioassays. However, historical tumour incidences are given by NTP and reproduced below. The applied NTP historical database contains all studies that use the NTP-2000 diet (i.e. starting from 1995) with histopathology findings completed up to the present NTP study of 4-methylimidazole.

Table 11. Summary table of animal studies on carcinogenicity

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
NTP, 2-year cancer bioassay, GLP, Similar to OECD TG 451 F344/N **rats**, 50/sex/dose Reliability 1	4-methylimidazole, > 99% pure, oral exposure for 106 weeks to 0, 625, 1250, or 2500 ppm (males) or 0, 1250, 2500, or 5000 ppm (females) in feed Calculated average doses based on food consumption of: 30, 55, 115 mg 4-methylimidazole /kg bw/day (males); 60, 120, 260 mg 4-methylimidazole /kg bw/day (females).	No significant effect on survival was observed. **General toxicity**: Lower mean terminal body weights of males in the 1250 and 2500 ppm groups and females in the 2500 and 5000 ppm groups compared to controls. Reduced feed consumption reported in high dose females (5,000 ppm). Clinical signs of neurological toxicity (clonic seizures, excitability, hyperactivity, and impaired gait) was observed in high dose females and some of these clinical findings were also observed in the lower exposed groups at greater frequencies than in the controls. **Non-neoplastic lesions:** Increased incidences of hepatic histiocytosis and chronic inflammation, in all exposed groups of male and female rats. Increased incidence of hepatocyte focal fatty change in males/females at doses ≥ 1250 ppm. Incidences of hepatocellular eosinophilic and mixed cell foci were increased in 2500 ppm males and 5000 ppm females. Increased incidences of inflammation in the prostate gland, focal hypertrophy in the pituitary gland (pars distalis), and follicular cystc in the thyroid gland of male rats at the high dose and also medium dose for pituitary lesions. In females, follicle mineralization in the thyroid gland at the high dose and lung inflammation, cardiomyopathy and focal atrophy of the acinar pancreas was observed in all exposure groups. These non-neoplastic lesions were graded as minimal to mild severity. **Neoplastic lesions:** *Mononuclear cell leukemia*: The incidence of mononuclear cell leukemia in high dose (5000 ppm) females was significantly greater than that in the controls, and the incidence exceeded the historical control range. Overall incidence rate: 18%, 14%, 32%, 40% in 0, 1250, 2500 and 5000 ppm exposure groups, respectively. Onset in high dose females was earlier (day 368) than in control females (day 624). Historical control data (HCD): 23.8% ± 9.1%; range 12-38%. Slight, non-significant increase in incidence of mononuclear cell leukemia in males (overall rates of 30%, 36%, 44%, 40% in 0, 625, 1250 and 2500 ppm exposure groups, respectively). No differences in time of onset was reported between control and treated groups. HCD: 46.8% ± 13.0%; range 30-68%. *Other neoplasms*: Significant reduction in neoplasms of the pituitary gland (pars distalis) and adrenal medulla in exposed groups of males	NTP, 2007; NTP 2007; Chan 2008

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	Test substance, dose levels duration of exposure	Results	Reference
		and of the pituitary gland (pars distalis), clitoral gland, mammary gland, and uterus in exposed groups of females. These incidences in the exposed groups were either below or at the lower end of the historical control ranges. <u>Decreased incidence of neoplasms (overall rates)</u>: Male rats: Adrenal medulla (benign, complex, or malignant pheochromocytoma (combined): In male rats 20, 12, 6 and 6% in 0, 625, 1250 and 2500 ppm. HCD11.6% ± 5.5%; range 5-20%. Male and female rats: Adenoma in pituitary gland: In male rats, overall rates 33, 27, 21 and 15%, and in female rats, overall rates 60, 38, 40, 18% in 0, 625, 1250 and 2500 ppm. HCD in males 22.6% ± 6.0%; range 17-33%. HCD in females 39,1% ± 10,9%; range 29-60%. Female rats: Adenoma in clitoral gland: 16, 2, 0, and 0% in 0, 625, 1250 and 2500 ppm. HCD 11.0% ± 6.5%; range 2-20% Fibroadenoma in mammary gland: 48, 12, 8, and 2% in in 0, 625, 1250 and 2500 ppm. HCD 44.8% ± 11.1%; range 28-55%. Stromal polyp in uterus: 32, 10, 4, and 4% in 0, 625, 1250 and 2500 ppm. HCD 17.9% ± 6.5%; range 12-32%	
NTP, 2-year cancer bioassay, GLP. Similar to OECD TG 451 B6C3F1 **mice**, 50/sex/dose Reliability 1	4-methylimidazole, > 99% pure, oral exposure f or 106 weeks to 0, 312, 625, or 1250 ppm in feed Calculated average doses based on food consumption of: 40, 80, or 170 mg 4-methylimidazole /kg bw (males/ females)	No significant effect on survival was observed. **General toxicity:** No clinical findings in exposed groups of male or female mice were considered to be related to treatment. Mean terminal body weights were reduced in the 1250 ppm group (males) and in all exposure groups (females). Feed consumption by exposed groups (males/females) was generally similar to the controls. **Non-neoplastic lesions:** The incidence of alveolar epithelium hyperplasia and of histiocytic cellular infiltration in 1250 ppm females was significantly greater than that in the controls. The incidence of histiocytic cellular infiltration, was slightly increased in 1250 ppm males. The incidence of thyroid follicular cyst in 1250 ppm females was significantly greater than that in the controls. There was a significant positive trend in the incidences of mammary gland hyperplasia in females (16/50, 10/50, 14/49, 24/49); however, none of the exposed groups differed	NTP, 2007; Chan 2008

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	Test substance, dose levels duration of exposure	Results	Reference
		significantly from the control group. **Neoplastic lesions:** In all exposed groups of females, the incidences of alveolar/bronchiolar adenoma were statistically significantly increased with a dose-related response up to the medium dose group (0, 16, 32 and 16% in the control, low, medium and high dose group, respectively), HCD: 3.7 ± 3.8%; range 0–10%. In exposed males, there was a non-significant trend seen as increased incidences of alveolar/bronchiolar adenoma (incidence of 16, 22, 26 and 30% in the control, low, medium and high dose group, respectively), HCD: 15.8 ± 6.3%; range 9–28%. In females, the incidences of alveolar/bronchiolar carcinoma in exposed groups were not statistically significantly different from the control group (6, 0, 4 and 14% in the control, low, medium and high dose group, respectively). HCD: 2.9 ± 2.5%; range 0–6%. In exposed males, there was significantly increased incidences of alveolar/bronchiolar carcinoma in the highest dose group compared to the control group (4, 8, 8 and 16% in the control, low, medium and high dose group, respectively). HCD: 7.8 ± 3.8%; range 4–14%. In females, the incidence of alveolar/bronchiolar adenoma or carcinoma combined was statistically significantly increased in the medium and high dose groups (6, 16, 34 and 28% in the control, low, medium and high dose group, respectively). HCD: 6.6 ± 4.2%; range 0–12%. In males, the incidence of alveolar/bronchiolar adenoma or carcinoma combined was statistically significantly increased in the high dose group (18, 26, 32 and 44% in the control, low, medium and high dose group, respectively). HCD: 22.2 ± 6.3%; range 14–32%. HCD is available from database over NTP-studies that use the NTP2000 diet, see above.	

Rat 2-year cancer bioassay

No significant effect on survival was reported, but lower terminal mean body weights of males in the 1250 and 2500 ppm groups and females in the 2500 and 5000 ppm groups compared to controls (95%, 87%, 81%, 65%, respectively) was observed. Clinical signs of neurological toxicity were observed in females, in particular at the two highest doses.

Mononuclear cell leukemia (Table 10): There was a slight, non-significant, increase in incidence of mononuclear cell leukemia in males (overall rates of 30%, 36%, 44%, 40% in the exposure groups 0, 625, 1250 and 2500 ppm, respectively). A mean incidence of 46.8% in the HCD was reported. There were no differences in time of onset in male rats.

The incidence of mononuclear cell leukemia in high dose females was significantly greater than that in the controls, and the incidence slightly exceeded the historical control range. The overall rates were: 18%, 14%, 32%, 40% in the 0, 1250, 2500 and 5000 ppm exposure groups, respectively. A mean incidence of 23.8% in the HCD was reported. Onset in high dose group in females was earlier (day 368) than in control females (day 624).

Table 12. Mononuclear cell leukemia in the rat 2-year cancer bioassay

Doses	0 ppm	625 ppm	1250 ppm	2500 ppm	5000 ppm	HCD, mean ± SD / range
Males	15/50 (30%)	18/50 (36%)	22/50 (44%)	20/50 (40%)		46.8 ± 13.0% / range 30-68%
Females	9/50 (18%)		7/50 (14%)	16/50 (32%)	20/50* (40%)	23.8 ± 9.1% / range 12-38%

HCD: males, total of 510 controls; females, total of 510 controls; *P<0.05, signicantly different from the concurrent control group by the Poly-3 test

Mouse 2-year cancer bioassay

No significant effect on survival was reported, but mean terminal body weights of males and females in the 1250 ppm groups were lower than those in the control groups (males, 86%; females, 81%).

Alveolar/bronchiolar adenoma and carcinoma:

The incidences of alveolar/bronchiolar adenoma in all exposed groups of females, alveolar/bronchiolar carcinoma in 1250 ppm males, and alveolar/bronchiolar adenoma or carcinoma (combined) in 1250 ppm males and 625 and 1250 ppm females were significantly greater than those in the control group.

In females, the incidences of alveolar/bronchiolar adenoma (all exposure groups) and alveolar/bronchiolar carcinoma (high dose) clearly exceeded the historical control range, whereas in males the incidences of alveolar/bronchiolar adenoma and carcinoma slightly exceeded the historical control range in the high dose.

In addition, the incidence of alveolar epithelium hyperplasia in 1250 ppm females was significantly greater than that in the controls. Histologically, this lesion was considered a morphologic continuum to adenoma.

Table 13. Lung tumours and hyperplasia in the mouse 2-year cancer bioassay

Doses	0 ppm	312 ppm	625 ppm	1250 ppm	HCD, mean ± SD / range
Males					
Alveolar/bronchiolar adenoma[a]	8/50 (16%)	11/50 (22%)	13/50 (26%)	15/50 (30%)	15.8 ± 6.3% / range 9–28%
Alveolar/bronchiolar carcinoma[a]	2/50 (4%)	4/50 (8%)	4/50 (8%)	8/50* (16%)	7.8 ± 3.8% / range 4–14%
Combined incidences	9/50 (18%)	13/50 (26%)	16/50 (32%)	22/50* (44%)	22.2 ± 6.3% / range 14–32%
Hyperplasia (alveolar epithelium)	7/50 (14%)	3/50 (6%)	1/50 (2%)	9/50* (18%)	
Females					
Alveolar/bronchiolar adenoma[a]	0/50 (0%)	8/50* (16%)	16/50* (32%)	8/50* (16%)	3.7 ± 3.8% / range 0–10%
Alveolar/bronchiolar carcinoma[a]	3/50 (6%)	0/50 (0%)	2/50 (4%)	7/50 (14%)	2.9 ± 2.5% / range 0–6%
Combined incidences	3/50 (6%)	8/50 (16%)	17/50* (34%)	14/50* (28%)	6.6 ± 4.2% / range 0–12%
Hyperplasia (alveolar epithelium)	3/50 (6%)	2/50 (4%)	3/50 (6%)	11/50* (22%)	

[a]includes multiple. Unadjusted adenoma/carcinoma rates given in parenthesis. HCD: males, total of 510 controls; females, total of 510 controls; *P<0.05, signicantly different from the concurrent control group by the Poly-3 test.

No human data on carcinogenicity of 4-methylimidazole was found by the DS.

Short summary and overall relevance of the provided information on carcinogenicity

Carcinogenicity of 4-methylimidazole has been investigated in two NTP 2-year cancer bioassay; one in rats and one in mice. The data are publicly available and has been evaluated by other bodies, including by IARC (IARC 2013). NTP in their report stated a "clear evidence of carcinogenic activity" of 4-methylimidazole in male and female mice based on increased incidences of combined alveolar/bronchiolar adenomas and carcinomas. Haseman (Haseman, 2013) has later argued that the NTP term ''some evidence'' rather than ''clear evidence'' of carcinogenic activity is a more correct interpretation of the data. IARC concluded in their evaluation that 4-methylimidazole is possibly carcinogenic to humans (Group 2B).

4-methylimidazole may induce mononuclear cell leukemia in female rats. NTP reported "equivocal evidence of carcinogenic activity" in female rats based on increased incidences of mononuclear cell leukemia and "no evidence of carcinogenic activity" in male rats. Mononuclear cell leukemia is a common tumour type in F344/N rats with variable indicences. 4-methylimidazole may possibly promote the occurrence of this lesion in females, as it occurred earlier in the high dose females and with a higher incidence in the mid and high dose groups, although only slightly exceeding the historical control range.

In mice, 4-methylimidazole increased the incidence of alveolar/bronchiolar adenoma in females in all dose groups, of alveolar/bronchiolar carcinoma in high dose males and of alveolar/bronchiolar adenoma and carcinoma combined in high dose males and in mid and high dose females. The female incidences of alveolar/bronchiolar adenoma (all exposure groups) and alveolar/bronchiolar carcinoma (high dose group) clearly exceeded the historical control range whereas the male incidences of alveolar/bronchiolar adenoma and carcinoma slightly exceeded the historical control range in the high dose group. Hyperplasia of alveolar epithelium is considered to be a precursor for neoplasia. However, no increase in hyperplasia was observed at lower doses and not in the 14 week study (NTP, 2004).

Significant reduction in neoplasms of the pituitary gland (pars distalis) and adrenal medulla in exposed groups of males and of the pituitary gland (pars distalis), clitoral gland, mammary gland, and uterus in exposed groups of females were observed in the rat study. The possible tumour preventing effect of 4-methylimidazole in the rat has been discussed further (Murray, 2011). The tumour incidences in the exposed groups were either below or at the lower end of the historical control ranges and were considered to be only partially explained by reduction in body weight. No 4-methylimidazole related decrease in tumours was observed in the mouse study.

4-methylimidazole is considered to act as a carcinogen via a non-genotoxic MoA based on an overall assessment of the available genotoxicity, mutagenicity and carcinogenicity data.

A search in the OECD QSAR Toolbox (2018) revealed a structural alert for carcinogenicity (non-genotoxic; for imidazole, benzimidazole) with reference to an ISS (The Italian National Institute of Health) profiler. The MoA, however is not certain. Clara cells in the terminal bronchiolar epithelium are considered to constitute a cell type from which alveolar/bronchiolar neoplasms arise. Clara cells are rich in cytochrome P450-enzymes and Dalvie *et al*. (2002) suggested that oxidative metabolism of imidazoles would lead to at least two reactive intermediates, an epoxide and dicarbonyl compound, and pyruvaldehyde. However, 4-methylimidazole has also been tested in bacterial mutagenicity assays with the use of exogenous metabolism provided by S9 from induced liver or lung from rat and mouse (Beevers *et al*., 2016). This study was also negative, thus there are no suggestions of a lung-specific metabolic activation of 4-methylimidazole to a genotoxic metabolite.

The human relevance of alveolar/bronchiolar adenoma and carcinoma in the mouse model has recently been questioned (Cohen *et al*., 2020; Smith *et al*., 2018). The activation of non-genotoxic substances to cytotoxic metabolites by CYP2F2 in Clara cells is considered a mouse specific MoA. One study (Cruzan *et al*., 2015) examined the hypothesis that 4-methylimidazole induces lung tumours by the same MoA as styrene, via CYP2F2 activation and/or induction of cell proliferation, but the hypothesis was not supported. It should be

noted that 4-methylimidazole has been shown to be an effective inhibitor of cytochromes P450 (Karangwa *et al*., 1990, Hargreaves *et al*., 1994).

The MoA leading to increase in mononuclear cell leukemia in female rats and in alveolar/bronchiolar tumours in mice is thus not clear. In addition, the reasons for the reduction of certain tumours in rats and the differences between rats and mice are currently unknown. Data are insufficient to confidently postulate a MoA. The animal tumour/cancer types induced are both presumed to be relevant for humans. However, F344 rats have a high and variable spontaneous incidence of mononuclear cell leukaemia reducing the strength of the evidence for 4-methylimidazole carcinogenicity in this species. On the other hand, the involvement of the mouse specific CYP2F2 Clara cell metabolisme MoA is not supported for 4-methylimidazole and the lung tumor are therefore considered by DS to be of human relevance.

Table 14. 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/N rats	Mononuclear cell leukemia. High spontaneous incidence. Incidence at high dose slightly exceeds the HCD range. Reduced tumor incidences observed in several other organs.	No	Malignant lesion	Yes	Single (female)	No	Oral	MoA unknown. Cancer type relevance for humans has been questioned (Maronpot *et al.*, 2016).
B6C3F1 mice	Alveolar/bronchiolar adenoma and carcinoma. Clear increase in incidence in particular of adenomas and combined adenomas and carcinomas.	No	Yes	Unknown	Both	No	Oral	MoA unknown. Tumour types considered relevant for humans as mouse specific MoA is not supported.

10.9.1 Comparison with the CLP criteria

It is recognised that genetic events are central in the overall process of cancer development. However, 4-methylimidazole is presumed to act via a non-genotoxic MoA based on an overall evaluation of the available genotoxicity and mutagenicity data.

Category 1A

Classification in category 1A concerns substances known to have carcinogenic potential for humans and is largely based on human evidence.

There are no human data on carcinogenicity of 4-methylimidazole available. Hence, classification of 4-methylimidazole in Carc. Cat. 1A is not justified.

Category 1B

Category 1B is for substances presumed to have carcinogenic potential for humans. Classification is largely based on animal evidence. According to the CLP Annex I, 3.6.2.2.3 an increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can provide sufficient evidence of carcinogenicity in experimental animals.

Two fairly recent, well conducted, NTP 2-year cancer bioassays in rats and mice have been performed with 4-methylimidazole. In the rat study, a slight induction of mononuclear cell leukemia was observed in females (significant different from the control in the highest dose group), just barely outside the range of the historical control data (40% vs. 38%). As mononuclear cell leukemia has a high spontaneous tumour incidence in F344 rats, this result is given little weight in the overall assessment of the strength and weight of evidence. However, it is interesting to note that the onset in 5000 ppm females was earlier (day 368) than in control females (day 624).

There was a reduced incidence of several tumours in both male and female rats. All controls were within the range of historical control incidences for these tumour types, although many in the high end of the range. Also most of the tumour incidences in the 4-methylimidazole exposed groups were within the historical control range, except for the incidences of mammary gland fibroadenoma and stromal poly in uterus which were decreased below the historical control range for all dose groups. Chan and colleagues discusses body weight loss as an explanation of the decreased incidence of tumours, but concludes that this alone can not explain the decrease (Chan *et al*., 2008).

In the mouse bioassay, a dose-related induction of alveolar/bronchiolar adenoma (significant in females), was seen in the low and medium dose groups compared to the controls. The incidence (females) was 0, 16, 32, and 16% in the control and the low-medium-high dose groups, and outside the HCD (3.7% ± 3.8%; range 0-10%. Alveolar/bronchiolar carcinoma was significantly increased in male mice in the high dosed-group (16% vs. 4% in the control; HCD: 7.8% ± 3.8%; range 4-14%) and showed a trend in the lower dose-groups (8% incidence in both dose-groups). A significant increase in the incidence of benign and malignant neoplasms combined was observed in both sexes, outside the HCD range for all dose groups in females and outside the HCD range for males in the high-dose group. No excessive toxicity was observed in the mouse cancer bioassay.

The DS considers the findings of statistically significantly increased incidences of alveolar/bronchiolar adenomas in female mice, alveolar/bronchiolar carcinomas in male mice and benign and malignant neoplasms combined in both sexes to constitute sufficient strength of evidence for classification in Carc. Cat. 1B, together with additional considerations. These considerations are findings of carcinogenic activity outside robust historical control data from NTP, progression to malignancy, response in both sexes, and to some extent response in two species, although as a cancer form with high spontanous tumour incidence in rats (mononuclear cell leukemia). Moreover, 4-methylimidazole has a structural alert for carcinogenicity in the OECD QSAR Toolbox (2018).

4-methylimidazole is considered a non-genotoxic carcinogen. The hypothesis that 4-methylimidazole induces lung tumours by the mouse specific CYP2F2 activation was not supported. Hyperplasia was only observed at the high dose and not observed in the 14-week repeated dose study suggesting that mitogenic effect or regenerative effects are not main contributers to the carcinogenic effects of 4-methylimidazole in mice. Currently 4-methylimidazole has an unknown carcinogenic MoA. Since the DS has found no data to conclusively conclude that the tumours are due to a MoA that is not relevant for humans, they are assumed to be relevant. Considering the clear dose-response in lung tumour incidences observed, a threshold for tumorigenic activity is not possible to establish based on available data.

Based on the NTP mouse study, 4-methylimidazole warrants a classification in Carc. Cat. 1B.

Category 2

Category 2 substances are suspected human carcinogens. Classification is based on evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B.

10.9.2 Conclusion on classification and labelling for carcinogenicity

Based on the arguments given above, DS propose that 4-methylimidazole warrants classification as carcinogenic, Cat 1B (H350). No SCL is proposed.

10.10 Reproductive toxicity

No human data are available for 4-methylimidazole. Two rodent experiments with high relevance for evaluation of reproductive toxicity have been reported. A NTP reproductive and developmental toxicity study in rats following a continuous breeding protocol has recently been performed (NTP 2019, Behl *et al*., 2020). In addition, there is a 14-week repeated dose toxicity study in rats and mice conducted by NTP (NTP 2004) that includes supportive information. Both male and female reproductive organs were examined including histopathology and sperm quality parameters and morphology determined in testis and epididymides.

10.10.1 Adverse effects on sexual function and fertility

Table 15. Summary table of animal studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
NTP reproductive and developmental continuous breeding (RACB) toxicity study. GLP compliant Hsd: Sprague Dawley SD rats Dose range-finding study: 0, 625, 1250, 2500, 5000, and 10 000 (males only at top dose) ppm. 8 rats/sex/group. Multigeneration study; F0: 23 rats/sex/group	4-Methylimidazole (CAS No. 822-36-6) purity > 99 %. Dietary study. 14-day pre-cohabitation exposure period of F0 animals. Dietary concentrations, 0, 750, 2500, and 5000 (only F0) ppm Mean delivered daily doses in mg/kg bw/day in the pre-mating period were F0: 47.9; 144.6 og 260.1 (males)	**Mortality**: Adult animals: The dose range-finding study showed excessive toxicity at 10 000 ppm (males only tested). In the main study, no treatment related mortality was noted for males. A number of females were dead/moribund in the mid (4) and high (11) dose groups and several of these were removed around the time of parturition with dystocia or retained placentas/fetuses. The high dose group (5000 ppm) was discontinued due to marked reduction in litters produced and toxicity to dams. Pups: Survival ratio in F0/F1-pups at PND1-4 was 96%/93% (controls), 98%/94% (750 ppm), 92%/84% (2500 ppm) and 44% (5000 ppm). **Body weights** were dose-dependently reduced. In adult rats, reductions in terminal bw in F0/F1 males were 5%/2% (750 ppm), 9%/11% (2500 ppm) and 10% (5000 ppm) compared to the control group. F0/F1 females at GD21 showed 4-9 % (750 ppm), 13-17% (2500 ppm) and 18-25% (5000 ppm) reductions in bw and 4%/4% (750 ppm), 13%/10% (2500 ppm) and 19% (5000 ppm) reduction in bw at termination. Pups: Body weights were dose-dependently reduced to F1: ≥ 97% (750 ppm), 89-97% (2500 ppm) and 67-73% (5000 ppm) at PND1 and 95% and 81% at PND28 (F1c) compared to controls across litters. For F2 pups there was no reduction in bodyweight in the 750	NTP, 2019 Behl *et al*., 2020

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	Test substance, dose levels duration of exposure	Results	Reference
F1: F1a and F1b euthanized at PND4. F1c used for breeding of F2 generation. F2a and F2b euthanized at PND4. F2c necpropsy at PND28. Cross-over mating groups included (F0) Reliability 1	and 46.8; 145.6 and 289.9 (females). F1 47.9; 144.6 og 260.1 and 206.9 (males); 66.3 and 225.4 (females).	ppm group, wherase in the 2500 ppm group body weights were ≥ 92% of controls. **Clinical signs**: Dose-related increase in female rats with convulsions; F0 4%, 0%, 9% and 39% in the 0, 750, 2500 and 5000 ppm groups, respectively; F1c: 0%, 1% and 16% in the 0, 750 and 2500 ppm groups, respectively. **Systemic toxicity:** Only modest effects on weights of non-reproductive organs were reported and most were considered related to body weight changes. Histopathology showed increased hepatocellular vacuolation in male rats of the 5000 ppm group and in mineralization in the kidney of F1 female rats. **Reproductive performance** Reduced number of litters/pair, mostly caused by reduced mating performance at the high dose (5000 ppm). Increased gestational length suggested in high dose F0a and significantly in F0b females (1.2 days) compared to control group. Dose-dependent reductions in total and live litter size in F0 and F1 pairings. **Reproductive organ toxicity** Males: No effect on absolute testis weight observed. Histopathological findings included a dose dependent increase in testicular germ cell degeneration in F0 males and a significant increase in spermatid retention at 5000 ppm. Reduced weights of the epididymis (absolute), dorsolateral prostate (absolute and relative in F0), ventral prostate (absolute and relative) and seminal vesicles (absolute and relative in F0) were reported. Histopathological findings in secondary reproductive organs included a significant increase in exfoliated germ cells in the epididymis in the 5000 ppm group (F0) and dose-dependent increases in ventral lobe atrophy (minimal-mild) in F0 and F1 males in the 750 and 2500 ppm groups with increased severity in the 5000 ppm group (F0). Sperm parameters: significantly reduced sperm count in 5000 ppm group. Dose-dependent reductions in % motile sperm were observed in F0 and F1c males. Females: Ovarian weights (absolute and relative) were significantly redued in the 5000 ppm dose group and some changes in follicular counts, extended diestrus and oestrus cycle length were reported. **Puberty**: Adjusted age at vaginal opening (VO) and balanopreputial separation (BPS) were significantly increased in alle exposure groups (750 and 2500 ppm, F1c). **Markers of sexual development**: No significant changes in anogenital distance (AGD) (body weight adjusted) was observed. Slight, but significant number of male pups showing areola/nipples in the 2500 ppm group (4.92% in F1c and 4.35% in F2c). Dose-dependent trend in delayed testis descent in F1 and F2.	
14-week feed	4-	**Mortality**:	NTP,

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
study in rats and mice; GLP compliant Similar to OECD TG 408 **Rat** (Fischer 344) 10 rats /sex/dose in core study groups Reliability 1	Methylimidazole (CAS No. 822-36-6) Purity: 99.0 ± 0.1% Dietary, 14 weeks Dietary concentrations: 0, 625, 1250, 2500, 5000, or 10000 ppm Mean delivered daily doses: 0, 40, 80, 160, 300, or 560 mg/kg bw/day 4-methylimidazole to males and females.	**80 mg/kg bw/day dose group**: One female rat was killed moribund during week 9 **560 mg/kg bw/day dose group**: One male rat died during week 1 **Clinical findings**: **160 mg/kg bw/day dose group**: Nasal and eye discharge in males and females **300 or 560 mg/kg bw/day dose group:** Abnormal breathing, nasal and eye discharge, ruffled fur, tremors, and ataxia were observed in males and females **Food consumption:** Reduced food intake (statistical non-significant) in the 300 or 560 mg/kg bw/day dose groups was observed. **Body weight:** Final mean bw and bw gains of males from 160, 300 and 560 mg/kg bw/day dose groups, and females from 300 and 560 mg/kg bw/day dose groups were statisticaly significantly lower than those of the controls. Final body weight relative to controls (%): • Males (95, 85 and 70 % of controls, in the 160, 300 and 560 mg/kg bw/day dose group) • Females (94 and 63 % of controls, in the 300 and 560 mg/kg bw/day dose group) **Reproductive organ toxicity** <u>Males</u> **80 mg/kg bw/day dose group**: No effects on absolute testis weight were reported. The spermatid heads per testis and mean spermatid count were significantly higher than in the control group. No similar increase was observed in the higher dose groups. However, a somewhat higher number of spermatid heads/g testis was was suggested in exposed groups (non-significant). **300 and 560 mg/kg bw/day dose group**: the absolute weight of the right testis was significantly lower than the controls, and the left absolute testis weight was significnalty lower than the controls for 300 mg/kg bw/day dose group. No data reported for 560 mg/kg bw/day dose group left absolute testis weight. **560 mg/kg bw/day dose group**: the relative weight of the right testis was significantly lower than the controls. The relative weight of the left testis was not reported. **300 mg/kg bw/day dose group**: Left epididymis and cauda epididymis weight were also significantly lower than the controls. The left epididymis and cauda epididymis weight were not reported for 40 and 560 mg/kg bw/day dose groups. **Gross Pathology**: small testis and small uteri in the 560 mg/kg bw/day dose group male and female rats reported (uterus weights	2004 Chan *et al*., 2006

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	Test substance, dose levels duration of exposure	Results	Reference
		were not recorded). **Histopathology:** Testicular degeneration: Significantly increase in the incidence of animals with testicular degeneration in the 300 and 560 mg/kg bw/day groups. Number of animal with testicular degeneration: 1, 1, 0, 4, 9** and 9** in the 0, 40, 80, 160, 300 and 560 mg/kg bw/day group, respectively. Severity was graded as minimal-moderate. Prostate gland atrophy: Significantly increase in the incidence of animals with prostate gland atrophy in the 300 and 560 mg/kg bw/day groups. Number of animal with prostate gland atrophy: 0, 1, 1, 2, 8** and 8** in the 0, 40, 80, 160, 300 and 560 mg/kg bw/day groups, respectively. Severity was graded as minimal. The incidences of epididymal hypospermia and prostate gland inflammation were significantly increased in the 560 mg/kg dose group (Epididymal hypospermia: 9/10 males compared to 0/10 in the control group. Prostate gland inflammation: 8/10 of the 560 mg/kg dose group and 2/10 of the control group). Sperm parameters: **80 mg/kg bw/day dose group**: The epididymal spermatozoal motility was significantly lower than the controls. Only 1 animal analysed in the 300 mg/kg bw/day group. **80 and 300 mg/kg bw/day dose group**: The epididymal spermatozoal concentrations were significantly higher than the controls. Females: No significant differences occurred in vaginal cytology parameters between exposed and control females. A slight, but non-significant increase in estrous cycle length was suggested in the 300 mg/kg bw/day group. No data for the 560 mg/kg bw/day group was presented.	
14-week feed study on rats and mice; GLP compliant Similar to OECD TG 408 **Mouse** (B6C3F1) 10 mice /sex/dose in core study groups Reliability 1	4-Methylimidazole (CAS No. 822-36-6) Purity: 99.0 ± 0.1% Dietary, 14 weeks Dietary concentrations: 0, 625, 1250, 2500, 5000, or 10000 ppm	**Mortality:** **3180 mg/kg bw/day dose group**: seven female mice were found dead during weeks 1, 2, and 3. **1840 mg/kg bw/day dose group**: One male mouse found dead during week 4 **Clinical findings:** clinical findings included ruffled fur and dull coats in the 3180 mg/kg bw/day dose group females. **Food consumption:** No significant effects were observed **Body weight:** The final mean body weights and body weight gains of males exposed to 240, 440, 915, or 1840 mg/kg bw/day dose group and all exposed groups of females were significantly lower than the control group. **Reproductive organ toxicity:** The absolute weight of the right and left testes in the 1840 mg/kg bw/day dose group was significantly lower than in the control group.	NTP, 2004 Chan *et al*., 2006

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	Test substance, dose levels duration of exposure	Results	Reference
	Mean delivered daily doses: Males: 0, 100, 240, 440, 915, or 1840 mg/kg bw/day Females: 0, 110, 250, 540, 1130, or 3180 mg/kg bw/day	No significant effects on spermatie counts or spermatide retention was observed. Left epididymis weight of the 1840 mg/kg bw/day dose group were significantly lower than control group. Weight of the right epididymis was not reported. No exposure-related gross or microscopic lesions were identified in male mice. No significant differences reported in sperm motility or sperm concentration or vaginal cytology parameters between exposed and control groups.	
Supportive MoA study (effects of imidazoles on testosterone secretion). Rat Sprague-Dawley, males 10 rats/group Reliability 2	Single dose (subcutaneous injection) exposures ranging between 10-300 mg/kg bw. 4-methylimidazole and related compounds. Purity of chemical was not stated	Subcutaneous 4-methylimidazole injections into 60 day old rats resulted in concentration dependent decreased testicular interstitial fluid (TIF) formation, decreased TIF and serum testosterone levels, as well as decreased serum luteinizing hormone (LH) secretion after 2 h at higher doses. At 50 mg/kg bw, 4-methylimidazole injection lead to immediately decreases in TIF volume, TIF and serum testosterone levels, and decreased serum LH after 4 h (not ot other time points). Study includes groups with co-exposures to several testicular stimulants including hCG. A direct effect of 4-methylimidazole on testicular testosterone secretion was shown.	Adams, 1998

10.10.1.1 Rat reproductive and developmental continuous breeding (RACB) study

The study design of the NTP reproductive assessment by continuous breeding protocol is outlined in Fig.1 below. The protocol is further specified by NTP (https://ntp.niehs.nih.gov/ntp/test_info/finalntp_reprospecsmay2011_508.pdf)

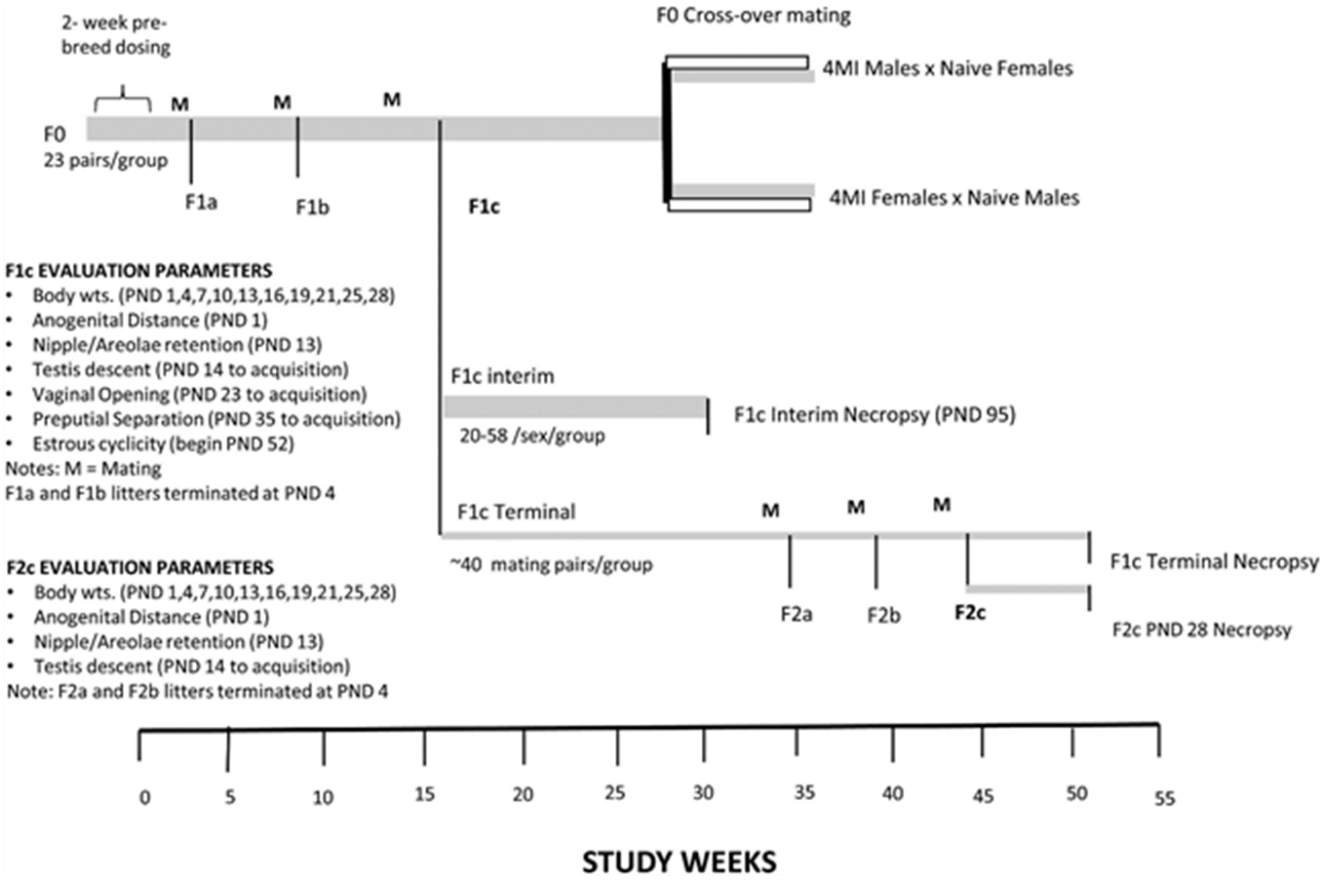


Fig. 1. Schematic of the Reproductive Assessment by Continuous Breeding (RACB) Design. From Behl *et al.*, 2020. Rats were administered 4-MI in the diet at 0, 750, 2500, or 5000 ppm ad libitum. The F0 adults were exposed during a 2-week prebreed exposure period, during cohabitation, gestation and lactation for the F1a, F1b, and F1c generations until necropsy. The F1c generation was exposed throughout life either indirectly via the mother during gestation and lactation and then by direct exposure to the dosed feed beginning on PND 28 until necropsy. The F2c generation was exposed to 4-MI via the mother during gestation and lactation. Reproductive and developmental performance was measured as described in the text. Additionally, a crossover mating was performed on the F0s following generation of the F1c to determine affected sex; during crossover mating, the 4-MI treated animals were crossed with naïve animals of the opposite sex.

The F0 5000 ppm group displayed a marked decrease in percent mated females/pair and reduced percent littered/pair relative to control. No reduction in reproductive performance was observed at lower doses (Table 16). Cross-over matings showed a reduction in mated/pair in the 5000 ppm exposed males mating with naïve females. All females that did not deliver were found to be non-pregnant indicating an effect on fertility in males. Potential effects on female fertility could not be assessed at the 5000 ppm dose in the cross-over study due to moribundity associated with parturition. There was no evidence of an exposure-related effect on reproductive performance in 2500 ppm group of animals in the cross-over study.

Table 16. Summary of reproductive performance in rat RACB study. Average values of the three pairings pairs and including crossover mating of exposed F0 males or F0 females with naïve partners.

Dose (ppm)	0	750	2500	5000
N (# of pairings)				
F0 (three pairings)	68	69	62	44
F1c terminal (three pairings)	118	126	111	-

Dose (ppm)	0	750	2500	5000
Mated/Pair (average three pairings)				
F0	97.0 %	98.6 %	96.8 %	48.1 % [a]
F1c	92.4 %	97.0 %	94.0 %	-
F0 (male x naïve female) [b]	87.0%**	–	80.0 %	33.3 %**
F0 (naïve male x female) [b]	68.2%	–	73.7 %	-
Littered/Mated (Average three pairings)				
F0	90.8%	98.6 %	86.2 %	75 % [a]
F1c	87.9 %	86.9 %	83.7 %	–
F0 (male x naïve female)	80.0 %	-	93.8 %	85.7 %
F0 (naïve male x female)	80.0 %	–	71.4 %	–
Number of Litters/Pair				
F0	2.6±0.2	2.9±0.1	2.4±0.2	0.1±0.1
F1c	2.5±0.1	2.5±0.1	2.3±0.1	-

[a] Paired only two times (A and B); removed from study prior to third pairing (C).
*p<0.05; ** p<0.01 for trend analysis and pair wise testing. Trend results appear in the 0 ppm column.

Total litter sizes were significantly reduced in the 2500 (F0 and F1c) and 5000 (F0) ppm groups and live litter size showed a dose dependent significant reducetion in the F1c group with a similar tendency observed for the F0 group (Table 17).

Table 17. Summary of live litter size and pup survival across pairings in rat RACB study.

Dose (ppm)	0	750	2500	5000
Average live litter size/pair				
F0	13.2±0.4	12.6±0.3	9.2±0.6	2.0±1.0
F1c	11.4±0.4**	10.1±0.5**	8.3±0.6	-
Survival ratio (PND1-PND4), each pair				
F0 - A	0.96±0.02	0.98±0.01	0.91±0.04	0.10±0.10
B	0.97±0.02	0.98±0.01	0.93±0.03	0.67[a)]
C	0.95±0.03	0.98±0.01	0.93±0.04	–
F1c – A	0.92±0.05**	0.93±0.04	0.72±0.08**	-
B	0.97±0.01*	0.97±0.01	0.91±0.03*	-
C	0.89±0.05	0.92±0.05	0.88±0.04	-
Survival ratio PND5-28, average				
F0	0.98±0.01	0.96±0.02	0.95±0.02	–
F1c	0.83±0.07	0.93±0.02	0.89±0.03	-

* p<0.05; ** p<0.01 for trend analysis and pair wise testing. Trend results appear in the 0 ppm column.
Testing for trend and pairwise differences was not performed for sample sizes of 1 or 2. * p<0.05, ** p<0.01. [a)] n = 1 litter.

Reproductive organ toxicity (Table 18):

No significant change in testis absolute weights were observed. Absolute epididymis weights were reduced in the 2500 and 5000 ppm groups of F0 and F1c males, whereas a slight increase in relative epididymis weights was suggested. Histopathological examinations showed testicular degeneration and testicular spermatid retention that was significant in the 5000 ppm group. The incidence of exfoliated germ cells in the epididymis was significantly increased in the F0 animals of the 5000 ppm group.

Absolute prostate and seminal vesicle weights were dose-dependently reduced in 4-methylimidazole exposed males. The relative prostate and seminal vesicle weights showed a similar pattern, but the findings were not significant for all doses/time-points for F1c animals. Histopathology revealed prostate gland atrophy of the ventral lobes in the F0 and F1 generations of treated animals. Prostate atrophy was generally of minimal to mild severity except in the 5000 ppm group, in which the lesions were generally of mild to moderate severity. Furthermore, absolute levator ani bulbocavernosus muscle complex (LABC) weights were significantly decreased in the F0 2500 and 5000 ppm groups compared to controls with decreasing trends observed also in the F1c generation. However, relative LABC weights were not significant difference from controls.

Female F0 rats showed a statistically significant decrease in ovarian weights (absolute and relative) in the 5000 ppm group. Lower absolute ovarian weights were also suggested in treated F1c interim and terminal rats.

Table 18. Summary of reproductive organ weights for male rats in RACB study:

Dose (ppm)	0	750	2500	5000
N (# of animals evaluated)				
F0	21-23	22-23	19-20	19-21
F1c interim	48-49	55-56	20	-
F1c terminal	40	44	39	-
Necropsy weight (g)				
F0	504.5±4.4**	477.5±5.9**	459.2±7.5**	455.2±5.5**
F1c interim	395.0±5.7**	383.4±4.9	338.9±4.4**	-
F1c terminal	497.3±7.7**	486.8±6.7	443.8±10.3**	-
Testis (g) (absolute)				
Right				
F0	2.089±0.023	2.089±0.030	2.086±0.028	2.066±0.029
F1c interim	1.928±0.029	1.928±0.023	1.897±0.047	-
F1c terminal	2.095±0.036	2.121±0.026	2.182±0.049	-
Left				
F0	2.075±0.026	2.074±0.026	2.096±0.026	2.062±0.029
F1c interim	1.931±0.029	1.911±0.023	1.884±0.038	-
F1c terminal	2.090±0.038	2.103±0.030	2.150±0.049	-
Epididymis (mg) (absolute)				
Right				
F0	681±9**	686±10	628±9**	600±9**
F1c interim	571±9**	564±8	513±12**	-
F1c terminal	697±9**	697±9	651±11**	-
Left				
F0	704±9**	718±13	657±11**	635±10**
F1c interim	579±10**	561±7	521±12**	-
F1c terminal	697±13**	701±9	648±12**	-
Epididymis (mg/g) (relative)				
Right	1.35±0.02	1.44±0.03*	1.37±0.03	1.32±0.02
F0	1.45±0.02	1.47±0.02	1.52±0.05	-
F1c interim	1.41±0.02*	1.44±0.02	1.47±0.02	-

Dose (ppm)	0	750	2500	5000
F1c terminal				
Left				
F0 F1c interim F1c terminal	1.40±0.02 1.47±0.02* 1.41±0.02	1.51±0.04* 1.47±0.01 1.45±0.02	1.44±0.03 1.54±0.04* 1.47±0.02	1.40±0.02 - -
Dorsolateral Prostate (mg) (absolute)				
F0 F1c interim F1c terminal	604±26** 402±11** 539±16**	492±23** 382±12 475±19*	469±15** 330±13** 449±19**	421±22** - -
Dorsolateral Prostate (mg/g) (relative)				
F0 F1c interim F1c terminal	1.20±0.05** 1.02±0.03 1.08±0.03	1.03±0.05** 1.00±0.03 0.98±0.04	1.02±0.03** 0.97±0.03 1.01±0.03	0.93±0.05** - -
Ventral Prostate (mg) (absolute)				
F0 F1c interim F1c terminal	935±28** 561±20** 825±22**	785±21** 446±14** 796±27	748±29** 355±21** 591±18**	515±27** - -
Ventral Prostate (mg/g) (relative)				
F0 F1c interim F1c terminal	1.85±0.05** 1.42±0.05** 1.67±0.05**	1.65±0.05** 1.16±0.03** 1.64±0.06	1.63±0.06** 1.05±0.06** 1.34±0.05**	1.13±0.06** - -
Seminal Vesicle (g) (absolute)				
F0 F1c interim F1c terminal	1.946±0.053** 1.304±0.032** 1.76±0.04**	1.647±0.045** 1.186±0.028* 1.69±0.04	1.520±0.045** 1.016±0.035** 1.46±0.05**	1.253±0.042** - -
Seminal Vesicle (mg/g) (relative)				
F0 F1c interim F1c terminal	3.87±0.10** 3.31±0.06* 3.56±0.08	3.44±0.11** 3.10±0.07 3.48±0.09	3.29±0.08** 3.00±0.09* 3.29±0.10	2.75±0.10** - -
LABC (g) (absolute)				
F0 F1c interim F1c terminal	1.438±0.028** 1.161±0.027* 1.341±0.029*	1.369±0.023 1.095±0.024 1.301±0.034	1.265±0.034** 1.052±0.035 1.222±0.041	1.236±0.022** - -
LABC (mg/g) (relative)				
F0 F1c interim F1c terminal	2.85±0.06 2.94±0.05 2.70±0.05	2.87±0.05 2.86±0.06 2.68±0.06	2.76±0.06 3.11±0.11 2.75±0.07	2.72±0.06 - -

*p<0.05; ** p<0.01 for trend analysis and pair wise testing. Trend results appear in the 0 ppm column.

Sperm and ovarian parameters (Table 19):

F0 rats exposed to 5000 ppm 4-methylimidazole displayed significantly reduced cauda epididymal sperm count and reduced % motile sperm compared to controls. Sperm/g cauda was decreased dose-dependently in the F0 and F1-interim groups, but the tendency did not reach significance for the F1 terminal group. There was

a significant trend toward a reduction in % motile sperm in the F0 and F1 terminal groups. Spermatid counts in the testis were not significantly affected by exposure in the F0 and F1c generations.

There were increases in primordial (77%), antral (82%) and atretic follicles (61%) in the F0 5000 ppm group of females, with a significant trend across doses. In addition, a significant increase in atretic follicles in the 750 ppm F0 group was reported. For the F1 animals, only data for the control and 2500 ppm groups are reported. An increase in primordial follicles (29%) is suggested also for the F1 animals, but the finding was not significant. No increase in atretic follicles was observed in the F1 animals at the 2500 ppm dose.

Table 19. Summary of sperm analysis and esterous cycling in rat RACB study

Dose (ppm)	0	750	2500	5000
Sperm/Cauda (10^6) F0 F1-interim F1-terminal	 180.6±8.2** 187.8±8.9 196.7±9.0	 206.6±7.9 168.8±6.5 190.2±7.1	 167.0±11.3 153.8±11.4 176.0±10.6	 135.1±7.3** - -
Concentration (10^6)/g cauda epididymal tissue) F0 F1-interim F1-terminal	 682.4±29.7* 856.4±26.1 759.6±27.3	 752.7±21.2 780.1±25.8* 723.0±26.2	 676.5±38.3 754.4±40.8* 721.2±35.0	 589.5±28.5 - -
% Motile sperm F0 F1-interim F1-terminal	 83.3 ± 2.1** 68.9 ± 1.8 80.1 ± 1.5**	 80.1 ± 1.6 68.7 ± 2.0 77.4 ± 1.2	 76.2 ± 1.8** 61.9 ± 1.2** 71.7 ± 2.9	 71.9 ± 2.5** - -
Oestrous cycle length (days) F0 F1c	 5.3 ± 0.21 5.0 ± 0.23	 5.4 ± 0.21 4.8±0.06	 5.9 ± 0.43 5.1±0.07*	 5.8 ± 0.68 -

*p<0.05; ** p<0.01 for trend analysis and pair wise testing. Trend results appear in the 0 ppm column.

Evaluation of developmental toxicity (Table 20):

No developmental study is available to DS, but some information may be derived from the RABC study. However, the distinction between fertility effects and developmental effects in this study are not necessarily clear-cut. The decrease in live litter size appears to a large degree to be related to a reduction in total litter size. However, whether the reduced litter size is related to pre- or postimplantation losses were not examined. Pup survival from PND1 to PND4 was reduced in F0 high dose group (5000 ppm) and in F1c in the 2500 ppm groups. Survival from PND5 to PND28 did not show significant exposure-related effects in the F0 and F1c 750 and 2500 groups relative to controls.

No consistent pattern of change was observed in male or female pup anogenital distance (AGD) or body weight adjusted AGD across litters. However, a small number of pups were observed to have areolae or nipples in the F1c and F2c generations in the 2500 ppm group. Furthermore, there was a significant trend toward delayed day of testicular descent in the F1 and F2 generation, and the delay in the 2500 ppm F2 males was significant by pairwise comparison to the controls.

Marked, dose-dependent delays in puberty (balanopreputial separation (BPS) and vaginal opening (VO)), were observed in male and female F1 offspring in the 750 and 2500 ppm groups relative to controls. These delayes remained significant after adjustment for body weight at weaning.

Table 20. Summary of effects on sexual developmental in the rat RACB study

Dose (ppm)	0	750	2500
No. examined Males (no. of litters)			
F1c	99 (18)	115 (22)	61 (15)
F2c	108 (25)	133 (32)	69 (20)
Pups with areolae/nipples (%)			
F1c	0 (0)*	0 (0)	3 (4.92)
F2c	0 (0)	0 (0)	3 (4.35)
Litters with areolae/nipples (%)			
F1c	0 (0)	0 (0)	2 (13.33)
F2c	0 (0)	0 (0)	1 (5.00)
Day of testis descent			
F1c	16.7 ± 0.2*	16.8 ± 0.2	17.1 ± 0.2
F2c [a)]	18.1 ± 0.3**	18.5 ± 0.4	19.4 ± 0.4*
F1c Examined, Males (litters)	89 (18)	100 (22)	60 (15)
Age at BPS (PND)	43.5 ± 0.4**	46.2 ± 0.4**	47.2 ± 0.6**
Adjusted age at BPS [b)]	44.3 ± 0.3*	46.4 ± 0.4**	46.4 ± 0.5*
F1c Examined, Females (litters)	96 (19)	111 (22)	67 (15)
Age at VO (PND)	33.8 ± 0.2**	37.2 ± 0.3**	39.4 ± 0.3**
Adjusted age at VO [b]	34.1 ± 0.2**	37.2 ± 0.3**	39.0 ± 0.3**

VO and BPS: Means of litter means for age at attainment are presented.
* $p<0.05$; ** $p<0.01$ for trend analysis and pair wise testing. Trend results appear in the 0 ppm column.
[a)] Number of animals (litters) examined for testicular descent was 107 (25), 132 (32), and 68 (20) respectively.
[b)] Means of adjusted age at BPS and VO were calculated as the mean of the litter means of the weaning weight-adjusted attainment age for individual pups.

10.10.1.2 **Rat 14 week repeated** dose toxicity study:

Indications of an adverse effect on fertility in rats exposed to 4-methylimidazole is supported by findings in the **NTP rat 14 week dietary repeated dose toxicity study**. In this study, absolute testis weights in the 300 mg/kg bw/day (5000 ppm) and 560 mg/kg bw/day dose groups were significantly lower than in controls (right testis: ~11% and left testis: ~15% less as compared to the controls; $p<0.01$). The absolute and the relative right testis weights of 560 mg/kg bw/day dose group were significantly lower than the controls (absolute: ~ 64% and relative: ~ 49% less as compared to the controls; $p<0.01$). The relative weight of the left testis was not reported. Further, the left epididymis and cauda epididymis weights were significantly lower than the controls (left epididymis: ~ 14% less as compared to the controls; $p<0.01$) in the 300 mg/kg bw/day dose group.

The incidences of epididymal hypospermia and prostate gland inflammation were significantly increased in 560 mg/kg bw/day dose group. The incidences of prostate gland atrophy was significantly increased in 300 and 560 mg/kg bw/day dose group. The number of animals with prostate gland atrophy were: 0, 1, 1, 2, 8** and 8** in the 0, 40, 80, 160, 300 and 560 mg/kg dose group, respectively. The incidences of testicular degeneration were significantly increased in 300 and 560 mg/kg bw/day dose group males. The number of animals with testicular degeneration: 1, 1, 0, 4, 9** and 9** in the 0, 40, 80, 160, 300 and 560 mg/kg dose group, respectively. The observed dose-dependent increase in the testicular degeneration and prostate gland atrophy was not considered to be explained by reduced body weight.

Table 21. Summary of reproductive organ weights for male rats in the 14 week study

Dose (mg/kg bw/day)	0	40	80	160	300	560
N (# of animals evaluated)	8	8	8	8	8	7
Necropsy weight (g)	352 ± 6	362 ± 8	353 ± 6	335 ± 4*	298 ± 4**	245 ± 4**
Testis (g) (absolute)[a)]						
Right	1.436	1.477	1.501	1.461	1.275**	0.511**
Left	1.51	-	1.561	1.480	1.291**	-
Testis (relative)						
Right	4.10	4.11	4.28	4.42	4.32	2.10**
Left [b)]	-	-	-	-	-	-
L epididymis (g)[c)]	0.508	-	0.524	0.511	0.438**	-
L cauda epididymis (g)[d)]	0.187	-	0.176	0.174	0.154**	-

*Significantly different (P< 0.05) from the control group by Williams' test
** Significantly different (P <0.01) from the control group by Williams' or Dunnett's test.
[a)] *Left absolute testis weight for 40 and 560 mg/kg bw/day dose groups were not reported.*
[b)] *Left relative testis weights were not reported.*
[c)] *Right epididymis for all dose groups and left epididymis for 40 and 560 mg/kg bw/day dose groups were not reported.*
[d)] *Right cauda epididymis for all dose groups and left cauda epididymis for 40 and 560 mg/kg bw/day dose groups were not reported.*

Sperm Motility and Vaginal Cytology Evaluations**:**
At the end of the studies spermatid heads per testis and per gram testis, spermatid count, and epididymal spermatozoal motility and concentration were evaluated. The left cauda, left epididymis, and left testis were also weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies. Oestrous cycle length and the percentage of time spent in the various oestrous stages were measured.

Table 22. Summary of sperm analysis and oesterous cycling in the rat 14 week study

Dose (mg/kg bw/day)	0	80	160	300
Spermatid measurements				
Spermatid heads (10^7 /g testis)	9.17 ± 0.24	9.81 ± 0.22	9.72 ± 0.34	9.97 ± 0.48
Spermatid heads (10^7 /testis)	13.78 ± 0.24	15.30 ± 0.40*	14.38 ± 0.50	12.81 ± 0.55
Spermatid counts (mean/ 10^{-4} mL suspension)	68.91 ± 1.18	76.50 ± 2.02*	71.88 ± 2.51	64.03 ± 2.76

Dose (mg/kg bw/day)	0	80	160	300
Epididymal spermatozoal measurements				
Motility (%)	91.34 ± 0.22	90.56 ± 0.21*	90.63 ± 0.20	90.00[a)]
Concentration (10^6/g cauda epididymal tissue)	406 ± 19	498 ± 41*	477 ± 21	504 ± 22*
Estrous cycle length (days)	4.70 ± 0.15	5.14 ± 0.24[b)]	5.40 ± 0.34	5.38 ± 0.24[c)]

*Significantly different (P< 0.05) from the control group by Dunn's test
[a)] *n=1*
[b)] *Oestrous cycle was longer than 12 days or unclear in 2 of 9 animals*
[c)] *Oestrous cycle was longer than 12 days or unclear in 6 of 10 animals*

In the 80 mg/kg bw/day dose group, the spermatid heads per testis and mean spermatid count were significantly higher than in the control group, while the epididymal spermatozoal motility was significantly lower than the controls. The epididymal spermatozoal concentrations of 80 and 300 mg/kg bw/day dose group were significantly higher than the controls.

Mean length of the oestrous cycle of 80, 160 and 300 mg/kg bw/day dose group were longer compared to the control group. However, the differences were not statistically significant. Further, there was no statistically significant difference in the time spent in each stage of the oestrous cycle between exposed and control groups.

Mouse 14 week repeated dose toxicity study:

The relative right testis weights of males exposed to 440, 915, or 1840 mg/kg bw/day of 4-methylimidazole were significantly higher than the control group. The absolute testis weight of the 1840 mg/kg bw/day dose group was significantly lower than control group. Absolute epididymis weight of the 1840 mg/kg bw/day dose group was significantly lower than control group. In contrast to in the rat study, no statistically significant effect on sperm count, sperm motility and oesterous cyling was recorded in the 14 week repeated dose toxicity study in mice (NTP, 2004) at dose levels up to ~1800 mg/kg bw/day.

Table 23. Summary of reproductive organ weights for male mice in the 14 week study

Dose (mg/kg bw/day)	0	100	240	440	915	1840
N (# of animals evaluated)	10	10	10	10	10	9
Necropsy body weight	35.3 ± 0.6	33.6 ± 0.9	32.6 ± 1.1*	31.8 ± 0.4**	29.6 ± 0.5**	28.0 ± 0.3**
Testis (g) (absolute)						
Right	0.124 ± 0.003	0.116 ± 0.003	0.121 ± 0.003	0.126 ± 0.002	0.120 ± 0.002	0.113 ± 0.002*
Left	0.1181 ± 0.0016	-	-	0.1198 ± 0.0018	0.1163 ± 0.0023	0.1077 ± 0.0020**
Testis (relative)						

Dose (mg/kg bw/day)	0	100	240	440	915	1840
Right	3.51 ± 0.11	3.47 ± 0.12	3.72 ± 0.09	3.95 ± 0.05**	4.05 ± 0.07**	4.02 ± 0.09**
Left [a)]	-	-	-	-	-	-
L epididymis (g)	0.0515 ± 0.0018	-	-	0.0476 ± 0.0009	0.0487 ± 0.0015	0.0439 ± 0.0019**
L cauda epididymis (g)	0.0176 ± 0.0008	-	-	0.0173 ± 0.0005	0.0176 ± 0.0005	0.0152 ± 0.0010

*Significantly different (P< 0.05) from the control group by Williams' test
** Significantly different (P <0.01) from the control group by Williams' or Dunnett's test
[a)] Left relative testis weight were not reported.

Table 24. Summary of sperm analysis in male mice in the 14 week study

Dose (mg/kg bw/day)	0	440	915	1840
Spermatid measurements				
Spermatid heads (10^7 /g testis)	16.89 ± 0.47	15.67 ± 0.50	16.25 ± 0.61	17.14 ± 0.70
Spermatid heads (10^7 /testis)	2.00 ± 0.07	1.88 ± 0.06	1.89 ± 0.06	1.84 ± 0.07
Spermatid counts (mean/ 10^{-4} mL suspension)	62.43 ± 2.31	58.60 ± 1.84	58.88 ± 1.87	57.58 ± 2.27
Epididymal spermatozoal measurements				
Motility (%)	90.36 ± 0.24	90.55 ± 0.34	90.00 ± 0.40	89.60 ± 0.27
Concentration (10^6/g cauda epididymal tissue)	894 ± 44	957 ± 49	899 ± 23	1007 ± 55

Table 25. Summary of oesterous cycling in female mice in the 14 week study

Dose (mg/kg bw/day)	0	250	540	1130
Oestrous cycle length (days)	4.60 ± 0.49	4.28 ± 0.12[a)]	4.55 ± 0.50	4.75 ± 0.27

[a)] Oestrous cycle was longer than 12 days in 1 of 10 animals

No human data on reproductive toxicity of 4-methylimidazole was found by the DS

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Data relevant for the evaluation of reproductive toxicity of 4-methylimidazole has been reported in two NTP studies; a 14 week repeated dose toxicity study in rats and mice and a multigeneration study in rats following the NTP continuous breeding protocol. The data from both studies is publicly available.

Rat multigeneration study

4-methylimidazole induced reproductive toxicity in rats in the multigenerational reproductive and developmental toxicity study following the NTP continuous breeding protocol (RACB). Markedly reduced fertility at the high dose (5000 ppm) and perturbed parturition in females in the 2500 and 5000 ppm dose groups was observed and the high dose was discontinued. The cross-over matings showed that male fertility was affected at the high dose. No high dose exposed females were included in the cross-over study due to moribundity associated with parturition. Total litter size was significantly and dose-dependently reduced in both F0 and F1 matings. Live litter size showed similar trends and was significant descreased in the F1 generation. Disturbed sexual development was reported in F1 and F2 generations as reflected by a modest increase in male pups with retained nipples at the high dose (2500 ppm) and delayed testicular descent (2500 ppm). In the F1 generation a clear dose-dependent delay in onset of puberty in both males (balanopreputial separation, BPS) and females (vaginal opening, VO) rats was observed.

Absolute testis weight was not affected by treatment. However, examination of testis pathology showed a dose-dependent increase in degeneration of the germinal epithelium of the testis (minimal-mild) in F0 males and a significant increase in spermatid retention in the 5000 ppm group. Markedly reduced absolute and relative weights of male secondary reproductive organs was reported at all exposure doses in F0 males with similar tendencies observed in the F1 animals. A significant increase in exfoliated germ cells in the epididymis in the 5000 ppm group and dose-dependent increases in ventral lobe atrophy (minimal-mild) in F0 and F1 males in the 750 and 2500 ppm groups and mild to moderate atrophy in the 5000 ppm group (F0) was reported. Furthermore, significantly reduced sperm counts in the 5000 ppm group and dose-dependent reductions in sperm mobility were observed in F0 and F1 males.

In females, the weights of the left and right ovaries (absolute and relative) were significantly redued in the 5000 ppm dose group, but were mostly not affected by treatment in the 750 and 2500 ppm groups. In addition, dose-dependent increases in the number of primordial ovarian follicles with increasing dose of 4-methylimidazole was reported in F0 animals and the findings seemed to be supported in the F1 females. Changes in other follicle classes appeared less consistent across doses and/or generations. Furthermore, oestrus cycle length appeard to be increased with higher doses of 4-methylimidazole in the F0 females.

In conclusion, evidence of male reproductive toxicity was observed at all doses of 4-methylimidazole and severity generally increased with increasing doses, but appears similar across generations. Reproductive toxicity in female rats was evident at the high dose (5000 ppm). The most sensitive endpoint in female rats was vaginal opening that was significant delayed at all exposure doses.

Toxicity in dams was high in the 5000 ppm group and appeared associated with disturbed parturition and dystocia. In the 750 and 2500 ppm groups, toxicity of parental animals appeared to be modest and mostly reflected by reductions in body weight. Reproductive and developmental toxities observed does thus not appear to be secondary to general toxicity.

14 week repeated dose toxicity study in rats and mice

Additional data on toxicity to reproductive organs in rats and mice can be derived from the NTP 14 week dietary study.

Among the main finding supporting an adverse effect of 4-methylimidazole on fertility in male rats is the reductions in absolute testis weights and increase in testicular degeneration in the 300 mg/kg bw/day (5000 ppm) and 560 mg/kg bw/day (10 000 ppm) dose groups compared to the controls. An increase in spermatide

retention was also suggested. In addition, the incidence of epididymal hypospermia was significantly increased in the 560 mg/kg bw/day dose group and the incidences of prostate gland atrophy was increased in 300 and 560 mg/kg bw/day dose group compared to controls.

In female rats, an increase in oestrous cycle length in exposed animals was suggested but the differences were not statistically significant. Further, there was no statistically significant difference in the time spent in each stage of the oestrous cycle between exposed and control groups.

In the mouse 14 week repeated dose toxicity study a decrease in absolute testis weight of the 1840 mg/kg bw/day dose group compared to the control group was reported. Absolute epididymis weight of the 1840 mg/kg bw/day dose group was significantly lower than control group, but no significant effects on sperm counts, sperm motility and oesterous cyling was observed.

Overall, the 14-week dietary study support the findings of reproductive organ toxicity as demonstrated in the rat multigeneration study.

Mode of action

Several lines of evidence indicates that 4-methylimidazole has anti-androgen effects in male rats. Although there was no consistent effect of 4-methylimidazole on AGD, an increase in areolas/nipple in male pups at the high dose (2500 ppm) and a dose-dependent delay of testis descent indicate perinatal androgen insuffiency. The number of pups observed to have retained nipples in the F1 and F2 generations were small. However, Behl and co-workers (Behl *et al.*, 2020) reports a historical incidence of retained nipples of 2/382 (0.5%) pups and litter incidence of 2/80 (2.5%). The incidences observed in the 4-methylimidazole study clearly exceed these numbers. Further evidence of anti-androgen effects of 4-methylimidazole stems from the reduced weight and atrophy of the androgen sensitive tissues prostate, seminal vesicles and levator ani-bulbocavernosus muscles (LABC). Furthermore, delayed puberty and impaired spermatogenesis support an anti-androgen mode of action. Organ weight decrease of the seminal vesicle and/or prostate is a sensitive indicator of low testosterone or anti-androgenic activity. Female rats also showed a dose-dependent delay of puberty supporting an impairment also of female steroidogenesis or steroid activity. In addition, dystocia in dams at higher doses was observed indicating disruption of the endocrine signalling regulating parturition.

In a non-guideline study with single injection in rats, 4-methylimidazole caused decreases in testosterone secretion and testicular interstitial fluid formation in a dose-dependent manner. Decreased serum luteinizing hormone (LH) was also reported (Adams *et al*., 1998). 4-methylimidazole has been shown to inhibit CYP enzyme activities and reduction of specific CYP activities may contribute to impaired testosterone and oestrogen syntehesis. This argument is supported by the known effects of structurally related azole fungicides, included the well studied fungicide ketoconazole (Feldman 1986). Furthermore, ketoconazole exposure has been shown to decreases plasma testosterone concentration also in humans (Pont *et al*. 1982).

10.10.3 Comparison with the CLP criteria

As no separate developmental study is available, the assessment of sexual function and fertility and development is presented together below. Some information on developmental toxicity could be found in the RABC study where the developmental endpoints included were testes descent, vaginal opening, and preputial separation.

10.10.3.1 Sexual function and fertility and development

Classified substances may be allocated to one of two categories – 1A/B or 2. In the CLP regulation the following is stated:

Category Repr. 1A Known human reproductive toxicant: The classification of a substance in Category 1A is largely based on evidence from humans.

No human data is available, so classification in category Repr. 1A is not justified

Category Repr. 1B Presumed human reproductive toxicant: The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary nonspecific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.

The database to assess sexual function and fertility of 4-methylimidazole in mammals is considered sufficient, and consists of one NTP multigeneration reproductive toxicity study in rats and supportive information from a NTP 14-week dietary repeated dose toxicity study in rats and mice.

In the multigeneration rat RACB study, marked effects on reproductive performance was observed at the high dose (5000 ppm), a dose that was discontinued due to severely reduced fertility and maternal toxicity including perturbed parturition. At lower doses clear toxicity to male reproductive primary and secondary organs was observed. In particular, absolute and relative ventral prostate weights were consistently reduced across generations in both the 750 and 2500 ppm dose groups and supported by histopatological changes. Furthermore, a significantly delayed male and female puberty onset was observed from the lowest dose group. These data points to a likely anti-antrogenic and anti-oestogenic mode of action for 4-methylimidazole. In rats, subcutaneous injection of 4-methylimidazole (and some other related imidazoles) has been show to disturb the hypothalamic–pituitary–gonadal axis (HPG axis; Adams *et al*., 1998). Following 4-methylimidazole injections concentration dependent decreased testicular interstitial fluid (TIF) formation, as well as serum testosterone and LH levels were observed.

It is highly unlikely that the reproductive toxicity observed in the multigeneration study is secondary to general or maternal toxicity as several adverse effects was observed in a dose-dependent manner already from the lowest exposure dose (750 ppm) that did only caused mild signs of toxicy in the form of slight reductions in body weights.

The 14 week repeated dose toxicity study supports the observations of adverse effects on the male rat reproductive organs, based on increased incidences of epididymal hypospermia and prostate gland inflammation in the 560 mg/kg bw/day dose group and increased incidences of prostate gland atrophy and testicular degeneration both in the 300 and 560 mg/kg bw/day dose group. Histopathological changes in male reproductive organs was not reported for mice, but changes in testicular weights were observed at the high dose. The 14 week study also lends some support to ovarian toxicity as the length of the oestrous cycle was increased (non-significantly) in female rats.

There are no prenatal development studies available for 4-methylimidazole. However, some information on developmental toxicity can be derived from the rat multigeneration RACB study (NTP 2019; Behl *et al*., 2020). These data are described above in section 10.10.1.1. In the RACB study, a slight but significant increase in male pups with areolas/nipple retention at PND13 at the 2500 ppm dose group as well as a dose-dependent delay in testicular descent was reported. The delay in day of testicular descent was also observed at lower doses not associated with maternal toxcity. In addition, reductions in litter size and in pup survival PND1-4 in the two highest dose groups was observed. Reductions in pup body weights were ≥ 97% (750 ppm) and 89-97% (2500 ppm) at PND1, and 95% and 81% at PND28 (F1) compared to controls.

In conclusion, we propose that 4-methylimidazole is classificed in category Repr. 1B (H360), on the basis of clear effects to male and female reproductive performance, effects on primary and secondary reproductive organs as well as on delayes in timing of sexual development markers indicating sex hormone insufficiensies and developmental toxicity. Developmental toxicity is further suggested by the reduction in pup survival at PND1-4.

Classification in Repr. Cat 1B - H360Fd is therefore warranted.

10.10.4 Adverse effects on development

There was no separate developmental study available and the assessment of sexual function and fertility and development is presented together in section 10.10.1 - 10.10.3.

10.10.5Adverse effects on or via lactation

DS is not aware of any study describing levels of 4-methylimidazole in human breast milk. No experimental data that gives information on potential adverse effects of 4-methylimidazole via lactation has been found. However, survival and growth of pups between PDN5 and PND28 did not appear to be reduced indicating that lactation as such was not impaired. 4-methylimidazol shows a wide distribution in the body and transfer into milk can be assumed based on studies with domesticated animals.To what degree the lactation exposure route contributes to postnatal developmental toxicities like delayed puberty onset is difficult to evaluated based on the available data.

10.10.6 Comparison with the CLP criteria

No classification for adverse effects via lactation is proposed.

10.10.7 Conclusion on classification and labelling for reproductive toxicity

Overall conclusion based on available data:

Based on the arguments given above, DS concludes that 4-methylimidazole warrants classification as Repr. Cat 1B (H360Fd). No SCL is proposed.

10.11 Specific target organ toxicity-single exposure

Hazard class not assessed in this dossier.

10.12 Specific target organ toxicity-repeated exposure

Hazard class not assessed in this dossier. However, findings from 14 weeks study in rats and mice are described in section 10.10 Reproductive Toxicity.

10.13 Aspiration hazard

Hazard class not assessed in this dossier.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Hazard class not assessed in this dossier.

12 EVALUATION OF ADDITIONAL HAZARDS

Hazard class not assessed in this dossier.

13 ADDITIONAL LABELLING

Not relevant.

14 REFERENCES

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15 ANNEXES

Annex 1 to the CLH report