Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

4-methylimidazole

EC Number: 212-497-3
CAS Number: 822-36-6

CLH-O-0000007050-88-01/F

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

Adopted

26 November 2021

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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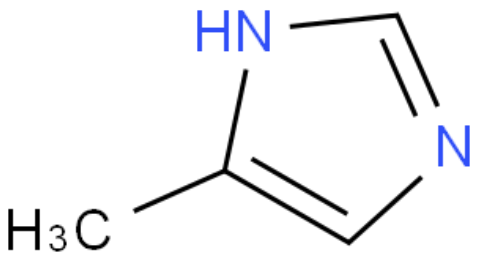
Version number: 3.0 **Date: 15.12.2020**

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.

RAC general comment
4-methylimidazole is used as an intermediate in the manufacture of chemicals and chemical products. It is also formed in heated foods by the Maillard reaction of D-glucose with ammonia.

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			

Property	Value	Reference	Comment (e.g. measured or estimated)
Flammability			
Explosive properties			
Self-ignition temperature			
Oxidising properties			
Granulometry			
Stability in organic solvents and identity of relevant degradation products			
Dissociation constant			
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	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
	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 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	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
	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 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	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
	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 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.

[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.

10.3 Acute toxicity - inhalation route

Hazard class not assessed in this dossier.

10.4 Skin corrosion/irritation

Hazard class not assessed in this dossier.

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,	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	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**
four lower doses were evaluated, down to 100 μg/plate. Deviation from OECD TG 471 by: 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	a) and by toxicity to 33 µg/plate in study b)			
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	Test material 4-methylimidazole (98 % purity).	Whole blood cells (lymphocytes) from four healthy donors (two males and two females) were used for	In 48 h treatment period 450, 600 µg/ml of 4-methylimidazole induced SCE. 4-Methylimidazole induced CA in	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
(MN) tests in human peripheral lymphocytes (non-guideline study) from 4 blood donors. CA test 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	Concentrations: 300, 450, 600 µg/ml for 24 h and 48 h periods.	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 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.	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 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
after the beginning of treatment; - Recommendation of counting at least 2000 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	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	NTP, 2007. Protocol according to Shelby *et al*., 1993.

Method, guideline, deviations if any, reliability	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
		species (n=5).	PCEs declined with increasing dose and were significantly depressed at the highest dose.	
Mouse peripheral blood micronucleus test. Food and Drug Administration Good Laboratory Practices Regulations (21 CFR, Part 58) Reliability 1	Test material 4-methylimidazole (99% purity). 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-week toxicity study (where peripheral blood for the micronucleus test were obtained in week 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 effects (no increases in 14-week peripheral blood micronucleus tests in male and female mice). No bone marrow toxicity observed.	NTP, 2007. MN peripheral blood assays 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	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	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**
abberations; - Non-recommended (i.p.) route of administration without justification; - 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		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 concentrations after 24 h,		

Method, guideline, deviations if any, reliability	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
100, 130 and 160 mg/kg bw); -No historical data available for comparison with negative and positive controls; - 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.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The dossier submitter (DS) presented three *in vitro* genotoxicity studies and three *in vivo* genotoxicity studies on the substance.

For *in vitro*, two bacterial reverse mutation assays (Ames, test, reliability 1) conducted in different *Salmonella typhimurium* strains were negative. 4-methylimidazole (up to 10000 µg/plate) was not mutagenic in *S. typhimurium* strains TA97, TA98, TA100, or TA1535, when tested with and without 10% or 30% hamster or rat liver S9 activation enzymes (NTP, 2007). In the second study, 4-methylimidazole (up to 5000 µg/plate) was not mutagenic in *S. 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. This in absence of cytotoxicity (Beevers and Adamson 2016). In the third study (Norizadeh Tazehkand *et al*., 2016) on sister chromatid exchanges, chromosomal aberrations and micronuclei induction in human primary lymphocytes a genotoxic chromosomal effect was observed, concurrently with cytotoxicity. The DS rated the study Klimisch 3, reported several deficiencies and guideline deviations and concluded that potentially the observed increased genotoxicity could be an indirect consequence of high cytotoxicity. This because 4-methylimidazole negatively affected the mitosis, the proliferation index and the nuclear division index at the genotoxic concentrations.

In vivo, two Micronucleus assays (reliability 1) were negative. In an NTP (2007a) study similar to OECD TG 475 no increases in the frequencies of micronucleated erythrocytes were seen in bone marrow of male F344/N male rats and male B6C3F1 mice was observed after *i.p.* administration three times at 24-hour intervals on three consecutive days in both species (n=5). Significant decrease in the percent of micronucleated polychromatic erythrocytes (PCEs) as indicator of bone marrow toxicity was observed only in rats. In a 14-week NTP (2007b) toxicity study on peripheral blood micronucleus induction with B6C3F1 mice exposed 7 days/week by feed no increases in peripheral blood micronuclei in male and female mice was observed, also no bone marrow toxicity. By contrast, in a chromosomal aberration test (Norizadeh Tazehkand *et al*., 2016) in the bone marrow cells of Swiss Albino mice (non-guideline study) comparable to OECD TG 475 with several deviations and considered by the DS as Klimisch 3, 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 the highest concentration for 12 h and at all concentrations for 24 h.

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).

No germ cell mutagenicity studies were 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 warranted.

Comments received during consultation

Three Member State Competent Authorities (MSCA) supported no classification. However, it was raised that there are uncertainties due to positive results on chromosomal aberrations *in vitro* and *in vivo* in Klimisch 3 studies, and inconsistent data in the NTP mouse bone marrow micronucleus test (1st trial positive, 2nd trial negative). One of the MSCAs considered no classification should be proposed based on inconclusive data or data lacking as no reliable *in vivo* negative chromosomal aberration test is available.

Assessment and comparison with the classification criteria

Three *in vitro* mutagenicity studies are available in the CLP report, two reliable Ames tests in *S. typhimurium* strains considered Klimisch 1 by the DS, and one non-guideline cytogenicity study analysing chromosomal aberration, micronuclei formation and sister chromatid exchanges in primary lymphocytes of four healthy donors, rated as Klimisch 3 by the DS.

***Table**: Overview on in vitro mutagenicity studies in bacteria and human lymphocytes*

Method, guideline, deviations if any, reliability	**Test substance & concentrations**	**Observations**
Bacterial Reverse Mutation Assay (NTP 2007). Similar to OECD TG 471. Reliability 1 Deviation: 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 TG 471.	4-methylimidazole (>99% purity) a) high dose: 0, 100, 333, 1000, 3333 and 10000 µg/plate (10000 µg/plate, exceeds the current OECD TG 471) b) lower doses: 1, 3.3, 10, 20, 33 µg/plate (limited by toxicity) +/- 10% or 30% hamster or rat liver S9 activation	4-methylimidazole (up to 10000 µg/plate) was **not mutagenic** in *S. typhimurium* strains TA97, TA98, TA100, or TA1535, +/- 10% or 30% hamster or rat liver S9.
Bacterial Reverse Mutation Assay (Beevers and Adamson, 2016). OECD TG 471 compliant. Reliability 1	4-methylimidazole (99% purity). plate incubation test: 0, 5, 15.81, 50, 158.1, 500, 1581, and 5000, 10000 µg/plate. (10000 µg/plate, exceeds the current OECD TG 471) pre-incubation test: 0, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate +/- rat 10% and mouse liver S9, to rat and mouse lung S9 activation enzymes.	4-methylimidazole (up to 5000 µg/plate) was **not mutagenic** in *S. typhimurium* strains TA98, TA1535, TA1537, TA100 and TA102. Consistent negative for +/- S9 **No cytotoxicity** was observed.

Non-guideline study (Celik & Topaktas, 2018) on chromosome aberration (comparable to OECD TG 473), micronucleus (comparable to OECD TG 487) and sister chromatid exchange (TG 479 is not a valid guideline because of a lack of understanding of the mechanism). Reliability 3 Deviations from TG 473 and TG 487: - Concentrations should cover a range producing cytotoxicity and moderate, little or no cytotoxicity; - Exposure to test chemical should include metabolic activation for 3-6 hrs, sampled at a time equivalent to about 1.5 normal cell cycle lengths; - 1000 binucleate cells were scored per concentration instead of at least 2000 for MN assessment; only 100 per donor instead of at least 300 well-spread metaphases scored per group; - Slides not blinded for scoring; - Negative control not included for the 48h treatments; - For SCE a total of 100 second metaphases per concentration (25 cells per donor) analysed.	4-methylimidazole (98% purity). concentrations: 300, 450, 600, 750 µg/mL for 24 h and 48 h. Whole blood lymphocytes (two male and two female healthy donors)	**Induced SCE** in 48 h treatment period 450, 600 µg/mL; **Induced CA** at all concentrations for 24 h and 48 h and chromatid and chromosome breakage and formation of fragments; **Induced MN** at 600 and 750 µg/mL at 24 h and 48 h; **Cytotoxicity all parameters**: Negatively affected mitosis at 24 h at 600 µg/mL, and at all concentrations after 48 h; Decreased the proliferation index at all concentrations in 24 h and at 600 and 750 µg/mL in 48 h; Significantly decreased nuclear division index at all concentrations for 24 h and 48 h. A genotoxic chromosomal effect was observed, concurrently with cytotoxicity. Potentially the observed increased genotoxicity could be an indirect consequence of high cytotoxicity.

CA: chromosome aberration; MN: micronucleus

The bacterial reverse mutation assays were unequivocally negative. The NTP assay (2007) did not cover the recommended five bacteria strains under the recent TG 471 , where either *E. coli WP2 uvrA*, or *E. coli WP2 uvrA* (pKM101), or *S. typhimurium* TA102 is recommended to be included. The second Ames test however, guideline compliant, from Beevers and Adamson (2016) does include test on TA102 and therefore should be capable to fully detect mutagenic potential in bacteria. This assay also included both the plate incubation and pre-incubation method. Both assays tested the substance with and without metabolic activation and results were negative regardless of S9 source (different species and organs). The concentrations deviated in the NTP study from the test guideline requirement as of the five concentrations the high dose of 10 mg/plate exceeded the recommended top concentration while in the second trial, cytotoxicity at comparable low levels limited the top concentration to 33 µg/plate. The Beevers and Adamson study covered at least six concentrations in the recommended range. Appropriate positive controls showed the sensitivity of the test system in both studies. RAC considers the substance as negative for bacterial gene mutation.

In an *in vitro* study on primary human lymphocytes studying chromosome aberration (CA), micronucleus (MN) formation and sister chromatid exchange (SCE) in 4 healthy donors for 24 h and 48 h testing intervals, genotoxic effects were reported for all three cytogenetic parameters (Celik & Topaktas, 2018). The concentration range tested was narrow with 300, 450, 600, 750 µg/mL. At all concentrations except the lowest, the substance induced SCE after

a 48-h treatment period, CA were induced in the cells at all concentrations both in the 24-h (statistically significant at the two highest) and 48-h treatment groups and led to chromatid and chromosome breakage and formation of fragments; the formation of MN was induced at the two highest concentrations (600 and 750 µg/mL) in the 24-h and 48-h treatment groups. The DS, however, reported several deviations from the relevant test guidelines on MN formation and CA. The concentration range should cover different degrees on cytotoxicity including little or no cytotoxicity to exclude secondary positive results (RAC notes that according to recent OECD test guidelines, cytotoxicity should be limited to 55 ± 5% and care should be taken to not markedly exceed 50% cytotoxicity). Three markers of cytotoxicity were measured, and all indices were reduced by treatment. The mitotic index considered as an appropriate marker for primary lymphocytes was negatively affected with a decrease by 45 – 70% at the 24-h top concentration and at all concentrations at 48 h. Thus, genotoxicity effects were largely observed at moderate to marked cytotoxic concentrations (see Annex to CLH report, tables 10-13). For CA however, all concentrations induced an effect, even the three lowest concentrations after 12 h, where only mild to moderate cytotoxicity was observed (9-37% ↓MI). Chromosomal aberrations therefore did not only occur at most toxic concentrations. Whether the values are within the confidence intervals of appropriate historical control data (HCD) for the testing facility cannot be assessed as such data are not available. Another limitation for the result validity, no negative control was included for the 48-h time interval, the trial where the cytogenetic response obtained was most prominent (assessed against the 24 h control). Furthermore, exposure time deviated from the test guideline (no short exposure of 3-6 h with metabolic activation and 1.5 of normal cell cycle length i.e., 30-36 h for primary lymphocytes), and finally the number of binucleated cells for MN and metaphase for CA evaluated was smaller than required. In summary, the reliability overall for this test seems limited based on these deficiencies, and the cytotoxicity is a confounding factor for these positive results. The trial was not repeated for confirmation of the result at different concentrations. Overall, the study seems not sufficiently valid for classification and labelling.

RAC notes that no studies on mammalian gene mutation (Mouse lymphoma assay (tk+/-locus) or hprt assay) are presented in the CLH report, which introduces uncertainties in concluding on the *in vitro* gene mutation potential.

Three *in vivo* studies are available that are in principle suitable to assess relevance of clastogenicity or induction of numerical aberrations, two micronucleus assays rated Klimisch 1 by the DS (NTP, 2007a,b), and one non-guideline chromosomal aberration study (Norizadeh Tazehkand *et al.*, 2016), however, rated as Klimisch 3 by the DS.

***Table**: Overview on in vivo mutagenicity studies rats and mice*

Method, guideline, deviations if any, reliability	**Test substance**	**Observations**
Micronucleated erythrocytes in rat and mouse bone marrow (NTP, 2007a, protocol according to Shelby *et al*., 1993) Reliability 1 Similar to OECD TG 475	4-methylimidazole (99.5% purity) Three consecutives *i.p.* doses at 24 h interval: 0, 25, 50, 100 mg/kg bw. PC = Cyclophosphamide Two species (N=5)	**Negative in rats.** No increases in the frequencies of micronucleated erythrocytes in bone marrow of rats. **Overall negative in mice**: In mice, at 50 and 100 mg/kg bw significant increases in the MN frequency in the first trial

	One trial in F344/N male rats / Two trials in B6C3F1 male mice	but not in second trial. **Bone marrow toxicity in rats only**: No significant alterations in %-micronucleated polychromatic erythrocytes in mouse. In rat bone marrow %-PCEs declined dose dependently and significantly depressed at high dose.
Mouse peripheral blood micronucleus test (NTP, 2007b, protocol according to MacGregor *et al*., 1990) Reliability 1 Food and Drug Administration Good Laboratory Practices Regulations (21 CFR, Part 58)	4-methylimidazole (99% purity) Daily feeding doses (ad libitum, 625, 1250, 2500, 5000, 10000 ppm): 100, 240, 440, 915 or 1840 mg/kg bw/d to males and 110, 240, 540, 1130 or 3180 mg/kg bw/d to females. 14-week toxicity study in B6C3F1 mice with peripheral blood for the micronucleus test obtained in week 14.	**Negative in mice**: No increases in 14-week peripheral blood micronuclei in male and female mice. **No bone marrow toxicity** observed. (scoring for N=5, except female high dose N=3)
Chromosomal aberration study in Swiss Albino mice bone marrow (Norizadeh Tazehkand *et al*., 2016) Reliability 3 Non-guideline, comparable to OECD TG 475, deviations: - No formal quality control and quality assurance procedures reported; - N=3 instead of minimum of 5 animals/sex/group; - Non-recommended (*i.p.*) route of administration without justification; - 24 h harvest time is earlier than the recommended 36-42 h second harvest time; - Negative control only included at one sample time point (24 h); - Dose level range to cover from the maximum to a dose producing little or no toxicity. When bone marrow target tissue toxicity is observed at all dose levels, further study at non-toxic doses is advisable, LD_{50} of 160 mg/kg bw was regarded as MTD instead of MTD identification, narrow dose spacing instead separated by a factor of 2; - 100 instead of 200 metaphase cells per	4-methylimidazole (98% purity) Single *i.p.* injection (0.5 mL/mouse dissolved in double-distilled water): 100, 130, 160 mg/kg bw (LD_{50}) N=3 males and females (not randomized). Body weight 33-40 g (unclear whether this was at arrival or at start of dosing). CA and mitotic index of bone marrow cells analysed 12 h and 24 h after treatment.	**Positive in mice**: Increased percentage of CA at all 12 h concentrations and at highest concentration after 24 h. **Cytotoxicity** based on decreased mitotic index in comparison with control for 12 h high dose and all 24 h doses.

animal examined, un-coded slides evaluated, - No HCD to compare NC & PC, no observations (clinical signs) reported for treated animals, no tabulated individual animal data.		

NC: negative control; PC: positive control; HCD: historical control data; MTD: maximum tolerated dose

In the bone marrow micronucleus study presented in NTP 2007, male B6C3F1 mice and male F344/N rats were treated three days in a row with the test substances by *i.p.* route of administration. In rats the dose levels 25, 50, and 100 mg/kg bw did not alter micronucleus induction in bone marrow erythrocytes. The percent PCEs declined with increasing dose of 4-methylimidazole and were significantly depressed at the highest dose, indicating target organ exposure, while the positive control showed the sensitivity of the test systems. In mice, two trials were conducted. In the first trial the two higher dose levels showed a statistical significant ($p < 0.008$) although small increase in micronucleated PCEs (micronucleated PCEs / 1000 PCEs for control, low, mid, high dose: 2.20 ± 0.44, 2.50 ± 0.22, 4.30 ± 1.08, 4.10 ± 0.58; PC cyclophosphamide 25 mg/kg bw: 31.30 ± 1.81), which was not confirmed in the second trial (micronucleated PCEs / 1000 PCEs for control, low, mid, high dose: 2.50 ± 0.22, 3.00 ± 0.27, 3.10 ± 0.66, 2.40 ± 0.56; PC cyclophosphamide 10 mg/kg bw: 12.90 ± 1.26) (see Annex to CLH report, table 15). The study used an unphysiological route of exposure generally not recommended by the test guideline. While the first mouse trial result introduces uncertainty in the study outcome as MN frequency was slightly increased, overall NTP concluded that the mouse bone marrow MN assay was negative. No significant alteration in percent micronucleated PCEs as measure for target organ exposure were seen in the mouse bone marrow or peripheral blood in either of the two trials. No HCD is reported to assist in result evaluation.

A second NTP MN study (reported in NTP 2004 and 2007) is available in male and female B6C3F1 mice. Peripheral blood micronuclei were measured at the end of a 14-week NTP dietary toxicity study. No effects are reported, including no MN induction in either males (N=5) or females (N=3) but also percentage of PCE as marker for bone marrow toxicity and indicator for target organ exposure was unchanged. For this study NTP reported that one of 10 males and seven of 10 females from the 10000 ppm groups died early, body weight gains of mice exposed to 1250 ppm or greater were significantly reduced. In addition, exposure concentration-related increases in relative liver weights, higher relative testis weights in males, and relative kidney weights in females were higher in groups exposed to 2500 ppm or greater. Furthermore, a minimal microcytic, normochromic, nonresponsive anaemia was observed in females at all exposure concentrations. The limit dose of OECD TG 408 (90-d) is 1000 mg/kg bw/d. In this NTP 14-week study dose levels up to 1840 mg/kg bw/d in males and 3180 mg/kg bw/d in females were employed, and the MTD was exceeded.

NTP considered the studies on *in vivo* micronucleus induction as negative. RAC notes that some uncertainty is evident due to absence of bone marrow toxicity in mice as an indicator of target organ exposure, but also due to a slight positive result in the first mouse trial in the *i.p.* study. For rats, bone marrow toxicity was evident, and no MN were induced. As regards the target organ exposure, toxicokinetic data presented in the CLH report show that 4-methylimidazole is rapidly absorbed, widely distributed, metabolised to a low degree in the liver, and eliminated in mammals without significant bioaccumulation after oral gavage and intravenous injection. The compound was excreted unchanged in urine, beginning

approximately 30 minutes after injection, and reached approximately 90% within 8 hours. In an *i.p*. study in rats, the uptake at 5 minutes after a single 216 mg/kg bw *i.p*. injection was highest in the intestines, followed by blood, liver, stomach, and kidney (Hidaka, 1976). The DS assumed 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.

Bone marrow is a highly perfused organ and based on the toxicokinetic data it may thus be reasonably assumed that the substance reached the blood and bone marrow after oral and *i.p*. administration. From the available data, no rapidly formed and highly reactive metabolites were produced in the liver after oral dosing; thus, systemic exposure with the toxic substance seems to be relevant. In the view of RAC the uncertainties regarding target organ exposure after *i.p.* and dietary administration are likely to be of a minor nature.

For the mouse *i.p.* trials, the second trial did not replicate the positive outcome of the first trial; thus, a weak but significant positive result not reproduced is likely not biologically relevant. Whether the values are within the confidence intervals of appropriate historical controls for the testing facility cannot be assessed as such data are not available.

Overall, RAC agrees that the MN data do not raise a concern for *in vivo* somatic cell mutagenicity / micronucleus induction.

In a third *in vivo* study (Norizadeh Tazehkand *et al*., 2016), according to DS conducted by the same research group as the *in vitro* cytogenicity study described above, chromosomal aberrations were investigated in Swiss Albino mouse bone marrow cells. In this non-guideline study groups of animals received a single *i.p.* dose over a rather narrow dose range of 100, 130, and 160 mg/kg bw. The substance increased CA at all 12-h dose levels and at the high dose 24 h concentration. As regards the acceptability criteria, the study failed with all criteria provided by OECD TG 475: no negative control was available for 24 h and no HCD are available to evaluate the acceptability of the NC and PC results. Too few cells have been analysed for three dose levels spanning not more than a range of 1.6-fold (instead of 2-4-fold per dose increment). Using an unphysiological route of administration, the high dose was pre-selected as LD_{50} (according to NTP 2007 the oral LD_{50} is 370 mg/kg bw and the *i.p.* LD_{50} is 165 mg/kg bw, while neurologic convulsant doses (CD_{50}) reported are 360 mg/kg bw orally and 155 mg/kg bw *i.p.*), instead of identifying an MTD. After 24 h, all dose levels produced toxicity based on decreased mitotic index. RAC agrees with the DS that the study is not sufficiently reliable for classification purposes.

RAC notes that no *in vivo* study capable of detecting gene mutations is available. To summarise, 4-methylimidazole was not mutagenic in *S. typhimurium* with or without metabolic activation using different S9 mix (NTP, 2007; Beevers and Adamson, 2016).
In vivo, 4-methylimidazole did not induce micronuclei in rat or mouse bone marrow cells from animals exposed to doses of up to 100 mg/kg bw by *i.p.* route, three times with 24-h intervals (NTP, 2007). Bone marrow toxicity was observed only in the study in rats where the percent of PCEs declined with increasing dose. Nor did the substance induce micronuclei *in vivo* in mouse peripheral blood erythrocytes sampled at the end of the 14week dietary study with feed concentrations up to 10000 ppm corresponding to 1840 and 3180 mg/kg bw/d in male and female mice, respectively. In the study no bone marrow cytotoxicity was shown.

Positive genotoxicity results were reported *in vitro* for CA, SCE and MN in a non-guideline study, rated Klimisch 3, with human peripheral blood lymphocytes from four donors. The genotoxicity result was obtained in presence of moderate and mainly marked cytotoxicity and

important deficiencies have been noted for the study. A follow up *in vivo* study of the same research group on CA in Swiss Albino mice was also reported positive, however, the study is considered not reliable for classification purpose (Norizadeh Tazehkand *et al*., 2016).

No studies on *in vitro* mammalian gene mutation are available in the absence of an *in vivo* study capable of detecting gene mutations. This introduces an uncertainty in the overall weight-of-evidence as gene mutation / point mutations have only been "preliminary" investigated based on bacteria. Despite, from these bacterial tests no concern arises in presence and absence of metabolic activation with S9 mix. The substance is largely excreted unchanged (see toxicokinetic data) and no reactive metabolites are formed: looking at the structure of this heterocyclic compound and *in silico* predictions on the substance and the four identified metabolites 4-hydroxymethylimidazole, its mono-glucuronide conjugate, 4-hydroxymethylimidazole glucuronide and 5-methylhydantoin, no alert for DNA binding is indicated (Howard and Choksi, 2020). The uncertainties arising from this data gap are therefore considered minor.

Conclusion on classification for germ cell mutagenicity

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…".

No epidemiological and germ cell mutagenicity studies are available and the two reliable *in vivo* micronucleus studies in rats and mice were negative overall and do not support Category 1B.

The third *in vivo* study concluded that 4-methylimidazole increased the percentage of chromosomal aberrations in mice; however, the non-guideline study is suffering from significant deficiencies failing to meet important reliability criteria so that the study is unsuitable for classification and labelling. This study was a follow-up study of an *in vitro* cytogenicity study conducted by the same research group.

The *in vitro* study presents a positive result for chromosomal aberrations (CA and MN); however, the study was equally of limited reliability and cytotoxicity is reported as a potentially confounding factor. The result has not been reproduced by the laboratory or by another independent study. Importantly, the available and reliable *in vivo* micronuclei studies conducted by NTP are considered appropriate follow-up studies for structural CA observed in an *in vitro* study.

The substance was unequivocally negative for bacterial mutagenicity in two reliable studies.

Taking these results together, the results obtained on CA in two studies of low reliability are insufficient for classification based on criteria in category 2 which is meant for "*Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans*".

RAC agrees with the DS on the overall assessment of *in vitro* and *in vivo* genotoxicity studies on 4-methylimidazole that no classification and labelling according to the CLP criteria is justified.

Despite remaining uncertainties related to the lack of mammalian gene mutation studies while the bacterial gene mutation tests are negative, RAC concludes that **no classification for germ cell mutagenicity is warranted**.

Supplemental information - In depth analyses by RAC

Summary of toxicokinetic data presented in the CLH report

The DS presented toxicokinetic data on 4-methylimidazole in rodents (F344/N rats and B6C3F1 mice) and livestock (cows, goats, and sheep) via oral, intravenous or intraperitoneal administration.

The toxicokinetics of 4-methylimidazole was similar in rats and mice. After oral (gavage) administration, it was readily absorbed and distributed systemically (with highest concentrations found in liver followed by kidney and lung) and was excreted largely unchanged without significant bioaccumulation. In the minor degree of metabolism *in vivo*, the identified metabolites were 4-hydroxymethylimidazole, its glucuronide, and other oxidised products, including methylhydantoin. No metabolites were detected after incubation with lung and liver microsomes or lung S9 fractions (Fennel *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).

The plasma concentration versus time data following a single i.v. dose of 10 mg/kg bw in rats and mice was described as a one-compartment model with first-order elimination. After oral (gavage) administration of 10, 50 or 100 mg/kg bw, the elimination half-lives ranged from 1–8 hours in rats and from 21 to 87 minutes in mice and increased with dose (NTP, 2007).

The estimated bioavailability of 4-methylimidazole after single oral (gavage) doses of 5, 50 or 150 mg/kg bw in male rats was ca. 60 to 70% (Yuan and Burka, 1995). The bioavailability was also similar in ewes given single oral (gavage) dose of 20 mg/kg bw (Karangwa *et al.*, 1990).

In goats and heifers, after a single oral or intravenous dose of 20 mg/kg bw, the metabolites were mainly excreted in urine, but also in milk and faeces. The metabolites identified included 5-methylhydantoic acid, an unidentified metabolite, and urea (Nielson *et al.*, 1993).

4-methylimidazole was found in milk following oral administration in pregnant and postpartum cows (1–2 cows). Toxic and even lethal doses in cows did not produce signs of toxicity in the calves. No effects were seen in mice receiving cow's milk (Morgan and Edwards, 1986). The DS questioned the validity of this study but did not provide any reasons for it.

Overall, the DS concluded that 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 administration.

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	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	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**
	(females).	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 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. Decreased incidence of neoplasms (overall rates): 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	4-methylimidazole, > 99% pure, oral exposure f or 106 weeks to 0, 312,	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.	NTP, 2007; Chan 2008

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
OECD TG 451 B6C3F1 **mice**, 50/sex/dose Reliability 1	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)	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 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	

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	Test substance, dose levels duration of exposure	Results	Reference
		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	No	Malignant lesion	Yes	Single (female)	No	Oral	MoA unknown. Cancer type relevance for humans has been questioned (Maronpot *et al.*, 2016).

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
	other organs.							
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.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS presented two studies on carcinogenicity of 4-methylimidazole conducted by NTP in rats and in mice.

NTP reported "equivocal evidence of carcinogenic activity" in female rats based on increased incidences of mononuclear cell leukaemia and "no evidence of carcinogenic activity" in male rats.

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. Hyperplasia of alveolar epithelium is considered to be a precursor for neoplasia but was not observed at lower doses and not in the 14-week study (NTP, 2004).

The mode of action (MoA) leading to an increase in mononuclear cell leukaemia in female rats and in alveolar/bronchiolar tumours in mice is unclear. Mononuclear cell leukaemia is a common tumour type in F344/N rats with variable indices and it was considered that the substance may possibly promote the occurrence of this lesion in female rats. Regarding lung tumours, the hypothesis of a mouse specific MoA leading to the induction of lung tumours by the same MoA as styrene via CYP2F2 activation and/or induction of cell proliferation was not supported by data. Therefore, the DS considered the tumours to be of human relevance.

Significant reduction in neoplasms either below or at the lower end of the historical control ranges 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. These reductions 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.

The data are publicly available and were evaluated by IARC (IARC, 2013) which concluded that 4-methylimidazole is possibly carcinogenic to humans (Group 2B).

The DS considered 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 as Carc. 1B. Additional considerations were findings of carcinogenic activity outside robust HCD from NTP, progression to malignancy, response in both sexes, and to some extent response in two species, although as a cancer form with high spontaneous tumour incidence in rats (mononuclear cell leukaemia).

Comments received during consultation

Three MSCAs provided comments and supported carcinogenicity classification. All MSCAs considered Category 1B justified; however, one of these MSCA also raised the possibility of Category 2 instead of Category 1B for several reasons. Mononuclear cell leukaemia in female rats could be a secondary effect linked to MTD exceedance or/and high background incidence in the species. A statistically significant progression of lesions to malignancy in mice were mostly noted for benign neoplastic lesions (adenomas), and the human relevance of alveolar/bronchiolar adenoma and carcinoma in mouse models has been questioned. The DS agreed that based on the available data, classification of 4-methylamidazole may be considered a borderline case between Cat. 1B and Cat. 2 and the DS proposal is primarily based on the significant increases of benign and/or malignant lung tumours observed in both males and females in the mouse study.

Assessment and comparison with the classification criteria

Carcinogenicity of 4-methylimidazole has been investigated by NTP in two 2-year-carcinogenicity studies similar to OECD TG 451 and according to GLP, one in F344/N rats and one in B6C3F1 mice, rated Klimisch 1 by the DS.

***Table**: Carcinogenicity studies available for 4-methylimidazole*

Method, guideline, deviations if any	**Results**
NTP, 2-year cancer bioassay in rats, GLP Similar to OECD TG 451 Reliability 1 F344/N **rats**, 50/sex/dose 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: 0, 30, 55, 115 mg/kg bw/d (males); 0, 60, 120, 260 mg/kg bw/d (females). (NTP, 2007; Chan 2008)	**General toxicity and survival:** No significant effect on survival was observed. 31/50, 34/50, 33/50, 32/50 males 43/50, 39/0, 34/50, 35/50 females ↓mean terminal body weights of males 1250 and 2500 ppm and females 2500 and 5000 ppm, ↓feed consumption high dose females (5000 ppm), Clinical signs of neurological toxicity (clonic seizures, excitability, hyperactivity, and impaired gait) in high dose females. **Non-neoplastic lesions (graded as minimal to mild):** ↑hepatic histiocytosis and chronic inflammation, hepatocyte focal fatty change, hepatocellular eosinophilic and mixed cell foci Males: Histiocytosis (38/50, 45/50, 50/50, 50/50); chronic inflammation (18/50, 32/50, 31/50, 36/50); hepatocyte, focal fatty change (21/50, 24/50, 37/50, 33/50); eosinophilic focus (4/50, 3/50, 7/50, 12/50); mixed cell focus (5/50, 7/50, 11/50, 27/50); Females: Histiocytosis (40/50, 50/50, 48/48, 50/50); chronic inflammation (17/50, 28/50, 34/48, 35/50); hepatocyte, focal fatty change (16/50, 29/50, 29/48, 32/50); eosinophilic focus (1/50, 2/50, 5/48, 11/50); mixed cell focus (10/50, 7/50, 6/48, 18/50); ↑inflammation in the prostate gland, focal hypertrophy in the pituitary gland (pars distalis), and follicular cysts in the thyroid gland of male rats at the high dose and also medium dose for pituitary lesions, ↑follicle mineralization in the thyroid gland at the high dose females and lung inflammation, cardiomyopathy and focal atrophy of the acinar pancreas in all exposure groups. **Neoplastic lesions:** ***Mononuclear cell leukaemia*** Incidence of **mononuclear cell leukaemia in high dose females** was significantly greater than that in the controls, and the incidence slightly **exceeded**

	the historical control range. **Reduced tumour onset**, in high dose group in females earlier (day 368) than in control females (day 624). Slight, non-significant, increase in incidence of mononuclear cell leukaemia in males. ***See table below*** ***<u>Other neoplasms decreased incidence (overall incidences)</u>:*** Either below or at the lower end of the historical control ranges. *Females* Adenoma in clitoral gland: 16, 2, 0, 0% in 0, 625, 1250, 2500 ppm (HCD 11.0% ± 6.5%; range 2-20%). Fibroadenoma in mammary gland: 48, 12, 8, 2% in 0, 625, 1250, 2500 ppm (HCD 44.8% ± 11.1%; range 28-55%). Stromal polyp in uterus: 32, 10, 4, 4% in 0, 625, 1250, 2500 ppm (HCD 17.9% ± 6.5%; range 12-32%). *Males* Adrenal medulla (benign, complex, or malignant pheochromocytoma (combined): In male rats 20, 12, 6 and 6% in 0, 625, 1250 and 2500 ppm (HCD 11.6% ± 5.5%; range 5-20%). *Males and females* Adenoma in pituitary gland: In male rats 33, 27, 21, 15% and in female rats 60, 38, 40, 18% in 0, 625, 1250, 2500 ppm (HCD in males 22.6% ± 6.0%; range 17-33%. HCD in females 39.1% ± 10.9%; range 29-60%).
NTP, 2-year cancer bioassay in mice, 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: 0, 40, 80, or 170 mg/kg bw/d (males/females) (NTP, 2007; Chan 2008)	**General toxicity and survival:** No significant effect on survival was observed. 45/50, 44/50, 42/50, 46/50 males 43/50, 40/50, 43/50, 40/50 females Mean terminal body weights reduced in the 1250 ppm group (males) and in all exposure groups (females). Feed consumption generally similar to the controls. No clinical findings considered treatment related. **Non-neoplastic lesions:** ↑incidences of alveolar epithelium hyperplasia and histiocytic cellular infiltration in 1250 ppm females significantly greater. Histiocytic cellular infiltration slightly increased in 1250 ppm males. ↑incidences of thyroid follicular cyst in 1250 ppm females significantly greater. ↑significant positive trend in incidences of mammary gland hyperplasia in females (16/50, 10/50, 14/49, 24/49). **Neoplastic lesions** ***<u>Alveolar/bronchiolar adenoma and carcinoma</u>*:** 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 higher 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. ***See table below***

In the rat study, 50 rats/sex/group received diets containing 0, 625, 1250, or 2500 ppm (males) or 0, 1250, 2500, or 5000 ppm (females) 4-methylimidazole for 106 weeks, corresponding to approx. 30, 55, and 115 mg/kg bw/d in males and 60, 120, and 260 mg/kg bw/d in females for low, mid and high dose, respectively. Dose levels were selected based on a preceding 14-week repeated dose toxicity study.

There was a slight, non-significant, increase in incidence of mononuclear cell leukaemia in males (overall rates of 30%, 36%, 44%, 40% for 0, 625, 1250 and 2500 ppm, respectively). A mean incidence of 46.8% in the HCD was reported, with a range of 30-68%. The time of onset in male rats was not reduced compared to control.

In females, the incidence of mononuclear cell leukaemia in high dose females was significantly higher compared to control; the incidence slightly exceeded the historical control range of 12-38% (mean 23.8 ± 9.1). Overall incidences of 18%, 14%, 32%, 40% in the 0, 1250, 2500 and 5000 ppm exposure groups, respectively, were reported. The onset in the high dose group females was earlier with day 368 compared to control females with day 624.

Survival was not significantly affected. Terminal mean body weights of males in the 1250 and 2500 ppm groups (95% and 87%, respectively) and in females in the 2500 and 5000 ppm groups (81% and 65%, respectively) were lower compared to controls. Clinical signs of neurological toxicity were observed in females, in particular at the two highest doses.

Table*: Incidences of mononuclear cell leukaemia observed in the NTP carcinogenicity study in rats*

Doses (ppm)	0	625	1250	2500	5000	Trend
Males						
Mononuclear cell leukaemia	15/50 (30%)	18/50 (36%)	22/50 (44%)	20/50 (40%)	-	-
HCD	46.8 ± 13.0% / range 30-68%					
Females						
Mononuclear cell leukaemia	9/50 (18%)	-	7/50 (14%)	16/50 (32%)	**20/50 (40%) p=0.013**	**p<0.001**
HCD	23.8 ± 9.1% / range 12-38%					

Mononuclear cell leukaemia is a very common finding with high and variable background incidences, as indicated by HCD, in F344/N rats. Only in high dose females the incidences were significantly increased. The trend was also tested statistically significant. In addition, the onset of tumours was markedly earlier in the high dose females. Incidences in males were only slightly increased and well within the HCD, while the current control was at the lower end of the HCD for the males. For interpretation of this tumour type, HCD play an important role, as also clearly stated by the CLP guidance (3.6.2.3.2.) Due to its high and variable background incidence, the tumour incidence in such case may not provide a reliable evidence of treatment related carcinogenicity. It has been discussed by the DS and IARC that 4-methylimidazole may have exacerbated the high and variable commonly observed background incidences. NTP has

introduced a switch in using the rat strain for different reasons, one of them being the high rates of mononuclear cell leukaemia; nowadays long-term toxicology and carcinogenicity studies in rodents usually involve the SD rat.

Another observation in females in this study was the marked decrease of several neoplastic findings to incidences at or below the lower end of HCD. The reason for this is unclear, the body weight gain reduction has been raised; however, the reductions are mild and thus cannot fully explain the dose-dependent reductions in neoplastic incidences.

Overall, the mechanism for increase in mononuclear cell leukaemia and for decreases of several neoplasias remains unclear. RAC considers that the increased incidence in the high dose females provides only very limited support for carcinogenicity classification due to the high and variable background incidence of the strain.

In the mouse study, 50 rats/sex/group received diets containing 0, 312, 625, and 1250 ppm 4-methylimidazole for 106 weeks, corresponding to approx. 40, 80, or 170 mg/kg bw/d in males and in females for low, mid and high dose, respectively. Dose levels were selected based on a preceding 14-week repeated dose toxicity study.

No significant effect on survival was reported. Mean terminal body weights of males and females in the 1250 ppm high dose groups were lower compared to control (males, 86%; females, 81%).

The incidences of alveolar/bronchiolar adenoma in all exposed groups of females, alveolar/bronchiolar carcinoma in high dose males, and alveolar/bronchiolar adenoma or carcinoma (combined) in high dose males and mid and high dose females were significantly increased compared to 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 increased compared to controls. Histologically, this lesion was considered a morphologic continuum to adenoma.

***Table**: Incidences of alveolar / bronchiolar neoplasia observed in the NTP carcinogenicity study in mice*

Dose level (ppm)	**0**	**312**	**625**	**1250**	**Trend**
Males					
Alveolar/bronchiolar adenoma	16%	22%	26%	**30%**	**-**
HCD	15.8 ± 6.3%; range 9–28%				
Alveolar/bronchiolar carcinoma	4%	8%	8%	**16%** **p=0.042**	**p=0.024**
HCD	7.8 ± 3.8%; range 4–14%				
Alveolar/bronchiolar adenoma or carcinoma combined	18%	26%	32%	**44%** **p=0.003**	**p<0.001**
HCD	22.2 ± 6.3%; range 14–32%				
Hyperplasia	14%	6%	2%	18%	-

Females					
Alveolar/bronchiolar adenoma	0%	**16%** **P=0.004**	**32%** **p<0.001**	**16%** **p=0.003**	**p=0.017**
HCD		3.7 ± 3.8%; range 0–10%			
Alveolar/bronchiolar carcinoma	6%	0%	4%	**14%**	**p=0.019**
HCD		2.9 ± 2.5%; range 0–6%			
Alveolar/bronchiolar adenoma or carcinoma combined	6%	**16%**	**34%** **p<0.001**	**28%** **p=0.002**	**p=0.002**
HCD		6.6 ± 4.2%; range 0–12%			
Hyperplasia	6%	4%	6%	**22%** **p<0.05**	-

No excessive toxicity was observed in the mouse cancer bioassay. The significant increase in the incidence of benign and malignant alveolar / bronchiolar 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.

The MoA and human relevance of alveolar/bronchiolar adenoma and carcinoma was discussed by the DS. No mechanistic data was presented in the CLH report. 4-methylimidazole is considered a non-genotoxic carcinogen. The hypothesis was raised that 4-methylimidazole induces lung tumours by activation of the mouse specific CYP2F2. The transformation of non-genotoxic substances to cytotoxic metabolites by CYP2F2 in Clara cells is considered a mouse specific MoA; however, the hypothesis was not supported, according to the DS, mentioning one study investigating this hypothesis. In this study, Cruzan *et al.* (2015) evaluated whether the substance induces mouse lung tumours by the same MoA as styrene via CYP2F2 metabolic activation and increased BrdU labelling (DNA synthesis / cell proliferation marker). With styrene as a positive control, bronchiolar region histopathology and DNA synthesis were analysed in a 5-d comparative toxicity study in C57BL/6 "wild type" and CYP2F2 "knock out" mice given the substance at the same dietary concentrations used in the NTP cancer bioassay, and in a 13-week comparative toxicity study of C57BL/6 and B6C3F1 mice. According to the authors the results do not support the hypothesis of 4-methylimidazole and styrene inducing lung tumours by the same MoA as no consistent effect on BrdU labelling or histopathology in the lungs of mice were seen. The DS further raised that 4-methylimidazole has been shown to be an effective inhibitor of cytochromes P450 (Karangwa *et al.*, 1990, Hargreaves *et al.*, 1994). Also, hyperplasia was only observed at the high dose and not observed in the 14-week repeated dose study suggesting that mitogenic or regenerative effects are not the main drivers for the carcinogenic effects of 4-methylimidazole in mice.

In both sexes, a rather clear dose-response is evident for adenoma/carcinoma combined incidences, while no MoA has been established. In addition, dose levels were rather low: 40, 80, and 170 mg/kg bw/d for low, mid and high dose, respectively. Adenomas were statistically significantly induced already in the low dose females with incidences exceeding the HCD. RAC thus agrees with the DS that the finding represents a clear carcinogenic response of which human relevance has to be assumed and that it is not possible to establish a threshold for tumorigenic activity based on the available data.

Conclusion on classification for carcinogenicity

Table*: relevant factors for classification can be summarized as follows (modifying table 14 of the CLH report):*

Species and strain	**B6C3F1 mice**	**F344/N rats**
Tumour type and background incidence	Alveolar/bronchiolar adenoma and carcinoma. Clear increase in incidence in particular of adenomas and combined adenomas and carcinomas. Clear dose-dependency, statistically significant and HCD exceeded.	Mononuclear cell leukaemia. High and variable spontaneous incidence in F344/N strain. CLP guidance: Tumour incidence in such case may not provide a reliable evidence of treatment related carcinogenicity Incidence at high doses statistically significant and slightly exceeds the HCD range. Dose-dependently reduced tumour incidences observed in several other organs.
Multi-site responses	No	No
Progression of lesions to malignancy	Yes, benign and malignant lesions	Yes, malignant lesion
Reduced tumour latency	Unknown	Yes
Responses in single or both sexes	Both males and females	Single, females
Confounding excessive toxicity?	No	MTD seems exceeded in high dose based on neurotoxicity and body weight reduction.
Route of exposure	Oral	Oral
MoA and relevance to humans	MoA unknown. Tumour types considered relevant for humans as mouse specific MoA is not supported. Not genotoxic. Threshold might exist but cannot be established	MoA unknown. Cancer type relevance for humans has been questioned (Maronpot *et al.*, 2016).
Study reliability	Klimisch 1 NTP carcinogenicity study similar to OECD TG 451, GLP	Klimisch 1 NTP carcinogenicity study similar to OECD TG 451, GLP

4-methylimidazole was tested in two reliable carcinogenicity studies.

In rats, the incidence of mononuclear cell leukaemia was increased with dose-dependent trend in females only, the high dose being significantly different from the control and slightly exceeding the HCD. At this dose, the MTD seems to be exceeded based on body weight gain reduction and neurotoxicity. Mononuclear cell leukaemia is a frequent tumour with high and variable background incidences in F334/N rats. Its relevance for humans has been questioned.

Considering the high background incidence, this tumour type may not provide reliable evidence for treatment related carcinogenicity of 4-methylimidazole. According to the CLP guidance, in such cases where only spontaneous tumours appear, downgrading from Category 1B to Category 2 or even no classification may be justified.

However, in the mouse study, 4-methylimidazole induced, dose-dependently in both sexes, an increased incidence in alveolar/bronchiolar adenoma and carcinoma that reached statistical significance in particular for adenomas in females, carcinoma in males, and combined adenomas and carcinomas in both sexes. Adenoma and carcinoma are considered a continuum in the neoplastic progression. The precursor lesion hyperplasia was only observed statistically significantly in high dose female animals. The incidences exceeded the concurrent and historical controls. No excessive toxicity was observed in the mouse cancer bioassay that could present a confounding factor in neoplastic progression. The MoA is unclear. A genotoxic mechanism is unlikely based on the available genotoxicity data. No plausible MoA hypothesis such as mouse specific CYP2F2 activation and cytotoxicity that would question human relevance has been proven. It is also not possible to derive a threshold for carcinogenicity considering the clear dose-response from low to high dose demonstrated in the mouse study.
RAC considers the benign and malignant alveolar/bronchiolar neoplasia clear evidence for 4-methylimidazole carcinogenicity and human relevance is assumed.

There are no human data on carcinogenicity of 4-methylimidazole available; hence, classification of 4-methylimidazole in Category 1A is not justified.

According to the criteria, 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 Practice, can provide sufficient evidence of carcinogenicity in experimental animals.

RAC considers this condition fulfilled as 4-methylimidazole increased the incidence of lung neoplasia in both sexes of a single species, the mouse, in a well-conducted NTP study following GLP.

RAC concludes that **classification and labelling of 4-methylimidazole as Carc. 1B (H350) is warranted.**

The DS did not propose an SCL, and RAC agrees that no SCL is necessary.

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 F1: F1a and F1b euthanized at PND4. F1c used for breeding of F2 generation. F2a and F2b euthanized at PND4. F2c necropsy at PND28. Cross-over mating groups included (F0) Reliability 1	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) 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).	**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 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	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**
		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 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	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 Mean delivered daily doses: 0, 40, 80, 160, 300, or 560 mg/kg bw/day 4-methylimidazole to males and	**Mortality**: **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)	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
	females.	• Females (94 and 63 % of controls, in the 300 and 560 mg/kg bw/day dose group) **Reproductive organ toxicity** Males **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 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.	

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		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 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	**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. 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.	NTP, 2004 Chan *et al*., 2006
Supportive MoA study (effects of imidazoles on testosterone secretion). Rat Sprague-Dawley, males	Single dose (subcutaneous injection) exposures ranging between 10-300 mg/kg bw. 4-methylimidazole and related	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	Adams, 1998

Method, guideline, deviations if any, species, strain, sex, no/group, reliability	Test substance, dose levels duration of exposure	Results	Reference
10 rats/group Reliability 2	compounds. Purity of chemical was not stated	testicular testosterone secretion was shown.	

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)

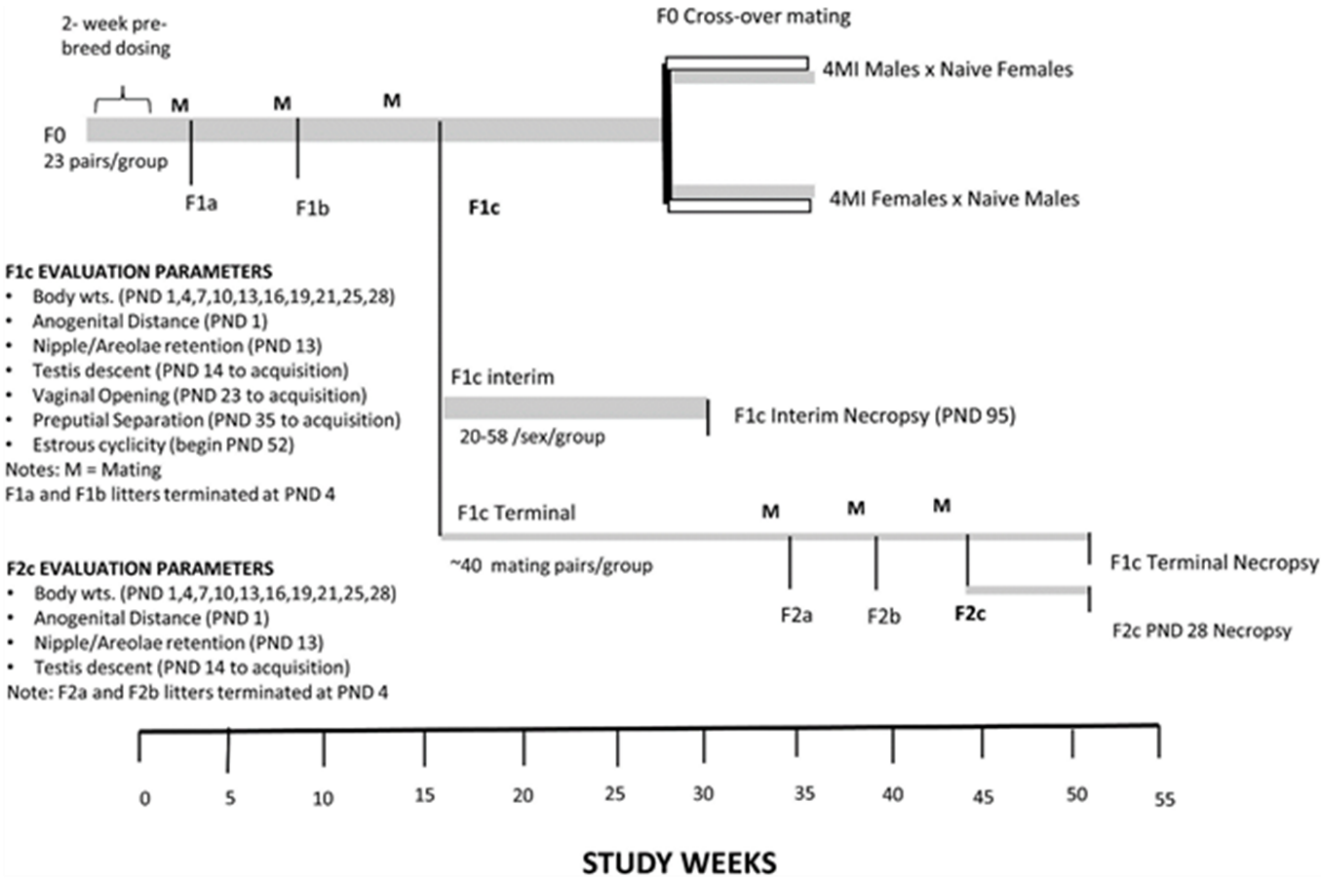


Fig. 1. Schematic of the Reproductive Assessment by Continuous Breeding (RACB) Design. From Behl *et al.*, 2020. Rats were administered 4-MI in the diet at 0, 750, 2500, or 5000 ppm ad libitum. The F0 adults were exposed during a 2-week prebreed exposure period, during cohabitation, gestation and lactation 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	-
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	0.96±0.02	0.98±0.01	0.91±0.04	0.10±0.10
	0.97±0.02	0.98±0.01	0.93±0.03	0.67[a)]

Dose (ppm)	0	750	2500	5000
F0 - A	0.95±0.03	0.98±0.01	0.93±0.04	–
B				
C	0.92±0.05**	0.93±0.04	0.72±0.08**	-
	0.97±0.01*	0.97±0.01	0.91±0.03*	-
F1c – A	0.89±0.05	0.92±0.05	0.88±0.04	-
B				
C				
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)				

Dose (ppm)	0	750	2500	5000
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				
F0	1.35±0.02	1.44±0.03*	1.37±0.03	1.32±0.02
F1c interim	1.45±0.02	1.47±0.02	1.52±0.05	-
F1c terminal	1.41±0.02*	1.44±0.02	1.47±0.02	-
Left				
F0	1.40±0.02	1.51±0.04*	1.44±0.03	1.40±0.02
F1c interim	1.47±0.02*	1.47±0.01	1.54±0.04*	-
F1c terminal	1.41±0.02	1.45±0.02	1.47±0.02	-
Dorsolateral Prostate (mg) (absolute)				
F0	604±26**	492±23**	469±15**	421±22**
F1c interim	402±11**	382±12	330±13**	-
F1c terminal	539±16**	475±19*	449±19**	-
Dorsolateral Prostate (mg/g) (relative)				
F0	1.20±0.05**	1.03±0.05**	1.02±0.03**	0.93±0.05**
F1c interim	1.02±0.03	1.00±0.03	0.97±0.03	-
F1c terminal	1.08±0.03	0.98±0.04	1.01±0.03	-
Ventral Prostate (mg) (absolute)				
F0	935±28**	785±21**	748±29**	515±27**
F1c interim	561±20**	446±14**	355±21**	-
F1c terminal	825±22**	796±27	591±18**	-
Ventral Prostate (mg/g) (relative)				
F0	1.85±0.05**	1.65±0.05**	1.63±0.06**	1.13±0.06**
F1c interim	1.42±0.05**	1.16±0.03**	1.05±0.06**	-
F1c terminal	1.67±0.05**	1.64±0.06	1.34±0.05**	-

Dose (ppm)	0	750	2500	5000
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	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** - -

Dose (ppm)	0	750	2500	5000
F1-terminal				
Oestrous cycle length (days)				
F0	5.3 ± 0.21	5.4 ± 0.21	5.9 ± 0.43	5.8 ± 0.68
F1c	5.0 ± 0.23	4.8±0.06	5.1±0.07*	-

*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
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)						
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

Dose (mg/kg bw/day)	0	440	915	1840
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.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Sexual function and fertility

For the evaluation of reproductive toxicity of 4-methylimidazole, the DS presented two U.S. NTP dietary studies with the substance – a Reproductive Assessment through Continuous Breeding protocol (the RATCB study) in rats (NTP, 2019; Behl *et al.*, 2020) and a 14-week repeated dose study (RDT) in rats and mice (NTP, 2004; Chan *et al.*, 2006). A supporting MoA study in male rats (Adams *et al.*, 1998) including single subcutaneous doses of 4-methylimidazole ranging from 10 – 300 mg/kg bw was also presented.

The DS proposed Cat. 1B for sexual function and fertility based on clear effects on male and female reproductive performance, effects on primary and secondary reproductive organs as well as on delays in timing of sexual developmental markers indicating sex hormone insufficiency in the RATCB study and supported by the effects on reproductive organs in the 14-week RDT study.

Developmental toxicity

No prenatal developmental toxicity studies are available for 4-methylimidazole. The DS proposed Cat. 2 for developmental toxicity based on the developmental effects (reductions in litter size, in pup survival and in pup body weights; delay in testicular descent and increased areolas/nipple retention in male pups) observed in the RATCB study (NTP, 2019; Behl *et al.*, 2020). In the CLH report, the DS presented a combined assessment of sexual function and fertility and development.

Adverse effects on or via lactation

The DS proposed no classification for adverse effects on or via lactation. The DS was not aware of any study describing levels of 4-methylimidazole in human breast milk. From the studies in domesticated animals, 4-methylimidazole shows transfer into the milk; however, the DS considered that is difficult to evaluate to what degree the lactation exposure contributes to postnatal developmental toxicities like delayed puberty onset, based on the available data. In the RATCB study (NTP, 2019; Behl *et al.*, 2020), there were no effects on survival and growth of pups between post-natal day (PND) 5 and 28 indicating no effects on or via lactation.

Comments received during consultation

Four MSCAs commented during the consultation and all supported the DS's proposal. Two of the MSCAs recommended further justification of the categorisation. One MSCA wondered if Cat. 1B for developmental toxicity could be considered due to read-across from 2-methylimidazole and 1-vinylimidazole that are classified as Cat. 1B for developmental toxicity based on dissecting aneurysm of the great vessels of the heart and pup mortality, and on vascular effects and pup mortality, respectively.

In response, the DS provided further reflections on Cat. 1B vs Cat. 2. The DS considered the classification for developmental toxicity as borderline between Cat. 1B and Cat. 2 and the distinction between fertility effects and developmental effects is not always well defined. The DS noted that it is not known whether the reduction in litter size is caused by impaired parental fertility or implantation loss, as implantation loss was not investigated in the RATCB study. Regarding read-across, the DS commented that in an earlier draft of the CLH report they indeed proposed read-across to 2-methylimidazole but did not consider it to be appropriate once they became aware of the RATCB study on 4-methylimidazole. According to the DS, the RATCB study does not include investigations of vascular effects/aneurysm in the offspring or developing foetus but gives information about lethality and other developmental effects. The DS considered that even if read-across were to be applied, information from the study on 4-methylimidazole would outweigh the read-across to a large extent (NTP, 2019; Behl *et al.*, 2020).

RAC notes that the classification of 2-methylimidazole and 1-vinylimidazole as Cat. 1B for developmental toxicity was based on screening studies (OECD TG 421/422) and no prenatal developmental toxicity studies are available for these substances either. For both these substances, dilated pericardial vessels were observed during gross pathological examination of pups. These gross pathological changes were then microscopically confirmed as aneurysms of the great vessels of the heart. RAC notes that in the RATCB study (NTP, 2019; Behl *et al.*, 2020) with 4-methylimidazole the pup gross pathology revealed no cardiovascular effects (i.e., dilated pericardial vessels were not observed). There are also differences in adverse effects on fertility between these substances. For example, there were no effects on the mating performance for 2-methylimidazole and 1-vinylimidazole, while that for 4-methylimidazole it was markedly reduced. Therefore, RAC considers that the read-across is not justified.

Assessment and comparison with the classification criteria

Sexual function and fertility

In the RATCB study, (NTP, 2019; Behl *et al.*, 2020) 4-methylimidazole (>99% purity) was administered to Hsd:Sprague Dawley SD rats via diet. Twenty-three pairs per group of rats (F0) received 0, 750, 2500 or 5000 ppm 4-methylimidazole. They were mated thrice to produce three litters (F1a, F1b and F1c). The F1a and F1b pups were terminated on PND 4. The treatment of F1c continued with an interim group being terminated at PND 95 and another group mated thrice to produce F2a, F2b and F2c. The F2a and F2b pups were terminated on PND 4, while the F2c on PND 28. The F0 generation in addition included cross-over mating groups (treated males were mated with untreated females (two groups, 750 and 2500 ppm) and vice versa (only one group, 2500 ppm)).

The mean doses in the premating period for F0 males/females corresponding to low (750 ppm), mid (2500 ppm) and high dose (5000 ppm) groups were approximately 48/47, 145/146 and 260/290 mg/kg bw/d, for m/f respectively. In the mid and high dose groups, 4 and 11 F0 females, respectively, were dead/moribund and several of these were removed around parturition with dystocia or retained placentas/foetuses (see table "Incidence of perturbed parturition…" below). The high dose F0 females group was subsequently discontinued; therefore, there were only two treatment groups (750 and 2500 ppm) in the F1. There was no treatment related mortality in F0 males.

The mean doses in the premating period for F1 males/females corresponding to 750 and 2500 ppm were approximately 64/66 and 207/225 mg/kg bw/d, m/f respectively.

In F0/F1 males the terminal body weights were reduced relative to controls by 5%/2% (750 ppm), 9%/11% (2500 ppm) and 10% (5000 ppm). The body weights of F0/F1 females at GD21 were reduced relative to controls by 4-9% (750 ppm), 13-17% (2500 ppm) and 18-25% (5000 ppm). The body weights of F1 pups (across litters) were reduced compared to controls to ≥ 97% (750 ppm), 89-97% (2500 ppm) and 67-73% (5000 ppm) at PND1; and to 95% (750 ppm) and 81% (2500 ppm) for F1c pups at PND28. The body weights of F2 pups were not affected at 750 ppm and were ≥ 92% at 2500 ppm compared to controls. For a summary of body weight changes compared to controls, please see table below ("Summary of body weight changes…"). Clinical signs included increase in convulsions in F0/F1 females (0 ppm: 4%/0%; 750 ppm: 0%/1%; 2500 ppm: 9%/16%; 5000 ppm: 39%). Histopathological changes were observed in liver (vacuolation) in F0 males of 5000 ppm and in kidney (mineralisation) in F1 females of 750 and 2500 ppm groups.

RAC considers the systemic toxicity observed in the F0 and F1 adults (even at the high dose for males and low and mid doses for females) as moderate and the reproductive effects described below are not a secondary non-specific consequence of the systemic effects.

***Table**: Incidence of perturbed parturition across the three pairings per generation in the RATCB study (Table 3 from Behl et al., 2020)*

Incidence of perturbed parturition across the three pairings per generation[a].

	Generation	0 ppm	750 ppm	2500 ppm	5000 ppm
Dystocia	F0	0	0	1	5
	F1	0	0	4	–
Retained Fetus/ Placentas	F0	0	0	1	1
	F1	1	1	1	0
Total Perturbed Parturition[b]	F0	0	0	2	6
	F1	1	1	5	–

[a] Number of females displaying evidence of mating across all three pairings: F0 n = 66, 68, 60, 21; F1 n = 109, 122, 104.
[b] Incidence of animals displaying dystocia or retained fetus/placentas.

***Table**: Summary of body weight changes compared to controls in the RATCB study*

Dose (ppm)	750 ppm	2500 ppm	5000 ppm
F0/F1 males at terminal	95%/98%	91%/89%	90%
F0/F1 females at GD21	91 – 96%	83 – 87%	82 – 75%
F0 females at LD1	94 – 95%	88%	81 – 82%
F1 pups at PND1	97 – 100%	89 – 97%	67 – 74%
F1 females at LD1	92 – 93%	84 – 85%	-
F2 pups at PND1	100 – 104%	92 – 95%	-
F0 females at LD4	93 – 95%	87%	69 – 75%
F1 pups at PND4	93 – 97%	79 – 91%	64%
F1 females at LD4	93%	83 – 85%	-
F2 pups at PND4	101 – 103%	88 – 90%	-
F1 pups at PND28	95%	81%	-
F2 pups at PND28	99%	80%	-

LD: lactation day

Reproductive performance

In the F0 5000 ppm group, there was a marked decrease in percent mated females/pair (48% vs 97% in controls) and percent littered/pair (75% vs 91% in controls). In the F0 cross-over mating with treated males and untreated females there was a marked reduction in mated/pair (33% vs 87% in controls) at 5000 ppm, while there were no significant effects at 2500 ppm. All the untreated females in 5000 ppm group that did not deliver were found to be non-pregnant implying an adverse effect on male fertility. Owing to high mortality of females at 5000 ppm there was no F0 cross-over mating with treated females and untreated males at this dose. There were no significant effects on female fertility though at 2500 ppm.

RAC considers the marked decrease in mating performance of F0 5000 ppm group as clear evidence of treatment related adverse effect on fertility. The high female mortality in this group was attributed to difficulty in parturition indicating severe effects on female fertility. In addition, in the F0 5000 ppm cross-over mating group of treated males vs untreated females, the marked decrease in mating performance indicates severe effects on male fertility.

***Table**: Summary of reproductive performance in the RATCB study (adapted from Table 16 of the CLH report)*

Dose (ppm)	0	750	2500	5000
N (# of pairings)				
F0 (three pairings)	68	69	62	44
F1c terminal (three pairings)	118	126	111	-
Mated/Pair (average of three pairings)				
F0	97.0%	98.6%	96.8%	48.1% [a]
F1c	92.4%	97.0%	94.0%	-
F0 (male x naïve female)	87.0%**	–	80.0%	33.3%**
F0 (naïve male x female)	68.2%	–	73.7%	-
Littered/Mated (average of 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%	–

[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.

Litter parameters

There was a dose-related statistically significant (trend with) lower number of total pups and live pups per litter on PND0 in the F0 and F1 matings. RAC considers this reduction in litter size as an adverse effect on fertility. However, as also pointed out by the DS, there is no examination of implantation sites in the RATCB study (NTP, 2019; Behl *et al.*, 2020), which raises an uncertainty that the reduction in litter sizes may also be due to adverse effect on development.

Pup survival on PND1-4 was significantly lower in the F0 5000 ppm group and after the first two matings of the F1 2500 ppm group. RAC considers these effects as adverse and relevant for classification for developmental toxicity. However, there were only 2 litters (first mating) and 1 litter (second mating) left in the F0 5000 ppm group. Also, the lower pup survival in the

2500 ppm group was not consistent (no effects at this dose level after any of the three matings of F0 and after the third mating of F1).

***Table**: Summary of litter parameters in the RATCB study (adapted from Behl et al., 2020)*

Dose (ppm)	0	750	2500	5000
Total litter size (PND0), each pair				
F0 - A	14.6 ± 0.4 (22)**	13.3 ± 0.6 (22)	9.9 ± 0.7 (17)**	5.7 ± 1.9 (6)**
B	14.2 ± 0.5 (19)**	13.5 ± 0.6 (23)	9.2 ± 0.7 (17)**	4.7 ± 0.9 (6)**
C	13.6 ± 0.6 (19)*	12.8 ± 0.6 (22)	10.6 ± 1.2 (16)*	–
F1c – A	13.2 ± 0.5 (33)**	11.0 ± 0.6 (34)*	8.9 ± 0.8 (31)**	-
B	15.3 ± 0.5 (34)**	12.3 ± 0.5 (37)**	10.6 ± 0.9 (29)**	-
C	10.7 ± 0.8 (28)	9.8 ± 0.7 (35)	9.4 ± 0.9 (23)	-
Live litter size (PND0), each pair				
F0 - A	14.0 ± 0.4 (22)**	12.7 ± 0.6 (22)	7.8 ± 1.0 (17)**	1.8 ± 1.6 (6)**
B	13.7 ± 0.5 (19)**	12.7 ± 0.5 (23)	8.5 ± 0.6 (17)**	0.5 ± 0.5 (6)**
C	12.2 ± 0.8 (19)	12.4 ± 0.5 (22)	9.8 ± 1.2 (16)	–
F1c – A	11.1 ± 0.7 (33)**	9.3 ± 0.7 (34)	7.2 ± 0.8 (31)**	-
B	13.6 ± 0.6 (34)**	11.9 ± 0.6 (37)	9.8 ± 0.8 (29)**	-
C	9.2 ± 0.7 (28)	8.8 ± 0.6 (35)	8.5 ± 0.9 (23)	-
Survival ratio (PND1-4), each pair				
F0 - A	0.96 ± 0.02 (22)	0.98 ± 0.01 (22)	0.91 ± 0.04 (15)	0.10 ± 0.10 (2)
B	0.97 ± 0.02 (19)	0.98 ± 0.01 (23)	0.93 ± 0.03 (17)	0.67 (1)
C	0.95 ± 0.03 (19)	0.98 ± 0.01 (22)	0.93 ± 0.04 (15)	–
F1c – A	0.92 ± 0.05 (33)**	0.93 ± 0.04 (32)	0.72 ± 0.08 (29)**	-
B	0.97 ± 0.01 (34)*	0.97 ± 0.01 (36)	0.91 ± 0.03 (29)*	-
C	0.89 ± 0.05 (28)	0.92 ± 0.05 (34)	0.88 ± 0.04 (23)	-
Survival ratio PND5-28				
F0c	0.98 ± 0.01 (19)	0.96 ± 0.02 (22)	0.95 ± 0.02 (15)	–
F1c	0.83 ± 0.07 (28)	0.93 ± 0.02 (33)	0.89 ± 0.03 (22)	-

Data are displayed as the means and standard errors of the litter means. In parentheses are the number of litters. *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.

Pup markers

In the 2500 ppm group, there was a statistically significant trend in increase of percentage of male pups with areolae/nipples and delay in day of testis descent in F1c. There was a non-significant increase in percentage of male pups with areolae/nipples and a statistically significant delay in the day of testis descent also in F2c. RAC considers these effects as adverse and relevant for classification for developmental toxicity. The number of pups with areolae/nipples (3 pups (4 – 5%) in each generation from 1 – 2 litters (5 – 13%)) was small but the incidence was high compared to historical control incidence (indicated in Behl *et al.*, 2020) of 2 pups (out of 382; 0.5%) and 2 litters (out of 80; 2.5%). Details on the HCD are

not available to assess its relevance. The delay in testis descent was also small; 0.4 days in F1c and 1.3 days in F2c compared to respective controls. This delay was less than the variation (1.4 days) between F1c and F2c controls.

Even after adjustment for weaning body weight, there was a dose-related significant delay in preputial separation and vaginal opening in the F1c. RAC considers these adverse effects on onset of puberty (sexual maturation) as relevant for classification for reproductive toxicity.

***Table**: Summary of developmental markers in the RATCB study (NTP, 2019; Behl et al., 2020) (Table 20 of the CLH report)*

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 (vaginal opening) and BPS (Balano-preputial separation): 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.

Sperm analysis and oestrous cycle

There were statistically significant reduced number of sperm per cauda in the F0 5000 ppm, reduced number of sperm per gram cauda in the F1 interim 750 and 2500 ppm groups. There were no effects in the F1 terminal groups. There was a statistically significant decrease in motile or progressively motile sperm in F0 2500 and 5000 ppm, and F1 interim 2500 groups. Extended dioestrus was observed in the F0 5000 ppm group and a statistically significant increase was observed in the total cycle length at the F1c 2500 ppm group.

RAC notes that the above effects are not severe or consistent across generations and there were no adverse effects on these parameters in the 14-week study either (neither in rats and mice).

***Table**: Summary of sperm analysis and oestrous cyclicity in the RATCB study (NTP, 2019; Behl et al., 2020) (Table 19 of the CLH report)*

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.

Organ weights

The following statistically significant effects were observed in the reproductive organ weights:

- dose-dependent decrease in absolute and relative weight of dorsolateral or ventral prostate in all F0 and F1 groups
- dose-dependent decrease in absolute weight of seminal vesicles in all F0 and F1 groups (except F1c-terminal 750 ppm group where the effect was not statistically significant but there was a trend). There was also a dose-dependent decrease in relative weight of seminal vesicles in all F0 groups and in F1c-interim 2500 ppm group
- dose-dependent decrease only in absolute weight of levator ani-bulbocavernosus muscle (LABC) in all F0 groups
- decreased absolute weight of left and right epididymis in the F0 2500 and 5000 ppm, and F1c 2500 ppm groups
- decreased absolute right ovary weight in all F0 groups (not dose-dependent) and in F1 interim 2500 ppm group. In the F0 5000 ppm, group there was also decreased weight of absolute right ovary and both absolute and relative left ovary weight.

RAC considers the above effects on prostate and seminal vesicles as severe and support the classification for fertility. These effects were consistent across generations with a clear dose-response and for prostate, were accompanied by clear dose-related histopathological changes (although only minimal to moderate in severity) in this study (see table "Summary of histopathologic lesions…" below) and in rats of the 14-week study (see table "Incidences of lesions in the reproductive organs" below).

***Table**: Summary of reproductive organ weights in the RATCB study (NTP, 2019; Behl et al., 2020) (adapted from Table 18 of the CLH report)*

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**
F1 interim	571 ± 9**	564 ± 8	513 ± 12**	-
F1 terminal	697 ± 9**	697 ± 9	651 ± 11**	-
Left				
F0	704 ± 9**	718 ± 13	657 ± 11**	635 ± 10**
F1 interim	579 ± 10**	561 ± 7	521 ± 12**	-
F1 terminal	697 ± 13**	701 ± 9	648 ± 12**	-
Epididymis (mg/g) (relative)				
Right				
F0	1.35 ± 0.02	1.44 ± 0.03*	1.37 ± 0.03	1.32 ± 0.02
F1 interim	1.45 ± 0.02	1.47 ± 0.02	1.52 ± 0.05	-
F1 terminal	1.41 ± 0.02*	1.44 ± 0.02	1.47 ± 0.02	-
Left				
F0	1.40 ± 0.02	1.51 ± 0.04*	1.44 ± 0.03	1.40 ± 0.02
F1 interim	1.47 ± 0.02*	1.47 ± 0.01	1.54 ± 0.04*	-
F1 terminal	1.41 ± 0.02	1.45 ± 0.02	1.47 ± 0.02	-
Dorsolateral Prostate (mg) absolute)				
F0	604 ± 26**	492 ± 23**	469 ± 15**	421 ± 22**
F1 interim	402 ± 11**	382 ± 12	330 ± 13**	-
F1 terminal	539 ± 16**	475 ± 19*	449 ± 19**	-
Dorsolateral Prostate (mg/g) (relative)				

F0 F1 interim F1 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 F1 interim F1 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 F1 interim F1 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 F1 interim F1 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 F1 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 F1 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 F1 interim F1 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 - -
Ovary (mg) (absolute)				
Right F0 F1 interim F1 terminal	 65.6 ± 3.9** 55.2 ± 1.4* 84.3 ± 4.3	 48.2 ± 3.7* 52.8 ± 2.2 73.6 ± 3.2	 61.3 ± 3.6 * 47.8 ± 1.6* 70.8 ± 3.3	 39.7 ± 3.4 ** – –
Left F0 F1 interim F1 terminal	 66.1 ± 4.4** 58.5 ± 1.5* 83.4 ± 4.1*	 53.2 ± 4.8 54.8 ± 2.4 75.4 ± 4.7	 66.0 ± 4.5 51.1 ± 2.7 71.1 ± 2.4	 38.9 ± 3.7** – –
Ovary (mg/g) (relative)				
Right F0 F1 interim F1 terminal	 0.19 ± 0.01 0.23 ± 0.01 0.24 ± 0.01	 0.17 ± 0.01 0.22 ± 0.01 0.23 ± 0.01	 0.20 ± 0.01 0.22 ± 0.01 0.23 ± 0.01	 0.15 ± 0.01* – –
Left F0 F1 interim F1 terminal	 0.20 ± 0.01 0.24 ± 0.01 0.24 ± 0.01	 0.18 ± 0.02 0.23 ± 0.01 0.23 ± 0.01	 0.22 ± 0.01 0.23 ± 0.01 0.23 ± 0.01	 0.14 ± 0.01* – –

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

Histopathology

Histopathology of reproductive organs showed testicular degeneration and testicular spermatid retention that was significant in the F0 5000 ppm group, but not dose-dependent across the groups or generations. The incidence of exfoliated germ cells in the epididymis was significantly increased in the F0 5000 ppm group, but there was no dose-response in terms of severity. Dose-dependent minimal to mild prostate gland atrophy was observed in all the F0 and F1 groups (except in the F0 5000 ppm group, where it was mild to moderate).

RAC notes that the testicular degeneration, prostate gland atrophy and histological changes in epididymis were also observed in rats of the 14-week study at the top doses with increasing severity (see table "Incidences of lesions in the reproductive organs" below). Therefore, RAC considers these effects as adverse and that they support the classification for fertility.

There were significant increases in primordial, atretic (also in F0 750 ppm group) and antral follicles in the F0 5000 ppm group but there was no clear pattern and the effects are considered not relevant.

***Table**: Summary of histopathologic lesions of reproductive organs in the RATCB study (adapted from Behl et al., 2020)*

Dose (ppm)	0	750	2500	5000
Prostate, ventral lobe – atrophy F0 F1 interim F1 terminal	 0%** 4 [1.0] 8%** 4 [1.0] 10%**	 9 [1.0][a] 39%** 25 [1.0] 45%** 10 [1.0] 23%	 20 [1.1] 87%** 17 [1.2] 85%** 35 [1.5] 88%**	 23 [2.4] 100%** – –
Testis – degeneration F0 F1 interim F1 terminal	 1 [1.0] 5%** 4 [1.3] 8% 2 [1.0] 5%	 0% 6 [1.0] 11% 5 [2.0] 11%	 4 [1.8] 17% 1 [2.0] 5% 5 [1.2] 13%	 8 [1.6] 35%* – –
Testis – spermatid retention F0 F1 interim F1 terminal	 2 [1.0] 9%* 0% 0%	 3 [1.0] 13% 3 [1.0] 5% 5 [1.2] 11%	 1 [1.0] 4% 4 [1.0] 20% 4 [1.0] 10%	 8 [1.3] 35%* – –
Epididymis – exfoliated germ cells F0 F1 interim F1 terminal	 1 [1.0] 5%** 3 [1.3] 6% 0%	 0% 5 [1.2] 9% 5 [1.2] 11%	 3 [1.7] 13% 4 [1.3] 20% 4 [1.3] 10%	 7 [1.3] 30%* – –

[a] Incidence with [avg. severity score] and percent incidence; Severity scores: 1 = minimal, 2 = mild, 3 = moderate, 4 = severe. Average severity scores were not used in statistical significance calculations. *p < 0.05; **p < 0.01; NE = not examined (read-down); "- "= no animals examined due to early removal.

In the 14-week dietary study with rats (Fischer 344) and mice (B6C3F1), equivalent to OECD TG 408 and in compliance with GLP, 10 animals/sex/dose received 0, 625, 1250, 2500, 5000 or 10000 ppm of 4-methylimidazole (purity: 99 ± 0.1%) (corresponding to approx. doses in male and female rats: 40, 80, 160, 300 or 560 mg/kg bw/d; and in male/female mice: 100/110, 240/250, 440/540, 915/1130 or 1840/3180 mg/kg bw/d).

Rats

One male in the 560 mg/kg bw/d group died during week 1 and one female in the 80 mg/kg bw/d group was killed moribund during week 9. Clinical findings in the 300 and 560 mg/kg bw/d group included abnormal breathing, nasal and eye discharge (also in 160 mg/kg bw/d group), ruffled fur, tremors and ataxia in males and females.

There was a statistically non-significant decrease in food intake in the 300 and 560 mg/kg bw/d groups. The final mean body weight and body weight gains were statistically significantly lower than controls in the 160, 300 and 560 mg/kg bw/d groups in males (% final weight relative to controls: 95, 85 and 70%, respectively) and in the 300 and 560 mg/kg bw/d groups in females (% final weight relative to controls: 94 and 63%, respectively).

The right testis weights were significantly lower in the 300 (absolute) and 560 (absolute and relative) mg/kg bw/d groups. The absolute weights of left testis, left epididymis and cauda epididymis were significantly lower in the 300 mg/kg bw/d group. No data for these was reported for 560 mg/kg bw/d group.

RAC considers the above effects on testis and epididymis as adverse and relevant for classification for fertility as these organ weight changes were accompanied by histopathological changes at the top dose levels (see table "Incidences of lesions in the reproductive organs" below).

***Table**: Summary of reproductive organ weights for male rats in the 14-week study (Table 21 of the CLH report)*

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) (absolute)[c)]	0.508	-	0.524	0.511	0.438**	-
L cauda epididymis (g) (absolute) [d)]	0.187	-	0.176	0.174	0.154**	-

*p < 0.05 (Williams' test); **p < 0.01 (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.

There were no adverse effects on sperm parameters and oestrous cyclicity.

The following statistically significant effects were observed during histopathology:

- Increase in the incidence of animals with minimal to slightly marked testicular degeneration in the 300 and 560 mg/kg bw/d groups (9 animals in each group vs 1 in control)
- Increase in the incidence of animals with prostate gland atrophy (minimal to mild) in the 300 and 560 mg/kg bw/d groups (8 animals in each group vs 0 in control)
- Significant increase in the incidences of epididymal hypospermia (9 animals vs 0 in control) and prostate gland inflammation (8 animals vs 2 in control) in the 560 mg/kg bw/d group. Other dose groups were not examined for epididymal hypospermia.

Histopathological changes in the above organs were also observed in the RATCB study. Thus, RAC considers these effects as adverse and that they support the classification for fertility.

***Table**: Incidences of lesions in the reproductive organs of male rats in the 14-week study (from Table 13 of NTP, 2004)*

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Epididymis	10	—	—	—	—	10
Hypospermia	0	—	—	—	—	9**
Prostate Gland	10	1	10	10	10	10
Atrophy	0	1 (1.0)	1 (1.0)	2 (1.0)	8** (1.1)	8** (1.9)
Inflammation	2 (1.5)	0	3 (1.0)	0	1 (2.0)	8* (1.5)
Testes	10	1	10	10	10	10
Degeneration	1 (2.0)	1 (1.0)	0	4 (1.0)	9** (1.3)	9** (3.1)

"–" Not examined at this exposure concentration, *p ≤ 0.05 (Fisher exact test); **p ≤ 0.01. Top row: Number of animals with organ examined microscopically, 2nd and 3rd rows: Number of animals with lesions (severity in parentheses); 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

Mice

One male was found dead during week 4 in the 1840 mg/kg bw/d group and seven females were found dead during weeks 1, 2 and 3 in the 3180 mg/kg bw/d group. Clinical findings in the 3180 mg/kg bw/d group included ruffled fur and dull coats in females.

There was no significant effect on food consumption. The final mean body weight and body weight gains of males were significantly lower in the 240, 440, 915 and 1840 mg/kg bw/d groups (% final weight relative to controls: 93, 90, 84 and 79, respectively). The final mean

body weight and body weight gains of females were significantly lower in all dose groups (% final weight relative to controls in the 110, 250, 540, 1130 and 3180 mg/kg bw/d groups was 90, 88, 80, 77 and 74, respectively).

The absolute weight of right and left testes and absolute weight of left epididymis (right epididymis weight not reported) were significantly lower in the 1840 mg/kg bw/d group. There were no effects observed in mice on histopathology of reproductive organs, sperm parameters or oestrous cyclicity.

In the MoA study, single subcutaneous doses of 4-methylimidazole (purity not stated) ranging from 10 – 300 mg/kg bw bw/d were injected to 10 male Sprague-Dawley rats per group. 4-methylimidazole caused dose dependent decrease in testicular interstitial fluid formation and serum testosterone levels and decrease in serum luteinizing hormone after 2 hours at higher doses. Co-exposure of 4-methylimidazole with known testicular stimulants such as hCG showed a direct effect of 4-methylimidzole on testis resulting in suppression of testosterone secretion.

The DS pointed out that several lines of evidence indicates that 4-methylimidazole has anti-androgenic effects in male rats. Such evidence includes the above MoA study and the effects observed in the RATCB study (increase in areolae/nipples in male pups, delay in day of testis descent, delay in preputial separation, effects on androgen sensitive tissues – prostate, seminal vesicles and LABC). Delay in vaginal opening and dystocia observed in the RATCB study also indicate impairment of steroidogenesis and disruption of endocrine signalling in females (NTP, 2019; Behl *et al.*, 2020). 4-methylimidazole has also been shown to inhibit CYP enzyme activities and reduction of specific CYP activities may contribute to impaired testosterone and oestrogen synthesis. The DS mentioned that this argument is supported by the known effects of structurally related azole fungicides, including the well-studied ketoconazole.

Comparison with the classification criteria for sexual function and fertility and development

Substances are classified in Cat. 1A largely based on evidence from humans. Since no human data is available for 4-methylimidazole, Cat. 1A is not applicable.

Substances are classified in Cat. 1B when the data 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 then the adverse effect is considered not to be a secondary non-specific consequence of other toxic effects.

Substances are classified in Cat. 2 based on some evidence of an adverse effect on sexual function and fertility or on development and where the evidence is not sufficiently convincing to place the substance in Cat. 1.

Sexual function and fertility

In the RATCB study (NTP, 2019; Behl *et al.*, 2020),

- There was a significant reduction in mating in the high-dose group and smaller litter sizes in all treatment groups. The female moribundity/mortality in the high dose group was associated with dystocia.
- It is apparent from the high-dose cross-over mating group (treated males with untreated females) that male fertility is severely affected, in the absence of systemic toxicity.

- There was a dose-dependent significant delay in preputial separation and vaginal opening.
- There was a dose-dependent decrease in prostate, seminal vesicles, and epididymis weights. There were also histopathological changes in prostate, epididymis and testes. Similar adverse effects on the reproductive organs were also observed in rats in the 14-week study.

RAC considers the above data as providing clear evidence of adverse effects on sexual function and fertility. Therefore, **RAC agrees with the DS and concludes that 4-methylimidazole warrants classification as Repr. 1B; H360F**.

Developmental toxicity

There are no prenatal developmental toxicity studies available for 4-methylimidazole. The following adverse effects on development were observed in the RATCB study (NTP, 2019; Behl *et al.*, 2020):

- There was a significant decrease in total litter and live litter size on PND0 in the F0 and F1 generations.
- Pup survival ratio on PND1-4 was lower in the F1 and F2 generation.

According to the CLP criteria (Annex I: 3.7.1.3), adverse effects on onset of puberty are regarded as effects that has the potential to interfere with sexual function and fertility. Therefore, the adverse effects on preputial separation and vaginal opening observed in the RATCB study are considered under the section on sexual function and fertility above.

Since there was no examination of implantation sites in the RATCB study, there is an uncertainty if the decrease in litter sizes may also be due to adverse effects on development and not just due to adverse effects on fertility. However, also given other serious effects on fertility, RAC considers this uncertainty as not sufficient ground to propose classification for developmental toxicity based on decreases in the litter sizes.

The pup survival ratio on PND1-4 was lower in the F0 5000 ppm group but there were just 1 – 2 litters left for evaluation. The effects on the pup survival in the 2500 ppm group were not consistent as pup survival ratio was statistically significantly lower only after the first two matings of the F1 generation and not after the third mating of the F1 or after all the three matings of the F0 generation.

The effects in male pups (nipple retention and delay in testis descent) are consistent with the anti-androgenic activity of 4-methylimidazole. However, the incidence of areolae/nipples in male pups was low (3 pups in each generation from 1 – 2 litters). The delay in testis descent, although statistically significant, was small (1.3 days) and was slightly less than the variation between the two generations control values. RAC considers these effects as adverse but small changes in developmental delay.

Overall, RAC considers the above effects on pup survival to provide some evidence of developmental toxicity. The changes in developmental landmarks (nipple retention and delay in testis descent, although small) support the classification for developmental toxicity.

Therefore, **RAC agrees with the DS and concludes that 4-methylimidazole warrants classification as Repr. 2; H361d.**

Comparison with the classification criteria for lactational effects

Classification for effects on or via lactation can be assigned based on:

a) human evidence indicating a hazard to babies during the lactation period; and/or
b) results of one- or two-generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk; and/or
c) absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk.

No human data is available for 4-methylimidazole indicating hazard to babies during the lactation period.

In the RATCB study, there were no effects on survival of pups between PND5 and PND28. RAC considers that the effects on developmental landmarks and decrease in pup body weight in the RATCB study do not provide clear evidence of adverse effect due to transfer in the milk or on the quality of the milk.

4-methylimidazole was found in the milk of goats and cows but there is no data showing that the levels are potentially toxic.

Therefore, RAC agrees with the DS and concludes that **4-methylimidazole does not warrant classification for adverse effects on or via lactation**.

Overall, RAC agrees with the DS and proposes **classification as Repr. 1B; H360Fd (May damage fertility. Suspected of damaging the unborn child)** for 4-methylimidazole.

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

Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

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

International Chemical Identification:

4-methylimidazole

EC Number: 212-497-3

CAS Number: 822-36-6

Index Number:

Contact details for dossier submitter: **Norwegian Environment Agency**

PB 5672 Torgarden,

N-7485 Trondheim

Version number: 2.0 **Date: 15 December 2020**

CONTENTS

1 PHYSICAL HAZARDS

This was not evaluated.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

2.1 [Study 1]

Study 1 reference:

NTP, 2007:

NTP, TR 535, 2007, TOXICOLOGY AND CARCINOGENESIS STUDIES OF 4-METHYLIMIDAZOLE

Test type

- Single-dose toxicokinetic studies in F344/N rats and B6C3F1 mice (no information about guidelines in NTP, TR535, 2007)

Detailed study summary and results:

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier
- 99% purity
- Unknown impurities less than 1%.
- Lot number 116H0901, from Sigma Chemical Company (St. Louis, MO)

Test animals

- F344/N RATS, males and females, Taconic Farms, Inc. (Germantown, NY)
- $B6C3F_1$ mice, males and females, Taconic Farms, Inc. (Germantown, NY)
- Age of the rats were 14 weeks, body weight males ranged from 231.2 to 322.4 and females from 166.6 to 197.1 grams at the day of dosing
- Age of male and female mice were 12 or 13 weeks old and body weight ranged from 19.3 to 35.6 (males) and 16.3 to 26.3 grams (females)
- 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

Administration/exposure

- Route of administration – gavage (male and female F344/N rats and B6C3F1 mice received a single dose of 10, 50, or 100 mg/kg body weight formulated in 0.05 M phosphate-buffered saline, pH 7.4 ± 0.1)
- Route of administration – intravenous (male and female F344/N rats and B6C3F1 mice received a single intravenous injection of 10 mg 4-methylimidazole/kg body weight)

Results and discussion

According to NTP (2007), 4-Methylimidazole was rapidly absorbed when administered by gavage to male and female F344/N rats and B6C3F$_1$ mice such that measurable concentrations of 4-methylimidazole were observed within 5 minutes of administration. The plasma concentration versus time data can be described by a one-compartment model with no lag phase and first-order absorption and elimination for both males and females. Absorption rate constants were larger than the elimination rate constants and were similar in males and females of each species. The absorption half-life values ranged from 5 to 23 minutes in rats and 2 to 5 minutes in mice and declined with dose. Elimination half-life values ranged from 65 to 499 minutes (1-8 hours) in rats and from 21 to 87 minutes in mice but increased with dose in both sexes of both species. Clearance across the dosed groups decreased with dose. These data indicate that the 100 mg/kg dose is approaching the upper limit of the linear dosing range and that higher doses would result in higher internal doses than expected based on extrapolation from the lower doses.

The plasma concentration versus time data following intravenous administration of 10 mg/kg 4-methylimidazole in rats and mice was described as a one-compartment model with first-order elimination.

There appears to be a species difference in 4-methylimidazole kinetics and metabolism. In rats, the uptake at 5 minutes after a single 216 mg/kg intraperitoneal injection of 4-methylimidazole 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% of the administered dose within 8 hours. In ewes, the absorption and elimination of a single oral dose of 4-methylimidazole followed first-order kinetics. An oral dose (20 mg/kg) of 4-methylimidazole was absorbed in about 27 minutes, and the maximum plasma level was reached 5 hours after oral administration (Karangwa *et al.*, 1990). 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 of the oral dose was recovered in urine unchanged. Metabolites of 4-methylimidazole were not detected by high-performance liquid chromatography (HPLC). In goats and heifers, the mean residence time of 4-methylimidazole administered orally or intravenously was about 5 hours, and the volume of distribution was 0.9 L/kg body weight in both goats and heifers (Nielsen *et al.*, 1993). 4-Methylimidazole and its metabolites were excreted mainly in urine, but also in milk and feces. 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. In pregnant and postpartum cows and mice, 4-methylimidazole was found in milk following oral administration (Morgan and Edwards, 1986).

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 (Yuan and Burka, 1995). At 150

mg/kg, the plasma concentration of [^{14}C]-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. Only one minor hydrophilic metabolite was present in urine and plasma. Fecal, biliary, or respirated elimination of radioactivity was negligible.

Fennell *et al*. (2019) report that of orally (gavage) administered 4-methylimidazole, 41–70% and 79–89% of the radioactivity were excreted in the urine of mice and rats, respectively. Most of the radioactivity (71–88%) in urine was unchanged 4-methylimidazole. Renal clearance was the major excretion pathway. Additional radioactive peaks (the largest metabolite was 8–18% of the dose) were characterized as 4-hydroxymethylimidazole, its glucuronide, and other oxidized products, including methylhydantoin. This minor degree of metabolism (4-methylimidazole was largely excreted unchanged in rats and mice with limited oxidative metabolism and conjugation) was similar between rats and mice. No metabolites were detected in rat or mouse lung and liver microsomes, or lung S-9 fractions. Tissue recovery of ^{14}C-radiolabeled 4-methylimidazole in mice was 0.067-0.14% 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 rats, tissue recoveries were 0.051-0.086% 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. These data show that 4-methylimidazole was readily absorbed and distributed systemically, and was excreted largely unchanged without significant bioaccumulation in mice and rats.

Yuan and Burka (1995) showed that metabolism and renal clearance of 4-methylimidazole were saturated by a 50 mg/kg oral dose. Hargreaves et al. (1994) reported that 4-methylimidazole was a strong inhibitor of p-nitrophenol hydrolase in rat liver. p-Nitrophenol is a cytochrome P450 2E1 substrate. 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). Binding of 4-methylimidazole by heme may therefore prolong its half-life. The phenomenon is well illustrated by the present toxicokinetic study data in rats and mice (NTP, 2007), which show that plasma concentrations of 4-methylimidazole increase as dose concentrations are increased. The elimination half-life of 4-methylimidazole is long enough to allow the manifestation of 4-methylimidazole toxicity.

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3 HEALTH HAZARDS

The endpoints/hazard classes of acute toxicity, skin corrosion/irritation, serious eye damage/eye irritation, respiratory and skin sensitisation are not assessed in this dossier.

3.1 Germ cell mutagenicity

No available data found.

3.2 In vitro data

3.2.1.1 Salmonella typhimurium mutagenicity test

Study reference:

NTP (2007), TOXICOLOGY AND CARCINOGENESIS STUDIES OF 4-METHYLIMIDAZOLE

Detailed study summary and results:

Test type

Salmonella typhimurium mutagenicity test using strains TA97, TA98, TA100, and TA1535, with and without hamster or rat liver metabolic activation enzymes (The study is included in NTP, 2007. The detailed protocol is described by Zeiger *et al*., 1988).

Test substance

- Test material used in the study (4-methylimidazole) is equivalent to the substance identified in the CLH dossier
- >99 % pure
- Unknown impurities less than 1%
- Lot number 116H0901, from Sigma Chemical Company (St. Louis, MO)

Administration/exposure

- The genetic toxicity of 4-methylimidazole was assessed by testing the ability of the chemical to induce mutations in *Salmonella typhimurium* strains TA97, TA98, TA100, and TA1535. Doses tested were 0, 1, 3.3, 10, 20, 33, 100, 333, 1000, 3333 and 10000 µg/plate, with and without hamster or rat liver S9 metabolic activation enzymes.

Results and discussion

4-Methylimidazole (doses up to 10,000 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 (Table 1 and 2). Positive controls showed the sensitivity of the test system.

Table 1: Mutagenicity of 4-Methylimidazole in Salmonella typhimurium (reproduced from Table E1 in NTP, 2007). High concentration results, study performed at SRI International [a]

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9 Trial 1	–S9 Trial 2	+hamster S9 10%	+hamster S9 30%	+rat S9 10%	+rat S9 30%
Study performed at SRI International							
TA100	0	136 ± 2.9	131 ± 3.5	131 ± 3.0	160 ± 4.7	135 ± 5.3	155 ± 3.5
	100	153 ± 7.0	119 ± 4.3	133 ± 9.5	166 ± 4.0	133 ± 2.8	163 ± 10.2
	333	143 ± 4.7	127 ± 0.0	129 ± 2.3	164 ± 4.4	125 ± 1.2	156 ± 5.2
	1,000	152 ± 14.4	121 ± 6.4	133 ± 2.6	171 ± 4.1	149 ± 5.5	158 ± 2.6
	3,333	149 ± 0.3	121 ± 8.4	131 ± 1.8	169 ± 5.2	128 ± 7.6	157 ± 4.1
	10,000	144 ± 8.4	115 ± 3.2	126 ± 1.2	151 ± 7.8	123 ± 3.0	150 ± 12.4
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[c]		959 ± 5.8	991 ± 51.4	426 ± 13.1	562 ± 16.6	334 ± 16.9	817 ± 34.8
TA1535	0	11 ± 1.8	14 ± 2.9	13 ± 1.0	13 ± 0.9	13 ± 2.4	11 ± 1.0
	100	13 ± 0.6	15 ± 0.7	13 ± 2.1	15 ± 1.5	13 ± 0.3	11 ± 0.3
	333	15 ± 1.7	16 ± 1.5	12 ± 0.3	11 ± 0.9	12 ± 1.5	13 ± 3.2
	1,000	12 ± 2.5	13 ± 2.5	16 ± 2.8	13 ± 1.5	9 ± 0.3	12 ± 1.5
	3,333	14 ± 0.9	17 ± 0.3	9 ± 0.3	13 ± 0.6	13 ± 2.4	12 ± 1.5
	10,000	12 ± 1.2	9 ± 0.6	13 ± 1.7	12 ± 2.5	13 ± 1.9	15 ± 2.6
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		858 ± 15.0	830 ± 12.6	136 ± 5.8	145 ± 4.4	137 ± 6.4	143 ± 11.1
TA97	0	177 ± 9.1	151 ± 3.7	170 ± 9.4	176 ± 9.5	143 ± 3.3	168 ± 9.3
	100	158 ± 6.1	156 ± 2.8	153 ± 3.3	168 ± 10.3	160 ± 9.2	155 ± 7.0
	333	177 ± 10.1	156 ± 1.5	160 ± 4.7	172 ± 2.2	167 ± 11.1	149 ± 10.4
	1,000	186 ± 5.6	155 ± 12.9	162 ± 9.0	178 ± 4.7	170 ± 3.0	165 ± 4.3
	3,333	165 ± 14.6	163 ± 1.2	149 ± 13.9	165 ± 9.3	168 ± 12.5	152 ± 12.8
	10,000	151 ± 11.7	168 ± 6.2	133 ± 14.0	169 ± 8.7	145 ± 4.4	176 ± 4.0

Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		361 ± 21.4	461 ± 29.7	453 ± 13.3	487 ± 12.2	363 ± 8.9	466 ± 18.8
TA98	0	15 ± 0.9	19 ± 0.9	24 ± 1.7	20 ± 0.7	22 ± 1.8	16 ± 1.9
	100	15 ± 1.8	21 ± 2.0	18 ± 1.8	19 ± 3.3	21 ± 3.1	16 ± 2.4
	333	20 ± 0.7	23 ± 4.3	20 ± 0.7	19 ± 3.6	18 ± 1.8	18 ± 0.6
	1,000	19 ± 1.2	22 ± 2.9	23 ± 3.0	20 ± 0.3	23 ± 2.3	21 ± 2.6
	3,333	18 ± 1.5	20 ± 2.4	22 ± 0.3	17 ± 0.3	23 ± 1.2	18 ± 0.6
	10,000	15 ± 1.2	22 ± 2.0	18 ± 0.7	17 ± 1.2	21 ± 1.7	14 ± 0.3
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		337 ± 25.3	349 ± 26.6	333 ± 23.3	424 ± 22.5	321 ± 16.8	407 ± 3.4

Table 2: Mutagenicity of 4-Methylimidazole in Salmonella typhimurium (NTP TR 2007). Low concentration results. Study performed at Environmental Health Research and Testing, Inc.

Strain	**Dose (µg/plate)**	**Revertants/Plate**					
		–S9		**+hamster S9**		**+rat S9**	
		Trial 1	**Trial 2**	**10%**	**30%**	**10%**	**30%**
Study performed at Environmental Health Research and Testing, Inc.							
TA100	0	127 ± 0.9	128 ± 2.1	128 ± 1.2	151 ± 1.5	136 ± 2.3	137 ± 1.5
	1	138 ± 1.7	130 ± 1.8	135 ± 1.8	149 ± 2.0	133 ± 2.3	139 ± 1.5
	3.3	133 ± 1.5	132 ± 1.5	138 ± 1.8	148 ± 1.3	139 ± 1.5	138 ± 1.5
	10	131 ± 2.1	135 ± 0.9	145 ± 2.4	143 ± 1.5	128 ± 1.5	140 ± 2.0
	20	136 ± 1.5	137 ± 2.3	139 ± 2.1	153 ± 0.9	131 ± 2.7	141 ± 2.1
	33	134 ± 2.1	134 ± 2.7	134 ± 2.3	151 ± 1.8	136 ± 2.1	137 ± 1.5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		531 ± 5.2	863 ± 14.3	985 ± 2.0	729 ± 3.5	900 ± 5.5	882 ± 4.6
TA1535	0	18 ± 0.9	15 ± 1.5	19 ± 0.6	18 ± 1.2	16 ± 0.9	20 ± 0.7
	1	17 ± 1.2	13 ± 0.9	19 ± 0.9	18 ± 1.5	16 ± 0.9	22 ± 1.2
	3.3	19 ± 0.7	13 ± 1.3	17 ± 1.2	20 ± 2.3	17 ± 1.5	18 ± 0.6
	10	17 ± 1.5	16 ± 1.0	17 ± 1.5	18 ± 1.5	18 ± 1.2	18 ± 1.5
	20	18 ± 2.1	13 ± 1.5	18 ± 1.0	18 ± 0.6	17 ± 1.5	20 ± 1.2
	33	20 ± 2.0	15 ± 1.5	18 ± 1.9	19 ± 0.9	17 ± 0.6	19 ± 1.2
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		804 ± 18.2	511 ± 5.4	241 ± 2.3	152 ± 2.3	190 ± 3.5	202 ± 6.7
TA97	0	117 ± 1.5	129 ± 1.8	139 ± 3.8	125 ± 1.5	138 ± 2.4	143 ± 0.3
	1	121 ± 1.8	133 ± 2.0	147 ± 4.4	139 ± 0.9	129 ± 2.0	156 ± 3.2
	3.3	123 ± 2.0	138 ± 1.5	146 ± 2.7	138 ± 1.2	135 ± 2.0	160 ± 1.5
	10	125 ± 1.5	127 ± 2.0	149 ± 4.6	141 ± 1.7	136 ± 1.8	158 ± 1.5
	20	126 ± 1.7	126 ± 1.7	140 ± 2.3	137 ± 1.5	142 ± 1.2	149 ± 2.3
	33	127 ± 1.3	128 ± 1.8	136 ± 3.5	141 ± 1.8	141 ± 1.9	148 ± 1.5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		348 ± 6.7	296 ± 4.6	814 ± 14.8	708 ± 17.3	795 ± 4.9	535 ± 7.8
TA98	0	47 ± 0.9	22 ± 1.2	28 ± 1.5	29 ± 0.6	41 ± 1.5	35 ± 2.0
	1	47 ± 0.9	24 ± 1.2	32 ± 2.4	36 ± 0.9	41 ± 1.5	39 ± 1.2
	3.3	50 ± 2.1	29 ± 1.8	37 ± 1.3	39 ± 1.5	39 ± 1.8	40 ± 2.0
	10	50 ± 2.1	29 ± 1.5	40 ± 0.3	39 ± 0.6	40 ± 2.4	41 ± 0.7
	20	50 ± 1.0	27 ± 1.8	42 ± 1.2	40 ± 0.9	44 ± 2.1	44 ± 1.5
	33	46 ± 1.8	27 ± 1.0	32 ± 1.2	38 ± 1.2	45 ± 0.9	39 ± 2.0
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		285 ± 3.8	345 ± 4.2	849 ± 9.5	829 ± 2.6	460 ± 4.1	442 ± 3.8

[a] The detailed protocol is presented by Zeiger *et al.* (1988). 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA97), and 4-nitro-o-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

3.2.1.2 Salmonella typhimurium mutagenicity test

Study reference:

Beevers, C., and Adamson, R.H. (2016). Evaluation of 4-methylimidazole, in the Ames/Salmonella test using induced rodent liver and lung S9. Environ Mol Mutagen. 57: 51-57.

Detailed study summary and results:

Test type: Bacterial Reverse Mutation Assay

TA98, TA1535, TA1537 (UK National Culture of Type Collections), TA100 and TA102 (Covance Laboratories, USA). OECD 471-compliant.

Test substance

- Test material: 4-methylimidazole (CAS Number 822- 36-6) from Sigma–Aldrich, UK
- >99 % purity

Administration/exposure

The genetic toxicity of 4-methylimidazole was assessed by testing the ability of the chemical to induce mutations in *Salmonella typhimurium* strains TA98, TA1535, TA1537, TA100 and TA102. Concentrations tested by the plate incubation methodology were 0, 5, 15.81, 50, 158.1, 500, 1581, and 5000 10000 µg/plate, and 0, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate in the pre-incubtion test, both with and without rat and mouse liver S9, in addition to rat and mouse lung S9 (supplied by Celsis In Vitro, Baltimore, Maryland, prepared from either male F344 rats or male B6C3F1 mice, and induced with Aroclor 1254).

Results and discussion

4-Methylimidazole (10,000 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. (Table 3 - 9). Positive controls showed the sensitivity of the test system.

Table 3: Mutagenicity of 4-Methylimidazole in the absence of exogenous metabolism, plate incorporation methodology (Beevers and Adamson, 2016).

Metabolic activation	Test article	Concentration (µg plate^{-1})	Revertants/plate (mean ± standard deviation)				
			TA98	TA100	TA1535	TA1537	TA102
Without Activation	Purified water	0	18 ± 7	94 ± 4	20 ± 5	18 ± 3	266 ± 22
	4-Methylimidazole	5	19 ± 7	104 ± 7	22 ± 2	18 ± 4	295 ± 17
	4-Methylimidazole	15.81	18 ± 2	99 ± 12	14 ± 2	12 ± 3	287 ± 12
	4-Methylimidazole	50	18 ± 3	105 ± 19	14 ± 3	15 ± 1	272 ± 6
	4-Methylimidazole	158.1	24 ± 2	105 ± 9	19 ± 4	15 ± 1	290 ± 15
	4-Methylimidazole	500	16 ± 6	102 ± 11	27 ± 7	16 ± 3	278 ± 15
	4-Methylimidazole	1581	18 ± 4	108 ± 10	19 ± 7	17 ± 7	275 ± 16
	4-Methylimidazole	5000	12 ± 2	102 ± 6	17 ± 2	12 ± 4	268 ± 13
	2-Nitrofluorene (2NF)	5	639 ± 74	NT	NT	NT	NT
	Sodium azide (NaN_3)	2	NT	613 ± 64	642 ± 35	NT	NT
	9-Aminoacridine (AAC)	50	NT	NT	NT	121 ± 11	NT
	Mitomycin C (MMC)	0.2	NT	NT	NT	NT	672 ± 33

NT: Not tested.

Table 4: Mutagenicity of 4-Methylimidazole in the presence of rat liver S9, plate incorporation methodology (Beevers and Adamson, 2016).

Metabolic activation	Test article	Concentration (µg plate^{-1})	Revertants/plate (mean ± standard deviation)				
			TA98	TA100	TA1535	TA1537	TA102
With Activation (Rat liver)	Purified water	0	36 ± 5	118 ± 10	19 ± 8	21 ± 6	213 ± 25
	4-Methylimidazole	5	35 ± 6	121 ± 10	23 ± 6	28 ± 8	218 ± 12
	4-Methylimidazole	15.81	41 ± 7	120 ± 5	21 ± 5	26 ± 6	223 ± 15
	4-Methylimidazole	50	40 ± 4	123 ± 7	15 ± 2	22 ± 5	233 ± 18
	4-Methylimidazole	158.1	37 ± 2	118 ± 9	20 ± 7	18 ± 3	234 ± 7
	4-Methylimidazole	500	40 ± 11	115 ± 6	20 ± 5	17 ± 2	213 ± 11
	4-Methylimidazole	1581	40 ± 9	100 ± 23	17 ± 3	24 ± 8	228 ± 9
	4-Methylimidazole	5000	34 ± 3	103 ± 8	10 ± 0	18 ± 7	213 ± 19
	Benzo[a]pyrene (B[a]P)	10	163 ± 67	NT	NT	NT	NT
	2-Aminoanthracene (AAN)	5	NT	1191 ± 163	173 ± 22	72 ± 14	NT
	2-Aminoanthracene (AAN)	20	NT	NT	NT	NT	1225 ± 19

NT: Not tested.

Table 5: Mutagenicity of 4-Methylimidazole in the presence of rat liver S9, preincubation methodology (Beevers and Adamson, 2016).

Metabolic activation	Test article	Concentration (µg plate^{-1})	Revertants/plate (mean ± standard deviation)				
			TA98	TA100	TA1535	TA1537	TA102
	Purified water	0	37 ± 7	119 ± 22	16 ± 5	18 ± 3	231 ± 17
With	4-Methylimidazole	156.3	31 ± 8	122 ± 2	13 ± 3	24 ± 2	215 ± 6
Activation	4-Methylimidazole	312.5	32 ± 2	106 ± 13	16 ± 8	21 ± 5	239 ± 10
(Rat liver)	4-Methylimidazole	625	36 ± 12	104 ± 11	14 ± 4	17 ± 3	240 ± 12
	4-Methylimidazole	1250	25 ± 10	103 ± 13	16 ± 3	23 ± 4	239 ± 8
	4-Methylimidazole	2500	29 ± 8	97 ± 15	18 ± 1	15 ± 1	236 ± 8
	4-Methylimidazole	5000	20 ± 7	96 ± 6	10 ± 1	12 ± 2	199 ± 15
	Benzo[a]pyrene (B[a]P)	10	135 ± 13	NT	NT	NT	NT
	2-Aminoanthracene (AAN)	5	NT	1446 ± 76	71 ± 13	64 ± 1	NT
	2-Aminoanthracene (AAN)	20	NT	NT	NT	NT	1182 ± 51

NT: Not tested.

Table 6: Mutagenicity of 4-Methylimidazole in the presence of rat lung S9, plate incorporation methodology (Beevers and Adamson, 2016).

			Revertants/plate (mean ± standard deviation)				
Metabolic activation	Test article	Concentration (μg plate^{-1})	TA98	TA100	TA1535	TA1537	TA102
With Activation (Rat lung)	Purified water	0	21 ± 4	111 ± 17	18 ± 5	16 ± 5	221 ± 6
	4-Methylimidazole	5	28 ± 1	119 ± 11	18 ± 3	14 ± 4	192 ± 11
	4-Methylimidazole	15.81	27 ± 2	110 ± 4	22 ± 6	12 ± 4	211 ± 4
	4-Methylimidazole	50	30 ± 6	112 ± 16	19 ± 2	11 ± 4	214 ± 14
	4-Methylimidazole	158.1	25 ± 3	105 ± 11	20 ± 5	10 ± 4	216 ± 15
	4-Methylimidazole	500	28 ± 3	114 ± 17	23 ± 1	12 ± 2	215 ± 3
	4-Methylimidazole	1581	27 ± 7	103 ± 9	18 ± 5	11 ± 1	229 ± 2
	4-Methylimidazole	5000	23 ± 6	112 ± 2	18 ± 3	13 ± 8	213 ± 17
	Benzo[a]pyrene (B[a]P)	10	55 ± 3	NT	NT	NT	NT
	2-Aminoanthracene (AAN)	5	NT	1265 ± 390	180 ± 28	192 ± 6	NT
	2-Aminoanthracene (AAN)	20	NT	NT	NT	NT	1268 ± 328

NT: Not tested.

Table 7: Mutagenicity of 4-Methylimidazole in the presence of rat lung S9, preincubation methodology (Beevers and Adamson, 2016).

			Revertants/plate (mean ± standard deviation)				
Metabolic activation	Test article	Concentration (μg plate^{-1})	TA98	TA100	TA1535	TA1537	TA102
	Purified water	0	29 ± 4	88 ± 4	15 ± 5	17 ± 4	236 ± 13
With Activation (Rat lung)	4-Methylimidazole	156.3	29 ± 5	83 ± 6	21 ± 5	16 ± 8	222 ± 24
	4-Methylimidazole	312.5	31 ± 5	88 ± 19	18 ± 7	11 ± 2	192 ± 45
	4-Methylimidazole	625	30 ± 0	93 ± 17	12 ± 7	16 ± 4	246 ± 15
	4-Methylimidazole	1250	28 ± 3	70 ± 14	19 ± 3	10 ± 5	224 ± 3
	4-Methylimidazole	2500	25 ± 10	83 ± 8	18 ± 7	11 ± 3	217 ± 15
	4-Methylimidazole	5000	22 ± 9	76 ± 5	13 ± 3	11 ± 3	206 ± 19
	Benzo[a]pyrene (B[a]P)	10	57 ± 4	NT	NT	NT	NT
	2-Aminoanthracene (AAN)	5	NT	999 ± 144	184 ± 33	195 ± 68	NT
	2-Aminoanthracene (AAN)	20	NT	NT	NT	NT	1029 ± 170

NT: Not tested.

Table 8: Mutagenicity of 4-Methylimidazole in the presence of mouse liver S9, plate incorporation methodology (Beevers and Adamson, 2016).

			Revertants/plate (mean ± standard deviation)				
Metabolic activation	Test article	Concentration (μg plate^{-1})	TA98	TA100	TA1535	TA1537	TA102
	Purified water	0	28 ± 4	104 ± 22	15 ± 2	12 ± 7	235 ± 28
With Activation (Mouse liver)	4-Methylimidazole	5	34 ± 7	113 ± 17	20 ± 1	17 ± 8	248 ± 23
	4-Methylimidazole	15.81	23 ± 3	112 ± 15	20 ± 4	18 ± 7	260 ± 10
	4-Methylimidazole	50	25 ± 11	117 ± 5	12 ± 2	21 ± 1	261 ± 35
	4-Methylimidazole	158.1	32 ± 2	128 ± 6	10 ± 2	24 ± 5	256 ± 13
	4-Methylimidazole	500	27 ± 8	124 ± 10	16 ± 4	13 ± 2	277 ± 5
	4-Methylimidazole	1581	28 ± 9	109 ± 8	18 ± 3	18 ± 8	260 ± 27
	4-Methylimidazole	5000	28 ± 5	110 ± 3	16 ± 4	19 ± 3	245 ± 13
	Benzo[a]pyrene (B[a]P)	10	133 ± 29	NT	NT	NT	NT
	2-Aminoanthracene (AAN)	5	NT	1031 ± 73	489 ± 41	114 ± 10	NT
	2-Aminoanthracene (AAN)	20	NT	NT	NT	NT	638 ± 14

NT: Not tested.

Table 9: Mutagenicity of 4-Methylimidazole in the presence of mouse liver S9, preincubation methodology (Beevers and Adamson, 2016).

			Revertants/plate (mean ± standard deviation)				
Metabolic activation	Test article	Concentration (μg plate^{-1})	TA98	TA100	TA1535	TA1537	TA102
	Purified water	0	24 ± 8	99 ± 2	14 ± 7	16 ± 5	234 ± 26
With	4-Methylimidazole	156.3	25 ± 7	92 ± 9	15 ± 3	13 ± 3	238 ± 15
Activation	4-Methylimidazole	312.5	23 ± 12	93 ± 11	13 ± 3	10 ± 3	234 ± 8
(Mouse liver)	4-Methylimidazole	625	28 ± 8	83 ± 24	14 ± 3	11 ± 1	223 ± 27
	4-Methylimidazole	1250	29 ± 4	95 ± 7	11 ± 4	12 ± 6	226 ± 15
	4-Methylimidazole	2500	22 ± 3	84 ± 17	15 ± 7	10 ± 1	185 ± 11
	4-Methylimidazole	5000	21 ± 9	81 ± 9	7 ± 4	9 ± 5	168 ± 19
	Benzo[a]pyrene (B[a]P)	10	164 ± 17	NT	NT	NT	NT
	2-Aminoanthracene (AAN)	5	NT	1248 ± 100	368 ± 17	191 ± 12	NT
	2-Aminoanthracene (AAN)	20	NT	NT	NT	NT	724 ± 43

NT: Not tested.

References

Beevers, C., and Adamson, R.H. (2016). Evaluation of 4-methylimidazole, in the Ames/Salmonella test using induced rodent liver and lung S9. Environ Mol Mutagen. 57: 51-7.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., and Mortelmans, K. (1988). Salmonella mutagenicity tests: IV. Results from the testing of 300 chemicals. Environ. Mol. Mutagen. 11 (Suppl. 12), 1-158.

3.2.1.1 Sister chromatid exchange (SCE), chromosome aberration (CA) and micronucleus (MN) tests in human peripheral lymphocytes

Study reference:

Celik, R., and Topaktas, M. (2018). Genotoxic effects of 4-methylimidazole on human peripheral lymphocytes in vitro, Drug and Chemical Toxicology 41: 27-32.

Detailed study summary and results:

Test type

In vitro sister chromatid exchange (SCE), chromosome aberration (CA) and micronucleus (MN) tests were used. The methods of Evans (1984) and Perry & Thomson (1984) were followed for preparation of the CA and the SCE tests, with minor modifications, and MN test according to Rothfuss et al. (2000). Non guideline.

Test substance

- Test material used in the study (4-methylimidazole) was supplied from Sigma-Aldrich (CAS No: 822-36-6)
- Purity 98 %

Administration/exposure

- The genetic toxicity of 4-methylimidazole was assessed by testing the ability of the chemical to induce SCE, CA and MN formation in human peripheral lymphocytes *in vitro*. For this purpose, the cells were treated with 300, 450, 600 µg/ml for 24 h and 48 h periods
- Whole blood from four healthy donor (two males and two females) were used for the SCE, CA and the MN test.

Results and discussion

Effects on SCE

- After a 48 h treatment period, except the lowest, all concentrations of 4-methylimidazole induced SCE.

Table 10. Effect of 4-methylimidazole on SCE in human peripheral lymphocytes (Celik and Topaktas, 2018).

	Treatment			
Test substance	Time (h)	Conc. (µg/mL)	Min–Max. SCE	SCE/cell ± SE
Control	–	–	0–14	4.55 ± 0.44
MMC	24	0.25	8–80	23.18 ± 1.9
4MEI	24	300	0–22	4.76 ± 0.18 b_3
4MEI	24	450	0–24	6.04 ± 0.60 b_3
4MEI	24	600	0–64	7.74 ± 0.19 b_3
4MEI	24	750	1–40	8.24 ± 0.29 b_3
P. control	48	0.25	5–89	31.31 ± 1.94
4MEI	48	300	2–46	6.39 ± 0.35 b_3
4MEI	48	450	3–31	9.04 ± 0.56 a_1 b_3
4MEI	48	600	0–42	11.24 ± 0.90 a_3 b_3
4MEI	48	750	2–39	12.54 ± 0.41 a_3 b_3

Data are expressed as the mean values (±SE) obtained from four donors (N=4). aSignificant from control in which a_1 shows $p<0.05$, a_2 shows $p<0.01$ and a_3 shows $p<0.001$. bSignificant from positive control in which b_1 shows $p<0.05$, b_2 shows $p<0.01$ and b_3 shows $p<0.001$.

- 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 (Table 11).

Table 11. Effect of 4-methylimidazole on CA in human peripheral lymphocytes (Celik and Topaktas, 2018).

Test substance	Treatment		Structural CA		Percentage of cells with aberrations ± SE	CA/cell ± SE
	Time (h)	Conc. (µg/mL)	Chromatid type	Chromosome type		
Control	–	–	10	13	5.75 ± 1.03	0.06 ± 0.009
P. control	24	0.25	71	90	33.50 ± 2.63	0.41 ± 0.034
4MEI	24	300	39	27	14.25 ± 2.02 b_2	0.17 ± 0.027
4MEI	24	450	45	55	19.50 ± 1.71 b_1	0.25 ± 0.011
4MEI	24	600	54	55	19.75 ± 1.44 a_1 b_1	0.28 ± 0.023 a_1 b_2
4MEI	24	750	84	84	35.75 ± 6.17 a_3	0.43 ± 0.067 a_3 b_1
P. control	48	0.25	97	128	46.75 ± 8.98	0.58 ± 0.110
4MEI	48	300	66	45	22.50 ± 1.04 a_1 b_2	0.29 ± 0.011 a_1 b_1
4MEI	48	450	66	52	23.75 ± 2.56 a_1 b_2	0.31 ± 0.040 a_2 b_1
4MEI	48	600	70	56	26.50 ± 3.88 a_2 b_1	0.35 ± 0.060 a_2 b_1
4MEI	48	750	88	66	32.25 ± 0.63 a_3	0.42 ± 0.014 a_3

Data are expressed as the mean values (±SE) obtained from four donors (N=4). aSignificant from control in which a_1 shows $p<0.05$, a_2 shows $p<0.01$ and a_3 shows $p<0.001$. bSignificant from positive control in which b_1 shows $p<0.05$, b_2 shows $p<0.01$ and b_3 $p<0.001$.

- 4-Methylimidazole induced the formation of MN at the two highest concentrations (600 and 750 µg/ml) in 24 h and 48 h treatment groups (Table 12)

Table 12. Frequency of micronucleated binuclear (MNBN) cells and MN % in cultured human peripheral lymphocytes treated with 4-Methylimidazole (Celik and Topaktas, 2018).

Test substance	Treatment		Percentage of MNBN cell ± SE	MN % ± SE
	Time (h)	Conc. (µg/mL)		
Control	–	–	0.225 ± 0.048	0.225 ± 0.048
P. control	24	0.25	0.450 ± 0.087	0.500 ± 0.071
4MEI	24	300	0.450 ± 0.132	0.475 ± 0.155
4MEI	24	450	0.325 ± 0.063	0.325 ± 0.063
4MEI	24	600	1.025 ± 0.075 a_2	1.050 ± 0.096 a_2
4MEI	24	750	1.450 ± 0.362 a_3 b_2	1.475 ± 0.382 a_3 b_2
P. control	48	0.25	1.075 ± 0.125	1.075 ± 0.125
4MEI	48	300	0.475 ± 0.075 b_2	0.500 ± 0.091 b_2
4MEI	48	450	0.675 ± 0.075 b_1	0.700 ± 0.091
4MEI	48	600	0.950 ± 0.065 a_2	0.975 ± 0.075 a_2
4MEI	48	750	1.375 ± 0.125 a_3	1.400 ± 0.147 a_3

Data are expressed as the mean values (± SE) obtained from four donors (N=4). aSignificant from control in which a_1 shows $p<0.05$, a_2 shows $p<0.01$ and a_3 shows $p<0.001$. bSignificant from positive control in which b1 shows $p<0.05$, b_2 shows $p<0.01$ and b_3 shows $p<0.001$.

Cytotoxicity:

- 4-Methylimidazole negatively affected the mitosis in 24 h treatment group at the highest concentration, while the same effect was seen at all concentrations after 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

Table 13. MI, PI and NDI in human peripheral lymphocytes treated with 4-Methylimidazole (Celik and Topaktas, 2018).

	Treatment				
Test substance	Time (h)	Conc. (µg/mL)	MI ± SE	PI ± SE	NDI ± SE
Control	–	–	5.73 ± 0.46	1.97 ± 0.02	1.496 ± 0,060
P. control	24	0.25	2.62 ± 0.41	1.58 ± 0.08	1.297 ± 0,020
4MEI	24	300	5.22 ± 0.85 b_1	1.72 ± 0.08 a_1	1.251 ± 0,020 a_3
4MEI	24	450	3.63 ± 0.59	1.72 ± 0.09 a_1	1.225 ± 0,030 a_3
4MEI	24	600	4.12 ± 0.34	1.54 ± 0.07 a_3	1.166 ± 0.020 a_3 b_1
4MEI	24	750	2.86 ± 0.43 a_2	1.42 ± 0.03 a_3	1.151 ± 0.020 a_3b_2
P. control	48	0.25	1.47 ± 0.37	1.54 ± 0.04	1.246 ± 0.050
4MEI	48	300	3.18 ± 0.79 a_1	1.79 ± 0.05 b_2	1.186 ± 0.020 a_3
4MEI	48	450	2.42 ± 0.55 a_3	1.60 ± 0.02 a_3	1.113 ± 0.020 a_3
4MEI	48	600	2.51 ± 0.49 a_2	1.51 ± 0.07 a_3	1.097 ± 0.007 a_3 b_1
4MEI	48	750	1.57 ± 0.43 a_3	1.41 ± 0.01 a_3	1.061 ± 0.006 a_3 b_2

Data are expressed as the mean values (±SE) obtained from four donors (N=4). aSignificant from control in which a_1 shows $p<0.05$, a_2 shows $p<0.01$ and a_3 $p<0.001$. bSignificant from positive control in which b_1 shows $p<0.05$, b_2 shows $p<0.01$ and b_3 shows $p<0.001$.

Cytotoxicity was observed at concentration levels where also indications of genotoxicity were observed, and the number of blood donors (N=4) are quite low for SCE, CA and the MN test. The authors conclude that 4-methylimidazole has a genotoxic effect, shown as induced SCE, CA and MN formation, which is in contrast to the in vivo and in vitro genotoxicity studies reported in NTP TR 2007. This academic study has major deviations compared to OECD test guidelines (e.g. cytotoxicity at dose levels where genotoxicity were observed), are of low reliability, and are not suitable for comarisons with classification criteria. Potentially the observed increased genotoxicity could be an indirect consequence of high cytotoxicity.

3.2.1.2 Micronucleated erythrocytes in rat and mouse bone marrow

Study reference:

NTP, 2007, TOXICOLOGY AND CARCINOGENESIS STUDIES OF 4-METHYLIMIDAZOLE

Detailed study summary and results:

Test type

- Micronucleated erythrocytes in rat and mouse bone marrow (detailed protocol is presented by Shelby *et al*., 1993).

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier
- 99.5% purity
- Unknown impurities less than 1%.
- Lot number 116H0901, from Sigma Chemical Company (St. Louis, MO)

Test animals

- F344/N rats, Taconic Farms, Inc. (Germantown, NY)
- $B6C3F_1$ male mice, Taconic Farms, Inc. (Germantown, NY)

- Mice age 9 to 14 weeks, weighing within a 2 g range of a mean body weight between 25 and 33 g were used
- Number of male rats with erythrocytes scored: 5

Administration/exposure

- Route of administration – intraperitoneal (three times at 24-hour intervals on three consecutive days) with 4-methylimidazole (0, 25, 50 100 mg/kg body weight)

Results and discussion

- No effects (no increases in the frequencies of micronucleated erythrocytes) were seen in bone marrow of male rats (Table 14). In mice, 4-methylimidazole at 50 and 100 mg/kg produced significant increases in the frequency of micronucleated PCEs in the bone marrow in the first trial, however, no evidence of MN induction was observed at the same doses of 50 and 100 mg/kg in the second trial (Table 15). NTP critically evaluated all the data, and ultimately concluded that the mouse bone marrow MN assay was negative overall.
- No significant alterations in percent micronucleated polychromatic erythrocytes (PCEs), a rough indicator of bone marrow toxicity, were seen in the mouse bone marrow or peripheral blood tests, but in bone marrow of male rats, percent PCEs declined with increasing dose of 4-methylimidazole and were significantly depressed at the highest dose.
- A positive control showed the sensitivity of the test systems.

Table 14: Induction of Micronuclei in Bone Marrow Erythrocytes of Male Rats Treated with 4-Methylimidazole by Intraperitoneal Injection[a] (NTP TR 2007).

Compound	Dose (mg/kg)	Number of Male Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	Pairwise P Value[c]	PCEs[b] (%)
Phosphate-buffered saline[d]					
	0	5	1.70 ± 0.25		47.80 ± 3.04
4-Methylimidazole	25	5	1.60 ± 0.19	0.5692	42.6 ± 3.5
	50	5	1.40 ± 0.29	0.7051	40.5 ± 3.6
	100	4	0.88 ± 0.24	0.9341	30.8 ± 2.5
			P = 0.939[e]		
Cyclophosphamide[f]	7.5	5	22.30 ± 1.62	0.0000	33.0 ± 3.5

[a] Study was performed at Environmental Health Research and Testing, Inc. The detailed protocol is presented by Shelby *et al.* (1993). PCE=polychromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the solvent control; dosed groups significant at P#0.008; positive control significant at P#0.05 (ILS, 1990)

[d] Solvent control

[e] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test, significant at P#0.025 (ILS, 1990)

[f] Positive control

Table 15: Induction of Micronuclei in Bone Marrow Erythrocytes of Male Mice treated with 4-Methylimidazole by Intraperitoneal Injection[a] (NTP TR 2007).

Compound	Dose (mg/kg)	Number of Male Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	Pairwise P Value[c]	PCEs[b] (%)
Trial 1					
Phosphate-buffered saline[d]	0	5	2.20 ± 0.44		54.4 ± 0.8
4-Methylimidazole	25	5	2.50 ± 0.22	0.3307	51.4 ± 2.3
	50	5	4.30 ± 1.08	0.0045	53.8 ± 2.9
	100	5	4.10 ± 0.58	0.0083	48.7 ± 2.3
			P = 0.003[e]		
Cyclophosphamide[f]	25	5	31.30 ± 1.81	0.0000	44.0 ± 1.5
Trial 2					
Phosphate-buffered saline	0	5	2.50 ± 0.22		48.1 ± 3.6
4-Methylimidazole	25	5	3.00 ± 0.27	0.2498	51.8 ± 5.7
	50	5	3.10 ± 0.66	0.2110	46.8 ± 3.3
	100	5	2.40 ± 0.56	0.5569	53.4 ± 2.3
			P = 0.614		
Cyclophosphamide	10	5	12.90 ± 1.26	0.0000	49.0 ± 1.9

[a] Study was performed at Environmental Health Research and Testing, Inc. The detailed protocol is presented by Shelby *et al.* (1993). PCE=polychromatic erythrocyte

[b] Mean ± standard error Pairwise comparison with the solvent control; dosed groups significant at P≤0.008; positive control significant at P≤0.05 (ILS, 1990)

[d] Solvent control

[e] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test, significant at P≤0.025 (ILS, 1990)

[f] Positive control

Reference

Shelby, M.D., Erexson, G.L., Hook, G.J., and Tice, R.R. (1993). Evaluation of a three-exposure mouse bone marrow micronucleus protocol: Results with 49 chemicals. Environ. Mol. Mutagen. 21, 160-179.

3.2.1.3 Mouse peripheral blood micronucleus test

Detailed study summary and results:

Test type

- NTP Mouse peripheral blood micronucleus test (detailed protocol presented by MacGregor et al., 1990). The 14-week toxicity study of 4-methylimidazole (where peripheral blood for the micronucleus test were obtained from) were conducted in compliance with Food and Drug Administration Good Laboratory Practices Regulations (21 CFR, Part 58). GLP

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier
- 99% purity
- Unknown impurities less than 1%.
- Lot no. 08302BF supplied by Aldrich Chemical Company (Milwaukee, WI)

Test animals

- $B6C3F_1$ MICE, Taconic Farms, Inc. (Germantown, NY)
- Age 7 weeks at start of exposure

Administration/exposure

- Exposure 7 days/week by feed, available *ad libitum*
- 14-week toxicity study
- 65, 170, or 500 mg/kg body weight to males and females
- Number of male and female mice with erythrocytes scored: 5

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- 4-methylimidazole produced no effects in 14-week peripheral blood micronucleus tests in male and female mice (Table 16).

Table 16: Frequency of Micronuclei in Peripheral Blood Erythrocytes in Mice Following Treatment with 4-Methylimidazole in Feed for 14 weeks[a] (NTP, 2007).

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	Pairwise P Value[c]	PCE[b] (%)
Male					
NIH-07 feed[d]	0	5	1.90 ± 0.56		7.3 ± 0.7
4-methylimidazole	625	5	1.70 ± 0.20	0.6307	9.0 ± 0.8
	1,250	5	1.90 ± 0.33	0.5000	8.2 ± 0.7
	2,500	5	2.10 ± 0.24	0.3758	7.2 ± 1.0
	5,000	5	2.50 ± 0.59	0.1826	6.7 ± 1.2
	10,000	3	1.83 ± 0.33	0.5376	8.0 ± 1.3
			P = 0.326[e]		
Female					
NIH-07 feed	0	5	2.30 ± 0.25		8.1 ± 1.6

4-methylimidazole	625	5	2.40 ± 0.43	0.4419	5.8 ± 0.8
	1,250	5	2.50 ± 0.35	0.3863	7.3 ± 0.5
	2,500	5	1.70 ± 0.44	0.8289	7.5 ± 1.1
	5,000	5	2.50 ± 0.32	0.3863	6.7 ± 0.4
	10,000	5	2.90 ± 0.70	0.2024	6.6 ± 0.4
			P = 0.153		

[a] Study was performed at Environmental Health Research and Testing, Inc. The detailed protocol is presented by MacGregor *et al.* (1990). NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte

[b] Mean ± standard error Pairwise comparison with the vehicle control; significant P≤0.005 (ILS, 1990)

[d] Vehicle control

[e] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at P≤0.025 (ILS, 1990)

Reference

MacGregor, J.T., Wehr, C.M., Henika, P.R., and Shelby, M.D. (1990). The in vivo erythrocyte micronucleus test: Measurement at steady state increases assay efficiency and permits integration with toxicity studies. Fundam. Appl. Toxicol. 14, 513-522.

Dalvie, D.K., Kalgutkar, A.S., Khojasteh-Bakht, S.C., Obach, R.S., and O'Donnell, J.P. (2002). Biotransformation reactions of five-membered aromatic heterocyclic rings. Chem. Res. Toxicol. 15, 269-299.

3.2.1.4 Chromosomal aberration test in mouse bone marrow cells

Study reference:

Norizadeh Tazehkand, M., Topaktas, M., Yilmaz, M.B. (2016). Assessment of chromosomal aberration in the bone marrow cells of Swiss Albino mice treated by 4-methylimidazole. Drug Chem Toxicol. 39, 307-311.

Detailed study summary and results:

Test type

- Chromosomal aberration test in mouse bone marrow cells (Norizadeh Tazehkand et al. 2016). Non-guideline study.

Test substance

- Test material is 4-methylimidazole (CAS Number: 822-36-6)
- 98 % purity

Test animals

- Male and female adult Swiss Albino mice (Medical Sciences, Experimental Research and Application Center of Cukurova University,Turkey)
- Body weight 33-40 g (not reported whether this was at arrival or at start of dosing)

Administration/exposure

- 4-Methylimidazole was dissolved in double distilled water and administered as a single dose of 0.5 mL per mouse by intraperitoneal administration
- 100, 130 and 160 mg/kg body weight to males and females (three females and three males per dosing group)
- CA and mitotic index (MI) of the mouse bone marrow cells were analyzed after treating the animals with 4-methylimidazole for 12 h and 24 h.

Results and discussion

4-Methylimidazole increased the percentage of CAs at all concentrations for 12 h and at highest concentration for 24 h treatment periods (Table 17). The mitotic index decreased in comparison with control at highest concentration for 12 h and at all concentrations for 24 h (Table 18). This academic study has major deviations compared to OECD test guidelines, are of low reliability, and are not suitable for comarisons with classification criteria.

Table 17. CA in bone marrow cells of Swiss Albino mice (Norizadeh Tazehkand *et al*., 2016).

	Treatment		Structural CA		Percentage of cells with aberrations ± SE
Test substance	Time (h)	Conc. (mg/kg)	Chromatid type	Chromosome type	
Control	–	–	1	5	1.00 ± 0.258 b_3
EMS	12	240	6	17	3.83 ± 0.401 a_3
4-MEI	12	100	7	7	2.33 ± 0.333 a_1b_2
4-MEI	12	130	8	6	2.33 ± 0.333 a_1b_3
4-MEI	12	160	7	8	2.50 ± 0.500 a_1b_1
EMS	24	240	5	13	3.00 ± 0.447 a_2
4-MEI	24	100	3	3	1.00 ± 0.258 b_3
4-MEI	24	130	4	4	1.33 ± 0.422 b_1
4-MEI	24	160	5	9	2.33 ± 0.333 a_1

Data are expressed as the mean values (±SE) obtained from six mice bone marrow cells (N=6). a: significant from negative control; b: significant from positive control (EMS). a_1b_1: $p<0.05$; a_2b_2: $p<0.01$; a_3b_3: $p<0.001$.

The MI at highest concentration for 12 h and at all concentrations for 24 h decreased in comparison with control (Table 18).

Table 18. MI in bone marrow cells of Swiss Albino mice (Norizadeh Tazehkand *et al*., 2016).

	Treatment		
Test substance	Time (h)	Conc. (mg/kg)	MI ± SE
Control	–	–	5.532 ± 0.315
EMS	12	240	2.217 ± 0.294 a_3
4-MEI	12	100	4.512 ± 0.437 b_2
4-MEI	12	130	4.965 ± 0.351 b_2
4-MEI	12	160	2.222 ± 0.438 a_3
EMS	24	240	2.683 ± 0.273 a_3
4-MEI	24	100	2.983 ± 0.445 a_2
4-MEI	24	130	2.863 ± 0.432 a_2
4-MEI	24	160	1.862 ± 0.191 a_3b_1

Data are expressed as the mean values (±SE) obtained from six mice bone marrow cells (N=6). a: significant from negative control; b: significant from positive control (EMS). a_1b_1: $p<0.05$; a_2b_2: $p<0.01$; a_3b_3: $p<0.001$.

3.3 Animal data

No data available.

3.4 Human data

No data available.

3.5 Carcinogenicity

3.5.1 Animal data

3.5.1.1 Rat 2-year study

Study reference:

NTP, 2007, TOXICOLOGY AND CARCINOGENESIS STUDIES OF 4-METHYLIMIDAZOLE. The study is also described by Chan PC, Hills GD, Kissling GE, Nyska A, 2008. Toxicity and carcinogenicity studies of 4-methylimidazole in F344/N rats and B6C3F1 mice. Arch Toxicol, 82:45–53.

Detailed study summary and results:

Test type

- NTP 2-year cancer bioassay, GLP

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier
- EC number 212-497-3
- Cas number 822-36-6
- > 99% pure
- Impurities not identified, still assumed not to affect the classification due to high purity
- Lot number 116H0901, from Sigma Chemical Company (St. Louis, MO)

Test animals

- F344/N RATS, Taconic Farms, Inc. (Germantown, NY)
- 50 animals per sex per dose
- Average age 6 weeks, average weight per group of 123-124 g (m) and 98-100 g (f)

Administration/exposure

- Route of administration – oral (via feed)

- 106 weeks duration
- 0, 625, 1,250, or 2,500 ppm (males) or 0, 1,250, 2,500, or 5,000 ppm (females) in feed; equivalent to average daily doses of approximately 30, 55, and 115 mg 4-methylimidazole/kg body weight to males and 60, 120, and 260 mg/kg to females). The highest dose was selected based on the reduced body weights observed in the 14-week toxicity study.
- Exposure daily by feed, available *ad libitum*
- Historical control data; from database over NTP-studies that use the NTP-2000 diet
- Chemical stability was monitored during the 2-year studies; no degradation of the bulk chemical was detected. Homogeneity and stability of the dose formulations was confirmed. The dose formulations were prepared every 2 weeks.

Results and discussion

Describe the relevant findings. If no effects occurred, explicitly note "No effects".

- No significant effect on survival were reported. There were 50 animals per sex per dose. Control group: 31(m) and 43 (f) survived to study termination, mean survival days of 701 (m) and 697 (f); 625 ppm (males only): 34 survived to study termination, mean survival days of 681; 1,250 ppm: 33 (m) and 39 (f) survived to study termination, mean survival days of 695 (m) and 701(f); 2,500 ppm: 32 (m) and 34 (f) survived to study termination, mean survival days of 689 (m) and 684 (f); 5000 ppm (female only): 35 survived to study termination, mean survival days of 691.
- 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.
- Lower mean body weights of males in the 1,250 and 2,500 ppm groups and females in the 2,500 and 5,000 ppm groups compared to controls. Reduced feed consumption reported in high dose females (5,000 ppm).
- The complete data was not available via the public information from NTP nor in Chan et al. (2008). However, the NTP report states that at the same exposure concentrations in the 14-week NTP toxicity study, there were minimal effects in hematology and clinical chemistry. In males of the 2500 ppm group, liver weights were increased and vacuolization was observed in hepatocytes. In females of the 5000 ppm group, spleen weights were reduced compared to controls.
- **Neoplastic lesions**: The incidence of mononuclear cell leukemia in 5,000 ppm females was significantly greater than that in the controls (Poly-3 stat. test on adjusted rate data), and the incidence exceeded the historical range in study controls given the NTP-2000 diet. Overall rate: 18%, 14%, 32%, 40% in 0, 1250, 2500 and 5000 ppm exposure, respectively. Onset in 5,000 ppm females was earlier (day 368) than in control females (day 624).

- 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 5000 ppm exposure, respectively. A mean incidence of 46.8% in historical control data was reported. No differences in time of onset.
- 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. These incidences in the exposed groups were either below the historical control ranges or at the lower end of the historical control ranges in study controls given the NTP-2000 diet.
- 4-methylimidazole was mostly negative in the genotoxicity assays available (refere section 3.1/3.2).
- Non-neoplastic lesions: Significant (Poly-3 test) increases in minimal to mild non-neoplastic liver lesions (histiocytosis, chronic inflammation and fatty change) in males and females, chronic inflammation of the prostate gland (m), hyperthtophy of the pituitary gland (m) and increase in number of males with follicle cysts in the thyroid gland. In females minimal to mild lesions in the thyroid gland (follicle minerilisation in the high dose group), lung (chronic, focal inflammation), heart (cardiomyopathy) and pancreas (acinus, atrophy, focal).

3.5.1.2 Mouse 2-year study

Study reference:

NTP, 2007, TOXICOLOGY AND CARCINOGENESIS STUDIES OF 4-METHYLIMIDAZOLE. The study is also described by Chan PC, Hills GD, Kissling GE, Nyska A, 2008. Toxicity and carcinogenicity studies of 4-methylimidazole in F344/N rats and B6C3F1 mice. Arch Toxicol, 82:45–53.

Detailed study summary and results:

Test type

NTP 2 year cancer bioassay, GLP.

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier
- EC number 212-497-3
- CAS number 822-36-6
- > 99% pure
- Impurities not identified, still assumed not to affect the classification due to high purity
- Lot number 116H0901, from Sigma Chemical Company (St. Louis, MO)

Test animals

- B6C3F1 mice, 50 animals per sex per dose, Taconic Farms, Inc. (Germantown, NY)
- Average age of 6 weeks at study initiation, average weight per group of 21.1-21.3 g (m) and 17.3-17.4 g (f)

Administration/exposure

- Route of administration – oral (via feed)
- 106 weeks duration
- 0, 312, 625, or 1,250 ppm 4-methylimidazole (equivalent to average daily doses of approximately 40, 80, and 170 mg 4-methylimidazole/kg body weight to males and females)
- Exposure daily by feed, available *ad libitum*
- Historical control data; from database over NTP-studies that use the NTP-2000 diet

Results and discussion

- No significant effect on survival compared to controls. There were 50 animals per sex per dose. Control group: 45/43 (m/f) survived to study termination, mean survival days of 717 (m) and 702 (f); 312 ppm: 44/40 (m/f) survived to study termination, mean survival days of 714/716 (m/f); 625 ppm: 42/43 (m/f) survived to study termination, mean survival days of 700 (m) and 717(f); 1250 ppm: 46/40 (m/f) survived to study termination, mean survival days of 721 (m) and 703 (f).
- No clinical findings in exposed groups of male or female mice were considered to be related to chemical exposure.
- Mean body weights of males and females in the 1,250 ppm groups were less than those in the control groups. Mean body weights of 312 and 625 ppm females were lower than controls after weeks 85 and 65, respectively.
- Feed consumption by exposed groups (m and f) was generally similar to that by the controls.
- The complete data was not available via the public information from NTP nor in Chan et al. (2008).
- **Neoplastic lesions:** The incidences of alveolar/bronchiolar adenoma in all exposed groups of females, alveolar/bronchiolar carcinoma in 1,250 ppm males, and alveolar/bronchiolar adenoma or carcinoma (combined) in 1,250 ppm males and 625 and 1,250 ppm females were significantly greater than those in the control groups as assessed byt the Poly-3 test. Incidences of alveolar/bronchiolar adenoma and carcinoma combined were as followes: 0 ppm, 18%/6% (m/f); 312 ppm, 26%/16% (m/f); 625 ppm, 32%/34% (m/f); 1250 ppm, 44%/28% (m/f). Mean (range) of the historical control data were 22,2% +/- 6.3% (14-32%) / 6.6% +/- 4.2% (0-12%) in males and females, respectively.
- Local or multi-site responses: No significant increases at other sites than in lungs were reported.
- Non-neoplastic lesions: The incidence of alveolar epithelium hyperplasia and of histiocytic cellular infiltration in 1,250 ppm females was significantly greater than that in the controls. The incidence of histiocytic cellular infiltration, was slightly increased in 1,250 ppm males.
- The incidence of thyroid follicular cyst in 1,250 ppm females was significantly greater than that in the controls (0 ppm, 20/50; 312 ppm, 22/49; 625 ppm, 29/50; 1,250 ppm, 30/48.

- There was a significant positive trend in the incidences of mammary gland hyperplasia in females (16/50, 10/50, 14/49, 24/49; P=0.013); however, none of the exposed groups differed significantly from the control group.
- Genetoxicity studies are mainly negative (refer section 3.1/3.2)

Table 19 Incidences of non-neoplastic and neoplastic lesions in the lungs of mice in the NTP 2-year cancer bioassay (Table from NTP 2008 report).

Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Mice in the 2-Year Feed Study of 4-Methylimidazole

	0 ppm	312 ppm	625 ppm	1,250 ppm
Male				
Number Examined Microscopically [a]	50	50	50	50
Alveolar Epithelium, Hyperplasia [b]	7 (2.0)	3 (1.0)	1 (2.0)	9 (1.9)
Infiltration Cellular, Histiocyte	5 (2.2)	6 (1.7)	5 (1.8)	11 (1.7)
Alveolar/bronchiolar Adenoma (includes multiple) [c]	8	11	13	15
Alveolar/bronchiolar Carcinoma (includes multiple) [d]				
Overall rate [e]	2/50 (4%)	4/50 (8%)	4/50 (8%)	8/50 (16%)
Adjusted rate [f]	4.1%	8.3%	8.8%	16.7%
Terminal rate [g]	1/45 (2%)	3/44 (7%)	4/42 (10%)	8/46 (17%)
First incidence (days)	513	613	729 (T)	729 (T)
Poly-3 test [h]	P=0.024	P=0.332	P=0.307	P=0.042
Alveolar/bronchiolar Adenoma or Carcinoma (combined) [i]				
Overall rate	9/50 (18%)	13/50 (26%)	16/50 (32%)	22/50 (44%)
Adjusted rate	18.4%	26.9%	35.0%	46.0%
Terminal rate	8/45 (18%)	11/44 (25%)	16/42 (38%)	22/46 (48%)
First incidence (days)	513	613	729 (T)	729 (T)
Poly-3 test	P<0.001	P=0.226	P=0.053	P=0.003

Female				
Number Examined Microscopically	50	50	50	50
Alveolar Epithelium, Hyperplasia	3 (1.7)	2 (2.5)	3 (1.7)	11* (1.9)
Infiltration Cellular, Histiocyte	1 (1.0)	5 (1.4)	1 (1.0)	8* (2.0)
Alveolar/bronchiolar Adenoma (includes multiple)[j]				
Overall rate	0/50 (0%)	8/50 (16%)	16/50 (32%)	8/50 (16%)
Adjusted rate	0.0%	16.6%	33.2%	17.4%
Terminal rate	0/43 (0%)	7/40 (18%)	15/43 (35%)	8/40 (20%)
First incidence (days)	—[k]	632	684	729 (T)
Poly-3 test	P=0.017	P=0.004	P<0.001	P=0.003
Alveolar/bronchiolar Carcinoma (includes multiple)[l]	3	0	2	7
Alveolar/bronchiolar Adenoma or Carcinoma (combined)[m]				
Overall rate	3/50 (6%)	8/50 (16%)	17/50 (34%)	14/50 (28%)
Adjusted rate	6.4%	16.6%	35.3%	30.3%
Terminal rate	3/43 (7%)	7/40 (18%)	16/43 (37%)	13/40 (33%)
First incidence (days)	729 (T)	632	684	687
Poly-3 test	P=0.002	P=0.109	P<0.001	P=0.002

* Significantly different ($P \leq 0.05$) from the control group by the Poly-3 test
(T) Terminal sacrifice
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Historical incidence for 2-year feed study controls given NTP-2000 diet (mean ± standard deviation): 75/510 (15.8% ± 6.3%); range, 9%-28%
[d] Historical incidence: 40/510 (7.8% ± 3.8%); range, 4%-14%
[e] Number of animals with neoplasm per number of animals with lung examined microscopically
[f] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[g] Observed incidence at terminal kill
[h] Beneath the control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.
[i] Historical incidence: 108/510 (22.2% ± 6.3%); range, 14%-32%
[j] Historical incidence: 19/509 (3.7% ± 3.8%); range, 0%-10%
[k] Not applicable; no neoplasms in animal group
[l] Historical incidence: 16/509 (2.9% ± 2.5%); range, 0%-6%
[m] Historical incidence: 35/509 (6.6% ± 4.2%); range, 0%-12%

3.5.2 **Human data**

No human data available

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

See section 3.1 Germ cell mutagenicity

3.6 Reproductive toxicity

3.6.1 Animal data

Two studies have been reported by the National Toxicology Program (NTP) with high relevance for the evaluation of reproductive toxicity of 4-methylimidazole.

1) A 14-week repeated dose toxicity study with rats and mice conducted by the NTP (NTP 2004) that includes reproductive organ histopathology and sperm quality analyses.
2) A reproductive and developmental toxicity study in rats following a continuous breeding protocol (NTP 2019; Behl et al., 2020).

3.6.1.1 NTP 14-week feed study of 4-methylimidazole in rats and mice

Study references:

NTP technical report 67 on the toxicity studies of 2- and 4-Methylimidazole (CAS No. 693-98-1 and 822-36-6) administered in feed to F344/N rats and B6C3F1 mice, 2004.

Chan et al., 2006. Induction of thyroid lesions in 14-week toxicity studies of 2 and 4-methylimidazole in Fischer 344/N rats and B6C3F1 mice. Arch. Toxicol. 80: 169-80.

Detailed study summary and results:

NTP 14-week feed study of 4-methylimidazole:

The 14-week studies of 2- and 4-methylimidazole was conducted in compliance with Food and Drug Administration Good Laboratory Practices Regulations (21 CFR, Part 58). Detailed study summary and results of the 14-weeks study on 4-methylimidazole is presented here in the annex.

- In the 14-week study, groups of 10 male and 10 female rats and mice were fed diets containing 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm 4-methylimidazole. Rats and female mice were housed five per cage and male mice were housed individually. Feed and water were available ad libitum. Clinical findings were recorded and animals were weighed initially, weekly, and at the end of the studies. Functional observation batteries - parameters as e.g. body position and activity level - were performed at weeks 5 and 12 on rats exposed to 0, 2,500, 5,000, or 10,000 ppm 4-methylimidazole. Feed consumption was measured weekly.

Test substance

- Test material used in the study is equivalent to the substance identified in the CLH dossier
- Purity: 99.0 ± 0.1%
- Impurities: One impurity peak with area to 0.1 % relative to major peak. This was not identified, but is assumed by the DS not to affect the classification due to the low concentration.

- Batch number: Lot 08302BF supplied by Aldrich Chemical Company (Milwaukee, WI).

Test animals

- Male and female F344/N rats and B6C3F1 mice were from Taconic Farms (Germantown, NY).
- 10 animals per sex per dose
- Average age 7 weeks; initial body weight rats; male (144 -146 g) and female (116 – 119 g) and initial body weight mice; male (22.2 – 23.9 g) and female (18.3 – 19.0 g).

Administration/exposure

- Route of administration – oral (feed)
- 14 weeks duration
- Doses/concentration levels, rationale for dose level selection
    - 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm in feed of 4-methylimidazole to male and female rats and mice, corresponding doses per kg bw/day are given below for each species.
- Exposure daily by feed, available ad libitum
- Dose formulations were prepared at the beginning of the studies, weekly for the first 4 weeks of the studies, and every 2 weeks thereafter. Homogeneity and stability studies of 4-methylimidazole (167, 300, 625, 1,500, 2,500, and 10,000 ppm) formulations were performed by the study laboratory using high-performance liquid chromatography. Homogeneity was verified. Stability was confirmed for up to 28 days. All dose formulations were within 10% of the target concentrations; 14 of 15 animal room samples for rats and 12 of 15 for mice were also within 10% of the target concentrations.
- The statistical evaluation was done using parametric one-way analysis of variance, and with the corresponding non-parametric test when appropriate. Duncans multiple range test was used to test the difference between test groups. Chi-square test was used to test independence between proportions, when appropriate.

Results and discussion

RATS

14-week study:

Dietary concentrations of 625, 1,250, 2,500, 5,000, or 10,000 ppm in the feed estimated to give daily doses of approximately 40, 80, 160, 300, or 560 mg/kg bw/day of 4-methylimidazole to males and females.

Mortality: One male rat died in the 10,000 ppm group during week 1 and one female rat in the 1,250 ppm group was killed moribund during week 9.

Clinical findings: nasal/eye discharge in males and females administered 2,500 ppm or greater; ruffled fur in males and females administered 5,000 or 10,000 ppm; and thinness, ataxia (females only), and abnormal breathing in males and females in the 10,000 ppm groups.

Food consumption: Reduced food intake was observed for 5,000 and 10,000 ppm groups.

Body weight: The final mean body weights and body weight gains of males from 2,500 ppm, 5,000 ppm, 10,000 ppm groups, and females from 5,000 and 10,000 ppm groups were significantly lower than those of the controls.

Table 20 (copy of Table 7 from NTP, 2004)

TABLE 7
Survival, Body Weights, and Feed Consumption of Rats in the 14-Week Feed Study of 4-Methylimidazole

Dose (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 14
Male							
0	10/10	144 ± 3	352 ± 6	208 ± 5	—	14.7	17.4
625	10/10	143 ± 4	362 ± 8	219 ± 7	103	14.3	16.6
1,250	10/10	146 ± 4	353 ± 6	207 ± 5	100	14.1	16.6
2,500	10/10	145 ± 4	335 ± 4*	190 ± 4**	95	13.7	16.4
5,000	10/10	144 ± 4	298 ± 4**	154 ± 2**	85	11.7	14.4
10,000	9/10[d]	144 ± 3	245 ± 4**	101 ± 3**	70	7.7	14.3
Female							
0	10/10	117 ± 2	201 ± 3	84 ± 2	—	11.6	10.6
625	10/10	116 ± 2	207 ± 3	91 ± 3	103	11.0	11.4
1,250	9/10[e]	116 ± 1	204 ± 2	88 ± 2	101	10.9	10.9
2,500	10/10	119 ± 2	198 ± 4	79 ± 3	98	10.0	9.9
5,000	10/10	117 ± 2	189 ± 6*	72 ± 5*	94	8.6	9.7
10,000	10/10	118 ± 2	127 ± 5**	9 ± 4**	63	5.0	7.6

* Significantly different (P≤0.05) from the control group by Williams' test
** P≤0.01
[a] Number of animals surviving at 14 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.
[c] Feed consumption is expressed as grams per animal per day.
[d] Week of death: 1
[e] Week of death: 9

Functional observations: On days 29 and 82; in 5,000 and 10,000 ppm rats included labored or increased respiration, mild tremors, walking on tiptoes, hunched posture, piloerection, crouching over, impaired coordination of movement, ataxia, and pupillary constriction.

Hematology and clinical chemistry: 4-Methylimidazole induced a transient erythrocytosis and a minimal, exposure concentration-related, microcytic, normochromic, nonresponsive anemia in male and female rats.

On day 8, there was evidence of a transient erythrocytosis; increased automated and manual hematocrit values, hemoglobin concentrations, and erythrocyte counts of 5,000 ppm males and 10,000 ppm males and females. On day 8, there was a minimal decrease in reticulocyte counts of 2,500 ppm males and 5,000 and 10,000 ppm males and females; this effect was transient and absent at the later time points. Further, decreases in mean cell volumes, mean cell hemoglobin values in males and females exposed to 5,000 or 10,000 ppm, and mean cell hemoglobin concentrations for males exposed to 2,500, 5,000 or 10,000 ppm at week 14, and females exposed to 2,500, 5,000 or 10,000 ppm on day 29. Transient decreased platelet counts on day 8 in males exposed to 2,500 ppm and males and females exposed to 5,000 or 10,000 ppm. By week 14, decreased platelet counts occurred only in 10,000 ppm females. The total protein and albumin concentrations of 10,000 ppm males and females on day 29 and 5,000 and 10,000 ppm females at week 14 were decreases. Further, increase in alkaline phosphatase activities of 10,000 ppm males and females on day 29 and of males and females exposed to 2,500 ppm or greater at week 14 were observed.

Necropsy findings: Microscopic liver analysis identified a significant increase in the incidences of cytoplasmic hepatocyte vacuolization in males exposed to 2,500 ppm or greater and 10,000 ppm females compared to the controls. The incidences of epididymal hypospermia and prostate gland inflammation were significantly increased in 10,000 ppm males. The incidences of prostate gland atrophy and testicular degeneration were significantly increased in 5,000 and 10,000 ppm males.

Gross Pathology: small testis and small uteri in the 560 mg/kg bw/day dose group male and female rats (uterus weights were not recorded).

Table 21: (Copy of Table 13 in NTP, 2004)

TABLE 13
Incidences of Selected Nonneoplastic Lesions in Rats in the 14-Week Feed Study of 4-Methylimidazole

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Male						
Liver[a]	10	—[c]	10	10	10	9
Hepatocyte, Vacuolization Cytoplasmic[b]	1 (1.0)[d]	—	3 (1.0)	10** (2.2)	10** (3.0)	9** (3.0)
Epididymis	10	—	—	—	—	10
Hypospermia	0	—	—	—	—	9**
Prostate Gland	10	1	10	10	10	10
Atrophy	0	1 (1.0)	1 (1.0)	2 (1.0)	8** (1.1)	8** (1.9)
Inflammation	2 (1.5)	0	3 (1.0)	0	1 (2.0)	8* (1.5)
Testes	10	1	10	10	10	10
Degeneration	1 (2.0)	1 (1.0)	0	4 (1.0)	9** (1.3)	9** (3.1)
Female						
Liver	10	1	2	10	10	10
Hepatocyte, Vacuolization Cytoplasmic	0	0	0	0	1 (1.0)	8** (1.4)

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesion
[c] Not examined at this exposure concentration
[d] Average severity of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Organ weight effects: In males, the absolute and relative liver weights of the 2,500, 5,000 and 10,000 ppm groups were significantly higher than controls. In females, the absolute liver weight of the 10,000 ppm group was significantly lower than controls, and the relative liver weights of the 5,000 and 10,000 ppm groups were significantly higher than controls. The absolute and relative spleen weights of females exposed to 2,500, 5,000 and 10,000 ppm were significantly lower than the control group. In males, the absolute right kidney weight of 10,000 ppm and the relative right kidney weights of 5,000 and 10,000 ppm were significantly higher than the controls.

Table 22: (Copy of Table 12 in NTP, 2004)

TABLE 12
Selected Organ Weight Data for Rats in the 14-Week Feed Study of 4-Methylimidazole[a]

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Male						
n	8	8	8	8	8	7
Necropsy body wt[b]	352 ± 6	362 ± 8	353 ± 6	335 ± 4*	298 ± 4**	245 ± 4**
R. Kidney						
Absolute	1.296 ± 0.031	1.282 ± 0.027	1.332 ± 0.036	1.260 ± 0.023	1.218 ± 0.035	1.165 ± 0.026**
Relative	3.70 ± 0.05	3.56 ± 0.05	3.78 ± 0.06	3.82 ± 0.06	4.12 ± 0.09**	4.78 ± 0.06**
Liver						
Absolute	11.935 ± 0.448	12.569 ± 0.272	12.644 ± 0.301	13.919 ± 0.404**	18.811 ± 0.645**	16.823 ± 0.632**
Relative	33.96 ± 0.70	34.96 ± 0.61	35.96 ± 0.82	42.12 ± 1.07**	63.73 ± 2.28**	68.92 ± 1.70**
R. Testis						
Absolute	1.436 ± 0.047	1.477 ± 0.042	1.501 ± 0.023	1.461 ± 0.027	1.275 ± 0.042**	0.511 ± 0.027**
Relative	4.10 ± 0.14	4.11 ± 0.08	4.28 ± 0.10	4.42 ± 0.09	4.32 ± 0.13	2.10 ± 0.10**
Female						
n	8	8	7	8	8	10
Necropsy body wt	201 ± 3	207 ± 3	204 ± 2	198 ± 4	189 ± 6*	127 ± 5**
Liver						
Absolute	7.062 ± 0.256	7.702 ± 0.158	7.383 ± 0.200	6.987 ± 0.243	7.152 ± 0.298	6.038 ± 0.243**
Relative	35.37 ± 1.22	37.12 ± 0.64	36.07 ± 0.93	35.56 ± 0.76	38.55 ± 0.77*	47.54 ± 1.02**
Spleen						
Absolute	0.501 ± 0.019	0.519 ± 0.019	0.506 ± 0.015	0.443 ± 0.013*	0.436 ± 0.019**	0.292 ± 0.011**
Relative	2.51 ± 0.10	2.50 ± 0.07	2.47 ± 0.08	2.26 ± 0.05*	2.35 ± 0.04*	2.30 ± 0.03*

* 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] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] For body weights n=10

Reproductive tissue evaluation:

The absolute weight of the both testes in the 5,000 ppm dose group and the right testis in the 10,000 ppm group was decreased. The relative weight of the right testis of 10,000 ppm were significantly lower than the controls (see table 22 above). No information is given about the absolute (or relative) weight of the left testis in the 10,000 ppm group. The incidences of epididymal hypospermia and prostate gland inflammation were significantly increased in 10,000 ppm males. The incidences of prostate gland atrophy and testicular degeneration were significantly increased in 5,000 and 10,000 ppm males (see table 21 above).

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

In 1,250 ppm group, the spermatid heads per testis and mean spermatid count were significantly higher than the control group, while the epididymal spermatozoal motility was significantly lower than the controls. The epididymal spermatozoal concentrations of 1,250 and 5,000 ppm males were significantly higher than the controls. No significant differences occurred in vaginal cytology parameters between exposed and control females. The estrous cycle was longer than 12 days or unclear in two females (2 out of 9 animals) of 1,250 ppm rats and 6 females (6 out of 10 animals) of 5,000 ppm group, see table 23 below.

Table 23: (Copy of Tables E3 and E4 in NTP, 2004)

TABLE E3
Summary of Reproductive Tissue Evaluation for Male Rats in the 14-Week Feed Study of 4-Methylimidazole[a]

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
n	8	8	8	8
Weights (g)				
Necropsy body wt	351 ± 8	352 ± 8	330 ± 3*	296 ± 5**
L. cauda epididymis	0.1873 ± 0.0067	0.1763 ± 0.0100	0.1742 ± 0.0077	0.1544 ± 0.0043**
L. epididymis	0.5079 ± 0.0172	0.5241 ± 0.0116	0.5111 ± 0.0191	0.4381 ± 0.0175**
L. testis	1.5100 ± 0.0427	1.5605 ± 0.0324	1.4801 ± 0.0268	1.2914 ± 0.0407**
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 count (mean/10^{-4} mL suspension)	68.91 ± 1.18	76.50 ± 2.02*	71.88 ± 2.51	64.03 ± 2.76
Epididymal spermatozoal measurements				
Motility (%)	91.34 ± 0.22	90.56 ± 0.21*[b]	90.63 ± 0.20	90.00[c]
Concentration (10^6/g cauda epididymal tissue)	406 ± 19	498 ± 41*	477 ± 21	504 ± 22*

* Significantly different ($P \leq 0.05$) from the control group by Williams' test (body weight) or Dunn's test (spermatid and epididymal spermatozoal measurements)
** Significantly different ($P \leq 0.01$) from the control group by Williams' test
[a] Data are presented as mean ± standard error. Differences from the control group for spermatid heads per testis were not significant by Dunn's test.
[b] n=7
[c] n=1; no standard error calculated

TABLE E4
Summary of Estrous Cycle Characterization for Female Rats in the 14-Week Feed Study of 4-Methylimidazole[a]

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
n	10	9	10	10
Necropsy body weight (g)	201 ± 2	204 ± 2	198 ± 4	189 ± 6
Estrous cycle length (days)	4.70 ± 0.15	5.14 ± 0.24[b]	5.40 ± 0.34	5.38 ± 0.24[c]
Estrous stages (% of cycle)				
Diestrus	41.7	53.7	47.5	58.3
Proestrus	15.0	11.1	16.7	15.0
Estrus	23.3	19.4	19.2	15.0
Metestrus	20.0	15.7	16.7	11.7

[a] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (body weight) or Dunn's test (estrous cycle length). By multivariate analysis of variance, exposed females do not differ significantly from the control females in the relative length of time spent in the estrous stages.
[b] Estrous cycle was longer than 12 days or unclear in two of nine animals.
[c] Estrous cycle was longer than 12 days or unclear in 6 of 10 animals.

Conclusion on reproductive toxicity data from the 14-week study is given below.

MICE (NTP, 2004)

14-week study:

Dietary concentrations of 625, 1,250, 2,500, 5,000, or 10,000 ppm in the feed estimated to give daily doses of approximately 100, 240, 440, 915, or 1,840 mg/kg bw 4-methylimidazole to males and 110, 250, 540, 1,130, or 3,180 mg/kg bw to females.

Mortality: During the 4-methylimidazole study, one 10,000 ppm male during week 4 and seven 10,000 ppm females during weeks 1, 2, and 3 were found dead.

Clinical findings: clinical findings in the 4-methylimidazole study included ruffled fur and dull coats in the 10,000 ppm females.

Food consumption: No significant effects was observed

Body weight: In the 4-methylimidazole study, the final mean body weights and body weight gains of males exposed to 1,250, 2,500, 5,000 and 10,000 ppm and all exposed groups of females were significantly lower than the control groups.

Table 24: (Copy of Table 18 from NTP, 2004)

TABLE 18
Survival, Body Weights, and Feed Consumption of Mice in the 14-Week Feed Study of 4-Methylimidazole

Dose (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 14
Male							
0	10/10	22.7 ± 0.4	35.3 ± 0.6	12.6 ± 0.5	—	4.4	4.3
625	10/10	22.5 ± 0.6	33.6 ± 0.9	11.1 ± 0.7	95	4.6	4.3
1250	10/10	22.2 ± 0.6	32.6 ± 1.1*	10.4 ± 0.8**	93	5.7	4.7
2500	10/10	23.9 ± 0.6	31.8 ± 0.4**	7.8 ± 0.3**	90	5.1	4.7
5000	10/10	22.9 ± 0.5	29.6 ± 0.5**	6.8 ± 0.5**	84	5.2	4.4
10000	9/10[d]	23.0 ± 0.4	28.0 ± 0.3**	5.1 ± 0.3**	79	5.4	4.0
Female							
0	10/10	18.3 ± 0.3	29.1 ± 1.1	10.8 ± 0.9	—	4.7	3.7
625	10/10	18.8 ± 0.4	26.3 ± 0.7*	7.5 ± 0.6**	90	4.2	3.8
1250	10/10	19.0 ± 0.4	25.7 ± 1.0**	6.7 ± 0.7**	88	4.5	4.6
2500	10/10	18.8 ± 0.5	23.4 ± 0.4**	4.6 ± 0.3**	80	4.4	4.7
5000	10/10	19.0 ± 0.3	22.5 ± 0.6**	3.5 ± 0.4**	77	4.9	4.5
10000	3/10[e]	18.0 ± 0.3	21.6 ± 0.3**	3.0 ± 0.4**	74	5.5	7.1

* Significantly different ($P \leq 0.05$) from the control group by Williams' test
** $P \leq 0.01$
[a] Number of animals surviving at 14 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.
[c] Feed consumption is expressed as grams per animal per day.
[d] Week of death: 4
[e] Week of death: 1, 1, 1, 1, 2, 2, 3

Functional observations: No significant effects was observed

Hematology and clinical chemistry: Administration of 4-methylimidazole resulted in minimal erythron decreases only in exposed females; decreased automated and manual hematocrit values and hemoglobin concentrations in all exposed groups; erythrocyte counts were unaffected.

The thyroid gland hormone data: On day 8 and 29, transient decrease compared to the control grouop (not observed on day 86) in thyroxine concentrations in 5,000 and 10,000 ppm males, and on day 29, in 10,000 ppm females were observed. On day 29, triiodothyronine concentration was increased in 5,000 ppm females; on day 86, triiodothyronine concentrations were increased in 5,000 and 10,000 ppm males.

Table 25: (Copy of table 22 in NTP, 2004, data also published in Chan et al., 2006)

TABLE 22
Selected Hematology and Clinical Chemistry Data for Mice in the 14-Week Feed Study of 4-Methylimidazole[a]

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
n	10	10	10	10	10	10
Male						
Hematology						
Automated hematocrit (%)	48.4 ± 0.8	47.0 ± 0.3	47.1 ± 0.5	47.9 ± 0.5	46.7 ± 1.0	46.8 ± 0.9
Manual hematocrit (%)	49.1 ± 0.6[b]	48.3 ± 0.3[b]	47.3 ± 0.5	48.9 ± 0.6[c]	48.8 ± 0.6[b]	47.4 ± 0.4[d]
Hemoglobin (g/dL)	15.4 ± 0.2	15.1 ± 0.1	15.1 ± 0.2	15.5 ± 0.1	15.0 ± 0.2	15.2 ± 0.1
Erythrocytes ($10^6/\mu L$)	9.56 ± 0.16	9.19 ± 0.09	9.37 ± 0.12	9.60 ± 0.13	9.26 ± 0.20	9.57 ± 0.16
Clinical Chemistry						
Triiodothyronine (ng/dL)						
Day 8	137.0 ± 5.4[e]	139.8 ± 3.5[f]	132.8 ± 3.7[f]	140.3 ± 3.8[g]	125.5 ± 2.5[h]	137.7 ± 5.8[g]
Day 29	142.3 ± 6.1[b]	152.5 ± 5.2[b]	141.1 ± 6.0[b]	148.0 ± 5.9[f]	163.8 ± 7.6[f]	168.0 ± 8.0[f]
Day 86	128.8 ± 3.7	130.3 ± 4.4[b]	133.4 ± 2.4	137.6 ± 4.9[b]	148.2 ± 3.8**[d]	176.7 ± 6.6**[f]
Thyroxine (μg/dL)						
Day 8	5.93 ± 0.24	6.14 ± 0.22	6.15 ± 0.27	5.55 ± 0.25	4.95 ± 0.15**	4.70 ± 0.18**[i]
Day 29	5.95 ± 0.22	6.18 ± 0.21	6.03 ± 0.14	5.57 ± 0.23	4.97 ± 0.12**	3.70 ± 0.09**
Day 86	4.47 ± 0.17	4.62 ± 0.25	4.67 ± 0.14	4.72 ± 0.09	4.62 ± 0.21	3.98 ± 0.18

TABLE 22
Selected Hematology and Clinical Chemistry Data for Mice in the 14-Week Feed Study of 4-Methylimidazole[a]

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
n	10	10	10	10	10	10
Female						
Hematology						
Automated hematocrit (%)	47.9 ± 0.4	46.5 ± 0.3*	46.0 ± 0.3**	45.3 ± 0.6**	45.7 ± 0.3**	45.0 ± 0.7**
Manual hematocrit (%)	49.4 ± 0.3[b]	48.3 ± 0.5[b]	48.1 ± 0.3*[b]	47.6 ± 0.6*[c]	48.0 ± 0.4*	45.0[j]
Hemoglobin (g/dL)	15.7 ± 0.1	15.2 ± 0.1**	15.2 ± 0.1**	14.9 ± 0.1**	14.9 ± 0.1**	14.8 ± 0.2**
Erythrocytes ($10^6/\mu L$)	9.51 ± 0.12	9.25 ± 0.07	9.22 ± 0.08	9.15 ± 0.14	9.17 ± 0.09	9.32 ± 0.20
Clinical Chemistry						
Triiodothyronine (ng/dL)						
Day 8	139.0 ± 6.4[f]	134.3 ± 3.9[f]	124.0 ± 2.3[c]	132.8 ± 3.1[i]	136.0 ± 11.0[g]	—[k]
Day 29	130.8 ± 3.5	132.5 ± 5.0[b]	130.8 ± 3.0	140.9 ± 6.7[c]	150.8 ± 5.1**[b]	148.5 ± 7.5[h]
Day 86	128.1 ± 4.9[c]	116.3 ± 3.4[i]	131.0 ± 3.8	149.7 ± 8.5[g]	141.0 ± 8.1[d]	—
Thyroxine (μg/dL)						
Day 8	7.14 ± 0.41	6.99 ± 0.31	7.30 ± 0.31	7.78 ± 0.31	7.19 ± 0.42	6.56 ± 0.42[i]
Day 29	6.98 ± 0.15	6.76 ± 0.15	7.19 ± 0.18	6.80 ± 0.18	7.51 ± 0.35	5.56 ± 0.14*[d]
Day 86	6.95 ± 0.40	6.45 ± 0.16	6.53 ± 0.22	6.91 ± 0.22	5.90 ± 0.28	5.25 ± 0.55[h]

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test
** Significantly different ($P \leq 0.01$) from the control group by Shirley's test
a Mean ± standard error. Statistical tests were performed on unrounded data.
b n=8
c n=7
d n=5
e n=4
f n=6
g n=3
h n=2
i n=9
j n=1; no standard error calculated
k Not analyzed

Necropsy findings: No exposure-related gross or microscopic lesions were identified in male mice. In females, the decreased incidence of periportal cytoplasmic vacuolization of the liver in the 10,000 ppm group was considered to be secondary to glycogen depletion and the poor nutritional status of this group according to study report.

Organ weights:The relative liver weights of all exposed groups of males were significantly higher than the control group. The absolute liver weight of the 10,000 ppm group was decreased. In females, the absolute heart, right kidney, and liver weights of the 5,000 and 10,000 ppm groups and absolute liver weight of 2,500 ppm females were significantly lower than the control group. While, the relative heart and right kidney weights of the females exposed to 2,500, 5,000 and 10,000 ppm and the relative liver weight of 625, 2,500 and 10,000 ppm females were significantly higher than the control group.

Table 26: Copy of Table 23 from NTP, 2004)

TABLE 23
Selected Organ Weight Data for Mice in the 14-Week Feed Study of 4-Methylimidazole[a]

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Male						
n	10	10	10	10	10	9
Necropsy body wt	35.3 ± 0.6	33.6 ± 0.9	32.6 ± 1.1*	31.8 ± 0.4**	29.6 ± 0.5**	28.0 ± 0.3**
Liver						
Absolute	1.568 ± 0.034[b]	1.581 ± 0.050	1.558 ± 0.052	1.568 ± 0.035	1.449 ± 0.047	1.427 ± 0.032*
Relative	44.20 ± 0.62[b]	47.10 ± 0.65**	47.75 ± 0.48**	49.33 ± 0.72**	48.85 ± 0.94**	50.89 ± 0.70**
R. Testis						
Absolute	0.124 ± 0.003	0.116 ± 0.003	0.121 ± 0.003	0.126 ± 0.002	0.120 ± 0.002	0.113 ± 0.002*
Relative	3.51 ± 0.11	3.47 ± 0.12	3.72 ± 0.09	3.95 ± 0.05**	4.05 ± 0.07**	4.02 ± 0.09**
Female						
n	10	10	10	10	10	3
Necropsy body wt	29.1 ± 1.1	26.3 ± 0.7*	25.7 ± 1.0**	23.4 ± 0.4**	22.5 ± 0.6**	21.6 ± 0.3**
Heart						
Absolute	0.128 ± 0.003	0.125 ± 0.003	0.120 ± 0.002	0.121 ± 0.003	0.109 ± 0.002**	0.105 ± 0.003**
Relative	4.42 ± 0.14	4.77 ± 0.10	4.73 ± 0.17	5.16 ± 0.08**	4.86 ± 0.09**	4.85 ± 0.19*
R. Kidney						
Absolute	0.191 ± 0.005	0.189 ± 0.003	0.181 ± 0.003	0.190 ± 0.008	0.168 ± 0.003**	0.166 ± 0.004*
Relative	6.60 ± 0.21	7.19 ± 0.11	7.13 ± 0.24	8.09 ± 0.27**	7.49 ± 0.12**	7.69 ± 0.28**
Liver						
Absolute	1.154 ± 0.031	1.166 ± 0.042	1.114 ± 0.031	1.042 ± 0.028*	0.932 ± 0.036**	1.011 ± 0.026**
Relative	40.03 ± 1.51	44.32 ± 1.07*	43.65 ± 1.29	44.47 ± 0.76*	41.42 ± 0.64	46.87 ± 1.78*

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test
** Significantly different ($P \leq 0.01$) from the control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n=9

Reproductive tissue evaluation: The relative weight of the right testis of males exposed to 2,500, 5,000 and 10,000 ppm was significantly higher than the control group, while the absolute weight of the testis in the 10,000 ppm group was significantly lower than control group.

No significant differences occurred in sperm motility or vaginal cytology parameters between exposed and control groups (see tables below). However, significant decrease in the left epididymis, and left testis weight were observed in the 10,000 ppm group.

Table 27: (Copy of Table E7 and E8 from NTP, 2004)

TABLE E7
Summary of Reproductive Tissue Evaluations for Male Mice in the 14-Week Feed Study of 4-Methylimidazole[a]

	0 ppm	2,500 ppm	5,000 ppm	10,000 ppm
n	10	10	10	9
Weights (g)				
Necropsy body wt	35.3 ± 0.6	31.8 ± 0.4**	29.6 ± 0.5**	28.0 ± 0.3**
L. cauda epididymis	0.0176 ± 0.0008	0.0173 ± 0.0005	0.0176 ± 0.0005	0.0152 ± 0.0010
L. epididymis	0.0515 ± 0.0018	0.0476 ± 0.0009	0.0487 ± 0.0015	0.0439 ± 0.0019**
L. testis	0.1181 ± 0.0016	0.1198 ± 0.0018	0.1163 ± 0.0023	0.1077 ± 0.0020**
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 count (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	1,007 ± 55

** Significantly different (P≤0.01) from the control group by Williams' test (body and testis weight) or Dunnett's test (l. epididymis weight)
[a] Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (l. cauda epididymis weight) or Dunn's test (spermatid and epididymal spermatozoal measurements).

TABLE E8
Summary of Estrous Cycle Characterization for Female Mice in the 14-Week Feed Study of 4-Methylimidazole[a]

	0 mg/kg	1,250 mg/kg	2,500 mg/kg	5,000 mg/kg
n	10	10	10	10
Necropsy body wt (g)	29.1 ± 1.1	25.7 ± 1.0**	23.4 ± 0.4**	22.5 ± 0.6**
Estrous cycle length (days)	4.60 ± 0.49	4.28 ± 0.12[b]	4.55 ± 0.50	4.75 ± 0.27
Estrous stages (% of cycle)				
Diestrus	34.2	29.2	30.0	28.3
Proestrus	12.5	20.0	26.7	24.2
Estrus	31.7	27.5	22.5	25.8
Metestrus	21.7	23.3	20.8	21.7

** Significantly different (P≤0.01) from the control group by Williams' test.
[a] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group for estrous cycle length are not significant by Dunn's test. By multivariate analysis of variance, exposed females do not differ significantly from the control females in the relative length of time spent in the estrous stages.
[b] Estrous cycle was longer than 12 days or unclear in 1 of 10 animals.

The current 14-week studies demonstrated that 10,000 ppm 4-methylimidazole induced tremors and ataxia in F344/N rats. Based on clinical findings, rats seemed to be more sensitive to the neurobehavioral effects of 4-methylimidazole than mice.

Conclusion on reproductive toxicity in the 14-week study: 4-methylimidazole seems to exert some toxicity to primary and secondary reproductive organs in rats and mice.

RATS

4-methylimidazole induced degeneration of the seminiferous tubules of the testes and atrophy of the prostate gland in male rats. The animals receiving 5,000 ppm and 10,000 ppm had decreased absolute weights of the right testis. The relative weight of the right testis of the 10,000 ppm group was significantly lower than the controls.

In female rats, a slight increase in estrous cycle was suggested.

MICE

The relative weights of the right testis of male mice exposed to 2,500 ppm or higher were significantly higher than the control group probably related to reduced body weights. The absolute weight of the left testis and epididymis were decreased in the group exposed to 10,000 ppm. No significant differences was observed in sperm motility or vaginal cytology parameters between exposed and control groups.

3.6.1.2 NTP reproductive and developmental continuous breeding (RACB) toxicity study of 4-methylimidazole in rats

Study references:

NTP web-page (data Tables): DOI: https://doi.org/10.22427/NTP-DATA-002-01511-0000-0000-0

Behl M et al., 2020. Multigenerational reproductive assessment of 4-methylimidazole administered in the diet to Hsd:Sprague Dawley SD rats. Reprodutive Toxicology (available online from 27 March 2020). https://doi.org/10.1016/j.reprotox.2020.03.005

A **dose range-finding study** was conducted with dietary doses of 0, 625, 1250, 2500, 5000, and 10,000 (males only) ppm prior to the main RACB study. 8 rats/sex/group. A 10 weeks prebreed exposure was used for the parental F0 male to encompass the complete spermatogenic cycle prior to breeding.

Detailed study summary and results:

Test substance

Two lots of 4-methylimidazole (one from Sigma-Aldrich St Lois, MO; lot# 119H5114 and one from Alfa Aesar Ward Hill, MA; lot# C07T016) were combined to make one homogeneous lot lot# 051410 and used in studies. The identity of the combined lot was confirmed by infrared spectroscopy, and 1H and 13C nuclear magnetic resonance spectroscopy. The purity was > 99 %. Prior to study start, stability of 4-methylimidazole in feed was confirmed for up to 42 days at ambient temperature.

Test animals

Adult Hsd:Sprague Dawley SD male and female rats were from Harlan Laboratories.

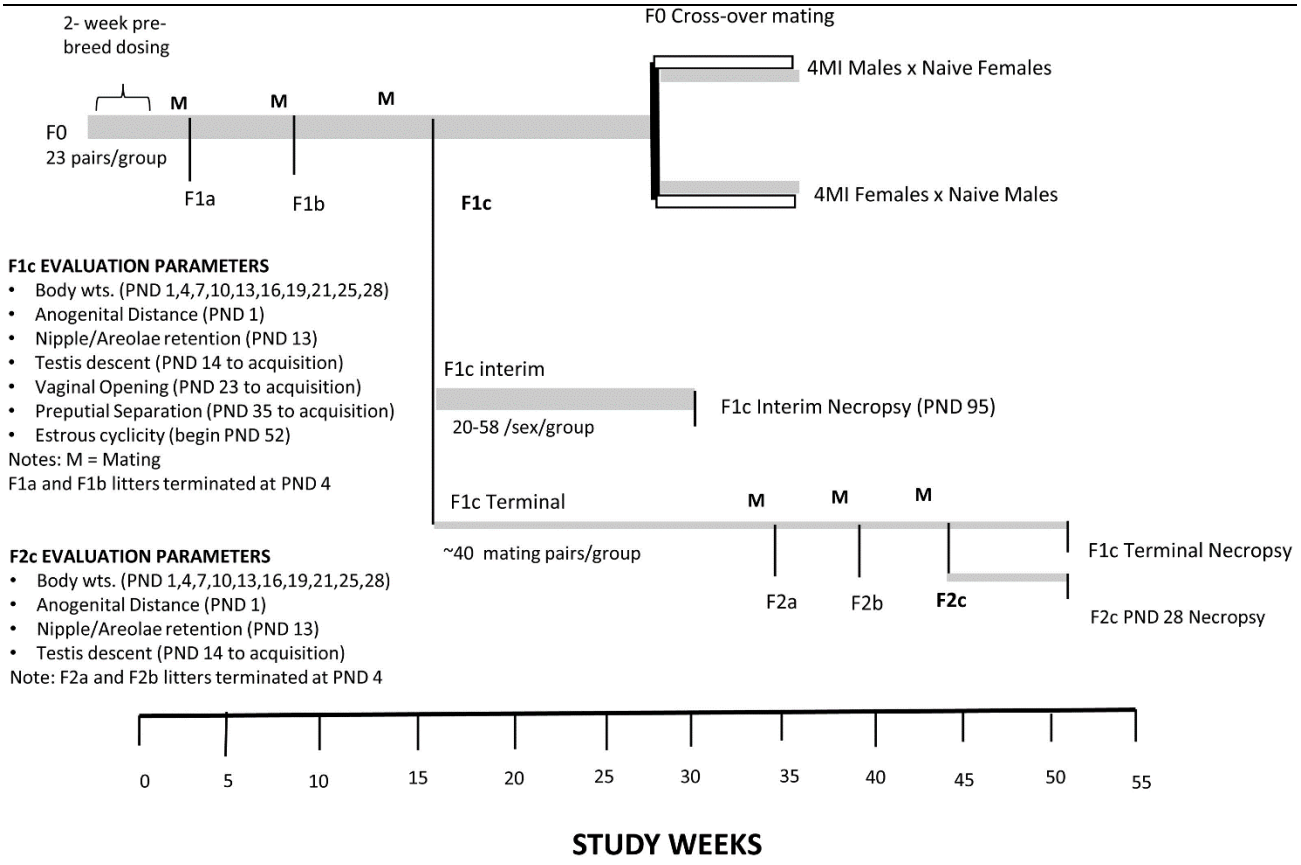
Administration/exposure

Feed and water were available ad libitum. F0: 23 rats/sex/group, age 10-12 weeks, were exposed to 4-methylimidazole in diet at 0, 750, 2500, and 5000 ppm from two weeks prior to the first cohabitation. Reproductive performance of the animals in the high dose group was severely impaired and thus the F1 and F2 generations were exposed only to the 0, 750 and 2500 ppm doses.

Study protocol

The reproductive and developmental study of 4-methylimidazole was conducted in compliance with GLP. The study protocol followed the reproductive assessment by continuous breeding (RACB) study design developed by NTP. Multiple breedings of the F0 and F1 generation were conducted with a continuous exposure to 4-methylimidazole via the diet beginning two weeks prior to cohabitation of the F0 generation animals. The animals were mated three times with the same partner to produce the F1a, F1b and F1c litters. F1a and F1b litters were euthanized on PND4. The F1c litters were retained until they were sexually mature (~PND 95) and paired to produce three litters (F2a, F2b, F2c). The F2a and F2b litters were euthanized on PND 4. The F2c litter and corresponding dams were evaluated through PND 28. Due to a decrease in litter size in the F1 generation, a crossover mating of F0 animals in the control, 2500 and 5000 ppm groups was conducted following generation of the F1c generation to investigate which sex was affected by the exposure. Offspring of the cross-over mating were euthanized on PND 4.

Fig.1 (Copy of Fig.1. from Behl et al., 2020)



Adult animals: Vaginal smears were collected from the F0 and F1c- terminal females for 16 consecutive days for evaluation of estrous cyclicity.

Histopathology was performed on selected tissues in F0 and F1c control and high dose animals and also lower dose groups if treatment related effects were observed. The data was subjected to additional NTP pathology peer-review procedures. For F2c offspring on PND 28, a complete gross evaluation was performed, and only gross lesions of the kidneys or testes were evaluated for histopathology.

Cauda epididymal sperm motility and sperm concentration as well as testicular spermatide head counts were evaluated in F0 and F1c males. Ovarian follicle counts was performed for F0 and F1c females.

Pups/pubertal animals: Body weight adjusted anogential distance (AGD) was measured at PND1 for all pups. Male pups were evaluated for retention of areolae/nipples on PND 13 and for testes descent beginning on PND 14. The acquisition of vaginal opening (VO) was evaluated in all F1c females beginning on PND 23 and the acquisition of preputial separation (PPS) was evaluated in F1c males beginning on PND 35.

Statistical analyses: Statistical methods differed for F0 and F1 animals, since methods for the F1 animals needed to account for within litter correlation where present. Statistical methods used are noted in the original Tables from Behl et al, 2020 that are presented below.

Results and discussion

Dose range-finding study*:*

Excessive toxicity in the 10 000 ppm group. Number of litters and live litter size clearly reduced in the 5000 ppm group.

Main study:

Survival**:** F0: No treatment related mortality noted for males. Reduced treatment related survival for females (22, 22, 19 and 12 in the 0, 750, 2500 and 5000 ppm groups, respectively). Several F0 females dead/moribound in the 2500 and 5000 groups likely due to difficulties in parturition. The 5000 ppm dose was thus terminated and no F1c litter was produced at the top dose. Several F1c females in the 2500 ppm group also displayed perturbed parturition and dystocia.

Table 28 (Copy of Table 3 from Behl et al., 2020)

Table 3
Incidence of perturbed parturition across the three pairings per generation[a].

	Generation	0 ppm	750 ppm	2500 ppm	5000 ppm
Dystocia	F0	0	0	1	5
	F1	0	0	4	–
Retained Fetus/ Placentas	F0	0	0	1	1
	F1	1	1	1	0
Total Perturbed Parturition[b]	F0	0	0	2	6
	F1	1	1	5	–

[a] Number of females displaying evidence of mating across all three pairings: F0 n = 66, 68, 60, 21; F1 n = 109, 122, 104.
[b] Incidence of animals displaying dystocia or retained fetus/placentas.

Food consumption and body weight**:** Food consumption of F0 females was up to 9% lower in the 5000 ppm group during pre-breed exposure and gestation of the F1 litters compared to the control group and sporadically lower in the 2500 ppm dose group at various stages of the study. Estimated intake of 4-methylimidazole in male and female rats during different study phases is shown below.

Table 29 (Copy of Table 1 from Behl et al., 2020)

Table 1
Mean 4-MI intake (mg/kg/day) during the various phases of the RACB study.

	750 ppm	2500 ppm	5000 ppm
F0			
Prior to Pairing (M, Study Days 0–14)	47.9	144.6	260.1
Prior to Pairing (F, Study Days 0–14)	46.8	145.6	289.9
Gestation (GD 1–21)[a]	48.3	151.2	307.0
Lactation (PND 1–4)[a]	79.1	231.9	274.0
Lactation (PND 1–13)[b]	101.4	319.0	–
F1c			
Prior to Pairing (M)	63.7	206.9	–
Prior to Pairing (F)	66.3	225.4	–
Gestation (GD 1–21)[a]	48.7	161.2	–
Lactation (PND 1–4)[a]	79.6	278.7	–

Lactation (PND 1–13)[b]	93.8	306.5	–

[a] Average intake (mg/kg/day) of dams across the three breeding (A, B, C) periods.
[b] Intake of the dams (mg/kg/day) during lactation period of the C litter.
"- "= No 5000 ppm data due to early removal from the study.

At necropsy, F0 male body weights in exposed groups were lower compared to respective controls. The terminal body weights in F0 and F1c were 2–5 % lower in the 750 ppm dose groups and 9–11 % lower in the 2500 ppm groups relative to controls. For females, there was a statistically significant decrease in terminal body weight in all dose-groups in the F0, F1-interim, and F1-terminal groups relative to controls. Weights were 4–14 %, 10–11 %, and 19 % lower , and 5000 ppm groups. At gestational day 21 (GD21) the body weights were reduced in F0 dams by 4-5%, 14-16 % and 19-25% in the 750, 2500, and 5000 ppm groups. For F1 dams the body weight reductions at gestational day 21 were 8-9 % and 13-17 % in the 750 and 2500 ppm groups.The F1 and F2 male and female pup body weights were not reduced PND 1 in the 750 ppm group, but reduced by 3-11% in the 2500 ppm group. In the F1c and F2c pups by the end of lactation (PND28) body weights were 5% lower than controls for F1c, whereas no change was observed for F2c in the 750 ppm group. In the 2500 ppm group body weights were ap. 20% lower than controls at PND28.

Clinical findings: Dose-related increase of 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.

Reproductive toxicity:
Fertility: 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 30).

Table 30 (Copy of Table 4 from Behl et al., 2020)

Table 4
Average reproductive performance of the three pairings pairs (A, B, C) of the F0 and F1c following 4-MI exposure, including crossover mating of exposed F0 males or F0 females with naïve partners.

	Generation	0 ppm	750 ppm	2500 ppm	5000 ppm
Mated/Pair					
Average of A, B, C Pairings	F0	97.0	98.6 %	96.8 %	48.1 %[a]
Average of A, B, C Pairings	F1c	92.4 %	97.0 %	94.0 %	–
4-MI Male x Naïve Female[b]	F0	87.0%**	–	80.0 %	33.3 %**
4-MI Female x Naïve Male[b]	F0	68.2%	–	73.7 %	–
Littered/Pair					
Average of A, B, C Pairings	F0	88.2%	97.1 %	83.4 %	32.4 %[a]
Average of A, B, C Pairings	F1c	81.8 %	84.3 %	78.8 %	
4-MI Male x Naïve Female[b]	F0	69.6%**	–	75.0 %	28.6 %*
4-MI Female x Naïve Male[b]	F0	54.5%	–	52.6 %	–
Littered/Mated					
Average of A, B, C Pairings	F0	90.8%	98.6 %	86.2 %	75 % [a]
Average of A, B, C Pairings	F1c	87.9 %	86.9 %	83.7 %	
4-MI Male x Naïve Female[b]	F0	80.0 %		93.8 %	85.7 %
4-MI Female x Naïve Male[b]	F0	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).

[b] Analysis of crossover mating was performed by Cochran-Armitage (trend) and Fisher Exact (pairwise) 2-sided tests. Trend results appear in the 0 ppm column.

*p < 0.05; ** p < 0.01.

Cross-over matings showed a reduction mated/pair in the 5000 ppm exposed males mating with naïve females. All females that did not deliver were found to be non-pregnant suggesting an effect on fertility in males. Potential effects on female fertility cound not be assessed as the 5000 ppm females were not included in the cross-over mating due to moribundity associated with parturition. There was no evidence of a exposure-related effect on reproductive performance in 2500 ppm group of animals in the cross-over study.

Total and live litter sizes were mostly significantly reduced at 2500 (F0 and F1c) and 5000 (F0) ppm groups and both parameters showed a statistically significant trend of smaller total litter size with increasing doses.

Table 31 (Copy of Table 5 from Behl et al., 2020)

Table 5
Average litter size across the pairings in each generation (average ± SEM).

	F0				F1c		
	0 ppm	750 ppm	2500 ppm	5000 ppm	0 ppm	750 ppm	2500 ppm
Total Litter Size (PND 0)[a]							
A	14.6 ± 0.4**	13.3 ± 0.6	9.9 ± 0.7**	5.7±1.9**	13.2 ± 0.5**	11.0 ± 0.6*	8.9 ± 0.8**
B	14.2 ± 0.5**	13.5 ± 0.6	9.2 ± 0.7**	4.7±0.9**	15.3 ± 0.5**	12.3 ± 0.5**	10.6 ± 0.9**
C	13.6 ± 0.6*	12.8 ± 0.6	10.6 ± 1.2*	–	10.7 ± 0.8	9.8 ± 0.7	9.4 ± 0.9
4-MI Male x Naïve Female	13.8 ± 0.5	–	11.6 ± 1.1	11.7 ± 1.8	–	–	–
4-MI Female x Naïve Male	11.4 ± 1.5	–	11.3 ± 1.7	–	–	–	–
Live Litter Size (PND 0)							
A	14.0 ± 0.4**	12.7 ± 0.6	7.8 ± 1.0**	1.8±1.6**	11.1 ± 0.7**	9.3 ± 0.7	7.2 ± 0.8**
B	13.7 ± 0.5**	12.7 ± 0.5	8.5 ± 0.6	0.5±0.5**	13.6 ± 0.6**	11.9 ± 0.6	9.8 ± 0.8**
C	12.2 ± 0.8	12.4 ± 0.5	9.8 ± 1.2	–	9.2 ± 0.7	8.8 ± 0.6	8.5 ± 0.9
4-MI Male x Naïve Female	13.4 ± 0.4	–	10.6 ± 1.1	11.5 ± 1.9	–	–	–
4-MI Female x Naïve Male	10.7 ± 1.4	–	11.0 ± 1.7	–	–	–	–
Survival Ratio (PND 1–4)							
A	0.96 ± 0.02	0.98 ± 0.01	0.91 ± 0.04	0.10±0.10	0.92 ± 0.05**	0.93 ± 0.04	0.72±0.08**
B	0.97 ± 0.02	0.98 ± 0.01	0.93 ± 0.03	0.67[b]	0.97 ± 0.01*	0.97 ± 0.01	0.91 ± 0.03*
C	0.95 ± 0.03	0.98 ± 0.01	0.93 ± 0.04	–	0.89 ± 0.05	0.92 ± 0.05	0.88 ± 0.04
4-MI Male x Naïve Female	0.97 ± 0.01	–	0.96 ± 0.02	0.99±0.01	–	–	–
4-MI Female x Naïve Male	0.99 ± 0.01*	–	0.82 ± 0.10*	–	–	–	–
Survival Ratio (PND 5–28)	0.98 ± 0.01	0.96 ± 0.02	0.95 ± 0.02	–	0.83 ± 0.07	0.93 ± 0.02	0.89 ± 0.03
Average Live Litter Size/Pair	13.2 ± 0.4	12.6 ± 0.3	9.2 ± 0.6	2.0 ± 1.0	11.4 ± 0.4**	10.1 ± 0.5**	8.3 ± 0.6

[a]F0 litter size and survival endpoints were analyzed using Jonckheere's test for trend (0 ppm column) and Shirley's or Dunn's methods for pairwise comparison of controls to dose groups. F1c data were analyzed using the bootstrapped Jonckheere test for trend; pairwise comparisons used the Datta-Satten modified Wilcoxon test with the Hommel adjustment for multiple comparisons. Testing for trend and pairwise differences was not performed for sample sizes of 1 or 2. * p < 0.05, ** p < 0.01.

[b] n = 1 litter.

Sperm parameters and oestrus cycle:

F0 rats exposed to 5000 ppm displayed significantly reduced cauda epididymal sperm count and reduced % motile sperm compared to controls. Sperm/g cauda was decreased dose-dependently in the F1-interim group, 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.

Table 32 (Copy of Table 7 from Behl et al., 2020)

Table 7
Epididymal sperm parameters (average ± SEM) of the F0, F1c Interim, and F1c Terminal male rats after 4-MI exposure.

Endpoint	Generation	0 ppm	750 ppm	2500 ppm	5000 ppm
Sperm/Cauda (10^6)	F0	180.6 ± 8.2**	206.6 ± 7.9	167.0 ± 11.3	135.1 ± 7.3**
	F1-interim	187.8 ±8.9	168.8 ±6.5	153.8 ±11.4	–
Sperm/g Cauda (10^6)	F1-terminal	196.7 ±9.0	190.2 ±7.1	176.0 ±10.6	–
	F0	682.4 ± 29.7*	752.7 ± 21.2	676.5 ±38.3	589.5 ± 28.5
	F1-interim	856.4 ±26.1	780.1 ± 25.8*	754.4 ± 40.8*	–
% Motile	F1-terminal	759.6 ±27.3	723.0 ± 26.2	721.2 ±35.0	–
	F0	83.3 ±2.1**	80.1 ± 1.6	76.2 ±1.8**	71.9 ± 2.5**
	F1-Interim	68.9 ± 1.8	68.7 ±2.0	61.9 ± 1.2**	–
% Progressively Motile	F1-terminal	80.1 ± 1.5**	77.4 ±1.2	71.7 ±2.9	–
	F0	70.0 ± 1.9*	68.9 ±1.3	67.2 ±1.6	65.3 ± 2.5
	F1-interim	57.6 ±1.6	57.4 ±1.7	51.4 ± 1.4*	–
	F1-terminal	66.9 ±1.3	66.5 ±1.2	64.4 ± 2.9	–

Statistical analysis for F0 data performed by Jonckheere trend (0 ppm column) and Shirley or Dunn pairwise tests. For F1 animals with littermates, a bootstrapped Jonckheere trend test was used, with pairwise comparisons using the Datta-Satten modified Wilcoxon test with a Hommel adjustment. F0 n = 23, 23, 20, 21; F1 – interim n = 49, 56, 20; F1 – terminal n = 40, 44, 39. * $p < 0.05$, ** $p < 0.01$.

Oestrus cycle length appeard to be increased with higher doses of 4-methylimidazole in the F0 females.

Reproductive organs (Tables 33-35):
No significant change in testis absolute weights were observed. Absolute epididymis weights were reduced in the 2500 and 5000 groups for 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 at the 5000 ppm dose. The incidence of exfoliated germ cells in the epididymis was significantly increased in the F0 animals of the 5000 ppm dose 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 in 4-methylimidazole treated animals. Prostate atrophy was generally of minimal to mild severity except in the 5000 ppm group, which was generally of mild to moderate severity (Table xx).

Absolute levator ani bulbocavernosus muscle complex (LABC) weights were significantly decreased in F0 2500 and 5000 ppm exposure groups compared to controls with decreasing trends observed in the F1c timepoints. However, relative LABC weights were not significant difference from controls.

Table 33 (Copy of Table 8 from Behl et al., 2020)

Table 8
Male rat body and reproductive organ weights (average ± SEM) for each generation after 4-MI exposure.

Endpoint	Generation	0 ppm	750 ppm	2500 ppm	5000 ppm
Body Weight (g)	F0	504.5 ± 4.4**	477.5 ± 5.9**	459.2 ± 7.5**	455.2 ± 5.5**

	F1-c interim	395.0 ±5.7**	383.4 ±4.9	338.9 ± 4.4**	–
Right Testis Absolute (g)	F1-c terminal	497.3 ±7.7**	486.8 ±6.7	443.8 ± 10.3**	–
	F0	2.089 ±0.023	2.089 ± 0.030	2.086 ± 0.028	2.066 ± 0.029
	F1-c interim	1.928 ±0.029	1.928 ±0.023	1.897 ±0.047	–
Left Testis Absolute (g)	F1-c terminal	2.095 ±0.036	2.121 ±0.026	2.182 ±0.049	–
	F0	2.075 ±0.026	2.074 ±0.026	2.096 ±0.026	2.062 ± 0.029
	F1-c interim	1.931 ±0.029	1.911 ±0.023	1.884 ±0.038	–
Right Epididymis Absolute (mg)	F1-c terminal	2.090 ±0.038	2.103 ±0.030	2.150 ±0.049	–
	F0	681 ± 9**	686 ± 10	628 ± 9**	600 ± 9**
	F1-c interim	571 ±9**	564 ±8	513 ±12**	–
Right Epididymis relative (mg/g)	F1-c terminal	697 ±9**	697 ±9	651 ±11**	–
	F0	1.35 ± 0.02	1.44 ± 0.03*	1.37 ±0.03	1.32 ± 0.02
	F1-c interim	1.45 ± 0.02	1.47 ±0.02	1.52 ±0.05	–
Left Epididymis Absolute (mg)	F1-c terminal	1.41 ± 0.02*	1.44 ±0.02	1.47 ±0.02	–
	F0	704 ± 9**	718 ± 13	657 ±11**	635 ± 10**
	F1-c interim	579 ±10**	561 ±7	521 ±12**	–
Left Epididymis Relative (mg/g)	F1-c terminal	697 ±13**	701 ±9	648 ±12**	–
	F0	1.40 ±0.02	1.51 ± 0.04*	1.44 ±0.03	1.40 ± 0.02
	F1-c interim	1.47 ± 0.02*	1.47 ±0.01	1.54 ± 0.04*	–
Dorsolateral Prostate Absolute (mg)	F1-c terminal	1.41 ±0.02	1.45 ±0.02	1.47 ±0.02	–
	F0	604 ±26**	492 ±23**	469 ±15**	421 ± 22**
	F1-c interim	402 ±11**	382 ± 12	330 ±13**	–
Dorsolateral Prostate Relative (mg/g)	F1-c terminal	539 ±16**	475 ± 19*	449 ±19**	–
	F0	1.20 ± 0.05**	1.03 ± 0.05**	1.02 ± 0.03**	0.93 ± 0.05**
	F1-c interim	1.02 ±0.03	1.00 ±0.03	0.97 ±0.03	–
Ventral Prostate Absolute (mg)	F1-c terminal	1.08 ±0.03	0.98 ±0.04	1.01 ±0.03	–
	F0	935 ±28**	785 ±21**	748 ±29**	515 ± 27**
	F1-c interim	561 ±20**	446 ± 14**	355 ±21**	–
Ventral Prostate Relative (mg/g)	F1-c terminal	825 ±22**	796 ± 27	591 ±18**	–
	F0	1.85 ± 0.05**	1.65 ± 0.05**	1.63 ± 0.06**	1.13 ± 0.06**
	F1-c interim	1.42 ±0.05**	1.16 ± 0.03**	1.05 ±0.06**	–
Seminal Vesicle Absolute (g)	F1-c terminal	1.67 ±0.05**	1.64 ± 0.06	1.34 ±0.05**	–
	F0	1.946 ± 0.053**	1.647 ± 0.045**	1.520 ± 0.045**	1.253 ± 0.042**
	F1-c interim	1.304 ± 0.032**	1.186 ± 0.028*	1.016 ± 0.035**	–
Seminal Vesicle Relative (mg/g)	F1-c terminal	1.76 ±0.04**	1.69 ± 0.04	1.46 ±0.05**	–
	F0	3.87 ±0.10**	3.44 ± 0.11**	3.29 ±0.08**	2.75 ± 0.10**
	F1-c interim	3.31 ± 0.06*	3.10 ±0.07	3.00 ± 0.09*	–
	F1-c terminal	3.56 ± 0.08	3.48 ±0.09	3.29 ± 0.10	
LABC Absolute (g)	F0	1.438 ± 0.028**	1.369 ± 0.023	1.265 ± 0.034**	1.236 ± 0.022**
	F1-c interim	1.161 ±0.027*	1.095 ±0.024	1.052 ±0.035	–
LABC Relative (mg/g)	F1-c terminal	1.341 ±0.029*	1.301 ±0.034	1.222 ±0.041	–
	F0	2.85 ± 0.06	2.87 ± 0.05	2.76 ± 0.06	2.72 ± 0.06
	F1-c interim	2.94 ±0.05	2.86 ±0.06	3.11 ±0.11	–
	F1-c terminal	2.70 ±0.05	2.68 ±0.06	2.75 ±0.07	–

Statistical analysis for F0 data performed by Jonckheere trend (0 ppm column) and Williams or Dunnett pairwise tests. Statistical analysis for F1 animals with littermates was performed by mixed models with a random litter effect and a Dunnett-Hsu adjustment for both trend and pairwise analyses. * $p < 0.05$, ** $p < 0.01$, "- "= no animals examined due to early removal.
F0 n = 20–23/group; F1-interim n = 20–56/group; F1-terminal n = 39–44/group.

Table 34 (Copy of Table 10 from Behl et al., 2020)

Table 10
Incidences of selected histopathologic lesions of the F0, F1c Interim, and F1c terminal rats after 4-MI exposure.

	Generation	0 ppm	750 ppm	2500 ppm	5000 ppm
MALES					
Number of animals examined (litters)	F0	(23)	(23)	(23)	(23)
	F1-interim	49 (18)	56 (22)	20 (8)	–
	F1-terminal	40 (18)	44 (22)	40 (15)	–
Prostate, Ventral Lobe – Atrophy	F0	0%**	9 [1.0][a] 39 %**	20 [1.1] 87 %**	23 [2.4] 100 %**
	F1-interim	4 [1.0] 8%**	25 [1.0] 45 %**	17 [1.2] 85 %**	–
	F1-terminal	4 [1.0] 10 %**	10 [1.0]23 %	35 [1.5] 88 %**	–
Testis – Degeneration	F0	1 [1.0] 5%**	0%	4 [1.8] 17 %	8 [1.6] 35 %*
	F1-interim	4 [1.3] 8%	6 [1.0] 11 %	1 [2.0] 5%	–
	F1- terminal	2 [1.0] 5%	5 [2.0] 11 %	5 [1.2] 13 %	–
Testis – Spermatid Retention	F0	2 [1.0] 9%*	3 [1.0] 13 %	1 [1.0] 4%	8 [1.3] 35 %*
	F1- interim	0%	3 [1.0] 5%	4 [1.0] 20 %	–
	F1- terminal	0%	5 [1.2] 11 %	4 [1.0] 10 %	–
Epididymis – Exfoliated Germ Cells	F0	1 [1.0] 5%**	0	3 [1.7] 13 %	7 [1.3] 30 %*
	F1- interim	3 [1.3] 6%	5 [1.2] 9%	4 [1.3] 20 %	–
	F1- terminal	0%	5 [1.2] 11 %	4 [1.3] 10 %	–
Liver, Centrilobular Hepatocyte – Vacuolation	F0	0%*	NE	0%	19 [1.9] 83 %**
	F1- interim	0%	NE	0%	–
	F1- terminal	0%	NE	1 [1.0] 3%	–
FEMALES					
Number of animals examined (litters)	F0	23	NE	23	23
	F1-interim	47	58	27	
	F1-terminal	40	43	40	
Kidney – Mineral	F0	1 [1.0] 4%	NE	2 [1.0] 9%	1 [2.0] 4%
	F1- interim	9 [1.0] 19 %**	44 [1.3] 76 %**	20 [1.2] 74 %**	–
	F1- terminal	8 [1.0] 20 %**	21 [1.1] 49 %*	27 [1.1] 68 %**	–

[a] Incidence with [avg. severity score] and percent incidence; Severity scores: 1 = Minimal, 2 = Mild, 3 = Moderate, 4 = Severe. Average severity scores were not used in statistical significance calculations.
Statistical analysis for the F0 animals was performed using the Poly-3 trend (0 ppm column) and pairwise statistics. Statistical analysis for F1 animals was performed using a Cochran-Armitage test with a poly-3 adjustment for survival and a Rao-Scott modification for litter effect. All tests were one-sided. * $p < 0.05$, ** $p < 0.01$, NE = not examined (read-down) "- "= no animals examined due to early removal.

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 35 (Copy of Table 9 from Behl et al., 2020)

Table 9
Mean body and ovarian weights (± SEM) of F0 and F1 females.

	Generation	0 ppm	750 ppm	2500 ppm	5000 ppm
Body weight (g)	F0	339.3 ± 6.3 **	291.7 ± 2.3**	300.7 ± 5.1**	275.0 ± 4.1**
	F1-interim	242.6 ± 3.9 **	233.8 ± 2.7	217.8 ±4.4 **	–
	F1-terminal	349.9 ± 6.5**	325.5 ± 4.1 **	311.9 ±3.8 **	–
Right Ovary Absolute (mg)	F0	65.6 ± 3.9**	48.2 ± 3.7*	61.3 ± 3.6 *	39.7 ± 3.4 **
	F1-interim	55.2 ± 1.4*	52.8 ±2.2	47.8 ± 1.6*	–
	F1-terminal	84.3 ± 4.3	73.6 ±3.2	70.8 ± 3.3	–
Right Ovary Relative (mg/g)	F0	0.19 ± 0.01	0.17 ± 0.01	0.20 ± 0.01	0.15 ± 0.01*
	F1-interim	0.23 ±0.01	0.22 ±0.01	0.22 ±0.01	–
	F1-terminal	0.24 ±0.01	0.23 ±0.01	0.23 ±0.01	–
Left Ovary Absolute (mg)	F0	66.1 ± 4.4**	53.2 ± 4.8	66.0 ± 4.5	38.9 ± 3.7**
	F1-interim	58.5 ±1.5*	54.8 ±2.4	51.1 ±2.7	–
	F1-terminal	83.4 ±4.1*	75.4 ±4.7	71.1 ±2.4	–
Left Ovary Relative(mg/g)	F0	0.20 ±0.01	0.18 ± 0.02	0.22 ± 0.01	0.14 ± 0.01*
	F1-interim	0.24 ±0.01	0.23 ±0.01	0.23 ±0.01	–
	F1-terminal	0.24 ±0.01	0.23 ±0.01	0.23 ±0.01	–

Statistical analysis for F0 data performed by Jonckheere trend (0 ppm column) and Williams or Dunnett pairwise tests. Statistical analysis for F1 animals with littermates was performed by mixed models with a random litter effect and a Dunnett-Hsu adjustment for both trend and pairwise analyses. * $p < 0.05$, ** $p < 0.01$, "- "= no animals examined due to early removal.
F0 n = 12–22/group; F1-interim n = 27–58/group; F1-terminal n = 23–34/group.

There were statistically significant increases in primordial (77%), antral (82%) and atretic follicles (61%) in the F0 5000 ppm group, with a significant dose-trend. 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 was not significant. No increase in atretic follicles was observed in the F1 animals at the 2500 ppm dose.

Systemic toxicity: No clear effects of 4-methylimidazole in the male and female non-reproductive tissues examined were reported. The changes described in liver and kidney weights were considered secondary to body weight reductions. Liver histopathology showed hepatocellular vacuolation in high dose (5000 ppm) F0 males. Female F1 rats had increased mineralization in the kidney. No apparent treatment related effects on the adrenal, thyroid or pituitary glands were reported.

Developmental toxicity:
Pup survival PND1-4: lower in F= 5000 ppm and F1c 2500 ppm groups. Survival from PND5-28 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 AGD or body weight adjusted AGD across litters. A small number of pups were observed to have areolae or nipples in the F1c and F2c generations in the 2500 ppm group.

Testicular descent: There was a significant trend toward delayed day of testicular descent in the F1c and F2c generation, and the delay in the 2500 ppm F2c males was significant by pairwise comparison to the controls

Statistically significant delays in PPS VO, markers of pubertal development, were seen in male and female F1c offspring in the 750 and 2500 ppm groups relative to controls. These delayes were still significant after adjustment for body weight at weaning.

Table 36 (Copy of Table 6 from Behl et al., 2020)

Table 6

Developmental markers (mean ± SEM) in male and female rats after 4-MI exposure.

	0 ppm	750 ppm	2500 ppm
F1c Examined, Males (no. of litters)	99 (18)	115 (22)	61 (15)
Areolae/nipples per litter	0	0	0.14 ± 0.10
Pups with areolae/nipples (%)	0 (0)*	0 (0)	3 (4.92)
Litters with areolae/nipples (%)	0 (0)	0 (0)	2 (13.33)
Day of testis descent	16.7 ± 0.2*	16.8 ± 0.2	17.1 ± 0.2
F2c Examined,Males (litters)	108 (25)	133 (32)	69 (20)
Areolae/nipples per litter	0	0	0.17 ± 0.17
Pups with areolae/nipples (%)	0	0	3 (4.35)
Litters with areolae/nipples (%)	0	0	1 (5.00)
Day of testis descent [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 PPS (PND)	43.5 ± 0.4**	46.2 ± 0.4**	47.2 ± 0.6**
Body Weight at PPS (g)	195.8 ± 2.7	205.6 ± 2.9*	190.4 ± 3.4
Body Weight at PND 28 (g)	85.4 ± 1.6**	80.4 ± 1.7	73.1 ± 2.9**
Adjusted age at PPS[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**
Body Weight at VO (g)	106.2 ± 2.0**	117.2 ± 2.0**	117.1 ± 1.8**
Body Weight at PND 28 (g)	76.7 ± 1.6**	71.3 ± 1.5*	64.3 ± 2.3**
Adjusted age at VO[b]	34.1 ± 0.2**	37.2 ± 0.3**	39.0 ± 0.3**

Means of litter means for age at attainment are presented. Trend (0 ppm column) and pairwise tests for age at attainment were based on mixed models with dose as a covariate and a random effect for litter, with a Dunnett-Hsu adjustment for multiple comparisons. For PPS and VO, mixed models included weaning weight as a covariate. Mixed models for body weight at attainment and body weight at weaning included dose as covariate and a random effect for litter, with a Dunnett-Hsu adjustment for multiple comparisons. * $p < 0.05$, ** $p < 0.01$.

[a] Number of animals (litters) examined for testicular descent was 107 (25), 132 (32), and 68 (20) respectively.

[b] Means of adjusted age at PPS and VO were calculated as the mean of the litter means of the weaning weight-adjusted attainment age for individual pups.

3.6.2 **Human data**

No data available.

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

3.6.3.1 Imidazoles and effects on testosterone and Testicular Interstitial Fluid Formation (TIF) in rats

Study reference:

Adams ML *et al*., 1998. Imidazoles suppress rat testosterone secretion and testicular interstitial fluid formation in vivo. Biol Reprod 59: 248-254.

Detailed study summary and results:

Test substances

Several imidazoles were included in the study. 4-methylimidazole was obtained from Sigma Chemical Company (St. Louis, MO). No information on purity was provided in the manuscript.

Test animals

Adult (60 days old) male Sprague-Dawley-derived rats originally derived from Sprague-Dawley, Inc. (Indianapolis, IN) rats were used for most of the experiments with 4-methylimidazole. Adolescent (42 days old) rats were used for the co-exposure study with NMA.

Exposure and study protocol

Dose-response experiment: 2 hour exposure, 10 rats/group. Doses of imidazoles from 10–300 mg/kg were administered by subcutaneous injection. Saline was used as vehicle control.

Time-response experiment: A dose of 50 mg/kg (609 µmol/kg) of 4-methylimidazole or saline control were administered by subcutaneous injection and serum and testicular interstitial fluid (TIF) were collected at 0.5, 1, 2, 4, 6, 8, 16, and 24 h after injection. 10 rats/group.

Co-exposure experiment: 4 hour exposure to 4-methylimidazole (50 mg/kg) in combination with injections of saline (control) or different testicular stimulants (human chorionic gonadotropin (hCG, 20 IU/kg)f N-methyl-D,L-aspartate (NMA, 70 mg/kg); N^G-nitro-L-arginine methyl ester (NAME, 100 mg/kg), or naltrexone (5 mg/kg)). 10 rats/group.

Testicular Interstitial Fluid (TIF) collection: Immediately after serum collection from trunk blood, both testes were removed, small holes were cut in the caudal end of each testis, and each testis was then suspended in a tube to allow TIF drainage overnight. TIF volumes were then measured with a pipette.

Hormonal measurements: Testestorone and LH were measured by RIA. Serum testosteron was measured following ethanol extraction whereas testosterone was measured in TIF and LH in serum without prior extraction.

Statistics: ANOVA followed by post hoc analysis with Fisher's protected least-significant difference tests was used to de-termine significant differences between groups.

Results and discussion

4-methylimidazole dose-dependently decreased serum and TIF testosterone as well as TIF volume 2 hours after subcutaneous injection. Serum LH was reduced compared to controls at the 2 hour time point in the dose-response experiment but only reduced at the 4 hour timepoint in the time-trend experiment. A dose of 50 mg/kg significantly decreased serum testosterone levels at 2–6 hours, TIF testosterone levels at 2–4 hours and TIF volumes at 1–8 hours after injection of 4-methylimidazole. Following these decreases, significant ''rebound'' increases in serum testosterone and TIF testosterone was observed and the levels were comparable to controls at the 24 hour time point. Co-exposure of 4-methylimidazole and hCG blocked the hCG-induced increase in testosterone secretion indicating that the anti-androgenic action of 4-methyimidazole mainly occurs at the level of the testis although a certain effect also on LH release is

suggested. Furthermore, 4-methylimidazole inhibited the increase in testosterone secretion observed in response to the nitric oxide synthetase inhibitor NAME, the opioid antagonist naltrexone and the excitatory amino acid NMA.

3.6.3.2 Non-animal tests:

4-methylimidazole is inactive in the ToxCast models CERAPP and COMPARA for (anti)oestrogen and (anti)androgen activities. In addition it is negative in 22 of the 22 EDSP21 assays performed.
CompTox Chemicals Dashboard axcessed 19th October 2020.

3.7 Specific target organ toxicity – single exposure

This endpoint was not evaluated.

3.8 Specific target organ toxicity – repeated exposure

This endpoint was not evaluated.

3.9 Aspiration hazard

This endpoint was not evaluated.

4 ENVIRONMENTAL HAZARDS

This endpoint was not evaluated.