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:

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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.

References

Fennell T., Watson S., Dhungana S., Snyder R.., 2019. Metabolism of 4-methylimidazole in Fisher 344 rata and B6C3F1 mice. Food Chem Toxicol. 123: 181-194.

Hargreaves, M.B., Jones, B.C., Smith, D.A., and Gescher, A. (1994). Inhibition of p-nitrophenol hydroxylase in rat liver microsomes by small aromatic and heterocyclic molecules. Drug Metab. Dispos. 22, 806-810.

Hidaka, M. (1976). Physiological activity of 4-methylimidazole. III. Absorbance and excretion rate of 4-methylimidazole in the organ. *Okayama Igakkai Zasshi* **88**, 665-671.

Karangwa, E., Mitchell, G.E., Jr., and Tucker, R.E. (1990). Pharmacokinetics of 4-methylimidazole in sheep. *J. Anim. Sci.* **68**, 3277-3284.

Morgan, S.E., and Edwards, W.C. (1986). Pilot studies in cattle and mice to determine the presence of 4-methylimidazole in milk after oral ingestion. *Vet. Hum. Toxicol.* **28**, 240-242.

Nielsen, P., Friis, C., Kraul, I., and Olsen, C.E. (1993). Disposition of 4-methylimidazole in goats and heifers. *Res. Vet. Sci.* **54**, 72-79.

NTP (2007). Toxicology and Carcinogenesis studies of 4-methylimidazole in F344/N rats and B6C3F1 mice (feed studies), NTP TR 535.

Yuan, J.H., and Burka, L.T. (1995). Toxicokinetics of 4-methylimidazole in the male F344 rat. *Xenobiotica* **25**, 885-894.

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	50	50	50	50
Alveolar Epithelium, Hyperplasia[a]	7 (2.0)[b]	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≤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 ($P \le 0.05$) from the control group by Dunn's or Shirley's test
** Significantly different ($P \le 0.01$) 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≤0.05) from the control group by Williams' or Dunnett's test
** Significantly different (P≤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 ($P \leq 0.01$) 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 ($P \leq 0.01$) 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

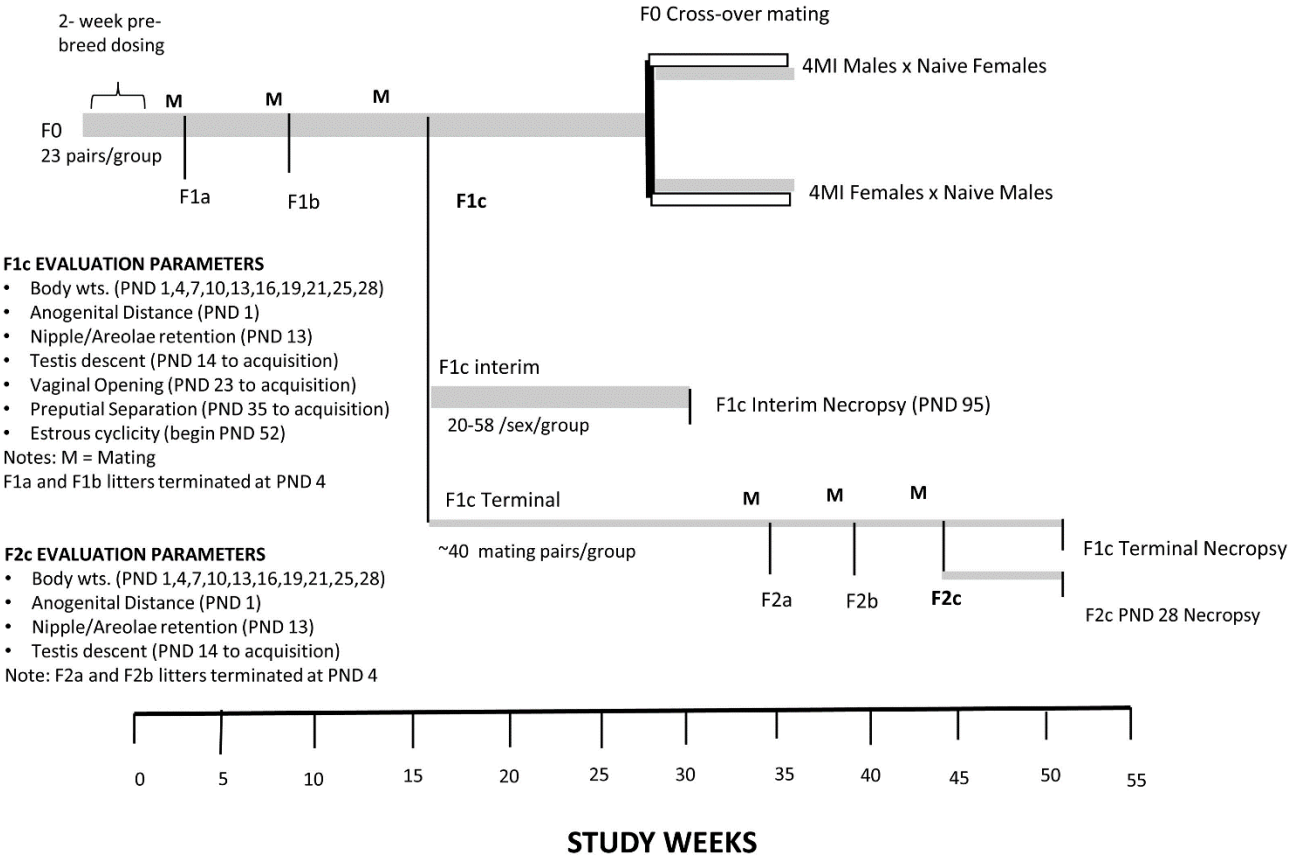
Feed and water were available ad libitum. F0: 23 rats/sex/group, age 10-12 weeks, were exposed to 4-methylimidazole in diet at 0, 750, 2500, and 5000 ppm from two weeks prior to the first cohabitation. Reproductive performance of the animals in the high dose group was severely impaired and thus the F1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