Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

4-methylimidazole

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

CLH-O-0000007050-88-01/F

Adopted

26 November 2021

26 November 2021

CLH-O-0000007050-88-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **4-methylimidazole**

EC Number: **212-497-3**

CAS Number: **822-36-6**

The proposal was submitted by **Norway** and received by RAC on **15 December 2020.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Norway has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **8 February 2021**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **9 April 2021**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Ifthekhar Ali Mohammed**

Co-Rapporteur, appointed by RAC: **Ruth Moeller**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **26 November 2021** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	4-methylimidazole	212-497-3	822-36-6	Carc. 1B Repr. 1B	H350 H360Fd	GHS08 Dgr	H350 H360Fd			
RAC opinion	TBD	4-methylimidazole	212-497-3	822-36-6	Carc. 1B Repr. 1B	H350 H360Fd	GHS08 Dgr	H350 H360Fd			
Resulting Annex VI entry if agreed by COM	TBD	4-methylimidazole	212-497-3	822-36-6	Carc. 1B Repr. 1B	H350 H360Fd	GHS08 Dgr	H350 H360Fd			

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

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

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

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

For *in vitro*, two bacterial reverse mutation assays (Ames, test, reliability 1) conducted in different *Salmonella typhimurium* strains were negative. 4-methylimidazole (up to 10000 µg/plate) was not mutagenic in *S. typhimurium* strains TA97, TA98, TA100, or TA1535, when tested with and without 10% or 30% hamster or rat liver S9 activation enzymes (NTP, 2007). In the second study, 4-methylimidazole (up to 5000 µg/plate) was not mutagenic in *S. typhimurium* strains TA98, TA1535, TA1537, TA100 and TA102. Consistent negative results were obtained both in the absence and presence of exogenous metabolism, regardless of whether metabolic activity was provided by S9 from induced rat liver or lung, or mouse liver or lung. This in absence of cytotoxicity (Beevers and Adamson 2016). In the third study (Norizadeh Tazehkand *et al*., 2016) on sister chromatid exchanges, chromosomal aberrations and micronuclei induction in human primary lymphocytes a genotoxic chromosomal effect was observed, concurrently with cytotoxicity. The DS rated the study Klimisch 3, reported several deficiencies and guideline deviations and concluded that potentially the observed increased genotoxicity could be an indirect consequence of high cytotoxicity. This because 4-methylimidazole negatively affected the mitosis, the proliferation index and the nuclear division index at the genotoxic concentrations.

In vivo, two Micronucleus assays (reliability 1) were negative. In an NTP (2007a) study similar to OECD TG 475 no increases in the frequencies of micronucleated erythrocytes were seen in bone marrow of male F344/N male rats and male B6C3F1 mice was observed after *i.p.* administration three times at 24-hour intervals on three consecutive days in both species (n=5). Significant decrease in the percent of micronucleated polychromatic erythrocytes (PCEs) as indicator of bone marrow toxicity was observed only in rats. In a 14-week NTP (2007b) toxicity study on peripheral blood micronucleus induction with B6C3F1 mice exposed 7 days/week by feed no increases in peripheral blood micronuclei in male and female mice was observed, also no bone marrow toxicity. By contrast, in a chromosomal aberration test (Norizadeh Tazehkand *et al*., 2016) in the bone marrow cells of Swiss Albino mice (non-guideline study) comparable to OECD TG 475 with several deviations and considered by the DS as Klimisch 3, 4-methylimidazole increased the percentage of chromosomal aberrations at all concentrations after 12 h and at highest concentration after 24 h. The mitotic index decreased in comparison with control at the highest concentration for 12 h and at all concentrations for 24 h.

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

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

Comments received during consultation

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

Assessment and comparison with the classification criteria

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

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

Method, guideline, deviations if any, reliability	**Test substance & concentrations**	**Observations**
Bacterial Reverse Mutation Assay (NTP 2007). Similar to OECD TG 471. Reliability 1 Deviation: The study did not include strain TA 102 or *E. coli* WP2*uvrA* since protocols used were developed and in place prior to the 1997 OECD TG 471.	4-methylimidazole (>99% purity) a) high dose: 0, 100, 333, 1000, 3333 and 10000 µg/plate (10000 µg/plate, exceeds the current OECD TG 471) b) lower doses: 1, 3.3, 10, 20, 33 µg/plate (limited by toxicity) +/- 10% or 30% hamster or rat liver S9 activation	4-methylimidazole (up to 10000 µg/plate) was **not mutagenic** in *S. typhimurium* strains TA97, TA98, TA100, or TA1535, +/- 10% or 30% hamster or rat liver S9.
Bacterial Reverse Mutation Assay (Beevers and Adamson, 2016). OECD TG 471 compliant. Reliability 1	4-methylimidazole (99% purity). plate incubation test: 0, 5, 15.81, 50, 158.1, 500, 1581, and 5000, 10000 µg/plate. (10000 µg/plate, exceeds the current OECD TG 471) pre-incubation test: 0, 156.3, 312.5, 625, 1250, 2500, and 5000 µg/plate +/- rat 10% and mouse liver S9, to rat and mouse lung S9 activation enzymes.	4-methylimidazole (up to 5000 µg/plate) was **not mutagenic** in *S. typhimurium* strains TA98, TA1535, TA1537, TA100 and TA102. Consistent negative for +/- S9 **No cytotoxicity** was observed.

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

CA: chromosome aberration; MN: micronucleus

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

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

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

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

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

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

Method, guideline, deviations if any, reliability	**Test substance**	**Observations**
Micronucleated erythrocytes in rat and mouse bone marrow (NTP, 2007a, protocol according to Shelby *et al*., 1993) Reliability 1 Similar to OECD TG 475	4-methylimidazole (99.5% purity) Three consecutives *i.p.* doses at 24 h interval: 0, 25, 50, 100 mg/kg bw. PC = Cyclophosphamide Two species (N=5) One trial in F344/N male rats / Two trials in B6C3F1 male mice	**Negative in rats.** No increases in the frequencies of micronucleated erythrocytes in bone marrow of rats. **Overall negative in mice**: In mice, at 50 and 100 mg/kg bw significant increases in the MN frequency in the first trial but not in second trial. **Bone marrow toxicity in rats only**: No significant alterations in %-micronucleated polychromatic erythrocytes in mouse. In rat

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

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

In the bone marrow micronucleus study presented in NTP 2007, male B6C3F1 mice and male F344/N rats were treated three days in a row with the test substances by *i.p.* route of

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

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

NTP considered the studies on *in vivo* micronucleus induction as negative. RAC notes that some uncertainty is evident due to absence of bone marrow toxicity in mice as an indicator of target organ exposure, but also due to a slight positive result in the first mouse trial in the *i.p.* study. For rats, bone marrow toxicity was evident, and no MN were induced. As regards the target organ exposure, toxicokinetic data presented in the CLH report show that 4-methylimidazole is rapidly absorbed, widely distributed, metabolised to a low degree in the liver, and eliminated in mammals without significant bioaccumulation after oral gavage and intravenous injection. The compound was excreted unchanged in urine, beginning approximately 30 minutes after injection, and reached approximately 90% within 8 hours. In an *i.p*. study in rats, the uptake at 5 minutes after a single 216 mg/kg bw *i.p*. injection was highest in the intestines, followed by blood, liver, stomach, and kidney (Hidaka, 1976). The DS assumed that toxicity of 4-methylimidazole stems from the parent chemical itself, and not from a metabolite since metabolism is almost absent in the available studies.

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

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

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

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

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

Positive genotoxicity results were reported *in vitro* for CA, SCE and MN in a non-guideline study, rated Klimisch 3, with human peripheral blood lymphocytes from four donors. The genotoxicity result was obtained in presence of moderate and mainly marked cytotoxicity and important deficiencies have been noted for the study. A follow up *in vivo* study of the same research group on CA in Swiss Albino mice was also reported positive, however, the study is considered not reliable for classification purpose (Norizadeh Tazehkand *et al*., 2016).

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

Conclusion on classification for germ cell mutagenicity

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

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

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

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

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

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

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

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

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

NTP reported "equivocal evidence of carcinogenic activity" in female rats based on increased incidences of mononuclear cell leukaemia and "no evidence of carcinogenic activity" in male rats. In mice, 4-methylimidazole increased the incidence of alveolar/bronchiolar adenoma in females in all dose groups, of alveolar/bronchiolar carcinoma in high dose males and of alveolar/bronchiolar adenoma and carcinoma combined in high dose males and in mid and high dose females. Hyperplasia of alveolar epithelium is considered to be a precursor for neoplasia but was not observed at lower doses and not in the 14-week study (NTP, 2004).

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

Significant reduction in neoplasms either below or at the lower end of the historical control ranges of the pituitary gland (pars distalis) and adrenal medulla in exposed groups of males and of the pituitary gland (pars distalis), clitoral gland, mammary gland, and uterus in exposed groups of females were observed in the rat study. These reductions were considered to be only partially explained by reduction in body weight. No 4-methylimidazole related decrease in tumours was observed in the mouse study.

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

The DS considered the findings of statistically significantly increased incidences of alveolar/bronchiolar adenomas in female mice, alveolar/bronchiolar carcinomas in male mice and benign and malignant neoplasms combined in both sexes to constitute sufficient strength of evidence for classification as Carc. 1B. Additional considerations were findings of carcinogenic activity outside robust HCD from NTP, progression to malignancy, response in both sexes, and to some extent response in two species, although as a cancer form with high spontaneous tumour incidence in rats (mononuclear cell leukaemia).

Comments received during consultation

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

Assessment and comparison with the classification criteria

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

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

Method, guideline, deviations if any	**Results**
NTP, 2-year cancer bioassay in rats, GLP Similar to OECD TG 451 Reliability 1 F344/N **rats**, 50/sex/dose 4-methylimidazole (> 99% pure) Oral exposure for 106 weeks to 0, 625, 1250, or 2500 ppm (males) or 0, 1250, 2500, or 5000 ppm (females) in feed Calculated average doses based on food consumption of: 0, 30, 55, 115 mg/kg bw/d (males); 0, 60, 120, 260 mg/kg bw/d (females). (NTP, 2007; Chan 2008)	**General toxicity and survival:** No significant effect on survival was observed. 31/50, 34/50, 33/50, 32/50 males 43/50, 39/0, 34/50, 35/50 females ↓mean terminal body weights of males 1250 and 2500 ppm and females 2500 and 5000 ppm, ↓feed consumption high dose females (5000 ppm), Clinical signs of neurological toxicity (clonic seizures, excitability, hyperactivity, and impaired gait) in high dose females. **Non-neoplastic lesions (graded as minimal to mild):** ↑hepatic histiocytosis and chronic inflammation, hepatocyte focal fatty change, hepatocellular eosinophilic and mixed cell foci Males: Histiocytosis (38/50, 45/50, 50/50, 50/50); chronic inflammation (18/50, 32/50, 31/50, 36/50); hepatocyte, focal fatty change (21/50, 24/50, 37/50, 33/50); eosinophilic focus (4/50, 3/50, 7/50, 12/50); mixed cell focus (5/50, 7/50, 11/50, 27/50); Females: Histiocytosis (40/50, 50/50, 48/48, 50/50); chronic inflammation (17/50, 28/50, 34/48, 35/50); hepatocyte, focal fatty change (16/50, 29/50, 29/48, 32/50); eosinophilic focus (1/50, 2/50, 5/48, 11/50); mixed cell focus (10/50, 7/50, 6/48, 18/50); ↑inflammation in the prostate gland, focal hypertrophy in the pituitary gland (pars distalis), and follicular cysts in the thyroid gland of male rats at the high dose and also medium dose for pituitary lesions, ↑follicle mineralization in the thyroid gland at the high dose females and lung inflammation, cardiomyopathy and focal atrophy of the acinar pancreas in all exposure groups. **Neoplastic lesions:** ***Mononuclear cell leukaemia*** Incidence of **mononuclear cell leukaemia in high dose females** was significantly greater than that in the controls, and the incidence slightly **exceeded the historical control range**. **Reduced tumour onset**, in high dose group in females earlier (day 368) than in control females (day 624). Slight, non-significant, increase in incidence of mononuclear cell leukaemia in males. ***See table below*** ***Other neoplasms decreased incidence (overall incidences):*** Either below or at the lower end of the historical control ranges. *Females*

Method, guideline, deviations if any	Results
	Adenoma in clitoral gland: 16, 2, 0, 0% in 0, 625, 1250, 2500 ppm (HCD 11.0% ± 6.5%; range 2-20%). Fibroadenoma in mammary gland: 48, 12, 8, 2% in 0, 625, 1250, 2500 ppm (HCD 44.8% ± 11.1%; range 28-55%). Stromal polyp in uterus: 32, 10, 4, 4% in 0, 625, 1250, 2500 ppm (HCD 17.9% ± 6.5%; range 12-32%). *Males* Adrenal medulla (benign, complex, or malignant pheochromocytoma (combined): In male rats 20, 12, 6 and 6% in 0, 625, 1250 and 2500 ppm (HCD 11.6% ± 5.5%; range 5-20%). *Males and females* Adenoma in pituitary gland: In male rats 33, 27, 21, 15% and in female rats 60, 38, 40, 18% in 0, 625, 1250, 2500 ppm (HCD in males 22.6% ± 6.0%; range 17-33%. HCD in females 39.1% ± 10.9%; range 29-60%).
NTP, 2-year cancer bioassay in mice, GLP Similar to OECD TG 451 B6C3F1 **mice**, 50/sex/dose Reliability 1 4-methylimidazole (> 99% pure) Oral exposure f or 106 weeks to 0, 312, 625, or 1250 ppm in feed Calculated average doses based on food consumption of: 0, 40, 80, or 170 mg/kg bw/d (males/females) (NTP, 2007; Chan 2008)	**General toxicity and survival:** No significant effect on survival was observed. 45/50, 44/50, 42/50, 46/50 males 43/50, 40/50, 43/50, 40/50 females Mean terminal body weights reduced in the 1250 ppm group (males) and in all exposure groups (females). Feed consumption generally similar to the controls. No clinical findings considered treatment related. **Non-neoplastic lesions:** ↑incidences of alveolar epithelium hyperplasia and histiocytic cellular infiltration in 1250 ppm females significantly greater. Histiocytic cellular infiltration slightly increased in 1250 ppm males. ↑incidences of thyroid follicular cyst in 1250 ppm females significantly greater. ↑significant positive trend in incidences of mammary gland hyperplasia in females (16/50, 10/50, 14/49, 24/49). **Neoplastic lesions** ***Alveolar/bronchiolar adenoma and carcinoma*:** The incidences of alveolar/bronchiolar adenoma in all exposed groups of females, alveolar/bronchiolar carcinoma in 1250 ppm males, and alveolar/bronchiolar adenoma or carcinoma (combined) in 1250 ppm males and 625 and 1250 ppm females were significantly higher than those in the control group. In females, the incidences of alveolar/bronchiolar adenoma (all exposure groups) and alveolar/bronchiolar carcinoma (high dose) clearly exceeded the historical control range, whereas in males the incidences of alveolar/bronchiolar adenoma and carcinoma slightly exceeded the historical control range in the high dose. ***See table below***

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

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

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

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

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

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

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

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

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

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

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

The incidences of alveolar/bronchiolar adenoma in all exposed groups of females, alveolar/bronchiolar carcinoma in high dose males, and alveolar/bronchiolar adenoma or carcinoma (combined) in high dose males and mid and high dose females were significantly increased compared to control group.

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

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

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

Dose level (ppm)	**0**	**312**	**625**	**1250**	**Trend**
Males					
Alveolar/bronchiolar adenoma	16%	22%	26%	**30%**	**-**
HCD			15.8 ± 6.3%; range 9–28%		
Alveolar/bronchiolar carcinoma	4%	8%	8%	**16%** **p=0.042**	**p=0.024**
HCD			7.8 ± 3.8%; range 4–14%		
Alveolar/bronchiolar adenoma or carcinoma combined	18%	26%	32%	**44%** **p=0.003**	**p<0.001**
HCD			22.2 ± 6.3%; range 14–32%		
Hyperplasia	14%	6%	2%	18%	-
Females					
Alveolar/bronchiolar adenoma	0%	**16%** **P=0.004**	**32%** **p<0.001**	**16%** **p=0.003**	**p=0.017**
HCD			3.7 ± 3.8%; range 0–10%		
Alveolar/bronchiolar carcinoma	6%	0%	4%	**14%**	**p=0.019**
HCD			2.9 ± 2.5%; range 0–6%		
Alveolar/bronchiolar adenoma or carcinoma combined	6%	**16%**	**34%** **p<0.001**	**28%** **p=0.002**	**p=0.002**
HCD			6.6 ± 4.2%; range 0–12%		
Hyperplasia	6%	4%	6%	**22%** **p<0.05**	-

No excessive toxicity was observed in the mouse cancer bioassay. The significant increase in the incidence of benign and malignant alveolar / bronchiolar neoplasms combined was observed in both sexes, outside the HCD range for all dose groups in females and outside the HCD range for males in the high-dose group.

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

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

Conclusion on classification for carcinogenicity

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

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

4-methylimidazole was tested in two reliable carcinogenicity studies.

In rats, the incidence of mononuclear cell leukaemia was increased with dose-dependent trend in females only, the high dose being significantly different from the control and slightly exceeding the HCD. At this dose, the MTD seems to be exceeded based on body weight gain reduction and neurotoxicity. Mononuclear cell leukaemia is a frequent tumour with high and variable background incidences in F334/N rats. Its relevance for humans has been questioned. Considering the high background incidence, this tumour type may not provide reliable evidence for treatment related carcinogenicity of 4-methylimidazole. According to the CLP guidance, in

such cases where only spontaneous tumours appear, downgrading from Category 1B to Category 2 or even no classification may be justified.

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

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

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

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

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

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

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Sexual function and fertility

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

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

Developmental toxicity

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

Adverse effects on or via lactation

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

Comments received during consultation

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

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

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

Assessment and comparison with the classification criteria

Sexual function and fertility

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

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

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

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

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

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

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

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

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

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

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

LD: lactation day

Reproductive performance

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

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

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

Dose (ppm)	0	750	2500	5000
N (# of pairings) F0 (three pairings) F1c terminal (three pairings)	 68 118	 69 126	 62 111	 44 -
Mated/Pair (average of three pairings) F0 F1c F0 (male x naïve female) F0 (naïve male x female)	 97.0% 92.4% 87.0%** 68.2%	 98.6% 97.0% – –	 96.8% 94.0% 80.0% 73.7%	 48.1% [a] - 33.3%** -
Littered/Mated (average of three pairings) F0 F1c F0 (male x naïve female) F0 (naïve male x female)	 90.8% 87.9% 80.0% 80.0%	 98.6% 86.9% - –	 86.2% 83.7% 93.8% 71.4%	 75% [a] – 85.7% –

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

Litter parameters

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

Pup survival on PND1-4 was significantly lower in the F0 5000 ppm group and after the first two matings of the F1 2500 ppm group. RAC considers these effects as adverse and relevant for classification for developmental toxicity. However, there were only 2 litters (first mating) and 1 litter (second mating) left in the F0 5000 ppm group. Also, the lower pup survival in the 2500 ppm group was not consistent (no effects at this dose level after any of the three matings of F0 and after the third mating of F1).

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

Dose (ppm)	0	750	2500	5000
Total litter size (PND0), each pair F0 - A B C F1c – A B C	 14.6 ± 0.4 (22)** 14.2 ± 0.5 (19)** 13.6 ± 0.6 (19)* 13.2 ± 0.5 (33)** 15.3 ± 0.5 (34)** 10.7 ± 0.8 (28)	 13.3 ± 0.6 (22) 13.5 ± 0.6 (23) 12.8 ± 0.6 (22) 11.0 ± 0.6 (34)* 12.3 ± 0.5 (37)** 9.8 ± 0.7 (35)	 9.9 ± 0.7 (17)** 9.2 ± 0.7 (17)** 10.6 ± 1.2 (16)* 8.9 ± 0.8 (31)** 10.6 ± 0.9 (29)** 9.4 ± 0.9 (23)	 5.7 ± 1.9 (6)** 4.7 ± 0.9 (6)** – - - -
Live litter size (PND0), each pair F0 - A B C F1c – A	 14.0 ± 0.4 (22)** 13.7 ± 0.5 (19)** 12.2 ± 0.8 (19) 11.1 ± 0.7 (33)**	 12.7 ± 0.6 (22) 12.7 ± 0.5 (23) 12.4 ± 0.5 (22) 9.3 ± 0.7 (34)	 7.8 ± 1.0 (17)** 8.5 ± 0.6 (17)** 9.8 ± 1.2 (16) 7.2 ± 0.8 (31)**	 1.8 ± 1.6 (6)** 0.5 ± 0.5 (6)** – -

Dose (ppm)	0	750	2500	5000
B	13.6 ± 0.6 (34)**	11.9 ± 0.6 (37)	9.8 ± 0.8 (29)**	-
C	9.2 ± 0.7 (28)	8.8 ± 0.6 (35)	8.5 ± 0.9 (23)	-
Survival ratio (PND1-4), each pair				
F0 - A	0.96 ± 0.02 (22)	0.98 ± 0.01 (22)	0.91 ± 0.04 (15)	0.10 ± 0.10 (2)
B	0.97 ± 0.02 (19)	0.98 ± 0.01 (23)	0.93 ± 0.03 (17)	0.67 (1)
C	0.95 ± 0.03 (19)	0.98 ± 0.01 (22)	0.93 ± 0.04 (15)	–
F1c – A	0.92 ± 0.05 (33)**	0.93 ± 0.04 (32)	0.72 ± 0.08 (29)**	-
B	0.97 ± 0.01 (34)*	0.97 ± 0.01 (36)	0.91 ± 0.03 (29)*	-
C	0.89 ± 0.05 (28)	0.92 ± 0.05 (34)	0.88 ± 0.04 (23)	-
Survival ratio PND5-28				
F0c	0.98 ± 0.01 (19)	0.96 ± 0.02 (22)	0.95 ± 0.02 (15)	–
F1c	0.83 ± 0.07 (28)	0.93 ± 0.02 (33)	0.89 ± 0.03 (22)	-

Data are displayed as the means and standard errors of the litter means. In parentheses are the number of litters. *p < 0.05; **p < 0.01 for trend analysis and pair wise testing. Trend results appear in the 0 ppm column. Testing for trend and pairwise differences was not performed for sample sizes of 1 or 2.

Pup markers

In the 2500 ppm group, there was a statistically significant trend in increase of percentage of male pups with areolae/nipples and delay in day of testis descent in F1c. There was a non-significant increase in percentage of male pups with areolae/nipples and a statistically significant delay in the day of testis descent also in F2c. RAC considers these effects as adverse and relevant for classification for developmental toxicity. The number of pups with areolae/nipples (3 pups (4 – 5%) in each generation from 1 – 2 litters (5 – 13%)) was small but the incidence was high compared to historical control incidence (indicated in Behl *et al.*, 2020) of 2 pups (out of 382; 0.5%) and 2 litters (out of 80; 2.5%). Details on the HCD are not available to assess its relevance. The delay in testis descent was also small; 0.4 days in F1c and 1.3 days in F2c compared to respective controls. This delay was less than the variation (1.4 days) between F1c and F2c controls.

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

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

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

Dose (ppm)	0	750	2500
F1c Examined, Females (litters)	96 (19)	111 (22)	67 (15)
Age at VO (PND)	33.8 ± 0.2**	37.2 ± 0.3**	39.4 ± 0.3**
Adjusted age at VO [b]	34.1 ± 0.2**	37.2 ± 0.3**	39.0 ± 0.3**

VO (vaginal opening) and BPS (Balano-preputial separation): means of litter means for age at attainment are presented. *p < 0.05; **p < 0.01 for trend analysis and pair wise testing. Trend results appear in the 0 ppm column.

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

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

Sperm analysis and oestrous cycle

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

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

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

Dose (ppm)	0	750	2500	5000
Sperm/Cauda (10^6)				
F0	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	-
F1-terminal	196.7 ± 9.0	190.2 ± 7.1	176.0 ± 10.6	-
Concentration (10^6)/g cauda epididymal tissue)				
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*	-
F1-terminal	759.6 ± 27.3	723.0 ± 26.2	721.2 ± 35.0	-
% Motile sperm				
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**	-
F1-terminal	80.1 ± 1.5**	77.4 ± 1.2	71.7 ± 2.9	-
Oestrous cycle length (days)				
F0	5.3 ± 0.21	5.4 ± 0.21	5.9 ± 0.43	5.8 ± 0.68
F1c	5.0 ± 0.23	4.8 ± 0.06	5.1 ± 0.07*	-

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

Organ weights

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

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

- decreased absolute weight of left and right epididymis in the F0 2500 and 5000 ppm, and F1c 2500 ppm groups
- decreased absolute right ovary weight in all F0 groups (not dose-dependent) and in F1 interim 2500 ppm group. In the F0 5000 ppm, group there was also decreased weight of absolute right ovary and both absolute and relative left ovary weight.

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

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

Dose (ppm)	**0**	**750**	**2500**	**5000**
N (# of animals evaluated)				
F0	21-23	22-23	19-20	19-21
F1c interim	48-49	55-56	20	-
F1c terminal	40	44	39	-
Necropsy weight (g)				
F0	504.5 ± 4.4**	477.5 ± 5.9**	459.2 ± 7.5**	455.2 ± 5.5**
F1c interim	395.0 ± 5.7**	383.4 ± 4.9	338.9 ± 4.4**	-
F1c terminal	497.3 ± 7.7**	486.8 ± 6.7	443.8 ± 10.3**	-
Testis (g) (absolute)				
Right				
F0	2.089 ± 0.023	2.089 ± 0.030	2.086 ± 0.028	2.066 ± 0.029
F1c interim	1.928 ± 0.029	1.928 ± 0.023	1.897 ± 0.047	-
F1c terminal	2.095 ± 0.036	2.121 ± 0.026	2.182 ± 0.049	-
Left				
F0	2.075 ± 0.026	2.074 ± 0.026	2.096 ± 0.026	2.062 ± 0.029
F1c interim	1.931 ± 0.029	1.911 ± 0.023	1.884 ± 0.038	-
F1c terminal	2.090 ± 0.038	2.103 ± 0.030	2.150 ± 0.049	-
Epididymis (mg) (absolute)				
Right				
F0	681 ± 9**	686 ± 10	628 ± 9**	600 ± 9**
F1 interim	571 ± 9**	564 ± 8	513 ± 12**	-
F1 terminal	697 ± 9**	697 ± 9	651 ± 11**	-
Left				
F0	704 ± 9**	718 ± 13	657 ± 11**	635 ± 10**
F1 interim	579 ± 10**	561 ± 7	521 ± 12**	-
F1 terminal	697 ± 13**	701 ± 9	648 ± 12**	-
Epididymis (mg/g) (relative)				
Right				
F0	1.35 ± 0.02	1.44 ± 0.03*	1.37 ± 0.03	1.32 ± 0.02
F1 interim	1.45 ± 0.02	1.47 ± 0.02	1.52 ± 0.05	-
F1 terminal	1.41 ± 0.02*	1.44 ± 0.02	1.47 ± 0.02	-
Left				

Dose (ppm)	0	750	2500	5000
N (# of animals evaluated)				
F0	21-23	22-23	19-20	19-21
F1c interim	48-49	55-56	20	-
F1c terminal	40	44	39	-
Necropsy weight (g)				
F0	504.5 ± 4.4**	477.5 ± 5.9**	459.2 ± 7.5**	455.2 ± 5.5**
F1c interim	395.0 ± 5.7**	383.4 ± 4.9	338.9 ± 4.4**	-
F1c terminal	497.3 ± 7.7**	486.8 ± 6.7	443.8 ± 10.3**	-
Testis (g) (absolute)				
Right				
F0	2.089 ± 0.023	2.089 ± 0.030	2.086 ± 0.028	2.066 ± 0.029
F1c interim	1.928 ± 0.029	1.928 ± 0.023	1.897 ± 0.047	-
F1c terminal	2.095 ± 0.036	2.121 ± 0.026	2.182 ± 0.049	-
Left				
F0	2.075 ± 0.026	2.074 ± 0.026	2.096 ± 0.026	2.062 ± 0.029
F1c interim	1.931 ± 0.029	1.911 ± 0.023	1.884 ± 0.038	-
F1c terminal	2.090 ± 0.038	2.103 ± 0.030	2.150 ± 0.049	-
F0	1.40 ± 0.02	1.51 ± 0.04*	1.44 ± 0.03	1.40 ± 0.02
F1 interim	1.47 ± 0.02*	1.47 ± 0.01	1.54 ± 0.04*	-
F1 terminal	1.41 ± 0.02	1.45 ± 0.02	1.47 ± 0.02	-
Dorsolateral Prostate (mg) absolute)				
F0	604 ± 26**	492 ± 23**	469 ± 15**	421 ± 22**
F1 interim	402 ± 11**	382 ± 12	330 ± 13**	-
F1 terminal	539 ± 16**	475 ± 19*	449 ± 19**	-
Dorsolateral Prostate (mg/g) (relative)				
F0	1.20 ± 0.05**	1.03 ± 0.05**	1.02 ± 0.03**	0.93 ± 0.05**
F1 interim	1.02 ± 0.03	1.00 ± 0.03	0.97 ± 0.03	-
F1 terminal	1.08 ± 0.03	0.98 ± 0.04	1.01 ± 0.03	-
Ventral Prostate (mg) (absolute)				
F0	935 ± 28**	785 ± 21**	748 ± 29**	515 ± 27**
F1 interim	561 ± 20**	446 ± 14**	355 ± 21**	-
F1 terminal	825 ± 22**	796 ± 27	591 ± 18**	-
Ventral Prostate (mg/g) (relative)				
F0	1.85 ± 0.05**	1.65 ± 0.05**	1.63 ± 0.06**	1.13 ± 0.06**
F1 interim	1.42 ± 0.05**	1.16 ± 0.03**	1.05 ± 0.06**	-
F1 terminal	1.67 ± 0.05**	1.64 ± 0.06	1.34 ± 0.05**	-
Seminal Vesicle (g) (absolute)				
F0	1.946 ± 0.053**	1.647 ± 0.045**	1.520 ± 0.045**	1.253 ± 0.042**
F1 interim	1.304 ± 0.032**	1.186 ± 0.028*	1.016 ± 0.035**	-
F1 terminal	1.76 ± 0.04**	1.69 ± 0.04	1.46 ± 0.05**	-
Seminal Vesicle (mg/g) (relative)				
F0	3.87 ± 0.10**	3.44 ± 0.11**	3.29 ± 0.08**	2.75 ± 0.10**
F1 interim	3.31 ± 0.06*	3.10 ± 0.07	3.00 ± 0.09*	-
F1c terminal	3.56 ± 0.08	3.48 ± 0.09	3.29 ± 0.10	-
LABC (g) (absolute)				
F0	1.438 ± 0.028**	1.369 ± 0.023	1.265 ± 0.034**	1.236 ± 0.022**
F1c interim		1.095 ± 0.024		

Dose (ppm)	0	750	2500	5000
N (# of animals evaluated)				
F0	21-23	22-23	19-20	19-21
F1c interim	48-49	55-56	20	-
F1c terminal	40	44	39	-
Necropsy weight (g)				
F0	504.5 ± 4.4**	477.5 ± 5.9**	459.2 ± 7.5**	455.2 ± 5.5**
F1c interim	395.0 ± 5.7**	383.4 ± 4.9	338.9 ± 4.4**	-
F1c terminal	497.3 ± 7.7**	486.8 ± 6.7	443.8 ± 10.3**	-
Testis (g) (absolute)				
Right				
F0	2.089 ± 0.023	2.089 ± 0.030	2.086 ± 0.028	2.066 ± 0.029
F1c interim	1.928 ± 0.029	1.928 ± 0.023	1.897 ± 0.047	-
F1c terminal	2.095 ± 0.036	2.121 ± 0.026	2.182 ± 0.049	-
Left				
F0	2.075 ± 0.026	2.074 ± 0.026	2.096 ± 0.026	2.062 ± 0.029
F1c interim	1.931 ± 0.029	1.911 ± 0.023	1.884 ± 0.038	-
F1c terminal	2.090 ± 0.038	2.103 ± 0.030	2.150 ± 0.049	-
F1 terminal	1.161 ± 0.027*	1.301 ± 0.034	1.052 ± 0.035	-
	1.341 ± 0.029*		1.222 ± 0.041	-
LABC (mg/g) (relative)				
F0	2.85 ± 0.06	2.87 ± 0.05	2.76 ± 0.06	2.72 ± 0.06
F1 interim	2.94 ± 0.05	2.86 ± 0.06	3.11 ± 0.11	-
F1 terminal	2.70 ± 0.05	2.68 ± 0.06	2.75 ± 0.07	-
Ovary (mg) (absolute)				
Right				
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	–
Left				
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	–
Ovary (mg/g) (relative)				
Right				
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				
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	–

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

Histopathology

Histopathology of reproductive organs showed testicular degeneration and testicular spermatid retention that was significant in the F0 5000 ppm group, but not dose-dependent across the

groups or generations. The incidence of exfoliated germ cells in the epididymis was significantly increased in the F0 5000 ppm group, but there was no dose-response in terms of severity. Dose-dependent minimal to mild prostate gland atrophy was observed in all the F0 and F1 groups (except in the F0 5000 ppm group, where it was mild to moderate).

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

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

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

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

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

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

Rats

One male in the 560 mg/kg bw/d group died during week 1 and one female in the 80 mg/kg bw/d group was killed moribund during week 9. Clinical findings in the 300 and 560 mg/kg bw/d

group included abnormal breathing, nasal and eye discharge (also in 160 mg/kg bw/d group), ruffled fur, tremors and ataxia in males and females.

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

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

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

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

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

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

a) Left absolute testis weight for 40 and 560 mg/kg bw/day dose groups were not reported.

b) Left relative testis weights were not reported.

c) Right epididymis for all dose groups and left epididymis for 40 and 560 mg/kg bw/day dose groups were not reported.

d) Right cauda epididymis for all dose groups and left cauda epididymis for 40 and 560 mg/kg bw/day dose groups were not reported.

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

The following statistically significant effects were observed during histopathology:

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

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

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

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

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

Mice

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

There was no significant effect on food consumption. The final mean body weight and body weight gains of males were significantly lower in the 240, 440, 915 and 1840 mg/kg bw/d groups (% final weight relative to controls: 93, 90, 84 and 79, respectively). The final mean body weight and body weight gains of females were significantly lower in all dose groups (% final weight relative to controls in the 110, 250, 540, 1130 and 3180 mg/kg bw/d groups was 90, 88, 80, 77 and 74, respectively).

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

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

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

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

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

Substances are classified in Cat. 1B when the data provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects or if occurring together with other toxic effects then the adverse effect is considered not to be a secondary non-specific consequence of other toxic effects.

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

Sexual function and fertility

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

- There was a significant reduction in mating in the high-dose group and smaller litter sizes in all treatment groups. The female moribundity/mortality in the high dose group was associated with dystocia.
- It is apparent from the high-dose cross-over mating group (treated males with untreated females) that male fertility is severely affected, in the absence of systemic toxicity.
- There was a dose-dependent significant delay in preputial separation and vaginal opening.
- There was a dose-dependent decrease in prostate, seminal vesicles, and epididymis weights. There were also histopathological changes in prostate, epididymis and testes. Similar adverse effects on the reproductive organs were also observed in rats in the 14-week study.

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

Developmental toxicity

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

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

According to the CLP criteria (Annex I: 3.7.1.3), adverse effects on onset of puberty are regarded as effects that has the potential to interfere with sexual function and fertility. Therefore, the

adverse effects on preputial separation and vaginal opening observed in the RAtCB study are considered under the section on sexual function and fertility above.

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

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

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

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

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

Comparison with the classification criteria for lactational effects

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

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

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

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

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

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

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

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).