Section or Page in CLH proposal	Comment
Page 158 – Section 2.6.5.2	*"It is acknowledged that MITC is only found to be carcinogenic at the highest dose(s) used in the lifetime rat bioassay, and the characteristics associated with doses exceeding the MTD as outlined above are present, this could be an indication of a confounding effect of excessive toxicity. This may support either a classification of MITC in Category 2 or no classification. Under CLP, when such a mechanism is identified then classification may not be appropriate."* As detailed below, the 20-ppm dose in the rat carcinogenicity study was clearly above the maximal tolerated dose (MTD). Also, new data significantly strengthens the case for a corrosive mode of action (MOA) for MITC ([redacted] 2020). The threshold MOA supports that the 20-ppm exposure is not relevant for humans given they cannot tolerate a lifetime exposure at 20 ppm. **Maximum Tolerated Dose** There is clear evidence that the 20-ppm concentration group, in addition to carcinogenicity, had overt toxicity that was unrelated to the nasal tumors. Toxicity was manifested as decreased body weight throughout the study, including a 34% decrease in males and a 20% decrease in females. Body weight gain from study week 0 to 103 was 54% of controls in males and 73% of controls in females. Further, the incidence of rales, thin appearance, and labored respiration was increased significantly in males and females. Rales was observed in 51 and 34 of the animals in the 20 ppm for group for males and females, respectively, and the first observation was at 47 days. The body weight decreases also occurred very early in the study, long before tumors developed. Even after the first week, there was a statistically significant decrease in body weights compared to the control. As noted in the CLP guidance in the section on "possibility of a confounding effect of excessive toxicity at test doses", it is stated "*Of these doses, the highest dose needs to induce minimal toxicity, such as characterised by an approximately 10% reduction in body weight gain (maximal tolerated dose, MTD dose). The MTD is the highest dose of the test agent during the bioassay that can be predicted not to alter the animal's normal longevity from effects other than*

carcinogenicity. While longevity was not affected in the 20-ppm group, the body weight reduction for the 20-ppm group was far greater than the 10% reduction as defined for the MTD dose.

MOA for Nasal Carcinogenicity

Recent work by the [redacted]) has substantially strengthened the case for a threshold for carcinogenicity for MITC. A recent mechanistic study ([redacted] 2020) included inhalation exposures of MITC at 0, 0.5, 5, or 20 ppm for 1 day, 5 days, or 4 weeks and showed increased DNA synthesis detected by nuclear incorporation of BrdU in nasal tissue at 5 and 20 ppm.

[redacted] (2020) further supports a threshold MOA for MITC tumorgenicity. The data supporting this MOA were evaluated under the framework recommended by the International Programme on Chemical Safety (IPCS) ([redacted] 2022). The proposed MOA has four key events: (1) direct cytotoxicity of MITC in the nasal mucosa (e.g., degeneration, necrosis/apoptosis) because of its irritating properties, (2) cell proliferation to compensate for MITC-induced cytotoxicity, (3) onset and persistence of squamous cell metaplasia as an adaptive response to the cytotoxic insult, and (4) development of tumors, primarily squamous cell carcinomas.

[redacted] (2022) contains the full details on the MOA evidence and applies the Bradford-Hill criteria to the evidence. The report is provided with this document.

<u>Key Event No. 1: Direct Cytotoxicity of MITC in the Nasal Mucosa</u>

Several inhalation studies in rats support Key Event No. 1. The most prominent tissue damage has been observed in the most forward of the respiratory epithelial-lined nasal passages (just distal to the normal squamous epithelium) of rats following inhalation exposure to MITC, where the highest exposure to MITC is expected to occur. The parts of the nasal passages more distal from the point of entry showed progressively less severe lesions. This indicates the importance of the portal of entry irritation and cytotoxicity effects of MITC. Observations of cytotoxicity included nasal epithelial degeneration, necrosis, and/or acute inflammation, and were consistently reported in the rat studies as early as one day post-inhalation exposure ([redacted] 2020; [redacted] 2011). These changes persisted to 104 weeks of inhalation exposure ([redacted] 2015).

The [redacted] (2020) study demonstrated the following findings at the 20 ppm MITC exposure: histopathologic changes in the nasal airways consisting of acute/subacute inflammation, epithelial degeneration and necrosis, olfactory sensory neuron apoptosis (after one day of exposure), and olfactory sensory neuron loss resulting in atrophy/loss of olfactory epithelium and respiratory metaplasia (after four weeks of exposure). Exposure to 5 ppm MITC resulted in

less severe cytotoxicity in the respiratory epithelium, minimal to mild nasal tissue inflammation, no histopathological abnormality of olfactory epithelium, and no evidence of squamous cell metaplasia, unlike the findings present in 20 ppm MITC-exposed rats. Animals exposed to 0.5 ppm MITC had no exposure-related histopathology.

Across studies of varying duration, the cytotoxicity observations are clearly dose- and exposure time-dependent in rat nasal tissue. Altogether, these findings support Key Event 1 and indicate that the irritant and cytotoxic effects of MITC persist in the nasal tissue with repeated and prolonged exposures with the severity of the effects dependent on the exposure level.

Key Event No. 2 – Cell Proliferation to Compensate for MITC-Induced Cytotoxicity

Evidence for Key Event 2 comes directly from the [redacted] (2020) study, in which rats exposed to 5 ppm and 20 ppm MITC were observed to have increased nasal cavity cell proliferation compared to control animals. This was demonstrated through both microscopic observations of nasal cavity epithelial hyperplasia and measurements of DNA synthesis detected by nuclear incorporation of bromodeoxyuridine (BrdU) in nasal tissue. BrdU incorporation is a common method used to assess DNA replication (Wood et al., 2015). Quantitative BrdU measurements from [redacted] (2020) showed increased DNA synthesis in the transitional epithelium lining of the maxilla turbinates at 5 ppm and 20 ppm. As shown in Table 1, the highest BrdU incorporation epithelial cells were observed after one day of MITC exposure at these dose levels. However, cell proliferation was still found to be significantly increased compared to controls at the 5-day and 4-week timepoints at both 5 and 20 ppm, *albeit* at significantly lower levels compared to their respective 1-day levels.

Table 1. Unit Length Labelling Index of BrdU-labeled nuclei in transitional epithelium lining the maxilloturbinates (T1 nasal section) of male rats

MITC dose (ppm)	Approximate mean BrdU-labeled nuclei/mm basal lamina length (mean ± SEM)			
	1 Day exposure	5 Days exposure	4 Weeks exposure	Recovery (4 weeks)
0	4.2 ± 0.63	3.2 ± 0.47	2.3 ± 0.24[#]	1.6 ± 0.3
0.5	3.0 ± 0.50	2.4 ± 0.45	3.2 ± 0.38	1.3 ± 0.3

5	33.5 ± 3.46*	12.0 ± 0.80*,#	9.7 ± 0.99*,#	1.3 ± 0.3
20	53.8 ± 1.88*	12.8 ± 1.88*,#	19.3 ± 3.31*,#	1.5 ± 0.2

* Statistically significant compared to respective control group at exposure day 1, 5, or 20; $p < 0.05$.
# Statistically significant compared to 1-day respective dose group, $p < 0.05$

Across the full suite of MITC inhalation studies, the data indicate that cell regeneration and increased cell proliferation effects of MITC occur in the nasal cavity tissue with repeated exposures and aligned with the observations of persistent cytotoxicity. These findings also indicate the presence of sustained cell proliferation in the nasal tissue with repeated exposures for the entire time of MITC exposure, up to two years. Results of the recovery experiment in [redacted] (2020) further indicate that MITC-induced cell proliferation does not persist following cessation of exposure.

Key Event 3: Onset and Persistence of Squamous Metaplasia as an Adaptive Response to the Cytotoxic Insult

Evidence for Key Event 3 is supported by the observation of increased squamous cell metaplasia in rats following repeat dose exposures. This finding was reported in rats exposed to the high dose of 20 ppm that induced nasal tumors in the rat carcinogenicity study. The findings were observed at 5 days and 4 weeks in [redacted] (2020) up to 2 years in the [redacted] (2015).

Squamous metaplasia is the usual response of cytotoxicity of various columnar epithelia such as the respiratory epithelium of the nasal cavity, but also occurs in the respiratory tract epithelium (Renne et al., 2009) of the larynx, trachea, and bronchi, as well as in some other tissues, such as the uterine cervix (Malpica, 2014). This is a typical response to toxicity in these columnar epithelial tissues, with the squamous metaplasia persisting as long as the toxic insult persists.

It should be noted that squamous epithelium, whether normally present or metaplastic, has a greater rate of proliferation than the corresponding columnar epithelium, such as the respiratory epithelium of the nasal cavity (Johnson et al., 1990; Harkema et al., 2006) (See Figure 2). This adds to the proportion of increased cell proliferation, as there is not only an increase in rate, but there is an increase in the number of cells as evident by hyperplasia, as well as the increase in squamous epithelium versus respiratory columnar epithelium.

Key Event 4: Development of Tumors

	Male rats developed nasal tumors following two years of exposure to 20 ppm MITC ([redacted] 2015). Male rats from the 20-ppm group showed a significant trend for nasal adenomas ($p < 0.05$); pairwise comparisons were significant for squamous cell papillomas ($p < 0.05$) and squamous cell carcinomas ($p < 0.01$); and all nasal tumors combined (adenomas, papillomas, and carcinomas) were significantly increased ($p < 0.01$) by both trend and pairwise comparisons. Tumors occurred only at the 20-ppm dose level, corresponding to the dose with severe cytotoxicity, increased regenerative cell proliferation and hyperplasia, and squamous cell metaplasia. The onset and persistence of squamous cell metaplasia, at a dose level exceeding the necessary threshold, leads to the eventual development of tumors at the 20-ppm exposure level. **Human Applicability of the Proposed MOA** *Is the Weight of Evidence Sufficient to Establish a MOA in the Rat?* The answer is "yes". The weight of evidence is sufficient to establish a MOA in the rat. While the complete mechanistic details of the MOA may not all be available, data from the available inhalation studies with MITC as well as other irritant compounds reinforce confidence in the cytotoxicity-based MOA for nasal tumors in rats. *Can Human Relevance of the MOA be Reasonably Excluded on the Basis of Fundamental Differences in Key Events Between Rats and Humans?* The answer is "no". In general, the proposed MOA for MITC-induced rat nasal tumors is potentially applicable to humans as well, since MITC is known to be an irritant for humans. Accordingly, tumorigenicity is expected in humans if they are chronically exposed to high enough concentrations of MITC that can induce excessive cytotoxicity leading to continuous reparative increased cell proliferation. *Can Human Relevance of the MOA be Reasonably Excluded on the Basis of Quantitative Differences Either Kinetic or Dynamic Factors Between Rats and Humans?* The answer is "yes". Chronic exposures to high levels of MITC leading to excessive cytotoxicity in the nasal passages are not expected to occur in real life situations in humans. For example, the estimated health protective concentration per irritant effects of acute (4-8 hours) MITC exposure is in the range of 0.2 to 0.8 ppm and is based on the sensory effects (perceived magnitude of irritation,

blinking, and tearing) observed in a human study by Russell and Rush (1996) ([redacted], 2010; U.S. EPA, 2008). Thus, the concentration required to induce tumors in the rat chronic toxicity is at least 25X higher and for a lifetime exposure, compared to the health protective value for acute irritation in humans. Tumorigenic consequences are a product of prolonged exposure to high, and excessively irritating, concentrations of MITC which individuals would not be expected to tolerate for any length of time. At exposure concentrations and durations that do not induce successive sensory irritancy, cytotoxicity and the consequent sustained increase reparative cell proliferation, MITC would not be expected to induce tumors in humans.

References

[redacted] 2022. Mode of Action (MOA) for Nasal Tumors Induced by Methyl Isothiocyanate (MITC) in Sprague Dawley Rats. Report for the MITC Task Force.

Harkema, J. R., Carey, S. A., Wagner, J. G. The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. Toxicol. Pathol. 34(3):252-269, 2006. DOI: 10.1080/01926230600713475.

Johnson, N. F., Hotchkiss, J. A., Harkema, J. R., Henderson, R. F. Proliferative responses of rat nasal epithelia to ozone. Toxicol. Appl. Pharmacol. 103(1):143-155, 1990. DOI: 10.1016/0041-008x(90)90270-5.

[redacted] D.T. 2011. Acute inhalation toxicity/neurotoxicity study of methyl isothiocyanate (MITC) in rats with histopathology and bronchoalveolar lavage evaluation. Report for [redacted] by [redacted]. Report no. [redacted]-824001.

[redacted] D.T. 2015. A combined inhalation chronic toxicity and 24-month carcinogenicity study of methyl isothiocyanate (MITC) in rats. Report for [redacted] by [redacted]. Report no. [redacted]-824015.

Malpica, A. Cervical benign and non-neoplastic conditions. IN: Mutter, G. L., Prat, J. Pathology of the Female Reproductive Tract. Churchill Livingstone, Elsevier, chapter 8, pp. 160-187, 2014.

Renne, R., Brix, A., Harkema, J., Herbert, R., Kittel, B., Lewis, D., March, T., Nagano, K., Pino, M., Rittinghausen, S., Rosenbruch, M., Tellier, P., Wohrmann, T. Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. Toxicol. Pathol. 37(7 Suppl):5S-73S, 2009. DOI: 10.1177/0192623309353423.

Russell, M.J.; Rush, T.I. 1996. Methyl isothiocyanate: determination of human olfactory detection threshold and human no observable effect level for eye

	irritation. Report prepared by Sensory Testing Laboratory at the University of California, Davis for Zeneca Ag Products. Report no. RR 96-049B. [redacted] J.T. 2020. A Histopathological Assessment of Nasal Tissues from Sprague Dawley Rats Exposed to Methyl Isothiocyanate (MITC) for 1 Day, 1 Week, 4 Weeks, and 4 Weeks followed by a 4-Week Recovery Period. Report for [redacted] by [redacted] and [redacted] Wood, C. E., Hukkanen, R. R., Sura, R., Jacobson-Kram, D., Nolte, T., Odin, M., Cohen, S. M. Scientific and Regulatory Policy Committee (SRPC) review: interpretation and use of cell proliferation data in cancer risk assessment. Toxicol. Pathol. 43(6):760-775, 2015. DOI: 10.1177/0192623315576005.