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Comparison of Acute Respiratory Epithelial Toxicity for 4-Methylimidazole and Naphthalene Administered by Oral Gavage in B6C3F1 Mice

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Abstract

4-Methylimidazole (4MEI)[1] is a contaminant in food and consumer products. Pulmonary toxicity and carcinogenicity following chronic dietary exposures to 4MEI is a regulatory concern based on previous rodent studies. This study examined acute pulmonary toxicity in B6C3F1 mice from 6 hours to 5 days after oral gavage with a single dose of 150 mg/kg 4MEI, a double dose delivered 6 hours apart, or vehicle controls. Oral gavage of 150 mg/kg naphthalene, a prototypical Club cell toxicant, was used as a positive control. Intrapulmonary conducting airway cytotoxicity was assessed in fixed-pressure inflated lungs using qualitative histopathology scoring, quantitative morphometric measurement of vacuolated and exfoliating epithelial cells, and immunohistochemistry. 4MEI treatment did not change markers of cytotoxicity including the mass of vacuolated epithelium, the thickness of the epithelium, or the distributions of epithelial proteins: secretoglobin 1A1, proliferating cell nuclear antigen, calcitonin gene-related peptide, and myeloperoxidase. 4MEI and vehicle controls caused slight cytotoxicity with rare vacuolization of the epithelium relative to the severe bronchiolar epithelial cell toxicity found in the naphthalene exposed mice at terminal bronchioles, intrapulmonary airways, or airway bifurcations. In summary, 4MEI caused minimal airway epithelial toxicity without characteristic Club Cell toxicity when compared to naphthalene, a canonical Club Cell toxicant.

Keywords

4-Methylimidazole; naphthalene; respiratory; acute toxicity; Club cell toxicity

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Conflicts of interest:

None of the authors have any financial interest in the manufacture or use of 4MEI but this study was performed under a contract with the American Beverage Association.

1. Introduction:

4-Methylimidazole (4MEI)[1] is a chemical byproduct of caramelization found in consumer products such as caramel-colored beverages, coffee, soy sauce, wine, vinegar, Worcestershire sauce, and ammoniated molasses (Hengel and Shibamoto, 2013). 4MEI concentrations range from <5 ng/mL up to 2,000 ng/mL in caramelized foods (Fierens et al., 2018; Folmer et al., 2018; Goscinny et al., 2014; Hengel and Shibamoto, 2013; Smith et al., 2015; Wang et al., 2015). Even though the major sources of dietary 4MEI exposure vary, 4MEI is consistently found in foods globally with mean intakes of 4MEI measured for high exposure scenarios at 93 μg/$kg_{bodyweight}$/day in America (Folmer et al., 2018), 3.7 μg/$kg_{bodyweight}$/day in Belgium (Fierens et al., 2018), and 5.2 μg/$kg_{bodyweight}$/day in China (Liang et al., 2019). The International Agency for Research on Cancer classified 4MEI as a Group 2B carcinogen–possibly carcinogenic to humans (International Agency for Research on Cancer (IARC), 2012). The California Office of Environment Health Hazard Assessment (OEHHA) set a No Significant Risk Level (NSRL) at 29 μg/person/day for 4MEI. Estimates of American soda consumption alone place daily 4MEI exposure at 5.7 μg/$kg_{body\ weight}$/day or 400 μg/day for a 70 kg person (Cunha et al., 2011) – over 10 times greater than the OEHHA NSRL but about 25,000 times less than the doses chosen for our study.

Regulatory concern about 4MEI carcinogenicity is largely based on the higher incidence of lung lesions found in male and female B6C3F1 mice after 106 weeks of 4MEI dietary exposure (625 and 1,250 ppm in chow consumed *ad libitum*) compared to unexposed controls (Chan et al., 2008; National Toxicology Program, 2007). At a cellular level, a 1.9-fold increase actively dividing lung cells labeled with bromodeoxyuridine was identified in male B6C3F1 mice after 6 days of dietary 4MEI exposure (2,500 ppm in chow consumed *ad libitum*) with corresponding slight epithelial hyperplasia in the preterminal and terminal bronchioles (Cruzan et al., 2015). A similar dietary exposure to 1,250 ppm 4MEI for five days caused a significant 2.8-fold increase in bromodeoxyuridine labeling of actively dividing airway cells in males, but not in females, of another mouse strain (C57Bl6) (Cruzan et al., 2015). However, the potential for 4MEI to cause acute lung injury, the mechanisms of lung specific pathology from chronic 4MEI exposure, and a specific target cell for lung carcinogenesis remain undefined (Chan et al., 2008; Cruzan et al., 2015; National Toxicology Program, 2007).

4MEI metabolism in multiple species and the lung-specific lesion sites in mice resemble the mechanisms of action involving airway Club cell generation of toxic metabolites for other rodent carcinogens, such as naphthalene and styrene (Cruzan et al., 2002; Plopper et al., 1992). Toxicant bioactivation by cytochrome P450 monooxygenase enzymes expressed within Club cells is a common cytotoxic mechanism. Club cells, also known as nonciliated bronchiolar epithelial cells, are a common cell type for human and mouse lung cancer initiation. Abundant distribution throughout the airway tree makes mice an ideal model to study Club cell toxicity, while human Club cells are more restricted to distal airways. 4MEI metabolism was demonstrated in mice and rats (22 to 40% of 50 or 150mg/kg oral gavage ^{14}C 4MEI) (Fennell et al., 2019)), goats (85% of the 20 mg/kg orally administered and 80% of the 20 mg/kg intravenous 4MEI) (Nielsen et al., 1993), and in heifers (25% of 20 mg/kg intravenous 4MEI) (Nielsen et al., 1993). Comparison of pulmonary injury after chronic

4MEI dietary exposure and intraperitoneal injection of the known Club cell toxicant, styrene, did not identify a cellular target for 4MEI toxicity (Cruzan et al., 2015). Further investigation of acute responses to 4MEI are needed to further clarify whether Club cells are targeted since bioactivated toxicant exposures can induce resistance to lung epithelial injury which potentially masks acute toxicity when assessing chronic exposures (Sutherland et al., 2012).

This study was designed to determine whether acute 4MEI toxicity targets specific sites or cellular targets in the mouse lung. To clarify the role of the metabolically active Club cells in 4MEI toxicity, mice were acutely exposed to high 4MEI doses and site-specific conducting airway epithelial toxicity was observed at high resolution using resin sections. Rather than conducting a feeding study, as in NTP exposures (Chan et al., 2008; National Toxicology Program, 2007), the acute 4MEI exposure by oral gavage was completed to allow administration of a known bolus dose and detection of any potential acute respiratory toxicity. 4MEI toxicity was also compared to the classic Club cell toxicant, naphthalene, given by the same route. Naphthalene has a well-characterized toxicity pattern targeting Club cells in conducting airways that is characterized by Club cell vacuolization and loss from the conducting airway epithelium in the early phases of toxicity irrespective of route of exposure (Buckpitt et al., 1992; Plopper et al., 1992; Van Winkle et al., 1995). The extent of cell loss and location is dependent on the route of exposure and the dose with systemic exposure resulting in distal airway toxicity and involving larger conducting airways as the dose increases. This pattern is readily detected using high resolution histopathology.

2. Materials and Methods:

2.1. Chemical Sources:

4(5)-Methylimidazole was obtained from Sigma (CAS- 822-36-6, 97.5% purity, Product #199885) and was dissolved in double-distilled water to 37.5 mg/mL for oral gavage. Naphthalene was purchased from Fisher (CAS- 91-20-3) and dissolved in corn oil (Mazola) to 37.5 mg/mL for administration. Araldite 502 epoxy resin, dodecenyl succinic anhydride, and DMP-30 were obtained from Ted Pella, Inc. All other chemicals were reagent grade or better.

2.2. Animals:

B6C3F1 mice (Taconic Bioscience) were acclimated for 7 days prior to study. Sentinel mice were housed in the same facility and tested negative for respiratory virus for the duration of the study as monitored using measurement of serum antibodies for the following pathogens: mouse hepatitis virus, mouse metapneumovirus, minute virus of mice, mycoplasma pulmonis, mycoplasma arthritis, theiler's encephalomyelitis virus (GDVII), ectromelia virus, epizootic diarrhea of infant mice virus, and sendai virus. Additionally, sentinel mice and their bedding were negative for mouse parvovirus, pinworms, and protozoa fur mites. All animal experiments were performed under protocols approved by the University of California Davis IACUC in accordance with National Institutes of Health guidelines (NIH Publications No. 8023, revised 1978). All mice were maintained in a barrier facility with filtered air in AAALAC approved conditions on a 12 hour light/dark cycle with food and

water *ad libitum* and animal health monitored by campus veterinarians and serology in sentinel animals. All animals were in good health and did not have any adverse pathogens or respiratory infections. The diet consisted of irradiated NTP 2000 open formula food, consistent with the NTP exposures (Chan et al., 2008; National Toxicology Program, 2007). Mice were weighed before each dosing and necropsy with no observed difference between treated and vehicle mice. Male mice were housed individually or in small groups <4 and female mice were housed 4 to 5 per cage. All mice were first exposed at 6 weeks of age. Mice were organized using unique access numbers. The sample size for each exposure group was between 3 and 5.

2.3. Oral gavage exposure:

Mice were not fasted before treatment. Total volume of each oral gavage was 4 μL/kg. The following experiments were conducted to evaluate the timing of 4MEI acute pulmonary epithelial toxicity and the relative severity of 4MEI toxicity compared to naphthalene toxicity (Figure 1, Table 1). Oral gavage was used to deliver a known amount of 4MEI and to establish a time-scale for potential injury after a bolus dose. The dose of 150 mg/kg was chosen based on a previous study demonstrating metabolism of 4MEI at this dose within 24 hours (Fennell et al., 2019). Two doses of 150 mg/kg 4MEI were used to determine whether an even higher dose could cause respiratory epithelial toxicity instead of a single dose to avoid the potential impact of delivering a high dose rate of 4MEI using a single dose of 300 mg/kg. Single dose 4MEI: Mice were orally gavaged with a single dose of 150 mg/kg 4MEI or water vehicle control. The mice were sacrificed and the lungs were collected at 6 hours, 1 day, or 5 days post-exposure. Double dose 4MEI: Mice were orally gavaged with two doses of 150 mg/kg 4MEI (total of 300 mg/kg) or water vehicle control administered at 6 hours apart. Mice were sacrificed 1 day, 3 days, or 5 days after the first treatment. Single dose naphthalene: Mice were orally gavaged with a single dose of 150 mg/kg naphthalene or corn oil vehicle control and sacrificed 1 day post-exposure.

2.4. Tissue Fixation and Embedding:

Mice were euthanized with intraperitoneal pentobarbital overdose (Fatal Plus at 4,000mg/kg, MWI Animal Health Amerisource Bergen 015199) and the lungs were removed according to previously established protocol (Van Winkle et al., 2017). For 1 hour at 30 cm constant pressure, the right lung was inflated with Karnovsky's fixative (1% glutaraldehyde and 0.5% paraformaldehyde in cacodylate buffer, 330mOsM, pH 7.4) and the left lobe was separately inflated with 1% paraformaldehyde. The right cranial lobe or right middle lobe of the fixed mouse lung was embedded in Araldite 502 epoxy resin as described previously (Van Winkle et al., 1995) for qualitative and quantitative morphological assessment of 1μm thick sections stained with Methylene Blue/Azure II. High resolution images of terminal bronchioles, intrapulmonary airways, and airway bifurcations in resin sections were captured using an Olympus BH-2 microscope in bright field mode. The left lung lobe was stored overnight in 1% paraformaldehyde and transferred to 70% ethanol within 1 day before paraffin embedding within 2 weeks. 5 μm thick sections of the paraffin embedded left lobes were used for immunohistochemistry.

2.5. Histopathology Scoring:

Tissue sections were scored using high resolution light microscopy on resin embedded sections at 200X and 400X magnification. Cytotoxicity was defined as cells that were vacuolated, swollen, or with a lightly stained cell interior. After initial screening for morphologic changes of the lung epithelia, cytotoxicity was found near terminal bronchioles and airway bifurcations of 4MEI exposed mice. Subsequent scoring of cytotoxicity for each mouse focused on these suspected target zones. Scoring of each mouse was based on between 3 and 6 unique sites per mouse for terminal bronchiolar epithelium, intrapulmonary airway epithelium (larger bronchioles), and airway bifurcations. Terminal bronchioles were defined as the last conducting airway generation before the gas exchange region of the lung. Intrapulmonary airways were defined as conducting airways between the lobar bronchi and terminal bronchioles. Airway bifurcations were defined as branches in airways that form an acute angle at any airway generation. Each mouse was scored in a blinded manner in a random order unrelated to treatment grouping by a single observer. Epithelial toxicity scores were defined as follows: 0: no swelling or vacuolization, 1: minor swelling of one cell, 2: one cell swollen or vacuolated (devoid of stain), 3: two or three cells vacuolated or swollen, 4: more than three vacuolated or swollen cells or squamation, and 5: exposed basement membrane.

2.6. Quantitative Assessment:

Quantitative measurements of epithelial toxicity were made using the Stereology Toolbox program (Morphometrix) as previously described (Van Winkle et al., 1995). A cycloid test system of known length per point (L/p) was used to perform point (P) and intercept (I) counting on vertical uniform random sections. The volume density (Vv), representing the fraction of vacuolated and swollen epithelial cells, was calculated by Vv=Pd/Pt where Pd is the number of test points hitting structures of interest (vacuolated or swollen epithelial cells) and Pt is the total number of points hitting structures of interest (epithelial cells). Reference volume per surface area of epithelial basement membrane (1/Sv), representing total airway thickness, was calculated by 1/Sv=2(L/p)(Pt/I)/3.14 where L/p is the length of cycloid per point at the level of the tissue, Pt is the number of test points hitting structures of interest (epithelial cells), and I is the number of intersections with the epithelial basal lamina. A minimum of 200 points per region of interest (terminal bronchiole and intrapulmonary airway epithelium) was counted. Cytotoxicity was defined as airway epithelial cells attached to the basal lamina that were vacuolated, swollen, or devoid of staining within cell interior. Exfoliated cells that were not attached to the basal lamina were not counted. Stereology was accomplished in a blinded manner by a single observer with a random order unrelated to treatment grouping.

2.7. Immunohistochemistry:

Paraffin sections of the left lobe were deparaffinized, treated with 9% hydrogen peroxide to block endogenous peroxidase activity, and treated with bovine serum albumin to block non-specific binding. The primary antibodies summarized in Table 2 were incubated on the slides overnight at 4°C. Slides were washed with phosphate buffered saline then treated with Peroxidase Rabbit IgG Vectastain ABC Kit (Pk-4001, Vector Laboratories) prior to a 5

minute 3,3′-Diaminobenzidine staining (SK-4100, Vector Laboratories). Tissues were dehydrated and coverslipped. Images of terminal bronchioles and intrapulmonary airways were captured using an Olympus BH-2 microscope in bright field mode.

2.8. Statistical Analysis:

For the histopathology scoring, Kruskal-Wallis testing was performed to compare epithelial toxicity scores between groups with significance set at $p<0.05$ using R Commander Version 2.1-2 (Fox, 2016; R Core Team, 2016). For the quantitative assessment, outliers were identified using the Grubs Test ($\alpha=0.01$) available from Graphpad 8.4.3 and removed from the data set. For quantitative assessments, one-way ANOVA with Tukey post-hoc test was performed to identify treatment differences for all 1 day treatments and two-way ANOVA with Tukey post-hoc test was performed to identify differences in morphology related to time after exposure and treatment. There was no difference between the mice with a single dose or a double oral gavage dose of water, so these groups were pooled together for the final statistical analysis. After this pooling of water vehicle control groups, the death of one female exposed to the double dose of 4MEI, and the exclusion of underinflated tissues each group analyzed contained a sample size of 4 to 10.

3. Results:

One female mouse died prematurely within 1 day after the administration of a second dose of 150 mg/kg 4MEI. No clear cause of death or gross respiratory damage was identified in this mouse, the cause of death was attributed to an improper injection into a major organ. No alternate cause of death could be identified.

3.1. Lack of Biologically Relevant 4MEI Lung Epithelial Toxicity:

Damaged airway epithelial cells were sporadically found at a similar frequency in mice exposed to 4MEI or water by oral gavage at the terminal bronchioles (Figure 2), intrapulmonary airways (Figure 3), or airway bifurcations (Supplemental Figure 1). The occasional injured cells were either swollen, vacuolated, or stained lightly with methylene blue. Histopathology scoring of terminal bronchioles, intrapulmonary airways, and airway bifurcations showed no significant difference between 4MEI exposed airways and water vehicle controls (Figure 4).

Quantitative assessment of terminal bronchioles and intrapulmonary airways detected slight airway epithelial cytotoxicity in 4MEI exposed airways that is not biologically significant (Figure 5). The only 4MEI exposed group with statistically significant differences in the average vacuolated cell volume density (Vv) and the volume of epithelial toxicity per basal lamina surface area (Vs) compared to water vehicle control group was the female group 3 days after exposure to 300 mg/kg 4MEI in the terminal bronchioles. The cytotoxicity detected through stereology in this 4MEI treated group was about 5-fold lower than the terminal bronchiole toxicity detected in the female mice exposed to naphthalene and was similar to the levels measured in corn oil vehicle controls. Vv ranged from 0 to 0.05 for terminal bronchioles and intrapulmonary airways after water or 4MEI exposures (Supplemental Figure 2). Vs for terminal bronchioles and intrapulmonary airways for water

or 4MEI exposed mice ranged from 0.1 to 1.8 $\mu m^3/\mu m^2$ (Figure 5). A significant site-specific difference in total airway thickness (1/Sv) unrelated to treatment was observed and expected based on the known shortening of the epithelial height as airways become more distal. The average total airway thickness (1/Sv) for water or 4MEI exposed terminal bronchioles ranged from 17 to 24 $\mu m^3/\mu m^2$ and intrapulmonary airways ranged from 19 to 27 $\mu m^3/\mu m^2$. Quantitative assessment of airway bifurcation toxicity was not completed due to limitations of tissue availability since bifurcations are relatively rare on tissue sections. Immunohistochemistry was also negative for any significant cellular responses after 4MEI exposure at terminal bronchioles or intrapulmonary airways including a lack of CC10 Club cell marker changes (Figures 6 and 7, respectively). Neither the 150 mg/kg nor the 300 mg/kg doses of 4MEI led to consistent cytotoxicity at any time point from 6 hours to 5 days post-exposure, so dose-response and sex differences were not evaluated. The absence of biologically significant 4MEI epithelial toxicity relative to water controls was consistent terminal bronchioles, intrapulmonary airways, and airway bifurcations.

3.2. Naphthalene Lung Epithelial Toxicity:

The average epithelial toxicity scores of the corn oil exposed mice were consistent with the water exposed mice, falling between scores of 0: no swelling or vacuolization and 2: one damaged cell (Figure 8). The average qualitative epithelial toxicity after naphthalene oral gavage was higher than the corn oil controls, falling between scores of 2: one damaged cell and 4: greater than three damaged cells or squamation (Figure 8). Exfoliation of cells was not observed at the terminal bronchioles (Figure 2), intrapulmonary airways (Figure 3), or airway bifurcations (Supplemental Figure 1). Histopathology scoring of airway toxicity was significantly higher than corn oil controls for most naphthalene exposed groups.

Quantitative assessment of the average vacuolated cell volume density (Vv) and the volume of epithelial toxicity per basal lamina surface area (Vs) were significantly higher for both terminal and proximal airway epithelium exposed to naphthalene when compared to corn oil controls (Figure 9). Total airway volume (1/Sv) for terminal bronchiolar and intrapulmonary airway epithelium showed no significant difference between naphthalene exposed airways and corn oil controls. The average volume density (Vv) for naphthalene exposed airways ranged from 0.1 to 0.3, while corn oil exposed airway Vv was significantly lower and ranged from 0.004 to 0.01 (Supplemental Figure 3). The volume of epithelial toxicity per basal lamina surface area (Vs) for naphthalene exposed airways ranged from 1.8 to 7 $\mu m^3/\mu m^2$, while Vs for corn oil exposed airways were significantly lower and ranged from 0.09 to 0.3 $\mu m^3/\mu m^2$ (Figure 9). No sex difference or site-specific difference in naphthalene toxicity was significant. Naphthalene caused significant airway toxicity for all tested airway sites, while 4MEI did not cause any biologically significant airway toxicity.

4. Discussion

Early features of cytotoxicity caused by metabolically activated toxicants, epithelial cell vacuolization and swelling, were not observed in the intrapulmonary airways of 4MEI exposed mice. The sparse cytotoxicity was slight in vehicle control and 4MEI treated mice compared to the airway epithelial cytotoxicity caused by known Club cell toxicants:

naphthalene (reported here) and styrene (Cruzan et al., 2015). Our data excludes 4MEI as a Club cell specific toxicant through stereological and immunohistochemical methods. The measurements of the cytotoxicity of Club cells and the distribution of ciliated cells, neuroendocrine cells, and inflammatory cells in this study did not identify a specific 4MEI cellular target.

Current 4MEI regulatory debate involves arguments that 4MEI regulation is too conservative (Morita and Uneyama, 2016; Murray, 2011) and conversely that a genotoxic mechanism of carcinogenesis action exists for 4MEI with no safe exposure threshold (Smith et al., 2015). Although this study did not confirm a mechanism of action, we confidently agree with Cruzan et al. (2015): the mechanism of action of 4MEI is not analogous to naphthalene (reported here) or styrene (as reported in Cruzan et al., 2015) which exhibit genotoxic and non-genotoxic mechanisms of action dependent on bioactivation and toxicity in Club cells. Our study added higher resolution stereology as a more sensitive measure of epithelial cell toxicity than the previous assessment of paraffin sections and further assessed airway epithelial cytotoxicity in response to an acute bolus dose administered by oral gavage instead of diet (Chan et al., 2008; Cruzan et al., 2015; National Toxicology Program, 2007). Oral gavage of bolus doses in this study did not replicate the normal exposure via diet, but instead allowed assessment of acute respiratory toxicity in response to a large defined bolus dose. We further note that several of the prior chronic studies were also far above environmentally relevant doses.

Naphthalene toxicity observed in this study was consistent with previous acute naphthalene exposure studies using intraperitoneal exposures to 100 mg/kg and 200 mg/kg doses of naphthalene (West et al., 2001). The 150 mg/kg dose of naphthalene used in this study was selected to be consistent with the 150 mg/kg 4MEI dose administered. Relative to intraperitoneal administration the oral gavage in our study resulted in a similarly potent dose of naphthalene in the lung over a one day post-exposure period (West et al., 2001). The naphthalene dose used here was about a quarter of the acute oral lethal dose for 50% of exposed mice previously established over a 2 week period after a single oral gavage of naphthalene in CD-1 mice: 533 and 710 mg/kg in male and female mice respectively (Shopp et al., 1984). One day after exposure, the severity of airway epithelial toxicity observed after 150 mg/kg naphthalene oral gavage in adult B6C3F1 mice was similar to adult Male Swiss Webster mice exposed to inhalation doses of 2 ppm naphthalene for 4 hours (West et al., 2001). In a previous study conducted in our lab and scored by the same observer, the average volume density (Vv) representing the fraction of epithelial cells vacuolated and swollen measured in the conducting airways of C57Bl6 mice one day after an inhalation exposure to 5 ppm naphthalene was about double the cytotoxicity produced in this study one day after 150 mg/kg naphthalene oral gavage (Kovalchuk et al., 2017). A site-specific difference in naphthalene toxicity did not occur for the single oral gavage dose administered in this study. For intraperitoneal naphthalene exposures, naphthalene is a more potent toxicant in terminal bronchiolar airways relative to larger proximal airways (Van Winkle et al., 1995). The injury target sites differ for naphthalene inhalation which causes more cytotoxicity in proximal airways relative to terminal bronchioles (Carratt et al., 2019; West et al., 2001). Consistent with previous intraperitoneal studies, the bifurcations did not demonstrate significant cytotoxicity after naphthalene oral gavage exposure in this study (Plopper et al., 1992;

Reynolds et al., 2000). Previous naphthalene inhalation exposures were able to cause bifurcation toxicity similar to toxicity in the other parts of the conducting airway epithelium (West et al., 2001). In addition to administration differences, mouse strain differences may contribute to the differences in severity and distribution of naphthalene toxicity observed in airway epithelium (Plopper et al., 2001). However, this study does confirm that naphthalene administered by oral gavage continues to cause a characteristic Club cell toxicity.

The scope of this study was limited to determine potential Club Cell toxicity caused by acute 4MEI exposure. Sufficiently sensitive methodologies and/or molecular and cellular approaches are required for future studies of the mechanisms of acute 4MEI toxicity and to identify potential cellular targets. No biologically significant toxicity was detected at either 4MEI dose selected to be over 25,000 times higher than expected daily human dietary exposures (Cunha et al., 2011; Fierens et al., 2018; Folmer et al., 2018; Liang et al., 2019) and a wider dose range, or a longer period of exposure, may be required to trigger acute airway toxicity. The limited doses and sample sizes used for this study did not highlight any sex differences or timescale differences for pulmonary toxicity after 4MEI exposure. Increased sample size or a wider range of doses will be required to further investigate sex differences in response to 4MEI or naphthalene administered by oral gavage. Additionally, the pathologic changes in the airways after 4MEI exposure in mice which eventually lead to lesion formation (Chan et al., 2008; National Toxicology Program, 2007) may occur outside of the timescale tested here and most likely occur through a mechanism that was not evaluated in this study and so future studies should consider later timepoints than 24 hours and subchronic dosing.

While the results here do not show biologically significant epithelial toxicity in response to 4-MEI exposure, one caveat must be considered. If the airway epithelial cell targeted is especially rare or is in a novel location, the approach used in this study may not have been sufficient. The low level of toxicity detected in many 4MEI studies may be consistent with a low abundance cell type target, like neuroendocrine cells. Future studies designed to evaluate the toxicity of 4MEI in low abundance cell types, that examine cell type specific responses such as apoptosis or that use *in situ* detection of permeable cells may be needed to further evaluate the mechanism of 4MEI respiratory toxicity. For example, given the low abundance of even mild epithelial changes future studies may need to include site-specific assessment of airway bifurcations. Further, 4MEI toxicity may possibly involve a pathway that only comes into play with repeated exposure and thus could not be detected in the current study. Metabolic activity studies using airway microsomes enriched with Phase I metabolic enzymes from multiple species were not able to inform the whole animal metabolism of 4MEI, so microdissected tissues or organ slices may be required to evaluate whether reactive 4MEI metabolites are produced in the lung under acute or chronic exposure conditions or in rare locations (Beevers and Adamson, 2016; Fennell et al., 2019).

5. Conclusions:

This study does not support Club cells as the target cell of 4MEI toxicity acutely after administration of high bolus doses. Further studies will be required to clarify the mechanism of acute 4MEI toxicity and to further evaluate whether Club cell metabolism is involved in

4MEI toxicity, perhaps affecting an alternate cell type. No 4MEI toxicity was detected after acute exposure to 150 mg/kg or 300mg/kg oral gavage doses. As a positive control, naphthalene pulmonary epithelial toxicity at 150 mg/kg oral gavage was consistent with previous studies demonstrating significant Club cell specific cytotoxicity after naphthalene inhalation or injection. This study demonstrates a lack of acute 4MEI airway epithelial toxicity at doses previously used to demonstrate 4MEI metabolism in rodents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

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4MEI	4-Methylimidazole
CC10	secretoglobin 1A1
CGRP	calcitonin gene-related peptide
MPO	myeloperoxidase
NSRL	No Significant Risk Level
NTP	National Toxicology Program
OEHHA	California Office of Environment Health Hazard Assessment
PCNA	proliferating cell nuclear antigen

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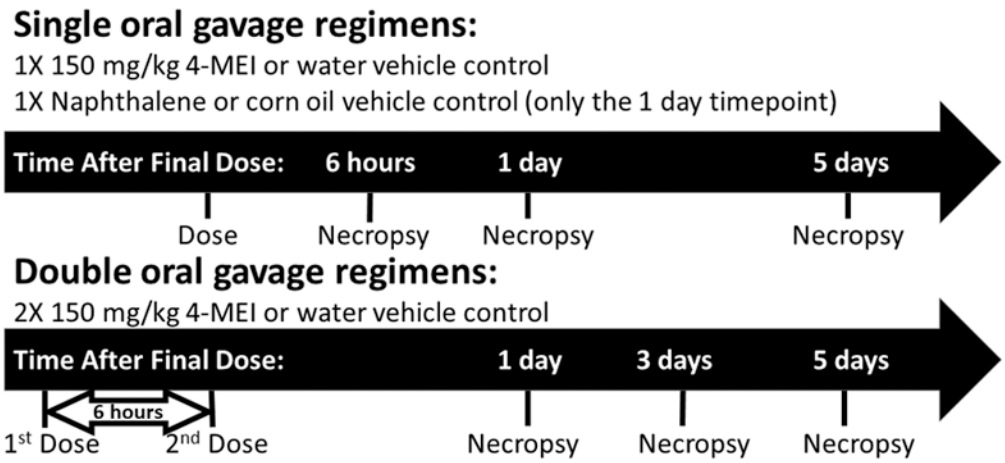

Figure 1. Experimental Timeline.

Mice were either exposed once or twice with exposures to 150 mg/kg of 4MEI or naphthalene by oral gavage. 5 females and 5 males were necropsied for each group.

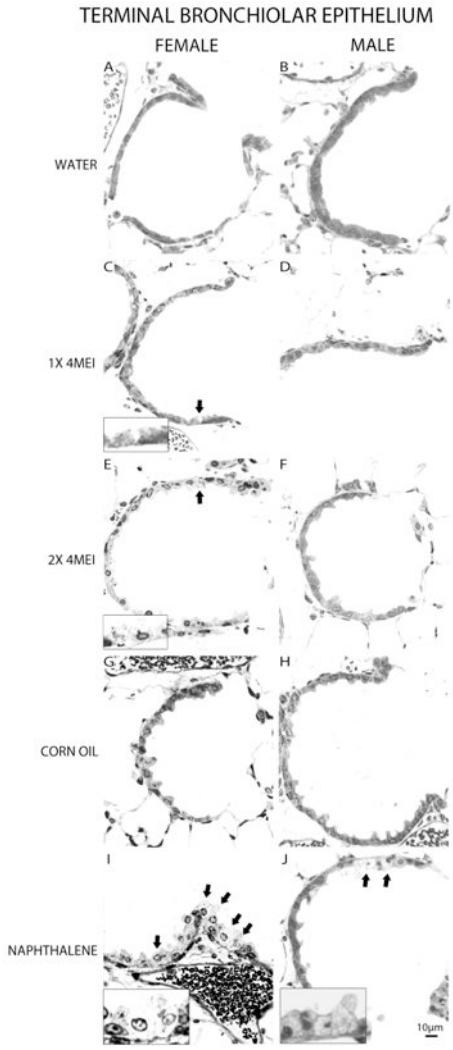

Figure 2. Representative light microscopic images of epithelial toxicity at terminal bronchioles of conducting airways after 4MEI or naphthalene oral gavage in female and male mice.

Terminal bronchioles were defined as the last conducting airway generation before the gas exchange region of the lung. Mouse lung tissue was fixed at constant pressure 1 day after oral gavage exposure to 150 mg/kg 4MEI, 2 doses of 150 mg/kg 4MEI (300 mg/kg 4MEI), or 150 mg/kg of naphthalene. Water (A-B) was the vehicle control for 4MEI exposures (C-F) and corn oil (G-H) was the vehicle control for naphthalene exposure (I-J). Images were taken at 400X of methylene blue/azure II stained resin sections of lung tissue and converted to grayscale. Injured epithelial cells are indicated with an arrow. Insets showing cytotoxicity are magnified to 800X.

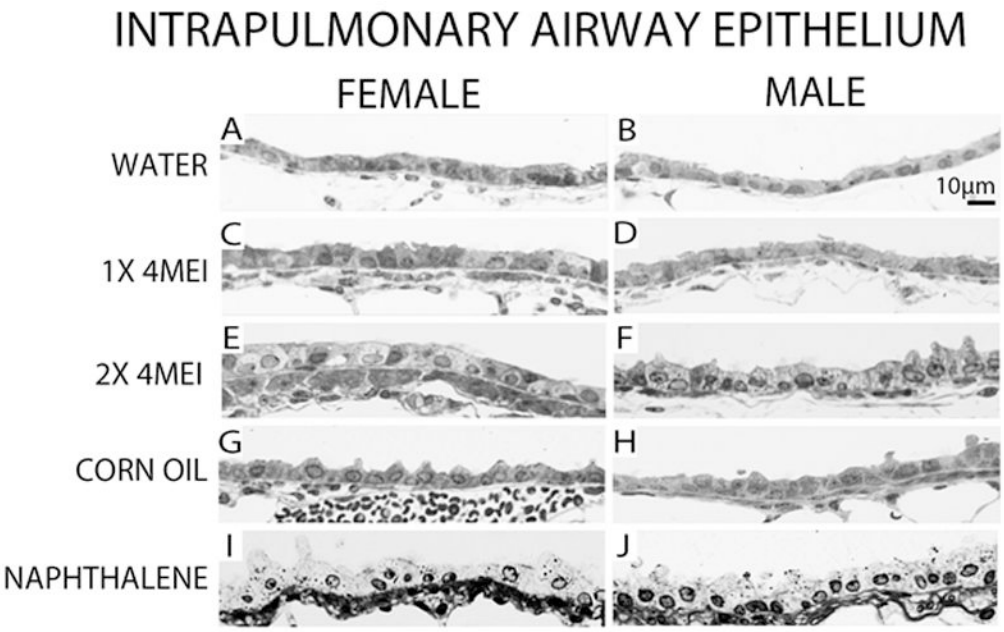

Figure 3. Representative light microscopic images of epithelial toxicity at larger intrapulmonary airways after 4MEI or naphthalene oral gavage in female and male mice.

Intrapulmonary airways were defined as conducting airways between the lobar bronchi and terminal bronchi, typically arising within a few generations of the lobar bronchus. Mouse lung tissue was fixed at constant pressure 1 day after oral gavage exposure to 150 mg/kg 4MEI, 2 doses of 150 mg/kg 4MEI (300 mg/kg 4MEI), or 150 mg/kg of naphthalene. Water (A-B) was the vehicle control for 4MEI exposures (C-F) and corn oil (G-H) was the vehicle control for naphthalene exposure (I-J). Images were taken at 400X of methylene blue/azure II stained resin sections of lung tissue and converted to grayscale. The naphthalene exposed airways consisted primarily of injured cells that were lightly stained, while the vehicle control and 4MEI exposed epithelium contained minimal cytotoxicity.

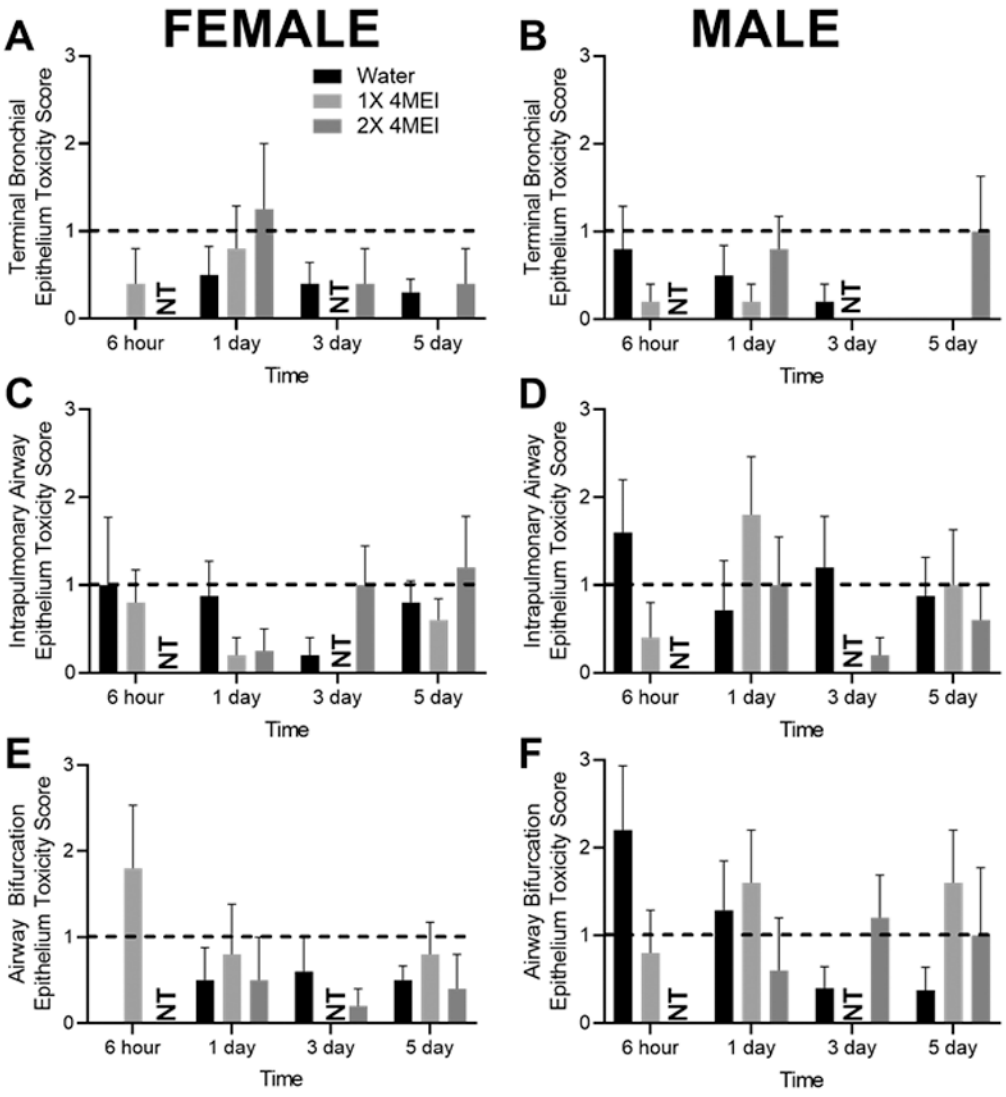

Figure 4. Histologic scoring of epithelial toxicity at terminal bronchioles, intrapulmonary airways, and airway bifurcations over time after 4MEI oral gavage in female and male mice.

An epithelial toxicity score was assigned to epithelial cells at 3-6 sites located at terminal bronchioles, intrapulmonary airways, or airway bifurcations after oral gavage with single dose of 150 mg/kg 4MEI or 2 doses of 150 mg/kg 4MEI (300 mg/kg 4MEI). Water was the vehicle control for 4MEI exposures. Epithelial toxicity scores were **0**: no swelling or vacuolization, **1**: minor swelling of one cell, **2**: one cell swollen or vacuolated (devoid of stain), **3**: two or three cells vacuolated or swollen, **4**: greater than three vacuolated or swollen cells or squamation, and **5**: exposed basement membrane. The threshold for suspected epithelial toxicity was set at average score of 1, as indicated with the dashed line. Bars represent the average score for at least 4 mice and error bars represent standard error. NT=not tested. No significant difference occurred between 4MEI treated groups and vehicle controls ($p<0.05$).

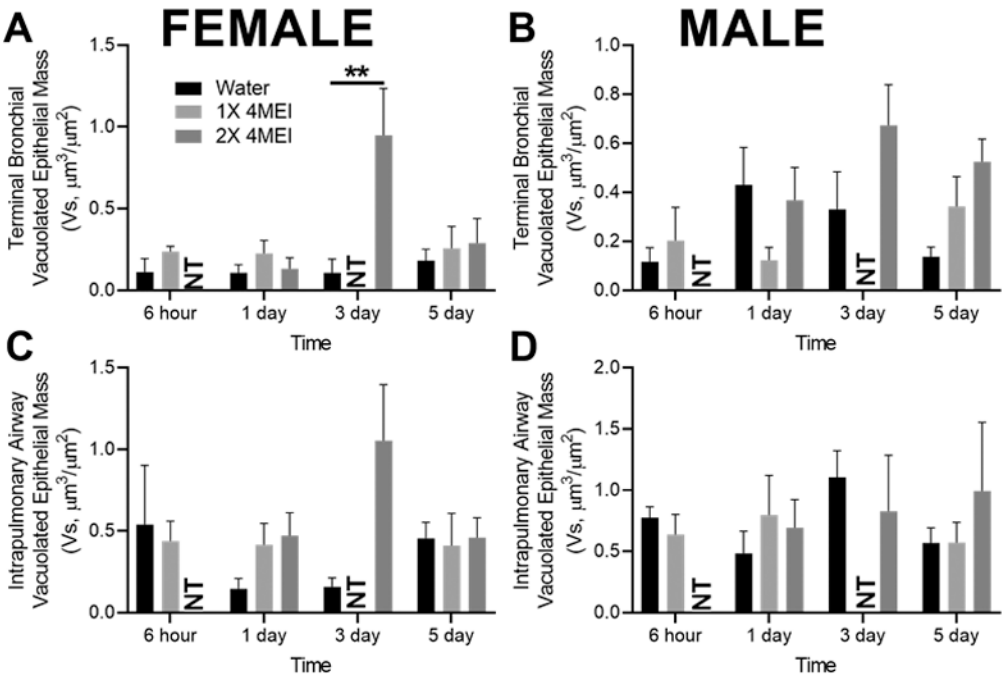

Figure 5. Morphometric measurement of the volume of vacuolated and swollen airway epithelial cells per basal lamina surface area over time after 4MEI oral gavage in female and male mice.

The average volume of epithelial toxicity per basal lamina surface area (Vs) was measured for female and male mice in terminal bronchiolar and intrapulmonary airway epithelium after oral gavage with single dose of 150 mg/kg 4MEI or 2 doses of 150 mg/kg 4MEI (300 mg/kg 4MEI). Water was the vehicle control for 4MEI exposures. At least 4 mice were included in each treatment group. Bars represent the average value and error bars represent standard error. NT=not tested. Significant differences between average score of treated mice compared to vehicle control are represented with ** ($p<0.01$).

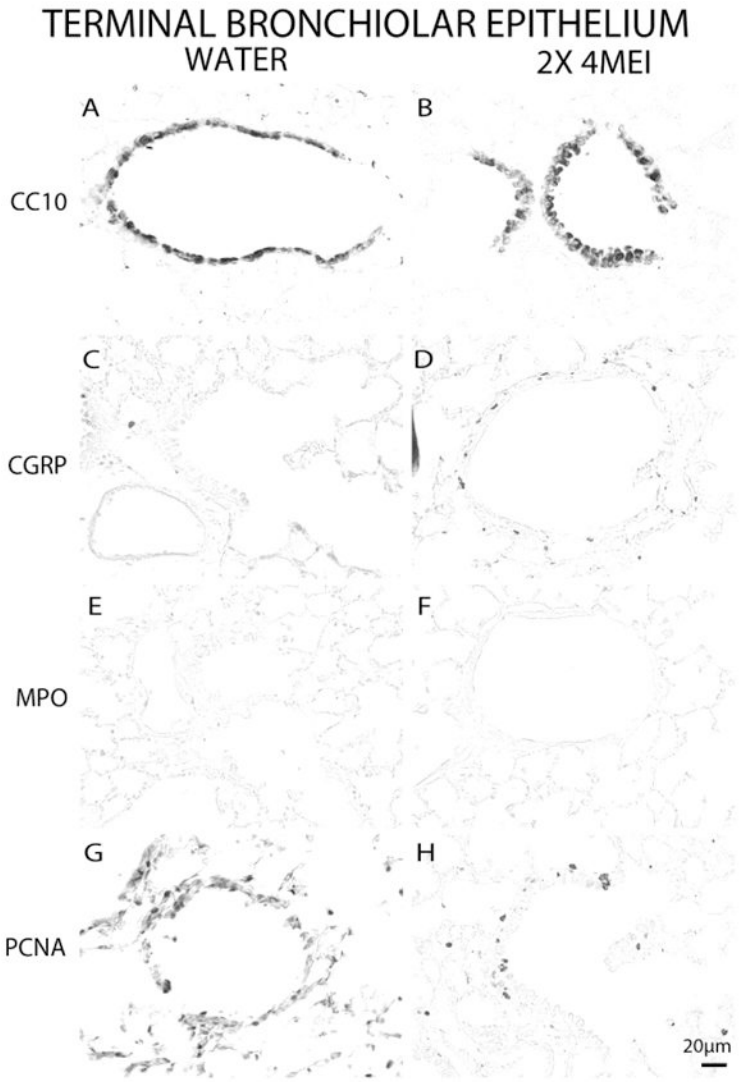

Figure 6. Representative images of immunohistochemistry at terminal bronchioles of conducting airways after 4MEI oral gavage in female mice.

Terminal bronchioles were defined as the last conducting airway generation before the gas exchange region of the lung. Mouse lung tissue was fixed at constant pressure 1 day after oral gavage exposure to 2 doses of 150 mg/kg 4MEI (300 mg/kg 4MEI). Water was the vehicle control for 4MEI exposures. Immunohistochemistry was conducted with anti-secretoglobin 1A1 (CC10), anti- calcitonin gene-related peptide (CGRP), anti-myeloperoxidase (MPO), and anti- proliferating cell nuclear antigen (PCNA) antibodies. Images were taken at 400X of lung tissue and converted to grayscale.

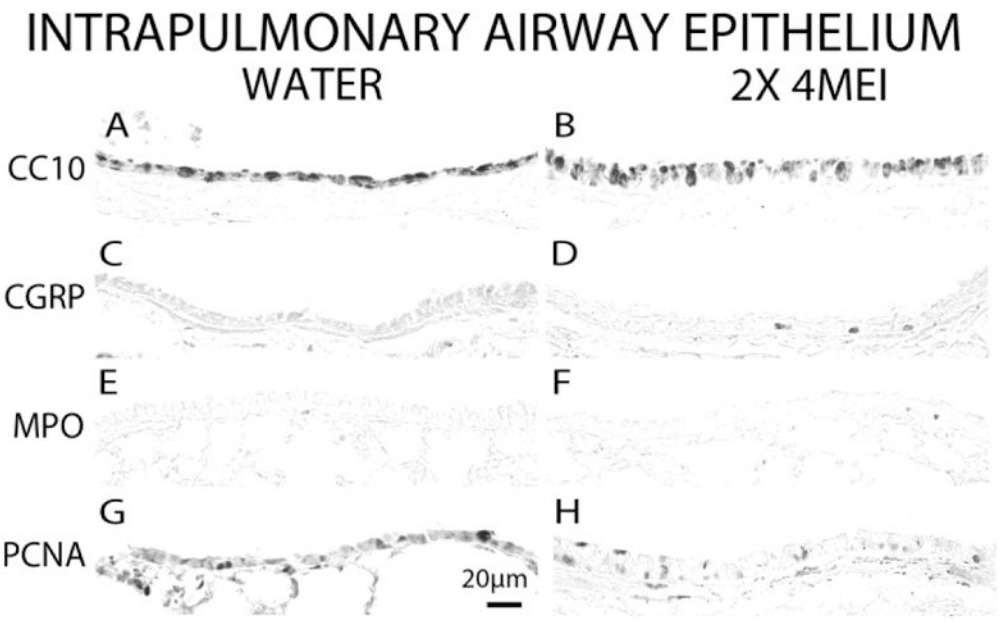

Figure 7. Representative images of immunohistochemistry at intrapulmonary conducting airways after 4MEI oral gavage in female mice.

Intrapulmonary airways were defined as conducting airways between the lobal bronchi and terminal bronchi. Mouse lung tissue was fixed at constant pressure 1 day after oral gavage exposure to 2 doses of 150 mg/kg 4MEI (300 mg/kg 4MEI). Water was the vehicle control for 4MEI exposures. Immunohistochemistry was conducted with anti- secretoglobin 1A1 (CC10), anti- calcitonin gene-related peptide (CGRP), anti- myeloperoxidase (MPO), and anti- proliferating cell nuclear antigen (PCNA) antibodies. Images were taken at 400X of lung tissue and converted to grayscale.

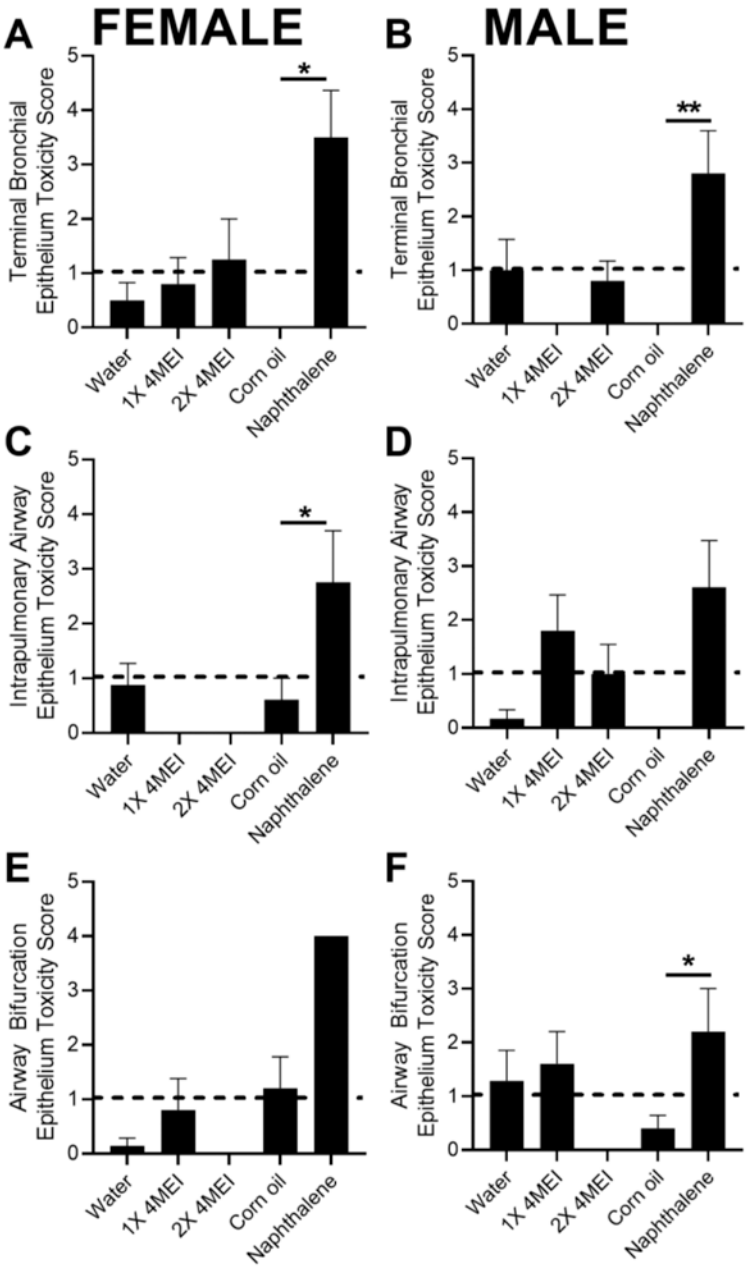

Figure 8. Histologic scoring of epithelial toxicity at terminal bronchioles, intrapulmonary airways, and airway bifurcations after 4MEI or naphthalene oral gavage in female and male mice.

An epithelial toxicity score was assigned to epithelial cells at 3-6 sites located at terminal bronchioles, intrapulmonary airways, or airway bifurcations fixed 1 day after oral gavage with single dose of 150 mg/kg 4MEI, 2 doses of 150 mg/kg 4MEI (300 mg/kg 4MEI), or 150 mg/kg of naphthalene. Water was the vehicle control for 4MEI exposures and corn oil is the vehicle control for naphthalene exposure. Vehicle treatments were not statistically different for any endpoints. Epithelial toxicity scores were **0**: no swelling or vacuolization, **1**: minor swelling of one cell, **2**: one cell swollen or vacuolated (devoid of stain), **3**: two or three cells vacuolated or swollen, **4**: greater than three vacuolated or swollen cells or

squamation, and **5**: exposed basement membrane. The threshold for suspected epithelial toxicity was set at average score of 1, as indicated with the dashed line. Bars represent the average score for at least 4 mice and error bars represent standard error. Significant differences between average score of treated mice compared to respective vehicle control at the same time point are represented with * ($p<0.05$) and ** ($p<0.01$).

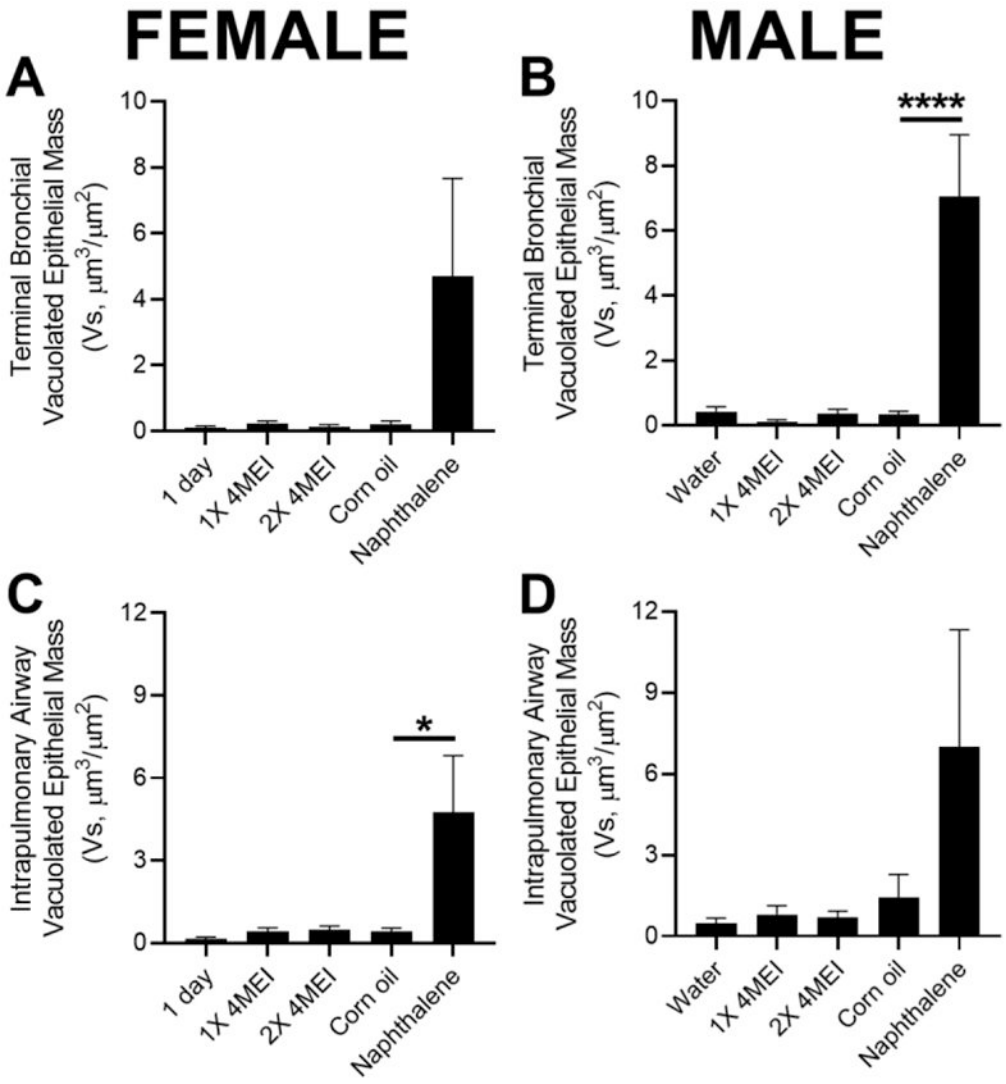

Figure 9. Morphometric measurement of the volume of vacuolated and swollen airway epithelial cells per basal lamina surface area after 4MEI or naphthalene oral gavage in female and male mice.

The average volume of epithelial toxicity per basal lamina surface area (Vs) was measured for female and male mice in terminal bronchiolar epithelium or intrapulmonary airway epithelium 1 day after oral gavage with single dose of 150 mg/kg 4MEI, 2 doses of 150 mg/kg 4MEI (300 mg/kg 4MEI), or 150 mg/kg of naphthalene. Water was the vehicle control for 4MEI exposures and corn oil was the vehicle control for naphthalene exposure. Vehicle treatments were not statistically different for any endpoints. At least 4 mice were included in each treatment group. Bars represent the average value and error bars represent standard error. Significant differences between average of treated mice compared to respective vehicle control at the same time point are represented with * ($p<0.05$) and **** ($p<0.0001$).

Table 1:

Study design table

Group	Treatment	Number of Doses	Oral Gavage Dose (mg/kg)	Total Dose
1	Water	1	0	0
2	4MEI	1	150	150
3	Water	2	0	0
4	4MEI	2	150	300
5	Corn oil	1	0	0
6	Naphthalene	1	150	150

Table 2:

List of primary antibodies used for immunohistochemistry

Biological target	Primary antibody	Primary antibody working dilution	Source
Club cells	anti- secretoglobin 1A1 (CC10)	1:16,000	Abcam 40873
Actively dividing cells	anti- proliferating cell nuclear antigen (PCNA)	1:1,300	Abcam ab18197
Neuroepithelial cells	anti- calcitonin gene-related peptide (CGRP)	1:15,000	Peninsula Laboratories T-4032
Neutrophils and macrophages	anti- myeloperoxidase (MPO)	1:200	Biogenex AR496-10R