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Evaluation of 4-methylcyclohexanemethanol (MCHM) in a combined irritancy and Local Lymph Node Assay (LLNA) in mice

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Abstract

4-Methylcyclohexanemethanol (MCHM) is a flotation reagent used in fine coal beneficiation. On January 9, 2014, crude MCHM, a mixture containing predominantly MCHM, was inadvertently released into the Elk River, a municipal water source that serves about 300,000 people in the Charleston, WV area, resulting in temporary contamination of 15 percent of the state's tap water and causing significant dermal exposure. The current studies were undertaken to determine whether crude MCHM or MCHM has the potential to produce dermal irritancy and/or sensitization. BALB/c female mice were treated daily for 3 consecutive days by direct epicutaneous application of 25 μ L of various concentrations of crude MCHM or MCHM to the dorsum of each ear. A mouse ear-swelling test was used to determine irritancy potential and was undertaken in combination with the standardized Local Lymph Node Assay (LLNA) to determine skin sensitizing potential. MCHM was found to produce skin irritation at concentrations above 20% and did not produce sensitization. Crude MCHM also produced irritation, although weaker, and in addition was found to be a weak to moderate skin sensitizer. The results are discussed in terms of potential human health hazard.

Keywords

4-Methylcyclohexanemethanol; Local Lymph Node Assay (LLNA); Allergic contact; Dermatitis (ACD); Dermal hypersensitivity; Dermal irritancy; Elk River spill

1. Introduction

4-Methylcyclohexanemethanol (MCHM; CASRN: 34885-03-5) is a flotation reagent used in fine coal beneficiation and was the primary chemical contaminant spilled from a storage tank into the West Virginia Elk River on January 9, 2014. The Elk River is a municipal water

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source that serves about 300,000 people in the Charleston area. The chemical spill temporarily contaminated 15 percent of the state's tap water. The leaking tank contained crude MCHM, a commercial mixture that is mostly MCHM (e.g. 68–88%), but also contains other related chemicals [detailed lists of chemical constituents is provided on the National Toxicology Program (NTP) website (NTP, 2015)]. The greatest measured concentration of MCHM in the spilled liquid entering or leaving the water treatment facility was 3.35 mg/L (Whelton et al., 2015). Concentrations of crude MCHM in tap water following the spill and prior to flushing were much lower, ranging from <10 to 420 ppb (Whelton et al., 2015); the upper level being slightly below the short-term drinking water limit of 1 ppm for a 10 kg child established by the Centers for Disease Control and Prevention (CDC) in response to the spill (Schade et al., 2015). In the days immediately following January 9, 2014, the West Virginia Poison Center received calls from over 1900 local residents reporting various health effects (West Virginia Poison Center Fact Sheet, 2014). Based upon syndromic surveillance records conducted through telephone interviews (Schade et al., 2015) and household surveys (CDC, 2014a), dermal effects were the most common reported symptom manifested by transient skin irritation ($p < 0.001$) and mild rash ($p < 0.002$). CDC further grouped symptoms based upon three exposure scenarios (more than one exposure may have been reported for a given subject): bathing, showering or other skin contact (52.6%); eating, drinking or other oral exposure (43.9%); and inhalation from vapor or mist (14.6%). These findings of dermal irritation are consistent with several experimental animal studies conducted on crude MCHM by Eastman Chemical Co. (Eastman Chemical Co, 1997). In these studies, acute dermal exposure in rabbits or Sprague-Dawley rats, as well as 24-day dermal exposure in rats showed that crude MCHM produced skin irritancy characterized by erythema and desquamation at the application site. Skin hypersensitivity testing, (i.e. allergic contact dermatitis, ACD), was also conducted on crude MCHM and was negative in a guinea pig model.

The Local Lymph Node Assay (LLNA) is a murine model used extensively to predict the potential for a chemical to induce hypersensitivity. The acceptance of the LLNA as a stand-alone alternative to the Guinea Pig Maximization Test/Buehler Assay by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) demonstrates the commitment of using more humane testing models for screening chemicals that may elicit contact hypersensitivity in humans (ICCVAM, 2011). Measurement of irritancy is incorporated into the LLNA protocol used by the NTP. Sensitization potential is evaluated by measuring the differential induction of lymphocyte proliferation in the draining lymph nodes relative to appropriate controls following dermal application of the chemical. Similar to other animal models used to assess dermal sensitization, the LLNA is approximately 72% predictive of skin sensitization in humans when compared with results from human skin prick or patch test data (ICCVAM, 1999), however, a recent evaluation suggests that one-third of strong human sensitizers may be under classified as weaker sensitizers by the LLNA method (ICCVAM, 2011).

The objective of the current study was to evaluate and compare the irritancy and sensitization potential of crude MCHM and MCHM in a combined irritancy/sensitization LLNA model. For dermal irritancy assessment, mouse ear swelling was measured on days 3 and 6 during the in-life phase of the study and punch biopsy ear weights were determined at

termination. Sensitization was assessed by measuring lymphocyte proliferation in the draining lymph node.

2. Materials and methods

These studies were conducted in compliance with the U.S. Food and Drug Administration Good Laboratory Practices for Nonclinical Laboratory Studies (Title 21 of the Code of Federal Regulations, Part 58).

2.1. Chemicals and dose formulations

MCHM (lot KDY3F) was supplied by TCI America (Portland, OR) and crude MCHM (lot TP14044373) was supplied by Eastman Chemical Company (Kingsport, TN). The chemical identity and purity was determined at MRI Global (Kansas City, MO). The identity of MCHM was confirmed by Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectrometry. The purity of MCHM estimated by gas chromatography (GC) with flame ionization detection (FID) was >99% with respect to *cis*- (~68%) and *trans*- (~32%) isomers of MCHM. The identity and purity of crude MCHM were determined by GC with mass spectrometry detection. Two major peaks representing *cis*- (~33%) and *trans*-isomer (~57%) of MCHM accounted for ~ 90% of the chemical composition of crude MCHM.

Dose formulation development and stability validation studies of crude MCHM were conducted at RTI International (Research Triangle Park, NC) using GC-FID. Crude MCHM (1%, v/v) in acetone:olive oil (4:1, v/v) (AOO) was stable for up to 14 days when stored ambient or refrigerated in glass amber vials. Assessment of the crude MCHM dosing solution under simulated dosing conditions (exposed to air and light) for 3 days revealed no significant loss of the test chemical in either case.

MCHM and crude MCHM were tested for solubility and ability to be delivered through a syringe in AOO vehicle. Both forms were determined to be soluble at 50% in AOO and the viscosity of the 50% solutions was acceptable based on lack of resistance when drawing or dispensing formulations using a syringe. Formulations were prepared in AOO from 0.45% to 20% and concentrations were analyzed. Dilutional linearity ($r = 0.9990$) was demonstrated for crude MCHM with an average percent recovery of 100%. Dilution verification of 40%, 80%, and neat formulations were also acceptable (relative error <10% for recovery). All dose formulations for MCHM and crude MCHM were analyzed for concentration and met acceptance criteria. Two studies were conducted. For Study 1, formulations of MCHM and crude MCHM were prepared at nominal concentrations of 0 (vehicle), 2, 20, 50 or 100% (v/v) and 0 (vehicle), 1, 2, 5, 20, 40, or 100% (v/v), respectively, in AOO by the study laboratory (Burleson Research Technologies). Due to excessive toxicity of neat (100%) MCHM and crude MCHM on study Day 1, the concentrations for the high dose group were reduced to 50% and 80%, respectively, for the remainder of the study. An aliquot of each of the dose formulations that were prepared on Day 3 was shipped to RTI International for analysis. Formulation concentrations, determined by GC-FID, were within 77–105% of target for MCHM and 76.8–103% of target for crude MCHM. For Study 2 only crude MCHM was tested. Formulations of crude MCHM were prepared by RTI International at

nominal concentrations of 0 (vehicle), 1, 5, 25, 50, and 75% (v/v) in AOO and shipped to Burleson Research Technologies. Formulation concentrations of crude MCHM were 95.2–100% of target concentration.

2.2. Test system

Female BALBc mice were purchased from Taconic Biosciences Inc., (Hudson, NY) and were 8–12 weeks of age at the start of treatment. The National Toxicology Program has historically used BALB/c mice for hypersensitivity testing and has an extensive database on the performance of this model (ICCVAM, 2009, 2011). Recent studies have compared responses in BALB/c and CBA/J mice in the LLNA using bromodeoxyuridine with flow cytometry and shown that both strains provide comparable results (Lee et al., 2017).

Based upon the vendor health reports and the study sentinel health assessments, the mice were deemed specific pathogen free. The animals were quarantined/acclimated for ~8 days and randomized by body weight 1–3 days prior to the start of test article application. Mice were group housed in individually ventilated cages with up to 5 mice from the same treatment group per cage. The cages contained irradiated Sanichip® woodchip bedding. The environmental conditions of the animal room, where all animals on study were housed, were recorded daily and had a temperature range of 69–75 °F, relative humidity range of 35–65%, and a 12-h light/dark cycle. Food, (irradiated NTP-2000 diet) and tap water were provided *ad libitum*. Body weights were recorded on study Days 1 and 6, and the animals were monitored twice daily (once before 10am and once after 2pm) for signs of toxicity. The studies were reviewed and approved by the Institutional Animal Care and Use Committee for adherence to the *Guide* and the applicable policies of the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were conducted in compliance with Nonclinical Laboratory Studies Good Laboratory Practice Regulations issued by the U.S. Food and Drug Administration (Title 21 of the Code of Federal Regulations, Part 58).

2.3. Treatment

Treatment was conducted in accordance with the performance standards established by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2009, 2011). Mice were restrained by hand and 25 µL of test or control article was applied daily for three consecutive days (24 h ± 2 h between applications) to the dorsum of each ear using a calibrated pipette.

Following acclimation, mice were randomized to treatment groups using body weight as the metric. In Study 1, formulations of MCHM were tested at 100%, 20%, and 2% while crude MCHM formulations were tested at 100%, 40%, 20%, 5%, 2%, and 1%. Two of the 5 mice treated with 100% (neat) MCHM on Day 1 showed clinical signs of toxicity and were euthanized moribund resulting in the dose subsequently being lowered to 50% for Days 2 and 3 (the group is referred to as 100/50%). Mice treated with 100% (neat) crude MCHM demonstrated similar, although less severe, clinical signs of toxicity after the first day of dosing resulting in the concentration being lowered to 80% for Days 2 and 3 (the group is referred to as 100/80%). Details of the clinical signs observed are provided in the results section. Study 2 was conducted only with crude MCHM to clarify the dose response curve

and help confirm the initial findings. The concentrations of crude MCHM tested in Study 2 were 75%, 50%, 25%, 5%, and 1%. The controls for all studies included the vehicle (AOO) and a positive control; 0.15% 1-fluoro-2,4-dinitrobenzene (DNFB), which was also prepared in AOO. DNFB is considered a potent sensitizer and irritant and commonly used as a positive control for skin sensitization studies in experimental models (ICCVAM, 2009, 2011).

2.4. Dermal irritation

A modification of a method described by Gerberick and Ryan (Gerberick et al., 2001) was used to determine dermal irritancy potential. Ear thickness measurements were taken on both ears using a calibrated micrometer 2 h following the last test chemical treatment on Day 3 and again on Day 6, prior to injection of ^{125}I iododeoxuridine ($^{125}\text{IUdR}$; Perkin Elmer, Inc., Melville, NY). Following euthanasia on Day 6, a 6 mm punch biopsy was taken from each ear and weighed as an additional quantitative measure of irritancy. Ear swelling data are presented as relative % change in ear thickness and ear punch biopsy weight of each test group relative to the AOO control group.

2.5. Local lymph node assay (LLNA)

On Day 6, a stock of 8 $\mu\text{Ci}/\text{mL}$ of $^{125}\text{IUdR}$ was prepared in Hanks' balanced salt solution containing 25 mM HEPES (HBSS/H) and each mouse was administered an intravenous injection of 2 μCi (0.25 mL of stock solution) into the lateral tail vein. Five hours after the $^{125}\text{IUdR}$ injections were completed, mice were euthanized by CO_2 asphyxiation and death was confirmed by severing the diaphragm. The cervical draining lymph nodes were collected and placed into a 15 mL conical tube containing HBSS/H. Lymph nodes collected from an individual animal were pooled in a single tube. The lymph nodes were then placed between the frosted ends of two glass microscope slides and macerated to produce a cell suspension. The cell suspensions were transferred to centrifuge tubes containing HBSS/H and processed through two centrifugations and wash cycles to remove any residual $^{125}\text{IUdR}$ not associated with live cells. Trichloroacetic acid (TCA, 5%) was added to the tubes containing the cell pellet following the final centrifugation. The tubes were vortexed and then wrapped in lead foil and refrigerated overnight (16–24 h). On the following day, tubes containing the cell suspensions were removed from the refrigerator, vortexed, and centrifuged to pellet the DNA. The resulting pellets were resuspended with 5% TCA and added to a gamma counter tube (1 tube per mouse). Radioactivity in the samples was determined using a Cobra II[®] Auto-Gamma[®] Counting System (Packard Instrument Company).

2.6. Data calculations & statistical analysis

Irritation was evaluated by measuring changes in ear swelling (thickness) and ear punch biopsy weights. Ear thickness changes were calculated as percent change from the mean of the vehicle control group on Days 3 and 6. Changes in ear punch biopsy weights were calculated as percent change from the mean of the vehicle control group on Day 6 as a supplemental measure of irritation.

Sensitization was evaluated by quantifying lymphocyte proliferation in the draining lymph nodes as measured by the incorporation of $^{125}\text{IUdR}$ into the DNA of the proliferating cells. The results from each cell suspension counted on the gamma counter were recorded in counts per minute (CPM). The CPMs were converted to disintegrations per minute (DPMs) by dividing by the gamma counter efficiency and multiplying by 100. After the DPM values were calculated, the mean blank DPM were subtracted from the individual DPM values for each mouse to obtain corrected DPM values. The mean corrected DPM and standard error of the mean (SE) were determined for each group. The stimulation index (SI) was calculated by dividing the individual animal DPM by the control (vehicle) group mean DPM. The SI data are expressed as mean $\pm$ SE for each group.

In accordance with the performance standards established by ICCVAM (ICCVAM, 2009, 2011), a compound is usually considered sensitizing if it has an SI value equal to or greater than 3. A one-sample *t*-test was performed to determine if the individual untransformed SI values for each dose level of each test article and DNFB were greater than 3. The natural log transformed DPM values for each compound were compared against the vehicle control by performing a Bartlett's Chi-Square test for variance homogeneity. If found to be non-significant, the results of a one-way analysis of variance (ANOVA) and Dunnett's *t*-test were used to evaluate for dose relationship and/or dose response. If the Bartlett's Chi-Square value was found to be significant, the results of the Kruskal-Wallis test and Jonckheere-Terpstra test were used to evaluate for dose relationships and/or dose-response. Statistical methods have been described by Salsburg (1986). A statistical significance of $p < 0.05$ was used in these evaluations.

For each test article, a fitted quadratic equation (a linear term and a square term of concentration) was used to determine the concentration of test article required to elicit a stimulation index of 3 (EC-3). If the quadratic term for this model was not statistically significant ($p < 0.05$), linear regression was used to determine the test article EC-3. All calculations were performed using Microsoft® Excel and SAS®.

3. Results

3.1. Study 1

Body weights for study animals following euthanasia on Day 6 were not significantly affected by any test article treatment (data not shown). However, mice treated with 100/80% crude MCHM had a slight, but significant reduction in body weight gain (-0.22 ± 0.1 g) from Days 1–6 relative to body weight gain observed in the vehicle control group (0.74 ± 0.18 g). Mice treated with 100/80% crude MCHM or 100/50% MCHM showed mild clinical signs of toxicity including hypoactivity (Days 1–2), eye squinting (Days 1–3), and isolation from their cage mates (Days 1–2). No clinical changes were observed in mice treated with lower concentrations of crude MCHM or MCHM. Mice treated with DNFB developed clinical signs similar to the high dose of MCHM including hypoactivity, eye squinting, and isolation from cage mates. In addition, dermal application of 0.15% DNFB resulted in mild-to-moderate erythema observed on Days 3–6. These effects observed with DNFB are consistent with the known properties for this positive control.

The potential for MCHM to produce dermal irritation in mice was determined by measuring relative changes in ear thickness and punch biopsy weights (data not shown). For crude MCHM, a modest, relative to the positive control group, but statistically significant increase in ear swelling was observed on Days 3 and 6 only at the 20% test concentration, which was not dose-responsive (Fig. 1A and B). For MCHM, modest but significant increased ear swelling was observed at both the 20 and 50% test formulations on test Days 3 and 6 with significant dose response trends occurring on both study days. Ear punch biopsy weights (data not shown) were not affected by any MCHM treatment. Following treatment with the positive control (0.15% DNFB), increased ear swelling occurred on Day 3, and both ear swelling (Fig. 1A and B) and punch biopsy weights (data not shown) were increased by 48.3% on Day 6 relative to the vehicle control group.

None of the mice treated with MCHM had mean group DPM or SI values that were significantly increased from the AOO vehicle control indicating a negative LLNA response. For crude MCHM, the DPM values and SIs were both significantly increased in mice treated with 20% test concentration with a statistically significant dose-response trend observed (Fig. 2). SI values > 3.0 occurred in mice treated with crude MCHM at 40% test concentration with a maximum SI value of 4.83 in the 100/80% treatment group. A substance is usually classified as a sensitizer if at least one concentration of the test material results in an SI of 3 or more. Since crude MCHM achieved SI values > 3.0, an EC-3 was calculated and determined to be 34.6%. Treatment with the positive control 0.15% DNFB resulted in highly significant increases in DPMs and the SI value (SI = 10.02).

3.2. Study 2

In order to clarify the lack of dose response seen in Study 1 for the irritancy test with crude MCHM and better define the dermal hypersensitivity dose-response curve, a second experiment was conducted with crude MCHM at concentrations of 1%, 5%, 25%, 50%, and 75%. The highest concentration of 75% was selected based on the overt clinical toxicity observed in mice treated with 100% MCHM in the first study. All animals survived the study period and there were no significant effects on body weight or body-weight gain associated with treatment (data not shown). Treatment with 50 or 75% crude MCHM produced similar, but less severe and less frequent transient clinical signs as observed in the 100/80% crude MCHM treatment group in Study 1, including hypoactivity, isolation from cage mates, and eye squinting. Mice treated with DNFB also developed mild clinical signs similar to those observed in Study 1. None of the other treatment groups demonstrated clinical changes during the study period.

The potential for crude MCHM to produce dermal irritation was assessed by measuring changes relative to the AOO vehicle control group in ear swelling on Days 3 and 6 and punch biopsy weights collected at the scheduled euthanasia on Day 6. There was a statistically significant increase in ear swelling relative to the AOO control group on Days 3 (1.63%) and 6 (2.40%) in mice treated with the 75% concentration of MCHM, but not in any of the other MCHM treatment groups (Fig. 3A and B). Ear punch biopsy weights were not affected by MCHM treatment when compared to the AOO vehicle control group (data not

shown). An increase in ear swelling and ear punch biopsy weights (38.0% relative to the vehicle control group) occurred on Day 6 in the DNFB positive control group.

The DPM values in the draining lymph node, an indication of lymphocyte proliferation, and the SI values showed significant dose-response increases in mice treated with crude MCHM (Fig. 4). DPM values were significantly increased at 50% MCHM concentrations compared to the AOO vehicle control group while the SI value was significantly increased in the draining lymph nodes of mice treated with 75% MCHM. An SI value of 4.2 (significantly >3) occurred in mice treated with crude MCHM at the 75% concentration with an EC-3 value of 60%. Treatment with 0.15% DNFB resulted in a high degree of cell proliferation with an SI of 76 compared to the vehicle control group.

4. Discussion

The current studies indicate that both crude MCHM and MCHM can be considered dermal irritants as classified by the Globally Harmonized System of Classification and Labelling of Chemicals (Occupational Safety and Health Administration (OSHA), 2015). As recently reviewed by Paustenbach et al. (2015), these findings are also consistent with previous animal studies conducted by the Eastman Chemical Co (Eastman Chemical Co, 1997; Shepard, 1990), and surveillance data collected from residents in districts with contaminated tap water at the time of the Elk River chemical spill (Schade et al., 2015; CDC, 2014a). On a percent weight basis, MCHM appears to be a more potent irritant than crude MCHM, suggesting that MCHM isomers are responsible for the irritant properties that are diluted by impurities contained in the crude mixture.

The levels of MCHM, which were found in residential tap water immediately after the spill, were several orders of magnitude lower than the lowest observed effect levels (LOELs) we report for either skin irritation or sensitization, but were above the short-term drinking water screening level of 1 ppm established by the Centers for Disease Control and Prevention at the time of the spill (Centers for Disease Control and Prevention (CDC), 2014). However, as mentioned previously, health effect surveys conducted following the spill indicated an elevated incidence of dermal irritation in the population. The clinical symptoms observed in the present study support the potential for high doses to cause mild irritation, likely sensory, although this did not result in significant changes in ear thickness indicative of dermal irritation. While this could be due to differences in species sensitivity, it should be noted that our test method uses limited applications; once per day for three consecutive days, over a small surface area of the skin. In contrast, human dermal exposure can occur over a longer period and involve large surface areas, as would occur from bathing or showering. Repeated exposures are also expected throughout the day due to hand and face washing. Clothes washing with contaminated water also represented a potential source of extended dermal exposure.

In contrast to the earlier findings reported by Eastman Chemical Co. (Eastman Chemical Co, 1997), the present study determined that crude MCHM is a skin sensitizer, with an EC-3 value in the 35–60% concentration range. The failure of the Eastman Chemical Co. study to detect sensitization is likely related either to the low concentration tested (1% for

sensitization and 10% for the challenge dose) or a result of the test method used for determination of sensitization potential; the guinea pig footpad test (the standard method per the 1981 version of OECD Test 406 at the time the studies were performed) versus the LLNA, which involve very different methodologies (ICCVAM, 2009, 2011).

That only crude MCHM, and not MCHM, caused skin sensitization in the LLNA suggests that sensitization involves chemical characteristics that are unique to crude MCHM. According to the Eastman Safety Data Sheet (Eastman Chemical Co, 2015), crude MCHM contains only 68–88% MCHM while according to Freedom Industries, the leaking tank contained 88.5% crude MCHM, 7.3% propylene glycol phenyl ether (PPH) stripped basic, and 4.2% water. PPH stripped is reported to be primarily composed of dipropylene glycol phenyl ether (DiPPH) and PPH (Foreman et al., 2015). While based upon available data propylene glycols are considered skin irritants, they have not been shown to be sensitizers (ATSDR, 1997). Furthermore, PPH was not part of the crude MCHM tested in the current studies.

Analysis (performed by MRI Global for the NTP) of the MCHM used in the present studies indicated that the purity of MCHM was >99% with *cis* and *trans* isomers representing approximately 68% and 32%, respectively. The lot of the crude MCHM used in the present studies was approximately 90% MCHM and the same two isomers were identified. However, the abundances of the *cis* and *trans* isomers were approximately 33% and 57%, respectively, which are opposite to the relative abundances observed in MCHM. Similar differences in isomer ratios for MCHM and crude MCHM have been reported previously (Foreman et al., 2015; Dietrich et al., 2015). Thus, it is possible that the MCHM *trans* isomer drives the sensitization potential of crude MCHM. In addition to differences in diastereomers, there were several components in the unidentified fraction (~10%) of crude MCHM at concentrations that might be considered significant. Several of those components were tentatively identified, by comparison to the National Institute of Standards and Technology Mass Spectral Library, as cyclohexanemethanol (1.82%), a mix of the *cis* and *trans* isomers of 1,4-cyclohexanedimethanol (7.15%), 2-ethyl-1-hexanol (0.06%), *cis*-octahydroisobenzofuran (0.13%), 4-methylcyclohexanecarboxylic acid methyl ester (0.50%), and 1,4-cyclohexanedicarboxylic dimethyl ester. For the most part, limited toxicological data are available for these chemicals, although the limited data available suggest that some are skin and/or eye irritants (Eastman Chemical Co, 2014). Structure activity relationship (SAR) analysis performed by NTP suggests that in at least one SAR model many of the chemicals in crude MCHM may be dermal sensitizers and/or irritants (NTP, 2016). In any case, these findings suggest that components other than MCHM could contribute to the sensitizing potential of crude MCHM and may warrant further testing.

Although the magnitude of proliferation in the draining lymph node cells closely correlates with chemical sensitization it has been reported that irritants can induce proliferation in the draining lymph nodes, and, if potent enough, induce false positives in the LLNA (ICCVAM, 2009; Gerberick et al., 2001; Dearman et al., 1999). Since crude MCHM produced both a positive skin irritancy and sensitization response, there is some concern that the LLNA response is providing a false signal. In the first study, dermal treatment with crude MCHM at 20% and 100/80% resulted in significantly increased ear swelling. Mice treated with 50%

crude MCHM did not show evidence of irritation in study 1 indicating the lack of a concentration-related trend. Study 2 confirmed the irritation at the high dose (75% crude MCHM) but clearly demonstrated a lack of change in ear swelling at all other concentrations. These results suggest that the changes in ear swelling observed in study 1 in mice treated with 20% crude MCHM may be anomalous. In addition, the severity of ear swelling observed following treatment with crude MCHM was less than 25% above the control group, a value which is not considered excessive and not expected to induce non-specific proliferation in the draining lymph node (ICCVAM, 2009, 2011). In this respect, significant increases in cell proliferation occurred in mice treated with MCHM at doses below those that produced ear swelling. Together, these findings indicate that crude MCHM was most likely correctly identified as a skin sensitizer.

In summary, both MCHM and crude MCHM were found to produce skin irritation although the latter appears to be a considerably weaker irritant. In addition, crude MCHM, but not MCHM, was identified as a skin sensitizer in the LLNA. The differences between the crude MCHM and MCHM in the LLNA response are postulated to be due to either variation in the concentration of their isomer or the presence of other chemicals in crude MCHM that have the potential to cause sensitization. In any case, although an increased incidence of skin irritation was observed in the residents with contaminated water, whether skin sensitization actually occurred is unknown as patch testing was not conducted, nor warranted, as residents are unlikely to have further exposure to MCHM. From a public health standpoint, the concentrations found to produce either irritation or sensitization are well above levels found in the tap water following the Elk River chemical spill.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACD	allergic contact dermatitis
AOO	acetone:olive oil (4:1, v/v)
CDC	Centers for Disease Control and Prevention
DNFB	0.15% 1-fluoro-2,4-dinitrobenzene
DPM	disintegrations per minute

ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
¹²⁵IUdR	¹²⁵ iodo-deoxuridine
LLNA	local lymph node assay
MCHM	4-methylcyclohexanemethanol
NTP	National Toxicology Program
SI	stimulation index

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Fig. 1. Impact of dermal treatment with MCHM on ear swelling in BALB/c mice. (Study 1) Treatment induced changes in ear thickness were measured using a calibrated micrometer approximately 2 h following the final application of MCHM on Day 3 (A) and again prior to euthanasia on Day 6 (B). Data are expressed as relative change from the vehicle control group mean ear thickness. DNFB - 1-Fluoro-2,4-dinitrobenzene; MCHM - 4-methylcyclohexanemethanol; AOO – acetone:olive oil (4:1 v/v) Each bar represents the mean $\pm$ SE (N = 5 except 100/50% MCHM where N = 3). *Significantly different from AOO control group ($P < 0.05$, Dunnett's T-Test). ¹Significant dose-response trend for crude MCHM and MCHM ($P < 0.05$, Jonckheere's Trend Test). ²Significant dose-response trend for MCHM ($P < 0.05$, Jonckheere's Trend Test).

Fig. 2. Impact of dermal treatment with MCHM on lymphocyte proliferation in the draining lymph node (Study 1)

Lymphocyte proliferation in the draining lymph node was determined by quantifying the incorporation of $^{125}\text{IUdR}$ into the DNA of the proliferating cells and data are presented at DPM values. The Stimulation Index (SI) was determined for each animal relative to the mean lymphocyte proliferation in the vehicle control group and was used to determine the concentration of MCHM that increased proliferation 3-fold (EC3). SI values for the AOO vehicle control group were 1.00 ± 0.22 . SI values for crude MCHM treatments were 1.83 ± 0.64 , 1.73 ± 0.15 , 2.29 ± 0.27 , 2.95 ± 0.33 , 3.09 ± 0.58 , and 4.83 ± 0.81 for groups treated with 1%, 2%, 5%, 20%, 40%, and 100/80%, respectively. SI values for MCHM treatments were 0.11 ± 0.05 , 0.28 ± 0.06 , and 0.88 ± 0.29 , respectively for groups treated with 2%, 20%, and 100/50%, respectively. SI values for the DNFB positive control group were 10.02 ± 1.19 . DNFB - 1-Fluoro-2,4-dinitrobenzene; MCHM - 4-methylcyclohexanemethanol; AOO - acetone:olive oil (4:1 v/v). Each bar represents the mean $\pm$ SEM (N = 5 except 100/50% MCHM where N = 3). *Significantly different from AOO control group ($P < 0.05$, Dunnett's T-Test). ¹Significant dose-response trend for crude MCHM ($P < 0.05$, Jonckheere's Trend Test).

Fig. 3. Impact of dermal treatment with crude MCHM on ear swelling in BALB/c mice. (Study 2) Treatment induced changes in ear thickness were measured using a calibrated micrometer approximately 2 h following the final application of MCHM on Day 3 (A) and again prior to euthanasia on Day 6 (B). Data are expressed as relative change from the vehicle control group mean ear thickness. DNFB - 1-Fluoro-2,4-dinitrobenzene; MCHM - 4-methylcyclohexanemethanol; AOO - acetone:olive oil (4:1 v/v). Each bar represents the mean $\pm$ SE (N = 8). *Significantly different from AOO control group ($P < 0.05$, Dunnett's T-Test). ¹Significant dose-response trend for crude MCHM ($P < 0.05$, Jonckheere's Trend Test).

Fig. 4. Impact of dermal treatment with crude MCHM on lymphocyte proliferation in the draining lymph node (Study 2)

Lymphocyte proliferation in the draining lymph node was determined by quantifying the incorporation of $^{125}\text{IUdR}$ into the DNA of the proliferating cells and data are presented at DPM values. The Stimulation Index (SI) was determined for each animal relative to the mean lymphocyte proliferation in the vehicle control group and was used to determine the concentration of MCHM that increased proliferation 3-fold (EC3). SI values for the AOO vehicle control group were 1.00 ± 0.24 . SI values for crude MCHM treatments were 1.10 ± 0.28 , 0.73 ± 0.10 , 1.45 ± 0.27 , 2.24 ± 0.46 , and 4.22 ± 0.54 for groups treated with 1%, 5%, 25%, 50%, and 75%, respectively. SI values for the DNFB positive control group were 76.02 ± 12.50 . DNFB - 1-Fluoro-2,4-dinitrobenzene at 0.15%; MCHM - 4-methylcyclohexanemethanol; AOO - acetone:olive oil (4:1 v/v). Each bar represents the mean $\pm$ SEM ($N = 8$). *Significantly different from AOO control group ($P < 0.05$, Dunnett's T-Test). ¹Significant dose-response trend ($P < 0.05$, Jonckheere's Trend Test).