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National Toxicology Program

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NTP IMMUNOTOXICITY  
TECHNICAL REPORT ON THE  
DERMAL HYPERSENSITIVITY AND  
IRRITANCY STUDIES OF

4-METHYLCYCLOHEXANEMETHANOL  
(CASRN 34885-03-5)

AND CRUDE

4-METHYLCYCLOHEXANEMETHANOL  
ADMINISTERED TOPICALLY TO  
FEMALE BALB/C MICE

NTP IMM 01

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**NTP Immunotoxicity Technical Report on the  
Dermal Hypersensitivity and Irritancy Studies of  
4-Methylcyclohexanemethanol  
(CASRN 34885-03-5) and  
Crude 4-Methylcyclohexanemethanol  
Administered Topically to Female BALB/c Mice**

Immunotoxicity Report 01

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## Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Immunotoxicity Report series began in 2017. The studies described in the Immunotoxicity Report series are designed and conducted to characterize and evaluate the potential of selected substances to affect the immune system in laboratory animals. Substances selected for NTP immunotoxicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in the Immunotoxicity Reports are derived solely from the results of these NTP studies and extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's immunotoxicologic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and Food and Drug Administration [Good Laboratory Practice Regulations](#) and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the [Public Health Service Policy on Humane Care and Use of Laboratory Animals](#). Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

The NTP Immunotoxicity Study Reports are available free of charge on the [NTP website](#) and cataloged in [PubMed](#), a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's [Chemical Effects in Biological Systems](#) database.

For questions about the reports and studies, please email [NTP](#) or call 984-287-3211.

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The draft *NTP Immunotoxicity Technical Report on the Dermal Hypersensitivity and Irritancy Studies of 4-Methylcyclohexanemethanol (CASRN 34885-03-5) and Crude 4-Methylcyclohexanemethanol Administered Topically to Female BALB/c Mice* was evaluated by the reviewers listed below. These reviewers served as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determined if the design and conditions of these NTP studies were appropriate and ensured that this Immunotoxicity Report presents the experimental results and conclusions fully and clearly.

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## Abstract

4-Methylcyclohexanemethanol (MCHM) and crude MCHM were nominated to the National Toxicology Program (NTP) for toxicity evaluation by the Centers for Disease Control and Prevention and the Agency for Toxic Substances and Disease Registry following the Elk River, West Virginia, chemical spill. The 10,000-gallon spill was a mixture of chemicals that contained approximately 75% MCHM. Due to reports of reddened skin and erythema following dermal exposure to contaminated water from the spill, NTP conducted studies in female BALB/c mice to assess the potential dermal hypersensitivity and irritancy of MCHM and crude MCHM.

MCHM is a clear, colorless oil with low estimated water solubility and a reported alcohol or licorice-like odor. MCHM is commonly sold as a crude mixture, which can contain 68% to 89% MCHM with additional components. It is used as a flotation reagent in fine coal beneficiation. Human exposure can occur during handling or use of the chemical.

In the initial study evaluating whether MCHM and crude MCHM have similar immunotoxicologic profiles, groups of five mice were administered a vehicle control (acetone/olive oil, 4:1 v/v [AOO]); one of two positive controls (1 fluoro-2,4-dinitrobenzene [0.15% in AOO] or isoeugenol [5% in AOO]); 2%, 20%, or 100/50% formulations of MCHM in AOO; or 1%, 2%, 5%, 20%, 40%, or 100/80% formulations of crude MCHM in AOO. The 100/50% group was administered 100% on day 1 and 50% on days 2 and 3; the 100/80% group was administered 100% on day 1 and 80% on days 2 and 3. The 100% dose was lowered to 50% (MCHM) and 80% (crude MCHM) on days 2 and 3 due to signs of morbidity. A repeat study of crude MCHM was undertaken to more clearly establish levels at which the test article induced sensitization in the absence of clinical toxicity. In the repeat study of crude MCHM, groups of female mice were administered the AOO vehicle control; a positive control (1 fluoro-2,4-dinitrobenzene [0.15% in AOO]); or crude MCHM at concentrations of 1%, 5%, 25%, 50%, or 75% in AOO. The mice were treated for three consecutive days by direct epicutaneous application of 25  $\mu$ L of the test or vehicle control article to the dorsum of each ear.

In the MCHM study, all mice administered 2% or 20% survived to study termination with no clinical observations of toxicity. Mice in the 100/50% group demonstrated signs of morbidity on exposure day 1, resulting in two mice being euthanized moribund. Clinical observations noted in surviving 100/50% mice included eye squinting and hypoactivity. Concentration-dependent increases in mean ear thickness were observed in the 20% and 100/50% groups on day 3 and day 6, relative to the vehicle control group. Treatment with MCHM did not significantly modulate mean group disintegrations per minute (DPM), compared to that of the vehicle control group or the stimulation index (SI) (ratio of treated group mean DPM/vehicle control group mean DPM) values in the local lymph node assay.

In the initial study of crude MCHM, all mice survived to study termination. Clinical signs of toxicity were only observed in the 100/80% group and included eye squinting, hypoactivity, and isolation from cage mates. Mean ear thickness was significantly increased on day 6 in the 20% group relative to the vehicle control group. It was also increased in the 100/80% group on day 3, though not significantly, and there was a significant concentration-related trend on day 3. In the local lymph node assay, mice treated with 5% crude MCHM or greater had significantly increased DPM values relative to vehicle controls, and this proliferative response followed a significant concentration-related trend. Groups of mice treated with 5% crude MCHM or greater

had significantly increased mean SI values, with SI values of more than 3.0 occurring at 40% and 100/80%. The concentration that would result in a SI of 3.0 (EC3) was calculated to be 34.6%.

In the repeat study of crude MCHM, all mice survived to study termination. Clinical signs of toxicity were limited to the 50% and 75% groups and included eye squinting and isolation from cage mates. Ear thickness in the 75% group was significantly increased relative to that of the vehicle control group on days 3 and 6. No changes in ear thickness were observed in the remaining dosed groups. Significant increases in lymph node cell proliferation as measured by DPM and SI values were observed at 50% and 75% crude MCHM. The concentration that would result in a SI of 3.0 (EC3) was calculated to be 60%.

Under the conditions of these dermal hypersensitivity and irritancy studies, MCHM induced dermal irritation in female mice, as indicated by increases in ear swelling at concentrations of 20% and 100/50%. Crude MCHM was identified as a skin sensitizer, with an EC3 value in the concentration range of 35% to 60%. Dermal exposure to crude MCHM produced minimal irritant effects, indicated by increases in ear swelling at concentrations of 20% and 75%, which were inconsistent across repeat studies.

Synonyms: Cyclohexanemethanol, 4-methyl-; 4-methyl-1-cyclohexanemethanol; (4-methylcyclohexyl)methanol

**Summary of Findings Considered Immunotoxicologically Relevant in Female BALB/c Mice Topically Administered 4-Methylcyclohexanemethanol and Crude 4-Methylcyclohexanemethanol**

	Pure 4-Methylcyclohexanemethanol	Crude 4-Methylcyclohexanemethanol	Crude 4-Methylcyclohexanemethanol (Repeat Study) <sup>a,b</sup>
<b>Concentrations by Topical Application</b>	0%, 2%, 20%, or 100/50%	0%, 1%, 2%, 5%, 20%, 40%, or 100/80%	0%, 1%, 5%, 25%, 50%, or 75%
<b>Survival Rates</b>	5/5, 5/5, 5/5, 3/5	5/5, 5/5, 5/5, 5/5, 5/5, 5/5, 5/5	13/13, 13/13, 13/13, 13/13, 13/13, 13/13
<b>Body Weights</b>	Treated groups similar to the vehicle control group	Treated groups similar to the vehicle control group	Treated groups similar to the vehicle control group
<b>Clinical Observations</b>	Isolation; eye shut, bilateral; hunched posture; and hypoactivity in 100/50% group	Isolation; eye shut, bilateral; and hypoactivity in 100/80% group	Isolation in 50% and 75% groups and eye shut, bilateral in 75% group
<b>Irritancy</b>	↑ Mean ear thickness in the 20% and 100/50% groups	↑ Mean ear thickness in the 20% group	↑ Mean ear thickness in the 75% group
<b>Hypersensitivity</b>	None	↑ DPM values in the 5%, 20%, 40%, and 100/80% groups ↑ SI values in the 5%, 20%, 40%, and 100/80% groups	↑ DPM values in the 50% and 75% groups ↑ SI values in the 75% group
	Calculated EC3 = N/A	Calculated EC3 = 34.6%	Calculated EC3 = 60.0%
<b>SI &gt; 3</b>	No	Yes (40% and 100/80% groups)	Yes (75% group)

DPM = disintegrations per minute (as measured by <sup>125</sup>I-UdR incorporation); SI = stimulation index; N/A = not applicable because SI did not reach 3.

<sup>a</sup>Irritancy studies were conducted using groups 1 through 7 (as described in the Materials and Methods section).

<sup>b</sup>Hypersensitivity studies were conducted using groups 8 through 14 (as described in the Materials and Methods section).

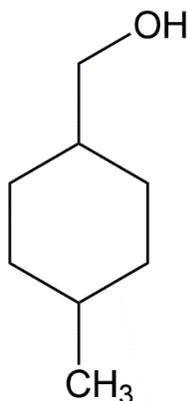
## Overview

The Centers for Disease Control and Prevention and the Agency for Toxic Substances and Disease Registry nominated chemicals associated with the Elk River spill in West Virginia to the National Toxicology Program (NTP) for toxicology studies. In response, NTP performed research to evaluate the toxicity of 4-methylcyclohexanemethanol (MCHM) and the additional chemical components of crude MCHM through various short-term studies. The goals of this research program were to: (1) evaluate the teratogenic, immunotoxic, and genotoxic potential of MCHM; (2) identify sensitive biological effects of the spill chemicals and provide additional information about the levels at which there are no adverse effects; and (3) use efficient, medium-, and high-throughput methods to predict qualitative and quantitative toxicological properties of all chemicals spilled into the Elk River.

These goals were addressed utilizing prenatal developmental toxicity studies in rats, dermal hypersensitivity and irritancy studies in mice, short-term toxicogenomic studies in rats, medium-throughput screening assessments in lower animal models, and high-throughput screening assays. The information and results presented in this Immunotoxicity Report are specific to the dermal hypersensitivity and irritancy studies on MCHM and crude MCHM, however, further information about the NTP research program related to the Elk River spill in West Virginia is available at: <http://ntp.niehs.nih.gov/results/areas/wvspill/index.html>.

This report is a comprehensive presentation of all data and information related to the studies of the dermal hypersensitivity and irritancy work on MCHM.

## Introduction



**Figure 1. MCHM (CASRN 34885-03-5; Chemical Formula: C<sub>8</sub>H<sub>16</sub>O; Molecular Weight: 128.21) and Crude MCHM (CASRN N/A)**

**Synonyms:** Cyclohexanemethanol, 4-methyl-; 4-methyl-1-cyclohexanemethanol; (4-methylcyclohexyl)methanol.

## Chemical and Physical Properties

4-Methylcyclohexanemethanol (MCHM) is an organic compound with a molecular weight of 128.21 g/mol and a density of 0.9074 g/cm<sup>3</sup>. MCHM has estimated boiling and melting points of 203.7°C and -12.0°C, respectively, and a vapor pressure of 0.0588 mm Hg at 25°C. It has an estimated water solubility of 2.024 × 10<sup>3</sup> mg/L (at 25°C) and an estimated log K<sub>ow</sub> of 2.55. MCHM is a clear, colorless oil and has been reported to have an alcohol or licorice-like odor.<sup>1-3</sup> MCHM exists as two distinct isomers, *cis*-MCHM and *trans*-MCHM; the *trans*-isomer, believed to be the source of the licorice odor, has an air odor threshold concentration of 0.060 ppb/v.<sup>4; 5</sup>

MCHM is commonly sold as a crude mixture, which can contain 68% to 89% MCHM with additional components including 4-(methoxymethyl)cyclohexanemethanol (CASRN 98955-27-2; 4% to 22%), water (4% to 10%), methyl 4-methylcyclohexanecarboxylate (CASRN 51181-40-9; 5%), dimethyl 1,4-cyclohexanedicarboxylate (CASRN 94-60-0; 1%), 1,4-cyclohexanedimethanol (CASRN 105-08-8; 1% to 2%), and methanol (1%).<sup>1</sup> Crude MCHM has a boiling point of 180°C, a freezing point of 0°C, and a vapor pressure of 18.0 mm Hg at 20°C.<sup>1</sup>

4-(Methoxymethyl)cyclohexanemethanol has a molecular weight of 158.24 g/mol, and methyl 4-methylcyclohexanecarboxylate has a molecular weight of 156.22 g/mol.<sup>6; 7</sup> No information is available on additional chemical or physical properties of 4-(methoxymethyl)cyclohexanemethanol or methyl 4-methylcyclohexanecarboxylate.

Dimethyl 1,4-cyclohexanedicarboxylate is a partially crystallized liquid with a molecular weight of 200.23 g/mol, a boiling point of 259°C (at 760 mm Hg), and a melting point of either 14°C (*cis*-isomer) or 71°C (*trans*-isomer). The vapor pressure of dimethyl 1,4-cyclohexanedicarboxylate is 1 mm Hg at 85.1°C.<sup>8</sup>

1,4-Cyclohexanedimethanol is a white, waxy solid with a molecular weight of 144.21 g/mol and a density of 1,023 g/L (at 50°C). It has a melting point range of 41°C to 61°C, a boiling point range of 284°C to 285°C, and a freezing point of 24°C.<sup>9</sup>

## Production, Use, and Human Exposure

Information regarding the production volume of MCHM is currently unavailable. MCHM is used as a flotation reagent in fine coal beneficiation. Human exposure can occur during handling or use of the chemical.

On January 9, 2014, approximately 10,000 gallons of a mixture of chemicals containing predominantly MCHM was leaked into the Elk River upstream of the intake for West Virginia American Water Company's Elk River plant, a municipal water source serving approximately 300,000 people in Charleston, West Virginia.<sup>10</sup> A number of chemicals were identified in the spill, including crude MCHM (estimated at 88.5%), propylene glycol ether, and dipropylene glycol phenyl ether.<sup>11</sup> Of the crude MCHM in the mixture, MCHM alone was estimated to be 75% of the entire 10,000-gallon spill.<sup>12</sup> This spill temporarily contaminated 15% of the state's tap water, and prior to flushing water lines, concentrations of MCHM in tap water ranged from less than 10 ppb up to 420 ppb; levels of the other components of crude MCHM were not measured.<sup>13</sup> Concentrations of MCHM were also measured at the intake (up to 3.35 ppm) and posttreatment (up to 2.4 ppm).<sup>10</sup> Exposure to 2.4 ppm is approximately equivalent to 0.07 mg/kg body weight per day for an adult (70 kg) consuming 2 L of water a day and 0.24 mg/kg per day for a child (10 kg) consuming 1 L of water a day. Levels of the other components of crude MCHM were not measured.

## Regulatory Status

Workplace exposure limits for MCHM are currently unavailable. Immediately following the Elk River spill, the Center for Disease Control and Prevention (CDC) and the Agency for Toxic Substances and Disease Registry (ATSDR) established a short-term drinking water limit of 1 ppm based on the approximate weight (10 kg) and drinking water intake (1 L) of a child.<sup>12</sup> Evaluation of the same data using differing adjustment factors resulted in a calculated health advisory level of 120 ppb.<sup>14</sup>

## Absorption, Distribution, Metabolism, and Excretion

### Experimental Animals

There are no studies in the literature on the absorption, distribution, metabolism, or excretion of MCHM in experimental animals.

Radiolabeled [<sup>14</sup>C] 1,4-cyclohexanedimethanol (70% *trans*, 30% *cis*-isomers), a component of crude MCHM, was rapidly absorbed from the gastrointestinal tract in male and female Charles River Sprague Dawley rats.<sup>15</sup> Of the administered dose (40 or 400 mg/kg), 95% was eliminated in the urine and 2.5% in the feces 48 hours postexposure. The half-life of 1,4-cyclohexanedimethanol in plasma following a dose of 400 mg/kg was approximately 13 minutes. The metabolites of 1,4-cyclohexanedimethanol were evaluated in the urine of exposed rats and determined to be cyclohexanedicarboxylic acid (68%) and 4-hydroxymethylcyclohexanecarboxylic

acid (31%). The ratio of *trans*- to *cis*-metabolites in the urine remained the same as that of the initial dose.

## Humans

There are no studies in the literature on the absorption, distribution, metabolism, or excretion of MCHM in humans.

## General Toxicity

### Experimental Animals

*4-Methylcyclohexanemethanol*: The acute dermal toxicity of MCHM was evaluated in male and female rats [CrI:CD(SD)BR] administered a single topical dose (0, 2, 6, or 20 mL/kg).<sup>16</sup> A dermal LD<sub>50</sub> value of 3.6 mL/kg was determined for both males and females.

Oral LD<sub>50</sub> values for MCHM were determined from an acute oral toxicity study in rats [CrI:CD(SD)BR] administered a single dose (0, 625, 1,250, or 2,500 mg/kg) by oral gavage.<sup>16</sup> LD<sub>50</sub> values of 1,768 and 884 mg/kg were calculated for male and female rats, respectively.

Male and female rats exposed to MCHM (0, 25, 100, or 400 mg/kg per day) by oral gavage 5 days per week for 4 weeks exhibited increased liver weights, liver inflammation, and kidney tubular degeneration at 400 mg/kg per day.<sup>17</sup> A no-observed-effect level was set at 100 mg/kg per day. The results of this study were used to establish the short-term drinking water limit of 1 ppm for MCHM.

The acute dermal toxicity of crude MCHM was evaluated in male and female rats administered a single dose of 2,000 mg/kg.<sup>18</sup> Mortality occurred in two female rats, and clinical signs of toxicity in females included weakness, prostration, stumbling, red urine, and a lack of feces. Dermal effects observed in both male and female rats included erythema and desquamation. Dermal induration was observed only in female rats.

*Crude 4-methylcyclohexanemethanol*: Oral LD<sub>50</sub> values for crude MCHM were established for both male (933 mg/kg) and female (707 mg/kg) Sprague Dawley rats (SAS:VAF[SD]) exposed to a single dose (0, 500, 1,000, or 2,000 mg/kg) by oral gavage and observed for 14 days following exposure.<sup>19</sup> Mortality occurred within 24 hours in all animals treated with 2,000 mg/kg and three males and four females treated with 1,000 mg/kg. Additional clinical signs of toxicity were noted during the 14-day observation period and included weakness, prostration, stumbling, dehydration, and red urine.

Female Sprague Dawley rats [CrI:CD(SD)IGS BR] were administered a single dose (500 mg/kg) of crude MCHM via oral gavage to assess its potential to induce hematuria in this rat strain.<sup>20</sup> Neither hematuria nor any additional clinical signs of toxicity were observed during a 14-day observation period.

Dimethyl 1,4-cyclohexanedicarboxylate (0%, 0.1%, 0.3%, or 1.0% in feed), a component of crude MCHM, did not induce any clinical signs of toxicity in male or female Sprague Dawley rats exposed for 4 weeks. A no-observed-adverse-effect level (NOAEL) was established at 1.0% (871 mg/kg in males, 894 mg/kg in females).<sup>21</sup> An oral LD<sub>50</sub> value for this chemical was reported to be greater than 5,000 mg/kg in rats.<sup>22</sup>

Acute exposure to 1,4-cyclohexanedimethanol (400 to 6,400 mg/kg), a component of crude MCHM, for 14 days by oral gavage induced weakness, prostration, and vasodilation in rats.<sup>23</sup> Male and female Sprague Dawley rats administered 1,4-cyclohexanedimethanol in drinking water (0, 4, 8, or 12.5 mg/L) for 13 weeks demonstrated reduced survival rates, red urine, decreased food consumption, and losses in both body weight and body weight gain at 12.5 mg/L. NOAELs were established at 479 mg/kg per day for male rats and at 754 mg/kg per day for female rats. An oral LD<sub>50</sub> value for 1,4-cyclohexanedimethanol was calculated at 3,200 to 6,400 mg/kg.

## Humans

No studies were identified on the general toxicity of MCHM in humans in the scientific literature. Immediately following the Elk River spill, residents of Charleston, West Virginia, reported symptoms of chemical exposures including reddened skin and erythema from dermal exposure and gastrointestinal distress (nausea, vomiting, and/or diarrhea) from ingestion of contaminated water.<sup>24</sup>

## Immunotoxicity

### Experimental Animals

*4-Methylcyclohexanemethanol:* Dermal irritation was observed in male guinea pigs [CrI:(HA)BR Hartley] 24 hours after topical administration of a single dose of 0.5 mL MCHM.<sup>16</sup> Repeated dermal administration (9 applications over 11 days) of 0.5 mL MCHM in the same guinea pig model resulted in an aggravated response, as indicated by moderate erythema, moderate edema, moderate necrosis, and moderate to strong eschars.<sup>16</sup>

MCHM was determined to be a moderate eye irritant in New Zealand white rabbits administered a single dose (0.1 mL) to the eyes.<sup>16</sup> Signs of irritation were observed 1 hour postexposure and included moderate erythema and edema of the eyelids, conjunctivae, and nictitating membranes, as well as moderate discharges.

*Crude 4-methylcyclohexanemethanol:* Acute dermal exposure (4 hours) to a mixture containing predominantly crude MCHM caused skin irritation, including erythema and edema, in albino rabbits [Hra:(NZW)SPF].<sup>25</sup> Very slight to well-defined erythema and very slight to moderate edema were observed as early as 1 hour postexposure, and the degree of irritation increased in severity up to 72 hours postexposure.

Dermal irritation was observed at all dose levels in male and female rats exposed dermally to crude MCHM (0, 100, 500, or 2,000 mg/kg per day) for 13 days.<sup>26</sup> A NOAEL for systemic toxicity was established at 2,000 mg/kg crude MCHM. Dermal exposure to a single dose of 2,000 mg/kg crude MCHM induced dermal irritation in male and female rats during the 14-day postexposure observation period.<sup>18</sup> Dermal irritation was indicated by the presence of focal necrosis and eschar formation on the skin of both males and females.

Crude MCHM did not induce skin hypersensitivity (i.e., allergic contact dermatitis) when evaluated in male guinea pigs [CrI:(HA)BR VAF/Plus<sup>®</sup>] using the footpad method.<sup>27</sup>

Structure-activity relationship analysis predicted that MCHM would be positive in models for skin and eye irritation and corrosion.<sup>28</sup>

## **Humans**

No studies were available on the immunotoxicity of MCHM in humans in the scientific literature. Immediately following the Elk River chemical spill, residents of Charleston reported symptoms of chemical exposures including mild rashes and reddened skin from dermal exposure.<sup>11</sup>

## **Study Rationale**

MCHM and crude MCHM were nominated to the National Toxicology Program (NTP) for toxicity evaluation by the CDC and ATSDR following the Elk River, West Virginia, chemical spill. Due to structural alerts from in silico models of potential sensitizers and reports of reddened skin and erythema following dermal exposure to contaminated water from the spill, NTP conducted studies in mice to assess the potential immunotoxicity, specifically dermal irritancy and hypersensitivity of MCHM and crude MCHM.

## Materials and Methods

### Procurement and Characterization

#### 4-Methylcyclohexanemethanol

4-Methylcyclohexanemethanol (MCHM) was obtained from TCI America (Portland, OR) in one lot (KDY3F). Crude MCHM was obtained from Eastman Chemical Company (Kingsport, TN) in one lot (TP14044373). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at MRIGlobal (Kansas City, MO) (Appendix A). Reports on analyses performed in support of the MCHM studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

Lot KDY3F of the pure chemical, a clear, colorless liquid, was identified as MCHM using Fourier transform infrared (FTIR) spectroscopy, Fourier transform proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography (GC) with mass spectrometry (MS) detection. In addition, boiling point, density, and octanol:water partition coefficient were determined. Karl Fischer titration was used to determine the water content of lot KDY3F. Purity of the test article was determined by elemental analyses for carbon, hydrogen, and nitrogen, and by determining the purity profile using GC with flame ionization detection (FID) and two columns with differing polarities.

For lot KDY3F, Karl Fischer titration indicated 0.21% water. Elemental analyses for carbon, hydrogen, and nitrogen were consistent with the theoretical values for MCHM. GC/FID analysis detected two major peaks with a combined area of 99.97% of the total peak area and no impurities with areas greater than or equal to 0.05% of the total peak area. The relative areas of the two major peaks indicated that MCHM consisted of 67.99% *cis*- and 31.98% *trans*-isomers. GC/FID by a second system detected two major peaks with a combined relative area of 99.83% (with relative areas of 67.80% [*cis*] and 32.03% [*trans*] isomers), and two minor impurities totaling 0.13% of the total peak area. The overall purity of lot KDY3F was determined to be greater than or equal to 99.8%.

A chemical identity and purity screen of lot TP14044373 of the crude test chemical, a clear liquid, was performed using GC/MS. The test article was found to be a 90.35% mix of the *cis*- (33.45%) and *trans*- (56.90%) isomers of MCHM on the basis of the relative areas of the two major peaks (Table 1). Six impurity components with a relative percent total area greater than or equal to 0.05% were observed, with a combined area of 9.66%. Three of these impurity components (each with an individual relative percent area greater than 1%) were tentatively identified as cyclohexanemethanol (1.82%), and a mix of the *cis*- and *trans*- isomers of 1,4-cyclohexanedimethanol (7.15%). The purity of lot TP14044373, relative to MCHM isomers, was estimated to be greater than 90%.

**Table 1. Constituents of Crude 4-Methylcyclohexanemethanol**

Chemical Name	CASRN	Concentration (%)
4-Methylcyclohexanemethanol	34885-03-5	90.35
<i>Trans</i> -isomer	–	56.90
<i>Cis</i> -isomer	–	33.45
Other Components Tentatively Identified	–	10.0 (approximately)
1,4-Cyclohexanedimethanol ( <i>cis</i> - and <i>trans</i> - isomers)	105-08-8	7.15
Cyclohexanemethanol	100-49-2	1.82
Methyl 4-methylcyclohexanecarboxylate	51181-40-9	0.50
<i>Cis</i> -octahydroisobenzofuran	13149-01-4	0.13
2-Ethyl-1-hexanol	104-76-7	0.06

Stability studies of lot KDY3F were conducted using GC/FID. These studies indicated that MCHM was stable as a bulk chemical for at least 14 days when stored in amber glass vials under an inert headspace at temperatures up to 60°C. To ensure stability, both bulk chemicals were stored at room temperature in amber glass bottles, and MCHM was stored under an inert headspace.

## Isoeugenol

Isoeugenol was obtained from Sigma-Aldrich, Inc. (St. Louis, MO) in one lot (05622BEV) that was used as a positive control in the initial dermal hypersensitivity and irritancy study. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Research Triangle Institute (RTI) (Research Triangle Park, NC).

The identity of lot 05622BEV, received as a clear liquid, was determined using FTIR and proton and carbon-13 NMR spectroscopy, GC/MS spectrometry, and ultra-high-performance liquid chromatography (UHPLC)/time-of-flight (TOF) MS. Karl Fischer titration was used to determine the water content of lot 05622BEV. Purity of the test article was determined by elemental analyses for carbon, hydrogen, and oxygen, and by determining the purity profile using GC/FID and UHPLC with photodiode array (PDA) detection.

For lot 05622BEV, Karl Fischer titration indicated 0.23% water. Elemental analyses for carbon, hydrogen, and oxygen were in agreement with the theoretical values for isoeugenol. GC/FID analysis indicated two major isomer peaks (totaling 98.7% of the total peak area) and two impurities (0.7% and 0.4% of the total peak area). UHPLC/PDA analysis indicated two major isomer peaks (totaling 99.1% of the total peak area) and two impurities (0.6% and 0.1% of the total peak area). The overall purity of lot 05622BEV was determined to be greater than or equal to 98.7%.

Stability studies of lot 05622BEV conducted using GC/FID indicated that isoeugenol was stable as a bulk chemical for at least 14 days when stored in amber glass vials sealed with Teflon<sup>®</sup>-lined caps at temperatures up to 60°C. To ensure stability, the bulk chemical was stored at room temperature under nitrogen.

## **1-Fluoro-2,4-dinitrobenzene**

1-Fluoro-2,4-dinitrobenzene was obtained from Sigma-Aldrich, Inc. in one lot (BCBN2339V) that was used as a positive control in the initial and repeat dermal hypersensitivity and irritancy studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at RTI.

The identity of lot BCBN2339V, a clear liquid, was determined using FTIR and proton, carbon-13, and fluorine-19 NMR spectroscopy, GC/MS spectrometry, and UHPLC/TOF MS. The water content of lot BCBN2339V was determined using Karl Fischer titration. Purity of the test article was determined by elemental analyses for carbon, hydrogen, nitrogen, oxygen, and fluorine, and by determining the purity profile using GC/FID and UHPLC with PDA detection.

For lot BCBN2339V, the water content was less than the limit of quantitation of 0.1%. Elemental analyses for carbon, hydrogen, nitrogen, and oxygen were in agreement with the theoretical values for 1-fluoro-2,4-dinitrobenzene; fluorine was not accurately quantitated due to interference with oxygen. GC/FID indicated one major peak (99.6% of the total peak area), and two impurity peaks (0.2% each of the total peak area). UHPLC/PDA analysis indicated one major peak (99.4% of the total peak area) and three impurity peaks (0.1%, 0.2%, and 0.3% of the total peak area). The overall purity of lot BCBN2339V was determined to be greater than or equal to 99.4%.

Stability studies of lot BCBN2339V conducted using GC/FID indicated that 1-fluoro-2,4-dinitrobenzene was stable as a bulk chemical for at least 14 days when stored in amber glass vials sealed with Teflon-lined caps at temperatures up to 60°C. To ensure stability, the bulk chemical was stored at room temperature under nitrogen.

## **Preparation and Analysis of Dose Formulations**

### **Formulation Materials**

For the initial study, all dose formulations were prepared by the study laboratory, Burlison Research Technologies, Inc. (Morrisville, NC), and analyzed by the analytical chemistry laboratory at RTI. For the repeat study, formulations were both prepared and analyzed by RTI.

Acetone and olive oil were obtained from multiple suppliers in multiple lots and these materials were combined with a 4:1 ratio to prepare the vehicle for the initial and repeat studies. For the repeat study, J.T. Baker Acetone (lot K30J01; Thermofisher Scientific, Waltham, MA) was analyzed using GC/FID indicating a purity of 99.9%, acceptable for use in the study. Olive oil (lot 0240-0545; Welch, Holme, and Clark Co., Newark, NJ) was analyzed to determine peroxide levels using potentiometric titration. Peroxide levels were within the acceptable limit of 5 mEq/kg.

Dose formulations of the test articles and positive controls for the initial study were prepared daily by mixing the appropriate amounts of each chemical with acetone:olive oil (4:1) to achieve the required concentrations. In the repeat study the dose formulations were stored in amber glass vials sealed with Teflon-lined caps at room temperature or refrigerated temperatures for up to 14 days.

Stability studies of the 1% (v/v) dose formulation of crude MCHM and the 0.15% (v/v) dose formulation of 1-fluoro-2,4-dinitrobenzene were performed by RTI using two different GC/FID systems. Stability was confirmed for at least 14 days for these dose formulations stored in amber glass vials with minimal headspace sealed with Teflon-lined lids at room temperature and for 3 hours under simulated animal room conditions.

Analyses of the dose formulations for the initial and repeat studies were conducted by RTI using GC/FID by one system for MCHM and crude MCHM and by a second system for the positive controls isoeugenol and 1-fluoro-2,4-dinitrobenzene. During the initial dermal hypersensitivity and irritancy study, the dose formulations were analyzed once; two of three MCHM dose formulations were within 15% of the target concentrations and five of six crude MCHM dose formulations were within 15% of the target concentrations (Table A-3). Single dose formulations of the positive controls isoeugenol and 1-fluoro-2,4-dinitrobenzene were each found to be within 15% of the target concentrations. During the repeat dermal hypersensitivity and irritancy study, the dose formulations were analyzed once, and animal room samples of these dose formulations were also analyzed (Table A-4). All five crude MCHM dose formulations were within 15% of the target concentrations; all five animal room samples were also within 15% of the target concentrations. A single dose formulation of 1-fluoro-2,4-dinitrobenzene and its associated animal room sample were both found to be within 15% of the target concentrations.

## **Animal Source**

Female BALB/c mice were obtained from Taconic Biosciences, Inc. (Germantown, NY). The National Toxicology Program (NTP) notes that the CBA mouse is the strain of choice for the local lymph node assay (LLNA). NTP began using BALB/c mice for hypersensitivity testing prior to the development of the OECD 429 Guidance. To maintain consistency with a large body of historical data, NTP continues to use the BALB/c mouse. NTP and others have conducted studies to examine the sensitivity of this mouse strain as compared with the CBA and multiple groups have concluded that the performance of LLNA with BALB/c mice is comparable with that of the CBA mouse.<sup>29-31</sup>

## **Animal Welfare**

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Studies were approved by the Burleson Research Technologies, Inc., Animal Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

## **Dermal Hypersensitivity and Irritancy Studies**

### **Study Conduct**

Female mice were 5 to 7 weeks old upon receipt. Animals were quarantined for 8 (initial MCHM and crude MCHM study) or 17 (crude MCHM repeat study) days and were 8 weeks old on the first day of dosing. Before the studies began, five mice were randomly selected for parasite evaluation and gross observation for evidence of disease. The health of the animals was

monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix C). All test results were negative.

In the initial study evaluating whether MCHM and crude MCHM have similar immunotoxicologic profiles, groups of five mice were administered a vehicle control (acetone/olive oil, 4:1 v/v [AOO]); one of two positive controls (1-fluoro-2,4-dinitrobenzene [0.15% in AOO] or isoeugenol [5% in AOO]); 2%, 20%, or 100/50% formulations of MCHM in AOO; or 1%, 2%, 5%, 20%, 40%, or 100/80% formulations of crude MCHM in AOO. The 100/50% group was administered 100% on day 1 and 50% on days 2 and 3; the 100/80% group was administered 100% on day 1 and 80% on days 2 and 3. Doses of MCHM and crude MCHM were selected on the basis of dermal immunotoxicity studies previously performed by the sponsor, Eastman Kodak Company.<sup>25; 18; 26</sup> To determine if the crude mixture of MCHM was responsible for any potential dermal immunotoxicity, three concentrations (2%, 20%, and 100%) were chosen for comparison between MCHM and crude MCHM.

A repeat study of crude MCHM was undertaken to more clearly establish levels in which the test article induced sensitization in the absence of clinical toxicity. In the repeat study of crude MCHM, groups of eight (groups 1 through 7) or five (groups 8 through 14) female mice were administered the AOO vehicle control; a positive control (1-fluoro-2,4-dinitrobenzene [0.15% in AOO]); or crude MCHM at concentrations of 1%, 5%, 25%, 50%, or 75% in AOO (Table 2).

The mice were treated for 3 consecutive days by direct epicutaneous application of 25  $\mu$ L of the test or control article to the dorsum of each ear. Feed and water were available ad libitum. The animals were housed up to five mice from the same treatment group per cage. Animals were weighed on days 1 and 6. Mice were observed twice daily for local irritation at the application site and for signs of systemic toxicity. Details of the study design and animal maintenance are summarized in Table 3. Information on feed composition and contaminants is provided in Appendix B.

**Table 2. Study Design for the Repeat Study of Crude 4-Methylcyclohexanemethanol**

Treatment	Group <sup>a,b</sup>													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	AOO	1%	5%	25%	50%	75%	DNFB	AOO	1%	5%	25%	50%	75%	DNFB
<b>Endpoint</b>														
Body Weight	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Clinical Observations	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Ear Thickness	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Lymph Node Cell Proliferation	x	x	x	x	x	x	x	–	–	–	–	–	–	–
Ear Punch Biopsy Weight	–	–	–	–	–	–	–	x	x	x	x	x	x	x
Ear Histopathology	–	–	–	–	–	–	–	x	x	x	x	x	x	x

X indicates groups for which the endpoint listed was assessed.

AOO = acetone olive oil; DNFB = 1-fluoro-2,4-dinitrobenzene.

<sup>a</sup>Groups 1 through 7 received <sup>125</sup>I-UdrR on day 6 for evaluation of lymph node cell proliferation. Ear punch biopsy weights were obtained only for groups 8 through 14 following euthanasia on day 6.

<sup>b</sup>n = 8 for groups 1–7; n = 5 for groups 8–14.

## Assessment of Irritancy

Dermal irritancy potential was assessed by measuring changes in ear swelling following application of MCHM, crude MCHM, or vehicle controls. For all mice, ear thickness measurements for both ears were taken prior to dosing on day 1, after dosing on day 3, and again on day 6. Ear thickness measurements were performed using a calibrated gage (Dyer model 304-196; S/N 4183012). The thickness gage and the gage block were calibrated against NIST-traceable thickness standards by an independent certification laboratory that is ISO/IEC 17025-accredited. The precision of the calibrated gage allowed for ear thickness to be measured to 0.1 mm. Mice were euthanized by carbon dioxide asphyxiation on day 6. A 6 mm punch biopsy was obtained from each ear of mice in the initial MCHM and crude MCHM groups in the repeat study of crude MCHM (groups 8 through 14) following terminal euthanasia. The biopsies were weighed; however, technical limitations prevented the measurement of usable data.

## Assessment of Hypersensitivity

Dermal hypersensitivity was evaluated using the LLNA, which provides an indication for the skin sensitization potential of a chemical.<sup>32-34</sup> In the assay, skin sensitization potential was measured as a function of lymphocyte proliferation within the auricular lymph nodes following dermal application of MCHM, crude MCHM, or vehicle controls to both ears.

On day 6, after ear thickness measurements and 5 hours before terminal euthanasia, mice in the initial study of MCHM and crude MCHM groups 1 through 7 were administered 2 µCi <sup>125</sup>I-iododeoxyuridine (<sup>125</sup>I-UdrR) (0.25 mL of a stock solution of 8 µCi/mL) by intravenous injection into the lateral tail vein using a tuberculin syringe. The auricular lymph nodes were collected after terminal euthanasia into a 15 mL conical tube containing Hanks' balanced salt solution with HEPES (HBSS/H). The lymph nodes collected from an individual animal were pooled in a single tube. The lymph nodes were placed between the frosted ends of two glass microscope slides and macerated to produce a cell suspension. The cell suspensions were placed into centrifuge tubes containing HBSS/H and processed through centrifugations and washes.

Trichloroacetic acid (TCA, 5%) was added to the tubes containing the final centrifugation pellet. The tubes were vortexed and then wrapped in lead foil and refrigerated overnight (16 to 24 hours). Subsequently, the tubes were removed from refrigeration, vortexed, and centrifuged. The resulting pellets were resuspended with 5% TCA and added to a gamma counter tube (1 gamma counter tube per mouse). Each tube was counted on the gamma counter for 5 minutes and counts per minute (CPM) were recorded. A 1:500 dilution of the 8  $\mu\text{Ci/mL}$   $^{125}\text{I}$ -UdR stock solution was prepared and counted. Four background/blank tubes, each containing 2 mL of 5% TCA were also counted. CPM values were converted to disintegrations per minute (DPM) by dividing by the gamma counter efficiency and multiplying the results by 100. Individual DPM values were corrected by subtracting the mean DPM for the blanks. The stimulation index (SI) was calculated by dividing the group mean DPM by the mean vehicle control DPM. SI is the accepted standard for quantification of skin sensitization potential, and an SI of 3 (a threefold increase in lymphocyte proliferation) has previously been determined to be a consistent distinguishing factor between sensitizers and nonsensitizers.<sup>35</sup> The EC3 value, or effective concentration for an SI of 3, has further been successfully used to directly determine the skin sensitization potential of chemicals.<sup>34</sup> The EC3 values were calculated via a linear regression model; all of the study data were used for the linear interpolation.

**Table 3. Experimental Design and Materials and Methods in the Dermal Hypersensitivity and Irritancy Studies of 4-Methylcyclohexanemethanol and Crude 4-Methylcyclohexanemethanol**

Study Design
<b>Study Laboratory</b>
Burleson Research Technologies, Inc. (Morrisville, NC)
<b>Strain and Species</b>
BALB/c mice
<b>Animal Source</b>
Taconic Biosciences, Inc. (Germantown, NY)
<b>Time Held Before Studies</b>
MCHM and crude 4-methylcyclohexanemethanol: 8 days
Crude MCHM (repeat study): 17 days
<b>Average Age When Studies Began</b>
8 weeks
<b>Date of First Dose</b>
MCHM and crude 4-methylcyclohexanemethanol: December 10, 2014
Crude MCHM (repeat study): March 20, 2015
<b>Duration of Dosing</b>
Once daily for 3 days
<b>Date of Last Dose</b>
MCHM and crude 4-methylcyclohexanemethanol: December 12, 2014
Crude MCHM (repeat study): March 22, 2015
<b>Terminal Euthanasia</b>
MCHM and crude 4-methylcyclohexanemethanol: December 15, 2014
Crude MCHM (repeat study): March 25, 2015

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**Study Design**

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**Average Age at Terminal Euthanasia**

9 weeks

**Size of Study Groups**

MCHM and crude 4-methylcyclohexanemethanol: 5

Crude MCHM (repeat study): 8 (groups 1 through 7) or 5 (groups 8 through 14)

**Method of Distribution**

Animals were distributed randomly into groups of approximately equal initial mean body weights.

**Animals per Cage**

5

**Method of Animal Identification**

Tail tattoo

**Diet**

Irradiated NTP-2000 open formula pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available ad libitum

**Water**

Tap water (Morrisville municipal supply) (Techniplast caging system, Techniplast, West Chester, PA), available ad libitum

**Cages**

Techniplast Sealsafe, individually ventilated caging system (Techniplast)

**Bedding**

Sani-Chips® (P.J. Murphy Forest Products Corp., Montville, NJ), changed twice weekly

**Animal Room Environment**

Temperature: 72°F ± 3°F

Relative humidity: 50% ± 15%

Room fluorescent light: 12 hours/day

Room air changes: 10/hour

**Concentrations**

MCHM and crude MCHM: 0%, 2%, 20%, or 100/50% formulations of MCHM; 1%, 2%, 5%, 20%, 40%, or 100/80% formulations of crude MCHM; 0.15% 1-fluoro-2,4-dinitrobenzene or 5% isoeugenol

Crude MCHM (repeat study): 1%, 5%, 25%, 50%, or 75% crude MCHM; or 0.15% 1-fluoro-2,4-dinitrobenzene  
Acetone:olive oil vehicle (AOO, 4:1 ratio) was used for all studies

**Type and Frequency of Observation**

Observed twice daily for toxicity and skin irritation at the application site; animals were weighed on days 1 and 6.

**Method of Euthanasia**

Carbon dioxide asphyxiation

**Ear Thickness Measurements**

Ear thickness measurements were taken for both ears of all mice on days 1, 3, and 6. On day 6, 6 mm punch biopsies were taken from each ear of MCHM and crude MCHM groups and crude MCHM (repeat study) groups 8 through 14 and weighed.

**Local Lymph Node Assay**

On day 6, 5 hours before terminal euthanasia, <sup>125</sup>I-UdR (2 µCi) was administered by intravenous injection into the lateral tail vein of MCHM and crude MCHM groups and crude MCHM (repeat study) groups 1 through 7. After terminal kill, auricular lymph nodes were collected and disintegrations per minute and stimulation indices were determined.

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## **Statistical Methods**

All p values are two-sided and considered statistically significant if less than 0.05.

### **Analysis of Continuous Variables**

For dosed groups compared to the vehicle control groups, two approaches were employed to assess the significance of pairwise comparisons in the analysis of continuous variables. Body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett<sup>36</sup> and Williams.<sup>37; 38</sup> Ear thickness, lymph node counts, and stimulation indices, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley<sup>39</sup> (as modified by Williams<sup>40</sup>) and Dunn.<sup>41</sup> Jonckheere's test<sup>42</sup> was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than would be a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey<sup>43</sup> were examined by NTP personnel, and implausible values were eliminated from the analysis.

### **Quality Assurance Methods**

The dermal hypersensitivity and irritancy studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations.<sup>44</sup> In addition, as records from the studies were submitted to the NTP Archives, they were audited retrospectively by an independent quality assessment contractor. Separate audits covered both completeness and accuracy of the data and a draft of this NTP Immunotoxicity Study Report. Audit procedures and findings are presented in the audit report and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Immunotoxicity Study Report.

## Results

### Data Availability

The National Toxicology Program (NTP) evaluated all study data. Data relevant for evaluating toxicological findings are presented here. All study data are available in the NTP Chemical Effects in Biological Systems (CEBS) database: <https://doi.org/10.22427/NTP-DATA-IMM-01>.<sup>45</sup>

### 4-Methylcyclohexanemethanol

4-Methylcyclohexanemethanol (MHCM) was initially tested at concentrations of 2%, 20%, and 100%. All mice administered 2% or 20% survived to the end of the study with no clinical observations of toxicity (Table 4, Table 5, and Table 6). Mice in the 100% group demonstrated signs of morbidity on day 1, including eye squinting, hypoactivity, hunched posture, and isolation from cage mates, resulting in two mice being euthanized moribund (Table 6). Consequently, the 100% dose was lowered to 50% on days 2 and 3 (this group is referred to hereafter as the 100/50% group). Clinical observations noted in surviving 100/50% mice included eye squinting and hypoactivity, albeit to a lesser extent than was observed prior to lowering the 100% dose. All clinical signs were observed during the afternoon observation period after treatment, but none was observed during morning observation the subsequent day. Group mean body weights were not significantly affected by treatment (Table 5).

**Table 4. Disposition of Female Mice in the Dermal Hypersensitivity and Irritancy Study of 4-Methylcyclohexanemethanol**

	Vehicle Control	2%	20%	100/50%	0.15% DNFB	5% ISO <sup>a</sup>
Animals Initially in Study	5	5	5	5	5	5
Euthanized Moribund	0	0	0	2	0	0
Terminal Removal	5	5	5	3	5	5

DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Positive control.

**Table 5. Survival and Mean Body Weights of Female Mice in the Dermal Hypersensitivity and Irritancy Study of 4-Methylcyclohexanemethanol<sup>a</sup>**

Concentration	Survival <sup>b</sup>	Initial Body Weight (g)	Final Body Weight (g)	Absolute Weight Gain or Loss
Vehicle Control	5/5	17.2 ± 0.6	17.9 ± 0.6	0.7 ± 0.2
2%	5/5	17.3 ± 0.6	18.0 ± 0.7	0.7 ± 0.2
20%	5/5	17.9 ± 0.6	18.1 ± 0.5	0.2 ± 0.2
100/50%	3/5 <sup>d</sup>	17.3 ± 0.6	18.4 ± 0.5	0.6 ± 0.2
0.15% DNFB <sup>c</sup>	5/5	17.4 ± 0.6	18.3 ± 0.7	0.9 ± 0.3
5% ISO	5/5	17.0 ± 0.4	17.5 ± 0.3	0.5 ± 0.2

DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Weights are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.

<sup>b</sup>Number of animals surviving on day 6/number initially in group.

<sup>c</sup>Positive control.

<sup>d</sup>Two moribund animals were removed on study day 1.

**Table 6. Clinical Observations for Female Mice in the Dermal Hypersensitivity and Irritancy Study of 4-Methylcyclohexanemethanol**

	Vehicle Control	2%	20%	100/50%	0.15% DNFB <sup>b</sup>	5% ISO <sup>c</sup>
<b>n</b>	5	5	5	5	5	5
Animal Behavior, Isolation <sup>a</sup>	0	0	0	5 (1)	5 (1)	0
Erythema, Ear, Bilateral, Mild	0	0	0	0	5 (4)	0
Erythema, Ear, Bilateral, Moderate	0	0	0	0	5 (3)	0
Eye Shut, Bilateral	0	0	0	5 (1)	5 (1)	0
Hunched	0	0	0	2 (1)	0	0
Hypoactivity	0	0	0	5 (1)	5 (3)	0

DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Number of animals with observation (study day first observed).

<sup>b</sup>Positive control.

<sup>c</sup>Positive control (did not perform as expected).

The irritancy properties of MCHM were assessed by evaluating changes in ear swelling, which were determined by measuring treatment-related changes in ear thickness, relative to vehicle control animals. Treatment resulted in concentration-dependent increases in mean ear thickness in the 20% and 100/50% groups on day 3 (4.44% and 6.47%, respectively) and day 6 (3.54% and 5.30%, respectively), relative to the vehicle control (Table 7).

**Table 7. Summary of Ear Thickness Measurement Data for Female Mice in the Dermal Hypersensitivity and Irritancy Study of 4-Methylcyclohexanemethanol<sup>a</sup>**

Concentration	Day 1 (mm × 10 <sup>-2</sup> )	Day 3 (mm × 10 <sup>-2</sup> )	Day 6 (mm × 10 <sup>-2</sup> )	Day 3 (%)	Day 6 (%)	Ear Weight (mg)
Vehicle Control	15.58 ± 0.17 (5)	15.66 ± 0.14 <sup>▲▲</sup> (5)	15.56 ± 0.12 <sup>▲▲</sup> (5)	-0.04 ± 0.96 <sup>▲▲</sup> (5)	0.02 ± 0.78 <sup>▲▲</sup> (5)	7.78 ± 0.42 (5)
2%	15.52 ± 0.08 (5)	15.98 ± 0.02 (5)	15.62 ± 0.07 (5)	2.04 ± 0.16 (5)	0.34 ± 0.39 (5)	8.94 ± 0.38 (5)
20%	15.60 ± 0.10 (5)	16.34 ± 0.12 <sup>**</sup> (5)	16.10 ± 0.10 <sup>**</sup> (5)	4.44 ± 0.78 <sup>**</sup> (5)	3.54 ± 0.64 <sup>**</sup> (5)	8.62 ± 0.11 (5)
100/50%	15.56 ± 0.06 (5)	16.67 ± 0.33 <sup>**</sup> (3)	16.40 ± 0.06 <sup>**</sup> (3)	6.47 ± 2.13 <sup>**</sup> (3)	5.30 ± 0.46 <sup>**</sup> (3)	8.57 ± 0.64 (3)
0.15% DNFB <sup>b</sup>	15.34 ± 0.04 (5)	16.94 ± 0.13 <sup>**</sup> (5)	16.98 ± 0.09 <sup>**</sup> (5)	8.12 ± 0.78 <sup>**</sup> (5)	8.98 ± 0.60 <sup>**</sup> (5)	11.60 ± 0.79 <sup>**</sup> (5)
5% ISO <sup>b</sup>	15.40 ± 0.10 (5)	16.92 ± 0.05 <sup>**</sup> (5)	16.60 ± 0.19 <sup>*</sup> (5)	7.96 ± 0.39 <sup>**</sup> (5)	6.74 ± 1.20 <sup>*</sup> (5)	8.10 ± 1.02 (5)

<sup>▲▲</sup>Significant trend (p ≤ 0.01) by Jonckheere's test.

<sup>\*</sup>Significantly different (p ≤ 0.05) from the vehicle control group by Shirley's or Dunn's test.

<sup>\*\*</sup>p ≤ 0.01.

DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Ear thickness data are presented as mean ± standard error (n); percentages are calculated as ear swelling (difference from vehicle control) using the equation: [mean measurement for both ears for each animal (day 3 or 6) – vehicle control mean ear thickness (day 3 or 6)]/[vehicle control mean ear thickness (day 3 or 6) × 100].

<sup>b</sup>Positive control.

The local lymph node assay (LLNA) was used to evaluate the ability of MCHM to induce dermal sensitization. The LLNA uses incorporation of <sup>125</sup>I-iododeoxyuridine (<sup>125</sup>I-UdR) as a measure of DNA synthesis and lymphocyte proliferation. On day 6, 3 days after final dosing, mice were injected intravenously with <sup>125</sup>I-UdR and rested for 5 hours to allow for incorporation of the labeled material into proliferating cells in the draining lymph nodes. Treatment with MCHM did not significantly modulate mean group disintegrations per minute (DPM) compared to that of the

vehicle control group or the stimulation index (SI; treated group mean DPM/vehicle control group mean DPM) values (Table 8 and Figure 2). No EC3 value was calculated for MCHM as all SI values were below 3.

**Table 8. Summary of  $^{125}\text{I}$ -UdR Incorporation and Stimulation Index Data for Female Mice in the Dermal Hypersensitivity and Irritancy Study of 4-Methylcyclohexanemethanol<sup>a</sup>**

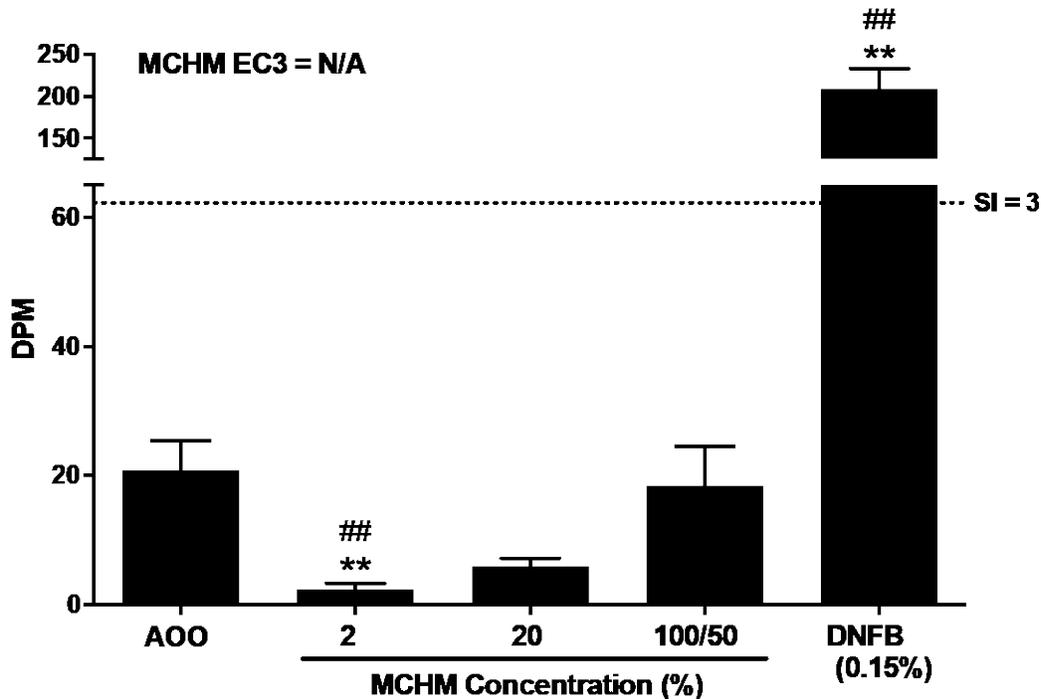
Concentration	n	Lymph Node Counts (DPM)	Stimulation Index
Vehicle Control	5	20.76 ± 4.67	1.00 ± 0.22
2%	5	2.33 ± 0.97**	0.11 ± 0.05**
20%	5	5.86 ± 1.33	0.28 ± 0.06
100/50%	3	18.39 ± 6.10	0.88 ± 0.29
0.15% DNFB <sup>b</sup>	5	208.37 ± 5.25**	10.02 ± 1.19**
5% ISO <sup>b</sup>	5	34.07 ± 5.25	1.64 ± 0.25

\*\*Significantly different ( $p \leq 0.01$ ) from the vehicle control group by Shirley's or Dunn's test.

DPM = disintegrations per minute; stimulation index = ratio of treated group mean DPM/vehicle control group mean DPM; DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Data are presented as mean ± standard error.

<sup>b</sup>Positive control.



**Figure 2.  $^{125}\text{I}$ -UdR Incorporation and Stimulation Index Data in the Dermal Hypersensitivity and Irritancy Study of 4-Methylcyclohexanemethanol**

AOO = acetone/olive oil vehicle control; DNFB = 1-fluoro-2,4-dinitrobenzene positive control; DPM = disintegrations per minute; MCHM = 4-methylcyclohexanemethanol; SI = stimulation index; EC3 = concentration of test article that results in an SI = 3; N/A = not applicable; \*\* = significantly different ( $p \leq 0.01$ ) from the vehicle control group by Shirley's or Dunn's test; ## = significantly different ( $p \leq 0.01$ ) SI value from the vehicle control group by Shirley's or Dunn's test.

Two positive controls were utilized in the study of MCHM: 1-fluoro-2,4-dinitrobenzene (0.15%), a strong sensitizer, and isoeugenol (5%), a weak sensitizer. 1-Fluoro-2,4-dinitrobenzene performed as expected; however, isoeugenol did not elicit the anticipated sensitizing response.

## Crude 4-Methylcyclohexanemethanol

Crude MCHM was initially administered at concentrations of 1%, 2%, 5%, 20%, 40%, and 100%. All mice survived to the end of the study (Table 9 and Table 10). Clinical signs of toxicity, including eye squinting, hypoactivity, and isolation from cage mates, were observed on day 1 in mice in the 100% group (Table 11). Consequently, the concentration was lowered to 80% (this group is referred to hereafter as the 100/80% group) for exposure days 2 and 3. Clinical signs of toxicity in the 100/80% group included eye squinting (days 1 through 3), hypoactivity (days 1 and 2), and isolation from cage mates (days 1 and 2). All clinical signs of toxicity were observed during the afternoon observation period after treatment, but not during morning observation the subsequent day. No clinical signs of toxicity were observed in the groups administered 40% or less. Group mean body weights were not significantly affected by treatment (Table 10).

**Table 9. Disposition of Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol**

	Vehicle Control	1%	2%	5%	20%	40%	100/80%	0.15% DNFB <sup>a</sup>	5% ISO <sup>a</sup>
Animals Initially in Study	5	5	5	5	5	5	5	5	5
Terminal Removal	5	5	5	5	5	5	5	5	5

DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Positive control.

**Table 10. Survival and Mean Body Weights of Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol<sup>a</sup>**

Concentration	Survival <sup>b</sup>	Initial Body Weight (g)	Final Body Weight (g)	Absolute Weight Gain or Loss
Vehicle Control	5/5	17.2 ± 0.6	17.9 ± 0.6	0.7 ± 0.2
1%	5/5	17.1 ± 0.7	18.3 ± 0.7	1.2 ± 0.2
2%	5/5	17.7 ± 0.8	18.3 ± 0.9	0.6 ± 0.2
5%	5/5	17.4 ± 0.5	18.3 ± 0.5	0.9 ± 0.1
20%	5/5	17.2 ± 0.6	18.2 ± 0.5	1.0 ± 0.2
40%	5/5	18.2 ± 0.8	19.0 ± 0.8	0.9 ± 0.2
100/80%	5/5	17.8 ± 0.7	17.6 ± 0.7	-0.2 ± 0.1**
0.15% DNFB <sup>c</sup>	5/5	17.4 ± 0.6	18.3 ± 0.7	0.9 ± 0.3
5% ISO <sup>c</sup>	5/5	17.0 ± 0.4	17.5 ± 0.3	0.5 ± 0.2

\*\*Significantly different ( $p \leq 0.01$ ) from the vehicle control group by Shirley's or Dunn's test.

DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Weights are given as mean ± standard error.

<sup>b</sup>Number of animals surviving on day 6/number initially in group.

<sup>c</sup>Positive control.

**Table 11. Clinical Observations for Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol**

	Vehicle Control	1%	2%	5%	20%	40%	100/80%	0.15% DNFB <sup>b</sup>	5% ISO <sup>c</sup>
<b>n</b>	5	5	5	5	5	5	5	5	5
Animal Behavior, Isolation <sup>a</sup>	0	0	0	0	0	0	5 (1)	5 (1)	0
Erythema, Ear, Bilateral, Mild	0	0	0	0	0	0	0	5 (4)	0
Erythema, Ear, Bilateral, Moderate	0	0	0	0	0	0	0	5 (3)	0
Eye Shut, Bilateral	0	0	0	0	0	0	5 (1)	5 (1)	0
Hypoactivity	0	0	0	0	0	0	5 (1)	5 (3)	0

DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Number of animals with observation (study day first observed).

<sup>b</sup>Positive control.

<sup>c</sup>Positive control (did not perform as expected).

Compared to that of the vehicle control group, mean ear thickness was significantly increased on day 6 (3.86%) in the 20% group (Table 12) only. There was a significant concentration-related trend for increased ear swelling on day 3.

**Table 12. Summary of Ear Thickness Measurement Data for Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol<sup>a</sup>**

Concentration	Day 1 (mm × 10 <sup>-2</sup> )	Day 3 (mm × 10 <sup>-2</sup> )	Day 6 (mm × 10 <sup>-2</sup> )	Day 3 (%)	Day 6 (%)	Ear Weight (mg)
Vehicle Control	15.58 ± 0.17 (5)	15.66 ± 0.14 <sup>▲▲</sup> (5)	15.56 ± 0.1 <sup>▲▲</sup> (5)	-0.04 ± 0.96 <sup>▲▲</sup> (5)	0.02 ± 0.78 <sup>▲▲</sup> (5)	7.78 ± 0.42 (5)
1%	15.78 ± 0.08 (5)	15.54 ± 0.07 (5)	15.72 ± 0.10 (5)	-0.84 ± 0.39 (5)	0.98 ± 0.60 (5)	8.10 ± 0.52 (5)
2%	15.52 ± 0.08 (5)	15.50 ± 0.00 (5)	15.50 ± 0.00 (5)	-1.00 ± 0.00 (5)	-0.30 ± 0.00 (5)	8.42 ± 0.46 (5)
5%	15.82 ± 0.09 (5)	15.98 ± 0.09 (5)	15.74 ± 0.09 (5)	1.88 ± 0.60 (5)	1.14 ± 0.53 (5)	8.40 ± 1.14 (5)
20%	15.22 ± 0.10 (5)	16.56 ± 0.17 (5)	16.16 ± 0.10 <sup>**</sup> (5)	5.72 ± 1.06 (5)	3.86 ± 0.64 <sup>**</sup> (5)	8.98 ± 0.43 (5)
40%	15.46 ± 0.16 (5)	15.88 ± 0.05 (5)	15.56 ± 0.06 (5)	1.24 ± 0.39 (5)	0.02 ± 0.32 (5)	8.32 ± 0.12 (5)
100/80%	15.42 ± 0.13 (5)	16.14 ± 0.19 (5)	15.72 ± 0.10 (5)	3.00 ± 1.24 (5)	0.98 ± 0.60 (5)	8.66 ± 1.10 (5)
0.15% DNFB <sup>b</sup>	15.34 ± 0.04 (5)	16.94 ± 0.13 <sup>**</sup> (5)	16.98 ± 0.09 <sup>**</sup> (5)	8.12 ± 0.78 <sup>**</sup> (5)	8.98 ± 0.60 <sup>**</sup> (5)	11.60 ± 0.79 <sup>**</sup> (5)
5% ISO <sup>b</sup>	15.40 ± 0.10 (5)	16.92 ± 0.05 <sup>**</sup> (5)	16.60 ± 0.19 <sup>*</sup> (5)	7.96 ± 0.39 <sup>**</sup> (5)	6.74 ± 1.20 <sup>*</sup> (5)	8.10 ± 1.02 (5)

<sup>▲▲</sup>Significant trend (p ≤ 0.01) by Jonckheere's test.

<sup>\*</sup>Significantly different (p ≤ 0.05) from the vehicle control group by Shirley's or Dunn's test.

<sup>\*\*</sup>p ≤ 0.01.

DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Ear thickness data are presented as mean ± standard error (n); percentages are calculated as ear swelling (difference from vehicle control) using the equation: [mean measurement for both ears for each animal (day 3 or 6) – vehicle control mean ear thickness (day 3 or 6)]/[vehicle mean ear thickness (day 3 or 6) × 100].

<sup>b</sup>Positive control.

In the 5% or greater groups, incorporation of <sup>125</sup>I-UdR (expressed as mean DPM) into lymphocytes from the draining lymph nodes was significantly increased relative to vehicle controls, and this proliferative response followed a significant concentration-related trend. Mean DPM values ranged from 47.53 to 100.41 DPM in the 5% or greater groups (Table 13 and Figure 3). Mice treated with 5% or greater had significantly increased mean SI values, with SI values of more than 3.0 occurring at 40% (3.09) and 100/80% (4.83). The concentration that would result in an SI of 3.0 (EC3), was calculated to be 34.6% (Figure 3).

**Table 13. Summary of  $^{125}\text{I}$ -UdR Incorporation and Stimulation Index for Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol<sup>a</sup>**

Concentration	n	Lymph Node Counts (DPM)	Stimulation Index
Vehicle Control	5	20.76 ± 4.67 <sup>▲▲</sup>	1.00 ± 0.22 <sup>▲▲</sup>
1%	5	38.16 ± 13.29	1.83 ± 0.64
2%	5	35.96 ± 3.05	1.73 ± 0.15
5%	5	47.53 ± 5.68*	2.29 ± 0.27*
20%	5	61.35 ± 6.82**	2.95 ± 0.33**
40%	5	64.32 ± 12.07**	3.09 ± 0.58**
100/80%	5	100.41 ± 16.89**	4.83 ± 0.81**
0.15% DNFB <sup>b</sup>	5	208.37 ± 5.25**	10.02 ± 1.19**
5% ISO <sup>b</sup>	5	34.07 ± 5.25	1.64 ± 0.25

<sup>▲▲</sup>Significant trend ( $p \leq 0.01$ ) by Jonckheere's test.

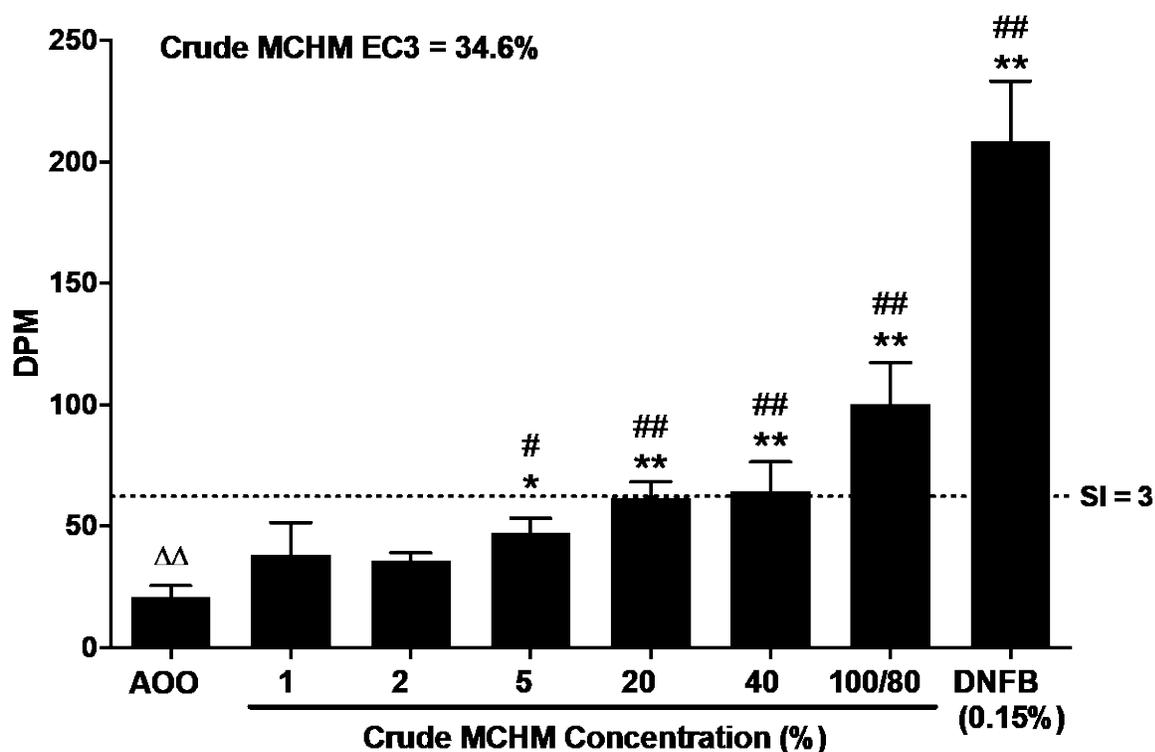
\*Significantly different ( $p \leq 0.05$ ) from the vehicle control group by Shirley's or Dunn's test.

\*\* $p \leq 0.01$ .

DPM = disintegrations per minute; stimulation index = ratio of treated group mean DPM/vehicle control group mean DPM; DNFB = 1-fluoro-2,4-dinitrobenzene; ISO = isoeugenol.

<sup>a</sup>Data are presented as mean ± standard error.

<sup>b</sup>Positive control.

**Figure 3.  $^{125}\text{I}$ -UdR Incorporation and Stimulation Index Data in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol**

AOO = acetone/olive oil vehicle control; DNFB = 1-fluoro-2,4-dinitrobenzene positive control; DPM = disintegrations per minute; MCHM = 4-methylcyclohexanemethanol; SI = stimulation index; EC3 = concentration of test article that results in an SI 3; ΔΔ = significant trend ( $p \leq 0.01$ ) for DPM by Jonckheere's test; \* = significantly different ( $p \leq 0.05$ ) from the vehicle control group by Shirley's or Dunn's test; \*\* = ( $p \leq 0.01$ ); # = significantly different ( $p \leq 0.05$ ) SI value from the vehicle control group by Shirley's or Dunn's test; ## = ( $p \leq 0.01$ ).

The positive controls utilized for the study of crude MCHM, 1-fluoro-2,4-dinitrobenzene (0.15%), and isoeugenol (5%) were shared controls with the study of MCHM. 1-Fluoro-2,4-dinitrobenzene performed as expected; however, isoeugenol did not elicit the anticipated sensitizing response.

## Crude 4-Methylcyclohexanemethanol (Repeat Study)

A repeat study of crude MCHM was conducted to confirm the results observed in the initial study and more clearly establish levels at which the test article induced sensitization in the absence of clinical toxicity. Crude MCHM was administered at concentrations of 1%, 5%, 25%, 50%, and 75%. All mice survived to the end of the study (Table 14 and Table 15). Clinical signs of toxicity were observed on days 1 through 3 in the 75% group and included eye squinting (in 8 of 13 mice) and isolation from cage mates (all 13 mice); isolation from cage mates was also observed on day 1 in all 13 mice in the 50% group (Table 16). All clinical signs of toxicity were observed during the afternoon after treatment, and none was observed during morning observation the subsequent day. No clinical signs of toxicity were observed in the 1%, 5%, or 25% groups. Group mean body weights were not significantly affected by treatment (Table 15).

**Table 14. Disposition of Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol (Repeat Study)**

	Vehicle Control	1%	5%	25%	50%	75%	0.15% DNFB <sup>a</sup>
Animals Initially in Study	13	13	13	13	13	13	13
Terminal Removal	13	13	13	13	13	13	13

DNFB = 1-fluoro-2,4-dinitrobenzene.

<sup>a</sup>Positive control.

**Table 15. Survival and Mean Body Weights of Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol (Repeat Study)<sup>a</sup>**

Concentration	Survival <sup>b</sup>	Initial Body Weight (g)	Final Body Weight (g)	Absolute Weight Gain or Loss
Vehicle Control	13/13	16.9 ± 0.3	17.2 ± 0.2	0.2 ± 0.1 <sup>▲▲</sup>
1%	13/13	17.2 ± 0.4	17.3 ± 0.3	0.1 ± 0.1
5%	13/13	17.3 ± 0.3	17.8 ± 0.3	0.5 ± 0.1
25%	13/13	17.2 ± 0.3	17.5 ± 0.3	0.3 ± 0.1
50%	13/13	17.3 ± 2.0	17.7 ± 0.3	0.4 ± 0.1
75%	13/13	17.1 ± 1.0	17.6 ± 0.3	0.5 ± 0.1
0.15% DNFB <sup>c</sup>	13/13	17.4 ± 0.3	17.7 ± 0.3	0.3 ± 0.1

<sup>▲▲</sup>Significant trend ( $p \leq 0.01$ ) by Jonckheere's test.

<sup>a</sup>Weights are given as mean ± standard error.

<sup>b</sup>Number of animals surviving on day 6/number initially in group.

<sup>c</sup>Positive control; DNFB = 1-fluoro-2,4-dinitrobenzene.

**Table 16. Clinical Observations for Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol (Repeat Study)**

	Vehicle Control	1%	5%	25%	50%	75%	0.15% DNFB <sup>b</sup>
<b>n</b>	13	13	13	13	13	13	13
Animal Behavior, Isolation <sup>a</sup>	0	0	0	0	13 (1)	13 (1)	13 (1)
Erythema, Ear, Bilateral, Mild	0	0	0	0	0	0	13 (5)
Eye Shut, Bilateral	0	0	0	0	0	8 (1)	4 (1)

DNFB = 1-fluoro-2,4-dinitrobenzene.

<sup>a</sup>Number of animals with observation (study day first observed).<sup>b</sup>Positive control.

Ear thickness in the 75% group was significantly increased relative to that of the vehicle control group on days 3 (1.63%) and 6 (2.39%) (Table 17). Ear swelling was also increased in the 100/80% group in the initial study of crude MCHM, however, not to a significant extent (Table 12). No changes in ear thickness were observed in the remaining dosed groups (Table 17).

**Table 17. Summary of Ear Thickness Measurement Data for Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol (Repeat Study)<sup>a</sup>**

Concentration	Day 1 (mm × 10 <sup>-2</sup> )	Day 3 (mm × 10 <sup>-2</sup> )	Day 6 (mm × 10 <sup>-2</sup> )	Day 3 (%)	Day 6 (%)	Ear Weight (mg)
Vehicle Control	18.01 ± 0.03 <sup>▲▲</sup> (13)	17.92 ± 0.04 <sup>▲▲</sup> (13)	17.56 ± 0.04 <sup>▲▲</sup> (13)	0.02 ± 0.24 <sup>▲▲</sup> (13)	0.02 ± 0.23 <sup>▲▲</sup> (13)	8.62 ± 0.10 (5)
1%	17.66 ± 0.07 <sup>**</sup> (13)	17.88 ± 0.06 (13)	17.50 ± 0.01 (13)	-0.20 ± 0.31 (13)	-0.31 ± 0.09 (13)	8.08 ± 0.42 (5)
5%	17.52 ± 0.06 <sup>**</sup> (13)	17.82 ± 0.06 (13)	17.60 ± 0.05 (13)	-0.52 ± 0.36 (13)	0.24 ± 0.26 (13)	8.9 ± 0.58 (5)
25%	17.55 ± 0.06 <sup>**</sup> (13)	17.89 ± 0.05 (13)	17.58 ± 0.04 (13)	-0.15 ± 0.30 (13)	0.13 ± 0.23 (13)	9.28 ± 0.35 (5)
50%	17.47 ± 0.05 <sup>**</sup> (13)	17.96 ± 0.06 (13)	17.65 ± 0.08 (13)	0.28 ± 0.03 (13)	0.52 ± 0.47 (13)	9.60 ± 0.63 (5)
75%	17.40 ± 0.04 <sup>**</sup> (13)	18.21 ± 0.06 <sup>**</sup> (13)	17.99 ± 0.06 <sup>**</sup> (13)	1.63 ± 0.30 <sup>**</sup> (13)	2.39 ± 0.33 <sup>**</sup> (13)	8.96 ± 0.52 (5)
0.15% DNFB <sup>b</sup>	17.35 ± 0.06 <sup>**</sup> (13)	18.00 ± 0.02 (13)	18.77 ± 0.14 <sup>**</sup> (13)	0.50 ± 0.14 (13)	6.77 ± 0.79 <sup>**</sup> (13)	11.88 ± 0.66 <sup>**</sup> (5)

<sup>▲▲</sup>Significant trend ( $p \leq 0.01$ ) by Jonckheere's test.

<sup>\*\*</sup>Significantly different ( $p \leq 0.01$ ) from the vehicle control group by Shirley's or Dunn's test.

DNFB = 1-fluoro-2,4-dinitrobenzene.

<sup>a</sup>Ear thickness data are presented as mean ± standard error (n); percentages are calculated as ear swelling (difference from vehicle control) using the equation: [mean measurement for both ears for each animal (day 3 or 6) - vehicle control mean ear thickness (day 3 or 6)]/[vehicle control mean ear thickness (day 3 or 6) × 100].

<sup>b</sup>Positive control.

Generally, concentration-related increases in DPM values occurred, and the increases were significant at 50% (68.29 DPM) and 75% (128.43 DPM) (Table 18 and Figure 4). Increased DPM values correlated with concentration-related increases in SI values that were also significant in the 50% (2.24) and 75% (4.22) groups. The increase in DPM in the 50% group occurred in the absence of irritation, suggesting that crude MCHM is a sensitizer. The positive control, DNFB (0.15%), performed as expected. The concentration that would result in an SI of 3.0 (EC3) was calculated to be 60.0% (Figure 4).

**Table 18. Summary of <sup>125</sup>I-Udr Incorporation and Stimulation Index Data for Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol (Repeat Study)<sup>a</sup>**

Concentration	n	Lymph Node Counts (DPM)	Stimulation Index
Vehicle Control	8	30.46 ± 7.28 <sup>▲▲</sup>	1.00 ± 0.24 <sup>▲▲</sup>
1%	8	33.42 ± 8.63	1.10 ± 0.28
5%	8	22.25 ± 2.98	0.73 ± 0.10
25%	8	44.14 ± 8.23	1.45 ± 0.27
50%	8	68.29 ± 14.10*	2.24 ± 0.46*
75%	8	128.43 ± 16.45**	4.22 ± 0.54**
0.15% DNFB <sup>b</sup>	8	2,315.52 ± 380.62**	76.02 ± 12.50**

<sup>▲▲</sup>Significant trend ( $p \leq 0.01$ ) by Jonckheere's test.

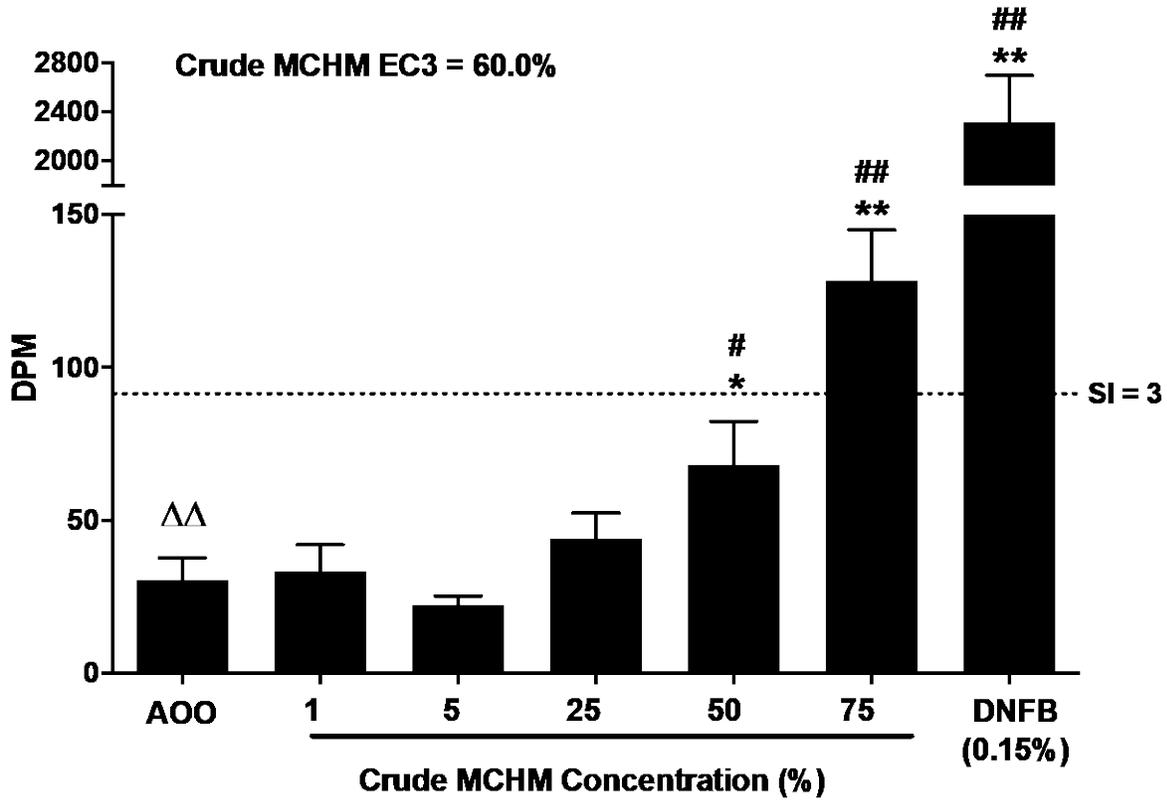
\*Significantly different ( $p \leq 0.05$ ) from the vehicle control group by Shirley's or Dunn's test.

\*\* $p \leq 0.01$ .

DNFB = 1-fluoro-2,4-dinitrobenzene.

<sup>a</sup>Data are presented as mean ± standard error; DPM = disintegrations per minute; stimulation index = ratio of treated group mean DPM/vehicle control group mean DPM.

<sup>b</sup>Positive control.



**Figure 4. <sup>125</sup>I-UdR Incorporation and Stimulation Index Data in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol (Repeat Study)**

AOO = acetone/olive oil vehicle control; DNFB = 1-fluoro-2,4-dinitrobenzene positive control; DPM = disintegrations per minute; MCHM = 4-methylcyclohexanemethanol; SI = stimulation index; EC3 = concentration of test article that results in an SI = 3; ΔΔ = significant trend ( $p \leq 0.01$ ) for DPM by Jonckheere's test; \* = significantly different ( $p \leq 0.05$ ) from the vehicle control group by Shirley's or Dunn's test; \*\* = ( $p \leq 0.01$ ); # = significantly different ( $p \leq 0.05$ ) SI value from the vehicle control group by Shirley's or Dunn's test; ## = ( $p \leq 0.01$ ).

## Discussion

The primary concern for human exposure to 4-methylcyclohexanemethanol (MCHM) is via occupational routes, primarily during handling or use of the chemical. However, on January 9, 2014, approximately 10,000 gallons of a chemical mixture containing predominantly MCHM was spilled into the Elk River, upstream of a municipal water source serving approximately 300,000 people in Charleston, West Virginia.<sup>10</sup> Immediately following the spill, there were numerous reports of mild rashes and reddened skin due to dermal exposure to the contaminated water.<sup>24</sup> Following nominations by the Centers for Disease Control and Prevention and the Agency for Toxic Substances and Disease Registry, the National Toxicology Program (NTP) conducted dermal hypersensitivity and irritancy studies of both MCHM and crude MCHM in female BALB/c mice.

In the initial study, MCHM (2%, 20%, and 100%) and crude MCHM (1%, 2%, 5%, 20%, 40%, and 100%) formulations were applied to the ears of female BALB/c mice for 3 consecutive days via direct epicutaneous application. Two mice administered 100% MCHM were euthanized moribund on day 1 of the study due to signs of morbidity, and the dose was subsequently lowered to 50%. Also, due to clinical signs of toxicity in mice administered 100% MCHM, this dose was lowered to 80%. A repeat study of crude MCHM was performed to confirm the observed dermal hypersensitivity response and more clearly establish levels at which the test article induced sensitization in the absence of clinical toxicity. Formulations of 1%, 5%, 25%, 50%, and 75% crude MCHM were utilized in the repeat study.

MCHM induced dermal irritation at concentrations greater than 20%. Administration of 20% and 100/50% MCHM induced significant concentration-dependent increases in mean ear thickness on day 3 (4.44% and 6.47%, respectively) and day 6 (3.54% and 5.30%, respectively). For crude MCHM, there was minimal evidence of irritation and the results were inconsistent between studies. In the initial study a statistically significant increase in irritation was observed on day 6 in the 20% group only (3.86%) and not at doses of 40 or 100/80%. In the repeat study significant changes in ear thickness occurred only at 75%, with no evidence of a concentration-dependent response. The results from these studies indicate that MCHM should be considered a dermal irritant on the basis of the Globally Harmonized System of Classification and Labelling of Chemicals.<sup>46</sup> These results are consistent with those from studies previously conducted by Eastman Kodak Company, which demonstrated that topical administration of MCHM and crude MCHM caused strong dermal irritation in guinea pigs (MCHM), albino rabbits (crude MCHM), and rats (crude MCHM).<sup>16; 25; 27; 18; 26</sup> The results of the studies described in this Immunotoxicity Report suggest that MCHM is likely a more potent irritant than crude MCHM. These results also suggest that MCHM itself, and not the other chemicals found in crude MCHM, was responsible for the dermal irritation reported following the Elk River spill.

The local lymph node assay (LLNA) is the standard assay for evaluating the potential of a compound to act as a skin sensitizer.<sup>32-34</sup> Skin sensitization potential is assessed as a measurement of lymph node cell proliferation in response to application of the compound in question, and a compound is classified as a skin sensitizer if one or more concentrations causes a threefold or greater increase in lymph node cell proliferation relative to vehicle control (stimulation index [SI]  $\geq 3$ ); the effective concentration of a compound that induces an SI equal to 3 is known as the EC3. In these studies, crude MCHM was determined to be a sensitizer, with

an EC3 in the concentration range of 35% to 60%. This is in contrast to sensitization results using the guinea pig footpad test and previously reported by Eastman Kodak Company<sup>27</sup>; however, this is likely due to the difference in testing methodologies.

MCHM was not deemed to be a skin sensitizer in these studies. These results suggest that although MCHM is a dermal irritant, other chemical components of crude MCHM are likely responsible for the observed stimulation in the LLNA. This is supported by results from structure-activity relationship analyses performed by NTP that indicated some of the minor components of crude MCHM were potential sensitizers.<sup>47</sup> Inconsistencies were observed in the studies of crude MCHM. In the initial study, administration of 20% crude MCHM significantly increased ear swelling, whereas administration of 50% crude MCHM did not. The lack of a concentration-dependent trend suggests that the effects observed at 20% MCHM might not have been treatment related. Similarly, in the repeat study of crude MCHM, treatment with 75% crude MCHM significantly increased ear swelling but treatment with other concentrations did not. It has been reported that dermal irritants could also induce lymph node cell proliferation, possibly resulting in false positives in the LLNA<sup>48; 32; 33</sup>; however, the irritancy responses were not considered to be strong enough to cause additional proliferation in the draining lymph nodes, and, therefore, support the identification of crude MCHM as a skin sensitizer.

A decrease in the SI was observed following treatment with MCHM (2%, 20%, and 100/50%) relative to vehicle control; this decrease was significant in the 2% MCHM group and lessened with increasing MCHM concentration. The SI value for the vehicle control animals ( $1.00 \pm 0.22$ ) was similar to that of the vehicle controls for the repeat study of crude MCHM ( $1.00 \pm 0.24$ ), indicating that the vehicle was likely not the reason for the observed decrease in SI. The reason for the low SI values in mice treated with MCHM is unclear; however, it could be due to the weak sensitizing ability of MCHM as only crude MCHM was determined to be a skin sensitizer in these studies.

Two positive controls were used in this study, 1-fluoro-2,4-dinitrobenzene and isoeugenol. 1-Fluoro-2,4-dinitrobenzene and isoeugenol are standard positive controls for dermal hypersensitivity and irritancy studies, with 1-fluoro-2,4-dinitrobenzene acting as a strong sensitizer and isoeugenol acting as a weak sensitizer. In these studies, 1-fluoro-2,4-dinitrobenzene performed as expected; however, isoeugenol did not elicit the anticipated sensitizing response. SI and EC3 values for the different categories of sensitizers (nonsensitizing, weak, moderate, strong, and extreme) have a wide range<sup>49</sup> and SI values for isoeugenol, in particular, are historically variable. Despite the unexpected weak response of isoeugenol, the SI values for 1-fluoro-2,4-dinitrobenzene (10.02 and 76.02) and isoeugenol (1.64) fell within the ranges of historical values for both the NTP's and the study laboratory's designations of potent and weak sensitizers. In addition, as both the crude MCHM and 1-fluoro-2,4-dinitrobenzene LLNA results were repeated, the lack of response from isoeugenol was considered an isolated result.

The results of these studies indicate that the estimated lowest-observed-effect levels for MCHM (20% based on irritancy) and crude MCHM (5% based on dermal sensitization) were significantly higher than those levels found in the area's residential tap water immediately following the Elk River spill (up to 3.35 ppm), which, in turn, were higher than that of the Centers for Disease Control and Prevention's short-term drinking water screening level of 1 ppm.<sup>12</sup> Despite the low levels of MCHM detected in the area's tap water following the spill,

reports of dermal irritation were increased in the population using the contaminated water. The dosing pattern utilized in the studies reported here was a single, daily administration, whereas the pattern of human exposure following the spill was potentially more extensive due to daily showering, bathing, and hand washing habits.

Under the conditions of these dermal hypersensitivity and irritancy studies, MCHM induced dermal irritation in female mice, as indicated by increases in ear swelling at concentrations of 20% and 100/50%. Crude MCHM was identified as a skin sensitizer, with an EC3 value in the concentration range of 35% to 60%. Dermal exposure to crude MCHM produced minimal irritant effects, indicated by increases in ear swelling at concentrations of 20% and 75%, which were inconsistent across repeat studies.

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## Appendix A. Chemical Characterization and Dose Formulation Studies

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## A.1. Procurement and Characterization

### A.1.1. 4-Methylcyclohexanemethanol

4-Methylcyclohexanemethanol (MCHM) was obtained from TCI America (Portland, OR) in one lot (KDY3F). Crude MCHM was obtained from Eastman Chemical Company (Kingsport, TN) in one lot (TP14044373). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at MRIGlobal (Kansas City, MO). Reports on analyses performed in support of the MCHM studies are on file at the National Institute of Environmental Health Sciences.

Lot KDY3F of the pure chemical, a clear, colorless liquid, was identified as MCHM using Fourier transform infrared (FTIR) spectroscopy, Fourier transform (FT) proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography (GC) with mass spectrometry (MS) detection. In addition, the boiling point, density, and octanol:water partition coefficient were determined. All spectra were consistent with isomers of the proposed structure and literature spectra of MCHM.<sup>50-53</sup> Representative FTIR and FT proton NMR spectra are presented in Figure A-1 and Figure A-2, respectively. Two components tentatively identified as *cis*- and *trans*- isomers of MCHM were observed for the test article using GC/MS. The boiling point of the test chemical was 199.4°C (consistent with a literature reference value of 202°C),<sup>54</sup> the relative density was 0.9203 g/mL, and the octanol:water partition coefficient, log P, was 2.55.

Karl Fischer titration was used to determine the water content of lot KDY3F. Elemental analyses for carbon, hydrogen, and nitrogen were conducted by Galbraith Laboratories, Inc. (Knoxville, TN). The purity profile was determined using GC with flame ionization detection (FID) and two columns with differing polarities.

For lot KDY3F, Karl Fischer titration indicated 0.21% water. Elemental analyses for carbon, hydrogen, and nitrogen were consistent with the theoretical values for MCHM. GC/FID analysis by system A (Table A-1) detected two major peaks with a combined area of 99.97% of the total peak area and no impurities with areas greater than or equal to 0.05% of the total peak area. The relative areas of the two major peaks indicated that MCHM consisted of 67.99% *cis*- and 31.98% *trans*-isomers. GC/FID by system B detected two major peaks with a combined relative area of 99.83% (with relative areas of 67.80% [*cis*] and 32.03% [*trans*] isomers), and two minor impurities totaling 0.13% of the total peak area. The overall purity of lot KDY3F was determined to be greater than or equal to 99.8%.

A chemical identity and purity screen of lot TP14044373 of the crude test chemical, a clear liquid, was performed using GC/MS. The test article was found to be a 90.35% mix of the *cis*- (33.45%) and *trans*- (56.90%) isomers of MCHM on the basis of the relative areas of the two major peaks. Six impurity components with a relative percent total area greater than or equal to 0.05% were observed, with a combined area of 9.66%. Three of these impurity components (each with an individual relative percent area greater than 1%) were tentatively identified as cyclohexanemethanol (1.82%), and a mix of the *cis*- and *trans*-isomers of 1,4-cyclohexanedimethanol (7.15%). Three minor impurities (each with an individual relative percent area between 0.05% and 1.0%) were tentatively identified as methyl 4-methylcyclohexanecarboxylate (0.50%), *cis*-octahydroisobenzofuran (0.13%), and 2-ethyl-1-

hexanol (0.06%). The purity of lot TP14044373, relative to MCHM isomers, was estimated to be greater than 90%.

Stability studies of lot KDY3F were conducted using GC/FID by system A. These studies indicated that MCHM was stable as a bulk chemical for at least 14 days when stored in amber glass vials under an inert headspace at temperatures up to 60°C. To ensure stability, both bulk chemicals were stored at room temperature in amber glass bottles, and MCHM was stored under an inert headspace.

### **A.1.2. Isoeugenol**

Isoeugenol was obtained from Sigma-Aldrich, Inc. (St. Louis, MO), in one lot (05622BEV) that was used as a positive control in the initial dermal hypersensitivity and irritancy study. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Research Triangle Institute (RTI) (Research Triangle Park, NC).

The identity of lot 05622BEV, received as a clear liquid, was determined using FTIR and proton and carbon-13 NMR spectroscopy and GC/MS spectrometry. All spectra were consistent with the structure of isoeugenol and literature spectra, where available.<sup>55-59</sup> An accurate molecular mass was measured using ultra-high-performance liquid chromatography (UHPLC)/time-of-flight (TOF) MS; results were within 1.2 ppm of the theoretical mass (165.0910 Da) for isoeugenol.

Karl Fischer titration was used to determine the water content of lot 05622BEV. Elemental analyses for carbon, hydrogen, and oxygen were conducted by Galbraith Laboratories, Inc. The purity was determined using GC/FID and UHPLC with photodiode array (PDA) detection. The Acquity<sup>®</sup> UHPLC/PDA system consisted of a UHP liquid chromatograph, a PDA detector (254 nm), and an ethylene bridged hybrid C18 column (2.1 mm × 50 mm, 1.7 μm particle size), all from Waters Corporation (Milford, MA). The mobile phases consisted of A) 10:90:0.1 acetonitrile:deionized water:acetic acid and B) 90:10:0.1 acetonitrile:deionized water:acetic acid, programmed with a gradient starting at 100% A for 1 minute, then to 50% B in 7 minutes, then to 100% B in 1 minute, then reversed to 100% A in 1 minute, and held for 2 minutes; the flow rate was 0.6 mL/minute.

For lot 05622BEV, Karl Fischer titration indicated 0.23% water. Elemental analyses for carbon, hydrogen, and oxygen were in agreement with the theoretical values for isoeugenol. GC/FID analysis by system C indicated two major isomer peaks (totaling 98.7% of the total peak area) and two impurities (0.7% and 0.4% of the total peak area). UHPLC/PDA analysis indicated two major isomer peaks (totaling 99.1% of the total peak area) and two impurities (0.6% and 0.1% of the total peak area). The overall purity of lot 05622BEV was determined to be greater than or equal to 98.7%.

Stability studies of lot 05622BEV conducted using GC/FID by system C indicated that isoeugenol was stable as a bulk chemical for at least 14 days when stored in amber glass vials sealed with Teflon-lined caps at temperatures up to 60°C. To ensure stability, the bulk chemical was stored at room temperature under nitrogen.

### A.1.3. 1-Fluoro-2,4-dinitrobenzene

1-Fluoro-2,4-dinitrobenzene was obtained from Sigma-Aldrich, Inc., in one lot (BCBN2339V) that was used as a positive control in the initial and repeat hypersensitivity and irritancy studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at RTI.

The identity of lot BCBN2339V, a clear liquid, was determined using FTIR and proton, carbon-13, and fluorine-19 NMR spectroscopy and GC/MS spectrometry. All spectra were consistent with the structure of 1-fluoro-2,4-dinitrobenzene and literature spectra, where available.<sup>60; 55; 61; 59</sup> An accurate molecular mass was measured using UHPLC/TOF MS; results were within 2.2 ppm of the theoretical mass for 1-fluoro-2,4-dinitrobenzene.

The water content of lot BCBN2339V was determined using Karl Fischer titration. Elemental analyses for carbon, hydrogen, nitrogen, oxygen, and fluorine were conducted by Galbraith Laboratories, Inc. The purity was determined using GC/FID and UHPLC with PDA detection. The Acquity<sup>®</sup> UHPLC/PDA system consisted of a UHP liquid chromatograph, a PDA detector (240 nm), and an ethylene bridged hybrid C18 column (2.1 mm × 50 mm, 1.7 μm particle size), all from Waters Corporation. The mobile phases consisted of A) water and B) acetonitrile, programmed with a gradient starting at 10% B for 1 minute, then to 30% B in 7 minutes, then to 100% B in 0.5 minutes, held for 0.5 minutes, then reversed to 10% B in 0.5 minutes, and held for 2.5 minutes; the flow rate was 0.6 mL/minute.

For lot BCBN2339V, the water content was less than the limit of quantitation of 0.1%. Elemental analyses for carbon, hydrogen, nitrogen, and oxygen were in agreement with the theoretical values for 1-fluoro-2,4-dinitrobenzene; fluorine was not accurately quantitated due to interference with oxygen. GC/FID using system C indicated one major peak (99.6% of the total peak area), and two impurity peaks (0.2% each of the total peak area). UHPLC/PDA analysis indicated one major peak (99.4% of the total peak area) and three impurity peaks (0.1%, 0.2%, and 0.3% of the total peak area). The overall purity of lot BCBN2339V was determined to be greater than or equal to 99.4%.

Stability studies of lot BCBN2339V conducted using GC/FID by system C indicated that 1-fluoro-2,4-dinitrobenzene was stable as a bulk chemical for at least 14 days when stored in amber glass vials sealed with Teflon-lined caps at temperatures up to 60°C. To ensure stability, the bulk chemical was stored at room temperature under nitrogen.

## A.2. Preparation and Analysis of Dose Formulations

### A.2.1. Formulation Materials

Acetone and olive oil were obtained from multiple suppliers in multiple lots and these materials were combined with a 4:1 ratio to prepare the vehicle for the initial and repeat studies. For the repeat study, J.T. Baker Acetone (lot K30J01; Thermofisher Scientific, Waltham, MA) was analyzed using GC/FID by system D indicating a purity of 99.9%, acceptable for use in the study. Olive oil (lot 0240-0545; Welch, Holme, and Clark Co., Newark, NJ) was analyzed to determine peroxide levels using potentiometric titration (platinum combination redox electrode) using a Mettler Toledo pH meter (Mettler Toledo, Columbus, OH) and AOAC method 965.33 (AOAC, 1990). Peroxide levels were within the acceptable limit of 5 mEq/kg.

Dose formulations of the test articles and positive controls were prepared daily by mixing the appropriate amounts of each chemical with acetone:olive oil (4:1) to achieve the required concentrations (Table A-2). The dose formulations were stored in amber glass vials sealed with Teflon-lined caps at room temperature or refrigerated temperatures (1-fluoro-2,4-dinitrobenzene formulations in the repeat study only) for up to 14 days.

Stability studies of the 1% dose formulation of crude MCHM and the 0.15% dose formulation of 1-fluoro-2,4-dinitrobenzene were performed by RTI using GC/FID by systems E and C, respectively (Table A-1). Stability was confirmed for at least 14 days for these dose formulations stored in amber glass vials with minimal headspace sealed with Teflon-lined lids at room temperature and for 3 hours under simulated animal room conditions.

Analyses of the dose formulations for the initial and repeat studies were conducted by RTI using GC/FID by system E (for MCHM and crude MCHM) and system C (for the positive controls isoeugenol and 1-fluoro-2,4-dinitrobenzene). During the initial hypersensitivity and irritancy study, the dose formulations were analyzed once; two of three MCHM dose formulations were within 15% of the target concentrations and five of six crude MCHM dose formulations were within 15% of the target concentrations (Table A-3). Single dose formulations of the positive controls isoeugenol and 1-fluoro-2,4-dinitrobenzene were each found to be within 15% of the target concentrations. During the repeat dermal hypersensitivity and irritancy study, the dose formulations were analyzed once, and animal room samples of these dose formulations were also analyzed (Table A-4). All five crude MCHM dose formulations were within 15% of the target concentrations; all five animal room samples were also within 15% of the target concentrations. A single dose formulation of 1-fluoro-2,4-dinitrobenzene and its associated animal room sample were both found to be within 15% of the target concentrations.

**Table A-1. Gas Chromatography Systems Used in the Dermal Hypersensitivity and Irritancy Studies of 4-Methylcyclohexanemethanol<sup>a</sup>**

Detection System	Column	Carrier Gas	Oven Temperature Program
<b>System A</b>			
Flame Ionization	Zebron™ ZB-624, 30 m × 0.53 mm, 3.0 μm film (Phenomenex, Inc., Torrance, CA)	Helium at 5 mL/minute	50°C for 2 minutes, then 10°C/minute to 240°C, held for 4 (purity studies) or 9 (stability studies) minutes
<b>System B</b>			
Flame Ionization	DB-1, 30 m × 0.53 mm, 1.5 μm film (Agilent J&W, Folsom, CA)	Helium at 5 mL/minute	50°C for 2 minutes, then 10°C/minute to 240°C, held for 4 minutes
<b>System C</b>			
Flame Ionization	HP-5, 30 m × 0.32 mm, 0.25 μm film (Agilent Technologies, Inc., Santa Clara, CA)	Helium at 1.5 mL/minute	50°C for 2 minutes, then 10°C/minute to 280°C, held for 5 minutes

Detection System	Column	Carrier Gas	Oven Temperature Program
<b>System D</b>			
Flame Ionization	DB WAX, 30 m × 0.53 mm, 1.5 µm film (Agilent)	Helium at 10 mL/minute	40°C for 5 minutes, then 10°C/minute to 220°C, held for 5 minutes
<b>System E</b>			
Flame Ionization	DB-5, 30 m × 0.53 mm, 1.5 µm film (J&W Scientific, Folsom, CA)	Helium at 6 mL/minute	50°C to 200°C at 10°C/minute, held for 5 minutes

<sup>a</sup>The gas chromatographs were manufactured by Agilent Technologies, Inc. (Santa Clara, CA).

**Table A-2. Preparation and Storage of Dose Formulations in the Dermal Hypersensitivity and Irritancy Studies of 4-Methylcyclohexanemethanol**

4-Methylcyclohexanemethanol and Crude 4-Methylcyclohexanemethanol (Initial Study)	Crude 4-Methylcyclohexanemethanol (Repeat Study)
<b>Preparation</b>	
The vehicle was prepared by adding the appropriate amounts of acetone and olive oil into a clear mixing bottle to obtain a 4:1 ratio. The mixing bottle was then capped and shaken for approximately 30 seconds. Dose formulations were prepared by transferring the appropriate amounts of neat or crude 4-methylcyclohexanemethanol, isoeugenol, or 1-fluoro-2,4-dinitrobenzene into volumetric flasks and bringing the flasks to final volume with the vehicle. The flasks were capped, and the contents were mixed by inversion. The dose formulations were prepared daily during the study.	The vehicle was prepared by adding the appropriate amounts of acetone and olive oil into an amber mixing bottle to obtain a 4:1 ratio. The mixing bottle was then capped and mixed by inversion. Dose formulations were prepared by transferring the appropriate amounts of neat crude 4-methylcyclohexanemethanol or 1-fluoro-2,4-dinitrobenzene into volumetric flasks and bringing the flasks to final volume with the vehicle. The flasks were capped, and the contents were mixed by inversion. The dose formulations were prepared daily during the study.
<b>Chemical Lot Numbers</b>	
KDY3F (4-Methylcyclohexanemethanol)	TP14044373 (Crude 4-methylcyclohexanemethanol)
TP14044373 (Crude 4-methylcyclohexanemethanol)	BCBN2339V (1-Fluoro-2,4-dinitrobenzene; positive control)
05622BEV (Isoeugenol; positive control)	–
BCBN2339V (1-Fluoro-2,4-dinitrobenzene; positive control)	–
<b>Maximum Storage Time</b>	
N/A	14 days
<b>Storage Conditions</b>	
The dose formulations were stored in amber glass vials sealed with Teflon-lined lids at room temperature.	The dose formulations were stored in amber glass vials sealed with Teflon-lined lids at room temperature (crude 4-methylcyclohexanemethanol) or at refrigerated temperatures (1-fluoro-2,4-dinitrobenzene).
<b>Study Laboratory</b>	
Burleson Research Technologies, Inc. (Morrisville, NC)	Burleson Research Technologies, Inc. (Morrisville, NC)

N/A = not applicable.

**Table A-3. Results of Analyses of Dose Formulations Administered to Female Mice on Day 3 in the Dermal Hypersensitivity and Irritancy Study of 4-Methylcyclohexanemethanol and Crude 4-Methylcyclohexanemethanol (Initial Study)**

Date Prepared	Date Analyzed	Target Concentration (%)	Determined Concentration <sup>a</sup> (%)	Difference from Target (%)
<b>4-Methylcyclohexanemethanol</b>				
December 12, 2014	December 12–16, 2014	2	1.54	-23 <sup>b</sup>
		20	21.0	+5
		50	44.8	-10
<b>Crude 4-Methylcyclohexanemethanol</b>				
December 12, 2014	December 12–16, 2014	1	0.896	-10
		2	2.01	+1
		5	3.84	-23 <sup>b</sup>
		20	19.2	-4
		40	37.1	-7
		80	82.0	+3
<b>Isoeugenol (Positive Control)</b>				
December 12, 2014	December 12–16, 2014	5	5.30	+6
<b>1-Fluoro-2,4-dinitrobenzene (Positive Control)</b>				
December 12, 2014	December 12–16, 2014	0.150	0.172	+15

<sup>a</sup>Results of triplicate analyses.

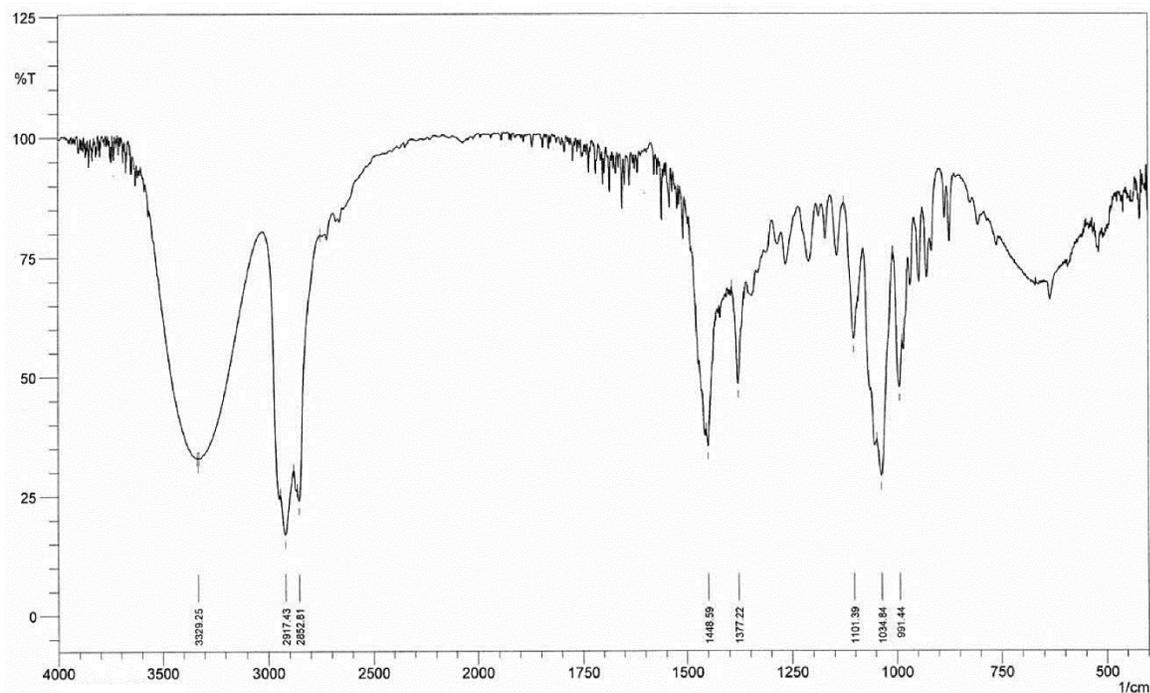
<sup>b</sup>Formulation was outside the acceptable range of  $\pm 15\%$  of target concentration. Formulations were prepared at the study laboratory and sent for chemical analysis after the study had already been initiated.

**Table A-4. Results of Analyses of Dose Formulations Administered to Female Mice in the Dermal Hypersensitivity and Irritancy Study of Crude 4-Methylcyclohexanemethanol (Repeat Study)**

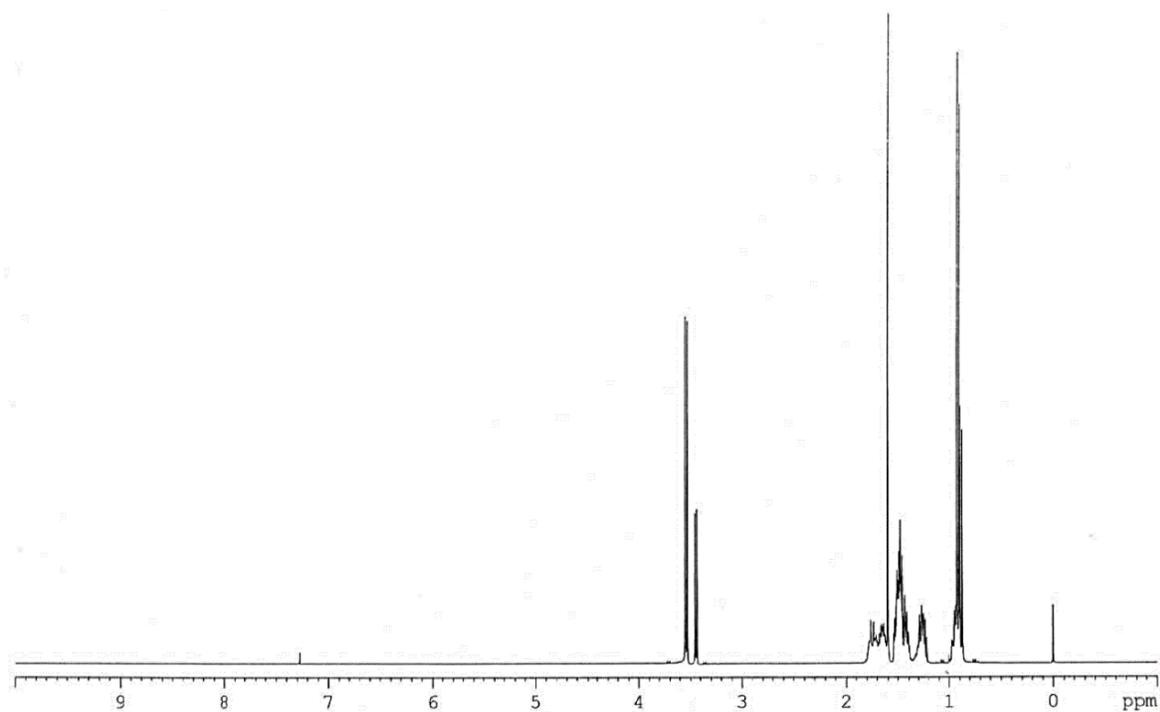
Date Prepared	Date Analyzed	Target Concentration (%)	Determined Concentration <sup>a</sup> (%)	Difference from Target (%)
<b>Crude 4-Methylcyclohexanemethanol</b>				
March 9–10, 2015	March 10, 2015	1	0.985	-2
		5	5.05	+1
		25	24.4	-2
		50	47.6	-5
		75	72.5	-3
	March 23, 2015 <sup>b</sup>	1	1.03	+3
		5	5.12	+2
		25	24.6	-2
		50	46.6	-7
		75	72.4	-3
<b>1-Fluoro-2,4-dinitrobenzene (Positive Control)</b>				
March 9, 2015	March 9, 2015	0.150	0.150	0
	March 23, 2015 <sup>b</sup>	0.150	0.156	+4

<sup>a</sup>Results of triplicate analyses.

<sup>b</sup>Animal room samples.



**Figure A-1. Fourier Transform Infrared Absorption Spectrum of 4-Methylcyclohexanemethanol**



**Figure A-2. Fourier Transform Proton Nuclear Magnetic Resonance Spectrum of 4-Methylcyclohexanemethanol**

## **Appendix B. Ingredients, Nutrient Composition, and Contaminant Levels in NTP-2000 Rat and Mouse Ration**

### **Tables**

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**Table B-1. Ingredients of NTP-2000 Rat and Mouse Ration**

Ingredients	Percentage by Weight
Ground Hard Winter Wheat	22.26
Ground #2 Yellow Shelled Corn	22.18
Wheat Middlings	15.0
Oat Hulls	8.5
Alfalfa Meal (Dehydrated, 17% Protein)	7.5
Purified Cellulose	5.5
Soybean Meal (49% Protein)	5.0
Fish meal (60% Protein)	4.0
Corn Oil (without Preservatives)	3.0
Soy Oil (without Preservatives)	3.0
Dried Brewer's Yeast	1.0
Calcium Carbonate (USP)	0.9
Vitamin Premix <sup>a</sup>	0.5
Mineral Premix <sup>b</sup>	0.5
Calcium Phosphate, Dibasic (USP)	0.4
Sodium Chloride	0.3
Choline Chloride (70% Choline)	0.26
Methionine	0.2

USP = United States Pharmacopeia.

<sup>a</sup>Wheat middlings as carrier.

<sup>b</sup>Calcium carbonate as carrier.

**Table B-2. Vitamins and Minerals in NTP-2000 Rat and Mouse Ration<sup>a</sup>**

	Amount	Source
<b>Vitamins</b>		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
$\alpha$ -Tocopheryl Acetate	100 IU	–
Niacin	23 mg	–
Folic Acid	1.1 mg	$\alpha$ -Calcium pantothenate
d-Pantothenic Acid	10 mg	–
Riboflavin	3.3 mg	Thiamine mononitrate
Thiamine	4 mg	–
B <sub>12</sub>	52 $\mu$ g	–
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	$\alpha$ -Biotin

	Amount	Source
<b>Minerals</b>		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

<sup>a</sup>Per kg of finished product.

**Table B-3. Nutrient Composition of NTP-2000 Rat and Mouse Ration**

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by Weight)	14.9 ± 0.26	14.6–15.1	3
Crude Fat (% by Weight)	8.2 ± 0.17	8.1–8.4	3
Crude Fiber (% by Weight)	9.4 ± 0.14	9.2–9.5	3
Ash (% by Weight)	6.6 ± 2.72	4.9–9.7	3
<b>Amino Acids (% of Total Diet)</b>			
Arginine	0.794 ± 0.070	0.670–0.970	26
Cystine	0.220 ± 0.022	0.150–0.250	26
Glycine	0.700 ± 0.038	0.620–0.800	26
Histidine	0.344 ± 0.074	0.270–0.680	26
Isoleucine	0.546 ± 0.041	0.430–0.660	26
Leucine	1.092 ± 0.063	0.960–1.240	26
Lysine	0.700 ± 0.110	0.310–0.860	26
Methionine	0.408 ± 0.043	0.260–0.490	26
Phenylalanine	0.621 ± 0.048	0.470–0.720	26
Threonine	0.508 ± 0.040	0.430–0.610	26
Tryptophan	0.153 ± 0.027	0.110–0.200	26
Tyrosine	0.413 ± 0.063	0.280–0.540	26
Valine	0.663 ± 0.040	0.550–0.730	26
<b>Essential Fatty Acids (% of Total Diet)</b>			
Linoleic	3.95 ± 0.242	3.49–4.55	26
Linolenic	0.31 ± 0.030	0.21–0.35	26
<b>Vitamins</b>			
Vitamin A (IU/kg)	4,670 ± 46	4,220–5,130	3
Vitamin D (IU/kg)	1,000 <sup>a</sup>	–	–
α-Tocopherol (ppm)	79.0 ± 20.42	27.0–124.0	26

## MCHM Hypersensitivity, NTP IMM 01

<b>Nutrient</b>	<b>Mean ± Standard Deviation</b>	<b>Range</b>	<b>Number of Samples</b>
Thiamine (ppm) <sup>b</sup>	8.2 ± 0.69	7.4–8.6	3
Riboflavin (ppm)	8.1 ± 2.91	4.20–17.50	26
Niacin (ppm)	78.9 ± 8.52	66.4–98.2	26
Pantothenic Acid (ppm)	26.7 ± 11.63	17.4–81.0	26
Pyridoxine (ppm) <sup>b</sup>	9.7 ± 2.09	6.44–14.3	26
Folic Acid (ppm)	1.59 ± 0.45	1.15–3.27	26
Biotin (ppm)	0.32 ± 0.10	0.20–0.704	26
Vitamin B <sub>12</sub> (ppb)	51.8 ± 36.6	18.3–174.0	26
Choline (ppm) <sup>b</sup>	2,665 ± 631	1,160–3,790	26
<b>Minerals</b>			
Calcium (%)	0.918 ± 0.026	0.894–0.946	3
Phosphorus (%)	0.553 ± 0.029	0.535–0.586	3
Potassium (%)	0.669 ± 0.030	0.626–0.733	26
Chloride (%)	0.386 ± 0.037	0.300–0.474	26
Sodium (%)	0.193 ± 0.024	0.160–0.283	26
Magnesium (%)	0.216 ± 0.057	0.185–0.490	26
Sulfur (%)	0.170 ± 0.029	0.116–0.209	14
Iron (ppm)	190.5 ± 38.0	135–311	26
Manganese (ppm)	50.7 ± 9.72	21.0–73.1	26
Zinc (ppm)	58.2 ± 26.89	43.3–184.0	26
Copper (ppm)	7.44 ± 2.60	3.21–16.3	26
Iodine (ppm)	0.514 ± 0.195	0.158–0.972	26
Chromium (ppm)	0.674 ± 0.265	0.330–1.380	25
Cobalt (ppm)	0.235 ± 0.157	0.094–0.864	24

<sup>a</sup>From formulation.

<sup>b</sup>As hydrochloride (thiamine and pyridoxine) or chloride (choline).

**Table B-4. Contaminant Levels in NTP-2000 Rat and Mouse Ration<sup>a</sup>**

	Mean ± Standard Deviation <sup>b</sup>	Range	Number of Samples
<b>Contaminants</b>			
Arsenic (ppm)	0.22 ± 0.011	0.21–0.23	3
Cadmium (ppm)	0.05 ± 0.004	0.046–0.053	3
Lead (ppm)	0.12 ± 0.027	0.09–0.14	3
Mercury (ppm)	<0.02	–	3
Selenium (ppm)	0.2 ± 0.033	0.17–0.23	3
Aflatoxins (ppb)	<5.00	–	3
Nitrate Nitrogen (ppm) <sup>c</sup>	11.51 ± 2.18	10.0–14.0	3
Nitrite Nitrogen (ppm) <sup>c</sup>	0.61	–	3
BHA (ppm) <sup>d</sup>	1.41 ± 0.72	1.0–2.24	3
BHT (ppm) <sup>d</sup>	<1.0	–	3
Aerobic Plate Count (CFU/g)	<10	–	3
Coliform (MPN/g)	3.0 ± 0.0	3.0–3.0	3
<i>Escherichia coli</i> (MPN/g)	<10	–	3
<i>Salmonella</i> (MPN/g)	Negative	–	3
Total Nitrosoamines (ppb) <sup>e</sup>	14.2 ± 0.07	14.1–14.2	3
<i>N</i> -Nitrosodimethylamine (ppb) <sup>e</sup>	6.5 ± 2.05	5.0–7.9	3
<i>N</i> -Nitrosopyrrolidine (ppb) <sup>e</sup>	7.7 ± 2.12	6.2–9.2	3
<b>Pesticides (ppm)</b>			
α-BHC	<0.01	–	3
β-BHC	<0.02	–	3
γ-BHC	<0.01	–	3
δ-BHC	<0.01	–	3
Heptachlor	<0.01	–	3
Aldrin	<0.01	–	3
Heptachlor Epoxide	<0.01	–	3
DDE	<0.01	–	3
DDD	<0.01	–	3
DDT	<0.01	–	3
HCB	<0.01	–	3
Mirex	<0.01	–	3
Methoxychlor	<0.05	–	3
Dieldrin	<0.01	–	3
Endrin	<0.01	–	3
Telodrin	<0.01	–	3

MCHM Hypersensitivity, NTP IMM 01

	Mean ± Standard Deviation <sup>b</sup>	Range	Number of Samples
Chlordane	<0.05	–	3
Toxaphene	<0.10	–	3
Estimated PCBs	<0.20	–	3
Ronnel	<0.01	–	3
Ethion	<0.02	–	3
Trithion	<0.05	–	3
Diazinon	<0.10	–	3
Methyl Chlorpyrifos	0.060 ± 0.044	0.020–0.107	3
Methyl Parathion	<0.02	–	3
Ethyl Parathion	<0.02	–	3
Malathion	0.017 ± 0.017	0.021–0.247	3
Endosulfan I	<0.01	–	3
Endosulfan II	<0.01	–	3
Endosulfan Sulfate	<0.03	–	3

CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride.

<sup>a</sup>All samples were irradiated.

<sup>b</sup>For values less than the limit of detection, the detection limit is given as the mean.

<sup>c</sup>Sources of contamination: alfalfa, grains, and fish meal.

<sup>d</sup>Sources of contamination: soy oil and fish meal.

<sup>e</sup>All values were corrected for percent recovery.

## Appendix C. Sentinel Animal Program

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### Table

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## C.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that could affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicological evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera or feces from extra (sentinel) or dosed animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

In the dermal hypersensitivity and irritancy studies of MCHM and crude MCHM, blood samples were collected, allowed to clot and the serum was separated. All samples were processed appropriately with serology testing performed by IDEXX BioResearch (formerly Research Animal Diagnostic Laboratory, University of Missouri), Columbia, MO for determination of the presence of pathogens. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Blood was collected from five female mice per time point.

**Table C-1. Laboratory Methods and Agents Tested for in the Sentinel Animal Program**

Method and Test	Time of Collection
<b>Mice</b>	
<b>Three-day Study (2014)</b>	
Multiplex Fluorescent Immunoassay (MFI)	
Ectromelia virus	1 week postarrival
EDIM	1 week postarrival
Lymphocytic choriomeningitis virus (LCMV)	1 week postarrival
Mycoplasma pulmonis	1 week postarrival
Mouse hepatitis virus (MHV)	1 week postarrival
Mouse norovirus (MNV)	1 week postarrival
Mouse parvovirus (MPV)	1 week postarrival
Minute virus of mice (MVM)	1 week postarrival
Pneumonia virus of mice (PVM)	1 week postarrival
Reovirus 3 (REO3)	1 week postarrival
Theiler's murine encephalomyelitis virus (TMEV)	1 week postarrival
Sendai	1 week postarrival
<b>Three-day Study (2015)</b>	
Multiplex Fluorescent Immunoassay (MFI)	
Ectromelia virus	1 week postarrival
EDIM	1 week postarrival

Method and Test	Time of Collection
LCMV	1 week postarrival
<i>M. pulmonis</i>	1 week postarrival
MHV	1 week postarrival
MNV	1 week postarrival
MPV	1 week postarrival
MVM	1 week postarrival
PVM	1 week postarrival
REO3	1 week postarrival
TMEV	1 week postarrival
Sendai	1 week postarrival

## C.2. Results

All test results were negative.

## **Appendix D. Supplemental Data**

Tables with supplemental data can be found here: <https://doi.org/10.22427/NTP-DATA-IMM-01>.

### **D.1. MCHM Pure – I14001**

I01 – Animal Removal Summary (32 KB)

I04 – Mean Body Weight Summary (51 KB)

I04G – Mean Body Weight Gain (45 KB)

I05 – Clinical Observations Summary (58 KB)

M01 – Irritancy Assay Summary (39 KB)

M02 – Hypersensitivity Assay Summary (32 KB)

### **D.2. MCHM Pure – I14001 Individual Animal Data**

Individual Animal Body Weight Data (5 MB)

Individual Animal Clinical Observation Data (24 KB)

Individual Animal Hypersensitivity Data (11 KB)

Individual Animal Irritancy (15 KB)

Individual Animal Removal Reasons (10 KB)

### **D.3. MCHM Crude – I14013**

I01 – Animal Removal Summary (31 KB)

I04 – Mean Body Weight Summary (65 KB)

I04G – Mean Body Weight Gain (53 KB)

I05 – Clinical Observations Summary (71 KB)

M01 – Irritancy Assay Summary (44 KB)

M02 – Hypersensitivity Assay Summary (35 KB)

### **D.4. MCHM Crude – I14013 Individual Animal Data**

Individual Animal Body Weight Data (5 MB)

Individual Animal Clinical Observation Data (32 KB)

Individual Animal Hypersensitivity Data (13 KB)

Individual Animal Irritancy (15 KB)

Individual Animal Removal Reasons (11 KB)

#### **D.5. MCHM Crude – I14013B**

I01 – Animal Removal Summary (29 KB)

I04 – Mean Body Weight Summary (54 KB)

I04G – Mean Body Weight Gain (47 KB)

I05 – Clinical Observations Summary (50 KB)

M01 – Irritancy Assay Summary (40 KB)

M02 – Hypersensitivity Assay Summary (32 KB)

#### **D.6. MCHM Crude – I14013B Individual Animal Data**

Individual Animal Body Weight Data (19 KB)

Individual Animal Clinical Observation Data (55 KB)

Individual Animal Death Details (13 KB)

Individual Animal Hypersensitivity (13 KB)

Individual Animal Irritancy (21 KB)

Individual Animal Removal Reasons (100 KB)



# National Toxicology Program

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