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Toxicity Study No. S.005223.2-19, March 2020
Protocol No. 0FMA-92-iv17-03-01S, 2758-70-iv19-04-01F and 49-iv19-03-01E

Tiered *In Vitro* Toxicity Testing of the Novel Energetic 1-methyl-2,4,5-trinitroimidazole (MTNI) April–September 2019

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General Medical: 500C, Specialty; Toxicity Study

APHC FORM 432-E, JAN 18

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
03/24/2020	Toxicology Study	April 2019-September 2019

4. TITLE AND SUBTITLE
Toxicity Study No. S.005223.2-19, March 2020
Protocol No. 0FMA-92-iv17-03-01S, 2758-70-iv19-04-01F and 49-iv19-03-01E
Tiered In Vitro Toxicity Testing of the Novel Energetic 1-methyl-2,4,5-trinitroimidazole (MTNI)

5a. CONTRACT NUMBER
MIPR W74RDV5291790

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER
WP18-C3-1203

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Public Health Center, Toxicology Directorate
8252 Blackhawk Rd, Aberdeen Proving Ground, MD 21010-5403

8. PERFORMING ORGANIZATION REPORT NUMBER
S.0058223.2-19

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Strategic Environmental Research and Development Program (SERDP)
4800 Mark Center Drive, Suite 17D03
Alexandria, Virginia 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)
CCDC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution A: Approved for public release

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The Army is identifying replacements for substances causing environmental and/or occupational risks. IMTNI was evaluated in several in vitro test systems, including the Ames mutagenicity assay, the Microtox® Acute Toxicity Test System, and the human cell line activation test (h-CLAT) for skin sensitization. Data from the h-CLAT were also utilized to predict a Hazard Category for acute oral toxicity. MTNI was found to be extremely toxic in all assays completed, mutagenic in the Ames, and a sensitizer in h-CLAT. The high aquatic toxicity combined with high solubility of MTNI causes concern for environmental and groundwater contamination. Additional aquatic testing should be conducted. Skin contact with MTNI should be avoided.

15. SUBJECT TERMS
Ames, genotoxicity, mutagenicity, Microtox, aquatic toxicity, skin sensitization, MTNI

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Valerie H Adams
					19b. TELEPHONE NUMBER *(Include area code)*
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Sponsor

Strategic Environmental Research and Development Program (SERDP)
4800 Mark Center Drive, Suite 17D03
Alexandria, Virginia 22350-3605

Study Title

Toxicology Study No. S.0058223.2-19 - Tiered In Vitro Toxicity Testing of the Novel Energetic 1-Methyl-2,4,5-trinitroimidazole (MTNI)

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Study Completed

December 2019

Performing Laboratory

U.S. Army Public Health Center
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Laboratory Project ID

Protocol No. 0FMA-92-iv17-03-01S
Protocol No. 2758-70-iv19-04-01F
Protocol No. 49-iv19-03-01E

Good Laboratory Practice Compliance Statement

The studies described in this report were conducted in compliance with Title 40, Code of Federal Regulations, Part 792, Good Laboratory Practice Standards, except for the following:

1. The test article characterization (purity) was conducted by the manufacturer, and it is not known whether the testing was done in compliance with the above regulation.

2. Due to time constraints, the method of analysis for these compounds could not be validated by the Laboratory Sciences Directorate (LAB) prior to the study start in compliance with study protocol and modification requirements. At the time this report was finalized, LAB had not achieved the capability to provide concentration verification for MTNI.

No deviations from the aforementioned regulation affected the quality or integrity of the study or the interpretation of the results.

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Standard Acronyms & Abbreviations

AFNOR	Association Française de Normalisation
Bp	boiling point
DA	Department of the Army
DIN	Deutsches Institut für Normung
DOD	Department of Defense
ECOSAR	Ecological Structure Activity Relationship
EC_{50}	median (50%) effect concentration
ESOH	environmental safety and occupational health
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
GHS	Global Harmonization System
GLP	Good Laboratory Practice
K_H	Henry's law constant
h-CLAT	human cell line activation test
IC_{50}	median (50%) inhibitory concentration
ISO	International Organization for Standardization
kg	kilogram
L	liter
LD_{50}	median (50%) lethal (oral) dose
log K_{OC}	Log Organic carbon partition coefficient
log K_{OW}	Log Octanol-water partition coefficient
LOAEL	lowest-observed adverse effect level

MFI	mean fluorescence intensity
µg	micrograms
µL	microliter
µM	micromolar
mg	milligram
mL	milliliter
mM	millimolar
MW	molecular weight
MRL	minimum risk level
NOAEL	no-observed adverse effect level
NOEL	no-observed effect level
NVN	Nederlandse voornorm
OECD	Organization for Economic Co-operation and Development
PI	propidium iodide
QSAR	Quantitative Structure-Activity Relationship
RDT&E	research, development, technology, and evaluation
RfD	reference dose
RFI	relative fluorescence
SD	standard deviation
USEPA	U.S. Environmental Protection Agency
USFDA	U.S. Food and Drug Administration

TOXICOLOGY STUDY NO. S.0058223.2-19
TIERED *IN VITRO* TOXICITY TESTING OF THE NOVEL ENERGETIC
1-METHYL-2,4,5-TRINITROIMIDAZOLE (MTNI)
APRIL–SEPTEMBER 2019

1 SUMMARY

1.1 Overview

The energetic and toxicological properties of 1-methyl-2,4,5-trinitroimidazole (MTNI) are under assessment as a replacement for energetics in current use, such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). This study evaluated the toxicity of MTNI in several *in vitro* test systems, including the Ames mutagenicity assay, the Microtox® Acute Toxicity Test System, and the human cell line activation test (h-CLAT) for skin sensitization. Data from the h-CLAT were also utilized to predict a Hazard Category (UNECE 2015) for acute oral toxicity. Data from this study are used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for further development of new munition compounds.

1.2 Purpose

This study provides toxicology data to support environmental and occupational health assessment on MTNI as a new or replacement energetic compound for military use. This information is critical to the research, development, testing, and evaluation (RDT&E) of munition formulation alternatives. This study addresses, in part, the environmental safety and occupational health (ESOH) requirements outlined in Department of the Army Regulation (AR) 200-1 (DA 2007b); AR 40-5 (DA 2007a); and AR 70-1 (DA 2018); Department of Defense Instruction (DoDI) 4715.4 (DoDI 2018); and Army Environmental Research and Technology Assessment (AERTA) requirement PP-3-02-05 (AERTA 2018). This program is under the direction of the Department of Defense (DOD) Strategic Environmental Research and Development Program (SERDP).

Research, development, testing, training, and use of substances potentially less hazardous to human health and the environment is vital to the readiness of the U.S. military. Safeguarding the health of Soldiers, Civilians, and the environment requires an assessment of alternatives before they are fielded. Continuous phased assessments, begun early in the research development test & evaluation (RDT&E) process, can save significant time and effort during RDT&E, as well as over the life cycle of the items developed. Residues of pyrotechnics, propellants, explosives, and incendiaries have been found in soil, air, surface, and groundwater samples, creating environmental problems and interfering with training activities.

The DOD is identifying replacements for substances causing environmental and/or occupational health hazards. This toxicology study evaluated MTNI mutagenicity, acute aquatic toxicity, and skin sensitization hazard using *in vitro* methods and following Good Laboratory Practice (GLP) regulations.

Use of trademarked name(s) does not imply endorsement by the U.S. Army but is intended only to assist in identification of a specific product.

1.3 Conclusions

This study reports the *in vitro* toxicity evaluation for the new energetic compound MTNI in the Ames assay for mutagenicity, the Microtox Acute Aquatic Toxicity assay, and the h-CLAT skin sensitization assay. MTNI was found to be mutagenic via Ames assay, extremely toxic with an EC_{50} of 0.035 mg/L for acute aquatic toxicity and is considered a skin sensitizer following positive results in the h-CLAT. Using *in silico* methods, the predicted acute oral toxicity was moderate—Category 3 by the GHS. As such, MTNI is considered a hazard for mammalian and aquatic life and likely to be mutagenic. Table 1 provides key toxicity values for TNT, DNP, and MTNI, although not all equivalent toxicity endpoints have been collected for these compounds.

Table 1. Comparative Toxicity for MTNI, DNP, and TNT

Compound	Genotoxicity	Skin sensitization		Acute mammalian toxicity (confidence)	Acute aquatic toxicity
MTNI	positive	positive		III (low))	I
DNP	positive	positive		IV (high)	III
TNT	positive	negative		IV (high)	II

1.4 Recommendations

MTNI was found to be extremely toxic in all assays completed, mutagenic in the Ames, and a sensitizer in h-CLAT. The high aquatic toxicity combined with high solubility of this compound in water causes concern for environmental release and transport into groundwater. These data are also supported by QSAR modeling. MTNI material should be handled with care. Conduct acute and 14-day rat studies and aquatic toxicity tests (fish, algae, and invertebrate) to reduce uncertainty and better characterize toxicity.

2 REFERENCES

See Appendix A for list of references.

3 AUTHORITY

Military Interdepartmental Purchase Request No. W74RDV5291790. This toxicology report addresses, in part, the ESOH requirements outlined in DoDI 4715.4 (DoDI 2018), AR 200-1 (DA 2007b); AR 40-5 (DA 2007a); and AR 70-1 (DA 2018); and AERTA Requirement PP-3-02-05 (AERTA 2018). It was conducted as part of an on-going effort by SERDP.

4 BACKGROUND

Current regulations require the assessment of human health and environmental effects arising from exposure to substances in soil, surface water, and ground water. Applied after an item has been fielded, these assessments can reveal the existence of adverse environmental and human

health effects that must be addressed, often at substantial cost. It is more efficient to begin the assessment of exposure, effects, and environmental transport of military-related compounds/substances early in the RDT&E process to avoid unnecessary costs, conserve physical resources, and sustain the health of those potentially exposed. A goal of this program is to investigate new compounds for operational and/or environment, safety, and occupational health issues. The candidates under development for high-density energetics include MTNI.

National defense requires the development of unique energetic compounds to perform specialized mission requirements. These requirements include the sustainable use of these materials in the environment, particularly during training operations. The use of RDX and TNT in warheads is a concern due to their ability to contaminate groundwater and, thus, enter into the drinking water supply. Unexploded ordnance and low-order detonations have become sources of ground water contamination and have affected drinking water resources.

The Centers for Disease Control and Prevention, Agency for Toxic Substances and Disease Registry (ATSDR), has developed an acute oral MRL for RDX of 60 µg/kg-day based on its epileptiform seizure neurotoxicity in humans and rodents (Burdette et al. 1988; Kasuske et al. 2009; Stone et al. 1969; Williams et al. 2011). The USEPA has derived a chronic RfD of 3 µg/kg-day based prostatic inflammation in rodents. RDX is also classified as a possible carcinogen (USAMRDC 1984; Parker et al. 2006).

TNT is acutely toxic to rats causing ataxia, tremors, and mild convulsions; the LD_{50} values range from 660 to 1,320 mg/kg. The subchronic and chronic oral RfD is 0.5 µg/kg-day based on a LOAEL of 0.5 mg/kg-day for liver effects in dogs. TNT is classified in weight-of-evidence Group C, possible human carcinogen (Lima et al. 2011; RAIS 2012).

The SERDP is dedicated to finding replacements for RDX and TNT that will reduce or eliminate ESOH risks and decrease potential impacts on readiness and the costs associated with training (USACHPPM 2007). The energetic and toxicological properties of MTNI are being evaluated as potential replacements for TNT and RDX. Toxicity tests can be conducted *in vivo* and *in vitro*. *In vitro* methods have the advantage of being relatively inexpensive, high-throughput, and capable of addressing many mechanistic issues at the cellular and molecular level. *In vitro* tests are ideally suitable and effective toxicity screening tools, especially when limited quantities of a compound are available. By identifying ESOH effects early in the acquisition process, unacceptable, or “regrettable,” replacement compounds can be identified. The U.S. Army Public Health Center (APHC) Toxicology Directorate (TOX) has been tasked with generating *in vitro* toxicity data for MTNI to determine its potential negative human and environmental effects. The data from these studies inform recommendations for the continued development and additional toxicity testing of MTNI that supports the appropriate hazard classification and exposure guidance.

The Ames test for mutagenicity (Ames et al. 1975) is a method widely accepted for evaluating mutagenic potential by the USEPA (2012), the USFDA (2007), and the OECD 1997). Historically, the mutagenicity of test materials has been evaluated in the agar plate-based Ames assay (Ames et al. 1975). The modified bacteria used in this test, *Salmonella typhimurium (S.t.)* and *Escherichia coli (E.c.)*, have point mutations in the histidine (*his*) and tryptophan (*trp*) operons, rendering the bacteria incapable of producing the amino acids histidine or tryptophan,

respectively. A chemical's mutagenic potential is assessed by exposing modified *S.t*. and *E.c.* organisms to varying concentrations of a test chemical and selecting for reversion events. Reversion occurs primarily through two molecular mechanisms: single base substitutions or frameshift mutations within the *his* or *trp* operons. Xenometrix has developed a proprietary MPF™ Ames test that provides a convenient, high-throughput capability for mutagenicity testing (Xenometrix 2012). The test uses the same strains of bacteria required by the USEPA (*S.t.*TA98, TA100, TA1535, TA1537, and a composite of *E.c.* pKM101/*uvrA* strains) and reduces the assay to a simple, non-agar, 384-well plate methodology that is expedient and cost effective.

The Microtox test system uses a strain of naturally occurring bioluminescent bacteria, *Aliivibrio fischeri*. *A fischeri* was formerly named *Vibrio fischeri;* the reagent supplier (Modern Water) still refers to it as *V. fischeri*. The marine bacterial bioluminescence is tied directly to cellular respiration, which is fundamental to cellular metabolism and associated life processes. These nonpathogenic, marine, bioluminescent bacteria are sensitive to a broad range of toxicants resulting in a decreased rate of respiration and a corresponding decrease in the rate of luminescence. Reducing the microorganism's light emission is proportional to the toxicity expressed as EC_{50}. This test has been shown to be an effective screening tool in assessing toxicity of varied chemical compounds comparing with other bioassays. The bacterial bioluminescence aquatic toxicity test has been validated by the industrial, academic, and governmental testing communities; the test achieved official "Standards Status" in several countries including an ASTM Standard (D-5660; withdrawn), ISO 11348-3 and Standard Method 8050 in the United States, AFNOR T90-320 in France, NVN 6516 (withdrawn) in the Netherlands, and DIN 38412 (Germany).

During a skin sensitizing reaction, activated dendritic cells migrate to the lymph node where the major histocompatibility complexes, which they are presenting, activate T-cells and T-cell proliferation. Secondary exposure to the chemical will then result in inflammation and an allergic reaction. Using adverse outcome pathway analysis, four key events for skin sensitization have been identified (OECD 2012). *In vitro* assays for each step have been developed and validated. The h-CLAT is an *in vitro* assay for key event 2 that measures the test chemical mediated dendritic cell activation via increased expression of CD54 and CD86 on the cell surface (OECD 2012). The presence of CD54 and CD86 proteins on the cell surface is detected with flow cytometry using fluorescently labelled antibodies specific for CD54 and CD86 (Ashikaga et al. 2010; ECVAM DB-ALM 2014; OECD 2018). The threshold criteria for a positive reaction in h-CLAT requires a 2-fold induction of CD54 and/or a 1.5-fold induction of CD86 compared to solvent controls. Multiple skin sensitization assays can be utilized to determine skin sensitization hazard in a tiered testing strategy that forms a defined approach. USEPA has recently accepted two defined approaches for submission and registration to predict hazard, in one, the h-CLAT is the first in a tiered strategy, where a positive result allows for a hazard determination to be made and no further testing is required (USEPA 2018). The h-CLAT can also be utilized to predict acute oral toxicity from the cytotoxicity data produced in the course of conducting the assay.

This report describes the toxic effect of MTNI in the Ames mutagenicity assay, the Microtox assay, and the h-CLAT. Table 2 identifies the critical events and dates of these studies.

Table 2. Critical Events

Critical Event	Date of Event (Ames)	Date of Event (Microtox)	Date of Event (h-CLAT)
Type-Protocol Modification Approved	1 July 2019	25 May 2019	21 May 2019
Study Start Date	2 July 2019	25 May 2019	21 May 2019
Experimental Start Date	2 July 2019	10 June 2019	21 May 2019
Experimental Completion Date	6 July 2019	19 June 2019	18 June 2019
Study Completion Date	December 2019	December 2019	December 2019

5 MATERIALS

5.1 Quality Assurance

APHC policy requires that all experiments and studies conducted by any element of APHC will be compliant with the applicable GLP Standard guideline (Memorandum 2018). For this study, the test article dictates that the following GLP guideline applies (CFR 1989):

Code of Federal Regulations (CFR). 1989. Title 40: Protection of Environment, Part 792, Good Laboratory Practice Standards

According to this policy and so that these results may be used in regulatory decisions involving the USEPA, these assays were conducted in compliance with GLP standards and followed the appropriate regulatory testing guidelines.

In compliance with the GLP requirements, the APHC Quality Systems Office audited critical phases of this study. Appendix B provides the Quality Assurance Statements, which include the dates of these audits, the audited phases, and the audited dates that the results were reported to Management and the Study Director. Appendix C provides the additional Quality Assurance/GLP requirement of archives location as well as the names of personnel contributing to the performance of this study.

5.2 Test Substance

Synthesis of MTNI (Chemical Abstracts Service Registry Number [CASRN] not found) was performed at Picatinny Arsenal, New York. Purity analysis for MTNI was not available. Figure 1 shows the MTNI molecular structures. MTNI is a nitroimdazole, which is a class of compounds that have antibiotic activity through a DNA damage mode of action (Lamp et al. 1999).

MTNI was readily soluble at 500 mg/mL in dimethyl sulfoxide (DMSO), solubility was likely higher; however, 500 mg/mL is the test concentration limit for the assays described herein. Aqueous solubility was determined for the Ames assay and used for setting the high dose in the Microtox and h-CLAT tests (APHC 2019b, 2017b). Concurrent with each test, the most dilute serial dilution was frozen at -80°C for concentration verification by the APHC Method Development Section Client Services Division (APHC-MDV-CSD).

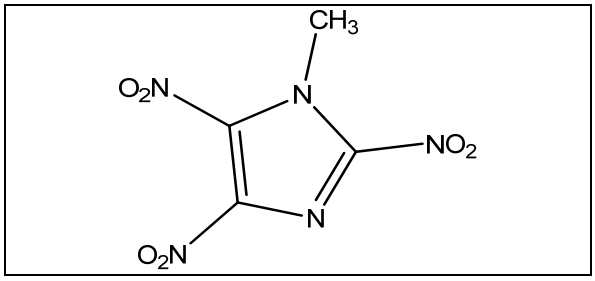

Figure 1. Molecular Structure of MTNI

5.3 Test System (Ames)

Xenometrix MPF™ kits were obtained from ANIARA (Mason, Ohio). The kit contained a quality assurance certificate for *S.t.* TA100. Appendix D provides a list of the kit reagents and supplementary reagents with expiration dates. All reagents were stored in refrigerators and freezers according to directions in the kit as described in the Toxicology Standing Operating Procedure (TOX SOP) 068 (APHC 2017b). BacTiter-Glo™ kits were obtained from Promega Corporation (Madison, Wisconsin) and contain a reagent that becomes luminescent when activated by incubation with adenosine triphosphate (ATP) in lysed bacteria.

5.4 Test System (Microtox)

The Microtox Acute Toxicity Test reagent and associated media and solutions were obtained from Modern Water, Inc., New Castle, Delaware. The reagent is a freeze-dried preparation of a specially selected strain of the marine bacterium *A. fischeri*. Appendix D provides a list of media, solutions, and other necessary test materials with expiration dates and lot numbers. All reagents were stored according to manufacturer instructions as described in the Toxicology Standing Operating Procedure (TOX SOP) 037 and study protocol (APHC 2017a, 2017c).

Zinc sulfate is the recommended standard or positive control for the test system. The zinc sulfate standard was purchased from Sigma-Aldrich (St. Louis, Missouri). Each vial of lyophilized *A. fischeri* was tested against the standard following reconstitution. Only vials with a calculated EC_{50} of 2–10 mg/L at 15 minutes were qualified for further use.

5.5 Test System (h-CLAT)

THP-1 cells were acquired from the American Type Tissue Collection (Manassas, Virginia). Appendix D provides a list of media, solutions, and other necessary test materials with expiration dates and lot numbers. All tissue culture reagents were acquired from Gibco, a subsidiary of ThermoFisher (Waltham, Massachusetts). Cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 µ/mL penicillin, 10 µg/mL streptomycin, and 0.05 mM 2-mercaptoethanol. All cells, reagents and chemicals were stored according to manufacturer's instructions (APHC 2017c). Dinitrochlorobenzene (DNCB) and nickel sulfate ($NiSO_4$) are the preferred positive control chemicals for the reactivity check, while DNCB is the positive control for the full test. Lactic acid (LA) is the negative control for the reactivity check. All chemicals were obtained from Sigma Aldrich.

6 METHODS

6.1 Ames

The experimental design and general procedures of this study were conducted under the APHC TOX SOP for the Xenometrix MPF Ames Test Kit (APHC 2017b). The test kit is designed to determine the mutagenicity of a test material in compliance with the USEPA (2012) and OECD guidelines for the Bacterial Reverse Mutation Test (OECD 1997), APHC TOX Type Protocol: "*The Ames Test For Mutagenicity # 2758-70-iv19-04-01*" (APHC 2019b) and modifications. The modifications to the protocol are approved and signed by the Study Director. The electronic and hard copy versions of the protocol modifications are saved and archived with the protocol and the raw data.

6.1.1 Sample Preparation

Solutions of 50-mg/mL MTNI in DMSO were first serially diluted in DMSO [six (6) half-log, (1:3.12), serial dilutions] and then further diluted 25X in Ames Exposure Medium without apparent precipitation. These final diluted solutions represent the exposure concentrations with final concentrations ranging from 6.8 to 2,000 µg/mL (nominal).

6.1.2 Validation of Cell Growth

The frozen TA100 bacterial stock was thawed according to TOX SOP 068 (APHC 2017b) and expanded in Xenometrix growth medium with shaking at approximately 37°C overnight with or without ampicillin as appropriate for each strain. The following morning, evidence of sufficient bacterial growth was assessed measuring the optical density at 600 nanometers (nm) (OD_{600}; BioMate™ 3S spectrophotometer, Fisher Scientific). $OD_{600} \geq 2.0$ indicated sufficient density for the Xenometrix Ames Assay, although lower OD_{600} values may also be acceptable. Per personal conversation with the manufacturer, sub-threshold growth could be corrected by increasing the volume of each bacterial suspension to the final incubation solution proportionately to provide an appropriate density of bacteria.

6.1.3 Compound Incubation

The bacterial suspension was diluted with Xenometrix incubation media and aliquoted to tissue culture wells containing serial dilutions of 25X concentrates of the test material. The suspensions were incubated with shaking for 90 minutes at approximately 37°C with and without the inclusion of S9 liver extract (±S9); inclusion of S9 determines if a mutagen is generated from the parent compound due to metabolism by liver enzymes. At the end of the incubation, each well was diluted 11-fold with purple Indicator Medium and 50 µL aliquots distributed appropriately into 384-well plates. Plates were incubated for 2 days at approximately 37°C and then scored for presence of revertant colonies (i.e., evidence of a positive mutagenic event). Positive wells were indicated by a color change (purple to yellow) or a visible bacterial colony. The indicator media turn from purple to yellow due to a pH change resulting from active metabolism of revertant bacteria.

6.1.4 Criteria for a Positive Mutagenic Event

A positive control appropriate for TA100 was included alongside the test material to assure the assay was valid for each run; positive controls are run in triplicate for each strain with and without the S9 microsomal fraction. The assay as a whole is considered valid if both the number of control background reversions and the number of positive control reversions are within prescribed limits (APHC 2019b, 2017b).

The criteria to determine a positive result include:

- The number of reversions induced by the test compound is at least 2-fold above the background control;
- A concentration-related increase over the range tested; and/or
- A reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system.

Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response. Results from test substances that do not meet the above criteria are considered nonmutagenic (Xenometrix 2012).

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgment about the activity of the test substance. Results may remain equivocal or questionable regardless of the number of times the experiment is repeated. Positive results from the bacterial reverse mutation test indicate that a substance induces point mutations by base substitutions and/or frame shifts in the genome of *Salmonella.* Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species.

6.1.5 Determination of Cytotoxicity

Coincident with the test incubation, a plate is prepared for determination of cytotoxicity of the test material using ATP luminescence. After a 90-minute incubation at approximately 37°C, samples from the cytotoxicity plate are aliquoted to a 96-well plate. An equal volume of luminescent reagent is added to each well according to the method described for the BacTiter-Glo Microbial Cell Viability Assay, and bioluminescence is measured via plate reader.

6.1.6 Data Analysis

Raw scores for color change (purple to yellow) were entered into an Excel® spreadsheet containing statistical algorithms to calculate mean, SD baseline, and fold change. These data were then graphed using GraphPad Prism® version 5.04.

6.2 Microtox Assay

6.2.1 Experimental Design

The experimental design and general procedures of this study were conducted under the APHC TOX SOP for the Microtox Acute Toxicity Assay (APHC 2017a). The test kit is designed to determine the aquatic toxicity of a test material in compliance with the APHC TOX Type Protocol: "Microtox Toxicity Testing System" (APHC 2017c), and modifications. The modifications to the protocol are approved and signed by the Study Director. The electronic and hard copy versions of the protocol modifications are saved and archived with the protocol and the raw data.

6.2.2 Range Finding

MTNI was dissolved in DMSO at the assay test limit (200 mg/mL). The solubility of MTNI was determined previously in the Ames test (APHC 2017b). MTNI was serially diluted 1:2 in DMSO and further diluted 1:100 in diluent. A total of eight concentrations were tested for the range finding. Reconstituted *A. fischeri* was added to each test concentration (10 µL), and samples were incubated and tested for luminescence at 5, 15, and 30 minutes using the Microtox Model 500 Analyzer (Modern Water, Inc.). The EC_{50} from the range finding determined the final test concentration range (see Appendix F for final chemical specific ranges).

6.2.3 Cytotoxicity Test

Following the range finding, MTNI was tested in duplicate on three separate days. On each testing day, 0.1 mg/mL MTNI in DMSO was prepared as the stock solution for the main test. Eight serial 1:2 dilutions into DMSO were made, and then each of these were diluted 1:100 into the diluent for testing. The highest tested concentration was approximately 33-fold higher than the EC_{50} determined in the range finding. Ten microliters reconstituted *A. fischeri* were added to each sample and luminescence measured at 5, 15, and 30 minutes as above.

6.2.4 Data Analysis

Raw luminescence data were recorded at 5, 15, and 30 minutes by the Microtox analyzer. The EC_{50} values at 5, 15, and 30 minutes were given by the MicrotoxOmni® software and further fitted to the Hill function using GraphPad PRISM 5.04. All data (prints and files) were archived.

6.3 h-CLAT

The assay was conducted in compliance with the APHC TOX Type Protocol: *In Vitro* Skin Sensitization Parts 1-3 (APHC 2019a). In the absence of an SOP, testing was performed according to ECVAM DB-ALM protocol number 158 and OECD Guideline 442E (ECVAM DB-ALM 2014; OECD 2018).

6.3.1 Buffers

The FACS buffer was prepared with phosphate-buffered saline (PBS) and 0.1% (weight/volume (w/v)) bovine serum albumin (BSA), the day before use and stored at +4 ± 2°C. Blocking solution was made up in 1% (w/v) globulins in PBS stocks as needed, with stock being used within 1 week and stored at +4°C. Blocking solution for use on the day of the experiment was diluted to a 0.1% solution in FACS buffer immediately prior to use. Propidium iodide (PI) was diluted to 12.5 µg/mL in PBS on the day of the experiment and maintained on ice.

6.3.2 Tissue Culture

Tissue culture media was prepared as described in section 5.5 and maintained at +4 ± 2°C. Media was pre-warmed at room temperature prior to use for each cell plating and passage. Cells were maintained at 1.5 x 10^5 – 8 x 10^5 cells/mL 37°C, 5% carbon dioxide (CO_2). Cells were passaged every 2–3 days for no more than 30 passages or 60 days. Prior to passage or test plating, cell density was determined by counting with the TC-20 automated cell counter (Bio-Rad, Inc., Hercules, California). Cell viability was determined by Trypan blue staining (Bio-Rad, Inc.). For all testing (i.e., reactivity check, range finding, and h-CLAT), cells were plated into 24-well plates at a density of 1 x 10^6 cells/well in 0.5 mL (i.e., 2 x 10^6 cells/mL). For maintenance, cells were plated at 1.5–2.0 x 10^5 cells/mL in 25–40-mL media depending on the timing of subsequent tests.

6.3.3 Reactivity Check

The reactivity check prior to full testing is used to confirm cell viability and induction of CD54 and CD86. Two weeks post thaw, a reactivity check of cells sampled from each propagation flask was performed using the control compounds: DNCB, $NiSO_4$, and LA. DNCB was prepared as a 20-mg/mL stock solution in DMSO and stored at +4°C in the dark. $NiSO_4$ was prepared as a 10-mg/mL stock solution in saline and stored at room temperature protected from light. LA was freshly prepared as a 100-mg/mL solution in saline. From these stock solutions, additional dilutions were made so that the tested concentrations were 3.3–4-µg/mL DNCB, 100-µg/mL $NiSO_4$, and 1,000 µg/mL for LA. A 100% cytotoxic DNCB concentration (0.2 mg/mL) was added to one well as a positive control. Negative controls (diluted DMSO and saline) were also included. After all dosing solutions were distributed to the test wells, the test plate was incubated (approximately 37°C / 5% CO_2) for 24 hours. Cells were then collected; processed; stained with PI and FITC labeled antibodies [anti-IgG1 (isotype control), anti-CD54, and anti-CD86]; and analyzed by flow cytometry (see sections 6.3.6 and 6.3.7). Criteria for a successful reactivity check requires the positive controls DNCB and $NiSO_4$ induce CD54 and CD86 (RFI criteria exceed: CD54 ≥ 200 and CD86 ≥ 150) and the negative control, LA, does not induce CD54 or CD86 or reduce viability by more than 50% [target ~75% viability (CV75)]. When the cell sample meets these criteria, the remainder of cells from its propagation flask are used for testing chemicals. Propagation flasks can be resampled if the first sample fails the reactivity check to confirm no or poor reactivity. A second fail is cause to discard that flask and thaw a new lot of cells.

6.3.4 Range Finding

The range finding test is used to bracket the appropriate dose range for the full test using only the percent viability endpoint. MTNI (200 mg/mL in DMSO) was prepared as the stock for eight serial dilutions (1:2, diluent = DMSO). Each dilution was subsequently diluted 1:250 into tissue culture media. Pooled THP-1 cells were plated at 1 x 10^6 cells/well (24-well plate). An equal volume (0.5 mL) of each final dilution was added to the appropriate test wells. Negative (vehicle) control and cytotoxicity ("dead cell") positive controls were also included on each test plate. Cells were incubated for 24 hours (approximately 37°C /5% CO_2). After the 24-hour incubation, cells were collected and processed for staining with PI. Briefly, cells were transferred to 5-mL tubes, centrifuged (200 x g; +4°C), and supernatants were discarded. Each pellet was resuspended in 0.6-mL cold FACS buffer, and 0.2 mL of each sample was transferred to new tubes and washed by centrifuging (200 x g; +4°C), decanting the supernatant, and resuspending the pellet in 0.2 mL FACS buffer. The wash step was repeated one time. The final pellets were resuspended in 0.4 mL FACS buffer and stained with 20 µL 12.5 µg PI/mL solution. Samples were maintained on ice in the dark and analyzed by flow cytometry (see section 6.3.7). Percent viability (ratio of live cells to total acquired cells) was utilized to determine the 75% cell viability (CV75). Where CV75 was not achieved due to compound toxicity, additional range finding tests with lower compound concentrations were conducted until the CV75 was identified. If cytotoxicity was not observed at the maximum concentration; then, by default, the maximum dose is used as the highest dose in the h-CLAT.

6.3.5 h-CLAT Test

In the full test, the CV75 from the range finding test is used to develop the dose range and represents the 2nd highest dose of an eight-dose treatment. For the MTNI range-finding assay, cytotoxicity was observed; therefore, the experimentally determine CV75 (0.009 mg/mL MTNI) was used to calculate the top dose (0.011 mg/mL final). MTNI was solubilized in DMSO at 5.5 mg/mL (500x). Eight 1:1.2 serial dilutions of MTNI were subsequently diluted 1:250 in complete media and added in equal volume to test wells containing 0.5 mL medium and 1 x 10^6 cells per well (24-well plate). Three concentrations of DNCB were prepared from the 20 mg/mL stock solution and added to the appropriate wells containing 1 x 10^6 cells (final concentrations 0.003, 0.004, and 0.0048 mg/mL DNCB in medium). A DMSO vehicle control was prepared as was a "dead cell" control containing 10 µL of the 20 mg/mL DMSO stock. Cells were incubated for 24 hours and processed for IgG1, CD54, and CD86 staining and analysis by flow cytometry (see sections 6.3.6 and 6.3.7).

6.3.6 Antibody Staining

Cells from each test well were transferred to individual 5-mL tubes and collected by centrifugation (250 x g/5 min/+4°C). The supernatants were discarded, and the pellets were resuspended in 1 mL-cold FACS buffer and washed 2x. Cells were then incubated with blocking solution (0.6 mL 0.1% blocking buffer) for 15 minutes at +4 ± 2°C. Following blocking, samples were prepared in triplicate (i.e., split into 3 aliquots) of 180–200 µL each in a round-bottom 96-well plate, centrifuged (250 x g/5 min/+4°C), and blocking buffer decanted. Samples were resuspended in 50-µL FACS buffer containing either IgG1, CD54, or CD86 antibodies as per the ECVAM protocol and gently vortexed, incubated at +4 ± 2°C in the dark for 30 minutes, and

washed twice in FACS buffer (ECVAM DB-ALM 2014). Samples were transferred to FACS analysis tubes between washes. Following the final wash, all samples were resuspended in 0.4-mL FACS buffer, stained with 20-µL PI, and mixed by vortexing. All samples were maintained on ice or at +4°C throughout the staining process.

6.3.7 Flow Cytometry

The fluorescence intensities of the labeled cells were analyzed by flow cytometry, using a BD FACSVerse™ flow cytometer, and captured/analyzed with BD FACSuite™ v1.0.5. The acquisition channels were FITC and PI. PI stained untreated cells were used to determine the correct voltages for the forward scatter and side scatter channels. The dead cell and media controls were used to gate live (PI negative) versus dead (PI positive) cells. For each sample, 10,000 live or 30,000 total counts (whichever count was acquired first) in the PI channel were acquired, and the geometric MFI for FITC was calculated. The cell viability for each test concentration was determined from the isotype (IgG1) stained sub-populations, which were co-stained with PI as per the section 6.3.6.

6.3.8 Data Analysis

If the RFI for any concentration exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150), the EC_{200} and EC_{150} were calculated using the validated calculation spreadsheet. If the EC_{200} or EC_{150} fell below the lowest dose, the values were extrapolated according to the ECVAM protocol (ECVAM DB-ALM 2014). Two independent experiments were completed for MTNI; the data from these two experiments were sufficient to determine sensitization, and a third experiment was not necessary.

6.3.9 Criteria for a Valid Assay

For a test to be acceptable, the following criteria were met:

- Cell viability of medium and DMSO controls was more than 90%.
- RFI values for the DNCB control for both CD54 and CD86 exceeded the positive criteria by ≥ 200% (CD54) and ≥ 150% (CD86).
- RFI values for the DMSO solvent control did not exceed positive criteria.
- The MFI ratio of both CD54 and CD86 to isotype control for DMSO and media controls exceeded 105%.
- The cell viability of at least 4 doses was greater than 50%.

6.3.10 Calculation of Acute Oral Hazard Category

The IC_{50} (level at which viability was reduced by 50%) was extrapolated from the cell viability of the range-finding experiments. From this IC_{50}, the following prediction model was used to predict the acute rodent toxicity:

$$\log LD_{50}\ (mg/kg) = 0.372 \log IC_{50}\ (\mu g/mL) + 2.024$$

This prediction model is based upon a rat-only weight regression as demonstrated in the validation project for the Neutral Red Uptake assay, an alternative cytotoxicity assay (ICCVAM 2006). This model was applied to the THP-1 cells of the uptake to determine a hazard category and not to provide an LD_{50} point estimate of rodent acute oral toxicity. The calculated LD_{50} was compared to the GHS categories of acute toxicity data and a category assigned (UNECE 2015; ICCVAM 2006).

6.4 Concentration Verification of MTNI

At the end of each test day for each assay, samples of the final serial dilution were collected and stored at -80°C for analysis by the APHC Client Services Division Method Development group. At the time of this report, verified test concentrations were not available so the nominal value has been used for reporting.

7 RESULTS AND DISCUSSION

7.1 Ames Assay

7.1.1 Cytotoxicity of MTNI

The cytotoxicity of MTNI to Salmonella had been previously reported (ARDEC 2008). TA100 was sensitive to MTNI and demonstrated reduced viability above 21.1 µg/mL. Presumptive metabolites of MTNI were of equal toxicity.

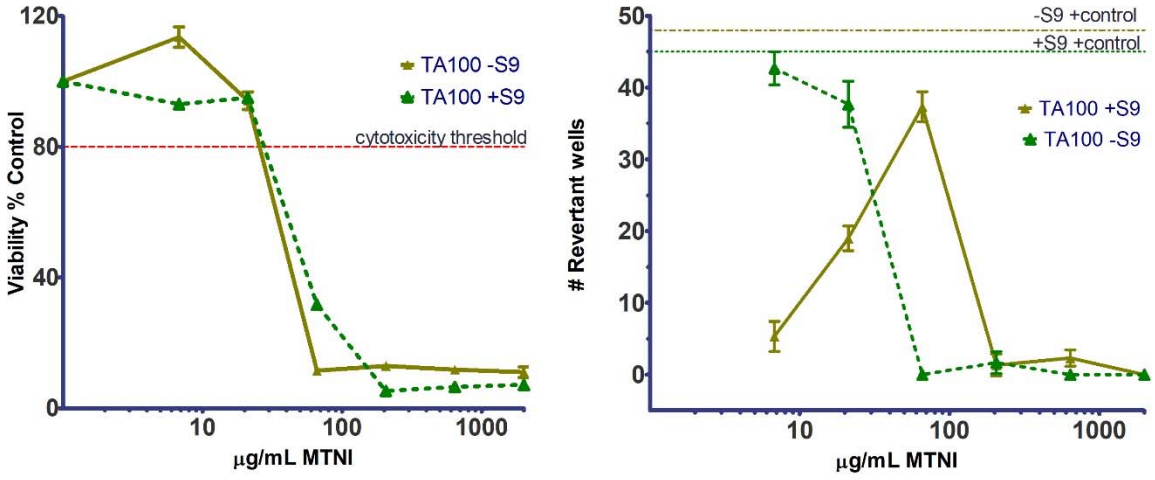

Figure 2. Cytotoxicity/Mutagenicity of MTNI to TA100 Left panel: Cytotoxicity of MTNI to TA100, defined as less than 80% viability (horizontal spark line) was observed at concentrations above 21.1 µg/mL in both test conditions. Right panel: Mutagenicity of MTNI to TA100 was observed at the lowest concentration tested (6.8 µg/mL) for TA100 –S9 and second lowest concentration (21.1 µg/mL) in the presence of S9 (+S9). The decline in revertants per well above 21.1 µg/mL is a result of the cytotoxicity of MTNI.

7.1.2 Mutagenicity of MTNI

The mutagenicity of MTNI was previously reported (ARDEC 2008). Data presented in this report confirmed the previous finding of mutagenicity. Appendix E provides the data (transcribed data from the hard copy, hand written raw data in the lab book) for the mutagenicity tests and calculations. MTNI was mutagenic in TA100 in both the –S9 or +S9 exposures. MTNI mutagenicity was reduced in the presence of +S9, suggesting that it is partially detoxified by liver enzymes. The threshold of mutagenicity was not identified for the –S9 condition. The threshold for mutagenicity in the +S9 condition was 21.1 µg/mL. These data are comparable to the ARDEC reported mutagenicity at 5 µg/plate (equivalent to ~1.8 µg/mL) in the –S9 condition.

7.2 Microtox Toxicity and Risk Assessment

Toxicity of MTNI to marine bacteria, *A. fischeri*, was measured by the Microtox acute toxicity test system at 5, 15, and 30 minutes. For each test compound, three individual experiments were performed in duplicate. Table 3 presents the toxicity data (EC_{50} and the 95% Confidence Interval) and risk assessment. Appendix F presents the analyzed data for Microtox.

Comparisons of toxicity results using these methods for a variety of compounds found that *A. fischeri* were, in most cases, more sensitive than other aquatic organisms (Dutka and Kwan 1981; McFeters et al. 1983; Riva et al. 2007). Thus, the results with Microtox tests are often useful screens in the assessment of relative toxicity to aquatic organisms. We used the aquatic toxicity criteria of the USEPA, the OECD, and the GHS to categorize the potential ecotoxicity of these new compounds (Table 4) (USEPA 2017; OECD 2001; UNECE 2015). This evaluation suggests that MTNI is extremely toxic according to USEPA categories (Acute Category 1 by GHS, Table 3).

Table 3. Microtox Toxicity and Risk Assessment

Compound	Microtox EC_{50} (mg/L) [95% CI]			USEPA Hazard Categories	OECD Hazard Classes	GHS Acute Aquatic Toxicity
	5 min	15 min (used for risk assessment)	30 min			
MTNI	0.064 [0.047-0.085]	0.035 [0.025-0.049]	0.033 [0.024-0.046]	Extremely Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. 1

Table 4. Ecotoxicity Assessment Scale

LC_{50} or EC_{50} Concentration Range (mg/L)	USEPA Hazard Categories	OECD Hazard Classes	GHS Acute Aquatic Toxicity
< 0.01	Super Toxic	Acute Toxicity I (very toxic to aquatic life)	Acute Cat. 1
0.01 to 0.1	Extremely Toxic		
0.1 to 1	Highly Toxic		
1 to 10	Moderately Toxic	Acute Toxicity II (toxic to aquatic life)	Acute Cat. 2
10 to 100	Slightly Toxic	Acute Toxicity III (harmful to aquatic life)	Acute Cat. 3
100 to 1000	Practically Nontoxic	—	—
> 1000	Relatively Harmless	—	—

7.3 h-CLAT

7.3.1 Reactivity Check

The THP-1 cells were checked and verified for reactivity to DNCB and $NiSO_4$ as well as a lack of reactivity to LA. Cells reacted as expected, with DNCB and $NiSO_4$ eliciting positive reactions for both CD54 and CD86, while LA was negative (see Appendix G for data).

7.3.2 Range-finding Test

Two independent dose-finding assays were completed to determine the CV75 of MTNI in THP-1 cells. Cytotoxicity was observed in the dosing range; therefore, a CV75 could be experimentally determined (0.009 mg/mL). These data were used to determine the top dose for the full assay (0.011 mg/mL). Appendix G shows the raw data for the Range Finding.

7.3.3 Full Test

Two independent h-CLAT assessments were completed for MTNI. MTNI was positive for both CD86 and CD54 expression, indicating a positive test and that MTNI is a skin sensitizer according to the defined approach. Appendix G presents the raw data. QSAR analysis predicted that MTNI is a skin sensitizer; no additional testing in other skin assays will need to be completed. Currently, data are reported for the nominal concentrations of the compounds because concentration verification has not yet been completed by Laboratory Sciences, Method Development Section [LS-MDV].

7.3.4 Acute Oral Hazard Designation

Mammalian acute oral toxicity was predicted using data collected in the h-CLAT. The estimated LD_{50} was 260.7 mg/kg, suggesting that MTNI has moderate oral toxicity (GHS Category 3; (UNECE 2015)).

8 CONCLUSIONS

In this study, MTNI was evaluated for mutagenicity, acute aquatic toxicity, and skin sensitization using QSAR and *in vitro* approaches. MTNI mutagenicity was predicted positive using TOPKAT™ and confirmed in the bacterial mutagenicity test. The ECOSAR prediction for the 96-hour fish LC_{50} was 135.7 mg/L, and acute category 1 (extremely toxic) (0.035 mg/L) for acute aquatic toxicity in the Microtox. MTNI is a nitroimidazole, which is a class of antibacterial compounds. The antimicrobial mechanism of 5-nitroimidazole-based compounds is from DNA strand breakage (Lamp et al. 1999). The observed toxicity in both the Ames and Microtox assays is consistent with the known action of the nitroimidazole chemical class. TOPKAT modeled MTNI as a skin sensitizer, which the h-CLAT result confirmed. Additionally, the hazard category algorithm for acute oral toxicity is predicted to be Category 3 by GHS. Therefore, MTNI is considered a hazard for mammalian and aquatic life and likely to be genotoxic.

MTNI was found to be extremely toxic in all assays completed, with positive results in the Ames assay for mutagenicity and in h-CLAT for skin sensitization. The high aquatic toxicity combined with high water solubility causes concern for environmental release and transport into groundwater. These data are also supported by QSAR modeling.

9 RECOMMENDATIONS

Although current data are exploratory and preliminary, any testing with this substances should include providing personnel with appropriate personal protective equipment to prevent skin, oral, and respiratory exposure to MTNI. Environmental releases should be avoided. Additional testing is recommended, especially for *in vivo* genotoxicity and aquatic toxicity (e.g., fish, invertebrates, and algae) concerns. With the current available toxicity information, it is unlikely that MTNI will reduce ESOH risk compared with currently used high explosives.

10 POINT OF CONTACT

Dr. Emily N. Reinke and Dr. Valerie H. Adams, Study Directors are the points of contact for this project. They may be reached at DSN 584-3980 or commercial 410-436-3980.

Toxicology Study No. S.0058223.2-19, April–September 2019

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APPENDIX A

REFERENCES

Army Environmental Requirements and Technology Assessments (AERTA). 2018. *Requirement PP-3-02-07, Compliant Ordnance Lifecycle for Warfighter Readiness.* U.S. Army Environmental Command. Aberdeen Proving Ground, Maryland. https://eqtp2.army.mil/programs/oep/

Ames, BN, J McCann, and E Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat Res* 31 (6):347-64.

U.S. Army Public Health Center (APHC). 2017a. TOX SOP 037*, Operation and Maintenance of the Microtox Analyzer*. Aberdeen Proving Ground, Maryland.

APHC. 2017b. TOX SOP 068*, Xenometrix AMES Test Kit*. Aberdeen Proving Ground, Maryland.

APHC. 2017c. Type Protocol Report No. 0FMA-92-iv-17-03-01, *Microtox Toxicity Testing System*. Aberdeen Proving Ground, Maryland.

APHC. 2019a. Type Protocol Report No. 49-iv19-03-01, *In Vitro Skin Sensitization Parts 1-3*. Aberdeen Proving Ground, Maryland.

APHC. 2019b. Type Protocol Report No. 70-iv19-04-01, *The Ames Test for Mutagenicity*. Aberdeen Proving Ground, Maryland.

Army Research Development Engineering Center (ARDEC). 2008. Evaluation of a Test Article in the *Salmonella typhimuriuml Escherichia coli* Plate Incorporation Mutation Assay in the Presence and Absence of Induced Rat Liver S-9 - MTNI. Prepared by: SITEK Research Laboratories, POC: P. Samuels Picatinny Arsenal, New Jersey.

Ashikaga, T, H Sakaguchi, S Sono, N Kosaka, M Ishikawa, Y Nukada, M Miyazawa, Y Ito, N Nishiyama, and H Itagaki. 2010. A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). *Altern Lab Anim* 38(4):275-84.

Burdette, LJ, LL Cook, and RS Dyer. 1988. Convulsant properties of cyclotrimethylenetrinitramine (RDX): spontaneous audiogenic, and amygdaloid kindled seizure activity. *Toxicol Appl Pharmacol* 92 (3):436-44.

Code of Federal Regulations (CFR). 1989. Title 40 CFR Part 792, Good Laboratory Practice Standards. U.S National Archives and Records Administration, Washington, DC.

Department of the Army (DA). 2007a. Regulation 40-5, *Preventive Medicine*. https://armypubs.army.mil/

DA. 2007b. Regulation 200-1, *Environmental Protection and Enhancement*. https://armypubs.army.mil/

DA. 2018. Regulation 70-1, *Army Acquisition Policy.* https://armypubs.army.mil/

Department of Defense Instruction (DoDI). 2018. Department of Defense Instruction 4715.23, *Integrated Recycling and Solid Waste Management (Cancels DoDI 4715.4 Pollution Prevention)*. http://www.dtic.mil/whs/directives/corres/pdf/471504p.pdf

Dutka, BJ and KK Kwan. 1981. Comparison of three microbial toxicity screening tests with the Microtox test. *Bull Environ Contam Toxicol* 27(6):753-7.

ECVAM DB-ALM. 2014. human Cell Line Activation Test (h-CLAT) DB-ALM Protocol No 158. Prepared by: European Union Reference Laboratory for Alternatives to Animal Testing,

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). 2006. *In Vitro Cytotoxicity Test Methods for Estimating Acute Oral Systemic Toxicity*. NIH Publication No 07-4518. http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_brd.htm

Kasuske, L, JM Schofer, and K Hasegawa. 2009. Two marines with generalized seizure activity. *J Emerg Nurs* 35(6):542-3.

Lamp, KC, CD Freeman, NE Klutman, and MK Lacy. 1999. Pharmacokinetics and pharmacodynamics of the nitroimidazole antimicrobials. *Clin Pharmacokinet* 36(5):353-73.

Lima, DR, ML Bezerra, EB Neves, and FR Moreira. 2011. Impact of ammunition and military explosives on human health and the environment. *Reviews on Environmental Health* 26(2):101-10.

McFeters, GA, PJ Bond, SB Olson, and YT Tchan. 1983. A comparison of microbial bioassays for the detection of aquatic toxicants. *Water Research* 17(12):1757-1762.

Memorandum, APHC Director (MCHB-PH-OOD), 6 December 2018, subject: Director's Policy Notice No. 70-2, Good Laboratory Practices.

Organization for Economic Cooperation and Development (OECD). 1997. *Guideline for testing of chemicals No. 471/472: Bacterial reverse mutation assay*. Prepared by: Paris, France, 471. www.oecd.org/dataoecd/18/31/1948418.pdf.

OECD. 2012. ENV/JM/MONO(2012)10/Part 1, *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins Part 1: Scientific Evidence*. Environment Directorate Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology. Paris, France https://read.oecd-ilibrary.org/environment/the-adverse-outcome-pathway-for-skin-sensitisation-initiated-by-covalent-binding-to-proteins_9789264221444-en#page1

OECD. 2018. Test Guideline 442E: In Vitro Skin Sensitisation: In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation. Paris, FR. https://www.oecd-ilibrary.org/content/publication/9789264264359-en

Parker, GA, G Reddy, and MA Major. 2006. Reevaluation of a twenty-four-month chronic toxicity/carcinogenicity study of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in the B6C3F1 hybrid mouse. *Int J Toxicol* 25(5):373-8.

Risk Assessment Information System (RAIS). 2012. Formal Toxicity Summary for 2,4,6-TRINITROTOLUENE. Prepared by: D M Opresko, Oak Ridge National Laboratory, Tennessee. http://rais.ornl.gov/tox/profiles/2_4_6_trinitrotoluene_f_V1.html.

Riva, MC, J Ribo, G Gilbert, and P Alanon. 2007. Acute toxicity of leather processing effluents on Vibrio fisheri and Brachydanio rerio. *Afinidad* 528:182-188.

Stone, WJ, TL Paletta, EM Heiman, JI Bruce, and JH Knepshield. 1969. Toxic effects following ingestion of C-4 plastic explosive. *Arch Intern Med* 124(6):726-30.

United Nations Economic Commission for Europe (UNECE). 2015. *The Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*. New York and Geneva. ISBN 978-92-1-117087-0. http://www.osha.gov/dsg/hazcom/ghs.html

U.S. Army Center for Health Promotion and Preventative Medicine (USACHPPM). 2007. Toxicology Report No. MA03N3-CA0700, *Environmental Health Evaluation for CA 07-00 (IM Fill DEMN) Formulation of New, Environmentally Benign Missile Propellants To Eliminate TNT-Based Fills*. Prepared by: Johnson, MS and HT Bausum, Aberdeen Proving Ground, Maryland.

U.S. Army Medical Research and Development Command (USAMRDC). 1984. Twenty-Four Month Chronic Toxicity/Carcinogenicity Study of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) in the B6C3F1 Hybrid Mouse, ADA No. 160774. [IITRI Project No L6121 study No 7 Contract No. DAMD17-79-C-9161.] Prepared by: Lish, PM, BS Levine, EM Marianna-Furedi, JM Sagartz, and VS Rac.

U.S. Environmental Protection Agency (USEPA). 2012. *Title 40 Code of Federal Regulations, Part 798, Health Effects Testing Guidelines, Subpart F, Sec. 798.5265, the Salmonella typhimurium reverse mutation assay.* Washington D.C. http://www.gpo.gov/fdsys/pkg/CFR-2012-title40-vol33/xml/CFR-2012-title40-vol33-sec798-5265.xml

USEPA. 2017. *Technical Overview of Ecological Risk Assessment, Analysis phase: Ecological effects characterization, ecotoxicity categories for terrestrial and aquatic organisms.* Office of Prevention, Pesticides, and Toxic Substances. Washington D.C.

USEPA. 2018. *Interim Science Policy: Use of Alternative Approaches for Skin Sensitization as a Replacement for Laboratory Animal Testing*. Office of Chemical Safety and Pollution Prevention. Washington, D.C.

U.S. Food and Drug Adminstration (USFDA). 2007. *Toxicological Principles for the Safety Assessment of Food Ingredients* Center for Food Safety and Applied Nutrition. 5001 Campus Drive, College Park, MD 20740.

Williams, LR, V Aroniadou-Anderjaska, F Qashu, H Finne, V Pidoplichko, DI Bannon, and MF Braga. 2011. RDX Binds to the GABAA Receptor-Convulsant Site and Blocks GABAA Receptor-Mediated Currents in the Amygdala: A Mechanism for RDX-Induced Seizures. *Environmental Health Perspectives* 119(3):357-63.

Xenometrix. 2012. *Ames MPF™ Penta I Microplate Format Mutagenicity Assay Instructions for use. Version 4.5_S*. Aniara Diagnostica, 7768 Service Center Drive, West Chester, OH 45069
https://www.aniara.com/mm5/PDFs/IFU/IFU_AC10-512.pdf

APPENDIX B

QUALITY ASSURANCE STATEMENT
AMES ASSAY

For: Toxicology Study No. S.0058223.2-19, Protocol No. 2758-70-iv19-04-01F Ames mutagenesis screen of 1-Methyl-2,4,5-trinitroimidazole (MTNI) the following critical phases were inspected/audited by the Quality Systems Office (QSO):

QUALITY ASSURANCE STATEMENT
MICROTOX ASSAY

For: Toxicology Study No. S.0058223.2-19, Protocol No. 0FMA-92-iv17-03-01S, Microtox Toxicity Testing of the Novel Energetic 1-Methyl-2,4,5-trinitroimidazole (MTNI) the following critical phases were inspected/audited by the Quality Systems Office (QSO):

QUALITY ASSURANCE STATEMENT
H-CLAT ASSAY

For: Toxicology Study No. S.0058223.2-19, Protocol No. 49-iv19-03-01E, h-CLAT Skin Sensitization Testing of the Novel Energetic 1-Methyl-2,4,5-trinitroimidazole (MTNI) the following critical phases were inspected/audited by the Quality Systems Office (QSO):

APPENDIX C

ARCHIVES AND STUDY PERSONNEL

C-1. ARCHIVES

All raw data, documentation, records, protocols, contributing scientist reports, and a copy of the final report generated as a result of this study will be archived in the storage facilities of the U.S. Army Public Health Center (APHC) Toxicology Directorate (TOX), for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

Records on the test system will be archived by TOX for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

The present study used the Toxicology Study No. S.0002728-15, Protocol Nos. 0FMA-92-iv1703-01S, 2758-70-iv19-04-01F, 49-iv19-03-01E.

The protocol, raw data, summary data, and the final report pertaining to this study will be physically maintained within Building E-2100, APHC. These data may be scanned to a computer disk. Scanned study files will be stored electronically with the study data in the archive.

Archived Standing Operating Procedures can be found in the APHC Master Control database. Maintenance and calibration logbooks may be found in Room 1026, Building E-2100, APHC, Aberdeen Proving Ground, MD 21010-5403.

Archivist: Martha Thompson

C-2. PERSONNEL

Management: Mark Johnson, Ph.D., D.A.B.T., Toxicology Director; Michael J. Quinn, Ph.D., Division Chief, Health Effects Division (HEF)

Study Directors: Valerie H. Adams Ph.D., D.A.B.T and Emily N. Reinke, Ph.D., D.A.B.T HEF

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Quality Assurance: Michael P. Kefauver, Chemist, Quality Systems Office

APPENDIX D

STUDY REAGENTS

Table D-1. Ames Test Reagents

Ames Reagents	Source	Lot #	Exp Date
AG-TA98 *hisD3052 Salmonella typhimurium*	Xenometrix	20a	2/1/2019
AG-TA100 *hisG46 Salmonella typhimurium*	Xenometrix	23b	1/1/2021
AG-TA1535 his G46 *Salmonella typhimurium*	Xenometrix	12c	2/1/2019
AG-TA1537 hisC3076 *Salmonella typhimurium*	Xenometrix	11d	5/1/2019
E. coli wp2 [pKM101] *trpE65 Escherichia coli*	Xenometrix	U16	6/1/2018
E. coli wp2 *uvrA trpE65 Escherichia coli*	Xenometrix	P14	6/1/2018
S9-Postmitochondrial Supernatant (Aroclor)	Xenometrix	150212E	3/1/2020
Ampicillin	Xenometrix	162	6/1/2018
N4-aminocytidine	Xenometrix	NA079541202	n/a
2-aminoanthracene	Xenometrix	6417AA	7/1/2019
2-nitrofluorene	Xenometrix	2276NF	9/1/2019
4-nitroquinoline-N-oxide	Xenometrix	182NQ	7/1/2019
9-aminoacridine	Xenometrix	1029AAHC	7/1/2016
Ames MPF Exposure Medium	Xenometrix	LA01342P	1/1/2019
Ames MPF Reversion Indicator Medium	Xenometrix	K08853P	8/1/2018
Ames MPF Growth Medium	Xenometrix	K05672P	5/1/2018
E.Coli Reversion Indicator Medium	Xenometrix	J06075P	6/1/2019
E.Coli Exposure Medium	Xenometrix	LA06662P	6/1/2019
Ames MPF S9 Buffer Salts, ,	Xenometrix	PCO-12511	11/1/2019
S9-100/1537 Booster Solution	Xenometrix	N/A	11/1/2019
Glucose-6-phosphate/ S9-G-6-P	Sigma-Aldrich	X1510SPGA	10/31/2017
NADP/ S9-NADP	Sigma-Aldrich	X0915SNAA	4/30/2018

Table D-2. Microtox Test Reagents

Microtox Reagents	Source	Lot #	Date Expiration
Modern Water Microtox Diluent	Modern Water	17E4130	05/2020
Modern Water Microtox Acute Reagent	Modern Water	17H4227	09/2019
Dimethyl sulfoxide	Sigma-Aldrich	RNBG1729 RNBG8238	07/2019 06/2020
Zinc Sulfate	Sigma-Aldrich	SLBC2469V	---
Phenol	Sigma-Aldrich	BCBW8224	---
Modern Water Microtox Reconstitution Solution	Modern Water	18C4048	3/2021

Table D-3. h-CLAT Test Reagents

Reagent	Supplier	Product Number	Lot Number	Expiration Date
THP-1	ATCC	TIB-202	62996831	N/A
RPMI-1640	Gibco	22400	1967406 2052771 2065544	04-30-2019* 03-30-2020 03-30-2020
FBS	Gibco	16140	1939739	11-30-2022
2-Mercaptoethanol	Gibco	21985	1922541	11-30-2020
Penicillin-Streptomycin	HyClone	SV30010	J170027	06-30-2019
Saline	Sigma	S8776	RNBD7305	N/A
DMSO (TC)	Sigma	D2438	RNBG4916	02-2020
Globulins	Sigma	G2388	017K7650V	N/A
BSA Fraction V	EMD Millipore	12660	3035346	N/A
D-PBS	Gibco	14190	1897013 1855254	07-30-2020 03-30-2020
Propidium Iodide	Sigma	P4864	MKBR1007V	N/A
CD54 Antibody, ICAM-1 Clone 6.5B5, FITC	Dako	F714301-8	20051521	09-2020
CD86 Antibody, Hu Fun-1, FITC	BD	555657	6348610	11-30-2021
IgG1 (mouse), FITC	Dako	X092701-2	20036430	07-31-2019
Flow Cytometer Beads	BD	650622	71259 81165	05-31-2019 05-31-2020
Sheath Fluid	BD	342003	0000197124	03-29-2021
2,4-dinitrochlorobenzene (DNCB)	Sigma	237329	BCBN7826V	N/A
Nickel Sulfate (NiSO4)	Sigma	656895	MKBT0269V	N/A
Lactic Acid (LA)	Sigma	W261106	MKBR4746V	N/A

Note:
RPMI-1640 did have an expiration date preceding the usage in the testing during the reactivity check and early growth of the cell stock; however, media stock had been appropriately temperature-maintained and was within a month of expiration. Professional experience has shown that this reagent maintains full effectiveness past the expiration date.

APPENDIX E

MTNI AMES ASSAY RAW DATA

TA100

Compound:	MTNI		
TA 100 -S9			
Conc. ()	Replicate #1	Replicate #2	Replicate #3
6.8	40	44	44
21.1	34	39	40
65.9	0	0	0
205.5	2	0	3
641.0	0	0	0
2000	0	0	0
Pos. Control	48	48	48

Spontaneous	
TA 100 -S9	
8	7
4	7
6	7
4	
8	
7	

Compound:	MTNI		
TA 100 +S9			
Conc. ()	Replicate #1	Replicate #2	Replicate #3
6.8	6	3	7
21.1	20	17	20
65.9	39	38	35
205.5	1	0	3
641.0	3	1	3
2000	0	0	0
Pos. Control	48	48	48

Spontaneous	
TA 100 +S9	
3	6
7	0
6	3
6	
15	
6	

MTNI

TA 100 -S9 — Assay Date: 7/3/2019

Conc. ()	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)	
0	9	6.44		1.51	7.95				
6.76483215	3	42.67		2.31		6.62	5.36	0.0000	
21.1062763	3	37.67		3.21		5.84	4.74	0.0000	
65.8515821	3	0.00		0.00		0.00	0.00	0.0000	Cytotoxic effect?
205.456936	3	1.67		1.53		0.26	0.21	0.0004	Cytotoxic effect?
641.025641	3	0.00		0.00		0.00	0.00	0.0000	Cytotoxic effect?
2000	3	0.00		0.00		0.00	0.00	0.0000	Cytotoxic effect?
Pos. Control	3	48.00		0.00					

MTNI

TA 100 +S9 — Assay Date: 7/3/2019

Conc. ()	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)	
0	9	5.78		4.12	9.89				
6.76483215	3	5.33		2.08		0.92	0.54	0.4321	
21.1062763	3	19.00		1.73		3.29	1.92	0.0002	
65.8515821	3	37.33		2.08		6.46	3.77	0.0000	
205.456936	3	1.33		1.53		0.23	0.13	0.0527	
641.025641	3	2.33		1.15		0.40	0.24	0.0974	
2000	3	0.00		0.00		0.00	0.00	0.0202	Cytotoxic effect?
Pos. Control	3	48.00		0.00					

APPENDIX F

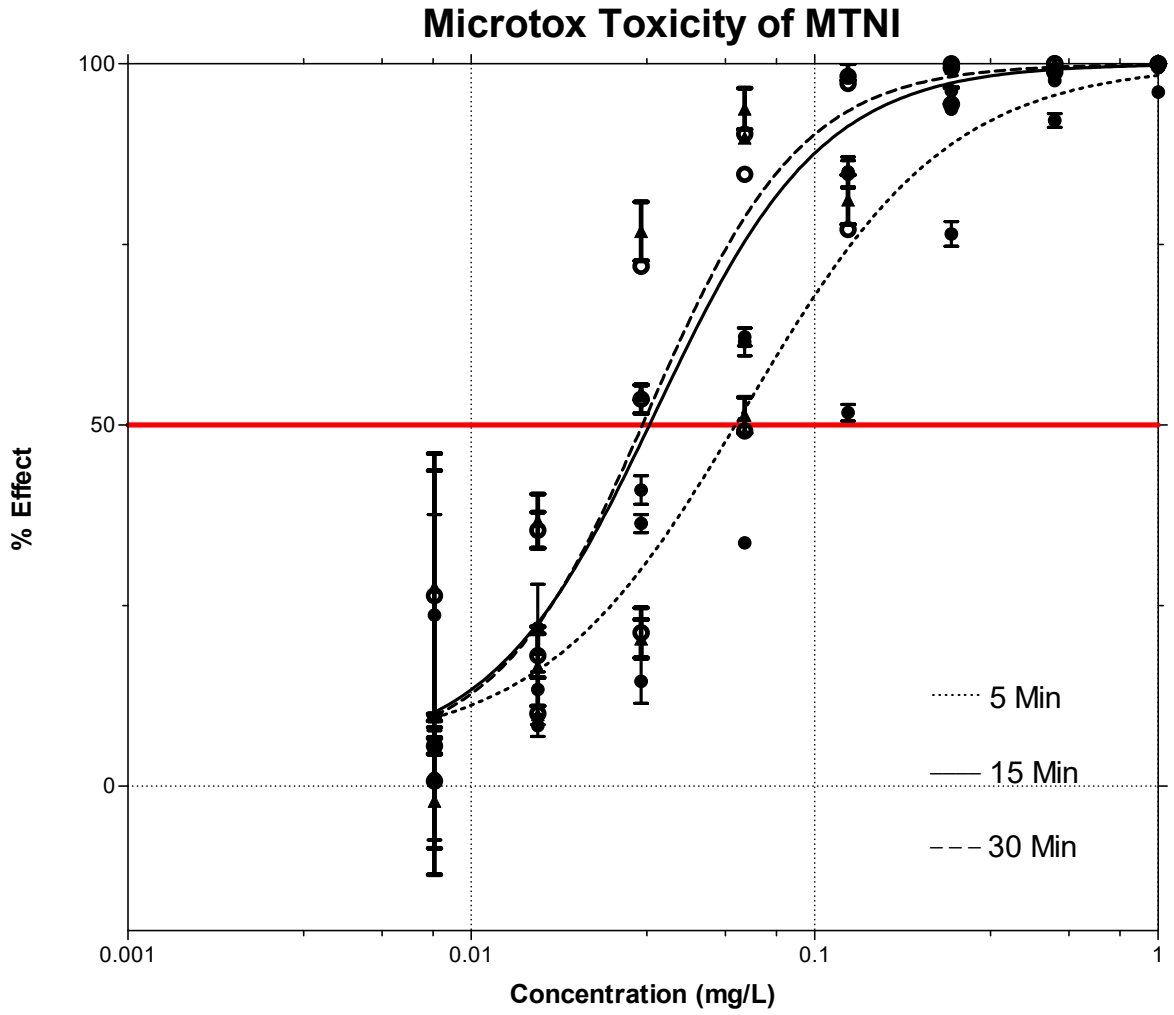
MTNI MICROTOX TEST RAW DATA

MTNI Microtox Test Data Tables and Calculations

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration* (mg/mL; 100x test concentration)		
	Test 1	Test 2	Test 3
0.00078125			
0.0015625			
0.003125			
0.00625			
0.0125			
0.025			
0.05			
0.1			

Note:
Corrected Working Concentrations were unavailable at the time of this report. Concentration verification had not been performed by APHC MDV.

MTNI EC_{50} (mg/L; 95% CI)		
5 minute	15 minute	30 minute
0.064 [0.047-0.085]	0.035 [0.025-0.049]	0.033 [0.024-0.046]


Microtox Toxicity of MTNI
100
50
0
% Effect
0.001
0.01
0.1
1
Concentration (mg/L)
5 Min
15 Min
30 Min

APPENDIX G

h-CLAT DATA

All figures in Appendix G are images of the raw data PDFs generated by the BD FacsSuite software.

Table G-1. Reactivity Check

Test article	Concentration (mg/mL)	Viability (% alive)	Percent Change (CD86)	Percent Change (CD54)	Positive (CD86/CD54)	Pass/Fail
Media		95.64	1	1	N/N	Pass
Saline		95.68	1	1	N/N	Pass
DMSO		95.9	1	1	N/N	Pass
DNCB	0.0033	83.58	335.80	518.64	Y/Y	Pass
	0.0040	79.72	223.15	664.07	Y/Y	Pass
	0.0048	65.56	119.08	227.63	Y/Y	
$NiSO_4$	0.10	78.01	297.63	2887.9	Y/Y	Pass
Lactic Acid	1	95.64	83.66	95.31	N/N	Pass

Figure G-1. Reactivity Check Raw Data

Experiment:
Reactivity Check 5-21-19
Protocol 49-iv19-03-01 A-E

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Dead:All Events	10,000	***	***	100.00	1,982
Dead:Live Cells	609	6.09	***	6.09	1,120
Live Cells:All Events	10,000	***	***	100.00	950
Live Cells:Live Cells	9,612	96.12	***	96.12	913
IgG Media:All Events	10,456	***	***	100.00	978
IgG Media:Live Cells	10,000	95.64	***	95.64	939
IgG Saline:All Events	10,451	***	***	100.00	967
IgG Saline:Live Cells	10,000	95.68	***	95.68	923
IgG DMSO:All Events	10,427	***	***	100.00	923
IgG DMSO:Live Cells	10,000	95.90	***	95.90	884
IgG Lactic Acid:All Events	10,456	***	***	100.00	931
IgG Lactic Acid:Live Cells	10,000	95.64	***	95.64	892
IgG Nickel Sulfate:All Events	12,817	***	***	100.00	1,027
IgG Nickel Sulfate:Live Cells	9,999	78.01	***	78.01	929
IgG DNCB 1:All Events	14,585	***	***	100.00	1,437
IgG DNCB 1:Live Cells	10,000	68.56	***	68.56	1,149
IgG DNCB 2:All Events	12,544	***	***	100.00	1,277
IgG DNCB 2:Live Cells	10,000	79.72	***	79.72	1,129
IgG DNCB 3:All Events	11,965	***	***	100.00	1,206
IgG DNCB 3:Live Cells	10,000	83.58	***	83.58	1,075
CD86 Media:All Events	10,504	***	***	100.00	1,799
CD86 Media:Live Cells	10,003	95.23	***	95.23	1,638
CD54 Media:All Events	10,503	***	***	100.00	1,241
CD54 Media:Live Cells	10,000	95.21	***	95.21	1,181
CD86 Saline:All Events	10,539	***	***	100.00	1,828
CD86 Saline:Live Cells	10,000	94.89	***	94.89	1,682
CD54Saline:All Events	10,520	***	***	100.00	1,235
CD54Saline:Live Cells	10,000	95.06	***	95.06	1,179
CD86 DMSO:All Events	10,597	***	***	100.00	1,992
CD86 DMSO:Live Cells	10,000	94.37	***	94.37	1,817
CD54 DMSO:All Events	10,521	***	***	100.00	1,235
CD54 DMSO:Live Cells	10,000	95.05	***	95.05	1,179
CD86 Lactic Acid:All Events	10,483	***	***	100.00	1,667
CD86 Lactic Acid:Live Cells	10,000	95.39	***	95.39	1,527
CD54 Lactic Acid:All Events	10,579	***	***	100.00	1,192
CD54 Lactic Acid:Live Cells	10,000	94.53	***	94.53	1,136
CD86 Nickel Sulfate:All Events	13,362	***	***	100.00	3,954
CD86 Nickel Sulfate:Live Cells	10,014	74.94	***	74.94	3,188
CD54 Nickel Sulfate:All Events	16,274	***	***	100.00	7,606
CD54 Nickel Sulfate:Live Cells	9,985	61.36	***	61.36	8,322
CD86 DNCB 1:All Events	15,254	***	***	100.00	3,965
CD86 DNCB 1:Live Cells	10,000	65.56	***	65.56	2,260
CD54 DNCB 1:All Events	15,883	***	***	100.00	2,210
CD54 DNCB 1:Live Cells	10,002	62.97	***	62.97	1,968
CD86 DNCB 2:All Events	13,327	***	***	100.00	4,070
CD86 DNCB 2:Live Cells	10,000	75.04	***	75.04	3,211
CD54 DNCB 2:All Events	13,597	***	***	100.00	2,992
CD54 DNCB 2:Live Cells	9,995	73.51	***	73.51	3,088
CD86 DNCB 3:All Events	12,327	***	***	100.00	4,736
CD86 DNCB 3:Live Cells	10,009	81.20	***	81.20	4,208
CD54 DNCB 3:All Events	12,469	***	***	100.00	2,648
CD54 DNCB 3:Live Cells	10,000	80.20	***	80.20	2,605

Operator: Emily Reinke, Ph.D.

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Software: BD FACSuite v1.0.5
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Table G-2. Range Finding

5/23/2019		PI- Dose Finding			
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	CV75	mg/mL
DMSO		0	93.2		
MTNI Run #1	1.56	0.00313	93.7		0.017
	3.13	0.00625	93.2		
	6.25	0.0125	92.1	-1.77	
	12.5	0.025	53.1		
	25	0.05	2.80		
	50	0.1	0.17		
	100	0.2	0.12		
	200	0.4	0.30		
5/29/2019		PI- Dose Finding			
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	CV75	mg/mL
DMSO		0	97.71		
MTNI Run #2	1.56	0.00313	72.23	-2.88	0.00131
	3.13	0.00625	70.02		
	6.25	0.0125	8.46		
	12.5	0.025	0.03		
	25	0.05	0.04		
	50	0.1	0.14		
	100	0.2	0.06		
	200	0.4	0.04		
6/05/2019		PI- Dose Finding			
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	CV75	mg/mL
DMSO		0	97.8		
MTNI Run #3	0.195	0.000391	97.4		0.0015
	0.391	0.000781	96.7	-2.8	
	0.781	0.00156	74.4		
	1.56	0.00313	63.0		
	3.13	0.00625	7.24		
	6.25	0.0125	0.64		
	12.5	0.025	0.3		
	25	0.05	0.1		

Note:
Average CV75 = 0.009 mg/mL

Figure G-2. Range Finding Experiment 1 Raw Data

Experiment:
hCLAT range finding MTNI OFHDA OC(NAH3)2 OFHMBS #1 05 Cytometer: BD FACSVerse Cytometer SN: Z6511530048
Protocol 49-IV19-03-01 mods A-E

Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Geo Mean
Live:All Events	10,000	***	***	100.00	407
Dead:Live	10,000	90.98	***	90.98	299
DMSO:Live	10,005	93.21	***	93.21	332
MTNI 1:Live	83	0.30	***	0.30	461
MTNI 2:Live	24	0.12	***	0.12	610
MTNI 3:Live	37	0.17	***	0.17	754
MTNI 4:Live	587	2.80	***	2.80	709
MTNI 5:Live	10,000	53.09	***	53.09	546
MTNI 6:Live	10,000	92.14	***	92.14	334
MTNI 7:Live	10,000	93.20	***	93.20	321
MTNI 8:Live	10,000	93.67	***	93.67	327
OFHDA 1:Live	10,000	90.62	***	90.62	433
OFHDA 2:Live	10,000	91.89	***	91.89	397
OFHDA 3:Live	10,000	93.14	***	93.14	370
OFHDA 4:Live	10,000	93.36	***	93.36	354
OFHDA 5:Live	10,000	93.60	***	93.60	337
OFHDA 6:Live	10,000	95.10	***	95.10	328
OFHDA 7:Live	10,000	93.41	***	93.41	333
OFHDA 8:Live	10,000	93.69	***	93.69	327
OC(NaH3)2 1:Live	10,000	93.00	***	93.00	364
OC(NaH3)2 2:Live	10,000	93.21	***	93.21	345
OC(NaH3)2 3:Live	10,000	92.94	***	92.94	338
OC(NaH3)2 4:Live	10,000	93.68	***	93.68	336
OC(NaH3)2 5:Live	10,000	93.45	***	93.45	332
OC(NaH3)2 6:Live	10,000	93.48	***	93.48	331
OC(NaH3)2 7:Live	10,000	93.49	***	93.49	325
OC(NaH3)2 8:Live	10,000	93.45	***	93.45	328
OFHMBS 1:Live	10,000	91.07	***	91.07	364
OFHMBS 2:Live	10,000	92.74	***	92.74	357
OFHMBS 3:Live	10,000	93.10	***	93.10	365
OFHMBS 4:Live	10,000	93.80	***	93.80	343
OFHMBS 5:Live	10,000	93.64	***	93.64	338
OFHMBS 6:Live	10,000	92.85	***	92.85	333
OFHMBS 7:Live	10,000	93.42	***	93.42	332
OFHMBS 8:Live	10,018	93.21	***	93.21	325

Operator: Lindsay A. Holden, PhD Printed: 5/24/2019 11:23:57 AM Software: BD FACSuite v1.0.5
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Figure G-3. Range Finding Experiment 2 Raw Data

Experiment:
Range Finding MTNI, Carbohydrazide, OFHDA, OFHM
Protocol 49-iv19-03-01 Mods A-E

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total
Dead cells A:All Events	10,000	***	***	100.00
Dead cells A:Live Cells	9,369	93.69	***	93.69
Live Cells A:All Events	10,000	***	***	100.00
Live Cells A:Live Cells	9,757	97.57	***	97.57
Dead Cells B:All Events	10,000	***	***	100.00
Dead Cells B:Live Cells	9,532	95.32	***	95.32
Live Cells B:All Events	10,000	***	***	100.00
Live Cells B:Live Cells	9,798	97.98	***	97.98
Plate A Media:All Events	10,234	***	***	100.00
Plate A Media:Live Cells	10,000	97.71	***	97.71
Plate A DMSO:All Events	10,234	***	***	100.00
Plate A DMSO:Live Cells	10,000	97.71	***	97.71
MTNI 1:All Events	13,845	***	***	100.00
MTNI 1:Live Cells	10,000	72.23	***	72.23
MTNI 2:All Events	14,281	***	***	100.00
MTNI 2:Live Cells	10,000	70.02	***	70.02
MTNI 3:All Events	13,848	***	***	100.00
MTNI 3:Live Cells	1,171	8.46	***	8.46
MTNI 4:All Events	18,721	***	***	100.00
MTNI 4:Live Cells	6	0.03	***	0.03
MTNI 5:All Events	17,329	***	***	100.00
MTNI 5:Live Cells	7	0.04	***	0.04
MTNI 6:All Events	14,224	***	***	100.00
MTNI 6:Live Cells	20	0.14	***	0.14
MTNI 7:All Events	15,896	***	***	100.00
MTNI 7:Live Cells	10	0.06	***	0.06
MTNI 8:All Events	17,172	***	***	100.00
MTNI 8:Live Cells	7	0.04	***	0.04
Carbohydrazide 1:All Events	10,307	***	***	100.00
Carbohydrazide 1:Live Cells	10,000	97.02	***	97.02
Carbohydrazide 2:All Events	10,240	***	***	100.00
Carbohydrazide 2:Live Cells	10,000	97.66	***	97.66
Carbohydrazide 3:All Events	10,261	***	***	100.00
Carbohydrazide 3:Live Cells	9,999	97.45	***	97.45
Carbohydrazide 4:All Events	10,271	***	***	100.00
Carbohydrazide 4:Live Cells	10,000	97.36	***	97.36
Carbohydrazide 5:All Events	10,262	***	***	100.00
Carbohydrazide 5:Live Cells	10,000	97.45	***	97.45
Carbohydrazide 6:All Events	10,298	***	***	100.00
Carbohydrazide 6:Live Cells	10,000	97.11	***	97.11
Carbohydrazide 7:All Events	10,273	***	***	100.00
Carbohydrazide 7:Live Cells	10,000	97.34	***	97.34
Carbohydrazide 8:All Events	10,292	***	***	100.00
Carbohydrazide 8:Live Cells	10,000	97.16	***	97.16
Plate B Media:All Events	10,252	***	***	100.00
Plate B Media:Live Cells	10,000	97.54	***	97.54
Plate B DMSO:All Events	10,281	***	***	100.00
Plate B DMSO:Live Cells	10,000	97.27	***	97.27
OFHDA 1:All Events	10,309	***	***	100.00
OFHDA 1:Live Cells	10,000	97.00	***	97.00
OFHDA 2:All Events	10,288	***	***	100.00
OFHDA 2:Live Cells	10,000	97.20	***	97.20
OFHDA 3:All Events	10,333	***	***	100.00
OFHDA 3:Live Cells	10,000	96.78	***	96.78
OFHDA 4:All Events	10,291	***	***	100.00
OFHDA 4:Live Cells	10,000	97.17	***	97.17
OFHDA 5:All Events	10,358	***	***	100.00
OFHDA 5:Live Cells	10,000	96.54	***	96.54
OFHDA 6:All Events	10,335	***	***	100.00
OFHDA 6:Live Cells	10,000	96.76	***	96.76
OFHDA 7:All Events	10,337	***	***	100.00
OFHDA 7:Live Cells	10,000	96.74	***	96.74
OFHDA 8:All Events	10,412	***	***	100.00
OFHDA 8:Live Cells	10,000	96.04	***	96.04

Statistics

Name	Events	% Parent	% Grandparent	% Total
OFH-MBS 1:All Events	10,293	***	***	100.00
OFH-MBS 1:Live Cells	10,000	97.15	***	97.15
OFH-MBS 2:All Events	10,266	***	***	100.00
OFH-MBS 2:Live Cells	9,999	97.40	***	97.40
OFH-MBS 3:All Events	10,318	***	***	100.00
OFH-MBS 3:Live Cells	10,000	96.92	***	96.92
OFH-MBS 4:All Events	10,330	***	***	100.00
OFH-MBS 4:Live Cells	10,000	96.81	***	96.81
OFH-MBS 5:All Events	10,318	***	***	100.00
OFH-MBS 5:Live Cells	10,000	96.92	***	96.92
OFH-MBS 6:All Events	10,361	***	***	100.00
OFH-MBS 6:Live Cells	10,000	96.52	***	96.52
OFH-MBS 7:All Events	10,434	***	***	100.00
OFH-MBS 7:Live Cells	10,000	95.84	***	95.84
OFH-MBS 8:All Events	11,103	***	***	100.00
OFH-MBS 8:Live Cells	10,000	90.07	***	90.07

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Software: BD FACSuite v1.0.5
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Figure G-4. Range Finding Experiment 3 Raw Data

Experiment:
h-CLAT Range-Finding MTNI #3 6-
Protocol 49-iv19-03-01 mod E

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total
Live Cells:All Events	10,000	***	***	100.00
Live Cells:Live cells	9,745	97.45	***	97.45
Dead Cells:All Events	10,000	***	***	100.00
Dead Cells:Live cells	12	0.12	***	0.12
Media:All Events	10,258	***	***	100.00
Media:Live cells	10,000	97.48	***	97.48
DMSO:All Events	10,225	***	***	100.00
DMSO:Live cells	10,000	97.80	***	97.80
MTNI 1:All Events	10,266	***	***	100.00
MTNI 1:Live cells	10,000	97.41	***	97.41
MTNI 2:All Events	10,338	***	***	100.00
MTNI 2:Live cells	10,000	96.73	***	96.73
MTNI 3:All Events	13,434	***	***	100.00
MTNI 3:Live cells	10,000	74.44	***	74.44
MTNI 4:All Events	15,866	***	***	100.00
MTNI 4:Live cells	10,000	63.03	***	63.03
MTNI 5:All Events	16,275	***	***	100.00
MTNI 5:Live cells	1,179	7.24	***	7.24
MTNI 6:All Events	16,401	***	***	100.00
MTNI 6:Live cells	106	0.65	***	0.65
MTNI 7:All Events	17,202	***	***	100.00
MTNI 7:Live cells	51	0.30	***	0.30
MTNI 8:All Events	18,860	***	***	100.00
MTNI 8:Live cells	18	0.10	***	0.10

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Software: BD FACSuite v1.0.5
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Figure G-5. h-CLAT Experiment 1 Raw Data

Experiment:
h-CLAT Carbohydrazide and MTNI 5-10-19
Protocol 49-iv19-03-01, B+E

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	950
Live Cells:Live Cells	9,488	94.88	***	94.88	911
Dead Cells:All Events	8,277	***	***	100.00	2,627
Dead Cells:Live Cells	300	3.62	***	3.62	1,340
IgG Media:All Events	10,741	***	***	100.00	928
IgG Media:Live Cells	10,145	94.45	***	94.45	873
IgG DMSO:All Events	10,635	***	***	100.00	911
IgG DMSO:Live Cells	10,141	95.35	***	95.35	860
IgG DNCB 1:All Events	13,245	***	***	100.00	1,242
IgG DNCB 1:Live Cells	10,000	75.50	***	75.50	1,068
IgG DNCB 2:All Events	10,473	***	***	100.00	1,366
IgG DNCB 2:Live Cells	7,348	70.16	***	70.16	1,126
IgG DNCB 3:All Events	14,246	***	***	100.00	1,428
IgG DNCB 3:Live Cells	10,000	70.20	***	70.20	1,134
IgG MTNI 1:All Events	10,549	***	***	100.00	894
IgG MTNI 1:Live Cells	10,000	94.80	***	94.80	852
IgG MTNI 2:All Events	10,571	***	***	100.00	901
IgG MTNI 2:Live Cells	10,000	94.60	***	94.60	859
IgG MTNI 3:All Events	10,609	***	***	100.00	919
IgG MTNI 3:Live Cells	10,000	94.26	***	94.26	872
IgG MTNI 4:All Events	10,690	***	***	100.00	923
IgG MTNI 4:Live Cells	10,000	93.55	***	93.55	873
IgG MTNI 5:All Events	12,042	***	***	100.00	1,085
IgG MTNI 5:Live Cells	10,000	83.04	***	83.04	953
IgG MTNI 6:All Events	15,200	***	***	100.00	1,338
IgG MTNI 6:Live Cells	10,000	65.79	***	65.79	1,057
IgG MTNI 7:All Events	15,009	***	***	100.00	1,472
IgG MTNI 7:Live Cells	8,331	55.51	***	55.51	1,109
IgG MTNI 8:All Events	14,467	***	***	100.00	1,666
IgG MTNI 8:Live Cells	8,480	58.62	***	58.62	1,277
CD86 Media:All Events	10,568	***	***	100.00	1,514
CD86 Media:Live Cells	10,000	94.63	***	94.63	1,387
CD54 Media:All Events	10,582	***	***	100.00	1,107
CD54 Media:Live Cells	10,000	94.50	***	94.50	1,044
CD86 DMSO:All Events	10,578	***	***	100.00	1,586
CD86 DMSO:Live Cells	10,000	94.54	***	94.54	1,445
CD54 DMSO:All Events	10,585	***	***	100.00	1,120
CD54 DMSO:Live Cells	10,000	94.47	***	94.47	1,044
CD86 DNCB 1:All Events	12,674	***	***	100.00	4,724
CD86 DNCB 1:Live Cells	9,166	72.32	***	72.32	3,989
CD54 DNCB 1:All Events	14,105	***	***	100.00	2,651
CD54 DNCB 1:Live Cells	9,998	70.88	***	70.88	2,702
CD86 DNCB 2:All Events	14,683	***	***	100.00	4,271
CD86 DNCB 2:Live Cells	9,538	64.96	***	64.96	3,188
CD54 DNCB 2:All Events	10,925	***	***	100.00	2,804
CD54 DNCB 2:Live Cells	6,776	62.02	***	62.02	2,857
CD86 DNCB 3:All Events	15,258	***	***	100.00	3,494
CD86 DNCB 3:Live Cells	9,929	65.07	***	65.07	1,714
CD54 DNCB 3:All Events	15,313	***	***	100.00	1,968
CD54 DNCB 3:Live Cells	9,679	63.21	***	63.21	1,623
CD86 MTNI 1:All Events	10,719	***	***	100.00	1,617
CD86 MTNI 1:Live Cells	10,000	93.29	***	93.29	1,467
CD54 MTNI 1:All Events	10,683	***	***	100.00	1,088
CD54 MTNI 1:Live Cells	10,000	93.61	***	93.61	1,023
CD86 MTNI 2:All Events	10,777	***	***	100.00	1,602
CD86 MTNI 2:Live Cells	10,000	92.79	***	92.79	1,467
CD54 MTNI 2:All Events	10,817	***	***	100.00	1,106
CD54 MTNI 2:Live Cells	9,997	92.42	***	92.42	1,028
CD86 MTNI 3:All Events	10,754	***	***	100.00	1,695
CD86 MTNI 3:Live Cells	10,000	92.99	***	92.99	1,542
CD54 MTNI 3:All Events	10,893	***	***	100.00	1,161
CD54 MTNI 3:Live Cells	10,000	91.80	***	91.80	1,081
CD86 MTNI 4:All Events	10,782	***	***	100.00	1,728
CD86 MTNI 4:Live Cells	10,000	92.75	***	92.75	1,570
CD54 MTNI 4:All Events	10,898	***	***	100.00	1,185
CD54 MTNI 4:Live Cells	10,000	91.76	***	91.76	1,099
CD86 MTNI 5:All Events	12,738	***	***	100.00	3,375
CD86 MTNI 5:Live Cells	9,992	78.44	***	78.44	2,524
CD54 MTNI 5:All Events	12,966	***	***	100.00	1,618
CD54 MTNI 5:Live Cells	10,000	77.12	***	77.12	1,418
CD86 MTNI 6:All Events	16,004	***	***	100.00	4,675
CD86 MTNI 6:Live Cells	9,991	62.43	***	62.43	2,941
CD54 MTNI 6:All Events	16,471	***	***	100.00	2,058
CD54 MTNI 6:Live Cells	9,967	60.51	***	60.51	1,839
CD86 MTNI 7:All Events	15,514	***	***	100.00	5,331
CD86 MTNI 7:Live Cells	7,721	49.77	***	49.77	2,821
CD54 MTNI 7:All Events	13,210	***	***	100.00	2,221
CD54 MTNI 7:Live Cells	6,555	49.62	***	49.62	1,987
CD86 MTNI 8:All Events	12,486	***	***	100.00	4,871
CD86 MTNI 8:Live Cells	6,583	52.72	***	52.72	2,577
CD54 MTNI 8:All Events	11,087	***	***	100.00	1,971
CD54 MTNI 8:Live Cells	6,332	57.11	***	57.11	1,660

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Software: BD FACSuite v1.0.5
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Table G-3. h-CLAT Experiment 1 Data Analysis

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
Media		0	94.45	873	1387	1	100		1044	1	100	
DMSO		0	95.53	860	1445	1.14	114		1044	1.08	108	
DNCB Control	1.67	0.00333	75.5	1068	3989	4.99	499		2702	9.56	956	
	2	0.004	70.2	1126	3188	3.52	352		2857	10.1	1012	
	2.4	0.0048	70.2	1134	1714	0.99	99.2		1623	2.86	286	
MTNI 6/10/2019	1.53	0.0031	94.8	852	1467	1.05	105		1023	0.93	92.9	
	1.84	0.0037	94.6	859	1467	1.04	104		1028	0.92	91.9	
	2.21	0.0044	94.3	872	1542	1.15	115		1081	1.14	113	
	2.65	0.0053	93.6	873	1570	1.19	119		1099	1.23	123	
	3.18	0.0064	83.0	953	2524	2.69	269	0.0055	1418	2.53	253	0.0059
	3.82	0.0076	65.8	1057	2941	3.22	322		1839	4.25	425	
	4.58	0.0092	55.5	1109	2821	2.93	293		1987	4.77	477	
	5.5	0.0110	58.6	1277	2577	2.22	222		1660	2.08	208	

Figure G-6. h-CLAT Experiment 2 Raw Data

Experiment:
hCLAT Carbohydrazide and MTNI 2A 6-18-19
Protocol 49-iv19-03-01 R E

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	1,089
Live Cells:Live Cells	9,497	94.97	***	94.97	1,038
Dead Cells:All Events	5,577	***	***	100.00	2,660
Dead Cells:Live Cells	183	3.28	***	3.28	2,142
IgG Media:All Events	10,746	***	***	100.00	1,074
IgG Media:Live Cells	10,216	95.07	***	95.07	1,012
IgG DMSO:All Events	10,760	***	***	100.00	1,047
IgG DMSO:Live Cells	10,238	95.15	***	95.15	992
IgG DNCB 1:All Events	13,067	***	***	100.00	1,389
IgG DNCB 1:Live Cells	10,635	81.39	***	81.39	1,210
IgG DNCB 2:All Events	12,930	***	***	100.00	1,328
IgG DNCB 2:Live Cells	10,665	82.48	***	82.48	1,151
IgG DNCB 3:All Events	14,250	***	***	100.00	1,411
IgG DNCB 3:Live Cells	10,734	75.33	***	75.33	1,182
IgG MTNI 1:All Events	18,383	***	***	100.00	1,647
IgG MTNI 1:Live Cells	10,817	58.84	***	58.84	1,251
IgG MTNI 2:All Events	18,232	***	***	100.00	1,807
IgG MTNI 2:Live Cells	8,768	48.09	***	48.09	1,317
IgG MTNI 3:All Events	18,350	***	***	100.00	1,928
IgG MTNI 3:Live Cells	8,055	43.90	***	43.90	1,331
IgG MTNI 4:All Events	16,517	***	***	100.00	2,148
IgG MTNI 4:Live Cells	5,784	35.02	***	35.02	1,467
IgG MTNI 5:All Events	15,834	***	***	100.00	2,070
IgG MTNI 5:Live Cells	8,814	55.67	***	55.67	1,525
IgG MTNI 6:All Events	16,293	***	***	100.00	2,200
IgG MTNI 6:Live Cells	8,458	51.91	***	51.91	1,594
IgG MTNI 7:All Events	17,285	***	***	100.00	2,266
IgG MTNI 7:Live Cells	8,183	47.34	***	47.34	1,543
IgG MTNI 8:All Events	12,592	***	***	100.00	3,179
IgG MTNI 8:Live Cells	2,500	19.85	***	19.85	1,848
CD 86 Media:All Events	10,580	***	***	100.00	2,249
CD 86 Media:Live Cells	10,000	94.52	***	94.52	2,031
CD54 Media:All Events	10,597	***	***	100.00	1,333
CD54 Media:Live Cells	10,000	94.37	***	94.37	1,253
CD86 DMSO:All Events	10,590	***	***	100.00	2,388
CD86 DMSO:Live Cells	10,007	94.49	***	94.49	2,143
CD54 DMSO:All Events	10,639	***	***	100.00	1,392
CD54 DMSO:Live Cells	10,000	93.99	***	93.99	1,299
CD86 DNCB 1:All Events	13,196	***	***	100.00	7,771
CD86 DNCB 1:Live Cells	10,000	75.78	***	75.78	6,491
CD54 DNCB 1:All Events	13,286	***	***	100.00	3,431
CD54 DNCB 1:Live Cells	10,000	75.27	***	75.27	3,481
CD86 DNCB 2:All Events	12,993	***	***	100.00	6,384
CD86 DNCB 2:Live Cells	10,000	76.96	***	76.96	5,210
CD54 DNCB 2:All Events	13,196	***	***	100.00	3,520
CD54 DNCB 2:Live Cells	10,000	75.78	***	75.78	3,618
CD86 DNCB 3 Skipped:All Events	***	***	***	***	***
CD86 DNCB 3 Skipped:Live Cells	***	***	***	***	***
CD54 DNCB 3 Skipped:All Events	***	***	***	***	***
CD54 DNCB 3 Skipped:Live Cells	***	***	***	***	***
CD86 MTNI 1:All Events	10,250	***	***	100.00	8,101
CD86 MTNI 1:Live Cells	10,000	97.56	***	97.56	7,883
CD54 MTNI 1:All Events	16,799	***	***	100.00	3,197
CD54 MTNI 1:Live Cells	9,103	54.19	***	54.19	3,605
CD86 MTNI 2:All Events	18,957	***	***	100.00	10,181
CD86 MTNI 2:Live Cells	8,473	44.70	***	44.70	4,972
CD54 MTNI 2:All Events	15,513	***	***	100.00	3,371
CD54 MTNI 2:Live Cells	6,659	42.93	***	42.93	4,070
CD86 MTNI 3:All Events	17,913	***	***	100.00	9,511
CD86 MTNI 3:Live Cells	6,842	38.20	***	38.20	4,234
CD54 MTNI 3:All Events	14,446	***	***	100.00	3,095
CD54 MTNI 3:Live Cells	5,377	37.22	***	37.22	3,294
CD86 MTNI 4:All Events	15,757	***	***	100.00	9,857
CD86 MTNI 4:Live Cells	4,629	29.38	***	29.38	3,293
CD54 MTNI 4:All Events	14,862	***	***	100.00	2,865
CD54 MTNI 4:Live Cells	4,365	29.37	***	29.37	2,562
CD86 MTNI 5:All Events	17,489	***	***	100.00	5,793
CD86 MTNI 5:Live Cells	8,158	46.65	***	46.65	2,683
CD54 MTNI 5:All Events	16,085	***	***	100.00	2,470
CD54 MTNI 5:Live Cells	7,107	44.18	***	44.18	1,770
CD86 MTNI 6:All Events	15,207	***	***	100.00	7,767
CD86 MTNI 6:Live Cells	6,444	42.38	***	42.38	3,521
CD54 MTNI 6:All Events	13,008	***	***	100.00	2,705
CD54 MTNI 6:Live Cells	5,315	40.86	***	40.86	2,055
CD86 MTNI 7:All Events	16,083	***	***	100.00	10,285
CD86 MTNI 7:Live Cells	6,438	40.03	***	40.03	4,246
CD54 MTNI 7:All Events	14,660	***	***	100.00	2,551
CD54 MTNI 7:Live Cells	5,843	39.86	***	39.86	2,197
CD86 MTNI 8:All Events	12,569	***	***	100.00	19,055
CD86 MTNI 8:Live Cells	2,286	18.19	***	18.19	3,551
CD54 MTNI 8:All Events	11,009	***	***	100.00	3,142
CD54 MTNI 8:Live Cells	1,980	17.99	***	17.99	2,418
CD86 MTNI 1 Repeat:All Events	17,120	***	***	100.00	7,348
CD86 MTNI 1 Repeat:Live Cells	10,000	58.41	***	58.41	4,471

Software: BD FACSuite v1.0.5

Operator: Emily Reinke, Ph.D.

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Table G-4. h-CLAT Experiment 2 Data Analysis

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
Media		0	95.07	1012	2031	1	100		1253	1	100	
DMSO		0	95.15	992	2143	1.13	113		1299	1.27	127	
DNCB Control	1.67	0.00333	81.39	1210	6491	9.03	903		3481	13.28	1328	
	2.0	0.004	82.48	1151	5210	6.94	694		3618	14.43	1443	
MTNI	1.53	0.0031	58.84	1251	4471	5.50	550		3605	7.67	767	
6/18/2019	1.84	0.0037	48.09	1317	4972	6.25	625		4070	8.97	897	
	2.21	0.0044	43.9	1331	4234	4.96	496		3294	6.39	639	
	2.65	0.0053	35.02	1467	3293	3.12	312		2562	3.57	357	
	3.18	0.0064	55.67	1525	2683	1.98	198		1770	0.80	79.8	
	3.82	0.0076	51.91	1594	3521	3.29	329	0.0059	2055	1.50	150	
	4.58	0.0092	47.34	1543	4246	4.62	462		2197	2.13	213	0.0089
	5.5	0.0110	19.85	1848	3551	2.91	291		2418	1.86	186	

Table G-5. Acute Oral Hazard Estimation Example

Test #2			
Concentration (ug/mL)	Log Conc	Viability	1000-viability
3.13	0.495	72.2	927.8
6.25	0.796	70.0	930
12.5	1.10	8.46	991.5
25	1.40	0.03	999.97
50	1.70	0.04	999.96
100	2	0.14	999.86
200	2.30	0.06	999.94
400	2.60	0.04	999.96
	Log Concentration	Viability	Desired LD
>50%	0.796	70.02	50
<50%	1.10	8.46	
	Slope =	-205	
	Intercept	233	
X	0.894		
IC50	7.83		
LOG LD50 (mg/kg)	2.36		
LD50 (mg/kg)	227		