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In Silico and *In Vitro* Toxicity Testing of the Novel
Energetic 1-(5-tetrazolyl)-4-guanyltetrazene hydrate (Tetrazene, TTZ)

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**Toxicology Study No. S.0042847-16- *In silico* and *in vitro* toxicity testing of the Novel
Energetic 1-(5-tetrazolyl)-4-guanyltetrazene hydrate (Tetrazene, TTZ)**

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Toxicology Directorate
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Good Laboratory Practice Compliance Statement

The studies described in this report were conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice (GLP) 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 Portfolio (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 TTZ.
3. At the time the study was conducted, there was not an APHC-approved GLP protocol for the h-CLAT. The method has been validated and a formal report documenting the protocol and local changes has been completed. The current study followed the methodology as described in the formal report and all steps were documented and are found in the archives. No deviations from the aforementioned regulation affected the quality or integrity of the study or the interpretation of the results.

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Toxicology Study No. S.0042847-16
***In Vitro* Toxicity Testing of the Novel Energetic**
1-(5-tetrazolyl)-4-guanyltetrazene hydrate (Tetrazene, TTZ)

1 SUMMARY

1.1 Overview

This study evaluated the toxicity of 1-(5-tetrazolyl)-4-guanyltetrazene (tetrazene, TTZ) in several *in vitro* test systems, including the Ames mutagenicity assay, the Microtox[®] Acute Toxicity Test System, and a skin sensitization tiered prediction model utilizing h-CLAT and DPRA. Data from the h-CLAT were also utilized to predict a Hazard Category (United Nations Economic Commission for Europe (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 environmental and occupational health information on new or replacement energetic compounds 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 (DA) Regulation 200-1 (DA 2007b); Army Regulation (AR) 40-5 (DA 2020); and AR 70-1 (DA 2018); Department of Defense (DOD) Instruction 4715.4 (DOD 1998); and Army Environmental Research and Technology Assessment (AERTA) requirement PP-3-02-07 (AERTA 2018), Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces.

Research, development, testing, training, and use of replacement substances that are potentially less hazardous to human health and the environment are vital to the readiness of the U.S. military. Safeguarding the health of Soldiers, Civilians, and the environment requires an assessment of alternatives before new substances are fielded. Continuous toxicological hazard assessments begun early in the 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. The purpose of this toxicology study was to evaluate tetrazene (TTZ) for mutagenicity, acute aquatic toxicity, and skin sensitization hazards using *in vitro* methods and following Good Laboratory Practice (GLP) regulations.

The mention of any non-federal entity and/or its products is not to be construed or interpreted, in any manner, as federal endorsement of that non-federal entity or its products.
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1.3 Conclusions

This study reports the *in vitro* toxicity for the energetic compound TTZ via the Ames assay for mutagenicity, the Microtox Acute Aquatic Toxicity assay, and the h-CLAT skin sensitization assay. TTZ mutagenicity in the Ames assay was inconclusive (i.e., equivocal). TTZ was found to be moderately toxic with a median (50%) effect concentration (EC₅₀) of 4.8 mg/L for acute aquatic toxicity, and is a skin sensitizer, confirming the Toxicity Prediction Komputer Assisted Technology (TOPKAT) prediction. Using *in silico* methods, the predicted acute oral toxicity was low—Category 5 in the Globally Harmonized System (GHS). As such, TTZ is identified as a potential hazard for aquatic life (OECD 2001) with a prediction of low acute toxicity to mammalian systems.

1.4 Recommendations

Based on the likelihood of environmental release, additional aquatic toxicity testing is warranted. The equivocal data for mutagenicity suggests further testing is needed and should be included along with repeated-dose rodent bioassays that are conducted in the future.

2 REFERENCES AND COMMONLY USED TERMS AND ACRONYMS

See Appendix A for list of references in this report. See the Glossary for commonly used terms and acronyms.

3 AUTHORITY

Military Interdepartmental Purchase Request No. W6DTAA0010872493. This technical report addresses, in part, the ESOH requirements outlined in DoD Instruction 4715.4 (DoD 1998); AR 200-1, Environmental Protection and Enhancement (DA 2007b); AR 40-5, Army Public Health Program (DA 2020); and AR 70-1, Army Acquisition Policy (DA 2018); DoD Instruction 4715.4, Pollution Prevention (DoD 1998); and AERTA Assessment Requirement PP-3-02-07, Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces. It was conducted as part of an on-going effort by Strategic Environmental Research and Development Program (SERDP). The Principal Investigator for this project is Ms. Cathleen Freeman, Combat Capabilities Development Command (CCDC).

4 BACKGROUND

Current Army guidance requires the assessment of human health and environmental effects prior to acquisition of a system. Previously, health and environmental evaluations were conducted only after an item was fielded. Any post-acquisition utilization of a system with adverse environmental and human health effects creates additional, substantial costs to the lifecycle of the system. 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 these new or replacement compounds for operational and/or ESOH issues.

National defense requires specialized energetic compounds to meet mission requirements. These requirements also include the sustainable use of these materials in the environment, particularly during training operations. Unexploded ordnance and low-order detonations have become sources of ground water contamination and have affected drinking water resources. Examples of compounds that have caused concern include RDX (1,3,5-hexahydro-1,3,5-trinitrotriazine) and TNT (2,4,6-Trinitrotoluene) due to their ability to contaminate groundwater and, thus, enter into the drinking water supply.

TTZ is a primary explosive being investigated as a constituent of replacements for lead azide and lead styphinate. By identifying ESOH effects early in the acquisition process, unacceptable, or “regrettable,” replacement compounds can be identified. 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. Given that limited amounts of the newly developed materials are synthesized, the *in vitro* tests are ideally suitable and effective toxicity screening tools.

The Toxicology Directorate (TOX) of the U.S. Army Public Health Center (APHC) has been tasked with reviewing the available toxicity data for TTZ (see Appendix B for TTZ toxicity profile) and generating *in vitro* toxicity data for TTZ to determine its potential negative human and environmental effects. The data from these studies informs recommendations for the continued development and additional toxicity testing of TTZ that supports the appropriate hazard classification and exposure guidance.

The Ames test for mutagenicity (Ames et al. 1975) is a method widely accepted for evaluation of mutagenic potential by the U.S. Environmental Protection Agency (USEPA) (USEPA 2012), the U.S. Food and Drug Administration (USFDA 2007), and the Organization for Economic Co-operation and Development (OECD 1997). Historically, the mutagenicity of test materials has been evaluated in the agar plate-based Ames assay (Ames et al. 1975). The bacteria used in this test, *Salmonella typhimurium* and *Escherichia coli*, 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 histidine or tryptophan negative (*his*- or *trp*-) 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 (*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and a composite of *Escherichia coli* (*E. coli*) pKM101/*uvrA* strains), and reduces the assay to a simple, non-agar, 384-well plate methodology that is expedient and cost effective.

Microtox® is a toxicity testing system that uses a strain of naturally occurring bioluminescent bacteria, *Aliivibrio fischeri* (formerly *Vibrio fischeri* and still referred to as *V. fischeri* by the supplier of the reagents (Modern Water); also known as *Photobacterium phosphoreum*, NRRL number B-11177). 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. Reduction of the microorganism's light emission is proportional to the toxicity expressed as EC₅₀. This test has been shown to be an effective screening tool in assessing toxicity of varied chemical compounds compared with other bioassays. The bacterial bioluminescence aquatic toxicity test has been validated by the industrial, academic, and governmental testing communities and achieved official "Standards Status" in several countries including an ASTM Standard (D-5660; withdrawn), ISO 11348-3 and Standard Method 8050 in the U.S., AFNOR T90-320 in France, NVN 6516 (withdrawn) in the Netherlands, and DIN 38412 (Germany).

There are multiple skin sensitization assays that can be utilized to determine skin sensitization hazard in a tiered testing strategy that forms a defined approach. The EPA has recently accepted two defined approaches for submission and registration to predict hazard. In one approach, 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). 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 3 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 2010; ECVAM DB-ALM 2014; OECD 2018). The threshold criteria for a positive reaction in h-CLAT requires either a 2-fold induction of CD54 or a 1.5-fold or induction of CD86 compared to solvent controls. The h-CLAT can also be utilized to predict acute oral toxicity from the cytotoxicity data produced in the course of conducting the assay.

In the absence of a positive result in the h-CLAT, a second assay should be conducted, either in a tiered approach or as a best-of-three approach (Kleinstreuer 2018; USEPA 2018). The direct peptide reactivity assay (DPRA) fulfills either of these approaches. The DPRA assesses the first key event in the adverse outcome pathway, formation of haptens through covalent binding of chemicals to proteins, which induces an immune response, leading to downstream effects such as the activation of dendritic cells as described in the previous paragraph. The DPRA is an *in chemico* assay that measures the decay of cysteine or lysine containing polypeptide sequences via high-performance liquid chromatography with ultraviolet detection (HPLC-UV). The threshold for a positive reaction requires a decrease in amino acid signal of approximately 6.5% as compared to control samples.

This report describes the toxic effect of TTZ in the Ames mutagenicity assay, the Microtox bacterial bioluminescent acute toxicity assay, and skin sensitization testing suite. Table 1 identifies the critical events and dates of these studies.

Table 1. Critical Events

Critical Event	Date of Event (Ames)	Date of Event (Microtox)	Date of Event (h-CLAT)	Date of Event (DPRA)
Non-Animal Use Protocol Approved	15 Feb 2018	9 August 2018	N/A	N/A
Study Start Date	9 August 2018	9 August 2018	9 August 2018	9 August 2018
Experimental Start Date	9 August 2018	9 August 2018	9 September 2018	1 September 2020
Experimental Completion Date	17 May 2019	13 August 2018	1 October 2018	14 September 2020
Study Completion Date	August 2021	August 2021	August 2021	August 2021

5 MATERIALS

5.1 Test Substance

Synthesis of TTZ (Chemical Abstracts Service Registry Number (CASRN) 31330-63-9) was performed at the U.S. Army Armament Research, Development, and Engineering Center (ARDEC), Aberdeen Proving Ground, Maryland. Purity analysis for TTZ was not available. The molecular structure of TTZ is shown in Figure 1.

TTZ was soluble at 28.5 mg/mL in DMSO. 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 (PHC-MDV-CSD).

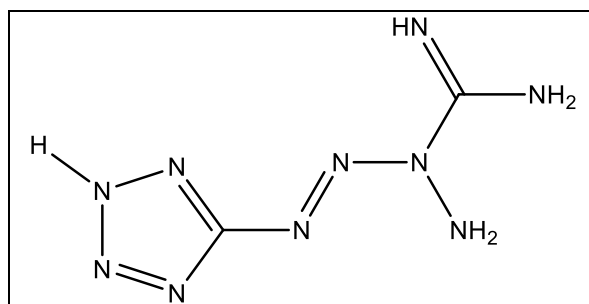


Figure 1. Molecular Structure of Tetrazene

5.2 Test System (Ames)

Xenometrix MPF™ kits were obtained from ANIARA, Mason, Ohio. Each kit contained Quality Assurance certificates for the bacterial strains provided: *Salmonella* TA98, TA100, TA1535, TA1537, and a composite mix of *E. coli* pKM101/*uvrA* strains. A list of the kit reagents and supplementary reagents with expiration dates are provided in Appendix D. All reagents were

stored in refrigerators and freezers in accordance with 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 contained a reagent that becomes luminescent when activated by incubation with adenosine triphosphate (ATP) in lysed bacteria.

5.3 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*. A list of media, solutions, and other necessary test materials with expiration dates and lot numbers is provided in Appendix D. All reagents were stored according to manufacturer instructions as described in the TOX SOP 037 and the 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 EC₅₀ of 2–10 mg/L at 15 min were qualified for further use.

5.4 Skin Sensitization Assays

5.4.1 Test System (h-CLAT)

THP-1 cells were acquired from the American Type Tissue Collection (Manassas, Virginia). A list of media, solutions, and other necessary test materials with expiration dates and lot numbers is provided in Appendix D. All tissue culture reagents were acquired from Gibco, a subsidiary of Thermo Fisher® (Waltham, Massachusetts). Cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 U/mL penicillin, 10 µg/mL streptomycin, and 0.05 mM 2-mercaptoethanol. All cells, reagents, and chemicals were stored according to manufacturer's instructions. Dinitrochlorobenzene (DNCB) and nickel sulfate (NiSO₄) 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 test chemicals were obtained from Sigma Aldrich. FITC-tagged antibodies against CD86, CD54, and IgG control were obtained from BD (San Jose, CA) and Dako-Agilent (Santa Clara, CA) respectively. Propidium iodide (PI) was obtained from Sigma Aldrich. PI was used for cytotoxicity estimations. Bovine serum albumin (BSA) Fraction V (EMD Millipore, Burlington, MA) was used for cell viability maintenance after exposure and globulins (Sigma-Aldrich) were used as a blocking agent prior to antibody staining.

5.4.2 Test System (DPRA)

Polypeptide sequences were synthesized and obtained from RS Synthesis (Lexington, KY). Sequences were as followed: Ac-RFAAKAA-COOH (molecular weight 776.2) and AC-RFAACAA-COOH (molecular weight 751.9). A list of chemicals, buffers, and other necessary test materials with expiration dates and lot numbers are provided in Appendix D. All reagents were stored according to manufacturer instructions as described in the study protocol and were

obtained from Sigma-Aldrich, Inc. (APHC 2019a). Cinnamaldehyde (100mM stock) was the positive control for the assay.

5.5 Quality Assurance

The APHC policy requires that all experiments and studies conducted by any element of the APHC TOX will be compliant with the applicable GLP Standard guideline (APHC 2016). For this study, the test article dictates that the following GLP guideline applies (CFR 1989):

CFR, Title 40, Protection of Environment, Part 792-Good Laboratory Practice Standards.

According to this policy and 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. A Quality Assurance Statement is provided in Appendix B, which provides the dates of these audits along with the audited phases and the dates that the results of the audits were reported to Management and the Study Director. The additional Quality Assurance/GLP requirement of archives location is provided in Appendix C, as well as the names of personnel contributing to the performance of this study.

6 METHODS

6.1 Mutagenicity – Ames Assay

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 (USEPA 2012) and OECD guidelines for the Bacterial Reverse Mutation Test (OECD 1997), APHC TOX Type Protocol: *The Ames Test For Mutagenicity # 2758-70-IV16-03-01* (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 28 mg/mL TTZ 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 (visually observed) precipitation. These final diluted solutions represent the exposure concentrations with final concentrations ranging from 3.8 to 1120 µg/mL (nominal).

6.1.2 Validation of Cell Growth

Five different bacterial strains were used to test the mutagenicity of TTZ. The frozen bacterial stocks were 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 absorbance at 600 nm (OD₆₀₀; BioMate™ 3S spectrophotometer, Fisher Scientific.) OD₆₀₀ ≥ 2.0 indicated sufficient density for the Xenometrix Ames assay, although lower OD₆₀₀ values may also be acceptable. Per a personal conversation with the manufacturer, subthreshold 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. OD₆₀₀ for *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* pKM101/*uvrA* strains were all above 2.0.

6.1.3 Compound Incubation

The bacterial suspension was diluted with Xenometrix incubation media and aliquoted into tissue culture wells containing serial dilutions of 25x concentrates of the test material. The suspensions were incubated with shaking for 90 minutes (or 20 minutes for *E. coli* + S9) 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 turns 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 each strain was included alongside the test material to assure the assay was valid for each run; positive controls were run in duplicate 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).

There are several criteria for determining a positive result:

- The number of reversions induced by the test compound is at least 2-fold above the background control,
- Concentration-related increase over the range tested, and/or,
- 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 either *Salmonella* and/or *E. coli*. 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 mutagenic incubation step, a plate was prepared for determination of cytotoxicity of the test material using ATP luminescence. After a 90-minute incubation at approximately 37°C, (20-minute incubation for *E. coli* + S9), samples from the incubation plate were aliquoted into a 96-well plate. An equal volume of luminescent reagent was added to each well according to the method described for the BacTiter-Glo™ Microbial Cell Viability Assay and bioluminescence was measured via plate reader.

6.2 Aquatic Toxicity/Luminescent Bacteria - 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) with modifications. The modifications to the protocol are approved and signed by the Study Director. The electronic and hard copy versions of the protocol modifications were saved and archived with the protocol and the raw data.

6.2.2 Range Finding

The TTZ was dissolved in DMSO at its solubility limit (28 mg/mL); due to the sensitive nature of this compound, one master stock was prepared and all subsequent dilutions were prepared from this master stock on each testing day. The solubility of each test article was determined previously in the Ames test (APHC 2017b). TTZ was serially diluted 1:2 in DMSO and further diluted 1:100 in diluent. A total of 8 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 EC₅₀ 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, each test article was tested in duplicate on 3 separate days. On each testing day, 7.13 mg/mL TTZ 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. Ten µL reconstituted *A. fischeri* were added to each sample and luminescence was 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 EC₅₀ values at 5, 15, and 30 minutes were given by the Microtox® Omni software and further fitted to the Hill function using GraphPad PRISM 5.04®. All data (prints and files) were archived.

6.2.5 Derivation of Ecotoxicity Hazard Values

Data from Microtox tests were aligned with the aquatic toxicity criteria of the USEPA, the OECD and the GHS to categorize the potential ecotoxicity of TTZ (Table 2) (USEPA 2017; OECD 2001; UNECE 2015).

Table 2. Ecotoxicity Assessment Scale

LC ₅₀ or EC ₅₀ Concentration Range (mg/L)	Hazard Categories (USEPA 2017)	Hazard Classes (OECD 2001)	Acute Aquatic Toxicity (UNECE 2015)
< 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	—	—

6.3 Skin Sensitization Assays

6.3.1 h-CLAT

The h-CLAT has undergone validation testing within the APHC to verify that the assay performs as expected with APHC equipment when compared to published results (APHC 2016). In the absence of a GLP *in vitro* protocol for this assay, testing was performed according to ECVAM DB-ALM protocol number 158 and OECD Guideline 442E (ECVAM DB-ALM 2014; OECD 2014).

6.3.1.1 Buffers

Fluorescence-activated cell sorting (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 the 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.

6.3.1.2 Tissue Culture

Tissue culture media was prepared as described in section 5.4 and maintained at 4 ± 2 °C. Media was pre-warmed to room temperature prior to use for each cell plating and passage. Cells were maintained at 1.5×10^5 – 8×10^5 cells/mL and were routinely passaged every 2–3 days. Cells were maintained at 37°C, 5% CO₂. Cells for the assay were in culture 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 (reactivity check, range finding and h-CLAT), cells were plated into 24-well plates at a density of 1×10^6 cells/well in 0.5 mL (i.e., 2×10^6 cells/mL). For maintenance, cells were plated at 1.5 – 2.0×10^5 cells/mL in 25–40 mL media depending on the timing of subsequent tests.

6.3.1.3 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, NiSO₄ and LA. DNCB was prepared as a 20 mg/mL stock solution in DMSO and stored at 4 °C in the dark. NiSO₄ was prepared as a 10 mg/mL stock solution in saline and stored at room temperature protected from light. Lactic acid (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 NiSO₄, 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% CO₂) for 24 hours. Cells were then collected, processed, stained with PI and 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 NiSO₄ 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.

6.3.1.4 Range Finding

The range-finding test is used to bracket the appropriate dose range for the full test using only the percent viability endpoint. TTZ (28 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 (final serial dilution). Pooled THP-1 cells were plated at 1×10^6 cells/well in a 24 well plate. An equal volume (0.5 mL) of each final serial 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% CO₂). After the 24-hour incubation, cells were collected and processed for staining with PI. Briefly, cells were transferred into 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.

6.3.1.5 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 8-dose treatment. For the TTZ range-finding assay, cytotoxicity was not observed at the highest tested concentration; therefore, the solubility limit (28-mg/mL TTZ) was the high dose (tested concentration of 0.056 mg/mL). The TTZ was solubilized in DMSO at the solubility limit (28 mg/mL) overnight. Eight 1:1.2 serial dilutions of TTZ were subsequently diluted 1:250 in complete media and added in equal volume to test wells containing 0.5 mL medium and 1 x 10⁶ cells per well in a 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 10⁶ 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).

6.3.1.6 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 twice. 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.

6.3.1.7 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 FL1 (FITC emission wavelength 530 ± 15 nm) and FL2 (PI emission wavelength >650 nm). 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.

6.3.1.8 Data Analysis

If the RFI for any concentration exceeded the positive criteria ($CD54 \geq 200$ and $CD86 \geq 150$), 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 TTZ; the data from these two experiments were sufficient to determine sensitization and a third experiment was not necessary.

6.3.1.9 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 ($CD54 \geq 200$ and $CD86 \geq 150$).
- 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 percent.
- The cell viability of at least 4 doses was greater than 50%.

6.3.1.10 Cell-based Acute Oral Toxicity Estimation (CAOTE)

The IC_{50} (level at which cytotoxicity was induced by 50 percent) 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:

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

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. This model was applied to the THP-1 cells of the uptake in order 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).

6.3.2 DPRA

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 154 and OECD Guideline 442C (ECVAM 2012; OECD 2015).

6.3.2.1 Buffers

A 100 mM, pH 7.5 sodium phosphate buffer was prepared using both sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate one day prior to use and stored at +4 °C. Ammonium acetate (100 mM, pH 10.2) buffer was also prepared one day prior to use and stored for up to two weeks at +4 °C. All buffers were prepared with NanoPure water (ThermoScientific, Waltham, MA).

6.3.2.2 Sample Preparation

100 mM solutions of cinnamaldehyde (0.5 mL for lysine or 1.25 mL for cysteine experiments) were prepared in acetonitrile. The TTZ and peptide controls were prepared in 50% DMSO:Acetonitrile v/v at 100 mM. Peptides were prepared in a stock solution of 0.667 mM in sodium phosphate (cysteine) or ammonium acetate (lysine) buffers. Controls consisted of peptide and buffer only and co-elution (test chemical only). Samples were prepared in triplicate according to Table 3.

Table 3. Sample Preparation*

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM test chemical	1:50 Ratio, Lysine Peptide 0.5 mM Peptide, 25 mM test chemical
• 750 µL Cysteine peptide solution (or pH 7.5 phosphate buffer for Co-elution controls) • 200 µL Acetonitrile/DMSO: Acetonitrile • 50 µL Test chemical solution (or solvent for reference controls)	• 750 µL Cysteine peptide solution (or pH 10.2 ammonium acetate buffer for Co-elution controls) • 250 µL Test chemical solution (or solvent for reference controls)

Note:

* ECVAM DB-ALM, *Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing DB-ALM Protocol No. 154*. 2012: European Union Reference Laboratory for Alternatives to Animal Testing.

A standard curve was prepared using a 20 % acetonitrile:buffer solution, samples are prepared in a 25 % acetonitrile:buffer solution. This is according to the protocol. The dilution buffer is prepared in advance and the peptide is diluted to 0.534 mM (1:0.8 dilution) and a serial 1:2 dilution was performed for a total of six standards plus a buffer-only sample.

Standard curve and reference controls were incubated in the dark at room temperature for approximately 21 hours prior to the start of sample analysis, this allowed for test chemical and positive control sample analysis to begin approximately 24 hours after incubation started. Each peptide was analyzed 2 times.

6.3.2.3 Sample Analysis

The sample analyses were performed by HPLC in conjunction with UV-Visible spectrophotometry detection. Chromatographic separation was achieved using a reverse phase C-18 HPLC column under gradient conditions. The aqueous mobile phase consists of 0.1 % trifluoroacetic acid (TFA) in deionized water; the organic mobile phase consists of 0.085 % TFA in acetonitrile. Column flow was maintained at 0.35 mL/min; the gradient was linear from 10 to 25 % acetonitrile over 10 minutes, followed by a step to 90 % acetonitrile. Injection volume was 10 μ L. Cysteine and Lysine calibration standards, ranging in concentration from 0.000 to 0.534 mM, were prepared at seven concentrations by serial dilution of a 0.667 mM stock standard, in phosphate or ammonium acetate buffer, respectively. Quantitation was performed at 220 nm.

6.3.2.4 Data Analysis

The concentration of the peptide was determined from the absorbance at 220 nm by determining the area under the curve (AUC) and matching it to a standard curve. Depletion of the peptide was determined by dividing the peak area of the test chemical by the mean peak area of the reference controls. The percent depletion was calculated by subtracting from 1 and multiplying by 100.

6.3.2.5 Criteria for a Valid Assay

- Standard curve $r^2 > 0.990$
- Mean peptide concentration in Reference Control A = 0.50 ± 0.05 mM
- Percent depletion in the positive control must fall into the following boundaries:
 - Cysteine: 60.8 to 100 %
 - Lysine: 40.2 to 69.4 %
 - Maximum standard deviations for positive control and test chemical replicates cannot exceed the following:
 - Cysteine: 14.9 %
 - Lysine: 11.6 %
- The CV of the peptide peak areas for Reference Controls B and C must be < 15.0 %

6.4 Concentration Verification of Tetrazene

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 TTZ

The five strains of bacteria were differentially sensitive to TTZ (Figure 2). The *E. coli* strain was least sensitive and had increased growth relative to the untreated controls in both the -S9 and +S9 test conditions. The TA100 strain was most sensitive and had significantly reduced viability at the highest concentration tested (1120 µg/mL) when tested in the absence of S9. Reduced viability (i.e., > 20% change) was observed for TA98, TA100, TA1535, and TA1537 at concentrations at and above 115 µg/mL when tested in the presence of S9. In the +S9 incubations, presumptive metabolites of TTZ were apparently more toxic than the parent as toxicity was increased and affected more *Salmonella* strains compared to the -S9 incubated samples.

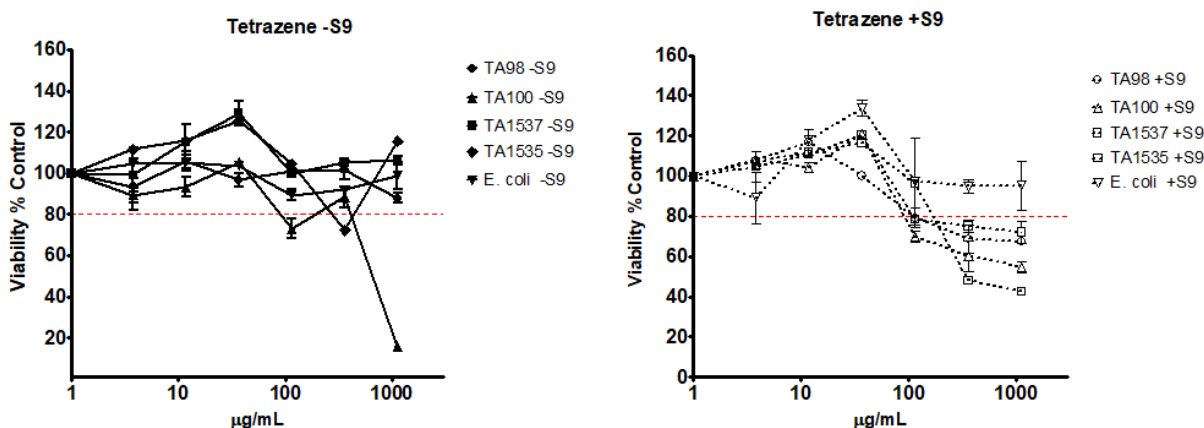


Figure 2. Cytotoxicity of Tetrazene to Bacterial Tester Strains

The five tester strains were exposed to tetrazene in either the absence (-S9; solid line; **left panel**) or presence (+S9; dashed line; **right panel**) of liver microsomes. Cytotoxicity, defined as less than 80% viability (horizontal spark line) was observed in two concentrations (316 and 1120 µg/mL) without S9 for TA100. Cytotoxicity was also observed in TA98, 100, 1535 and 1537 in the presence of S9 at ≥ 115 µg/mL tetrazene.

7.1.2 Mutagenicity of TTZ

The data (transcribed data from the hard copy, hand written raw data in the lab book) for the mutagenicity tests, and calculations are provided in Appendix E. The TTZ was not mutagenic in TA98, TA1535, and TA1537 in either the -S9 or +S9 exposures; TTZ was also not mutagenic in the TA100 +S9 test. The TTZ was mutagenic in TA100 (-S9) but only at concentrations associated with cytotoxicity; therefore, this response is considered nonmutagenic. Tetrazene

was tested on 2 separate days for mutagenicity in *E. coli*. In the first experiment, TTZ was mutagenic in *E. coli*, without S9, and at concentrations without cytotoxicity. In the +S9 test, the background reversion rate was too high to permit a valid measurement. When the assay with *E. coli* was repeated, TTZ was not mutagenic in *E. coli* (-S9 treatment), and the +S9 treatment did not meet criteria for scoring. It is not clear why there is a discrepancy between the results for *E. coli*. One potential explanation is that the TTZ molecule is inherently unstable, and there were differences between the two TTZ samples. Tetrazene was not readily soluble in DMSO, and it was allowed to incubate overnight in DMSO at room temperature to improve solubility.

7.2 Microtox Toxicity and Risk Assessment

Historically, data from *A. fischeri* toxicity tests demonstrated in most cases, the dose response will be protective (i.e., more sensitive) of other aquatic organisms (Dutka and Kwan 1981; McFeters et al. 1983; Riva et al. 2007). Toxicity of TTZ 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. The toxicity data (EC₅₀ and the 95% CI) and risk assessment are presented in Table 3. Analyzed data are presented in Appendix F: Figures—Microtox. This evaluation suggests that TTZ is moderately toxic according to USEPA categories (Acute Category II by GHS, Table 3).

Table 4. Microtox Toxicity and Risk Assessment

Compound	Microtox EC ₅₀ (mg/L) [95 % CI]			Hazard Categories (USEPA 2017)	Hazard Classes (OECD 2001)	Acute Aquatic Toxicity (GHS 2005)
	5 min	15 min*	30 min			
Tetrazene	10.28 [8.3-12.7]	4.842 [3.8-6.2]	1.1 [0.5731-2.28]	Moderately Toxic	Acute Toxicity 2 (toxic to aquatic life)	Acute Cat. II

Note:

*The value of EC₅₀ at 15 minutes is used for the risk assessment.

7.3 Skin Sensitization Assays

7.3.1 h-CLAT

7.3.1.1 Reactivity Check

The THP-1 cells were checked and verified for reactivity to DNCB and NiSO₄ and lack of reactivity to LA. Cells reacted as expected, with DNCB and NiSO₄ eliciting positive reactions for both CD54 and CD86, while LA was negative. (See Appendix H for data.)

7.3.1.2 Range finding Assay

Two independent dose-finding assays were completed in order to determine the CV75 of TTZ in THP-1 cells. Cytotoxicity was not observed at the highest concentration tested. The extrapolated CV75 was greater than the solubility limit; therefore, the maximum solubility (28 mg/mL TTZ in DMSO) was used as the highest concentration in the h-CLAT. Raw data for the Range Finding are in Appendix H.

7.3.1.3 h-CLAT

Two independent h-CLAT assessments were completed for TTZ. For all of these tests, neither CD54 nor CD86 cell surface expression was stimulated by TTZ. The TTZ was not a sensitizer in the h-CLAT. The raw data are in Appendix H. QSAR analysis predicted that TTZ is a skin sensitizer, therefore additional testing in other skin assays will need to be completed. Data are reported for the nominal concentrations of TTZ due to the fact that as of the writing of this report, concentration verification has not yet been completed by LS-MDV.

7.3.1.4 CAOTE

Mammalian acute oral toxicity was predicted using data collected in the h-CLAT. The poor water solubility of TTZ limited the usefulness of this prediction since the highest concentration tested was not cytotoxic. Using this incomplete data set, the estimated LD₅₀ was > 2000 mg/kg, suggesting that TTZ has low oral toxicity.

7.3.2 DPRA

Two independent experiments were completed for TTZ for both cysteine and lysine. Compared to reference controls, TTZ did significantly deplete both cysteine (20%) and lysine (40%) peptides. Based on the prediction model, TTZ is a skin sensitizer. The raw data are in Appendix H.

8 CONCLUSIONS

In this study, TTZ was evaluated for mutagenicity, acute aquatic toxicity, and skin sensitization using QSAR and *in vitro* approaches. The TTZ mutagenicity was predicted negative using TOPKAT and was equivocal in the Ames assay. Ecological structure activity relationship (ECOSAR) prediction for 96-hour LC₅₀ for fish was 3.46×10^5 mg/L and TTZ was moderately toxic (4.8 mg/L) for acute aquatic toxicity in the Microtox assay. TOPKAT modeled TTZ as a skin sensitizer, while the h-CLAT result was negative for sensitization, DPRA did confirm that TTZ is likely to be a sensitizer. Additionally, the hazard category algorithm for acute oral toxicity is predicted to be Category 5 by GHS. As such, TTZ is considered a moderate hazard for aquatic life (OECD 2001) and unlikely to be mutagenic. The poor solubility of TTZ contributes to uncertainty in these data as it was not tested to the routine upper limits of 500 mg/mL for h-CLAT and 50 mg/mL for mutagenicity. The TTZ is not anticipated to adsorb to soil, and due to its low solubility, it is not expected to transport in groundwater, except in acidic conditions. For additional information on TTZ toxicology and ecotoxicology predictions, see Appendix B.

9 RECOMMENDATIONS

It is recommended that further aquatic toxicity testing be considered based on the moderate toxicity exhibited with the Microtox assay and the likelihood of environmental release. The equivocal data for mutagenicity suggests further testing is needed and should be included in any short-term or repeat-dose rodent bioassays conducted in the future. The TTZ skin sensitization was predicted to be positive by TOPKAT, tested negative in the h-CLAT and positive in the DPRA – yielding a weight of evidence for TTZ as a skin sensitizer. The low solubility of TTZ could impact the interpretation of the h-CLAT data. However, TTZ has been used as an explosive for 30+ years, and there have been no reports of occupational exposures to solely TTZ that resulted in sensitization.

10 POINT OF CONTACT

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

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Appendix A

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Toxicology Study No. S.0042847-16, August 2021

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Appendix B

Substance Toxicity Profile
(E)-4-(2H-tetrazol-5-yl)tetraaz-3-ene-2-carboximidamide
[Tetrazene, TTZ]
CASRN: 31330-63-9

B-1. GENERAL INFORMATION

Tetrazene is a colorless, to pale yellow amorphous fluffy solid (Olin Winchester Ammunition 2017). There is a tautomeric form where there is a double bond between the 5-carbon on the tetrazole ring and the first nitrogen in the side chain. The molecule typically has a single water of hydration that is associated with it. Synonyms include: TTZ, tetracene, 1-(5-tetrazolyl)-4-guanyltetrazene hydrate, guanyl nitrosoaminoguanyltetrazene, and 4-(1H-tetrazol-5-yl)-3-tetrazene-2-carboximidamide monohydrate.

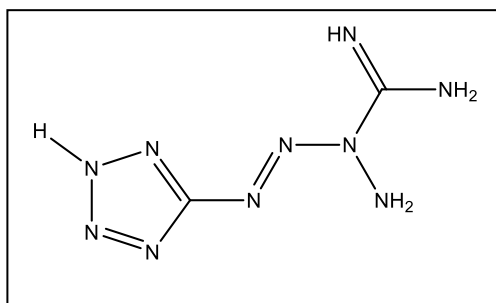


Figure B-1. Tetrazene [TTZ]
SMILES: NC(N(N)/N=N/C1=NN([H])N=N1)=N

B-2. TOXICOLOGY DATA

Few experimental data were located for TTZ. In the 1970s and 80s wastewater from the manufacture of primers was evaluated for toxicity (USAMRDC 1975). TTZ was one of the chemicals evaluated; however, only “desensitized” TTZ (boiled) and wastewater from TTZ production were tested. An additional report with “desensitized” TTZ discusses aquatic toxicity (USAMRDC 1981). These data are not included in this assessment due to inadequate chemical purity and the presence of multiple chemical species. The manufacturer’s SDS provides some analysis, but omits the primary information in most cases. The SDS indicates TTZ will produce transient organ target effects from a single exposure to unidentified organs. Additional hazards noted are eye irritation consisting of redness, swelling, and pain, with conjunctivitis from repeated exposures. Inhalation of high concentrations may cause respiratory and nasal irritation, coughing, and difficulty breathing. Ingestion may cause nausea, vomiting, constipation, cramps, or stomach pain (Olin Winchester Ammunition 2017).

B-3. ORAL

TOPKAT modeling predicts a rat oral LD₅₀ of 1100 mg/kg at high confidence and a chronic lowest-observed adverse effect level (LOAEL) of 220.5 mg/kg-day at moderate confidence. This would classify TTZ in GHS Category 4 for acute oral toxicity. The manufacturer's SDS also indicates TTZ is classified in GHS Category 4 (Olin Winchester Ammunition 2017). Using the CAOTE, the estimated acute mammalian toxicity was > 2,000 mg/kg.

B-4. INHALATION

The manufacturer's SDS indicates TTZ is classified in GHS inhalation Category 4 for dust/mist, but provides no quantitative information (Olin Winchester Ammunition 2017).

TOPKAT modeling predicts an inhalation LC₅₀ of 0.626 g/m³-hour at moderate confidence. Due to low vapor pressure, TTZ is expected to exist in the atmosphere as a dust or particulate; this LC₅₀ value places TTZ in GHS Category 3 for acute inhalation toxicity.

A case report of a worker developing a machine to charge percussion caps with an explosive mixture discussed the development of asthma that was attributed to the TTZ in the mixture following testing (Burge et al. 1984).

B-5. DERMAL

TOPKAT modeling predicts TTZ to be a possible dermal irritant and a probable sensitizer. The manufacturer's SDS reports no data available for sensitization (Olin Winchester Ammunition 2017). A case report indicates that TTZ may have caused contact dermatitis following repeated occupational exposure to TTZ (Burge et al. 1984). TTZ was negative for sensitization in the hCLAT and positive for sensitization in the DPRA.

B-6. OCULAR

The manufacturer's SDS indicates TTZ is a GHS Category 2 eye irritant, meaning it has reversible adverse effects (7 to 21 days) on the cornea, iris, or conjunctiva of the eye (Olin Winchester Ammunition 2017).

B-7. DEVELOPMENT AND REPRODUCTION

The manufacturer's SDS report TTZ is not known to cause developmental or reproductive effects (Olin Winchester Ammunition 2017). TOPKAT modeling predicts TTZ will not be a developmental or reproductive toxicant at moderate confidence.

B-8. GENOTOXICITY

The manufacturer's SDS reports TTZ is not known to be mutagenic (Olin Winchester Ammunition 2017). TOPKAT modeling predicts TTZ will be mutagenic in the Ames test at low confidence. In the Ames assay, TTZ was not reproducibly mutagenic—the stability of TTZ in the test solution may have contributed to these inconsistent data (APHC, internal test results).

B-9. CARCINOGENICITY

TOPKAT modeling of TTZ for carcinogenicity is indeterminate. The manufacturer's SDS indicates TTZ is not listed as a carcinogen by the NTP, IARC, OSHA, ACGIH®, or the USEPA.

B-10. ECOTOXICOLOGY

The manufacturer's SDS indicates TTZ has not been evaluated for environmental toxicity or fate and transport or bioaccumulation (Olin Winchester Ammunition 2017).

B-11. FATE AND TRANSPORT

TTZ is practically insoluble in water under neutral and alkaline conditions; however, it is soluble in dilute acids, so there is a greater likelihood that movement into water is possible from acidic soil (USAMRDC 1981). Otherwise, it is unlikely to adsorb to soil. Partitioning to the atmosphere from wet or dry surfaces is unlikely due to the very small value of the Henry's Law constant and vapor pressure. There is little risk of bioaccumulation due to the small value of the log K_{ow} (USEPA 2012). It also is unlikely to hydrolyze unless exposed to water temperatures greater than 80°C.

B-12. ECOTOXICITY

No experimental data were found. TOPKAT modeling predicts an EC_{50} in *Daphnia* of 1600 mg/L at low confidence; no suitable model was available to make a prediction for fathead minnows.

USEPA's ECOSAR program (USEPA 2012) models TTZ as an aliphatic amine. The predicted 96-hour EC_{50} in green algae is 6.82×10^4 mg/L, the predicted 48-hour LC_{50} in *Daphnia* is 2.02×10^4 mg/L, and the predicted 96-hour LC_{50} in fish is 3.46×10^5 mg/L. All values indicating low toxicity in the GHS system.

B-13. DEGRADATION/TREATMENT

EPI Suite™ 4.11 modeling predicts TTZ will not be readily biodegradable, with environmental persistence of days to weeks.

TTZ is predicted to be poorly removed (<2%) by physical wastewater treatment processes, primarily by sludge adsorption.

B-14. DATA

Table B-1. Physical and Chemical Properties

Molar Mass (g/mol)	Melting Point (°C)	Boiling Point (°C)	Aqueous solubility (mg/L) @ 25°C	log K _{ow}	log K _{oc}	Henry's Law Constant (atm-m ³ /mol) @ 25°C	Vapor Pressure mmHg @ 25°C
188.15 ^a	100 ^c (dec)	128 ^c (explodes)	Insoluble in cold water; decomposes in boiling water ^c	-4.13 ^b	-0.90 ^b	3.58E-19 ^b	7.13E-07 ^b

Legend:

a = (Pub Chem 2015)

b = EPI Suite 4.11 model prediction

c = Olin Winchester Ammunition 2017

Table B-2. TOPKAT Toxicity Data

Acute Oral (mg/kg)	Chronic Oral LOAEL (mg/kg-d)	Inhalation (g/m ³ -h)	Dermal	Ocular	Genotoxicity	Carcinogenicity
1100 ^a	220.5 ^a	0.626 ^a	Possible irritant; probable sensitizer ^a	Possible irritant ^a	Probable ^a	Indeterminate ^a

Legend:

a = TOPKAT model prediction (BIOVIA 2015)

Table B-3. Toxicity Assessment

Oral	Inhalation	Dermal	Ocular	Carcinogenicity	Comments
Mod	Mod	Mod	Mod	Unk	

Table B-4. Ecotoxicity Assessment

Aquatic	Terrestrial Invertebrates	Terrestrial Plants	Mammals	Birds	Comments
Low	Unk	Unk	Mod	Unk	

B-15. DISCUSSION

B-15-1. Regulations and Standards

No regulations or standards relating to tetrazene were found. Exposure limits for tetrazene have not been established (Olin Winchester Ammunition 2017).

The TTZ is not listed under the right-to-know statutes in the states of California, New Jersey, Pennsylvania, Massachusetts, or Michigan. The TTZ is not listed under California Proposition 65 (Olin Winchester Ammunition 2017).

B-15.2 Summary

TTZ is assessed to have low to moderate oral, inhalation, ocular, and dermal toxicity, posing a moderate hazard in an occupational health environment, primarily due to possible dermal sensitization. QSAR modeling predicts TTZ will not be a developmental or reproductive toxicant and the carcinogenicity prediction is indeterminate.

Ecotoxicity predictions are for low toxicity. Mobility in groundwater is expected to be low, with moderate environmental persistence. Bioaccumulation is not expected to be a hazard.

B-16. REFERENCES

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Toxicology Study No. S.0042847-16, August 2021

Appendix C

Toxicology Study No. S.0042847, April 2020

Appendix B

QUALITY ASSURANCE STATEMENT AMES ASSAY

For: Toxicology Study No. S.0042847-16, Protocol No. 2758-70-iv16-03-01 Q Ames mutagenesis screen of 1-(5-tetrazolyl)-4-guanyltetrazene hydrate (Tetrazene, TTZ) the following critical phases were inspected/audited by the Quality Systems and Regulatory Compliance Office (QSARC):

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Type Protocol Good Laboratory Practice Standard Review	03/31/2016	03/31/2016
Test Article Specific Protocol Modification Review	02/15/2018	02/15/2018

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Analytical Chemistry Support – QA review of Dosing Solution Concentration Verification	12/06/2016	12/06/2016
In-Vitro AMES Assay - Reagents, Working Solutions Bacteria Storage	05/02/2018	05/05/2018
In-Vitro AMES Assay - Reagents, Working Solutions Bacteria Storage	05/02/2018	05/05/2018
In-Vitro AMES Assay – Compliance with GLP requirements for Test Facility SOPs	05/02/2018	05/05/2018
In-Vitro AMES Assay – Calibration Verification of Equipment Used during assay	05/02/2018	05/05/2018
Study Raw Data Good Laboratory Practice Standard Review	10/29/2019	10/29/2019
Final Study Good Laboratory Practice Standard Report Review	10/29/2019	10/29/2019

Note 1: All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.

Note 2: This report has been audited by the Quality Assurance Unit (QSARC), and is considered to be an accurate account of the data generated and of the procedures followed

Note 3: In addition to the study specific critical phase inspections listed here, general facility and process based inspections not specifically related to this study are done monthly and are also listed here in accordance with QA Standard Operating Procedure.

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Michael P. Kefauver
GLP Quality Assurance Specialist, QSARC

09/21/2021
Date

Toxicology Study No. S.0042847-16, August 2021

**Quality Assurance Statement
Microtox Assay**

For: Toxicology Study No. S.0042847-16, Protocol No. 0FMA-92-iv16-06-01L Microtox Toxicity Testing of the Novel Energetic 1-(5-tetrazolyl)-4-guanyltetrazene hydrate (Tetrazene, TTZ) the following critical phases were inspected/audited by the Quality Safety Accreditation and Regulatory Compliance Office (QSARC):

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Type Protocol Good Laboratory Practice Standard Review	03/01/2018	03/01/2018
Test Article Specific Type Protocol Modification Review	04/25/2019	04/25/2019
Analytical Chemistry Support – QA review of Dosing Solution Concentration Verification	12/06/2016	12/06/2016
Microtox - Reagent and Test System Storage and Labeling requirements	05/02/2018	05/05/2018
Microtox - Data Processing and Raw Data Documentation Procedures	05/02/2018	05/05/2018
Microtox - Compliance with GLP requirements for Test Facility SOPs	05/02/2018	05/05/2018
Microtox - Calibration Verification of Equipment - Balance and Pipettes	05/02/2018	05/05/2018
Microtox Test Study Endpoint Criteria Compliance	10/28/2018	10/28/2018
Study Raw Data Good Laboratory Practice Standard Review	10/29/2019	10/29/2019
Final Study Good Laboratory Practice Standard Report Review	10/29/2019	10/29/2019

Note: All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table. A review of the raw data and records indicates the above mentioned report accurately reflects the raw data and study as it was conducted. In addition to the study specific critical phase inspections listed here, general facility and process based inspections not specifically related to this study are done monthly and are also listed here in accordance with QA Standard Operating Procedure.

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Michael P. Kefauver
GLP Quality Assurance Specialist, QSARC

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Toxicology Study No. S.0042847-16, August 2021

Quality Assurance Statement Skin Sensitization Assay

For: Toxicology Study No. S.0042847-16, Skin Sensitization Testing of the Novel Energetic 1-(5-tetrazolyl)-4-guanyltetrazene hydrate (Tetrazene, TTZ) the following critical phases were inspected/audited by the Quality Safety Accreditation and Regulatory Compliance Office (QSARC):

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Test Article Specific Protocol Modification Review	N/A	N/A

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
In-Vitro Skin Sensitization h-CLAT Assay - Preparation of Stock and Working Solutions	7/10/2019	9/23/2019
In-Vitro Skin Sensitization h-CLAT Assay - Cell Suspension and Exposure	7/10/2019	9/24/2019
In-Vitro Skin Sensitization h-CLAT Assay - Reagents, Working Solutions and Cell Suspension Storage	7/10/2019	9/23/2019
In-Vitro Skin Sensitization h-CLAT Assay – Compliance with GLP requirements for Test Facility SOPs	7/10/2019	9/24/2019
In-Vitro Skin Sensitization h-CLAT Assay – Calibration Verification of Equipment Used during assay	7/10/2019	9/23/2019
Study Raw Data Good Laboratory Practice Standard Review	9/14/2019	9/14/2019
Final Study Good Laboratory Practice Standard Report Review	10/29/2019	10/29/2019

Note: All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table. A review of the raw data and records indicates the above mentioned report accurately reflects the raw data and study as it was conducted. In addition to the study specific critical phase inspections listed here, general facility and process based inspections not specifically related to this study are done monthly and are also listed here in accordance with QA Standard Operating Procedure.

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Michael P. Kefauver
GLP Quality Assurance Specialist, QSARC

9/17/2021

Date

Appendix D

Archives and Study Personnel

D-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 Toxicology Portfolio, APHC, for a minimum of 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 the Toxicology Portfolio for a minimum of 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.0042847-16, Protocol Nos. 0FMA-92-iv17-03-01L and 2758-70-iv16-03-01.

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 SOPs can be found in the Master Control database at APHC. Maintenance and calibration logbooks may be found in Room 1026, Building E-2100, PHC, APG, MD, 21010.

Archivist: Martha Thompson

D-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

Technical staff: Tariq Armstead, ORISE Fellow

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

Appendix E

Reagents List

Table E-1. Ames Test Reagents

Ames Reagents	Source	Lot #	Exp Date
<u>AG-TA98 hisD3052 Salmonella typhimurium</u>	Xenometrix	20a	2/1/2019
<u>AG-TA100 hisG46 Salmonella typhimurium</u>	Xenometrix	23b	1/1/2021
<u>AG-TA1535 his G46 Salmonella typhimurium</u>	Xenometrix	12c	2/1/2019
<u>AG-TA1537 hisC3076 Salmonella typhimurium</u>	Xenometrix	11d	5/1/2019
<i>E. coli</i> wp2 [pKM101] <i>trpE65 Escherichia coli</i>	Xenometrix	U16	6/1/2018
<i>E. coli</i> wp2 <i>uvrA trpE65 Escherichia coli</i>	Xenometrix	P14	6/1/2018
<u>S9-Postmitochondrial Supernatant (Aroclor)</u>	Xenometrix	150212E	3/1/2020
Ampicillin	Xenometrix	162	6/1/2018
N4-aminocytidine	Xenometrix	NA079541202	n/a
<u>2-aminoanthracene</u>	Xenometrix	6417AA	7/1/2019
<u>2-nitrofluorene</u>	Xenometrix	2276NF	9/1/2019
<u>4-nitroquinoline-N-oxide</u>	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
<u>E.Coli Reversion Indicator Medium</u>	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

Table E-2. Microtox Test Reagents

Microtox Reagents	Source	Lot #	Date Expiration
Modern Water Microtox Diluent	Modern Water	16C4015	07/2019
Modern Water Microtox Acute Reagent	Modern Water	17C4076	03/2019
Dimethyl sulfoxide	Sigma	RNBG1729	07/2019
Zinc Sulfate	Sigma-Aldrich	SLBC2469V	---
Modern Water Microtox Reconstitution Solution	Modern Water	16D4031	4/2019
Modern Water Microtox Diluent	Modern Water	16C4015	07/2019

Table E-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	1930207	01-30-2019
FBS	Gibco	16140	1891436	05-30-2022
2-Mercaptoethanol	Gibco	21985	1922541	11-30-2020
Penicillin-Streptomycin	Gibco	15140	1881459	05-31-2018*
Saline	Sigma	S8776	RNBD7305	N/A
DMSO (TC)	Sigma	D2438	RNBF9410	03-2019
Globulins	Sigma	G2388	017K7650V	N/A
BSA Fraction V	Calbiochem	12660	D00150383	N/A
D-PBS	Gibco	14190	1897013	07-30-2020
Propidium Iodide	Sigma	P4864	MKBR1007V	N/A
CD54 Antibody, ICAM-1 Clone 6.5B5, FITC	Dako	F714301-8	20030876	01-2019
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	05-31-2019
Sheath Fluid	BD	342003	0000197124	03-29-2021
2,4-dinitrochlorobenzene (DNCB)	Sigma	237329	BCBN7826V	N/A
Nickel Sulfate (NiSO ₄)	Sigma	656895	MKBT0269V	N/A
Lactic Acid (LA)	Sigma	W261106	MKBR4746V	N/A

Note: *Penicillin-Streptomycin did have an expiration date preceding the usage in the testing, however, stock had been frozen immediately upon receipt and had been maintained frozen until use. Professional experience has shown that this reagent maintains full effectiveness past the expiration date and that good aseptic technique is additional protection against contamination of the media should the reagent not perform as expected.

Appendix F

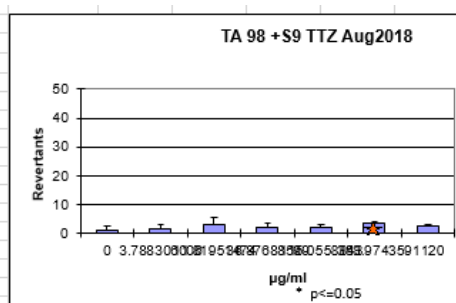
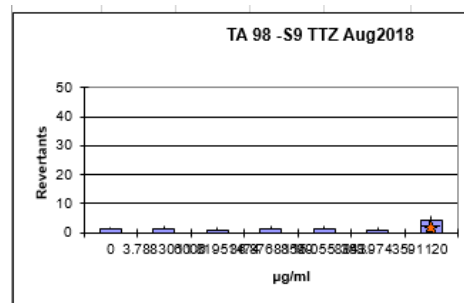
Ames Data

TA98

Compound:	TTZ Aug2018			
TA 98 -S9				Spontaneous
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3	TA 98 -S9
3.8	0	2	2	2
11.8	0	2	0	1
36.9	2	0	2	1
115.1	0	2	2	
359.0	2	1	0	
1120	5	5	2	
Pos. Control	48	48	48	
Compound:	TTZ Aug2018			
TA 98 +S9				Spontaneous
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3	TA 98 +S9
3.8	3	0	2	1
11.8	2	1	6	3
36.9	1	4	1	0
115.1	1	3	2	
359.0	3	4	4	
1120	2	3	3	
Pos. Control	45	46	45	

TA 98 -S9					Assay Date:			
Conc. (µg/ml)	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	3	1.33		0.58	1.91			
3.78830601	3	1.33		1.15		1.00	0.70	0.5000
11.8195147	3	0.67		1.15		0.50	0.35	0.2108
36.876886	3	1.33		1.15		1.00	0.70	0.5000
115.055884	3	1.33		1.15		1.00	0.70	0.5000
358.974359	3	1.00		1.00		0.75	0.52	0.3217
1120	3	4.00		1.73		3.00	2.09	0.0323
Pos. Control	3	48.00		0.00				

TTZ Aug2018 TA 98 +S9					Assay Date:			
Conc. (µg/ml)	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	3	1.33		1.53	2.86			
3.78830601	3	1.67		1.53		1.25	0.58	0.4012
11.8195147	3	3.00		2.65		2.25	1.05	0.1991
36.876886	3	2.00		1.73		1.50	0.70	0.3217
115.055884	3	2.00		1.00		1.50	0.70	0.2807
358.974359	3	3.67		0.58		2.75	1.28	0.0343
1120	3	2.67		0.58		2.00	0.93	0.1151
Pos. Control	3	45.33		0.58				



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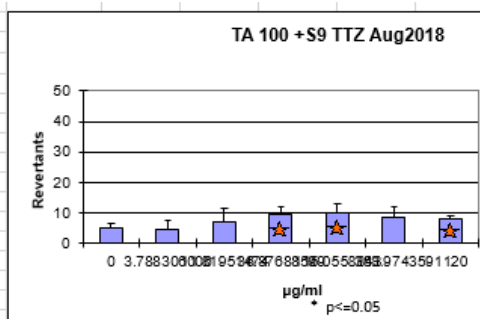
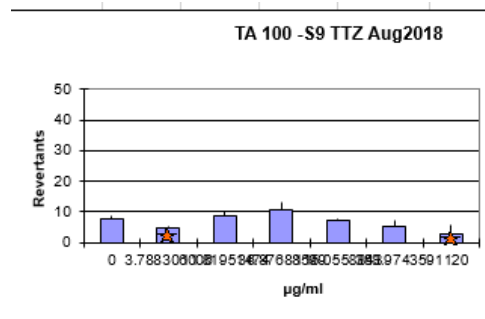
TA100

Compound: TTZ Aug2018				Spontaneous
TA 100 -S9				
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3	TA 100 -S9
3.8	5	5	4	9
11.8	10	7	9	7
36.9	8	12	12	7
115.1	7	7	8	
359.0	7	5	3	
1120	1	2	6	
Pos. Control	47	47	47	

Compound: TTZ Aug2018				Spontaneous
TA 100 +S9				
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3	TA 100 +S9
3.8	4	2	8	5
11.8	12	6	3	7
36.9	10	7	12	4
115.1	10	8	13	
359.0	11	5	10	
1120	8	9	8	
Pos. Control	40	41	41	

TTZ Aug2018									
TA 100 -S9				Assay Date:					
Conc. (µg/ml)	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	3	7.67		1.15	8.82				
3.78830601	3	4.67		0.58		0.61	0.53	0.0079	Cytotoxic effect?
11.8195147	3	8.67		1.53		1.13	0.98	0.2084	
36.876886	3	10.67		2.31		1.39	1.21	0.0572	
115.055884	3	7.33		0.58		0.96	0.83	0.3389	
358.974359	3	5.00		2.00		0.65	0.57	0.0581	
1120	3	3.00		2.65		0.39	0.34	0.0244	Cytotoxic effect?
Pos. Control	3	47.00		0.00					

TTZ Aug2018				Assay Date:				
TA 100 +S9								
Conc. (µg/ml)	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	3	5.33		1.53	6.86			
3.78830601	3	4.67		3.06		0.88	0.68	0.3762
11.8195147	3	7.00		4.58		1.31	1.02	0.2911
36.876886	3	9.67		2.52		1.81	1.41	0.0317
115.055884	3	10.33		2.52		1.94	1.51	0.0212
358.974359	3	8.67		3.21		1.63	1.26	0.0900
1120	3	8.33		0.58		1.56	1.21	0.0167
Pos. Control	3	40.67		0.58				



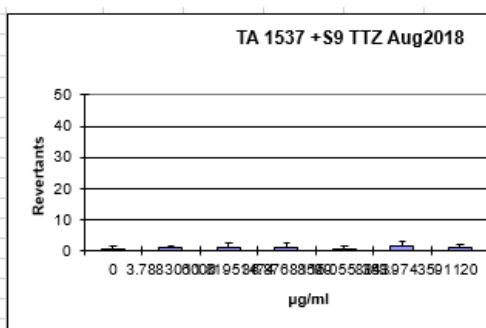
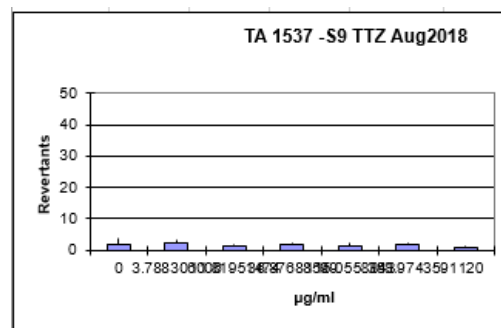
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TA1537

Compound: TTZ Aug2018				Spontaneous
TA 1537 -S9				
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3	TA 1537 -S9
3.8	3	1	3	0
11.8	1	1	2	3
36.9	2	1	2	3
115.1	2	0	2	
359.0	1	2	2	
1120	1	1	0	
Pos. Control	48	48	48	

Compound: TTZ Aug2018				Spontaneous
TA 1537 +S9				
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3	TA 1537 +S9
3.8	1	2	1	0
11.8	0	0	3	2
36.9	0	1	3	0
115.1	2	0	0	
359.0	3	2	0	
1120	2	0	1	
Pos. Control	27	25	25	

TA 1537 -S9					Assay Date:		
Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	t-test p-value (unpaired, 1 sided)
0	3	2.00		1.73	3.73		
3.78830601	3	2.33		1.15		1.17	0.3976
11.8195147	3	1.33		0.58		0.67	0.2807
36.876886	3	1.67		0.58		0.83	0.3838
115.055884	3	1.33		1.15		0.67	0.3043
358.974359	3	1.67		0.58		0.83	0.3838
1120	3	0.67		0.58		0.33	0.1373
Pos. Control	3	48.00		0.00			
TTZ Aug2018					Assay Date:		
TA 1537 +S9					Assay Date:		
Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	SD	Base-line	Fold increase (over zero value)	t-test p-value (unpaired, 1 sided)
0	3	0.67	1.00	1.15	1.82		
3.78830601	3	1.33		0.58		1.33	0.2108
11.8195147	3	1.00		1.73		1.00	0.3976
36.876886	3	1.33		1.53		1.33	0.2895
115.055884	3	0.67		1.15		0.67	0.5000
358.974359	3	1.67		1.53		1.67	0.2084
1120	3	1.00		1.00		1.00	0.3623
Pos. Control	3	25.67		1.15			



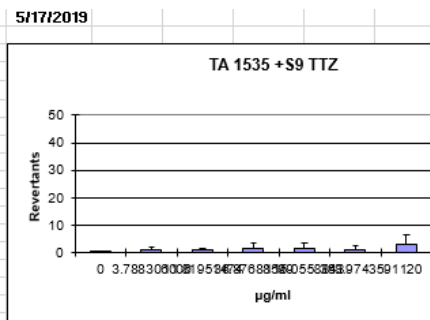
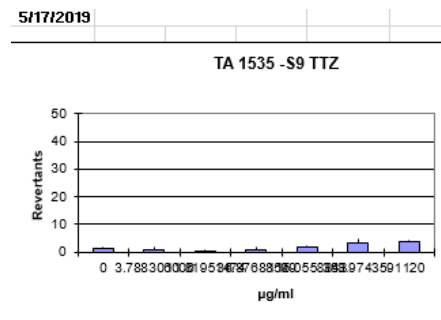
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TA1535

Compound:	TTZ				
TA 1535 -S9					Spontaneous
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3		TA 1535 -S9
3.8	1	2	0		2
11.8	0	1	0		1
36.9	1	0	2		1
115.1	1	2	2		
359.0	2	5	3		
1120	4	3	4		
Pos. Control	48	48	48		
Compound:	TTZ				
TA 1535 +S9					Spontaneous
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3		TA 1535 +S9
3.8	2	0	1		0
11.8	1	1	2		0
36.9	4	1	0		1
115.1	1	0	4		
359.0	2	2	0		
1120	1	7	1		
Pos. Control	27	34	37		

TTZ											
TA 1535 -S9						Assay Date:	5/17/2019				
Conc. (µg/ml)	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	3	1.33		0.58	1.91						
3.78830601	3	1.00		1.00		0.75	0.52	0.3217			
11.8195147	3	0.33		0.58		0.25	0.17	0.0506			
36.876886	3	1.00		1.00		0.75	0.52	0.3217			
115.055884	3	1.67		0.58		1.25	0.87	0.2593			
358.974359	3	3.33		1.53		2.50	1.74	0.0506			
1120	3	3.67		0.58		2.75	1.92	0.0039			
Pos. Control	3	48.00		0.00							

TTZ											
TA 1535 +S9						Assay Date:	5/17/2019				
Conc. (µg/ml)	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	3	0.33	1.00	0.58	1.00						
3.78830601	3	1.00		1.00		1.00	1.00	0.1870			
11.8195147	3	1.33		0.58		1.33	1.33	0.0506			
36.876886	3	1.67		2.08		1.67	1.67	0.1726			
115.055884	3	1.67		2.08		1.67	1.67	0.1726			
358.974359	3	1.33		1.15		1.33	1.33	0.1254			
1120	3	3.00		3.46		3.00	3.00	0.1294			
Pos. Control	3	32.67		5.13							



E.Coli

Compound: TTZ Jan2019					
EC Combo -S9				Spontaneous	
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3	EC Combo - S9	
3.8	2	1	1	4	
11.8	0	3	1	3	
36.9	2	3	2	2	
115.1	0	3	2		
359.0	3	2	0		
1120	0	3	2		
Pos. Control	33	35	38		

Compound: TTZ Jan2019					
EC Combo +S9				Spontaneous	
Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3	EC Combo +S9	
3.8	0	1	0	1	
11.8	0	2	0	4	
36.9	2	0	1	3	
115.1	1	3	2		
359.0	4	5	2		
1120	1	3	3		
Pos. Control	4	2	1		

Compound: TTZ Aug2018					
EC Combo -S9				Spontaneous	
Conc. ()	Replicate #1	Replicate #2	Replicate #3	EC Combo - S9	
3.8	5	8	9	2	✓
11.8	15	12	15	9	✓
36.9	15	15	8	7	✓
115.1	13	9	20		✓
359.0	7	3	12		✓
1120	5	3	6		✓
Pos. Control	35	38	41		

Compound: TTZ Aug2018					
EC Combo +S9				Spontaneous	
Conc. ()	Replicate #1	Replicate #2	Replicate #3	EC Combo +S9	
3.8	14	17	21	13	✓
11.8	10	16	15	24	✓
36.9	15	16	24	17	✓
115.1	16	17	21		✓
359.0	16	13	21		✓
1120	8	12	13		✓
Pos. Control	26	30	31		

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TTZ Jan2019					Assay Date:			
EC Combo -S9								
Conc. (µg/ml)	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	3	3.00		1.00	4.00			
3.78830601	3	1.33		0.58		0.44	0.33	0.0334
11.8195147	3	1.33		1.53		0.44	0.33	0.0945
36.876886	3	2.33		0.58		0.78	0.58	0.1870
115.055884	3	1.67		1.53		0.56	0.42	0.1373
358.974359	3	1.67		1.53		0.56	0.42	0.1373
1120	3	1.67		1.53		0.56	0.42	0.1373
Pos. Control	3	35.33		2.52				
TTZ Jan2019					Assay Date:			
EC Combo +S9								
Conc. (µg/ml)	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	3	2.67		1.53	4.19			
3.78830601	3	0.33		0.58		0.13	0.08	0.0343
11.8195147	3	0.67		1.15		0.25	0.16	0.0724
36.876886	3	1.00		1.00		0.38	0.24	0.0945
115.055884	3	2.00		1.00		0.75	0.48	0.2807
358.974359	3	3.67		1.53		1.38	0.87	0.2338
1120	3	2.33		1.15		0.88	0.56	0.3890
Pos. Control	3	2.33		1.53				

Cytotoxic effect?

Cytotoxic effect?

TTZ Aug2018					Assay Date:			
EC Combo -S9								
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	3	6.00		3.61	9.61			
3.78830601	3	7.33		2.08		1.22	1.83	0.0157
11.8195147	3	14.00		1.73		2.33	3.50	0.0003
36.876886	3	12.67		4.04		2.11	3.17	0.0079
115.055884	3	14.00		5.57		2.33	3.50	0.0140
358.974359	3	7.33		4.51		1.22	1.83	0.0897
1120	3	4.67		1.53		0.78	1.17	0.0945
Pos. Control	3	38.00		3.00				
TTZ Aug2018					Assay Date:			
EC Combo +S9								
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	3	18.00		5.57	23.57			
3.78830601	3	17.33		3.51		0.96	4.13	0.0013
11.8195147	3	13.67		3.21		0.76	3.26	0.0029
36.876886	3	18.33		4.93		1.02	4.37	0.0031
115.055884	3	18.00		2.65		1.00	4.29	0.0005
358.974359	3	16.67		4.04		0.93	3.97	0.0025
1120	3	11.00		2.65		0.61	2.62	0.0046
Pos. Control	3	29.00		2.65				

Appendix G
Microtox Test Data Tables and Calculations

Table G-1. Microtox Test Dilutions

Nominal Concentration (mg/mL; 100x test concentration)	Corrected Working Concentration* (mg/mL; 100x test concentration)		
	Test 1	Test 2	Test 3
0.056			
0.11			
0.22			
0.45			
0.89			
1.78			
3.56			
7.13			

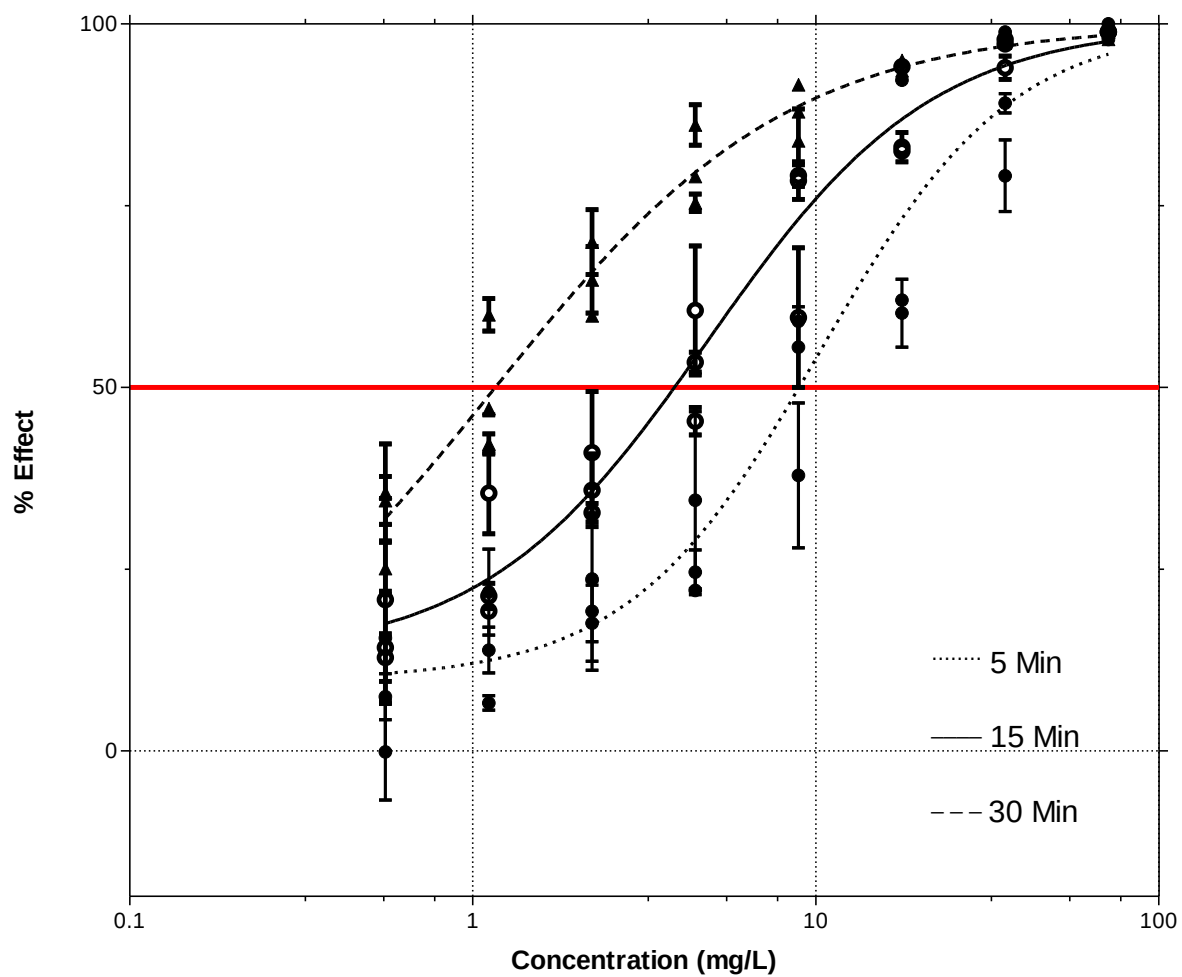
Note:

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

Table G-2. Tetrazene EC₅₀ Estimates

Tetrazene EC ₅₀ (mg/L; 95% CI)		
5 minute	15 minute	30 minute
10.28 [8.3-12.7]	4.842 [3.8-6.2]	1.1 [0.5731-2.28]

Microtox Toxicity of Tetrazene



Appendix H

h-CLAT Data

Table H-1. Reactivity Check

Test article	Concentration (mg/mL)	Viability (% alive)	RFI (CD86)	RFI (CD54)	Positive (CD86/CD54)	Pass/Fail
Saline		93.98	1	1	N/N	Pass
DMSO		93.03	1	1	N/N	Pass
DNCB	0.0033	79.9	395.05	578.17	Y/Y	Pass
	0.0040	73.12	160.07	459.39	Y/Y	Pass
NiSO ₄	0.10	77.09	342.92	4909.4	Y/Y	Pass
Lactic Acid	1	94.16	86.45	99.28	N/N	Pass

Reactivity Check Raw Data

Experiment:
h-CLAT Reactivity check 9-11-18

Cytometer: BD FACSVers

Cytometer SN: Z6511530048

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	1,103
Live Cells:Live Cells	9,016	90.16	***	90.16	990
Dead Cells:All Events	10,000	***	***	100.00	2,221
Dead Cells:Live Cells	103	1.03	***	1.03	1,486
IgG Media:All Events	10,399	***	***	100.00	966
IgG Media:Live Cells	9,883	95.04	***	95.04	908
IgG Saline:All Events	10,641	***	***	100.00	969
IgG Saline:Live Cells	10,000	93.98	***	93.98	895
IgG DMSO:All Events	10,746	***	***	100.00	949
IgG DMSO:Live Cells	9,997	93.03	***	93.03	870
IgG Lactic Acid:All Events	10,620	***	***	100.00	942
IgG Lactic Acid:Live Cells	10,000	94.16	***	94.16	864
IgG DNCB 1:All Events	12,516	***	***	100.00	1,295
IgG DNCB 1:Live Cells	10,000	79.90	***	79.90	1,062
IgG DNCB 2:All Events	13,677	***	***	100.00	1,339
IgG DNCB 2:Live Cells	10,000	73.12	***	73.12	1,028
IgG DNCB 3:All Events	15,285	***	***	100.00	1,442
IgG DNCB 3:Live Cells	10,010	65.49	***	65.49	1,016
IgG N2S:All Events	12,972	***	***	100.00	1,111
IgG N2S:Live Cells	10,000	77.09	***	77.09	953
CD86 Media:All Events	10,320	***	***	100.00	1,579
CD86 Media:Live Cells	9,944	96.36	***	96.36	1,463
CD54 Media:All Events	10,738	***	***	100.00	1,163
CD54 Media:Live Cells	10,000	93.13	***	93.13	1,075
CD86 Saline:All Events	10,705	***	***	100.00	1,562
CD86 Saline:Live Cells	10,000	93.41	***	93.41	1,382
CD54 Saline:All Events	10,786	***	***	100.00	1,158
CD54 Saline:Live Cells	9,946	92.21	***	92.21	1,034
CD86 DMSO:All Events	11,174	***	***	100.00	1,729
CD86 DMSO:Live Cells	10,000	89.49	***	89.49	1,476
CD54 DMSO:All Events	10,901	***	***	100.00	1,179
CD54 DMSO:Live Cells	10,000	91.73	***	91.73	1,067
CD86 Lactic Acid:All Events	10,800	***	***	100.00	1,466
CD86 Lactic Acid:Live Cells	10,000	92.59	***	92.59	1,285
CD54 Lactic Acid:All Events	11,015	***	***	100.00	1,131
CD54 Lactic Acid:Live Cells	10,000	90.79	***	90.79	1,002
CD86 DNCB 1:All Events	13,508	***	***	100.00	4,620
CD86 DNCB 1:Live Cells	10,000	74.03	***	74.03	3,456
CD54 DNCB 1:All Events	13,065	***	***	100.00	2,633
CD54 DNCB 1:Live Cells	9,273	70.98	***	70.98	2,201
CD86 DNCB 2:All Events	13,008	***	***	100.00	3,237
CD86 DNCB 2:Live Cells	9,797	75.32	***	75.32	1,998
CD54 DNCB 2:All Events	14,129	***	***	100.00	2,309
CD54 DNCB 2:Live Cells	10,000	70.78	***	70.78	1,933
CD86 N2S:All Events	13,402	***	***	100.00	3,562
CD86 N2S:Live Cells	10,000	74.62	***	74.62	2,623
CD54 N2S:All Events	13,606	***	***	100.00	7,090
CD54 N2S:Live Cells	9,830	72.25	***	72.25	7,777

Table H-2. Range Finding

9/14/2018		PI- Dose Finding			
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	CV75	mg/mL
DMSO		0	98.2		
Tetrazene Run #1	0.219	0.000438	99.1		> 0.056 mg/mL
	0.438	0.000875	97.7		
	0.875	0.00175	97.3		
	1.75	0.0035	97.9		
	3.5	0.007	97.7		
	7	0.014	97.4		
	14	0.028	97.2		
	28	0.056	97.5	N/A	
9/18/2018		PI- Dose Finding			
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	CV75	mg/mL
DMSO		0	98.0		
Tetrazene Run #2	0.219	0.000438	98.8		> 0.056 mg/mL
	0.438	0.000875	96.4		
	0.875	0.00175	98.1		
	1.75	0.0035	97.6		
	3.5	0.007	97.8		
	7	0.014	97.6		
	14	0.028	98.2		
	28	0.056	97.8		

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Range Finding Experiment 1 Raw Data

Experiment:
h-CLAT Range-Finding MDA50, TTZ #1 9-14-18

Cytometer: BD FACSVe

Cytometer SN: Z6511530048

Statistics					
Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	360
Live Cells:Live Cells	9,834	98.34	***	98.34	353
Dead cells:All Events	10,000	***	***	100.00	28,272
Dead cells:Live Cells	1,470	14.70	***	14.70	608
DMSO:All Events	10,170	***	***	100.00	359
DMSO:Live Cells	9,988	98.21	***	98.21	349
MDA50 1:All Events	10,194	***	***	100.00	446
MDA50 1:Live Cells	9,989	97.99	***	97.99	435
MDA50 2:All Events	10,233	***	***	100.00	516
MDA50 2:Live Cells	9,992	97.64	***	97.64	501
MDA50 3:All Events	10,268	***	***	100.00	624
MDA50 3:Live Cells	9,993	97.32	***	97.32	608
MDA50 4:All Events	10,100	***	***	100.00	859
MDA50 4:Live Cells	9,964	98.65	***	98.65	825
MDA50 5:All Events	14,501	***	***	100.00	1,359
MDA50 5:Live Cells	10,061	69.38	***	69.38	1,084
MDA50 6:All Events	30,000	***	***	100.00	2,413
MDA50 6:Live Cells	9,283	30.94	***	30.94	1,338
MDA50 7:All Events	20,868	***	***	100.00	4,747
MDA50 7:Live Cells	5,176	24.80	***	24.80	1,755
MDA50 8:All Events	14,328	***	***	100.00	9,991
MDA50 8:Live Cells	367	2.56	***	2.56	897
TTZ 1:All Events	10,091	***	***	100.00	359
TTZ 1:Live Cells	9,997	99.07	***	99.07	342
TTZ 2:All Events	10,236	***	***	100.00	345
TTZ 2:Live Cells	10,000	97.69	***	97.69	338
TTZ 3:All Events	10,280	***	***	100.00	347
TTZ 3:Live Cells	10,000	97.28	***	97.28	344
TTZ 4:All Events	10,214	***	***	100.00	343
TTZ 4:Live Cells	10,000	97.90	***	97.90	340
TTZ 5:All Events	10,232	***	***	100.00	358
TTZ 5:Live Cells	10,000	97.73	***	97.73	347
TTZ 6:All Events	10,288	***	***	100.00	353
TTZ 6:Live Cells	10,017	97.37	***	97.37	345
TTZ 7:All Events	10,292	***	***	100.00	371
TTZ 7:Live Cells	10,000	97.16	***	97.16	356
TTZ 8:All Events	10,255	***	***	100.00	365
TTZ 8:Live Cells	10,000	97.51	***	97.51	355

Toxicology Study No. S.0042847-16, August 2021

Range Finding Experiment 2 Raw Data

Experiment:
h-CLAT MDA50 and TTZ Range Finding #2 9-18-18

Cytometer: BD FACSVerser

Cytometer SN: Z6511530048

Statistics					
Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	347
Live Cells:Live Cells	9,826	98.26	***	98.26	332
Dead Cells:All Events	10,000	***	***	100.00	35,215
Dead Cells:Live Cells	1,070	10.70	***	10.70	582
DMSO:All Events	10,303	***	***	100.00	339
DMSO:Live Cells	10,097	98.00	***	98.00	328
MDA50 #1:All Events	10,282	***	***	100.00	466
MDA50 #1:Live Cells	10,000	97.26	***	97.26	440
MDA50 #2:All Events	10,420	***	***	100.00	538
MDA50 #2:Live Cells	10,000	95.97	***	95.97	500
MDA50 #3:All Events	10,785	***	***	100.00	693
MDA50 #3:Live Cells	10,000	92.72	***	92.72	638
MDA50 #4:All Events	13,364	***	***	100.00	919
MDA50 #4:Live Cells	10,000	74.83	***	74.83	771
MDA50 #5:All Events	30,000	***	***	100.00	1,425
MDA50 #5:Live Cells	19,325	64.42	***	64.42	1,082
MDA50 #6:All Events	11,648	***	***	100.00	2,473
MDA50 #6:Live Cells	8,888	76.30	***	76.30	1,874
MDA50 #7:All Events	28,582	***	***	100.00	4,583
MDA50 #7:Live Cells	1,211	4.24	***	4.24	1,029
MDA50 #8:All Events	15,118	***	***	100.00	10,526
MDA50 #8:Live Cells	241	1.59	***	1.59	722
TTZ #1:All Events	10,117	***	***	100.00	347
TTZ #1:Live Cells	10,000	98.84	***	98.84	330
TTZ #2:All Events	10,233	***	***	100.00	343
TTZ #2:Live Cells	9,864	96.39	***	96.39	335
TTZ #3:All Events	10,135	***	***	100.00	349
TTZ #3:Live Cells	9,938	98.06	***	98.06	338
TTZ #4:All Events	10,245	***	***	100.00	344
TTZ #4:Live Cells	9,999	97.60	***	97.60	331
TTZ #5:All Events	10,225	***	***	100.00	343
TTZ #5:Live Cells	10,000	97.80	***	97.80	334
TTZ #6:All Events	10,196	***	***	100.00	354
TTZ #6:Live Cells	9,951	97.60	***	97.60	346
TTZ #7:All Events	10,122	***	***	100.00	353
TTZ #7:Live Cells	9,941	98.21	***	98.21	339
TTZ #8:All Events	10,224	***	***	100.00	347
TTZ #8:Live Cells	10,000	97.81	***	97.81	338

Operator: Emily Reinke, Ph.D.

Printed: 9/18/2018 9:37:42 AM

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

Experiment:

h-CLAT TTZ #1 9-20-18

Software: BD FACSuite v1.0.5

Cytometer SN: Z6511530048

Statistics						Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean	Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live:All Events	10,000	***	***	100.00	1,045	CD86 Tetrazene 6:All Events	10,467	***	***	100.00	2,169
Live:Live Cells	9,275	92.75	***	92.75	949	CD86 Tetrazene 6:Live Cells	9,889	94.48	***	94.48	1,887
Dead:All Events	10,000	***	***	100.00	54,060	CD54 Tetrazene 6:All Events	11,088	***	***	100.00	1,246
Dead:Live Cells	336	3.36	***	3.36	9,407	CD54 Tetrazene 6:Live Cells	10,421	93.98	***	93.98	1,127
IgG Media:All Events	10,363	***	***	100.00	1,032	CD86 Tetrazene 7:All Events	10,935	***	***	100.00	2,043
IgG Media:Live Cells	9,927	95.79	***	95.79	952	CD86 Tetrazene 7:Live Cells	10,000	91.45	***	91.45	1,745
IgG DMSO:All Events	10,791	***	***	100.00	1,031	CD54 Tetrazene 7:All Events	10,989	***	***	100.00	1,258
IgG DMSO:Live Cells	9,999	92.66	***	92.66	911	CD54 Tetrazene 7:Live Cells	9,959	90.63	***	90.63	1,099
IgG DNCB 1:All Events	12,226	***	***	100.00	1,339	CD86 Tetrazene 8:All Events	10,779	***	***	100.00	2,222
IgG DNCB 1:Live Cells	9,992	81.73	***	81.73	1,125	CD86 Tetrazene 8:Live Cells	9,877	91.63	***	91.63	1,895
IgG DNCB 2:All Events	12,693	***	***	100.00	1,386	CD54 Tetrazene 8:All Events	10,917	***	***	100.00	1,194
IgG DNCB 2:Live Cells	10,000	78.78	***	78.78	1,111	CD54 Tetrazene 8:Live Cells	9,998	91.58	***	91.58	1,052
IgG DNCB 3:All Events	15,231	***	***	100.00	2,515						
IgG DNCB 3:Live Cells	3,203	21.03	***	21.03	1,347						
IgG Tetrazene 1:All Events	10,677	***	***	100.00	1,070						
IgG Tetrazene 1:Live Cells	9,875	92.49	***	92.49	950						
IgG Tetrazene 2:All Events	10,880	***	***	100.00	1,053						
IgG Tetrazene 2:Live Cells	10,001	91.92	***	91.92	926						
IgG Tetrazene 3:All Events	10,881	***	***	100.00	1,018						
IgG Tetrazene 3:Live Cells	10,000	91.90	***	91.90	897						
IgG Tetrazene 4:All Events	10,842	***	***	100.00	1,046						
IgG Tetrazene 4:Live Cells	10,000	92.23	***	92.23	921						
IgG Tetrazene 5:All Events	10,819	***	***	100.00	1,010						
IgG Tetrazene 5:Live Cells	9,999	92.42	***	92.42	907						
IgG Tetrazene 6:All Events	10,675	***	***	100.00	990						
IgG Tetrazene 6:Live Cells	10,000	93.68	***	93.68	905						
IgG Tetrazene 7:All Events	10,779	***	***	100.00	1,051						
IgG Tetrazene 7:Live Cells	9,934	92.16	***	92.16	935						
IgG Tetrazene 8:All Events	10,799	***	***	100.00	1,013						
IgG Tetrazene 8:Live Cells	10,000	92.60	***	92.60	897						
CD86 Media:All Events	10,762	***	***	100.00	2,280						
CD86 Media:Live Cells	9,959	92.54	***	92.54	1,940						
CD54 Media:All Events	10,781	***	***	100.00	1,177						
CD54 Media:Live Cells	9,998	92.74	***	92.74	1,055						
CD86 DMSO:All Events	10,618	***	***	100.00	2,336						
CD86 DMSO:Live Cells	9,892	93.16	***	93.16	2,017						
CD54 DMSO:All Events	10,539	***	***	100.00	1,223						
CD54 DMSO:Live Cells	9,852	93.48	***	93.48	1,099						
CD86 DNCB 1:All Events	11,452	***	***	100.00	5,481						
CD86 DNCB 1:Live Cells	9,764	85.26	***	85.26	4,129						
CD54 DNCB 1:All Events	12,846	***	***	100.00	2,327						
CD54 DNCB 1:Live Cells	9,995	77.81	***	77.81	1,981						
CD86 DNCB 2:All Events	12,989	***	***	100.00	3,981						
CD86 DNCB 2:Live Cells	10,000	76.99	***	76.99	2,561						
CD54 DNCB 2:All Events	12,968	***	***	100.00	2,519						
CD54 DNCB 2:Live Cells	10,000	77.11	***	77.11	2,139						
CD86 Tetrazene 1:All Events	10,875	***	***	100.00	2,081						
CD86 Tetrazene 1:Live Cells	10,000	91.95	***	91.95	1,784						
CD54 Tetrazene 1 :All Events	10,449	***	***	100.00	1,291						
CD54 Tetrazene 1 :Live Cells	9,889	94.64	***	94.64	1,162						
CD86 Tetrazene 2:All Events	10,762	***	***	100.00	2,088						
CD86 Tetrazene 2:Live Cells	9,912	92.10	***	92.10	1,780						
CD54 Tetrazene 2:All Events	10,451	***	***	100.00	1,235						
CD54 Tetrazene 2:Live Cells	9,891	94.64	***	94.64	1,114						
CD86 Tetrazene 3:All Events	10,738	***	***	100.00	2,051						
CD86 Tetrazene 3:Live Cells	9,892	92.12	***	92.12	1,760						
CD54 Tetrazene 3:All Events	11,051	***	***	100.00	1,251						
CD54 Tetrazene 3:Live Cells	10,000	90.49	***	90.49	1,071						
CD86 Tetrazene 4:All Events	10,965	***	***	100.00	2,019						
CD86 Tetrazene 4:Live Cells	10,000	91.20	***	91.20	1,689						
CD54 Tetrazene 4:All Events	11,014	***	***	100.00	1,268						
CD54 Tetrazene 4:Live Cells	10,000	90.79	***	90.79	1,095						
CD86 Tetrazene 5:All Events	10,915	***	***	100.00	2,029						
CD86 Tetrazene 5:Live Cells	10,000	91.62	***	91.62	1,706						
CD54 Tetrazene 5:All Events	10,893	***	***	100.00	1,196						
CD54 Tetrazene 5:Live Cells	10,000	91.80	***	91.80	1,061						

Operator: Emily Reinke, Ph.D.

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Software: BD FACSuite v1.0.5

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Table H-3. h-CLAT Experiment 1 Data Analysis

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	HTC IgG	HTC CD86	RFI	% change	EC150	HTC CD54	RFI	% change	EC200
9/20/2018												
Media		0	95.8	952	1940	1	100		1055	1	100	
DMSO		0	92.7	911	2017	1.12	112		1099	1.83	183	
DNCB Control	1.67	0.00333	81.7	1125	4129	2.72	272		1981	8.31	831	
	2	0.004	78.8	1111	2561	1.31	131		2139	9.98	998	
	2.4	0.0048	21.0	1347	---				--			
TTZ	7.81	0.0156	92.5	950	1784	0.754	75.4	N/A	1162	2.06	206	N/A
	9.38	0.0188	91.9	926	1780	0.772	77.2		1114	1.83	183	
	11.3	0.0225	91.9	897	1760	0.780	78.0		1071	1.69	169	
	13.5	0.0270	92.2	921	1689	0.694	69.4		1095	1.69	169	
	16.2	0.0324	92.4	907	1706	0.722	72.2		1061	1.50	150	
	19.4	0.0389	93.7	905	1887	0.888	88.8		1127	2.16	216	
	23.3	0.0467	92.2	935	1745	0.732	73.2		1099	1.59	159	
	28.0	0.0560	92.6	897	1895	0.902	90.2		1052	1.50	150	

h-CLAT Experiment 2 Raw Data

Experiment:
h-CLAT TTZ #2 9-27-18

Cytometer: BD FACSVerser

Cytometer SN: Z6511530048

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
CD54 TTZ 9:All Events	11,491	***	***	100.00	1,321
Media:All Events	10,000	***	***	100.00	955
Media:Live Cells	9,330	93.30	***	93.30	866
IgG Media:All Events	10,742	***	***	100.00	941
IgG Media:Live Cells	10,000	93.09	***	93.09	855
IgG DMSO:All Events	10,849	***	***	100.00	984
IgG DMSO:Live Cells	10,000	92.17	***	92.17	863
IgG DNCB 1:All Events	14,095	***	***	100.00	1,538
IgG DNCB 1:Live Cells	10,000	70.95	***	70.95	1,245
IgG DNCB 2:All Events	12,581	***	***	100.00	1,544
IgG DNCB 2:Live Cells	9,849	78.28	***	78.28	1,314
IgG TTZ 1:All Events	10,907	***	***	100.00	949
IgG TTZ 1:Live Cells	10,000	91.68	***	91.68	851
IgG TTZ 2:All Events	10,760	***	***	100.00	981
IgG TTZ 2:Live Cells	9,793	91.01	***	91.01	869
IgG TTZ 3:All Events	10,896	***	***	100.00	953
IgG TTZ 3:Live Cells	10,000	91.78	***	91.78	841
IgG TTZ 4:All Events	10,360	***	***	100.00	981
IgG TTZ 4:Live Cells	9,952	96.06	***	96.06	914
IgG TTZ 5:All Events	11,011	***	***	100.00	993
IgG TTZ 5:Live Cells	10,000	90.82	***	90.82	862
IgG TTZ 6:All Events	10,942	***	***	100.00	939
IgG TTZ 6:Live Cells	9,997	91.36	***	91.36	831
IgG TTZ 7:All Events	11,034	***	***	100.00	957
IgG TTZ 7:Live Cells	9,995	90.58	***	90.58	841
IgG TTZ 8:All Events	10,890	***	***	100.00	934
IgG TTZ 8:Live Cells	10,000	91.83	***	91.83	832
IgG TTZ 9:All Events	10,579	***	***	100.00	976
IgG TTZ 9:Live Cells	9,874	93.34	***	93.34	875
CD86 Media:All Events	10,847	***	***	100.00	1,935
CD86 Media:Live Cells	10,129	93.38	***	93.38	1,692
CD54 Media:All Events	10,782	***	***	100.00	1,134
CD54 Media:Live Cells	10,000	92.75	***	92.75	1,018
CD86 DMSO:All Events	10,973	***	***	100.00	1,993
CD86 DMSO:Live Cells	10,000	91.13	***	91.13	1,689
CD54 DMSO:All Events	11,036	***	***	100.00	1,165
CD54 DMSO:Live Cells	10,000	90.61	***	90.61	1,017
CD86 DNCB 1:All Events	13,226	***	***	100.00	6,358
CD86 DNCB 1:Live Cells	8,811	66.62	***	66.62	4,084
CD54 DNCB 1:All Events	11,539	***	***	100.00	2,539
CD54 DNCB 1:Live Cells	7,412	64.23	***	64.23	2,068
CD86 DNCB 2:All Events	14,005	***	***	100.00	5,362
CD86 DNCB 2:Live Cells	8,572	61.21	***	61.21	3,337
CD54 DNCB 2:All Events	13,645	***	***	100.00	2,613
CD54 DNCB 2:Live Cells	8,678	63.60	***	63.60	2,232
CD86 TTZ 1:All Events	11,196	***	***	100.00	1,880
CD86 TTZ 1:Live Cells	10,000	89.32	***	89.32	1,543
CD54 TTZ 1 :All Events	11,132	***	***	100.00	1,194
CD54 TTZ 1 :Live Cells	10,000	89.83	***	89.83	1,030
CD86 TTZ 2:All Events	11,218	***	***	100.00	1,841
CD86 TTZ 2:Live Cells	10,000	89.14	***	89.14	1,506
CD54 TTZ 2:All Events	11,152	***	***	100.00	1,167
CD54 TTZ 2:Live Cells	10,000	89.67	***	89.67	1,011
CD86 TTZ 3:All Events	11,074	***	***	100.00	1,912
CD86 TTZ 3:Live Cells	10,000	90.30	***	90.30	1,601
CD54 TTZ 3:All Events	11,137	***	***	100.00	1,160
CD54 TTZ 3:Live Cells	9,992	89.72	***	89.72	1,012
CD86 TTZ 4:All Events	11,150	***	***	100.00	1,867
CD86 TTZ 4:Live Cells	10,000	89.69	***	89.69	1,541
CD54 TTZ 4:All Events	10,960	***	***	100.00	1,150
CD54 TTZ 4:Live Cells	10,000	91.24	***	91.24	1,020
CD86 TTZ 5:All Events	11,208	***	***	100.00	1,755
CD86 TTZ 5:Live Cells	10,000	89.22	***	89.22	1,454
CD54 TTZ 5:All Events	11,060	***	***	100.00	1,139
CD54 TTZ 5:Live Cells	9,982	90.25	***	90.25	1,003
CD86 TTZ 6:All Events	11,394	***	***	100.00	1,859
CD86 TTZ 6:Live Cells	10,655	93.51	***	93.51	1,607
CD54 TTZ 6:All Events	11,305	***	***	100.00	1,172
CD54 TTZ 6:Live Cells	10,000	88.46	***	88.46	1,002
CD86 TTZ 7:All Events	11,164	***	***	100.00	1,721
CD86 TTZ 7:Live Cells	10,000	89.57	***	89.57	1,448
CD54 TTZ 7:All Events	11,011	***	***	100.00	1,131
CD54 TTZ 7:Live Cells	10,000	90.82	***	90.82	997

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
CD86 TTZ 8:All Events	11,040	***	***	100.00	1,751
CD86 TTZ 8:Live Cells	9,993	90.52	***	90.52	1,512
CD54 TTZ 8:All Events	11,132	***	***	100.00	1,118
CD54 TTZ 8:Live Cells	10,000	89.83	***	89.83	986
CD86 TTZ 9:All Events	11,359	***	***	100.00	2,199
CD86 TTZ 9:Live Cells	10,000	88.04	***	88.04	1,816
CD54 TTZ 9:All Events	11,491	***	***	100.00	1,321
CD54 TTZ 9:Live Cells	10,000	87.02	***	87.02	1,124

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

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	HTC IgG	HTC CD86	RFI	% change	EC ₁₅₀	HTC CD54	RFI	% change	EC ₂₀₀
9/27/2018												
Media		0	93.1	855	1692	1	100		1018	1	100	
DMSO		0	92.2	863	1689	0.987	98.7		1017	0.945	94.5	
DNCB Control	1.67	0.00333	71.0	1245	4084	3.44	344		2068	7.99	799	
	2	0.004	78.3	1314	3337	2.45	245		2232	8.91	891	
	2.4	0.0048	---		---				---			
TTZ	7.81	0.0156	91.7	851	1543	0.837	83.7	N/A	1030	1.16	116	N/A
	9.38	0.0188	91.0	869	1506	0.771	77.1		1011	0.922	92.2	
	11.3	0.0225	91.8	841	1601	0.920	92.0		1012	1.11	111	
	13.5	0.0270	96.1	914	1541	0.759	75.9		1020	0.688	68.8	
	16.2	0.0324	90.8	862	1454	0.717	71.7		1003	0.916	91.6	
	19.4	0.0389	91.4	831	1607	0.940	94.0		1002	1.11	111	
	23.3	0.0467	90.6	841	1721	1.07	107		997	1.01	101	
	28.0	0.0560	91.8	832	1512	0.823	82.3		986	1.00	100	
	28.0	0.0840	93.3	875	1816	1.14	114		1124	1.62	161	

Cell-based Acute Oral Hazard Estimation (CAOTE) Example

Test #1			
Concentration (ug/mL)	Log Conc	Viability	1000- viability
0.438	-0.359	99.1	900.9
0.875	-0.0580	97.7	902.3
1.75	0.243	97.3	902.7
3.5	0.544	97.9	902.1
7	0.845	97.7	902.3
14	1.15	97.4	902.6
28	1.45	97.2	902.8
56	1.75	97.5	902.5
	Log Concentration	Viability	Desired LD
>50%	1.15	97.4	50
<50%	1.45	97.2	
	Slope =	-0.698	
	Intercept	98.2	
X	69.0		
IC50	1.12E+69		
LOG LD50 (mg/kg)	27.7		
LD ₅₀ (mg/kg)	5.13E+27		

DPRA Data

Raw Data Cysteine #1

Instrument: U3000_SYS_1B Sequence: DP-DPRA-TTZ_CBZ_PCL_C_Run3-11Sept20

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Summary

Sequence Details

Name:	DP-DPRA-TTZ_CBZ_PCL_C_Run3-11Sept20	Created On:	26/Feb/19 14:30:42
Directory:	DNK1Data	Created By:	US Army
Data Vault:	ChromleonLocal	Updated On:	12/Sep/20 11:59:38
No. of Injections:	41	Updated By:	US Army

By Component

Cysteine

No.	Injection Name	Ret Time min Cysteine UV_VIS_1	Area mAU*min Cysteine UV_VIS_1	Height mAU Cysteine UV_VIS_1	Amount mM peptide Cysteine UV_VIS_1	Rel Area % Cysteine UV_VIS_1	Peak Type Cysteine UV_VIS_1
1	ACN blk	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	STD 7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	STD 6	10.937	1.204	12.192	0.013	100.00	M
5	STD 5	10.928	2.428	24.435	0.033	100.00	M
6	STD 4	10.935	4.702	47.633	0.070	100.00	M
7	STD 3	10.933	8.432	87.790	0.133	100.00	BMB*
8	STD 2	10.930	16.336	159.978	0.267	100.00	BMB*
9	STD 1	10.912	31.482	289.445	0.534	100.00	BMB*
10	ref control A	10.913	30.911	284.101	0.524	100.00	BMB*
11	ref control A	10.907	30.993	285.523	0.525	100.00	BMB*
12	ref control A	10.907	30.888	285.413	0.523	100.00	BMB*
13	ref control B	10.900	30.828	282.150	0.522	100.00	BMB*
14	ref control B	10.913	30.886	283.849	0.523	100.00	BMB*
15	ref control B	10.910	30.742	280.684	0.521	100.00	BMB*
16	ref control C	10.917	27.497	253.930	0.462	100.00	BMB*
17	DMSO_AcN	10.920	19.659	189.875	0.324	100.00	BMB*
18	Cinnamaldehyde	10.930	6.887	69.865	0.107	100.00	BMB*
19	TTZ	10.693	14.187	113.040	0.230	100.00	M
20	CBZ	10.915	10.850	106.370	0.173	100.00	BMB*
21	PCL	10.918	22.666	214.433	0.377	100.00	BMB*
22	reference control C	10.908	26.570	247.371	0.446	100.00	BMB*
23	DMSO_AcN	10.922	18.685	179.731	0.307	100.00	BMB*
24	Cinnamaldehyde	10.933	7.218	70.725	0.112	100.00	M
25	TTZ	10.700	14.441	113.765	0.234	100.00	M
26	CBZ	10.928	9.075	91.024	0.143	100.00	BMB*
27	PCL	10.912	21.683	206.912	0.360	100.00	BMB*
28	reference control C	10.908	25.794	240.372	0.432	100.00	BMB*
29	DMSO_AcN	10.913	17.597	170.041	0.289	100.00	BMB*
30	Cinnamaldehyde	10.910	7.105	69.877	0.110	100.00	M
31	TTZ	10.690	13.521	109.876	0.219	100.00	M
32	CBZ	10.938	7.707	76.306	0.121	100.00	BMB*
33	PCL	10.917	20.794	198.747	0.344	100.00	BMB*
34	reference control C	10.903	24.831	232.903	0.415	100.00	BMB*
35	DMSO_AcN	10.910	16.583	159.881	0.271	100.00	BMB*
36	Cinnamaldehyde	10.925	6.860	68.351	0.106	100.00	BMB*
37	Reference control B	10.895	28.950	265.933	0.489	100.00	BMB*
38	Reference control B	10.912	29.040	268.496	0.490	100.00	BMB*
39	Reference control B	10.915	29.050	266.292	0.490	100.00	BMB*
40	10% ACN	10.937	0.397	1.662	n.a.	100.00	M
41	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

DP-DPRA-TTZ_CBZ_PCL_C_Run3-11Sept20/Summary

Chromleon (c) Dionex
Version 7.1.1.1127

Toxicology Study No. S.0042847-16, August 2021

Raw Data Lysine #1

Instrument: UH3000_SYS_18 Sequence: DP-DPRA-TTZ_IAMMDAS2_CBZ_Run1-K-2_Sep-20

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Summary							
Sequence Details							
Name:		DP-DPRA-TT2_IAMMDAS2_CBZ_Run1-K-2_Si			Created On: 26/Feb/19 14:30:42		
Directory:		DNK/Data			Created By: US Army		
Data Vault:		ChromleonLocal			Updated On: 03/Sep/20 08:23:56		
No. of Injections:		45			Updated By: US Army		
By Component		Lysine					
No.	Injection Name	Ret. Time min Lysine UV_VIS_1	Area mAU*min Lysine UV_VIS_1	Height mAU Lysine UV_VIS_1	Amount mM peptide Lysine UV_VIS_1	Rel Area % Lysine UV_VIS_1	Peak Type Lysine UV_VIS_1
1	ACN bK	11.088	0.188	0.493	0.007	100.00	M
2	10% ACN	11.168	0.335	0.682	0.009	100.00	M
3	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	STD 7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	STD 6	8.348	0.029	12.033	0.018	81.77	BMB*
6	STD 5	8.346	1.979	24.419	0.033	100.00	BMB*
7	STD 4	8.337	4.246	46.840	0.068	100.00	BMB*
8	STD 3	8.327	8.788	93.663	0.133	100.00	BMB*
9	STD 2	8.312	17.820	169.848	0.267	100.00	BMB*
10	STD 1	8.293	35.428	266.210	0.534	100.00	BMB*
11	ref control A	8.287	33.158	279.258	0.499	100.00	BMB*
12	ref control A	8.280	33.387	280.767	0.503	100.00	BMB*
13	ref control A	8.287	33.381	282.099	0.502	100.00	BMB*
14	ref control B	8.280	33.958	287.420	0.511	100.00	BMB*
15	ref control B	8.272	33.251	281.093	0.501	100.00	BMB*
16	ref control B	8.292	33.213	282.523	0.500	100.00	BMB*
17	ref control C	8.285	33.709	285.052	0.508	100.00	BMB*
18	DMSO_AcN	8.303	33.328	277.214	0.502	100.00	BMB*
19	Co. Elut. TT2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
20	Co. Elut. IAMMDAS2	10.627	0.227	1.997	0.008	30.50	M
21	Co. Elut. CBZ	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
22	Cinnamaldehyde	8.325	7.367	79.322	0.112	91.93	BMB*
23	TT2	8.310	19.247	181.632	0.289	99.81	BMB*
24	IAMMDAS2	8.318	13.323	129.947	0.200	72.80	BMB*
25	CBZ	8.282	32.608	274.363	0.496	100.00	BMB*
26	reference control C	8.283	32.684	280.123	0.497	100.00	BMB*
27	DMSO_AcN	8.278	33.531	278.454	0.506	99.48	BMB*
28	Cinnamaldehyde	8.318	7.547	81.761	0.115	92.90	BMB*
29	TT2	8.297	19.403	183.342	0.291	99.81	BMB*
30	IAMMDAS2	8.320	12.605	126.736	0.191	100.00	BMB*
31	CBZ	8.292	33.024	275.185	0.497	100.00	BMB*
32	reference control C	8.283	32.949	278.737	0.495	100.00	BMB*
33	DMSO_AcN	8.293	33.324	277.461	0.502	100.00	BMB*
34	Cinnamaldehyde	8.327	8.192	86.750	0.124	98.24	BMB*
35	TT2	8.302	19.951	187.038	0.299	99.86	BMB*
36	IAMMDAS2	8.305	12.868	126.241	0.194	98.21	BMB*
37	CBZ	8.282	33.029	274.049	0.497	100.00	BMB*
38	reference control C	8.287	33.064	278.244	0.498	99.71	BMB*
39	DMSO_AcN	8.285	33.475	278.422	0.504	100.00	BMB*
40	Cinnamaldehyde	8.333	8.480	90.741	0.129	97.82	BMB*
41	Reference control B	8.290	33.831	281.637	0.506	100.00	BMB*
42	Reference control B	8.286	33.778	283.848	0.509	100.00	BMB*
43	Reference control B	8.280	33.658	286.352	0.507	100.00	BMB*

DP-DPRA-TTZ_IAMMDAS2_CBZ_Run1-K-2_Sep-20/Summary

Chromleon (c) Dionex
Version 7.1.1.1127

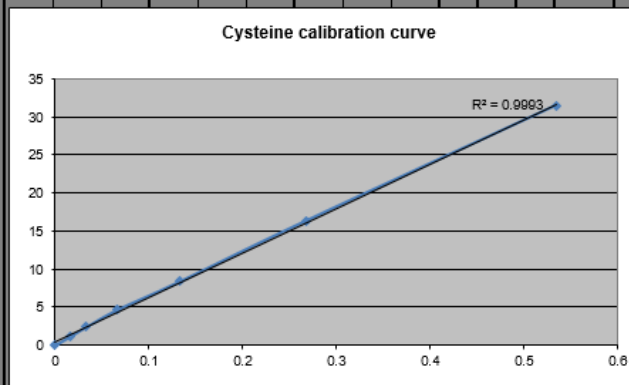
Instrument: UH3000_SYS_18 Sequence: DP-DPRA-TTZ_IAMMDAS2_CBZ_Run1-K-2_Sep-20

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44	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
45	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

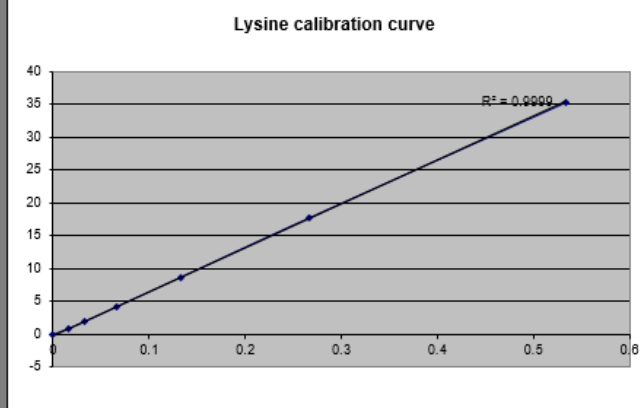
DPRA Cysteine #1 Calculations

Vehicle	Code	Conc/replicate	Cysteine																		
			Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area			Peptide Conc			Peptide Depl.			Precipitates	Re-solub. Or Centrif.	Absorbs at 220 nm	Retent. Time similar to pept.	Co-elution	If available: Peak Area at 258 nm
	STANDARD																				
		STD1	31	0.5																	
		STD2	16	0.3																	
		STD3	8.4	0.1																	
		STD4	4.7	0.1																	
		STD5	2.4	0																	
		STD6	12	0																	
		Dil Buff	0	0																	
	REF CTRL A	r1	30.911	0.520461964						0.52	0.00	0.00									
		r2	31	0.521862097																	
		r3	31	0.520069244																	
	REF CTRL B	r1	30.828	0.519044757																	
		r2	30.896	0.520035094																	
		r3	30.742	0.517576325			30.8	0.07	0	0.26	0.29	1.13									
		r4		-0.0073369																	
		r5		-0.0073369																	
		r6		-0.0073369																	
2M in DMS	REF CTRL C	r1	18.685	0.31170557			17.6	1.05	0.06	0.29	0.02	0.06									
		r2	18	0.293128197																	
		r3	17	0.275814359																	
Acetonitrile	POSITIVE CONTROL																				
	Cinnamic Aldehyde	r1	6.887	0.110257185	0.609174312	0.609174312															
		r2	7	0.115908941	0.590390618	0.590390618	7.07	0.17	0.02	0.11	0	0.03	0.6	0.01	0.02						
		r3	7	0.11397949	0.596803178	0.596803178															
2M in DMS	CHEMICAL 1																				
	Tetrazene	r1	14.187	0.23490316	0.194911567	0.194911567	14	0.48	0.03	0.23	0.01	0.03	0.2	0.03	0.13	Y/N	Y/N	Y/N	Y/N	Y/N	XX
		r2	14	0.239240157	0.180497494	0.180497494															XX
		r3	14	0.223531349	0.232705949	0.232705949															XX



DPRA Lysine #1 Calculations

Vehicle		Code	Concl replicate	Lysine																	If available: Peak Area at 258 nm			
				Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area			Peptide Conc			Peptide Depl.			Precipitates	Re-solub. Or Centrif.	Absorbs at 220 nm	Retent. Time similar to pept.		Co-elution		
STANDARD																								
		STD1	35	0.5																				
		STD2	18	0.3																				
		STD3	8.8	0.1																				
		STD4	4.2	0.1																				
		STD5	2	0																				
		STD6	0.9	0																				
		Dil Buff	0	0																				
REF CTRL A																								
		r1	33.158	0.499152915																				
		r2	33	0.502586026						0.501	0.002	0.004												
		r3	33	0.50219624																				
REF CTRL B																								
		r1	33.958	0.511146314																				
		r2	33.251	0.500547148																				
		r3	33.213	0.499977461				33.6	0.29	0	0.51	0	0.01											
		r4	33.631	0.506244012																				
		r5	33.776	0.508417815																				
		r6	33.658	0.506648789																				
2M in DMS	REF CTRL C																							
		r1	33.531	0.504744837				33.3	0.23	0.01	0.5	0	0.01											
		r2	33	0.501641545																				
		r3	33	0.497743691																				
Acetonitrile	POSITIVE CONTROL Cinnamic Aldehyde																							
		r1	7.367	0.112500743	0.778810837	0.778810837																		
		r2	8	0.115199258	0.773406459	0.773406459	7.7	0.43	0.06	0.12	0.01	0.06	0.77	0.01	0.02									
		r3	8	0.124868935	0.754040773	0.754040773																		
2M in DMS	CHEMICAL 1 Tetrazene																							
		r1	19.247	0.290602709	0.422121919	0.422121919	19.5	0.38	0.02	0.29	0.01	0.02	0.41	0.01	0.03	N	Y/N	Y	N	N				
		r2	19	0.292941422	0.417438125	0.417438125																		
		r3	20	0.301306817	0.400684554	0.400684554																		



QC Data Cysteine and Lysine #1

Criterion	Criterion met?
Lysine: $r^2 > 0,99$	Lys R2 0.99993632 YES
	Lys Intercept -0.137177861
Cysteine: $r^2 > 0,99$	Lys Slope 66.70336253
	Cys R2 0.999327253 YES
	Cys Intercept 0.429691949
	Cys Slope 58.56587061
Range Mean Conc Cys [0.45 to 0.55]	YES
Range Mean Conc Lys [0.45 to 0.55]	YES
Peak Area CV ctrl B < 15% - Cys	YES
Peak Area CV ctrl B < 15% - Lys	YES
Peak Area CV ctrl C < 15% - Cys	YES
Peak Area CV ctrl C < 15% - Lys	YES
Range Mean Conc Cys [0.45 to 0.55]	Not Met
Range Mean Conc Lys [0.45 to 0.55]	YES
60,8 < Mean % Depl Cys < 96,6	Not Met
40,2 < Mean % Depl Lys < 69,4	Not Met
SD % Depl Cys < 14,9	YES
SD % Depl Lys < 11,6	YES
SD % Depl Cys < 14,9	YES
SD % Depl Lys < 11,6	YES

Cinnamic aldehyde depletion for Lysine was greater than the preferred mean percentage range. Still consider that this was a successful assay. Additionally, lysine is not required to make a call for sensitization.

DPRA Cysteine #2 Raw Data

Instrument: U3000_SYS_1B Sequence: DP-DPRA-TTZ_CBZ_C_Run4-12Sept20

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Summary							
Sequence Details							
Name:		DP-DPRA-TTZ_CBZ_C_Run4-12Sept20		Created On:		26/Feb/19 14:30:42	
Directory:		DNK1Data		Created By:		US Army	
Data Vault:		ChromeleonLocal		Updated On:		14/Sep/20 08:25:18	
No. of Injections:		42		Updated By:		US Army	
By Component		Cystene					
No.	Injection Name	Ret.Time min Cystene UV_VIS_1	Area mAU*min Cystene UV_VIS_1	Height mAU Cystene UV_VIS_1	Amount mM peptide Cystene UV_VIS_1	Rel.Area % Cystene UV_VIS_1	Peak Type Cystene UV_VIS_1
1	ACN blk	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	STD 7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	STD 6	10.942	1.540	14.288	0.014	100.00	M
6	STD 5	10.935	2.930	28.988	0.032	100.00	M
7	STD 4	10.930	5.653	56.138	0.068	97.61	M
8	STD 3	10.925	10.542	104.299	0.135	99.09	M
9	STD 2	10.922	19.452	185.389	0.266	98.44	M
10	STD 1	10.900	34.543	313.311	0.534	96.09	M
11	ref control A	10.913	33.927	305.058	0.522	96.41	M
12	ref control A	10.910	33.538	304.712	0.514	96.21	M
13	ref control A	10.913	33.585	303.893	0.515	96.58	M
14	ref control B	10.910	32.983	298.100	0.503	96.27	M
15	ref control B	10.900	32.849	297.626	0.500	96.88	M
16	ref control B	10.903	32.916	297.194	0.501	96.64	M
17	ref control C	10.898	33.797	305.246	0.519	96.80	M
18	DMSO_AcN	10.933	21.592	204.918	0.300	94.36	M
19	Cinnamaldehyde	10.932	7.265	69.566	0.090	99.18	M
20	TTZ	10.703	10.481	80.856	0.134	100.00	M
21	CBZ	10.933	11.322	112.077	0.146	94.42	M
22	TTZ for Compar	10.708	21.491	167.893	0.299	100.00	M
23	reference control C	10.923	33.364	302.352	0.510	96.34	M
24	DMSO_AcN	10.928	20.415	196.132	0.282	95.23	M
25	Cinnamaldehyde	10.927	7.185	68.258	0.089	97.31	M
26	TTZ	10.707	10.216	78.629	0.130	99.21	M
27	CBZ	10.933	9.042	90.638	0.114	95.29	M
28	TTZ for Compar	10.712	21.627	168.113	0.301	100.00	M
29	reference control C	10.908	32.780	297.654	0.499	96.33	M
30	DMSO_AcN	10.925	19.331	185.398	0.265	93.77	M
31	Cinnamaldehyde	10.928	6.860	66.850	0.084	96.09	M
32	TTZ	10.697	9.796	77.440	0.124	98.94	M
33	CBZ	10.925	7.385	73.819	0.091	91.94	M
34	TTZ for Compar	10.713	21.595	169.526	0.301	100.00	M
35	reference control C	10.910	32.013	293.536	0.484	97.35	M
36	DMSO_AcN	10.918	18.169	175.643	0.247	93.71	M
37	Cinnamaldehyde	10.928	6.761	66.131	0.083	98.47	M
38	Reference control B	10.902	30.535	280.591	0.456	96.23	M
39	Reference control B	10.918	30.613	281.648	0.457	96.59	M
40	Reference control B	10.912	30.402	280.337	0.453	96.63	M
41	10% ACN	10.938	0.093	1.038	n.a.	100.00	M
42	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

DP-DPRA-TTZ_CBZ_C_Run4-12Sept20/Summary

Chromeleon (c) Dionex
Version 7.1.1.1127

DPRA Lysine #2 Raw Data

Instrument: U3000_SYS_15 Sequence: DP-DPRA-TTZ_IAMMDA52_CBZ_Run2-K_3_Sep-20

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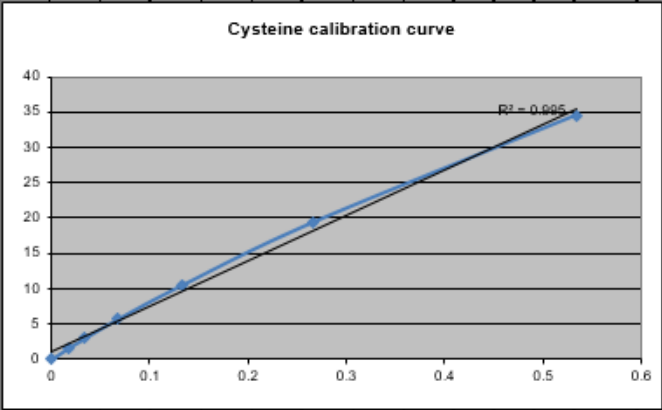
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Sequence Details							
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Directory:		DNKIData			Created By:	US Army	
Data Vault:		ChromeleonLocal			Updated On:	04/Sep/20 08:07:53	
No. of Injections:		42			Updated By:	US Army	
By Component		Lysine					
No.	Injection Name	Ret Time min Lysine UV_VIS_1	Area mAU*min Lysine UV_VIS_1	Height mAU Lysine UV_VIS_1	Amount mM peptide Lysine UV_VIS_1	Rel Area % Lysine UV_VIS_1	Peak Type Lysine UV_VIS_1
1	ACN blk	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	STD 7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	STD 6	8.353	0.877	11.357	0.021	100.00	BMB*
6	STD 5	8.340	1.642	22.092	0.032	100.00	BMB*
7	STD 4	8.342	4.004	48.282	0.066	100.00	BMB*
8	STD 3	8.318	8.568	93.012	0.132	98.24	BMB*
9	STD 2	8.308	17.815	171.430	0.268	100.00	BMB*
10	STD 1	8.273	35.476	294.292	0.534	99.65	BMB*
11	ref control A	8.283	33.495	288.166	0.504	100.00	BMB*
12	ref control A	8.282	33.236	288.539	0.500	100.00	BMB*
13	ref control A	8.273	31.971	277.460	0.480	100.00	BMB*
14	ref control B	8.277	33.579	288.073	0.505	100.00	BMB*
15	ref control B	8.295	32.636	278.469	0.490	100.00	BMB*
16	ref control B	8.292	32.399	279.144	0.487	100.00	BMB*
17	ref control C	8.292	33.250	279.984	0.500	100.00	BMB*
18	DMSO_AcN	8.295	33.650	276.530	0.506	100.00	BMB*
19	Cinnamaldehyde	8.328	7.152	75.655	0.112	94.45	BMB*
20	TTZ	8.297	29.017	249.593	0.435	99.25	BMB*
21	IAMMDA52	8.335	3.281	39.719	0.056	100.00	BMB*
22	CBZ	8.297	33.072	274.713	0.497	100.00	BMB*
23	reference control C	8.290	33.196	283.321	0.499	100.00	BMB*
24	DMSO_AcN	8.292	33.051	275.387	0.497	100.00	BMB*
25	Cinnamaldehyde	8.340	7.136	76.675	0.111	96.89	BMB*
26	TTZ	8.290	28.912	250.149	0.434	100.00	BMB*
27	IAMMDA52	8.337	3.269	39.424	0.056	94.27	BMB*
28	CBZ	8.282	33.137	275.631	0.498	100.00	BMB*
29	reference control C	11.387	0.080	0.224	0.010	100.00	M
30	DMSO_AcN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
31	Cinnamaldehyde	8.345	7.380	79.312	0.115	98.35	BMB*
32	TTZ	8.295	28.857	248.372	0.433	100.00	BMB*
33	IAMMDA52	8.330	3.200	38.736	0.055	80.59	BMB*
34	CBZ	8.288	32.961	275.426	0.495	100.00	BMB*
35	reference control C	8.297	33.579	283.165	0.505	100.00	BMB*
36	DMSO_AcN	8.295	33.138	275.595	0.498	100.00	BMB*
37	Cinnamaldehyde	8.340	8.451	84.165	0.130	100.00	BMB*
38	Reference control B	8.290	33.687	283.473	0.506	100.00	BMB*
39	Reference control B	8.285	33.778	282.675	0.508	100.00	BMB*
40	Reference control B	8.275	33.762	284.203	0.508	100.00	BMB*
41	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
42	10% ACN	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

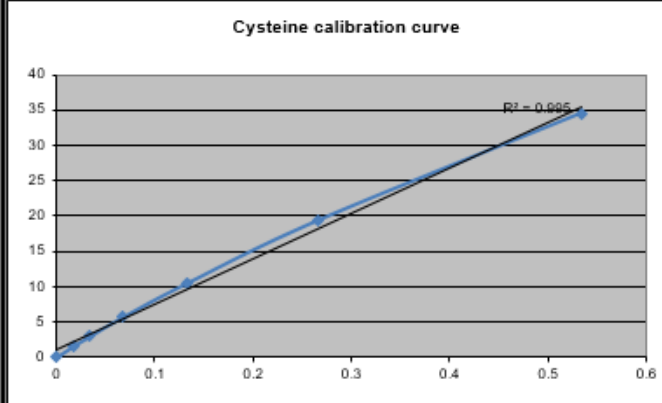
* Observed some pps DP

DP-DPRA-TTZ_IAMMDA52_CBZ_Run2-K_3_Sep-20/Summary

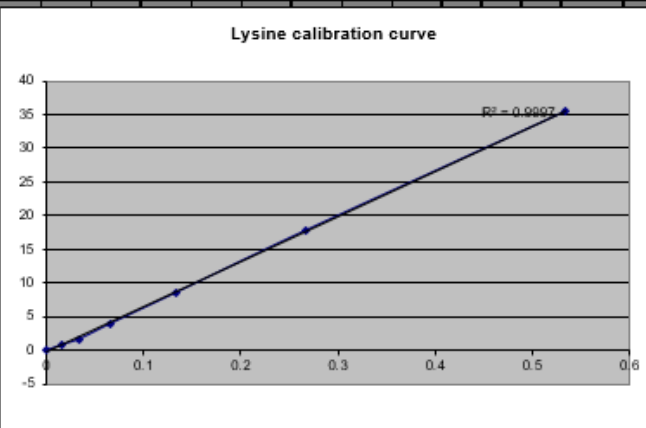
Chromeleon (c) Dionex
Version 7.1.1.1127

DPRA Cysteine #2 Data Calculations

Vehicle		Code	Concl replicate	Cysteine																											
				Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area			Peptide Conc			Peptide Depl.			Precipitates	Re-solub. Or Centrif.	Absorb at 220 nm	Retent. Time similar to pept.	Co-elution										
		STANDARD						mean	SD	CV	mean	SD	CV	mean	SD	CV															
			STD1	35	1																										
			STD2	19	0																										
			STD3	11	0																										
			STD4	6	0																										
			STD5	3	0																										
			STD6	2	0																										
			Dil Buff	0	0																										
		REF CTRL A	r1	33.327	0.51078637																										
			r2	34	0.50475626						0.51	0.00	0.01																		
			r3	34	0.50548483																										
		REF CTRL B	r1	32.383	0.43615289																										
			r2	32.316	0.43511428																										
			r3	33.797	0.50877116			32	1.5	0	0.48	0.02	0.05																		
			r4	30.55	0.45843758																										
			r5	30.613	0.45341417																										
			r6	30.402	0.45614334																										
2M in DMF		REF CTRL C	r1	20.415	0.3013292																										
			r2	22	0.31957454																										
			r3	19	0.2845255			20	1.1	0.1	0.3	0	0.1																		
Acetonitrile		POSITIVE CONTROL Cinnamic Aldehyde	r1	7.265	0.0974836	0.64467377	0.644673775																								
			r2	7	0.09624348	0.64858652	0.648586521	7.1	0.2	0	0.1	0	0	0.7	0	0															
			r3	7	0.09120547	0.66448205	0.66448205																								
2M in DMF		CHEMICAL 1 Tetrazene	r1	10.481	0.14733664	0.48738139	0.487381395	10	0.3	0	0.1	0	0	0.5	0	0	Y/N	Y/N	Y/N	Y/N	Y/N										
			r2	10	0.14322872	0.50034237	0.500342365																								
			r3	10	0.13671807	0.52088428	0.520884281																								



DPRA Cysteine #2 Data Calculations

Vehicle	Code	Concl replicate	Lysine																		
			Peak Area at 220 nm	Peptide Concentration (mM)	Peptide Depletion (%)	CORRECTED Peptide Depletion (%)	Peak Area			Peptide Conc			Peptide Depl.			Precipitates	Re-solub. Or Centrif.	Absorb at 220 nm	Relent. Time similar to pept.	Co-elution	
	STANDARD							mean	SD	CV	mean	SD	CV	mean	SD	CV					
		STD1	35	1																	
		STD2	18	0																	
		STD3	9	0																	
		STD4	4	0																	
		STD5	2	0																	
		STD6	1	0																	
		Dil Buff	0	0																	
		Lysine calibration curve 																			
	REF CTRL A	r1	33.495	0.50377505							0.43	0.01	0.02								
		r2	33	0.49991519																	
		r3	32	0.48106297																	
	REF CTRL B	r1	33.579	0.5050269																	
		r2	32.636	0.49097342				33	0.6	0	0.5	0	0								
		r3	32.399	0.48744142																	
		r4	33.687	0.50663641																	
		r5	33.778	0.50799258																	
		r6	33.762	0.50775413																	
2M in DMF	REF CTRL C	r1	33.65	0.50608501				33	0.3	0	0.5	0	0								
		r2	33	0.49715814																	
		r3	33	0.4984547																	
Acetonitrile	POSITIVE CONTROL Cinnamic Aldehyde	r1	7.152	0.11118684	0.785094	0.785094001															
		r2	7	0.1109484	0.78557478	0.785574775	7.2	0.1	0	0.1	0	0	0.8	0	0						
		r3	7	0.11458471	0.77824297	0.778242971															
2M in DMF	CHEMICAL 1 Tetrazene	r1	29.017	0.43703967	0.12808622	0.128086219	29	0.1	0	0.4	0	0	0.1	0	0	N	Y/N	Y	N	N	
		r2	29	0.43547487	0.1312413	0.131241298															
		r3	29	0.4346552	0.13289396	0.132893959															

QC Data Cysteine and Lysine #2

Criterion	Criterion met?
Lysine: $r^2 > 0,99$	Lys R2 0.993737764 YES
	Lys Intercept -0.308731071
Cysteine: $r^2 > 0,99$	Lys Slope 67.10064419
	Cys R2 0.99500148 YES
	Cys Intercept 0.976370896
	Cys Slope 64.50960979
Range Mean Conc Cys [0.45 to 0.55]	YES
Range Mean Conc Lys [0.45 to 0.55]	YES
Peak Area CV ctrl B < 15% - Cys	YES
Peak Area CV ctrl B < 15% - Lys	YES
Peak Area CV ctrl C < 15% - Cys	YES
Peak Area CV ctrl C < 15% - Lys	YES
Range Mean Conc Cys [0.45 to 0.55]	Not Met
Range Mean Conc Lys [0.45 to 0.55]	YES
60,8 < Mean % Depl Cys < 96,6	YES
40,2 < Mean % Depl Lys < 69,4	Not Met
SD % Depl Cys < 14,9	YES
SD % Depl Lys < 11,6	YES
SD % Depl Cys < 14,9	YES
SD % Depl Lys < 11,6	YES

Note:

Cinnamic aldehyde depletion for Lysine was greater than the preferred mean percentage range. Still consider that this was a successful assay. Additionally, lysine is not required to make a call for sensitization.

GLOSSARY

ACGIH®

American Conference of Governmental Industrial Hygienists

AERTA

Army Environmental Research and Technology Assessment

AFNOR

Association of Francaise de Normalisation

APHC

U.S. Army Public Health Center

AR

Army Regulation

ARDEC

U.S. Army Armament Research, Development, and Engineering Center

atm-m₃/mol

air to moles per cubic meter per mole

ATP

Adenosine triphosphate

BSA

Bovine serum albumin

CAOTE

Cell-based Approximate Oral Toxicity Estimation

CASRN

Chemical Abstracts Service Registry Number

CCDC

Combat Capabilities Development Command

CFR

Code of Federal Regulations

CI

Confidence interval

CO₂

Carbon dioxide

CV₇₅

75% cell viability

DA

Department of the Army

DIN

Deutsches Institut für Normung

DMSO

Dimethyl sulfide

DNCB

Dinitrochlorobenzene

DOD

Department of Defense

DPRA

Direct Peptide Reactivity Activity

EC₅₀

Median (50%) effect concentration

ECOSAR

Ecological Structure Activity Relationship

ECVAM DB-ALM

European Centre for the Validation of Alternative Methods
Database on Alternative Methods for Animal Experimentation

ESOH

Environmental safety and occupational health

FACS

Fluorescence-activated cell sorting

FITC

Fluorescein isothiocyanate

g/m³

grams per cubic meter

g/mol

grams per mole

GHS

Globally Harmonized System

GLP

Good Laboratory Practice

h-CLAT

Human cell line activation test

IARC

International Agency for Research on Cancer

IC₅₀

median (50%) inhibitory concentration

ICCVAM

Interagency Coordinating Committee on the Validation Alternative Methods

ISO

International Organization of Standardization

L

Liter

LA

Lactic acid

LC₅₀

median (50%) lethal (oral) concentration

LD₅₀

median (50%) lethal (oral) dose

LOAEL

Lowest-observed adverse effect level

log K_{oc}

Organic carbon partition coefficient

log K_{ow}

Octanol-water partition coefficient

MFI

Mean Florescence Intensity

µg/mL

microgram per milliliter

Toxicology Study No. S.0042847-16, August 2021

μL
microliter

mg/kg
milligram per kilogram

mg/kg-day
milligram per kilogram per day

mg/L
milligram per liter

μM
micromolar

mL
milliliter

min
minute

mm Hg
millimeter of Mercury

mM
millimolar

NiSO₄
Nickel sulfate

nm
nanometer

NTP
National Toxicology Program

NVN
Nederlandse voornorm

OECD
Organization of Economic Cooperation and Development

OSHA
Occupational Safety and Health Administration

Toxicology Study No. S.0042847-16, August 2021

PBS

Phosphate buffered saline

PI

Propidium iodide

QSAR

Quantitative Structure Activity Relationship

RDT&E

Research, development, technology, and education

RDX

1,3,5-hexahydro-1,3,5-trinitrotriazine

RFI

Relative Fluorescence Intensity

SDS

Safety Data Sheets

SERDP

Strategic Environmental Research and Development Program

SOP

Standing operating procedure

TNT

2,4,6-Trinitrotoluene

TOPKAT

Toxicity Prediction Komputer Assisted Technology

TOX

Toxicology Directorate

TTZ

1-(5-tetrazolyl)-4-guanyltetrazene (tetrazene)

U/mL

Units per milliliter

UNECE

United Nations Economic Commission for Europe

USAMRDC

U.S. Army Medical Research and Development Command

Toxicology Study No. S.0042847-16, August 2021

USEPA

U.S. Environmental Protection Agency

USFDA

U.S. Food and Drug Administration

v/v

volume/volume

w/v

weight/volume