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Human Cell Line Activation Test of the novel energetic N,N'-bis(4-nitro-1,2,5-oxadiazol-3-yl)-methanediamine (MBANF) February–March 2020

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The energetic and toxicological properties of n,n'-bis(4-nitro-1,2,5-oxadiazol-3-yl)-methanediamine (MBANF) are under assessment as replacements for energetics in current use, such as such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). This study evaluated the skin sensitization hazard of MBANF through the h-CLAT, an in vitro approach to assess activation of dendritic cells. Data from the h-CLAT were also utilized to predict a Hazard Category (UNECE 2015) for acute oral toxicity. Data from this study are used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for development of new munition compounds.

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Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:
1. The test article characterization (purity) was conducted by the manufacturer and it is not known whether the testing was done in compliance with the above regulation.

2. Due to time constraints, the method of analysis for these compounds could not be validated by the Laboratory Sciences Directorate (LAB) prior to the study start in compliance with Good Laboratory Practice (GLP) requirements. Because of this the dosing solutions used for all strains were verified after being frozen (at - 80 degrees C) until the method could be validated by the LAB after the study was completed.

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

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Emily N. Reinke, Ph.D., D.A.B.T. Date
Study Director
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Standard Acronyms & Abbreviations

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

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

TOXICOLOGY STUDY NO. S.0070548-19
HUMAN CELL LINE ACTIVATION TEST OF THE NOVEL ENERGETIC N,N'-BIS(4-NITRO-1,2,5-OXADIAZOL-3-YL)-METHANEDIAMINE (MBANF)
FEBRUARY–MARCH 2020

1 SUMMARY

1.1 Overview

The energetic and toxicological properties of n,n'-bis(4-nitro-1,2,5-oxadiazol-3-yl)-methanediamine (MBANF) are under assessment as replacements for energetics in current use, such as such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and trinitrotoluene (TNT). This study evaluated the skin sensitization hazard of MBANF through the h-CLAT, an *in vitro* approach to assess activation of dendritic cells, which is a critical step in the elicitation of a sensitizing response. Data from the h-CLAT were also utilized to predict a Hazard Category (UNECE 2015) for acute oral toxicity. Data from this study are used to assist in making environment and health-based decisions regarding the design and selection of formulas and materials for further development of new munition compounds.

1.2 Purpose

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

Research, development, testing, training, and use of substances potentially less hazardous to human health and the environment is vital to the readiness of the U.S. military. Safeguarding the health of Soldiers, Civilians, and the environment requires an assessment of alternatives before they are fielded. Continuous assessments that begin 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 and is creating environmental problems and interfering with training activities.

The DOD is identifying replacements for substances causing environmental and/or occupational health hazards. This toxicology evaluated MBANF skin sensitization hazard using the h-CLAT assay and following GLP regulations.

1.3 Conclusions

The MBANF was found to elicit positive reactions for both sensitization markers in the THP-1 monocytic leukemia cell line, which is a dendritic cell surrogate. Both CD54 and CD86 expression levels were increased as a result of 24-hour exposure to MBANF. According to the defined approach for skin sensitization (Kleinstreuer et al. 2018; USEPA 2018), a positive test in the h-CLAT indicates that a compound is a skin sensitizer.

1.4 Recommendations

The MBANF is a skin sensitizer based on the weight of evidence from both *in vitro* results and QSAR analysis (Accelrys Inc.). The h-CLAT can be used as a definitive test to predict skin sensitization, especially when QSAR analysis supports this prediction (USEPA 2018; Kleinstreuer et al. 2018; Strickland et al. 2018). No further skin sensitization tests are necessary. Previously conducted testing estimated MBANF to be a GHS category 3 for oral toxicity, to have low mutagenic potential in the Ames, and to be a GHS category 1 for aquatic toxicity (USAPHC 2013a, 2013b). With MBANF also predicted to be a skin sensitizer, general laboratory precautions should be taken when handling the compound. Release into the environment should be avoided due to its moderate water solubility and high predicted aquatic toxicity.

2 REFERENCES

See Appendix A for list of references.

3 AUTHORITY

The authority for this report is from the Military Interdepartmental Purchase Request No. W74RDV92544618. This toxicology report addresses, in part, the ESOH requirements outlined in DoDI 4715.4 (DoDI 2018), AR 200-1 (DA 2007b); AR 40-5 (DA 2007a); AR 70-1 (DA 2018); and AERTA Requirement PP-3-02-05 (AERTA 2018). It was conducted as part of an on-going effort by Strategic Environmental Research and Development Program (SERDP).

4 BACKGROUND

Current regulations require the assessment of human health and environmental effects arising from exposure to substances in soil, surface water, and groundwater. Applied after an item has been fielded, these assessments can reveal the existence of adverse environmental and human health effects that must be addressed, often at substantial cost. It is more efficient to begin the assessment of exposure, effects, and environmental transport of military-related compounds/substances early in the RDT&E process to avoid unnecessary costs, conserve physical resources, and sustain the health of those potentially exposed. A goal of this program is to investigate new compounds for operational and/or environment, safety, and occupational health issues. The candidates under development for high-density energetics include MBANF.

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

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

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

During a skin sensitizing reaction, activated dendritic cells migrate to the lymph node where the major histocompatibility complexes present on the cell surface (e.g., CD54 and CD86), activate T-cells and T-cell proliferation. Secondary exposure to the chemical will 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 the second key event that measures the test chemical-mediated dendritic cell activation via increased expression of CD54 and CD86 on the cell surface (OECD 2018). The presence of CD54 and CD86 proteins on the cell surface is detected with flow cytometry using fluorescently labelled antibodies specific for these proteins (Ashikaga et al. 2010; OECD 2018). The threshold criteria for a positive reaction in h-CLAT requires a 2-fold induction of CD54 and/or a 1.5-fold induction of CD86 compared to solvent controls. Multiple skin sensitization assays can be utilized to determine skin sensitization hazard in a tiered testing strategy that forms a defined approach. The USEPA has recently accepted two defined approaches for submission and registration to predict hazard; the h-CLAT is the first in a tiered strategy, where a positive result allows for a hazard determination to be made and no further testing is required (USEPA 2018). The h-CLAT can also be utilized to predict acute oral toxicity from the cytotoxicity data produced in the course of conducting the assay.

This report describes the toxic effect of MBANF in the h-CLAT. Table 1 identifies the critical events and dates of this study.

Table 1. Critical Events

Critical Event	Date of Event (h-CLAT)
Type-Protocol Modification Approved	18 February 2020
Study Start Date	13 February 2020 (Reactivity check)
Experimental Start Date	21 February 2020
Experimental Completion Date	10 March 2020
Study Completion Date	April 2020

5 MATERIALS

5.1 Quality Assurance

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

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

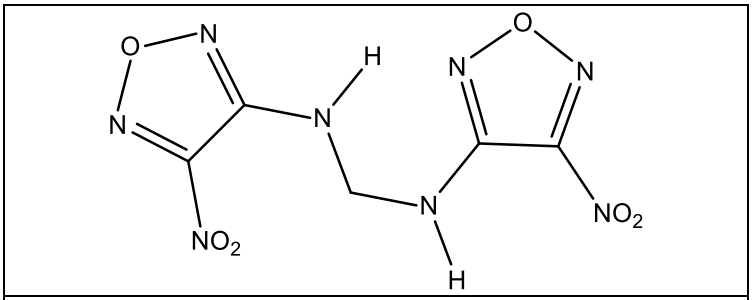
In compliance with the GLP requirements, the APHC Quality Systems Office audited critical phases of this study. Appendix B provides the Quality Assurance Statements, which include the dates of these audits, the audited phases, and the audited dates that the results were reported to Management and the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

5.2 Test Substance

Synthesis of MBANF (Chemical Abstracts Service Registry Number [CASRN] 146859-30-5) was completed by BAE (HSAAP). The sample purity was provided by the study sponsor and was 98%. Figure 1 shows the molecular structures of the compound.

The MBANF was readily soluble at 500 mg/mL in dimethyl sulfoxide (DMSO) and solubility was likely higher; however, 500 mg/mL is the test concentration limit for the assay described herein. Aqueous solubility was determined for the Ames assay and used for setting the high dose in h-CLAT tests (APHC 2019b, 2017b). Concurrent with each test, the most dilute serial dilution

was frozen at -80°C for concentration verification by the APHC Method Development Section Client Services Division (APHC-MDV-CSD).



N,N'-bis(4-nitro-1,2,5-oxadiazol-3-yl)-methanediamine
[MBANF]

Figure 1. Molecular Structure

5.3 Test System

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

6 METHODS

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

6.1 Buffers

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

diluted to a 0.1% solution in FACS buffer immediately prior to use. Propidium iodide (PI) was diluted to 12.5 µg/mL in PBS on the day of the experiment and maintained on ice.

6.2 Tissue Culture

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

6.3 Reactivity Check

The reactivity check prior to full testing is used to confirm cell viability and induction of CD54 and CD86. Two weeks post thaw, a reactivity check of cells sampled from each propagation flask was performed using the control compounds: DNCB, $NiSO_4$, and LA. DNCB was prepared as a 20 mg/mL stock solution in DMSO and stored at +4°C in the dark. The $NiSO_4$ was prepared as a 10 mg/mL stock solution in saline and stored at room temperature protected from light. LA was freshly prepared as a 100 mg/mL solution in saline. From these stock solutions, additional dilutions were made so that the tested concentrations were 3.3–4 µg/mL DNCB, 100-µg/mL $NiSO_4$, and 1,000 µg/mL for LA. A 100% cytotoxic DNCB concentration (0.2 mg/mL) was added to one well as a positive control. Negative controls (diluted DMSO and saline) were also included. After all dosing solutions were distributed to the test wells, the test plate was incubated (approximately 37°C / 5% CO_2) for 24 hours. Cells were then collected; processed; stained with PI and FITC labeled antibodies [anti-lgG1 (isotype control), anti-CD54, and anti-CD86]; and analyzed by flow cytometry (see sections 6.6 and 6.7). Criteria for a successful reactivity check requires the positive controls DNCB and $NiSO_4$ to induce CD54 and CD86 (RFI criteria exceed: CD54 ≥ 200 and CD86 ≥ 150); 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.

6.4 Range Finding

The range finding test is used to bracket the appropriate dose range for the full test using only the percent viability endpoint. The MBANF (targeted 500 mg/mL in DMSO; actual weighed resulted in 496 mg/mL; run 2 targeted 100 mg/mL; actual weighed was 99 mg/mL) was prepared as the stock for eight serial dilutions (1:2, diluent = DMSO). Each dilution was subsequently diluted 1:250 into tissue culture media. Pooled THP-1 cells were plated at 1 x 10^6 cells/well (24-well plate). An equal volume (0.5 mL) of each final dilution was added to the

appropriate test wells. Negative (vehicle) control and cytotoxicity ("dead cell") positive controls were also included on each test plate. Cells were incubated for 24 hours (approximately 37°C /5% CO_2). After the 24-hour incubation, cells were collected and processed for staining with PI. Briefly, cells were transferred to 5 mL tubes, centrifuged (200 x g; +4°C), and supernatants were discarded. Each pellet was resuspended in 0.6 mL cold FACS buffer, and 0.2 mL of each sample was transferred to new tubes and washed by centrifuging (200 x g; +4°C), decanting the supernatant, and resuspending the pellet in 0.2 mL FACS buffer. The wash step was repeated one time. The final pellets were resuspended in 0.4 mL FACS buffer and stained with 20 µL 12.5 µg PI/mL solution. Samples were maintained on ice in the dark and analyzed by flow cytometry (see section 6.7). 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.

6.5 h-CLAT Test

In the full test, the CV75 from the range finding test is used to develop the dose range and represents the 2nd highest dose of an eight-dose treatment. For the MBANF range-finding assay, cytotoxicity was observed; therefore, the experimentally determined CV75 (9E-05 mg/mL MBANF) was used to calculate the top dose (1E-04 mg/mL final). The MBANF was solubilized in DMSO at 100 mg/mL (2000x), diluted 1:100, and then diluted 1:20. Eight 1:1.2 serial dilutions of MBANF were subsequently diluted 1:250 in complete media and added in equal volume to test wells containing 0.5 mL medium and 1 x 10^6 cells per well (24-well plate). This dilution process resulted in no cytotoxicity at the predicted level, so a subsequent assay utilized top dose of 4.2E-04 mg/mL. The targeted stock concentration was again 100 mg/mL (actual 105.6 mg/mL; 200x) with 1:10 and 1:20 dilutions prior to the dilution series. Twelve total concentrations were assayed. Again, no cytotoxicity was observed, invalidating the test. For the third and fourth assays, MBANF was again solubilized in DMSO at a targeted concentration of 100 mg/mL (actual 99.1 and 92.5 mg/mL respectively, 40x). Subsequent dilutions were 1:20 followed by a 1:2 dilution. A total of 24 concentrations were setup ranging from ~0.038 to 2.5 mg/mL. For all assays, three concentrations of DNCB were prepared from the 20 mg/mL stock solution and added to the appropriate wells containing 1 x 10^6 cells (final concentrations 0.003, 0.004, and 0.0048 mg/mL DNCB in medium). A DMSO vehicle control was prepared as well as 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 6.6 and 6.7). For assays 3 and 4, immediately following the 24-hour incubation period, viability was assayed across the 24-concentrations, an 8-concentration range was selected to assay by flow cytometry, and the range was the same for both assays 3 and 4, ~0.45–1.65 mg/mL.

6.6 Antibody Staining

Cells from each test well were transferred to individual 5 mL tubes and collected by centrifugation (250 x g/5 min/+4°C). The supernatants were discarded, and the pellets were resuspended in 1 mL-cold FACS buffer and washed 2x. Cells were then incubated with blocking solution (0.6 mL 0.1% blocking buffer) for 15 minutes at +4 ± 2°C. Following blocking,

samples were prepared in triplicate (i.e., split into 3 aliquots) of 180–200 µL each in a round-bottom 96-well plate, centrifuged (250 x g/5 min/+4°C), and blocking buffer decanted. Samples were resuspended in 50 µL FACS buffer containing either IgG1, CD54, or CD86 antibodies as per the ECVAM protocol and gently vortexed, incubated at +4 ± 2°C in the dark for 30 minutes, and washed twice in FACS buffer (ECVAM DB-ALM 2012). 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.

6.7 Flow Cytometry

The fluorescence intensities of the labeled cells were analyzed by flow cytometry, using a BD FACSVerse™ flow cytometer, and captured/analyzed with BD FACSuite™ v1.0.5. The acquisition channels were FITC and PI. The 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 6.6.

6.8 Data Analysis

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

6.9 Criteria for a Valid Assay

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

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

6.10 Calculation of Acute Oral Hazard Category

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

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

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

6.11 Concentration Verification of MBANF

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-MDV-CSD. 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 Reactivity Check

The THP-1 cells were checked and verified for reactivity to DNCB and $NiSO_4$ as well as a lack of reactivity to LA. Cells reacted as expected by DNCB and $NiSO_4$ eliciting positive reactions for both CD54 and CD86, while LA was negative; this indicates appropriate reactivity responses in the cells (see Appendix E for data).

7.2 Range Finding Assay

Two independent dose-finding assays were completed to determine the CV75 of MBANF in THP-1 cells. Cytotoxicity was observed in the dosing range; therefore, a CV75 could be experimentally determined (0.0009 mg/mL). These data were used to determine the top dose for the full assay (0.001 mg/mL). Appendix E shows the raw data for the Range Finding.

7.3 Full Test

Four independent h-CLAT assessments were completed for MBANF. Four assays were necessary as the first full test did not result in any cytotoxicity; according to acceptance criteria, if a test is not at the assay limit (final test concentration of 1 mg/mL) and there is no cytotoxicity, it is not a valid assay. A second assay with an increased top dose (4.2E-4 mg/mL) also did not elicit cytotoxicity. For the third and fourth assay, a top concentration of ~ 0.003 mg/mL was utilized successfully. The MBANF was positive for both CD86 and CD54 expression, indicating a positive test and that MBANF is a skin sensitizer according to the defined approach. Appendix E presents the raw data. Because QSAR analysis predicted that MBANF is a skin sensitizer, no additional testing in other skin assays will need to be completed. Currently, data are reported for the nominal concentrations of the compounds because concentration

verification has not yet been completed by Laboratory Sciences, Method Development Section [LS-MDV].

7.4 Acute Oral Hazard Designation

Mammalian acute oral toxicity was predicted using data collected from the h-CLAT. The estimated LD_{50} was 114 mg/kg, suggesting that MBANF has moderate oral toxicity (GHS Category 3; (UNECE 2015)).

8 CONCLUSIONS

The MBANF was found to elicit positive reactions for both sensitization markers in the THP-1 monocytic leukemia cell line, a dendritic cell surrogate. Both CD54 and CD86 expression levels were increased as a result of 24-hour exposure to MBANF. According to the defined approach for skin sensitization, a positive test in the h-CLAT indicates that a compound is a skin sensitizer.

9 RECOMMENDATIONS

The MBANF is a skin sensitizer based on the weight of evidence from both *in vitro* and QSAR analysis (Accelrys Inc.). The h-CLAT can be used as a definitive test to predict skin sensitization, especially when QSAR analysis supports this prediction (USEPA 2018; Kleinstreuer et al. 2018; Strickland et al. 2018). No further skin sensitization tests are necessary. Previously conducted testing estimated MBANF to be a GHS category 3 for oral toxicity, to have low mutagenic potential in the Ames, and to be a GHS category 1 for aquatic toxicity (USAPHC 2013a, 2013b). With MBANF predicted to be a skin sensitizer, general laboratory precautions should be taken with handling the compound. Release into the environment should be avoided due to its moderate water solubility and high predicted aquatic toxicity.

10 POINT OF CONTACT

Dr. Emily N. Reinke, the principal investigator, is the point of contact for this project. She may be reached at DSN 584-3980 or commercial 410-436-3980.

Toxicology Study No. S.0070548-19, February–March 2020

Submitted by: U.S. Army Public Health Center
Health Effects Division, MCHB-PH-HEF
Toxicology Directorate
Aberdeen Proving Ground, MD 21010-5403
410-436-3980

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Approved by:

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Mark S. Johnson, Ph.D., D.A.B.T.
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U.S. Army Public Health Center

Date

APPENDIX A

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Appendix B

QUALITY ASSURANCE STATEMENT
H-CLAT ASSAY

For: Toxicology Study No. S.0070548-19, Protocol No. 49-iv19-03-01M Human Cell Line Activation Test of the N,N'-bis(4-nitro-1,2,5-oxadiazol-3-yl)-methanediamine (MBANF) 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	03/18/2019	03/18/2019
Test Article Specific Protocol Modification Review	02/18/2020	02/18/2020

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	4/16/2020	4/16/2020
Final Study Good Laboratory Practice Standard Report Review	4/16/2020	4/16/2020

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

4/20/2020
Date

APPENDIX C

ARCHIVES AND STUDY PERSONNEL

C-1 ARCHIVES

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

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

The present study used the Toxicology No. S.0070548-19, March 2020, Protocol No. 49-iv17-02-01M. 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, APHC, APG, MD, 21010.

Archivist: Martha Thompson

C-2 PERSONNEL

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

Study Director: Emily N. Reinke, Ph.D., D.A.B.T., HEF.

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

APPENDIX D

REAGENTS USED

Reagent	Supplier	Product Number	Lot Number	Expiration Date
THP-1	ATCC	TIB-202	62996831	N/A
RPMI-1640	Gibco	22400	2085154	04-30-2020
FBS	Gibco	16140	1982139	4-30-23
2-Mercaptoethanol	Gibco	21985	1922541	11-30-2020
Penicillin-Streptomycin	Gibco	15140	2068816	1-30-2020
Saline	Sigma	S8776	RNBD7305	N/A
DMSO (TC)	Sigma	D2438	RNBG82238	06-20
Globulins	Sigma	G2388	017K7650V	N/A
BSA Fraction V	EMD Chemicals	12660	D00150383	N/A
D-PBS	Glbco	14190	1897013	07-20
Propidium Iodide	Sigma	P4864	MKBR1007V	N/A
CD54 Antibody, ICAM-1 Clone 6.5B5, FITC	Dako	F714301-8	20051521	09-20
CD86 Antibody, Hu Fun-1, FITC	BD	555657	8115611	10-31-23
IgG1 (mouse), FITC	Dako	X092701-2	200406402	04-30-20
Flow Cytometer Beads	BD	650622	81165	05-31-20
Sheath Fluid	BD	342003	0000221830	02-04-22
2,4-dinitrochlorobenzene (DNCB)	Sigma	237329	BCBN7826V	N/A
Nickel Sulfate (NiSO4)	Sigma	656895	MKBT0269V	N/A
Lactic Acid (LA)	Sigma	W261106	MKBR4746V	N/A

APPENDIX E

h-CLAT DATA

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

Table E-1. Reactivity Check

Test Article	Concentration (mg/mL)	Viability (% alive)	Percent Change (CD86)	Percent Change (CD54)	Positive (CD86/CD54)	Pass/Fail
Media		95.58	100	100	N/N	Pass
Saline		94.65	100	100	N/N	Pass
DMSO		93.89	100	100	N/N	Pass
Lactic Acid	1.0	95.02	81.77	103.7	N/N	Pass
Nickel	0.10	70.1	181.0	2399	Y/Y	Pass
DNCB	0.0033	76.86	298.1	319.6	Y/Y	Pass
	0.0040	73.8	325.9	442.6	Y/Y	Pass
	0.0048	68.62	139.6	400.7	N/Y	N/A

Figure E-1. Reactivity Check Raw Data

Experiment:
h-CLAT Reactivity Check 2-13-20 Mods L,M
Protocol 49-iv19-03-01

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	993
Live Cells:Live Cells	9,524	95.24	***	95.24	932
Dead Cells:All Events	10,000	***	***	100.00	3,695
Dead Cells:Live Cells	60	0.60	***	0.60	2,143
IgG Media:All Events	10,462	***	***	100.00	998
IgG Media:Live Cells	10,000	95.58	***	95.58	938
IgG Saline:All Events	10,567	***	***	100.00	992
IgG Saline:Live Cells	10,002	94.65	***	94.65	923
IgG DMSO:All Events	10,651	***	***	100.00	967
IgG DMSO:Live Cells	10,000	93.89	***	93.89	899
IgG Lactic Acid:All Events	10,524	***	***	100.00	944
IgG Lactic Acid:Live Cells	10,000	95.02	***	95.02	879
IgG Nickel Sulfate:All Events	14,266	***	***	100.00	1,211
IgG Nickel Sulfate:Live Cells	10,000	70.10	***	70.10	1,064
IgG DNCB 1:All Events	13,010	***	***	100.00	1,468
IgG DNCB 1:Live Cells	10,000	76.86	***	76.86	1,212
IgG DNCB 2:All Events	13,546	***	***	100.00	1,439
IgG DNCB 2:Live Cells	9,997	73.80	***	73.80	1,202
IgG DNCB 3:All Events	14,574	***	***	100.00	1,574
IgG DNCB 3:Live Cells	10,000	68.62	***	68.62	1,265
CD86 Media:All Events	10,547	***	***	100.00	2,639
CD86 Media:Live Cells	10,000	94.81	***	94.81	2,383
CD54 Media:All Events	10,568	***	***	100.00	1,104
CD54 Media:Live Cells	10,000	94.63	***	94.63	1,027
CD86 Saline:All Events	10,563	***	***	100.00	2,787
CD86 Saline:Live Cells	10,011	94.77	***	94.77	2,481
CD54 Saline:All Events	10,593	***	***	100.00	1,116
CD54 Saline:Live Cells	10,000	94.40	***	94.40	1,030
CD86 DMSO:All Events	10,662	***	***	100.00	2,874
CD86 DMSO:Live Cells	10,000	93.79	***	93.79	2,549
CD54 DMSO:All Events	10,684	***	***	100.00	1,133
CD54 DMSO:Live Cells	10,000	93.60	***	93.60	1,047
CD86 Lactic Acid:All Events	10,582	***	***	100.00	2,470
CD86 Lactic Acid:Live Cells	9,959	94.11	***	94.11	2,153
CD54 Lactic Acid:All Events	10,566	***	***	100.00	1,068
CD54 Lactic Acid:Live Cells	10,000	94.64	***	94.64	990
CD86 Nickel Sulfate:All Events	14,494	***	***	100.00	7,618
CD86 Nickel Sulfate:Live Cells	10,000	68.99	***	68.99	3,884
CD54 Nickel Sulfate:All Events	15,376	***	***	100.00	3,172
CD54 Nickel Sulfate:Live Cells	10,000	65.04	***	65.04	3,631
CD86 DNCB 1:All Events	12,974	***	***	100.00	9,058
CD86 DNCB 1:Live Cells	10,001	77.08	***	77.08	6,130
CD54 DNCB 1:All Events	13,203	***	***	100.00	1,926
CD54 DNCB 1:Live Cells	9,995	75.70	***	75.70	1,685
CD86 DNCB 2:All Events	13,887	***	***	100.00	10,119
CD86 DNCB 2:Live Cells	10,000	72.01	***	72.01	6,579
CD54 DNCB 2:All Events	14,041	***	***	100.00	2,065
CD54 DNCB 2:Live Cells	10,000	71.22	***	71.22	1,857
CD86 DNCB 3:All Events	14,810	***	***	100.00	6,860
CD86 DNCB 3:Live Cells	10,000	67.52	***	67.52	3,568
CD54 DNCB 3:All Events	15,347	***	***	100.00	2,134
CD54 DNCB 3:Live Cells	10,000	65.16	***	65.16	1,858

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

2/21/2020		PI- Dose Finding		
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	mg/mL
Media			97.72	
DMSO			97.88	
MBANF	3.875	0.00775	6.79	Experiment stopped due to low viability
Run #1	7.75	0.0155	3.44	
	15.5	0.031	1.47	
	31	0.062		
	62	0.124		
	124	0.248		
	248	0.496		
	496	0.992		
2/26/2020		PI- Dose Finding		
	Stock (mg/mL)	Test Concentration in DMSO(mg/mL)	Viability	mg/mL
Media			97.74	
DMSO			97.53	
MBANF	0.0387	7.73E-05	86.69	9.74E-05
Run #2	0.0773	0.000155	51.51	
	0.155	0.000309	25.78	
	0.309	0.000619	18.45	
	0.619	0.00124	10.2	
	1.24	0.00249	2.98	
	2.48	0.00495	1.97	
	4.95	0.0099	1.42	

Figure E-2. Range Finding Experiment 1 Raw Data

Experiment:
Range-FindingKDNP MBANF #1 2-21-20 Mods LM

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	394
Live Cells:Live Cells	9,727	97.27	***	97.27	373
Dead Cells:All Events	10,000	***	***	100.00	62,325
Dead Cells:Live Cells	20	0.20	***	0.20	506
Media:All Events	10,233	***	***	100.00	393
Media:Live Cells	10,000	97.72	***	97.72	376
DMSO:All Events	10,178	***	***	100.00	386
DMSO:Live Cells	9,962	97.88	***	97.88	366
KDNP 1:All Events	10,288	***	***	100.00	382
KDNP 1:Live Cells	10,000	97.20	***	97.20	360
KDNP 2:All Events	10,278	***	***	100.00	382
KDNP 2:Live Cells	9,999	97.29	***	97.29	359
KDNP 3:All Events	10,301	***	***	100.00	393
KDNP 3:Live Cells	10,000	97.08	***	97.08	368
KDNP 4:All Events	10,314	***	***	100.00	391
KDNP 4:Live Cells	10,000	96.96	***	96.96	369
KDNP 5:All Events	10,333	***	***	100.00	405
KDNP 5:Live Cells	9,993	96.71	***	96.71	386
KDNP 6:All Events	10,416	***	***	100.00	424
KDNP 6:Live Cells	10,000	96.01	***	96.01	397
KDNP 7:All Events	10,364	***	***	100.00	433
KDNP 7:Live Cells	10,000	96.49	***	96.49	407
KDNP 8:All Events	10,969	***	***	100.00	597
KDNP 8:Live Cells	10,000	91.17	***	91.17	493
MBANF 1:All Events	23,016	***	***	100.00	39,489
MBANF 1:Live Cells	1,563	6.79	***	6.79	823
MBANF 2:All Events	24,249	***	***	100.00	45,326
MBANF 2:Live Cells	835	3.44	***	3.44	851
MBANF 3:All Events	15,633	***	***	100.00	71,977
MBANF 3:Live Cells	232	1.48	***	1.48	967
MBANF 4:All Events	***	***	***	***	***
MBANF 4:Live Cells	***	***	***	***	***
MBANF 5:All Events	***	***	***	***	***
MBANF 5:Live Cells	***	***	***	***	***
MBANF 6:All Events	***	***	***	***	***
MBANF 6:Live Cells	***	***	***	***	***
MBANF 7:All Events	***	***	***	***	***
MBANF 7:Live Cells	***	***	***	***	***
MBANF 8:All Events	***	***	***	***	***
MBANF 8:Live Cells	***	***	***	***	***

Operator: Emily Reinke, Ph.D.

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Software: BD FACSuite v1.0.5
Page 1 of 1

Figure E-3. Range Finding Experiment 2 Raw Data

Experiment:
h-CLAT_Range-Finding_KDNP_MBANF_2-26-20
Protocol 49-iv19-03-01 Mods L, M

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	Propidium Iodide-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	377
Live Cells:Live Cells	9,769	97.69	***	97.69	359
Dead Cells:All Events	10,000	***	***	100.00	32,935
Dead Cells:Live Cells	74	0.74	***	0.74	709
Media:All Events	10,240	***	***	100.00	381
Media:Live Cells	10,009	97.74	***	97.74	359
DMSO:All Events	10,270	***	***	100.00	370
DMSO:Live Cells	10,016	97.53	***	97.53	349
KDNP 1:All Events	10,258	***	***	100.00	371
KDNP 1:Live Cells	10,120	98.65	***	98.65	353
KDNP 2:All Events	10,257	***	***	100.00	384
KDNP 2:Live Cells	10,000	97.49	***	97.49	360
KDNP 3:All Events	10,240	***	***	100.00	378
KDNP 3:Live Cells	10,000	97.66	***	97.66	354
KDNP 4:All Events	10,273	***	***	100.00	387
KDNP 4:Live Cells	10,000	97.34	***	97.34	361
KDNP 5:All Events	10,237	***	***	100.00	396
KDNP 5:Live Cells	10,000	97.68	***	97.68	378
KDNP 6:All Events	10,306	***	***	100.00	408
KDNP 6:Live Cells	10,000	97.03	***	97.03	382
KDNP 7:All Events	10,480	***	***	100.00	535
KDNP 7:Live Cells	10,000	95.42	***	95.42	476
KDNP 8:All Events	10,932	***	***	100.00	651
KDNP 8:Live Cells	10,000	91.47	***	91.47	517
MBANF 1:All Events	11,149	***	***	100.00	688
MBANF 1:Live Cells	10,000	89.69	***	89.69	498
MBANF 2:All Events	19,450	***	***	100.00	3,368
MBANF 2:Live Cells	10,000	51.41	***	51.41	726
MBANF 3:All Events	30,000	***	***	100.00	8,985
MBANF 3:Live Cells	7,733	25.78	***	25.78	892
MBANF 4:All Events	30,000	***	***	100.00	20,670
MBANF 4:Live Cells	5,534	18.45	***	18.45	825
MBANF 5:All Events	30,000	***	***	100.00	46,580
MBANF 5:Live Cells	3,059	10.20	***	10.20	993
MBANF 6:All Events	30,000	***	***	100.00	55,671
MBANF 6:Live Cells	894	2.98	***	2.98	902
MBANF 7:All Events	30,000	***	***	100.00	63,273
MBANF 7:Live Cells	592	1.97	***	1.97	931
MBANF 8:All Events	30,000	***	***	100.00	60,118
MBANF 8:Live Cells	425	1.42	***	1.42	958

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Software: BD FACSuite v1.0.5
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Figure E-4. h-CLAT Experiment 1 Raw Data

Experiment: h-CLAT KDNP MBANF #1 2-28-20
Protocol 49-iv19-03-01 Mods L,M

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	871
Live Cells:Live Cells	9,678	96.78	***	96.78	856
Dead Cells:All Events	10,000	***	***	100.00	2,637
Dead Cells:Live Cells	41	0.41	***	0.41	1,301
IgG Media:All Events	10,376	***	***	100.00	836
IgG Media:Live Cells	10,000	96.38	***	96.38	820
IgG DMSO:All Events	10,329	***	***	100.00	829
IgG DMSO:Live Cells	10,000	96.81	***	96.81	809
IgG DNCB 1:All Events	12,171	***	***	100.00	1,050
IgG DNCB 1:Live Cells	9,679	79.53	***	79.53	970
IgG DNCB 2:All Events	12,873	***	***	100.00	1,151
IgG DNCB 2:Live Cells	9,918	77.04	***	77.04	1,006
IgG DNCB 3:All Events	3,456	***	***	100.00	3,133
IgG DNCB 3:Live Cells	59	1.71	***	1.71	1,149
IgG MBANF 1:All Events	10,385	***	***	100.00	851
IgG MBANF 1:Live Cells	10,009	96.38	***	96.38	835
IgG MBANF 2:All Events	10,366	***	***	100.00	841
IgG MBANF 2:Live Cells	10,000	96.47	***	96.47	818
IgG MBANF 3:All Events	10,353	***	***	100.00	852
IgG MBANF 3:Live Cells	10,000	96.59	***	96.59	830
IgG MBANF 4:All Events	10,427	***	***	100.00	870
IgG MBANF 4:Live Cells	10,000	95.90	***	95.90	848
IgG MBANF 5:All Events	10,359	***	***	100.00	831
IgG MBANF 5:Live Cells	10,000	96.53	***	96.53	808
IgG MBANF 6:All Events	10,333	***	***	100.00	838
IgG MBANF 6:Live Cells	10,000	96.78	***	96.78	816
IgG MBANF 7:All Events	10,454	***	***	100.00	846
IgG MBANF 7:Live Cells	10,000	95.66	***	95.66	816
IgG MBANF 8:All Events	10,430	***	***	100.00	845
IgG MBANF 8:Live Cells	10,000	95.88	***	95.88	819
CD86 Media:All Events	10,382	***	***	100.00	2,318
CD86 Media:Live Cells	10,000	96.32	***	96.32	2,199
CD54 Media:All Events	10,386	***	***	100.00	961
CD54 Media:Live Cells	10,000	96.28	***	96.28	935
CD86 DMSO:All Events	10,344	***	***	100.00	2,141
CD86 DMSO:Live Cells	10,004	96.71	***	96.71	2,058
CD54 DMSO :All Events	10,326	***	***	100.00	965
CD54 DMSO :Live Cells	10,000	96.84	***	96.84	942
CD86 DNCB 1:All Events	13,428	***	***	100.00	8,059
CD86 DNCB 1:Live Cells	9,973	74.27	***	74.27	6,650
CD54 DNCB 1:All Events	13,366	***	***	100.00	2,135
CD54 DNCB 1:Live Cells	9,997	74.79	***	74.79	2,224
CD86 DNCB 2:All Events	14,349	***	***	100.00	7,194
CD86 DNCB 2:Live Cells	10,007	69.74	***	69.74	5,105
CD54 DNCB 2:All Events	14,134	***	***	100.00	2,284
CD54 DNCB 2:Live Cells	10,000	70.75	***	70.75	2,416
CD86 DNCB 3:All Events	***	***	***	***	***
CD86 DNCB 3:Live Cells	***	***	***	***	***
CD54 DNCB 3:All Events	***	***	***	***	***
CD54 DNCB 3:Live Cells	***	***	***	***	***
CD86 MBANF 1:All Events	10,375	***	***	100.00	2,255
CD86 MBANF 1:Live Cells	10,000	96.39	***	96.39	2,161
CD54 MBANF 1:All Events	10,363	***	***	100.00	989
CD54 MBANF 1:Live Cells	10,000	96.50	***	96.50	966
CD86 MBANF 2:All Events	10,492	***	***	100.00	2,053
CD86 MBANF 2:Live Cells	9,998	95.29	***	95.29	1,971
CD54 MBANF 2:All Events	10,415	***	***	100.00	972
CD54 MBANF 2:Live Cells	10,000	96.02	***	96.02	945
CD86 MBANF 3:All Events	10,402	***	***	100.00	2,151
CD86 MBANF 3:Live Cells	10,000	96.14	***	96.14	2,056
CD54 MBANF 3:All Events	10,468	***	***	100.00	978
CD54 MBANF 3:Live Cells	10,000	95.53	***	95.53	948
CD86 MBANF 4:All Events	10,436	***	***	100.00	2,163
CD86 MBANF 4:Live Cells	10,000	95.82	***	95.82	2,075
CD54 MBANF 4:All Events	10,467	***	***	100.00	967
CD54 MBANF 4:Live Cells	10,000	95.54	***	95.54	938
CD86 MBANF 5:All Events	10,378	***	***	100.00	2,311
CD86 MBANF 5:Live Cells	10,000	96.36	***	96.36	2,206
CD54 MBANF 5:All Events	10,515	***	***	100.00	974
CD54 MBANF 5:Live Cells	10,000	95.10	***	95.10	943
CD86 MBANF 6:All Events	10,591	***	***	100.00	2,349
CD86 MBANF 6:Live Cells	10,000	94.42	***	94.42	2,240
CD54 MBANF 6:All Events	10,470	***	***	100.00	960
CD54 MBANF 6:Live Cells	10,000	95.51	***	95.51	947
CD86 MBANF 7:All Events	10,506	***	***	100.00	2,577
CD86 MBANF 7:Live Cells	10,000	95.18	***	95.18	2,447
CD54 MBANF 7:All Events	10,475	***	***	100.00	996
CD54 MBANF 7:Live Cells	10,000	95.47	***	95.47	961
CD86 MBANF 8:All Events	10,518	***	***	100.00	2,658
CD86 MBANF 8:Live Cells	10,000	95.08	***	95.08	2,512
CD54 MBANF 8:All Events	10,484	***	***	100.00	992
CD54 MBANF 8:Live Cells	10,000	95.38	***	95.38	959

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

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
Media		0	96.38	820	2199	1	100		935	1	100	
DMSO		0	96.81	809	2058	0.906	90.6		942	1.16	116	
DNCB Control	1.67	0.0033	79.53	970	6650	4.55	455		2224	10.90	1090	
	2	0.004	77.04	1006	5105	3.28	328		2416	12.26	1226	
	2.4[a]	0.0048	1.71	1149	--	--	--		--	--	--	
MBANF 2/28/2020	0.0140	0.0000279	96.38	835	2161	1.06	106		966	0.98	98.5	
	0.0167	0.0000335	96.47	818	1971	0.92	92.3		945	0.95	95.6	
	0.0204	0.0000402	96.59	830	2056	0.98	98.2		948	0.89	88.7	
	0.0241	0.0000482	95.9	848	2075	0.98	98.2		938	0.68	67.7	
	0.0289	0.0000579	96.53	808	2206	1.12	112		943	1.02	102	
	0.0347	0.0000694	96.78	816	2240	1.14	114		947	0.98	98.5	
	0.0417	0.0000833	95.66	816	2447	1.31	131		961	1.09	109	
	0.05	0.0001000	95.88	819	2512	1.36	136		959	1.05	105	

Note:
[a]Viability too low for analysis

Figure E-5. h-CLAT Experiment 2 Raw Data

Experiment:
h-CLAT KDNP MBANF #2 3-4-2020
Protocol 49-iv19-03-01 Mods L,M

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	895
Live Cells:Live Cells	9,578	95.78	***	95.78	870
Dead cells:All Events	10,000	***	***	100.00	2,079
Dead cells:Live Cells	69	0.69	***	0.69	1,189
IgG Media:All Events	10,509	***	***	100.00	924
IgG Media:Live Cells	10,071	95.83	***	95.83	895
IgG DMSO:All Events	10,215	***	***	100.00	883
IgG DMSO:Live Cells	9,985	97.75	***	97.75	868
IgG DNCB 1:All Events	11,876	***	***	100.00	1,040
IgG DNCB 1:Live Cells	10,154	85.50	***	85.50	989
IgG DNCB 2A:All Events	11,289	***	***	100.00	1,005
IgG DNCB 2A:Live Cells	9,622	85.23	***	85.23	972
IgG DNCB 3:All Events	14,876	***	***	100.00	1,407
IgG DNCB 3:Live Cells	10,175	68.40	***	68.40	1,161
IgG DNCB 2B:All Events	12,318	***	***	100.00	1,033
IgG DNCB 2B:Live Cells	10,000	81.18	***	81.18	941
IgG MBANF 1 :All Events	10,351	***	***	100.00	864
IgG MBANF 1 :Live Cells	10,009	96.70	***	96.70	839
IgG MBANF 2:All Events	10,471	***	***	100.00	867
IgG MBANF 2:Live Cells	10,000	95.50	***	95.50	836
IgG MBANF 3:All Events	10,421	***	***	100.00	850
IgG MBANF 3:Live Cells	10,000	95.96	***	95.96	822
IgG MBANF 4:All Events	10,544	***	***	100.00	885
IgG MBANF 4:Live Cells	10,000	94.84	***	94.84	841
IgG MBANF 5:All Events	10,481	***	***	100.00	900
IgG MBANF 5:Live Cells	9,998	95.39	***	95.39	875
IgG MBANF 6:All Events	10,419	***	***	100.00	929
IgG MBANF 6:Live Cells	9,955	95.55	***	95.55	891
IgG MBANF 7:All Events	10,490	***	***	100.00	897
IgG MBANF 7:Live Cells	10,000	95.33	***	95.33	861
IgG MBANF 8:All Events	10,441	***	***	100.00	893
IgG MBANF 8:Live Cells	10,000	95.78	***	95.78	859
IgG MBANF 9:All Events	10,484	***	***	100.00	918
IgG MBANF 9:Live Cells	10,000	95.38	***	95.38	880
IgG MBANF 10:All Events	10,460	***	***	100.00	931
IgG MBANF 10:Live Cells	10,000	95.60	***	95.60	896
IgG MBANF 11:All Events	10,527	***	***	100.00	939
IgG MBANF 11:Live Cells	10,222	97.10	***	97.10	920
IgG MBANF 12:All Events	10,526	***	***	100.00	917
IgG MBANF 12:Live Cells	10,000	95.00	***	95.00	879
CD86 Media:All Events	10,503	***	***	100.00	2,575
CD86 Media:Live Cells	10,000	95.21	***	95.21	2,423
CD54 Media:All Events	10,435	***	***	100.00	1,131
CD54 Media:Live Cells	10,000	95.83	***	95.83	1,096
CD86 DMSO:All Events	10,454	***	***	100.00	2,723
CD86 DMSO:Live Cells	10,000	95.66	***	95.66	2,599
CD54 DMSO:All Events	10,413	***	***	100.00	1,174
CD54 DMSO:Live Cells	10,000	96.03	***	96.03	1,135
CD86 DNCB 1:All Events	11,982	***	***	100.00	10,312
CD86 DNCB 1:Live Cells	9,987	83.35	***	83.35	9,532
CD54 DNCB 1:All Events	11,850	***	***	100.00	1,895
CD54 DNCB 1:Live Cells	10,000	84.39	***	84.39	1,866
CD86 DNCB 2A:All Events	12,661	***	***	100.00	11,039
CD86 DNCB 2A:Live Cells	9,875	78.00	***	78.00	9,961
CD54 DNCB 2A:All Events	12,382	***	***	100.00	2,220
CD54 DNCB 2A:Live Cells	9,986	80.65	***	80.65	2,289
CD86 DNCB 3:All Events	15,580	***	***	100.00	6,310
CD86 DNCB 3:Live Cells	9,975	64.02	***	64.02	2,181
CD54 DNCB 3:All Events	15,907	***	***	100.00	1,700
CD54 DNCB 3:Live Cells	10,590	66.57	***	66.57	1,417
CD86 DNCB 2B:All Events	13,276	***	***	100.00	11,297
CD86 DNCB 2B:Live Cells	9,997	75.30	***	75.30	10,207
CD54 DNCB 2B:All Events	12,902	***	***	100.00	2,291
CD54 DNCB 2B:Live Cells	10,002	77.52	***	77.52	2,326
CD86 MBANF 1:All Events	10,600	***	***	100.00	2,896
CD86 MBANF 1:Live Cells	10,000	94.34	***	94.34	2,728
CD54 MBANF 1:All Events	10,278	***	***	100.00	1,212
CD54 MBANF 1:Live Cells	9,890	96.22	***	96.22	1,191
CD86 MBANF 2:All Events	10,619	***	***	100.00	3,002
CD86 MBANF 2:Live Cells	10,000	94.17	***	94.17	2,825
CD54 MBANF 2:All Events	10,493	***	***	100.00	1,183
CD54 MBANF 2:Live Cells	10,000	95.30	***	95.30	1,137
CD86 MBANF 3:All Events	10,528	***	***	100.00	2,867
CD86 MBANF 3:Live Cells	10,000	94.98	***	94.98	2,715
CD54 MBANF 3:All Events	10,463	***	***	100.00	1,171
CD54 MBANF 3:Live Cells	10,207	97.55	***	97.55	1,148
CD86 MBANF 4:All Events	10,735	***	***	100.00	2,955
CD86 MBANF 4:Live Cells	10,000	93.15	***	93.15	2,777
CD54 MBANF 4:All Events	10,529	***	***	100.00	1,222
CD54 MBANF 4:Live Cells	10,000	94.98	***	94.98	1,175
CD86 MBANF 5:All Events	10,797	***	***	100.00	2,996
CD86 MBANF 5:Live Cells	10,000	92.62	***	92.62	2,857
CD54 MBANF 5:All Events	10,528	***	***	100.00	1,195
CD54 MBANF 5:Live Cells	10,000	94.98	***	94.98	1,154

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
CD86 MBANF 6:All Events	10,514	***	***	100.00	2,929
CD86 MBANF 6:Live Cells	10,000	95.11	***	95.11	2,810
CD54 MBANF 6:All Events	10,506	***	***	100.00	1,226
CD54 MBANF 6:Live Cells	10,000	95.18	***	95.18	1,180
CD86 MBANF 7:All Events	10,733	***	***	100.00	2,957
CD86 MBANF 7:Live Cells	9,998	93.15	***	93.15	2,808
CD54 MBANF 7:All Events	10,492	***	***	100.00	1,181
CD54 MBANF 7:Live Cells	10,000	95.31	***	95.31	1,147
CD86 MBANF 8:All Events	10,605	***	***	100.00	3,073
CD86 MBANF 8:Live Cells	10,000	94.30	***	94.30	2,901
CD54 MBANF 8:All Events	10,533	***	***	100.00	1,192
CD54 MBANF 8:Live Cells	10,000	94.94	***	94.94	1,150
CD86 MBANF 9:All Events	10,533	***	***	100.00	3,110
CD86 MBANF 9:Live Cells	9,995	94.89	***	94.89	2,965
CD54 MBANF 9:All Events	10,545	***	***	100.00	1,246
CD54 MBANF 9:Live Cells	10,000	94.83	***	94.83	1,196
CD86 MBANF 10:All Events	10,682	***	***	100.00	3,053
CD86 MBANF 10:Live Cells	10,000	93.62	***	93.62	2,878
CD54 MBANF 10:All Events	10,548	***	***	100.00	1,210
CD54 MBANF 10:Live Cells	10,000	94.80	***	94.80	1,160
CD86 MBANF 11:All Events	10,592	***	***	100.00	3,018
CD86 MBANF 11:Live Cells	9,939	93.83	***	93.83	2,846
CD54 MBANF 11:All Events	10,615	***	***	100.00	1,265
CD54 MBANF 11:Live Cells	10,000	94.21	***	94.21	1,202
CD86 MBANF 12:All Events	10,700	***	***	100.00	3,258
CD86 MBANF 12:Live Cells	9,998	93.44	***	93.44	3,080
CD54 MBANF 12:All Events	10,563	***	***	100.00	1,215
CD54 MBANF 12:Live Cells	10,000	94.67	***	94.67	1,167

Operator: Emily Reinke, Ph.D.

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Software: BD FACSuite v1.0.5
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Table E-4. h-CLAT Experiment 2 Data Analysis

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
Media		0	95.83	895	2423	1	100		1096	1	100	
DMSO		0	97.75	969	2599	1.07	107		1174	1.02	102	
DNCB Control	1.67	0.0033	85.5	989	9532	6.84	684		1895	7.88	788	
	2	0.004	85.23	972	11039	8.06	806		2289	11.45	1145	
	2	0.004	81.18	941	10207	7.42	742		2326	12.04	1204	
	2.4	0.0048	68.4	1161	2181	0.82	81.7		1417	2.23	223	
MBANF	0.0284	0.00006	96.7	839	2728	1.16	116		1191	1.72	172	
3/4/2020	0.0341	0.00007	95.5	836	2825	1.22	122		1137	1.47	147	
	0.0409	0.00008	95.96	822	2715	1.16	116		1148	1.59	159	
	0.0491	0.00010	94.84	841	2777	1.19	119		1175	1.63	163	
	0.0589	0.00012	95.39	875	2857	1.22	122		1154	1.36	136	
	0.0707	0.00014	95.55	891	2810	1.18	118		1180	1.41	141	
	0.0849	0.00017	95.33	861	2808	1.19	119		1147	1.40	140	
	0.102	0.00020	95.78	859	2901	1.25	125		1150	1.42	142	
	0.122	0.00024	95.38	880	2965	1.28	128		1196	1.54	154	
	0.147	0.00029	95.6	896	2878	1.22	122		1160	1.29	129	
	0.176	0.00035	97.4	920	2846	1.18	118		1202	1.38	138	
	0.211	0.00042	95	879	3080	1.35	135		1167	1.40	141	

Figure E-6. h-CLAT Experiment 3 Raw Data

Experiment: h-CLAT MBANF 3 3-6-2020
Protocol 49-iv19-03-01 Mod M

Cytometer: BD FACSVerse

Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	836
Live Cells:Live Cells	9,703	97.03	***	97.03	821
Dead Cells:All Events	10,000	***	***	100.00	1,218
Dead Cells:Live Cells	6,822	68.22	***	68.22	989
IgG Media:All Events	10,362	***	***	100.00	854
IgG Media:Live Cells	9,978	96.29	***	96.29	845
IgG DMSO:All Events	10,406	***	***	100.00	835
IgG DMSO:Live Cells	10,000	96.10	***	96.10	808
IgG DNCB 1:All Events	12,212	***	***	100.00	994
IgG DNCB 1:Live Cells	9,967	81.62	***	81.62	877
IgG DNCB 2:All Events	13,203	***	***	100.00	1,065
IgG DNCB 2:Live Cells	9,888	74.89	***	74.89	901
IgG DNCB 3:All Events	15,291	***	***	100.00	1,245
IgG DNCB 3:Live Cells	9,979	65.26	***	65.26	966
IgG MBANF 15:All Events	10,565	***	***	100.00	977
IgG MBANF 15:Live Cells	9,941	94.09	***	94.09	938
IgG MBANF 16:All Events	10,703	***	***	100.00	1,033
IgG MBANF 16:Live Cells	9,949	92.96	***	92.96	971
IgG MBANF 17:All Events	11,387	***	***	100.00	1,099
IgG MBANF 17:Live Cells	10,000	87.82	***	87.82	1,004
IgG MBANF 18:All Events	12,594	***	***	100.00	1,192
IgG MBANF 18:Live Cells	10,000	79.40	***	79.40	1,052
IgG MBANF 19:All Events	16,151	***	***	100.00	1,385
IgG MBANF 19:Live Cells	10,000	61.92	***	61.92	1,141
IgG MBANF 20:All Events	25,939	***	***	100.00	1,621
IgG MBANF 20:Live Cells	12,026	46.36	***	46.36	1,436
IgG MBANF 21:All Events	30,000	***	***	100.00	1,764
IgG MBANF 21:Live Cells	5,080	16.93	***	16.93	1,502
IgG MBANF 22:All Events	30,000	***	***	100.00	1,932
IgG MBANF 22:Live Cells	1,551	5.17	***	5.17	1,634
CD86 Media:All Events	10,482	***	***	100.00	1,884
CD86 Media:Live Cells	10,031	95.70	***	95.70	1,770
CD54 Media:All Events	10,402	***	***	100.00	1,113
CD54 Media:Live Cells	9,998	96.12	***	96.12	1,084
CD86 DMSO:All Events	10,489	***	***	100.00	2,014
CD86 DMSO:Live Cells	10,000	95.34	***	95.34	1,888
CD54 DMSO:All Events	10,448	***	***	100.00	1,114
CD54 DMSO:Live Cells	10,000	95.71	***	95.71	1,078
CD86 DNCB 1:All Events	13,240	***	***	100.00	7,402
CD86 DNCB 1:Live Cells	9,999	75.52	***	75.52	6,126
CD54 DNCB 1:All Events	12,859	***	***	100.00	3,015
CD54 DNCB 1:Live Cells	10,002	77.78	***	77.78	3,236
CD86 DNCB 2:All Events	13,994	***	***	100.00	6,283
CD86 DNCB 2:Live Cells	10,049	71.81	***	71.81	4,936
CD54 DNCB 2:All Events	14,037	***	***	100.00	3,266
CD54 DNCB 2:Live Cells	9,989	71.16	***	71.16	3,802
CD86 DNCB 3:All Events	16,967	***	***	100.00	6,003
CD86 DNCB 3:Live Cells	10,000	58.94	***	58.94	2,728
CD54 DNCB 3:All Events	16,445	***	***	100.00	2,425
CD54 DNCB 3:Live Cells	10,027	60.97	***	60.97	2,411
CD86 MBANF 15:All Events	10,748	***	***	100.00	3,406
CD86 MBANF 15:Live Cells	10,000	93.04	***	93.04	3,148
CD54 MBANF 15:All Events	10,703	***	***	100.00	1,319
CD54 MBANF 15:Live Cells	10,000	93.43	***	93.43	1,251
CD86 MBANF 16:All Events	10,957	***	***	100.00	3,556
CD86 MBANF 16:Live Cells	10,000	91.27	***	91.27	3,228
CD54 MBANF 16:All Events	10,888	***	***	100.00	1,376
CD54 MBANF 16:Live Cells	10,000	91.84	***	91.84	1,315
CD86 MBANF 17:All Events	11,461	***	***	100.00	4,660
CD86 MBANF 17:Live Cells	10,000	87.25	***	87.25	4,180
CD54 MBANF 17:All Events	11,429	***	***	100.00	1,610
CD54 MBANF 17:Live Cells	9,965	87.19	***	87.19	1,508
CD86 MBANF 18:All Events	12,592	***	***	100.00	5,695
CD86 MBANF 18:Live Cells	10,000	79.42	***	79.42	4,810
CD54 MBANF 18:All Events	12,788	***	***	100.00	1,877
CD54 MBANF 18:Live Cells	10,000	78.20	***	78.20	1,748
CD86 MBANF 19:All Events	16,940	***	***	100.00	6,454
CD86 MBANF 19:Live Cells	10,000	59.03	***	59.03	5,155
CD54 MBANF 19:All Events	15,610	***	***	100.00	2,273
CD54 MBANF 19:Live Cells	9,495	60.83	***	60.83	2,207
CD86 MBANF 20:All Events	27,947	***	***	100.00	6,871
CD86 MBANF 20:Live Cells	10,000	35.78	***	35.78	4,586
CD54 MBANF 20:All Events	30,000	***	***	100.00	2,240
CD54 MBANF 20:Live Cells	10,000	33.33	***	33.33	2,386
CD86 MBANF 21:All Events	30,000	***	***	100.00	7,298
CD86 MBANF 21:Live Cells	4,473	14.91	***	14.91	3,246
CD54 MBANF 21:All Events	30,000	***	***	100.00	2,095
CD54 MBANF 21:Live Cells	4,198	13.99	***	13.99	2,467
CD86 MBANF 22:All Events	30,000	***	***	100.00	9,281
CD86 MBANF 22:Live Cells	902	3.01	***	3.01	3,150
CD54 MBANF 22:All Events	30,000	***	***	100.00	2,082
CD54 MBANF 22:Live Cells	743	2.48	***	2.48	1,740

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Table E-5. h-CLAT Experiment 3 Data Analysis

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
Media		0	96.29	845	1770	1	100		1084	1	100	
DMSO		0	96.1	808	1888	1.17	117		1078	1.13	113	
DNCB Control	1.67	0.0033	81.62	877	6126	4.20	420		3236	20.51	2051	
	2	0.004	74.89	901	4936	3.23	323		3802	25.23	2523	
	2.4	0.0048	65.26	966	2728	1.41	141		2411	12.57	1257	
MBANF	0.480	0.00096	94.09	938	3148	1.36	136		1251	1.53	153	
3/6/2020	0.576	0.00115	92.96	971	3228	1.38	138		1315	1.68	168	
	0.691	0.00138	87.82	1004	4180	1.95	195	0.0012	1508	2.46	246	0.0012
	0.829	0.00166	79.4	1052	4810	2.31	231		1748	3.40	340	
	0.995	0.00199	61.92	1151	5115	2.43	243		2207	5.15	515	
	1.19	0.00239	46.36	1436	4586	1.93	193		2386	4.63	463	
	1.43	0.00287	16.93	1502	3246	1.07	107		2467	4.71	471	
	1.72	0.00344	5.17	1634	3150	0.93	93.01		1740	0.52	51.7	

Figure E-7. h-CLAT Experiment 4 Raw Data

Experiment:
h-CLAT MBANF #4 3-10-20
Protocol 49-iv19-03-01 Mod M

Cytometer: BD FACSVerse Cytometer SN: Z6511530048

Statistics

Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
Live Cells:All Events	10,000	***	***	100.00	840
Live Cells:Live Cells	9,466	94.66	***	94.66	806
Dead Cells:All Events	7,224	***	***	100.00	1,932
Dead Cells:Live Cells	3,087	42.73	***	42.73	1,148
IgG Media:All Events	10,566	***	***	100.00	870
IgG Media:Live Cells	10,004	94.68	***	94.68	841
IgG DMSO:All Events	10,411	***	***	100.00	810
IgG DMSO:Live Cells	10,000	96.05	***	96.05	778
IgG DNCB 1:All Events	12,462	***	***	100.00	1,127
IgG DNCB 1:Live Cells	10,000	80.24	***	80.24	986
IgG DNCB 2:All Events	12,905	***	***	100.00	1,155
IgG DNCB 2:Live Cells	9,904	76.75	***	76.75	996
IgG DNCB 3:All Events	23,879	***	***	100.00	1,945
IgG DNCB 3:Live Cells	10,262	42.97	***	42.97	1,160
IgG MBANF 15:All Events	10,700	***	***	100.00	960
IgG MBANF 15:Live Cells	10,000	93.46	***	93.46	903
IgG MBANF 16:All Events	10,732	***	***	100.00	1,002
IgG MBANF 16:Live Cells	10,000	93.18	***	93.18	936
IgG MBANF 17:All Events	11,191	***	***	100.00	1,090
IgG MBANF 17:Live Cells	10,000	89.36	***	89.36	988
IgG MBANF 18:All Events	11,738	***	***	100.00	1,162
IgG MBANF 18:Live Cells	10,000	85.19	***	85.19	1,034
IgG MBANF 19:All Events	12,543	***	***	100.00	1,337
IgG MBANF 19:Live Cells	9,688	77.24	***	77.24	1,134
IgG MBANF 20:All Events	17,320	***	***	100.00	1,576
IgG MBANF 20:Live Cells	10,000	57.74	***	57.74	1,240
IgG MBANF 21:All Events	29,100	***	***	100.00	1,926
IgG MBANF 21:Live Cells	10,000	34.36	***	34.36	1,524
IgG MBANF 22:All Events	30,000	***	***	100.00	1,978
IgG MBANF 22:Live Cells	7,081	23.60	***	23.60	1,698
IgG MBANF 23 Did not assay:All Events	***	***	***	***	***
IgG MBANF 23 Did not assay:Live Cells	***	***	***	***	***
IgG MBANF 24 did not assay:All Events	***	***	***	***	***
IgG MBANF 24 did not assay:Live Cells	***	***	***	***	***
CD86 Media:All Events	10,691	***	***	100.00	2,669
CD86 Media:Live Cells	10,016	93.69	***	93.69	2,437
CD54 Media:All Events	10,596	***	***	100.00	1,055
CD54 Media:Live Cells	10,007	94.44	***	94.44	1,013
CD86 DMSO:All Events	10,571	***	***	100.00	2,678
CD86 DMSO:Live Cells	10,000	94.60	***	94.60	2,504
CD54 DMSO:All Events	10,473	***	***	100.00	1,064
CD54 DMSO:Live Cells	10,000	95.48	***	95.48	1,021
CD86 DNCB 1 :All Events	12,873	***	***	100.00	8,925
CD86 DNCB 1 :Live Cells	10,003	77.71	***	77.71	7,091
CD54 DNCB 1:All Events	12,835	***	***	100.00	2,138
CD54 DNCB 1:Live Cells	10,000	77.91	***	77.91	2,056
CD86 DNCB 2:All Events	13,804	***	***	100.00	8,154
CD86 DNCB 2:Live Cells	9,988	72.36	***	72.36	5,513
CD54 DNCB 2:All Events	13,869	***	***	100.00	2,550
CD54 DNCB 2:Live Cells	9,990	72.03	***	72.03	2,696
CD86 DNCB 3:All Events	23,484	***	***	100.00	21,976
CD86 DNCB 3:Live Cells	8,724	37.15	***	37.15	1,461
CD54 DNCB 3:All Events	20,585	***	***	100.00	2,230
CD54 DNCB 3:Live Cells	7,003	34.02	***	34.02	1,176
CD86 MBANF 15:All Events	11,028	***	***	100.00	3,862
CD86 MBANF 15:Live Cells	9,973	90.43	***	90.43	3,400
CD54 MBANF 15:All Events	10,835	***	***	100.00	1,298
CD54 MBANF 15:Live Cells	10,000	92.29	***	92.29	1,214
CD86 MBANF 16:All Events	10,857	***	***	100.00	3,901
CD86 MBANF 16:Live Cells	10,000	92.11	***	92.11	3,531
CD54 MBANF 16:All Events	10,852	***	***	100.00	1,318
CD54 MBANF 16:Live Cells	10,000	92.15	***	92.15	1,227
CD86 MBANF 17:All Events	11,331	***	***	100.00	5,214
CD86 MBANF 17:Live Cells	10,000	88.25	***	88.25	4,453
CD54 MBANF 17:All Events	11,307	***	***	100.00	1,445
CD54 MBANF 17:Live Cells	10,000	88.44	***	88.44	1,329
CD86 MBANF 18:All Events	11,943	***	***	100.00	6,661
CD86 MBANF 18:Live Cells	10,000	83.73	***	83.73	5,454
CD54 MBANF 18:All Events	12,064	***	***	100.00	1,556
CD54 MBANF 18:Live Cells	10,000	82.89	***	82.89	1,413
CD86 MBANF 19:All Events	13,417	***	***	100.00	8,051
CD86 MBANF 19:Live Cells	10,000	74.53	***	74.53	6,724
CD54 MBANF 19:All Events	13,578	***	***	100.00	1,713
CD54 MBANF 19:Live Cells	10,000	73.65	***	73.65	1,529
CD86 MBANF 20:All Events	18,032	***	***	100.00	10,044
CD86 MBANF 20:Live Cells	9,760	54.13	***	54.13	7,714
CD54 MBANF 20:All Events	20,645	***	***	100.00	1,992
CD54 MBANF 20:Live Cells	10,000	48.44	***	48.44	1,685
CD86 MBANF 21:All Events	30,000	***	***	100.00	10,130
CD86 MBANF 21:Live Cells	9,401	31.34	***	31.34	6,874
CD54 MBANF 21:All Events	30,000	***	***	100.00	2,068
CD54 MBANF 21:Live Cells	8,988	29.96	***	29.96	1,891
CD86 MBANF 22:All Events	30,000	***	***	100.00	10,491
CD86 MBANF 22:Live Cells	5,344	17.81	***	17.81	4,547
CD54 MBANF 22:All Events	30,014	***	***	100.00	2,130
CD54 MBANF 22:Live Cells	4,985	16.61	***	16.61	1,991

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Table E-6. h-CLAT Experiment 4 Data Analysis

	Stock Concentration (mg/mL)	Concentration (mg/mL)	Viability (IgG)	FITC IgG	FITC CD86	RFI	% change	EC150	FITC CD54	RFI	% change	EC200
Media		0	94.68	841	2437	1	100		1013	1	100	
DMSO		0	96.05	778	2504	1.08	108		1021	1.41	141	
DNCB Control	1.67	0.0033	80.24	986	7091	4.89	489		2056	9.30	930	
	2	0.004	76.75	996	5513	3.62	363		2696	14.78	1478	
	2.4	0.0048	42.97	1160	1461	0.24	24.1		1176	0.14	14.0	
MBANF 3/4/2020	0.449	0.00090	93.46	903	3400	1.53	153		1214	1.52	152	
	0.539	0.00108	93.18	936	3531	1.59	159		1227	1.42	142	
	0.647	0.00129	89.36	988	4453	2.13	213	0.0010	1329	1.66	166	
	0.776	0.00155	85.19	1034	5454	2.71	271		1413	1.85	185	
	0.932	0.00186	77.24	1134	6724	3.43	343		1529	1.93	193	
	1.12	0.00224	57.74	1240	7714	3.97	397		1685	2.17	217	0.0020
	1.34	0.00268	34.36	1524	6874	3.28	328		1891	1.79	179	
	1.61	0.00322	23.6	1698	4547	1.75	175		1991	1.43	143	

Table E-7. Acute Oral Hazard Estimation Example

Test #3				**Test #4**			
Concentration (ug/mL)	**Log Conc**	**Viability**	**1000-viability**	**Concentration (ug/mL)**	**Log Conc**	**Viability**	**1000-viability**
0.96	-0.01771	94.09	905.91	0.90	-0.04641	93.46	906.54
1.15	0.061471	92.96	907.04	1.08	0.032768	93.18	906.82
1.38	0.140652	87.82	912.18	1.29	0.11195	89.36	910.64
1.66	0.219833	79.4	920.6	1.55	0.191131	85.19	914.81
1.99	0.299015	61.92	938.08	1.86	0.270312	77.24	922.76
2.39	0.378196	46.36	953.64	2.24	0.349493	57.74	942.26
2.87	0.457377	16.93	983.07	2.68	0.428675	34.36	965.64
3.44	0.536558	5.17	994.83	3.22	0.507856	23.6	976.4
	Log Concentration	Viability	Desired LD		Log Concentration	Viability	Desired LD
>50%	0.299015	61.92	50	>50%	0.349493	57.74	50
<50%	0.378196	46.36		<50%	0.428675	34.36	
	Slope =	-196.511			Slope =	-295.272	
	Intercept	120.6797			Intercept	160.9356	
X	0.359673			X	0.375707		
IC50	2.289143			IC50	2.375235		
LOG LD50 (mg/kg)	2.157798			LOG LD50 (mg/kg)	2.163763		
LD50 (mg/kg)	143.8			LD50 (mg/kg)	145.8		