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Toxicology Study No. S.0024589d-15, April 2016
Toxicology Directorate

Human Cell Line Activation Test of the Novel Energetic 3,4-Dinitropyrazole

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PHC FORM 432-E (MCHB-CS-IP), NOV12

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ARIMS designation: 500c

ACKNOWLEDGEMENTS

I would like to acknowledge the support and encouragement provided to this effort by Mr. Erik Hangeland, Director, Environmental Acquisition and Logistics Program (EALSP), RDECOM, and John LaScala, Ph.D. Co-chair, EQT Pollution Prevention Technology Team (P2TT). I also acknowledge Dr. William Eck for his critical review and comments on this report.

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REPORT DOCUMENTATION PAGE					Form Approved OMB No. 0704-0188							
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1. REPORT DATE (DD-MM-YYYY) 17-05-2016		2. REPORT TYPE Technical Report			3. DATES COVERED (From - To) March 2016-April 2016							
4. TITLE AND SUBTITLE Toxicology Study No. S.0024589d-15 Human Cell Line Activation Test of the Novel Energetic 3,4-Dinitropyrazole				5a. CONTRACT NUMBER								
				5b. GRANT NUMBER								
				5c. PROGRAM ELEMENT NUMBER								
6. AUTHOR(S) Emily N. Reinke, Ph.D.				5d. PROJECT NUMBER								
				5e. TASK NUMBER								
				5f. WORK UNIT NUMBER								
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Army Public Health Center (Provisional), Toxicology Directorate 5158 Blackhawk Road, Aberdeen Proving Ground, MD 21010-5403					8. PERFORMING ORGANIZATION REPORT NUMBER S.0024589d-15							
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research, Development and Engineering Command Environmental Acquisition and Logistics Sustainment Program 3072 Aberdeen Blvd., Aberdeen Proving Ground, MD 21005					10. SPONSOR/MONITOR'S ACRONYM(S) RDECOM							
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)							
12. DISTRIBUTION/AVAILABILITY STATEMENT DISTRIBUTION A: Approved for public release; distribution is unlimited.												
13. SUPPLEMENTARY NOTES												
14. ABSTRACT The novel energetic 3,4-dinitropyrazole (DNP) is under consideration as a replacement for trinitrotoluene (TNT) in explosive formulations. The toxicological properties of DNP are under evaluation as part of this effort. This study assessed the skin sensitization potential of DNP through the human cell line activation test (h-CLAT), an in vitro approach to assess dendritic cell activation by chemicals, a critical step in the elicitation of a skin allergy. 3,4-DNP was found to be a sensitizer.												
15. SUBJECT TERMS 3,4-dinitropyrazole, skin sensitization, human cell line activation test, in vitro												
16. SECURITY CLASSIFICATION OF: <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 33%; padding: 2px;">a. REPORT</td> <td style="width: 33%; padding: 2px;">b. ABSTRACT</td> <td style="width: 33%; padding: 2px;">c. THIS PAGE</td> </tr> <tr> <td style="text-align: center; padding: 5px;">U</td> <td style="text-align: center; padding: 5px;">U</td> <td style="text-align: center; padding: 5px;">U</td> </tr> </table>			a. REPORT	b. ABSTRACT	c. THIS PAGE	U	U	U	17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 26	19a. NAME OF RESPONSIBLE PERSON Emily N. Reinke	
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					19b. TELEPHONE NUMBER(Include area code) 410-436-3980							

Sponsor

U.S. Army Research, Development and Engineering Command,
Environmental Acquisition and Logistics Sustainment Program
Aberdeen Proving Ground, MD 21005-5201

Study Title

Toxicology Study No. S.0024589d-15
Human Cell Line Activation Test of the Novel Energetic 3,4-Dinitropyrazole

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Study Completed

April 2016

Performing Laboratory

Army Public Health Center (Provisional)
Toxicology Directorate
Health Effects Research Program
MCHB-IP-THE
Aberdeen Proving Ground, MD 21010-5403

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Toxicology Study No. S.0024589d-15
Human Cell Line Activation (h-CLAT) Assay
of the Novel Energetic 3,4-Dinitropyrazole (DNP)

1 Summary

1.1 Overview

The novel energetic 3,4-dinitropyrazole (DNP) is under consideration as a replacement for trinitrotoluene (TNT) in explosive formulations. The toxicological properties of DNP are under evaluation as part of this effort. The following study assessed the skin sensitization potential of DNP through the human cell line activation test (h-CLAT), an *in vitro* approach to assess activation of dendritic cells, a critical step in the elicitation of a sensitizing response. DNP has already been assessed by the direct peptide reactivity assay (DPRA) and was found to be positive according to test criteria.

1.2 Purpose

The following study was initiated in order to provide environmental and occupational health information on new or replacement energetic compounds developed for military use. The information garnered by this and other studies is necessary for the research, development, testing and evaluation of alternative munition formulations. This program is under the direction of the U.S. Army Research, Development and Engineering Command (USARDEC) Environmental Acquisition Logistics & Sustainment Program (EALSP) and Environmental Quality Technology (EQT) Pollution Prevention pillar. The purpose of this study is to fill existing toxicity data gaps pertaining to human exposure to this compound.

1.3 Conclusions

DNP was found to elicit a positive reaction 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 DNP. Thus, DNP is a sensitizer according to the h-CLAT test.

1.4 Recommendations

DNP appears to be a skin sensitizer upon analysis of the two currently available skin sensitizing tests at the Army Public Health Center (Provisional) (APHC (Prov)) when combined with QSAR analysis and personal observation with the compound developers [1, 2]. Further *in vitro* or *in vivo* testing is recommended to more definitively determine DNP's sensitizing potential. The h-CLAT is one of many non-animal skin sensitizing tests, and it comprises part of an integrated testing strategy with two other *in vitro* approaches, the DPRA and the KeratinoSens assay [3-7]. A comprehensive assessment of skin sensitization potential requires results from all three assays along with specific *in silico* analysis provides a more robust estimation of skin sensitization than h-CLAT alone [8]. As testing has only occurred with DPRA and h-CLAT, it is not yet possible to provide a definitive response as to the sensitization potential. The third test is currently under validation by APHC (Prov) and should be available to complete the *in vitro* tests necessary for analysis. However these test results, when considered along with anecdotal evidence of increased incidence of sensitization to DNP (as reported by researchers at the U.S. Army Aviation and Missile Research Development and Engineering Center) would indicate DNP is a sensitizer.

2 References

See Appendix A for list of references

3 Authority

Military Interdepartmental Purchase Request No. 10688668. This technical report addresses, in part, the environment, safety and occupational health (ESOH) requirements outlined in Department of Defense Instruction (DODI) 4715.4 [9], Department of the Army Regulation (AR) 200-1, Environmental Protection and Enhancement[10]; AR 40-5, Preventive Medicine [11]; and AR 70-1, Army Acquisition Policy [12]; Department of Defense Instruction 4715.4, Pollution Prevention [9]; and Army Environmental Research and Technology Assessment Requirement PP-3-02-05, Compliant Ordnance Lifecycle for Readiness of the Transformation and Objective Forces . It was conducted as part of an on-going effort by the U.S. Army Research, Development and Engineering Command (RDECOM), Environmental Acquisition and Logistics Sustainment Program (EALSP, Mr. Erik Hangeland) and the Environmental Quality Technology (EQT) Pollution Prevention Team (P2TT), chaired by Dr. John LaScala.

4 Background

Historically, the development of novel munitions was primarily based on the efficacy of the compound to perform the mission, with secondary consideration given to the human health and environmental impacts of the munition. Trinitrotoluene (TNT) is a commonly used explosive with well documented negative health effects, such as ataxia, tremors, mild convulsions, blood and liver toxicity, as documented via toxicity studies in rats and dogs [13]. In humans, prolonged exposure to TNT has resulted in anemia and abnormal liver function. TNT is currently listed as a possible human carcinogen by the U.S. Environmental Protection Agency (USEPA) [14]. Additionally, TNT and its breakdown products have been found to contaminate surface and groundwater at munitions testing grounds. Due to the potential health and environmental impacts of TNT, there is a concerted effort to replace TNT with new munitions compounds which are both efficacious and minimize negative health effects of exposure.

DNP (Chemical Abstract Number (CASRN) 38858-92-3) is a novel energetic under evaluation as a replacement for TNT. Few toxicity data on the compound exist, however, Quantitative-Structure Activity Relationship (QSAR) analysis indicates that DNP may be a strong sensitizer [15]. The Toxicology Directorate of the Army Public Health Center (Provisional) has been tasked with evaluating the skin sensitization potential of DNP. Testing in the h-CLAT *in vitro* system constitutes the second evaluation of DNP using *in vitro* skin sensitization methods; multiple test systems are required to confirm results.

The h-CLAT is an *in vitro* approach to analyze dendritic cell activation of test chemicals via the expression of CD54 and CD86 on the cell surface. There are several key steps in the elicitation of a skin sensitizing reaction, including the activation of dendritic cells and the transformation from antigen processing to antigen presenting cells [16]. Multiple cell surface markers are expressed by dendritic cells, with CD54 and CD86 being just two examples. The increase in expression on the cellular surface of these proteins is measured by flow cytometry as a result of a fluorescent signal on the antibodies which bind to either CD54 or CD86 [17-19]. The criteria for a positive reaction in h-CLAT require either a 2-fold or a 1.5-fold induction of CD54 or CD86 respectively as compared to solvent controls. During a skin sensitizing reaction, activated dendritic cells migrate to the lymph

node where the major histocompatibility complexes which they are presenting activated T-cells and T-cell proliferation. Secondary exposure to the chemical will then result in inflammation and an allergic reaction.

5 Materials and Methods

5.1 Materials

5.1.1 Test Substance

Synthesis of DNP (CASRN: 38858-92-3) was completed by the Holston Army Ammunition Plant, Kingston, TN, batch number 1120-114R with a purity of 99.83 percent. The manufacturer expressed some concern about the level of residual sulfates (0.998 percent), but the levels would have no effect on the h-CLAT. The molecular structure of the compound is shown in Figure 1.

DNP was fully soluble in saline at a concentration of 100 mg/mL, the starting concentration for determining appropriate dosing levels for the assay.

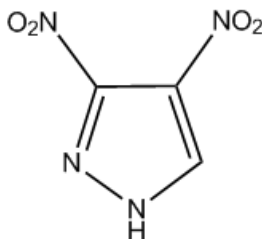


Figure 1. 3,4-Dinitropyrazole (DNP)

5.1.2 Cell Line, Chemicals and Reagents

The h-CLAT has undergone validation testing within the APHC (Prov) to verify that the assay performs as expected with APHC (Prov) equipment when compared to published results. THP-1 cells were acquired from the American Type Tissue Collection (ATCC, Manassas, VA). All tissue culture reagents were acquired from Gibco, a subsidiary of ThermoFisher (Waltham, MA). Cells were cultured in RPMI-1640 containing 10 percent fetal bovine serum, 100 U/mL penicillin, 10 µg/mL streptomycin and 0.05 mM 2-mercaptoethanol. Reagents for flow cytometry were as follows: physiological saline (Sigma-Aldrich, Inc., St. Louis, MO), dimethyl sulfoxide (DMSO, Sigma-Aldrich, Inc.), Dulbecco's phosphate buffered saline without calcium, magnesium or phenol red (Gibco, Inc.), bovine serum albumin fraction V (BSA, Calbiochem, Billerica, MA), globulins Cohn fraction II, II, human (Sigma-Aldrich, Inc.), and propidium iodide (PI, Sigma-Aldrich, Inc.). Control test chemicals were all obtained from Sigma-Aldrich, Inc., to include 2,4-dinitrochlorobenzene (DNCB, CASRN 97-00-7), nickel sulfate (NiSO₄, CASRN 7786-81-4), and lactic acid (LA, CASRN 50-21-5). Antibodies against IgG1 (control) and CD54 were obtained from Dako

(Carpinteria, CA) and antibodies against CD86 (Clone 2331, Fun-1) were obtained from BD Biosciences (San Jose, CA). All antibodies were tagged with the FITC fluorophore. All cells, reagents and chemicals were stored according to manufacturer's instructions.

5.1.3 Equipment

The assay reaction was analyzed by flow cytometry utilizing a BD FACSVerser flow cytometer (BD Biosciences).

5.2 Methods

All assay setup was performed according to ECVAM DB-ALM protocol number 158, OECD Guideline [18, 19].

5.2.1 Buffers

FACS buffer was prepared with PBS and 0.1 percent (w/v) BSA the day before use and stored at +4 °C. Blocking solution was made up in 1 percent (w/v) globulins in PBS stocks as needed, with stock being used within one week and stored at +4 °C. Blocking solution for use on the day of the experiment was diluted to a 0.1 percent solution in FACS buffer immediately prior to use. PI was diluted to 12.5 µg/mL in PBS on the day of the experiment and maintained on ice.

5.2.2 Tissue Culture

Tissue culture media was prepared as described in section 5.1.2 and maintained at +4 °C. Media was pre-warmed 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 percent CO₂. Cells for the assay had not been in culture for 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, CA). Cell viability was determined by Trypan blue staining (Bio-Rad, Inc.). For range finding and h-CLAT testing, cells were plated into 24-well plates at a density of 1×10^6 cells/well in 0.5 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.

5.2.3 Reactivity Check

Two weeks after cells were thawed, a reactivity check on the batch is carried out utilizing DNCB, NiSO₄ and LA. DNCB was prepared in 20 mg/mL stock solutions in DMSO and diluted to 2.4 mg/mL in DMSO. Stock solutions of DNCB were maintained at +4 °C. Serial dilutions of 1:1.2 were carried out for a total of 2 dosing levels and subsequently diluted 1:250 in 0.5 mL media. NiSO₄ was prepared in a 10 mg/mL solution in saline and diluted 1:50 into 0.5 mL media and 1:34.5 into 0.5 mL media. LA was prepared as a 100 mg/mL solution in saline and diluted 1:50 and 1:34.5 into 0.5 mL media. One 1:1.2 dilution was made. DNCB, NiSO₄ and LA were then diluted 1:2 into the 0.5 mL containing 1×10^6 cells. A dead cell well was prepared by diluting 10 µL of 20 mg/mL DNCB (final concentration 0.2 mg/mL) into 1 mL of media containing 1×10^6 cells in a 24-well plate. DMSO and saline control wells were also prepared. The plate was incubated for 24 hours and cells and were processed and stained for IgG1, CD54 and CD86 and analyzed by flow cytometry (section 5.2.6).

5.2.4 Range finding

In order to determine appropriate dosing levels, DNP was prepared in a 100 mg/mL stock solution in saline and 1:2 serial dilutions were prepared in saline for a total of 8 dilutions. Each dilution was further diluted 1:50 into 0.5 mL media and diluted 1:2 into 0.5 mL media containing 1×10^6 cells in a 24-well plate. As described in 5.2.3, a saline control and dead cell control were prepared. Cells were incubated for 24 hours. Following incubation, each sample was transferred to a 5 mL tube and spun down at $200 \times g$ at $+4^\circ\text{C}$. Pellets were resuspended in 0.6 mL cold FACS buffer and 0.2 mL transferred to flow cytometry sample tubes. Samples were washed twice in 0.2 mL FACS buffer, resuspended in 0.4 mL FACS buffer and stained with 20 μL of the 12.5 $\mu\text{g/mL}$ PI stock (final concentration 0.625 $\mu\text{g/mL}$). Samples were maintained on ice in the dark and assayed for viability by flow cytometry. The dead cell control and the saline control were used to gate out dead cells stained with PI and the flow cytometer was set to acquire 10,000 live cell hits (PI negative) or 30,000 total hits, whichever was achieved first. Percent viability (ratio of live cells to total acquired cells) was utilized to determine the 75 percent cell viability (CV75) by the following equation (see also Figure 2):

$$\log \text{CV75} = \frac{(75-c) \times \log b - (75-a) \times \log d}{a-c}$$

where:

a = Percent viability above 75 percent (nearest dose)

b = Dose level of a

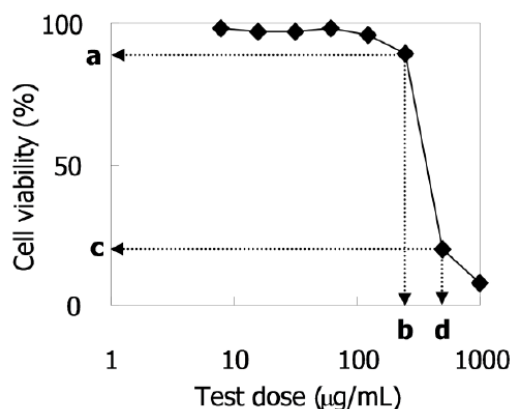
c = Percent viability below 75 percent (nearest dose)

d = Dose level of c

See Figure 1.

The CV75 is the value at which the second highest dose is set for the final test.

Figure 2- Example results range finding PI assay*



*ECVAM DB-ALM, *human Cell Line Activation Test (h-CLAT)*, DB-ALM Protocol No. 158. 2015: European Union Reference Laboratory for Alternatives to Animal Testing [18, 19].

The range finding assay was completed a minimum of two times to verify results, if results were similar after two tests, no more testing was completed.

5.2.5 h-CLAT Test

Once the CV75 was determined, a dosing scheme was setup such that the highest dose was 1.2-fold higher than the CV75. DNP was weighed and solubilized in saline at a concentration 100x of the 1.2 x CV75. The solution was then diluted in a 1.2 serial dilution for a total of 8 concentration levels and each concentration diluted 1:50 in 0.5 mL complete media. This 0.5 mL was then diluted 1:2 into 0.5 mL containing 1×10^6 cells in a 24-well plate. DNCB was prepared from the 20 mg/mL stock by diluting to 2.4 mg/mL in DMSO, serially diluting 1:1.2 for 3 dilutions and then diluting a further 250x into media. These were also diluted 1:2 into 0.5 mL media containing 1×10^6 cells. A saline and DMSO control were prepared as was a “dead cell” control containing 10 μ L 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 (section 5.2.6).

5.2.6 Antibody Staining and Flow Cytometry

Each well was transferred containing cells and treatment or treatment control was transferred to a 5 mL snap-cap tube and collected by centrifugation ($250 \times g$, 5 min, $+4^\circ\text{C}$) and washed twice in 1 mL cold FACS buffer. Cells were blocked in 0.6 mL 0.1 percent blocking buffer (prepared from the 1 percent stock in FACS buffer) for 15 min. at $+4^\circ\text{C}$. Following blocking, each sample was split into 3 aliquots of 180-200 μ L each in a round-bottom 96-well plate. Samples were spun as above and stained with antibodies. See Table 1 for antibody concentrations.

Table 1 – Antibody concentration

	Volume of antibody	Volume of FACS buffer	Total volume of working solution/sample
FITC labeled-mouse IgG1	6 μ L	44 μ L	50 μ L
Anti-CD54 antibody	3 μ L	47 μ L	50 μ L
Anti-CD86 antibody	3 μ L	47 μ L	50 μ L

A master-mix for each antibody was prepared immediately prior to use and added directly to each cell pellet after removal of the blocking buffer. Each plate was gently vortexed to resuspend the cells in the antibody mix and incubated at $+4^\circ\text{C}$ in the dark for 30 min. Following the 30 minute incubation, samples were again spun down and washed 2x in FACS buffer. Between the first and second wash, samples were transferred to FACS analysis tubes. Samples were maintained on ice throughout the transfer process. Following the final wash, samples were resuspended in 0.4 mL FACS buffer and stained with 20 μ L PI. Each sample was gently vortex to mix.

Samples were analyzed by flow cytometry under the following conditions. Acquisition channels should be set to read propidium iodide (PI) and fluorescein isothiocyanate (FITC). The following plots were captured for each sample: 2-dimensional plot of forward and side scatter, 2-dimensional dot plot of FITC vs PI and a histogram plot of both FITC and PI. Live cells were used to determine the correct voltages for the forward scatter and side scatter channels. Dead cells were gated out by PI using the dead cell control and the IgG1 saline control and total acquisition was determined by either 10,000 PI negative hits or 30,000 total hits on the PI channel. For each sample, the geometric mean fluorescence intensity (MFI) was captured for all hits and live/viable cell hits.

From the MFI, the relative fluorescence intensity (RFI) was determined by the following equation:

$$RFI = \frac{\text{MFI of chemical treated cells} - \text{MFI of chemical treated isotype cells}}{\text{MFI of solvent treated cells} - \text{MFI of solvent treated isotype cells}}$$

The cell viability for each concentration was also recorded from the isotype control population.

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

- Cell viability of medium and DMSO controls was more than 90 percent.
- RFI values for the DNCB control for both CD54 and CD86 exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 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 percent.

5.2.7 Data Analysis

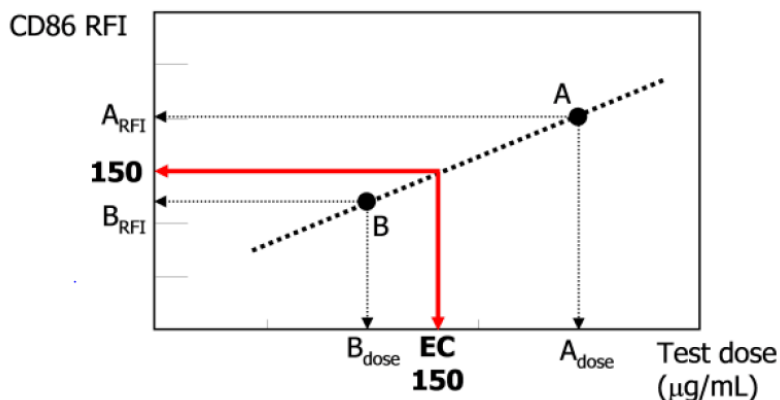
If the RFI for any concentration exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150), the EC200 and EC150 were calculated by the following equation:

$$EC_{200} (CD54) = B_{dose} + [(200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$

$$EC_{150} (CD86) = B_{dose} + [(150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times (A_{dose} - B_{dose})]$$

Where A_{dose} , B_{dose} , A_{RFI} and B_{RFI} were determined from the following chart (Figure 3):

Figure 3- Example dose response curve for CD86*



*ECVAM DB-ALM, *human Cell Line Activation Test (h-CLAT)*, DB-ALM Protocol No. 158. 2015: European Union Reference Laboratory for Alternatives to Animal Testing [18, 19].

If the EC200 or EC150 fell below the lowest dose, the values were extrapolated by the following equations.

$$EC_{200} (CD54) = 2 \exp[\log_2(B_{dose}) + (200 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times [\log_2(A_{dose}) - \log_2(B_{dose})]]$$

$$EC_{150} (CD86) = 2 \exp[\log_2(B_{dose}) + (150 - B_{RFI}) / (A_{RFI} - B_{RFI}) \times [\log_2(A_{dose}) - \log_2(B_{dose})]]$$

Three independent runs were completed for DNP.

6 Results and Discussion

6.1 Reactivity Check

The THP-1 cells were checked and verified for reactivity to DNCB, 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 in both (Table 2). The cells met criteria for further testing.

Table 2: Results of Reactivity Check

Test article	Concentration (mg/mL)	Viability	RFI (CD86)	RFI (CD54)	Positive (CD86/CD54)
Saline		90.12	100	100	N/N
DMSO		91.1	100	100	N/N
DNCB	0.0033	80.5	402.2	331.4	Y/Y
	0.0040	69.15	191.8	571.7	Y/Y
	0.0048	70.67	136.7	406.7	N/Y
NiSO ₄	0.10	60.25	280.1	4497.7	Y/Y
	0.14	60.66	192.1	4768.2	Y/Y
Lactic Acid	1	90.93	82.9	135.8	N/N
	1.4	91.67	70.4	106.9	N/N

6.2 Range finding Assay

Two independent dose finding assays were completed in order to determine the CD75 of DNP in THP-1 cells. The average CV75 between the two assays was 0.278 mg/mL DNP (Table 3).

Table 3: Results of Range finding Assays

	CV75 (mg/mL)	Average (mg/mL)
Assay 1	0.259	0.278
Assay 2	0.299	

6.3 CD54 and CD86 expression in response to DNP exposure of THP-1 cells

Three independent tests were completed for DNP for both CD54 and CD86. Due to lower than anticipated viability of the cells in the first two runs, despite determining a CD75 of 0.278 mg/mL, the third run had an expanded range of concentrations in order to attempt to capture a lower dosage at which the cells were not responding to DNP. In all three runs, CD54 was positive and in two of the three runs CD86 was positive (Table 4). At the higher dosing levels, the RFI did not exceed positive criteria, but this is commonly seen when cell viability is lower, even in the positive controls. At lower viability levels, there is a diffuse labeling of cytoplasmic structures which affects the background levels of stain and negates a positive response. The EC200 range for CD54 was 0.07 – 0.09 mg/mL and the EC150 range for CD86 was 0.084-0.094 mg/mL.

Table 4: Results of DNP Analysis

Compound	CD86 EC150	CD54 EC200	Positive Control (CD86/CD54)	Positive Test?
DNP	N/A	Cannot Extrapolate*	Y/Y	Yes
	0.085	0.069	Y/Y	Yes
	0.094	0.095	Y/Y	Yes

*The EC200 could not be extrapolated due to the RFI decreasing with each increase in concentration. The test is still considered positive but the EC200 cannot be determined.

6.4 Criteria for Valid Assay

All criteria were met for all the assays.

7 Conclusions

As determined by h-CLAT, DNP is considered positive by the test criteria. QSAR analysis by TOPKAT (BIOVIA, Inc.) predicted that DNP was potentially a strong sensitizer. These data combined with the previously recorded positive by DPRA indicate that DNP is most likely a skin sensitizer, but further testing and analysis is necessary.

8 Recommendations

DNP appears to be a skin sensitizer upon analysis of the two currently available skin sensitizing tests at APHC (Prov) when combined with QSAR analysis and personal observation with the compound developers [1, 2]. Further *in vitro* or *in vivo* testing is recommended to more definitively determine DNP's sensitizing potential. The h-CLAT is one of many non-animal skin sensitizing tests, and it comprises part of an integrated testing strategy with two other *in vitro* approaches, the DPRA and the KeratinoSens assay [3-7]. A comprehensive assessment of skin sensitization potential requires results from all three assays along with specific *in silico* analysis provides a more robust estimation of skin sensitization than h-CLAT alone [8]. As testing has only occurred with DPRA and h-CLAT, it is not yet possible to provide a definitive response as to the sensitization potential. The third test is currently under validation by APHC (Prov) and should be available to complete the *in vitro* tests necessary for analysis. However these test results, when considered along with anecdotal evidence of increased incidence of sensitization to DNP (as reported by researchers at the U.S. Army Aviation and Missile Research Development and Engineering Center), indicate that DNP is a sensitizer.

9 Point of Contact

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Toxicology Study No. S.0024589d-15, April 2016

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Appendix A

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Appendix B

Raw Data

Experiment 1*

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
IgG Saline:All Events	10,811	***	***	100.00	1,037
IgG Saline:Live Cells	10,008	92.57	***	92.57	983
IgG DMSO:All Events	10,589	***	***	100.00	983
IgG DMSO:Live Cells	10,000	94.44	***	94.44	927
IgG DNCB 1:All Events	12,798	***	***	100.00	1,459
IgG DNCB 1:Live Cells	9,998	78.12	***	78.12	1,317
IgG DNCB 2:All Events	13,474	***	***	100.00	1,822
IgG DNCB 2:Live Cells	10,000	74.22	***	74.22	1,738
IgG DNCB 3:All Events	13,070	***	***	100.00	1,871
IgG DNCB 3:Live Cells	9,857	75.42	***	75.42	1,728
IgG DNP 1:All Events	13,878	***	***	100.00	1,880
IgG DNP 1:Live Cells	9,725	70.07	***	70.07	1,697
IgG DNP 2:All Events	13,761	***	***	100.00	1,736
IgG DNP 2:Live Cells	9,806	71.26	***	71.26	1,528
IgG DNP 3:All Events	14,229	***	***	100.00	1,815
IgG DNP 3:Live Cells	9,858	69.28	***	69.28	1,658
IgG DNP 4:All Events	15,855	***	***	100.00	1,904
IgG DNP 4:Live Cells	10,000	63.07	***	63.07	1,662
IgG DNP 5:All Events	17,523	***	***	100.00	1,689
IgG DNP 5:Live Cells	9,737	55.57	***	55.57	1,472
IgG DNP 6:All Events	16,863	***	***	100.00	1,611
IgG DNP 6:Live Cells	9,848	58.40	***	58.40	1,376
IgG DNP 7:All Events	16,295	***	***	100.00	1,500
IgG DNP 7:Live Cells	9,747	59.82	***	59.82	1,230
IgG DNP 8:All Events	16,727	***	***	100.00	1,530
IgG DNP 8:Live Cells	10,001	59.79	***	59.79	1,237
CD54 Saline:All Events	10,816	***	***	100.00	1,183
CD54 Saline:Live Cells	9,999	92.45	***	92.45	1,102
CD86 Saline:All Events	10,806	***	***	100.00	3,143
CD86 Saline:Live Cells	10,000	92.54	***	92.54	2,861
CD54 DMSO:All Events	10,643	***	***	100.00	1,240
CD54 DMSO:Live Cells	10,041	94.34	***	94.34	1,166
CD86 DMSO:All Events	10,608	***	***	100.00	3,075
CD86 DMSO:Live Cells	10,000	94.27	***	94.27	2,830
CD54 DNCB 1:All Events	12,443	***	***	100.00	2,103
CD54 DNCB 1:Live Cells	9,679	77.79	***	77.79	1,997
CD86 DNCB 1:All Events	12,753	***	***	100.00	8,156
CD86 DNCB 1:Live Cells	10,000	78.41	***	78.41	7,071
CD54 DNCB 2:All Events	12,945	***	***	100.00	2,226
CD54 DNCB 2:Live Cells	9,732	75.18	***	75.18	2,031
CD86 DNCB 2:All Events	13,344	***	***	100.00	8,565
CD86 DNCB 2:Live Cells	9,798	73.43	***	73.43	7,376
CD54 DNCB 3:All Events	14,447	***	***	100.00	2,305
CD54 DNCB 3:Live Cells	9,991	69.16	***	69.16	2,228
CD86 DNCB 3:All Events	13,312	***	***	100.00	7,374
CD86 DNCB 3:Live Cells	9,645	72.45	***	72.45	5,994
CD54 DNP 1:All Events	14,400	***	***	100.00	3,104
CD54 DNP 1:Live Cells	10,000	69.44	***	69.44	3,022
CD86 DNP 1:All Events	14,934	***	***	100.00	6,626
CD86 DNP 1:Live Cells	10,000	66.96	***	66.96	4,041
CD54 DNP 2:All Events	14,448	***	***	100.00	2,741
CD54 DNP 2:Live Cells	9,694	67.10	***	67.10	2,679
CD86 DNP 2:All Events	15,426	***	***	100.00	6,176
CD86 DNP 2:Live Cells	10,518	68.18	***	68.18	3,557
CD54 DNP 3:All Events	14,898	***	***	100.00	2,665
CD54 DNP 3:Live Cells	10,000	67.12	***	67.12	2,552
CD86 DNP 3:All Events	14,217	***	***	100.00	5,188
CD86 DNP 3:Live Cells	9,744	68.54	***	68.54	3,070
CD54 DNP 4:All Events	16,291	***	***	100.00	2,380
CD54 DNP 4:Live Cells	10,000	61.38	***	61.38	2,327
CD86 DNP 4:All Events	16,704	***	***	100.00	6,352
CD86 DNP 4:Live Cells	10,001	59.87	***	59.87	3,291
CD54 DNP 5:All Events	19,182	***	***	100.00	2,052
CD54 DNP 5:Live Cells	9,993	52.10	***	52.10	1,684
CD86 DNP 5:All Events	18,748	***	***	100.00	6,609
CD86 DNP 5:Live Cells	10,000	53.34	***	53.34	3,038
CD54 DNP 6:All Events	17,301	***	***	100.00	1,855
CD54 DNP 6:Live Cells	9,687	55.99	***	55.99	1,456
CD86 DNP 6:All Events	18,825	***	***	100.00	5,706
CD86 DNP 6:Live Cells	10,054	53.41	***	53.41	2,637
CD54 DNP 7:All Events	17,171	***	***	100.00	1,919
CD54 DNP 7:Live Cells	9,769	56.89	***	56.89	1,546
CD86 DNP 7:All Events	16,831	***	***	100.00	5,484
CD86 DNP 7:Live Cells	9,654	57.36	***	57.36	2,496
CD54 DNP 8:All Events	17,571	***	***	100.00	1,782
CD54 DNP 8:Live Cells	9,912	56.41	***	56.41	1,339
CD86 DNP 8:All Events	17,086	***	***	100.00	5,475
CD86 DNP 8:Live Cells	9,760	57.12	***	57.12	2,547

*Page 2 of raw data did not contain any data, it was created automatically by the FACSverse software.

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Experiment 2

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
IgG Saline:All Events	11,841	***	***	100.00	1,002
IgG Saline:Live Cells	10,996	92.86	***	92.86	930
IgG DMSO:All Events	10,659	***	***	100.00	922
IgG DMSO:Live Cells	10,031	94.11	***	94.11	846
IgG DNCB 1:All Events	12,531	***	***	100.00	1,333
IgG DNCB 1:Live Cells	10,462	83.49	***	83.49	1,140
IgG DNCB 2:All Events	13,587	***	***	100.00	1,411
IgG DNCB 2:Live Cells	10,469	77.05	***	77.05	1,165
IgG DNCB 3:All Events	14,343	***	***	100.00	1,346
IgG DNCB 3:Live Cells	10,620	74.04	***	74.04	1,129
IgG DNP 1:All Events	14,709	***	***	100.00	1,316
IgG DNP 1:Live Cells	11,052	75.14	***	75.14	1,121
IgG DNP 2:All Events	13,593	***	***	100.00	1,400
IgG DNP 2:Live Cells	9,974	73.38	***	73.38	1,153
IgG DNP 3:All Events	14,176	***	***	100.00	1,485
IgG DNP 3:Live Cells	10,068	71.02	***	71.02	1,189
IgG DNP 4:All Events	14,404	***	***	100.00	1,508
IgG DNP 4:Live Cells	10,075	69.95	***	69.95	1,222
IgG DNP 5:All Events	15,176	***	***	100.00	1,532
IgG DNP 5:Live Cells	10,104	66.58	***	66.58	1,240
IgG DNP 6:All Events	21,468	***	***	100.00	1,552
IgG DNP 6:Live Cells	11,684	54.43	***	54.43	1,199
IgG DNP 7:All Events	20,695	***	***	100.00	1,354
IgG DNP 7:Live Cells	11,580	55.96	***	55.96	1,161
IgG DNP 8:All Events	18,918	***	***	100.00	1,388
IgG DNP 8:Live Cells	10,000	52.86	***	52.86	1,062
CD54 Saline:All Events	10,705	***	***	100.00	1,118
CD54 Saline:Live Cells	9,906	92.54	***	92.54	1,029
CD86 Saline:All Events	10,775	***	***	100.00	2,313
CD86 Saline:Live Cells	9,811	91.05	***	91.05	2,026
CD54 DMSO:All Events	11,000	***	***	100.00	1,115
CD54 DMSO:Live Cells	10,000	90.91	***	90.91	1,017
CD86 DMSO:All Events	11,100	***	***	100.00	2,509
CD86 DMSO:Live Cells	10,000	90.09	***	90.09	2,221
CD54 DNCB 1:All Events	13,251	***	***	100.00	1,918
CD54 DNCB 1:Live Cells	10,014	75.57	***	75.57	1,669
CD86 DNCB 1:All Events	12,312	***	***	100.00	6,546
CD86 DNCB 1:Live Cells	10,000	81.22	***	81.22	5,165
CD54 DNCB 2:All Events	15,060	***	***	100.00	2,308
CD54 DNCB 2:Live Cells	10,048	66.72	***	66.72	2,071
CD86 DNCB 2:All Events	13,357	***	***	100.00	6,569
CD86 DNCB 2:Live Cells	9,572	71.66	***	71.66	4,834
CD54 DNCB 3:All Events	12,883	***	***	100.00	2,212
CD54 DNCB 3:Live Cells	9,736	75.57	***	75.57	1,913
CD86 DNCB 3:All Events	14,574	***	***	100.00	5,928
CD86 DNCB 3:Live Cells	10,009	68.68	***	68.68	4,456
CD54 DNP 1:All Events	14,258	***	***	100.00	2,257
CD54 DNP 1:Live Cells	10,854	76.13	***	76.13	1,961
CD86 DNP 1:All Events	13,894	***	***	100.00	4,986
CD86 DNP 1:Live Cells	9,771	70.33	***	70.33	2,944
CD54 DNP 2:All Events	14,585	***	***	100.00	2,543
CD54 DNP 2:Live Cells	10,000	68.56	***	68.56	2,384
CD86 DNP 2:All Events	13,968	***	***	100.00	5,334
CD86 DNP 2:Live Cells	9,815	70.27	***	70.27	3,369
CD54 DNP 3:All Events	15,263	***	***	100.00	2,999
CD54 DNP 3:Live Cells	10,090	66.11	***	66.11	3,021
CD86 DNP 3:All Events	14,250	***	***	100.00	5,612
CD86 DNP 3:Live Cells	9,681	67.94	***	67.94	3,096
CD54 DNP 4:All Events	15,882	***	***	100.00	3,010
CD54 DNP 4:Live Cells	9,993	62.92	***	62.92	3,007
CD86 DNP 4:All Events	14,912	***	***	100.00	4,916
CD86 DNP 4:Live Cells	10,066	67.50	***	67.50	2,945
CD54 DNP 5:All Events	16,249	***	***	100.00	3,076
CD54 DNP 5:Live Cells	9,737	59.92	***	59.92	3,084
CD86 DNP 5:All Events	16,440	***	***	100.00	4,694
CD86 DNP 5:Live Cells	10,057	61.17	***	61.17	2,745
CD54 DNP 6:All Events	16,779	***	***	100.00	2,537
CD54 DNP 6:Live Cells	9,520	56.74	***	56.74	2,293
CD86 DNP 6:All Events	19,589	***	***	100.00	5,238
CD86 DNP 6:Live Cells	10,285	52.50	***	52.50	2,308
CD54 DNP 7:All Events	18,677	***	***	100.00	1,921
CD54 DNP 7:Live Cells	9,594	51.37	***	51.37	1,677
CD86 DNP 7:All Events	22,569	***	***	100.00	5,739
CD86 DNP 7:Live Cells	10,539	46.70	***	46.70	2,090
CD54 DNP 8:All Events	18,240	***	***	100.00	1,857
CD54 DNP 8:Live Cells	9,731	53.35	***	53.35	1,425
CD86 DNP8:All Events	20,079	***	***	100.00	5,709
CD86 DNP8:Live Cells	9,999	49.80	***	49.80	2,129

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Experiment 3

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
IgG Saline:All Events	11,020	***	***	100.00	930
IgG Saline:Live Cells	10,897	98.88	***	98.88	905
IgG DMSO:All Events	10,570	***	***	100.00	931
IgG DMSO:Live Cells	10,000	94.61	***	94.61	874
IgG DNCB #1:All Events	14,757	***	***	100.00	1,405
IgG DNCB #1:Live Cells	10,000	67.76	***	67.76	1,182
IgG DNCB #2:All Events	15,664	***	***	100.00	1,349
IgG DNCB #2:Live Cells	10,000	63.84	***	63.84	1,099
IgG DNCB #3:All Events	13,437	***	***	100.00	1,332
IgG DNCB #3:Live Cells	9,750	72.56	***	72.56	1,130
IgG DNP 1:All Events	10,880	***	***	100.00	1,052
IgG DNP 1:Live Cells	9,889	90.89	***	90.89	967
IgG DNP 2:All Events	11,115	***	***	100.00	1,058
IgG DNP 2:Live Cells	10,000	89.97	***	89.97	973
IgG DNP 3:All Events	11,536	***	***	100.00	1,115
IgG DNP 3:Live Cells	9,627	83.45	***	83.45	1,011
IgG DNP 4:All Events	11,334	***	***	100.00	1,153
IgG DNP 4:Live Cells	9,841	86.83	***	86.83	1,035
IgG DNP 5:All Events	12,095	***	***	100.00	1,219
IgG DNP 5:Live Cells	9,617	79.51	***	79.51	1,096
IgG DNP 6:All Events	13,963	***	***	100.00	1,272
IgG DNP 6:Live Cells	10,008	71.68	***	71.68	1,108
IgG DNP 7:All Events	12,536	***	***	100.00	1,299
IgG DNP 7:Live Cells	9,753	77.80	***	77.80	1,108
IgG DNP 8:All Events	12,954	***	***	100.00	1,327
IgG DNP 8:Live Cells	9,711	74.97	***	74.97	1,128
IgG DNP 9:All Events	16,050	***	***	100.00	1,403
IgG DNP 9:Live Cells	10,000	62.31	***	62.31	1,106
IgG DNP 10:All Events	16,268	***	***	100.00	1,366
IgG DNP 10:Live Cells	10,009	61.53	***	61.53	1,102
IgG DNP 11:All Events	18,115	***	***	100.00	1,415
IgG DNP 11:Live Cells	10,003	55.22	***	55.22	1,125
IgG DNP 12:All Events	19,728	***	***	100.00	1,375
IgG DNP 12:Live Cells	10,000	50.69	***	50.69	1,059
CD86 Saline:All Events	10,677	***	***	100.00	2,120
CD86 Saline:Live Cells	10,000	93.66	***	93.66	1,902
CD54 Saline:All Events	11,184	***	***	100.00	1,133
CD54 Saline:Live Cells	9,936	88.84	***	88.84	1,025
CD86 DMSO:All Events	11,288	***	***	100.00	2,176
CD86 DMSO:Live Cells	9,783	86.67	***	86.67	1,898
CD54 DMSO:All Events	11,223	***	***	100.00	1,161
CD54 DMSO:Live Cells	10,000	89.10	***	89.10	1,055
CD86 DNCB #2:All Events	13,480	***	***	100.00	6,344
CD86 DNCB #2:Live Cells	9,431	69.96	***	69.96	4,924
CD54 DNCB #2:All Events	16,061	***	***	100.00	2,262
CD54 DNCB #2:Live Cells	10,003	62.28	***	62.28	2,140
CD86 DNP 12:All Events	20,256	***	***	100.00	5,869
CD86 DNP 12:Live Cells	10,000	49.37	***	49.37	1,961
CD54 DNP 12:All Events	18,017	***	***	100.00	1,916
CD54 DNP 12:Live Cells	10,000	55.50	***	55.50	1,502
CD86 DNP 11:All Events	17,072	***	***	100.00	5,035
CD86 DNP 11:Live Cells	9,699	56.81	***	56.81	2,277
Cd54 DNP 11:All Events	18,459	***	***	100.00	2,194
Cd54 DNP 11:Live Cells	10,001	54.18	***	54.18	1,885
CD86 DNP 10:All Events	16,820	***	***	100.00	5,171
CD86 DNP 10:Live Cells	9,981	59.34	***	59.34	2,869
CD54 DNP 10:All Events	16,924	***	***	100.00	2,944
CD54 DNP 10:Live Cells	10,051	59.39	***	59.39	3,063
CD86 DNP 9:All Events	16,609	***	***	100.00	4,922
CD86 DNP 9:Live Cells	10,000	60.21	***	60.21	2,762
CD54 DNP 9:All Events	14,422	***	***	100.00	2,675
CD54 DNP 9:Live Cells	9,633	66.79	***	66.79	2,595
CD86 DNP 8:All Events	14,911	***	***	100.00	4,538
CD86 DNP 8:Live Cells	9,979	66.92	***	66.92	2,932
CD54 DNP 8:All Events	13,295	***	***	100.00	2,470
CD54 DNP 8:Live Cells	9,713	73.06	***	73.06	2,336
CD86 DNP 7:All Events	15,451	***	***	100.00	5,052
CD86 DNP 7:Live Cells	10,000	64.72	***	64.72	3,129
CD54 DNP 7:All Events	13,076	***	***	100.00	2,339
CD54 DNP 7:Live Cells	9,748	74.55	***	74.55	2,175
CD86 DNP 6:All Events	13,727	***	***	100.00	3,943
CD86 DNP 6:Live Cells	10,000	72.85	***	72.85	2,680
CD54 DNP 6:All Events	13,038	***	***	100.00	1,916
CD54 DNP 6:Live Cells	9,646	73.98	***	73.98	1,699
CD86 DNP 5:All Events	13,071	***	***	100.00	3,603
CD86 DNP 5:Live Cells	10,000	76.51	***	76.51	2,588
CD54 DNP 5:All Events	12,379	***	***	100.00	1,833
CD54 DNP 5:Live Cells	10,000	80.78	***	80.78	1,640

Statistics					
Name	Events	% Parent	% Grandparent	% Total	FITC-A Geo Mean
CD86 DNP 4:All Events	12,284	***	***	100.00	3,195
CD86 DNP 4:Live Cells	10,000	81.41	***	81.41	2,521
CD54 DNP 4:All Events	12,900	***	***	100.00	1,584
CD54 DNP 4:Live Cells	10,000	77.52	***	77.52	1,381
CD86 DNP 3:All Events	11,828	***	***	100.00	2,833
CD86 DNP 3:Live Cells	9,788	82.75	***	82.75	2,267
CD54 DNP 3:All Events	12,117	***	***	100.00	1,499
CD54 DNP 3:Live Cells	10,000	82.53	***	82.53	1,329
CD86 DNP 2:All Events	12,010	***	***	100.00	2,661
CD86 DNP 2:Live Cells	10,000	83.26	***	83.26	2,146
CD54DNP 2:All Events	11,883	***	***	100.00	1,389
CD54DNP 2:Live Cells	10,000	84.15	***	84.15	1,258
CD86 DNP 1:All Events	11,054	***	***	100.00	2,397
CD86 DNP 1:Live Cells	9,805	88.70	***	88.70	2,041
CD54 DNP 1:All Events	11,439	***	***	100.00	1,377
CD54 DNP 1:Live Cells	9,758	85.30	***	85.30	1,239
CD86 DNCB #1:All Events	14,079	***	***	100.00	5,192
CD86 DNCB #1:Live Cells	9,332	66.28	***	66.28	3,885
CD54 DNCB #1:All Events	17,272	***	***	100.00	2,123
CD54 DNCB #1:Live Cells	10,097	58.46	***	58.46	1,983
CD86 DNCB #3:All Events	14,713	***	***	100.00	5,517
CD86 DNCB #3:Live Cells	9,506	64.61	***	64.61	4,295
Cd54 DNCB #3:All Events	17,453	***	***	100.00	2,241
Cd54 DNCB #3:Live Cells	10,020	57.41	***	57.41	2,170

Appendix C

Data Analysis

Experiment 1

4/7/2016

	Concentration (mg/mL)	Viability (IgG)	MFI IgG1	MFI CD86	RFI	% change	EC150	MFI CD54	RFI	% change	EC200
Saline	0.00	92.57	983	2861	1.00	100.00		1102	1.00	100.00	
DMSO	0.00	94.44	927	2830	1.00	100.00		1166	1.00	100.00	
DNCB Control	0.0033	78.12	1459	7071	2.95	294.90		1997	2.25	225.10	
	0.0040	74.22	1822	7376	2.92	291.85		2031	0.87	87.45	
	0.0048	75.42	1728	5994	2.24	224.17		2228	2.09	209.21	
DNP	0.0931	70.07	1697	4041	1.25	124.81		3022	11.13	1113.45	
	0.1117	72.26	1528	3557	1.08	108.04		2679	9.67	967.23	
	0.1341	69.28	1658	3070	0.75	75.19		2552	7.51	751.26	
	0.1609	63.07	1662	3291	0.87	86.74		2327	5.59	558.82	
	0.1931	55.57	1472	3038	0.83	83.39		1684	1.78	178.15	
	0.2317	58.40	1376	2637	0.67	67.15		1456	0.67	67.23	
	0.2780	59.82	1230	2496	0.67	67.41		1546	2.66	265.55	
	0.3336	59.79	1237	2547	0.70	69.76		1339	0.86	85.71	

Experiment 2

4/8/2016

	Concentration (mg/mL)	Viability (IgG)	MFI IgG1	MFI CD86	RFI	% change	EC150	MFI CD54	RFI	% change	EC200
Saline	0.00	92.86	930	2026	1.00	100.00		1029	1.00	100.00	
DMSO	0.00	94.11	846	2221	1.00	100.00		1017	1.00	100.00	
DNCB Control	0.0033	83.49	1140	5165	2.93	292.73		1669	3.09	309.36	
	0.0040	77.05	1165	4834	2.67	266.84		2071	5.30	529.82	
	0.0048	74.04	1129	4456	2.42	241.96		1913	4.58	458.48	
DNP	0.0931	75.14	1121	2944	1.66	166.33	0.084621	1961	8.48	848.48	
	0.1117	73.38	1153	3369	2.02	202.19		2384	12.43	1243.43	
	0.1341	71.02	1189	3096	1.74	174.00		3021	18.51	1850.51	
	0.1609	69.95	1222	2945	1.57	157.21		3007	18.03	1803.03	
	0.1931	66.58	1240	2745	1.37	137.32		3084	18.63	1862.63	
	0.2317	54.43	1199	2308	1.01	101.19		2293	11.05	1105.05	
	0.2780	55.96	1161	2090	0.85	84.76		1677	5.21	521.21	
	0.3336	52.86	1062	2129	0.97	97.35		1425	3.67	366.67	

Extrapolation for DNP EC200

Concentration (ug/mL)	RFI	Log2 Conc	Extrap.	ug/mL
93.1	848.48	6.54	6.11	69.03468
111.7	1243.43	6.80		

Experiment 3

4/12/2016

	Concentration (mg/mL)	Viability (IgG)	MFI IgG1	MFI CD86	RFI	% change	EC150	MFI CD54	RFI	% change	EC200
Saline	0	98.88	905	1902	1.00	100.00		1025	1.00	100.00	
DMSO	0	94.61	874	1898	1.00	100.00		1055	1.00	100.00	
DNCB Control	0.0033	67.76	1182	3885	2.64	263.96		1983	4.43	442.54	
	0.0040	63.84	1099	4924	3.74	373.54		2140	5.75	575.14	
	0.0048	72.56	1130	4295	3.09	309.08		2157	5.67	567.40	
DNP	0.0449	90.89	967	2041	1.08	107.72	0.0939	1239	2.27	226.67	0.0952
	0.0539	89.97	973	2146	1.18	117.65		1258	2.38	237.50	
	0.0647	83.45	1011	2267	1.26	125.98		1329	2.65	265.00	
	0.0776	86.83	1035	2521	1.49	149.05		1381	2.88	288.33	
	0.0931	79.51	1096	2588	1.50	149.65		1640	4.53	453.33	
	0.1117	71.68	1108	2680	1.58	157.67		1699	4.93	492.50	
	0.1341	77.8	1108	3129	2.03	202.71		2175	8.89	889.17	
	0.1609	74.97	1128	2932	1.81	180.94		2336	10.07	1006.67	
	0.1931	62.31	1106	2762	1.66	166.10		2595	12.41	1240.83	
	0.2317	61.53	1102	2869	1.77	177.23		3063	16.34	1634.17	
	0.2780	55.22	1125	2277	1.16	115.55		1885	6.33	633.33	
	0.3336	50.69	1059	1961	0.90	90.47		1502	3.69	369.17	