



Correlating the structure and reactivity of a contact allergen, DNCB, and its analogs to sensitization potential[☆]

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ABSTRACT

We report a study that seeks to find a correlation between the overall sensitization potential quantified by the expression of IL-8 by stimulated monocytes and the chemical structure of a model contact allergen, 2,4-dinitrochlorobenzene (DNCB). We show that structure and reactivity of the chemical compounds play an important role in activation of the monocytes and subsequent inflammation in tissue. However, we observed a non-linear correlation between the rate of reaction and biological activity indicating a required balance of stability and reactivity.

1. Introduction

Elucidation of contact hypersensitivity is a longstanding goal in allergy and immune response. The concept of protein reactivity is commonly used to explain the initial step towards sensitization by small molecular weight compounds commonly called contact allergens.^{1,2} The physicochemical properties of the contact allergens that dictate this initial phase of the pathology includes reactivity, spatial geometry, and steric restraints.^{3,4} There have been multiple studies highlighting the relationship between chemical reactivity and an external sensitization index such as LLNA.^{5–8} However, limited studies encompass both the structural and electronic requirements of reactivity, with a direct comparison to *in-vivo* and *in-vitro* activity. DNCB (1) is a widely used reference molecule in sensitization models probing pathways and cell types involved in the sensitization process.^{9–11} Here, we investigate the specific structural requirements for the high sensitization potential of DNCB (1) using a two-pronged strategy encompassing a cell-based assay and examination of parameters defining chemical reactivity (Fig. 1).

We report that the rate of reactivity as well as steric elements contribute to a contact allergen's ability to stimulate IL-8 production in target human THP-1 cells and ultimately sensitivity *in vivo*. However, the relationship is not linear. Using these experiments, we also show that non-allergenic compounds can be made to activate skin inflammation by controlling their rate of aromatic substitution reaction – the reaction postulated to occur within the skin. In examining contact

allergen responses, we sought a reliable *in vitro* technique for quantifying activity of individual compounds. Historically, contact allergen identification was done by accessing the elicitation phase after painting the skin or injecting the allergen.^{12–15} However, in the past decades, the field has moved to murine local lymph node assay (LLNA)^{16–18} and cell-based assays.^{19–21} These cell-based assays, notably the activation of cultured THP-1 monocyte, minimize animal usage and are translatable to humans.¹⁹ DNCB is hypothesized to form covalent bonds through a nucleophilic substitution mechanism with nucleophilic residues of proteins. What intrigued us was that despite the widespread use of DNCB both in culture and *in vivo* as a model of allergy, very little work examines the mechanism of DNCB or its analogs.

2. Results and discussion

In the 1930's, Landsteiner and Jacobs reported a series of papers detailing their sensitization experiments on guinea pigs with DNCB and structurally-similar compounds.^{15,22–24} Since then, there have been many reports studying the effect of electrophilicity of contact allergens on sensitization potential.^{25–30} From these earlier experiments, we ascertained that although reactivity was a critical component to sensitization, sterics play a role in immunological activity and sought to clarify this further. For all initial experiments, we maintained consistency with the field of contact allergens by testing with the human monocyte cell line THP-1 for our cell model and measuring interleukin (IL)-8 expression^{31–33} as the proxy for the sensitization potential of

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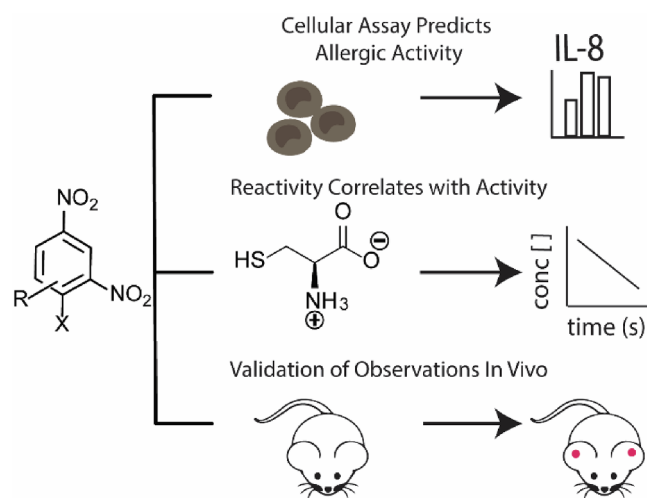


Fig. 1. The overview of this paper. The sensitization potential of DNCB and derivatives is measured employing a variety of techniques.

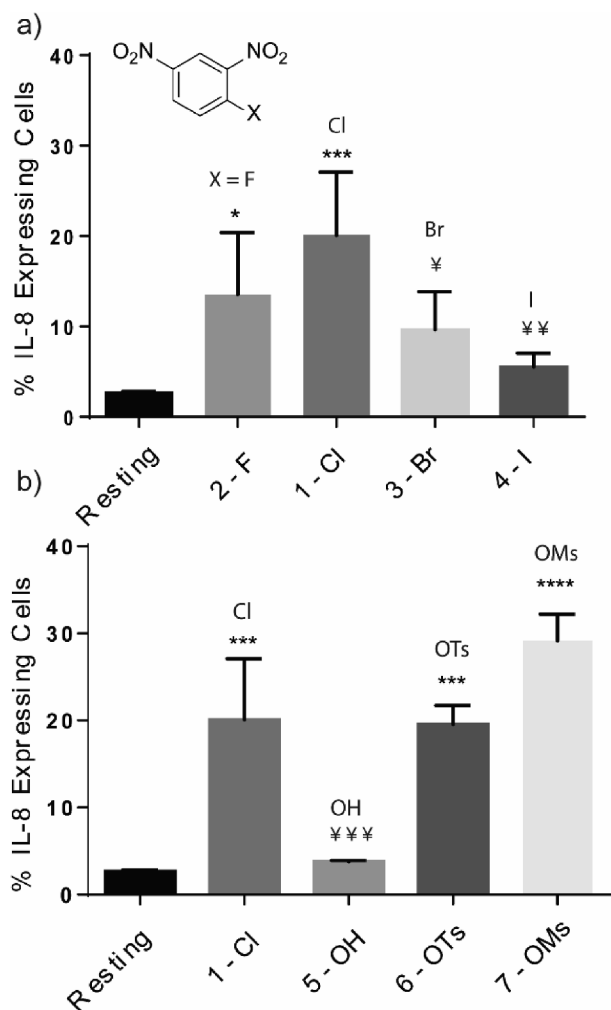


Fig. 2. The leaving group halides (a) and non-halide leaving groups (b) were explored. THP-1 cells were incubated with 50 μ M of labelled compounds for 10 h and the percentage of IL-8 expressing cells were measured via flow cytometry. * = significance from resting, ¥ = significance from DNCB. * $P \leq 0.05$ *** $P \leq 0.001$ **** $P \leq 0.0001$ ¥¥ $P \leq 0.01$.

DNCB and its derivatives. Working from the previous report by Landsteiner in 1935, we first tested the effect of the quality of leaving group of the aromatic compounds.²² The substitution reaction mechanism

occurs through a Meisenheimer complex that is stabilized by electro-negative leaving groups.³⁴ We chose halogen derivatives of DNCB as an initial examination of this phenomenon (Fig. 2a, 1–4). We expected that we would see a correlation between electronegativity and IL-8 expression where the most electronegative leaving group would lead to the highest expression. THP-1 cells were incubated with the compounds at 50 μ M concentration, the optimal condition for IL-8 release (Supplementary Fig. 1), and the percent of IL-8 expressing cells was determined through either cytokine staining or quantified using an ELISA assay.

Broadly, we observed reduction of IL-8 expression which correlated to the decrease of electronegativity of the leaving group halides. We detected a loss of activity with dinitrophenol (DNP, 5), which confirmed the requirement of a leaving group. Interestingly, we observed that the difference in IL-8 expression between dinitrofluorobenzene (DNFB, 2) and DNCB (1) was not significant (Fig. 2a). This could be due to the high reactivity of DNFB, which could result in loss of activity through hydrolysis. Based on our initial observations, we hypothesized that any leaving group which enabled a Meisenheimer complex would likely allow the activation of IL-8 in cells. To test this, we modified DNP with both tosyl (6) and mesyl (7) groups to create DNP based derivative capable of reacting (Fig. 2b). Both derivatives 6 and 7 activated cells resulting in IL-8 levels comparable to or higher than that of DNCB (Fig. 2). Interestingly, the mesyl 7 resulted in higher activity than the tosyl 6 and the parent compound 1, implying an additive positive effect of having a small and unhindered leaving group. As the leaving group experiments indicated aromatic nucleophilic substitution (S_NAr) mechanism, we tested the quality of electron withdrawing substituents. One important element of an S_NAr is the ability to correlate reactivity with the electron density at the point of reaction in the molecule.^{35,36} To test this hypothesis, we chose substituents with varying electron withdrawing capabilities³⁶ including cyano (8 and 12, σ_p 0.66), carboxylic acid (9 and 12, σ_p 0.45), and a methyl ester (10 and 13, σ_p 0.45) at the para and ortho position relative to the leaving group (Fig. 3a).

Using the same experimental procedure, IL-8 expression was measured. As expected, we observed a decrease in IL-8 expression as the compounds assayed were more electron-rich than DNCB (1, Fig. 3a). From this, we concluded that the electron withdrawing capability of the substituents are a determining factor for activity. This scaffold required substituents with electron withdrawing capabilities comparable to the nitro group at the para and ortho position as is the case for DNCB (1). With this in mind, we sought to elucidate the effect of increasing the number of electron withdrawing groups – balancing it with the activity of steric interactions with potential target proteins. To test overall electron withdrawing capacity, we examined a set of compounds which included derivatives with the two nitro groups of the parent compound 1 as well as carboxylic acid and ester groups (Fig. 3b, 14–17).

We observed comparable activity of 3,5-dinitro-4-chlorobenzoic acid (14), to DNCB (1). This similarity indicated a cumulative effect of the electron-withdrawing groups in this compound. However, similar derivatives (15–17) were not comparable in activity, implying that sterics and potentially charge density were also critical factors. Investigating this hypothesis further, we synthesized and assayed ester derivatives (Fig. 4, 18–21). Although these ester derivatives failed to elicit a response as strong as the one observed from the carboxylic acid (14), we observed an above resting activity with the ortho substituted esters. This suggests the importance of the carboxylic group or nitro group at the para position (Fig. 4, 18 and 19). One potential explanation for this difference is that the potential target protein/s has a binding pocket with an H-bonding acceptor.

Observing such clear relationships between structure and activity, we examined the mechanism/process by comparing the chemical reactivity of these compounds to the biological activity, both experimentally and with models. To simulate the reaction with a protein, we reacted the compounds with cysteine at physiological pH. This reaction

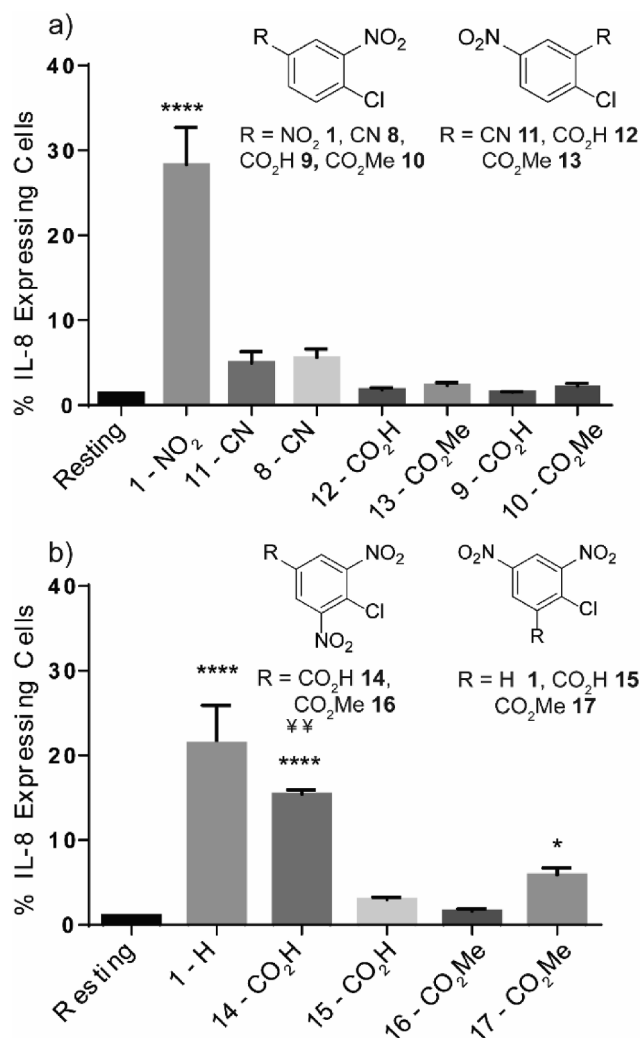


Fig. 3. The quality of electron-withdrawing groups was explored. A series of electron withdrawing groups were substituted in place of nitro group (a) or additional electron-withdrawing groups were substituted in ortho position (b). THP-1 cells were incubated with 50 μ M of labelled compounds for 10 h and the percentage of IL-8 expressing cells were measured via flow cytometry. * = significance from resting, Ψ = significance from DNCB. ** $P \leq 0.05$ *** $P \leq 0.01$ **** $P \leq 0.0001$.

is a common proxy for a contact allergen's ability to generate a response.³⁷ Plotting the log k_{obs} vs normalized IL-8 activity, we observed that there was no simple, linear correlation between electrophilicity of a compound and its cellular activity. High reactivity did not immediately translate to high cellular response. Our model compound (1) was comparable in biological reactivity to the tosyl (6) and 3,5-dinitro-4-chlorobenzoic acid (14). The ester derivatives (15, 17, and 19) showed the highest relative rate of reaction. These compounds, however, resulted in lower IL-8 expression when incubated with THP-1 cells compared to DNCB (1, Fig. 5). We also observed that there was a rate of reactivity window where a balance between compound stability and reactivity led to IL-8 expression (Fig. 5). We observed that the substituted esters (19–21) were not as potent as DNCB (1) in eliciting IL-8 activity (Figs. 3 and 4). By plotting the log k_{obs} vs IL-8, we see that the chemical reactivity of these esters is comparable to DNCB (1) but that they elicit low IL-8 expression suggesting steric hindrance at the site of reactivity (Fig. 5).

Using a molecular modelling software (Spartan), we calculated the molecular orbitals of the electrophiles using B3LYP/6-31 + G (d,p) basis set and generated an electrophilicity map. This was done by mapping

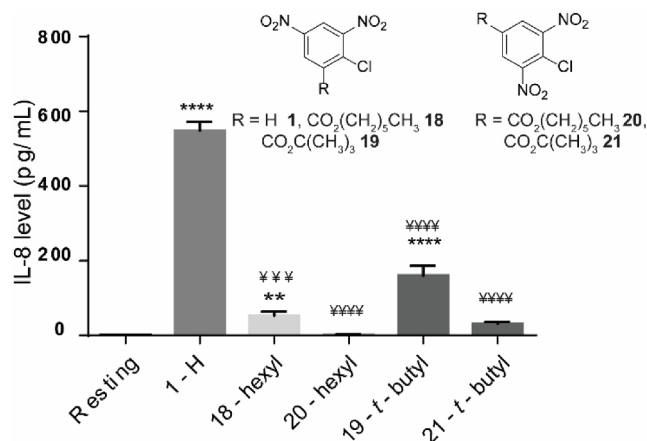


Fig. 4. IL-8 secretion by THP-1 cells stimulated with the parent compound (1) and the ester derivatives (18–21). THP-1 cells were incubated with 50 μ M of labelled compounds for 20 h and the IL-8 secreted to supernatant was quantified using sandwich ELISA * = significance from resting, Ψ = significance from DNCB. ** $P \leq 0.01$ *** $P \leq 0.001$ **** $P \leq 0.0001$.

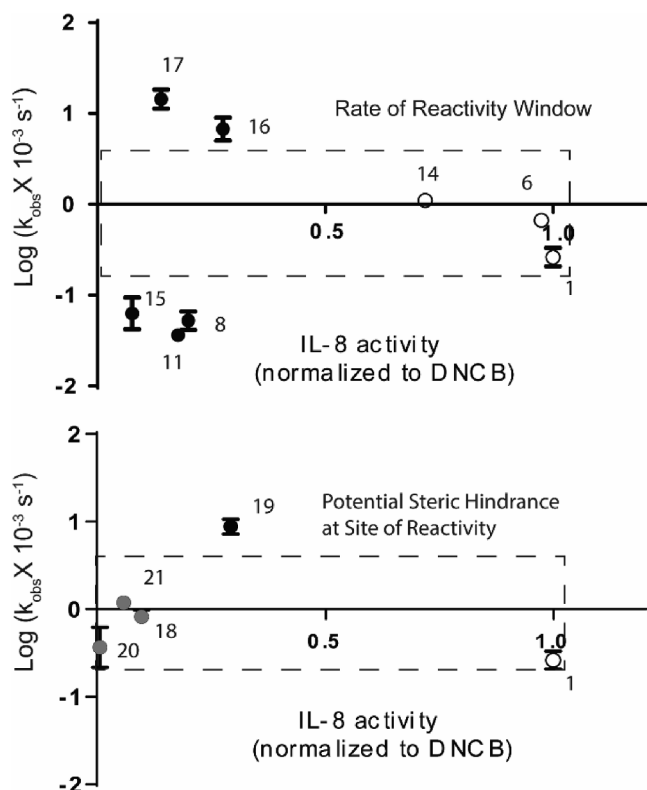


Fig. 5. Correlation IL-8 activity to the rate of reactivity to cysteine. IL-8 activity of compounds was normalized to DNCB (1).

the absolute value of the lowest-unoccupied molecular orbital (LUMO) on the electron density. We compared the nucleophilic reactivity of the derivatives at the site of nucleophilic substitution reaction. Plotting the $|LUMO|$ value vs log k_{obs} , we saw that there was a loose but direct correlation between the experimental and computational reactivity. Plotting IL-8 activity v $|LUMO|$ (Supplementary Fig. 7) indicated a similar trend as with the experimental plot (Fig. 5) where there appeared a required balance of reactivity and stability for optimal IL-8 expression.

Hapten formation is considered the initial step in the pathology of allergic contact dermatitis.³ There are ongoing studies that are targeted

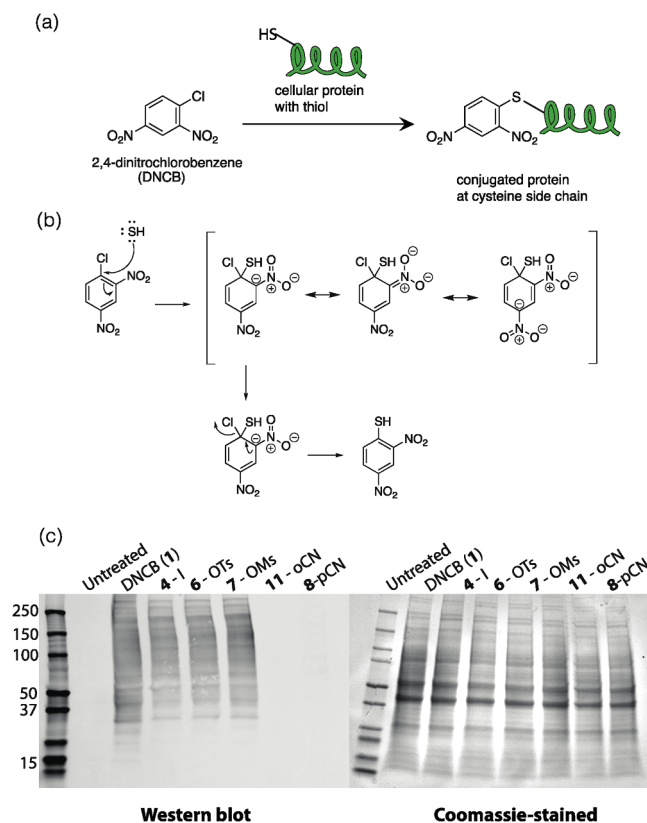


Fig. 6. Diagram showing DNCB modification to cysteine side chains of cellular protein (a) and the reaction mechanism (b). Proteins modified by DNCB (1) and its derivatives were analyzed by western blotting (c, left). Untreated lane shows no band, confirming that anti-DNP antibody is specific to DNP-like-moieties. Compounds 4, 6, and 7 show band intensity similar to that of DNCB. Proteins were separated using the same material and methods and Coomassie-stained to confirm that the same amount of proteins were used in all lanes (c, right).

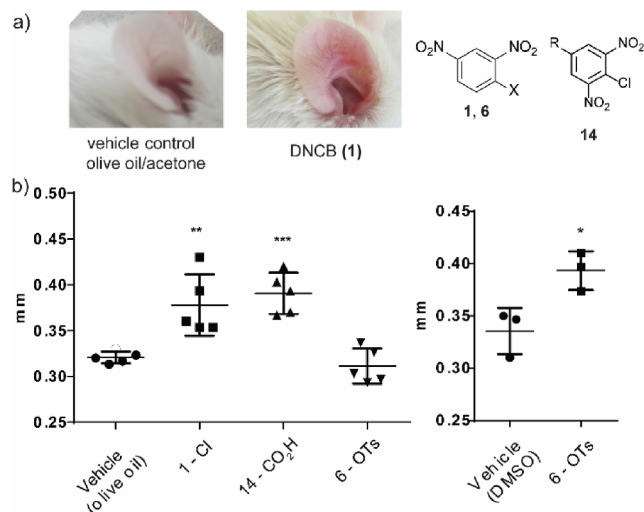


Fig. 7. Skin inflammation study on mouse dorsum. (a) Examples of unswollen (left) and swollen (right) mouse dorsum. Mouse was treated with 0.5% compound solution on day 0 and 1. The mouse was challenged with the same 0.3% compound solution on day 5, 6, 7 and 8. (b) Ear thickness measured on day 9. * = significance from resting, **P ≤ 0.01 ***P ≤ 0.001 ****P ≤ 0.0001.

to the identification of proteins that react with contact allergens and become immunogenic. Therefore, we wanted to compare proteins that were modified by DNCB to those of some of the derivatives. DNCB usually modifies nucleophilic side chains such as cysteines (Fig. 6A and

B). Proteins modified by DNCB and its derivatives were analyzed by western blotting with anti-DNP antibody that could be used to detect DNP-moieties on the proteins (Supplementary Fig. 3). We expected that this experiment would tell us which proteins are labelled and the relative amount of modification. Because compounds 8 and 11 would result in conjugation of a non-DNP moiety, we confirmed the specificity of the anti-DNP antibody with BSA modified with both 8 and 11 (SI). From the western blot, we saw that treatment with compounds 4–7 resulted in a series of gel staining pattern similar to that of DNCB (1, Fig. 6C), suggesting that similar proteins are modified. Compounds 8 and 11 however showed no modified proteins in the lysate. This result indicates that although the immune response seen after treating THP-1 cells with the derivatives 4–7 was varied, these compounds seemed to modify similar proteins. Treatment with compounds 8 and 11 led to low or resting IL-8 levels on THP-1 cells. These derivatives also showed slow reaction kinetics with cysteine. In this experiment, we also confirmed that these two derivatives did not modify any proteins when added to THP-1 cells, confirming that protein modification is a clear correlation for an immune response on the THP-1 cells.

Lastly, we sought to confirm that our cell-based assay results would translate into a mouse model. We obtained male Balb/c mice between 6 and 7 weeks old. Based on a previously published protocol,³⁸ we sensitized their shaven abdomen for two consecutive days. After a rest period, the mice were challenged on the ear dorsum for five consecutive days. To determine the degree of inflammation, we measured the ear thickness the day after the last compound exposure.

We observed a general inflammation of the mice dorsum with DNCB (1) that was comparable to that of benzoic acid (14). The tosyl (6) induced low inflammation which was unexpected based on the cell-based assay, where we saw comparable IL-8 expression to DNCB (1, Fig. 7). We speculated that the compound was unable to cause inflammation at a comparable level with DNCB due to its inability to cross the skin barrier. To investigate this hypothesis, we repeated the experiment using DMSO as a vehicle to ensure skin penetration. In this case, we did observe inflammation that was statistically significant when compared to the control mice – confirming our hypothesis (Fig. 7).

In this study, we show that there are various aspects to the degree of sensitization potential of DNCB and its derivatives. We show that understanding the reactivity of the compounds can be used to manipulate and predict the in vitro and in vivo response more broadly than screening each compound individually. However, the rate of covalent bond formation of a contact allergen is not linearly correlated with its sensitization potential. This result strongly implies that there are specific protein targets which rely on spatial information along with reactivity to determine activation of an allergic response. We hope to use these findings in the development of DNCB based contact allergen probes for the identification of protein targets in the overall elucidation of the contact allergen pathway. This work may also guide the use of DNCB analogs which elicit stronger reactions than DNCB improving the outcomes of immunological mechanisms probed via DNCB.

3. Methods

3.1. Intracellular cytokine flow cytometry staining

Cytofix/Cytoperm™ kit with GolgiPlug™ was purchased from BD Biosciences (San Jose, CA). Cytokine staining was performed according to BD Biosciences protocol with minor modifications. The test compounds were dissolved in DMSO to make 0.5 M or 1 M concentration and sterile-filtered using 0.22 μm syringe filters. THP-1 cells were harvested and plated in a 24-well plate at the concentration of 10⁶ cells/mL. The cells were subsequently incubated with 50 μM of compounds and 1 μL of BD GolgiPlug™ (protein transport inhibitor containing Brefeldin A). Lipopolysaccharide (LPS, 200 ng/mL) was used as positive control for immune activation. After 10 h incubation at 37 °C

with 5% CO₂, the cells were washed with FACS buffer (300 µL, 2X). The cell pellet was resuspended in 300 µL of Cytofix/Cytoperm™ solution and incubated on ice for 20 m. The cells were washed with 1X BD Perm/Wash™ Buffer (300 µL, 2X) and then resuspended in 50 µL of 10X BD Perm/Wash™ Buffer. For antibody staining, 5 µL of FITC anti-human IL-8 antibody (BioLegend) was added to each sample and vortexed. After 30 m incubation on ice with rigorous vortexing at the halfway mark, the cells were washed with 1X BD Perm/Wash™ Buffer (300 µL, 3X). IL-8 expression was analyzed using BD Accuri C6 cytometer and BD Accuri C6 software.

3.2. ELISA

Human IL-8 ELISA MAX™ Deluxe was purchased from BioLegend (San Diego, CA). The experiment was carried out according to the manufacturer's protocol. About 5×10^5 cells were plated in 500 µL of culture media containing 50 µM compounds. LPS at concentration of 200 ng/mL was used for positive control. After 20 h of incubation, the cells were harvested and the supernatant was collected for analysis.

3.3. Western blot analysis

Conjugation of target protein was performed according to previously published protocol.^{39–41} Bovine serum albumin (BSA, Millipore Sigma) and the selected compounds were dissolved in a mixture composed of 17 parts 1X PBS and 3 parts 100% acetonitrile (HPLC grade) and incubated in 37 °C for 7 days. To ensure conjugation, 10 µL of the sample was desalted and analyzed using MALDI. To analyze proteins modified by DNCB and its derivatives, THP-1 cells were incubated in RPMI supplemented with 10% FBS and 50 µM of compounds. After 30 m incubation, cells were lysed with 1x cell lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) supplemented with 1 tablet of cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma). Cell lysates were quantified using Pierce™ BCA Protein Assay Kit. For western blot analysis, proteins were first separated by 4–15% Mini-Protean® TGX™ Precast Protein Gels. Then, separated proteins were tank-transferred to PVDF membrane, and primarily stained with mouse anti-DNP antibody (Millipore) that could bind to protein-conjugated DNP moieties.⁴⁰ After staining with goat anti-mouse IgG (H + L) Secondary Antibody, DyLight 650, the membrane was imaged using Azure c600 Imaging System (Azure Biosystems). To ensure that equal amount of protein was used, cell lysates were separated again in the same condition, fixed in gel-fixing solution (50% (v/v) ethanol in water with 10% (v/v) acetic acid) for 1 h, washed in gel-washing solution (50% (v/v) methanol in water with 10% (v/v) acetic acid) overnight, stained in Coomassie solution (0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol in water with 10% (v/v) acetic acid) for 3 h, and then destained with gel-washing solution until desired color and band intensity appeared. Band intensity was calculated using ImageJ and normalized to the band intensity of DNCB-treated sample.

3.4. Quantifying sensitization through mice ear thickness experiments

This experiment was run according to literature protocol³⁸ with minor modifications. DNCB and its derivatives were dissolved into 0.5% (w/v) solutions using a 4:1 by volume acetone/olive mixture or DMSO. Male Balb/c mice was sensitized by exposing the shaven abdomen to 25 µL of the compounds utilizing a different animal for each compound. The negative control was run in tandem, utilizing the vehicle without any of the assayed compounds in solution. Sensitization was performed on day 0 and 1. On day 5, 6, 7, and 8, the mice were challenged on the dorsum of both ears with 10 µL of 0.3% (w/v) solutions of the compounds in the same vehicle as the sensitization phase. Ear thickness was measured 24 h after the last compound exposure using a digital micrometer.

3.5. General protocol for rate analysis

The compounds of interest were dissolved in 10% CH₃CN in PBS (1 mM, 1 mL). The reaction was initiated by adding 100 µL of cysteine (10 equiv., 10 mM) in PBS to the fully dissolved compounds. At each time point, 10 µL aliquots of solution were quenched with 0.1% TFA and the content was analyzed by HPLC. The area under the compound curve and the compound-cysteine curve were calculated and used to generate pseudo first-order rate.

3.6. Computational details

All computations were performed using Spartan software (Wavefun. Inc.). Ground state geometries and energies were optimized in gas phase using the B3LYP functional and 6-31 + G (d,p) basis set for all atoms. The electron density, and the composite surface of |LUMO| mapped onto the electron density were then calculated.

Hexyl-2-chloro-3,5-dinitrobenzoate (**18**), hexyl-4-chloro-2,5-dinitrobenzoate (**20**), *tert*-butyl-4-chloro-3,5-dinitrobenzoate (**19**), and *tert*-butyl-2-chloro-3,5-dinitrobenzoate (**21**) were truncated to propyl-2-chloro-3,5-dinitrobenzoate, propyl-4-chloro-2,5-dinitrobenzoate, isopropyl-4-chloro-3,5-dinitrobenzoate, isopropyl-2-chloro-3,5-dinitrobenzoate respectively for feasible calculation (Supplementary Table 1).

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Declaration of Competing Interest

No competing financial interests have been declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bmc.2019.05.017>.

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