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DR. DANIEL ANGUS RYAN (Orcid ID : 0000-0003-3971-5399)

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Rational Design of Peptide Derivatives for Inhibition of MyD88-Mediated TLR Signaling in Human PBMCs and Epithelial Cells Exposed to *F. tularensis*

Ryan, D. A.;[a] ‡ Degardin, M.;[a] ‡ Alam, S.;[b] Kissner, T. L.;[b] Hale, M;[b], Cameron, M. D.;[c] Rebek, M.;[a] Ajami, D.;[a] Saikh, K. U.;[b] and Rebek, J. Jr.[a*]

‡ These authors contributed equally.

* Corresponding author

E-mail address: jrebek@scripps.edu (Julius Rebek, Jr.)

[a] *Department of Chemistry,*

The Skaggs Institute for Chemical Biology,

The Scripps Research Institute, 10550 North Torrey Pines Rd,

La Jolla, California, 92037, USA.

[b] *Department of Immunology,*

Army Medical Research Institute of Infectious Diseases,

1425 Porter Street,

Frederick, MD 21702, USA.

[c] *Department of Molecular Therapeutics,*

The Scripps Research Institute, 130 Scripps Way,

Jupiter, Florida, 33458, USA.

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ABSTRACT

Small molecules were developed to attenuate proinflammatory cytokines resulting from activation of MyD88-mediated TLR signaling by *F. tularensis*. Fifty-three tripeptide derivatives were synthesized to mimic a key BB-loop region involved in toll-like/interleukin-1 receptor recognition (TIR) domain interactions. Compounds were tested for inhibition of TNF-α, IFN-γ, IL-6, and IL-1β in human peripheral blood mononuclear cells (PBMCs) and primary human bronchial epithelial cells exposed to LPS extracts from *F. tularensis*. From fifty-three compounds synthesized and tested, ten compounds were identified as effective inhibitors of *F. tularensis* LPS-induced cytokines. Compound stability testing in the presence of human liver microsomes and human serum resulted in the identification of tripeptide derivative **7** that was a potent, stable and drug-like small molecule. Target corroboration using a cell-based reporter assay and competition experiments with MyD88 TIR domain protein supported that the effect of **7** was through MyD88 TIR domain interactions. Compound **7** also attenuated proinflammatory cytokines in human peripheral blood mononuclear cells and bronchial epithelial cells challenged with a live vaccine strain of *F. tularensis* at a multiplicity of infection of 1:5. Small molecules that target TIR domain interactions in MyD88-dependent TLR signaling represent a promising strategy toward host-directed adjunctive therapeutics for inflammation associated with biothreat agent-induced sepsis.

1. Introduction

Systemic Inflammatory Response Syndrome (SIRS) is classified by a set of clinical manifestations of immunological disorder and inflammation that can be the result of trauma, infectious or non-infectious diseases.[1, 2] When an infection is the cause of the inflammatory response, the condition is normally termed sepsis.[3] In sepsis, an uncontrolled reaction of the innate immune system and broader immunological dysfunction may progress to tissue damage, organ damage or death.[4] Beyond primary infections, sepsis is a concern in many medical conditions when damaged or dysfunctional tissues and organs become predisposed to infection, or through opportunistic infections of immunocompromised hosts.[5, 6] Sepsis has been reported to affect more than 750,000 people per year in the United States, a rising number,[7] and mortality rates have been reported in the range of 20-50 %.[8, 9] In low-to-middle income countries the morbidity and mortality due to sepsis is considered to be higher.[10] Despite advances in treatments that include antibiotics, intravenous fluids injection

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and/or vasopressors, dialysis, blood sugar level maintenance, respiratory support and surgery,[11, 12] there is still no efficient therapy available.[13] A difficulty with treating sepsis is that a number of inflammatory pathways become active simultaneously such as cytokine network and coagulation system.[7] Under normal circumstances, bacterial infections are countered by host innate immunity, which identifies pathogens and pathogen-associated molecular patterns (PAMPs) leading to the release of proinflammatory cytokines that ultimately destroy the pathogens.[14] When there is excessive production of cytokines and other inflammatory mediators including danger-associated molecular patterns (DAMPs),[15] normal immune response is altered manifesting symptoms of sepsis with potentially dire consequences to the host.

Bacterial infections are the most common cause of sepsis.[16] One such pathogen is *Francisella tularensis* that is an endotoxin-positive, Gram-negative bacterium and the causative agent of tularemia, also known as 'rabbit fever' or 'tick fever'. Although the pathogenesis of *F. tularensis* is complex, an initial induction period where the pathogen largely evades the immune system precedes a 'cytokine storm' associated with severe, systemic sepsis.[17-20] Respiratory infections of *F. tularensis* leading to pneumonic tularemia and sepsis are considered among the most severe forms of infection with the highest mortality rate.[21] According to the Centers for Disease Control and Prevention (CDC), the incidence of tularemia in the United states has been in the range of 93-205 cases per year from 2005 through 2014.[22] Despite the relatively low incidence of tularemia, *F. tularensis* has been classified as one of six current Category A bioterrorism agents by the CDC due to its high virulence, potential for aerosolized infection and history as an investigational warfare agent.[20] An infection of only 10 bacteria have been reported as sufficient to cause tularemia in humans, although the minimal threshold for infection also has been shown to depend on the route of infection.[23, 24] First-line treatment of tularemia are antibiotics with streptomycin and gentamicin being the drugs of choice.[25] However, there has been renewed interest in developing more effective therapies and strategies to treat sepsis as a late-stage condition of tularemia.[26]

Toll-like receptors (TLRs) function as recognition elements of innate immune system and mediate the proinflammatory effects of sepsis at a cellular level.[27, 28] The proinflammatory response to *F. tularensis* in human cells is consistent with a TLR-mediated signaling mechanism, including induction of NF-κb and increased TNF-α and IFN-γ as observed in peripheral blood mononuclear cells (PBMCs) isolated from tularemia patients.[29] However, *F. tularensis* differs from other endotoxins, such as *E. coli*, in that lipopolysaccharide (LPS) from *F. tularensis* is modified and induces cytokines in both a TLR2- and TLR4-dependent manner, while being relatively more potent

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for TLR2 that other endotoxins.[30, 31] Signal transduction from a ligand-activated TLR proceeds through a cytosolic toll-like/interleukin-1 receptor recognition (TIR) domain that is common to TLR and IL-1 receptors. Myeloid differentiation primary response protein 88 (MyD88) is involved in intracellular signal transduction by binding of its TIR domain (*i.e.*, a MyD88 TIR domain) to the structurally analogous TIR domains of a TLR/IL-1 receptor or other adaptor proteins.[32, 33] MyD88-mediated signaling has been ascribed to all TLR receptor subtypes, except TLR3 that is dependent on a different adaptor protein (i.e., TIR domain-containing adaptor protein-inducing IFN-β, TRIF). A conserved 'BB-loop' region has been characterized as a surface-exposed β-strand decisively involved in TIR domain interactions and propagation of TLR/IL-1 receptor signaling with MyD88.[34-36] The importance of the 'BB-loop' region in TIR domain interactions has been supported by structure-function studies that exhibited dominant-negative effects on TLR receptor signaling. Point mutations in 'box 2' motif of TIR domains have been shown to block TLR signaling and mice carrying point mutations (e.g., P712H) in the TLR4 TIR domain were shown to be endotoxin resistant.[34, 37] The downstream effects of TLR and IL-1R activation through MyD88 include activation of mitogen-activated protein (MAP) kinases, activation of transcription factors NF-κb, AP-1 or IRF3 and generation of proinflammatory cytokines.[38] Thus, TIR domain interactions represent a critical junction for inflammatory signaling and pose a pharmacological target for attenuating late-stage sepsis in tularemia by modulating the host inflammatory response.[39, 40]

The goal of this study was to develop small molecule inhibitors of proinflammatory cytokines resulting from TLR signaling activation upon exposure to *F. tularensis*. Our group previously reported compound **AS1** (i.e., 'hydrocinnamoyl-L-valinoyl-pyrrolidine') that is a small molecule mimetic of the Phe-Val/Ile/Leu-Pro conserved 'box 2' motif in the TIR domain 'BB-Loop' region (Table 1). **AS1** was effective at blocking proinflammatory cytokines in response to IL-1 receptor activation.[39, 41] Dimeric analogs of **AS1** were also developed that more potently disrupt protein-protein interactions of MyD88.[42-44] **AS1** attenuated *in vitro* IL-1β signaling in murine lymphocytes and EL4 thymoma cells in a concentration range of 10-100 μM and reduced IL-1β-induced fever in mice at 200 mg/kg (*i.p.*). The compound did not reduce LPS-mediated activation of TLR signaling up to 100 μM, demonstrating relatively high specificity for IL-1 receptor signaling. Due to the high sequence homology among the TIR domains of the TLR/IL-1 receptors, we hypothesized that small molecule derivatives of 'box 2' motif peptides might be similarly prepared that attenuate proinflammatory signaling through TLR signaling pathways. Here, we report the development of tripeptide derivatives capable of decreasing proinflammatory cytokines in primary human cells exposed to *F. tularensis*. These small molecules represent early-stage candidates for use as an adjunctive therapy for TLR-mediated inflammation associated with sepsis.

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2. Methods and Materials

2.1. Materials and Instrumentation

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received without further purification unless noted below. Amino acids and peptide coupling agents (*i.e*., EDC) were purchased from Oakwood Products Inc. (Estill, SC). Compounds **11**, **14**, **19** and **23** were purchased from Enamine Ltd (Monmouth Jct., NJ). Solvents were purchased from Fisher Scientific (Waltham, MA) or J.T. Baker (Avantor Performance Materials Inc., Center Valley, PA). Solvents dichloromethane, tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF) and triethylamine were purified by passage through two columns packed with activated alumina under an argon atmosphere encased in a glass contour systems from SG Water, USA. Deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. For HPLC, water was obtained from a Milli-Q water delivery system (EMD Millipore), while CH_3CN and TFA were purchased in HPLC quality. Analytical thin-layer chromatography (TLC) was performed on Silicycle 60 F254 glass-backed plates. Hepatic microsomes were purchased from BioreclamationIVT (Chestertown, MD). Human serum was purchased from BioWorld (Dublin, OH) and stored frozen until use. The Meso Scale Discovery (MSD) multi spot array ultra-sensitive cytokine assay kit was purchased from MSD (Gaithersburg, MD). Ficoll-Hypaque was purchased from GE Healthcare Biosciences (Piscataway, NJ). HEK 293 (TLR4-MD2- NF-κB-SEAPorter™ transfected) stable cell line was purchased from Imgenex (San Diego, CA). Additional information on materials, instrumentation and methods can be found in the Supporting Information.

2.2. General Synthetic Procedures

2.2.1. 'Procedure A' for EDC-mediated amide bond formation:

To a solution of an *N*-Boc protected amino acid (1 equiv, 100 mM), 1-hydroxybenzotriazole hydrate (2 equiv) and amine coupling partner (1.2 equiv.) in dichloromethane was added 1(-3-dimethylamino)propyl-3-carbodiimide hydrochloride (EDC, 1.2 equiv) and triethylamine (1.2 equiv) at 21 °C. The reaction was stirred for 12-16 hours. At this time, the reaction was diluted with dichloromethane and washed with 0.5 N $HCl_{(aq)}$, a saturated solution of $NaHCO_{3(aq)}$ and water. The organic layer was dried (magnesium sulfate), filtered, and concentrated by rotary evaporation. For synthetic intermediates, the crude product thus obtained was of sufficient purity to advance to the next reaction. For final products, the crude product was purified by preparative HPLC and lyophilized to obtain compounds of suitable purity for biological screening.

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2.2.2. 'Procedure B' for removal of Boc group:

To a solution of an *N*-Boc protected amide (1 equiv, 142 mM) in dichloromethane was added trifluoroacetic acid (8 equiv) at 0°C. The reaction was stirred at this temperature and monitored by TLC for consumption of starting material (typically 5-8 hours). At this time, the reaction was concentrated by rotary evaporation. The oily residue was diluted with diethyl ether and then extracted with water (3x). The combined aqueous layers were neutralized to pH 8 by portionwise addition of solid $NaHCO_3$ and solid Na_2CO_3. The aqueous layer was re-extracted with dichloromethane and ethyl acetate multiple times using TLC (ninhydrin staining) to monitor recovery of the amine from the aqueous phase as necessary. The combined organic layers were dried (magnesium sulfate), filtered and concentrated by rotary evaporation and vacuum. The crude products (*e.g.*, compound **3**) thus obtained were of sufficient purity to advance to the next reaction.

2.2.3. 'Procedure C' for N-sulfonylation:

Benzenesulfonyl chloride or an aryl-substituted benzenesulfonyl chloride (1.2 equiv) was added to a solution of amine (1 equiv, 100 mM) and triethylamine (1.2 equiv) in dichloromethane at 21 °C. The reaction was stirred for 12-16 hours. At this time, the reaction was diluted with dichloromethane and washed with 0.5 N $HCl_{(aq)}$, a saturated solution of $NaHCO_{3(aq)}$ and water. The organic layer was dried (magnesium sulfate), filtered, and concentrated by rotary evaporation and vacuum. The crude product thus obtained was purified by preparative HPLC and then lyophilized to obtain compounds suitable for biological screening.

2.2.4. 'Procedure D' for N-acylation:

Benzoyl chloride or an aryl-substituted benzoyl chloride (1.2 equiv) was added to a solution of amine (1 equiv, 140 mM) and triethylamine (1.2 equiv) in dichloromethane at 21 °C. The reaction was stirred for 12-16 hours. At this time, the reaction was diluted with dichloromethane and washed with 0.5 N $HCl_{(aq)}$, a saturated solution of $NaHCO_{3(aq)}$ and water. The organic layer was dried (magnesium sulfate), filtered and concentrated by rotary evaporation and vacuum. The crude product thus obtained was purified by preparative HPLC and then lyophilized to obtain compounds suitable for biological screening.

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2.3. Compounds Synthesis and Characterization

All new compounds and compounds not previously characterized by comparable techniques in the literature were characterized. The synthesis and characterization of compound **7** and synthetic intermediates was described below; all other compound synthesis procedures and characterization data was reported in the Supporting Information. Compound **14** was reported previously.[45] Synthetic intermediates **3**, **S1**-**S7** and **S12** were previously described,[46] or have been indexed in the Chemical Abstracts Service.

(*S*)-*tert*-Butyl (4-methyl-1-oxo-1-(pyrrolidin-1-yl)pentan-2-yl)carbamate (Intermediate **S1**). Reaction of Boc-L-Leu (2.13 g, 9.21 mmol) and pyrrolidine by Procedure A provided compound **S1** (3.04 g) as a yellow oil in approximate 85-90 % purity by ^{1}H NMR. Compound characterization data (^{1}H NMR, ^{13}C NMR and mass spectrometry analysis) were consistent with the literature report.[47] Product **S1** was used in subsequent steps without further purification.

(*S*)-2-Amino-4-methyl-1-(pyrrolidin-1-yl)pentan-1-one (Intermediate **3**). Reaction of **S1** (3.04 g, 10.7 mmol) by Procedure B provided compound **3** (1.24 g, 6.73 mmol) in 73 % yield over two steps from Boc-L-Leu. Compound **3** was obtained as a yellow oil in approximate 95 % purity by ^{1}H NMR. Compound characterization data (^{1}H NMR, ^{13}C NMR and mass spectrometry analysis) were consistent with the literature report.[47] Compound **3** was used in subsequent steps without further purification.

(*S*)-4-Ethyl-*N*-(4-methyl-1-oxo-1-(pyrrolidin-1-yl)pentan-2-yl)benzamide (**7**). Reaction of **3** (202 mg, 1.10 mmol) and 4-ethylbenzoic acid by Procedure A provided compound **7** (219 mg, 0.69 mmol) in 63 % yield after preparative HPLC purification. Compound **7** was obtained as a white powder. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.73 (d, J = 8.1 Hz, 2H), 7.42 (s, 1H), 7.23 (d, J = 8.1 Hz, 2H), 5.04 (m, 1H), 3.91 (m, 1H), 3.53 (m, 2H), 3.44 (m, 1H), 2.67 (q, J = 7.6 Hz, 2H), 2.02 (m, 2H), 1.88 (m, 2H), 1.74 (m, 2H), 1.51 (m, 1H), 1.23 (t, J = 7.6 Hz, 3H), 0.99 (d, J = 6.4 Hz, 3H), 0.95 (d, J = 6.4 Hz, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.98, 167.81, 148.74, 130.77, 128.13, 127.43, 49.80, 46.99, 46.58, 41.55, 28.26, 26.02, 24.95, 24.21, 23.41, 21.96, 15.33 ppm. HRMS (ESI) m/z for $C_{19}H_{28}N_2O_2$ calculated $[M+H]^+$: 317.2229, found: 317.2222 $[M+H]^+$.

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2.4. Hepatic Microsomal Compound Stability Assay

Compound stability in the presence of 1 mg/mL hepatic microsomes was evaluated by incubating 1 μM compound in 100 mM potassium phosphate at pH 7.4. The reaction was initiated by addition of NADPH to give a 1 mM final concentration. Aliquots were removed at 0, 5, 10, 20, 40 and 60 minutes and added to acetonitrile (5x volume) to stop the reaction and precipitate proteins. NADPH dependence of the reaction was evaluated with the addition of no-NADPH control samples. At the end of the assay, the samples were centrifuged through a Millipore Multiscreen Solvinter 0.45 micron low binding PTFE hydrophilic filter plate and analyzed on a ABSciex 5500 LC-MS/MS. Data was log transformed and represented as half-life calculated by using the 0 minute sample as 100 % compound remaining. Carbamazapine was used as an analytical internal standard for the mass spectrometer. Sunitinib was run with the compounds as a historical standard for inter-assay reproducibility.

2.5. Serum Compound Stability Assay

Compound stability in the presence of human serum was evaluated by adaptation of a reported procedure.[48] Test compound was incubated at 200 μM in human serum at 37 °C in a sealable glass vial. Aliquots were removed at 0, 15, 30 min, 1, 2, 4, 8, 16, 32 and 64 h. Each aliquot was extracted with dichloromethane (5 x 0.5 mL) and the combined organic extracts were concentrated. A solution of 2 % DMSO in water/acetonitrile (1/1) containing 200 μM of 4-hydroxybenzoic acid internal standard was added, the sample was vortexed and analyzed by analytical HPLC. Quantification of peak areas normalized to the internal standard provided the concentration of test compound at each time point. *ortho*-Nitrobenezene trifluoroacetamide that was previously shown to be a substrate for human cholinesterases was analyzed to validate serum amidase activity in the samples used.[49]

2.6. Cell Isolation, Culture Conditions and Compound Treatment

Peripheral blood mononuclear cells (PBMC), obtained from consenting healthy donors in accordance with Institutional Review Board-approved research donor protocol, were isolated by standard density gradient centrifugation with Ficoll-Hypaque (GE Healthcare Biosciences AB, Uppsala, Sweden), harvested from the interface, washed, and suspended in RPMI 1640 medium. Purified hPBMCs were cryopreserved and on the day of use the cells were thawed and cultured for 2 hours prior to treatment.

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For application of test compounds to cell culture, stock solutions of compound in dimethyl sulfoxide (DMSO) were freshly prepared for experiments and administered to cell cultures such that the final concentration of DMSO vehicle was 1 % (v/v) at all concentrations tested. Control groups (i.e., in the presence or absence of LPS) received DMSO vehicle at the same final concentration. For application of LPS extract to cells, the LPS extract was administered as a solution in water 30 min after addition of test compound unless noted otherwise. Statistical comparisons among experimental groups were made using a one-way ANOVA with Bonferroni post-hoc test ($\alpha = 0.05$).

2.7. Isolation of LPS from F. tularensis

Bacteria were grown on modified Thayer-Martin agar plates prepared with ingredients: GC medium base (Difco Labs, 36 g/L), hemoglobin (GIBCO Labs, 10 g/L) and Isovitalex (Becton Dickinson, 10 mg/L). A frozen stock (NDBR 101) of *F. tularensis* was thawed and 0.2 mL was spread evenly on a plate. The inoculated plate was incubated at 37 °C for 2 days in an incubator with 5 % CO_2 and 95 % humidity. Colonies were plated on 3 additional plates and incubated for 16 h. Plates with confluent growth were plated on 30 additional plates and incubated for 3 days in culture conditions described above. Bacteria were harvested by adding 15 mL of PBS (pH 7.4) to each agar plate and gently scrapping the agar surface to dislodge the bacteria. The bacteria remaining on the agar surface were harvested by serial passage of the first 15 mL suspension. Bacteria were collected by centrifugation at 10,000 x g for 30 min at 4 °C, and washed 5 times with PBS. The suspension of bacteria was frozen at -70 °C and inactivated by gamma irradiation with 2.1 megarads absorbed dose. Inactivated bacteria were dialyzed against sterile deionized water using a 3500 MW restriction membrane and then lyophilized. The LPS fraction was obtained by extraction of whole bacteria in a mixture of sterile deionized water and phenol (Sigma Chemicals, molecular biology grade) in a modification of the method of Westphal and Jann.[50] The heat killed bacteria were suspended in a mixture of water/phenol (1.2/1.0 v/v) and placed at 68 °C for 15 min in screw capped Teflon® centrifuge tubes. Phase separation was accomplished by centrifugation at 10,000 x g for 30 min at room temperature. The aqueous phase was collected. An equivalent volume of sterile deionized water was added to the remaining phenol phase and phenol/water interface, placed at 68 °C for 10 min, and the second aqueous phase collected by centrifugation as described above. This procedure was repeated once more for a total of three aqueous phases. The three aqueous phases were pooled and dialyzed against sterile deionized water for 4 days at 4 °C with daily changes of dialysate. The dialyzed phenol-water extract was freeze dried. The yield of the phenol-water extract was 15 % of the whole cells calculated as dry weight. A 50 mg (dry weight) portion of the extract was treated at 37 °C for 16 hours in a 10 mL solution of 50 mM Tris (pH 7.4, BioRad Labs, Richmond, CA), 10 mM $MgC1_2$, 0.2 % sodium azide, 2 mg deoxyribonuclease and 3.5 mg ribonuclease (Sigma Chemicals).

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Proteins were degraded by the addition of 10 mg proteinase K (Sigma Chemicals) for 16 hours at 45 °C. LPS was extracted from the proteinase digest by phenol/water extraction using the same protocol as described above. The yield of purified LPS was 51.4 % (dry weight) of the phenol-water extract preparation. LPS sample purity was examined by Western blots with polyclonal antibody ABE 335 (Critical Reagents. Program, Frederick, MD). Each sample was estimated to be >99 % pure. LPS quantitation was performed using the LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA).

2.8. Cytokine Assay

Cell cultures in the presence or absence of test compound and LPS were incubated (37 °C, 5 % CO_2) for 16 h. Cytokines in culture supernatants were measured using a Meso Scale Discovery (MSD) multi spot array ultrasensitive cytokine kit for human TNF-α, IFN-γ, IL-6, and IL-1β per the manufacturer's protocol. Briefly, the 96-well cytokine assay plate was blocked with 'diluent 2' for 30 min at room temperature with constant shaking at 400 rpm. The calibrators were processed per manufacturer's protocol. Aliquots (25 µL) of both calibrator and sample solutions were separately added to the plate in triplicate and incubated for 2 h at room temperature with constant shaking at 400 rpm. After 2 h, the plates were washed 3 times with 1× PBS + 0.05 % Tween-20, and then 25 µL of the detection antibody solution was added to each well of the plate. The plate was incubated for 2 h at room temperature with constant shaking at 400 rpm. After 2 h, the plates were washed 3 times with 1×PBS + 0.05 % Tween-20. Finally, 150 µL of 2× Read Buffer T was added to each well of the plate and analyzed on the SECTOR Imager. The assay results were read using an MSD SECTOR Image 2400 incorporating a CCD. Sample cytokine concentrations were determined with Softmax Pro Version 4.6 software following manufacturer's specifications. Averaged cytokine levels for TNF-α, IFN-γ, IL-6, and IL-1β were expressed as percent inhibition relative to cytokine levels in untreated, LPS-induced cells (i.e., % Inhibition = 100 – {100* [cytokine concentration in compound-treated LPS-induced cells/cytokine concentration in LPS-induced cells]}). In certain examples, apparent activation of cytokines in the presence of a test compound and LPS was observed and this data was reported as having no inhibitory effect. Results were based on cytokine levels that occurred within the linear range of the LPS-induced cytokine response curve. Concentration (i.e., dose)-response curves were obtained by plotting the data using Graphpad Prism (La Jolla, CA) on a semi-log plot and IC_{50} values obtained by non-linear curve fitting.

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2.9. NF-κB Transcriptional SEAP-Reporter Assay

Stably-expressing TLR4/MD-2/NF-κB/SEAPorter™ HEK 293 cells (5×10^5 cells/ 0.5ml/well) were cultured in 24-well plates with varying concentrations of test compound, or test compound and TIR domain protein for competitive binding experiments. After 30 min, *F. tularensis* LPS extract (10 μg/mL final concentration) was added and the cells incubated at 37 °C for 16 h. The culture supernatant was collected and centrifuged to remove any cell debris. The Great EscAPe SEAP Assay from Clonetech was used to determine the amount of alkaline phosphatase secreted into the supernatant following the manufacturer's specifications. A 1× dilution buffer was prepared and a 75 μL aliquot was mixed with 25 μL of the supernatant, then incubated for 30 min at 65 °C to inactivate endogenous alkaline phosphatase. The samples were placed on ice for 3 min and then equilibrated at room temperature. SEAP substrate solution (100 μL) was added to each sample and light intensity was read at 10 minute intervals using a chemiluminescent plate reader. Averaged values were expressed as percent inhibition relative to cytokine levels in untreated, LPS-induced cells. Concentration (i.e., dose)-response curves were obtained by replotting the data using Graphpad Prism (La Jolla, CA) on a semi-log plot and IC_{50} values obtained by non-linear curve fitting.

2.10. Preparation of MyD88 TIR Domain Protein

Preparation of MyD88 TIR domain protein was adapted from a published protocol.[43] The open reading frame encoding the TIR domain of human MyD88 (residues 157–296) was amplified from pCDNA3-MyD88-GFP (Addgene plasmid 13026, Addgene, Cambridge, MA, USA) and inserted into pDONR201 (Invitrogen, Carlsbad, CA,). The gene was sequence verified and then inserted into the destination vector pDEST-HisMBP by Gateway recombinatorial cloning to generate expression vector pPS2218.[51]

The recombinant His-MBP-MyD88(157–296) fusion protein was expressed in E. coli BL21(DE3) Codon Plus-RIL cells (Stratagene, La Jolla, CA), which were grown in Luria broth and induced at mid-log phase with 1 mM IPTG for 4 h at 30 °C. The cells were harvested by centrifugation at 4 °C and frozen at -80 °C until use. The cell pellet was resuspended in 50 mM sodium phosphate pH 7.1, 150 mM NaCl, 5 % glycerol, 25 mM imidazole, and then the cells were disrupted using an APV Model G1000 homogenizer (Invensys, Roholmsvej, Denmark). The lysate was centrifuged at 15,000 rpm at 4 °C, filtered, and the fusion protein was purified by immobilized metal affinity chromatography (IMAC) as described.[52] Fractions containing the fusion protein were pooled, cleaved overnight with hexahistidine-tagged TEV protease, and then subjected to another

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round of IMAC as described.[52] The flow-through fractions were pooled, concentrated to 5 mL and applied to a 320 mL XK26/60 Sephacryl S-100 gel filtration column (GE Healthcare, Piscataway, NJ) equilibrated in 25 mM 2-(*N*-morpholino)ethanesulfonic acid pH 6.3, 150 mM NaCl, and 2 mM tris(2-carboxyethyl)phosphine hydrochloride. The peak fractions corresponding to MyD88 (157–296) were pooled and concentrated to approximately 1 mg/mL.

2.11. F. tularensis Live Vaccine Strain Infection of hPBMCs

For compound treatment pre-infection, test compound was added to hPBMCs and after a 30 min incubation the cells were infected with *F. tularensis* live vaccine strain (LVS) obtained from USAMRIID. Frozen aliquots of *F. tularensis* LVS were thawed and grown in chocolate blood agar plate for 48 hours at 37 °C. The bacterial colonies were then resuspended in 5-10 mL liquid medium BGEM (Lonza, MD). Optical Density (OD) was taken at 600 nm to calculate the Colony Forming Unit (CFU). Mononuclear cells at $1x10^6$/mL RPMI 1640 in the presence or absence of 5 % AB human serum per tube were prepared then treated with a concentration-range of test compound at 500 µM, 100 µM, 10 µM, 1 and 0 µM. After 30 min incubation at 37 °C, cells were infected with *F. tularensis* LVS at a MOI of 1:5 for 2 hours at 37 °C. After 2 hours, cells were isolated by centrifugation at 1200 rpm and the supernatant was carefully discarded. Fresh medium (1 mL) containing gentamycin (10 µg/mL) and the same test compound at specified concentration were added to the cells. Supernatants were collected at 48 hours and analyzed with the MSD cytokine assay as described above.

For post-infection experiments, cells were infected with aliquots of *F. tularensis* LVS as described above. After a 30 min incubation, the infected cells were isolated by centrifugation and resuspended in media as described above, then treated with a concentration-range of test compound. Supernatants were collected at 48 hours and analyzed with the MSD cytokine assay as described above.

2.12. F. tularensis Live Vaccine Strain Infection of Broncho Epithelial cells

Human bronchial epithelial cells (16HBE14o-) were obtained from Dr. D. C. Gruenert, California Pacific Medical Center Research Institute, San Francisco, CA. 16HBE14o- cells were grown to 80 % confluence in 24-well plates and sub-cultured in EMEM supplemented with 10 % FBS. All experiments in this study were performed on 16HBE14o- cells between 4-15 passage levels. For development of polarized 16HBE14o- cells, the cells were trypsinized with the Reagent Pack

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subculture system (Lonza Biological, Walkersville MD), then resuspended in bronchial epithelial growth medium (BEGM) at 5 x 10^5 cells/mL. Aliquots (500 µL) of the resulting cell suspension were added to collagen-coated inserts (1.0 mm, 24-well plate, BD Biosciences, Farming MA). Medium in both the apical and basal chambers was changed every 48 h. Cells were cultured until polarized monolayers developed (approximately 8 days in culture) and apical cell layers were used in these experiments.

For compound treatment pre-infection, media was removed and replaced with 300 µL of either BEGM or BEGM containing test compound (final concentration 100 µM, 10 µM, 1 and 0 µM). After a 30 min incubation, 300 µL of BEGM containing *F. tularensis* LVS (1µg/mL) was added to give a MOI of 1:5 and incubated for 2 hours at 37^o C. Cells were isolated by centrifugation 1200 rpm and the supernatant was discarded. Fresh medium (0.5 ml) containing gentamycin (10 µg/ml) and the same test compound at specified concentration were added to the cells. Supernatants were collected after 48 hours and analyzed by MSD cytokine assay as described above.

For post-infection experiments, human bronchial epithelial cells were infected with aliquots of *F. tularensis* LVS at a MOI of 1:5 as described above. After a 2 h incubation, the infected cells were isolated by centrifugation and resuspended in media as described above, then treated with a concentration-range of test compound. Supernatants were collected after 48 hours and analyzed by MSD cytokine assay as described above.

3. Results

3.1. Chemistry

Compounds were synthesized to mimic a key tripeptide sequence of the BB-Loop region of the TIR domains of human MyD88 and TLRs (Table 1). Three regions of the molecule were targeted for structural optimization: i) an N-terminal phenylalanine present in TLRs 1, 2 and 4, ii) a central region for leucine/isoleucine/valine present in TLRs and MyD88 and iii) a C-terminal proline present in TLRs 1, 2, 4 and MyD88. All analogs were purified to >98 % by preparative HPLC. Compounds were tested iteratively to identify structural features that conferred compound potency for inhibition of cytokines.

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Solution phase peptide coupling chemistry readily provided the target tripeptide derivatives (Figure 1). EDC-mediated coupling of *N*-Boc-L-Leu-OH (**2**) with secondary amines (e.g., pyrrolidine) provided a C-terminal amide proline surrogate without appreciable loss of stereochemistry. Following removal of the N-terminal Boc group to give amine **3**, diverse N-terminal substituents were introduced by reaction of amine **3** with either sulfonyl chlorides or carboxylic acids in the presence of EDC. A dihydrocinnamyl group present in **AS1** was incorporated in an analogous leucine scaffold (*i.e.*, **4**), as well as analogs **5** and **6** with hydroxylated dihydrocinnamyl groups. Alternatively, the ethylene tether present in dihydrocinnamyl was truncated in the preparation of benzoyl amides with alkyl (**7**), hydroxyl (**8**), or cyano (**9**, **10**) substituents on the aryl ring. Sulfonamides were also explored as alternatives to amide bonds in the N-terminal region. Benzenesulfonamide analogs were prepared that were either unsubstituted (**11**) or substituted with alkyl (**12**, **13**, **14**, **15**), chloro (**16**), cyano (**17**, **18**), alkoxy (**19**, **20**, **21**), acetamido (**22**) and methanesulfonyl (**23**) functional groups on the aryl ring. These compounds represented a range of N-termainl linkers, aryl substituents and hydrogen bonding groups designed to identify substructures in the N-terminal aryl region associated with compound potency for inhibition of LPS-induced cytokines.

In the central region of the scaffold, non-peptidic substructures were investigated as an alternative to the valine/leucine/isoleucine present in TIR domains of TLRs and MyD88 (Figure 2A). A series of piperidine 2-, 3-, and 4-carboxylic acids (*i.e.*, scaffolds A1, A2, and A3) were prepared to test how altering the position of the C-terminal pyrrolidine amide would affect compound potency. An octahydroindole scaffold (*i.e.*, scaffold B1) was prepared to provide a more hydrophobic side chain in the central region of the molecule. Tripeptide derivatives based on scaffolds A1, A2, A3 and B1 were synthesized using EDC-mediated peptide chemistry as described above using either N-Boc protected piperidine carboxylic acids or N-Boc protected octahydroindole carboxylic acid. Following removal of the N-terminal Boc group, N-terminal aromatic substituents were introduced in the last step to provide compounds **24**-**39** that contained either the dihydrocinnamyl group of **AS1** or diverse N-terminal aryl substituents (i.e., 'R' groups in Figure 2). A second series of compounds (*i.e.*, **40**-**56**) based on scaffolds A1, A2, A3 and B1 was prepared by substituting the C-terminal pyrrolidine amide for a larger and more lipophilic *N*-methyl-*N*-cyclohexylamide (Figure 2B). Together, fifty-three compounds were prepared that represented a range of substructures in the three regions of the **AS1** scaffold. Compounds were prepared and tested iteratively for inhibition of TLR-MyD88 signaling as described below.

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3.2. Biological Evaluation of Compounds

3.2.1. Effect of Compounds on Cytokines Levels in Human Peripheral Blood Mononuclear Cells

Compounds **4**-**56** were tested for inhibition of cytokines in primary human peripheral blood mononuclear cells (hPBMCs) treated with *F. tularensis* LPS extracts (10 μg/mL) using an adaptation of protocols previously described for other pathogens.[43, 44] Cells were pre-incubated with test compound for 30 minutes and then exposed to LPS extracts from *F. tularensis* (10 μg/mL) for 16 hours. Cytokine response was measured using a MSD human proinflammatory fourplex ultrasensitive kit (TNF-α, IFN-γ, IL-6, IL-1β) with electrochemiluminescent output.[44] The calibrated response curve demonstrated that all four cytokines gave a linear response in the range of 1 pg/mL to 10 μg/mL of LPS extracts. hPBMCs that were not treated with LPS averaged 51 pg/mL of TNF-α, 54 pg/mL of IFN-γ, 18 pg/mL of IL-1β and 44 pg/mL of IL-6 (i.e., baseline levels). hPBMCs treated with *F. tularensis* LPS extracts (10 μg/mL) averaged 9400 pg/mL of TNF-α, 470 pg/mL of IFN-γ, 13000 pg/mL of IL-6 and 3600 pg/mL of IL-1β. For compound-treated cells, percent inhibition of TNF-α, IFN-γ, IL-6 and IL-1β levels were expressed as percent inhibition of cytokine levels relative to LPS-treated cells in the absence of test compound (Figure 3).

Nineteen compounds were identified that provided greater than 10 % average inhibition of the four cytokines in *F. tularensis* LPS-induced hPBMCs. Among compounds with diverse N-terminal aryl substructures (*i.e.*, compounds from Figure 1), compound **7** with a 4-ethylbenzoyl amide was one of the most potent analogs and inhibited 99 % of TNF-α, 96 % of IFN-γ, 99 % of IL-6 and 21 % of IL-1β at 100 μM. By contrast, analogs of compound **7** that incorporated either a 4-hydroxy-substituent in **8** or cyano-substituents in **9** and **10** were less effective or inactive at this concentration. Substituted benzenesulfonamides were found to be effective alternatives for benzenecarboxamide substructures in the N-terminal region as observed for compounds **13**, **15**, **16**, **17** and **20**. Alkyl-substituted benzenesulfonamides **13** and **15** gave an average of 16 % and 19 % inhibition of the four cytokines in *F. tularensis* LPS-induced hPBMCs, respectively. Compound **16** with a 4-chloro aryl substituent emerged as the most potent benzenesulfonamide in this structural series and inhibited 98 % of TNF-α, 84 % of IFN-γ, >99 % of IL-6 and 4 % of IL-1β. Unsubstituted benzenesulfonamide **11** was inactive at this concentration (*i.e.*, 100 μM). Other substituted benzenesulfonamides were identified that inhibited LPS-induced cytokines in hPBMCs, including the 3-cyanobenzenesulfonamide **17** and 3,4-dimethoxybenezenesulfonamide **20**. Compound **17** was marginally more potent than **20** for average inhibition of cytokines, and **17** inhibited 55 % of TNF-α, 56 % of IFN-γ, 40 % of IL-6 and 47 % of IL-1β. From this compound series (i.e., Figure 1 compounds), compounds **7** and **16** provided the greatest attenuation of cytokine levels with an average

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79 % and 71 % inhibition of the four cytokines at 100 μM, respectively. The results supported that non-polar alkyl- and chloro-substituted aryl groups were a structural feature in the N-terminal region associated with compound potency, although this effect showed some dependency on substituent ring position. Neither **AS1** nor its leucine analog **4** attenuated LPS-induced cytokines in hPBMCs at these concentrations.

Non-natural scaffolds in the core region of the tripeptide (*i.e.*, scaffolds A1, A2, A3 and B1 from Figure 2) were tested for inhibition of cytokines in LPS-challenged hPBMCs. In the first series of compounds (i.e., Figure 2A compounds), the C-terminal region of these non-natural scaffolds was maintained as a pyrrolidine amide to mimic a proline amide in this region. For the 2-piperidinecarboxylic acid series (*i.e.*, scaffold A1), compounds **24** and **28** were identified as inhibitors of cytokines in *F. tularensis* LPS-challenged hPBMCs. Compound **28** with an N-terminal 4-chlorobenzenesulfonamide substructure was more potent than **24** with an N-terminal dihydrocinnamyl substructure, which was consistent with SAR trends observed in the N-terminal region. Compound **28** inhibited 66 % of TNF-α, 51 % of IFN-γ, 33 % of IL-6 and 47 % of IL-1β at 100 μM. For the 3-piperidinecarboxylic acid series (*i.e.*, scaffold A2), only compound **32** attenuated cytokines with a 21% average inhibition of the four cytokines. These results supported a positional dependency of the pyrrolidine amide on compound potency, where compounds with a pyrrolidine amide in either the 2- or 4-position were more potent than 3-substituted piperidine carboxamide analogs. In the 4-piperidinecarboxylic acid (*i.e.*, scaffold A3) series, both compound **30** that contained an N-terminal 4-chlorobenzenesulfonamide and compound **37** that contained an N-terminal 4-(*tert*-butyl)benzenesulfonamide inhibited cytokines. These results with **30** and **37** were consistent with prior structure-activity trends observed in the N-terminal region. Compound **30** was the most potent compound among the piperidine carboxylic acid analogs tested in this series and inhibited 99 % of TNF-α, 90 % of IFN-γ, 86 % of IL-6 and 97 % of IL-1β at 100 μM.

Compounds with non-natural core scaffolds (*i.e.*, scaffolds A1, A2, A3 and B1 from Figure 2) wherein the C-terminal pyrrolidine amide was changed to a more bulky and lipophilic *N*-methyl-*N*-cyclohexylamide were also tested (*i.e.*, Figure 2B compounds). A trend emerged that compounds with a C-terminal *N*-methyl-*N*-cyclohexylamide substructure were generally more potent than analogous C-terminal pyrrolidine amides. For the 2-piperidinecarboxylic acid series compounds (i.e., scaffold A1) incorporating a C-terminal *N*-methyl-*N*-cyclohexylamide, compound **40** with an N-terminal dihydrocinnamyl amide and compound **45** with an N-terminal 4-(methanesulfonyl)benzenesulfonamide were both potent inhibitors of cytokines, although analogous

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compounds in the pyrrolidine amide series (*i.e.*, **24** and **31**) were considerably less potent or inactive at the same concentration. Compound **40** inhibited >99 % of TNF-α, 93 % of IFN-γ, >99 % of IL-6 and 99 % of IL-1β levels. Compound **45** inhibited 79 % of TNF-α, 54 % of IFN-γ, 40 % of IL-6 and 51 % of IL-1β levels. Also in the 2-piperidinecarboxylic acid series, compound **53** with an N-terminal 4-cyanobenzoyl amide was identified as one of the most potent inhibitors of cytokines and inhibited >99 % of TNF-α, IFN-γ, IL-6 and 99% of IL-1β at 100 μM. For the 3-piperidinecarboxylic acid series compounds (*i.e.*, scaffold A2) incorporating a C-terminal *N*-methyl-*N*-cyclohexylamide, compounds **41**, **50** and **56** were identified as inhibitors, unlike their C-terminal pyrrolidine amide analogs. However, these compounds were of modest potency and inhibited TNF-α, IFN-γ, IL-6 and IL-1β in the range of 0-57 %. Again, the results supported that the 3-piperidinecarboxylic acid derivatives were less effective than the 2-piperidinecarboxylic acid analogs. Among 4-piperidinecarboxylic acid analogs, none of the compounds tested with a C-terminal *N*-methyl-*N*-cyclohexylamide provided greater than 10 % average inhibition of cytokines that marked a departure from structure-activity trends observed in the C-terminal pyrrolidine amide series. Compound **52** based on the octahydroindole scaffold inhibited cytokines at 100 μM in contrast to the analogous C-terminal pyrrolidine amide **39** that was inactive at this concentration. Compound **52** inhibited 40 % of TNF-α, 47 % of IFN-γ, 66 % of IL-6 and 13 % of IL-1β levels. Overall, tripeptide analogs with an *N*-methyl-*N*-cyclohexylamide in the C-terminal region were found to be among the most potent inhibitors of proinflammatory cytokines in hPBMCs challenged with *F. tularensis* LPS extracts. The 2-piperidinecarboxylic acid derivatives **40** and **53** identified in this structural series were the most potent compounds identified in this study.

From these studies, compounds **7**, **16**, **17**, **20**, **28**, **30**, **40**, **45**, **52** and **53** were identified as the most effective inhibitors of cytokines in *F. tularensis* LPS-challenged hPBMCs *in vitro*. These compounds showed the greatest average inhibition of the cytokines tested and were effective across TNF-α, IFN-γ, IL-6 and IL-1β. As **AS1** and analog **4** were ineffective at attenuating cytokines in response to exposure to *F. tularensis* LPS, the new compounds represented a significant advance in using small molecules to block MyD88-mediated TLR signaling by *F. tularensis* LPS.

3.2.2. Compound Stability in Human Microsomes and Serum

The stability of potent compounds identified above was tested via a time-course study in the presence of human liver microsomes.[53] Compounds were incubated in microsomal protein preparations and the change in compound concentration was determined at specified time points by

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mass spectrometry. The half-life of the compound in the presence of human microsomes was determined using a pseudo first-order kinetic analysis based on an observed decrease in compound peak area (Table 2).

Microsomal stability studies confirmed that **AS1** exhibited fast loss in the presence of human liver microsomes with a half-life of 9 minutes. Some of the new molecules resulted in a significant increase in compound half-lives in the presence of hepatic microsomes. Compounds **7** and **8** derived from substituted benzoic amides in the N-terminal region had half-lives of 57 min and 120 min, respectively. Among N-terminal sulfonamides, compound **20** with an N-terminal 3,4-dimethoxybenzenesulfonamide had a half-life of 50 minutes. Compound **17** with an N-terminal 3-cyanobenzenesulfonamide had a half-life of 26 minutes. However, many of the other potent compounds in this series, such as compounds **13**, **15**, and **16** derived from alkyl- or chloro-substituted benzenesulfonamides, were relatively unstable (*i.e.*, $t_{1/2}$ <16 min). Compounds derived from piperidinecarboxylic acid substructures (i.e., **28**, **30**, **40**, **50**, **52**, **53** and **56**) all exhibited rapid loss in the presence of human liver microsomes, irrespective of the position of the C-terminal amide on the piperidine ring. The octahydroindole-derived compound **45** with a C-terminal *N*-methyl-*N*-cyclohexylamide group was also rapidly lost in the presence of human liver microsomes (*i.e.*, half-life < 3 min). Thus, compound **7** emerged as the lead candidate due to its potent inhibition of all four cytokines (*i.e.*, TNF-α, IFN-γ, IL-6 and IL-1β) in LPS challenged hPBMCs, while showing good stability in the presence of human liver microsomes. Compounds **16**, **17** and **20** also exhibited improved stability in the presence of human liver microsomes, but these compounds were less potent than compound **7** for in vitro attenuation of cytokines. Subsequently, chemical stability of compound **7** was tested in human serum at 37 °C by adaptation of an established HPLC method.[48] No loss of **7** was observed for up to 64 h post incubation that confirmed excellent chemical stability of the compounds to serum degradation, which included positive controls for serum amidase activity.[49]

3.2.3. Validation of TLR-MyD88 Signaling Inhibition by Compound 7: F. tularensis LPS-Induced SEAP Reporter Gene Expression Driven by the Transcriptional Activation of NF-κB.

To further characterize the effect of compound **7** on blocking MyD88-mediated TLR signaling in response to LPS stimulation, a secreted alkaline phosphatase (SEAP) reporter gene under the transcriptional control of NF-κB was examined using an established reporter gene construct (TLR4/MD-2/NF-κB/SEAPorter™) in HEK-293 cells. SEAP activity was determined using a pro-chemiluminescent substrate (Figure 4). Cells treated with LPS extract from *F. tularensis* showed a

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significant induction of Nf-κB driven SEAP reporter expression compared to untreated cells (i.e., 3.7-fold; $p < 0.001$). In the presence of compound **7** (i.e., 0, 1, 10, 100 or 500 μM), a concentration-dependent inhibition of SEAP expression was observed (Figure 4A). Compound **7** was effective at concentrations as low as 1 μM that gave 7 % inhibition of SEAP expression ($p < 0.01$) and inhibited > 98 % of SEAP expression at 500 μM. Compound **7** had an IC_{50} of 27 μM for *F. tularensis* LPS-induced SEAP expression. Since the SEAP reporter construct expresses full length TLR4 and *F. tularensis* LPS was previously shown to agonize the TLR4 receptor,[31] the results suggest that compound **7** specifically blocked TLR4-MyD88-dependent signaling in *F. tularensis* LPS-challenged cells.

Specific inhibition of TIR domain interactions by compound **7** was tested in a competition experiment using MyD88 TIR domain protein. HEK-293 cells containing the NF-κB driven SEAP reporter gene construct (TLR4/MD-2/NF-κB/SEAPorter™) were incubated with *F. tularensis* LPS (10 μg/mL) and compound **7** that lowered SEAP expression to 45 % of the untreated LPS-induced cells (i.e., 55 % inhibition). TIR domain protein from MyD88 was then added in a concentration range (0-100 μg/mL) and the resulting SEAP activity was measured (Figure 4B). TIR domain protein concentration-dependently lessened the inhibitory effect of **7** on SEAP reporter expression induced by *F. tularensis* LPS. TIR domain protein at 100 μg/mL resulted in a return to 81 % SEAP expression (i.e., 21 % inhibition) that represented a 2.6-fold lessening in the inhibitory effect of **7** at this concentration. For comparison, bovine serum albumin (BSA) protein added in the same concentration range (0-100 μg/mL) showed no obvious concentration-dependent effects in the presence of compound **7** with 52 % of SEAP expression remaining (i.e., 48 % inhibition) at 100 μg/mL of BSA. Control experiments showed that TIR domain protein had no significant effect on SEAP expression induced by *F. tularensis* LPS in the absence of **7**. In addition, neither TIR domain protein nor compound **7** significantly affected basal levels of SEAP reporter expression in cells not treated with LPS. These results supported that compound **7** specifically interacts with TIR domain protein for inhibition of LPS-induced TLR signaling.

3.2.4. Investigation of Compound 7 with a Live Vaccine Strain of F. tularensis: Pre- and Post-Exposure Scenarios with hPBMCs and Human Bronchial Epithelial Cells

Compound **7** was tested in hPBMCs or human bronchial epithelial cells exposed to a *F. tularensis* LVS at a multiplicity of infection (MOI) of up to 1:5 (human cells: *F. tularensis*). A concentration range of compound **7** (i.e., 0, 1, 10, 100 or 500 μM) was administered to hPBMCs 30

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minutes prior to exposure to *F. tularensis* LVS and cytokine levels were measured 48 hours following exposure (Table 3). hPBMCs challenged with *F. tularensis* (MOI of 1:5) in the absence of **7** gave a large induction of TNF-α (250-fold), IFN-γ (130-fold), IL-6 by (240-fold) and IL-1β (330-fold). In the presence of compound **7**, a concentration-dependent inhibition of cytokines was observed with IC_{50} values for TNF-α of 49 μM, IFN-γ of 12 μM, IL-6 of 65 μM and IL-1β of 54 μM. Compound **7** was also effective at reducing cytokine levels in primary human bronchial epithelial cells. Human bronchial epithelial cells exposed to *F. tularensis* LVS (MOI of 1:5) gave a significant induction of cytokines, but the fold-change induction in cytokines was lower compared to hPBMCs. Human bronchial epithelial cells exposed to *F. tularensis* LVS (MOI of 1:5) increased TNF-α (4.5-fold), IFN-γ (3.0-fold), IL-6 (4.3-fold) and IL-1β (2.6-fold). In the presence of compound **7**, a concentration-dependent inhibition of cytokines was observed with IC_{50} values for TNF-α of 50 μM, IFN-γ of 21 μM, IL-6 of 12 μM and IL-1β of 7 μM. Accordingly, compound **7** inhibited TNF-α, IFN-γ, IL-6 and IL-1β levels in a narrow range for both cell types (*i.e.*, IC_{50} values ranged from 12-65 μM for hPBMCs and 7-50 μM for bronchial epithelial cells).

Compound **7** was also tested in a post-exposure scenario. Compound **7** was administered to hPBMCs or human bronchial epithelial cells after the cells had been exposed to *F. tularensis* LVS (MOI = 1:5) and cytokine levels were measured 48 hours following exposure (Table 4). For hPBMCs, compound **7** gave a concentration-dependent inhibition of cytokines albeit with lower potency than for a pre-treatment scenario. hPBMCs challenged with *F. tularensis* LVS (MOI of 1:5) and then treated with a concentration-range of **7** after 30 minutes provided IC_{50} values for TNF-α of 67 μM, IFN-γ of 210 μM, IL-6 of 99 μM and IL-1β of 170 μM. Primary human bronchial epithelial cells were challenged with *F. tularensis* LVS (MOI of 1:5) for a longer duration (i.e., 2h) to increase the relative induction of cytokine levels. Primary human bronchial epithelial cells challenged with *F. tularensis* LVS (MOI of 1:5) and then treated with a concentration-range of **7** after 2 hours provided IC_{50} values for TNF-α of 13 μM, IFN-γ of 11 μM, IL-6 of 12 μM and IL-1β of 5 μM. For bronchial epithelial cells, compound **7** inhibited TNF-α, IFN-γ, IL-6 and IL-1β cytokine levels in a comparable range as the pre-exposure scenario (*i.e.*, IC_{50} range of 7-50 μM for pre-exposure and 5-13 μM for post-exposure). The results may suggest some differences in the efficacy of compound **7** compared among hPBMCs and bronchial epithelial cells, however the induction of cytokines in bronchial epithelial cells was also generally lower than observed for hPBMCs. Collectively, these *in vitro* results support that targeting TIR domain interactions of the TLR/IL receptor and MyD88 was an effective approach to attenuating cytokines in either a pre- or post-exposure scenario to a live vaccine strain of *F. tularensis*.

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4. Discussion

Sepsis is a complex condition that poses challenges to therapeutic development in part because it remains unknown if targeting a single signaling pathway can lead to an effective treatment for the immunological effects.[54] Given the important role of TIR domain interactions in MyD88-dependent TLR/IL receptor signaling across multiple receptor subtypes, small molecules were developed to inhibit proinflammatory cytokines by targeting this signaling junction. This strategy targets a host's immune response to inflammatory pathogens rather than the pathogen itself and may have potential applications as an adjunctive therapy for sepsis.[39, 40, 55] With hPBMCs used in this study, exposure to *F. tularensis* induced a marked increase in NF-κb and TNF-α, IFN-γ, IL-1β and IL-6 cytokines that was consistent with the established role for MyD88-dependent TLR/IL receptor signaling for induction of proinflammatory cytokines.[29, 56, 57] Small molecule development was guided by mimicking TIR domain regions known to be important to TLR/IL receptor interactions with MyD88. Compound **7** emerged from testing several candidate tripeptide mimetics and **7** potently decreased proinflammatory cytokines in hPBMCs exposed to *F. tularensis* with comparable potency across the cytokines tested. Compound **7** was also effective in human bronchial epithelial cells that relate to the respiratory effects of sepsis that are considered a particularly severe form of tularemia.[21] While it remains to be seen if such a strategy can be effective in vivo, targeting the host inflammatory signaling pathways with small molecule **7** attenuated cytokines in a pre-exposure or post-exposure scenario for isolated cells.

Structural trends were identified among those compounds that most potently inhibited cytokines in hPBMCs exposed to *F. tularensis*. Substitution of the central valine residue in **AS1** for a leucine residue in compound **7** was a structural feature associated with biological potency. This structural modification was consistent with the presence of a leucine in the target region of the TIR domain of MyD88 or an isoleucine in the TIR domains of TLR2 and TLR4.[58, 59] Other substitutions for the central valine of **AS1** resulted in the identification of piperidinecarboxylate-derived compounds (*e.g.,* **30**, **40**, **50** or **53**) that potently inhibited proinflammatory cytokine levels. Although this represented an interesting structural development by changing the spatial relation of the C-terminal and N-terminal groups, these piperidinecarboxylate derivatives were rapidly lost in the presence of human liver microsomes and so were not further pursued. In the N-terminal region, alkyl- or chloro-substituted benzoyl amides or benzenesulfonamides were identified as structural features associated with a marked increase in potency. Lipophilic aryl substituents in this phenylalanine mimetic region may improve binding, but the positional-dependency of these substituents on compound potency suggested that the effects were not solely due to indiscriminate binding by lipophilic molecules. Based on both the structural resemblance of compound **7** to the target tripeptide sequence and interactions of

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7 with the MyD88 TIR domain, compound **7** may indeed function as a peptidomimetic inhibitor of TIR domain interactions. However, further resolution of the binding site of **7** is still needed. One approach might be to conduct competitive binding experiments using radiolabeled compounds and isolated TIR domain proteins, where MyD88 inhibitors that have established binding sites may be used to test for site-specificity of the new compounds. However, alternative binding modes for compound **7** cannot be excluded and detailed characterization of a TIR domain protein complex with compound **7** may be necessary to more precisely attribute the molecular basis for increased potency. Co-crystallization of TIR domains and compound **7**, or solution phase NMR binding experiments are two possibilities to obtaining this information. Compound **7** was a potent inhibitor of cytokines, but also resulted in much greater stability of the compound in the presence of human liver microsomes and so represented a significant advance in development a drug-like small molecule.

Compound **7** and most analogs prepared in this study have not been previously reported, however other classes of small molecules and glycolipids have been developed as modulators of proinflammatory signaling at the TLR level (*i.e.*, upstream in the signaling pathway).[60] Among TIR domain inhibitors, **AS1** that targeted the box 2 of TIR domain for inhibition of IL-1 receptor signaling represents a close structural comparison to compound **7** in that both compounds are small tripeptide derivatives intended to mimic similar regions of TIR domains.[39] The inhibitory effect of compound **7** on TLR signaling represents a key distinction among these molecules. Other molecules targeting box 2 of TIR domains have been developed that typically employed larger molecules designed to mimic larger regions of the TIR domain proteins, including some molecules potentially capable of mimicking dimeric interactions. Synthetic cell-penetrating oligopeptides based on TIR domain sequences were reported to inhibit TIR domain interactions for MyD88 homodimerization,[61] and MyD88-TLR heterodimerization,[62] for attenuation of proinflammatory signaling. Based on some of the peptide work, compound ST2825 was developed as a peptidomimetic inhibitor of the MyD88 TIR domain 'BB-Loop' region that encompassed the box 2 domain targeted by **AS1**.[63] Compound ST2825 was reportedly based on a heptapeptide sequence of this domain and blocked TIR domain interactions induced by IL-1β treatment and reduced IL-6 levels in C57BL/6 mice. Additionally, we have reported a dimerization strategy for **AS1** that gave compounds (i.e., **EM163** and **4210**) that were significantly more potent than **AS1**, and compound **4210** attenuated proinflammatory cytokines induced by several biothreat agents and was efficacious in a lethal shock challenge in BALB/c mice.[42, 44] More recently, *ortho*-vanillin and certain derivatives (e.g., compound C29) were identified as inhibitors of the TIR domain interactions of TLR2 and inhibited TLR2-mediated inflammation in mice.[64] Direct comparisons of potency among compound **7** and the above TIR domain inhibitors are difficult due to the different experimental conditions and proinflammatory stimulants used, but

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compound **7** appears to have comparable potency range as most of the larger peptide derivatives and *ortho*-vanillin (i.e., NF-κb reporter data indicated inhibition in a range of 1-200 μM for most molecules where data is available). Dimeric **AS1** analogs such as **4210** have been shown to be a strategy for increasing the potency of these small tripeptide mimetics.[44] Thus, the potency of compound **7** relative to size of the molecule represents a possibly advantageous property. More generally, the current work further supports that compounds that inhibit TIR domain interactions associated with specific TLR subtypes (e.g., TLR4) might be used as a scaffold for developing inhibitors of the related TIR domains based on structural analogy among these domains. Collectively, these studies show that disrupting protein-protein interactions associated with TIR domain interactions may be a promising strategy for controlling host-mediated inflammatory processes .

Conclusions

Small molecule inhibitors of MyD88-mediated TLR signaling were developed that target TIR domain interaction*s*. Compound **7** inhibited cytokines in both primary human PBMCs and bronchial epithelial cells challenged with *F. tularensis*, including a live vaccine strain. Compound **7** was also effective at lowering cytokine levels in cells post-exposure to *F. tularensis*. Small molecules, such as **7**, that inhibit proinflammatory signaling at the TLR-MyD88 signaling junction may represent a potential strategy for a host-directed, adjunctive treatment for severe inflammatory reactions of sepsis, including those associated with biothreat agents.

Disclosures

Peripheral blood mononuclear cells used in this study were obtained from healthy donors with written consents, in accordance with guidelines of the Human Use Committee (HUC) and an institutional (USAMRIID) review board-approved research donor protocol. Human bronchial epithelial cells (16HBE14o-) were obtained from Dr. D. C. Gruenert, California Pacific Medical Center Research Institute (San Francisco, CA) following institutionally approved protocols and in accordance with the HUC guidelines. The views expressed in this paper are those of the authors and do not purport to reflect official policy of the USAMRIID.

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Competing Financial Interests

The authors declare no competing financial interests.

Supporting Information

Supplementary data related to this article can be found online.

Abbreviations:

CDC, Centers for Disease Control and Prevention; DAMPs, damage-associated molecular patterns; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; hPBMCs, human peripheral blood mononuclear cells; IFN, interferon; IL, interleukin; IMAC, immobilized metal affinity chromatography; IRF, interferon regulatory factor; LPS, lipopolysaccharides; LVS, live vaccine strain; MAP, mitogen-activated protein; MOI, multiplicity of infection; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor κB; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; SIRS, systemic inflammatory response syndrome; TIR, toll-like/interleukin-1 receptor recognition; THF, tetrahydrofuran; TLR, toll-like receptor; TFA, trifluoroacetic acid; TNF, tumor necrosis factor.

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Figure Legends and Tables

Table 1. Sequence alignment of the target BB-Loop region of human TIR domains of MyD88, TLRs and IL-1 receptor.

Protein	Accession Number (UniProtKB)		Sequence*
MyD88	Q99836	196	R D **V L P** G T
TLR1	Q15399	671	R N **F V P** G K
TLR2	O60603	677	R D **F I P** G K
TLR4	O00206	710	R D **F I P** G V
IL-1R1	P14778	428	R D **D Y V** G E

* **AS1**-mimetic sequence in bold.

Figure 1. Synthesized tripeptide derivatives **4**-**23** with diverse N-terminal aryl substituents. i) pyrrolidine (1 equiv.), EDC (1.2 equiv.), HOBt (2.0 equiv.), CH_2Cl_2, 21 °C, 12-16 h, ii) TFA (8 equiv.), CH_2Cl_2, 0 °C, 5-8 h; $NaHCO_{3(s)}$ iii) RCO_2H (1.2 equiv.), EDC (1.2 equiv.), HOBt (2.0 equiv.), CH_2Cl_2, 21 °C, 12-16 h, iv) RSO_2Cl (1.2 equiv.), NEt_3 (1.2 equiv.), CH_2Cl_2, 21 °C, 12-16 h.

Figure 2. Synthesized tripeptide derivatives **24**-**56** with 2-, 3- or 4-piperidinecarboxylic acid substructures (i.e., scaffolds A1, A2 or A3) or a 2-substituted L-octahydroindolecarboxylic acid substructure (i.e., scaffold B1). **A**) Compounds synthesized with a C-terminal pyrrolidine amide, **B**) Compounds synthesized with a C-terminal *N*-methyl-*N*-cyclohexylamide (**B**).

Figure 3. Effects of compounds (100 μM) on cytokine levels in human peripheral blood mononuclear cells exposed to LPS extracts (10 μg/mL) from *F. tularensis*. Compounds were administered to cells 30 min prior to addition of LPS extracts and the concentration of TNF-α, IFN-γ, IL-6, and IL-1β was measured after 16 h. Data was expressed as percent inhibition of cytokine levels relative to LPS-treated cells in the absence of test compound, showing results for compounds that had greater than 10 % average inhibition of TNF-α, IFN-γ, IL-6, and IL-1β.

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Table 2. Half-lives of compounds in the presence of human hepatic microsomes.

Compound	$t_{1/2}$ (min)	Compound	$t_{1/2}$ (min)
AS1	9	**28**	5
7	57	**30**	2
8	120	**40**	2
13	5	**45**	2
15	2	**50**	7
16	16	**52**	2
17	26	**53**	2
20	50	**56**	7

Figure 4. Effect of compound **7** on SEAP reporter expression in HEK 293 cells expressing the TLR4/MD-2/NF-κB/SEAPorter™ construct induced by *F. Tularensis* LPS (10 μg/mL). **A.** Compound **7** concentration-range (0, 1, 10, 100 and 500 μM) was administered to cells for 30 min prior to addition of *F. tularensis* LPS (10 μg/mL) and SEAP activity was measured by chemiluminescence after 16 h. Data was expressed as a percentage of SEAP activity compared to LPS-treated cells in the absence of test compound. Values are the average (n = 3) showing error bars for standard deviation. Error bars were obscured by data symbols in several instances. **B.** Competitive binding experiment using MyD88 TIR domain protein with compound **7**. Human MyD88 TIR domain protein or bovine serum albumin protein control were administered in a concentration range (i.e., 0, 1, 5, 25 and 100 μg/mL) to cells incubated in the presence of compound **7** (100 μM), and *F. tularensis* LPS (10 μg/mL) was added after 30 min. Separately, TIR domain protein (100 mg/mL) was administered to cells in the absence of compound **7** and *F. tularensis* LPS (10 μg/mL) was added after 30 min. SEAP activity was measured by chemiluminescence after 16 h. Data was expressed as a percentage of SEAP activity compared to LPS-treated cells in the absence of test compound. Values are the average (n = 3) showing error bars for standard deviation. Error bars were obscured by data symbols in several instances.

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Table 3. Compound **7** IC_{50} values for cytokines in hPBMCs or human bronchial epithelial cells exposed to *F. tularensis* live vaccine strain 30 minutes after compound treatment.

Cytokine	**IC_{50} (μM)**	
	hPBMCs (MOI = 1:5)	Epithelial Cells (MOI = 1:5)
TNF-α	49	50
IFN-γ,	12	21
IL-6	65	12
IL-1β	54	7

Table 4. Compound **7** IC_{50} values for cytokines in hPBMCs or human bronchial epithelial cells exposed to *F. tularensis* live vaccine strain for 30 min (hPBMCs) or 2 h (epithelial cells) prior to compound treatment.

Cytokine	**IC_{50} (μM)**	
	hPBMCs (MOI = 1:5)	Epithelial Cells (MOI = 1:5)
TNF-α	67	13
IFN-γ,	210	11
IL-6	99	12
IL-1β	170	5

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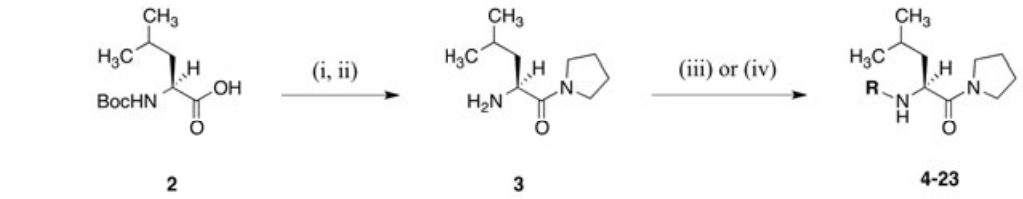

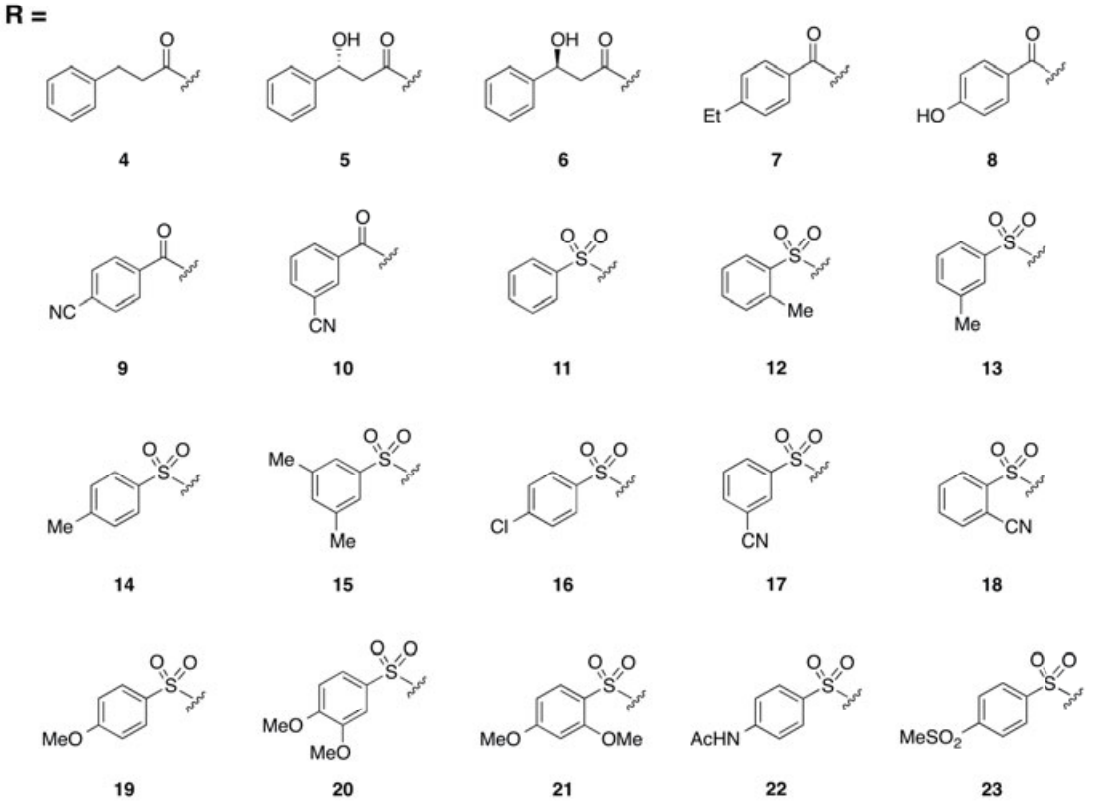

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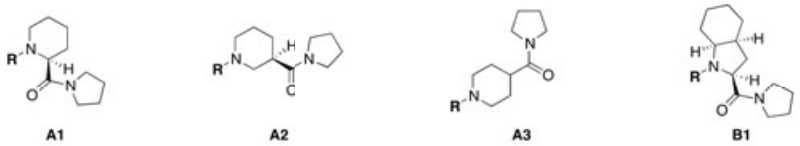

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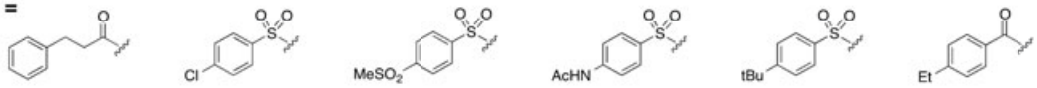

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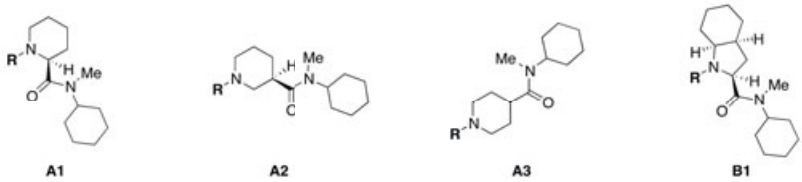

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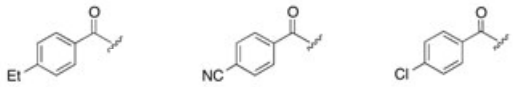

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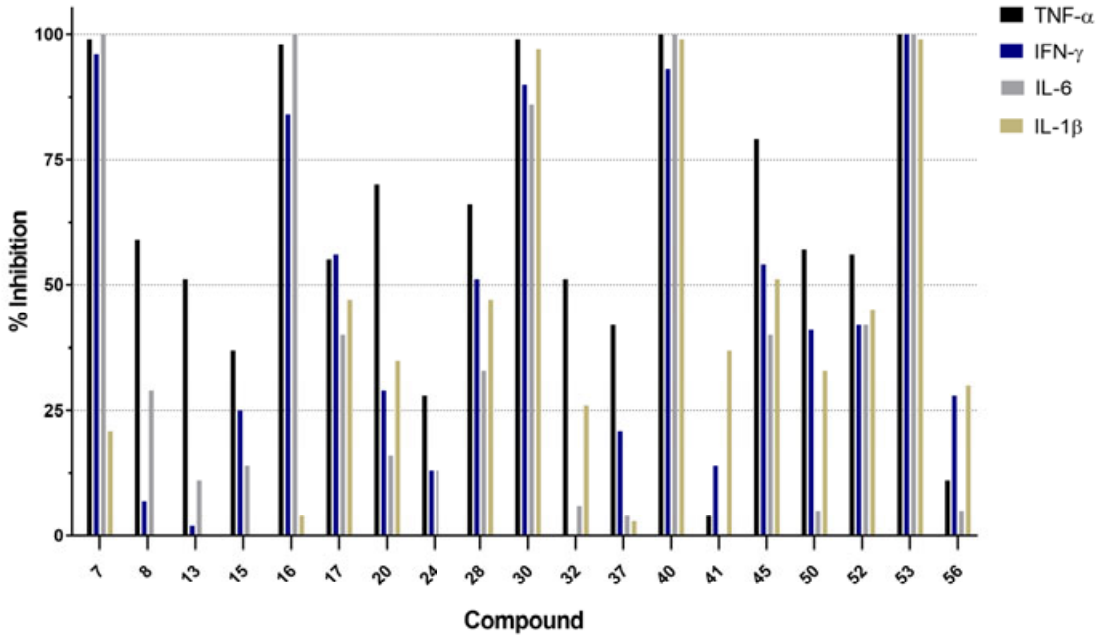

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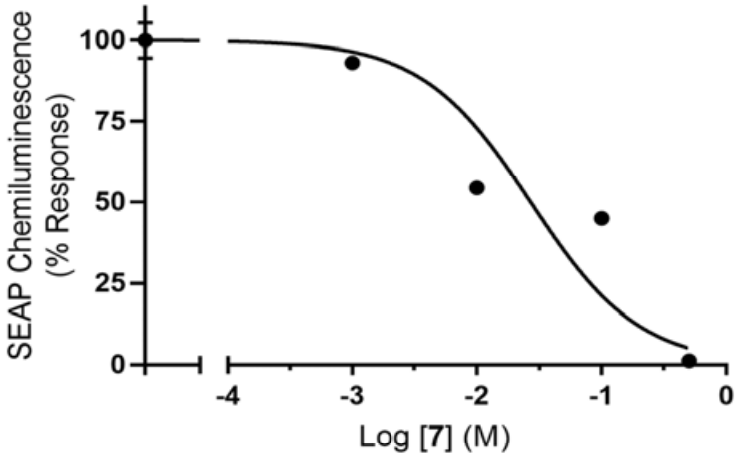

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