Probe Report

Project: High Throughput Screening for Toll-Like Receptors

Grant Number: 1R03MH081265-01

Screening Center: The Scripps Research Institute Molecular Screening Center

Principal Investigator of Screening Center: Hugh Rosen, MD PhD

Assay Submitter & Institution: Professor Peter Tobias, The Scripps Research Institute

PubChem Bioassay Identifer (AID): 811

Assay or Pathway Target: TLR4-MyD88 signaling

Assay Target: TLR4-MyD88 interaction

Probe PubChem Substance Identifier (SID): 26543390

Specific Aim: The goal of this project is to identify inhibitors of TLR4 signaling.

Significance: In atherosclerosis, kidney transplantation and other disease, inappropriate inflammatory responses contribute to poor patient outcomes. Toll-like receptor (TLR) signaling has been strongly implicated in the inflammatory response. Toll-like receptors (TLRs) recognition of microbial components and partner proteins signaling are important elements of the innate immune response[1]. TLRs are type I transmembrane proteins consisting of an extracellular domain and an IL1R homologous intracellular domain and are comprised of at least 9 members. One of the TLRs, TLR4, recognizes lipopolysaccharides and signals through the shared adapter protein, MyD88 leading to activation of the NFKB pathway[2]. TLR4 signals enhance the immune response to bacterial infections but excessive signaling leads to severe inflammation and septic shock.

Rationale: Small molecule inhibitors of TLR4-MyD88 will be useful in exploring the biology of TLR signaling, the NFKB pathway and innate immune responses. Such compounds may serve as starting points for development of therapeutic compounds for inflammatory diseases.

Assay Results

We screened the Maybridge HitFinder library consisting of 16,000 compounds and reported the results of the screen, counterscreening and followup characterization of the confirmed hits. [3]. Screening the 16,000 compound Maybridge Hitfinder collection resulted in a plate average Z' of 0.74. We selected 45 primary hits of which 10 confirmed in the primary assay(Table 1). Dry powder aliquots of these10 compounds were purchased from Maybridge and used for the remaining characterizations. A fluorescent microscope counterscreen using Hela cells containing full-length beta-lactamase was run with the 10 confirmed hits. The counterscreen assay eliminated 5 false positive compounds that directly inhibit beta-lactamase.(Figure 1)

Compounds were tested at 10 uM for inhibition of MyD88 co-immunoprecipitation with TLR4CD. In the time course study, 4 of the 5 compounds were active after 120 minutes (Figure 2). The expression levels of TLR4CD and MyD88 (input) were not changed by the addition of test compounds; indicating that the compounds directly affect the binding between TLR4 and MyD88.

Compounds were also tested for their inhibitory effects on TLR4 signaling (Fig. 3). LPS stimulation was blocked by 50% in the presence of 0.37 uM of SID 2653390.

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Prior Probes:
Anti-inflammatory drugs act via a wide variety of mechanisms, the end result of many is to inhibit NF-KB activation[4]. Several compounds that inhibit LPS mediated NF-KB activation have been previously reported. The synthetic analogue of bacterial lipid A E5531 and a cyclic cationic polypeptide antibiotic, polymyxin B (PMB) inhibit TLR signaling via interactions with LPS[5, 6]. Recently, the anti-inflammatories, Auranofin and Curcumin, at 10 and 20 micromolar, were reported to block TLR4 homodimerization[7, 8]. High concentrations (10 micromolar) of these compounds blocked LPS-mediated NF-KB activation. The high concentration required for biological activity these compounds and the supports the need for compounds that inhibit the TLR4/MyD88 interaction for investigating the role of TLR signaling in atherosclerosis, sepsis and other inflammatory processes.

Probe Summary
SID 26543390 is available in good quantity from Maybridge as NRB 00125. This compound attenuates LPS mediated signaling, and should be useful in elucidating molecular mechanisms of LPS activation of the immune system. Additional characterization of SID 26543390 is published in Lee et al. Mol. Pharm, 2007.

Table 1

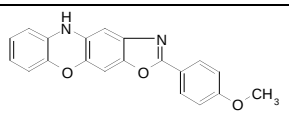
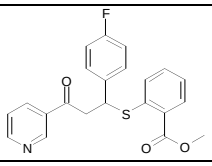
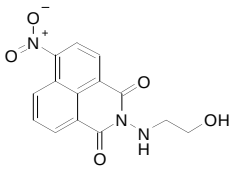
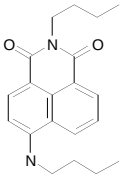
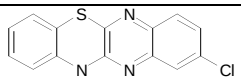
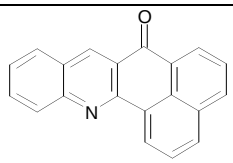
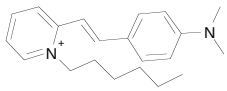
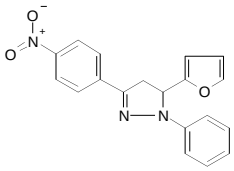
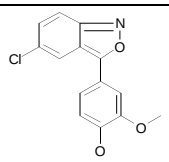
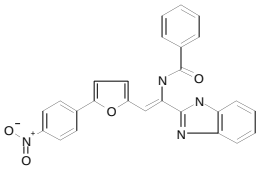
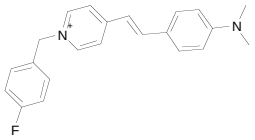
plate well	Maybridge ID	SID	Structure
1 L9	JFD 01932	26528267	
2 G5	PD 00285	26528376	
16 J19	PHG 00966	26530156	
20 G16	JFD 03094	26530642	
21 C19	RJC 01451	26530737	
21 E14	BTB 12965	26530760	
26 J10	NRB 00125	26543390	
27 N15	RDR 02308	26531575	
32 J10	RH 01473	26532248	
45 L14	S 05405	26533887	
50 F12	JFD 02841	26543649	

Figure 1. Elimination of false positive hit compounds using stable HeLa line expressing full-length β-lactamase. HeLa/CL3-4 or HeLa/full Bla cells were plated in 12-well plates. After 24 h, cells were treated with 10 μg/ml clavulanate or 10 μM different compounds as noted for 30 min. Cells were then incubated with CCF2/AM substrate for 1 h and analyzed by fluorescence microscopy. Five positive hit compounds are in bold.

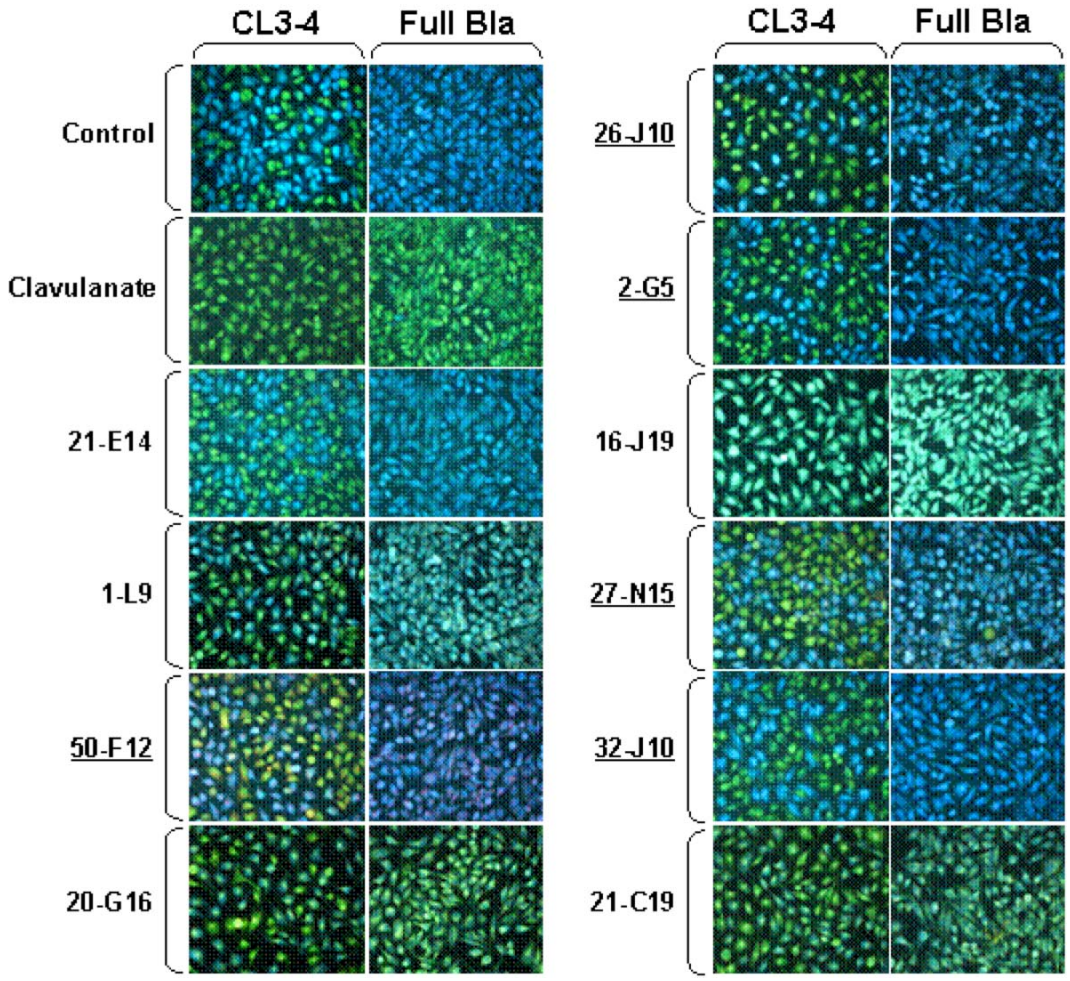


Figure 2
Inhibition of TLR4CD-MyD88 binding by the five positive compounds.
(A) For the coimmunoprecipitation assay, HEK293T cells were transiently transfected withFLAG-TLR4CD and HA-MyD88 vectors (0.5 μg/ml each). After 24 h, cells were treated with the compounds at 10 μM for the indicated length of time (an equivalent amount ofDMSO was used as the untreated control). Cells were then lysed and immunoprecipitated with anti-FLAG antibody. Western blotting was performed with both anti-FLAG (TLR4CD detection) and anti-HA (MyD88 detection) antibodies according to standard protocols. Input levels of MyD88 in the crude lysates were also analyzed using anti-HAantibody. The arrows indicated MyD88, TLR4 and IgG light chain (LC). (B) Percent binding of MyD88 to TLR4CD was analyzed using Quantity One software (Bio-Rad).

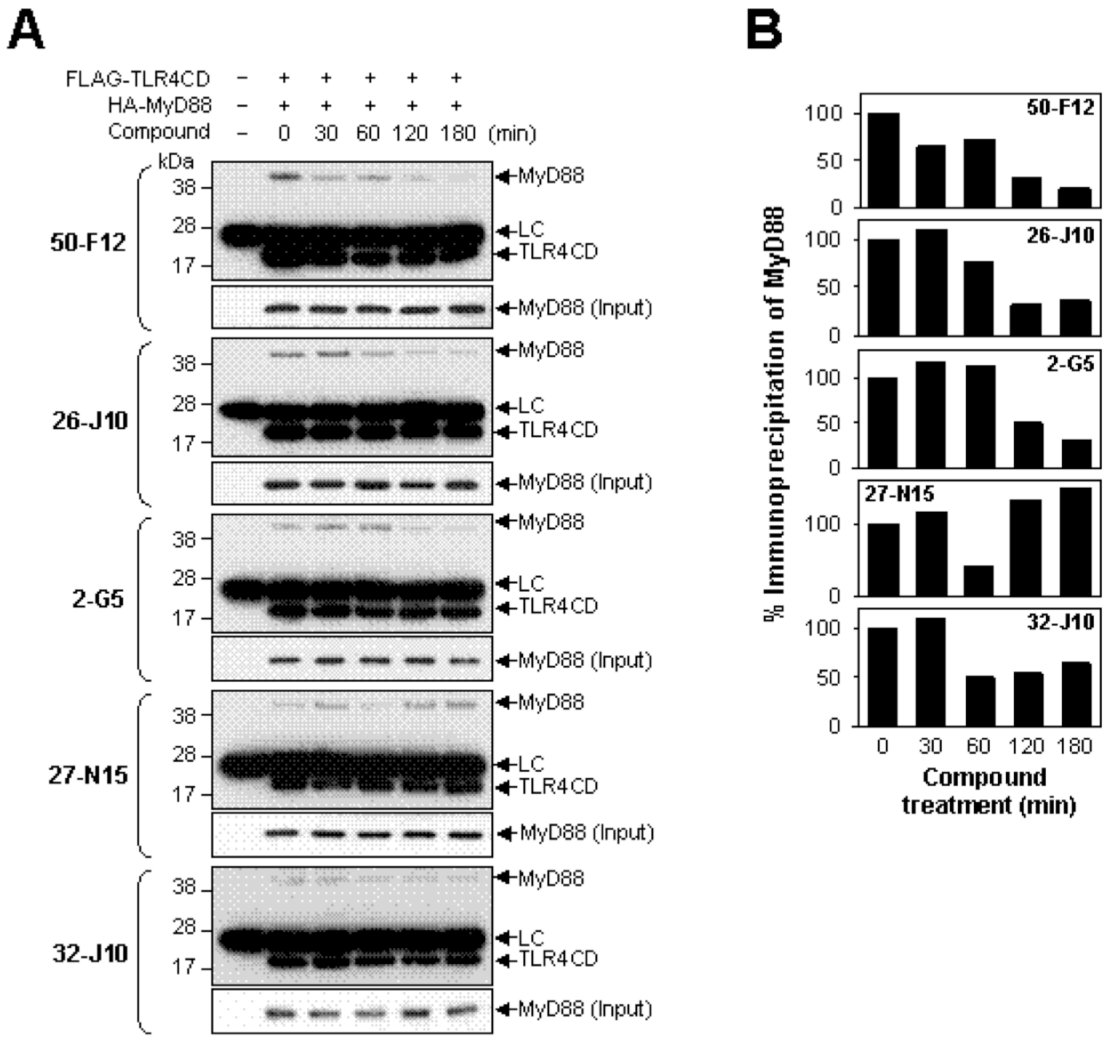
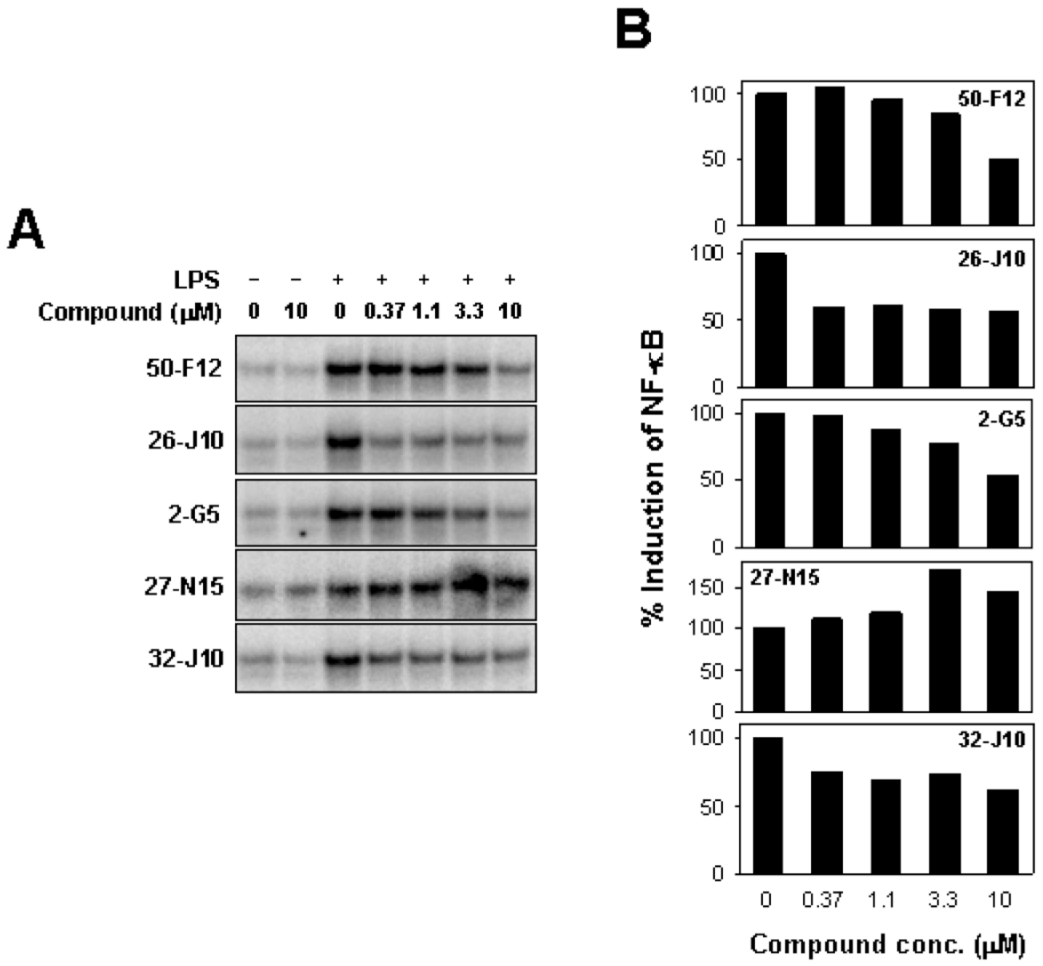


Figure 3
Inhibition of LPS-mediated NF-κB induction in RAW264.7 cells. (A) For the gelshift assay, cells were grown in 12-well tissue culture plates with DMEM/10% FCS for 24 h and pre-treated for 30 min with 0.37, 1.1, 3.3 or 10 μM inhibitory compound as indicated (DMSO was used as the untreated control). Cells were then stimulated with 0.1 μg/ml LPS for 1 h. Nuclear extracts were prepared and analyzed by gel-shift assay using an NF-κB oligonucleotide probe labeled with [γ-32P]ATP. (B) Percent activation of NF-κB translocation was analyzed using Quantity One Software (BioRad).



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