

Application of β -Lactamase Enzyme Complementation to the High-Throughput Screening of Toll-Like Receptor Signaling Inhibitors^[S]

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ABSTRACT

We describe a successful application of β -lactamase fragment complementation to high-throughput screening (HTS) for Toll-like receptor 4 (TLR4) signaling inhibitors. We developed a stable cell line, HeLa/CL3-4, expressing MyD88/Bla(a) and TLR4/Bla(b), in which the two β -lactamase fragments complement with each other by virtue of spontaneous MyD88-TLR4 binding via their Toll/IL-1R (TIR) domains. Inhibition of the MyD88-TLR4 binding leads to the disruption of the enzyme complementation and a loss of the lactamase activity. We used

a 384-well plate format to screen 16,000 compounds using this assay and obtained 45 primary hits. After rescreening these 45 hits and eliminating compounds that directly inhibited β -lactamase, we had five candidate inhibitors. We show that these five act as inhibitors of TLR4-MyD88 binding and are variously effective at inhibiting lipopolysaccharide-stimulated cytokine release from RAW264.7 cells. One compound is effective near 100 nM. None of the compounds showed any cytotoxicity at 20 μ M.

In the innate immune system, Toll-like receptors (TLRs) function indispensably as sensors of microbial molecules. Upon recognition of microbial components, TLRs quickly engage with their adaptors and initiate innate immune signals by sequentially recruiting downstream signaling mediators. The TLR-mediated innate immune signals generally provide immediate host protection and stimulate the host's adaptive immune response (Akira et al., 2001; Medzhitov, 2001; Hoebe et al., 2004). Since the first mammalian Toll homolog was discovered (Medzhitov et al., 1997), at least 11 mammalian TLRs have been identified (Ulevitch, 2004). All TLRs initiate cellular signaling through their cytoplasmic Toll/interleukin-1 receptor (TIR) domain, which triggers signaling pathways by TIR-TIR homophilic interaction with TIR domain

containing TLR adaptors. Myeloid differentiation primary response protein 88 (MyD88) is the most common TLR adaptor (Kawai et al., 1999). Others include MAL/TIRAP, TRAM and TRIF, but all TLRs use MyD88, with the exception of TLR3, which uses only TRIF (Akira et al., 2006).

Despite the fact that TLR-mediated innate immune signals are required to combat invading pathogens, when signaling is not well regulated, uncontrolled activation can result in the disruption of host homeostasis leading to long-term inflammation and septic shock. Although there are naturally occurring negative regulators of TLR signaling that function as part of cell homeostasis (Kobayashi and Flavell, 2004; Ulevitch, 2004; Han and Ulevitch, 2005; Miggin and O'Neill, 2006), these are not adequate in all situations. A number of authors have commented on the need for pharmacological means to regulate TLR signaling, and some efforts are underway (Cristofaro and Opal, 2006; Földes, 2006; Leaver et al., 2007). We have exploited a new approach to screen libraries for TLR signaling inhibitors. Based on our previous study of in vivo detection of TLR-TLR and TLR-adaptor association using β -lactamase enzyme fragment complementation (Lee

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ABBREVIATIONS: TLR, Toll-like receptor; HTS, high-throughput screening; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; TIR domain, Toll/interleukin-1 receptor domain; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; IL, interleukin; plpC, polyinosinic-polycytidylic acid; TNF- α , tumor necrosis factor α ; NF- κ B, nuclear factor κ B; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; HA, hemagglutinin; FCS, fetal calf serum; AM, acetoxymethyl ester; TIRAP, toll-interleukin 1 receptor domain containing adaptor protein; S/B, signal-to-background ratio; MALP-2, macrophage-activating lipopeptide 2 kDa; CpG, cytosine phosphate guanine; TLR4CD, Toll-like receptor 4 transmembrane-cytoplasmic domain; Bla, β -lactamase.

et al., 2004), we have developed an application of cell-based β-lactamase enzyme complementation to the high-throughput screening of small molecules. We found that high-throughput screening of 16,000 compounds resulted in five inhibitors of TLR4-MyD88 interaction and that they specifically inhibit the MyD88-dependent TLR signaling pathway.

Materials and Methods

Reagents and Antibodies. Potassium clavulanate as a lactamase inhibitor was from Sigma-Aldrich (St. Louis, MO), and the lactamase substrate, CCF2/AM, was from Invitrogen (Carlsbad, CA). The 16,000 target compounds used for screening were from Maybridge (Cornwall, England). For the experiments in Figs. 4 through 9, they were prepared at 10 mM in DMSO. Lipopolysaccharide (LPS) from *Escherichia coli* (O111:B4) was purchased from List Biological Laboratories (Campbell, CA), and polyinosinic-polycytidylic acid (pIpC) was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Anti-FLAG M2-agarose and antibody were obtained from Sigma-Aldrich, and anti-HA.11 antibody was obtained from Covance-Berkeley Antibody Company (Richmond, CA). Murine IL-1β and TNFα were purchased from PeproTech (Rocky Hill, NJ). Mouse TNFα and IL-6 ELISA kits were purchased from BD Biosciences (San Jose, CA).

Stable Cell Line and Culture Conditions. The stable HeLa line HeLa/CL3-4, which expresses two β-lactamase fusion proteins, MyD88-Bla(a) and TLR4-Bla(b), was made using two expression constructs, pCDNA3.1/MyD88/Bla(a) and pEF6/TLR4/Bla(b), as described previously (Lee et al., 2004). The stable HeLa/full-Bla line, which expresses a fusion protein of MyD88 with full-length β-lactamase, was made using pCDNA3.1/MyD88/full-length Bla. HeLa/CL3-4 were grown in DMEM with 10% FCS containing 200 μg/ml G418 and 10 μg/ml blasticidin and HeLa/full-Bla in the same medium containing 200 μg/ml G418 only. Both HeLa/CL3-4 and HeLa/full-Bla were selected using FACSsort (BD Biosciences) equipped with excitation at 408 nm and emission at 519/30 nm (CCF, green fluorescence) or 450/40 nm (cleaved CCF2, blue fluorescence). The primary blue-positive cells sorted by FACS were collected, grown in the media described above, and resorted. This sequential sorting step was repeated three times.

Assay Optimization. HeLa/CL3-4 cells were plated into 384-well plates in a series of different concentrations (1×10^6 , 0.5×10^6 , 0.25×10^6 , 0.125×10^6 , and 0.06×10^6 cells/ml) using the FlexDrop Precision Reagent Dispenser (PerkinElmer Life and Analytical Sciences, Waltham, MA). Each well contained 30 μl of cells. Cells were incubated at 37°C overnight. On the following day, cells were treated with different amounts of clavulanate (10, 3.3, 1.1, 0.37, 0.12, 0.04, 0.014, 0.0046, and 0.0015 μg/ml) for 30 min at 37°C and then loaded with 1 μM CCF2/AM for 2 h at room temperature. These two steps were performed using the BioRaptor FRD (Beckman Coulter, Fullerton, CA). The plates were then read using the EnVision multilabel reader (PerkinElmer Life and Analytical Sciences). The data were analyzed with Microsoft Excel (Redmond, WA) and Prism software (GraphPad Software, San Diego, CA). The response metric was the ratio of blue fluorescence intensity to green fluorescence intensity.

To assess assay uniformity across multiple plates, HeLa/CL3-4 cells ($30 \mu\text{l}$ of 0.125×10^6 cells/ml) were plated into three 384-well plates with DMEM/10% FCS using the FlexDrop Precision Reagent Dispenser. Cells were incubated at 37°C overnight. On the following day, the three plates were treated with 10 μg/ml (High clavulanate), 0.12 μg/ml (Medium clavulanate), or control PBS (Low clavulanate) with three plate layouts, specifically plate 1 (HML), plate 2 (LHM), and plate 3 (MLH). After 30 min, the plates were loaded with 1 μM CCF2/AM for 2 h at room temperature. The plates were then read using the EnVision multilabel reader (PerkinElmer Life and Analytical Sciences).

Compound Screening and Hit Selection. HeLa/CL3-4 cells in DMEM/10% FCS ($10 \mu\text{l}$ of 0.22×10^6 cells/ml) were dispensed into low-volume, black clear bottom 384-well plates (Greiner Bio-One, Longwood, FL) on the FlexDrop Precision Reagent Dispenser (PerkinElmer Life and Analytical Sciences). Cells were incubated at 37°C overnight. On the following day, cells were treated with 10 μM concentrations of various compounds (10 nl/well) delivered with a pintool (V&P Scientific, San Diego, CA) on a Beckman FX (Beckman Coulter, Fullerton, CA), except for the first two and the last two columns, which served as controls. The first two columns were treated with different amounts of clavulanate (10, 3.3, 1.1, 0.37, 0.12, 0.04, 0.014, 0.0046, and 0.0015 μg/ml), whereas the last two columns were left untreated. The plates were incubated at 37°C for 30 min and then loaded with 1 μM CCF2/AM for 2 h at room temperature. Thereafter, the plates were read using the EnVision multilabel reader (PerkinElmer Life and Analytical Sciences).

Z' was calculated as described previously (Zhang et al., 1999). Active wells were defined as those with percentage of inhibition greater than 3 S.D. from the low control. Percentage of inhibition was defined as $100 \times (1 - (\text{Well Median High Control})/(\text{Median Low Control} - \text{Median High Control}))$, where High Control was the wells treated with 10 μg/ml clavulanic acid, and Low Control was treated with vehicle alone. A total of 24 wells met this definition, and 21 more with borderline activity were selected for repeat. Cherry-picked compounds were tested in the primary screening assay in triplicate.

Microscopy. Microscopy was performed as described previously (Lee et al., 2004). In brief, cells were cultured in a 12-well plate overnight. Cells were washed twice with a modified physiological saline buffer (10 mM HEPES, 6 mM sucrose, 10 mM glucose, 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 1% probenecid, pH 7.35) and loaded with 1 μM CCF2/AM for 1 h at room temperature. Cells were washed with physiological saline buffer and analyzed by fluorescence microscopy [Zeiss Axiovert 100TV (Carl Zeiss, Thornwood, NJ) with a Diagnostic Instruments SPOT-cooled charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI)] using a filter set from Omega Optical XF12-2 (Brattleboro, VT): 405 nm excitation, 420 nm dichroic mirror, and 435 nm long-pass emission.

Electrophoretic Mobility Shift Assay. RAW264.7 cells (0.5×10^6 cells/ml) were grown in 12-well tissue culture plates with DMEM/10% FCS for 24 h and pretreated with 10 μM inhibitory compounds for 30 min. Cells were then stimulated with LPS (0.1 μg/ml), MALP-2 (50 ng/ml), pIpC (20 μg/ml), CpG (20 μg/ml), IL-1β (50 ng/ml), or TNFα (50 μg/ml) for 1 h. Cells were lysed with buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 1% Nonidet P-40) followed by centrifugation. The pellet was treated with buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride) to prepare nuclear extracts. Electrophoretic mobility shift assay was performed using NF-κB oligonucleotide probe (Promega, Madison, WI) labeled with [γ -³²P]ATP (GE Healthcare) using T4 polynucleotide kinase (New England BioLabs, Ipswich, MA) as described previously (Lee et al., 2006).

Luciferase Reporter Assay. The luciferase reporter assay was performed as described previously (Lee et al., 2004). In brief, HeLa cells (0.5×10^6 cells/ml) were grown in 12-well plates with DMEM/10% FCS. On the following day, cells were transiently transfected with 0.01 μg/ml TLR4, CD14, and MD-2 vectors, along with 0.05 μg/ml pIL8-promoter-Luc vector and pSV-β-galactosidase vector (Promega). After 24 h, cells were pretreated with different compounds for 30 min and then stimulated with LPS for 6 h. Cell extracts were prepared using the cell culture lysis buffer (Promega), and the luciferase activity was measured using a Luciferase Reporter Assay System (Promega), and β-galactosidase activity was measured using *O*-nitrophenyl-β-D-galactopyranoside as substrate. Luciferase activity reported in the figures is normalized for transfection efficiency using the β-galactosidase activity.

Immunoprecipitation and Western Blotting. HEK293T cells (0.5×10^6 cells/ml) were transiently transfected with 0.5 μ g of TLR4 transmembrane-cytoplasmic domain (TLR4CD) (Lee et al., 2004) and MyD88 vectors. Twenty-four hours after transfection, cells were treated with different compounds at the different time points as indicated in the figure legends. Cells were lysed with lysis buffer (150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.5) containing protease inhibitor cocktails (Roche Applied Science, Indianapolis, IN). The supernatants were incubated with anti-FLAG M2-agarose (Sigma-Aldrich) and incubated for 3 h at 4°C. The mixtures were washed four times with lysis buffer, separated on a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen) and transferred to nitrocellulose membrane. Western blotting was performed by probing the membrane with both anti-FLAG and anti-HA antibodies according to standard protocols.

Cytokine ELISA. RAW264.7 cells (0.5×10^6 cells/ml) were cultured in a 12-well plate for 24 h and preincubated with different concentrations of compounds for 1 h as indicated in the figures. Cells were then washed once with DMEM/10% FCS and stimulated with 0.05 μ g/ml LPS for 16 h. TNF α and IL-6 in the culture supernatants were measured by mouse TNF α and IL-6 ELISA according to the manufacturer's instructions (BD Biosciences).

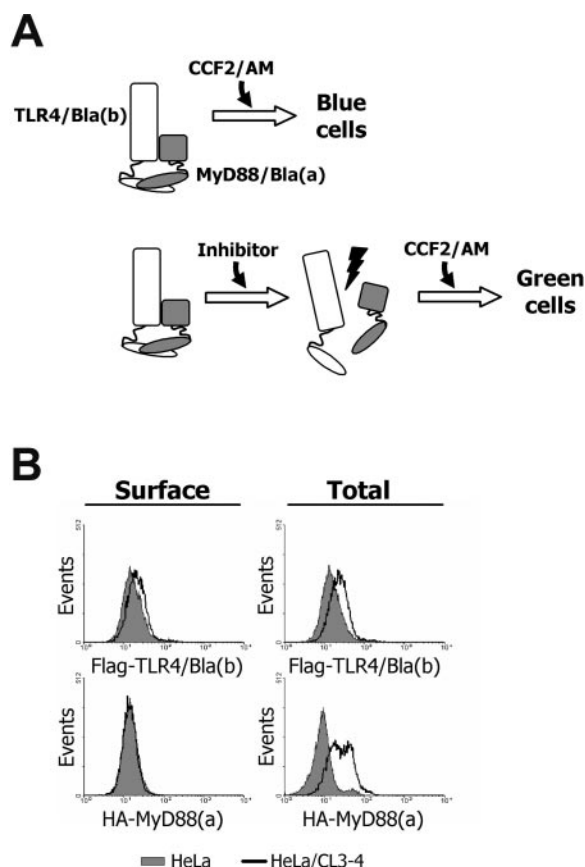


Fig. 1. Strategy for screening of TLR4 signaling inhibitors and protein expression patterns in HeLa/CL3-4 cell line. **A**, schematic representation of inhibitor screening strategy using β -lactamase fragment fusion proteins. TLR4 and MyD88 in HeLa/CL3-4 spontaneously interact with each other and bring two β -lactamase fragments into proximity, resulting in a functional form of β -lactamase. Upon the addition of β -lactamase substrate CCF2/AM and excitation at 409 nm, HeLa/CL3-4 cells emit blue fluorescence at 447 nm (A, top). If an inhibitor breaks TLR4-MyD88 binding resulting in disruption of β -lactamase fragment complementation, HeLa/CL3-4 cells emit green fluorescence at 520 nm (A, bottom). **B**, intact or permeabilized HeLa or HeLa/CL3-4 cells were stained with anti-FLAG [TLR4/Bla(b) detection] or anti-HA antibody [MyD88/Bla(a) detection] and analyzed by FACS.

Cell Viability Test. RAW264.7 cells (0.5×10^6 cells/ml) were cultured in a 12-well plate for 24 h. Cells were washed with PBS once and treated with different compounds at 20 μ M or DMSO only, or 70% ethanol for 1 h. Cells were then washed with PBS and treated with 2 μ M calcein AM and 4 μ M EthD-1 solution (Invitrogen) for 30 min. Cells were analyzed by fluorescence microscopy at 495 and 528 nm.

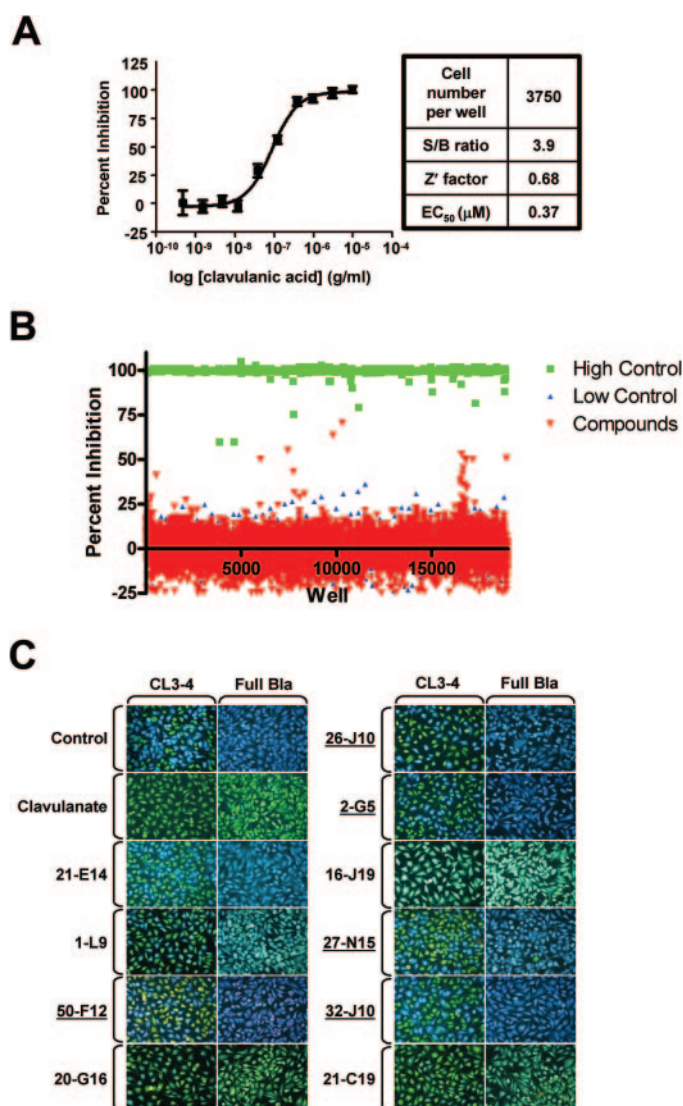


Fig. 2. Compound screening and hit selection. **A**, EC₅₀ evaluation using clavulanic acid as a model inhibitor. HeLa/CL3-4 cells in DMEM/10% FCS (0.125×10^6 cells/ml) were dispensed into 384-well plates using the FlexDrop Precision Reagent Dispenser. After 24 h, cells were treated with a series of different concentrations of clavulanic acid for 30 min and incubated with CCF2/AM substrate for 2 h. The plates were then read using the EnVision multilabel reader (PerkinElmer Life and Analytical Sciences). EC₅₀ value of clavulanic acid was analyzed using Prism software. The table beside the graph summarizes the S/B ratio and Z' factor achieved. **B**, the HeLa/CL3-4 cell line-based complementation assay was screened against 16,000 compounds. The figure includes controls and standards and represents 19,200 wells in total. The well number versus percentage of inhibition of the high (10 μ g/ml clavulanic acid) and low (vehicle alone) controls is shown. The average Z' for each plate of the screening campaign was 0.64. **C**, elimination of false-positive compounds in 10 positive hits 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 μ g/ml clavulanic acid or 10 μ M compound for 30 min as indicated. Cells were then incubated with CCF2/AM substrate for 1 h and analyzed by fluorescence microscopy. The five compounds kept for further study are underlined.

Results

Assay Design. Based on our previous work (Lee et al., 2004), we felt that a stable cell line expressing chimeras of TLR4 and MyD88 with fragments of β-lactamase would be useful for identifying inhibitors of the TIR-TIR interactions by which TLR4 and MyD88 associate (Fig. 1A). We hypothesized that such inhibitors might have some use for manipulating TLR activity.

HeLa cells were transfected with plasmids expressing chimeras as noted and antibiotic-selectable markers. After several rounds of FACS selection for cells expressing the two complementing chimeras, HeLa/CL3-4 was further characterized (Supplementary Fig. S1). The expression levels of the two proteins were explored by FACS analysis. As anticipated, MyD88/Bla(a) was expressed intracellularly, whereas TLR4/Bla(b) was expressed on the surface (Fig. 1B).

Before attempting to screen for inhibitory compounds, we first tested our HTS assay system using clavulanic acid. The β-lactamase inhibitor clavulanic acid was chosen to use as a model inhibitor during assay development because there was no drug known that inhibits TLR4-MyD88 binding. Assay testing included EC₅₀ value determination and plate uniformity assessment. Preliminary experiments suggested that 3750 cells/well with a 384-well plate gave a good, nearly confluent, monolayer of cells. Clavulanic acid inhibited the β-lactamase activity in HeLa/CL3-4 cells in a dose-dependent manner with an EC₅₀ of 0.37 μM (Fig. 2A). The signal-to-

background (S/B) ratio and the Z' value for this dose-response experiment were 3.9 and 0.68, respectively. Because we are not aware of a published value of the EC₅₀ of clavulanic acid for β-lactamase, it is difficult to compare the EC₅₀ value in this study with others. However, the minimum inhibitory concentrations of clavulanic acid against various bacteria ranged from 0.1 to 512 mg/l (Finlay et al., 2003). Although the assay systems are different, our EC₅₀ of 0.37 μM (0.088 mg/l) is comparable with those minimum inhibitory concentrations.

The plate uniformity assay was done with three different plate layouts as noted in Supplementary Fig. S2. Each layout resulted in a constant pattern with reliable Z' values ranging from 0.67 to 0.83 (Supplementary Fig. S2). Taken together, these data suggested that the proposed assay system could be used for library screening.

For library screening, a total of 16,000 compounds in 50 plates were used for the primary screening (Fig. 2B). Each plate contained a clavulanic acid dose-response control, which was used to determine the hit cutoff for each screening plate. The primary screening yielded a total of 45 hits. These consist of 24 hits selected by each cutoff and an additional 21 hits with borderline activities selected by the overall cutoff. These 45 hits were further tested by rescreening. A total of 10 of 45 compounds were reproducibly positive hits. To detect which compounds were inhibitory because they directly inhibit β-lactamase, we used a cell line expressing full-length

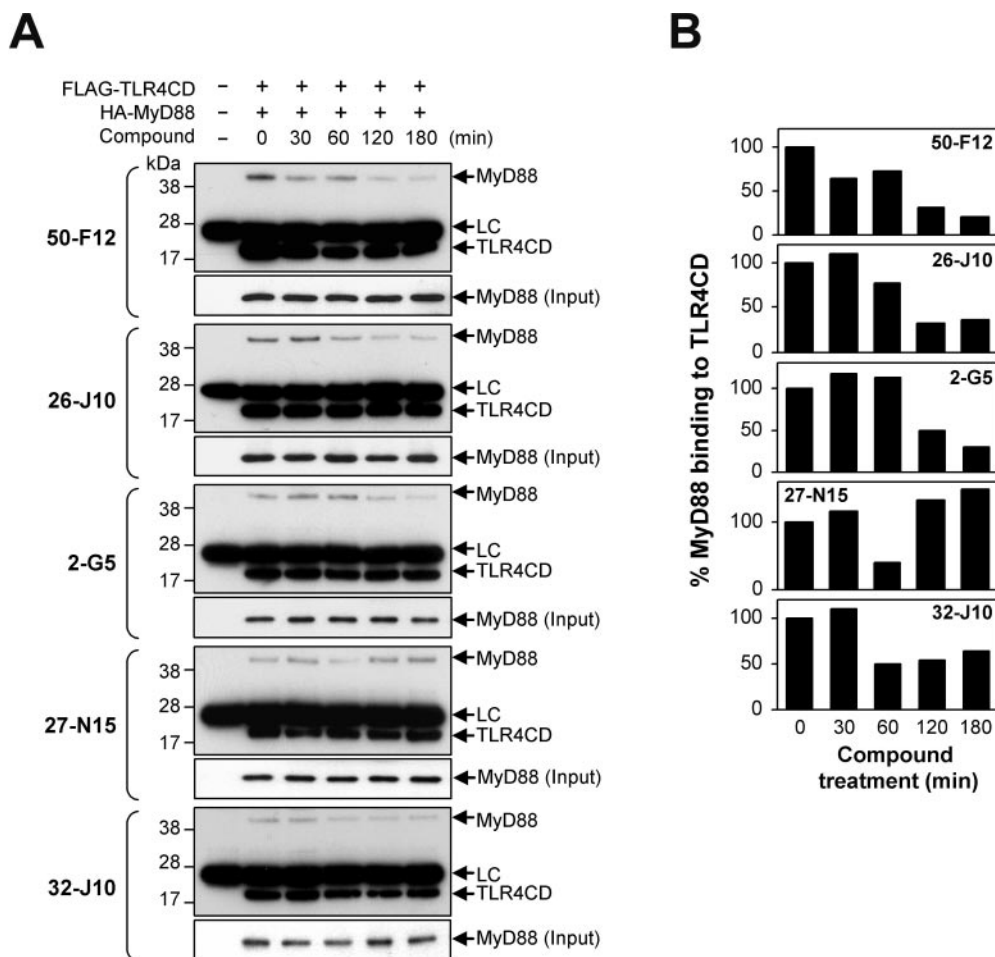


Fig. 3. Inhibition of TLR4CD-MyD88 binding by the five positive compounds. A, for the coimmunoprecipitation assay, HEK293T cells were transiently transfected with FLAG-TLR4CD and HA-MyD88 vectors (0.5 μg/ml each). After 24 h, cells were treated with the compounds at 10 μM for the indicated length of time (an equivalent amount of DMSO 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-HA antibody. The arrows indicate MyD88, TLR4, and IgG light chain (LC). B, percentage binding of MyD88 to TLR4CD was analyzed using Quantity One software (Bio-Rad, Hercules, CA).

β -lactamase. As shown in Fig. 2C, five of the tested compounds and clavulanic acid inhibited full-length β -lactamase. These compounds were eliminated from further study, although they might be useful as lactam antibiotic adjuvants, as is clavulanic acid (Finlay et al., 2003).

Inhibition of MyD88 Binding to TLR4CD. To define whether the selected compounds inhibit TLR4-MyD88 binding, we first studied coimmunoprecipitation of TLR4CD and MyD88. TLR4CD consists of TLR4 transmembrane and cytoplasmic domains. This construct was made previously and was shown to coprecipitate with MyD88 (Lee et al., 2004). HEK293T cells transiently transfected with TLR4CD and MyD88 vectors for 24 h were incubated with the five candidate compounds for different lengths of time. As shown in Fig. 3, most compounds directly inhibited the TLR4CD-MyD88 binding, although compound 27-N15 was the least effective. The time course of its effect suggests that it may be unstable, perhaps being metabolized. That the expression levels of TLR4CD and MyD88 (input) did not change before and after the addition of the compounds shows that the compounds directly affect the binding between TLR4 and MyD88 but not protein expression (Fig. 3A).

Inhibitory Effects of Positive Hit Compounds on TLR4 Signaling. We next investigated whether inhibition of TLR4-MyD88 binding by the compounds is correlated with TLR4 signaling. We first performed NF- κ B nuclear translocation experiments using a γ - 32 P-labeled NF- κ B oligonucleotide probe. RAW264.7 cells pretreated with different amounts of the compounds were stimulated with LPS. All of the compounds except for 27-N15 inhibited LPS-mediated NF- κ B induction in RAW264.7 cells (Fig. 4).

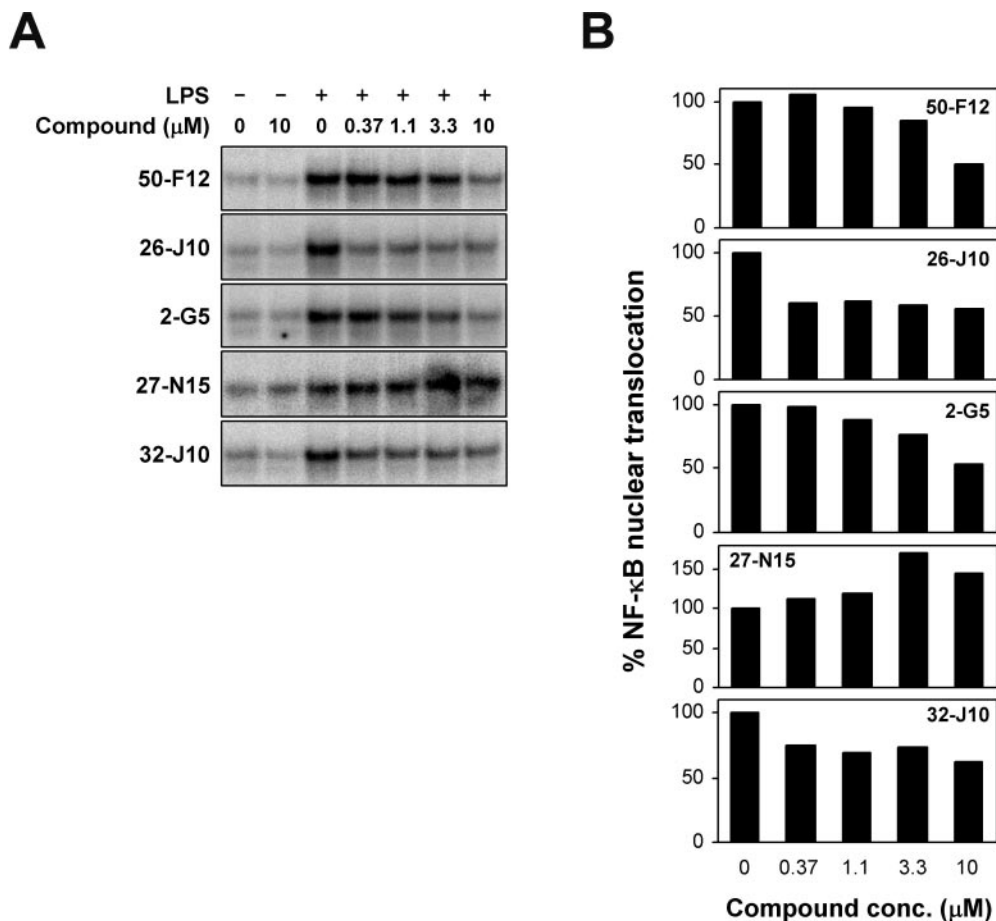


Fig. 4. Inhibition of LPS-mediated NF- κ B induction in RAW264.7 cells. A, for the gel-shift assay, cells were grown in 12-well tissue culture plates with DMEM/10% FCS for 24 h and pretreated for 30 min with 0.37, 1.1, 3.3, or 10 μ M inhibitory compound as indicated (DMSO was used as the untreated control). Cells were then stimulated with 0.1 μ g/ml LPS for 1 h. Nuclear extracts were prepared and analyzed by gel-shift assay using an NF- κ B oligonucleotide probe labeled with [γ - 32 P]ATP. B, percentage of activation of NF- κ B translocation was analyzed using Quantity One Software (Bio-Rad).

We also performed IL-8-promoter reporter gene assay using HeLa cells transiently transfected with TLR4, CD14, and MD-2 vectors plus the reporter constructs as noted in Fig. 5A. LPS-mediated IL-8 promoter activity was inhibited by all the compounds except for 27-N15 (Fig. 5A).

To determine whether this transcription factor inhibition really affects the production of cytokines, we next performed ELISA to measure TNF α and IL-6 in RAW264.7 cell cultures. Cells were preincubated with different concentrations of the compounds for 60 min, changed with fresh culture media to remove all existing DMSO, and stimulated with LPS for 16 h. As shown in Fig. 5, B and C, IL-6 production was generally more strongly inhibited than TNF α , even by compound 27-N15. The most inhibitory compound is 26-J10, which is inhibitory of both cytokines at 120 nM.

MyD88-Dependent Inhibition. We next investigated the inhibitory specificity of the compounds. Using the two most potent compounds (50-F12 and 26-J10), we performed an NF- κ B activation assay on RAW 264.7 cells stimulated with a variety of agonists that act through different receptors. In addition to LPS (TLR4 ligand), we used MALP-2 (TLR2 ligand), pIpC (TLR3 ligand), CpG (TLR9 ligand), IL-1 β and TNF α . It is interesting that both compounds inhibited MALP-2- and LPS-induced NF- κ B activation, whereas they only slightly inhibited CpG- or IL-1 β -mediated NF- κ B induction (Fig. 6). However, pIpC and TNF α mediated NF- κ B inductions were not affected. These results suggest that the inhibition is not only MyD88-dependent (Fig. 6) but could also be specific for the particular MyD88 partner.

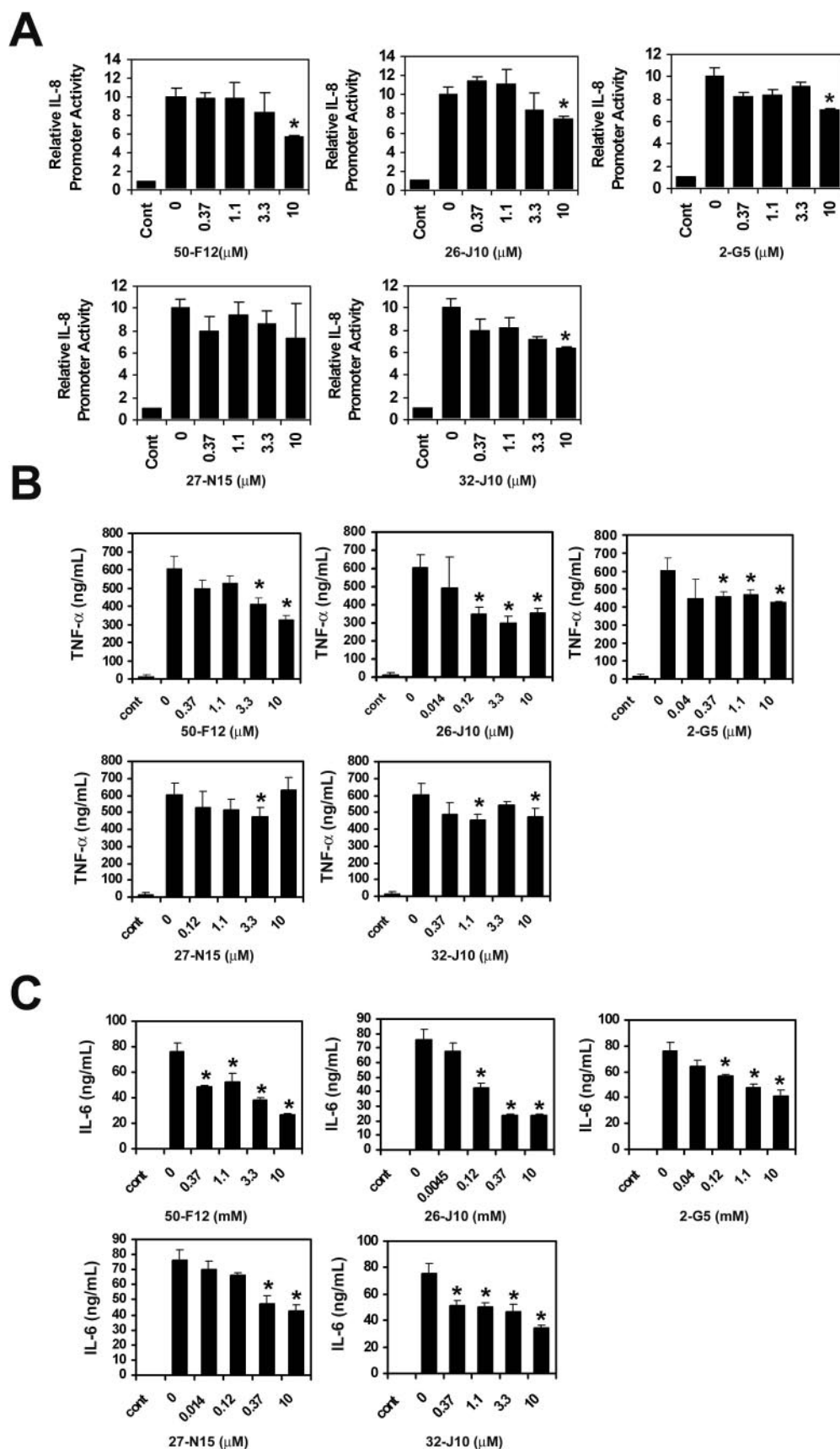


Fig. 5. Inhibition of LPS-stimulated cell activation. **A**, inhibition of LPS-mediated IL-8 promoter activity in HeLa cells. HeLa cells were transiently cotransfected with TLR4/CD14/MD-2 vectors (0.01 μ g/ml each) and pIL8-promoter-Luc and pSV- β -galactosidase vectors (0.05 μ g/ml each). After 24 h, cells were preincubated with increasing amounts of compounds for 30 min as indicated and stimulated with 0.1 μ g/ml LPS for 6 h. The luciferase activity was then measured, and all the luciferase activity was normalized with β -galactosidase activity. Results are shown as the mean \pm S.D. *, $p < 0.05$ versus LPS alone (Mann-Whitney U test). Cont, LPS-negative vehicle control. **B** and **C**, inhibition of LPS-mediated inflammatory cytokine production in RAW264.7 cells. RAW264.7 cells were preincubated with different concentrations of compound for 60 min as indicated. Cells were washed once with DMEM/10% FCS and then stimulated with LPS (0.05 μ g/ml) for 16 h. TNF α (**A**) or IL-6 (**B**) in the culture supernatants were measured by ELISA. Results are shown as the mean \pm S.D. Cont, LPS-negative control. *, $p < 0.05$ versus LPS alone (Mann-Whitney U test). Note that the compound concentrations used are not the same for all compounds (**B** and **C**). Cont, LPS-negative vehicle control.

Discussion

The data presented here verify that the β -lactamase fragment complementation assay is effectively applicable to the high-throughput screening of small molecules using a 384-well plate format. That β -lactamase can be split into two fragments that can complement with each other when they are properly presented was first shown by others (Galarneau et al., 2002; Wehrman et al., 2002). We introduced the use of this technique for type I transmembrane protein interactions such as TLR-TLR and TLR-adaptor interactions (Lee et al., 2004). The HeLa/CL3-4 cell line used in this study was made with the β -lactamase fragment-conjugated fusion constructs based on our previous study (Lee et al., 2004). This cell line has a β -lactamase-positive phenotype because the two β -lactamase fragments spontaneously complement with each other via the TIR domains of MyD88 and TLR4. The HTS using this HeLa/CL3-4 line enabled us to isolate five small inhibitor compounds out of a 16,000 compound library. Our data show that those five compounds, to varying degrees, interfere with the binding between MyD88 and TLR4 and lead to the inhibition of LPS-initiated TLR4 signaling.

During the development of the assay we used clavulanic acid, which is a known β -lactamase inhibitor, as a model for the inhibitors we were seeking because there was no known inhibitor for the TLR4-MyD88 binding. The utility of the

screening assay was validated with a reproducible dose-response to clavulanic acid and a reproducible uniformity of inhibition assessment that provided an average Z' factor of 0.75 (Supplementary Fig. S2). We also observed that the HeLa/CL3-4 cells tolerated 1% DMSO (data not shown).

Because the screening assay detects loss of β -lactamase activity, a secondary assay was necessary to detect those compounds that, like clavulanic acid, inhibit β -lactamase directly. To detect these compounds, we constructed another stable cell line, HeLa/full Bla, that expresses a MyD88-conjugated full β -lactamase fusion protein. Using this cell line enabled us to directly visualize and select out the β -lactamase inhibitors when these cells were pretreated with compound followed by CCF2/AM substrate treatment and observed by fluorescence microscopy (Fig. 2C).

All five compounds selected as "real" positive hits did not affect the cell viability at 20 μ M (Supplementary Fig. S3). Among the five compounds, two, 50-F12 and 26-J10, showed the most consistent inhibitory effects through the various assays. 50-F12 and 26-J10 showed dose-dependent inhibition of TNF α and IL-6 production in RAW264.7 cells activated by LPS, among which 26-J10 exhibited stronger inhibition (Fig. 5, B and C). The inhibitory activity of these compounds does not seem to be TLR4-dependent but rather MyD88-dependent because they inhibited TLR2-mediated signal transduction as well (Fig. 6). The results of Fig. 6 are suggestive of some selectivity for particular TLRs, but further data with a larger variety of agonists will be required for a full appreciation of the compounds' specificities. It is interesting that the possibility that the inhi-

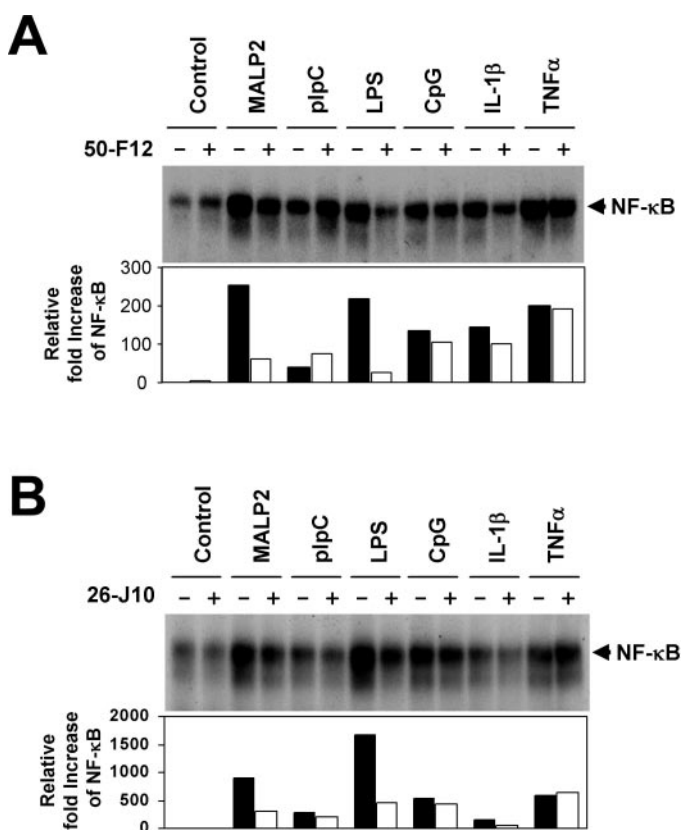


Fig. 6. Inhibition of MyD88-dependent NF- κ B induction. RAW264.7 cells were grown 12-well tissue culture plates with DMEM/10% FCS for 24 h and were pretreated with 10 μ M inhibitory compounds 50-F12 (A) and 26-J10 (B) for 30 min. Cells were then stimulated with MALP-2 (50 ng/ml), pIpC (20 μ g/ml), LPS (0.1 μ g/ml), CpG (20 μ g/ml), IL-1 β (50 ng/ml), or TNF α (50 μ g/ml) for 1 h. Nuclear extracts were then prepared, and gel-shift assay was performed using NF- κ B oligonucleotide probe labeled with [γ - 32 P]ATP.

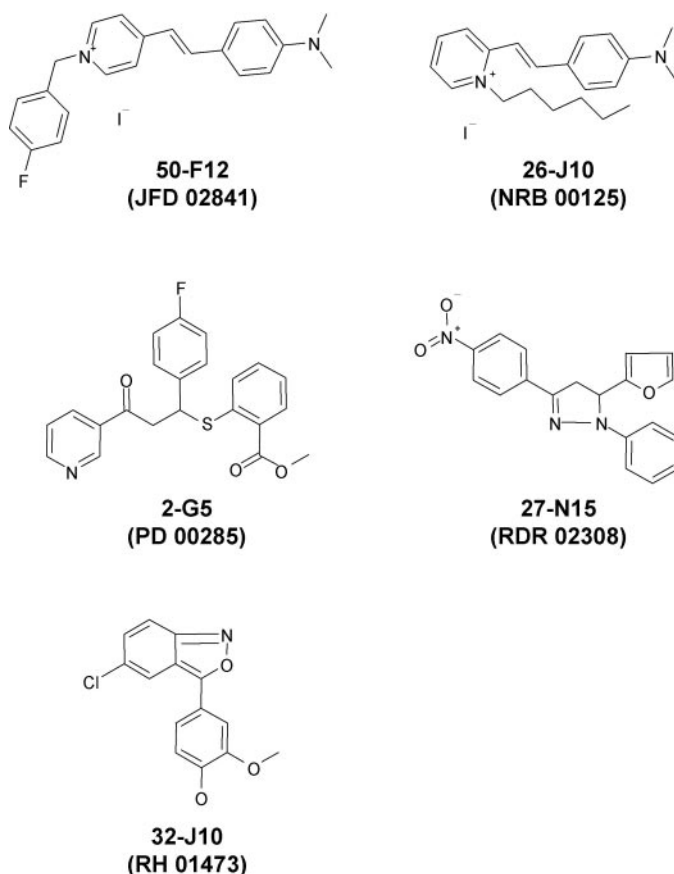


Fig. 7. The structures of the five positive hit compounds selected in this study. The compound codes (Maybridge) are shown in parentheses.

bition is more selective for TLR2 and TLR4 signaling might be correlated with the adaptor usage of these two receptors. According to previous studies, a coadaptor, TIRAP/Mal, is known to be essential in the MyD88-dependent signaling pathway shared by TLR2 and TLR4 but not by TLR3, TLR5, TLR7, TLR9, or IL-1R (Horng et al., 2002; Yamamoto et al., 2002). A recent study also shows that TIRAP functions to facilitate the MyD88 recruitment to TLR4 when TIRAP is recruited to the plasma membrane via its phosphatidylinositol 4,5-bisphosphate binding domain in the presence of phosphatidylinositol 4,5-bisphosphate (Kagan and Medzhitov, 2006). Whether our selected inhibitory compounds involve TIRAP adaptor remains to be further elucidated.

MyD88 is a universal adaptor that transmits signals initiated by TLR/IL-1R family members with the exception of TLR3 (O'Neill, 2003; Ulevitch, 2004). MyD88 contains an N-terminal death domain that is separated from a C-terminal TIR domain by a short intermediate domain. The interaction of MyD88 with given receptors commonly occurs via homophilic TIR-TIR domain association, whereas the death domain of MyD88 recruits signaling kinases, resulting in a series of signaling cascades downstream. It is interesting that a recent study demonstrated that three different point mutations in MyD88 generated by ethyl nitroso-urea-mediated mouse germ line mutagenesis differentially affected signaling among TLRs (Jiang et al., 2006), implying that the interactions between MyD88 and TLRs are specific to each TLR. This further implies that inhibitors that differentially affect the interactions of MyD88 with different TLRs should be definable.

The structures of the five compounds identified in this study are shown in Fig. 7. The two most active compounds, 50-F12 and 26-J10, share a similar core structure consisting of a pyridinium moiety linked via a transethylene bridge to a paradimethylaminophenyl group. Because the library screened was selected for diversity, limited structure-activity relationship is expected. We plan to screen additional, larger collections, such as the National Institutes of Health Molecular Libraries Screening Centers Network Library. These results will form a basis for the extension of observed structure-activity relationship by purchase and synthesis of compounds guided by pharmacophore modeling.

The β -lactamase complementation-based HTS described in this study is quite comparable with the HTS assays using β -galactosidase enzyme fragment complementation. The β -galactosidase complementation system has long been applied to cell-based assays. Unlike β -lactamase fragments, two split β -galactosidase fragments rapidly combine to form active enzyme in solution. In an assay strategy, β -galactosidase is often split into donor and acceptor; the donor is conjugated with a bait molecule so that a bait binding protein added in solution interrupts the donor-acceptor complementation. Hence, the screening of unknown target libraries is based on the competition between the bait molecule and unknown target molecules, and an HTS assay based on this strategy is often used for screening G protein-coupled receptor agonists or protease inhibitors (Golla, 2002; Naqvi et al., 2004). Together, these studies and our present study demonstrate that either β -galactosidase or β -lactamase complementation is a useful tool that can be applied to HTS assays.

Naturally occurring TLR signaling inhibitors in host cells normally are sufficient to maintain host homeostasis (Miggin and O'Neill, 2006). However, none of these have yet been

turned into useful drugs. Recent reports indicating TLR involvement in sterile inflammatory injury (Beutler, 2004; Mollen et al., 2006) and inflammation initiated by exogenous pathogens suggest the need for pharmacological means to regulate TLR activity. Therefore, we hope that the approach we have initiated and described herein may provide useful reagents for manipulating TLR activity.

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