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TITLE:

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

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RUNNING TITLE:

β-Lactamase Enzyme Complementation-Based HTS

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ABBREVIATIONS: TLR, Toll-like receptor; HTS, high-throughput screening; DMSO, dimethy sulfoxide; LPS, lipopolysaccharide; TIR domain, Toll/interleukin-1 receptor homology domain; EC50, effective concentration 50; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay.

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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 re-screening these 45 hits and eliminating compounds that directly inhibited β-lactamase we had five candidate inhibitors. We show that these five appear to act as inhibitors of TLR4-MyD88 binding and are variously effective at inhibiting LPS stimulated cytokine release from RAW264.7 cells. One compound is effective near 100 nM. None of the compounds showed any cytotoxicity at 20 μM.

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INTRODUCTION

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 also stimulate the host's adaptive immune response (Akira et al., 2001; Medzhitov, 2001; Hoebe et al., 2004). Since the first mammalian Toll homologue 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 utilize the 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 disruption of host homeostasis leading to chronic inflammation and septic shock. Although there are naturally occurring negative regulators of TLR signaling which 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

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TLR signaling, and some efforts are underway (Cristofaro and Opal, 2006; Foldes, 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 as well as TLR-adaptor association using β-lactamase enzyme fragment complementation (Lee 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.

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MATERIALS AND METHODS

Reagents and Antibodies. Potassium clavulanate as a lactamase inhibitor was from Sigma-Aldrich and the lactamase substrate, CCF2AM from Invitrogen (Carlsbad, CA). The 16,000 target compounds used for screening were from Maybridge (Cornwall, England) For the experiments in Figures 4-9, 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) from Amersham Biosciences (Piscataway, NJ). Anti-FLAG M2-agarose and antibody were obtained from Sigma-Aldrich (St. Louis, MO) 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 TNF-α and IL-6 ELISA kits were purchased from BD Biosciences.

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 previously described (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 FACSort (BD Biosciences) equipped with excitation at 408 nm and

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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 re-sorted. This sequential sorting step was repeated three times.

Assay Optimization. HeLa/CL3-4 cells were plated into regular 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 per ml) using the FlexDrop Precision Reagent Dispenser (PerkinElmer). Each well contained 30 µl of cells. Cells were incubated at 37^{o}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 per mL) for 30 min at 37^{o}C and then loaded with 1µM CCF2AM for 2h at room temperature. These two steps were performed using the BioRaptor FRD (Aurora). The plates were then read using the EnVision multi-label reader (PerkinElmer). The data were analyzed with Excel as well as Prism software. 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 µl 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^{o}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) with three plate layouts, specifically plate 1 (HML), plate 2 (LHM) and plate 3 (MLH). After 30 min, the plates

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were loaded with 1μM CCF2AM for 2h at room temperature. The plates were then read using the EnVision multi-label reader (PerkinElmer).

Compound Screening and Hit Selection. HeLa/CL3-4 cells in DMEM/10% FCS (10μl of 0.22×10^6 cells/ml) were dispensed into low-volume, black clear bottom 384-well plates (Greiner) on the FlexDrop Precision Reagent Dispenser (PerkinElmer). Cells were incubated at 37^{o}C overnight. On the following day, cells were treated with 10μM various compounds (10 nl/well) delivered with a pintool (V & P Scientific) on a Beckman FX, except 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 per mL) while the last two columns were left untreated. The plates were incubated at 37^{o}C for 30 min and then loaded with 1μM CCF2AM for 2h at room temperature. Thereafter, the plates were read using the EnVision multi-label reader (PerkinElmer).

Z' was calculated as described (Zhang et al., 1999). Active wells were defined as those with percent inhibition greater than 3 standard deviations from the low control. Percent inhibition was defined as 100*(1-(Well-Median High Control)/(Median Low Control-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. Cherrypicked compounds were tested in the primary screening assay in triplicate.

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Microscopy. Microscopy was performed as described (Lee et al., 2004). Briefly, 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 $CaCl_2$, 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 with a Diagnostics Instruments SPOT cooled CCD camera) using a filter set from Omega Optical XF12-2: 405 nm excitation, 420 nm dichroic mirror, 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 pre-treated 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 TNF-α (50 μg/ml) for 1 h. Cells were lysed with buffer A (10 mM HEPES [pH7.9], 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1% NP-40) followed by centrifugation. The pellet was treated with buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and 0.1 mM PMSF) to prepare nuclear extracts. EMSA was performed utilizing NF-κB oligonucleotide probe (Promega, Madison, WI) labeled with [γ-^{32}P]ATP (Amersham Biosciences) using T4 polynucleotide kinase (New England BioLabs) as previously described (Lee et al., 2006).

Luciferase Reporter Assay. The luciferase reporter assay was performed as previously described (Lee et al., 2004). Briefly, HeLa cells (0.5×10^6 cells/ml) were grown in 12-

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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, Madison, WI). After 24 h, cells were pre-treated with different compounds for 30 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 μg 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 P40, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) containing protease inhibitor cocktails (Roche Applied Science). The supernatants were incubated with anti-FLAG M2-agarose (Sigma-Aldrich) and incubated for 3 h at 4^{o}C. The mixtures were washed four times with lysis buffer, separated on a 4-12% 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.

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Cytokine ELISA. RAW264.7 cells (0.5×10^6 cells/ml) were cultured in a 12-well plate for 24 h and pre-incubated 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 μg/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).

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 μM or DMSO only, or 70% ethanol for 1h. Cells were then washed with PBS and treated with 2 μM calcein AM and 4 μM EthD-1 solution (Invitrogen) for 30 min. Cells were analyzed by fluorescence microscopy at 495 nm and 528 nm.

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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 utility for manipulating TLR activity.

HeLa cells were transfected with plasmids expressing chimeras as noted as well as antibiotic selectable markers. After several rounds of FACS selection for cells expressing the two complementing chimeras, HeLa/CL 3-4 was further characterized (Supplementary Fig. 1). The expression levels of the two proteins were explored by FACS analysis. As anticipated, MyD88/Bla(a) was expressed intracellularly while 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 EC_{50} value determination and plate uniformity assessment. Preliminary experiments suggested that 3750 cells/well with a 384 well plate gave a good, nearly confluent, mono layer of cells. Clavulanic acid inhibited the β-lactamase activity in HeLa/CL3-4 cells in a dose-dependent manner with an EC_{50} of 0.37 μM (Fig. 2A). The S/B ratio and the Z' value for this dose-response experiment were 3.9 and 0.68, respectively. Since we are not aware of a published value of the EC_{50} of clavulanic acid for β-lactamase, it is difficult to compare

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the EC_{50} value in this study with others. However, the Minimum Inhibitory Concentrations (MICs) 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 EC_{50} of 0.37 μM (= 0.088 mg/L) is comparable with those MICs.

The plate uniformity assay was done with three different plate layouts as noted in Supplementary Figure 2. Each layout resulted in a constant pattern with reliable Z' values ranging from 0.67 to 0.83 (Supplementary Fig. 2). 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 additional 21 hits with borderline activities selected by the overall cutoff. These 45 hits were further tested by re-screening. A total of 10 compounds out of 45 were reproducibly positive hits. To detect which compounds were inhibitory because they directly inhibit β-lactamase we utilized a cell line expressing full length β-lactamase. As shown in figure 2C, five of the tested compounds, as well as 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 TLR4 Cytoplasmic Domain (TLR4CD). To define whether the selected compounds inhibit TLR4-MyD88 binding, we first studied

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coimmunoprecipitation of TLR4CD and MyD88. TLR4CD consists of TLR4 transmembrane and cytoplasmic domains. This construct was previously made and shown to co-precipitate with MyD88 (Lee et al., 2004). HEK293T cells transiently transfected with TLR4CD and MyD88 vectors for 24 h were incubated with the 5 candidate compounds for different lengths of time. As shown in Figure 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 addition of the compounds, show 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-κB nuclear translocation experiments using a [γ-^{32}P]-labeled NF-κB oligonucleotide probe. RAW264.7 cells pretreated with different amounts of the compounds were stimulated with LPS. All the compounds except 27-N15 inhibited LPS-mediated NF-κB induction in RAW264.7 cells (Fig. 4).

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 Figure 5A. LPS-mediated IL-8 promoter activity was inhibited by all the compounds except 27-N15 (Fig. 5A).

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To determine if this transcription factor inhibition really effects production of cytokines, we next performed ELISA to measure TNF-α and IL-6 in RAW264.7 cell cultures. Cells were pre-incubated 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 Figures 5B and 5C. 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-κB activation assay on RAW 264.7 cells stimulated with a variety of agonists which act through different receptors. In addition to LPS (TLR4 ligand), we used MALP-2 (TLR2 ligand), pIpC (TLR3 ligand), CpG (TLR9 ligand), IL-1β and TNF-α. Interestingly, both compounds inhibited MALP-2 and LPS-induced NF-κB activation while they only slightly inhibited CpG- or IL-1β-mediated NF-κB induction (Fig. 6). However, pIpC and TNF-α mediated NF-κB 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.

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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 which 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 as well as 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 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 (see Supplementary Fig. 2). We also observed that the HeLa/CL3-4 cells tolerated 1% DMSO (data not shown).

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Because the screening assay detects loss of β-lactamase activity, a secondary assay was necessary to detect those compounds which, like clavulanic acid, inhibit β-lactamase directly. To detect these compounds we constructed another stable cell line, HeLa/full Bla, which 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 (see Fig. 2C).

All five compounds selected as "real" positive hits did not affect the cell viability at 20 μM (Supplementary Fig. 3). 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 (see Figs. 5B and 5C). The inhibitory activity of these compounds does not seem to be TLR4-dependent but MyD88-dependent since they inhibited TLR2-mediated signal transduction as well (see Fig. 6). The results of Figure 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 compound's specificities. Interestingly, the possibility that the inhibition 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-

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bisphosphate (PIP2) binding domain in the presence of PIP2 (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 (DD) which is separated from a C-terminal TIR domain by a short intermediate domain (ID). The interaction of MyD88 with given receptors commonly occurs via homophilic TIR-TIR domain association while the DD of MyD88 recruits signaling kinases resulting in a series of signaling cascades downstream. Interestingly, 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 which 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 Figure 7. The two most active compounds, 50-F12 and 26-J10, share a similar core structure consisting of a pyridinium moiety linked via a trans ethylene bridge to a para dimethylaminophenyl group. Since the library screened was selected for diversity, limited structure-activity relationship (SAR) is expected. We plan to screen additional, larger collections, such as the NIH MLSCN Library. These results will form a basis for extension of observed SAR by purchase and synthesis of compounds guided by pharmacophore modeling.

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The β-lactamase complementation-based HTS described in this study is quite comparable to 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, among which 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 GPCR agonists or protease inhibitors (Golla, 2002; Maqvi 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) as well as 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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REFERENCES

Akira S, Takeda K, and Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Rev Immunol* **2**:675-680.

Akira S, Uematsu S, and Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* **124**:783-801.

Beutler B (2004) Inferences, questions and possibilities in Toll-like receptor signaling. *Nature* **430**:257-263.

Cristofaro P and Opal SM (2006) Role of Toll-like receptors in infection and immunity: clinical implications. *Drugs* **66**:15-29.

Finlay J, Miller L, and Poupard JA (2003) A review of the antimicrobial activity of clavulante. *J Antimicrob Chemother* **52**:18-23.

Foldes G, von Haehling S, and Anker SD (2006) Toll-like receptor modulation in cardiovascular disease: a target for intervention? *Expert Opin Investig Drugs* **15**:857-871.

Galarneau A, Primeau M, Trudeau LE, and Michnick SW (2002) β-lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein-protein interactions. *Nat Biotechnol* **20**:619-622.

Golla R and Seethala R (2002) A homogeneous enzyme fragment complementation cyclic AMP screen for GPCR agonists. *J Biomol Screen* **7**:515-525.

Han J and Ulevitch RJ (2005) Limiting inflammatory responses during activation of innate immunity. *Nat Rev Immunol* **6**:1198-1205.

Hoebe K, Janssen E, and Beutler B (2004) The interface between innate and adaptive immunity. *Nat Immunol,* **5**:971-974.

Horng T, Barton GM, Flavell RA, and Medzhitov R (2002) The adaptor molecule TIRAP provides signaling specificity for Toll-like receptors. *Nature* **420**:329-333.

Jiang Z, Georgel P, Li C, Choe J, Crozat K, Rutschmann S, Du X, Bigby T, Mudd S, Sovath S, Wilson IA, Olson A, and Beutler B (2006) Details of Toll-like receptor:adaptor interaction revealed by germ-line mutagenesis. *Proc Natl Acad Sci USA* **103**:10961-10966.

Kagan JC and Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* **125**:943-955.

Kawai T, Adachi O, Ogawa T, Takeda K, and Akira S (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**:115-122.

Kobayashi KS and Flavell RA (2004) Shielding the double-edged sword: negative regulation of the innate immune system. *J Leukoc Biol* **75**:428-433.

Leaver SK, Finney SJ, Burke-Gaffney A, and Evans TW (2007) Sepsis since the discovery of Toll-like receptors: Disease concepts and therapeutic opportunities. *Crit Care Med* **35**:1404-1410.

Lee HK, Dunzendorfer S, Soldau K, and Tobias PS (2006) Double-stranded RNA-mediated TLR3 activation is enhanced by CD14. *Immunity* **24**:153-163.

Lee HK, Dunzendorfer S, and Tobias PS (2004) Cytoplasmic domain-mediated dimerizations of Toll-like receptor 4 observed by β-lactamase enzyme fragment complementation. *J Biol Chem* **279**:10564-10574.

Medzhitov R, Preston-Hurlburt P, and Janeway, CA Jr (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* **388**:394-397

Medzhitov R (2001) Toll-like receptors and innate immunity. *Nat Rev Immunol* **1**:135-145.

Miggin SM and O'Neill LAJ (2006) New insights into the regulation of TLR signaling. *J Leukoc Biol* **80**:220-226.

Mollen KP, Anand RJ, Tsung A, Prince JM, Levy RM, and Billiar TR (2006) Emerging paradigm: toll-like receptor 4-sentinel for the detection of tissue damage. *Shock* **26**:430-437.

Naqvi T, Lim A, Rouhani R, Singh R, and Eglen RM (2004) β-galactosidase enzyme fragment complementation as a high-throughput screening protease technology. *J Biomol Screen* **9**:398-408.

O'Neill LA (2003) The role of MyD88-like adapters in Toll-like receptor signal transduction. *Biochem Soc Trans* **31**:643-647.

Ulevitch RJ (2004) Therapeutics targeting the innate immune system. *Nat Rev Immunol* **4**:512-520.

Wehrman T, Kleaveland B, Her JH, Balint RF, and Blau HM (2002) Protein-protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments. *Proc Natl Acad Sci USA* **99**:3469-3474.

Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, Hoshino K, Takeuchi O, Kobayashi M, Fujita T, Takeda K, and Akira S (2002) Essential role for TIRAP in activation of the signaling cascade shared by TLR2 and TLR4. *Nature* **420**:324-329.

Zhang JH., Chung TDY, and Oldenburg KR (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* **4**:67-73.

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FOOTNOTES

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FIGURE LEGENDS

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 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.

Fig. 2. Compound screening and hit selection. (A) EC_{50} 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 multi-label reader (PerkinElmer). EC_{50} of clavulanic acid was analyzed using Prism software. The table beside the graph summarizes the signal-to-background (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

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represents 19,200 wells in total. The well number vs. percent inhibition of the high (10 μg/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 ten 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 μg/ml clavulanic acid or 10 μM 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.

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 indicated MyD88, TLR4 and IgG light chain (LC). (B) Percent binding of MyD88 to TLR4CD was analyzed using Quantity One software (Bio-Rad).

Fig. 4. Inhibition of LPS-mediated NF-κB 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 pre-treated for 30 min with 0.37, 1.1, 3.3 or 10 μM inhibitory compound as

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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-^{32}P]ATP. (B) Percent activation of NF-κB translocation was analyzed using Quantity One Software (Bio-Rad).

Fig. 5. Inhibition of LPS-stimulated cell activation. (A) Inhibition of LPS-mediated IL-8 promoter activity in HeLa cells. HeLa cells were transiently co-transfected with TLR4/CD14/MD-2 vectors (0.01 μg/ml each) as well as pIL8-promoter-Luc and pSV-β-galactosidase vectors (0.05 μg/ml each). After 24 h, cells were pre-incubated with increasing amounts of compounds for 30 min as indicated and stimulated with 0.1 μg/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 ± S.D. *$p < 0.05$ versus LPS alone (Mann-Whitney U test). Cont, LPS-negative vehicle control. (B, C) Inhibition of LPS-mediated inflammatory cytokine production in RAW264.7 cells. RAW264.7 cells were pre-incubated 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 μg/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 ± 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.

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Fig. 6. Inhibition of MyD88-dependent NF-κB induction. RAW264.7 cells were grown 12-well tissue culture plates with DMEM/10% FCS for 24 h and pre-treated with 10 μM inhibitory compounds 50-F12 (A) and 26-J10 (B) for 30 min. Cells were then stimulated with MALP-2 (50 ng/ml), pIpC (20 μg/ml), LPS (0.1 μg/ml), CpG (20 μg/ml), IL-1β (50 ng/ml), or TNF-α (50 μg/ml) for 1 h. Nuclear extracts were then prepared and gel-shift assay was performed using NF-κB 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, Cornwall, England) are parenthesized.

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FIG. 1

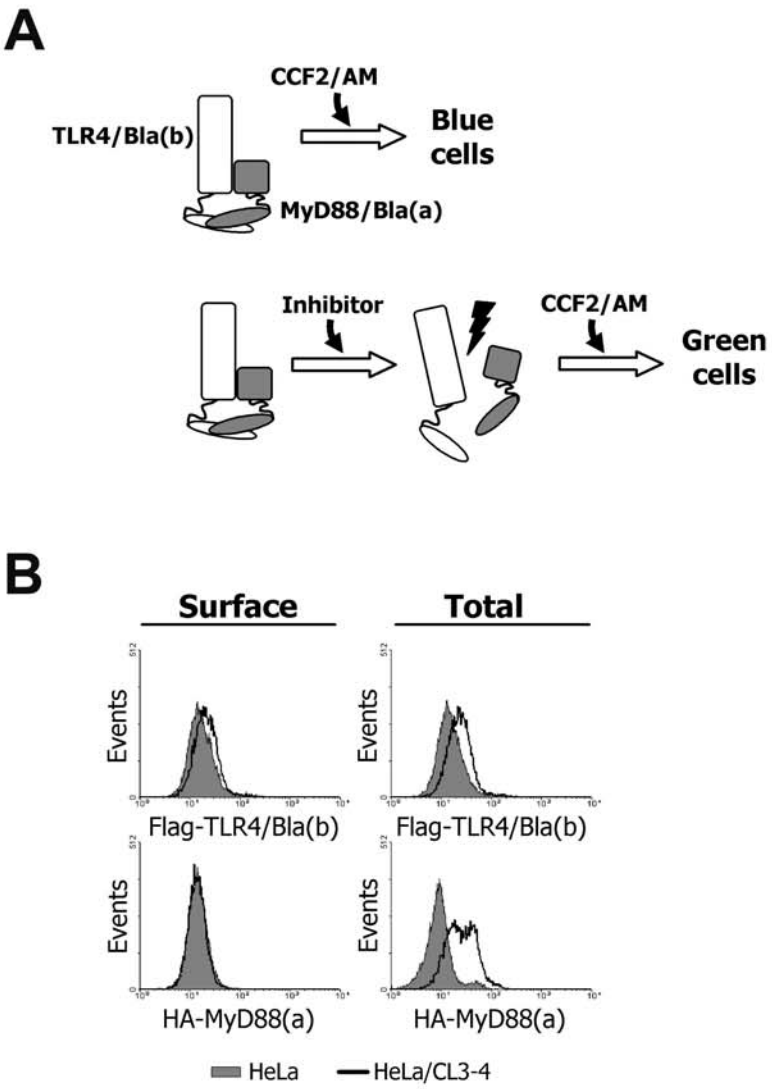

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Fig. 2

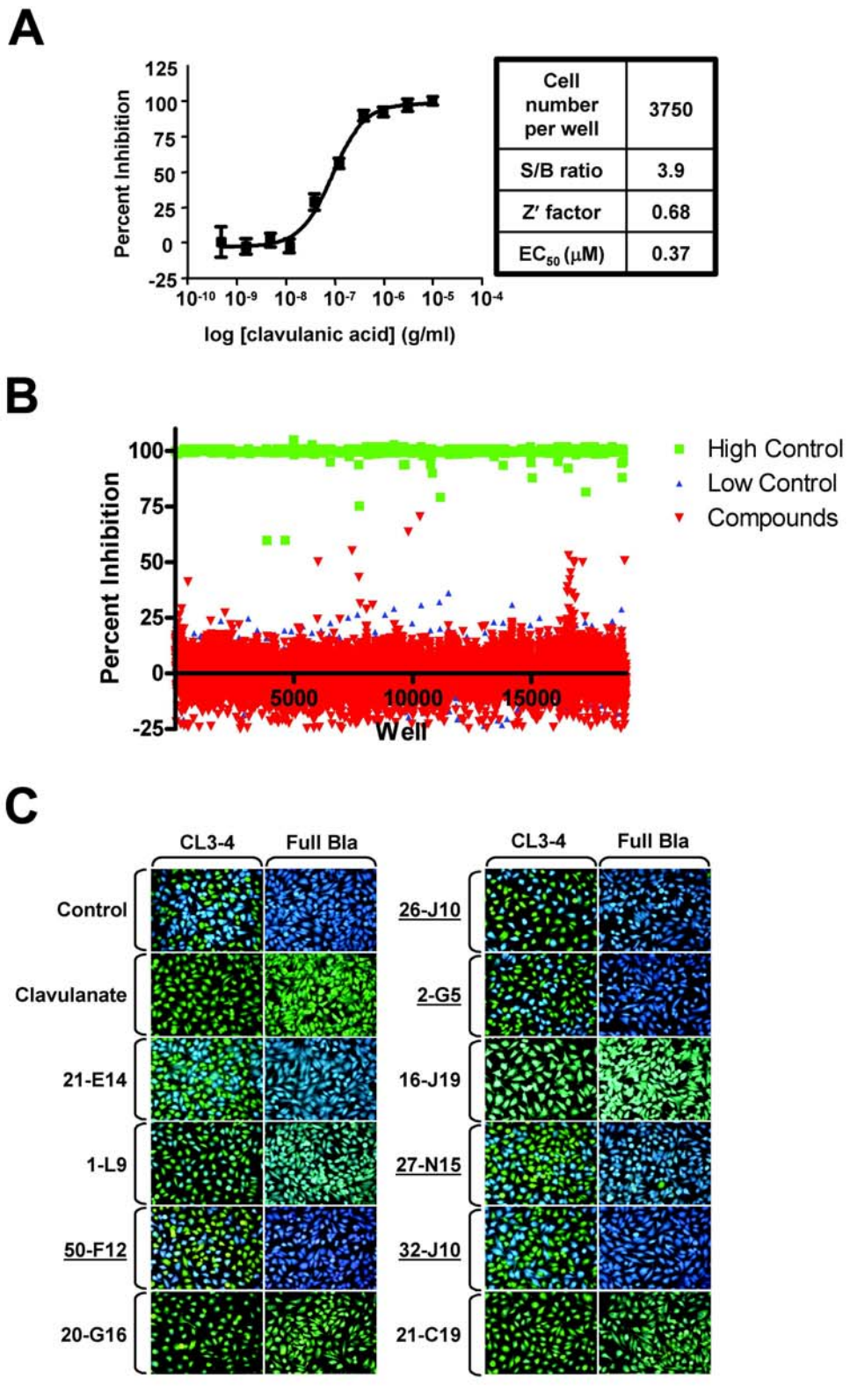

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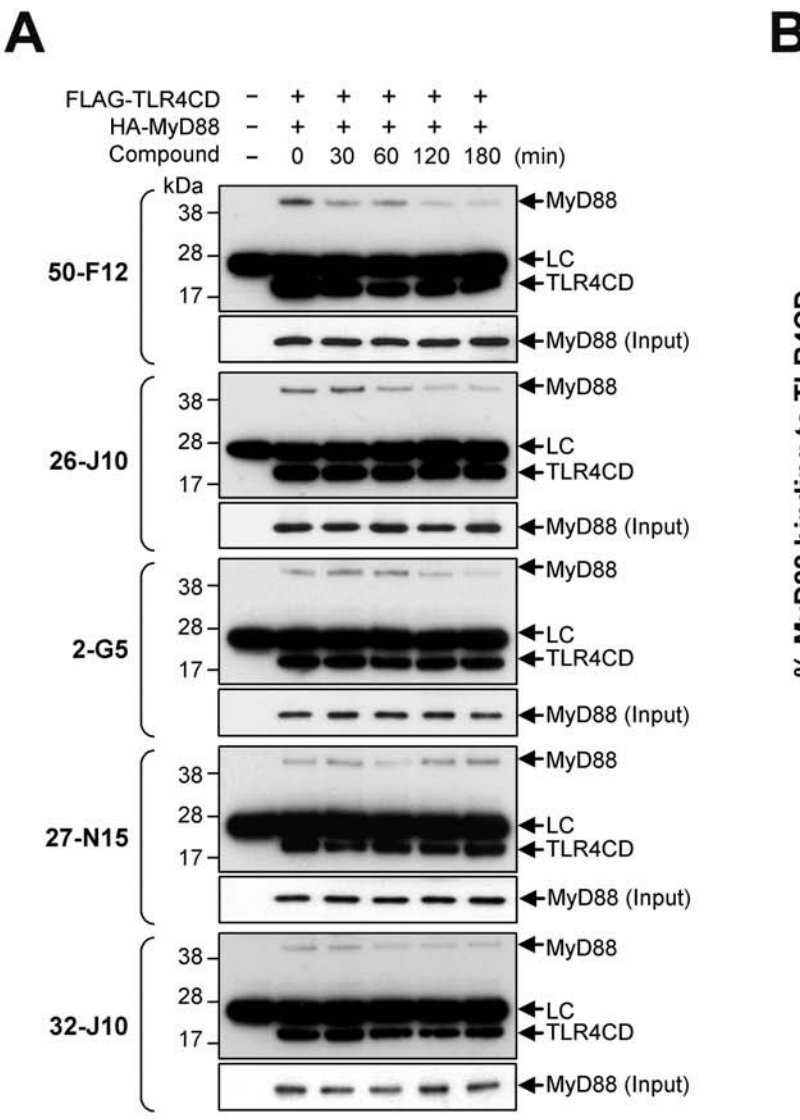
FIG. 3



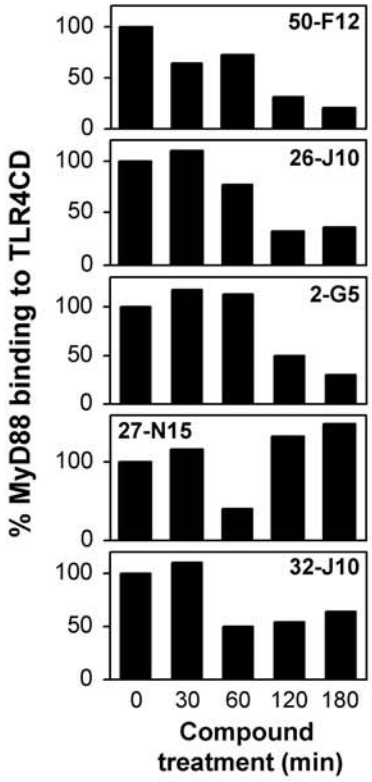


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FIG. 4

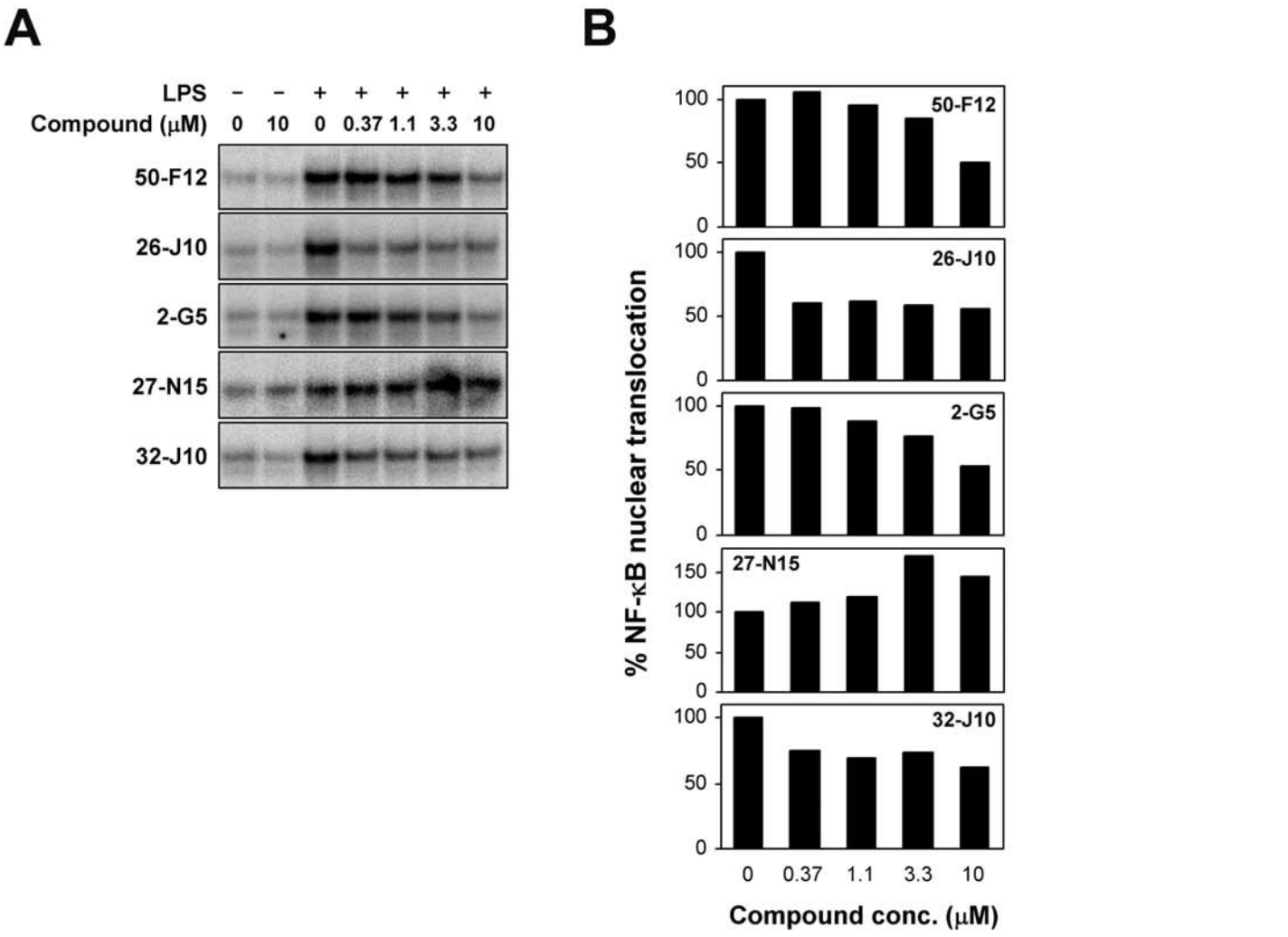

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FIG. 5

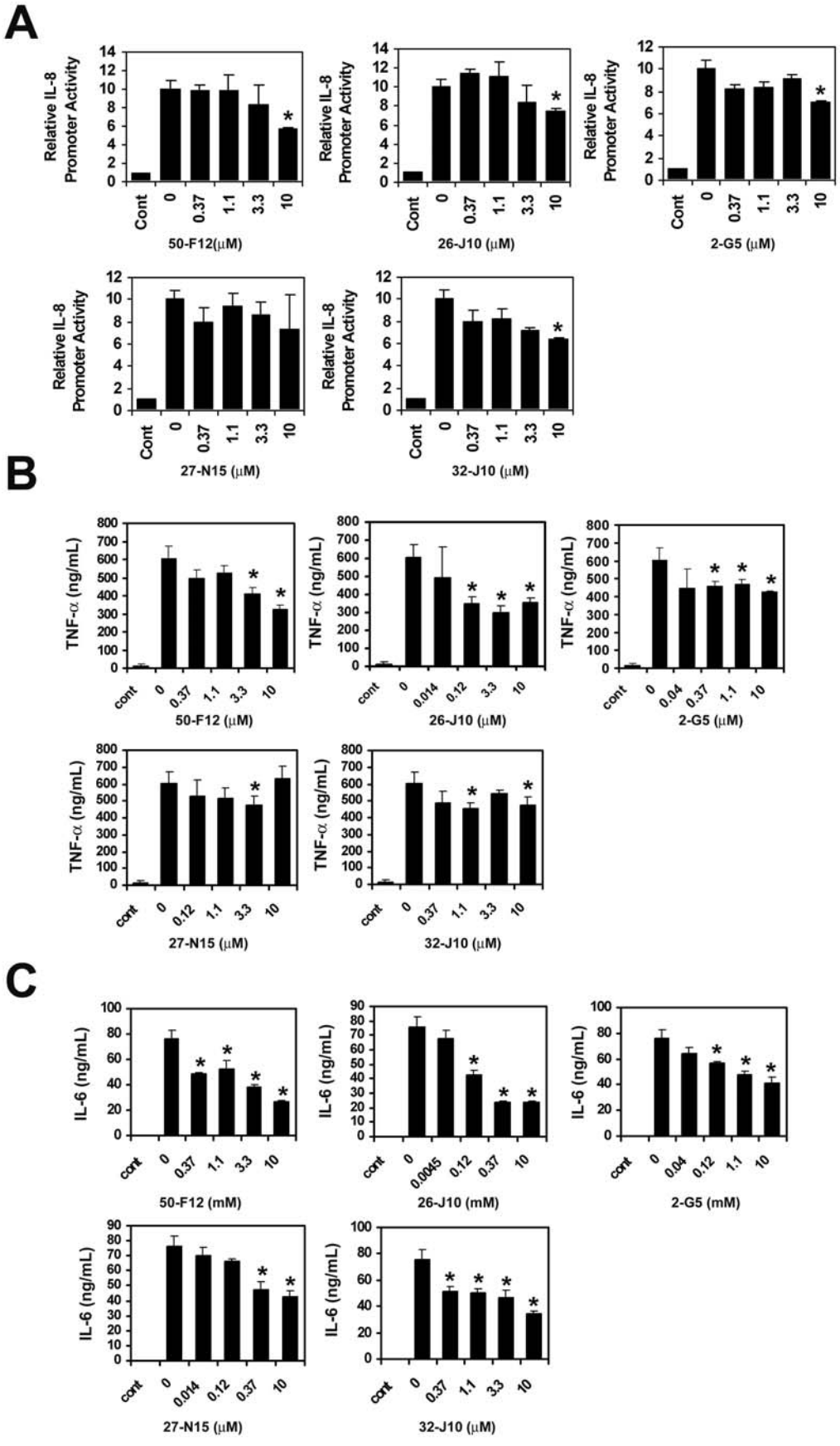

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FIG. 6

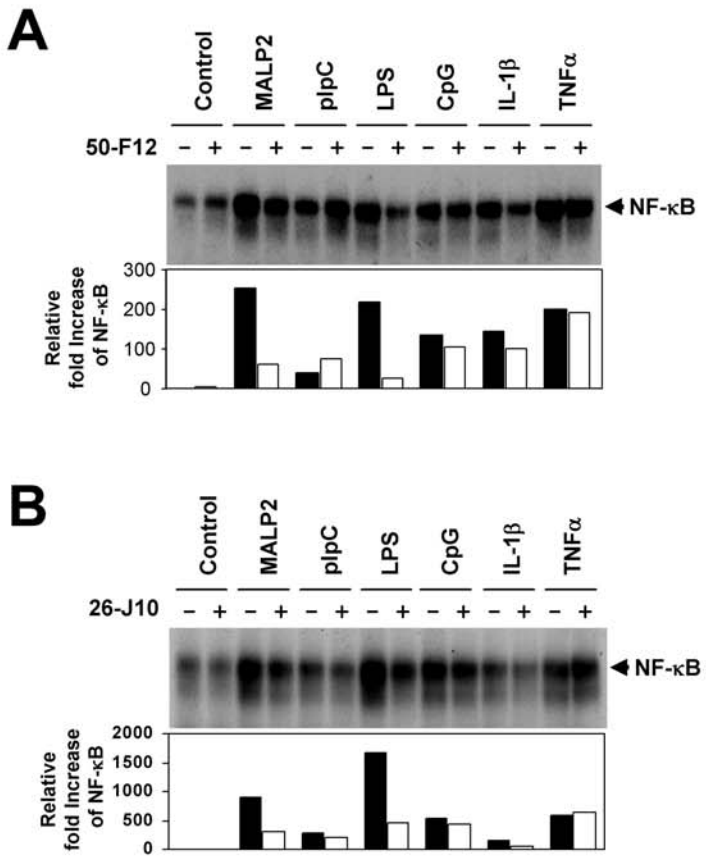

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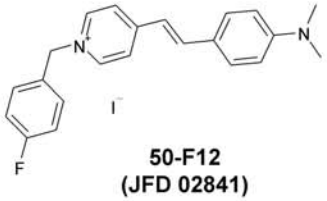
FIG. 7



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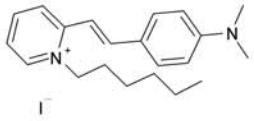

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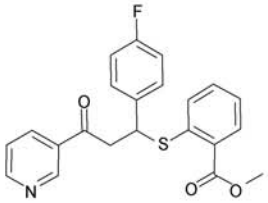

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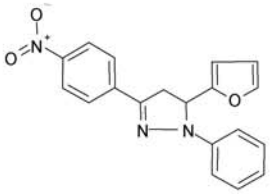

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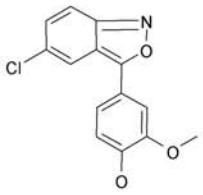

32-J10
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