Curcumin Analog L48H37 Prevents Lipopolysaccharide-Induced TLR4 Signaling Pathway Activation and Sepsis via Targeting MD2

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ABSTRACT

Endotoxin-induced acute inflammatory diseases such as sepsis, mediated by excessive production of various proinflammatory cytokines, remain the leading cause of mortality in critically ill patients. Lipopolysaccharide (LPS), the characteristic endotoxin found in the outer membrane of Gram-negative bacteria, can induce the innate immune system and through the myeloid differentiation protein 2 (MD2) and Toll-like receptor 4 (TLR4) complex, increase the production of inflammatory mediators. Our previous studies have found that a curcumin analog, L48H37 [1-ethyl-3,5-bis(3,4,5-trimethoxybenzylidene)piperidin-4-one], was able to inhibit LPS-induced inflammation, particularly tumor necrosis factor α and interleukin 6 production and gene expression in mouse macrophages. In this study, a series of biochemical experiments demonstrate L48H37 specifically targets MD2 and inhibits the interaction and signaling transduction of LPS-TLR4/MD2. L48H37 binds to the hydrophobic region of MD2 pocket and forms hydrogen bond interactions with Arg90 and Tyr102. Subsequently, L48H37 was shown to suppress LPS-induced mitogen-activated protein kinase phosphorylation and nuclear factor κB activation in macrophages; it also dose dependently inhibits the cytokine expression in macrophages and human peripheral blood mononuclear cells stimulated by LPS. In LPS-induced septic mice, both pretreatment and treatment with L48H37 significantly improved survival and protected lung injury. Taken together, this work identified a new MD2 specific inhibitor, L48H37, as a potential candidate in the treatment of sepsis.

Introduction

Sepsis, or systemic inflammatory response syndrome, is a severe condition marked by an overwhelming immune response to a serious infection and results in the excessive production of various proinflammatory cytokines and cellular injury. In critically ill patients, sepsis is the leading cause of mortality, with hospital mortality rates between 15–30% (Gayeski and Goyal, 2013), and is responsible globally for millions of deaths each year (Balk, 2014). Bacteria are the most common culprits in infections that develop into sepsis, particularly Gram-negative bacteria, owing to lipopolysaccharide (LPS), the major glycolipid found in their outer membrane. Following infection with Gram-negative bacteria, the host is exposed to microbial LPS through ancillary proteins, such as LPS-binding protein and CD14, which transport the microbial LPS to bind a surface receptor complex composed of Toll-like receptor 4 (TLR4) and myeloid differentiation protein 2 (MD2) in specific target cells. Subsequent TLR4 activation leads to the recruitment of myeloid differentiation primary-response gene 88 (MyD88), activating downstream nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways. It is well known that LPS from Gram-negative bacteria is a potent stimulant of the immune response (Rossol et al., 2011), and the activation of the NF-κB and MAPK pathways induces the upregulation and increased expression of proinflammatory genes, contributing to multiple organ dysfunction and systemic

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ABBREVIATIONS: bis-ANS, 1,1′-bis(anilino)-4,4′-bis(naphthalene)-8,8′-disulfonate; CRX-526, (2S)-2-[[3R]-3-hexanoyloxytetradecanoyl]amino]-3-[[2R,3R,4S,SS,6R]-3-[[3R]-3-hexanoyloxytetradecanoyl]amino]-4-[[3R]-3-hexanoyloxytetradecanoyl]oxy-6-hydroxymethyl]-5-phosphonoxyoxan-2-yl oxypropanoic acid; DMSO, dimethyl sulfoxide; E5531, (6-O-[2-deoxy-6-O-methyl-4-O-phosphono-3-O-[[3R]-3-Z-dodec-5-enoyloxydecyl]-2-[3-oxo-tetradecanoylamido]-β-O-phosphono-α-o-glucopyranose tetrasodium salt; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-related kinase; FITC, fluorescein isothiocyanate; IL, interleukin; JNK, c-Jun N-terminal kinase; JSH, 2,4-dihydroxy-6-isopentyl-oxochalcone; JT-T705, S-[2-[[1-2-ethylbutoxy(cyclohexyl)carbonyl]amino]phenyl] 2-methylpropanethiol; L48H37, 1-ethyl-3,5-bis(3,4,5-trimethoxybenzylidene)piperidin-4-one; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MPM, mouse primary peritoneal macrophage; NF, nuclear factor; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qPCR, quantitative PCR; rhMD2, recombinant human MD2; RT-qPCR, reverse transcription qPCR; SPR, surface plasmon resonance; TAK-242, ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate; TNF, tumor necrosis factor.
inflammatory response syndrome (Park et al., 2012; Cighetti et al., 2014).

Interestingly, rather than TLR4, it is MD2 that recognizes the lipid A moiety of LPS, and MD2 is absolutely required to trigger LPS-induced TLR4 activity and primarily responsible for determining the specificity of different LPS chemotypes (Park et al., 2012). Since MD2 plays a critical role in LPS recognition, increasing studies reveal that MD2 can be the potential therapeutic target of acute inflammatory disorders, including sepsis. So far, several MD2 inhibitors have been reported (Fig. 1A). For example, Peluso et al. (2010) showed that a chalcone derivative, xanthohumol, competitively displaced LPS from MD2, which inhibited LPS-induced TLR4 activity. Two natural compounds, caffeic acid phenethyl ester and JSH [2’,4-dihydroxy-6’-isopentyloxychalcone], containing the same moiety 3-(4-hydroxyphenyl) acrylaldehyde in the structures, were also found to inhibit LPS-induced TLR4 activation partly by interfering with LPS binding to MD2 (Roh et al., 2011; Kim et al., 2013). Curcumin, isolated from the natural spice turmeric, is a pleiotropic molecule that exhibits many pharmacological effects, including anti-inflammatory, antitumor, antioxidative, and cardiovascular protective effects (Prasad et al., 2014). It has been shown that curcumin is able to regulate a variety of molecular targets in cells (Hasima and Aggarwal, 2012). With the moiety 3-(4-hydroxyphenyl) acrylaldehyde in its structure, curcumin has been shown to bind at submicromolar affinity to MD2, competing with LPS for the same binding site and resulting in the pharmacological outcomes in suppression of the inflammation caused by LPS (Gradisar et al., 2007).

In the past decade, our laboratory has been engaged in medicinal chemistry, turning to natural products, such as curcumin, in efforts to discover new anti-inflammatory drugs. Our laboratory and others observed that owing to curcumin’s poor stability under physiologic conditions, its clinical application was extremely limited (Joe et al., 2004). Therefore, in efforts to increase the stability of curcumin, we designed and synthesized in our previous studies a series of mono-carbonyl curcumin analogs without the β-diketone moiety of curcumin, which showed enhanced chemical stability (Fig. 1B) (Wu et al., 2013). Among these curcumin analogs, 1-ethyl-3,5-bis(3,4,5-trimethoxybenzylidene)piperidin-4-one (L48H37; Fig. 1B), exhibited high chemical stability and strong anti-inflammatory ability. The aim of the present study was to find the molecular target of L48H37 as well as the underlying mechanism of its anti-inflammatory actions. On the basis of the structural similarity, we hypothesized that L48H37 exerted its anti-inflammatory actions by directly targeting MD2. This study identified L48H37 as a novel and specific MD2 inhibitor that can directly bind to MD2, block the LPS-TLR4/MD2 signaling activation, and suppress the expression of inflammatory cytokine in vitro and endotoxin-induced septic shock in vivo.

Materials and Methods

Cells, Materials, and Reagents. Mouse primary peritoneal macrophages (MPMs) were prepared and cultured from C57BL/6 mice using the method described in our previous paper (Pan et al., 2013). Human peripheral blood mononuclear cells (PBMCs) were purified as described by Goodall et al. (2014). Briefly, the whole blood...
was overlaid on Ficoll Hypaque (GE Healthcare, Buckinghamshire, UK) at a 2:1 ratio of blood to Ficoll before separation via centrifugation at 1600 rpm without braking for 30 minutes at room temperature. After the layers were separated, the PBMC layer was directly removed from above the Ficoll layer and washed three times with phosphate-buffered saline (PBS). Collected PBMCs were resuspended in RPMI for further analysis, Curcumin, LPS (from Salmonella typhosa), TLR2 agonist Pam3CCK, and fluorochrome isothiocyanate (FITC)-labeled LPS (LPS-FITC; from Escherichia coli 055:B5) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against p65, p-p65, c-Jun N-terminal kinase (JNK), p-JNK, extracellular signal-related kinase (ERK), p-ERK, Lkβ1, and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX). Recombinant human TLR4 protein was purchased from Sino Biologic Inc. (Beijing, China). Anti-human MD2 antibody and mouse tumor necrosis factor (TNF)-α and interleukin (IL)-6 enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBioscience (eBioscience, San Diego, CA).

**Synthesis of L48H37.** The procedure for synthesis of compound L48H37 is briefly described: The compounds 1-ethylpiperidin-4-one (2 mmol) and 3,4,5-trimethoxybenzaldehyde (4 mmol) were dissolved in water with macrogol 15 hydroxystearate (a nonionic solubilizer for injection from BASF, Florham Park, NJ). The rhMD2 mutations R90A and Y102A were introduced into the pET28a vector by polymerase chain reaction (PCR)-based mutagenesis (primers for mutations were listed in Table II). Three expression vectors were cloned into E. coli BL21(DE3). One millimolar isopropl-β-D-thiogalactoside was added to induce protein expression, and the culture was allowed to incubate at 28°C for 8 hours. After extraction from E. coli cells using a combination of lysozyme and sonication, the inclusion bodies were harvested and dissolved with 50 mM Tris-HCl, 0.6 M NaCl, and 8 M urea (pH 8.0), and kept at room temperature for 12 hours. Centrifugation at 12,000 rpm for 30 minutes removed any residual insoluble matter, and the supernatant was filtered through a 0.22-μm filter (EMD Millipore, Billerica, MA). The diluted supernatant was applied to Ni-IDA Sepharose 6 Fast Flow (General Electric, Fairfield, CT) according to the manufacturer's instructions. Refolding of rhMD2, rhMD2/R90A, or rhMD2/Y102A (4 μg/mL) was then treated for 5 minutes, and the relative fluorescence units emitted at 430–590 nm were measured.

**Docking of L48H37 to the MD2 Structural Model.** Docking simulation of L48H37 with MD2 protein (PDB ID 2E59) was carried out with the Tripos molecular modeling package Sybyl-2.0 (Tripos, St. Louis, MO). The ligand–receptor complex went through energy minimization using the Tripos force field and Gasteiger-Hückel electrostatic charges, according to the protocol previously indicated. To allow for flexible docking and production of over 100 structures, the ligand-binding groove on MD2 was kept fixed, whereas all torsible bonds of L48H37 were kept flexible. Final docked conformations were clustered within the tolerance of 1 Å root-mean-square deviation.

**Surface Plasmon Resonance Analysis.** The binding affinity of L48H37 was determined using a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) with an ECL sensor chip (ProteOn). Briefly, rhMD2, rhTLR4, rhMD2/R90A, or rhMD2/Y102A (in acetate and buffer pH 5.5) was loaded to the sensor, which was activated with 10 mM NISodium, and the L48H37 samples (at 100, 50, 25, 12.5, and 6.25 μM) were prepared with running buffer (PBS, 0.1% SDS, 5% DMSO). Sensor and sample plates were placed on the instrument. The L48H37 samples were then captured in the first flow cells, and the second flow cell was left as a blank. Five concentrations...
were simultaneously injected at a flow rate of 30 μM/min for 120 seconds of association phase, followed by 120 seconds of dissociation phase at 25°C. The final graphs represent the difference between the duplex or quadruplex sensorgrams and the blank sensorgrams. Data analysis was done using the ProteOn Manager Software, and the KD was calculated by aligning the kinetic data from various concentrations of L48H37 to the 1.1 Langmuir binding model.

Western Blot. Collected cells were lysed, and 30 mg of lysates was separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Following preincubation for 1 hour at room temperature in Tris-buffered saline (pH 7.6) with 0.05% Tween-20 and 5% nonfat milk, each membrane was then incubated with specific antibodies. Following incubation with a secondary antibody conjugated with horseradish peroxidase, the membrane was visualized using enhanced chemiluminescence reagents (Bio-Rad), and the immunoreactive bands were then detected. The protein levels were analyzed using ImageJ software version 1.38e (NIH, Bethesda, MD) and normalized to their respective control.

Assay of Cellular NF-κB p65 Translocation. Using a Cellular NF-κB p65 Translocation Kit (Beyotime Biotech, Nantong, China), the cells were immunofluorescence-labeled in accordance with the manufacturer's instructions. Briefly, cultured MPMs were pretreated with L48H37 (10 μM) or the vehicle control (DMSO) for 2 hours, and then stimulated with LPS (0.5 μg/ml) for 1 hour. After 1 hour of treatment, the cells were incubated with p65 antibody and Cy3 fluororescin-conjugated secondary antibody, and nuclei were stained with 2-(4-aminophenyl)-1H-indole-6-carboxamidine. The images (200× magnification) were obtained by fluorescence microscopy. The experiment was repeated independently three times with similar results, and the results were quantified.

ELISA. ELISA kits (eBioscience) were used to measure the protein levels of TNF-α and IL-6 in the culture medium. The total amount of cytokines in the cell medium was normalized to the total amount of protein in the viable cell pellet. The experiments were performed in triplicate.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction. Cells were homogenized in Trizol (Invitrogen) according to the manufacturer's protocol for extraction of RNA. A two-step M-MLV Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) was used for both quantitative (qPCR) and reverse transcription qPCR (RT-qPCR), and Eppendorf's Mastercycler replex detection system (Eppendorf, Hamburg, Germany) was used for qPCR analysis. The primers of genes used are shown as follows:

- mouse TNF-α forward 5’-TCTCATCCTGGTGGTGTGGCAG-3’ and reverse 5’-TCTCAAGTTGTTGCTGATCAG-3’
- mouse IL-6 forward 5’-CCAAAGGAGGTGGACTGCCTTCC-3’ and reverse 5’-CTGGTTGTCACCTGCCTCCT-3’
- mouse IL-1β forward 5’-ACTCCCTATGCTCGCGCCCA-3’ and reverse 5’-CCATCAGGCAACAGGAGGAA-3’
- mouse IL-10 forward 5’-GGTTGGCAAACCGTTATCAAGGA-3’ and reverse 5’-ACCTGTCCACTGCTGCTG-3’
- mouse COX-2 forward 5’-TCTGTGCCTGGTTCCTGATG-3’ and reverse 5’-ACGTGAAAGCTGCTGCTG-3’
- mouse iNOS forward 5’-GCTGGGTGGTGTCAGACAACTC-3’ and reverse 5’-CACCAGTGCTGGTGTGGG-3’
- mouse β-actin forward 5’-TGCACCAACGACTGCTTAGG-3’ and reverse 5’-GGATGACGGAGGTGGTTC-3’
- human TNF-α forward 5’-CCCAAGGAGAGGTTCTTCACT-3’ and reverse 5’-ATGGGCCCTACACGTGTGTTCC-3’
- human IL-6 forward 5’-GCACTGCCAAGAAAACACCT-3’ and reverse 5’-TCAAACTCTAGAAGACCGTGGA-3’
- human β-actin forward 5’-GCTGGACACCAGACCAATAC-3’ and reverse 5’-GCAGGATCCACAGGAGTACT-3’

All primers were synthesized and purchased from Invitrogen (Shanghai, China). The gene expression levels were normalized to the amount of β-actin.

Treatment of Mice with L48H37 in Endotoxic Mouse Model. Male C57BL/6 mice weighing 18–22 g were injected with 200 μl of LPS (at 20 mg/kg i.v. and through tail vein) 15 minutes before (for treatment) or after (for prevention) injection of L48H37 (at 10 mg/kg i.v. and through tail vein), respectively. LPS was used for intravenous injection in a 0.9% saline. Body weight change and mortality were recorded for 7 days. In another experiment, male C57BL/6 mice weighing 18–22 g were injected with 200 μl of LPS (at 20 mg/kg i.v. and through tail vein) 15 minutes after injection of L48H37 (at 10 mg/kg i.v. and through tail vein). Two or eight hours after LPS injection, mice were anesthetized with diethyl ether and sacrificed. Lung samples were harvested and fixed in 10% formalin for 24 hours. The formalin-fixed lung samples were then embedded in paraffin, sectioned, stained with H&E, and observed 20× under a light microscope. In the above in vivo studies, mice in both the vehicle control group and LPS alone group received 100 μl solution of 7.5% macrogol 15 hydroxy stearate in water, and mice in vehicle control group also received 200 μl saline.

Statistical Analysis. All values are represented as means ± S.E.M. from three independent experiments. Data analysis, including statistical analysis with the student’s t test or one-way analysis of variance, was done using GraphPad Prism 5.0. A P value of <0.05 was considered to be statistically significant.

Results

Chemical Stability of L48H37 Was Improved In Vitro. The chemical stability of L48H37 and curcumin was tested using an absorption spectrum assay. Figure 1C shows that the UV-visible absorption spectrum of curcumin displayed a significant peak with a maximum absorption of close to 425 nm. However, the intensity of curcumin’s absorption spectrum significantly decreased over time in the phosphate buffer (pH 7.4). In contrast, L48H37 showed no degradation under the same conditions (Fig. 1C), suggesting that a chemical modification to L48H37 significantly increased curcumin’s stability and attenuated in vitro degradation.

L48H37 Blocks the Interaction between MD2 and LPS. We first determined the effect of L48H37 on LPS-induced TLR4/MD2 complex conformation by immunoprecipitation assay. As shown in Fig. 2A, the complex of TLR4/MD2 profoundly increased in LPS-stimulated macrophages, whereas treatment with L48H37 significantly inhibited LPS-induced TLR4/MD2 complex. It is unclear whether L48H37 directly affects the interaction of LPS-MD2 or that of TLR4-MD2. Since MD2 is mainly located in the cell membrane, where LPS can interact with MD2, we further tested if L48H37 is able to reduce the LPS-MD2 binding in cell surface. MPMs, with or without L48H37, were incubated with FITC-marked LPS (FITC-LPS) and then subjected to flow cytometry analysis. Figure 2B shows that FITC-LPS binds to the cell surface with a median fluorescence intensity of 17.4, and treatment with L48H37 dose dependently reduced the interaction of FITC-LPS with the receptor on the cell surface. To validate the effects of L48H37 on the interaction between LPS and MD2, we established a biotin-streptavidin–based ELISA system at the molecular level. The results in Fig. 2C show that biotin-marked LPS was able to bind to recombinant human MD2 (rhMD2) protein in the plates, whereas coincubation with L48H37 significantly blocked the interaction of biotin-marked LPS and rhMD2.

L48H37 Directly Binds to MD2 Protein. The direct interaction of L48H37 and rhMD2 protein was determined using fluorescence spectroscopy and surface plasmon resonance (SPR) assay. As shown in Fig. 2D, fluorescence values of bis-ANS, a fluorescent probe used to map the hydrophobic
**Fig. 2.** Antagonistic effect of L48H37 on LPS binding to MD2. (A) Coimmunoprecipitation of MD2. MPMs were pretreated with L48H37 (10 μM) or DMSO for 30 minutes and then incubated with LPS (1 μg/ml) for 5 minutes. Cells were lysed and the total protein was collected. Four hundred micrograms of the total protein was incubated with beads and anti-MD2 antibody overnight at 4°C. The immunoprecipitated proteins and precipitated MD2 proteins, were resolved by SDS-PAGE and detected using anti-TLR4 antibody. The column figures represent the mean optical density ratio of three independent experiments (**P, 0.01). (B) Flow cytometric analysis. HUVEC304 cells were incubated with media alone (Ctrl), L48H37 (10 μM), LPS-FITC (50 μg/ml), and LPS-FITC (50 μg/ml) plus L48H37 (0.1, 1, and 10 μM), respectively. These cells were subjected to flow cytometry analysis, in which the values for the median fluorescence intensity were also provided. Conc., concentration. (C) In vitro assays for LPS binding to MD2. rhMD2 antibody was coated to a 96-well plate at 4°C overnight. rhMD2 (4 μg/ml) in 10 mM Tris-HCl buffer was added to the precoated plate for 1.5 hours at room temperature. After washing with PBS–TWEEN-20, biotin-labeled LPS was added to the plate with or without the presence of L48H37 (1.5 μM). LPS ability to bind to rhMD2 was determined using ELISA, represented by absorbance values at 450 nm (A450). Data are mean values (± S.E.M.) of three separate experiments, each performed in duplicate. **P < 0.01 versus buffer alone–added group. (D) Fluorescence measurements. bis-ANS (5 μM) was preincubated with rhMD2 (5 nM) to reach stable fluorescence values under excitation at 380 nm and to reach stable relative fluorescence units emitted at 430–590 nm under excitation at 385 nm. Nonfluorescent L48H37 (at 2.5, 5, 10, 20, 30 μM) was then treated for 5 minutes, and the relative fluorescence units emitted at 430–590 nm were measured. (E) SPR analysis showed that L48H37 could not directly bind to rhTLR4 protein. (F) The binding affinity of L48H37 with rhMD2 was determined using a SPR assay. Conc., concentration; IB, immunoblot; IP, immunoprecipitation; MFI, mean fluorescence intensity; RU, response unit.
binding sites in proteins, were markedly enhanced upon binding to cell-free rhMD2 protein, whereas incubation with L48H37 dose dependently decreased the fluorescence intensity of bis-ANS, suggesting that L48H37 competitively binds to rhMD2. Next, the SPR experiments showed no interaction between L48H37 and recombinant human TLR4 proteins (Fig. 2E), whereas Fig. 2F shows that L48H37 directly binds rhMD2 protein in a dose-dependent manner and with a very high affinity ($K_D = 0.000113 \text{ M}$). These data indicate that L48H37 is a novel and MD2-specific inhibitor.

**L48H37 Acts on Arg$^{90}$ and Tyr$^{102}$ Residues in MD2 Protein Pocket.** We further predict the underlying binding

![Diagram of LPS binding to MD2](image-url)

**Fig. 3.** Antagonistic mechanism of L48H37 on LPS binding to MD2. (A) Molecular docking of L48H37 with rhMD2 (PDB ID 2E56) was analyzed with the Sybyl-2.0 molecular modeling software from Tripos. hTLR4 and rhMD2 are shown in white and green, respectively; (B and C) Surface plasmon resonance analysis. rhMD2$^{R90A}$ or rhMD2$^{Y102A}$ was biotinylated with biotin, and L48H37 was diluted to 100, 50, 25, 12.5, or 6.25 μM. The binding affinity of L48H37 was determined using a fortéBio Octet Red (Menlo Park, CA) equipped with a super streptavidin sensor; (D and E) In vitro assay for LPS binding to MD2 variants. rhMD2 antibody was coated on a 96-well plate at 4°C overnight. rhMD2$^{R90A}$ or rhMD2$^{Y102A}$ (4 μg/ml) in 10 mM Tris-HCl buffer was added to the precoated plate for 1.5 hours at room temperature. After washing with PBS–Tween-20, biotin-labeled LPS was added to the plate with or without the L48H37 treatment (1.5 μM). LPS binding to rhMD2 was determined by ELISA and represented by absorbance values at 450 nm ($A_{450}$). Data are mean values (±S.E.M.) of three separate experiments, each performed in duplicate. RU, response unit.
mode of L48H37 in MD2 protein using a molecular simulation of L48H37-MD2 complex. As shown in Fig. 3A, L48H37 was fitted into the hydrophobic pocket of MD2, interacting with the residues, including Tyr102, Phe121, Leu61, Cys133, and Arg90, in the most energetically favorable configuration (Fig. 3A). The whole molecule of L48H37 is buried inside the lipid-binding pocket and overlaps to a large extent with the binding sites of LPS, indicating the structural mechanism behind L48H37's observed competitive inhibition of LPS. The computer-assisted simulation also shows that the two amino residues Arg90 and Tyr102 are the most probable candidates to form hydrogen bonds with L48H37 (Fig. 3A). Thus, to confirm the importance of Arg90 and Tyr102 in L48H37 binding to rhMD2, two new rhMD2 mutations, rhMD2R90A or rhMD2Y102A, were prepared, respectively. SPR assay indicated that L48H37 no longer binds to these two mutations (Fig. 3, B and C), and the ELISA method also found that L48H37 could not inhibit the binding of Biotin-LPS with either rhMD2R90A or rhMD2Y102A (Fig. 3, D and E). These results present the possible binding sites of L48H37 in the MD2 protein pocket, which we believe will be helpful in the design of new MD2 inhibitors.

**L48H37 Inhibited LPS-Induced MAPKs and NF-κB Activation in Macrophages.** We then determined the effects of L48H37 on LPS-activated downstream signaling in the TLR4/MD2 cascade, including the representative MAPKs pathway and the transcriptional factor NF-κB. The MAPK family consists of ERK, p38, and JNK. Figure 4A shows that all three pathways were activated by LPS stimulation in MPMs, whereas the LPS-induced phosphorylations of ERK, p38, and JNK were markedly decreased following pretreatment with L48H37 in a dose-dependent manner. After IκB degradation, NF-κB p65 translocates from the cytoplasm to the nucleus, binds to the target promoters, and induces transcription. Using Western blotting, we first evaluated the effect of L48H37 on IκB degradation in total cell protein extracts. LPS exposure for 1 hour induced an 84% degradation of IκBα. Thus, we treated MPMs with LPS (0.5 μg/ml) for 1 hour in the presence of L48H37 (0.5 μM) or DMSO (vehicle control) followed by Western blotting with a p65 antibody. The column figures represent the mean optical density ratio in three independent experiments. *P < 0.05, **P < 0.01, versus the LPS-treated group. (C) Cultured MPMs were pretreated with L48H37 (10 μM) or vehicle control (DMSO) for 2 hours, and then stimulated with LPS (0.5 μg/ml) for 1 hour. After 1 hour of treatment, the cells were incubated with p65 antibody and Cy3 fluorescein-conjugated secondary antibody (red), and the nuclei were stained with 2-(4-aminophenyl)-1H-indole-6-carboxamidine (blue). The images (200 × magnification) were obtained by fluorescence microscope and overlay. Similar results were obtained for three independent experiments. The column figure for the p65 translocation represents the mean optical density ratio in three independent experiments. *P < 0.05, **P < 0.01, versus LPS-treated group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
of IκB, whereas pretreatment with L48H37 reversed LPS-induced IκB degradation in MPMs in a dose-dependent manner (Fig. 4B). Consequently, as shown in Fig. 4C, LPS stimulation could increase NF-κB p65 nuclear translocation (red point within blue nucleus, as indicated by arrows), whereas in L48H37 pretreated cells, LPS-induced nuclear levels of p65 were significantly decreased.

**L48H37 Strongly Inhibits LPS-Induced Inflammatory Cytokine Expression in Macrophages.** MPMs and human PBMCs were used to examine the anti-inflammatory activity of L48H37. As shown in Fig. 5, A and B, the LPS-induced increases in TNF-α and IL-6 levels were dose dependently inhibited by L48H37 in MPMs. Here, a TLR2 agonist Pam3CK was used as a comparison. Interestingly, although 0.1 μg/ml Pam3CK significantly induced TNF-α overexpression in MPMs, L48H37 could not inhibit the inflammatory response induced by Pam3CK (Fig. 5A). Since MD2 is not required in TLR2 signaling pathway activation, the fact that L48H37 failed to fight TLR2-related inflammation validates the specificity of L48H37 as an MD2 inhibitor.

**Fig. 5.** L48H37 inhibited LPS-induced inflammatory cytokine expression in mouse macrophages and human PBMCs. (A and B) MPMs were pretreated with the vehicle control (DMSO) or L48H37 (1, 5, or 10 μM) for 2 hours followed by incubation with LPS (0.5 μg/ml) or Pam3CK (0.1 μg/ml) for 22 hours. The protein levels of TNF-α (A) and IL-6 (B) in the culture medium were measured by ELISA. The total amount of cytokines in the cell medium was normalized to the total amount of protein in the viable cell pellet. The results are expressed as a percentage of the LPS-alone group (solid dark bar). Each bar represents mean ± S.E.M. of three to five independent experiments. (C) MPMs were pretreated with vehicle control (DMSO) or L48H37 (10 μM) for 2 hours followed by incubation with LPS (0.5 μg/ml) for 6 hours. The mRNA levels of inflammatory cytokines, including TNF-α, IL-6, IL-1β, IL-10, COX-2, and iNOS were quantified by RT-qPCR. The mRNA values were normalized to the internal control β-actin mRNA and are expressed as a percentage of the LPS control. Each bar represents mean ± S.E.M. of three to five independent experiments. *P < 0.05, **P < 0.01, versus LPS-treated group; ns, not significant.

(D) Human PBMCs were pretreated with the vehicle control (DMSO) or L48H37 (1, 5, or 10 μM) for 2 hours followed by incubation with LPS (0.5 μg/ml) for 6 hours. The mRNA levels of inflammatory cytokines, including TNF-α and IL-6 were quantified by RT-qPCR. The mRNA values were normalized to the internal control β-actin mRNA and are expressed as a ratio of the LPS-alone group (solid dark bar). Each bar represents mean ± S.E.M. of three to five independent experiments. *P < 0.05, **P < 0.01, versus LPS-treated group; ns, not significant.
The anti-inflammatory activity of L48H37 was also observed at the mRNA level. MPMs treated with LPS (0.5 μg/ml) for 6 hours were examined through qPCR for the expression of proinflammatory genes in the presence or absence of L48H37. As shown in Fig. 5C, L48H37 at 10 μM potently inhibited LPS-induced upregulation of TNF-α (54.7%, P < 0.01), IL-6 (82.3%, P < 0.01), IL-1β (91.2%, P < 0.01), cyclooxygenase-2 (COX-2, 57.5%, P < 0.05), and iNOS (50.9%, P < 0.01) transcripts in MPMs. As expected, L48H37 upregulated the expression of the anti-inflammatory cytokine IL-10. Furthermore, similar results were observed in human PBMCs, and L48H37 also significantly and dose dependently suppressed LPS-increased TNF-α and IL-6 expression (Fig. 5D).

**L48H37 Effectively Protects Mice from LPS-Induced Septic Shock and Lung Injury.** Male C57BL/6 mice were injected with LPS (20 mg/kg i.v.) in the presence or absence of L48H37 pretreatment (intravenous), and the survival rates were monitored for 7 days. Figure 6A shows that animals treated with LPS alone all died within 48 hours. In contrast, treatment with L48H37 at 10 mg/kg either 15 minutes prior to LPS injection (prevention group) or 15 minutes after LPS injection (treatment group) significantly improved the survival rates compared with those of the control group (P < 0.01 in both groups versus LPS group). Also, the weight lost in both groups improved slowly 2–7 days after LPS injection (Fig. 6B).

We also examined the beneficial effects of L48H37 on lung injury in LPS-treated mice. Two or eight hours after administration with LPS (20 mg/kg i.v.), histopathological changes in the lungs of C57BL/6 mice were observed using H&E staining. L48H37 pretreatment at 10 mg/kg significantly improved pulmonary damage and amended the LPS-injured tissue structure of pulmonary lobules (Fig. 6C). These data demonstrate the anti-inflammatory effects of L48H37 in septic mice.

**Discussion**

Sepsis can be caused by trauma, infection, or burns and can lead to septic shock and organ failure. More than 30 pharmaceutical candidates for the treatment of sepsis have been developed or are currently in the developmental stage; however, most treatments brought to clinical use have failed because of the complicated nature of sepsis, which remains the most common cause of death in intensive care units (King et al., 2014). Xigris was used to treat sepsis as a recombinant human–activated protein C that attenuates the development...
of organ failure attributable to sepsis. However, no significant improvement was observed in clinical uses, and in 2012 use of Xigris was suspended (Opal et al., 2014). Although statins have some nonspecific anti-inflammatory effects, they are currently not being considered as therapeutic options for sepsis (Gazzarro et al., 2012; Ou et al., 2014). Therefore, there is an urgent need to find novel and effective therapeutic approaches for sepsis.

One potential approach to treating and preventing septic shock and its associated diseases is the intervention of the TLR4/MD2-mediated inflammatory response (Savva and Roger, 2013). LPS is presented to TLR4/MD2 complex via the LPS-binding protein and CD14 (Park and Lee, 2013). MD2 recognizes the lipid A domain of LPS, leading to the formation of the TLR4/MD2/LPS complex and activation of the downstream cellular response (Park et al., 2012; Oblak and Jerala, 2015). Both TLR4 and MD2 are essential for the LPS-induced inflammatory response and sepsis. Both TLR4<sup>−/−</sup> and MD2<sup>−/−</sup> mice fail to respond to LPS and survive endotoxic shock (Duan et al., 2014). Therefore, TLR4 and MD2 are proposed as potential targets for therapy that neutralizes the toxic effects of endotoxin.

The growth in our understanding of the structure and function of the TLR4/MD2 complex has provided a new direction in the development of new drug targets in the treatment and prevention of sepsis (Peri and Calabrese, 2014). Because of the seemingly higher importance of TLR4, researchers paid more attention to TLR4 than MD2 in the past decades (Wittebole et al., 2013). However, the clinical trials of TAK-242 [(6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate], a TLR4-specific inhibitor, have failed to treat severe sepsis and related respiratory disease in patients (Rice et al., 2010). In addition, blocking TLR can lead to severe side effects, “inappropriate” immune responses, such as allergic Th2 responses, or immunologic tolerance (Ishii et al., 2006; Nakamoto and Kunai, 2014). On the other hand, a series of MD2 antagonists with lipid A structure (mimics LPS) targeting the MD2 protein, such as fatty acid chain–containing E5531 [6-O-[2-deoxy-6-O-methyl-4-O-phosphono-3-O-[(R)-3-Z-dodec-5-enoylxydecyl]-2-[3-oxo-tetradecanoylamido]-β-O-phosphono-α-D-glucopyranose tetrasodium salt] (Bryant et al., 2007), CRX-526 [((2R,3S,4R,5S,8R)-3-[(3S)-3-hexanoyloxytetradecanoyl]aminol-3-[(2R,3R,4R,5S,8R)-3-[(3R)-3-hexanoyloxytetradecanoyl]amino]-4-[(3R)-3-hexanoyloxytetradecanoyl]oxy-6-(hydroxymethyl)-5-phosphonoxyoxan-2-yl]oxypropanoic acid] (Lin et al., 2013), and eritoran (Rallabhandi et al., 2012), have been evaluated in clinical and preclinical studies. Unfortunately, the most studied one, eritoran, failed in phase III clinical trials in 2011 because there was no significant improvement in eritoran-treated patients compared with the placebo group (Barochia et al., 2011).

Recently, some natural active compounds that do not contain the structure of lipid A or fatty acids have been found to be able to target MD2 directly (Fig. 1A). These small molecules, such as xanthohumol (Peluso et al., 2010), caffeic acid phenethyl ester (Kim et al., 2013), JSH (Roh et al., 2011), and curcumin (Gradinar et al., 2007), bind directly to the MD2 pocket, and block the TLR4/MD2’s recognition of LPS, resulting in the prevention of proinflammatory signaling and septic shock. Although their specificities for targeting other proteins remain to be defined, these natural compounds provide us the important structural information for the design and discovery of new synthetic MD2 inhibitors. As shown in Fig. 1A, the structures of the MD2 inhibitors share the same 3-(4-hydroxyphenyl) acrylaldehyde skeleton. Thus, it is hypothesized that our new synthetic compound, L48H37, which shows excellent anti-inflammatory activity and contains the structure of 3-(4-hydroxyphenyl) acrylaldehyde, may target MD2 and serve as an antisepsis candidate.

Hence, the interaction between L48H37 and MD2 was investigated at both cell-free molecular and cellular levels. Fluorescence spectroscopy and SPR assay demonstrated that L48H37 was able to dose dependently bind to rhMD2 protein (Fig. 2, D and E). The interaction of L48H37 with MD2 remarkably affected the LPS binding to rhMD2 (Fig. 2C), suggesting that the binding site for L48H37 in MD2 pocket overlaps that for LPS, which is also consistent with the molecular docking results (Fig. 3A). At the cellular level, flow cytometry (Fig. 2B) and immunoprecipitation (Fig. 2A) revealed the inhibitory effects of L48H37 on LPS-MD2 interactions and MD2-TLR4 complex formation, respectively. Interestingly, our data also showed that L48H37 is a specific MD2 inhibitor, since L48H37 could not inhibit the Pam3CK-induced TLR2 activation, which shares the MAPKs/NF-κB– involved proinflammatory signaling pathway with TLR4 (Fig. 5A) but is independent on MD2. There, this study demonstrates that MD2 is a molecular target of L48H37 and that L48H37 can downregulate TLR4 activation and inflammatory gene expression, as well as attenuate LPS-induced sepsis, by interrupting the association of LPS with MD2 (Fig. 6).

![Fig. 7. Proposed model of signaling pathway involved in L48H37 prevented LPS-induced TLR4 signaling pathway activation and sepsis.](image-url)
Molecular modeling of the crystal structure of MD2 provided further support for the binding of L48H37 to MD2. The X-ray diffraction–based structural information and exact binding mechanism for nonlipid compounds binding to MD2 protein are still unclear. The MD2 binding sites of nonlipid compounds have been predicted by computer-assisted simulation, and the Cys133 in the MD2 binding pocket is considered a molecular target of several natural inhibitors. Small-molecule inhibitors with α,β-unsaturated ketones are capable of forming covalent bonds with Cys133 via a Michael-type reaction. JTT-705 [S-2-{{[2-(1-ethylbutyl)cyclohexyl(carbonyl) amino]phenyl} 2-methylpropanethioate] (Mancek-Keber et al., 2009) and caffeic acid phenethyl ester (Kim et al., 2013) have been predicted to covalently bind Cys133 residue and showed an irreversible inhibition against MD2. However, the α,β-unsaturated ketone–containing curcumin interacts with MD2 via a noncovalent mechanism, a finding supported by studies showing that it can be removed from the complex bound to MD2 by chloroform extraction and that it can still inhibit LPS from binding to the mutant MD2 Cys133Phe in the same manner as the wild-type (Gradisar et al., 2007). In addition, some residues, Lys122, Tyr102, Gly123, Ser120, Lys130, and Phe138, in the MD2 pocket were predicted to play a possible role in the interaction between MD2 protein and natural small-molecule inhibitors such as JSH (Roh et al., 2011), taxanes (Resman et al., 2008), and xanthohumol (Peluso et al., 2010). In this study, we found the possible binding mechanism of L48H37-MD2 using the molecular docking method. The results indicated that the binding site for L48H37 in the MD2 pocket overlapped that for LPS, rather than TLR4 (Fig. 3A), which is also evidenced by the experimental data at the molecular and cellular levels. Using further molecular dynamics, we showed that L48H37 may form hydrogen bonds with two key residues, Arg90 and Tyr102, which also play a role in the binding of LPS (Fig. 3A). To validate this prediction, we replaced the two amino residues Arg90 and Tyr102 with Ala in rhMD2 mutations. As expected, the SPR analysis and ELISA showed that L48H37 could not interact with the rhMD2 mutations, indicating that Arg90 and Tyr102 play a critical role in L48H37-MD2 interactions. Although the Tyr102 residue has been predicted to be of importance in isoxanthohumol-MD2 interactions, the authors failed to demonstrate the possible hydrogen bond formation with Tyr102 (Peluso et al., 2010). In addition, this is the first time that Arg90 has been highlighted as an important molecular target for MD2 inhibitors. Thus, the results of this study provide the important structural information and understanding of the amino residue sites that support the use and further design of MD2 inhibitors as anti-inflammatory agents.

L48H37’s inhibition of MD2 resulted in a series of anti-inflammatory activities in macrophages. MAPKs and NF-κB have been demonstrated to be the main mediators in the LPS–TLR4/MD2 proinflammatory signaling pathway. L48H37 prevented TLR4-mediated MAPKs and NF-κB activation in LPS-stimulated macrophage, as evidenced by a dose-dependent decrease in the levels of ERK/p38/JNK phosphorylation, IκB degradation, and p65 translocation (Fig. 4). Figure 5 further shows the inhibitory effects of L48H37 on LPS-induced inflammatory cytokine overexpression in both mouse MPMs and human PBMCs. In vivo, either pretreatment or post-treatment with L48H37 significantly increased survival in the LPS-injected septic mice (Fig. 6A). Lung histologic changes in the LPS-injected mice were also suppressed by L48H37 pretreatment (Fig. 6C). These results validated the potential of the MD2-targeting L48H37 as a therapeutic agent in both the prevention and treatment of acute inflammatory diseases.

Collectively, our data reveal that MD2 is the anti-inflammatory target of novel compound L48H37 and can lead to the blockage of LPS-TLR4/MD2 complex formation and decrease of downstream signal activation and inflammatory mediator expression. A schematic for the protection of L48H37 from LPS-induced sepsis is illustrated in Fig. 7. Arg90 and Tyr102 in the MD2 protein play an important role in L48H37’s interaction with MD2 via two hydrogen bonds. In vivo, L48H37 improved survival and protected lungs against LPS-induced injury in septic mice. This study suggests that MD2 is an important therapeutic target against inflammatory disorders and proves that a new MD2 inhibitor, L48H37, can be developed as a potential agent in the treatment of sepsis.


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