Curcumin analog L48H37 prevents LPS-induced TLR4 signaling pathway activation and sepsis via targeting MD2

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L48H37, 1-ethyl-3,5-bis(3,4,5-trimethoxybenzylidene)piperidin-4-one; SIRS, systemic inflammatory response syndrome; LBP, LPS-binding protein; MD2, myeloid differentiation 2; MPMs, Mouse primary peritoneal macrophages; PBMCs, peripheral blood mononuclear cells.

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Abstract

Endotoxin-induced acute inflammatory diseases such as sepsis, mediated by excessive production of various pro-inflammatory 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 immunity system and through the myeloid differentiation 2 (MD-2) and toll-like receptor 4 (TLR4) complex, increase the production of inflammatory mediators. Our previous studies have found that a curcumin analog L48H37 was able to inhibit LPS-induced inflammation, particularly TNF-α and IL-6 production and gene expression in mouse macrophages. In this study, a series of biochemical experiments demonstrate L48H37 specifically targets MD-2 and inhibits the interaction and signaling transduction of LPS-TLR4/MD-2. L48H37 binds to the hydrophobic region of MD-2 pocket and forms hydrogen bond interactions with Arg^{90} and Tyr^{102}. Subsequently, L48H37 was shown to suppress LPS-induced MAPK phosphorylation and NF-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 pre-treatment 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 (SIRS), is a severe condition marked by an overwhelming immune response to a serious infection, and results in the excessive production of various pro-inflammatory cytokines and cellular injury. In critically ill patients, sepsis is the leading cause of mortality with hospital mortality rates between 15-30% (Gaieski 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 due to lipopolysaccharide (LPS), which is the major glycolipid found in its outer membrane. Following infection with Gram-negative bacteria, the host is exposed to microbial LPS through ancillary proteins, such as LPS-binding protein (LBP) and CD14, which transports the microbial LPS to specific target cells and a surface receptor complex composed of Toll-like receptor 4 (TLR4) and myeloid differentiation 2 (MD2). Subsequent TLR4 activation leads to the recruitment of myeloid differentiation primary-response gene 88 (MyD88), activating downstream NF-κB and 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 up-regulation and increased expression of pro-inflammatory genes, contributing to multiple organ dysfunction and SIRS (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 (Figure 1A). For example, Peluso et al. showed that a chalcone derivative, xanthohumol,
competitively displaced LPS from MD2, which inhibited LPS-induced TLR4 activity (Peluso et al., 2010). Two natural compounds caffeic acid phenethyl ester and JSH, containing the same moiety 3-(4-hydroxyphenyl) acrylaldehyde in the structures, were also found to inhibit LPS-induced TLR4 activation partly by interfering 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-inflammation, anti-tumor, anti-oxidation, and cardiovascular protection (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 lab has been engaged in the medicinal chemistry, turning to natural products, such as curcumin, in efforts to discover new anti-inflammatory drugs. Our lab and others observed that due to curcumin’s poor stability under physiological conditions, its clinical application of curcumin 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 (Figure 1B) (Wu et al., 2013). Among these curcumin analogs, 1-ethyl-3,5-bis(3,4,5-trimethoxybenzylidene)piperidin-4-one (L48H37, Figure 1B), exhibited high chemical stability and strong anti-inflammatory ability. The aim of the present study is to find the molecular target of L48H37 as well as the underlying mechanism of its anti-inflammatory actions. Based on 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) was purified as described by Goodall et al. (Goodall et al., 2014). Briefly, the whole blood was overlayered 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 min at room temperature. After the layers were separated, the PBMC layer was directly removed from above the Ficoll layer and washed three times with PBS. Collected PBMCs were resuspended in RPMI for further analysis. Curcumin, LPS (from Salmonella typhosa), TLR2 agonist Pam3CK, and LPS-FITC (from E. coli 055:B5) were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies against p-p38, p38, p-JNK, JNK, p-ERK, ERK, IκBα, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human TLR4 protein was purchased from Sino Biological Inc. (Beijing, China). Anti-human MD2 antibody and mouse TNF-α and IL-6 ELISA kits were obtained from eBioscience (Bioscience, San Diego, CA).

Synthesis of L48H37

The procedure for synthesis of compound L48H37 is briefly described as follows. The compounds 1-ethylpiperidin-4-one (2 mmol) and 3,4,5-trimethoxybenzaldehyde (4 mmol) were dissolved in ethanol and catalyzed by NaOH at 5 ~ 8°C. Silica gel TLC was used to monitor the reactions and at the end of the reaction, distilled water was added into the reaction mixture to precipitate the product. Further purification was accomplished through using column chromatography with PE/EA. The chemical structure of synthetic L48H37 was well characterized.
by $^1$HNMR and ESI-MS. Before use in biological experiments, HPLC was employed to determine the purity (99.12%). In *in vitro* experiments, L48H37 was dissolved in DMSO solution with DMSO as a vehicle control. In *in vivo* studies, L48H37 was first dissolved in water with macrogol 15 hydroxystearate (a nonionic solubilizer for injection from BASF). The concentration of L48H37 in the water solution was 2 mg/mL, while the concentration of solubilizer was ranged 7.5% in final solution. A 7.5% solubilizer/water solution was used as the vehicle control.

**Protein Expression and Purification of MD2 proteins**

Recombinant human MD2 (rhMD2, residue 17-160, PDB ID: 2E59) cDNA was synthesized from Invitrogen (*Shanghai, China*). The synthesized fragment was then ligated into pET28a vector (Invitrogen, *Carlsbad, CA*). The rhMD2 mutations R90A and Y102A were introduced into the pET28a vector by PCR-based mutagenesis (The primers for mutations were listed in supplementary data). The three expression vectors were cloned into *E. coli* BL21(DE3). 1 mM of IPTG was added to induce protein expression, and the culture was allowed to incubate at 28°C for 8 h. 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 h. Centrifugation at 12,000 rpm for 30 mins removed any residual insoluble matter, and the supernatant was filtered through a 0.22 μM filter (*Millipore, MA*). The diluted supernatant was applied to Ni-IDA Sepharose™ 6 Fast Flow (General Electric Company, *Fairfield, CT*) according to the manufacturer’s instruction. Refolding of rhMD2, rhMD2/R90A, rhMD2/Y102A peptides were carried out by gradient dialysis against 6 M ~ 0.5 M urea in 50 mM Tris-HCl and 0.6 NaCl (pH 8.0) at 4°C. Bradford method was used to assess the concentration of purified protein.
**Animals**

Male C57BL/6 (B6) mice (6–8 weeks age) were obtained from the Animal Center of Wenzhou Medical University (*Wenzhou, China*). Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. And all animal experimental procedures were approved by the Wenzhou Medical University Animal Policy and Welfare Committee (wydw2013-0042).

**UV-visible absorption spectra of curcumin and L48H37**

Absorbance readings from 250 to 600 nm were taken using a SpectraMax M5 (*Molecular Devices, Sunnyvale, CA*). A stock solution of 1 mM curcumin or L48H37 was prepared and diluted by phosphate buffer (pH 7.4) to a final concentration of 20 μM. The UV absorption spectra was collected for over 25 min at 5 min intervals at 25 °C. All spectral measurements were carried out in a 1 cm path-length quartz cuvette.

**Co-immunoprecipitation assay**

Mouse peritoneal macrophages (MPMs) were obtained as previous described (Pan et al., 2013). Before treatment, MPMs were cultured in 60-mm plates and incubated overnight at 37°C. After overnight incubation, MPMs were pretreated with L48H37 (10 μM) or vehicle control (DMSO) for 30 min and then incubated with LPS (1 μg/mL) for 5 min. Total cells were lysed in an extraction buffer (containing mammalian protein extraction reagent supplemented with protease and phosphatase inhibitor cocktails) and centrifuged at 12,000 rpm for 10min at 4°C. Anti-MD2
antibody was then added into 400 µg of protein and gently shaken at 4°C overnight. The immunocomplex was collected with protein A+G agarose, and the precipitates were washed five times with ice-cold PBS. Finally, proteins were released by boiling in sample buffer and analyzed through Western blotting with anti TLR-4 antibody.

**Flow Cytometric Analysis**

Cellular binding of fluorescein isothiocyanate-labeled LPS (LPS-FITC, from *E. coli* 055:B5, Sigma, *St. Louis, MO*) was measured as described previously (Roh et al., 2011). Briefly, HUVEC304 cells (1×10^5) were incubated with LPS-FITC (50 µg/mL) for 30 min with or without the presence of L48H37 (0.1, 1, or 10 µM). After washing, the cells with bound LPS-FITC were analyzed by flow cytometry.

**LPS binding assay**

Anti-human MD2 antibody (*eBioscience, San Diego, CA*) was coated to a 96-well plate overnight at 4°C in 10 mM Tris-HCl buffer (pH 7.5). The plate was washed with PBST and blocked with 3 % BSA for 1.5 h at room temperature. rhMD2, rhMD2/R90A, or rhMD2/Y102A (4 µg/mL, respectively) in 10 mM Tris–HCl buffer (pH 7.5) were added to a pre-coated plate and incubated for 1.5 h at room temperature. After washing with PBST, biotin-labeled LPS (*InvivoGen, San Diego, CA*) was incubated for 1 h at room temperature with or without the presence of compound L48H37 (1 µM). After further washing, streptavidin-conjugated horseradish peroxidase (*Beyotime, Shanghai, China*) was added for 1 h at room temperature. The horseradish peroxidase activity was determined using TMB substrate solution (*eBioscience, San Diego, CA*). The optical density of each well was measured at 450 nm.
Fluorescence measurements of Competition Displacement

Fluorescence measurements were performed with a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). Briefly, 1,1’-Bis(anilino)-4,4’-bis(naphthalene)-8,8’-disulfonate (bis-ANS, 5 μM) and rhMD2 protein (5 nM) were mixed in PBS (pH 7.4) and incubated until fluorescence values following excitation at 385 nm stabilized. Non-fluorescent L48H37 (at 2.5, 5, 10, 20, 30 μM) was then treated for 5 min, and the relative fluorescence units (RFUs) emitted at 430-590 nm were measured.

Docking of L48H37 to the MD2 structural model

Docking simulation of L48H37 with MD2 protein (PDB ID: 2E56) was carried out with the program Tripos molecular modeling packages 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, using the protocol previously indicated. To allow for flexible docking and production of over 100 structures, the ligand-binding groove on MD2 was kept fixed, while all torsible bonds of L48H37 were kept free. 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 a HTE sensor chip (ProteOn™, #176-5033). Briefly, rhMD2, rhTLR4, rhMD2/R90A, or rhMD2/Y102A (in acetate acid buffer pH 5.5) was loaded to the sensor, which was activated with 10 mM NiSO₄, 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, while the second flow cell was left as a blank. Five concentrations were simultaneously injected at a flow rate of 30 μM/min for 120 s of association phase, followed with 120 s of dissociation phase at 25 °C. The final graphs represent the difference the duplex or quadruplex sensorgrams and the blank sensorgrams. Data analysis was done using the ProteOn manager software, and the $K_D$ was calculated by aligning the kinetic data from various concentrations of L48H37 to the 1:1 Langmuir binding model.

**Western Blot**

Collected cells were lysated, and 30 mg of lysates were separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Following pre-incubation for 1 h at room temperature in Tris-buffered saline (pH 7.6) with 0.05% Tween 20 and 5% non-fat 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, Hercules, CA, USA), 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 p-65 translocation**

Using a Cellular NF-κB p-65 Translocation Kit (Beyotime Biotech, Nantong, China), the cells were immunofluorescence-labeled in accordance with the manufacturer’s instructions. Briefly, cultured MPMs were pretreated L48H37 (10 μM) or the vehicle control (DMSO) for 2 h, and then
stimulated with LPS (0.5 μg/mL) for 1 h. After 1 h of treatment, the cells were incubated with p65 antibody and Cy3 fluorescein-conjugated secondary antibody, and nuclei were stained with DAPI. The images (200×) were obtained by fluorescence microscope. The experiment was repeated independently three times, obtaining similar results, and the results were quantified.

**ELISA**

ELISA kits (Bioscience, San Diego, CA) 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**

Cells were homogenized in TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol for extraction of RNA. A two-step M-MLV Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA) was used for both reverse transcription and quantitative PCR, and Eppendorf’s Mastercycler realplex detection system (Eppendorf, Hamburg, Germany) was used for q-PCR analysis. The primers of genes used are shown as following: mouse TNF-α forward 5’-TCTCATTCCTGCTTGTGGCAG-3’ and reverse 5’-TCCACCTTGTTGGTGTACG-3’; mouse IL-6 forward 5’-CCAAGAGGTGAGTGCTTCCC-3’ and reverse 5’-CTGTTGTTCAGACTCTCTCCT-3’; mouse IL-1β forward 5’-ACTCCTTAGTCTCGGCA-3’ and reverse 5’-CCATCAGAGGGAAGGAAGA-3’; mouse IL-10 forward 5’-GGTTGCCAAGCCTTAATCGGA-3’ and reverse 5’-ACCTGCTCCACTGCCTTGCT-3’; mouse COX-2 forward 5’-TGGTCCTGCTGTATGATG-3’ and reverse 5’-GTGGTAACCGCTCAGGTGTT-3’; mouse iNOS forward
5'-CAGCTGGGCTGTACAAACCTT-3’ and reverse 5’-CATTGGAAGTGAAGCGTTTCG-3’; mouse 
β-actin forward 5’-TGCACCACCAACTGCTTAG-3’ and reverse 5’-GGATGCAGGGATGATGTC-3’; human TNF-α forward 5’-CCCAGGGACCTCTCTCTCTAATC-3’ and reverse 5’-ATGGGCTACAGGCTTGTCACT-3’; human IL-6 forward 5’-GCACGTGGCACAAAACACCT-3’ and reverse 5’-TCAAACCTCCCAAAGACCAGTGA-3’; human β-actin forward 5’-CCTGGCACCCAGCACAAT-3’ and reverse 5’-GCCGA TCCACACGGAGTACT-3’. All primers were synthesized and purchased from Invitrogen (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, intravenous and through tail vein) 15 min before (for treatment) or after (for prevention) intravenous (IV) injection of L48H37 (at 10 mg/kg, IV and through tail vein), respectively. LPS was used for IV 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 min after i.v. 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 h. The formalin-fixed lung samples were then embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and observed at 200× 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 hydroxystearate in water, and mice in vehicle control group also received 200 μL saline.
Statistical analysis

All values are represented as means ± SEM from three independent experiments. Data analysis, including statistic analysis with the student’s t-test or one-way ANOVA, was done using GraphPad Prism 5.0. A $p$-value <0.05 was considered to be statistical significant.
Results

The chemical stability of L48H37 was improved in vitro

The chemical stability of L48H37 and curcumin was tested using an absorption spectrum assay. Figure 1C showed 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 (Figure 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 Figure 2A, the complex of TLR4/MD2 profoundly increased in LPS-stimulated macrophages, while 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 an MFI 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 Figure 2C show that biotin-marked LPS (Biotin-LPS) was able to bind to recombinant human MD2 (rhMD2) protein in the plates, while co-incubation with L48H37 significantly blocked the interaction of Biotin-LPS and rhMD2.

**L48H37 directly binds to MD2 protein,**

The direct interaction of L48H37 and rhMD2 protein was determined using fluorescence spectroscopy and SPR assay. As shown in Figure 2D, fluorescence values of bis-ANS, a fluorescent probe used to map the hydrophobic binding sites in proteins, were markedly enhanced upon binding to cell-free rhMD2 protein, while 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 (Figure 2E), while Figure 2F exhibited that L48H37 directly binds rhMD2 protein in a dose-dependent manner and with a very high affinity (K_D value = 0.0000113M). These data indicate that L48H37 is a novel and MD2-specific inhibitor.

**L48H37 acts on Arg90 and Tyr102 residues in MD2 protein pocket**

We further predict the underlying binding mode of L48H37 in MD2 protein using a molecular simulation of L48H37-MD2 complex. As shown in Figure 3A, L48H37 was fitted into the hydrophobic pocket of MD2, interacting the residues including Tyr^{102}, Phe^{121}, Leu^{61}, Cys^{133}, and Arg^{90} in the most energetically favorable configuration (Figure 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 show that two amino residues Arg^{90} and Tyr^{102} are
most likely to form hydrogen bonds with L48H37 (Figure 3A). Thus, in order to confirm the importance of Arg$_{90}^{90}$ and Tyr$_{102}^{102}$ in L48H37 binding to rhMD2, two new rhMD2 mutations, rhMD2$^{R90A}$ or rhMD2$^{Y102A}$, were prepared respectively. SPR assay indicated that L48H37 no longer binds to these two mutations (Figure 3B and 3C), and the ELISA method also found that L48H37 could not inhibit the binding of biotin-LPS with either rhMD2$^{R90A}$ or rhMD2$^{Y102A}$ (Figure 3D and 3E). 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 TLR4/MD2 cascade, including the representative MAPKs pathway and the transcriptional factor NF-κB. MAPK family consists of ERK, p38, and JNK. Figure 4A shows that all of the three pathways were activated by LPS stimulation in MPMs, while the LPS-induced phosphorylations of ERK, p38 and JNK were markedly decreased following pre-treatment 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 h induced an 84% degradation of IκB, while pre-treatment with L48H37 reversed LPS-induced IκB degradation in MPMs in a dose-dependent manner (Figure 4B). Consequently, as shown in Figure 4C, LPS stimulation could increase NF-κB p65 nuclear translocation (red point in blue nucleus), while in L48H37 pre-treated cells, LPS-induced nuclear levels of p65 were significantly decreased.

**L48H37 strongly inhibits LPS-induced inflammatory cytokine expression in macrophages**

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MPMs and human PBMCs were used to examine the anti-inflammatory activity of L48H37. As shown in Figure 5A–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 (Figure 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 a MD2 inhibitor. The anti-inflammatory activity of L48H37 was also observed at the mRNA level. MPMs treated with LPS (0.5 μg/mL) for 6 h were examined through real-time quantitative PCR for the expression of pro-inflammatory genes in the presence or absence of L48H37. As shown in Figure 5C, L48H37 at 10 μM potently inhibited LPS-induced up-regulation 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 inducible nitric oxide synthase (iNOS, 50.9%, \( p<0.01 \)) transcripts in MPMs. As expected, L48H37 up-regulated 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 (Figure 5D).

**L48H37 effectively protects mice from LPS-induced septic shock and lung injury**

Male C57BL/6 mice were injected with LPS (i.v., 20 mg/kg) in the presence or absence of L48H37 pre-treatment (i.v.), and the survival rates were monitored for seven days. Figure 6A showed that animals treated with LPS alone all died within 48 h. In contrast, treatment with L48H37 at 10 mg/kg either 15 min prior to LPS injection (prevention group) or 15 min after LPS injection (treatment group) significantly improved the survival rates compared to that of the control
group ($p<0.01$ in both groups vs. LPS group). Also, the weight lost in both groups improved slowly 2-7 days after LPS injection (Figure 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 pre-treatment at 10 mg/kg significantly improved pulmonary damage and amended the LPS-injured tissue structure of pulmonary lobules (Figure 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. Despite there being more than 30 pharmaceutical candidates for the treatment of sepsis currently in the developmental stage, most of these treatments have failed due to the complicated nature of sepsis, and sepsis remains as 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 due 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 non-specific anti-inflammatory effects, they are currently not being considered as therapeutic options for sepsis (Gazzerro 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 TLR/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, 2014). Both TLR4 and MD2 are essential for the LPS-induced inflammatory response and sepsis. Both TLR4-/- and MD2-/- mice fail to respond to LPS and survive endotoxic shock (Duan et al., 2014). Therefore, TLR4 and MD2 are proposed as potential targets for the 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). Due to the seemingly higher importance of TLR4, researchers paid more attention to TLR4 than MD2 in the past decades (Wittebole et al., 2010; Svažiger et al., 2013). However, the clinical trials of TAK-242, 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 immunological tolerance (Ishii et al., 2006; Nakamoto and Kanai, 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 (Bryant et al., 2007), CRX-526 (Lin et al., 2013), and eritoran (Rallabhandi et al., 2012), have been evaluated in clinical and pre-clinical studies. Unfortunately, the most studied one, eritoran, has failed in phase III clinical trial in 2011 due to no significant improvement in eritoran-treated patients compared to 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 (Figure 1A). These small molecules, such as xanthohumol (Peluso et al., 2010), CAPE (Kim et al., 2013), JSH, (Roh et al., 2011) and curcumin (Gradisar et al., 2007), bind directly to the MD2 pocket, and block the TLR4/MD2’s recognition of LPS, resulting in the prevention of pro-inflammatory signaling and septic shock. Although their specificities for targeting other proteins remain to be defined, these natural compounds (1-4) provide us the important structural information for the design and discovery of new synthetic MD2 inhibitors. As shown in Figure 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 anti-sepsis 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 (Figure 2D and 2E). The interaction of L48H37 with MD2 remarkably affected the LPS binding to rhMD2 (Figure 2C), suggesting that the binding site for L48H37 in MD2 pocket overlaps that for LPS, which is also consistent with the molecular docking results (Figure 3A). At the cellular level, flow cytometry (Figure 2B) and immunoprecipitation (Figure 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-kB-involved pro-inflammatory signaling pathway with TLR4 (Figure 5A), but is independent on MD2. There, this study demonstrates that MD2 is a molecular target of L48H37 and that L48H37 can down-regulate TLR4 activation and inflammatory gene expression, as well as attenuate LPS-induced sepsis, by interrupting the association of LPS with MD2 (Figure 6).

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 non-lipid compounds binding to MD2 protein are still unclear. The MD2-binding sites of non-lipid compounds have been predicted by computer-assisted simulation, and the Cys\textsuperscript{133} in the MD2 binding pocket is considered a molecular target of several natural inhibitors. Small-molecule inhibitors with \(\alpha,\beta\)-unsaturated ketones are capable of forming covalent bonds with Cys\textsuperscript{133} via a Michael-type reaction. JTT-705 (Mancek-Keber et al., 2009) and CAPE (Kim et al., 2013) have been predicted to covalently bind Cys\textsuperscript{133} residue and showed an irreversible inhibition against MD2. However, the \(\alpha,\beta\)-unsaturated ketone-containing curcumin interacts with MD2 via a non-covalent
mechanism, 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 Cys\textsuperscript{133}Phe in the same manner as the wild-type (Gradisar et al., 2007). In addition, some residues Lys\textsuperscript{122}, Tyr\textsuperscript{102}, Gly\textsuperscript{123}, Ser\textsuperscript{120}, Lys\textsuperscript{130}, and Phe\textsuperscript{126} in 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 (Figure 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, Arg\textsuperscript{90} and Tyr\textsuperscript{102}, which also play a role in the binding of LPS (Figure 3A). To validate this prediction, we replaced these two amino residues Arg\textsuperscript{90} and Tyr\textsuperscript{102} with Ala in rhMD2 mutations. As expected, the SPR analysis and ELISA showed that L48H37 could not interact the rhMD2 mutations any more, indicating that Arg\textsuperscript{90} and Tyr\textsuperscript{102} play a critical role in L48H37-MD2 interactions. Although the Tyr\textsuperscript{102} residue has been predicted to be of importance in isoxanthohumol-MD2 interactions, the authors failed to demonstrate the possible hydrogen bond formation with Tyr\textsuperscript{102} (Peluso et al., 2010). In addition, this is the first time that Arg\textsuperscript{90} 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 as the main mediators in the LPS-TLR4/MD2 pro-inflammatory 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 (Figure 4). Figure 5 further showed the inhibitory effects of L48H37 on LPS-induced inflammatory cytokine overexpression in both mouse MPMs and human PBMCs. *In vivo*, either pre-treatment or post-treatment with L48H37 significantly increased survival in the LPS-induced septic mice (Figure 6A). Lung histological changes in the LPS-injected mice were also suppressed by L48H37 pre-treatment (Figure 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 Figure 7. Arg\(^{90}\) and Tyr\(^{102}\) 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.
Authorship Contributions

**Participated in research design:** Yi Wang, Guang Liang, Xiaou Shan, Guilong Guo

**Conducted experiments:** Yi Wang, Lili Jiang, Gaozhi Chen, Yali Zhang, Zhe Wang

**Contributed new reagents or analytic tools:** Lili Dong, Jianzhang Wu

**Performed data analysis:** Yi Wang, Yuanrong Dai, Guang Liang

**Wrote or contributed to the writing of the manuscript:** Yi Wang, Lili Jiang, Guang Liang
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**Footnotes**

Yi Wang and Xiaoou Shan contributed equally to this paper.

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Figure Legends

Figure 1. Design, synthesis and stability assay of curcumin analogue L48H37. (A) Structures of current MD2 inhibitors; (B) Design and synthesis of curcumin analog L48H37; (C) UV-visible absorption spectra of curcumin and L48H37. Curcumin or L48H37 were dissolved in phosphate buffer (pH 7.4) to a final concentration of 20 μM. Absorbance readings were taken from 250 to 600 nm using a spectraMax M5. The UV absorption spectra were collected for over 25 min at 5 min intervals at 25°C.

Figure 2. Antagonistic effect of L48H37 on LPS binding to MD2. (A) Co-immunoprecipitation of MD2. MPMs were pretreated with L48H37 (10 μM) or DMSO for 30 min and then incubated with LPS (1 μg/mL) for 5 min. Cells were lysed and the total protein was collected. 400 μg of the total protein were 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), 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 (MFI) were also provided; (C) In vitro assays for LPS binding to MD2. rhMD2 antibody was coated to a 96-well at 4°C overnight. rhMD2 (4 μg/mL) in 10 mM Tris-HCl buffer was added to the pre-coated plate for 1.5 h at room temperature. After washing with PBST, 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 (±SEM) of 3 separate experiments, each performed in duplication. *p<0.05, **p<0.01 vs. buffer alone-added group; (D) Fluorescence measurements. bis-ANS (5 μM) was pre-incubated with rhMD2 (5 nM) to reach stable fluorescence values under excitation at 380 nM and to reach stable relative fluorescence units (RFUs) emitted at 430–590 nm under excitation at 385 nm. Non-fluorescent L48H37 (at 2.5, 5, 10, 20, 30 μM) was then treated for 5 min, and the relative fluorescence units (RFUs) 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.

**Figure 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-C) Surface Plasmon resonance analysis. rhMD2R90A or rhMD2Y102A 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 equipped with a super streptavidin (SSA) sensor; (D-E) In vitro assay for LPS binding to MD2 variants. rhMD2 antibody was coated on a 96-well at 4°C overnight. rhMD2R90A or rhMD2Y102A (4 μg/mL) in 10 mM Tris-HCl buffer was added to the pre-coated plate for 1.5 h at room temperature. After washing with PBST, 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 (A450). Data are mean values (±SEM) of 3 separate experiments, each performed in duplication.
Figure 4. L48H37 inhibited LPS-induced MAPK phosphorylation and NF-κB activation. (A-B) MPMs were pretreated with the vehicle control (DMSO) or L48H37 (1, 2.5, 5, or 10 μM) for 2 h followed by incubation with LPS (0.5 μg/mL) for 1 h. The protein levels of p-ERK, ERK, p-p38, p38, p-JNK, JNK, I-κB were examined by Western blot. The column figures represent the mean optical density ratio of three independent experiments. * p<0.05, ** p<0.01, vs. the LPS-treated group; (C) Cultured MPMs were pretreated with L48H37 (10 μM) or vehicle control (DMSO) for 2 h, and then stimulated with LPS (0.5 μg/mL). After 1 h of treatment, the cells were incubated with p65 antibody and Cy3 fluorescein-conjugated secondary antibody (red), and the nuclei were stained with DAPI (blue). The images (200×) 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, vs. LPS-treated group.

Figure 5. L48H37 inhibited LPS-induced inflammatory cytokine expression in mouse macrophages and human PBMCs. (A-B) MPMs were pretreated with the vehicle control (DMSO) or L48H37 (1, 5, or 10 μM) for 2 h followed by incubation with LPS (0.5 μg/mL) or Pam3CK (0.1 μg/mL) for 22 h. 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 ± SEM of 3-5 independent experiments. * p<0.05, ** p<0.01, v.s. LPS-treated group. (C) MPMs were pretreated with vehicle control (DMSO) or L48H37 (10 μM) for 2 h followed by incubation with LPS (0.5 μg/mL) for 6 h. 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 values for the LPS control. Each bar represents mean ± SEM of 3-5 independent experiments. * p<0.05, ** p<0.01, vs. LPS-treated group. (D) Human PBMCs were pretreated with the vehicle control (DMSO) or L48H37 (1, 5, or 10 μM) for 2 h followed by incubation with LPS (0.5 μg/mL) for 6 h. 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 ± SEM of 3-5 independent experiments. * p<0.05, ** p<0.01, vs. LPS-treated group.

Figure 6. L48H37 improved survival and lung injury of mice subjected to a lethal dose of LPS. C57BL/6 mice (n=10/group) were treated with 10 mg/kg L48H37 15 min before or after injection of 20 mg/kg LPS (i.v.). Survival rates (A) and body weight (B) were recorded for 7 days after LPS injection at the interval of 12 h. ** p<0.01 vs. LPS-treated group. (C) C57BL/6 mice (n=10/group) were treated with 10 mg/kg L48H37 15 min before injection of 20 mg/kg LPS (i.v.). Two or eight hours after LPS injection, five mice were anesthetized with diethyl ether and sacrificed, respectively. Lung histopathological analysis was performed using H&E staining as described in Materials and Methods. The representative images are shown.

Figure 7. Proposed model of signaling pathway involved in L48H37 prevented LPS-induced TLR4 signaling pathway activation and sepsis.
Mono-carbonyl analogs of curcumin

Delete the beta-diketone moiety to obtain the structurally stable mono-carbonyl analogs

JSH Curcumin
Xanthohumol
Caffeic acid phenethyl ester
Curcumin

A

B

C

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**Figure 2**

A. IB: TLR4
IP: MD2

B. Relative density

C. LPS binding

D. Emission wavelength (nm)

E. L48H37-rhMD2

F. L48H37-rhTLR4

**Absorbance (A450)**

**Relative density**

**Emission wavelength (nm)**

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Figure 3

A

B

C

D

E

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Figure 4

A

LPS (0.5 μg/ml) - + + + + +
L48H37 (μM) - - 1.0 2.5 5 10
p-ERK1/2
ERK2
p-p38
p38
p-JNK
JNK

B

LPS (0.5 μg/ml) - + + + + +
L48H37 (μM) - - 2.5 5.0 10

C

DMSO
LPS

IκB
GAPDH

Density (compared to LPS %)

P65 translocation related to DAPI (%)

LPS (0.5 μg/ml) - + + + + +
L48H37 (μM) - - 2.5 5 10

*p  **p

**p This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5

A. TNF-α

B. IL-6

C. TNF-α, IL-6

D. TNF-α, IL-6, COX-2, iNOS
Figure 6

A

![Survival Rate vs. Hours](image)

- **Vehicle + LPS (20mg/kg)**
- **L48H37 (10mg/kg) + LPS (20mg/kg)** (Prevention)
- **LPS (20mg/kg) + L48H37 (10mg/kg)** (Treatment)

B

![Body Weight vs. Hours](image)

- **Vehicle + LPS (20mg/kg)**
- **L48H37 (10mg/kg) + LPS (20mg/kg)** (Prevention)
- **LPS (20mg/kg) + L48H37 (10mg/kg)** (Treatment)

C

![Histology Images](image)

- **Vehicle**
- **LPS**
- **LPS + L48H37**

2h

8h