Characterization of Sparstolonin B, a Chinese Herb-derived Compound, as a Selective Toll-like Receptor Antagonist with Potent Anti-inflammatory Properties

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Blockade of excessive Toll-like receptor (TLR) signaling is a therapeutic approach being actively pursued for many inflammatory diseases. Here we report a Chinese herb-derived compound, sparstolonin B (SsnB), which selectively blocks TLR2- and TLR4-mediated inflammatory signaling. SsnB was isolated from a Chinese herb, Sparganium stoloniferum; its structure was determined by NMR spectroscopy and x-ray crystallography. SsnB effectively inhibited inflammatory cytokine expression in mouse macrophages induced by lipopolysaccharide (LPS, a TLR4 ligand), Pam3CSK4 (a TLR1/TLR2 ligand), and Fsl-1 (a TLR2/TLR6 ligand) but not that by poly(I:C) (a TLR3 ligand) or ODN1668 (a TLR9 ligand). It suppressed LPS-induced cytokine secretion from macrophages and diminished phosphorylation of Erk1/2, p38α, IκBα, and JNK in these cells. In THP-1 cells expressing a chimeric receptor CD4–TLR4, which triggers constitutive NF-κB activation, SsnB effectively blunted the NF-κB activity. Co-immunoprecipitation showed that SsnB reduced the association of MyD88 with TLR4 and TLR2, but not that with TLR9, in HEK293T cells and THP-1 cells overexpressing MyD88 and TLRs. Furthermore, administration of SsnB suppressed splenocyte inflammatory cytokine expression in mice challenged with LPS. These results demonstrate that SsnB acts as a selective TLR2 and TLR4 antagonist by blocking the early intracellular events in the TLR2 and TLR4 signaling. Thus, SsnB may serve as a promising lead for the development of selective TLR antagonistic agents for inflammatory diseases.

Toll-like receptors (TLRs) are key components of innate immunity (1) expressed by macrophages, dendritic cells, and many other cell types (2, 3). TLRs serve as the first line of defense against invading pathogens such as bacteria and viruses. Currently more than a dozen TLRs have been identified, with the first nine being well characterized. Some TLRs, including TLR1, -2, -4, -5, and -6, are mainly located on the plasma membrane and recognize bacterial, fungal, and protozoan pathogens, whereas others, including TLR3, -7, -8, and -9, are mainly located on endosomal/lysosomal membranes where they bind viral RNAs or DNAs (4–6). All TLRs use leucine-rich repeats to sense the ligands and the Toll/IL-1 receptor homologue (TIR) domain to trigger downstream signaling by binding to adaptor proteins MyD88 (7–9), TIRAP/Mal (10, 11), or TRIF (12, 13). The signaling initiated by TLRs is a double-edged sword. On the one hand, it may lead to confining or eliminating the invading organisms (14, 15); on the other hand, a prolonged and exaggerated response can cause tissue damage or organ damage (16, 17). Moreover, TLR signaling triggered by exogenous or endogenous ligands contributes to the pathogenesis of many chronic inflammatory diseases (18). For example, TLR2 and TLR4 are involved in atherosclerosis (19, 20), autoimmune colitis (21), systemic lupus erythematosus (22, 23), diabetes (24, 25), and Alzheimer disease (26, 27). Thus, blockade of excessive TLR signaling is a therapeutic approach being actively pursued for these diseases (28, 29). However, the development of TLR antagonists as therapeutic agents has been surprisingly slow. No TLR antagonists are currently approved for clinical use, and only a few are in human clinical trials (30–32). The current approach for developing TLR antagonists is to screen the structural analogs of TLR agonists for their ability to bind to the receptor without triggering proinflammatory signaling. However, because the TLRs tolerate considerable structural variation in functional ligands (33, 34), the analogs often display agonistic effects. As a result, there are overwhelmingly more agonists than antagonists identified for TLRs (28).

A Chinese herb, Sparganium stoloniferum, is a perennial, aquatic plant grown in North and East China whose tubers have long been used in traditional Chinese medicine for the treatment of several inflammatory diseases (35, 36). Although much work has been done with extracts from this herb (37–40), no in-depth molecular investigation of its components has been performed. In this study, in an effort to isolate and functionally...
characterize compounds from *S. stoloniferum* tubers, we obtained a novel compound, designated Sparstolonin B (SsnB). We determined its structure, tested its anti-inflammatory activity in vitro and in vivo, and investigated the underlying molecular mechanism. We demonstrated that SsnB is a selective TLR2 and TLR4 antagonist. It may block the TLR2- and TLR4-triggered inflammatory signaling by inhibiting the recruitment of MyD88 to the TIR domains of TLR2 and TLR4.

**EXPERIMENTAL PROCEDURES**

**Plant Materials**—The tubers of *S. stoloniferum* were collected from Pan’an County (Zhjegang, China) in November 2008 and authenticated by Professor Qinan Wu, Nanjing University of Chinese Medicine. A voucher specimen (no. 2008112006) was deposited at Herbarium of Nanjing University of Chinese Medicine.

**Reagents and Cells**—Primers were customer-synthesized by Invitrogen. The THP-1 human monocyte cells (TIB-202™), HEK293T cells (CRL-1573), human umbilical vein endothelial cells (CRL-1730), and human aortic smooth muscle cell HASMCs (CRL-1999) were purchased from ATCC (Manassas, VA). Super pure LPS was purchased from Sigma. Pam3CSK4, Fsl-1, poly(I:C), and ODN1668 were from Invivogen (San Diego, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). RPMI 1640, t-glutamine, streptomycin, and penicillin were purchased from Mediatech (Herndon, VA). Dulbecco’s phosphate-buffered saline was purchased from HyClone (Logan, UT). Human umbilical vein endothelial cell growth medium (F12K) containing 20% FBS and growth supplements such as heparin and endothelial cell growth supplement was purchased from Sigma. All tissue culture plastic ware was purchased from Corning (Corning, NY). FLAG-CMV1-CD4/TLR4, FLAG-TLR4, and FLAG-TLR9 plasmids were kindly provided by Dr. Tianyi Wang at the University of Pittsburgh. The pcDNA-MyD88-CFP plasmid was purchased from Addgene (Cambridge, MA), Ready-To-Glow Secreted Luciferase Reporter System including the reporter vectors pCMV-MLuc and pNFκB-MLuc and the reporter reagents were purchased from Clontech (Mountain View, CA).

**Antibodies**—Rabbit anti-Erk1/2, anti-phospho-Erk1/2, and anti-phospho-p38α antibodies, mouse anti-p38α antibody, and chicken anti-GFP IgY were from Millipore (Billerica, MA). Rabbit anti-IκBα, anti-phospho-IκBα, anti-SAPK/JNK, and anti-phospho-SAPK/JNK antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit anti-β-actin antibody was from Sigma. Mouse monoclonal antibodies to human TLR4 and TLR9 were from Abcam (Cambridge, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Millipore. HRP-conjugated rabbit anti-chicken IgY was from Abcam.

**NMR Spectroscopy**—One-dimensional (1H and 13C) NMR experiments were performed on a Bruker DRX300 spectrometer (300.13 MHz for 1H and 75.47 MHz for 13C) equipped with a direct probe head of 5 mm. Two-dimensional NMR experiments were performed on a Bruker DRX500 spectrometer (500.13 MHz for 1H and 125.76 MHz for 13C) equipped with an inverse probe head of 5 mm. 1H NMR spectra were acquired with an spectral width in Hertz of 6.67 KHz, time domain data point of 64,000, and number of scans of 16. For 13C NMR spectra, an SWF of 26.32 kHz was used with a TD of 32K and NS of 1024. Heteronuclear single quantum correlation experiments were performed using standard pulse sequences supplied by the spectrometer manufacturer. Heteronuclear multiple bond coherence spectra were obtained in experiments with delay values optimized for 2J(H,C) = 8 Hz. All NMR experiments were performed at 300 K, and the concentration of the sample was 20 mg·ml⁻¹ in DMSO-d₆ with 0.03% TMS as an internal reference.

**X-ray Crystallography**—Yellow needle crystals of SsnB were grown from an ethanol-water solution. X-ray intensity data were collected at 150(2) K using a Bruker SMART™ APEX diffractometer equipped with molybdenum Kα radiation (λ = 0.71073 Å). The raw area detector data frames were reduced with the SAINT+ and SADABS programs.3 Final unit cell parameters were determined by least-squares refinement of 4141 reflections taken from the data set. Direct methods structure solution, difference Fourier calculations, and full-matrix least-squares refinement against F² were performed with SHELXTL (42). The crystals adopt the space group P2₁/n of the monoclinic system, with a = 7.1545(10) Å, b = 9.1716(12) Å, c = 16.735(2) Å, β = 93.620(3)°, V = 1095.9(3) Å³, and Z = 4. There is one molecule in the asymmetric unit of the crystal. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms bonded to carbon were located in Fourier difference maps before being placed in idealized positions and included as riding atoms. Hydrogen atoms bonded to oxygen were located and refined freely. The final residual factors are R₁ = 0.0425/wR₂ = 0.1054 for 1567 reflections with I > 2σ(I), and R₁ = 0.0537/wR₂ = 0.1129 for all 1935 data.

**Isolation and Treatment of Mouse Peritoneal Macrophages**—To collect primary mouse macrophages, 3 ml of 3% thioglycollate was injected into mice intraperitoneally. After 3 days, 10 ml of PBS was injected into the peritoneal cavity to collect cells. The cells were seeded in either 6- or 12-well dishes in DMEM with 10% FBS for 1 h and washed with serum-free DMEM twice to remove unattached cells. The attached macrophages were further cultured in appropriate medium. For LPS treatment, mouse macrophages were incubated for 6–24 h in the culture medium (DMEM with 0.25% FBS) with an addition of LPS and SsnB at the indicated concentrations. The cells were then washed with Dulbecco’s phosphate-buffered saline twice before being lysed for total RNA or protein extraction.

**MITT Assay**—Cytotoxicity of SsnB was determined using an MTT cell viability assay kit from Biotium (Hayward, CA) following the manufacturer’s instructions. The 96-well microplates were read using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

**Quantitative Real-time PCR**—Total RNA was extracted and purified using TRIzol reagent (Invitrogen) and RNeasy™ Mini kit (Qiagen) according to the manufacturer’s instructions before performing reverse transcription using a First-strand cDNA Synthesis System (Marligen Bioscience, MD). Quantita-

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1 SMART Version 5.630, SAD+ Version 6.45, and SADABS Version 2.10, Bruker Analytical X-ray Systems, Inc., Madison, WI.
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Isolation and Structural Determination of SsnB—The powdered plant material (20 kg) was soaked in 85% ethanol overnight and then extracted (×3) with the same solvent. After filtration and concentration under vacuum, the residue was suspended in water and extracted with petrol, EtOAc and n-butyralcohol sequentially. The EtOAc extract (60g) was subjected to a Si-gel CC eluted with petrol-EtOAc mixtures of increasing polarity and gave 300 mg of yellow needles 100:13 of petrol-EtOAc. SsnB (mp.258°C) produced a molecular ion peak at m/z 269.0449, corresponding to [M+H]+ in the positive high resolution electrospray ionization mass spectroscopy, establishing the molecular formula C_{15}H_8O_4, which indicates 12 double-bond equivalents. Its UV spectrum showed the typical pattern of conjugated phenyl groups at 288.5, 352.5, and 386.0 nm. Its IR spectrum indicated the presence of hydroxyl pattern of conjugated phenyl groups at 288.5, 352.5, and 386.0 nm. Its IR spectrum indicated the presence of hydroxyl groups are attached at C-8 and C-5

RESULTS

Enzyme-linked Immunosorbent Assay (ELISA)—Mouse TNFα and IL-6 protein concentrations were measured by ELISA using kits from eBioscience (San Diego, CA) following the manufacturer’s instructions. The macrophage-conditioned culture medium was diluted by 30–180-fold for the measurement. The 96-well microplates were read using a SpectraMax M5 microplate reader.

Co-immunoprecipitation—HEK293T cells or THP-1 cells were transfected with TLRs and MyD88 for co-immunoprecipitation experiments. Lipofectamine™ LTX reagent (Invitrogen) was used following the manufacturer’s instruction.

Western Blot Analysis—Samples were loaded onto SDS-PAGE gels for electrophoresis. The size separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences). Indicated primary antibodies and HRP-conjugated secondary antibodies were used to detect target proteins. Signal was detected using an ECL kit (Amersham Biosciences).

Results before Protein G beads (New England Biolabs, Ipswich, MA) were added and incubated for another hour at 4°C. Then the solution was applied to a magnetic separation rack, and the supernatant was removed and saved. After washing with PBS twice, SDS loading buffer was added to the beads to solubilize the precipitates. The solubilized precipitates and the supernatant samples were loaded to the SDS-PAGE for electrophoresis. Anti-GFP or anti-MyD88 antibody was used to detect MyD88-CFP protein in the precipitates and the supernatants.

Mice—Mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and housed in the University of South Carolina Animal Research Facility. All animal experiments were carried out in compliance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of South Carolina.

Statistical Analysis—One-way analysis of variance was used for multiple group comparison; if the data followed a Gaussian distribution, then Bonferroni’s multiple comparisons were further used as a post-test. Otherwise, non-parametric Kruskal Wallis test and Dunn’s multiple comparison post-test were used to analyze the data. When only two groups were compared, Student’s t test was performed. p values of <0.05 were considered statistically significant. GraphPad Prism 5 software was used to carry out all statistical analysis.

RESULTS

A selective real-time PCR analyses were carried out using Fast Start Universal SYBR Green Master (RoX) (Roche Applied Science) on an Eppendorf Realplex2 Mastercycler (Eppendorf, Hamburg, Germany). The primers used in quantitative real-time PCR are listed: mouse 18 S, RNA (internal control) 5′-CGCGGTTCATTTTTTGTGGT-3′ (forward) and 5′-AGTCGGCCATCGTTATAGGGT-3′ (reverse); mouse TNFα, 5′-CTCAGCCGATTGGACTGTA-3′ (forward) and 5′-CGGACTCCGC-AAAGTCTAA-3′ (reverse); mouse IL-6, 5′-AGTTGCTCTTCTTGGGACTGA-3′ (forward) and 5′-TCCACGATTTCCCCAGAACA-3′ (reverse); mouse IL-1β, 5′-GCCCATCTCTTGACTCAT-3′ (forward) and 5′-AGGCCACAGTTATTGTGCG-3′ (reverse); mouse TLR4, 5′-GCTTTCACTCTGCGTTTAC-3′ (forward) and 5′-GAAACTGCGATTTGAGCA-3′ (reverse). Samples were amplified using the following program: 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 68°C for 20 s, then a melting curve analysis from 60°C to 95°C every 0.2°C. The abundance of each gene was detected using an ECL kit (Amersham Biosciences).
the heteronuclear multiple bond coherence spectrum, correlations were also observed from H-3 to C-1, C-10, and C-1, from H-6 to C-8 and C-10, from H-4 to C-2 and C-6, and from H-6 to C-2 and C-4. From the above evidence, the structure of SsnB was elucidated as 8,5-dihydroxy-4-phenyl-5,2-oxidoisocoumarin (Fig. 1B), containing the core structures of both xanthone and isocoumarin (Fig. 1A). To the best of our knowledge, the isolation of the 4-phenyl-5,2-oxidoisocoumarin structure from plant material has not been described before. To further confirm the structure, crystals of SsnB were grown from an ethanol-water solution, and the x-ray crystallographic structure was determined using single crystals prepared as described under “Experimental Procedures.” The data confirmed the structure deduced from NMR spectroscopy as shown in Fig. 1C.

Cytotoxicity Measurement of SsnB—Before further investigating the biological activity of SsnB, we sought to determine its cytotoxicity using both trypan blue staining and the MTT assay. Several cell types were tested, including peritoneal mouse macrophages, human monocytes THP-1 cells, phorbol 12-myristate 13-acetate-differentiated THP-1 macrophages, human umbilical vein endothelial cells, and HASMCs. SsnB was dissolved in DMSO as a stock solution of 50 mg/ml and then diluted into appropriate media. The solubility of SsnB in most of the cell culture media is 150 g/ml (0.56 mM). Our data showed that at concentrations up to 100 M, SsnB did not cause apparent cytotoxicity to any of the above cell types (supplemental Fig. 1).

SsnB Inhibits TLR Ligand-induced Cytokine Expression in Macrophages—Thioglycollate-elicited mouse peritoneal macrophages were cultured in serum-free DMEM overnight before treatment was started. The cells were treated for 6 h with SsnB or TLR ligands alone or in combination at the concentrations indicated in the legend to Fig. 2. Quantitative real-time PCR was performed to measure the cytokine expression levels. As shown in Fig. 2, SsnB alone did not alter the expression levels of any of the three cytokines (TNFα, IL-6, and IL-1β), indicating there was no endotoxin contamination in the SsnB solution preparation and that SsnB itself had no effects on base-line levels of the cytokines. All TLR ligands, including TLR4 ligand LPS, TLR1/2 ligand Pam3CSK4, TLR2/6 ligand Fsl-1, TLR3 ligand poly(I:C), and TLR9 ligand ODN1668 at the concentra-
tions used significantly increased the expression of all 3 cyto-
kines compared with control (p < 0.01). SsnB significantly
inhibited the expression of the three cytokines induced by LPS,
Pam3CSK4, and Fsl-1 but had no effect on that induced by
poly(I:C) and ODN1668. In another set of experiments, macro-
phages were treated with SsnB and LPS for 16 h, and the media
were collected for measurement of cytokine concentrations
using ELISA. As shown in Fig. 3, in control and SsnB alone
groups, TNFα and IL-6 levels in the media were almost unde-
tectable. LPS (50 ng/ml) treatment dramatically increased the
cytokine levels, whereas co-treatment with SsnB significantly
reduced the levels in a dose-dependent manner. SsnB co-treat-
ment decreased LPS-induced TNFα levels in the medium by 3-
and 18-fold at 10 and 100 μM concentrations, respectively, and
reduced LPS-induced IL-6 by 2- and 10-fold at these two con-
tentions, respectively. We repeated these experiments with
phorbol 12-myristate 13-acetate-differentiated THP-1 macro-
phages and obtained similar results (data not shown). We also
examined the effects of SsnB on the expression of TLR4 in
macrophages. We found SsnB alone (both at 10 and 100 μM)
did not alter TLR4 expression in mouse macrophages; however, at
a 100 μM concentration, SsnB slightly but significantly
decreased LPS-induced TLR4 expression in macrophages (sup-

**FIGURE 4.** Mouse macrophages were treated with SsnB and LPS as indicated for 16 h. Cytokine levels in the medium were measured by ELISA. Bars represent the mean ± S.E. For each treatment, n = 5. *, p < 0.01, versus LPS, Student’s t test.

**FIGURE 3.** Mouse macrophages were treated with SsnB and LPS as indicated for 16 h. Cytokine levels in the medium were measured by ELISA. Bars represent the mean ± S.E. For each treatment, n = 5. *, p < 0.01, versus LPS, Student’s t test.

**SsnB Inhibits Phosphorylation of Multiple Signaling Proteins—**

TLR ligands activate various downstream intracellular signal-
ning cascades such as MAPK pathway and NF-κB pathway in
macrophages, leading to the induction of cytokine expression.
To test if SsnB blocks these TLR-mediated downstream signal-
ning pathways, we examined the effects of SsnB on the phospor-
ylation of Erk1/2, p38, IκBα, and JNK. Mouse macrophages
were cultured in FBS-free DMEM in 3.5-cm dishes overnight
before any treatments. For some dishes, the cells were pre-
treated with SsnB for 30 min and washed twice with FBS-free
DMEM to remove SsnB. At this point, fresh FBS-free DMEM
with or without LPS and SsnB (as indicated in Fig. 4) was added
to the wells; control dishes contained neither LPS nor SsnB.
After 30 min of treatment at 37 °C, the cells were lysed on ice in
radioimmune precipitation assay buffer with protease inhibitor
and phosphatase inhibitor cocktails, and the cell lysates were
subjected to Western blot analyses. Fig. 4 and supplemental Fig.
3 show that pretreatment with 10 or 100 μM SsnB similarly
inhibited LPS-induced phosphorylation of Erk1/2, p38, IκBα,
and JNK; however, when concomitantly added to the cells with
LPS, SsnB inhibited LPS-induced phosphorylation of all three
proteins in an apparent concentration-dependent manner.
Similar experiments using other TLR ligands showed that SsnB
inhibited the phosphorylation of the signaling proteins induced
by Pam3CSK4 and Fsl-1 but not that induced by poly(I:C) and
ODN1668 (supplemental Fig. 2B). We also found that SsnB had
no effects on phosphorylation of SHP-2 in cells treated with any
of the above TLR ligands (data not shown) and that SsnB had no
effects of phosphorylation of IκBα in macrophages induced by
TNFα and IL-1β (supplemental Fig. 2C). To test if the inhibi-
tory effect of SsnB on macrophage inflammatory signaling is
reversible, we treated the cells with 100 μM SsnB for 1 h and
further cultured the cells for 24 h in the absence of SsnB before

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LPS was used to stimulate the cells, and we found the inflammatory responses to LPS were completely recovered (data not shown).

SsnB Acts Intracellularly on TLR4—To test if SsnB acts on the ligand-TLR binding step or the intracellular signaling steps, we examined the effects of SsnB on the cells expressing a chimeric CD4–TLR4 receptor. An early seminal work showed that expression of the chimeric CD4–TLR4 receptor consisting of the ectodomain (Ig domain) of the CD4 and the transmembrane region and cytoplasmic domain of TLR4 renders THP-1 cells constitutively active (43) (Fig. 5A). These cells constantly express proinflammatory cytokines as a result of constitutive NF-κB activation enabled by CD4–TLR4 signaling even in the absence of TLR4 ligand. We first transfected HEK293T cells with FLAG-CMV1-CD4/CD4–TLR4 (CD4–TLR4) along with a NF-κB activity luciferase reporter vector pNFκB-MLuc or control pCMV-MLuc; subsequently, the cells were treated with SsnB for 6 h, and the media were collected for secreted luciferase activity luciferase reporter vector pNFκB-MLuc (supplemental Fig. 4), indicating that SsnB indeed suppressed the CD4–TLR4 chimeric receptor-enabled NF-κB activation by CD4–TLR4 signaling even in the absence of TLR4 ligand. We first transfected HEK293T cells with FLAG-CMV1-CD4/CD4–TLR4 (CD4–TLR4) along with a NF-κB activity luciferase reporter vector pNFκB-MLuc or control pCMV-MLuc; subsequently, the cells were treated with SsnB for 6 h, and the media were collected for secreted luciferase activity assay. It was shown that SsnB significantly decreased the luciferase activities in the cells transfected with CD4–TLR4 and pNFκB-MLuc plasmids but not in the cells transfected with CD4–TLR4 and control pCMV-MLuc (supplemental Fig. 4), indicating that SsnB indeed suppressed the CD4–TLR4 chimeric receptor-enabled NF-κB activation. Second, we transfected THP-1 cells with CD4–TLR4 and pNFκB-MLuc or pCMV-MLuc plasmids and treated the cells with 0, 10, or 100 μM SsnB for 16 h. Consistent with the findings in HEK293T cells, SsnB suppressed the secreted luciferase activities in the THP-1 cell culture media in a concentration-dependent manner (Fig. 5B).

SsnB Inhibits MyD88 Recruitment to TLR4 and TLR9—Based on above results, we speculated that SsnB may act on the early steps of the intracellular TLR4 signaling, possibly the early adaptor protein recruitment by the TLR TIR domain. To test this, we examined if SsnB blocks the recruitment of MyD88 by TLR4. We co-transfected HEK293T cells with pcDNA-MyD88-CFP and FLAG-TLR4 or FLAG-TLR9 plasmids; 16 h after the transfection, the cell culture media were changed to DMEM with 1% FBS containing 50 ng/ml LPS or 2.5 μM ODN1668 with or without 100 μM SsnB. After 30 min of treatment, the cells were lysed, and cell lysates were used for detecting the expression of MyD88-CFP (using anti-GFP antibody) and TLR4 or TLR9 as well as for detecting the association of MyD88-CFP with MyD88–CFP or with anti-TLR4 or anti-TLR9 antibody. The precipitates (P) and supernatants (S) were examined against anti-GFP antibody for detecting MyD88–CFP. Mouse Ig was used as a control for immunoprecipitation. Shown is the representative of three independent experiments. B, THP-1 cells (5 × 10⁵ cells/well in a 6-well plate) were transiently co-transfected with FLAG-TLR4 or FLAG-TLR9 and pcDNA-MyD88–CFP or used for co-immunoprecipitation (IP) by anti-TLR4 or anti-TLR9 antibody. The precipitates (P) and supernatants (S) were examined against anti-GFP antibody for detecting MyD88–CFP. Mouse Ig was used as a control for immunoprecipitation. Shown is the representative of three independent experiments. B, THP-1 cells (5 × 10⁵ cells/well in a 6-well plate) were transiently co-transfected with FLAG-TLR4 or FLAG-TLR9 and pcDNA-MyD88–CFP or used for co-immunoprecipitation (IP) by anti-TLR4 or anti-TLR9 antibody. The precipitates (P) and supernatants (S) were examined against anti-GFP antibody for detecting MyD88–CFP. Mouse Ig was used as a control for immunoprecipitation. Shown is the representative of three independent experiments. B, THP-1 cells (5 × 10⁵ cells/well in a 6-well plate) were transiently co-transfected with FLAG-TLR4 or FLAG-TLR9 and pcDNA-MyD88–CFP or used for co-immunoprecipitation (IP) by anti-TLR4 or anti-TLR9 antibody. The precipitates (P) and supernatants (S) were examined against anti-GFP antibody for detecting MyD88–CFP. Mouse Ig was used as a control for immunoprecipitation. Shown is the representative of three independent experiments.
precipitation indicated SsnB at a 100 ng/ml LPS and indicated concentration of SsnB. As shown in Fig. 6, HEK293T cells at 80% confluence in 10-cm dish were co-transfected with FLAG-TLR4, pcDNA-MyD88-CFP, and NFkB-Luc plasmids (5 μg each). 16 h after the transfection the cells were split in 24-well plates at 0.25 × 10^6/well. The culture medium was changed to DMEM with 1% FBS with 100 ng/ml LPS and indicated concentration of SsnB. The culture medium was collected 24 h later for luciferase report assay. Luciferase activity readings of the samples were shown in the left panel. Half-maximal inhibitory concentration (IC_{50}) was shown in the right panel. Data represent the mean ± S.E. For each treatment, n = 5. B, THP-1 cells were transiently transfected with NFkB-Luc plasmids (5 μg each 10-cm dish). 24 h after the transfection, the cells were split in 24-well plates at 0.1 × 10^6/well. The culture medium was changed to RPMI1640 with 1% FBS with 100 ng/ml LPS and indicated concentration of SsnB. The culture medium was collected 24 h later for luciferase report assay. Luciferase activity (relative luciferase units (RLU)) readings of the samples were shown in the left panel. Estimated IC_{50} was shown in the right panel. Data represent the mean ± S.E. For each treatment, n = 4.

association of MyD88-CFP with TLR9. Next, we tried to confirm this result using more relevant THP-1 cells. Preliminary experiments showed that TLR antibodies could not precipitate enough MyD88 protein detectable by Western blots in non-transfected THP-1 cells stimulated by TLR ligands; however, when THP-1 cells highly over-expressed TLRs and MyD88 via transient transfection, the base-line levels of TLRs-MyD88 association (in the absence of TLR ligands) were high, and the effect of SsnB was not significant even for TLR2 and TLR4 (data not shown). After careful adjustment of the amount of DNA for transfection to largely eliminate base-line TLR-MyD88 association (in the absence of TLR ligands), we transfected 5 × 10^5 THP-1 cells in each well of the 6-well plate with a total of 1 μg of DNA (1:1 of pFLAG-TLR4-pcDNA-MyD88-CFP). As shown in Fig. 6B, co-immunoprecipitation indicated SsnB at a 100 μM concentration substantially reduced the amount of MyD88 co-precipitated with TLR4 and TLR2 in the THP-1 cells stimulated with TLR4 ligand LPS (100 ng/ml) or TLR2 ligand Pam3CSK4 (500 ng/ml) but did not affects the TLR9-MyD88 association induced by TLR9 ligand ODN1668 (2.5 μM).

**SsnB Dose-dependently Attenuates TLR4-mediated NF-κB Activation**—To further quantitate the inhibitory effects of SsnB on TLR4 signaling, we co-transfected HEK293T cells with FLAG-TLR4, pcDNA-MyD88-CFP, and pNFkB-MLuc plasmids and treated the cells with LPS and different concentrations of SsnB. The cells were transfected with the above three plasmids (5 μg each) in a 10-cm culture dish for 16 h before the cells were split into 24-well plates. After a 24-h further incubation in DMEM with 10% FBS, the cells were treated with 50 ng/ml LPS and the indicated amount of SsnB in DMEM with 1% FBS for 24 h before the conditioned media were taken for secreted luciferase activity assay. Fig. 7A showed that SsnB concentration dependently inhibited the secreted luciferase activities. Because SsnB at 200 μM did not further inhibit NF-κB activation compared with that at 100 μM concentration, we considered that SsnB achieved its maximal inhibitory effect at 100 and 200 μM. We plotted the dose-dependent inhibitory effects of SsnB in Fig. 7A, right panel, indicating the half-maximal inhibitory concentration (IC_{50}) of SsnB is ~10 μM. We repeated this experiment using THP-1 cells transiently transfected with pNFkB-MLuc plasmid. The cells were treated with 50 ng/ml LPS and SsnB at various concentrations. As shown in Fig. 7B, SsnB reduced NF-κB activity dose dependently, and the IC_{50} was estimated as 1–10 μM.

**SsnB Suppresses LPS-provoked Inflammation in Mice**—To evaluate the anti-inflammatory effect of SsnB in vivo, we used an acute LPS-induced sepsis mouse model. To mimic acute endotoxemia, one dose (100 μg/mouse) of LPS from *Escherichia coli* 055:B5 (catalog #L4524, Sigma) was injected intraperitoneally into 5–6-week-old male C57Bl/6 mice (body weight 18–20 g). One hour before the LPS administration, SsnB (100 μg/mouse) or vehicle was intraperitoneally injected into the mice. Eight hours after the LPS administration, the mice were sacrificed, and the spleens were used for measuring the inflammatory cytokine expression by quantitative real-time PCR. As shown in Fig. 8, LPS administration...
significantly stimulated the expression of all three cytokines (TNFα, IL-6, and IL-1β) in the spleen; the SsnB pretreated group had significantly lower TNFα and IL-1β expression levels compared with LPS only group (n = 3, p < 0.01). The group also had a lower level of IL-6 expression but did not reach statistical significance.

DISCUSSION

TLR-mediated signaling plays detrimental roles in many inflammatory diseases, such as multiple sclerosis, autoimmune colitis, and systemic lupus erythematosus. For these diseases, there are very limited validated therapeutic options. Chinese traditional medicine modalities have long been proven effective in these refractory inflammatory diseases. However, the molecular mechanisms have not been elucidated. Here we report the isolation, structural determination, functional analysis, and mechanistic investigation of a Chinese herb-derived compound, SsnB. SsnB was isolated from a commonly used Chinese herb, S. stoloniferum, whose tubers have been used in traditional Chinese medicine for the treatment of several inflammatory diseases (35, 36). This study demonstrates that SsnB is a selective TLR antagonist, shedding light on the anti-inflammatory mechanism of this Chinese herb.

NMR and x-ray crystallography demonstrated SsnB is a new polyphenol with structural features of xanthone and isocoumarin. Polyphenols have anti-oxidation and anti-inflammatory property (44); xanthones exhibit anti-oxidation, immunomodulation, and cholesterol-lowering benefits (45), whereas isocoumarins are used as anti-coagulants, anti-tumor, and anti-inflammatory agents (46). Our data show that like all these three types of compounds, SsnB displays potent anti-inflammatory effects on mouse and human macrophages. More importantly, we demonstrated that SsnB selectively inhibited macrophage inflammatory responses to TLR2 and TLR4 ligands but not those to TLR3 and TLR9 ligands. Furthermore, we found SsnB suppressed multiple signaling pathways downstream of TLR2 and TLR4 activation, including MAPK and NF-κB pathways, leading us to speculate that SsnB may act on the early events of TLR2 and TLR4 signaling, possibly the ligand-TLR binding and immediate adaptor recruitment steps. The fact that SsnB had no effects on TNFα and IL-1β-induced phosphorylation of IκBα is also in favor of this assumption. SsnB did not affect the TLR4 expression in macrophages in the absence of LPS; thus, its effect on the inflammatory signal transduction is not likely due to the alteration of TLR expression. Although SsnB slightly attenuated the up-regulation of TLR4 mRNA levels by LPS, this might be secondary to the suppression of LPS-induced proinflammatory signaling. For example, it was reported that MAPK signaling pathway increases the stability of TLR4 mRNA in vascular smooth muscle cells (47). Using cells expressing a constitutively active chimeric receptor, CD4-TLR4, we found SsnB effectively attenuated the constitutive NF-κB activation mediated by CD4-TLR4 chimeric receptor, indicating that SsnB more likely acts intracellularly. Co-transfection and co-immunoprecipitation experiments further showed that SsnB inhibited ligand-induced TLR4-MyD88 and TLR2-MyD88 association, but not TLR9-MyD88 association, explaining the fact that SsnB diminished TLR4 and TLR2 signaling but had no effects on TLR9 signaling. In the meantime, this also suggests that SsnB can enter the cells, excluding the possibility that SsnB cannot enter the cells and, therefore, could not exert inhibitory effects on TLR3 and TLR9 signaling as these two receptors are mainly located in the endosomal membrane. Because SsnB had effects on TLR4 and TLR2 but not on TLR9, and MyD88 is involved in all these three TLRs signaling, it is likely that SsnB may act directly on TLR2 and TLR4 TIR domains or TIRAP/mal protein, which is an indispensable adaptor bridging TLR2/TLR4 TIR domain and MyD88 interaction but is not involved in TLR9 signaling. Further studies are warrant to locate the exact acting site(s) of SsnB.

To test if the TLR4 blocking activity of SsnB translates into in vivo anti-inflammatory effect, we used an LPS-induced sepsis model. In sepsis, LPS released from Gram-negative bacteria induces strong immune responses and causes severe complications (48, 49). Chronic low level blood LPS has been increasingly appreciated as a link between chronic infectious diseases such as periodontitis and other immune-related chronic inflammatory diseases such as atherosclerosis (50, 51). In both acute and chronic cases, LPS-TLR4 signaling-induced proinflammatory cytokine production is the primary pathogenic

![A Selective TLR Antagonist](image-url)
A Selective TLR Antagonist

cause (52, 53). Our data showed that SsnB could significantly blunt the expression of proinflammatory cytokines in LPS-challenged mice, indicating SsnB is able to block LPS-induced TLR4 signaling in vivo. Further studies will be directed to evaluate the bioavailability, biosafety, and other pharmacological properties of SsnB in vivo.

TLR2 and TLR4 have been demonstrated to be involved in many diseases such as sepsis (54), atherosclerosis (19), heart and brain ischemia/reperfusion injury (55, 56), autoimmune colitis (21), systemic lupus erythematosus (22), diabetes (24), and Alzheimer disease (26, 27). The potential that SsnB can be developed into a selective TLR2 and TLR4 antagonist will add it to the short list of TLR antagonists for further drug development for these diseases. It is important to note that SsnB is significantly different in structure from other small molecule TLR2 and TLR4 antagonists that are currently in clinical development, including AV411 (32), Eritoran (30, 57), and TAK-242 (41, 58). It is also notable that to our knowledge SsnB is the first natural small molecule TLR antagonists that are currently in clinical development, including AV411 (32), Eritoran (30, 57), and TAK-242 (41, 58).

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