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*J Immunol* 2014; 192:2778-2786; Prepublished online 14 February 2014; doi: 10.4049/jimmunol.1301931

http://www.jimmunol.org/content/192/6/2778
Zhankuic Acid A Isolated from *Taiwanofungus camphoratus* Is a Novel Selective TLR4/MD-2 Antagonist with Anti-Inflammatory Properties

Yu-Fon Chen,* Ai-Li Shiau,† Sheng-Hung Wang,‡ Jai-Sing Yang,§ Sue-Joan Chang,* Chao-Liang Wu,§ and Tian-Shung Wu*∥

TLR4, a membrane receptor that functions in complex with its accessory protein myeloid differentiation factor-2 (MD-2), is a therapeutic target for bacterial infections. *Taiwanofungus camphoratus* is highly valued as a medicinal mushroom for cancer, hypertension, and inflammation in traditional medicine. Zhankuic acid A (ZAA) is the major pharmacologically active compound of *T. camphoratus*. The mechanism of action of *T. camphoratus* or ZAA has not been fully elucidated. We analyzed the structure of human TLR4/MD-2 complex with ZAA by X-score and HotLig modeling approaches. Two Abs against MD-2 were used to verify the MD-2/ZAA interaction. The inflammation and survival of the mice pretreated with ZAA and injected with LPS were monitored. The modeling structure shows that ZAA binds the MD-2 hydrophobic pocket exclusively via specific molecular recognition; the contact interface is dominated by hydrophobic interactions. Binding of ZAA to MD-2 reduced Ab recognition to native MD-2, similar to the effect of LPS binding. Furthermore, ZAA significantly ameliorated LPS-induced endotoxemia and *Salmonella*-induced diarrhea in mice. Our results suggest that ZAA, which can compete with LPS for binding to MD-2 as a TLR4/MD-2 antagonist, may be a potential therapeutic agent for gram-negative bacterial infections. *The Journal of Immunology*, 2014, 192: 2778–2786.
Materials and Methods

Cells, bacteria, and mice

The RAW264.7 murine macrophage cell line and attenuated Salmonella enterica subsp. enterica serovar Choleraesuis (S. choleraesuis) (13) were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Male C3H/HeJ, C3H/HeN, and C57BL/6 mice (8–10 wk old) were obtained from the National Laboratory Animal Center, Taiwan (Taipei, Taiwan).

Plasmids and reagents

The NF-κB reporter plasmid p-NF-κB-Luc was purchased from Promega (Madison, WI). The pβ-actin-LacZ plasmid was derived from pRRL2 plasmid (14) by replacing the firefly luciferase expression cassette driven by the CMV promoter with the β-galactosidase expression cassette driven by the β-actin promoter. The pCMV-Luc reporter plasmid was obtained from Addgene (Cambridge, MA). The pEGFP-N1 (ΔEGFP) plasmid containing the kanamycin-resistant gene was derived from pEGFP-N1 by deletion of the EGFP coding region. Abs against COX2, iNOS, and TLR4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs against IkBa, ERK, JNK, Akt, and p38, as well as phospho (p)-IkB kinase (IKK) α/β (pIKKα/β), pNF-κBp65, pERK, pJNK, pAkt, and pp38 were obtained from Cell Signaling (Danvers, MA).

Extraction and isolation of fungal compounds

ZAA was isolated from T. camphoratus as previously described (10, 15). The compound was dissolved at a concentration of 2 mg/ml in 40% cy-clohexadrin (Sigma-Aldrich, St. Louis, MO) for use as stock solutions, stored at −20°C, and diluted with cell culture medium prior to each experiment. The final concentration of cyclohexadrin used in all experiments was <0.2%.

Assay of anti-inflammatory molecules

C57BL/6 mice were injected i.p. with 3% thioglycollate, and their peritoneal macrophages were collected 72 h later. Macrophages were cultured in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO2. Cells were pretreated with or without ZAA for 1 h and then incubated with LPS (Sigma-Aldrich; 0.5 μg/ml) or IFN-γ (PeproTech, Rocky Hill, NJ; 50 ng/ml) for 24 h. Cell lysates were subjected to SDS-PAGE for detection of COX2 and iNOS expression. The presence of nitrite (a metabolite of NO) in the culture medium was analyzed with the Griess assay (Sigma-Aldrich) as described previously (16).

Immunoblot analysis

RAW264.7 cells were treated with or without various concentrations of ZAA for 1 h, followed by stimulation with LPS (1 μg/ml) for 30 min and homogenization in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 8.0, 1 mM Na2VO4, 20 μg/ml leupeptin, 20 μg/ml apronin, 1 mM PMSF, and 50 mM NaF). Cell lysates were analyzed by immunoblotting with primary Abs against pIKKα/β, IkBa, pNF-κBp65, pERK, pJNK, pAkt, Akt, pp38, and p38, and β-actin, followed by appropriate secondary Abs. Immunoreactive protein bands were detected using an ECL kit (Pierce Biotechnology, Rockford, IL). Relative intensities of the protein bands were normalized to that of β-actin and quantified using Image J software (available at http://rsb.info.nih.gov/ij/).

FIGURE 1. ZAA inhibits the production of inflammation-related molecules in LPS- and IFN-γ–stimulated murine macrophages. (A) Murine peritoneal macrophages were pretreated with or without ZAA for 1 h, followed by incubation with LPS (0.5 μg/ml) for 24 h. Total cell lysates were subjected to immunoblotting for detecting COX2 and iNOS. Relative expression levels of COX2 and iNOS protein were quantified by densitometric analysis with ImageJ software and normalized according to the β-actin reference band. (B) Murine peritoneal macrophages were pretreated with ZAA for 1 h, followed by incubation with LPS (0.5 μg/ml) or IFN-γ (50 ng/ml) for 24 h. The presence of nitrite in the culture medium was analyzed with the Griess assay and used as an indicator of NO levels (n = 4, *p < 0.05, ***p < 0.001 versus LPS- or IFN-γ–stimulated cells). (C) Raw264.7 cells were cotransfected with pNFκB-Luc and pβ-actin-LacZ plasmids. After 48 h, the cells were treated with or without ZAA for 1 h and then treated with LPS (0.5 μg/ml) for 24 h. Total cell lysates were harvested, and their luciferase activities were determined and normalized on the basis of β-galactosidase activities. Values are means ± SD (n = 4, *p < 0.05, **p < 0.01 versus LPS-stimulated cells). (D) and (E) ZAA inhibits NF-κB, MAPK, and Akt signaling pathways in LPS-stimulated RAW264.7 cells. Cells were treated with ZAA for 1 h, followed by stimulation with LPS (0.5 μg/ml) for 30 min. Total cell lysates were examined for the indicated proteins by immunoblotting. Numbers below the blots in (D) and those shown in the table below the blots in (E) represent the relative expression levels quantified by densitometric analysis with ImageJ software and normalized according to the β-actin reference bands. Similar results were obtained in three independent experiments. N.D., not detectable.

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Reporter assay

Subconfluent Raw264.7 cells cultured in 24-well plates were cotransfected with p-NF-κB-Luc and pβ-actin-LacZ plasmids using the Neon Transfection System (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Forty-eight hours after transfection, cells were cultured in serum-free DMEM with or without ZAA (0.5 or 10 μM) for 1 h and then treated with LPS (1 μg/ml) for 24 h. Cell lysates were harvested and their luciferase activities were determined by a dual-light luciferase and β-galactosidase reporter gene assay system (Tropix, Bedford, MA). Relative luciferase activity was measured as luciferase activity divided by β-galactosidase activity to normalize transfection efficiency.

Molecular docking

Flexible molecular docking was performed using Dock 5.1 software (17). Kollam partial charges were applied to protein models for force field calculation. Energy-optimized three-dimensional coordinates of small molecules were generated by Marvin 5.2.2 (available at http://www.chemaxon.com) and Balloon 0.6 software (18). Additionally, the Gaussian partial charges were calculated by applying OpenBabel 2.2.3 software (19). The parameters for the Dock program were set to generate 1000 orientations and 200 conformers iteratively in the MD-2 binding pocket. The docked conformers were rescored and ranked with HotLig to predict the protein-ligand interactions. HotLig is a molecular surface-directed scoring function, which applies the Connolly surface of a protein for evaluation of molecular interactions. First, the Connolly surface of protein was calculated by PscanMS, a tool in the HotLig package, and then the docked ligand conformers were input for analysis of molecular interactions and calculation of binding energy scores. The rendering of figures for molecular modeling was performed using Chimera software (20).

Native PAGE

For in vitro binding analysis, predetermined amounts of LPS or ZAA were sonicated for 3 min and incubated with recombinant human MD-2 (R&D...
Systems, Minneapolis, MN; 0.15 μg) or recombinant human TLR4/MD-2 complex (R&D Systems; 1 μg) at 37°C for 3 h. Samples were subjected to native PAGE, and the levels of TLR4-associated or free MD-2 were detected by immunoblotting with two anti–MD-2 Abs, rabbit polyclonal Ab against MD-2 aas 110–160 (Abcam, Cambridge, MA) and mouse mAb against MD-2 aas 2–160 (Abcam). Signals were detected with an ECL kit.

**ELISA for cytokine expression**

Raw264.7 cells were incubated with or without various concentrations of ZAA for 1 h, followed by treatment with LPS (0.25 or 0.5 μg/ml) or *S. choleraesuis* (2 × 10^3 CFU/well) for 4 and 6 h for detection of TNF-α and IL-6 levels, respectively, in the supernatants by ELISA kits (R&D).

**Mouse models of LPS- or *S. choleraesuis*-induced inflammatory responses and diarrhea**

Mice were pretreated i.p. with ZAA (20 mg/kg) or the vehicle (0.2% cyclodextrin in normal saline) for 30 min, followed by i.p. injection with LPS (4 mg/kg). After 6 h, the expression levels of cytokines in the plasma were measured with ELISA. Ten hours after LPS treatment, mice were sacrificed, and the organs were resected and fixed in formalin. The lung and kidney were embedded in paraffin, sectioned, and stained with H&E. Serum samples were collected for determination of blood urea nitrogen (BUN) and serum creatinine levels. The pEGFP-N1 (ΔEGFP)-transformed *S. choleraesuis* (2 × 10^5 CFU/mouse) was administered orally to mice with or without oral pretreatment of ZAA (2 or 10 mg/kg). Serum samples were collected 6 h later for determining TNF-α and IL-6 levels with ELISA. Fecal samples were collected at 24-h intervals until 96 h after *S. choleraesuis* infection. *S. choleraesuis* in the feces was quantified by plating serial dilutions of fecal samples on kanamycin-containing agar plates and counting colonies after overnight incubation at 37°C.

**LPS-induced sepsis model**

C3H/HeJ and C3H/HeN mice were pretreated i.p. with ZAA (2 or 10 mg/kg) or the vehicle. After 30 min, they were administered i.p. with LPS (0.2% cydextrin in normal saline) for 30 min, followed by i.p. injection with LPS (0.25 or 0.5 mg/ml) or *S. choleraesuis* (2 × 10^8 CFU/m) in normal saline). The mice were monitored every 2–4 h until all C3H/HeN mice in the ZAA-untreated and LPS-treated groups expired.

**Luciferase-based noninvasive bioluminescence imaging**

C57BL/6 mice were given ZAA (2 mg/kg) or the vehicle orally, followed by oral administration of pCMV-Luc–transformed *S. choleraesuis* (2 × 10^8 CFU/mouse) 30 min later. After 48 h, mice were injected i.p. with α-luciferin potassium salt (Promega; 2.5 mg in 100 μl). They were then anesthetized with 2% isoflurane. In vivo bioluminescence imaging and quantitative analysis were performed using the IVIS-200 System and its integrated acquisition and analysis software (Living Image V. 2.50; Perkin Elmer, Foster City, CA).

**Statistical analysis**

Results are presented as means ± SD. Statistical differences were analyzed using Student unpaired t test and SigmaPlot software (Systat); p < 0.05 was considered statistically significant.

**Results**

**ZAA dose-dependently inhibits the production of iNOS, COX2, and NO**

We first investigated the anti-inflammatory properties of ZAA purified from *T. camphoratus*. During inflammation, large amounts of proinflammatory mediators, NO, and PGE2 are generated by iNOS and COX2. ZAA downregulated the levels of COX2 and iNOS induced by LPS in murine peritoneal macrophages (Fig. 1A), as well as inhibited LPS- and IFN-γ–induced NO production (Fig. 1B) in a dose-dependent manner. However, treatment of ZAA up to 30 μM for 72 h did not exert any cytotoxic effects on resident or LPS-activated macrophages, as determined by MTS and sulphorhodamine B assays (22), as shown in Supplemental Fig. 1.

**ZAA blocks LPS-induced NF-κB, MAPK, and Akt signaling pathways**

To investigate the inhibitory role of ZAA in LPS-stimulated NF-κB signaling, we first detected its effect on the transactivation of NF-κB. Fig. 1C shows that ZAA inhibited NF-κB–mediated transactivation in Raw264.7 cells, as determined by the luciferase reporter assay. Furthermore, LPS treatment stimulated NF-κBp65 phosphorylation, which was significantly prevented by ZAA (Fig. 1D). Similarly, LPS-induced phosphorylation of ERK, JNK, p38, and Akt was also inhibited by ZAA (Fig. 1E). Collectively, these results strongly suggest that ZAA can suppress LPS-stimulated NF-κB, MAPK, and Akt signaling pathways.

**ZAA interacts with the hydrophobic pocket of MD-2 to block LPS actions**

It has been demonstrated that MD-2 in association with the extracellular domain of TLR4 can trigger LPS-mediated responses (23, 24). To investigate whether ZAA interrupts TLR4 signaling by competing the binding of LPS to MD-2, we applied an in silico molecular docking analysis to simulate the interactions between ZAA and MD-2. Previous studies have shown that knowledge-based scoring functions are better methods for prediction of protein-ligand interactions, whereas empirically based scoring functions are more effective for predicting ligand-binding affinities (25, 26). Therefore, we used a new knowledge-based scoring program, HotLig, to predict the molecular interactions between ZAA and MD-2. Furthermore, we applied an empirically based scoring program, X-score, to predict their binding affinities. The HotLig showed ~85%–90% success rates for predicting ligand binding poses (12). On the other hand, the X-score was reported to
be the best scoring function for ranking protein-ligand affinities while comparing with many other well-known scoring programs (25, 26). Fig. 2A presents the clipped surface model of MD-2 (PDB entry: 3FXI) along with the ribbon model to depict the LPS-binding pocket buried within the MD-2 protein. Notably, ZAA assumes a matched configuration to fit into the previously identified LPS-binding pocket. Fig. 2B shows the interactions between ZAA and the hydrophobic amino acid residues of MD-2 (e.g., Ile, Val, Phe, Leu, and Tyr), which constitute the LPS-binding pocket. Clearly, ZAA contacts the pocket by hydrophobic interactions (radiating line-semicircle symbols in Fig. 2B), without hydrogen bonding. Additionally, as shown in Fig. 2C, the molecular superimposition of MD-2-bound ZAA (depicted in black) and LPS (cocrystallized ligand within the 3FXI MD-2 structure) implies that ZAA can occupy the space otherwise occupied by the terminal carbon chains of LPS.

FIGURE 3. ZAA inhibits TNF-α and IL-6 production in LPS- or S. choleraesuis-treated RAW264.7 cells and mice. RAW264.7 cells in a 96-well plate (2 × 10^4 cells/well) were incubated with ZAA for 1 h, followed by treatment with (A) LPS (0.25 or 0.5 μg/ml) or (C) S. choleraesuis (2 × 10^9 CFU/well). The supernatants collected after 4 and 6 h were assessed for TNF-α and IL-6 levels with ELISA, respectively. (B) C3H/HeJ and C3H/HeN mice were pretreated i.p. with 2 mg/kg ZAA for 30 min, followed by i.p. injection of 4 mg/kg LPS. (D) C57BL/6 mice were pretreated with 10 mg/kg ZAA for 30 min, followed by oral administration of S. choleraesuis (2 × 10^9 CFU/mouse). Levels of TNF-α and IL-6 were measured in the plasma after 6 h with ELISA. Values are means ± SD (n = 6–8). Similar results were obtained in at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. S.C., S. choleraesuis.
To evaluate the potential binding affinities of ZAA and LPS for MD-2, a consensus scoring analysis was performed using the X-Score scoring functions after generating binding pose predictions via HotLig (Table I). The predicted pKd (the average of the HPscore, HMScore, and HSScore) of ZAA was 7.83, whereas that of LPS was 5.83 (Table II). Therefore, we hypothesize that the matched molecular configuration of ZAA, coupled with the significant hydrophobic interaction effect, can provide a sufficient binding force to stabilize the MD-2/ZAA complex. Thus, ZAA might interfere with the recognition of anti-MD-2 Ab to MD-2 through competitive binding with the Ab or causing the conformational change of the MD-2 protein.

ZAA reduces LPS- and S. choleraesuis–induced proinflammatory cytokine production

As LPS induces proinflammatory cytokine production, we next compared the effects of ZAA on LPS- and S. choleraesuis–induced production of TNF-α and IL-6 in vitro and in vivo. ZAA inhibited LPS-induced TNF-α and IL-6 production in Raw264.7 macrophages at the two concentrations tested (Fig. 3A). To demonstrate whether such effects were also observed in vivo, ZAA was administered to mice 30 min before injection of LPS. Whereas ZAA significantly reduced TNF-α and IL-6 production in C3H/HeN mice, a much weaker response was observed in TLR4 signaling-defective C3H/HeJ mice (Fig. 3B), suggesting that the actions of ZAA are selective for the LPS/TLR4/MD-2 pathway.

We next explored the effects of ZAA on S. choleraesuis–mediated proinflammatory cytokine production both in vitro and in vivo. Our results show that pretreatment of ZAA for 1 h effectively suppressed S. choleraesuis–induced TNF-α and IL-6 production in RAW264.7 cells (Fig. 3C) and C57BL/6 mice (Fig. 3D).

FIGURE 4. ZAA reduces LPS-induced pathological changes in mice. (A–D) C3H/HeN and C3H/HeJ mice were pretreated i.p. with ZAA (20 mg/kg) or the vehicle for 30 min and then injected i.p. with LPS (4 mg/kg). After 10 h, mice were sacrificed, and their lung and kidney tissues were removed. (A) Representative microscopic images of hematoxylin-and-eosin-stained sections of lung and kidney tissues are shown. Scale bars, 20 μm. Original magnification ×200. (B) The number of infiltrated PMNs in each alveolus was observed by light microscopy. The number of PMNs was counted in four randomly chosen fields per slide for each mouse and normalized to the number of alveoli. (C and D) ZAA decreases the levels of BUN (C) and serum creatinine (D). Values shown in (B)–(D) are means ± SD (n = 10. **p < 0.01, ***p < 0.001). (E) C3H/HeN and C3H/HeJ mice that had been pretreated i.p. with ZAA (2 or 10 mg/kg) or the vehicle for 30 min were injected i.p. with a lethal dose of LPS (20 mg/kg). Survival time was monitored, and Kaplan-Meier survival curves were shown in four groups (n = 10. ***p < 0.001 versus LPS-treated C3H/HeN mice). Similar results were obtained in at least three independent experiments.
ZAA attenuates LPS-induced lung and renal injury and lethality

Because ZAA inhibited LPS-induced proinflammatory cytokine production and signaling pathways, we then explored ZAA to reduce organ pathology and lethality provoked by LPS in vivo. We determined the inflammation responses in lung and kidney tissues because of their constitutive TLR4 expression (30, 31). The infiltration of polymorphonuclear leukocytes (PMNs) was elevated in the lung after administration of LPS to C3H/HeN mice; however, ZAA treatment significantly prevented LPS-induced pulmonary accumulation of PMNs (Fig. 4A, 4B). Similarly, the extent of LPS-induced glomerulonephritis was significantly reduced in ZAA-pretreated C3H/HeN mice (Fig. 4A). Consistent with the functional failure of the kidney, BUN and serum creatinine levels were increased after LPS administration. ZAA markedly reduced the production of both kidney injury markers (Fig. 4C, 4D). Notably, LPS treatment did not induce any pathologic changes in TLR4 signaling-defective C3H/HeJ mice (Fig. 4A–4D).

To evaluate the protective efficacy of ZAA against LPS-induced lethality, C3H/HeN and C3H/HeJ mice were treated with ZAA or the vehicle and challenged with LPS. ZAA significantly protected C3H/HeN mice against lethality and improved survival during endotoxemia (Fig. 4E), suggesting that ZAA has therapeutic potential against LPS-induced sepsis and gram-negative bacterial infections in general.

**FIGURE 5.** ZAA ameliorates S. choleraesuis–induced diarrhea, body weight loss, and infection in the gastrointestinal tract. (A and B) C57BL/6 mice that had been pretreated i.p. with ZAA (2 or 10 mg/kg) or the vehicle for 30 min were orally administered with kanamycin-resistant S. choleraesuis (2 × 10^9 CFU/mouse). (A) Diarrhea was scored after 2 d on a 0–3 scale (0 = normal pellets, 1 = slightly loose feces, 2 = loose feces, and 3 = watery diarrhea). (B) Body weight was recorded every 2 d for 2 wk (n = 9–12; **p < 0.01, ***p < 0.001). (C) Fecal samples were collected at 24-h intervals until 96 h after S. choleraesuis infection and assessed for viable bacterial CFU counts (n = 10; **p < 0.01, ***p < 0.001). (D and E) C57BL/6 mice that were treated orally with ZAA (2 mg/kg) or vehicle for 30 min were orally administered pCMV-Luc-transformed S. choleraesuis (2 × 10^9 CFU/mouse). After 48 h, bioluminescence imaging of the mice was conducted after injection with β-luciferin. (D) Whole-body images are shown. The photon flux scale is shown on the right. (E) Quantification of bioluminescent imaging data. Radiance values are expressed as means ± SD (***p < 0.001). S.C., S. choleraesuis.

ZAA ameliorates clinical symptoms of mice infected with S. choleraesuis

TLR4 plays a significant role in host defense responses against Salmonella infections (32, 33). Previous studies have shown that mice lacking TLRs, especially TLR4, are more resistant to Salmonella infections (32, 34), suggesting that blockade of LPS on the outer membrane of the bacterium and hence the TLR4/MD-2 interaction is a promising antibacterial strategy. To further confirm the anti-inflammatory properties of ZAA in vivo, S. choleraesuis–infected C57BL/6 mice were treated with ZAA, and diarrhea and body weight was monitored for 2 d and 2 wk, respectively. Fig. 5A shows that ZAA pretreatment reduced the diarrhea score in the infected mice. ZAA treatment (10 mg/kg) dramatically attenuated body weight loss (Fig. 5B), resulting in a return to normal body weight within 2 wk. As shown in Fig. 5C, the bacterial load in the feces of the untreated mice gradually declined over time. Notably, fecal bacterial load was dramatically reduced in dose- and time-dependent manners in ZAA-treated mice. At 96 h after bacterial infection, fecal bacterial level was undetectable in mice treated with 10 mg/kg ZAA. Furthermore, mice infected with S. choleraesuis carrying the pCMV-Luc plasmid exhibited much weaker bioluminescence following oral treatment with ZAA than with the vehicle (Fig. 5D, 5E). Taken together, these results show that ZAA can suppress S. choleraesuis infection effectively and attenuate the clinical symptoms in mice.
**Discussion**

In this study, we used two different modeling approaches and Ab recognition to show that ZAA interacts with the hydrophobic pocket of MD-2 to block LPS actions. We found that ZAA can act as a ligand for MD-2, thereby suppressing the interaction of LPS with MD-2. We also showed that systemic administration of ZAA protects mice from LPS-induced lung and renal injury and *Salmonella*-induced enteritis and body weight loss. Our results indicate that ZAA possesses anti-inflammatory activity and may be a potential therapeutic agent for septic shock.

Mechanistic extracts of the fruiting body of *T. camphoratus* inhibit COX2, iNOS, and TNF-α production in LPS/IFN-γ-activated microglia (8), suggesting that their anti-inflammatory properties might be attributable to the suppression of ERK, JNK, and NF-κB phosphorylation. Given that ZAA is the major pharmacologically active compound in the fruiting body, this study investigated the anti-inflammatory properties of ZAA. Previous studies have revealed that ZAA inhibits reactive oxygen species production in iMLF- or PMA-activated peripheral human neutrophils (9). Similarly, we demonstrated that ZAA modulates NO production and attenuates the expression of proinflammatory mediators (e.g., iNOS, COX2, TNF-α, IL-6) in activated murine macrophages.

Abs against the TLR4/MD-2 complex have shown efficacy for the treatment of LPS-evoked acute inflammatory conditions (35, 36). Molecules capable of blocking TLR4/MD-2 heterodimer formation and the initiation of inflammation have also been explored recently. For example, eritoran, a synthetic tetra-acetylated lipid A, competes with LPS for the same binding site in MD-2 and impairs the formation of the LPS-activated receptor complex, which sequentially inhibits signal transmission across the plasma membrane (37). Unfortunately, the phase III study of eritoran showed no significant differences between the treatment and placebo groups (38). Recently, natural and synthetic chemicals that are unrelated to the structure of bacterial lipid A have also been reported to be MD-2–directed LPS antagonists (39). The mechanisms of binding of the MD-2 pocket can be divided into three general types: 1) competition for entry into the MD-2 pocket (e.g., bis-ANS [1-anilinonaphthalene 8-sulfonate] and paclitaxel), 2) covalent interaction with the Cys133 residue within the MD-2 pocket (e.g., N-pyrene maleimide, auranofin, JTT-705), and 3) linear alignment at the mouth of the bottom interior portion of the pocket (e.g., JSH, curcumin, xanthohumol, isoxanthohumol). ZAA competes with LPS for entry into the MD-2 pocket; therefore, the therapeutic efficacy of ZAA could possibly be improved by increasing its solubility in the blood, or by enhancing its ability to target the MD-2 pocket via LPS-binding protein recognition.

*Salmonella* infection is associated with bacteremia, typhoid fever, and enteritis in humans. Most studies on *Salmonella* pathogenesis have used the *S. enterica* subsp. *enterica* serovar Typhimurium (*S. typhimurium*) infection model in mice. *S. typhimurium* infection in mice results in a typhoid fever-like disease; it exclusively causes enteritis in humans (40). Since mice infected with *S. typhimurium* do not develop diarrhea, the mouse typhoid model is not a good model for investigating enteritis caused by *Salmonella* infection. On the other hand, *S. choleraesuis* produces both enteritis and bacteremia in swine, which is its natural host, but also causes diseases in humans (41). Moreover, infection of *S. choleraesuis* can lead to bacteremia and death in mice (42, 43). Therefore, mice infected with *S. choleraesuis* can serve as a suitable model of *Salmonella*-induced bacteremia and enteritis. Because we have used an LPS-induced sepsis model to show that pretreatment of ZAA prior to a lethal LPS challenge can improve the survival of mice (Fig. 4E), it is desirable to use *S. choleraesuis* infection model to investigate further the effects of ZAA on the amelioration of enteritis in mice. In this study, we used an attenuated strain of *S. choleraesuis*, which is a vaccine candidate for swine. Infection of mice with this strain leads to diarrhea and body weight loss, making it suitable for investigating the effects of ZAA on *Salmonella* infection. Our results revealed that ZAA pretreatment ameliorates clinical symptoms of *S. choleraesuis*-infected mice in terms of the severity of diarrhea, changes in body weight, and fecal bacterial loads.

In conclusion, we show that ZAA effectively ameliorates the inflammatory responses resulting from LPS- or *S. choleraesuis*–induced experimental endotoxemia, possibly because of its specific interaction with the MD-2 pocket in macrophages. Furthermore, alleviation of inflammation may be attributed to the reduced *Salmonella* levels in the feces. However, ZAA has no direct inhibitory effects on the growth of *Salmonella* (Supplemental Fig. 2). Moreover, our observations strongly support the hypothesis that ZAA can modulate *S. choleraesuis*–induced diarrhea without provoking adverse effects that are common to many antibiotics (i.e., harmful immune responses attributable to the killing of beneficial bacteria). Collectively, this study provides novel insights into the mechanisms of ZAA and its analogs as anti-inflammatory agents for LPS-mediated infections. Additional investigations including the safety issues should be explored.

**Disclosures**

The authors have no financial conflicts of interest.

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