

The Anti-Sepsis Effect of Isocorydine Screened from Guizhou Ethnic Medicine is Closely Related to Upregulation of Vitamin D Receptor Expression and Inhibition of NFκB p65 Translocation into the Nucleus

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Background: The anti-inflammatory application of Guizhou ethnic medicine in the Karst area of China is mainly based on folk medicine experience, and there has been a lack of systematic research, leading to limited application of Guizhou ethnic medicine.

Purpose: To evaluate the anti-inflammatory effects of compounds extracted from Guizhou ethnic medicine in the Karst area and investigate their molecular mechanisms.

Methods and Results: Preliminarily, the anti-inflammatory effects of 181 compounds extracted from Guizhou ethnic medicine were screened in lipopolysaccharide (LPS)-stimulated peritoneal macrophages and the 41 compounds with anti-inflammatory effects were selected. Then, these 41 compounds with anti-inflammatory effects were investigated for their druggability and 18 compounds were selected. Thirdly, compound Hx-150, named isocorydine, was selected as the candidate compound. In vitro and in vivo, isocorydine inhibited LPS-induced TNF-α and IL-6 release from LPS-treated mouse peritoneal macrophages. Isocorydine decreased TNF-α, IL-6, and IL-1β levels in the blood, lung, and spleen, and ameliorated lung tissue damage. Mechanistically, isocorydine had no effect on the mRNA expressions and protein levels of *Tlr4*, *Myd88*, and *Traf6*. Isocorydine also had no effect on the expression of *RelA* (encoding NFκB p65) mRNA, but inhibited phosphorylation of IκBα and NFκB p65 in the TLR4-mediated signaling pathway. Furthermore, isocorydine increased the cytoplasmic level of NFκB p65 and decreased its nuclear level in LPS-treated macrophages. Importantly, isocorydine upregulated *Vdr* mRNA (encoding the vitamin D receptor) expression and increased the nuclear VDR protein level.

Conclusion: Many compounds from Guizhou ethnic medicine had potential anti-inflammatory activities. Among them, isocorydine has a strong anti-sepsis effect, which is tightly related to its upregulation of VDR expression and inhibition of NFκB p65 translocation into the nucleus, leading to reduced pro-inflammatory cytokines release and protection for LPS-challenged mice.

Keywords: Guizhou ethnic medicine, LPS, Sepsis, Pro-inflammatory cytokines, NFκB, VDR

Introduction

Sepsis is a common clinical syndrome, with 48.9 million cases being reported globally; among which 11 million people died from sepsis in 2017, representing almost 20% of all global deaths.¹ Currently, the treatment of sepsis mainly adopts comprehensive therapy, including early and effective antimicrobial treatment, resuscitation (fluid resuscitation,

vasopressors), lung protective ventilation, nutrition and glucose management, molecular targeted sepsis therapies et al.^{2,3} However, there is no approved specific drug for sepsis. Therefore, it is of great significance to investigate effective drugs to treat sepsis. The pathophysiological process of sepsis is very complex and divided into two stages, including the cytokine storm that causes an excessive inflammatory response and immune cell dysfunction causing immunosuppression.² Controlling the early cytokine storm helps to maintain the body's homeostasis. In addition, the sepsis-induced uncontrolled cytokine storm is the main cause of mortality during the earlier stage. Therefore, the search for drugs to control the cytokine storm is of great significance.⁴

Lipopolysaccharide/endotoxin (LPS) is an important pathogenic molecule in the outer membrane of gram-negative bacteria, which induces excessive inflammation in the body, and corresponds to the most common cause of sepsis.^{5,6} Toll-like receptors (TLRs) are termed pathogen-associated molecular patterns (PAMPs) because they recognize various microbial structural components.⁷ LPS is recognized by Toll-like receptor 4 (TLR4), which then activates TNF receptor associated factor 6 (TRAF6) via the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway, which leads to activation of the nuclear transcription factor kappa B p65 (NFκB p65). This results in the production and release of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), thus initiating an inflammatory response.^{8,9} Therefore, focusing on downregulating the cytokine storm and promoting anti-inflammatory activity represents a significant therapy to treat sepsis.

Vitamin D receptor (VDR) is the receptor that recognizes and binds vitamin D₃ (VD₃), and is also a nuclear transcription factor. VDR is involved in multiple physiological functions of the body and plays a role in immune-inflammatory responses.^{10,11} VDR can interact with NFκB p65 in the cytoplasm and block the activation of the NFκB signaling pathway, thereby inhibiting NFκB p65 entry into the nucleus, resulting in reduced expression of proinflammatory cytokines.^{12–14} In addition, VDR, as a negative regulatory molecule, can directly enter the nucleus, leading to reduced proinflammatory cytokine release.¹⁵

More than 150,000 species of plants have been studied worldwide. The World Health Organization (WHO) estimates that 65% of the world's population incorporates traditional medicines (ethnobotanical uses) into their health care.¹⁶ Medicinal plants produce rich substances with biological effects because of their special living environments, and have become an important source of substances to develop new drugs.¹⁷ Studies have found that the active ingredients in medicinal plants are effective in reducing inflammation and have fewer side effects; therefore, increasing numbers of researchers are searching for anti-inflammatory drugs in medicinal plants.^{16,18} The natural environment is complex and diverse, with unique climate and ecological conditions. Among these environments, the Karst region of Guizhou contains abundant natural medicinal plant resources and is one of the four genuine production areas of Chinese medicinal materials. However, the anti-inflammatory application of Guizhou ethnic medicine is mainly based on folk medicine experience, and there is a lack of scientific and systematic research. Therefore, the present study aimed to explore whether there are anti-inflammatory compounds in Guizhou ethnic medicine, and to investigate their potential anti-inflammatory mechanisms, further providing the possibility of identifying drugs to treat sepsis.

Materials and Methods

Animals

Kunming (KM) mice (male, 7–8 weeks old, weighing 35–40 g) were obtained from Laboratory Animal Center of Zunyi Medical University (Zunyi, China). BALB/C mice (male, 6–7 weeks old, weighing 21–23 g) were obtained from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, China). All mice were housed in a pathogen-free facility at the Zunyi Medical University, with a 12 h artificial light–dark cycle and food and purified water provided ad libitum. All protocols and experimental procedures involving live animals were approved by the Animal Care Welfare Committee of Guizhou Medical University (License number: 1,804,102). Animal studies were performed according to National Institute of Health Guide for the Care and Use of Laboratory Animals.

Cell Lines, Culture, and Isolation of Peritoneal Macrophages from Mice

The murine macrophage-like cell line RAW264.7 was purchased from the American Type Culture Collection (Manassas, VA, USA), and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) Fetal

bovine serum (FBS) (Biological Industries, Cromwell, CT, USA) and 1% antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37°C in a humidified 5% CO₂ atmosphere.

Murine peritoneal macrophages were isolated and cultured from male KM mice.^{19,20} Each mouse was injected intraperitoneally with 3 mL of 3% thioglycolate medium (T9032, Sigma-Aldrich, St. Louis, MO, USA) on day 1 and killed using isoflurane on day 3. After each mouse was intraperitoneally injected with 10 mL of normal saline, the peritoneal macrophages were collected by aspiration, suspended in DMEM, added to cell culture dishes, and incubated in a humidified 5% CO₂ incubator for 3 h at 37 °C. Thereafter, the floating cells were removed by washing the cells with phosphate-buffered saline (PBS). The attached cells were considered to be peritoneal macrophages (their purity was about 90%) and were subjected to further experiments. All the reagents and utensils used in the experiment were endotoxin-free.

In vitro Analysis of the Anti-Inflammatory Effect of the Compounds

The 181 compounds extracted from specific medicinal herbs in Karst area were provided by the Hao Research Group of the key laboratory of Chemistry of Natural Products of Guizhou Province and Chinese Academy of Sciences. In the experiments, cells (1.0×10^6 cells/mL) were seeded in 100 µL of medium in 96-well plates. The cells were treated with the various compounds at 52.03 µM for 4 h both in the presence and absence of LPS (50 ng/mL) (*Escherichia coli* O55: B5, Sigma-Aldrich). The culture supernatants were harvested and processed for IL-6 detection using an enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific, Waltham, MA, USA). The compounds with an anti-inflammatory effect (reduced secretion of IL-6) were further confirmed via IL-6 and TNF-α detection using their respective ELISA kits (Thermo Fisher Scientific).

Establishment of LPS-Challenged Mouse Model and Isocorydine Treatment in vivo

For isocorydine treatment in vivo, an LPS-challenged mouse model was established. Isocorydine, purity ≥98%, was purchased from Absin Bioscience Inc. (Shanghai, China). Forty-eight BALB/C mice were divided into six groups (n = 8). The solvent group was given normal saline (0.2 mL/mouse) by intravenous injection and then 2.5% DMSO (0.2 mL/mouse) was given immediately by intraperitoneal injection. The LPS group was given LPS (30 mg/kg) by intravenous injection and then 2.5% DMSO (0.2 mL/mouse) was given immediately by intraperitoneal injection. The positive drug treatment group was given LPS (30 mg/kg) by intravenous injection and then Dexamethasone sodium phosphate injection (DEX, Southwest Pharmaceutical Co. LTD, Chongqing, China) (5 mg/kg) was given immediately by intraperitoneal injection. The isocorydine treatment groups were given LPS (30 mg/kg) by intravenous injection and then three different doses of isocorydine (1.67, 5, and 15 mg/kg) were given, respectively, by intraperitoneal injection at 0 h and 2 h. The mice were killed at 3 h after LPS injection, and then their blood, spleen, and lung tissues were collected.

Serum was collected from the separated blood samples, and one gram of spleen and lung tissues was homogenized (50 Hz, 30s, three times) and centrifuged ($1008 \times g$ for 10 min). The supernatants were collected and the levels of TNF-α, IL-6, and IL-1β in all samples were measured using their respective ELISA kits (Thermo Fisher Scientific).

Druggability Evaluation of Compounds

The medicinal properties of the compounds were evaluated by their anti-inflammatory effect, safe range of concentration, solubility, extraction amount, and Lipinski's rule of five (including a molecule with a molecular mass ≤ 500 Da, hydrogen bond donors ≤ 5, hydrogen bond acceptors ≤ 10, partition coefficient (log P) value ≤ 5, and rotatable bonds ≤ 10).^{21,22} The solubility and Lipinski's rules of compounds were obtained via referring their chemical structure in the Scifinder database (<https://www.cas.org/solutions/cas-scifinder-discovery-platform/cas-scifinder>). The partition coefficient (log P) value of some compounds was predicted using ChemDraw software 19.0 (Perkin-Elmer, Waltham, MA, USA). The extraction amount was provided by the Hao Research Group of the key laboratory of Chemistry of Natural Products of Guizhou Province and Chinese Academy of Sciences.

Quantitative Real-Time Reverse Transcription-PCR (qRT-PCR) Assays of Important Genes

Peritoneal macrophages (1.0×10^6 cells/mL) were seeded in 1 mL of medium in 12-well plates. The cells were treated with isocorydine (52.03 μ M) for 2 h, both in the presence and absence of LPS (50 ng/mL). The culture supernatants or cells were harvested and processed for further analyses. Total RNAs were extracted from treated peritoneal macrophages using RNeasy mini kits (Takara, Shiga, Japan), and reverse transcribed into cDNAs using PrimeScript™ RT Reagent Kit (Takara). The primers were purchased from Sangon Biotech (Shanghai, China) (Table 1). The cDNA was used as the template in a 15 μ L quantitative real-time PCR (qPCR) reaction (including 7.5 μ L of iQTM SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 0.5 μ L of primer, 4 μ L of DEPC-H₂O, and 3 μ L of cDNA), which was performed using the CFX Connect Real-Time System (Bio-Rad). The average expression levels of the genes were normalized to the expression of *Actb* mRNA (encoding β -actin). The relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method,²³ where C_t is the cycle threshold value.

Western Blotting (WB) Assays of Important Proteins

Peritoneal macrophages (1.0×10^6 cells/mL) were seeded in 2 mL of medium in 6-well plates. The cells were treated with isocorydine (52.03 μ M) for 2 h, both in the presence and absence of LPS (50 ng/mL). The culture supernatants or cells were harvested and processed for further analyses. Cell proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors (Solarbio, Beijing, China). The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Solarbio). The protein samples were separated by electrophoresis using 10% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with 5% non-fat powdered milk for 2 h at room temperature, then incubated with primary antibodies (Supplementary Table 1) overnight at 4°C (1:1000). The membranes were washed three times with TBS containing Tween 20 and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature (1:1000). Membranes were washed three times with TBS containing Tween 20, and then developed using the ECL chemiluminescent substrate (Meilunbio, Dalian, China) with a ChemiDoc™ Touch Imaging System (Bio-Rad). Then band intensities were analyzed using the Image Lab software (Bio-Rad).

ELISA of NF κ B p65 and Vitamin D Receptor Proteins

Peritoneal macrophages (1.0×10^6 cells/mL) were seeded in 2 mL of medium in 6-well plates. The cells were treated with isocorydine (52.03 μ M) in the presence and absence of LPS (50 ng/mL). After various times of incubation, the cells were processed for further analyses. The proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China), and the protein concentration was determined using a BCA protein assay kit (Solarbio). For the NF κ B p65 and Vitamin D receptor (VDR) protein levels in the nucleus and cytoplasm, proteins were detected using a mouse NF κ B p65 ELISA kit (Cell Signaling Technology (CST), Danvers, MA, USA) and a mouse VDR ELISA kit (FineTest, Wuhan, China), respectively.

Table 1 Primer Sequences Used for qRT-PCR

Genes	Species	Forward Primer	Reverse Primer
<i>Tlr4</i>	Mouse	aatccctgcatagaggtagttc	caaggggttgaagctcagat
<i>Myd88</i>	Mouse	ttgccaaggctttgtccctgcc	tgcatagcctgtgtgcca
<i>Traf6</i>	Mouse	accttgctttgctccgtgc	cgtttgagctgcccacgtaca
<i>RelA</i>	Mouse	ctctataggaactatggatactcgg	caccaaggatccacctcacc
<i>Vdr</i>	Mouse	gaatgtgcctcggatctgtgg	atgctggcaatctccattgaag
<i>Actb</i>	Mouse	agggtgtaaacgcagctca	gatcaagatcattgctcctcctg

Analysis of Cell Viability

Peritoneal macrophages or RAW264.7 cells (1.0×10^5 cells per well) was seeded in 96-well plates in regular medium. After 2 h, the peritoneal macrophages were incubated with the compounds (at 0 and 52.03 μM) for 4 h or were treated with the various concentrations of the compounds (0, 52, 104, 208, and 416 μM) for 4 h. In addition, the peritoneal macrophages were incubated with isocorydine (0 and 52.03 μM) for 24 h and RAW264.7 cells were incubated with isocorydine (0 and 52.03 μM) for 24 h and 48 h. Herein, isocorydine was extracted from *Aconitum apetalum* (Huth) B. Fedtsch, with a purity of not less than 98% and was provided by the Hao Research Group of the key laboratory of Chemistry of Natural Products of Guizhou Province and Chinese Academy of Sciences. In addition, our cooperation partner provided a high-performance liquid chromatography (HPLC) chromatogram of isocorydine used for early activity screening ([Supplementary Figure 1](#)). We also purchased commercial isocorydine from Absin Bioscience Inc (Shanghai, China); its purity was $\geq 98\%$. The HPLC chromatogram of commercial isocorydine is shown in [Supplementary Figure 2](#). As can be seen from [Supplementary Figure 1](#) and [Supplementary Figure 2](#), the mass spectrum behavior of the two compounds is the same. Then, 100 μL of diluted Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) solution was added to each well of the 96-well plates and incubated in a humidified 5% CO_2 incubator for 1 h at 37 $^\circ\text{C}$. Finally, the absorbance at 450 nm of the cells in the 96-well plates was recorded using a plate reader (Thermo Fisher Scientific).

Immunofluorescence Assay of NF κ B p65

Peritoneal macrophages (1.0×10^6 cells/mL) were seeded in 0.3 mL of medium in 24-well plates. The cells were treated with isocorydine (52.03 μM) for 2 h both in the presence and absence of LPS (50 ng/mL). The cells were then processed for further analyses. The cells on coverslips were fixed using 4% paraformaldehyde for 20 min and permeabilized using 0.3% Triton X-100 (Solarbio) in PBS for 20 min. Then, the cells on the coverslips were blocked for 1 h at 37 $^\circ\text{C}$ in PBS containing 3% bovine serum albumin (BSA) and incubated with primary antibodies recognizing NF κ B p65 (CST, #8242) at 4 $^\circ\text{C}$ overnight. Next day, the cells were incubated with FITC Goat anti-Rabbit IgG (BA1105, Boster, Wuhan, China) and counterstained with 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI) (Solarbio). Images were captured using a Zeiss LSM900 confocal microscope (Zeiss, Oberkochen, Germany) with a Plan-Apo Chromat 63 \times /1.40 oil objective.

Histological Examination of Lung Tissues

The harvested left lung tissues were fixed in 4% paraformaldehyde solution, embedded in paraffin, and cut into sections. The sections were stained using hematoxylin–eosin (H&E) by an ST5010 AutoStainer (Leica, Nussloch, Germany). Finally, the changes in pulmonary histopathology were observed using BX43 light microscopy (Olympus, Tokyo, Japan).

Statistical Analysis

Descriptive and analytical statistical analyses of all data were performed using GraphPad Prism 8.0.1 (244) software package (GraphPad Inc., La Jolla, CA, USA). Statistical analysis between groups was determined using a two-tailed Student's *t*-test. Each value was repeated at least three times. All results are expressed as the means \pm standard deviation (SD). For each parameter of the data presented, $p < 0.05$ indicates the statistical significance.

Results

One Hundred and Eighty-One Compounds Were Preliminarily Screened for Their Anti-Inflammatory Effect

IL-6 is a very important pro-inflammatory cytokine that plays a key role in LPS-induced early inflammation.²⁴ Therefore, IL-6 was selected as key marker to evaluate the effects of the compounds on LPS-induced inflammation. The effects of 181 compounds (at 52.03 μM) on IL-6 release from LPS-treated murine peritoneal macrophages were preliminarily screened. The results showed that the level of IL-6 in the untreated group and the groups treated with the compounds only was very low. Among 181 compounds ([Supplementary Table 2](#)), 41 compounds could inhibit IL-6 release from

Table 2 The 41 Compounds That Inhibited IL-6 Release from LPS-Treated Peritoneal Macrophages

Compounds	Inhibition	Inhibition Range (%)	Cell Viability Range (%)
Hx-127, Hx-92, Hx-44	+	60.0–70.0	107.0–117.1
Hx-91, Hx-89, Hx-176, Hx-177	+	50.0–60.0	100.6–116.4
Hx-24, Hx-76, Hx-86, Hx-42, Hx-105, Hx-74, Hx-150	+	40.0–50.0	100.8–112.0
Hx-118, Hx-50, Hx-77, Hx-25, Hx-16, Hx-22, Hx-15, Hx-10, Hx-23	+	20.0–40.0	96.7–128.4
Hx-124, Hx-182	±	50.0–60.0	101.2–109.9
Hx-67, Hx-69, Hx-129, Hx-112, Hx-71	±	40.0–50.0	100.6–113.8
Hx-99, Hx-82, Hx-80, Hx-100, Hx-109, Hx-138, Hx-31, Hx-113	±	30.0–40.0	99.2–109.3
Hx-119, Hx-32, Hx-168	±	10.0–30.0	102.0–116.3

Notes: Peritoneal macrophages were treated with different compounds (52.03 μ M) for 4 h both in the presence and absence of LPS (50 ng/mL). The compounds with anti-inflammatory effects were determined by testing their effect to inhibit LPS-induced IL-6 release ($n = 3$). + indicates a significant anti-inflammatory effect; \pm indicates an unstable anti-inflammatory effect.

LPS-treated cells (Table 2). Importantly, at a concentration of 52.03 μ M, the compounds had no influence on the viability of the peritoneal macrophages (Table 2).

Forty-One Compounds Were Confirmed to Have an Anti-Inflammatory Effect

TNF- α is a small molecule protein that is mainly generated by activated macrophages, T-lymphocytes, and natural killer cells. It has been identified as a major regulator of inflammatory responses and is involved in physiological and pathological processes, such as inflammatory response and cellular immunity.²⁵ To confirm the anti-inflammatory effect of the 41 compounds selected from the preliminary screening, their effect on both IL-6 and TNF- α release from LPS-treated murine peritoneal macrophages were observed. The results showed that 18 out of 41 compounds significantly inhibited LPS-induced IL-6 and TNF- α release (Figure 1A-E).

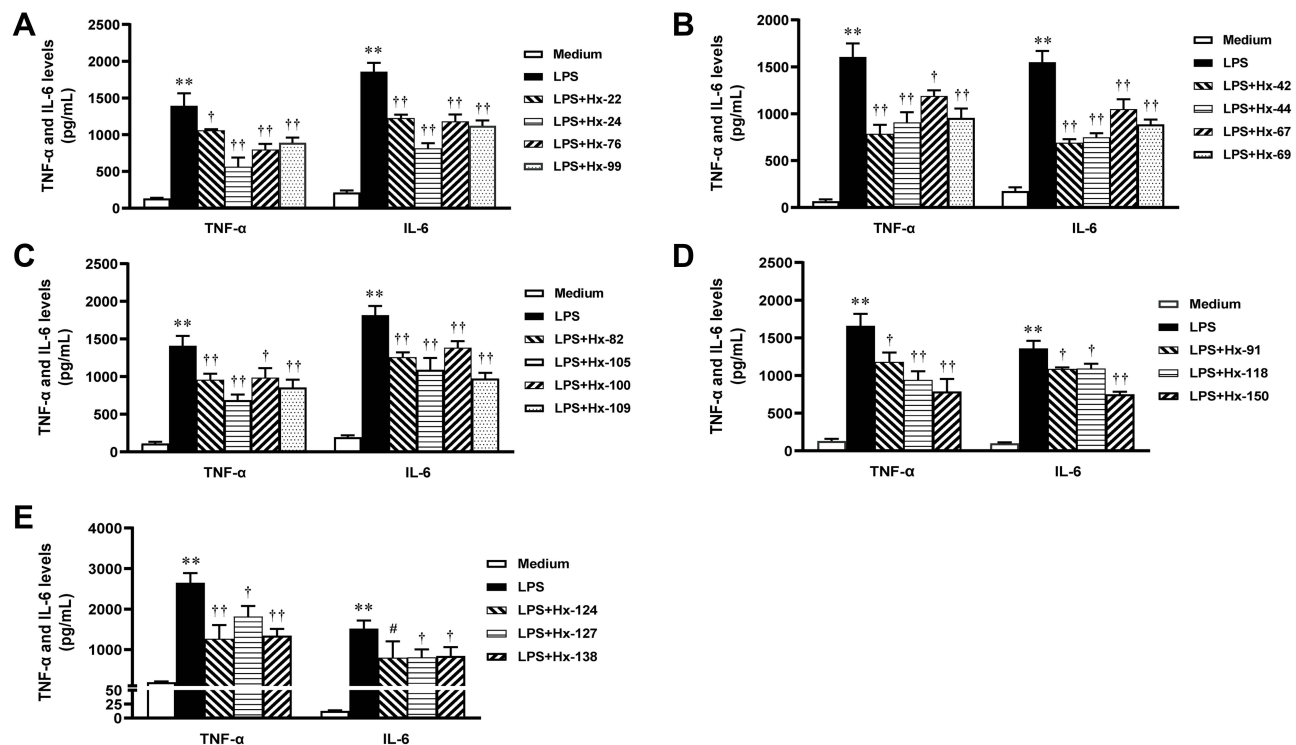


Figure 1 The compounds inhibit TNF- α and IL-6 release from LPS-treated peritoneal macrophages ($n = 3$). Peritoneal macrophages were treated with different compounds (52.03 μ M) and LPS (50 ng/mL) for 4 h. The levels of TNF- α and IL-6 in the cell culture supernatant were measured using respective ELISA kits (A-E). ** $p < 0.01$ vs Medium; † $p < 0.05$, †† $p < 0.01$, # $p > 0.05$ vs 50 ng/mL LPS.

Compound Hx-150 Was Selected as Candidate Compound After Preliminary Evaluation of Its Druggability

The activity, safety, and target of a compounds are the key factors to determine whether a compound becomes a drug. Lipinski's Rule of Five is an experimental and computational methodology that is used to estimate the solubility, membrane permeability, and efficacy in the drug-development setting.²⁶ Lipinski's Rule of Five is based on the chemical and physical properties of more than two thousand reference medicines, such that the druggability of compounds can be evaluated.²⁶ In addition, the solubility and accessibility of the compounds are also necessary to search for promising drugs. Herein, the Scifinder database was used to search the compound structure to assess Lipinski's Rule of Five (including molecular mass, hydrogen bond donors, hydrogen bond acceptors, partition coefficient (log P) value, and rotatable bonds), and the Mass Intrinsic Solubility of the compounds. In addition, ChemDraw software 19.0 was used to predict the log P of Hx-42, Hx-67, Hx-82, Hx-91, and Hx-100 (Table 3). The accessibility was provided by the Hao Research Group of the key laboratory of Chemistry of Natural Products of Guizhou Province and Chinese Academy of Sciences. Subsequently, the anti-inflammatory effect, safety, Lipinski's Rule of Five, solubility, and accessibility of the compounds were evaluated synthetically. The results showed that compound Hx-150 had a good anti-inflammatory effect, high safety, conformed to Lipinski's Rule of Five, and had easy accessibility, ranking first in our comprehensive evaluation (Table 4). Therefore, compound Hx-150 was identified as having a certain research value and was selected as a candidate compound for further investigation in subsequent experiments.

Isocorydine Inhibits Pro-Inflammatory Cytokine Release from LPS-Treated Macrophages Without Affecting Cell Viability

Compound Hx-150, called isocorydine, is an aporphine alkaloid with several pharmacological effects (Figure 2), and is widely present in many plants.^{27,30}

Dexamethasone (DEX) is a glucocorticoid that has been widely used to treat inflammatory-related diseases in the clinic.³¹ Therefore, DEX (100 ng/mL) was selected as a positive control drug in the present study, and the anti-inflammatory effect of

Table 3 Scifinder Database Referring to Lipinski's Rule of Five and Mass Intrinsic Solubility of the Compounds

Compounds	Molecular Mass (Da)	Log P	Hydrogen Bond Acceptors	Hydrogen Bond Donors	Rotatable Bonds	Mass Intrinsic Solubility (g/L)
Hx-22	454.7	7.6 ± 0.6	3	1	1	1.4 × 10 ⁻³
Hx-24	258.4	5.0 ± 0.3	1	0	2	2.6 × 10 ⁻³
Hx-42	270.0	3.5	2	1	3	2.1 × 10 ⁻¹
Hx-44	362.3	3.9 ± 0.5	2	1	3	2.1 × 10 ⁻¹
Hx-67	432.0	6.1	4	1	7	4.7 × 10 ⁻³
Hx-69	550.0	7.9 ± 0.5	9	0	10	3.8 × 10 ⁻⁵
Hx-76	510.6	4.0 ± 0.5	7	4	9	3.3 × 10 ⁻³
Hx-82	387.0	6.0	4	2	4	2.2 × 10 ⁻²
Hx-91	334.0	2.5	4	0	3	2.4 × 10 ⁻¹
Hx-99	422.5	1.5 ± 0.7	7	3	7	3.8
Hx-100	334.0	2.5	4	0	3	2.4 × 10 ⁻¹
Hx-105	344.5	5.4 ± 0.6	2	0	4	2.2 × 10 ⁻²
Hx-109	306.2	1.7 ± 1.5	8	3	5	6.1 × 10 ⁻²
Hx-118	300.3	4.6 ± 0.9	6	2	4	2.0 × 10 ⁻⁴
Hx-124	234.3	3.5 ± 0.5	2	1	3	4.5 × 10 ⁻¹
Hx-127	218.3	5.0 ± 0.3	1	0	1	4.1 × 10 ⁻²
Hx-138	292.4	3.8 ± 0.4	4	1	3	3.8 × 10 ⁻²
Hx-150	341.4	1.4 ± 0.4	5	1	4	6.5 × 10 ⁻¹

Notes: The data of Lipinski's rule of five and Mass Intrinsic Solubility of compounds were obtained from the Scifinder database. Lipinski's rule of five: molecular mass ≤ 500 Da, hydrogen bond donors ≤ 5, hydrogen bond acceptors ≤ 10, partition coefficient (log P) value ≤ 5, and rotatable bonds ≤ 10. ChemDraw software 19.0 was used to forecast the log P of Hx-42, Hx-67, Hx-82, Hx-91, and Hx-100.

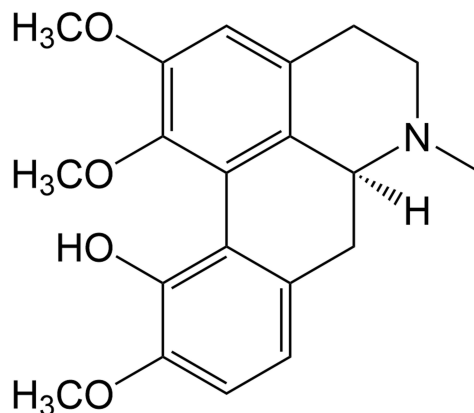
Table 4 Druggability Evaluation Table of 18 Active Compounds

Compounds	Activity	Cytotoxicity	Rule of Five	Intrinsic Solubility	Accessibility	Total Points	Integrated Rank
Hx-150	3	2	2	1	2	2.35	1
Hx-124	3	2	2	1	1	2.3	2
Hx-138	2	2	2	2	1	1.95	3
Hx-42	3	1	2	2	0	1.9	4
Hx-44	3	1	2	1	0	1.85	5
Hx-118	2	2	1	1	1	1.8	6
Hx-24	3	1	1	1	0	1.75	7
Hx-127	2	1	1	1	2	1.45	8
Hx-109	2	0	2	3	0	1.15	9
Hx-99	2	0	1	2	2	1.1	10
Hx-105	2	0	1	2	0	1	11
Hx-76	2	0	1	1	0	0.95	12
Hx-69	2	0	1	1	0	0.95	13
Hx-100	1	0	2	2	1	0.75	14
Hx-91	1	0	2	1	1	0.7	15
Hx-82	1	0	1	2	0	0.6	16
Hx-22	1	0	1	1	0	0.55	17
Hx-67	1	0	1	1	0	0.55	18

Note: Activity and cytotoxicity each accounted for 40%, rule of five accounted for 10%, solubility and accessibility accounted each for 5%. "3" indicated as high activity and slightly soluble; "2" indicated as general activity, wide range of safety, easy accessibility, congruent Lipinski's rule of five, and very slightly soluble. "1" indicated as poor activity, narrow range of safety, difficult accessibility, incongruent Lipinski's rule of five, and practically insoluble or insoluble; "0" indicated as no data.

isocorydine (52.03 μM) in vitro was reevaluated. The results demonstrated that isocorydine significantly inhibited LPS-induced TNF- α and IL-6 release, with an inhibition rate of 29.4% compared a rate of 50% by DEX (Figure 3A and B).

Meanwhile, cell viability experiments were carried out to determine whether the concentrations of isocorydine used in this study affected the viability of murine peritoneal macrophages and RAW264.7 cells. The results of CCK-8 assays showed that isocorydine (0, 52, 104, 208, and 416 μM) for 4 h had no effect on the viability of murine peritoneal macrophages (Figure 3C). Furthermore, the peritoneal macrophages were treated with isocorydine of 52.03 μM for 24 and RAW264.7 cells were treated with isocorydine of 52.03 μM for 24 h and 48 h, the results showed that there was no effect on the viability of murine peritoneal macrophages and RAW264.7 cells during long-term treatment (Figure 3D and E), indicating that its anti-inflammatory activity was not related to inhibition of cell activity, and it had better safety.

**Figure 2** The chemical structure of isocorydine.

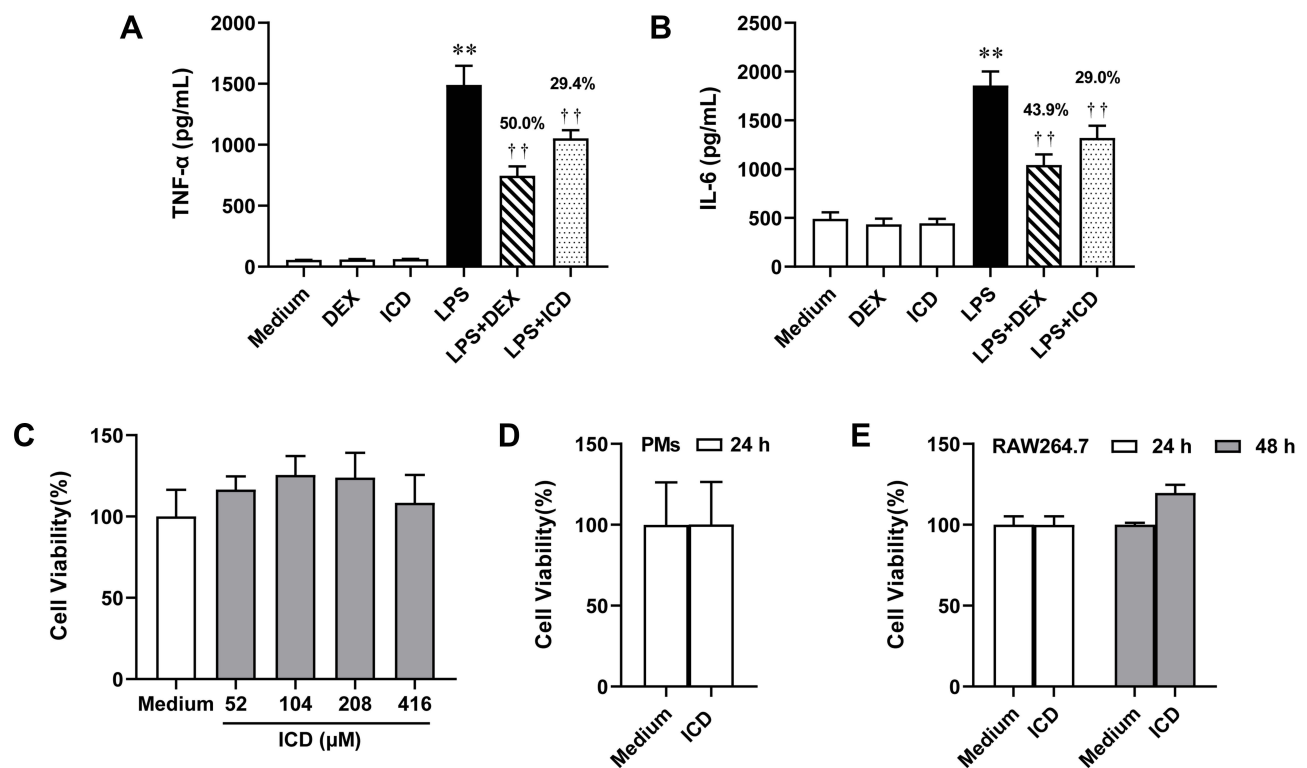


Figure 3 Isocorydine inhibits TNF- α and IL-6 release from LPS-treated peritoneal macrophages. Peritoneal macrophages were treated with isocorydine (ICD) (52.03 μ M) and LPS (50 ng/mL) for 4 h. The levels of TNF- α (A) and IL-6 (B) in the peritoneal macrophages cell culture supernatant were measured using respective ELISA kits (n = 3). Peritoneal macrophages were treated with ICD (0, 52, 104, 208, and 416 μ M) for 4 h, and the viability of the peritoneal macrophages was assessed (n = 3) (C). Peritoneal macrophages or RAW264.7 cells were treated with ICD at 52.03 μ M for 24 h or 48 h, and the viability of the peritoneal macrophages (n = 3) (D) and RAW264.7 (n = 4) (E) was determined using the CCK-8 assay. ***p* < 0.01 vs Medium; ††*p* < 0.01 vs 50 ng/mL LPS.

Isocorydine Significantly Decreases Pro-Inflammatory Cytokine Levels in LPS-Challenged Mice and Ameliorates Lung Tissue Damage

An LPS-challenged mouse model is the most commonly used sepsis model and is widely employed in the preliminary screening and evaluation of anti-inflammatory drugs.^{32,33} To determine isocorydine's anti-inflammatory effect *in vivo*, isocorydine was further investigated in LPS-challenged mice. The results showed that the levels of TNF- α , IL-6, and IL-1 β increased significantly in the serum, spleen, and lungs of the LPS-challenged mice, and DEX very significantly decreased the levels of these three pro-inflammatory cytokines, whereas 2.5% DMSO had no effect on the levels of the pro-inflammatory cytokines. In contrast, among the three doses of isocorydine (1.67, 5, and 15 mg/kg), the highest dose had best effect in terms of decreasing the levels of pro-inflammatory cytokines and there was no difference between isocorydine (15 mg/kg) and DEX (5 mg/kg) (*p* > 0.05), demonstrating that isocorydine possesses a strong anti-inflammatory effect (Figure 4A-C).

Lung tissue was the most severely damaged tissue in the LPS-challenged mice; therefore, histological changes in lung tissue were examined. The results showed substantial morphological alterations, including pulmonary alveoli and interstitium edema, pulmonary capillary hemorrhage, and alveolar collapse in the LPS-challenged mice, whereas isocorydine treatment markedly ameliorated these pathological changes and the structure of lung tissue recovered significantly (Figure 4D).

Isocorydine Inhibits Nuclear Translocation of NF κ B p65, but Has No Effect on the mRNA Expression and Protein Level of Important Molecules in the TLR4-Mediated Signaling Pathway

LPS induces the release of cytokines by inducing activation of the TLR4/MyD88/TRAF6/NF κ B pathway.³⁴ Thus, the expression levels of mRNAs encoding key molecules such as TLR4, MyD88, TRAF6, and NF κ B of the LPS/TLR4

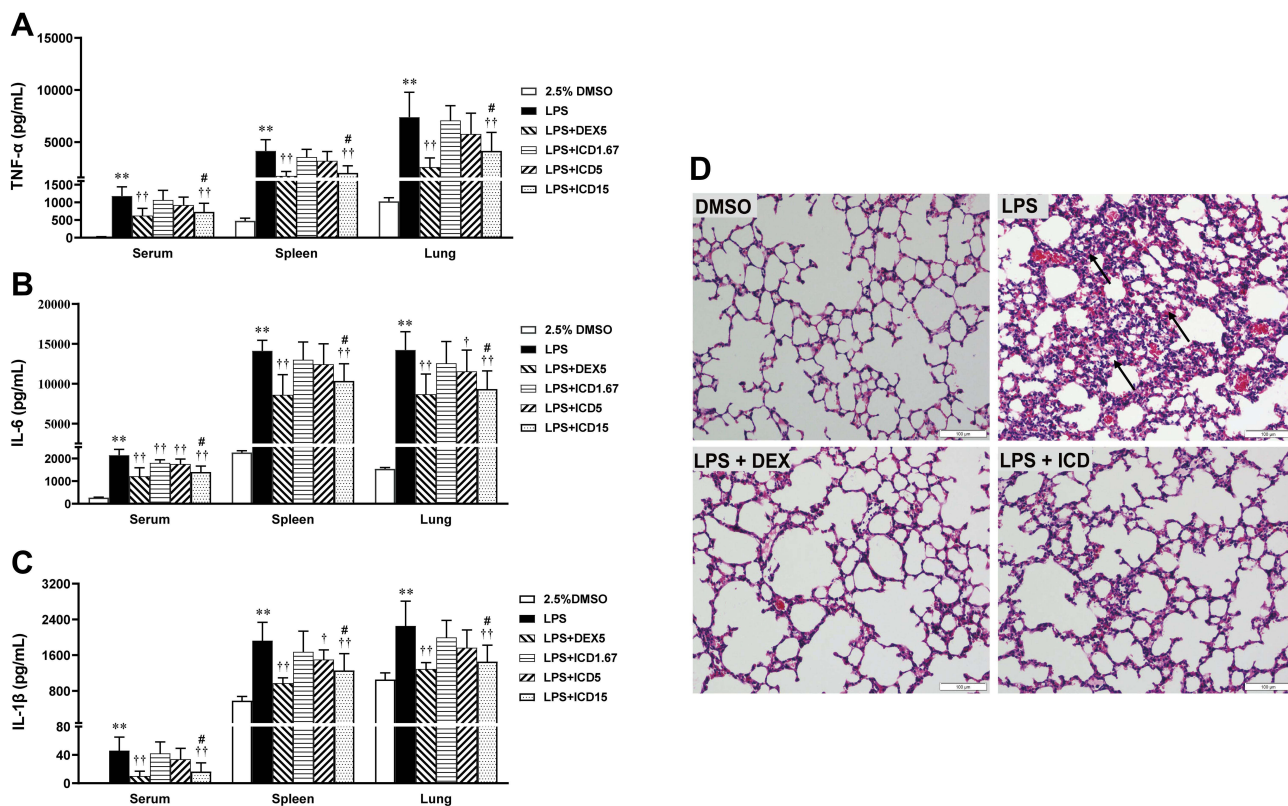


Figure 4 Isocorydine exerts an anti-inflammatory effect in LPS-challenged mice and changes in lung tissue ($n = 8$). Serum, spleen, and lung samples were collected from LPS-challenged mice at 3 h after isocorydine (ICD, 1.67, 5 and 15 mg/kg) injections. The levels of TNF- α (A), IL-6 (B), and IL-1 β (C) were determined in the serum, spleen, and lung using respective ELISA kits ($n = 8$). Lung histological changes were observed at 3 h after LPS injection. Representative images of hematoxylin and eosin-stained sections of lung tissues from the 2.5% DMSO, LPS, LPS + DEX and LPS + ICD groups are shown (D), indicating that treatment with ICD strongly ameliorates LPS-challenged lung edema, hemorrhage, and alveolar collapse. Bar = 100 μm ** $p < 0.01$ vs 2.5% DMSO; † $p < 0.05$, †† $p < 0.01$ vs 30 mg/kg LPS; # $p > 0.05$ vs 5 mg/kg DEX.

signaling pathway were assayed using qRT-PCR. The results showed that isocorydine had no significant influence on the mRNA expression levels of these proteins in LPS-treated macrophages (Figure 5A-D). Furthermore, the protein levels of these key molecules were assayed using Western blotting. The results showed that isocorydine had no significant influence on the protein levels of TLR4, MyD88, TRAF6, but significantly reduced the level of phosphorylated I κ B α and NF κ B p65 in LPS-treated macrophages (Figure 5E and F). Moreover, we observed that the NF κ B p65 level in LPS-treated macrophages increased in the nucleus, but decreased in the cytoplasm, whereas isocorydine + LPS treatment maintained the cytoplasmic level of NF κ B p65, but reduced its nuclear level (Figure 5G-I), suggesting that isocorydine inhibits NF κ B p65 translocation from the cytoplasm into the nucleus, but has no effect on *RelA* (NF κ B p65) mRNA expression.

To further confirm our speculation, the influence of isocorydine on NF κ B p65 distribution within cells was observed using laser scanning confocal microscopy. The results showed that isocorydine increased the distribution of NF κ B p65 in the cytoplasm and decreased its distribution in the nucleus (Figure 5J and K), which was consistent with protein level determination. Therefore, the results demonstrated that the isocorydine-induced inhibition of LPS-induced pro-inflammatory cytokine release is closely related to its inhibition of NF κ B p65 translocation into the nucleus.

Isocorydine Upregulates VDR Expression and Increases the VDR Protein Level in the Nucleus

VDR is a nuclear transcription factor,¹¹ and the results from our laboratory and previous studies indicated that VDR can block the canonical NF κ B activation pathway by interacting with NF κ B p65 in the cytoplasm to inhibit NF κ B p65

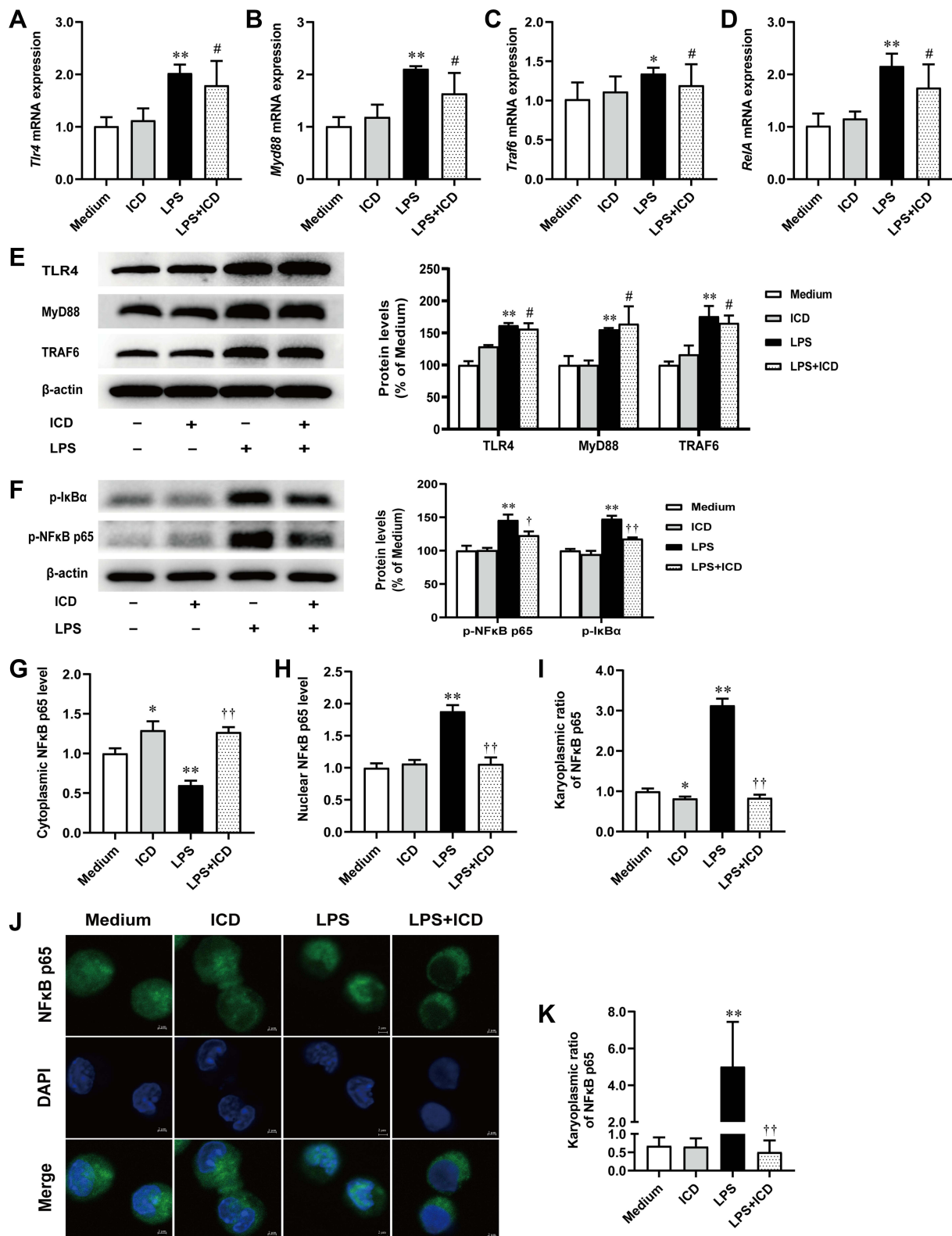


Figure 5 Isocorydine inhibits the nuclear translocation of NFκB p65. Peritoneal macrophages were treated with isocorydine (ICD, 52.03 μM) and with LPS (50 ng/mL) for 2 h. The mRNA expression levels of *Tlr4* (A), *Myd88* (B), *Traf6* (C) and *RelA* (D) were detected using qRT-PCR (n = 4). The protein levels of TLR, MyD88, and TRAF6 were detected using Western blotting (E) (n = 3). The protein levels of p-IκBα and p-NFκB p65 were detected using Western blotting (F) (n = 3). The NFκB p65 levels in the cytoplasmic (G) and nuclear (H) lysates were detected using ELISA kits (n = 3). The karyoplasmic ratio of NFκB p65 levels in the nucleus and cytoplasm was calculated (I). Meanwhile, the nuclear translocation of NFκB p65 was observed using immunofluorescence (J). NFκB p65 was probed using FITC (green). Representative images (Bar = 2 μm). The karyoplasmic ratio of NFκB p65 was quantified from 50 cells (K). **p* < 0.05, ***p* < 0.01 vs Medium; †*p* < 0.05, ††*p* < 0.01, #*p* > 0.05 vs 50 ng/mL LPS.

translocation into the nucleus.^{13,35} Reasonably, we speculated whether decreased NFκB p65 translocation into nucleus was related to upregulation of VDR. Therefore, the influence of isocorydine on *Vdr* mRNA and protein distribution was investigated.

Firstly, the results showed that *Vdr* mRNA expression was significantly upregulated in LPS-treated macrophages and was further upregulated by isocorydine treatment (Figure 6A). Secondly, the results from the quantitative analysis of VDR protein in the cytoplasm and nucleus showed that isocorydine could significantly increase the VDR protein level in the nucleus (Figure 6B), but did not significantly influence the VDR protein level in the cytoplasm (Figure 6C). These findings demonstrated that isocorydine increased the *Vdr* mRNA expression and VDR protein level in the nucleus.

Discussion

Our results demonstrated that there are many compounds with anti-inflammatory effects in Guizhou ethnic medicine from the Karst area in Guizhou, China. Among them, isocorydine was found to possess a marked anti-inflammatory effect, could decrease the release of pro-inflammatory cytokines, and ameliorated lung tissue damage in LPS-challenged mice. These effects are closely related to upregulation of *Vdr* mRNA expression and inhibition of NFκB p65 translocation into the nucleus.

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to bacterial, viral, fungal, and parasitic infection.³⁶ However, there is no specific and effective drugs that target sepsis. The pathophysiological process of sepsis is divided into two stages: the early stage of sepsis is mainly an excessive inflammatory response caused by a cytokine storm, and the later stage mainly comprises immune cell dysfunction causing immunosuppression.² The uncontrolled cytokine storm is the main cause of mortality in sepsis, similar to that in COVID-19. To date, there is no effective and safe drug to control the cytokine storm in sepsis and/or to ameliorate the resulting multiple organ dysfunction syndrome.³⁷ Therefore, it is important to screen drugs to control the cytokine storm of sepsis.

Many Guizhou ethnic medicines from the Karst area^{38,39} are used to treat inflammation. However, the identity of the anti-inflammatory compounds in Guizhou ethnic medicine has not been systematically investigated. Herein, we showed that 41 out of 181 compounds from Guizhou ethnic medicine had anti-inflammatory effects.

Isocorydine is an aporphine alkaloid that is widely present in many plants, such as Annonaceae, Fumariaceae, Lauraceae, Menispermaceae, Monimiaceae, Papaveraceae, and Ranunculaceae.²⁷ Investigations have reported that isocorydine has several pharmacological effects, such anti-arrhythmia, vasodilation, antitumor, and anti-inflammatory effects.^{28–30} In addition, isocorydine was reported to inhibit the release of pro-inflammatory cytokines from LPS-induced RAW264.7 cells;⁴⁰ however, whether it has anti-inflammatory effects in vivo has not been reported. Here, we investigated the anti-inflammatory effects of isocorydine in vitro and in vivo. Studies have reported that the half-life of isocorydine is short ($t_{1/2}=2.09 \pm 0.457$ h).⁴¹ Therefore, three different doses of isocorydine were given twice after 0 h and

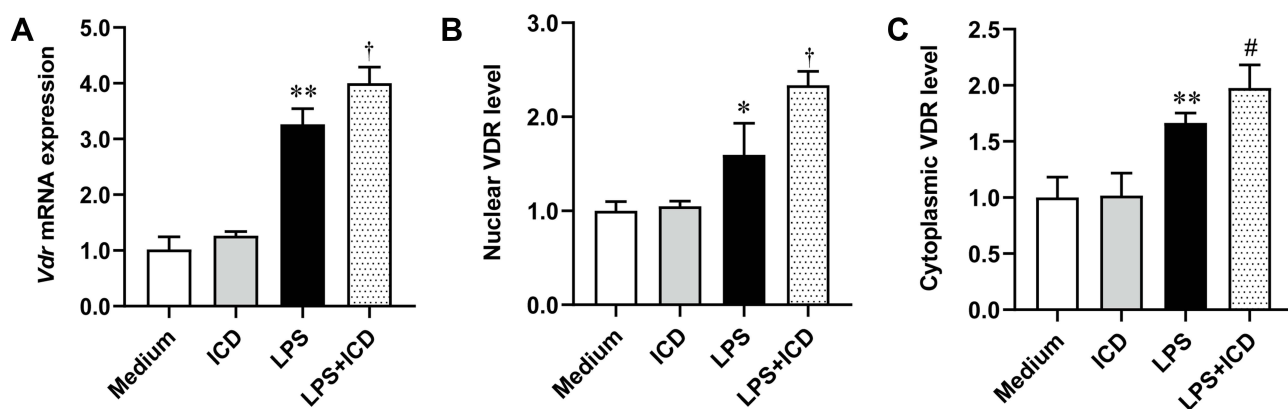


Figure 6 Isocorydine upregulates *Vdr* mRNA expression and changes the VDR distribution within cells. Peritoneal macrophages were treated with isocorydine (ICD, 52.03 μM) and with LPS (50 ng/mL) for 4 h (n = 4). The expression of *Vdr* mRNA in LPS-treated peritoneal macrophages was measured using qRT-PCR (A). The levels of VDR in the nucleus (B) and cytoplasm (C) were assayed using respective ELISA kits (n = 3). * $p < 0.05$, ** $p < 0.01$ vs Medium; † $p < 0.05$, # $p > 0.05$ vs 50 ng/mL LPS.

2 h of modeling to achieve an effective blood concentration. Moreover, in our previous studies, we studied the multi-organ function of mice during endotoxin challenge and in the cecum ligation and puncture (CLP) model. The results showed that the pathological changes of spleen and lung were the most obvious, and the changes of pro-inflammatory cytokines were the most significant.⁴² Therefore, serum, spleen, and lung tissues of mice were selected as samples in this investigation.

Herein, our results showed that isocorydine inhibited not only TNF- α and IL-6 release from LPS-treated peritoneal macrophages, but also decreased TNF- α , IL-6 and IL-1 β levels in LPS challenged mice, a widely used sepsis model, demonstrating that isocorydine has a strong anti-inflammatory effect and is worthy of further investigation.

LPS is an important pathogen-derived molecule that is involved in the pathogenesis of sepsis.⁴³ LPS is recognized by TLR4, an important pattern-recognition receptor (PRR) of macrophages.⁷ This recognition results in a conformational change of the receptor, which leads to recruitment of intracellular adaptor proteins, such as MyD88 and TIR domain - containing adaptor protein inducing interferon (IFN)- β (TRIF). Once recruited, these adaptors activate separately a series of signal transduction molecules including interleukin (IL)-1R-associated kinases (IRAKs) and transforming growth factor (TGF)- β -activated kinase (TAK1). Subsequently, the activation of IKK- α /IKK- β leads to the phosphorylation and degradation of I κ B α . Ultimately, these events lead to activation of NF κ B and interferon regulatory factor (IRF3). These transcription factors upregulate the expression of various oncogenes and pro-inflammatory cytokines.⁴⁴ In both MyD88-dependent and independent pathways, NF κ B p65 is phosphorylated in the cytoplasm and then translocated into nucleus, which is the most important step. The results of the present study showed that isocorydine had no effect on the mRNA expression and protein levels of *Tlr4*, *Myd88*, *Traf6*, and isocorydine also had no effect on mRNA expression of *RelA* (encoding NF κ B p65), but inhibited the phosphorylation of I κ B α and NF κ B p65 in the TLR4-mediated signaling pathway. Furthermore, isocorydine increased the NF κ B p65 level in cytoplasm and decreased the level in the nucleus of LPS-treated macrophages, suggesting that the anti-inflammatory effect of isocorydine is closely related to inhibition of NF κ B p65 translocation from the cytoplasm into the nucleus, but not decreased NF κ B p65 expression.

VDR is a member of nuclear receptor superfamily, and recognizes VD₃, an intrinsically active metabolite of vitamin D, thus mainly mediating the biological function of VD₃ in the body.⁴⁵ VDR is present in almost all nucleated cells. After binding to VDR in the body, VD₃ is widely involved in regulating cell proliferation, differentiation, inflammation, immune response, as well as inhibiting tumors.¹¹ VDR plays an important role in the anti-inflammatory response: the action of VDR-mediated VD₃ is involved in the process of inhibiting inflammation (ie, participating in anti-inflammatory effects), during which *Vdr* mRNA shows high expression.⁴⁶ VDR exerts its anti-inflammatory functions directly and indirectly: It directly regulates the transcription of downstream inflammation-related target genes via binding to their promoters, such as *ATG16L1* (encoding autophagy related 16 like 1) and those encoding many cytokines.¹⁵ Other studies reported that VDR mediates NF κ B signaling pathways, playing an important role in inhibiting inflammation. VD₃ plays a role by negatively regulating NF κ B through VDR. VDR can block the canonical NF κ B activation pathway by interacting with NF κ B p65 in cytoplasm and then inhibiting NF κ B p65 translocation into the nucleus, leading to inhibition of the expression and release of pro-inflammatory cytokines.^{12,14}

We observed that isocorydine had no significant influence on the mRNA expression levels of key molecules such as TLR4, MyD88, TRAF6, and NF κ B p65, but reduced the levels of phosphorylated I κ B α and NF κ B p65 in the LPS/TLR4 signaling pathway in LPS-treated macrophages. Moreover, isocorydine also inhibited NF κ B p65 translocation from the cytoplasm into the nucleus. Therefore, we speculated whether decreased NF κ B p65 translocation into nucleus was related to VDR. Our results showed that isocorydine upregulated *Vdr* mRNA expression, and interestingly, isocorydine increased the VDR protein level in the nucleus, but not in the cytoplasm. In theory, highly expressed VDR should result in high levels of cytoplasmic and nuclear protein distribution. Therefore, why did the level of VDR in the cytoplasm not increase significantly? Considering the results for NF κ B p65, we hypothesized that VDR might bind strongly to NF κ B p65 in the cytoplasm, resulting in decreased levels of free NF κ B p65 and consequently reduced levels of NF κ B p65 translocating into the nucleus. VDR can enter the nucleus directly from the cytoplasm; therefore, the protein level of VDR in the nucleus showed a significant increase. The above ideas are summarized in a diagram (Figure 7). Importantly, the above speculation needs to be investigated in a future study.

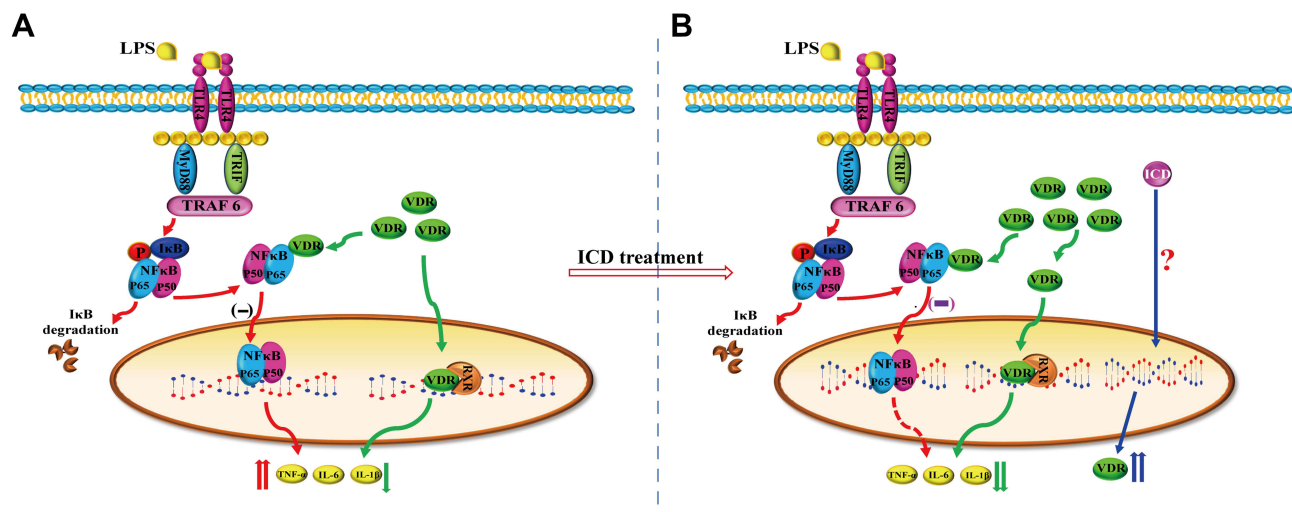


Figure 7 Flow chart of the possible mechanism of action of isocorydine. **(A)** LPS activates the TLR4-NFκB signaling pathway, leading to large amounts of NFκB translocation from the cytoplasm into the nucleus and subsequent expression of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β ; meanwhile, LPS can increase vitamin D receptor (VDR) translocation from the cytoplasm into the nucleus, leading to inhibition of the expression of pro-inflammatory cytokines. **(B)** On the one hand, isocorydine (ICD) increases *Vdr* mRNA expression and VDR distribution in the nucleus. On the other hand, ICD is presumed to inhibit NFκB p65 translocation from the cytoplasm into the nucleus, because highly expressed VDR strongly interacts with NFκB p65, leading to less NFκB p65 translocation into the nucleus and subsequently, reduced release of pro-inflammatory cytokines.

In conclusion, our results demonstrated that the compounds extracted from Guizhou ethnic medicine in the Karst area in Guizhou had good anti-inflammatory effects. Among them, isocorydine has good druggability. Isocorydine inhibited the LPS-induced release of pro-inflammatory cytokines in vitro and decreased the levels of pro-inflammatory cytokines in LPS-challenged mice, consequently ameliorating lung tissue damage. Mechanistically, isocorydine inhibits NFκB p65 translocation into the nucleus, and increases *Vdr* mRNA expression and VDR distribution in the nucleus, leading to reduced release of pro-inflammatory cytokines, thereby protecting LPS-challenged mice.

Ethics Approval and Informed Consent

In this study, all protocols and experimental procedures involving live animals were approved by the Animal Care Welfare Committee of Guizhou Medical University (License number: 1,804,102). Animal studies were performed according to National Institute of Health Guide for the Care and Use of Laboratory Animals.

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Disclosure

The authors declare no conflicts of interest in this study.

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