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Inhibitory Effects of JEUD-38, a New Sesquiterpene Lactone from *Inula japonica* Thunb, on LPS-Induced iNOS Expression in RAW264.7 Cells

Xiaoqing Wang,[1,2] Sheng-An Tang,[1] Ran Wang,[1] Yuling Qiu,[1] Meihua Jin,[1,4] and Dexin Kong[1,3,4]

ABSTRACT—We isolated JEUD-38, a new sesquiterpene lactone from *Inula japonica* Thunb. JEUD-38 dramatically attenuated lipopolysaccharide (LPS)-induced nitric oxide (NO) production. Consistent with this finding, the protein expression of inducible nitric oxide synthase (iNOS) was blocked by JEUD-38 in a concentration-dependent manner. To elucidate the mechanism, we examined the effect of JEUD-38 on LPS-stimulated nuclear factor-κB (NF-κB) nuclear translocation, inhibitory factor-κB (IκB) phosphorylation, and degradation. JEUD-38 reduced the translocation of p65, *via* abrogating IκB-α phosphorylation and degradation. In addition, JEUD-38 inhibited LPS-stimulated phosphorylation of mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun *N*-terminal kinase (JNK), and p38. Since iNOS as well as the upstream NF-κB and MAPKs are known to be closely involved in inflammation, these results suggest that JEUD-38 is a promising candidate for prevention and therapy of inflammatory diseases.

KEY WORDS: JEUD-38; sesquiterpene lactone; NO; NF-κB; MAPK.

INTRODUCTION

Chronic or acute inflammation is a multiple process mediated by activated inflammatory or immune cells [1, 2]. Macrophages play a key role in mediating many different immunopathological phenomena including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as nitric oxide (NO), prostaglandin (PG) E_2, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [3, 4].

NO is a reactive radical molecule generated *via* the oxidative deamination of L-arginine by a family of NO synthases (NOS) [5]. Among them, inducible NOS (iNOS) is expressed in response to lipopolysaccharide (LPS) and various pro-inflammatory cytokines [6]. NO production has beneficial effects for host innate immune response to pathogens such as viruses, bacteria, fungi, helminthes, and protozoa [7, 8]. However, excessive NO production can be harmful to the host, resulting in various inflammatory diseases [9].

NF-κB is a ubiquitous transcription factor which plays a crucial role in immune and inflammatory responses through regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, and inducible enzymes such as cyclooxygenase-2 (COX-2) and iNOS [10–12]. In resting cells, NF-κB is associated with the inhibitor IκB in the cytoplasm. Many stimuli activate NF-κB, mostly through IκB kinase (IKK)-dependent phosphorylation and subsequent degradation of IκB proteins. Following activation, NF-κB dimmers dissociate from the inhibitor and enter the nucleus [13].

The mitogen-activated protein kinases (MAPK) are known to play a crucial role in mediating inflammatory responses [14]. The MAPK family consists of three well-

[1] Tianjin Key Laboratory on Technologies Enabling Development of Clinical Therapeutics and Diagnostics, School of Pharmacy, Tianjin Medical University, Tianjin, 300070, China
[2] Department of Pharmacy, Second Hospital of Tianjin Medical University, Tianjin, 300211, China
[3] Research Center of Basic Medical Sciences, Tianjin Medical University, Tianjin, 300070, China
[4] To whom correspondence should be addressed to Meihua Jin at Tianjin Key Laboratory on Technologies Enabling Development of Clinical Therapeutics and Diagnostics, School of Pharmacy, Tianjin Medical University, Tianjin, 300070, China. E-mail: jinmeihua@tijmu.edu.cn; and Dexin Kong at Research Center of Basic Medical Sciences, Tianjin Medical University, Tianjin, 300070, China. E-mail: kongdexin@tijmu.edu.cn

characterized subfamilies including extracellular regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 MAP kinases. MAPK pathway is involved in the up-regulation of iNOS and pro-inflammatory cytokines in murine macrophages [15, 16]. Therefore, NF-κB and MAPKs are known as important targets for anti-inflammatory therapy.

The flowers of *Inula japonica* Thunb (*I.japonica*) have been used in traditional Chinese medicine, owing to their activities of relieving phlegm, detumescence, peptic, vermifuge, and anti-inflammation [17]. In our previous studies, we demonstrated that the flower extract of *I. japonica* showed anti-inflammatory activities, such as alleviation of ovalbumin (OVA)-induced airway inflammation in murine model of asthma [18], suppression of mast cell-mediated allergic reaction and mast cell activation [19], and reduction of pro-inflammatory cytokines release and NO production in RAW264.7 cells [20].

JEUD-38, 1-oxo-4*aH*-eudesma-5 (6),11 (13)-dien-12,8*β*-olide, is a new sesquiterpene lactone isolated from *I. japonica*. In a preliminary experiment, we found that JEUD-38 inhibited NO production in RAW264.7 cells. To investigate the mechanism, we evaluated the effect on iNOS expression, NF-κB activation, and MAPK phosphorylation in LPS-stimulated RAW264.7 cells.

MATERIALS AND METHODS

Plant Material

JEUD-38 was isolated from the ethanol extract of *I. japonica*. The plants of *I. japonica* were collected from Henan Province, China, and identified by Professor Y. Zhou (Department of Pharmacognosy, School of Pharmacy, Tianjin Medical University). A voucher specimen (IJ201105) was deposited at the School of Pharmacy, Tianjin Medical University, China. Prior to use, JEUD-38 was dissolved in dimethyl sulfoxide (DMSO).

Extraction and Isolation

The powdered and dried flowers of *I. japonica* (8.0 kg) were extracted with 75 % ethanol (15 L×3, 2 h each time) under reflux. The extracts were concentrated to give a residue (600 g), which was suspended in water and partitioned with petroleum ether (P.E.), ethyl acetate (EtOAc), and *n*-butyl alcohol (*n*-BuOH) successively.

The P.E.-partitioned extract (36 g) was chromatographed on a silica gel column (500 g, 300–400 mesh, 80×10 cm) and eluted with a gradient solvent system (P.E.-EtOAc, *v/v*, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, with EtOAc of 2000 mL) to produce 39 fractions (fractions 1–39). The fraction 15 (2.1 g) was re-chromatographed on a silica gel column (50 g, 300–400 mesh, 60×3 cm), eluted with a gradient solvent system (CH_2Cl_2-MeOH, *v/v*, 99:1, 97:3, 95:5, 1:1, with MeOH of 200 mL) to produce 22 fractions (fractions 15.1–15.22). The fraction 15.4 (725.5 mg) was then separated by gel permeation chromatography (GPC) (2×50 cm×2 cm, MeOH, flow rate 3 mL/min) to afford 12 fractions (fractions 15.4.1–15.4.12). Finally, the fraction 15.4.3 was separated by HPLC (ODS, 5 μm, 2×25 cm, MeOH-H_2O, *v/v*, 9:1, flow rate 3 mL/min, Rt =22 min) to afford compound JEUD-38 (12.0 mg).

Reagents

The antibodies specific for iNOS, phospho-IκB-α, IκB-α, phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK, β-actin, and the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies for p65 and lamin B were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The enhanced chemiluminescence (ECL) Western blot detection reagent was from Thermo Fisher Scientific (Rockford, IL, USA). The bacterial LPS was purchased from Sigma-Aldrich (Louis, MO, USA).

Cell Culture

The RAW264.7 macrophage cells were obtained from the Korea Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10 % FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

Determination of Cell Viability

MTT assay was used to measure cell viability. RAW 264.7 cells were treated with different concentrations of JEUD-38 for 24 h. Then, MTT (5 mg/mL) was added and further incubated for 4 h. The culture medium was discarded and the formazan blue formed in the cells was resolved with DMSO. The absorbance at 490 nm was measured with a microplate absorbance reader (BIO-RAD iMark, Hercules, CA, USA).

Measurement of NO

RAW264.7 cells (2×10^5 cells/mL) were pre-treated with or without JEUD-38 for 1 h and stimulated with LPS (200 ng/mL) for 18 h. NO production was evaluated by measuring nitrite level in the culture media using Griess

reagent (1 % sulfanilamide, 0.1 %*N*-1-naphthylenediamine dihydrochloride, and 2.5 % phosphoric acid). The signal was determined by measuring the absorbance at 570 nm with multi-mode microplate reader (Molecular Devices FilterMax F5, Sunnyvale, CA, USA). L-N6-(1-iminoethyl) lysine (L-NIL, a selective inhibitor of iNOS) was used as a positive control.

Extraction of Nuclear Protein

RAW264.7 cells pretreated with or without JEUD-38 or ammonium pyrrolidinedithiocarbonate (PDTC) for 1 h were incubated with LPS for 30 min. Then, the nuclear extracts were prepared as described in the manufacturer's protocol (Panomics Nuclear Extraction Kit, Fremont, CA, USA).

SDS-PAGE/Immunoblot Analysis

SDS-PAGE/immunoblot analysis was performed as described by us previously [21]. Equal amounts of protein were electrophoresed on 10 % SDS-polyacrylamide gels and blotted onto a PVDF membrane. Membranes were blocked with 5 % non-fat dry milk in TTBS (20 mM Tris–HCl, 150 mM NaCl, and 0.05 % Tween-20) and probed with various primary antibodies. After overnight incubation with primary antibody followed by three times of washes, the membrane was hybridized with HRP-conjugated secondary antibody for 1 h and washed three times with TTBS. The protein bands were then visualized with an ECL system.

Statistical Analysis

Results are expressed as mean±S.D. One-way analysis of variance (ANOVA) was utilized to determine the statistical significance.

RESULTS

Structure Elucidation of JEUD-38

JEUD-38 was isolated as a pale yellow solid, with the formula of $C_{15}H_{18}O_3$ as determined by HR ESI-MS at *m/z* 269.1150 $[M+Na]^+$, ^{1}H-, and ^{13}C-NMR spectral data. ^{13}C NMR and DEPT spectra exhibited 15 carbons in the molecule, involving two methyls, four methylenes, four methines, and five quaternary carbons. The low field region of the ^{13}C NMR spectrum revealed the presence of one carbonyl carbon [δ_C 213.2 (C-1)], one carboxyl carbon [δ_C 169.9 (C-12)], and four olefinic carbons [δ_C 145.7 (C-5), 139.1 (C-11), 122.3 (C-13), 121.7 (C-6)]. Its ^{1}H NMR spectral data (Table 1) exhibited two methyl proton signals [δ_H 1.26 (3H, d, J =7.2 Hz, H-15), 1.39 (3H, s, H-14)], three olefinic proton signals [δ_H 5.37 (1H, d, J =4.0 Hz, H-6), 5.68 (1H, d, J =1.6 Hz, H-13a), 6.25 (1H, d, J =1.6 Hz, H-13b)], and one oxygenated methines [δ_H 4.86 (1H, m, H-8)]. Two double bonds and two carbonyl groups derived from ^{13}C NMR analysis accounted for four of the seven degrees of unsaturation, thus implying a polycyclic nature for JEUD-38.

The ^{13}C NMR and DEPT spectra of JEUD-38 showed that this compound had 15 carbons for a sesquiterpene-type compound (Table 1), with a structure very similar to the known compound 1β-hydroxyalantolactone [22], except for the ketone group at C-1 instead of a hydroxyl group in reported compound. The protons at δ1.39 (CH3-14), 2.25 (H-2a), 1.97 (H-3a) revealed HMBC correlations with the ketone group at δ213.2, indicating that the ketone group is positioned at C-1. Finally, the structure of JEUD-38 was elucidated as 1-oxo-4αH-eudesma-5 (6),11 (13)-dien-12,8β-olide (Fig. 1).

The Cytotoxicity of JEUD-38 in RAW264.7 Cells

Firstly, the cytotoxic effect of JEUD-38 (Fig. 2) was measured on RAW264.7 cells by MTT assay. Treatment of RAW264.7 cells with JEUD-38 for 24 h showed that cell

Table 1. Spectroscopic Data of JEUD-38 [δ in ppm]

Position	^{1}H NMR	^{13}C NMR
1	–	213.2 s
2	2.75 (1H, m)	35.4 t
	2.25 (1H, m)	
3	1.97 (1H, m)	28.5 t
	1.77 (1H, m)	
4	2.63 (1H, m)	35.9 d
5	–	145.7 s
6	5.37 (1H, d, J =4.0 Hz)	121.7 d
7	3.58 (1H, m)	39.3 d
8	4.86 (1H, m)	75.2 d
9	2.47 (1H, dd, J =3.1, 15.6 Hz)	33.9 t
	1.83 (1H, d, J =3.4, 15.6 Hz)	
10	–	46.9 s
11	–	139.1 s
12	–	169.9 s
13	5.68 (1H, d, J =1.6 Hz)	122.3 t
	6.25 (1H, d, J =1.6 Hz)	
14	1.39 (3H, s)	28.3 q
15	1.26 (3H, d, J =7.2 Hz)	22.9 q

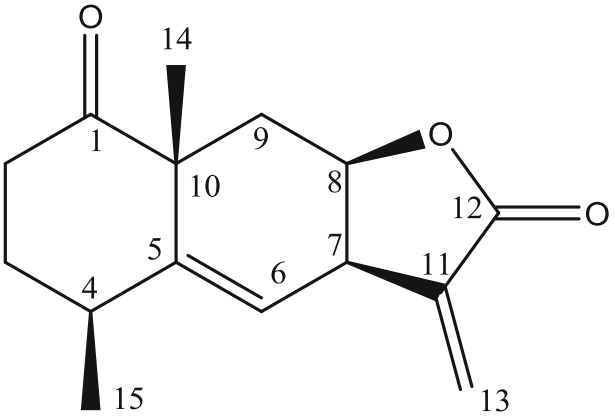

Fig. 1. Chemical structure of JEUD-38.

viability was affected by 25 μM but was a little changed by 10 μM (Fig. 2). Therefore, concentrations of equal to or lower than 10 μM were selected for subsequent experiments.

Effect of JEUD-38 on LPS-induced NO Production and iNOS Protein Expression in RAW264.7 Cells

To investigate the effects of JEUD-38 on LPS-induced NO production in RAW264.7 cells, cells were pretreated with various concentrations of JEUD-38 for 1 h and then stimulated with LPS (200 ng/mL) for 18 h. Upon stimulation with LPS, NO production increased approximately 11-fold, but these increases were inhibited in a dose-dependent manner by JEUD-38 with IC_{50} as 2.56 μM which was calculated by the use of GraphPad Prism 4 (GraphPad software, San Diego, CA, USA) (Fig. 3a). L-NIL (10 μM), an NO production inhibitor [23], was used as a positive control. In addition, to investigate if the inhibition of NO production by JEUD-38 is associated with iNOS protein expression, Western blotting was performed. The expression level of iNOS was significantly up-regulated in RAW264.7 cells when exposed to LPS, and JEUD-38 markedly suppressed the increased iNOS protein level (Fig. 3b).

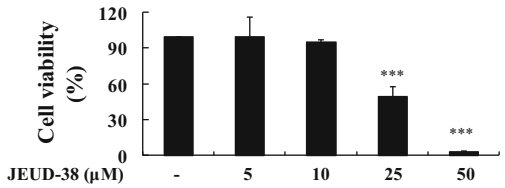

Fig. 2. Effect of JEUD-38 on RAW264.7 cell viability. RAW264.7 cells were treated with indicated concentrations of JEUD-38 for 24 h, and the cell viability was measured by determining the absorbance at 490 nm. ***P<0.001, compared with cells in the absence of JEUD-38.

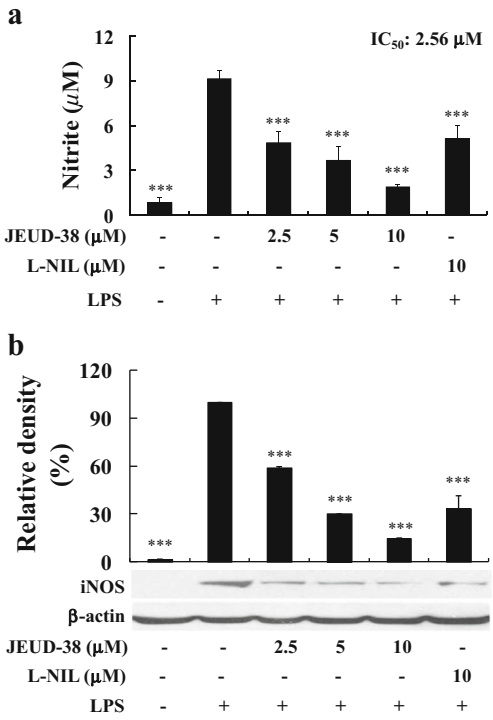

Fig. 3. Effect of JEUD-38 on NO production and iNOS protein expression in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with various concentrations of JEUD-38 for 1 h and then stimulated with 20 ng/mL of LPS for 18 h. **a** The amount of NO in the medium was measured using the Griess reagent. **b** iNOS protein expression levels were determined by Western blotting analysis. Data are shown as the mean ±S.D. of three different samples. ***P<0.001, compared with LPS-induced cells in the absence of JEUD-38.

Effect of JEUD-38 on LPS-induced Nuclear Translocation of NF-κB p65, Degradation and Phosphorylation of IκBα in RAW264.7 Cells

NF-κB regulates numerous genes, particularly those involved in immune and inflammatory responses. NF-κB is retained in the cytoplasm in an inactive form through binding to IκB proteins. Diverse cell stimuli induce phosphorylation and subsequent proteasomal degradation of IκB, leading to NF-κB release from the cytoplasmic IκBα/NF-κB complex and translocation to the nucleus [24].

The p65 is the major subunit of NF-κB. The p65 levels in nuclear extract and in cytoplasm were evaluated respectively by Western blotting analysis. As shown in Fig. 4a, b, after treatment by JEUD-38, the p65 level in the nucleus was decreased while that in the cytoplasm was increased, suggesting that LPS-induced nuclear translocation of p65 was strongly inhibited in a dose-dependent manner. Lamin B and β-actin were used as internal

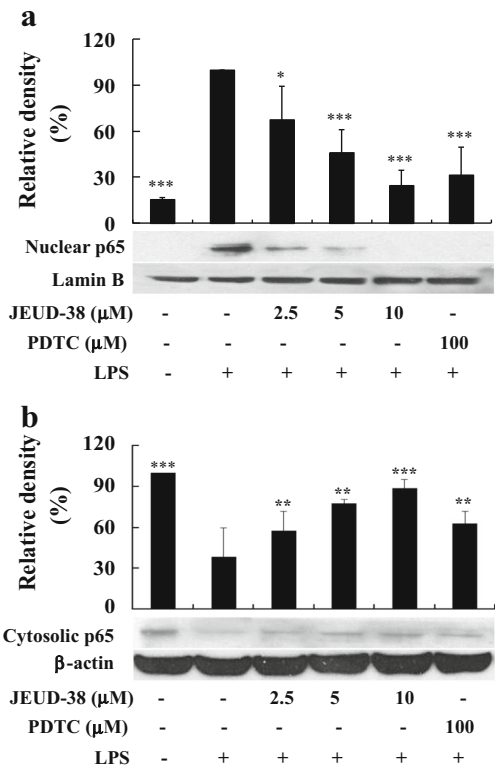

Fig. 4. Effect of JEUD-38 on LPS-induced p65 nuclear translocation in RAW264.7 cells. RAW264.7 cells were pre-incubated with indicated concentrations of JEUD-38 for 1 h, followed by stimulation with LPS (200 ng/mL) for 30 min. The nuclear (**a**) and cytosolic (**b**) extracts were prepared in accordance with the manufacturer's instructions and subjected to Western blotting analysis. As a positive control, a specific NF-κB inhibitor named PDTC was also applied. Data are expressed as the mean ±S.D. of three different samples. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with LPS-induced cells in the absence of JEUD-38.

controls. We also explored whether JEUD-38 inhibits the LPS-stimulated degradation of IκBα. As shown in Fig. 5, LPS-induced IκBα was suppressed by JEUD-38 pretreatment. To confirm that this IκBα degradation was associated with IκBα phosphorylation, we also examined the effect of JEUD-38 on LPS-induced p-IκBα. As shown in Fig. 5, IκBα phosphorylation was also significantly blocked in the presence of JEUD-38, consistent to the result for the IκBα degradation. As a positive control, we used PDTC which is a specific NF-κB inhibitor.

Effect of JEUD-38 on LPS-Induced Activation of MAPK in RAW264.7 Cells

MAPK pathway activates a variety of transcription factors and coordinates induction of many genes encoding inflammatory mediators [25]. Signal proteins of this

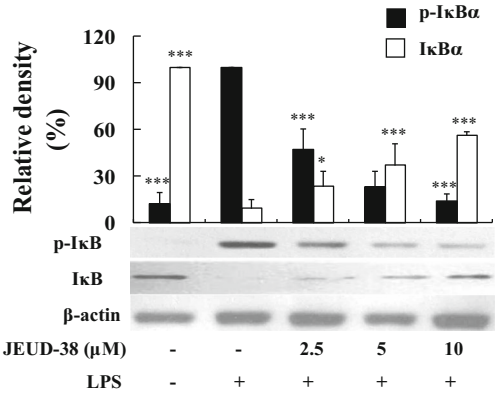

Fig. 5. Effect of JEUD-38 on LPS-induced degradation and phosphorylation of IκBα in RAW264.7 cells. RAW264.7 cells were stimulated with LPS in the presence or absence of JEUD-38 for 15 min (for detection of p-IκB) or 30 min (for detection of IκB). The phosphorylation and degradation of IκBα were determined by Western blotting analysis. Data are shown as the mean±S.D. of three different samples. *$P<0.05$, ***$P<0.001$, compared with LPS-induced cells in the absence of JEUD-38.

pathway such as ERK, JNK, and p38 are known to be closely involved in the expression of iNOS and NO production [12, 13, 15, 16, 26]. To investigate whether JEUD-38 inhibits the MAPK pathway, we tested the effects of JEUD-38 on LPS-induced phosphorylation of ERK, JNK, and p38 in RAW264.7 cells by Western blotting. As a result, the phosphorylation of ERK, JNK, and p38 were increased in cells treated with LPS alone. However, JEUD-38 inhibited phosphorylated ERK, JNK, and p38 levels in a dose-dependent manner (Fig. 6). PD98059, SP600125, and SB203580 were used as positive controls because they are known as ERK, JNK, and p38 pathway inhibitors, respectively [21, 27]. These findings suggest that phosphorylation of MAPKs might be involved in the JEUD-38-mediated suppression of LPS-induced iNOS expression.

DISCUSSION

The pro-inflammatory mediator NO plays critical roles in inflammatory diseases [28]. NO is synthesized from L-arginine after the activation of NOS. There are three isoforms of the NOS, including neuronal NOS (nNOS), endothelial NOS (eNOS) which is constitutive, and iNOS which is produced by activated macrophages [29]. In this study, we demonstrated that JEUD-38 suppresses LPS-induced NO production and iNOS protein expression in mouse macrophage RAW264.7 cells with an IC_{50} of 2.56 μM (Fig. 3).

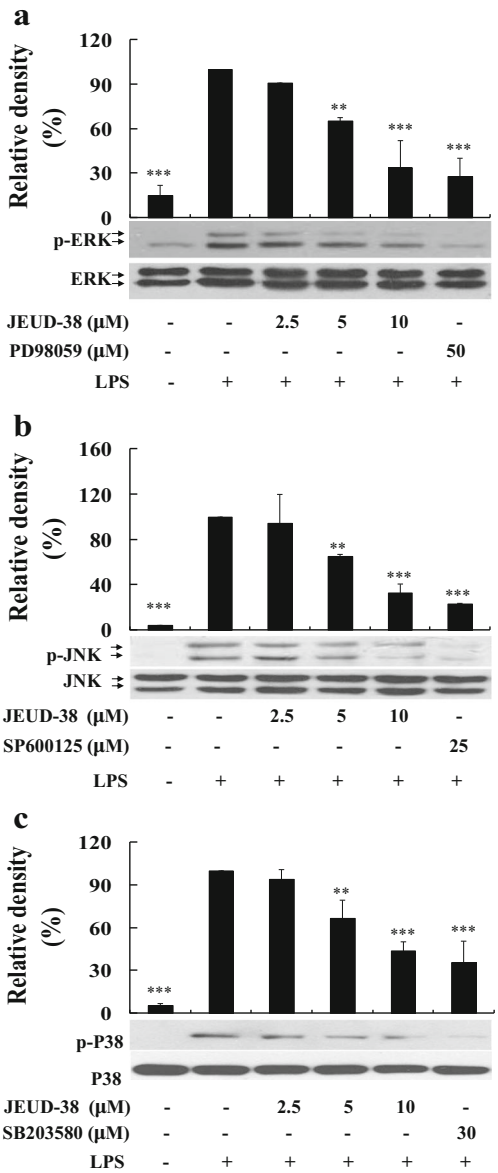

Fig. 6. Effects of JEUD-38 on LPS-induced MAPKs phosphorylation in RAW264.7 cells. RAW264.7 cells were pretreated with various concentrations of JEUD-38, 25 μM of SP600125 (JNK inhibitor), 30 μM of SB203580 (p38 inhibitor), or 50 μM of PD98059 (ERK inhibitor) for 1 h and then incubated for an additional 30 min with LPS (200 ng/mL). Cells were harvested, and the cell lysates were prepared. The phosphorylation of the MAPKs was analyzed by immunoblotting. All experiments were performed three times, and representative results are shown. Data are expressed as the mean±S.D. of three different samples. $^{**}P<0.01$, $^{***}P<0.001$, compared with LPS-induced cells in the absence of JEUD-38.

LPS stimulation induces inflammation through the activation of NF-κB and MAPK pathways [30, 31]. NF-κB plays a key role in controlling the expression of multiple inflammatory and immune genes and therefore leads to the development of inflammatory diseases including asthma, rheumatoid arthritis, type 1 diabetes, and Parkinson's disease [10, 32]. NF-κB is located in the cytoplasm in a quiescent form bound to IκB [33]. In the majority of cells, NF-κB is composed of a p50 and p65 subunit. NF-κB translocates into the nuclei after the degradation of IκB. Therefore, the amount of NF-κB protein in nuclear extracts could authentically reflect the activation status of NF-κB [13]. In the present study, we found that the translocation of activated NF-κB p65 to the nucleus was reduced by JEUD-38 (Fig. 4), and the degradation and phosphorylation of IκBα were also inhibited in a concentration-dependent manner by JEUD-38 (Fig. 5). Activated ERK, JNK, and p38 have been postulated to play important roles in controlling iNOS gene expression [34, 35]. In this study, active ERK, JNK, and p38 were significantly inhibited by JEUD-38 (Fig. 6). These results suggest that JEUD-38 reduction of LPS-induced iNOS expression could occur through blocking NF-κB signaling and MAPK activation.

The flower of *I. japonica* is well known in traditional Chinese herbal medicine. It is often used to treat emesis, chronic bronchitis, and acute pleurisy in China (Pharmacopoeia Commission of People's Rebublic of China, 2010). The extract of *I. japonica* has beneficial pharmacologic effects, including anti-diabetic and hypolipidemic effects [36, 37]. In the previous study, our group reported that *I. japonica* extract has anti-asthmatic and anti-inflammatory activities [18–20]. In this paper, we isolated and identified a new sesquiterpene lactone named JEUD-38 from *I. japonica*, which dramatically inhibited NO production in LPS-induced RAW264.7 cells.

In summary, the present study revealed the anti-inflammatory activities and the related mechanism of a new compound JEUD-38. We demonstrated that JEUD-38 inhibits LPS-induced NO production accompanied with down-regulation of iNOS protein expression in murine RAW264.7 macrophage cells. Moreover, JEUD-38 interferes the activation of NF-κB transcription factor by inhibiting IκBα phosphorylation and degradation. In addition, JEUD-38 also reduces MAPK activation. Based on these results, JEUD-38 might become a promising drug candidate for prevention and treatment of inflammatory diseases.

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Conflict of Interest. There is no conflict of interest for all authors of this manuscript.

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