Original Article

Lobolide, a diterpene, blockades the NF- κ B pathway and p38 and ERK MAPK activity in macrophages *in vitro*

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Aim: Recent studies have shown that constitutive activation of the nuclear factor κ B (NF- κ B) plays a key role in chronic inflammation and cancers. The aim of this study was to characterize lobolide, a cembrane diterpene, as a drug candidate targeting the NF- κ B signaling pathway.

Methods: A HEK 293/NF- κ B-Luc stable cell line was constructed to evaluate the effect of lobolide on NF- κ B activation. THP-1 human monocytes and peripheral blood mononuclear cells (PBMCs) from healthy volunteers were tested. Lipopolysaccharide (LPS)-induced TNF α and IL-1 β production and activation of the TAK1-IKK-NF- κ B pathway were studied using ELISA and Western blot analysis.

Results: In HEK 293/NF- κ B-Luc stable cells, lobolide (0.19–50 μ mol/L) inhibited NF- κ B activation in a concentration-dependent manner with an IC₅₀ value of 4.2 $\pm$ 0.3 μ mol/L. Treatment with lobolide (2.5–10 μ mol/L) significantly suppressed LPS-induced production of TNF α and IL-1 β in both THP-1 cells and PBMCs. In THP-1 cells, the suppression was partially caused by blockade of the translocation of NF- κ B from the cytoplasm to the nucleus via affecting the TAK1-IKK-NF- κ B pathway and p38 and ERK MAPK activity.

Conclusion: Lobolide is a potential inhibitor of the NF- κ B pathway, which blocks the translocation of NF- κ B from the cytoplasm to the nucleus. Lobolide inhibits LPS-stimulated TNF α and IL-1 β release, suggesting that the compound might be an anti-inflammatory compound.

Keywords: lobolide; diterpene; monocytes; lipopolysaccharide; NF- κ B; TNF α ; IL-1 β ; TAK1; Erk 1/2; p38; inflammation

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) have become one of the most widely used groups of drugs in the history of medicine. It has been estimated that more than 30 million people take NSAIDs daily for relief of symptoms of rheumatoid arthritis, osteoarthritis and other arthritides. The history of NSAIDs can be traced to ancient Egypt, where an extract of willow bark was used to treat inflammation. The active component of the extracts was subsequently identified as the glucoside of salicyl alcohol. Hydrolysis of the carbohydrate moiety produces salicyl alcohol, which can be oxidized to salicylic acid, the actual anti-inflammatory agent^[1,2]. Subsequently, a

number of other anti-inflammatory agents have been developed over the past 60 years. These fall into six distinct classes: salicylates, anthranilic acid derivatives, indomethacin and related derivatives, oxicams, phenylalkanoic acid and related derivatives, and pyrazole derivatives^[3]. The more traditional NSAIDs, such as aspirin, phenylbutazone, and indomethacin, have excellent anti-inflammatory efficacy and function primarily through a reduction in prostaglandin (PG) synthesis by inhibiting the enzyme prostaglandin endoperoxide synthase. This polypeptide enzyme has both cyclooxygenase and peroxidase activities. It occurs as two isoforms, designated as isoforms 1 and 2, which are referred to as cyclooxygenase (COX-1 and COX-2) in the current literature^[4]. COX-1, the constitutive enzyme, is involved primarily in housekeeping functions and is responsible for the production of PGs, while COX-2, the inducible isoform, exclusively modulates inflammatory reactions^[5]. NSAIDs inhibit cyclooxygenase activity mainly by

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inhibiting access of arachidonic acid to the COX active site^[6].

NSAIDs also share several unwanted side effects, with the induction of gastrointestinal injury, such as gastric and/or intestinal erosion and/or ulceration with consequent blood loss and anemia, being the most common^[7, 8]. These side effects appear to be related to the drugs' inhibition of COX-1 because constitutive expression of COX-2 in normal gastric mucosal tissue is low or absent^[9]. However, COX-2 inhibitors may increase the risk of cardiovascular complications, such as heart attack or stroke, especially if they are used for a prolonged time^[10]. In this study, we focused on finding NSAIDs that target the nuclear factor-kappa B (NF- κ B) signaling pathway, but not COXs.

As an evolutionary conserved family of transcription factors, NF- κ B was initially discovered as a regulator of inflammatory responses. In unstimulated immune cells, NF- κ B is inactive in the cytoplasm, forming a complex composed of p65 (Rel A), p50 (NF- κ B) and an inhibitor of NF- κ B α (I κ B α)^[11]. Through distinct signaling pathways, pro-inflammatory stimuli, such as cytokines and lipopolysaccharide (LPS), converge their signaling on activation of IKK α / β , then phosphorylation and degradation of I κ B α . This degradation exposes the nuclear localization signal (NLS) sequences of NF- κ B, allowing nuclear translocation of the p50/p65 complex and regulation of the expression of a vast array of genes^[12, 13]. Abnormal activation of the transcription factor NF- κ B by nuclear translocation of the cytoplasmic complexes plays an important role in some inflammatory diseases via its ability to induce expression of cytokines, chemokines, mediators and immune-related receptor genes. Inhibition of the NF- κ B signaling pathway would be an important approach for the treatment of inflammatory diseases.

Lobolide is a cembrane diterpene extracted from the soft coral *Lobophytum* sp of the Lingshui Bay, Hainan Province, China^[14]. Soft coral cembrane diterpenes are usually produced as a defense against predators and display cytotoxic, anti-inflammatory, antimicrobial and antiarthritic effects^[15]. In the present study, we employed a cell model using luciferase activity regulated by the NF- κ B transcription factor to search for new molecules that could suppress NF- κ B signaling. Among the candidates, lobolide was identified as an inhibitor of the NF- κ B signaling pathway in THP-1 cells. In addition, we further studied the mechanism underlying lobolide's inhibitory activity.

Materials and methods

Preparation of lobolide

Lobolide is a cembrane diterpene, isolated from the *Lobophytum* sp, with a molecular weight of 374 daltons. Its structure (Figure 1) was consistent with previous reports^[16]. The purity of this compound was more than 98%, as estimated by high-performance liquid chromatography analysis. Lobolide was dissolved in DMSO (Sigma, St Louis, MO, USA) and stored at -20°C. For *in vitro* experiments, lobolide was diluted in the culture media specific to the different cells utilized in this study, and the final concentration of DMSO was always 0.1%

Figure 1. Chemical structure of lobolide.

or lower.

Generation of a HEK 293/NF- κ B-Luc stable cell line

HEK 293 cells with 50%–80% confluence were co-transfected with the pNF κ B-TA-Luc vector (Clontech, Palo Alto, CA, USA) and the pcDNA3.1/*myc*-HisB (Invitrogen, Carlsbad, CA, USA) vector at a ratio of 10:1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. pNF κ B-TA-Luc is designed for monitoring NF- κ B signaling transduction pathways with four tandem copies of the κ B motif linked with the luciferase gene. When the NF- κ B transcription factor binds to the κ B motif, the luciferase gene is activated and transcribed for expression. Because there is no selectable marker on this plasmid, the pcDNA3.1/*myc*-HisB plasmid was used to supply a selectable marker. After transfection for 12 h, the cells were treated with geneticin (Merck, Darmstadt, Germany). The geneticin-resistant clones were further selected by a luciferase reporter assay, with 1 μ g/mL lipopolysaccharide (LPS from *Escherichia coli* O55:B5, Sigma, St Louis, MO, USA) used as a stimulator. The luciferase reporter assay was performed using the Luciferase Assay System (Promega, Madison, WI, USA). Briefly, the cells were lysed with the cell culture lysis reagent, and then, the cell lysates were transferred to 96-well LUMITRAC™ 200 flat bottom plates (Greiner Bio-one, Frickenhausen, Germany). The relative light units (RLUs) were measured immediately after the substrates were added to the cell lysates with a NOVOstar microplate reader (BMG LabTechnologies, Offenburg, Germany). The resultant HEK 293/NF- κ B-Luc stable cell lines were maintained in the presence of 0.8 mg/mL geneticin for approximately 2 months.

Short hairpin DNA (shDNA) preparation

shDNA sequences were designed and synthesized by GenePharma (GenePharma Co, Ltd, Shanghai, China) for knock-down of NF- κ B/p65 expression. The sequences shown in Table 1 were inserted into the pGPU/GFP/Neo plasmids (GenePharma Co, Ltd, Shanghai, China). The constructed

Table 1. shDNA sequences details of p65 and the negative control.

shDNA	shDNA sequences (sense strand)
Sh p65	5'-GAG TAC CCT GAG GCT ATA ACT-3'
Negative control	5'-GTT CTC CGA ACG TGT CAC GT-3'

pGPU/GFP/Neo-sh p65 plasmids and the negative control (NC) were then transfected into cells.

Reverse transcription-polymerase chain reaction (RT-PCR) and luciferase reporter assay

Transfection of shDNA into the HEK 293/NF- κ B-Luc cells (5×10^5 /mL in 24-well plates) was performed using Lipofectamine 2000. After transfection for 48 h, the cells were exposed to 1 μ g/mL LPS for 6 h. Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 1 μ g of RNA was reversely transcribed into cDNA, which was then subjected to 20–30 cycles of PCR (Applied Biosystems, Foster City, CA, USA). The PCR cycles consisted of a 30-s denaturation at 94°C, a 30-s annealing at 56°C, and a 30-s extension at 72°C. The sequences of the PCR primers and the sizes of PCR products are shown in Table 2. RT-PCR was performed in two steps following the instructions included with the RTase M-MLV (RNase H⁻) and Taq enzymes (Takara Shuzo, Kyoto, Japan).

Table 2. Primers of p65 and 18S rRNA and their PCR products sizes.

Gene	PCR primers	Products size (bp)
18S rRNA	5'-CGG CTA CCA CAT CCA AGG AA-3' 5'-GCT GGA ATT ACC GCG GCT-3'	187
p65	5'-CGA CCT GAA TGC TGT GCG GC-3' 5'-GAT CTC ATC CCC ACC GAG GC-3'	181

For the luciferase reporter assay, the same transfection protocol was used as that for RT-PCR, except that the cells were seeded in 96-well plates. The experiments were repeated thrice.

Cell culture conditions

THP-1 cells (ATCC, TIB-202) were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 0.05 mmol/L 2-mercaptoethanol and 10% heat-inactivated fetal bovine serum (FBS) (GIBCO-BRL, Grand Island, NY, USA). HEK 293/NF- κ B-Luc cells were maintained in Dulbecco's modified essential medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated FBS (GIBCO-BRL, Grand Island, NY, USA). Both cell lines were cultured with 100 U/mL penicillin and 100 g/mL streptomycin and incubated in a humidified atmosphere containing 5% CO₂ at 37°C.

NF- κ B activity inhibition analysis

HEK 293/NF- κ B-Luc cells were seeded in 96-well plates at a density of 3×10^5 cells/mL. The cells were first incubated with varying concentrations of lobolide for 15 min, and then, the cells were incubated with LPS (1 μ g/mL) for 6 h. Blank and LPS-only controls were also performed; both controls contained 0.1% DMSO (*v/v*), and each treatment was performed in

triplicate. The inhibition rate of the compound was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{RLU}_{\text{LPS control}} - \text{RLU}_{\text{Compound}}}{\text{RLU}_{\text{LPS control}} - \text{RLU}_{\text{Blank}}} \times 100\%$$

Cytotoxicity assay

THP-1 cells (1×10^4 cells/well) were seeded in 96-well plates and treated with varying concentrations of lobolide for 48 h. A blank control containing 0.1% DMSO (*v/v*) was used, and each experiment was performed triplicate. The cells were exposed to water-soluble WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (Dojindo Lab, Kumamoto, Japan) for 6 h. The absorbance values were then measured at 450 nm using a microplate reader (BioRad, Hercules, CA, USA). The cytotoxicity of lobolide on the cells was estimated by the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{OD}_{\text{Blank}} - \text{OD}_{\text{Compound}}}{\text{OD}_{\text{Blank}}} \times 100\%$$

Protein preparation and immunoblotting

THP-1 cells (2×10^6 cells/sample) were washed twice with ice-cold phosphate-buffered saline (PBS). Cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer (10 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.1% deoxycholate, 0.1% sodium dodecyl sulfate, 5 mmol/L ethylenediaminetetraacetic acid), containing 1 $\times$ complete protease inhibitor cocktail (Sigma, St Louis, MO, USA), 1 mmol/L sodium fluoride (NaF), and 1 mmol/L sodium vanadate (Na₃VO₄). Nuclear proteins were extracted with a CellLytic NuCLEAR Extraction Kit (Sigma, St Louis, MO, USA). The concentrations of whole-cell protein and nuclear protein were determined using the Bio-Rad protein assay kit. The proteins were subjected to SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes (Pall Life Sciences, Ann Arbor, MI, USA), and then probed with the following antibodies (Abs): anti-NF- κ B p65 (C22B4), anti-I κ B α , anti-IKK α , anti-IKK β , anti-TAK1, anti-phospho-I κ B α (Ser32/36) (5A5), anti-phospho-IKK α (Ser180)/IKK β (Ser181), anti-phospho-TAK1 (Thr184), anti-p38 MAP Kinase, anti-phospho-p38 MAP Kinase (Thr180/Tyr182), anti-Erk1/2 (137F5), anti-phospho-Erk1/2 (Thr202/Tyr204) (197G2), anti-histone H3 (all of the antibodies mentioned above were from Cell Signaling Technology Inc, Beverly, MA, USA) and anti β -actin (Sigma, St Louis, MO, USA). β -Actin was used as a loading control for whole cytoplasmic protein extracts. Histone H3 was used as a loading control for nuclear protein extracts. The blots were developed using Pierce's SuperSignal West Dura Extended Duration Substrate according to the manufacturer's instructions (Thermo Fisher Scientific, Nepean, CA, USA).

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples donated by healthy volunteers by Ficoll-Paque PLUS density centrifugation (Amersham Biosciences,

Uppsala, Sweden). After washing with PBS, PBMCs were ready for the enzyme-linked immunosorbent assay (ELISA).

ELISA

PBMCs were diluted in RPMI-1640, supplemented with 10% heat-inactivated FBS, to 3×10^5 cells/well in 96-well plates and incubated with different concentrations of lobolide for 15 min at 37°C. The cells were then stimulated with LPS (1 $\mu\text{g}/\text{mL}$) for 24 h, and the supernatants were collected and diluted for ELISA quantification. Blank and LPS-only controls, each containing 0.1% DMSO (*v/v*), were also used. The concentrations of the cytokines IL-1 β and TNF α were measured using ELISA kits (ExCell, Shanghai, China). The same method was used to determine the relative concentrations of cytokines released by THP-1 cells (1×10^5 cells/well in 96-well plates). Each experiment was repeated thrice.

Indirect immunofluorescence assay and laser scanning confocal microscopy (LSCM) analysis^[17]

THP-1 cells, differentiated on coverslips for 24 h by 20 $\mu\text{g}/\text{L}$ phorbol 12-myristate 13-acetate (PMA, Sigma, St Louis, MO, USA), were fixed with freshly prepared 4% paraformaldehyde (PFA) for 15 min and permeabilized with 0.3% NP-40 (Sigma, Fluka Chemie AG, Buchs, Switzerland) for 15 min. BSA (3%) was used to block the slides, which were then incubated at 4°C overnight with anti-NF- κB p65 (C22B4) antibody diluted 1:50 in the antibody dilution buffer (ADB) (1 $\times$ PBS, containing 0.3% Triton X-100 and 1% BSA). Then, the slides were incubated with FITC-conjugated goat anti rabbit IgG (Invitrogen, Carlsbad, CA, USA) (diluted 1:200 in ADB) for 1 h. To identify the nuclei, the FITC-labeled samples were further stained with 25 mg/L propidium iodide (PI) (Sigma, St Louis, MO, USA) for 2 min. Dual-color images were obtained by Olympus Fluoview 1000 laser scanning confocal microscopy (Olympus Fluoview, Mellville, NY, USA).

Statistical analysis

Independent values are expressed as the mean $\pm$ SD. Significant differences were determined using the two-tailed student's *t* test, with *P* values <0.05 considered significant.

Results

Lobolide blocked NF- κB -driven luciferase expression

HEK 293/NF- κB -Luc stable cell lines were constructed to evaluate the lobolide inhibitory effect on NF- κB activation. The luciferase activity in the stable cell line stimulated by LPS (1 $\mu\text{g}/\text{mL}$) was hundreds of times higher than that in unstimulated cells. To further confirm that the cell model worked well, the HEK 293/NF- κB -Luc stable cell line was transfected with shDNA targeting p65. Small interfering RNA (siRNA) could be synthesized in cells using expression vectors containing a short hairpin structure of DNA. The results demonstrated that luciferase activity was reduced when the expression of p65 was targeted, compared to the negative control (Figure 2). These data indicated that the cell model could be employed to evaluate NF- κB activity after treatment with different com-

Figure 2. shDNA targeting p65 downregulated p65 expression. (A) HEK 293/NF- κB -Luc cells seeded at a density of 5×10^5 cells/mL in 24-well plates were transiently transfected with shDNA targeting p65 and a random sequence as a negative control (NC) for 48 h and then stimulated with LPS (1 $\mu\text{g}/\text{mL}$) for 6 h. Total RNA was extracted and subjected to RT-PCR analysis to detect cellular levels of p65; 18S rRNA was used as reference. (B) HEK 293/NF- κB -Luc cells (5×10^5 cells/mL) seeded in 96-well plates were transiently transfected with shDNA targeting p65 and the negative control (NC) for 48 h and then induced with LPS (1 $\mu\text{g}/\text{mL}$) for 6 h. The cell lysate was subjected to the luciferase reporter assay. Luciferase expression is represented as relative light units (RLUs). Mean $\pm$ SD. *n*=3. ^b*P*<0.05 vs negative control group.

pounds. Thus, this cell model was used to screen new anti-inflammatory compounds. Lobolide was shown to have a significant effect on NF- κB activity. To determine the lobolide concentration that results in 50% inhibition (the IC₅₀ value), HEK 293/NF- κB -Luc cells were treated with different concentrations of lobolide (0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 $\mu\text{mol}/\text{L}$) for 15 min, followed by LPS (1 $\mu\text{g}/\text{mL}$) stimulation for 6 h. Cell extracts in the presence of lobolide were subjected to the luciferase reporter assay. The results showed that NF- κB activity induced by LPS was potently inhibited by lobolide; the IC₅₀ value was 4.2 ± 0.3 $\mu\text{mol}/\text{L}$ (Figure 3).

Lobolide attenuated NF- κB /p65 nuclear translocation

To ensure that NF- κB activity inhibition was not due to cytotoxic effects, the cytotoxic effects of varying concentrations of lobolide (0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 $\mu\text{mol}/\text{L}$) on THP-1 cells were assessed. Indeed, cell death was induced by lobolide at concentrations above 12.5 $\mu\text{mol}/\text{L}$ (Figure 4). THP-1 cells were almost completely killed by 50 $\mu\text{mol}/\text{L}$ lobolide. However, cell viability was not significantly affected when the lobolide concentration was equal or less than 12.5 $\mu\text{mol}/\text{L}$ (Figure 4). Hence, lobolide concentrations below 10 $\mu\text{mol}/\text{L}$ were used in subsequent studies.

The nuclear translocation of NF- κB in THP-1 cells is immediately induced by the bacterial endotoxin LPS. The subcellular localization of NF- κB /p65 was easily detected by indirect immunofluorescence assays and laser scanning confocal microscopy with FITC-labeled NF- κB /p65- (green color in Figure 5A) and PI-stained nuclei (red color in Figure 5A). As shown in Figure 5A, NF- κB /p65 was primarily located in the cytoplasm of the cell in the absence of exogenously provided LPS (as shown in Figure 5A-1a, 1b, 1c). However, the

Figure 3. Inhibition of luciferase activity by lobolide. HEK 293/NF- κ B-Luc cells (5×10^5 cells/mL) were incubated with varying concentrations of lobolide (0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 $\mu\text{mol/L}$) for 15 min and then exposed to LPS (1 $\mu\text{g/mL}$) for 6 h. Blank and LPS-only controls, each containing 0.1% DMSO (v/v), were used. Cell extracts treated with different concentrations of lobolide were subjected to the luciferase reporter assay. The inhibition rate was calculated using the formula presented in *Materials and methods*. The results were analyzed by the sigmoidal fit function of Origin software (Origin 7.0, Microcal Software, Northampton, MA, USA). Means $\pm$ SD. $n=3$.

translocation of NF- κ B/p65 proteins into the nucleus was significantly induced by LPS (as shown in Figure 5A-2a, 2b, 2c). Interestingly, with the same LPS stimulation, the translocation of NF- κ B/p65 from the cytoplasm to nucleus was significantly

Figure 4. The cytotoxic effects of lobolide on THP-1 cells. THP-1 cells (1×10^5 cells/mL) were incubated with varying concentrations of lobolide (0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50 $\mu\text{mol/L}$) for 48 h and then subjected to the WST-8 method, measuring optical density at 450 nm (OD_{450}). A blank containing 0.1% DMSO (v/v) was used as the control. The cytotoxicity of lobolide was calculated using the formula found in *Materials and methods*. Mean $\pm$ SD. $n=3$.

blocked by pretreatment with 10 $\mu\text{mol/L}$ lobolide (as shown in Figure 5A-3a, 3b, 3c) and 5 $\mu\text{mol/L}$ 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1, Merck, USA) (as shown in Figure 5A-4a, 4b, 4c). TPCA-1 is an inhibitor of IKK α/β and selectively inhibits IKK β 20-fold more than IKK α ^[18]. A final concentration of 0.1% (v/v) DMSO had no influence on LPS-stimulated NF- κ B/p65 translocation to the nucleus, as reported previously (as shown in Figure 5A-5a, 5b, 5c)^[19, 20]. Immunoblot analysis showed that the levels of

Figure 5. Inhibition of NF- κ B/p65 nuclear translocation by lobolide. (A) THP-1 cells were seeded at a density of 2.5×10^5 cells/mL and differentiated by 20 $\mu\text{g/L}$ PMA on coverslips for 24 h. Then, the cells were treated with 10 $\mu\text{mol/L}$ lobolide and 5 $\mu\text{mol/L}$ TPCA-1 for 15 min before being stimulated with 1 $\mu\text{g/mL}$ LPS for 1 h. Blank and LPS-only controls both contained 0.1% DMSO (v/v). The subcellular localization of NF- κ B/p65 was observed directly by LSCM. a) FITC-labeled NF- κ B/p65 in green. b) PI-stained nuclei in red. c) a merged with b. 1) Blank control, without LPS stimulation. 2) LPS-only control. 3) 10 $\mu\text{mol/L}$ lobolide with LPS stimulation. 4) 5 $\mu\text{mol/L}$ TPCA-1 with LPS stimulation. (B) THP-1 cells were seeded at a density of 2×10^6 cells/mL and pretreated with different concentrations of lobolide (2.5, 5, 7.5, and 10 $\mu\text{mol/L}$) for 15 min prior to treatment with 1 $\mu\text{g/mL}$ LPS for 1 h. Blank and LPS-only controls each contained 0.1% DMSO (v/v). Cell lysate was separated by SDS-PAGE and immunoblotted with p65- and histone H3-specific primary antibodies.

NF- κ B/p65 proteins in nuclear extracts treated by lobolide were markedly decreased compared to those treated only by LPS (Figure 5B). Furthermore, the NF- κ B/p65 nuclear translocation stimulated by LPS was impaired by lobolide in a dose-dependent manner (Figure 5B). These data confirmed that lobolide inhibited LPS-induced NF- κ B/p65 nuclear translocation.

Lobolide reduced LPS-induced cytokine production

In most studies, inflammatory cytokine production from mononuclear cells has been investigated by stimulating cells with the gram-negative bacterial component LPS. TNF α and IL-1 β are the two main cytokines produced by THP-1 cells and PBMCs in response to LPS stimulation. Their expression is regulated by the NF- κ B transcription factor^[21]. Because lobolide may inhibit NF- κ B translocation from the cytoplasm to the nucleus, we examined TNF α and IL-1 β production induced by LPS in the absence or presence of varying concentrations of lobolide. The results showed that lobolide significantly inhibited TNF α and IL-1 β production induced by LPS in THP-1 cells and PBMCs in a dose-dependent manner (2.5, 5, 7.5, and 10 μ mol/L) (Figure 6). The results also suggested that lobolide effectively blocks the transcription activity of NF- κ B. However, the mechanism responsible for this inhibition remains uncertain.

Lobolide attenuated phosphorylation of TAK1 and ERK induced by LPS

The exact mechanism underlying LPS-activated-NF- κ B signaling has not yet been fully addressed, but TGF β -activated kinase (TAK1) and I κ B kinases (IKKs) have been suggested to be two important factors^[22]. We considered whether lobolide inhibited NF- κ B translocation by blocking the TAK1-IKK-NF- κ B signaling pathway. To address this question, THP-1 cells were treated with 2.5, 5, and 10 μ mol/L lobolide, while cells treated with 2 μ mol/L and 5 μ mol/L TPCA-1 served as positive controls; Western blot analyses were subsequently performed. Similar to the effects observed in the positive con-

trol TPCA-1-treated cells, lobolide inhibited the LPS-activated phosphorylation of TAK1, IKK α / β , and I κ B α in a dose-dependent manner and consequently also impaired I κ B α degradation (Figure 7A). In addition to the TAK1-IKK-NF- κ B signaling pathway activated by LPS in monocytes, two major MAPK signaling pathways, which are the ERK1/2 (the extracellular signal-regulated kinases 1 and 2) and p38 kinase signaling pathways, are also involved in the inflammatory response^[23]. Indeed, lobolide also blocked the two MAPK signaling pathways by inhibiting the phosphorylation of ERK1/2 and p38 (Figure 7B).

Figure 7. Lobolide inhibited the phosphorylation of TAK1, IKK α / β , I κ B α , ERK1/2, and p38 stimulated by LPS and, accordingly, blocked I κ B α degradation induced by LPS. THP-1 cells were seeded at a density of 2×10^6 cells/mL and pretreated with different concentration of lobolide (2.5, 5, and 10 μ mol/L) and TPCA-1 (2 and 5 μ mol/L) for 15 min followed by stimulation with 1 μ g/mL LPS for 30 min. Blank and LPS-only controls each contained 0.1% DMSO (v/v). TPCA-1 (2 and 5 μ mol/L) was used as a positive control. The cell lysate was subjected to SDS-PAGE and probed with antibodies of TAK1, phosphorylated-TAK1, IKK α / β , phosphorylated IKK α / β , I κ B α , phosphorylated I κ B α , Erk1/2, phosphorylated Erk1/2, p38, and phosphorylated p38 and β -actin (as a loading control).

Figure 6. Effects of lobolide on LPS-induced cytokine production in THP-1 cells and PBMCs. THP-1 cells (A) were seeded at a density of 1×10^5 cells/well and pretreated with different concentration of lobolide (2.5, 5, 7.5, and 10 μ mol/L) for 15 min, and then stimulated with 1 μ g/mL LPS for 24 h. Blank and LPS-only controls each contained 0.1% DMSO (v/v). After centrifugation, the supernatants of the cultured media were collected for ELISA. PBMCs (B) were seeded at a density of 3×10^5 cells/well and treated as described in A, followed by ELISA quantitation. ^b $P < 0.05$, ^c $P < 0.01$ with respect to treatment with the LPS-only control.

Discussion

NF- κ B is an inducible transcription factor that plays an important role in several aspects of human health and disease. The dysregulation of NF- κ B and its related pathway is associated with many diseases, ranging from inflammation, cancer, and viral infection to genetic disorders^[24]. Because the molecular details are well elucidated, many studies have focused on the pharmacological intervention in the NF- κ B signaling pathway for new drug development. The present study focuses on the search for new bioactive molecules with the same purpose. As a member of the diterpenoid group, and extracted from the *Lobophytum* sp, lobolide has previously shown nearly no biological activities except its toxicity to fish. However, we demonstrated that lobolide significantly inhibited the production of the proinflammatory cytokines TNF α and IL-1 β at low concentrations (10 μ mol/L). These inhibitory effects were mainly caused by a concentration-dependent decrease of NF- κ B proteins in the nucleus translocated from the cytoplasm after LPS stimulation. Lobolide also impaired p38 and Erk1/2 MAPKs and TAK1 signaling activated by LPS in monocytes/macrophages. Our data suggest that lobolide might inhibit TNF α and IL-1 β products by blocking the NF- κ B signaling pathway by attenuating the phosphorylation of TAK, IKK, I κ B α , Erk1/2, and p38 as well as I κ B α degradation to interfere with p65 translocation from the cytoplasm to nuclei.

TAK1 was first reported as a regulator of MAPK signaling activated by TGF- β ^[25, 26] and is evolutionally conserved. Drosophila TAK1 is essential for antibacterial innate immunity^[27]. When TAK1 is kinase-negatively mutated, the LPS induced NF- κ B activation is inhibited^[28]. Thus, TAK1 is a critical mediator in the LPS-induced signaling pathway. With LPS stimulated, TAK1 is able to be phosphorylated and form a complex with TRAF6/TAB1/TAB2, which then together translocate from the membrane to the cytosol^[22]. The activated TAK1 then phosphorylates downstream targets, such as the IKKs, which leads to the degradation of I κ B α and consequent release of NF- κ B. TAK1 can also activate p38 by LPS stimulation^[29]. Interestingly, p38 also regulates NF- κ B-dependent gene transcription by modulating activation of TBP (a TATA-binding protein). TBP is very important for transcriptional regulation of NF- κ B by binding to the carboxyl terminus of p65^[30]. Thus, we might conclude that the low expression of IL-1 β and TNF α in cells treated by lobolide is the combined consequence of inhibition of both TAK1 and p38 phosphorylation and activation. Erk1/2 are other MAP kinases activated by LPS. The activation of Erk1/2 can activate the AP-1 transcription factor and thus lead to iNOS synthesis and NO release, which are well-known inflammatory mediators^[31]. Lobolide attenuates p38 and Erk1/2 phosphorylation and activation, the coordination of which is critical for the inflammatory response.

In summary, we showed that the production of the proinflammatory cytokines TNF α and IL-1 β was blocked by lobolide. The results revealed that lobolide affected multiple signaling pathways stimulated by LPS in THP-1 cells. Lobolide was implicated in the inhibition of the phosphorylation and activation of TAK1, p38 MAP kinase and Erk1/2 MAP kinase.

Thus, lobolide might be a lead compound for anti-inflammatory drug development.

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Author contribution

Xiao-fen LV: experimental design, data collection and analysis, manuscript writing; Si-han CHEN: data collection and assembly; Jie LI: data collection and assembly; Jian-ping FANG: conception and design; Yue-wei GUO: preparation of the compounds, conception and design; Kan DING: conception and design, manuscript revision.

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