Maackiapterocarpan B from *Sophora tonkinensis* Suppresses Inflammatory Mediators via Nuclear Factor-κB and Mitogen-Activated Protein Kinase Pathways

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Maackiapterocarpan B, one of the pterocarpan analogs found in *Sophora tonkinensis*, is known to display pharmacological activities. However, the anti-inflammatory effects of maackiapterocarpan B and its molecular mechanism have yet to be clearly elucidated. In the present study, the effects of maackiapterocarpan B on macrophage-mediated inflammation in vitro were assessed. Maackiapterocarpan B inhibited the production of nitric oxide, the expression of tumor necrosis factor α, colony stimulating factor 2, interleukin-1β, and interleukin-6, and the activation of nuclear factor-κB and mitogen-activated protein kinases in lipopolysaccharide-stimulated macrophages. These observations suggest the potential of maackiapterocarpan B in the treatment of inflammatory diseases.

**Key words** maackiapterocarpan B; macrophage; nitric oxide; nuclear factor kappa B; mitogen-activated protein kinase

Inflammation is a beneficial reaction to foreign challenge or injury that leads to restoration of function. ¹⁻³ In an immune response, active phase is followed by a maintained response, which fades and ends when the processes triggered by the initial responses are stopped. Thus, accurate coordination and timely resolution of the inflammatory response are critical in maintaining the balance between health and disease. ⁴⁻⁵ Macrophages play an important role in immune system that plays an indispensable role in homeostasis and defense. ⁶ Their differentiation, growth, and distribution have been studied extensively during development, in the ordinary people, and after various immunological stimuli. ⁷ Lipopolysaccharide (LPS), which is known as endotoxins, elicits several signaling pathways in macrophages by acting on toll like receptor 4 (TLR4) to induce the expression of inflammatory genes and the release of mediators such as cytokines and nitric oxide (NO), all of which are involved in the pathogenesis of many diseases. ⁸⁻¹⁰ Therefore, control of NO over-production may provide a measure to assess the effects of drugs on the inflammatory response.

Nuclear factor Kappa B (NF-κB) is an important transcription factor, controlling the gene expression of cytokines, chemokines, and growth factors in both normal and abnormal states. ¹¹ The activation of NF-κB subsequently promotes the transcription of a number of genes involved in inflammation including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). ¹² Inappropriate activation of NF-κB or mitogen-activated protein kinases (MAPKs) have been shown to be associated with the pathophysiological mechanisms of cancer and inflammation. ¹³ Therefore, the discovery of active compounds from natural products that can regulate the activation of NF-κB and MAPKs may lead to the development of anti-inflammatory therapeutics.

As part of our ongoing search to discover anti-inflammatory agents from medicinal plants, ¹⁴⁻¹⁵ *Sophora tonkinensis* was selected due to previous reports related to the modulation of inflammatory responses. ¹⁶⁻¹⁸ Currently, the proprietary extract from *S. tonkinensis* is under clinical trials in the Republic of Korea for the treatment of asthma. ¹⁹ All the compounds obtained from the former study ²⁰ were evaluated for their inhibitory activity against NO production in LPS-induced RAW264.7 cells. Of the tested compounds, maackiapterocarpan B was found to significantly inhibit NO production. Maackiapterocarpan B, a pterocarpan-type flavonoid, is known to display cytotoxic and interleukin (IL)-6 inhibitory activity, ²¹⁻²² however, its anti-inflammatory effect on macrophages remains unknown. In the present study, maackiapterocarpan B is evaluated for its inhibitory effect on inflammatory mediator production, activation of NF-κB and MAPKs in LPS-induced RAW264.7 cells, and mRNA expression of cytokines.

**MATERIALS AND METHODS**

**Preparation of Maackiapterocarpan B** The test compound, maackiapterocarpan B (Fig. 1), was isolated from the roots of *Sophora tonkinensis* according to a previous report, and was provided by one of the authors. ²³ The structure was confirmed by comparing the measured ¹H- and ¹³C-NMR data with the published values. The purity of this compound was determined to be over 95% using HPLC-photo diode array (PDA) detection.

**Cell Culture** Murine macrophages, RAW264.7, were obtained from the Korean Research Institute of Bioscience and Biotechnology (South Korea), and grown in RPMI medium containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin sulfate. Cells were incubated in a humidified 5% CO₂ atmosphere at 37°C.

**Drugs and Chemicals** RPMI, penicillin, and streptomycin were purchased from Hyclone (Logan, UT, U.S.A.). Bovine serum albumin and LPS were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-mouse IL-6 antibody and
biontinated anti-mouse IL-6 antibody were purchased from BD Biosciences (BD Pharmingen, San Diego, CA, U.S.A.). Antibodies against iNOS, COX-2, phospho-inhibitor of IκB kinase (p-IKK), IKK, Lamin B, IκBα, p-p65 (NF-κB), p65 (NF-κB), β-actin, phospho-extracellular signal-regulated kinase (p-ERK), ERK, phospho-c-Jun N-terminal kinase (p-JNK), JNK, p-p38, and p38 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Oligonucleotide primers for TNF-α, colony-stimulating factor (CSF2), IL-6, IL-1β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Bioneer Corp. (Daejeon, Korea).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay for Cell Viability Cells were seeded into 96-well plates at a density of 5×10^4 cells/well and incubated with serum-free media in the presence of different concentrations of maackiapterocarpan B. Following incubation for 24h, 10 µL MTT (5 mg/mL in saline) was added and incubation was continued for further 4h. Mitochondrial succinate dehydrogenase in live cells converts MTT into visible formazan crystals during incubation. The formazan crystals were then solubilized in dimethyl sulfoxide and the absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Benchmark, Bio-Rad Laboratories, CA, U.S.A.). Relative cell viability was calculated compared with the absorbance of the untreated control group. All experiments were performed in triplicate.

Measurement of NO Production The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction.20 RAW264.7 cells (2×10^5 cells/well) were cultured in 96-well plates using RPMI without phenol red, and pretreated with different concentrations of maackiapterocarpan B for 1h. Cellular NO production was induced by the addition of 100 ng/mL final concentration LPS and incubation for 24h. Following incubation, 100 µL conditioned media was mixed with the same volume of Griess reagent and incubated for 15 min. The absorbance of the mixture at 540 nm was measured with an ELISA microplate reader (Benchmark, Bio-Rad Laboratories, CA, U.S.A.). The values obtained were compared with those of standard concentrations of sodium nitrite dissolved in RPMI, and the concentrations of nitrite in the conditioned media of sample-treated cells were calculated.

Immunoblotting Analysis Protein expression was assessed by Western blotting analysis according to the standard procedure. Briefly, HMC-1 were cultured in 60-mm culture dishes (2×10^5/mL) and pretreated with various concentrations of maackiapterocarpan B (5, 10, and 20 µM). After a 30-min pretreatment, LPS was added to the culture medium, and the cells were then incubated at 37°C for 30 min. Following incubation, the cells were washed twice in ice cold phosphate buffered saline (PBS) (pH 7.4). The sample-treated cell pellets were then resuspended in lysis buffer on ice for 15 min, after which the cell debris was removed by centrifugation. Protein concentration was then determined using Bio-Rad protein assay reagent according to the manufacturer’s instructions. Protein (20–30 µg whole cell) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% sodium dodecyl sulfate (SDS), 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris [pH 6.8]), loaded onto an 8% or 15% SDS-polyacrylamide gel electrophoresis (PAGE) gel, and run at 150 V for 90 min. Cellular proteins were transferred to an Immobilon polyvinylidene difluoride membrane (Bio-Rad) using a Bio-Rad semidry transfer system according to the manufacturer’s instructions. The membrane was then incubated with primary antibody (dilutions 1:500–1:1000) in Tris-buffered saline with 0.1% Tween 20 and 5% milk overnight. The blots were washed three times with Tris-buffered saline with 0.1% Tween 20 and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary anti-immunoglobulin G (IgG) antibody (dilution 1:2000–1:20000). The blots were washed again three times with Tris-buffered saline with 0.1% Tween 20, and immunoreactive bands were developed using the chemiluminescent substrate ECL Plus (Amerham Biosciences, Piscataway, NJ, U.S.A.).

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total cellular RNA was isolated using a Trizol RNA extraction kit according to the manufacturer’s instructions. Briefly, total RNA (1 µg) was converted to cDNA by treatment with 200 units reverse transcriptase and 500 ng oligo-dT primer in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), and 1 mM deoxyribonucleotide triphosphates (dNTPs) at 42°C for 1 h. The reaction was then stopped by incubating the solution at 70°C for 15 min, after which 1 µL cDNA mixture was used for enzymatic amplification. PCR reactions were performed using 1 µL cDNA and 9 µL master mix, containing iQ SYBR Green Supermix (Bio-Rad), 5 pmol forward primer, and 5 pmol reverse primer, in a CFX384 Real-Time PCR Detection System (Bio-Rad) as follows: 3 min at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 55°C. The fluorescence signal generated with SYBR Green I DNA dye was measured during annealing steps. Specificity of the amplification was confirmed using a melting curve analysis. Data were collected and recorded by the CFX Manager Software (Bio-Rad) and expressed as a function of threshold cycle (Ct). The relative quantity of the gene of interest was then normalized to the relative quantity of hypoxanthine phosphoribosyltransferase (ΔΔCt). The sample mRNA abundance was calculated by the equation 2^-ΔΔCt. Specific primer sets used are as follows (5’ to 3’): IL-6 CAA AGC CAG AGT CCT TCA GAG (forward), GCC ACT CTT CTC TGT GAC TCC (reverse); GAPDH TGT TTC TAC CCC CAA ATG TGT (forward), TGT GAG GGA GAT GCT CAG TG (reverse); IL-1β GAC CTT CAC CGA GAT GGA ACA (forward), TGG TCA TCT CGG AGC CCT GT A (reverse); TNF-α CAA ATG GCC TCC CTC TCA T (forward), TGG GCT ACA GCG CT TCA (reverse); COX-2 GCC CTT GGA AGC ATG TGA AG (forward), TGG GCT ACA GCG CT TCA (reverse); iNOS TGA CAC AAG GCT CTA CAA AA (forward), CCA ACT TGA TGT GAC GGA (reverse). Gene-

![Fig. 1. Structure of Maackiapterocarpan B](image-url)
specific primers were custom-synthesized by and purchased from Bioneer, Daejeon, Korea.

**Immunofluorescence** RAW264.7 cells cultured on Permanox plastic chamber slides were fixed with ethanol for 30 min at 4°C. After washing with PBS and blocking with 3% bovine serum albumin in PBS for 30 min, samples were incubated overnight at 4°C with rabbit monoclonal anti-COX2, anti-iNOS, and anti-p65 (NF-κB) (1:200 dilution, Abcam, Cambridge, MA, U.S.A.). The excess primary antibody was removed, slides were washed with PBS, and the samples were incubated with Alexa 488-conjugated and Alexa 594-conjugated secondary antibodies (Invitrogen Molecular Probes, Burlington, ON, Canada) for 2 h at room temperature. After washing with PBS, slides were mounted using ProLong Gold
Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Thermo Scientific, U.S.A.) to visualize the nuclei. Specimens were covered with coverslips and evaluated under a confocal laser scanning microscope (LSM510 meta, Carl Zeiss, Germany).

Statistical Analysis Experimental data are presented as the mean±standard error of the mean (S.E.M.). The level of statistical significance was determined by ANOVA followed by Dunnett’s t-test for multiple comparisons. p Values less than 0.05 were considered significant.

RESULTS

Toxicity of Maackiapterocarpan B on Macrophages To determine the effect of maackiapterocarpan B on cell viability, various concentrations of maackiapterocarpan B were tested with the MTT assay using RAW264.7 cells grown in serum-free medium. As shown in Fig. 2, neither maackiapterocarpan B nor dimethyl sulfoxide (DMSO) exerted any significant toxic effect on RAW264.7 cells under the tested concentrations after 24 h of treatment. Therefore, non-toxic concentrations of maackiapterocarpan B were used in all the experiments.

NO Inhibitory Activity of Maackiapterocarpan B in RAW264.7 Cells The effect of maackiapterocarpan B on LPS-induced NO production in RAW264.7 cells was investigated by measuring the amount of nitrite released into the culture medium using the Griess reaction. Maackiain was used as a positive control since it has been known as potent inhibitor of nitric oxide in RAW264.7 cells.22) The three concentrations of maackiapterocarpan B were tested with the MTT assay using RAW264.7 cells grown in serum-free medium. As shown in Fig. 2, neither maackiapterocarpan B nor dimethyl sulfoxide (DMSO) exerted any significant toxic effect on RAW264.7 cells under the tested concentrations after 24 h of treatment. Therefore, non-toxic concentrations of maackiapterocarpan B were used in all the experiments.

Fig. 4. Inhibition of iNOS and COX-2 Protein Expression by Maackiapterocarpan B

(A) RAW264.7 cells were pretreated with different concentrations of maackiapterocarpan B for 30 min and stimulated with LPS (250 ng/mL) for a further 24 h. Equal amounts of protein in the cell lysates were electrophoresed and the protein expression levels of iNOS and COX-2 were determined using specific antibodies against iNOS and COX-2. (B) Effect of maackiapterocarpan B on the expression of iNOS and COX-2 mRNA in LPS-stimulated RAW264.7 cells by qRT-PCR. †Significant difference from normal, *significant difference from LPS, p<0.05, and **significant difference from LPS, p<0.01. (C) Expression of iNOS and COX-2 by maackiapterocarpan B. Cells were cultured for 24 h with LPS (250 ng/mL), fixed, permeabilized, and incubated with a rabbit polyclonal anti-iNOS antibody followed by an Alexa-488-conjugated anti-rabbit Ig, and with a mouse polyclonal anti-COX-2 antibody followed by an Alexa-594-conjugated anti-mouse Ig. The nuclei of the corresponding cells were visualized using DAPI, (A) and (B) staining. Normal, untreated control cells; LPS, only LPS (250 ng/mL) treatment; MB, maackiapterocarpan B (magnification: ×60, scale bars: 50 µm).
stituents of *Sophora tonkinensis* (trifolirhizin, maackiaain, and maackiapterocarpan B) were tested for their NO inhibitory activity, and the results are shown in Fig. 3. Maackiapterocarpan B suppressed nitrite production in a concentration-dependent manner with an IC$_{50}$ value of 8.12 µM.

### iNOS and COX-2 Expression Levels as a Result of Maackiapterocarpan B in RAW264.7 Cells

iNOS and COX-2 are the inflammatory factors associated with LPS stimulation. To investigate the anti-inflammatory activity of maackiapterocarpan B, we tested the effects of maackiapterocarpan B on LPS-induced iNOS and COX-2 upregulation in RAW264.7 cells by Western blotting analysis and immunofluorescence. As shown in Fig. 4A, in untreated cells, iNOS and COX-2 protein expression were not detected in unstimulated cells, but had markedly increased by 24 h after stimulation with 250 ng/mL LPS. Cells pretreated with maackiapterocarpan B (5–20 µM) showed a concentration-dependent inhibition of iNOS and COX-2 protein expression following LPS stimulation for 24 h. As shown in Fig. 4B, iNOS and COX-2 mRNA expression were not detected in normal cells, however, increased markedly after treatment with 250 ng/mL LPS for 24 h compared with the normal control. Cells pretreated with maackiapterocarpan B showed a concentration-dependent inhibition of iNOS and COX-2 mRNA expression following LPS stimulation for 24 h.

### Activation of the MAPK Pathway by Maackiapterocarpan B

In order to elucidate the underlying mechanisms of maackiapterocarpan B, we examined the effects of maack-
iapterocarpan B on the activation of mitogen-activated protein kinases. The stimulation of RAW264.7 cells with LPS resulted in an increased phosphorylation of all three types of MAPKs, p38, JNK, and ERK 30 min post-treatment. Maackiapterocarpan B suppressed the phosphorylation of ERK1/2 and JNK1/2, but had no effect on the phosphorylation of p38 MAPK (Fig. 5).

Modulation of the NF-κB Pathway by Maackiapterocarpan B

We also investigated whether maackiapterocarpan B prevents the translocation of the p65 subunits of NF-κB from the cytosol to the nucleus after their release from IκBα. Nuclear and cytosolic extracts were prepared and subjected to immunoblotting analysis. Lamin B1 (a nuclear protein) and β-actin (a cytosolic protein) were used as controls to confirm that there was no contamination during the extraction of each fraction. We found that p65 was distributed in the cytosolic compartment before LPS stimulation but accumulated in the nucleus after LPS stimulation (Fig. 6A). Confocal microscopy analyses revealed that in unstimulated cells, NF-κB (p65) was present mostly in the cytosol. Following LPS treatment, the majority of the intracellular p65 was translocated from the cytosol into the nucleus, as shown by the strong NF-κB (p65) staining in this organelle (Fig. 6B). The level of p65 in the nuclear fraction was significantly reduced by pretreatment with 20 µM maackiapterocarpan B, and was almost abolished by 20 µM maackiapterocarpan B in Western blotting and immunofluorescence analysis (Fig. 6). In addition, maackiapterocarpan B inhibited the induced degradation of IκBα as well as the LPS-induced increase in p65 in the nuclear fraction, indicating that the extract inhibits the degradation of IκBα, thereby preventing the expression of NF-κB-regulated genes.

Control of Cytokines by Maackiapterocarpan B

NF-κB and MAPKs act as the major regulators for the expression of cytokine genes following stimulation with LPS. We examined the effect of maackiapterocarpan B on the expression of TNF-α, CSF2, IL-1β, and IL-6 mRNA in macrophages. As shown in Fig. 7, pretreatment with maackiapterocarpan B significantly lowered the expression of TNF-α, CSF2, IL-1β, and IL-6 in LPS-induced RAW264.7 cells (Fig. 7).

DISCUSSION

iNOS expression, which catalyzes the oxidative deamination of L-arginine to produce NO, a potential immune mediator, is induced by LPS or cytokines in immune cells. Overproduction of NO appears to be connected to tissue damage and organ dysfunction. In the present study, we demonstrate that maackiapterocarpan B inhibited LPS-induced NO production in RAW264.7 cells. Our results show that the inhibition of NO production in LPS-stimulated RAW264.7 cells by maackiapterocarpan B occurred via the inhibition of iNOS protein expression in a dose-dependent manner. COX-2 is another crucial enzyme in the inflammation process. COX-2 is scarcely detectable under normal physiological conditions; however, it can be rapidly induced in macrophages by stimuli including endotoxin, and cytokine. Activated COX-2 converts
arachidonic acid to prostanoids (including prostaglandins, and thromboxanes) causing edema, and pain at the site of the inflammation.25,26) In our study, we demonstrate that maackiapterocarpan B also inhibited COX-2 protein and mRNA expression.

Since ERK, JNK, and p38 MAPKs are known to be associated with LPS-mediated induction of iNOS and COX-2 in macrophages,27,28) we investigated the effect of maackiapterocarpan B on the activation of MAPKs in LPS-induced macrophages. When cells were pretreated with maackiapterocarpan B and LPS for 30 min, maackiapterocarpan B was found to attenuate the LPS-stimulated activation of ERK and JNK, but did not suppress the expression of p38 MAPK. The expression of many inflammatory genes is regulated through the NF-κB pathway.29) Under normal conditions, NF-κB is impounded within the cytosol as an inactive complex bound to IκBa. Following activation by various stimuli, IκB is phosphorylated by the IκB kinases, IKKα and IKKβ, and then the IκB subunit is rapidly degraded by proteasomes.30,31) In our study, we examined NF-κB and IKK phosphorylation as well as the degradation of IκBa. Immunoblotting analysis shows that the inhibition of NF-κB activity by maackiapterocarpan B may be a result of the inhibition of IκBa degradation. Moreover, we found that LPS-induced NF-κB p65 translocation from the cytosol to the nucleus was strongly inhibited by maackiapterocarpan B treatment.

In conclusion, our results demonstrate that maackiapterocarpan B regulated the expression of nitric oxide, TNF-α, CSF2, IL-1β, and IL-6 mRNA in LPS-stimulated macrophages. Maackiapterocarpan B also decreased iNOS and COX-2 expression. Moreover, maackiapterocarpan B suppressed the ERK1/2, JNK1/2, and NF-κB pathways. Thus, maackiapterocarpan B appears to exert anti-inflammatory effects via the inhibition of NF-κB and MAPK activation in macrophages, thereby inhibiting the expression of iNOS and COX-2.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials (1H-NMR spectra and 13C-NMR spectral data for maackiapterocarpan B).

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