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Anti-inflammatory Mechanism of 15,16-Epoxy-3α-hydroxylabda-8,13(16),14-trien-7-one via Inhibition of LPS-Induced Multicellular Signaling Pathways

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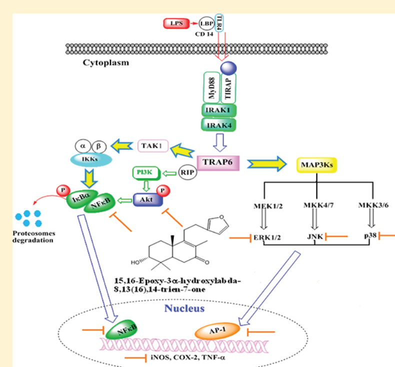
Supporting Information

ABSTRACT: Phytochemical investigation of *Leonurus japonicus* has led to the isolation of a labdane diterpene derivative, 15,16-epoxy-3α-hydroxylabda-8,13(16),14-trien-7-one (**1**), which was tested for its in vitro anti-inflammatory effects. The results demonstrated that **1** exhibits an inhibitory effect on LPS-stimulated RAW 264.7 macrophages. The anti-inflammatory action shown by **1** suppressed LPS-induced NF-κB activation, resulting in the down-regulation of iNOS and COX-2 protein expression, attributable to the inhibitory action of LPS-induced NO and PGE_2 production. Compound **1** inhibited LPS-induced phosphorylation and the degradation of inhibitory kappa B (IκBα) and decreased the nuclear translocation of p50 and p65. In addition, **1** exhibited an inhibitory effect on LPS-induced NF-κB-DNA and AP-1-DNA binding activity, using an electrophoretic mobility shift assay with NF-κB- and AP-1-specific ^{32}P-labeled probes. The LPS-induced mitogen-activated protein kinases (p-JNK, p-p38, and p-ERK) and p-Akt were inhibited after 30 and 10 min of LPS stimulation, respectively. In addition, TNF-α production was suppressed by **1**.



Inflammatory responses require the coordinated activation of various signaling pathways that regulate the expression of pro-inflammatory mediators.[1] Inflammation also has a significant role in priming the immune response to generate immunological memory.[2] The deregulation of this complex pathophysiological process may lead to chronic inflammation or inappropriate priming of adaptive immunity and autoimmune diseases.[1]

The NF-κB pathway has been implicated strongly in the pathogenesis of chronic inflammatory diseases.[1,2] NF-κB appears to play a pleiotropic role in the immune and inflammatory responses. However, the recent description of a second, evolutionarily conserved, NF-κB pathway has revealed new insights into the regulation of NF-κB activation and the role of this pathway in innate and adaptive immunity.[3−5] NF-κB and activator protein 1 (AP-1) are key transcription factors that coordinate expression of several inflammatory genes.[6] Even though NF-κB and AP-1 are regulated by different mechanisms, they appear to be activated concurrently by the same multitude of stimuli.[7] Numerous reports have demonstrated that these transcription factors appear to be regulated by the same intracellular signal transduction cascades. Various inflammatory and cancer mediators require the concomitant activation of AP-1 and NF-κB.[8] Moreover, the activation of other signaling pathways, such as PI3K/Akt and MAPK, is also involved in the activation of NF-κB.[5,9]

Akt, also known as protein kinase B (PKB), is a serine/threonine protein kinase that plays a key role in multiple cellular processes.[8] Numerous reports have confirmed the convergence of the NF-κB and Akt signaling pathways.[8,9] Indeed, IκB kinase is a substrate of Akt, involved in NF-κB activation; thus, activation of Akt stimulates NF-κB. The modulation of Akt activity can affect the pro-inflammatory mediators through NF-κB activation.[9,10]

The mitogen-activated protein kinases (MAPKs) are a group of signaling pathways that play a vital role in the regulation of cell differentiation and growth, as well as in the control of cellular responses to cytokines and stresses.[5,11] The MAPKs are the extracellular signal-regulated kinase (ERK), the p38 mitogen-activated protein kinase (p38 MAPK), and the c-Jun NH_2-terminal kinase (JNK).[6] The phosphorylation of MAPKs is known to be a critical component in the production of NO and pro-inflammatory cytokines in activated macrophages.[5,11]

Leonurus japonicus Miq. (Lamiaceae) is used widely as a traditional remedy for various therapeutic purposes, such as its purported antiarrhythmic, antimicrobial, anticoagulant, antioxidant, and anticancer effects.[12−15] Several metabolites have been isolated from *L. japonicus* that substantiate these recorded properties. Some labdane diterpenoids isolated from plants in the genus *Leonurus* are used for the treatment of cardiovascular

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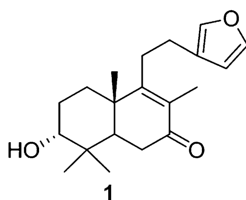
disease and for their sedative and uterotonic effects. Labdanes demonstrate a significant potential as new pharmacological agents.[16] Recently, some labdane diterpenes have been shown to exert anti-inflammatory,[17] neuroprotective,[18] antispasmodic,[19] and cytotoxic and trypanocidal[20] activities.

As part of a research project into the biological activities of terpenoids, the naturally occurring labdane compound 15,16-epoxy-3α-hydroxylabda-8,13(16),14-trien-7-one (**1**) has been investigated as a potential anti-inflammatory agent. To evaluate the mechanism of action of **1**, targets relevant to the regulation of the inflammatory response were studied. The present investigation was focused on the detailed anti-inflammatory mechanism of **1**, isolated from *L. japonicus*, in terms of its activity on LPS-stimulated macrophages that influence three different cellular signaling pathways.



■ RESULTS AND DISCUSSION

The cytotoxic effects of **1** on LPS-stimulated RAW 264.7 macrophages were determined initially. The results demonstrated that **1** did not affect cell viability at concentrations up to 50 μM. Therefore, nontoxic concentrations were used in subsequent experiments on **1**.

NO is involved in various biological processes, including inflammation and immunoregulation.[21,22] Therefore, the inhibition of nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) may have potential therapeutic value when related to inflammation-associated diseases. Compound **1** displayed an inhibitory effect on NO production, with an IC_{50} value of 27.0 μM.

To investigate whether the inhibitory effect of **1** on NO production was via the inhibition of the corresponding gene expression, the protein iNOS was evaluated by Western blot analysis (Figure S1, Supporting Information). Additionally, cyclooxygenase-2 (COX-2) protein expression was also evaluated (Figure S1, Supporting Information). In unstimulated RAW 264.7 cells, the iNOS and COX-2 protein expression levels were almost undetectable. However, after LPS treatment, the protein expression levels of iNOS and COX-2 were augmented markedly, and pretreatment of the cells with different concentrations of **1** attenuated LPS-induced iNOS and COX-2 protein and gene expression in a concentration-dependent manner. These data suggest that **1** can down-regulate LPS-induced iNOS and COX-2 expression at the transcriptional level.

NF-κB transcription factor has been shown to play a significant role in LPS-induced expression of pro-inflammatory mediators, including iNOS and COX-2.[23] To investigate the molecular mechanism of inhibition of iNOS and COX-2 transcription mediated by **1**, NF-κB transcriptional activity was investigated using a reporter gene assay system. RAW 264.7 cells were stably transfected with a pNF-κB-secretory alkaline phosphatase (SEAP)-NPT plasmid containing four copies of the κB sequence fused to SEAP as the reporter.[22−24] LPS treatment of the transfected cells for 18 h increased the SEAP expression approximately 6.6-fold over the basal level (Figure 1). The pretreatment of cells with **1** inhibited LPS-induced SEAP expression significantly in a concentration-dependent manner, corresponding to 20.9 ± 6.1% at 5 μM, 56.6 ± 4.6% at 15 μM, 69.1 ± 0.7% at 25 μM, and 90.3 ± 2.5% at 50 μM. As a positive control, *N*-*p*-Tosyl-L-phenylalanyl chloromethyl ketone (TPCK) also showed a significant inhibitory effect on NF-κB transcription activity (Figure 1).

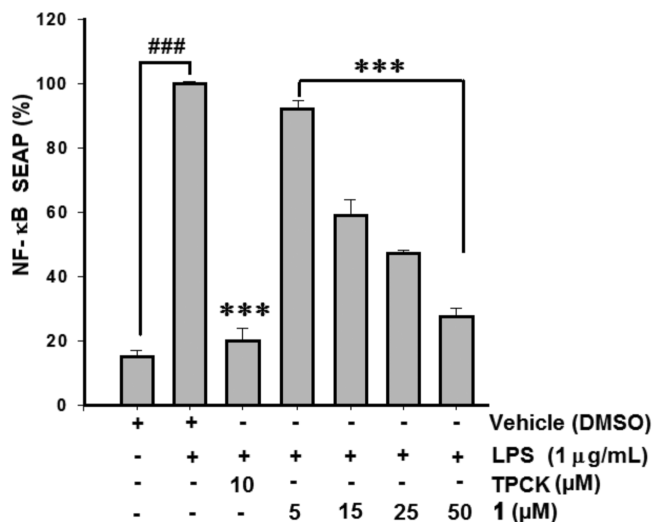


Figure 1. Effect of **1** on NF-κB-dependent alkaline phosphatase (SEAP) expression in transfected RAW 264.7 macrophages. Data were derived from three independent experiments and are expressed as the means ± SD. (***) $p < 0.001$ indicates a significant difference from the LPS (1 μg/mL)-challenged for 18 h incubation group. (###) $p < 0.001$ indicates a significant difference from the unstimulated control group. *N*-*p*-Tosyl-L-phenylalanyl chloromethyl ketone (TPCK 10 μM) was used as a positive control.

Since phosphorylation of inhibitory kappa B (IκB) and its subsequent degradation is a critical step in NF-κB activation by various stimuli,[2] the effect of **1** on LPS-induced degradation and phosphorylation of IκBα protein was investigated by immunoblot analysis. A time-course experiment showed that the phosphorylated forms of IκBα were barely detectable at 5 min in LPS-stimulated RAW 264.7 cells. However, on exposure to LPS (1 μg/mL) alone for 15 min, IκBα phosphorylation was manifested. LPS-mediated IκBα phosphorylation was inhibited by **1** after 15 and 20 min at 50 μM (Figure 2A). Furthermore,

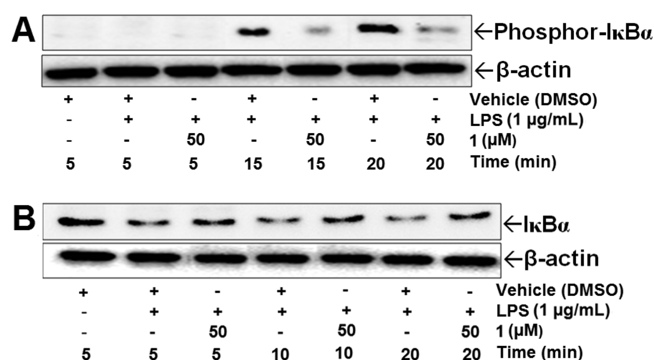


Figure 2. Effect of **1** on (A) the expression of phosphorylated IκBα and (B) IκBα protein in cytosolic extracts determined by Western blot analysis, as described in the Experimental Section. The RAW 264.7 cells were pretreated with 50 μM **1** for 2 h and treated with LPS (1 μg/mL) for the time periods specified.

the IκBα degradation was completely protected after 10 and 20 min of LPS (1 μg/mL) stimulation at 50 μM (Figure 2B).

To elucidate the inhibitory mechanism on NF-κB and AP-1 activation, the DNA binding activity of NF-κB and AP-1 of **1** in LPS-stimulated RAW 264.7 macrophages was determined, which was analyzed by electrophoretic mobility shift assay (EMSA) with NF-κB- and AP-1-specific ^{32}P-labeled oligonucleotides. The RAW 264.7 cells increased the DNA binding activity of NF-κB and AP-1 complexes significantly upon exposure to LPS alone for 1 h (Figures 3 and S2, Supporting Information). On the other hand, **1** suppressed the LPS-induced DNA binding activity of NF-κB and AP-1 complexes in a dose-dependent manner (Figures 3 and Figure S2, Supporting Information). For the competition assay, EMSA were performed with excess amounts of unlabeled NF-κB oligonucleotide. The results obtained showed that the LPS-stimulated nuclear extract with excess unlabeled NF-κB oligonucleotide before EMSA abolished NF-κB DNA binding (Figure S3, Supporting Information).

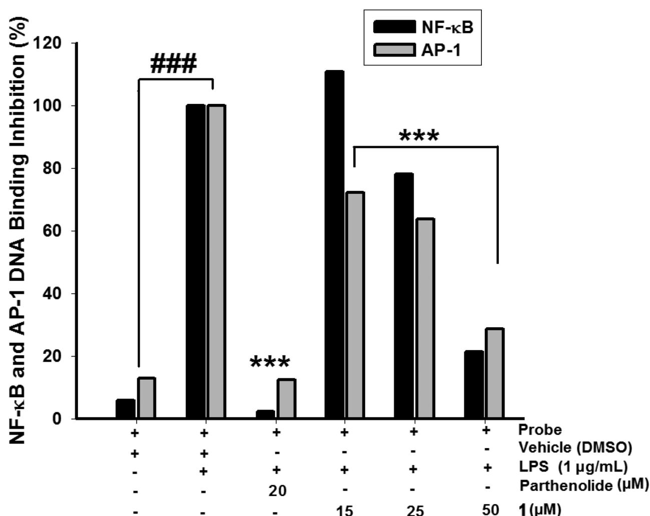


Figure 3. Effect of **1** on NF-κB and AP-1 DNA binding activity. An electrophoretic mobility shift assay (EMSA) was performed as described in the Experimental Section. RAW 264.7 macrophages were pretreated with the indicated concentrations of **1** for 2 h and stimulated with LPS (1 μg/mL) for 1 h. Then, 5 μg (NF-κB) and 3 μg (AP-1) from nuclear extracts were incubated with a ^{32}P-labeled oligonucleotides specific to NF-κB and AP-1 and electrophoresed on a 6% PAGE. Parthenolide (20 μM) was used as a positive control.

In order to evaluate more specifically whether **1** can affect the nuclear translocation of NF-κB, Western immunoblot analysis for NF-κB p50 and p65 was conducted with nuclear extracts of LPS-stimulated RAW 264.7 macrophages. The amounts of NF-κB p50 and p65 in the nucleus were increased significantly upon exposure to LPS alone (Figure S3, Supporting Information). The LPS-induced nuclear translocation of NF-κB p50 and p65 was inhibited by **1** in a dose-dependent manner (Figure S3, Supporting Information).

After the observation that **1** reduces the activation of the two critical pro-inflammatory transcription factors, NF-κB and AP-1, the potential effect was investigated of **1** on TNF-α, another important target mediator of inflammation.[25] Treatment of LPS-stimulated macrophages with **1** led to a reduction of TNF-α secretion in RAW 264.7 cells, with an IC_{50} value of 38.5 ± 1.63 μM. Therefore, **1** attenuated the release of a crucial mediator of inflammatory disease.[26]

To further explore the inhibitory mechanism of **1**, the Akt signaling pathway was also investigated. The phosphorylated form of Akt was evaluated using Western blotting. A time-course investigation conducted showed that **1** suppressed the phosphorylated form of Akt after 15 and 30 min of stimulation by LPS (Figure 4). These results demonstrate that the inhibition of LPS-induced Akt activation led to the suppression of NF-κB activation, resulting in down-regulation of pro-inflammatory enzymes (iNOS, COX-2) and therefore NO and TNF-α production. The activation of NF-κB requires phosphorylation of IκB, which then targets IκB for ubiquitination and degradation. Inhibition of Akt, which was demonstrated as suppressed Akt phosphorylation in the present study, caused reduced phosphorylation of IκB and attenuated IκB degradation. This might inhibit translocation of NF-κB to the nucleus, where it generally activates gene transcription.[8] Hence, since IκB kinase is a substrate of Akt,[8] activation of Akt might consequently stimulate NF-κB activation. Thus, increased inflammatory mediators may result from convergence of the Akt and NF-κB signaling pathways in LPS-stimulated RAW 264.7 macrophages.

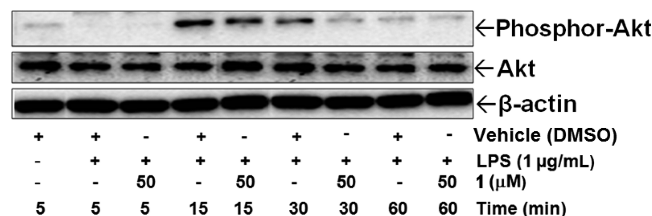


Figure 4. Effect of **1** on Akt. Phosphorylated Akt protein in the cytosolic extract was determined by Western blot analysis, as described in the Experimental Section. The RAW 264.7 cells were pretreated with 50 μM **1** for 2 h and treated with LPS (1 μg/mL) for the time periods specified.

When NF-κB and AP-1 are activated simultaneously, the increased level of the NF-κB downstream target gene *elk-1* can be further activated by ERK1/2, p38, and JNK for better induction of *fos* expression and AP-1 activation, which enhance the expression of AP-1 downstream target genes.[9] Several reports have demonstrated the importance of MAPKs in the transcriptional regulation of the LPS-induced inflammatory enzymes (iNOS) through the activation of transcription factors, especially NF-κB or AP-1.[26] To evaluate whether the inhibition of NF-κB activation and NO production by **1** is mediated through the MAPK pathway, the effect of **1** on LPS-induced phosphorylation of p38 MAPK, JNK, and ERK was investigated using Western blot analysis (Figure 5A–C). A time-course experiment demonstrated that **1** (50 μM) suppressed LPS-induced phosphorylation of p38 MAPK and JNK at 30 min, whereas LPS-mediated ERK phosphorylation inhibition began after 10 min and was suppressed after 30 min of LPS-stimulation (Figure 5A–C).

In conclusion, the present results suggest that 15,16-epoxy-3α-hydroxylabda-8,13(16),14-trien-7-one (**1**) may be a useful lead compound for development as an anti-inflammatory agent because of the significant suppression induced on crucial inflammatory mediators, i.e., iNOS, COX-2, and TNF-α secretion, via the inactivation of the NF-κB, MAPK, and Akt signaling pathways (Figure S5, Supporting Information). This

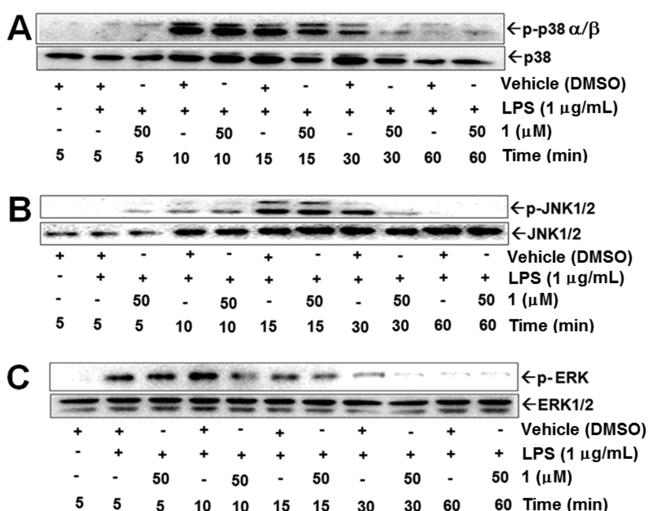


Figure 5. Effect of **1** on MAPKs (A) p-p38α/β, (B) p-JNK, and (C) p-ERK protein in cytosolic extracts determined by Western blot analysis, as described in the Experimental Section. RAW 264.7 cells were pretreated with 50 μM **1** for 2 h and treated with LPS (1 μg/mL) for the time periods specified.

inhibitory action most likely occurs in transcription because of the interaction of **1** with NF-κB and AP-1 transcription factors.

■ EXPERIMENTAL SECTION

General Experimental Procedures. RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained at subconfluence in a 95% air and 5% CO_2 humidified atmosphere at 37 °C. For routine subculturing, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) was used. The RAW 264.7 cells harboring the pNF-κB-secretory alkaline phosphatase (SEAP)-NPT reporter construct were cultured under the same conditions, except that the medium was supplemented with 500 μg/mL Geneticin.[24] All the samples were dissolved in dimethyl sulfoxide (DMSO) to make a 100 mM stock concentration and were then further diluted with DMSO for working concentrations. Final DMSO concentrations were <0.5% and did not interfere with the assays. *Escherichia coli* lipopolysaccharide (LPS) and parthenolide were purchased from Sigma-Aldrich (Steinheim, Germany). The ^{32}P-end-labeled 22-mer double-stranded NF-κB and AP-1 oligonucleotides were obtained from Promega (Madison, WI, USA).

Plant Material. The dried aerial parts of *Leonurus japonicus* were purchased from a herbal store at Gyeong-dong Market, Seoul, Korea, in 2008. The plant material was identified by Professor Je-Hyun Lee, Dongguk University, Korea. A voucher specimen (09E1001) was deposited in the Herbarium of the School of Oriental Medicine, Dongguk University, Korea.

Extraction and Isolation. The dried roots of *Leonurus japonicus* (15.0 kg) were extracted three times with 100% MeOH for 2 h each time, and a residue (1.18 kg) was obtained after removing the solvent under reduced pressure. This MeOH extract was suspended with distilled water and partitioned with *n*-hexane, EtOAc, and *n*-BuOH successively. The *n*-hexane fraction (241 g) was chromatographed on a silica gel column eluted with hexane−EtOAc (100:1 → 10:1) to give eight fractions (H1−H8). Fraction H2 was applied to a HP-20 resin column and eluted with 90% to 100% MeOH to afford three fractions (H2-1−H2-3). Using reversed-phase MPLC [MeOH−H_2O, 10:90 → 90:10, C18 360 g column (Redisep), 20 mL/min], fraction H2-1 (8.3 g) was separated to give seven fractions (H2-1-1−H2-1-7). Of these, fraction H2-1-6 (1.2 g) was separated by reversed-phase HPLC (MeOH−H_2O, 70:30, J'sphere ODS, 250 × 10 mm i.d. (YMC HPLC), 2 mL/min), to afford five fractions (H2-1-6-1−H2-1-6-5). Compound **1** (30 mg) was separated from fraction H2-1-6-3 (253 mg) by recrystallization. HPLC analysis was conducted, and its purity was determined as 95%. The identity of **1** was confirmed by comparison of ^{1}H NMR data with literature values for this compound.[27]

Cell Viability and Nitric Oxide Determination. To determine the cell viability of **1**, an MTT assay was carried out. Briefly, RAW 264.7 cells were plated at a density of 1×10^5 per well in a 24-well plate and incubated at 37 °C for 24 h. The cells were treated with various concentrations (5, 15, 25, 50, 75, and 100 μM) of **1** or vehicle alone for 2 h before LPS (1 μg/mL) stimulation and then incubated at 37 °C for an additional 18 h. After incubation for 18 h, 100 μL aliquots of the cell-free culture medium were taken for NO measurement according to the Griess reaction method, and cell viability was measured as described previously.[2] For this experiment, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine was used as a positive control.

NF-κB Secretory Alkaline Phosphatase Reporter Gene Assay. The NF-κB SEAP inhibitory activity of **1** was determined in LPS-stimulated RAW 264.7 macrophages. The NF-κB-dependent reporter gene transcription was analyzed by the SEAP assay, as previously described, with some modifications.[2] In brief, 1×10^5 RAW 264.7 macrophages transfected with pNF-κB-SEAP-NPT, encoding four copies of the -κB sequence and the SEAP gene as a reporter, were preincubated with different concentrations of **1** for 2 h and were then challenged with LPS (1 μg/mL) for an additional 18 h. TPCK (10 μM) was used as the positive control for this experiment.

Western Immunoblot Analysis. RAW 264.7 macrophages were pretreated with the indicated concentrations of **1** or vehicle for 2 h and then stimulated with LPS (1 μg/mL) for 5, 10, 15, 20, 30, and 60 min (phosphor-IκBα and IκBα, p-p38, p-JNK, p-ERK, p-Akt) and 18 h (COX-2 and iNOS). All of the primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ten micrograms of total protein for iNOS, IκBα, phosphor-IκBα, p-p38, p38, p-JNK, JNK, p-ERK, ERK, p-Akt, Akt, p65, and p50 and 5 μg for COX-2 were separated by SDS-PAGE, 8% (iNOS, and COX-2) and 10% (phosphor-IκBα, IκBα p-p38, p38, p-JNK, JNK, p-ERK, ERK, p-Akt, Akt, p65, p50, and β-actin). After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany), blocked with 5% nonfat milk in TBS-T buffer, and blotted with each primary antibody (1:1000) and its corresponding secondary antibody (1:5000), according to the manufacturer's instructions. The antibodies were detected with the WEST-SAVE Up luminol-based ECL reagent (LabFrontier, Seoul, Korea). The target bands were quantified using UN-SCAN-IT software version 6.1 (Silk Scientific Co., Orem, UT, USA).

Electrophoretic Mobility Shift Assay. EMSA was performed to investigate the inhibitory effect on NF-κB and AP-1 DNA binding, as previously described.[2] Briefly, nuclear extracts prepared from LPS (1 μg/mL)-treated cells were incubated with ^{32}P-end-labeled 22-mer double-stranded NF-κB and AP-1 consensus oligonucleotide (Promega, sequence: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and 5′-(CGC TTG ATG AGT CAG CCG GAA)-3′) for 30 min at room temperature. To verify the specificity for NF-κB, a 50-fold excess of unlabeled NF-κB oligonucleotide was added to the reaction mixture as a competitor, and the DNA protein complexes were then separated from the free oligonucleotides on 6% native polyacrylamide gels. The signals obtained from the dried gel were quantified with an FLA-3000 apparatus (Fuji), using the BAS reader version 3.14 and Aida Version 3.22 software (Amersham Biosciences, Piscataway, NJ). The binding conditions were optimized as reported earlier.[28]

Measurement of TNF-α Production. TNF-α production in the culture medium was determined using a commercially available TNF-α ELISA kit (eBioscience, Inc., San Diego, CA, USA).

Statistical Analysis. Unless otherwise stated, results are expressed as means ± standard deviations (SD) from three different experiments. One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was applied to assess the statistical significance of the differences between the study groups (SPSS version 10.0, Chicago, IL, USA). A value of $p < 0.05$ was chosen as the criterion for statistical significance.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Down-regulation of iNOS and COX-2 protein expressions using Western blotting, effect of **1** on NF-κB and AP-1 DNA binding activity, EMSA competition assay, effect of **1** on NF-κB subunits (p65 and p50) nuclear proteins using Western blot analysis, and a scheme for the targeted cellular signaling pathways affected by **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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