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# Blockade of IL-6 Secretion Pathway by the Sesquiterpenoid Atractylenolide III

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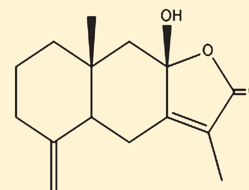
**ABSTRACT:** Atractylenolide III (**1**) is the major bioactive component of *Atractylodes lancea*. The aim of this study was to analyze the effect on the regulation of interleukin (IL)-6 secretion pathway caused by **1**. This sesquiterpenoid inhibited the secretion and expression of IL-6 in phorbol 12-myristate 13-acetate- and calcium ionophore A23187-stimulated human mast cells (HMC)-1. In addition, **1** inhibited histamine release in stimulated HMC-1 cells. In stimulated HMC-1 cells, **1** suppressed activation of p38 mitogen-activated protein kinase, C-Jun-N-terminal protein kinase, and nuclear factor-*κ*B. In addition, **1** suppressed the activation of caspase-1 and the expression of receptor interacting protein-2. These results provide new insights that atractylenolide III (**1**) may control immunological reactions by regulating the cellular functions of IL-6 in mast cells.

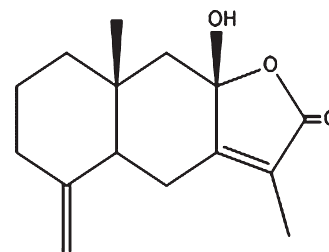



Mast cells are broadly distributed throughout mammalian tissues and play a critical role in a wide variety of biological responses. They have been implicated in diverse immune reactions including allergic inflammation,[1] rheumatoid arthritis,[2] and septic peritonitis.[3] Upon degranulation, mediators are released from mast cells including histamine and some cytokines.[4] In the inflammatory process, cytokines recruit activated immune cells to the site of lesions, thereby amplifying and perpetuating this condition.[5] IL-6 is a pleiotropic cytokine that is produced upon activation of T helper type 2 cells or mast cells,[6] which mediate physiological processes.[7] IL-6 secretion is regulated by mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPK, and nuclear factor-kB (NF-*κ*B).[8,9]

Caspase-1, originally designated IL-1-converting enzyme, is a member of the group of caspases,[10] and its activation regulates apoptosis and inflammation.[11] Specific adaptors have been reported to regulate the activation of caspase-1. They are receptor interacting protein (RIP)-2,[12] apoptosis-associated speck-like protein containing a caspase recruitment domain, a PYRIN-caspase recruitment domain protein,[13] and Ice-protease activating factor.[14] The activation of caspase-1 induces cytokine secretion such as IL-6.[15]

The rhizomes of *Atractylodes lancea* DC. (Asteraceae) have been used for various immune diseases in traditional Korean medicine for centuries.[16] As for the chemical constituents of this species, several sesquiterpenoids[17] and the components of the essential oil were reported.[18,19] Atractylenolide III (**1**) is the major component of *A. lancea* and exhibits significant inhibitory effects both on ear edema induced by xylene and on peritoneal capillary permeability induced by acetic acid in mice.[20] Wang et al. reported the gastroprotective activity of **1** on ethanol-induced gastric ulcers.[21] Atractylenolide III also inhibits lipopolysaccharide-induced tumor necrosis factor (TNF)-α and nitric oxide production in macrophages.[22]

In order to analyze the regulation of IL-6 secretion pathway by **1**, the effects of **1** on the secretion and expression of IL-6 and the activation of p38 and JNK were investigated. In addition, it was examined as to whether **1** can regulate histamine release and the caspase-1/RIP-2/NF-*κ*B activation in phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (PMACI)-stimulated human mast cells (HMC)-1.




**1**

## RESULTS AND DISCUSSION

Mast cells arise from pluripotential stem cells and mature in the tissue. They have the ability to generate immune reactions following exposure to a variety of receptor-mediated signals initiated by both innate and acquired immune response mechanisms. Activated mast cells release a broad spectrum of mediators including cytokines such as IL-6.[23,24] To determine whether **1** modulates PMACI-induced IL-6 secretion, the cells were pretreated with various concentrations of this compound for 1 h prior to PMACI stimulation. Dexamethasone was used as a reference compound. Culture supernatants were assayed for IL-6

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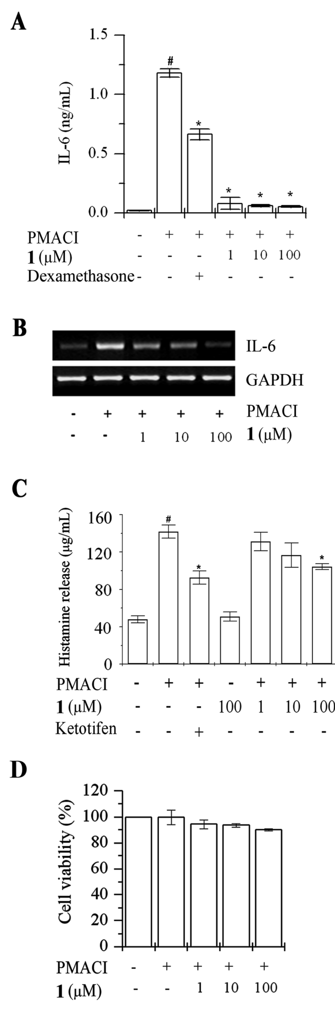


**Figure 1.** Effect of atractylenolide III (**1**) on IL-6 secretion and mRNA expression in HMC-1. (A) The cells were pretreated with various concentrations of **1** (1−100 $\mu$M) or dexamethasone (100 $\mu$M) for 1 h prior to PMACI stimulation for 8 h. Secreted IL-6 levels in culture supernatants of cells were measured by an ELISA method. (B) The cells were pretreated with **1** (1−100 $\mu$M) for 1 h prior to PMACI stimulation for 5 h. The total RNA was assayed by RT-PCR analysis, and results are representative of three independent experiments. (C) The cells were pretreated with various concentrations of **1** (1−100 $\mu$M) or ketotifen (2 $\mu$g/mL) for 1 h prior to PMACI stimulation for 6 h. Secreted histamine was assayed by using the histamine assay kit. (D) The cells were treated with **1** (1−100 $\mu$M) for 8 h. Cells were then collected and assessed for viability using MTT. Data represent mean ± SEM of three independent experiments. ($^*p < 0.05$: significantly different from the PMACI-stimulated cells. $^{\#}p < 0.01$: significantly different from unstimulated cells.)

levels by using an ELISA method. In PMACI-stimulated cells, IL-6 secretion was enhanced, and **1** inhibited increased IL-6 levels in a dose-dependent manner (Figure 1A). The effect of **1** exposure on IL-6 mRNA expression induced by PMACI was examined using RT-PCR, and cells were pretreated with **1** for 1 h and then treated with PMACI. As shown in Figure 1B, treatment of PMACI increased the mRNA expression of IL-6, whereas **1** suppressed this enhanced expression. To clarify the effect of **1** on the degranulation of mast cells, histamine was measured in HMC-1 cells. PMACI increased histamine release, whereas this increased histamine was significantly decreased by **1** (100 $\mu$M) in HMC-1 cells (Figure 1C, $p < 0.05$). Ketotifen, as a mast cell stabilizer, was used as a reference compound. Moreover, compound **1** did not affect cell viability (Figure 1D). These results suggest that **1** blocks the secretion and expression of IL-6 as well as histamine release in the activated mast cell.

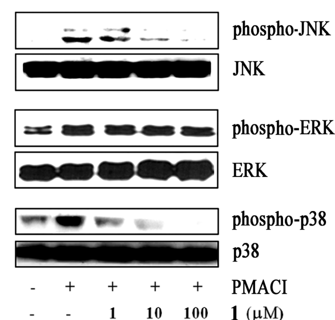


**Figure 2.** Effect of atractylenolide III (**1**) on MAPK phosphorylation in HMC-1. The cells were pretreated with **1** (1−100 $\mu$M) for 1 h prior to PMACI stimulation for 30 min. (A) The phosphorylated MAPKs level was assayed by Western blot analysis. Results are representative of three independent experiments.

To determine the effect of **1** on MAPK activation induced by PMACI, Western blot analysis for phosphorylated p38, JNK, and ERK was performed. Cells were pretreated with **1** for 1 h and then treated with PMACI. The phosphorylation of MAPKs was increased by PMACI, but **1** attenuated PMACI-induced p38 and JNK phosphorylation. However, compound **1** did not affect the phosphorylation of ERK (Figure 2). MAPKs represent an important point of convergence for multiple signaling pathways that are activated in immune reaction, cell death, and proliferation.[25] Activation of JNK and p38 MAPK induces cytokine secretion.[8] Thus, it is accepted widely that JNK and p38 MAPK represent a central component in the regulation of the immune response. The present observations demonstrate that the effect of **1** on the mast cell-mediated immune reaction, at least in part, might be derived through regulation of the JNK and p38 pathway.

As the suppression of NF-$\kappa$B activation has been linked with the inhibition of inflammation, it was postulated that **1** mediates its effects at least partly through suppression of NF-$\kappa$B activation. Since NF-$\kappa$B activation requires nuclear translocation of the *Rel A*/p65 subunit of NF-$\kappa$B, the effect of **1** was examined on the nuclear pool of *Rel A*/p65 protein by Western blot analysis. As shown in Figure 3A, PMACI treatment considerably increased the nuclear *Rel A*/p65 protein level, indicating nuclear translocation of *Rel A*/p65. Pretreatment with **1** inhibited the PMACI-stimulated increase of the nuclear *Rel A*/p65 levels. In addition, PMACI treatment effectively induced I$\kappa$B-$\alpha$ degradation. Compound **1** inhibited PMACI-induced I$\kappa$B-$\alpha$ degradation (Figure 3A). In order to confirm whether **1** is an inhibitor of NF-$\kappa$B-mediated gene transcription, HMC-1 was transfected with the NF-$\kappa$B-luciferase reporter plasmid. Transfected cells were incubated with **1** for 1 h and then stimulated by PMACI for 24 h. It was observed that **1** reduced the PMACI-induced luciferase activity (Figure 3B).

Activation by RIP-2 induces caspase-1 oligomerization and promotes caspase-1 activation, with the latter inducing cytokine stimulation. To address whether PMACI induces RIP-2 expression in HMC-1 cells, cells were treated with PMACI for various times. As shown in Figure 4A, RIP-2 expression was increased up to 6 h after PMACI treatment. To determine the effect of **1** on

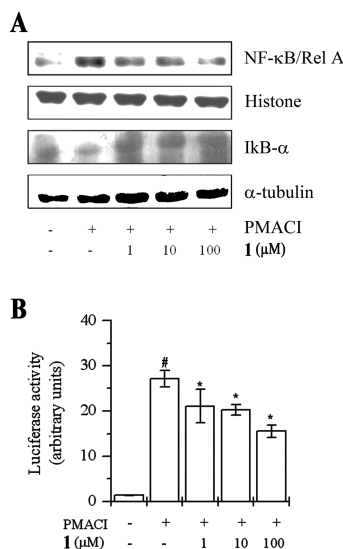


**Figure 3.** Effect of atractylenolide III (**1**) on NF-*κ*B activation in HMC-1. (A) Cells were pretreated with **1** (1−100 *μ*M) for 1 h prior to PMACI stimulation for 2 h. Nuclear protein and cytoplasmic protein were prepared and analyzed for NF-*κ*B and I*κ*B-α by Western blotting as described in the Experimental Section. (B) NF-*κ*B activity was assayed by a luciferase assay, and results are representative of three independent experiments. (*$p < 0.05$: significantly different from the PMACI-stimulated cells. $^{\#}p < 0.01$: significantly different from unstimulated cells.)

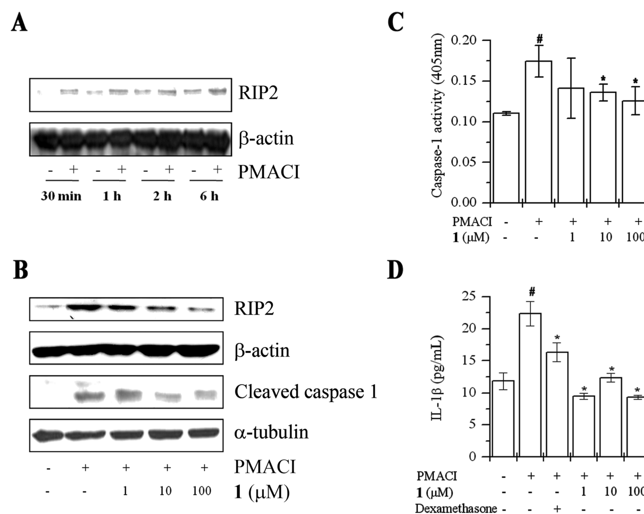


**Figure 4.** Effect of atractylenolide III (**1**) on RIP-2/caspase-1 activation in HMC-1. (A) The cells were pretreated with PMACI for various times. The level of RIP-2 was assayed by Western blot analysis. (B) The cells were pretreated with ATL-III (1−100 *μ*M) for 1 h prior to PMACI stimulation for 30 min. The level of RIP-2 and caspase-1 was assayed by Western blot analysis. (C) The enzymatic activity of caspase-1 was tested by a caspase colorimetric assay. (D) IL-1*β* concentrations were measured in cell supernatants using the ELISA method. Results are representative of three independent experiments. (*$p < 0.05$: significantly different from the PMACI-stimulated cells. $^{\#}p < 0.01$: significantly different from unstimulated cells.)

RIP-2 and caspase-1 activation induced by PMACI, cells were treated with PMACI for 30 min. The results indicated that compound **1** suppressed the RIP-2 activation induced by PMACI in a dose-dependent manner (Figure 4B). Next, the effect of **1** was investigated on caspase-1 activation induced by PMACI, and a Western blot analysis for caspase-1 was performed. Cells were pretreated with **1** for 1 h and then treated with PMACI. Caspase-1 activation was decreased by the treatment of **1** in a dose-dependent manner (Figure 4B). The effects of **1** were also measured on caspase-1 activation using a caspase-1 assay kit, and enhanced caspase-1 activity was decreased by **1** (Figure 4C). ProIL-1*β* is proteolytically processed to its active form by caspase-1. Compound **1** also decreased IL-1*β* secretion in HMC-1 (Figure 4D). IL-1*β* secretion induced by PMACI was also inhibited by dexamethasone (Figure 4D). It was reported that caspase-1−/− mice have a decreased secretion of both IL-1*β* and IL-6 after stimulation with LPS.[26] RIP-2 is a specific adaptor molecule and regulates the activation of caspase-1 and NF-*κ*B.[12] Another study has shown that RIP-2 knockout reduced secretion of IL-6.[27,28] These investigations have suggested that the activation of RIP2/caspase-1 is an important target for IL-6 regulation. Therefore, it was postulated that **1** mediates its effects partly through suppression of RIP2/caspase-1 activation. This compound suppressed RIP-2 and caspase-1 activation induced by PMACI and also inhibited IL-1*β* secretion. These results suggest that the inhibitory effect of **1** on IL-1*β* secretion might occur through the regulation of RIP-2 and caspase-1 activation.

In conclusion, it has been shown that atractylenolide III (**1**) can modulate the secretion and expression of IL-6 through the regulation of JNK, p38, and NF-*κ*B. In addition, this compound can suppress RIP2/caspase-1 activation induced by PMACI. This study provides new insights into the mechanism regarding the beneficial effects of **1** on the mast cell-mediated immune reaction.

## EXPERIMENTAL SECTION

**Materials.** Atractylenolide III (**1**) (99.0% purity as determined by HPLC) was obtained from Wako (Osaka, Japan). PMA, A23187, dimethyl sulfoxide (DMSO), ketotifen, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other reagents were purchased from Sigma (St. Louis, MO). Iacove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Anti-human IL-6 and IL-1*β*, biotinylated anti-human IL-6 and IL-1*β*, and recombinant human (rh) IL-6 and IL-1*β* were purchased from Pharmingen (San Diego, CA). Antibodies (Abs) for caspase-1, p38, pp38, JNK, pJNK, ERK, pERK, NF-*κ*B, I*κ*B-α, histone, *β*-actin, and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The caspase assay kit was supplied by R&D Systems, Inc. (Minneapolis, MN). The luciferase assay kit was purchased from Promega (Madison, WI).

**Cell Culture.** HMC-1 was grown in IMDM supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated FBS at 37 °C 5% $CO_2$, and 95% humidity.

**MTT Assay.** To test cell viability, the MTT colorimetric assay was performed. Briefly, HMC-1 cells ($5 \times 10^5$ cells/well) were incubated for 24 h after stimulation in the presence or absence of **1**. MTT solution (5 mg/mL) was added, and the cells were incubated at 37 °C for an additional 4 h. The crystallized MTT was dissolved in DMSO, and the absorbance measured at 540 nm using a microplate reader.

**Assay of IL-6 and IL-1*β* Secretion.** Secreted IL-6 and IL-1*β* levels in culture supernatants of cells were measured by an enzyme-linked immunosorbent assay (ELISA) method. Thus, 96-well plates were coated with 100 *μ*L aliquots of anti-human IL-6 and IL-1*β* monoclonal Abs at a concentration of 1.0 *μ*g/mL in PBS (pH 7.4), respectively, and incubated overnight at 4 °C. After additional washes, 100 *μ*L of cell medium, IL-6, or IL-1*β* standards was added and incubated at 37 °C for 2 h. After the wells were washed, biotinylated anti-human IL-6 (0.2 *μ*g/mL) was added and incubated at 37 °C for an additional 2 h. Next, the wells were washed, and


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then avidin-peroxidase was added and incubated for 30 min at 37 °C. After the wells were washed again, ABTS substrate was added. Color development at 405 nm was then measured using an automated microplate ELISA reader. In addition, a standard curve was generated for each assay plate by measuring the absorbance of serial dilutions of recombinant IL-6 and IL-1$\beta$ at 405 nm.

**RNA Isolation and RT-PCR.** Total RNA was isolated from HMC-1 according to the manufacturer's specifications using an easy-BLUE RNA extraction kit. The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.0 $\mu$g) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using a cDNA synthesis kit. RT-PCR was carried out with 1 $\mu$L of a cDNA mixture, in 20 $\mu$L final volume with 2.5 mM $MgCl_2$, 200 mM dNTPs, 25 pM cytokine primers, and 2.5 U of TaqDNA polymerase in the reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, and 0.1% Triton X-100). PCR was performed with the following primers for human IL-6 (5′-GAT GGA TGC TTC CAA TCT GGA T-3′ and 5′-AGT TCT CCA TAG AGA ACA ACA TA-3′) and GAPDH (5′-CAA AAG GGT CAT CAT CTC TG-3′ and 5′-CCT GCT TCA CCA CCT TCT TG-3′), which were used to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 50 °C for IL-6 and 62 °C for GAPDH, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

**Histamine Assay.** Histamine was measured from cells according to the manufacturer's specifications using a histamine assay kit supplied by Oxford Biomedical Research (Oxford, MI).

**Preparation of Cytoplasmic and Nuclear Extract.** After cell activation, cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in 60 $\mu$L of buffer A (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). The cells were allowed to swell on ice for 15 min, lysed gently with 2.5 $\mu$L of 10% Nonide P (NP)-40, and centrifuged at 2000*g* for 10 min at 4 °C. The supernatant was collected and used as cytoplasmic extract. The nuclei pellet was resuspended in 40 $\mu$L of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), left on ice for 20 min, and inverted. The nuclear debris was then spun down at 15000*g* for 15 min. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen, and stored at −70 °C until ready for analysis.

**Western Blot Analysis.** For analysis of the phosphorylated or total protein levels of indicated proteins in the text, stimulated cells were rinsed twice with ice-cold PBS and then lysed in ice-cold lysis buffer (PBS containing 0.1% SDS, 1% Triton, and 1% deoxycholate). Cell lysates were centrifuged at 15000*g* for 5 min at 4 °C; the supernatant was then mixed with an equal volume of 2× SDS sample buffer, boiled for 5 min, and then separated through a 12% denaturing protein gel. After electrophoresis, the protein was transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% skim milk for 2 h, rinsed, and incubated overnight at 4 °C with primary antibodies. After three washes in PBST/0.1% Tween 20, the membranes were incubated for 1 h with HRP-conjugated secondary antibodies. After three washes in PBST/0.1% Tween 20, the protein bands were visualized by an enhanced chemiluminescence assay following the manufacturer's instructions.

**Caspase-1 Activity.** The enzymatic activity of caspase-1 was assayed using a caspase colorimetric assay kit according to the manufacturer's protocol. The lysed cells were centrifuged at 15000*g* for 5 min. The protein supernatant was incubated with 50 $\mu$L of reaction buffer and 5 $\mu$L of caspase substrate at 37 °C for 2 h. The absorbance was measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a bicinchoninic acid protein (BCA) quantification kit.

**Transient Transfection and Luciferase Assay.** For the transfection, the HMC-1 cells ($1 \times 10^7$) were seeded in a 100 mm culture dish. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used to transfect transiently reporter gene constructs into HMC-1 cells. The cell extract (20 $\mu$L) and the luciferase assay reagent (100 $\mu$L) were mixed at room temperature. To measure the luciferase activity, a luminometer (1420 luminescence counter, Perkin-Elmer) was used, in accordance with the manufacturer's protocol. All transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of *firefly* luciferase activity to *renilla* luciferase activity.

**Statistical Analysis.** Results were expressed as the mean ± SEM of independent experiments, and statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey and a Duncan post hoc test to express the differences between groups. All statistical analyses were performed using SPSS v11.0 statistical analysis software. A value of $p < 0.05$ was considered to indicate statistical significance.

## AUTHOR INFORMATION

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