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Inhibition of IL-6-induced STAT3 Activation by Pimaradienoic Acid from *Aralia continentalis*

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Aralia continentalis Kitagawa (Araliaceae) is one of the most commonly used medicinal plants in China and Korea. The roots of the plant have been used for the treatment of inflammatory disorders, including rheumatism, lumbago, and lameness.[1] The major constituents of this plant include a variety of diterpenes, flavonoids, saponins and polyacetylenes.[2] Many studies reported that the extract or compounds isolated from *A. continentalis* possess a variety of biological activities, such as anti-inflammatory, anti-tumor, anti-Alzheimer, and antimicrobial properties.[3-6] In particular, diterpene compounds isolated from this plant have been reported to exhibit inhibitory activities towards cholinesterase, COX-1, and NO production.[3,5,7] In addition, the methanol extract of this plant has been shown to inhibit IL-8 production induced lipopolysaccharide (LPS) in peritoneal macrophages.[8] However, no reports of IL-6-induced STAT3 activation have been presented.

Interleukin-6 (IL-6) is pleiotropic cytokine, and is secreted in immune and inflammatory cells such as macrophages and lymphocytes.[9] It is a key molecule involved in the physiological and pathological processes of several inflammatory diseases and malignancies. In addition, it is excessively produced from a variety of external stimuli such as viral infections, trauma, other tissue damages, and excess IL-6 leading to the activation of STAT3 (signal transducer and activator of transcription 3).[10-12] STAT3 activated by IL-6 suppresses the anti-tumor immune response, and promotes the inflammatory procedure related to several human diseases such as rheumatoid arthritis, psoriasis, inflammatory bowel disease, osteoarthritis, and multiple myeloma.[13,14] Thus, IL-6 palys a central role in the pathogenesis of immune and inflammatory diseases. In particular, many researchers have been trying to find IL-6 inhibitors, and a number of natural products including curcumin and resveratrol have been reported to show inhibition of STAT3 activation by IL-6.[15,16] Therefore, the development of an IL-6–receptor antagonist or interference with STAT3 activation is one of the effective treatment methods to ease the pathogenesis of diseases related to IL-6. In current study, pimaradienoic acid was isolated from the MeOH extract of *Aralia continentalis* and investigated the potent effect of pimaradienoic acid on the response to IL-6.

In the ongoing search for IL-6 inhibitors from natural sources, the MeOH extract from the roots of *Aralia continentalis* exhibited 30% inhibitory activity at the concentration of 10 μg/mL on IL-6-induced STAT3 activity. The MeOH extract was suspended in H_2O and partitioned with *n*-hexane, EtOAc and *n*-BuOH. The *n*-hexane-soluble fraction was concentrated and separated using a silica gel column chromatography, followed by semi-preparative HPLC, resulting in the isolation of the pure compound **1**. The structure of compound **1** was identified as pimaradienoic acid (Figure 1) using spectroscopic methods (^{1}H-, ^{13}C-NMR, and ESI-MS) and by comparing the data obtained with those of previously reported values.[5]

The inhibitory effect of pimaradienoic acid was tested on STAT3-dependent gene transcription activity by IL-6 using Hep3B cells transformer with the pStat3-Luc plasmid.[17] The cells were seeded on 96-well culture plates, cultured for 24 h, and starved for 12 h. The cells were then stimulated with IL-6 (10 ng/mL) for 12 h in the presence or absence of pimaradienoic acid. The STAT3-dependent promoter activity was measured according to the manufacturer's protocol. The inhibitory activity of pimaradienoic acid was compared to genistein (Sigma Chemical Co., synthetic, purity: ≥ 98%), a tyrosine inhibitor, used as a positive control, which inhibited the STAT3-dependent luciferase activity with an IC_{50} value of 15 μM in this assay system.[18] Pimaradienoic acid showed inhibitory activity on STAT3 activation by IL-6 in a dose dependent manner with an IC_{50} value of 1.06 ± 0.11 μM (Figure 2(a)). In addition, Hep3B cell viability using MTT

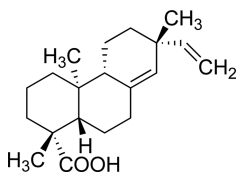

Figure 1. Chemical structure of pimaradienoic acid (**1**) isolated from *Aralia continentalis.*

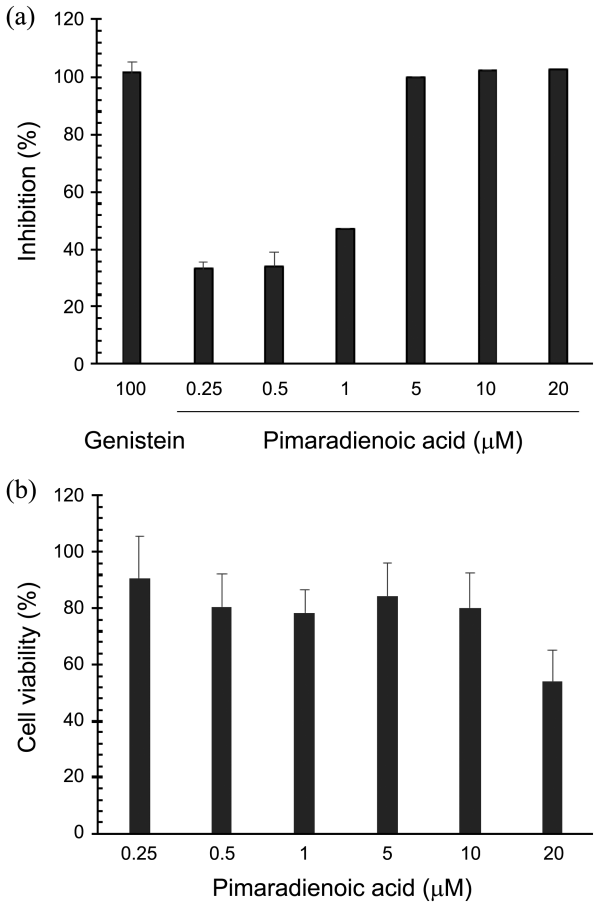

Figure 2. Inhibitory effects of pimaradienoic acid on IL-6-induced STAT3 activation (a) and cell viability (b). Hep3B cells expressing stably pStat3-Luc were seeded on to 96-well culture plates at 2×10^4 cells/well. Data are presented as the mean ± SE ($n = 3$).

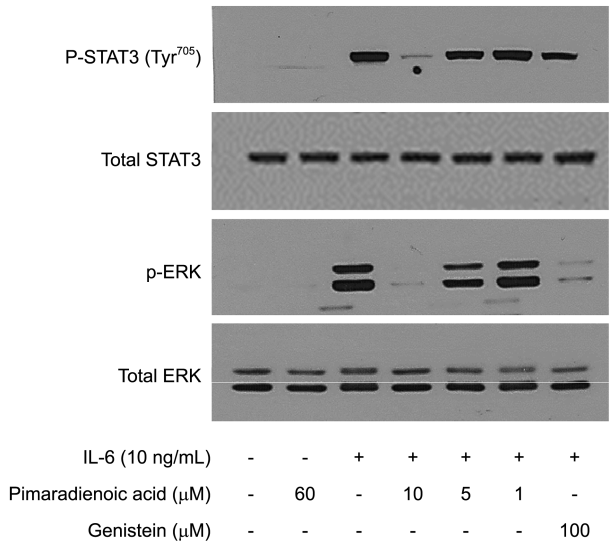

Figure 3. Effects of pimaradienoic acid on the STAT3 and ERK1/2 phosphorylation by IL-6.

followed the assays to confirm that the inhibitory activity of pimaradienoic acid was not due to any cytotoxic action of the compound. As shown in Figure 2(b), pimaradienoic acid exhibited 60% cell viability at the concentration of 20 μM, and it did not show any cytotoxicity at the other concentrations used in this study (Figure 2(b)).

After IL-6 binds to its receptor, it induces the phosphorylation and dimerization of gp130 (a signal transducing glycoprotein 130). This results in the activation and phosphorylation of three major downstream signaling cascades: JAK2/STAT3, MEK/ERK and PI3-K/Akt.[19] Thus, the activation of STAT3 is one of the most important steps in the pro-inflammatory signals induced by IL-6. Therefore, we investigated whether pimaradienoic acid show inhibitory effects on the phosphorylation of STAT3 induced by IL-6 in Hep3B cells. As shown in Figure 3, the IL-6 induced tyrosine phosphorylation of STAT3 was strongly inhibited by pretreatment of pimaradienoic acid at 10 μM for 1 h in the Hep3B cells compared with genistein (100 μM); although low concentrations of this compound barely inhibited STAT3 phosphorylation. In addition, pimaradienoic acid was tested for inhibitory activity on the phosphorylation of ERK1/2, another downstream signaling molecule involved in the IL-6 signaling pathway, induced by IL-6 in Hep3B cells. This compound inhibited the phosphorylation of ERK1/2 in a dose dependent manner (Figure 3). No cytotoxic activity by this compound was observed at the concentration used, indicating that the inhibitory effect of these substances on IL-6-induced STAT3 activation was not related to cytotoxicity (Figure 2(b)).

It has been reported that pimaradienoic acid exerted an anti-inflammatory effect through the inhibition of LPS-induced IL-6, NO, and PGE_2 production in RAW 264.7 macrophages.[20] Toll-like receptor 4 (TLR4) and CD14 detect LPS which is the membrane component of gram-negative bacteria and results in the induction of immune and inflammatory genes, including such important cytokines as tumor necrosis factor-α (TNF-α), IL-6, and type I interferon. Much evidence points to a role for TLRs including TLR4 in IL-6 production. Here, we the role of pimaradienoic acid in the inhibition of TLR activation was investigated. TLR activation was investigated by assessing NF-κB and AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) activity in THP1-Blue-CD14 cells. The THP1-Blue-CD14 cells were stimulated with TLR2 agonist (Pam3SCK4) and TLR9 agonist (ODN2006), respectively, in the presence or absence of pimaradienoic acid for 18 h. The cell suspension was mixed with QUANTI-Blue colorimetric assay reagent to detect SEAP, and incubated for 1 h. Color development was read at 655 nm using a microplate reader. Pimaradienoic acid exhibited a mild inhibitory effect on TLR2 and TLR9 activation (Figure 4). Although pimaradienoic acid effectively inhibits NF-κB activation and IL-6 production, it did not completely inhibit TLR activation, suggesting that the inhibitory target of pimaradienoic acid is NF-κB not TLR. Therefore, it can be hypothesized that this compound may be regulate the function of IL-6 in inflammatory sites by inflammatory stimulus. Detailed studies on IL-6 mediated cellular signaling pathway of pimaradienoic acid are now in

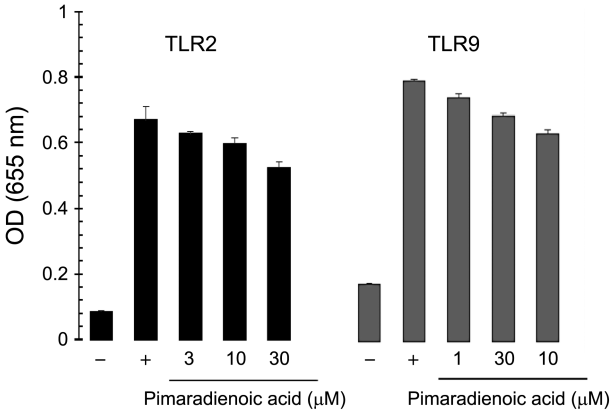

Figure 4. Effect of pimaradienoic acid on the TLR2 and TLR9 activity. THP1-Blue-CD14 cells were treated with Pam3SCK4 (10 ng/mL) and ODN2006 (10 μg/mL) in the presence or absence of pimaradienoic acid for 18 h, and the NF-κB-inducible SEAP activity was reported as relative activity compared to that in untreated cells.

progress.

In conclusion, pimaradienoic acid shows potent inhibitory activity on IL-6 signaling as well as IL-6 production. Pimaradienoic acid inhibits tyrosine phosphorylation of STAT3 as well as STAT3-dependent luciferase in Hep3B cells. This compound also inhibits the phosphorylation of ERK1/2. Therefore, this compound could be useful a candidate for treatment of inflammatory diseases related IL-6 and could be basis for the design of IL-6 inhibitors.

Experimental Section

Isolation and Purification. The roots of *Aralia continentalis* were purchased from a local market in Daejeon, Korea. A voucher specimen (NO. 1785) was deposited in the author's laboratory. The roots of *A. continentalis* (4 kg) were extracted with MeOH (1 L × 3) for 24 h at room temperature. The MeOH extract was evaporated in *vacuo*, and the dried MeOH extract was suspended with distilled water and partitioned sequentially with *n*-hexane, EtOAc and *n*-BuOH. Of the solvent fractions, the *n*-hexane-soluble fraction (85 g) was subjected to silica gel (230-400 mesh, 500 g, Merck) column chromatography eluted with a gradient solvent system using *n*-hexane-acetone (100:1 → 80:1 → 60:1 → 40:1 → 20:1 → 10:1 → 1:1→ 1:2 → 100% MeOH; each 1 L, v/v) to afford four fractions (Fr. 1-4) through the based on the TLC profile. Fr. 1 (10 g) was successively separated using a semi-preparative HPLC (YMC J'sphere ODS H-80, 4 μm, φ 20 × 150 mm; UV 210 nm; flow rate 1.8 mL/min) eluted with CH_3CN-H_2O (80:20, v/v), and gave the pure compound **1** (1000 mg).

Pimaradienoic Acid (1): ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.71 (1H, dd, *J* = 10.6, 17.2 Hz, H-15), 5.14 (1H, br d, *J* = 1.2 Hz, H-14), 4.94 (1H, dd, *J* = 2.2, 11.2 Hz, H-16b), 4.91 (1H, dd, *J* = 2.1, 17.1, H-16a), 2.34 (1H, ddd, *J* = 2.4, 4.2, 13.8 Hz, H-12b), 2.17 (1H, br d, *J* = 13.5 Hz, H-3b), 1.98 (1H, td, *J* = 5.1, 13.8, H-12a), 1.84-1.92 (2H, m, H-6), 1.78-1.82 (1H, m, H-2b,11b), 1.74 (1H, m, H-1b) 1.70 (1H, m, H-9), 1.5-1.6 (1H, m, H-7a), 1.46-1.51 (1H, m, H-11a), 1.22-1.32 (1H, m, H-2a), 1.28 (1H, dd, *J* = 3.0, 14.4, H-5), 1.26 (3H, s, H-18), 1.17-1.24 (1H, m, H-7b), 1.05 (1H, dt, *J* = 3.9, 13.5, H-1a, 3a), 1.00 (3H, s, H-17), 0.65 (3H, s, H-20); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 184.6 (COOH), 147.1 (C-15), 137.9 (C-8), 128.0 (C-14), 112.9 (C-16), 56.1 (C-5), 50.5 (C-9), 44.0 (C-4), 39.2 (C-1), 39.2 (C-10), 38.5 (C-13), 37.9 (C-3), 36.4 (C-12), 35.8 (C-7), 29.3 (C-17), 29.2 (C-18), 24.1 (C-6), 19.6 (C-11), 19.2 (C-2), 13.8 (C-20).

Reagents and Chemicals. Recombinant human IL-6 was purchased from R&D systems (Minneapolis, MN, USA). All reagents including genistein were obtained from Sigma-Aldrich Ltd (St Louis, MO, USA).

Cell line and Cell Culture. Human hepatoma Hep3B cells were obtained from American Type Culture Collection (ATCC No. HB-8064, Rockville, MD) and were maintained in a DMEM medium, supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin at 37 °C in a 5% CO_2 incubator. All cell culture reagents were obtained from GibcoBRL (Life Technologies, Cergy-Pontoise, France).

Establishment of Stable Cell Line Expressing pStat3-Luc. Hep3B cells were cotransfected with pStat3-Luc encoding the Stat3 binding site and pcDNA3.1 (+) carrying a hygromycin selection marker (Clontech laboratories, Palo Alto, CA) by using lipofectamin plus (Invitrogen, Carlsbad, CA, USA). Two days after the transfection, cells that stably expressed luciferase were selected by hygromycin treatment (100 μg/mL) and stable clones were expanded. The expression of luciferase in the clones stably expressing pStat3-Luc was confirmed by luciferase assays.

Luciferase Assay. Hep3B cells stably expressing pStat3-Luc were seeded on 96-well culture plates at 2 × 10^4 cells/well. After 24 h, the cells were starved for 12 h, and then treated with IL-6 (10 ng/mL) with or without compounds for 12 h. A luciferase assay was performed with the kit from Promega according to the manufacturer's protocol.

Western Blot Analysis. Total proteins were prepared from cells and subjected to Western blot with primary rabbit anti-phospho STAT3 (Tyr705) IgG, anti-total STAT3 IgG, anti-phospho ERK1/2, anti-total ERK (1:1000), and secondary antibody (1:2000).

Cell Viability. Hep3B cells were seeded at a plating density of 2 × 10^4 cells/well and cultured for 24 h to allow them to adhere to the plate. After 24 h, the culture medium changed to the serum free medium supplemented with the samples at the indicated dose. Following the culture with sample for 48 h, MTT (0.5 mg/mL) was added, and after 4 h of incubation at 37 °C, 200 μL of DMSO was added into each well. The absorbance of the samples at 540 nm was measured against a background control by using a 96-well plate reader. The percentage of viable cells under each treatment condition was determined according to the negative control.

TLR Activation Assay. THP1-Blue-CD14 cells express TLR1-10, overexpress CD14, and are transfected with a

reporter plasmid containing SEAP under the control of a NFκB- and AP-1-inducible promoter. TLR2 and 9 activations were determined by quantifying secreted SEAP. Briefly, THP1-Blue-CD14 cells were seeded on 96-well culture plates at 2×10^5 cells/well, and the cells were treated with pimaradienoic acid for 1 h before incubating with Pam3SCK4 (10 ng/mL) and ODN2006 (10 μg/mL). After 18 h of incubation at 37 °C in a 5% CO_2 atmosphere, 20 μL of the cell suspension was added to new 96-well plates and mixed with 200 μL of QUANTI-Blue colorimetric assay reagent to detect SEAP. After incubation for 1 h for color development at 37 °C, a quantitative reading was performed at 655 nm using a microplate reader.

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