A Doctoral Dissertation

# Anti-osteoclastogenic effect and action mechanism of the sargachromanol G isolated from *Sargassum siliquastrum*

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**August, 2010**

# *Sargassum siliquastrum* 로부터 분리한 sargachromanol G 의 파골세포형성 억제 효과 및 작용 기전

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이 논문을 의학 박사학위 논문으로 제출함

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# Anti-osteoclastogenic effect and action mechanism of the sargachromanol G isolated from *Sargassum siliquastrum*

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**Abstract**

Inflammatory bone diseases are characterized by the presence of pro-inflammatory cytokines that regulate bone turnover. The receptor activator of NF-κB ligand (RANKL) is a soluble osteoblast-derived protein that induces bone resorption through osteoclast differentiation and activation. Sargachromanol G is isolated from *Sargassum siliquastrum* and has cytotoxicity, antioxidant, and antiviral activities. The active components and underlying mechanisms of its anti-osteoclastogenic activity remain largely unknown. In the present study, we investigated the anti-osteoclastogenic effects of sargachromanol G isolated from *S. siliquastrum* on the expression of IL-1β-induced osteoclastogenic factors (RANKL, IL-6, $PGE_2$ and COX-2) in human osteoblast MG-63 cells, as well as LPS or RANKL-induced pro-inflammatory factors and osteoclastogenic factors (nitric oxide (NO), cytokines (TNF-α, IL-1β and IL-6), TRAP, CTR, TRAF6, Cath-K, and MMP-9) in murine macrophage RAW 264.7 cells. We also examined the role of nuclear factor-κB (NF-κB) and mitogen activated protein kinase (MAPK) signaling induced by IL-1β in MG-63 and LPS or RANKL in RAW 264.7 cells. Sargachromanol G dose-dependently inhibited the production of osteoclastogenic factors in MG-63 and RAW 264.7 cells. Sargachromanol G also inhibited phosphorylation of NF-κB (IκB-α, p65 and p50) and MAPK (ERK1/2, JNK and p38). These results suggest that the anti-osteoclastogenic activity of sargachromanol G isolated from *S. siliquastrum* may result from modulation of osteoclastogenic factors and cytokines via suppression of phospholrylated MAPK and NF-κB activation.

Key word: RANKL, sagachromanol G, *Sargassum siliquastrum*, MAPK, NF-κB



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# 1. Introduction

Bones are constantly remodeled through the synthesis of bone matrix by osteoblasts and the resorption of bone by osteoclasts (Theill *et al*., 2002, Boyle *et al*., 2003, Karsenty *et al*., 2002). Perturbations in inflammatory cytokines, growth factors, and hormones cause an imbalance between osteoclast and osteoblast activities and can result in skeletal abnormalities such as osteoporosis and osteopetrosis (Theill *et al*., 2002, Boyle *et al*., 2003, Karsenty *et al*., 2002). Osteoporosis is a devastating disease characterized by lower bone density, frequently found in older people (especially women) (Theill *et al*., 2002, Boyle *et al*., 2003, Karsenty *et al*., 2002), immobilized patients, or even astronauts as a result of experiencing zero gravity (Boyle *et al*., 2003, Wronski *et al*., 1983), and ultimately results in bone fractures. In contrast, osteopetrosis or abnormally increased bone density occurs mainly as a result of rare hereditary disorders.

Inflammatory conditions such as osteoporosis and periodontal or osteoarthritic disease are associated with local loss of bone tissue, mainly due to activation of osteoclastic bone resorption. Based on the bone resorptive effects of cytokines such as interleukin-1 (IL-1), IL-6, IL-11, IL-17, tumor necrosis factor-α (TNF-α), TNF-β, leukemia inhibitory factor (LIF), and oncostatin M (OSM), and the inhibitory effect on bone resorption by cytokines such as IL-4, IL-10, IL-12, IL-13, IL-18, and interferon-γ (Horowitz and Lorenzo, 2002), inflammation-induced bone resorption may be mediated by stimulatory and inhibitory cytokines (Firestein, 2003, Walsh and Gravalles, 2004). Inflammatory cytokines are typically found in elevated concentrations in diseased tissue adjacent to sites of bone resorption, potentially contributing to osteoclastogenesis.

Inflammatory cytokines are typically found in elevated concentrations in diseased tissue adjacent to sites of bone resorption. These cytokines play an important role in activating



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osteoblast-osteoclast interactions that culminate in net bone resorption. Osteoblasts regulate osteoclast activity via cell-cell contact whereby osteoblast cell-surface receptor activator of NF-κB ligand (RANKL) engages osteoclast precursor cell or mature cell receptors, receptor activator of NF-κB (RANK). The interaction of RANKL with RANK is the key terminal factor in inducing osteoclast precursor cell differentiation, as well as inducing activation of mature osteoclasts (Udagawa *et al*., 2000, Khosla, 2001). However, osteoblasts also secrete osteoprotegerin (OPG), a bone-protective soluble decoy receptor for RANKL (Udagawa *et al*., 2000, Khosla, 2001). Compelling evidence indicates that the ratio of RANKL/OPG production by osteoblasts is a crucial determinant of osteoclast differentiation and activation (Udagawa *et al*., 2000, Aubin and Bonnelye, 2000). In essence, at sites of strong osteoblast-derived OPG secretion, bone resorption will diminish (Hofbauer *et al*., 2000, Teitelbaum, 2000)

Factors that control osteoclasts include RANK, its ligand RANKL (Anderson *et al*., 1997, Wong *et al*., 1997, Yasuda *et al*., 1998) and OPG (Yasuda *et al*., 1998, Tsuda *et al*., 1997, Simonet *et al*., 1997). RANKL binding to RANK drives osteoclast development from haematopoietic progenitor cells and activates mature osteoclasts. OPG negatively regulates RANKL binding to RANK and therefore inhibits bone turnover by osteoclasts.

Parathyroid hormone (PTH), prostaglandin $E_2$ ($PGE_2$), dexamethason, lipopolysaccharide (LPS), 1, 25 dihydroxyvitamin D3, and inflammatory cytokines such as IL-1 and TNF-α can stimulate RANKL expression. In contrast, estrogen or transforming growth factor-β (TGF-β) attenuate RANKL expression. OPG and RANKL expression are often oppositely regulated by these factors (Thrill *et al*., 2002, Suda *et al*., 2003, Walsh and Choi, 2003). RANKL expression is also upregulated in malignant tumor cells and involved in bone destruction in cancer (Wittrant *et al*., 2004).

Recently, it has been shown that expression of a TNF-related cytokine, RANKL, is a



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crucial factor in bone resorption (Teitelbaum, 2003, Boyle *et al*., 2003). RANKL is mainly expressed as a transmembrane protein on osteoblasts in the periosteum and on stromal cells in bone marrow, but also as a soluble cytokine. The activation of RANK on osteoclast progenitor cells by RANKL leads to stimulate of TNF receptor–associated factors (TRAFs) and then activates several downstream signaling molecules, including NF-κB, MAP kinases (MAPKs), activating protein 1 (AP-1), nuclear factor of activated T cells 2 (NFAT-2), and phosphatidylinositol 3-kinase, resulting in differentiation of the osteoclast progenitor cells to cells that finally fuse to multinucleated, bone-resorbing osteoclasts (Teitelbaum, 2003, Boyle et al., 2003, Lerner, 2004).

RANKL activates nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) (Lee *et al*., 1997, Matsumoto *et al*., 2000, Zhang *et al*., 2001), and agents that suppress RANKL signaling can suppress osteoclastogenesis-induced bone loss. The inflammatory cytokines IL-1β and TNF-α or lipopolysaccharide (LPS) are strong inducers of NF-κB and mitogen activated protein kinase (MAPK) signal transduction cascades in osteoblasts or monocyte/macrophage cell lines. RANK signaling activates NF-κB and c-Jun N-terminal kinase (JNK), which correlated with TRAF6 interactions (Hsu *et al*., 1999, Galibert *et al*., 1998). In addition, mice with a disrupted TRAF6 gene show an osteopetrotic phenotype due to a defect in bone resorption (Lomaga *et al*., 1999, Naito *et al*., 1999). Therefore, JNK might play an important role in osteoclast differentiation **(Figure 1)**.

TNF-receptor-associated factor (TRAF) proteins associate with the cytoplasmic domain of RANK and relay RANK stimulation to NF-κB. TRAF6 is required for osteoclast formation and osteoclast activation (Lomaga *et al*., 1999). NF-κB activation is also important for RANK–RANKL signaling and osteoclastogenesis. In mice, IKKβ is required for RANKL-induced osteoclastogenesis both *in vitro* and *in vivo*, whereas IKKα appears to be required only *in vitro* but not *in vivo* (Ruocco *et al*., 2005).



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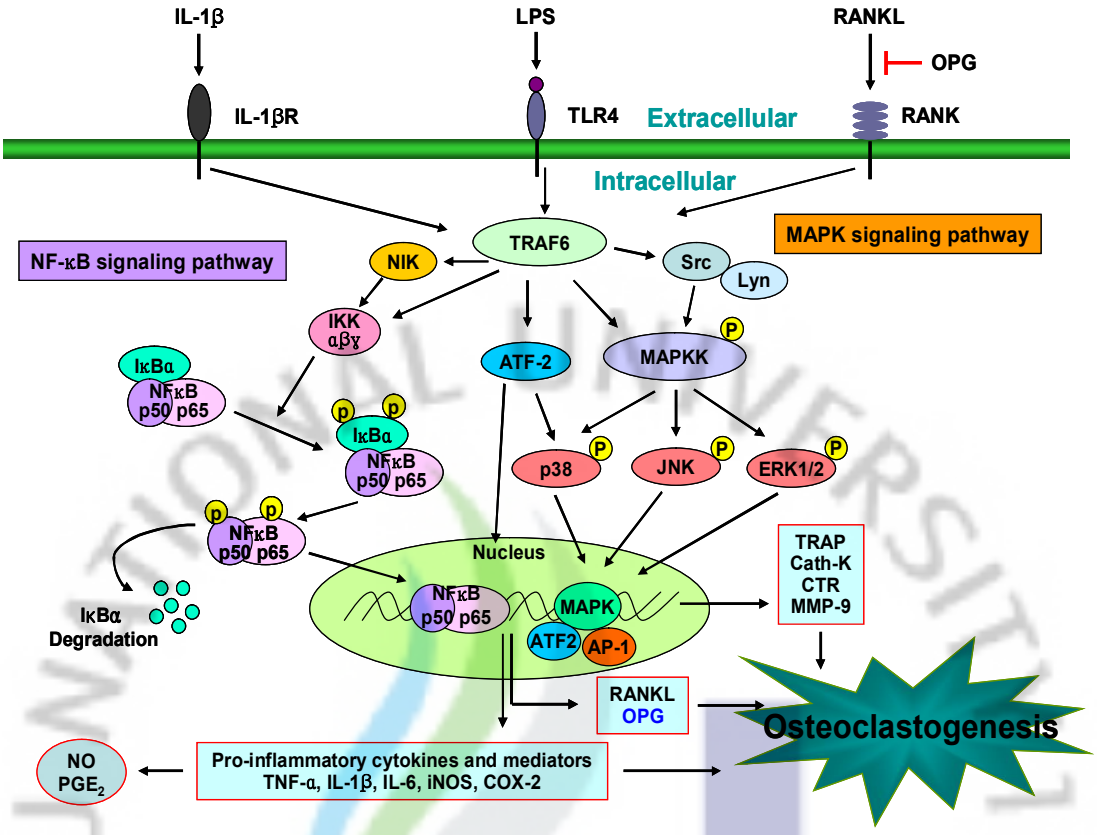


**Figure 1. Intracellular signaling pathway of osteoclastogenesis**



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Mitogen-activated protein (MAP) kinases are proline-directed serine/threonine kinases that are important in cell growth, differentiation, and apoptosis (Nishida and Gotoh, 1993, Avruch *et al*., 1994, Davis, 1994, Iwasaki *et al*., 1996). They become activated by phosphorylation on threonine and tyrosine in response to external stimuli. Three major subfamilies of MAP kinase have been identified in mammalian cells: p38-MAPKs (p38-MAPKα, β, γ and δ), c-Jun N-terminal kinases (JNK1, 2 and 3), extracellular signal-regulated kinases (ERK1 and ERK2), and the big MAPKs ERK5, ERK7 and ERK8 (Wada and Penninger, 2004, Chang and Karin, 2001), all of which play important roles in osteoclastogenesis. Many of these kinases are activated downstream of RANK and can mediate the final cellular response. p38-MAPKs, JNKs (also called SAPKs: stress activated protein kinases), and their direct upstream kinase, MKKs, are involved in osteoclastogenesis *in vitro* (David *et al*., 2002, Yamamoto *et al*., 2002). AP-1 transcription factors under the control of JNKs are also involved in RANK-regulated osteoclastogenesis. The AP-1 family members c-Jun, JunB, c-Fos, and Fra but not JunD are essential for efficient osteoclastogenesis (David *et al*., 2002, Kenner *et al*., 2004, Wagner, 2002). Src family kinases (SFKs), including c-Src and Lyn, are nonreceptor tyrosine kinases that influence growth, differentiation, cytoskeletal organization, and survival (Lowell and Soriano, 1996). SFKs and ERK are also activated by RANK. SFKs and inhibition of MEKs (ERK kinases) by PD98059 or U0126 does not, however, attenuate osteoclast differentiation (Matsumoto *et al*., 2000), but rather increases osteoclastogenesis (Hotokezaka *et al*., 2002). Stimulation of p38 activates the microphthalmia/microphthalmia transcription factor to regulate genes encoding tartrate-resistant acid phosphatase (TRAP) and cathepsin K, indicating the importance of p38 signaling cascades (Boyle *et al*., 2003).

There are various species of marine algae in Jeju Island, Korea. Useful secondary metabolites are obtained from marine algae. So, study of the marine algae is very valuable.



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We aimed to screen marine algae products for their ability to suppress osteoclastogenesis factors. We found that *Sargassum siliquastrum* had inhibitory effects on osteoclastogenesis factors. *S. siliquastrum* wae studied of activities such as cytotoxicity, antioxidant and antiviral. Sargachromanol G was isolated from the brown alga *S. siliquastrum* collected from Jeju Island, Korea. In this study, we examined the effect of sagachromanol G on IL-1β and RANKL-induced osteoclastogenesis related factors, the role of MAPK and NF-κB activation on RANKL expression in osteoblast (MG-63) cells. Also, we examined the effect of sagachromanol G on LPS and RANKL-induced osteoclastogenesis factors, the role of MAPK and NF-κB activation on osteoclastogenesis related factors in RAW 264.7 cells.



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## 2. Material and Methods

### 2-1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY). $PGE_2$ and IL-6 ELISA kits were purchased from R&D systems, Inc. (St. Louis, MO) and BD biosciences (San Diego, CA). COX-2 and iNOS antibodies were purchased from BD Biosciences (San Diego, CA, USA) and Calbiochem (San Diego, CA, USA). NF-κB (anti-p50, anti-p65 and anti-IκBα), MAPKs (anti-ERK1/2, anti-JNK and anti-p38) and osteoclastogenic factors (TRAP, MMP-9, TRAF6, anti-SFK, anti-Lyn, anti-MEK and anti-ATF2) mouse or rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. PD98059 (a specific inhibitor of ERK1/2), SB203580 (a specific inhibitor of p38), SP600125 (a specific inhibitor of JNK), PDTC (a specific inhibitor of NF-κB), and all other reagents were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

### 2-2. Isolation of sagachromanol G

The marine alga *Sargassum siliquastrum* was collected along the coast of Jeju Island, Korea, between October 2005 and March 2006. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water, and maintained in a medical refrigerator at -20 °C. Frozen samples were lyophilized and homogenized with a grinder prior to extraction. The powdered *S. siliquastrum* (400 g) was extracted three times with 80% aqueous methanol (MeOH) and filtered. The filtrate was then evaporated under vacuum to obtain the 80% MeOH extract, which was dissolved in water, and then partitioned with dichloromethane ($CH_2Cl_2$) The



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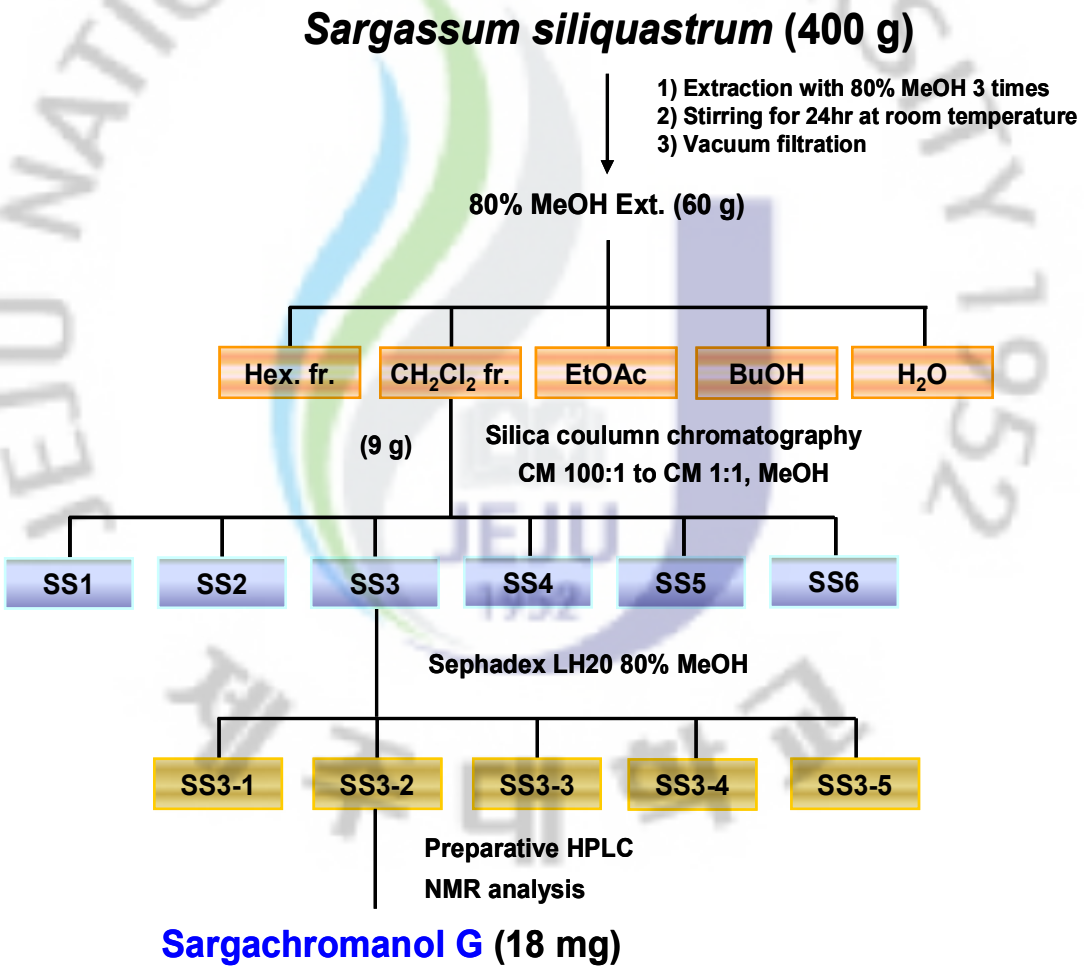
$CH_2Cl_2$ fraction (9 g) was fractionated by silica column chromatography with stepwise elution of $CH_2Cl_2$–MeOH mixture (100:1–1:1) to afford separated active fractions. A combined active fraction from the silica gel chromatography was fractionated by a Sephadex LH-20 column with 80% aqueous MeOH, and was finally purified with reversed-phase HPLC to give the compound **(Scheme 1)**.




**Scheme 1**. **Isolation procedure of compounds from *Sargassum siliquastrum***



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**2-3. Cell culture**

The human osteoblast cell line, MG-63, and the murine macrophage cell line, RAW 264.7, were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO Inc.), supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). These cells were maintained at subconfluence in a 95% air, 5% $CO_2$ humidified atmosphere at 37 ℃ and were subcultured every 3-4 days. Cells were counted with a hemocytometer and the number of viable cells was determined through trypan blue dye exclusion.

**2-4. Cell viability**

Cell viability was measured by Lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays. LDH leakage measures membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol into the medium. LDH activity was determined following the production of NADH during the conversion of lactate to pyruvate. Briefly, culture medium was centrifuged at 12,000 rpm for 3 min at room temperature to remove cells. The cell-free culture medium (50 μL) was collected and then incubated with 50 μL of the reaction mixture from the cytotoxicity detection kit for 30 min at room temperature in the dark. 50 μL of HCl (1N) was added to each well to stop the enzymatic reaction. The optical density was then measured with an ELISA plate reader at 490 nm. The percent cytotoxicity was determined relative to the control group (Fernandez et al., 2006).

Cell viability was measured by a conventional MTT assay. Cells were seeded on 96-well plates and cultured for 24 h, followed by 24 h in medium with horse placenta, MTT solution (10 mg/mL in phosphate buffered-saline, pH 7.4) was added at 50 μL per well (0.5 mg/mL



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final concentration), and plates were incubated for 4 h at 37 °C to completely dissolve the formazan crystals. The incubation was stopped by addition of 15% sodium dodesyl sulfate into each well for solubilization of formazans, and the optical density (OD) at 570 nm ($OD_{570-630}$) was measured with a microplate reader.

**2-5. Measurement of $PGE_2$ production**

MG-63 cells ($1.5\times10^5$ cells/mL) and RAW 264.7 cells ($1.5\times10^5$ cells/mL) were stimulated with IL-1β (10 ng/mL) and LPS (1 μg/mL) for 24 h, respectively. $PGE_2$ levels in the culture supernatant were determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The assay is based on competition between unlabelled $PGE_2$ and a fixed quantity of peroxidase-labeled $PGE_2$ for a limited number of binding sites on a $PGE_2$-specific antibody. The optical density of the solution was then measured with a microplate reader at 450 nm. All experiments were performed in triplicate.

**2-6. Measurement of cytokines (TNF-α, IL-1β and IL-6) production**

Cytokine production was determined using a sandwich ELISA. Sargachromanol G was diluted with EtOH (less than 0.1%) in DMEM before treatment. MG-63 cells ($1.0\times10^5$ cells/mL) were incubated for 18 h in 24-well plates, followed by IL-1β (10 ng/mL) and Sargachromanol G for 24 h. RAW 264.7 cells ($2.0\times10^5$ cells/mL) were incubated for 18 h followed by LPS (1 μg/mL) and sargachromanol G for 24 h. Media levels of TNF-α, IL-1β, and IL-6 assays were performed using mouse ELISA kits (R & D Systems Inc, MN, USA) and measured at 450 nm. All experiments were performed in triplicate.



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**2-7. Measurement of NO production**

RAW 264.7 cells ($1.5\times10^5$ cells/mL) were pre-incubated for 18 h and then treated with LPS (1 μg/mL) and sargachromanol G for 24 h. Nitrite in culture supernatants was measured by mixing 100 μL of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μL medium. The concentration of $NO_2$- was calculated by comparison with a standard curve prepared using $NaNO_2$. All measurements were performed in triplicate.

**2-8. Immunoblotting analysis**

The cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonident P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 g/mL leupeptin] and kept on ice for 30 minutes. The cell lysates were centrifuged at 12,000 × g at 4 ℃ for 15 minutes and supernatants were stored at -70 °C until use. Protein concentrations were measured using the Bradford method. Aliquots of the lysates (30-40 μg of protein) were separated on an 8-12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking nonspecific sites with 5% nonfat dried milk, the membrane was incubated with primary antibody (1:1000) at 4 °C overnight. The membrane was further incubated for 60 minutes with horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (1:5000, Amersham Pharmacia Biotech, Little Chalfont, UK). The proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersharm Pharmacia Biotech., NY, USA).



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**2-9. RT-PCR analysis**

Total RNA from cells was prepared by adding TRIzol Reagent (Gibco BRL), according to the manufacturer's protocol and stored at -70 °C until use. Semiquantitative RT reactions were performed with MuLV reverse transcriptase. Total RNAs (1 μg) were incubated with oligo-dT15 at 70 °C for 5 minutes and mixed with 5x first strand buffer, 10 mM dNTP, and 0.1 M DTT at 37 °C for 5 minutes, and for 60 minutes after the addition of MuLV reverse transcriptase (2 U). The reactions were terminated at 70 °C for 10 minutes and RNA was depleted by adding RNase H. The PCR mixture [2 μL cDNA, 4 μM 5' and 3' primers, 10x buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1 % Triton X-100), 250 μM dNTP, 25 mM $MgCl_2$, and 1 unit Taq polymerase (Promega, USA)] was run with a 30 second denaturation time at 94 °C, an annealing time of 60 seconds at 55 to 62 °C, an extension time of 60 seconds at 72 °C, and final extension of 7 minutes at 72 °C at the end of 30 cycles. Primers (Bioneer, Seoul, Korea) are shown in **Table 1**.

**Table 1. Sequences of primers and product lengths of the genes in RT-PCR analysis**

| Gene | | Primer sequences | Fragment size(bp) |
|---|---|---|---|
| TRAP | F | 5'-AAATCACTCTTTAAGACCAG-3' | 317 |
| | R | 5'-TTATTGAATAGCAGTGACAG-3' | |
| Cath-K | F | 5'-CCTCTCTTGGTGTCCATACA-3' | 490 |
| | R | 5'-ATCTCTCTGTACCCTCTGCA-3' | |
| MMP-9 | F | 5'-CTGTCCAGACCAAGGGTACAGCCT-3' | 383 |
| | R | 5'-GTGGTATAGTGGGACACATAGTGG-3' | |
| CTR | F | 5'-ACCGACGAGCAACGCCTACGC-3' | 272 |
| | R | 5'-GCCTTCACAGCCTTCAGGTAC-3' | |
| β-Actin | F | 5'-GTGGGCCGCCCTAGGCACCAG-3' | 603 |
| | R | 5'-GGAGGAAGAGGATGCGGCAGT-3' | |



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### 2-10. Tartrate-resistant acid phosphatase (TRAP) staining and assay

RAW 264.7 cells were plated at $2.0 \times 10^4$ cells/well using a 48-well tissue culture plate in the presence of RANKL (100 ng/mL) for 96 h. Cells were fixed in 3.7% formalin for 10 min. Fixed cells were washed with PBS twice and permeabilized with 0.1% Triton X-100 for 1 min. Cells were stained for TRAP activity using a kit (Sigma-Aldrich Chemical Co., St Louis, MO, USA) according to the manufacturer's instructions. After incubation at 37 °C in a humid and light-protected incubator for 1 h, cells were washed with distilled water three times. TRAP-positive cells appeared dark red, and TRAP-positive cells containing three or more nuclei were determinded as osteoclasts. RAW 264.7 cells ($1.0 \times 10^5$ cells/mL) were incubated for 18 h in 48-well plates with the same conditions. RANKL (100 ng/mL) and the sargachromanol G were then added to the cultured cells for 72 h incubation. The medium was used for TRAP activity using TRAP assay kit. TRAP assay was then measured by using an ELISA plate reader at 450 nm.

### 2-11. Statistical analyses

Student's t-test and one-way ANOVA were used for statistical analysis. Data are expressed as means ± standard deviation (SD) of at least three independent experiments performed in triplicate. P-values of 0.005 or less were considered statistically significant.



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# 3. Results

## 3-1. Results

**Isolation of sargachromanol G and effect of sagachromanol G in IL-1β-stimulated osteoblast**

### 3-1-1. Sargachromanol G isolated from *Sargassum siliquastrum*

In screening marine algae products for suppression of osteoclastogenic factors, we found that *Sargassum siliquastrum* inhibited osteoclastogeneic and inflammatory factors, especially the $CH_2Cl_2$ fraction. We fractionated the $CH_2Cl_2$ fraction with silica gel and Sephadex LH-20 open column chromatography and purified the active compounds by reversed-phase HPLC. Sargachromanol G showed an HREIMS $[M]^+$ ion peak at m/z 426.2774 for a molecular formula of $C_{27}H_{38}O_4$ (calcd 426.2770). The $^{13}C$ NMR data for this compound were very similar to sargachromanol lineage, with the replacement of an oxymethine with a 67 carbonyl carbon at δC 201.3 as the most significant difference. Combined analyses of 2D NMR data located the carbonyl and remaining hydroxyl group at C-12 and C-13, respectively **(Figures 3, 4) (Table 2)**. An 18 mg of sargachromanol G was yielded from *S. siliquastrum* dried powder (400 g) and the purity was confirmed by NMR and HPLC as 99.9%.

**Sargachromanol G** : colorless gum; $[\alpha]^{20}D$ – 79.2° (*c* 0.12, MeOH); IR (NaCl) *ν*max 3400-3300, 2970, 2930, 1665, 1470, 1220 $cm^{-1}$; UV (MeOH): $\lambda_{max}$ nm (log ε) 230 (3.80) nm; $^1H$ and $^{13}C$ NMR, see **Table 2**; HREIMS *m/z* 426.2774 $[M]^+$ (calcl for $C_{27}H_{38}O_4$, 426.2770).



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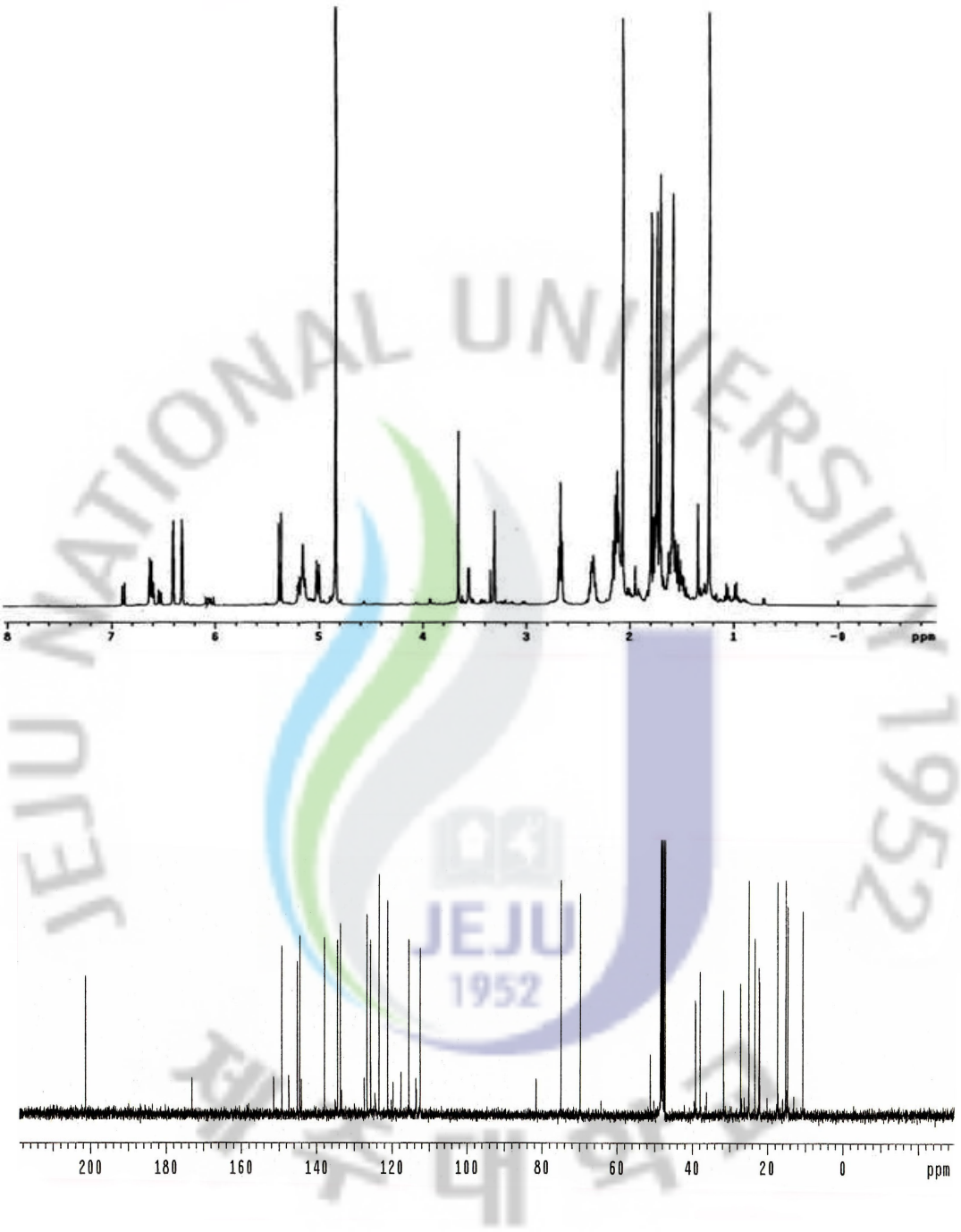


**Figure 2. Proton and Carbon NMR spectrum of sargachromanol G**



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Table 2. $^{1}H$ and $^{13}C$ NMR assignments for sargachromanol G

| Position | $^{13}C$ | $^{1}H$ (mult. *J*=Hz) |
|---|---|---|
| 1 | 22.2 | 2.65 ($^{2}H$, *J*=7.2) |
| 2 | 31.6 | 1.79 ($^{1}H$, m) |
| | | 1.72 ($^{1}H$, m) |
| 3 | 75 | |
| 4 | 39.1 | 1.63 ($^{1}H$, m) |
| | | 1.48 ($^{1}H$, m) |
| 5 | 22.1 | 2.12 ($^{2}H$, m) |
| 6 | 125.6 | 5.14 ($^{1}H$, *J*=7.3) |
| 7 | 133.7 | |
| 8 | 37.9 | 2.00 ($^{2}H$, t, *J*=7.8) |
| 9 | 27.1 | 2.34 ($^{2}H$, m) |
| 10 | 144.5 | 6.55 ($^{1}H$, *J*=7.3) |
| 11 | 134.4 | |
| 12 | 201.3 | |
| 13 | 69.8 | 6.55 ($^{1}H$, d, *J*=9.6) |
| 14 | 123.4 | 5.00 ($^{1}H$, dh, *J*=9.6, 1.5) |
| 15 | 137.9 | |
| 16 | 24.9 | 1.71 ($^{3}H$, S) |
| 17 | 17.3 | 1.82 ($^{3}H$, S) |
| 18 | 10.7 | 1.80 ($^{3}H$, S) |
| 19 | 14.6 | 1.59 ($^{3}H$, S) |
| 20 | 23.3 | 1.25 ($^{3}H$, S) |
| 1' | 145.1 | |
| 2' | 121.1 | |
| 3' | 112.4 | 6.30 ($^{1}H$, d, *J*=2.8) |
| 4' | 149.2 | |
| 5' | 115.4 | 6.39 ($^{1}H$, d, *J*=2.8) |
| 6' | 126.5 | |
| 7' | 15.2 | 2.05 ($^{3}H$, s) |

* 400 MHz for $^{1}H$ and 100 MHz for $^{13}C$



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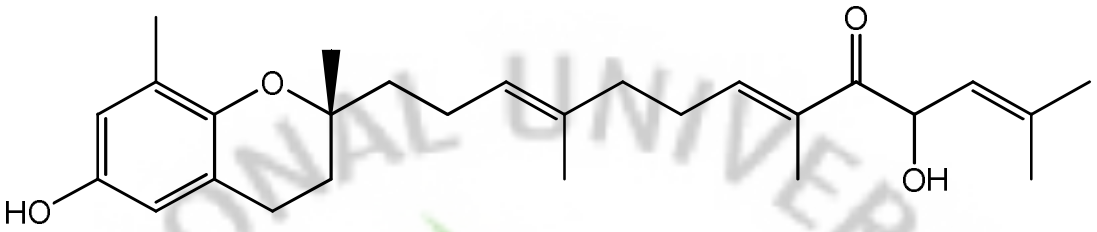


**Figure 3. The Structure of sargachromanol G isolated from *Sargassum siliquastrum***



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**3-1-2. Effects of solvent fractions and sargachromanol G from *S. siliquastrum* on the cell viability in MG-63 cells**

MG-63 cells were treated with various concentrations of solvent fractions and sargachromanol G isolated from *S. siliquastrum* for 24 h and cell viability was assessed using an LDH and MTT assay. Solvent fractions (50 μg/mL) and Sargachromanol G (40 μM) exhibited no cytotoxic effect on cells in comparison to control cells that received no treatment. The highest concentration of solvent fractions and sargachromanol G that did not cause more than 20% loss in cell viability were 50 μg/mL and 40 μM, respectively. Therefore, 40 μM sargachromanol G was chosen for further studies **(Figure 4-5-2)**.



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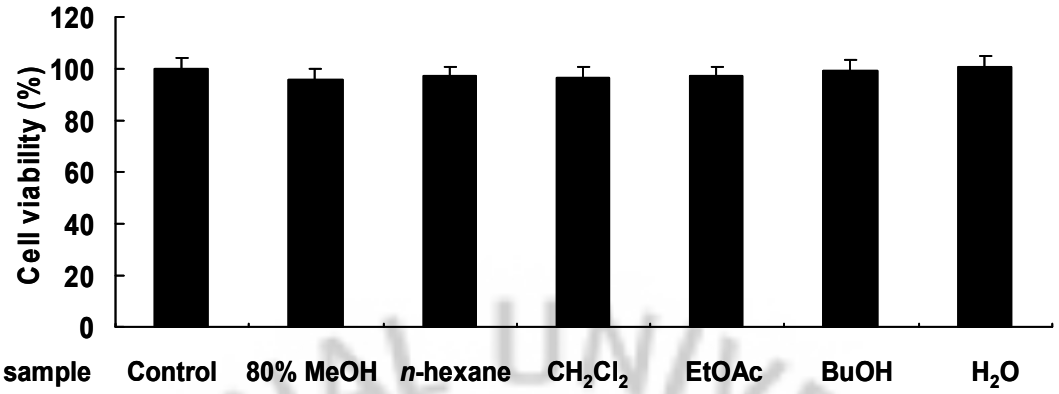


**Figure 4. Effects of 80% MeOH extract and solvent fractions of *S. siliquastrum* on the cell viability in MG-63 cells.** An MTT assay was performed after incubation of MG-63 cells with the 80% MeOH extract and solvent fractions of *S. siliquastrum* (50 μg/mL) for 24 h at 37 °C in a 5% $CO_2$ atmosphere.



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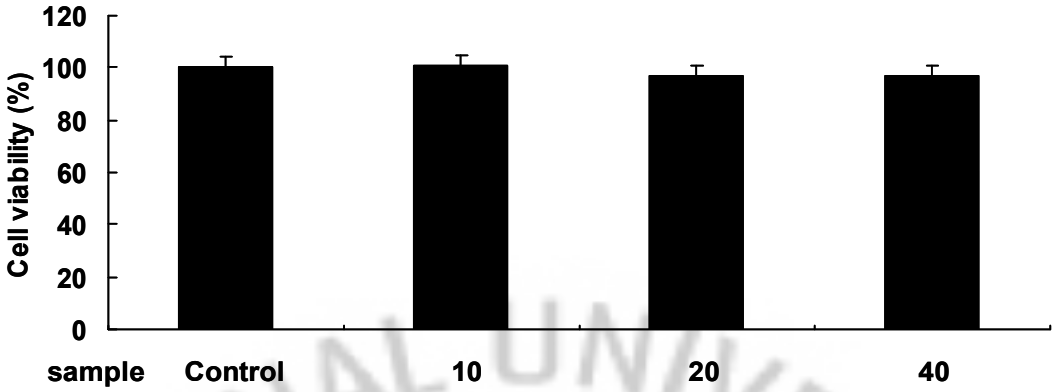


**Figure 4-1. Effect of sargachromanol G on the cell viability in MG-63 cells.** An MTT assay was performed after incubation of MG-63 cells with sagachromanol G (10, 20 and 40 μM) isolated from *S. siliquastrum* for 24 h at 37 °C in a 5% $CO_2$ atmosphere.



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### 3-1-3. Effects of solvent fractions and sargachromanol G on $PGE_2$ production in IL-1β-stimulated MG-63 cells

$PGE_2$ is an inflammatory mediator produced from the conversion of arachidonic acid by cyclooxygenase (COX). COX-2 is induced by cytokines and other activators, such as IL-1β, to release $PGE_2$ at inflammatory sites. IL-1β (10 ng/mL) treatment of MG-63 cells for 24 h increased $PGE_2$ levels in the medium, and both the $CH_2Cl_2$ fraction of *S. siliquastrum* and sargachromanol G dose-dependently blocked this effect **(Figure 5-5-2)**. The MAPK and NF-κB inhibitors PD98059, SB203580, SP600125, and PDTC also reduced $PGE_2$ levels.



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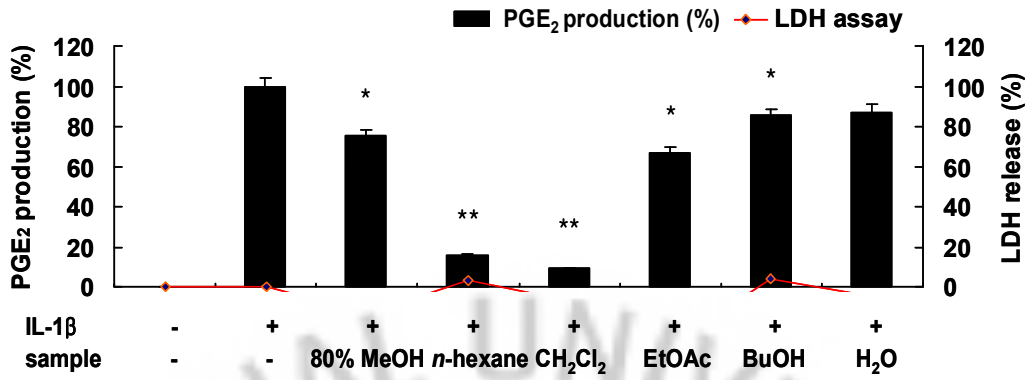


**Figure 5. Effects of 80% MeOH extract and solvent fractions of *S. siliquastrum* on $PGE_2$ production and cytotoxicity in IL-1β-stimulated MG-63 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with IL-1β (10 ng/mL) for 24 h in the presence of 80% EtOH extract and solvent fractions of *S. siliquastrum* (50 μg/mL). Supernatants were collected and the $PGE_2$ concentration in the supernatants was determined by ELISA. Cytotoxicity was determined using the LDH method. Values are the mean ± SEM of triplicate experiments. *, *P<0.05; **, P<0.01*



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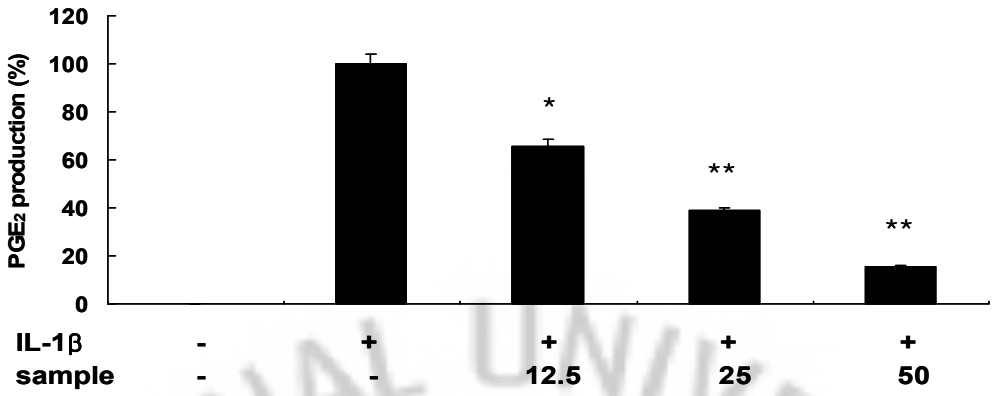


**Figure 5-1. Inhibitory effect of $CH_2Cl_2$ fraction of *S. siliquastrum* on $PGE_2$ production in IL-1β-stimulated MG-63 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with IL-1β (10 ng/mL) for 24 h in the presence of $CH_2Cl_2$ fraction of *S. siliquastrum* (12.5, 25 and 50 μg/mL). Supernatants were collected, and the $PGE_2$ concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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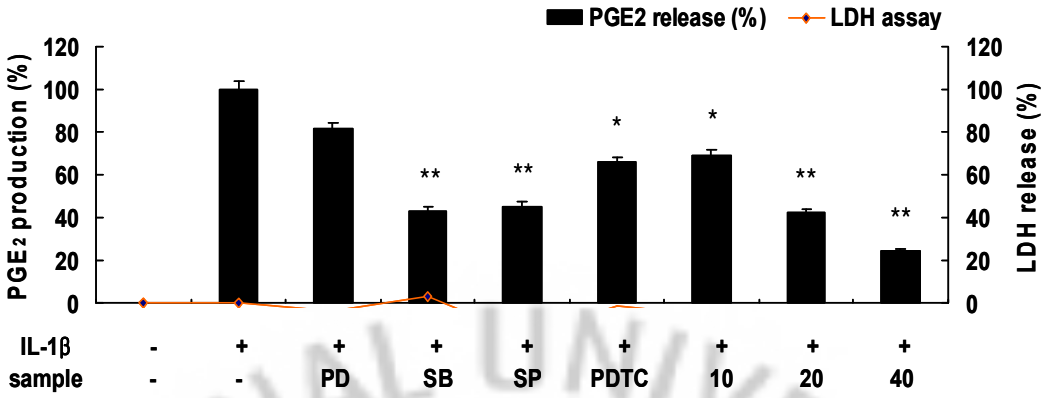


**Figure 5-2. Effects of sargachromanol G on $PGE_2$ production and cytotoxicity in IL-1β-stimulated MG-63 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with IL-1β (10 ng/mL) for 24 h in the presence of sargachromanol G (10, 20 and 40 μM), PD: PD98059 (20 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM). Supernatants were collected, and the $PGE_2$ concentration in the supernatants was determined by ELISA. Cytotoxicity was determined using the LDH method. Values are the mean ± SEM of triplicate experiments. *, *P<0.05; **, P<0.01*



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**3-1-4. Effects of solvent fractions and sargachromanol G on IL-6 production in IL-1β-stimulated MG-63 cells**

IL-1β and $PGE_2$ stimulate IL-6 production in osteoblasts. IL-1β (10 ng/mL) for 24 h increased IL-6 production in MG-63 cells, but both the $CH_2Cl_2$ fraction of *S. siliquastrum* and sargachromanol G dose-dependently this IL-6 production **(Figure 6-6-2)**. MAPK and NF-κB inhibitors PD98059, SB203580, SP600125, and PDTC also reduced IL-6 levels.



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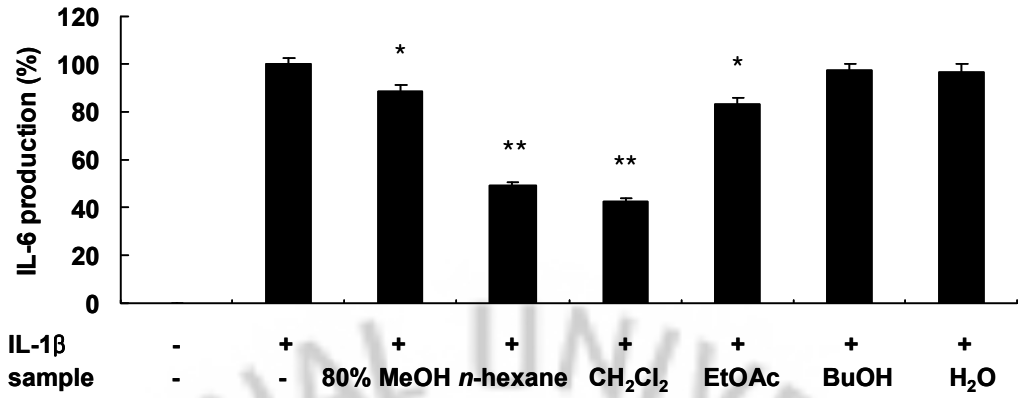


**Figure 6. Inhibitory effects of 80% MeOH extract and solvent fractions of *S. siliquastrum* on IL-6 production in IL-1β-stimulated MG-63 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with IL-1β (10 ng/mL) for 24 h in the presence of 80% MeOH extract and solvent fractions of *S. siliquastrum* (50 μg/mL). Supernatants were collected, and the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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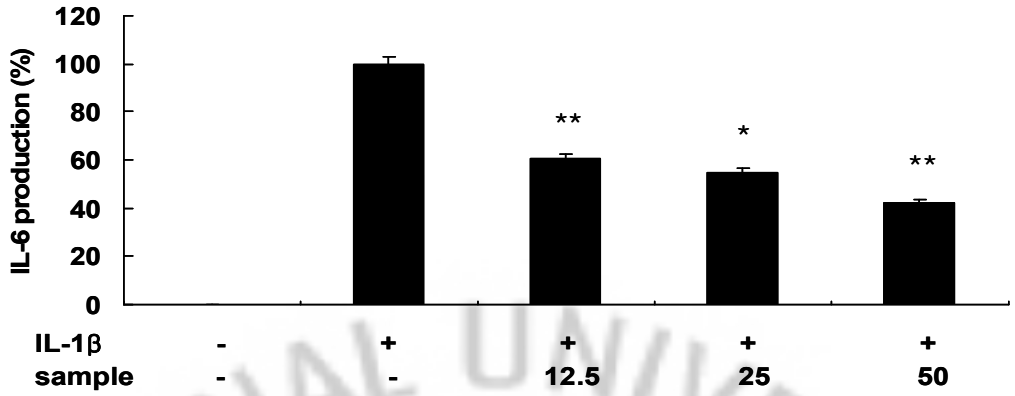


**Figure 6-1. Inhibitory effect of $CH_2Cl_2$ fraction of *S. siliquastrum* on IL-6 production in IL-1β-stimulated MG-63 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with IL-1β (10 ng/mL) for 24 h in the presence of $CH_2Cl_2$ fraction of *S. siliquastrum* (50 μg/mL). Supernatants were collected, and the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. *, *P<0.05; **, P<0.01*



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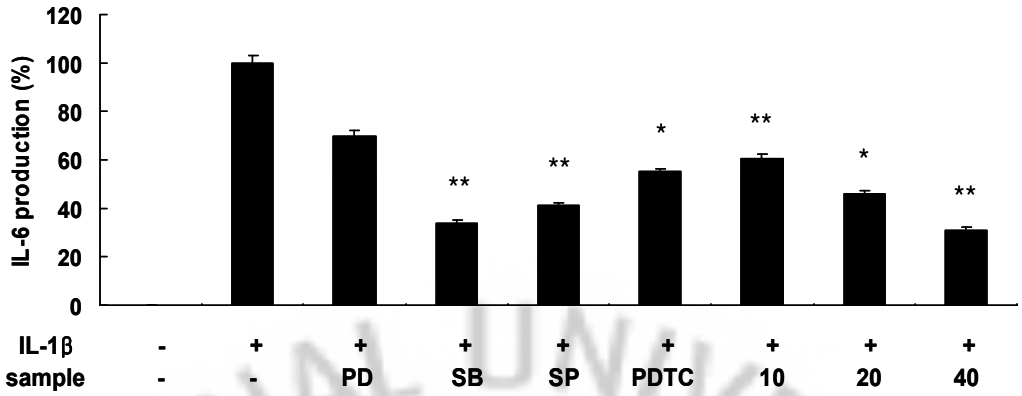


**Figure 6-2. Inhibitory effect of sargachromanol G on IL-6 production in IL-1β-stimulated MG-63 cells.** Cells ($1.5 \times 10^5$cells/mL) were stimulated with IL-1β (10 ng/mL) for 24 h in the presence of sargachromanol G (10, 20 and 40 μM), PD: PD98059 (20 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM). Supernatants were collected, and the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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### 3-1-5. Effects of sargachromanol G on protein levels of COX-2, RANKL and OPG in IL-1β-stimulated MG-63 cells

RANKL binding to RANK drives osteoclast development from haematopoietic progenitor cells and activates mature osteoclasts. OPG negatively regulates RANKL binding to RANK and therefore inhibits bone turnover by osteoclasts. RANKL activates NF-κB, JNK, p38, and p44/p42 mitogen-activated protein kinase (Lomaga *et al*., 1999, Naito *et al*., 1999, Rucoco *et al*., 2005) to suppress osteoclastogenesis-induced bone loss. We therefore tested whether sargachromanol G affected COX-2 levels using immunoblotting. Sargachromanol G dose-dependently reduced COX-2 levels **(Figure 7)**. RANKL deficiency and OPG over-expression increase bone mass due to decreased osteoclast formation and activation, whereas OPG deficiency induces osteoporosis due to enhanced osteoclast formation. Sargachromanol G reduced RANKL and increased OPG protein levels **(Figure 7)**.



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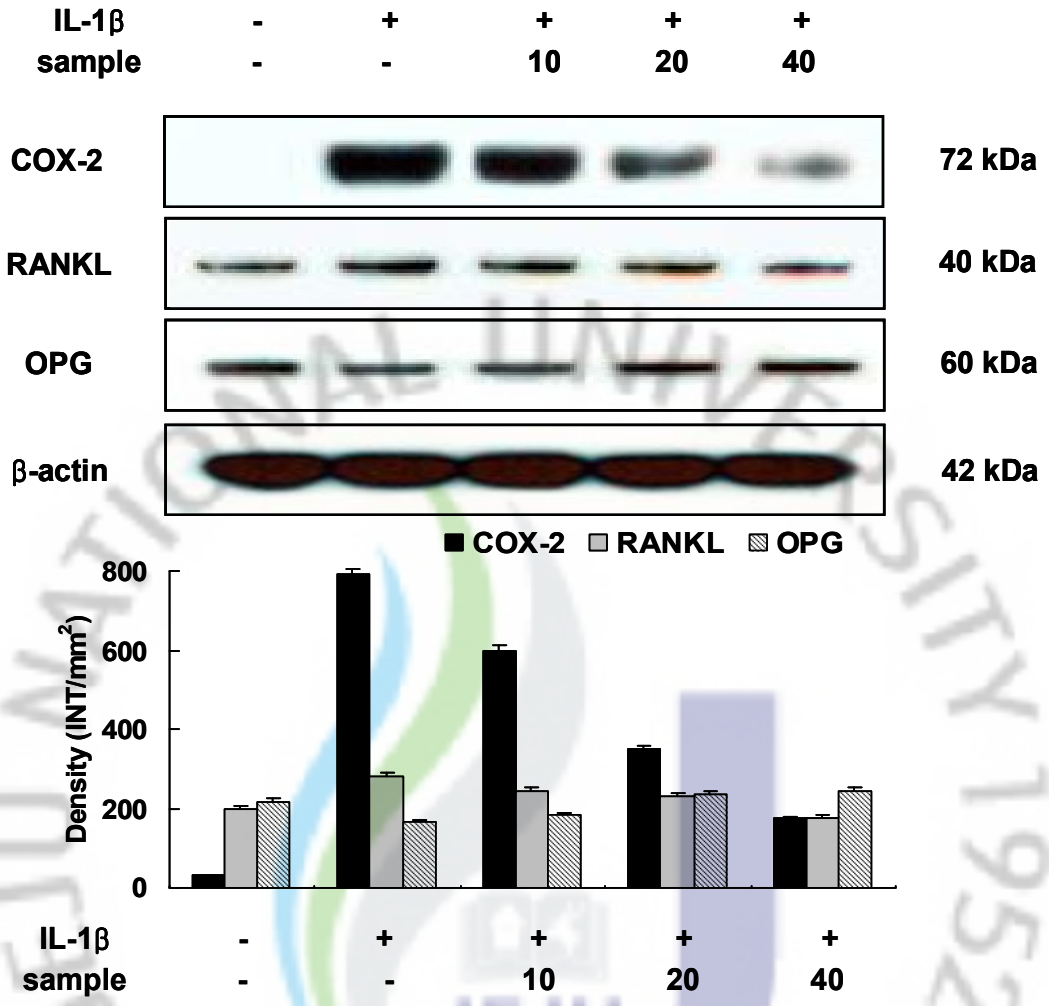


**Figure 7. Effects of sargachromanol G isolated from *S. siliquastrum* on the protein levels of COX-2, RANKL and OPG in IL-1β-stimulated MG-63 cells.** MG-63 cells ($1.0 \times 10^6$ cells/mL) were pre-incubated for 18 h, and the cells were stimulated with IL-1β (10 ng/mL) in the presence of sargachromanol G (10, 20 and 40 μM) for 72 h. Protein level was determined using immunoblotting method.



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### 3-1-6. Effect of sargachromanol G on NF-κB signaling pathway in IL-1β-stimulated MG-63 cells

It has been demonstrated that IL-1β activates NF-κB, a transcription factor that leads to the induction of the expression of many immediate early genes. To clarify the inhibitory mechanism of action of sargachromanol G for IL-1β-induced NF-κB, the translocation of p65 and p50 as well as IκB-α degradation were examined. As a control, we applied PDTC, a specific NF-κB inhibitor. As shown in Figure **(Figure 9-10)**, treatment with IL-1β increased p65 and p50. In the presence of sargachromanol G and PDTC, nuclear translocation of p65 and p50 was inhibited in a dose-dependent manner in MG-63 cells. Moreover, sargachromanol G inhibited the IL-1β-induced degradation of IκB-α **(Figure 8)**. These results indicate that sargachromanol G may inhibit the IL-1β-induced activation of NF-κB via an inhibition of IκB-α degradation as well as a translocation of p65 and p50 into the nucleus, resulting in the inhibition of IL-1β-induced osteoclastogenic factors production.



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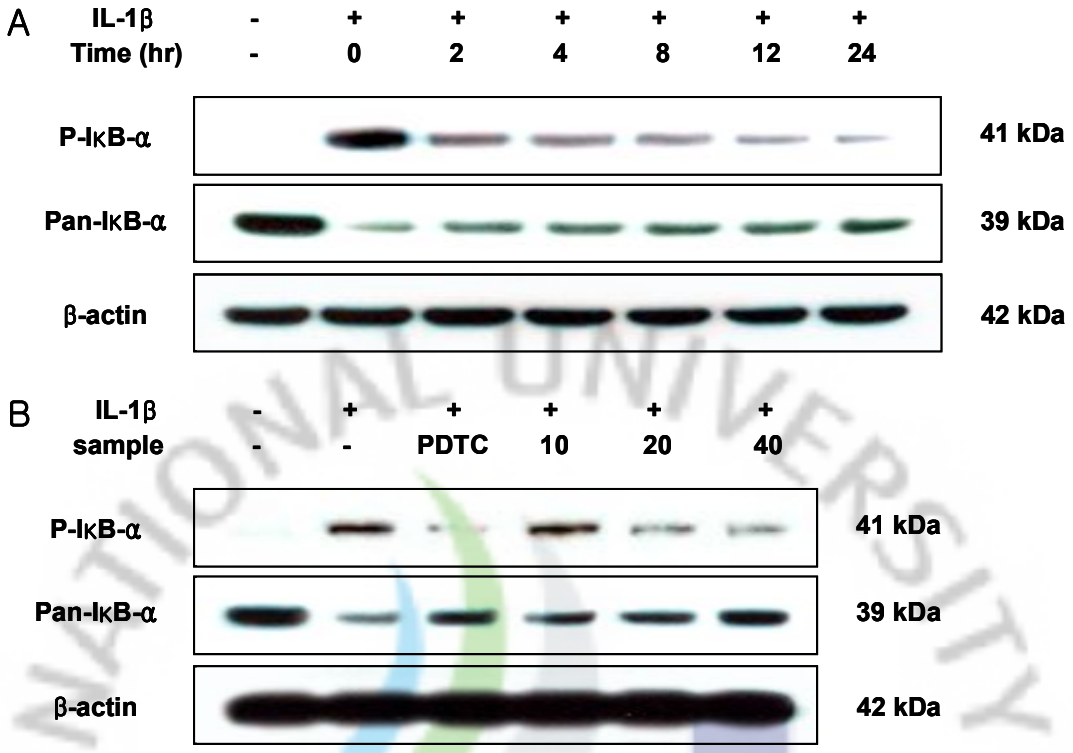


**Figure 8. Inhibitory effect of sargachromanol G on IκB-α protein level in IL-1β-stimulated MG-63 cells. A** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (40 μM) for 0-24 h, and then stimulated with IL-1β (10 ng/mL) for 5 min. **B** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and PDTC (10 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 5 min. The levels of p-IκB-α (phosphorylated-IκB-α) and pan-IκB-α were determined using immunoblots.



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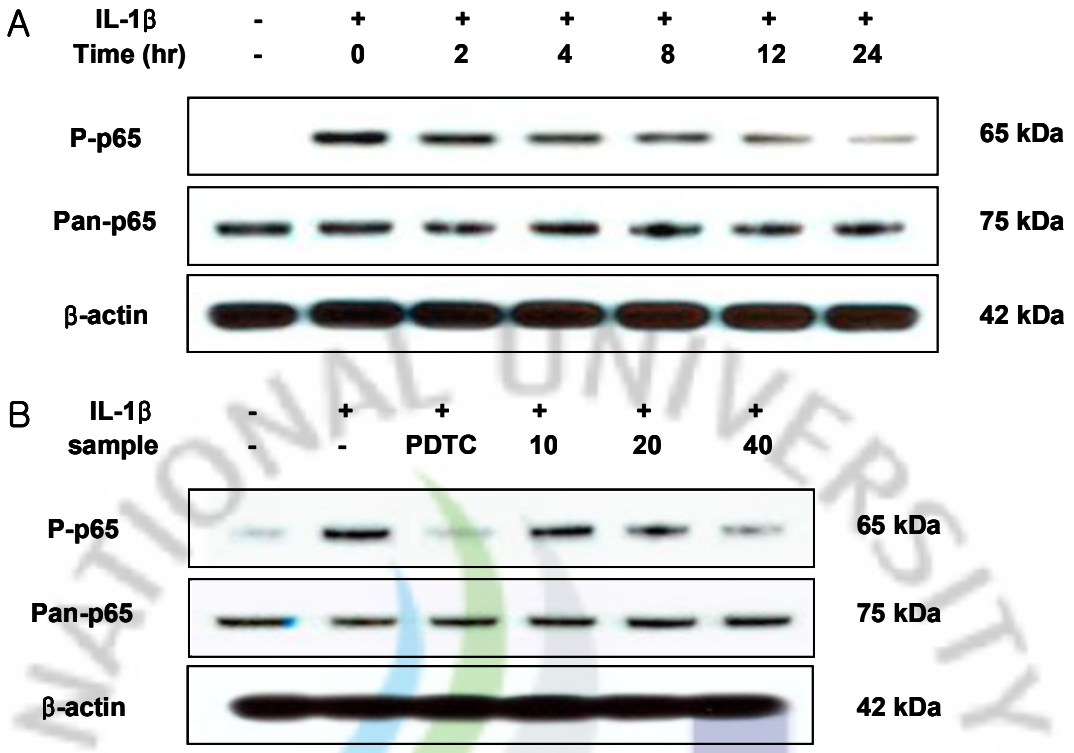


**Figure 9. Inhibitory effect of sargachromanol G on p65 protein level in IL-1β-stimulated MG-63 cells. A** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (40 μM) for 0-24 h, and then stimulated with IL-1β (10 ng/mL) for 5 min. **B** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and PDTC (10 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 5 min. The levels of p-p65 (phosphorylated-p65) and pan-p65 were determined using immunoblotting method.



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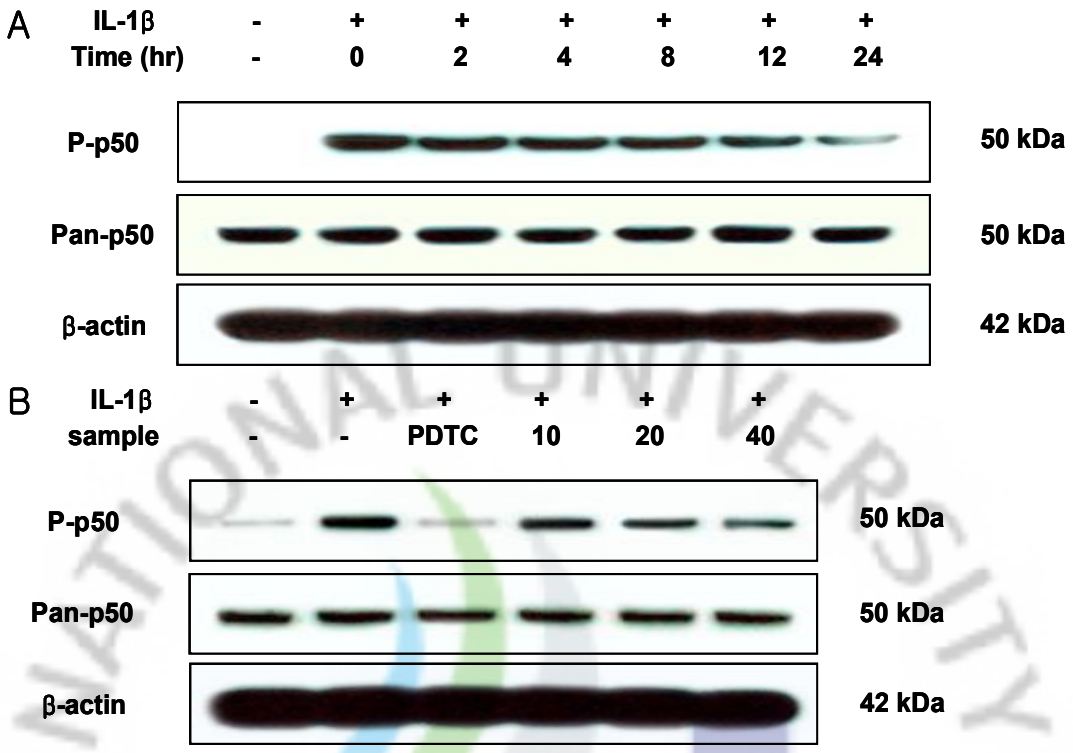


**Figure 10. Inhibitory effect of sargachromanol G on p50 protein level in IL-1β-stimulated MG-63 cells. A** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (40 μM) for 0-24 h, and then stimulated with IL-1β (10 ng/mL) for 5 min. **B** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and PDTC (10 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 5 min. The levels of p-p50 (phosphorylated-p50) and pan-p50 were determined using immunoblotting method.



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### 3-1-7. Effects of sargachromanol G on MAPKs signaling pathway in IL-1β-stimulated MG-63 cells

MAP kinase is known to be important in the expression of osteoclastogenic factors. MAPK regulate cell growth and differentiation, as well as responses to cytokines and stresses, and contribute to the expression of osteoclastogenic factors. We next tested whether sargachromanol G affected IL-1β-induced phosphorylation of ERK, JNK and p38 in MG-63 cells using immunoblotting. Indeed, sargachromanol G (10, 20 and 40 μM) inhibited ERK, JNK and p38 MAP kinase activation, but did not change total protein levels **(Figure 11-13)**. IL-1β-induced MAPK expression was significantly suppressed by the MAPK inhibitors PD98059, SB203580, and SP600125. Thus, sargachromanol G may inhibit NF-κB activity by reducing the phosphorylation of ERK, JNK and p38.



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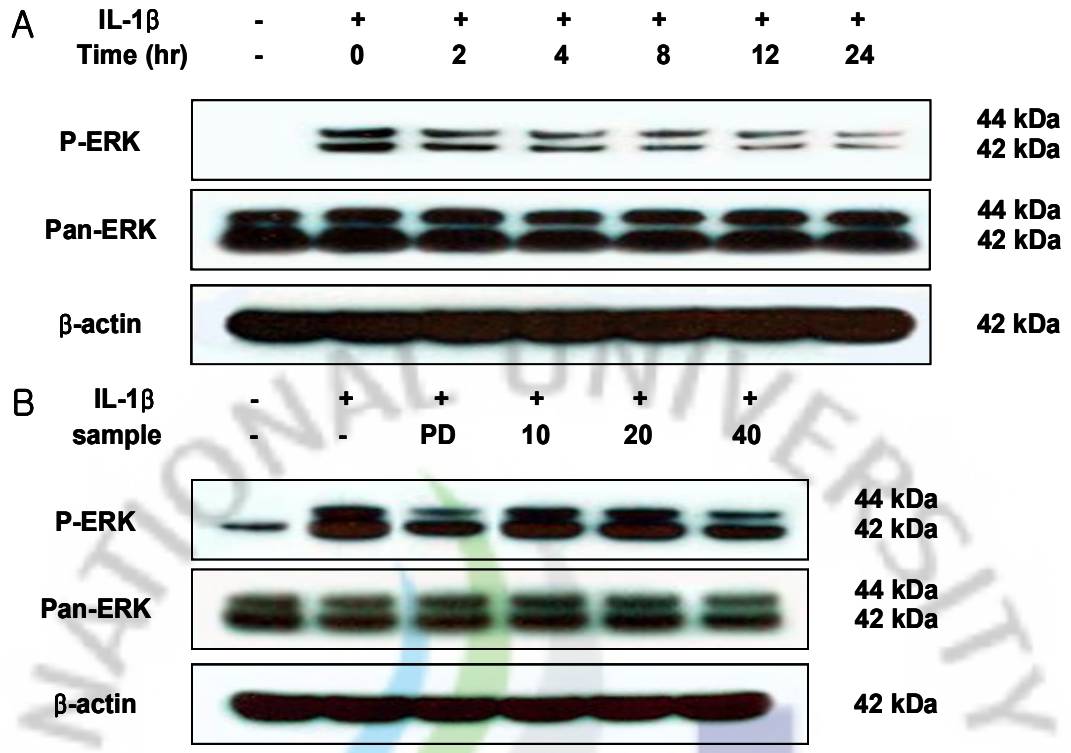


**Figure 11. Inhibitory effect of sargachromanol G on ERK protein level in IL-1β-stimulated MG-63 cells. A** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (40 μM) for 0-24 h, and then stimulated with IL-1β (10 ng/mL) for 10 min. **B** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and PD : PD98059 (40 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 10 min. The levels of p-ERK (phosphorylated-ERK) and pan-ERK were determined using immunoblotting method.



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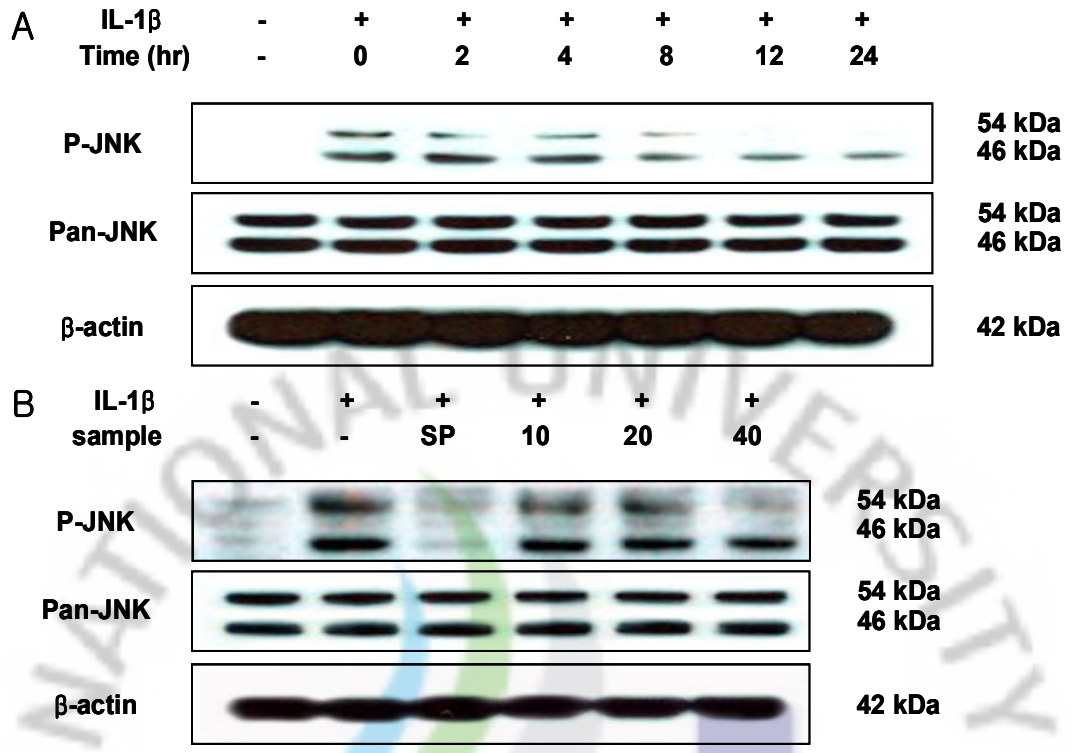


**Figure 12. Inhibitory effect of sargachromanol G on JNK protein level in IL-1β-stimulated MG-63 cells. A** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (40 μM) for 0-24 h, and then stimulated with IL-1β (10 ng/mL) for 20 min. B : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and SP : SP600125 (10 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 20 min. The levels of p-JNK (phosphorylated-JNK) and pan-JNK were determined using immunoblotting method.



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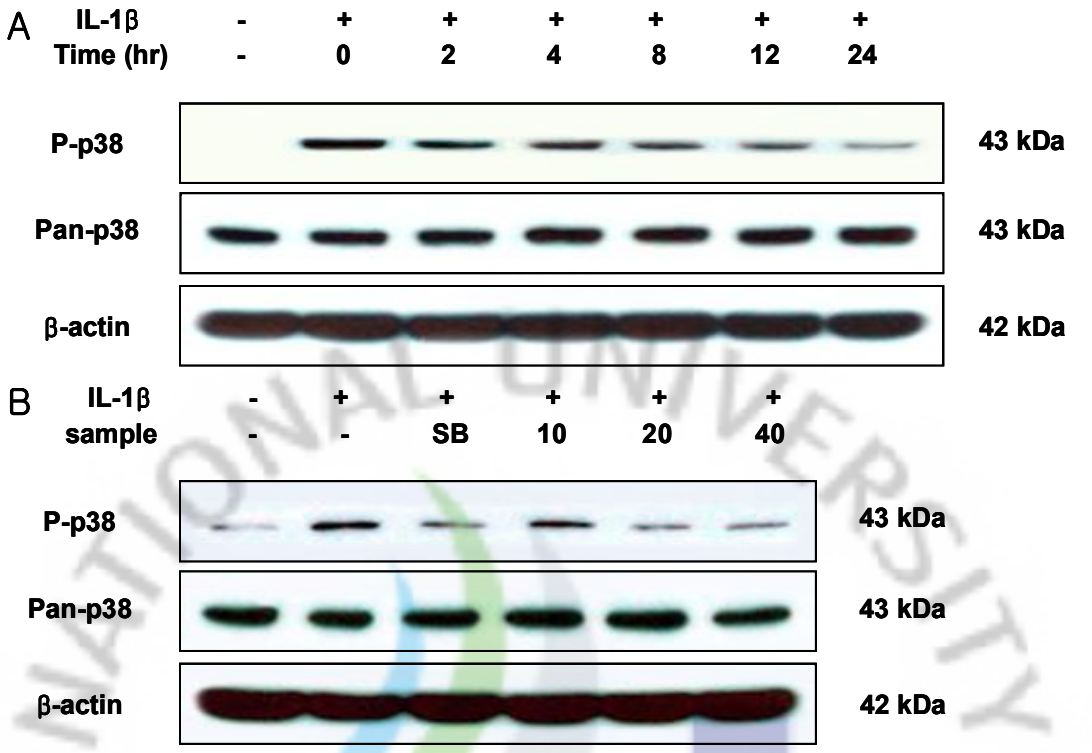


**Figure 13. Inhibitory effect of sargachromanol G on p38 protein level in IL-1β-stimulated MG-63 cells. A** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (40 μM) for 0-24 h, and then stimulated with IL-1β (10 ng/mL) for 10 min. **B** : MG-63 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and SB : SB203580 (20 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 10 min. The levels of p-p38 (phosphorylated-p38) and pan-p38 were determined using immunoblotting method.



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## 3-2. Results

### Effect of sargachromanol G in LPS-stimulated macrophage

#### 3-2-1. Effects of solvent fractions and sargachromanol G from *S. siliquastrum* on the cell viability in RAW 264.7 cells

RAW 264.7 cells were treated with various concentrations of solvent fractions and sargachromanol G isolated from *S. siliquastrum* for 24 h, and cell viability was assessed using an LDH and MTT assay. Solvent fractions (50 μg/mL) and Sargachromanol G (40 μM) exhibited no cytotoxic effect on cells in comparison to control cells that received no treatment. The highest concentration of solvent fractions and sargachromanol G that did not cause more than 20% loss in cell viability was 50 μg/mL and 40 μM, respectively. Therefore, 40 uM sargachromanol G was chosen for further studies **(Figure 14, 14-1, 15-15-2)**.



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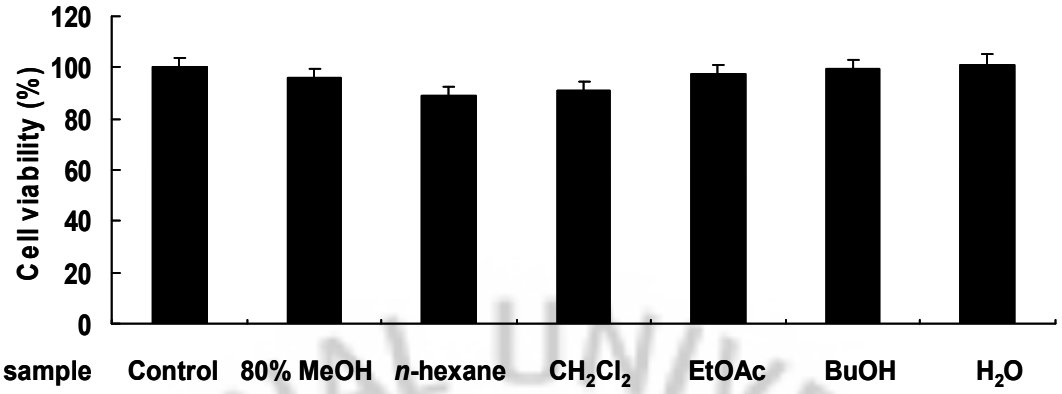


**Figure 14. Effects of 80% MeOH extract and solvent fractions of *S. siliquastrum* on the cell viability in RAW 264.7 cells.** An MTT assay was performed after incubation of RAW 264.7 cells with the 80% MeOH extract and solvent fractions of *S. siliquastrum* (50 μg/mL) for 24 h at 37 °C in a 5% $CO_2$ atmosphere.



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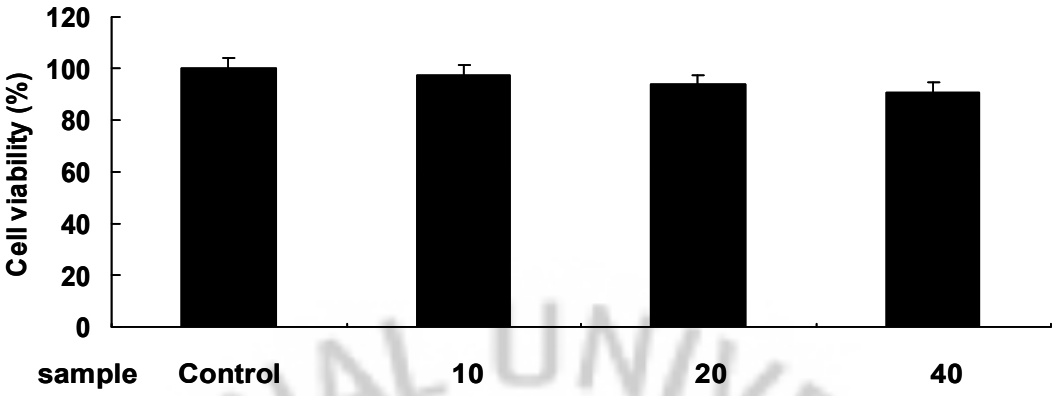


**Figure 14-1. Effect of sargachromanol G on the cell viability in RAW 264.7 cells.** An MTT assay was performed after incubation of RAW 264.7 cells with sagachromanol G (10, 20 and 40 μM) isolated from *S. siliquastrum* for 24 h at 37 °C in a 5% $CO_2$ atmosphere.



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**3-2-2. Effects of solvent fractions and sargachromanol G from *S. siliquastrum* on NO production and cytotoxicity in LPS-stimulated RAW 264.7 cells**

LPS-induced production of NO from macrophages occurs in the inflammatory response. Nitrite levels, as measured with Greiss reagent, are used as a measure of NO production due to the short half-life of NO. The solvent fractions and sargachromanol G dose-dependently inhibited LPS (1 μg/mL)-induced increases in NO in RAW 264.7 cells **(Figure 15-15-2)**, SB203580 and PDTC reduced LPS-induced NO production, but PD98059 and SP600125 did not.



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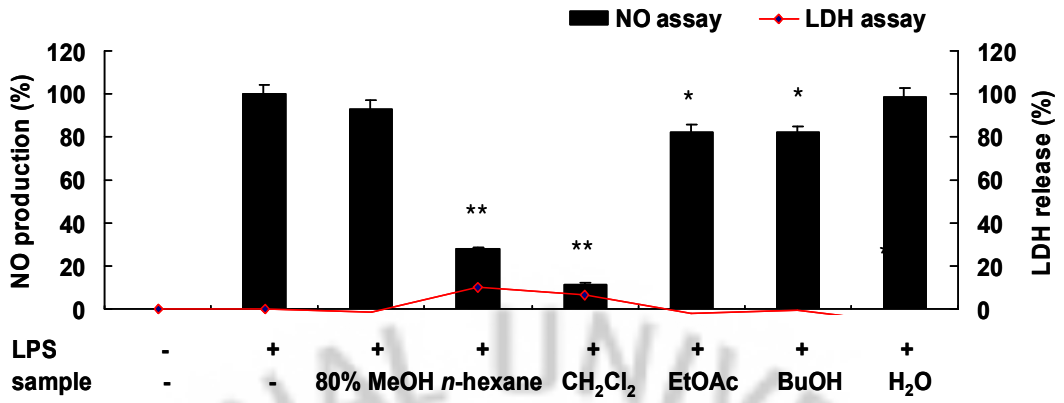


**Figure 15. Effects of 80% MeOH extract and solvent fractions of *S. siliquastrum* on nitric oxide production and cytotoxicity in LPS-stimulated RAW 264.7 cells.** The production of nitric oxide was assayed in the culture medium of cells stimulated with LPS (1 μg/mL) for 24 h in the presence of 80% MeOH extract and solvent fractions of *S. siliquastrum* (50 μg/mL). Cytotoxicity was determined using the LDH method. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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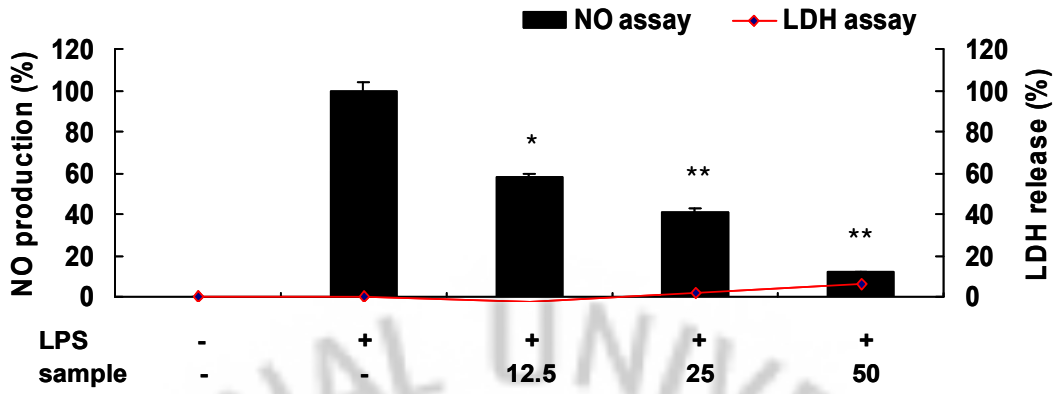


**Figure 15-1. Effects of $CH_2Cl_2$ fraction of *S. siliquastrum* on nitric oxide production and cytotoxicity in LPS-stimulated RAW 264.7 cells.** The production of nitric oxide was assayed in the culture medium of cells stimulated with LPS (1 μg/mL) for 24 h in the presence of $CH_2Cl_2$ fraction of *S. siliquastrum* (12.5, 25 and 50 μg/mL). Cytotoxicity was determined using the LDH method. Values are the mean ± SEM of triplicate experiments. *, *P<0.05; **, P<0.01*



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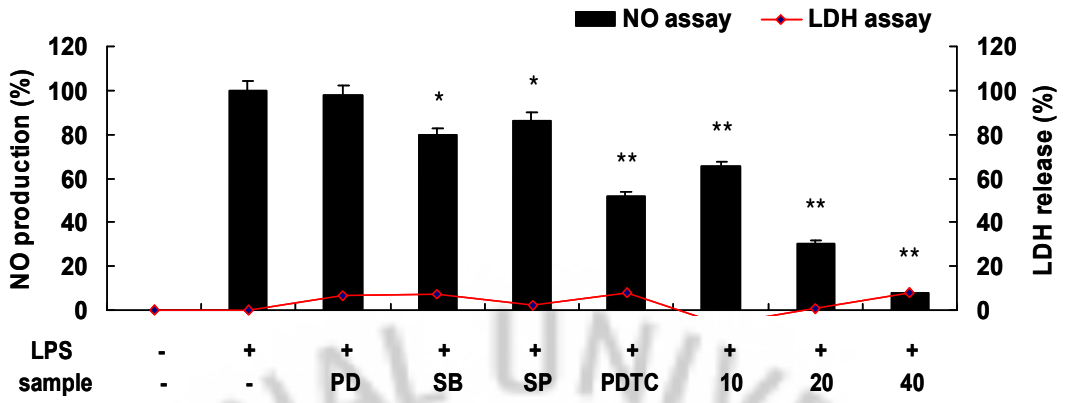


**Figure 15-2. Effects of sargachromanol G on nitric oxide production and cytotoxicity of sargachromanol in LPS-stimulated RAW 264.7 cells.** The production of nitric oxide was assayed in the culture medium of cells stimulated with LPS (1 μg/mL) for 24 h in the presence of sargachromanol G (10, 20 and 40 μM), PD: PD98059 (20 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM). Cytotoxicity was determined using the LDH method. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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### 3-2-3. Effects of solvent fractions and sargachromanol G from *S. siliquastrum* on $PGE_2$ production in LPS-stimulated RAW 264.7 cells

LPS was used to stimulate the release of $PGE_2$ from macrophage cells. $PGE_2$ is an inflammatory mediator produced from the conversion of arachidonic acid by cyclooxygenase. In a variety of inflammatory cells, including macrophages, COX-2 is induced by cytokines and other activators, such as LPS, resulting in the release of a large amount of $PGE_2$ at inflammatory sites. LPS can stimulate $PGE_2$ release from RAW 264.7 macrophages. LPS (1 μg/mL) treatment for 24 h increased $PGE_2$ levels in the culture medium. Solvent fractions and sargachromanol G dose-dependently suppressed this LPS-induced $PGE_2$ production **(Figure 16-16-2)**. SB203580, SP600125, and PDTC significantly reduced LPS-induced $PGE_2$ production, but PD98059 did not.



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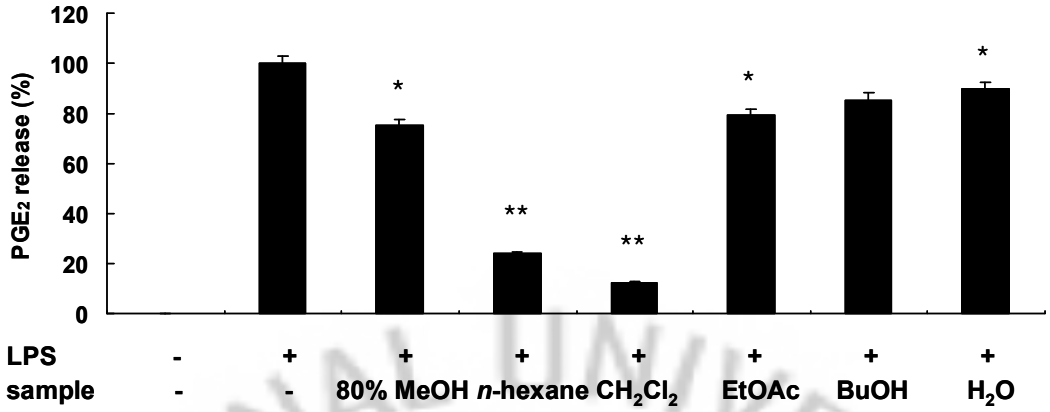


**Figure 16. Inhibitory effects of 80% MeOH extract and solvent fractions of *S. siliquastrum* on $PGE_2$ production in LPS-stimulated RAW 264.7 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with LPS (1 μg/mL) for 24 h in the presence of 80% MeOH extract and solvent fractions of *S. siliquastrum* (50 μg/mL). Supernatants were collected and the $PGE_2$ concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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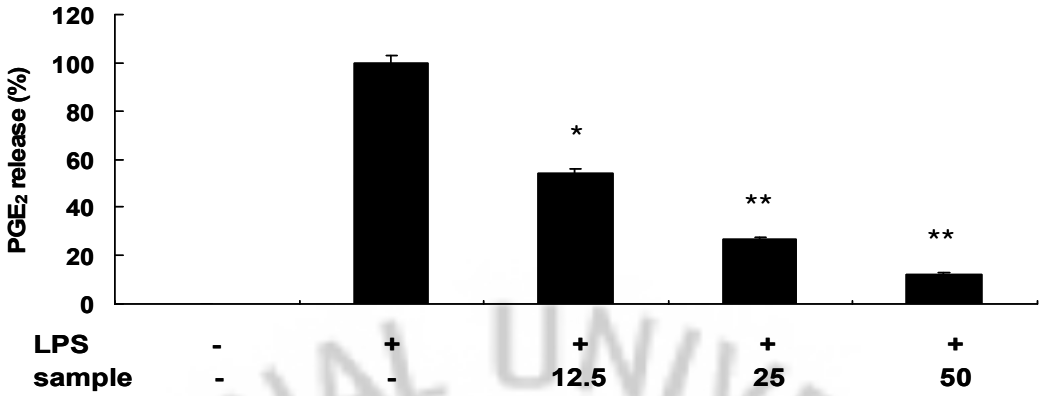


**Figure 16-1. Inhibitory effect of $CH_2Cl_2$ fraction of *S. siliquastrum* on $PGE_2$ production in LPS-stimulated RAW 264.7 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with LPS (1 μg/mL) for 24 h in the presence of $CH_2Cl_2$ fraction of *S. siliquastrum* (12.5, 25 and 50 μg/mL). Supernatants were collected, and the $PGE_2$ concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. *, *P<0.05;* **, *P<0.01*



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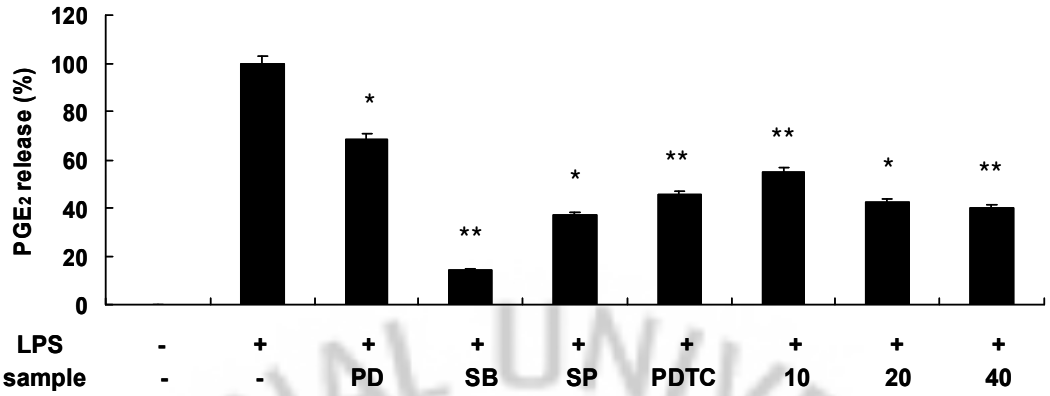


**Figure 16-2. Inhibitory effect of sargachromanol G on $PGE_2$ production in LPS-stimulated RAW 264.7 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with LPS (1 μg/mL) for 24 h in the presence of sargachromanol G (10, 20 and 40 μM), PD: PD98059 (20 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM). Supernatants were collected, and the $PGE_2$ concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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### 3-2-4. Effects of sargachromanol G on protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells

Decreased NO and $PGE_2$ production may result from lower iNOS and COX-2 enzymatic activity or decreased expression. We therefore tested the effects of sargachromanol G on iNOS and COX-2 levels after LPS induction. Sargachromanol G (10, 20 and 40 μM) dose-dependently inhibited the LPS-induced increases in iNOS and COX-2 levels **(Figure 17)**.



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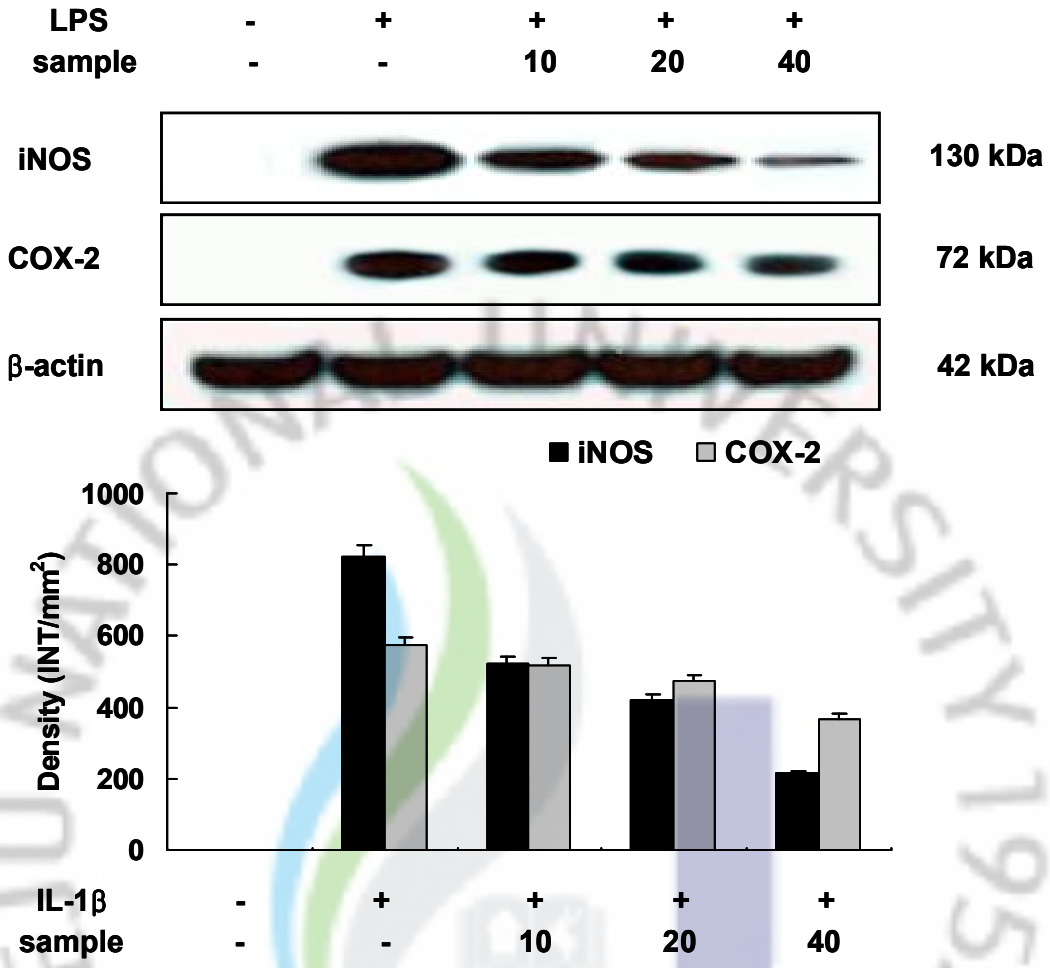


**Figure 17. Effects of sargachromanol G on protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were pre-incubated for 18 h, and the cells were stimulated with LPS (1 μg/mL) in the presence of sargachromanol G (10, 20 and 40 μM) for 24 h. The levels of iNOS and COX-2 were determined using immunoblotting method.



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**3-2-5. Effects of sargachromanol G on pro-inflammatory cytokines production in LPS-stimulated RAW 264.7 cells**

Osteoclasts induced by TNF-α form resorption pits on dentine slices only in the presence of IL-1β. TNF-α together with IL-1 may regulate bone resorption during inflammatory diseases (Kobayashi *et al*., 2000). IL-1, a pleiotropic cytokine, induces the expression of multiple proinflammatory molecules (Dinarello *et al*., 1994) and hematopoietic cytokines such as leukaemia inhibitory factor (LIF) and IL-6. LPS (1 μg/mL) stimulation of RAW 264.7 cells for 24 h increased TNF-α, IL-1β, and IL-6 levels in supernatants, as measured by ELISA **(Figure 18-18-2)**. Sargachromanol G treatment for 24 h dose-dependently inhibited TNF-α, IL-1β, and IL-6 production, as did SB203580 and SP600125, but PD98059 and PDTC did not.



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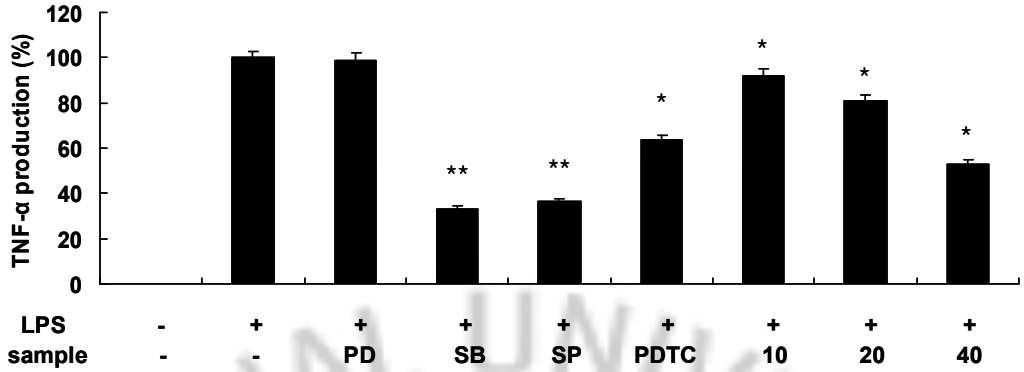


**Figure 18. Inhibitory effect of sargachromanol G on TNF-α production in LPS-stimulated RAW 264.7 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with LPS (1 μg/mL) in the presence of sargachromanol G (10, 20 and 40 μM), PD: PD98059 (20 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) for 24 h. Supernatants were collected, and the TNF-α concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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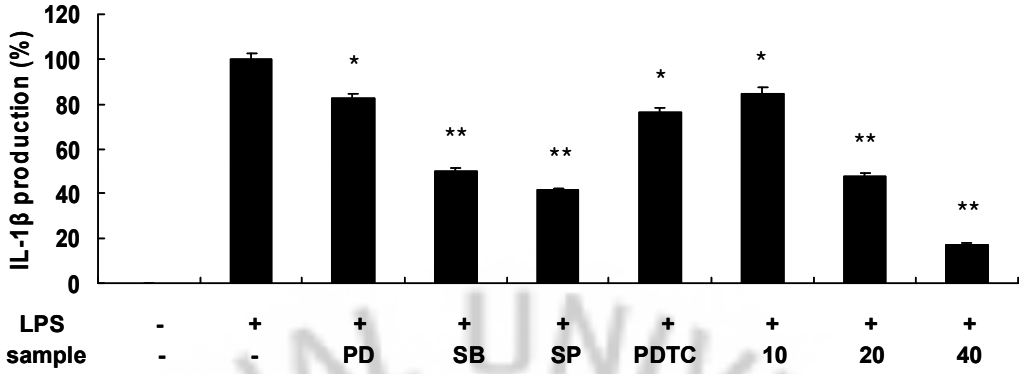


**Figure 18-1. Inhibitory effect of sargachromanol G on IL-1β production in LPS-stimulated RAW 264.7 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with LPS (1 μg/mL) in the presence of sargachromanol G (10, 20 and 40 μM), PD: PD98059 (20 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) for 24 h. Supernatants were collected, and the IL-1β concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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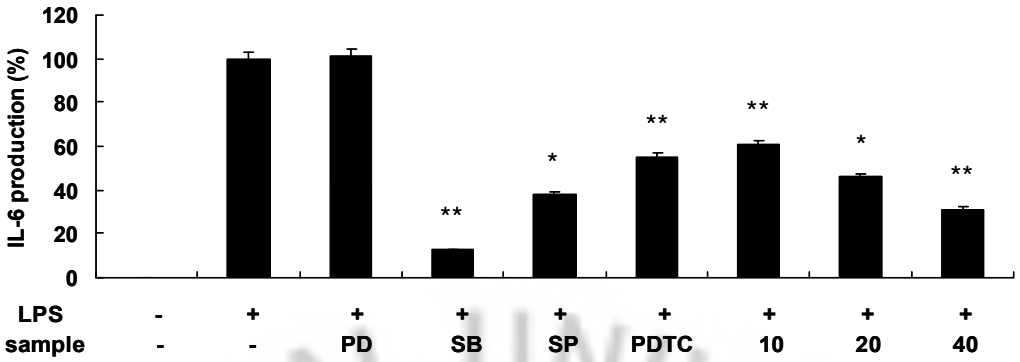


**Figure 18-2. Inhibitory effect of sargachromanol G on IL-6 production in LPS-stimulated RAW 264.7 cells.** Cells ($1.5 \times 10^5$ cells/mL) were stimulated with LPS (1 μg/mL) in the presence of sargachromanol G (10, 20 and 40 μM), PD: PD98059 (20 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) for 24 h. Supernatants were collected, and the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SEM of triplicate experiments. **, P<0.05; **, P<0.01*



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**3-2-6. Effect of sargachromanol G on NF-κB signaling pathway in LPS-stimulated RAW 264.7 cells**

LPS also activated NF-κB in RAW 264.7 cells via phosphorylatin of p65 and p50 **(Figure 20-20-1)**. Sargachromanol G (10, 20 and 40 μM) and PDTC (10 μM) dose-dependently inhibited this translocation, and also inhibited phophorylation and degradation of IκB-α **(Figure 19, 19-1)**. This inhibition of translocation and IκB-α degradation may mediate the effects on LPS-induced NO and $PGE_2$ production as well as iNOS and COX-2 expression.



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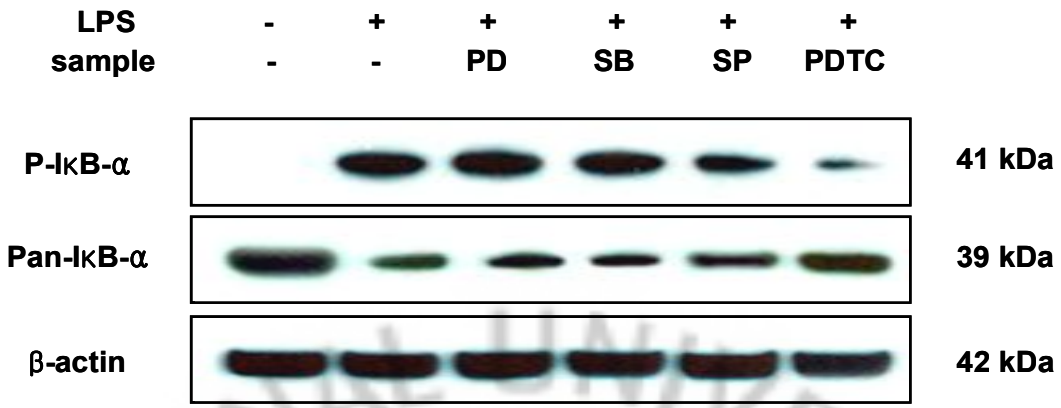


**Figure 19. Effects of inhibitors on the IκB-α protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^5$ cells/mL) were pre-incubated for 18 h, and the cells were pre-incubated with PD: PD98059 (40 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) at indicated concentrations for 24 h and then stimulated with LPS (1 μg/mL) for 10 min. The levels of p-IκB-α (phosphorylated-IκB-α) and pan-IκB-α were determined using immunoblotting method.



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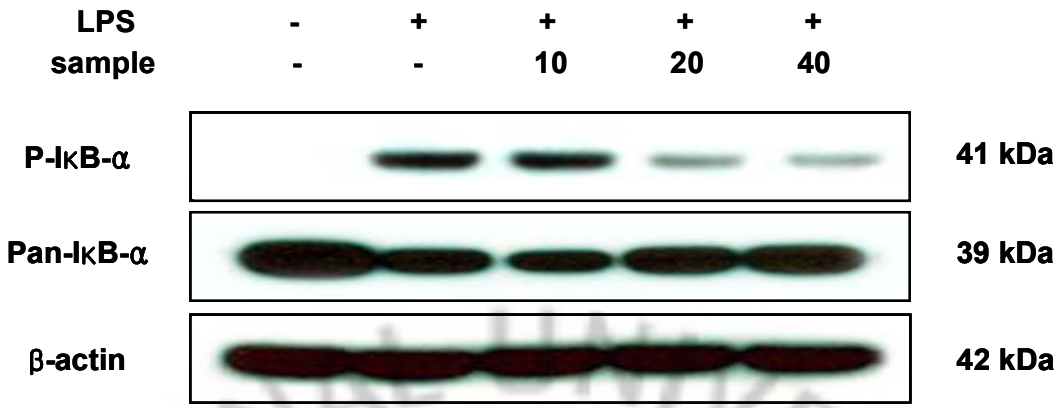


**Figure 19-1. Inhibitory effect of sargachromanol G on IκB-α protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (10, 20 and 40 μM) at indicated concentrations for 24 h, and then stimulated with LPS (1 μg/mL) for 10 min. The levels of p-IκB-α (phosphorylated-IκB-α) and pan-IκB-α were determined using immunoblots.



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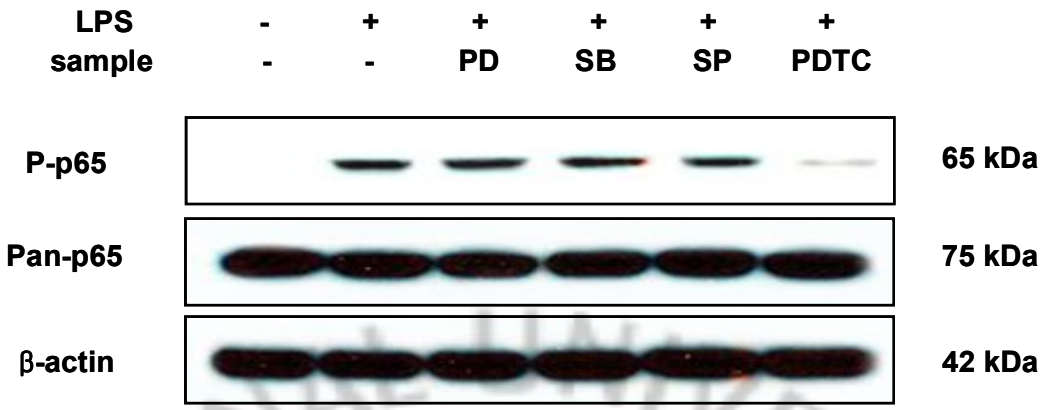


**Figure 20. . Effects of inhibitors on the p65 protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were pre-incubated for 18 h, and the cells were pre-incubated with PD: PD98059 (40 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) at indicated concentrations for 24 h and then stimulated with LPS (1 μg/mL) for 10 min. The levels of p-p65 (phosphorylated-p65) and pan-p65 were determined using immunoblotting method.



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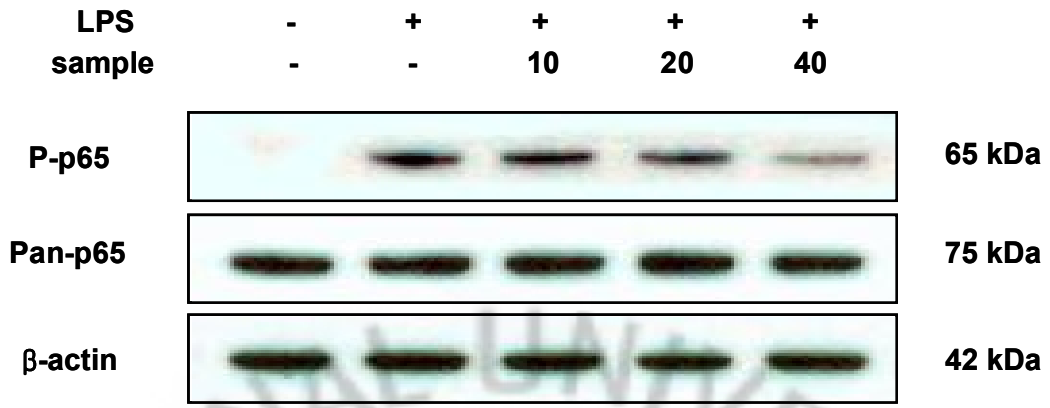


**Figure 20-1. Inhibitory effect of sargachromanol G on p65 protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (10, 20 and 40 μM) at indicated concentrations for 24 h, and then stimulated with LPS (1 μg/mL) for 10 min. The levels of p-p65 (phosphorylated-p65) and pan-p65 were determined using immunoblots.



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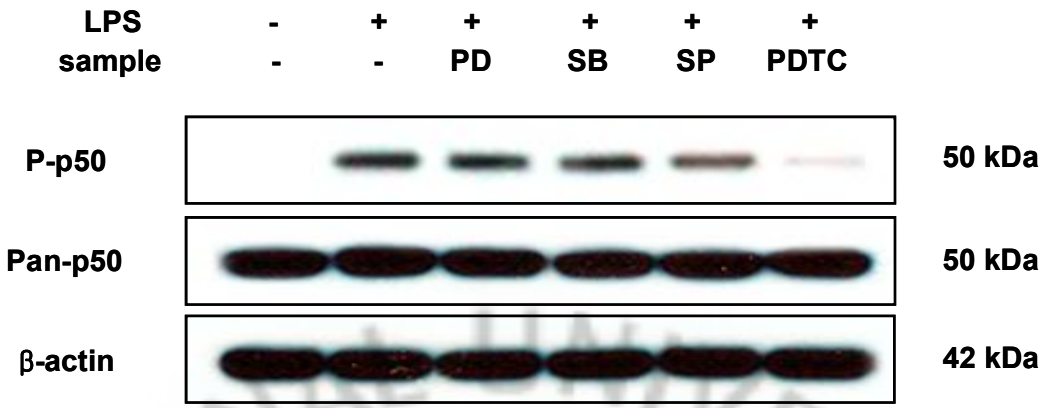


**Figure 21. Effects of inhibitors on the p50 protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were pre-incubated for 18 h, and the cells were pre-incubated with PD: PD98059 (40 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) at indicated concentrations for 24 h and then stimulated with LPS (1 μg/mL) for 10 min. The levels of p-p50 (phosphorylated-p50) and pan-p50 were determined using immunoblotting method.



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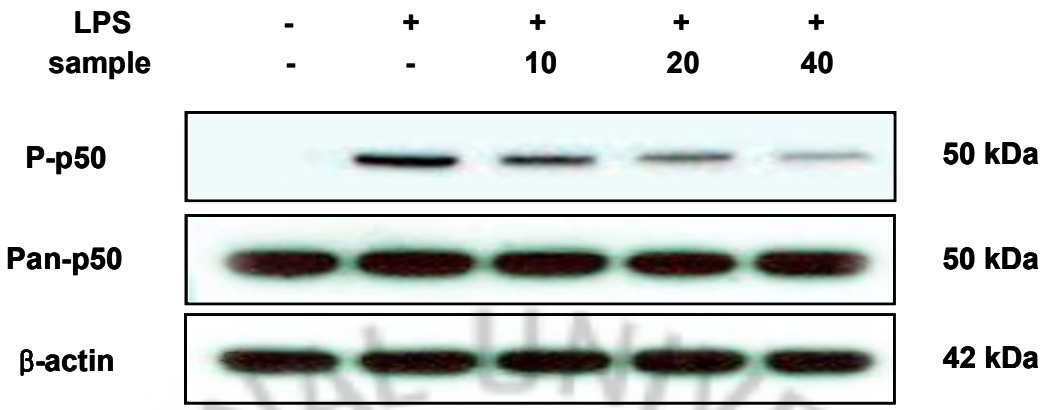


**Figure 21-1. Inhibitory effect of sargachromanol G on p50 protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (10, 20 and 40 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 10 min. The levels of p-p50 (phosphorylated-p50) and pan-p50 were determined using immunoblots.



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### 3-2-7. Effects of sargachromanol G on MAPKs signaling pathway in LPS-stimulated RAW 264.7 cells

MAPKs also regulate iNOS and COX-2 expression. We therefore examined the effect of sargachromanol G on LPS-induced phosphorylation of ERK, JNK and p38 in RAW 264.7 cells using immunoblotting. LPS stimulated phosphorylation of ERK, JNK and p38, and sargachromanol G blocked this activation **(Figure 22-24-1)** but did not change phosphorylation of MAPK. PD98059, SP200615, and SB203580 significantly suppressed LPS-induced phophorylation of MAPK.



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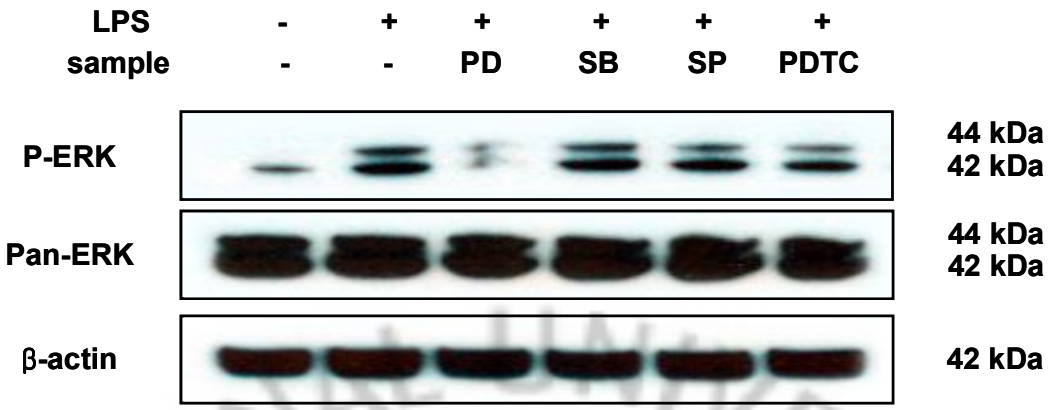


**Figure 22. Effects of inhibitors on the ERK protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were pre-incubated for 18 h, and the cells were pre-incubated with PD: PD98059 (40 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) at indicated concentrations for 24 h and then stimulated with LPS (1 μg/mL) for 20 min. The levels of p-pERK (phosphorylated-ERK) and pan-ERK were determined using immunoblotting method.



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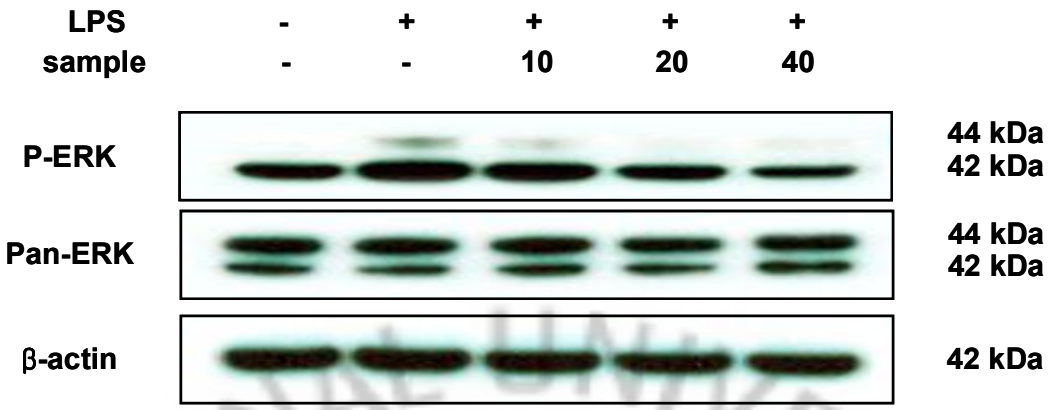


**Figure 22-1. Inhibitory effect of sargachromanol G on ERK protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (10, 20 and 40 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 20 min. The levels of p-ERK (phosphorylated-ERK) and pan-ERK were determined using immunoblots.



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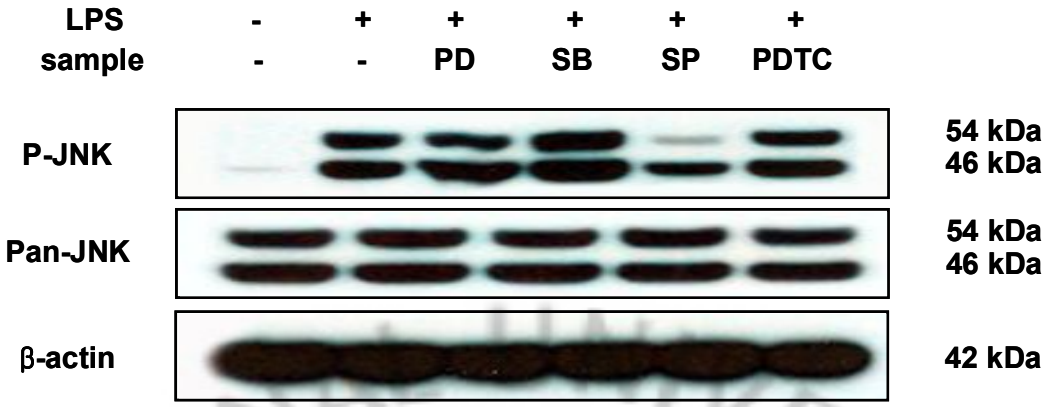


**Figure 23. Effects of inhibitors on the JNK protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were pre-incubated for 18 h, and the cells were pre-incubated with PD: PD98059 (40 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) at indicated concentrations for 24 h and then stimulated with LPS (1 μg/mL) for 30 min. The levels of p-pJNK (phosphorylated-JNK) and pan-JNK were determined using immunoblotting method.



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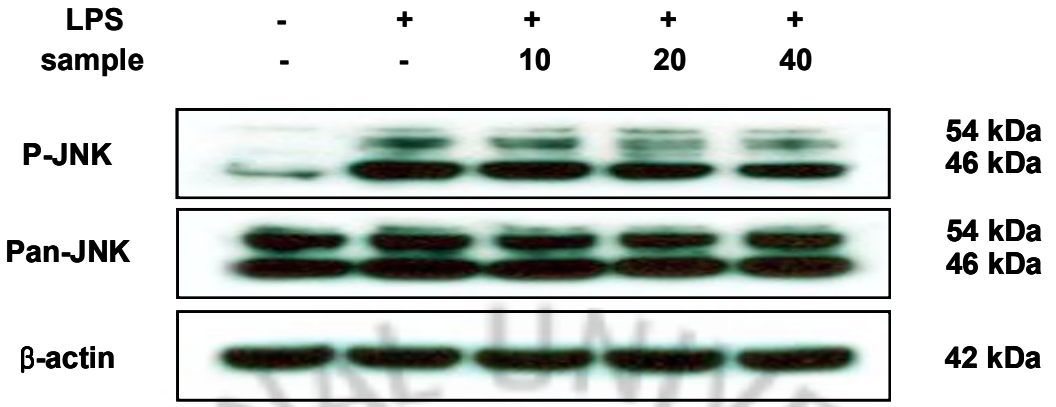


**Figure 23-1. Inhibitory effect of sargachromanol G on JNK protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (10, 20 and 40 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 30 min. The levels of p-JNK (phosphorylated-JNK) and pan-JNK were determined using immunoblots.



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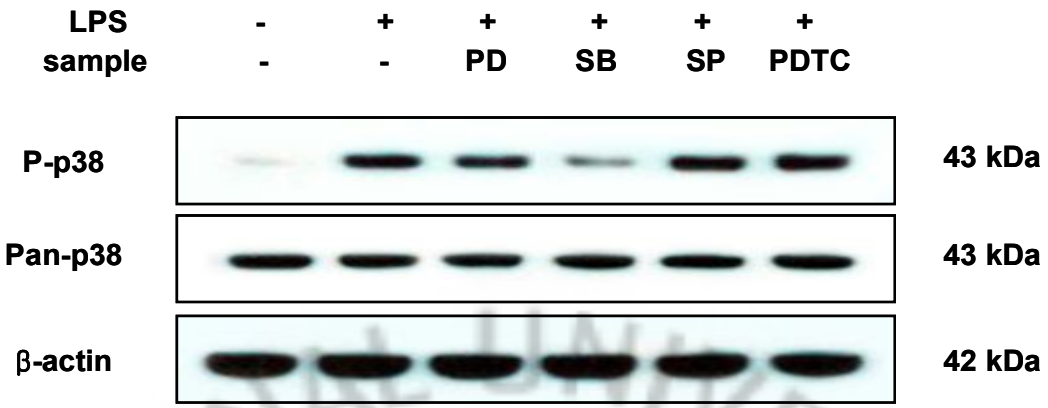


**Figure 24. Effects of inhibitors on the p38 protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were pre-incubated for 18 h, and the cells were pre-incubated with PD: PD98059 (40 μM), SB: SB203580 (20 μM), SP: SP600125 (10 μM) and PDTC (10 μM) at indicated concentrations for 24 h and then stimulated with LPS (1 μg/mL) for 30 min. The levels of p-p38 (phosphorylated-p38) and pan-p38 were determined using immunoblotting method.



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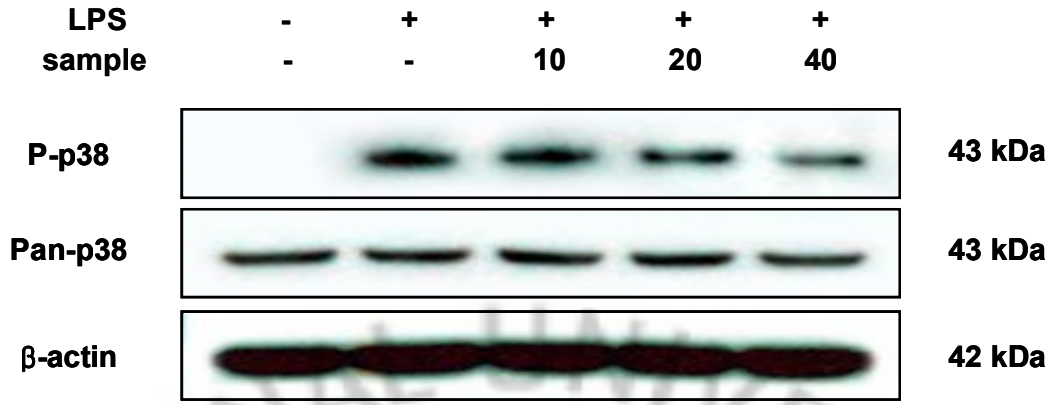


**Figure 24-1. Inhibitory effect of sargachromanol G on p38 protein level in LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-treated with sargachromanol G (10, 20 and 40 μM) at indicated concentrations for 24 h, and then stimulated with IL-1β (10 ng/mL) for 30 min. The levels of p-p38 (phosphorylated-p38) and pan-p38 were determined using immunoblots.



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## 3-3. Results

**Effect of sargachromanol G in RANKL- stimulated preosteoclast**

### 3-3-1. Effects of sargachromanol G on osteoclastogenic factors in RANKL-stimulated RAW 264.7 cells

TRAF6 proteins associate with the cytoplasmic domain of RANK and relay RANK stimulation to NF-κB, and TRAF6 is required for osteoclast formation and osteoclast activation (Lomaga *et al*., 1999). NF-κB activation is also important for RANK–RANKL signaling and osteoclastogenesis. RAW 264.7 cells were incubated in the presence or absence of RANKL and sargachromanol G for 72 h, and changes in the expression of osteoclastoenic factors were assessed by RT-PCR and Western blotting. Differentiated RAW 264.7 cells express high levels of osteoclastogenic factors like TRAF6, TRAP, Cath-K, MMP-9, and CTR (Rahman *et al*., 2003), whereas sargachromanol G alone had no effect (data not shown). However, sargachromanol G reduced mRNA levels of these osteoclastogenic factors (TRAP, Cath-K, MMP-9 and CTR) after RANKL treatment, and reduced protein levels of osteoclastogenic factors (TRAF6, TRAP, and MMP-9) **(Figure 25, 26)**.



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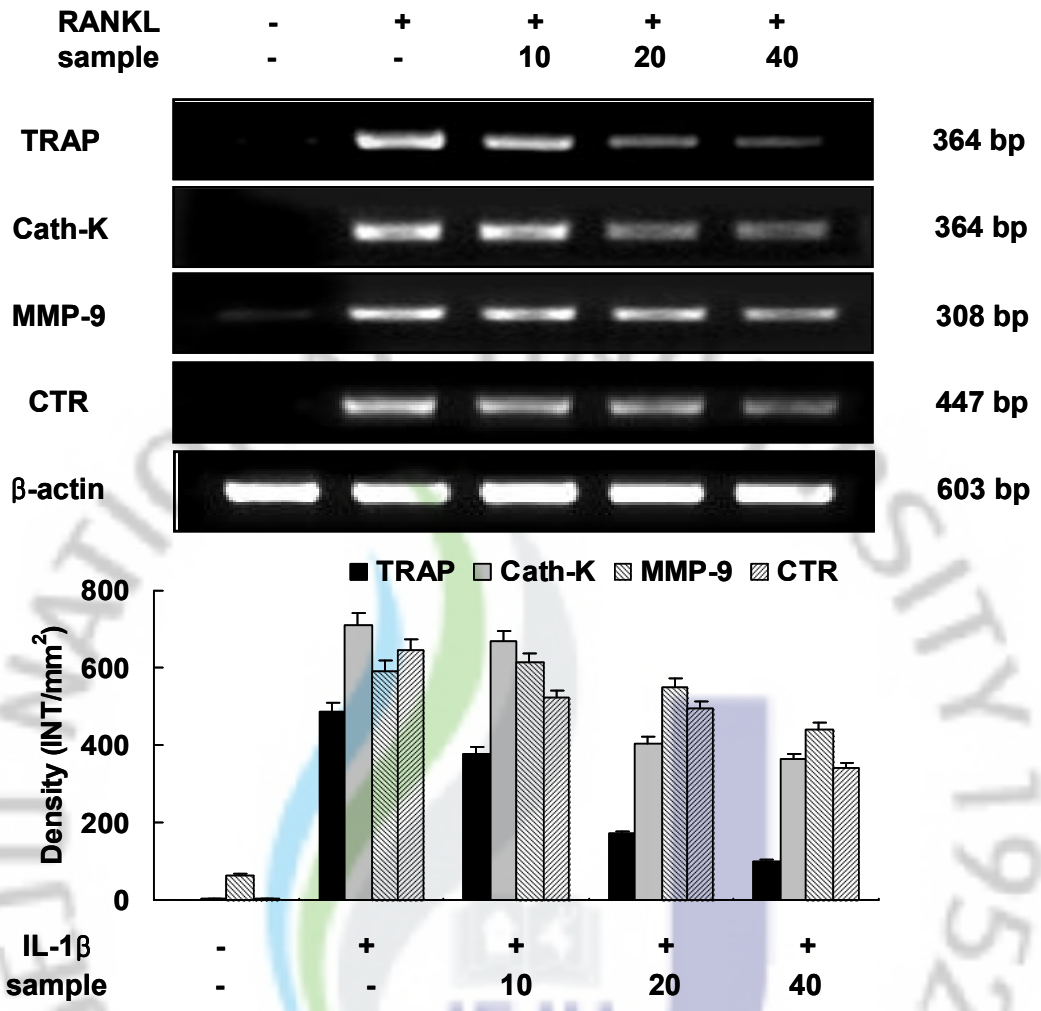


**Figure 25. Effects of sargachromanol G on mRNA expression of osteoclastogenic factors in RANKL- stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^5$ cells/mL) were pre-incubated for 18 h, and the cells were stimulated with RANKL (100 ng/mL) in the presence of sargachromanol G (10, 20 and 40 μM) for 72 h. mRNA expressions of osteoclastogenic factors were determined using RT-PCR.



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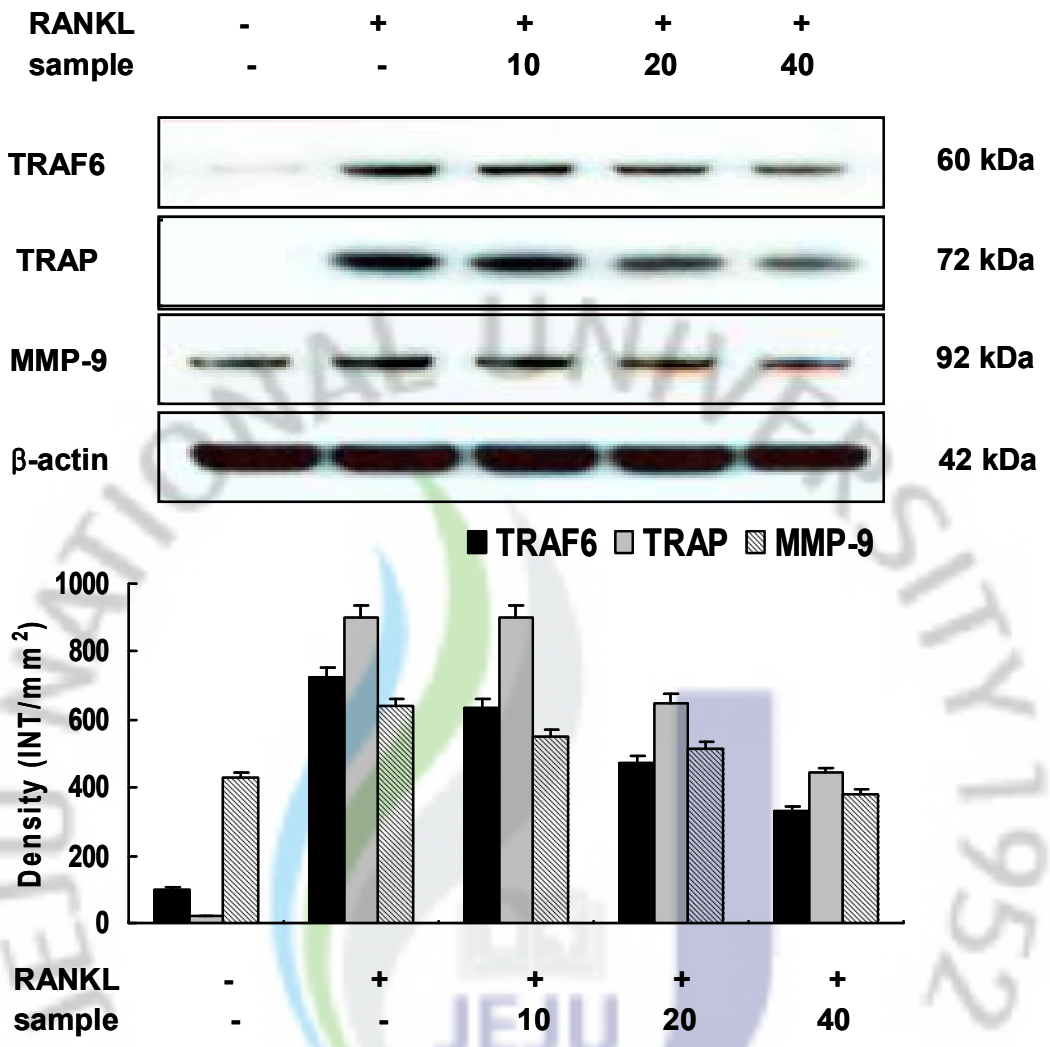


**Figure 26. Effects of sargachromanol G on the protein levels of osteoclastogenic factors in RANKL-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^5$ cells/mL) were pre-incubated for 18 h, and the cells were stimulated with RANKL (100 ng/mL) in the presence of sargachromanol G (10, 20 and 40 μM) for 72 h. Protein levels of osteoclastogenic factors was determined using immunoblotting method.



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### 3-3-2. Effect of sargachromanol G on osteoclast differentiation from RANKL-stimulated RAW 264.7 cells

During bone resorption, mononuclear perfusion osteoclasts fuse to form mature multinucleated osteoclasts. To determine the effects of sargachromanol G on osteoclast formation from macrophage, we used murine macrophage cell line RAW 264.7 cells. RAW 264.7 cells were incubated with sargachromanol G in the presence of RANKL and/or LPS, and allowed to grow and differentiate into osteoclasts. RANKL induced osteoclast formation in RAW 264.7 cells. Sargachromaol G inhibited the osteoclast formation induced by RANKL at concentrations of 10, 20 and 40 μM **(Figure 27, 28)**.



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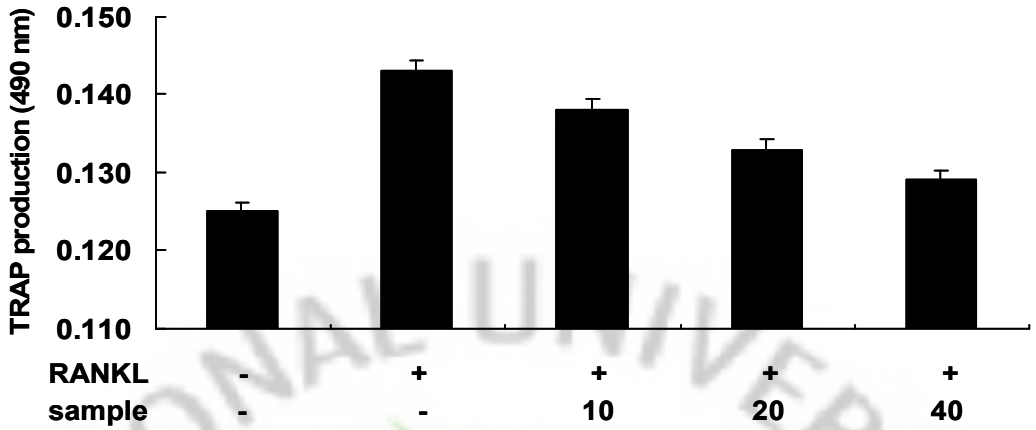


**Figure 27. Inhibitory effect of sargachromanol G on TRAP production in RANKL-stimulated RAW 264.7 cells.** Cells ($1.0 \times 10^5$ cells/mL) were stimulated with RANKL (100 ng/mL) for 72 h in the presence of sargachromanol G (10, 20 and 40 μM). Supernatants were collected, and the TRAP concentration in the supernatants was determined in 540 nm by ELISA reader. Values are the mean ± SEM of triplicate experiments.



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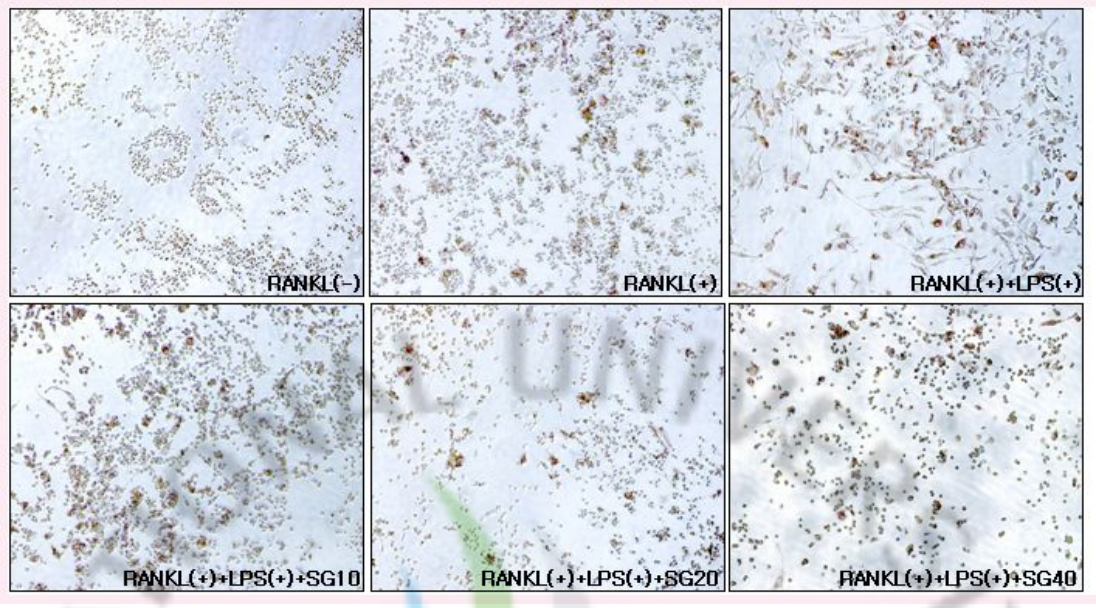


**Figure 28. Sargachromanol G inhibits osteoclastogenesis through TRAP in RAW 264.7 cells.** RAW 264.7 cells ($2.0 \times 10^4$ cells/mL) were pre-incubated for 18 h, incubated for 96 h with sargachromanol G (10, 20, and 40 μM), RANKL (100 ng/mL), or LPS (0.5 μg/mL). Cells were stained for TRAP activity and TRAP-positive cells were identified with a microscope (×200).



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### 3-3-3. Effect of sargachromanol G on NF-κB signaling pathway in RANKL-stimulated RAW 264.7 cells

RANKL binding to RANK activates NF-κB and osteoclastogenesis. Treatment with RANKL in RAW 264.7 cells stimulated phophorylation of p65 and p50 as well as phophorylation and degradation of IκB-α **(Figure 30, 31)**. Sargachromanol G and PDTC dose-dependently blocked this nuclear translocation, as well as the degradation of IκB-α **(Figure 29)**, which could explain how sargachromanol G inhibits RANKL-induced production of osteoclastogenic factors.



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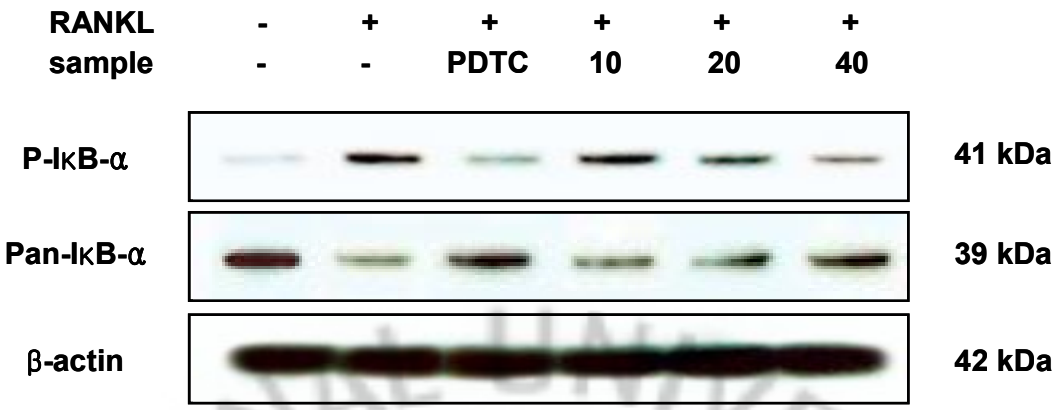


**Figure 29. Inhibitory effect of sargachromanol G on IκB-α protein level in RANKL-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and PDTC (10 μM) at indicated concentrations for 24 h, and then stimulated with RANKL (100 ng/mL) for 10 min. The levels of p-IκB-α (phosphorylated-IκB-α) and pan-IκB-α were determined using immunoblots.



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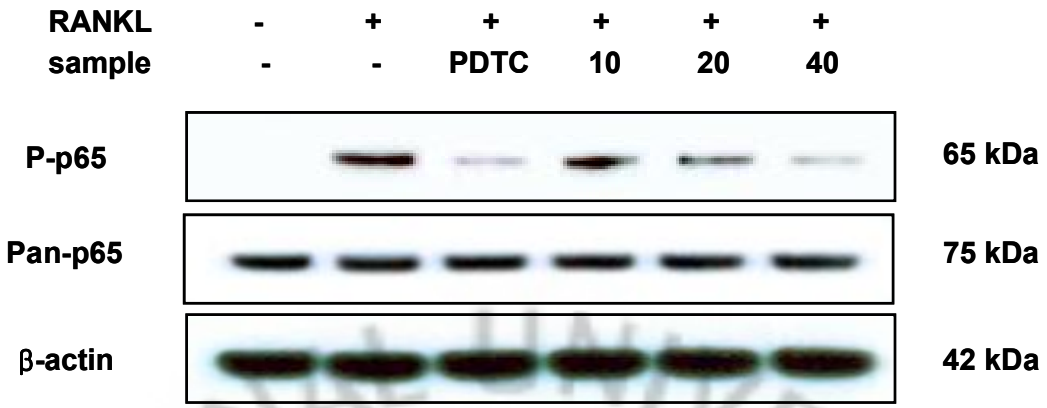


**Figure 30. Inhibitory effect of sargachromanol G on p65 protein level in RANKL-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and PDTC (10 μM) at indicated concentrations for 24 h, and then stimulated with RANKL (100 ng/mL) for 10 min. The levels of p-p65 (phosphorylated-p65) and pan-p65 were determined using immunoblots.



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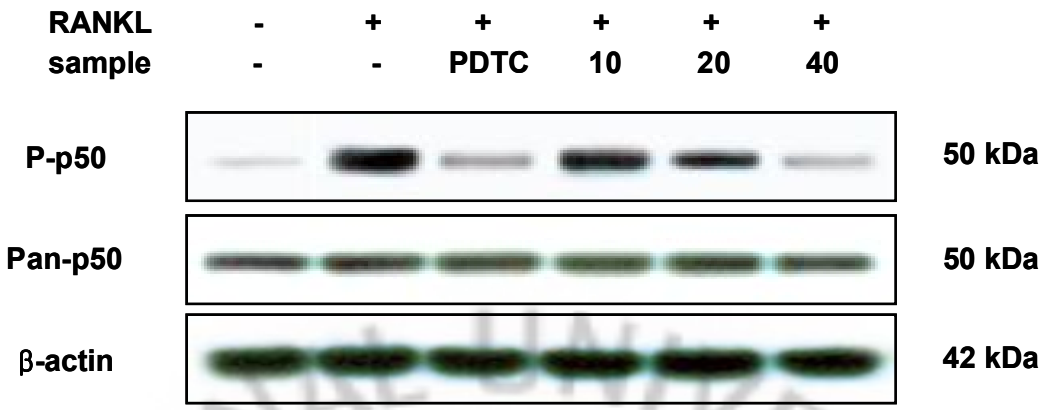


**Figure 31. Inhibitory effect of sargachromanol G on p50 protein level in RANKL-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and PDTC (10 μM) at indicated concentrations for 24 h, and then stimulated with RANKL (100 ng/mL) for 10 min. The levels of p-p50 (phosphorylated-p50) and pan-p50 were determined using immunoblots.



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### 3-3-4. Effects of sargachromanol G on MAPKs signaling pathway in RANKL-stimulated RAW 264.7 cells

RANKL is known to activate mitogen-activated protein kinase (MAPK). MAPKs play important roles in differentiation and osteoclastogensis of osteoclast. RANKL activates and phosphorylates MAPKs to induce NF-κB activation **(Figure 32-34)**. Sargachromanol G markedly inhibited ERK, JNK and p38 MAP kinase activation, but did not change total protein levels, providing a mechanism whereby sargachromanol G can inhibit RANKL-induced NF-κB binding. PD98059, SB203580, and SP600125 also blocked MAPK activation.



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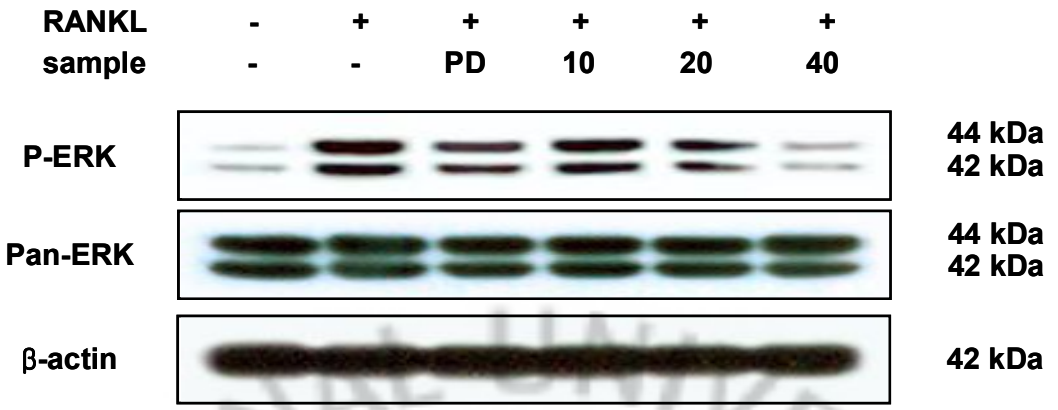


**Figure 32. Inhibitory effect of sargachromanol G on ERK protein level in RANKL-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and PD : PD98059 (40 μM) at indicated concentrations for 24 h, and then stimulated with RANKL (100 ng/mL) for 20 min. The levels of p-ERK (phosphorylated-ERK) and pan-ERK were determined using immunoblots.



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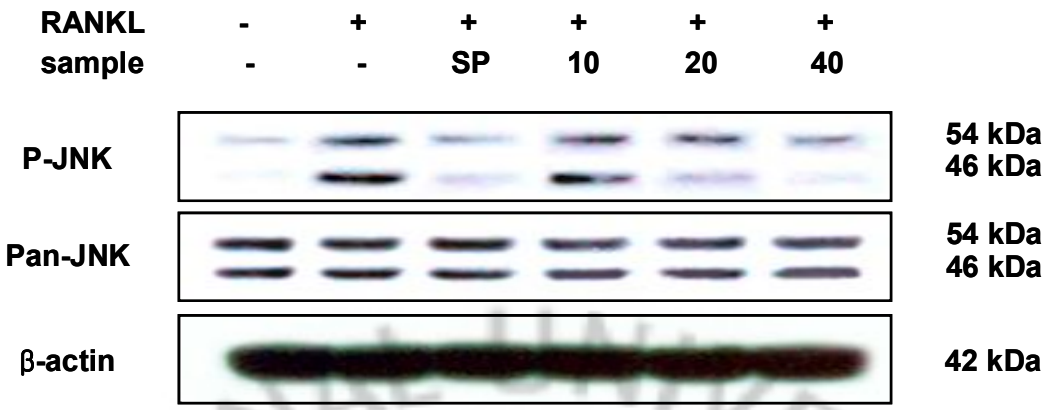


**Figure 33. Inhibitory effect of sargachromanol G on JNK protein level in RANKL-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and SP : SP600125 (10 μM) at indicated concentrations for 24 h, and then stimulated with RANKL (100 ng/mL) for 20 min. The levels of p-JNK (phosphorylated-JNK) and pan-JNK were determined using immunoblots.



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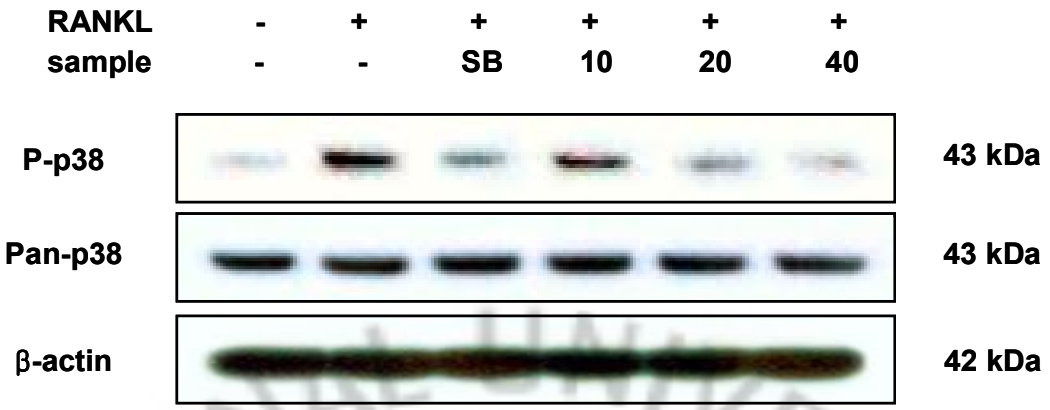


**Figure 34. Inhibitory effect of sargachromanol G on p38 protein level in RANKL-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) and SB : SB203580 (20 μM) at indicated concentrations for 24 h, and then stimulated with RANKL (100 ng/mL) for 20 min. The levels of p-p38 (phosphorylated-p38) and pan-p38 were determined using immunoblots.



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### 3-3-5. Effects of sargachromanol G on transcription factors related-osteoclastogenesis in RANKL-stimulated RAW 264.7 cells

Src family kinases (SFKs) influence cell growth, differentiation, cytoskeletal organization, and survival (Lowell and Soriano, 1996). c-Src regulates osteoclast function and immune receptors in lymphocytes and macrophages. SFKs and MEK negatively regulate RANKL-stimulated osteoclastogenesis and bone resorption. RANKL induces phosphorylation of MKK3/6 and ATF2 to osteoclast differentiation in macrophages and osteoclasts. We therefore tested the effects of sargachromanol G on the RANKL-induced phosphorylation of these osteoclastogenic factors (SFKs, MEK, MKK3/6 and ATF2) in RAW 264.7 cells. Sargachromanol G dose-dependently inhibited the RANKL-induced phosphorylation of SFKs, MEK, MKK3/6 and ATF2 **(Figure 35)**.



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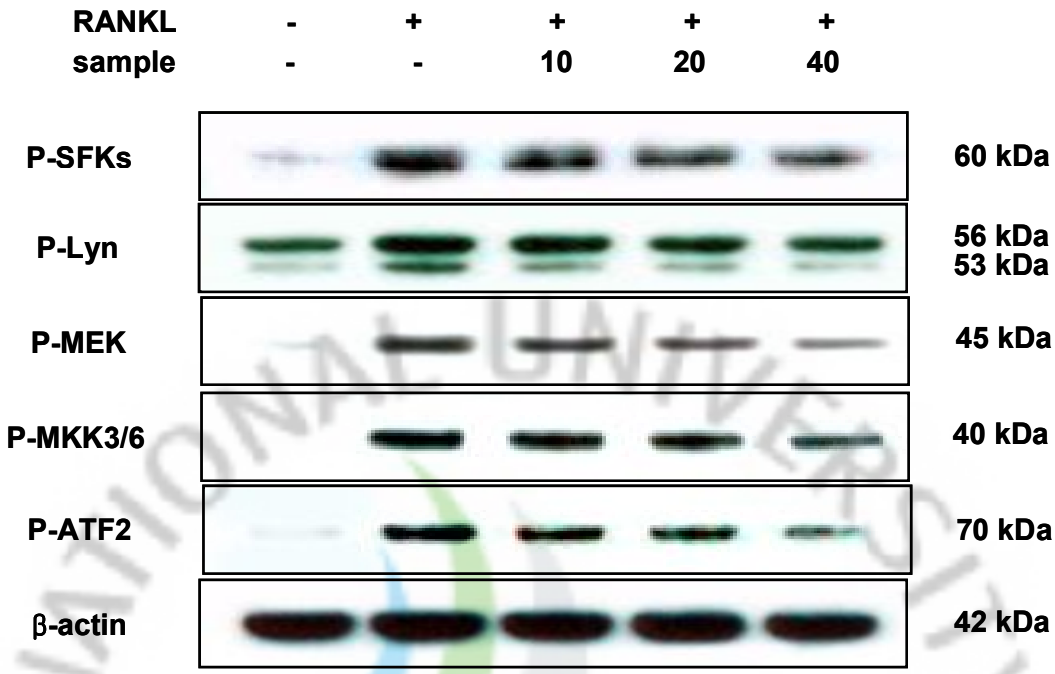


**Figure 35. Effects of sargachromanol G on transcription factors related-osteoclastogenesis in RANKL-stimulated RAW 264.7 cells.** RAW 264.7 cells ($1.0 \times 10^6$ cells/mL) were cultured for 18 h, pre-incubated with sargachromanol G (10, 20 and 40 μM) at indicated concentrations for 24 h, and then stimulated with RANKL (100 ng/mL) for 10-30 min. The protein levels of were determined using immunoblotting method.



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## 4. Discussion

Sargachromanol G was isolated and purified from the brown alga *Sargassum siliquastrum* as novel anti-osteoclastogenic activity substances. We tested whether sargachromanol G could block osteoclastogenesis induced by interleukin-1β (IL-1β) in human osteoblast MG-63 cells as well as LPS and RANKL in murine macrophage RAW 264.7 cells. We also examined the role of NF-κB and MAPK signaling in this induction.

$PGE_2$, downstream of COX-2, increases both osteoblast proliferation and osteoclast differentiation, confirming that disruption of COX-2 expression causes defective osteoblast secretion of RANKL and impaired osteoclast formation in response to hormones (Okada *et al*., 2000). The COX-2 pathway has also been implicated in osteoclast formation and bone destruction in bone metastases of mammary carcinoma cells (Ono *et al*., 2002). Previous work with $PGE_2$ and the RANK/RANKL/OPG system used isolated cultures of either osteoclasts or osteoblasts (Okada *et al*., 2000, Li *et al*., 2002). IL-6 induced pro-inflammatory cytokines to stimulate inflammatory bone resorption (Manolagas and Jilka 1995). One such pro-inflammatory cytokine, IL-1β, is elevated in inflamed tissues adjacent to bone and induces IL-6 production by osteoblasts. IL-1β-stimulated IL-6 expression in human osteoblast (MG-63) cells, as previously reported (Joo *et al.,* 2003). The presence of IL-6 in the immediate vicinity of bone surfaces leads to the recruitment of osteoclast precursor cells (derived from monocyte cell lineage) and differentiation of these precursor cells. Mature and multi-nucleated osteoclasts dissolve bone, so secretion of IL-6 and increasing osteoclast precursor cells allows osteoblasts to increase bone resorption (Webb *et al*., 2002). In addition, IL-6 significantly induces the expression of COX-2 mRNA (Yucel-Lindberg *et al*., 1999). We showed that IL-6 induced COX-2 transcription and $PGE_2$ synthesis in osteoblasts. Osteoclast formation induced by IL-6 was accompanied by $PGE_2$



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production in co-cultures of bone marrow cells and osteoblastic cells, this was blocked completely by a selective COX-2 inhibitor. Osteoclast formation, induced by IL-1 and/or IL-6, appears to depend on their ability to induce COX-2 gene transcription in osteoblasts (Tai *et al*., 1997). Here, sargachromanol G dose-dependently reduced the IL-1β-induced expression of $PGE_2$, IL-6, and COX-2 in MG-63 cells **(Figure 5-2, 6-2, 7)**.

RANKL is a soluble, osteoblast-derived protein that induces bone resorption through osteoclast differentiation and activation (Teitelbaum *et al*., 2006). RANKL expression is induced in osteoblasts/mesenchymal cells by a bone resorption factor (Yasuda, *et al*., 1998). Osteoclast precursors respond to RANKL after adherence to osteoblast/mesenchymal cells, causing differentiation into osteoclasts (Anderson, *et al*., 1997, Lacey, *et al*., 1998, Nakagawa, *et al*., 1998). OPG is a decoy receptor that binds directly to RANKL to block binding to RANK; it inhibits the differentiation, survival, and bone resorption of osteoclasts (Simonet *et al*., 1997, Tsuda, *et al*., 1997, Yasuda, *et al*., 1998). RANKL and OPG both regulate bone resorption, and an imbalance between them leads to increased resorption (Yasuda, *et al*., 1998, Anderson, *et al*., 1997, Simonet *et al*., 1997, Mizuno, *et al*., 1998, Kong, *et al*., 1999). Sargachromanol G inhibited RANKL and increased OPG protein levels in IL-1β-stimulated MG-63 cells **(Figure 7)**.

IL-1β and TNF-α activate NF-κB. TRAF proteins relay RANK stimulation to NF-κB. Mice lacking both the NF-κB p50 and p52 proteins are osteopetrotic (Iotsova *et al*., 1997). The upstream subunits that mediate NF-κB activation, the catalytic subunits IκB kinase α (IKKα) and IKKβ and the non-catalytic subunit IKKγ (also called NEMO), are also important for RANK–RANKL signaling and osteoclastogenesis. Importantly, patients with X-linked osteopetrosis, lymphedema, anhidrotic ectodermal dysplasia, and immunodeficiency (OLEDA-ID syndrome) carry a X420W point mutation in IKKγ and have osteopetrosis (Doffinger *et al*., 2001). Thus, the NF-κB pathway is relevant for RANKL–



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RANK-regulated osteoclast development and osteoclast function in humans. Sargachromanol G and PDTC dose-dependently reduced phosphorylation of p65 and p50 in MG-63 cells **(Figure 9, 10)**. Moreover, sargachromanol G inhibited the IL-1β-induced degradation of IκB-α, putatively providing a mechanism to explain how sargachromanol G may block the production of IL-1β-induced osteoclastogenic factors **(Figure 8)**.

IL-1β induces $PGE_2$ and IL-6 secretion into MG-63 culture supernatants via MAPK activation, as measured by phosphorylation of MAPKs by its activators (MEK, MKK4 (MAP Kinase Kinase 4) and MKK3/6) (Webb *et al*., 2002). MAPK inhibitors (PD98059, SB203580 and SP600125) inhibited the IL-1β-stimulated release of $PGE_2$. p38 and JNK inhibitors blocked COX-2 and $PGE_2$ increases, but ERK did not. These findings are consistent with a previous report, demonstrating that cotreatment of MG-63 cells with IL-1β enhances phosphorylation of p38 and JNK, but not ERK (Brechter and Lerner, 2007).

Modulating MAP kinase-dependent IL-6 production by osteoblastic cells has therapeutic potential. For example, the p38 MAP kinase inhibitor, SB242235, improved joint integrity in rats with adjuvant-induced arthritis, as reflected by improvements in inflammation and bone mineral density and lower serum levels of IL-6 (Badger *et al.*, 2000). Similarly, the induction of IL-6 production and bone resorption by IL-1β and TNF-α in osteoblasts and chondrocytes could be inhibited by pre-treatment with SB203580 (Kumar *et al.,* 2001), indication a connection between p38 activity and cytokine responses. Sargachromanol G markedly inhibited ERK, p38, and JNK activation but did not change total protein levels, providing a mechanism for how sargachromanol G inhibits IL-1β-induced NF-κB binding **(Figure 11-13)**.

LPS stimulates macrophages such as RAW 264.7 to produce pro-inflammatory mediators, including the potent vasodilator, NO, and the cytokines, TNF-α, IL-1, and IL-6 (Lapa *et al*., 2000) to activate macrophages and contribute to acute and chronic inflammation (Mehra *et*



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*al*., 2005; Kofler *et al*., 2005). We tested the effects of sargachromanol G on LPS-induced production of inflammatory mediators and cytokines in RAW 264.7 cells.

NO regulates bone formation, resorption, remodeling, mechanotransduction, and repair in physiological or pathophysiological conditions, often through targeting osteoclast formation, activity, or survival (van't Hof and Ralston 2001, Blair *et al*., 2002). LPS induces osteoclast formation in mouse bone marrow cultures (Park *et al*., 2007) and stimulates osteoclast-mediated bone resorption through COX-2 induction and $PGE_2$ production in vivo (Sakuma *et al*., 2000). Binding of LPS to toll-like receptor 4 activates NF-κB and AP-1, which induce the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α (Medzhitov *et al*., 2001, Aderem *et al*., 2000) that promote osteoclast differentiation and activation (Kong et al., 1999, Gravallese *et al*., 2000, Takayanagi *et al*., 2000).

NO and $PGE_2$ are important mediators of inflammation (Ahmad *et al*., 2002; Murakami *et al*., 2007). Inhibitors of iNOS and COX-2 may help prevent inflammatory diseases. Sargachromanol G dose-dependently inhibited LPS-induced NO and $PGE_2$ production via down-regulation of iNOS and COX-2 expression **(Figure 15-2, 16-2, 17)**.

TNF-α, IL-1β, and IL-6 abnormalities are important in inflammatory lesions (Feldmann *et al*., 1991). TNF-α can stimulate the production or expression of IL-6, IL-1β, $PGE_2$, collagenase, and adhesion molecules, and can cause septic shock, inflammation, and cytotoxicity (Kim and Moudgil, 2008; Feldmann, 2008; Sugita, 2009). IL-6 is secreted by T cells and macrophages to stimulate the immune response to trauma, especially burns or other tissue damage leading to inflammation (Ding *et al*., 2009; Kim *et al*., 2009). IL-1β is primarily released by macrophages and plays an important role in the pathophysiology of rheumatoid arthritis (Fogal and Hewett, 2008; Ren and Torres, 2009). Thus, inhibiting cytokine production or function is important in controlling inflammation. Sargachromanol G inhibited LPS-induced production of TNF-α, IL-1β, and IL-6, indicating that it has useful



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anti-inflammatory activity **(Figure 18-18-2)**.

NF-κB activation regulates the production of iNOS, COX-2, and cytokines in macrophages in response to LPS (Ghosh and Hayden, 2008; Wong and Tergaonkar, 2009; Edwards *et al*., 2009). Sargachromanol G blocks NF-κB activation and these downstream pro-inflammatory mediators **(Figure 19-1-21-1)**.

LPS regulates iNOS and COX-2 expression through ERK, JNK, and p38, leading to NF-κB activation in macrophages. MAPK activation mediates NF-κB-induced changes in iNOS and COX-2 (Malemud *et al*., 2008; Brown and Sacks, 2008; Ji *et al*., 2009). Sargachromanol G reduced LPS-stimulated phosphorylation of ERK, JNK and p38, indicating that this pathway may mediate changes in iNOS and COX-2 expression via NF-κB activation **(Figure 22-1-24-1)**.

Sargachromanol G dose-dependently inhibited RANKL-induced osteoclast differentiation, particularly TRAP-positive formation, due to the large number of TRAP-positive cells in sargachromanol G-treated cultures **(Figure 27, 28)**. TRAP, MMP-9, cathepsin K, and CTR are markers of osteoclast differentiation (Faccio *et al*., 2005), and sargachromanol G reduced levels of these markers, as TRAP positive cells are also positive for these genes **(Figure 25, 26)**.

RANKL-induced osteoclast differentiation requires NF-κB, JNK, and p38 MAPKs (Ikeda *et al*., 2004, Takayanagi *et al*., 2002, Teitelbaum *et al*., 2000). NF-kB knockout mice and transgenic mice expressing dominant-negative c-Jun or dominant-negative JNK1 exhibited osteopetrosis (Ikeda *et al*., 2004, Iotsova *et al*., 1997), and SP600125 and SB203580 inhibit osteoclastogenesis (Ikeda *et al*., 2004, Takayanagi *et al*., 2002). Sargachromanol G inhibits LPS-induced inflammatory events in RAW 264.7 cells by negatively regulating inflammatory mediators and NF-κB activation. In contrast, NF-κB activation by the sargachromanol G was observed in MG-63 cells (Chung *et al*., 2005). These discrepancies



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might be attributable to isomer and/or tissue specificity. Interestingly, sargachromanol G inhibited RANKL-induced NF-κB activation in RAW 264.7 cells **(Figure 29-31)**. NF-κB is considered to be involved not only in the differentiation but also the maturation of osteoclasts (Kobayashi *et al*., 2001, Wada *et al*., 2006). Sargachromanol G also inhibited phosphorylation of ERK, JNK and p38 in RANKL-induced RAW 264.7 cells **(Figure 32-34)**.

TRAF6 binding is necessary for RANK-induced NF-κB activation and *in vitro* osteoclastogenesis (Darnay *et al*., 1998), and TRAF6-deficient mice develop severe osteopetrosis (Lomaga *et al*., 1999). IL-1, RANKL, and LPS, improved osteoclast survival but did not induce p38, MKK3/6, or ATF2 phosphorylation, nor did SB203580 change survival, indicating that the p38 MAPK signaling pathway is nonfunctional in osteoclasts. In contrast, LPS and RANKL activated ERK and JNK.

c-Src is specifically recruited to the terminal 3 amino acids of the β3 subunit of the αvβ3 integrin (Zou *et al*., 2007) and to Y559 of the macrophage-colony stimulating factor (M-CSF) receptor (Faccio *et al* 2007), c-Fms, to modulate cytoskeletal changes. c-Src also binds activated RANK (Wong et al., 1999) to recruit TRAF6 and Grb2-associated binder 2 (Gab2), followed by phosphorylation of IκB-α and JNK (Wada *et al*., 2005), and ultimately NF-κB and AP-1 activation (Teitelbaum et al., 2003, Tanaka *et al*., 2005, David *et al*., 2002). Sargachromanol G inhibited phosphorylation of SFKs, MEK, Lyn, MKK3/6 and ATF2 **(Figure 35)**.

In summary, sargachromanol G treatment of MG-63 cells blocked the induction of osteoclastogenic factors ($PGE_2$, COX-2 and IL-6) following stimulation by IL-1β. Sargachromanol G suppressed the production of pro-inflammatory mediators (NO, iNOS, $PGE_2$ and COX-2) and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in LPS-stimulated RAW 264.7 cells. Also, sargachromanol G was inhibited the induction of osteoclastogenic factors (TRAP, CTR, Cath-k and TRAF6) and osteoclastogenesis in



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RANKL-stimulated preosteoclast. Sargachromanol G inhibits production of osteoclastogenic factors and cytokines through blocking NF-κB and the MAPK pathway **(Figure 36)**. Sargachromanol G activity was mediated by the down-regulation of NF-κB and MAPKs. Sargachromanol G may therefore have therapeutic potential for osteoprotic diseases.

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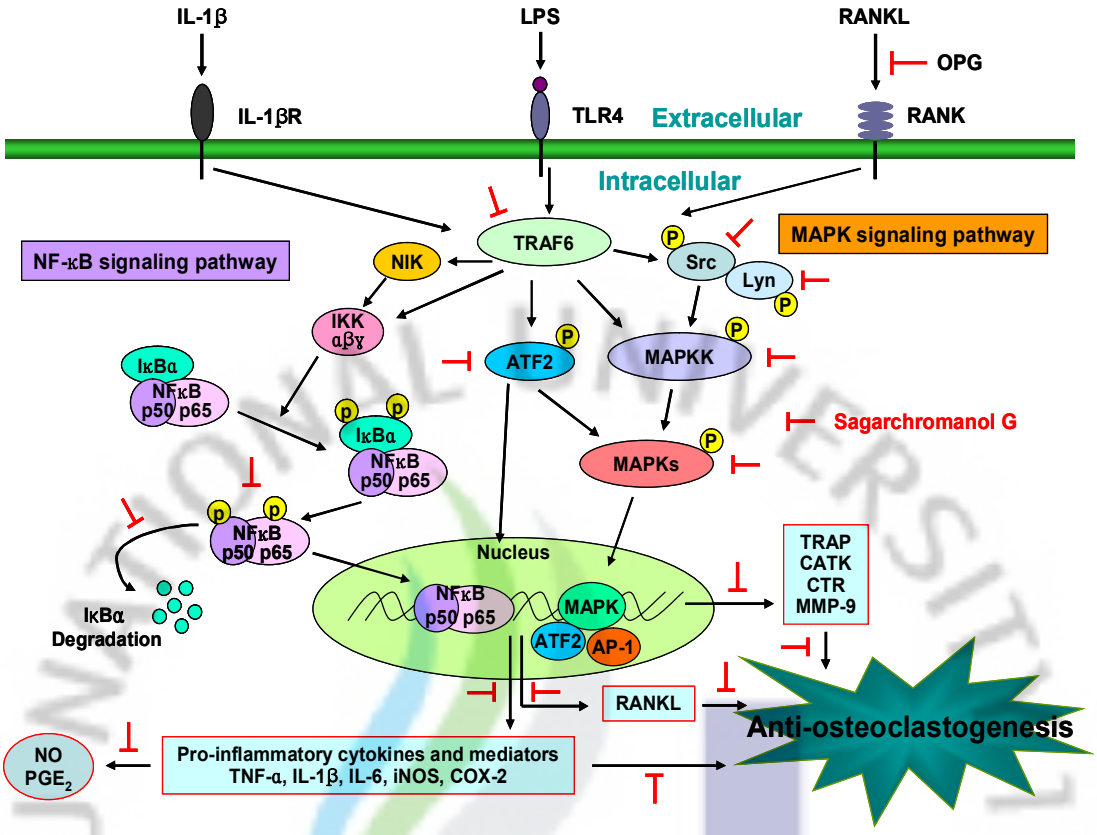


**Figure 36. Effects of sagachromanol G on osteoclastogenic factors**



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## 5. Reference


Aderem A., and Ulevitch R.J., Toll-like receptors in the induction of the innate immune response. *Nature*, 406(6797), 782-787 (2000).

Ahmad, N., Chen, L.C., Gordon, M.A., Laskin, J.D., and Laskin, D.L., Regulation of cyclooxygenase-2 by nitric oxide in activated hepatic macrophages during acute endotoxemia. *J Leukoc Biol*, 71(6), 1005-1011 (2002).

Anderson, D.M., Maraskovsky, E., Billingsley, W.L., Dougall, W.C., Tometsko, M.E., Roux, E.R., Teepe, M.C., DuBose, R.F., Cosman, D., and Galibert, L., A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature*, 390(6656), 175-179. (1997).

Aubin, J.E., and Bonnelye, E., Osteoprotegerin and its ligand: a new paradigm for regulation of osteoclastogenesis and bone resorption. *Osteoporos Int*, 11(11), 905-1013 (2000).

Avruch, J., Zhang, X.F., and Kyriakis, J.M., Raf meets Ras: completing the framework of a signal transduction pathway. *Trends Biochem Sci*, 19(7), 279-283 (1994).

Badger, A.M., Griswold, D.E., Kapadia, R., Blake, S., Swift, B.A., Hoffman, S.J., Stroup, G.B., Webb, E., Rieman, D.J., Gowen, M., Boehm, J.C., Adams, J.L., and Lee, J.C., Disease-modifying activity of SB 242235, a selective inhibitor of p38 mitogen-activated protein kinase, in rat adjuvant-induced arthritis. Arthritis Rheum, 43(1), 175-183 (2000).

Blair, H.C., Zaidi, M., and Schlesinger, P.H., Mechanisms balancing skeletal matrix synthesis and degradation. *Biochem J*, 364(Pt 2), 329-41 (2002).

Boyle, W.J., Simonet, W.S., and Lacey, D.L., Osteoclast differentiation and activation. *Nature*, 423(6937), 337-342 (2003).

Brechter, A.B., and Lerner, U.H., Bradykinin potentiates cytokine-induced prostaglandin biosynthesis in osteoblasts by enhanced expression of cyclooxygenase-2, resulting in




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increased RANKL expression. *Arthritis Rheum*, 56(3), 910-923 (2007).

Brown, M.D., and Sacks, D.B., Compartmentalised MAPK pathways. *Handb Exp Pharmacol*, (186), 205-235 (2008).

Chang, L., and Karin, M., Mammalian MAP kinase signaling cascades. *Nature*, 410(6824), 37-40 (2001).

Chung, S., Brown, J.M., Provo, J.N., Hopkins, R., and McIntosh, M.K., Conjugated linoleic acid promotes human adipocyte insulin resistance through NFkappaB-dependent cytokine production. *J Biol Chem*, 280(46), 38445-38456 (2005).

Darnay, B.G., Haridas, V., Ni, J., Moore, P.A., and Aggarwal, B.B., Characterization of the intracellular domain of receptor activator of NF-κB (RANK): interaction with tumor necrosis factor receptor-associated factors and activation of NF-κB and c-Jun N-terminal kinase. *J Biol Chem*, 273(32), 20551-20555 (1998).

David, J.P., Sabapathy, K., Hoffmann, O., Idarraga, M.H., and Wagner, E.F., JNK1 modulates osteoclastogenesis through both c-Jun phosphorylation-dependent and –independent mechanisms. *J Cell Sci*, 115(Pt 22), 4317-4325 (2002).

Davis, R.J., MAPKs: new JNK expands the group. *Trends Biochem Sci*, 19(11), 470-473(1994).

Dinarello, C.A., The interleukin-1 family: 10 years of discovery. *FASEB J*, 8(15), 1314-25 (1994).

Ding, C., Cicuttini, F., Li, J., and Jones, G., Targeting IL-6 in the treatment of inflammatory and autoimmune diseases. *Expert Opin Investig Drugs*, 18(10), 1457-1466 (2009).

Döffinger, R., Smahi, A., Bessia C., Geissmann, F., Feinberg, J., Durandy, A., Bodemer, C., Kenwrick, S., Dupuis-Girod, S., Blanche, S., Wood, P., Rabia, S.H., Headon, D.J., Overbeek, P.A., Le Deist, F., Holland, S.M., Belani, K., Kumararatne, D.S., Fischer, A., Shapiro, R., Conley, M.E., Reimund, E., Kalhoff, H., Abinun, M., Munnich, A., Israël, A.,



Collection @ jeju

Courtois, G., and Casanova, J.L., X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. *Nat Genet*, 27(3), 277-285 (2001).

Edwards, M.R., Bartlett, N.W., Clarke, D., Birrell, M., Belvisi, M., and Johnston, S.L., Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease. *Pharmacol Ther*, 121(1), 1-13 (2009).

Faccio, R., Takeshita, S., Colaianni, G., Chappel, J., Zallone, A., Teitelbaum, S.L., and Ross, F.P., M-CSF regulates the cytoskeleton via recruitment of a multimeric signaling complex to c-Fms Tyr-559/697/721. *J Biol Chem*, 282(26), 18991-18999 (2007).

Faccio, R., Teitelbaum, S.L., Fujikawa, K., Chappel, J., Zallone, A., Tybulewicz, V.L., Ross, F.P., and Swat W., Vav3 regulates osteoclast function and bone mass. *Nat Med*, 11(3), 284-290 (2005).

Feldmann, M., Brennan, F.M., Chantry, D., Haworth, C., Turner, M., Katsikis, P., Londei, M., Abney, E., Buchan, G., Barrett, K., Corcoran, A., Kissonerghis, M., Zheng, R., Grubeck-Loebenstein, B., Barkley, D., Chu, C.Q., Field, M. and Maini, R.N. Cytokine assays: role in evaluation of the pathogenesis of autoimmunity. *Immunol Rev*, 119, 105-123 (1991).

Feldmann, M., Many cytokines are very useful therapeutic targets in disease. *J Clin Invest*, 118(11), 3533-3536 (2008).

Fernandez, M., Rios, J.C., Jos, A., and Repetto, G., Comparative cytotoxicity of alachlor on RTG-2 trout and SH-SY5Y human cells. *Arch Environ Contam Toxicol*, 51(4), 515-520 (2006).

Firestein GS., Evolving concepts of rheumatoid arthritis. *Nature*, 423(6937), 356–61 (2003).

Fogal, B., and Hewett, S.J., Interleukin-1beta: a bridge between inflammation and excitotoxicity? *J Neurochem*, 106(1), 1-23 (2008).

Galibert, L., Tometsko, M.E., Anderson, D.M., Cosman, D., and Dougall, W.C., The



Collection @ jeju

involvement of multiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NF-kappaB, a member of the TNFR superfamily. *J Biol Chem*, 273(51), 34120-34127 (1998).

Ghosh, S., Hayden, M.S., New regulators of NF-kappaB in inflammation. *Nat Rev Immunol*, 8(11), 837-848 (2008).

Gravallese, E.M., Manning, C., Tsay, A., Naito, A., Pan, C., Amento, E., and Goldring, S.R., Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. *Arthritis Rheum*, 43(2), 250-258 (2000).

Hofbauer, L.C., Khosla, S., Dunstan, C.R., Lacey, D.L., Boyle, W.J., Riggs, B.L., The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *J Bone Miner Res*, 15(1), 2-12 (2000).

Horowitz, M.C., and Lorenzo, J.A., Local regulators of bone: IL-1, TNF, lymphotoxin, interferon-γ, IL-8, IL-10, IL-4, the LIF/IL-6 family, and additional cytokines. Principles of bone biology, San Diego: Academic Press, 961–978 (2002).

Hotokezaka, H., Sakai, E., Kanaoka, K., Saito, K., Matsuo, K., Kitaura, H., Yoshida, N., and Nakayama, K., U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. *J Biol Chem*, 277(49), 47366-47372. (2002).

Hsu, H., Lacey, D.L., Dunstan, C.R., Solovyev, I., Colombero, A., Timms, E., Tan, H.L., Elliott, G., Kelley, M.J., Sarosi, I., Wang, L., Xia, X.Z., Elliott, R., Chiu, L., Black, T., Scully, S., Capparelli, C., Morony, S., Shimamoto, G., Bass, M.B., and Boyle, W.J., Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA*, 96(7), 3540-3545 (1999).

Ikeda, F., Nishimura, R., Matsubara, T., Tanaka, S., Inoue, J., Reddy, S.V., Hata, K.,



Collection @ jeju

Yamashita, K., Hiraga, T., Watanabe, T., Kukita, T., Yoshioka, K., Rao, A., and Yoneda, T., Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. *J Clin Invest*, 114(4), 475-484 (2004).

Iotsova, V., Caamano, J., Loy, J., Yang, Y., Lewin, A., and Bravo, R., Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat Med*, 3(11), 1285-1289 (1997).

Iwasaki, S., Hattori, A., Sato, M., Tsujimoto, M., and Kohno, M., Characterization of the bone morphogenetic protein-2 as a neurotrophic factor. Induction of neuronal differentiation of PC12 cells in the absence of mitogen-activated protein kinase activation. *J Biol Chem*, 271(29), 17360-17365(1996).

Ji, R.R., Gereau, R.W. 4th, Malcangio, M., and Strichartz, G.R., MAP kinase and pain. *Brain Res Rev*, 60(1), 135-148 (2009).

Joo, S.S., Kang, H.C., Lee, M.W., Choi, Y.W., and Lee, D.I., Inhibition of IL-1β and IL-6 in osteoblast-like cell by isoflavones extracted from Sophorae fructus. *Arch Pharm Res*, 26(12), 1029-1035 (2003).

Karsenty, G. and Wagner, E.F., Reaching a genetic and molecular understanding of skeletal development. *Dev Cell*, 2(4), 389-406 (2002).

Kenner, L., Hoebertz, A., Beil, T., Keon, N., Karreth, F., Eferl, R., Scheuch, H., Szremska, A., Amling, M., Schorpp-Kistner, M., Angel, P., and Wagner, E.F., Mice lacking JunB are osteopenic due to cell-autonomous osteoblast and osteoclast defects. *J Cell Biol*, 164(4), 613-623 (2004).

Khosla, S., Minireview: the OPG/RANKL/RANK system. *Endocrinology*, 142(12), 5050-5055 (2001).

Kim, E.Y., and Moudgil, K.D., Regulation of autoimmune inflammation by pro-inflammatory cytokines. *Immunol Lett*, 120(1-2), 1-5 (2008).

Kim, J.H., Bachmann, R.A., and Chen, J., Interleukin-6 and insulin resistance. *Vitam Horm*,



Collection @ jeju

80, 613-633 (2009).

Kobayashi, K., Takahashi, N., Jimi, E., Udagawa, N., Takami, M., Kotake, S., Nakagawa, N., Kinosaki, M., Yamaguchi, K., Shima, N., Yasuda, H., Morinaga, T., Higashio, K., Martin, T.J., and Suda, T., Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *J Exp Med*, 191(2):275-286 (2000).

Kobayashi, N., Kadono, Y., Naito, A., Matsumoto, K., Yamamoto, T., Tanaka, S., and Inoue, J., Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. *EMBO J*, 20(6), 1271-1280 (2001).

Kofler, S., Nickel, T., and Weis, M., Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. *Clin Sci.*, 108(3), 205-213 (2005).

Kong, Y.Y., Yoshida, H., Sarosi, I., Tan, H.L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A.J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C.R., Lacey, D.L., Mak, T.W., Boyle, W.J., and Penninger, J.M., OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*, 397(6717), 315-323 (1999).

Kumar, S., Votta, B.J., Rieman, D.J., Badger, A.M., Gowen, M., and Lee, J.C., IL-1- and TNF-induced bone resorption is mediated by p38 mitogen activated protein kinase. *J Cell Physiol*, 187(3), 294-303 (2001).

Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y.X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., Boyle, W.J., Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, 93(2), 165-176 (1998).

Lapa e Silva, J.R., Possebon da Silvia, M.D., Lefort, J., and Vargaftig, B.B., Endotoxin,



Collection @ jeju

asthma, and allergic immune responses. *Toxicology*, 152(1-3), 31-35 (2000).

Lee, S.Y., Reichlin, A., Santana, A., Sokol, K.A., Nussenzweig, M.C., and Choi, Y., TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity*, 7(5), 703-713 (1997).

Lerner, U.H., Inflammation-induced bone remodelling in periodontal disease and the influence of post-menopausal osteoporosis. *J Dent Res*, 85(7), 596-607 (2006).

Lerner, U.H., New molecules in the tumor necrosis factor ligand and receptor superfamilies with importance for physiological and pathological bone resorption. *Crit Rev Oral Biol Med*, 15(2), 64-81 (2004).

Li, X., Pilbeam, C.C., Pan, L., Breyer, R.M., and Raisz, L.G., Effects of prostaglandin E2 on gene expression in primary osteoblastic cell from prostaglandin receptor knockout mice. *Bone*, 30(4), 567-573 (2002).

Lomaga, M.A., Yeh, W.C., Sarosi, I., Duncan, G.S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z., Penninger, J.M., Paige, C.J., Lacey, D.L., Dunstan, C.R., Boyle, W.J., Goeddel, D.V., and Mak, T.W., TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev*, 13(8), 1015-1024 (1999).

Lowell, C.A., and Soriano, P., Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories. *Genes Dev*, 10(15), 1845-1857 (1996).

Malemud, C.J., and Miller, A.H., Pro-inflammatory cytokine-induced SAPK/MAPK and JAK/STAT in rheumatoid arthritis and the new anti-depression drugs. *Expert Opin Ther Targets*, 12(2), 171-183 (2008).

Manolagas, S.C., and Jilka, R.L., Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *N Engl J Med*, 332(5), 305-311 (1995).

Matsumoto, M., Sudo, T., Saito, T., Osada, H., and Tsujimoto, M., Involvement of p38



dCollection @ jeju

mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). *J Biol Chem*, 275(40), 31155-31161 (2000).

Medzhitov, R., Toll-like receptors and innate immunity. *Nat Rev Immunol*, 1(2), 135–145 (2001).

Mehra, V.C., Ramgolam, V.S., and Bender, J.R., Cytokines and cardiovascular disease. *J Leukoc Biol*, 78(4), 805-818 (2005).

Mizuno, A., Amizuka, N., Irie, K., Murakami, A., Fujise, N., Kanno, T., Sato, Y., Nakagawa, N., Yasuda, H., Mochizuki, S., Gomibuchi, T., Yano, K., Shima, N., Washida, N., Tsuda, E., Morinaga, T., Higashio, K., Ozawa, H., Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochem Biophys Res Commun*, 247(3), 610-615 (1998).

Murakami, A., and Ohigashi, H., Targeting NOX, INOS and COX-2 in inflammatory cells: chemoprevention using food phytochemicals. *Int J Cancer*, 121(11), 2357-2363 (2007).

Naito, A., Azuma, S., Tanaka, S., Miyazaki, T., Takaki, S., Takatsu, K., Nakao, K., Nakamura, K., Katsuki, M., Yamamoto, T., and Inoue, J., Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells*, 4(6), 353-362 (1999).

Nakagawa, N., Kinosaki, M., Yamaguchi, K., Shima, N., Yasuda, H., Yano, K., Morinaga, T., Higashio, K., RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem Biophys Res Commun*, 253(2), 395-400 (1998).

Nishida, E., and Gotoh, Y., The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem Sci*, 18(4), 128-131 (1993).

Okada, Y., Lorenzo, J.A., Freeman, A.M., Tomita, M., Morham, S.G., Raisz, L.G., and Pilbeam, C.C., Prostaglandin G/H synthase-2 is required for maximal formation of



Collection @ jeju

osteoclast-like cells in culture. *J Clin Invest*, 105(6), 823-832 (2000).

Ono, K., Akatsu, T., Murakami, T., Kitamura, R., Yamamoto, M., Rokutanda, M., Nagata, N., Kugai, N., Involvement of cyclo-oxygenase-2 in osteoclast formation and bone destruction in bone metastasis of mammary carcinoma cell lines. *J Bone Miner Res*, 17(5), 774–781 (2002).

Park, C.K., Kim, H.J., Kwak, H.B., Lee, T.H., Bang, M.H., Kim, C.M., Lee, Y., Chung, D.K., Baek, N.I., Kim, J., Lee, Z.H., and Kim, H.H., Inhibitory effects of Stewartia koreana on osteoclast differentiation and bone resorption. *Int Immunopharmacol*, 7(12), 1507-1516 (2007).

Rahman, M.M., Kukita, A., Kukita, T., Shobuike, T., Nakamura, T., and Kohashi, O., Two histone deacetylase inhibitors, trichostatin A and sodium butyrate, suppress differentiation into osteoclasts but not into macrophages. *Blood*, 101(9), 3451-3459 (2003).

Ren, K., and Torres, R., Role of interleukin-1beta during pain and inflammation. *Brain Res Rev*, 60(1), 57-64 (2009).

Ruocco, M.G., Maeda, S., Park, J.M., Lawrence, T., Hsu, L.C., Cao, Y., Schett, G., Wagner, E.F., and Karin, M., I{kappa}B kinase (IKK){beta}, but not IKK{alpha}, is a critical mediator of osteoclast survival and is required for inflammation-induced bone loss. *J Exp Med*, 201(10), 1677-1687 (2005).

Sakuma, Y., Tanaka, K., Suda, M., Yasoda, A., Natsui, K., Tanaka, I., Ushikubi, F., Narumiya, S., Segi, E., Sugimoto, Y., Ichikawa, A., and Nakao, K., Crucial involvement of the EP4 subtype of prostaglandin E receptor in osteoclast formation by proinflammatory cytokines and lipopolysaccharide. *J Bone Miner Res*, 15(2), 218-227 (2000).

Simonet, W.S., Lacey, D.L., Dunstan, C.R., Kelley, M., Chang, M.S., Lüthy, R., Nguyen, H.Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H.L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L.,



Collection @ jeju

Hughes, T.M., Hill, D., Pattison, W., Campbell, P., Sander, S., Van, G., Tarpley, J., Derby, P., Lee, R., and Boyle, W.J., Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*, 89(2), 309-319 (1977).

Suda, T., Ueno, Y., Fujii, K., and Shinki, T., Vitamin D and bone. *J Cell Biochem*, 88(2), 259-266 (2003).

Sugita, T., Targeting therapy for inflammatory diseases by anti-TNF-alpha biologics. *Yakugaku Zasshi*, 129(1), 19-24 (2009).

Tai, H., Miyaura, C., Pilbeam, C.C., Tamura, T., Ohsugi, Y., Koishihara, Y., Kubodera, N., Kawaguchi, H., Raisz, L.G., and Suda, T. Transcriptional induction of cyclooxygenase-2 in osteoblasts is involved in interleukin-6-induced osteoclast formation. *Endocrinology*, 138(6), 2372-2379 (1997).

Takayanagi, H., Iizuka, H., Juji, T., Nakagawa, T., Yamamoto, A., Miyazaki, T., Koshihara, Y., Oda, H., Nakamura, K., and Tanaka, S., Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum*, 43(2), 259-269 (2000).

Takayanagi, H., Kim, S., Koga, T., Nishina, H., Isshiki, M., Yoshida, H., Saiura, A., Isobe, M., Yokochi, T., Inoue, J., Wagner, E.F., Mak, T.W., Kodama, T., and Taniguchi, T., Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell*, 3(6), 889-901 (2002).

Tanaka, S., Nakamura, K., Takahasi, N., and Suda, T., Role of RANKL in physiological and pathological bone resorption and therapeutics targeting the RANKL-RANK signaling system. *Immunol Rev*, 208, 30-49 (2005).

Teitelbaum, S., Osteoclasts; culprits in inflammatory osteolysis. *Arthritis Res Ther*, 8(1), 201-208 (2006).

Teitelbaum, S.L., and Ross, F.P., Genetic regulation of osteoclast development and function.



Collection @ jeju

*Nat Rev Genet*, 4(8), 638-649 (2003).

Teitelbaum, S.L., Bone resorption by osteoclasts. *Science*, 289(5484), 1504-1508 (2000).

Theill, L.E., Boyle, W.J., and Penninger, J.M., RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol*, 20, 795-823 (2002).

Tsuda, E., Goto, M., Mochizuki, S., Yano, K., Kobayashi, F., Morinaga, T., and Higashio, K., Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophys Res Commun*, 234(1), 137-142 (1997).

Udagawa, N., Takahashi, N., Yasuda, H., Mizuno, A., Itoh, K., Ueno, Y., Shinki, T., Gillespie, M.T., Martin, T.J., Higashio, K., Suda, T., Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. *Endocrinology*, 141(9), 3478-3784 (2000).

van't Hof, R.J., and Ralston, S.H., Nitric oxide and bone. *Immunology*, 103(3), 255-261. (2001).

Wada, T., and Penninger, J.M., Mitogen-activated protein kinases in apoptosis regulation. *Oncogene*, 23(16), 2838-2849 (2004).

Wada, T., Nakashima, T., Hiroshi, N., and Penninger, J.M., RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med*, 12(1), 17-25 (2006).

Wada, T., Nakashima, T., Oliveira-dos-Santos, A.J., Gasser, J., Hara, H., Schett, G., and Penninger, J.M., The molecular scaffold Gab2 is a crucial component of RANK signaling and osteoclastogenesis. *Nat Med*, 11(4), 394-399 (2005).

Wagner, E.F., Functions of AP1 (Fos/Jun) in bone development. *Ann Rheum Dis*, 61 Suppl 2, ii40-42 (2002).

Walsh, M.C., and Choi, Y., Biology of the TRANCE axis. *Cytokine Growth Factor Rev*, 14(3-4), 251-263 (2003).

Walsh, N.C., and Gravallese, E.M., Bone loss in inflammatory arthritis: mechanisms and



Collection @ jeju

treatment strategies. *Curr Opin Rheumatol*, 16(4), 419-427 (2004).

Webb, S.J., McPherson, J.R., Pahan, K., and Koka, S., Regulation of TNF-alpha-induced IL-6 production in MG-63 human osteoblast-like cells. *J Dent Res*, 81(1), 17-22 (2002).

Wittrant, Y., Théoleyre, S., Chipoy, C., Padrines, M., Blanchard, F., Heymann, D., and Rédini, F., RANKL/RANK/OPG: new therapeutic targets in bone tumours and associated osteolysis. *Biochim Biophys Acta*, 1704(2), 49-57 (2004).

Wong, B.R., Besser, D., Kim, N., Arron, J.R., Vologodskaia, M., Hanafusa, H., and Choi, Y., TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell*, 4(6), 1041-1049 (1999).

Wong, B.R., Rho, J., Arron, J., Robinson, E., Orlinick, J., Chao, M., Kalachikov, S., Cayani, E., Bartlett, F.S. 3rd., Frankel, W.N., Lee, S.Y., and Choi, Y., TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem*, 272(40), 25190-25194 (1997).

Wong, E.T., and Tergaonkar, V., Roles of NF-kappaB in health and disease: mechanisms and therapeutic potential. *Clin Sci*, 116(6), 451-465 (2009).

Wronski, T.J. and Morey, E.R., Alterations in calcium homeostasis and bone during actual and simulated space flight. *Med Sci Sports Exerc*, 15(5), 410-414 (1983).

Yamamoto, A., Miyazaki, T., Kadono, Y., Takayanagi, H., Miura, T., Nishina, H., Katada, T., Wakabayashi, K., Oda, H., Nakamura, K., and Tanaka, S., Possible involvement of IkappaB kinase 2 and MKK7 in osteoclastogenesis induced by receptor activator of nuclear factor kappaB ligand. *J Bone Miner Res*, 17(4), 612-621 (2002).

Yasuda, H., Shima, N., Nakagawa, N., Mochizuki, S.I., Yano, K., Fujise, N., Sato, Y., Goto, M., Yamaguchi, K., Kuriyama, M., Kanno, T., Murakami, A., Tsuda, E., Morinaga, T., and Higashio, K., Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in



Collection @ jeju

vitro. *Endocrinology*, 139(3), 1329-1337 (1998).

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N., and Suda, T., Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA*, 95(7), 3597-3602 (1998).

Yucel-Lindberg, T., Ahola, H., Carlstedt-Duke, J., and Modeer, T. Involvement of tyrosine kinases on cyclooxygenase expression and prostaglandin E2 production in human gingival fibroblasts stimulated with interleukin-1β and epidermal growth factor. *Biochem Biophys Res Commun*, 257(2), 528-532 (1999).

Zhang, Y.H., Heulsmann, A., Tondravi, M.M., Mukherjee, A., and Abu-Amer, Y., Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. *J Biol Chem*, 276(1), 563-568 (2001).

Zou, W., Kitaura, H., Reeve, J., Long, F., Tybulewicz, V.L., Shattil, S.J., Ginsberg, M.H., Ross, F.P., and Teitelbaum, S.L., Syk, c-Src, the alphavbeta3 integrin, and ITAM immunoreceptors, in concert, regulate osteoclastic bone resorption. *J Cell Biol*, 176(6), 877-888 (2007).



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