Journal of Medicinal Plants Research Vol. 5(6), pp. 903-909, 18 March, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Inhibitory effect of ursane-type triterpenoids from *Weigela subsessilis* on RANKL-induced osteoclast differentiation

Young Ju Bae[1], Phuong Thien Thuong[2], Nacksung Kim[3], Won Keun Oh[4], Jin Hee Kim[5] and Junwon Lee[1]*

[1]Department of life Science and Genetic Engineering, Paichai University, Daejeon, Korea.
[2]Vietnam National Institute of Medicinal Materials, 3B-Quang Trung, Hoan Kiem, Hanoi, Vietnam.
[3]National Research Laboratory for Regulation of Bone Metabolism and disease, Chonnam National University Medical School, Gwangju, Korea.
[4]College of Pharmacy, Chosun University, Gwangju, Korea.
[5]Department of Herbal Skin care, Daegu Hanny University, Gyeongsan, Korea.

Accepted 4 October, 2010

Osteoclasts play an important role in bone metabolism by resorbing the bone matrix. Thus, the compounds inhibiting osteoclasts can treat bone diseases such as osteoporosis. Among the 8 triterpenoids tested, we show that Ilekudinol B isolated from the plant *Weigela subsessilis* inhibits receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis from bone marrow-derived monocyte/macrophage cells in a dose-dependent manner, whereas it has no significant effect on osteoblast differentiation. Furthermore, Ilekudinol B attenuates the induction of nuclear factor of activated T cells (NFAT) c1 and osteoclast-associated receptor (OSCAR) expression. Our results indicate that Ilekudinol B has the potential to inhibit osteoclast formation by attenuating the signaling cascades associated with RANKL.

Key words: Triterpenoids, osteoclastogenesis, receptor activator of NF-κB ligand (RANKL), inhibitory activity.

INTRODUCTION

Osteoclasts are multinucleated cells that present only in bone and play an important role in bone metabolism. These cells are formed by fusion of the precursors from hematopoietic origin and differentiated by expression of osteotropic factors (Suda et al., 1999; Lorenzo et al., 2008; Roodman, 2004). Accordingly, osteoclasts remove bone tissue by removing its mineralized matrix, which is known as bone resorption process. Osteoblasts support osteoclast formation from monocyte precursors of hematopoietic origin in response to osteotropic factors such as two essential cytokines, macrophage colony-stimulating factor (M-CSF) and tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE; also called RANKL, OPGL, and ODF) (Suda et al., 1999; Lorenzo et al., 2008; Del, Fattore et al., 2008). Therefore, stimulation of osteoclast differentiation will increase the bone resorption that may lead to bone loss (Teitelbaum, 2000).

The plant *Weigela subsessilis* L. H. BAILEY is an endemic species that grows throughout Korea (Thuong et al., 2005, 2006). Flavonoids and coumarins have been isolated from the leaf and flower of this edible plant (Thuong et al., 2005). The eight ursane-type triterpenoids, including Ilekudinol B isolated from this plant, showed an anti-complement activity against the classical pathway of the complement system in innate immune defense. Although the role of Ilekudinol B have established on inflammatory injuries *in vitro* model, the effect of Ilekudinol B on osteoclast differentiation has yet to be revealed. We demonstrate that Ilekudinol B inhibits RANKL-induced osteoclast differentiation. Ilekudinol B blocks the induction of nuclear factor of activated T cells (NFAT) c1 and osteoclast-associated receptor (OSCAR)

*Corresponding author. E-mail: junwon@pcu.ac.kr. Tel: + 82 42 5205914. Fax: + 82 42 5205385.

expression. Thus, this paper reports the characterization of Ilekudinol B as a potential therapeutic agent for the treatment of bone diseases such as osteoporosis.

MATERIALS AND METHODS

Isolation of ursane-type triterpenoids and Ilekudinol B from *W. subsessilis*

The leaf and stem of *W. subsessilis* were collected at Gyeryong Mountain, Chungnam province, Korea in April, 2008. The plant material was identified by Prof. Won Keun Oh. The triterpenoids (1–8) were isolated from the plant *W. subsessilis* and identified as described previously (Thuong et al., 2006). In brief, dried and powdered plant material (5 kg) was extracted with MeOH at room temperature and filtered with gauze. The concentrated extract (520 g) was suspended in 1 L of water and partitioned sequentially against hexane (1 LX2), EtOAc (1 LX2), and BuOH (0.5 LX2). The EtOAc fraction (125 g) was subjected to silica gel column chromatography (10 X 30 cm) eluted with gradient hexane-EtOAc (10:1, 9:1, 8:1 to 0:1) and separated into 8 fraction. Fraction 1-2 (28 g) was chromatographed on a silica gel column (5 X 40 cm) using hexane-EtOAc (1:4) to give compound 2 (205 mg). Fraction 3 (11 g) was rechromatographed on a Sephadex LH-20 column (3 X 30 cm) eluted with MeOH-H_2O (20:1) and separated into 5 fraction, fraction 3a, fraction 3b (compound 3 and 10 mg), fraction 3c, fraction 3d (compound 2 and 21 mg) and fraction 3e. Fraction 3a (3.6 g) was subjected to an RP-18 column (2.5 X 30 cm) eluted with MeOH-H_2O (20:1) to yield Ilekudinol B (164 mg) and compound 4 (120 mg). Fraction 4 (5.2 g) was rechromatographed on an RP-18 column (2.5 X 30 cm) eluted with MeOH-H_2O (20:1) to yield compound 6 (5.3 mg). Fraction 6 (23 g) was rechromatographed on an RP-18 column (4 X 30 cm) eluted with MeOH-H_2O (2:1) to yield compound 7 (5.9 mg) and compound 8 (17 mg). Fraction 7 (11 g) was rechromatographed on an RP-18 column (4X30 cm) eluted with MeOH-H_2O (2:1) to yield compound 1 (10.1 mg). The positive control, silibinin, was purchased from SigmaAldrich (USA) with approximately 98% purity.

Cell culture of osteoclasts

Murine osteoclasts were prepared from bone marrow cells as previously described (Lee et al., 2006; Kim et al., 2009). In brief, bone marrow cells were cultured in α–minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) with M-CSF (5 ng/ml, R and D Systems Inc., MN) for 16 h. Nonadherent cells were harvested and cultured for 3 days with M-CSF (30 ng/ml). Floating cells were removed and attached cells were used as osteoclast precursors (BMMs). To generate osteoclasts, BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml, R and D Systems Inc., MN) for 3 days.

TRAP staining and solution assay

Cultured cells were fixed with 10% formaldehyde, treated with ethanol/acetone (50:50) and stained for tartrate-resistant acid phosphatase (TRAP) as previously described. TRAP-positive multinuclear cells ($TRAP^+$ MNCs), which contain more than 3 nuclei, were counted and were photographed using Leica DM inverted microscope system (Leica, Wetzlar, Germany) (Lee et al., 2006). To determine the TRAP activities of osteoclasts, cultured cells were washed with phosphate-buffered saline (PBS) and lysed in TRAP buffer (120 mM sodium acetate [pH 5.2], 0.012% sodium tartrate, and 1% Triton X-100). Cell lysates were incubated with *P*- nitrophenyl phosphate (pNPP) solution (Sigma-Aldrich) at 37°C for 20 min. The reactions were stopped with 1 M NaOH and optical density (OD) values were determined at 405 nm (Lee et al., 2006).

Survival assay

To determine cell proliferation, an MTT assay was performed. The cultured cells were incubated with 25 µl/well of MTT solution (5 mg/ml) for 2 h at 37°C. Formazan crystals were dissolved in 100 µl extraction buffer (20% SDS/50% DMF). After incubation for 24 h, the absorbance was measured at 570 nm using a microplate reader (Lee et al., 2006).

Formation of osteoblasts and alkaline phosphatase activity measurement

Primary osteoblast were cultured in α–MEM containing 10% FBS as previously described (Lee et al., 2007). On the 3rd day of culture, the plating medium was replaced with α–MEM containing 10% FBS, ascorbic acid (25 µg/ml), and β–glycerophosphate (10 mM). After 7 days of culture, cells were washed with PBS and lysed by incubating with 400 µl of lysis buffer (0.5 M Tris, pH 9.0, 150 mM NaCl, 1% Triton X-100) for 16 h at 4°C. A volume of 50 µl lysates were incubated with 100 µl of p-nitrophenyl phosphate substrate (Sigma-Aldrich) for 1 h at room temperature. The absorbance was measured at 405 nm using a microplate reader.

Isolation of total RNA and semiquantitative RT-PCR

Total RNA was isolated with TRIzol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. The concentrations of total RNA were calculated from the absorbance at 260 and 280 nm with BioPhotometer (Eppendorf AG, Hamburg, Germany). RT-PCR was performed as previously described (Kim et al., 2007). The following primers were used: RANK-sense, 5'-TAC TAC AGG AAG GGA GGG AAA G-3'; RANK-antisense, 5'-CCT GCT GGA TTA GGA GCA GTG-3'; NFATc1-sense, 5'-CTC GAA AGA CAG CAC TGG AGC AT-3'; NFATc1-antisense, 5'-CGG CTG CCT TCC GTC TCA TAG-3'; OSCAR-sense, 5'-CTG CTG GTA ACG GAT CAG CTC CCC AGA-3'; OSCAR-antisense, 5'-CCA AGG AGC CAG AAC CTT CGA AAC T-3'; TRAP-sense, 5'-CAG TTG GCA GCA GCC AAG GAG GAC-3'; TRAP-antisense, 5'-GTC CCT CAG GAG TCT AGG TAT CAC-3'; HPRT-sense, 5'-GTA ATG ATC AGT CAA CGG GGG AC-3'; HPRT-antisense, 5'-CCA GCA AGC TTG CAA CCT TAA CCA-3'.

Statistical analysis

The data were expressed as means ±SD of triplicate samples. Statistical analyses were performed by an unpaired two-tailed student's *t*-test, assuming unequal variances. Values of $p < 0.05$ were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Bone remodeling is tightly regulated by the balance between bone formation by osteoblasts and bone resorption by osteoclasts. When bone resorption exceeds bone formation, an imbalance of skeletal turnover causes bone-resorbing diseases such as osteoporosis, Paget's disease, and periodontal disease. Because activation of

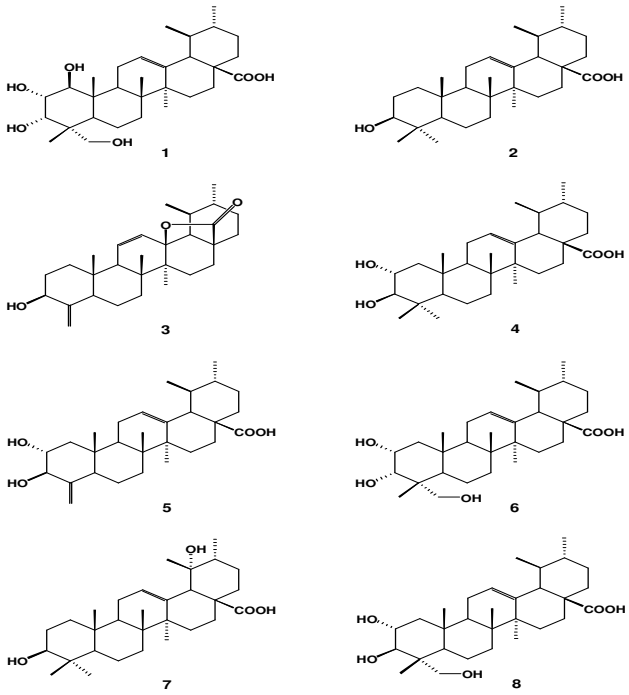


Figure 1. The structure of the tested compounds isolated from *W. subsessilis.* (1: Weigelic acid. 2: Ursolic acid. 3: Ilekudinol A. 4; Corosolic acid. 5: Ilekudinol B. 6: Esculentic acid. 7: Pomolic acid. 8: Asiatic acid).

Table 1. Inhibitory effects of isolated compounds 1-8 on osteoclast differentiation.

Compound[a]	Inhibitory effect (IC_{50}, µg/ml)[b]	Proliferation ratio (%)[c]
1	20.5 ±1.4	78.2
2	>80	100.0
3	20.3 ±2.1	94.5
4	>80	100
5	8.1 ± 1.2	100
6	>80	100
7	30.2 ±1.8	92
8	40.3 ±1.5	82.4
Silibinin	9.2±1.4	100

[a] The purity of compounds for assay was purified by HPLC over 96%. [b] Values are mean ±SD from three separate experiments. Seven concentration points were set for establishment of the inhibition curve to calculate the IC_{50} value. [c] Values are mean from three separate experiments at the concentration of 10 µg/ml. Silibinin was used as a positive control.

osteoclast may cause osteoporosis, the modulation of the differentiation and function of osteoclasts has been considered as therapeutic approach in the treatment of osteoporosis (Teitelbaum, 2000; Boyle et al., 2003; Lorenzo et al., 2008). There are number of agents that have been found to exhibit inhibitory effect on osteoclast differentiation, such as peptides (Kwak et al., 2003), endogenous components (Maran et al., 2006), and natural compounds (Han et al., 2007; Kim et al., 2004). In the course of searching inhibitors of osteoclast differentiation from natural sources, we examined the activities of eight triterpenoids (1-8) isolated from the plant *W. subsessilis* (Thuong et al., 2006).

To examine the effects among these triterpenoids on osteoclasts, eight triterpenoids (Figure 1) were added to bone marrow-derived macrophage cells cultures in the presence of M-CSF and RANKL. Ilekudinols A (3) and Ilekudinol B (5) exhibited no significant effects on osteoclast proliferation and had considerably inhibitory effects on osteoclast differentiation (Table 1), whereas weigelic acid (1) had significant effects on osteoclast proliferation. This indicated that the double bond at

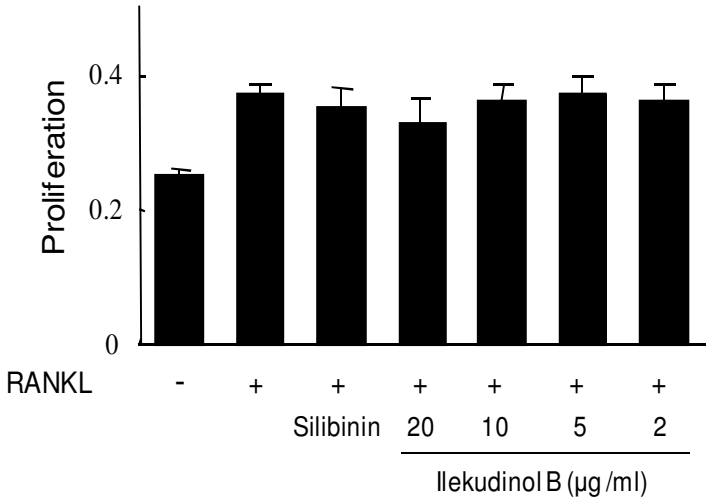


Figure 2. Toxic effect of Ilekudinol B on bone marrow-derived macrophage. MTT assay results were determined by measuring OD values at 570 nm. Data represent the mean of triplicate samples. Silibinin was used as a positive control (10 μg/ml) described in "materials and methods".

(C4/C23) contributes to increase the inhibitory effect of triterpenoids on osteoclast differentiation without cytotoxic effect. These triterpenoids were further tested for inhibitory effect on RANKL-induced osteoclast differentiation using an *in vitro* assay. The result presented that only Ilekudinol B exhibited considerably inhibitory effects on osteoclast differentiation. As depicted in Figure 2, Ilekudinol B showed a very weak cytotoxic activity against osteoclast cells at the concentration of 20 μg/ml and no cytotoxicity at 10 and 5 μg/ml, indicating that this compound inhibited only the osteoclast differentiation. Consistent with previous data (Lee et al., 2006; Kim et al., 2007), RANKL induced formation of TRAP-positive multinucleated osteoclasts ($TRAP^+$ MNCs) (Figure 3B). Figures 3A and B, displays the strong suppressive effect of Ilekudinol B on osteoclast formation in bone marrow-derived macrophage in a dose-dependent manner with an IC_{50} value of 8.1 μg/ml (Table 1), comparable to those of silibinin (IC_{50} = 9.2 μg/ml) used as a positive control (Kim et al., 2009). The very weak activity of Ilekudinol A against osteoclast formation suggests that the carboxylic group plays an important role in the inhibitory effect.

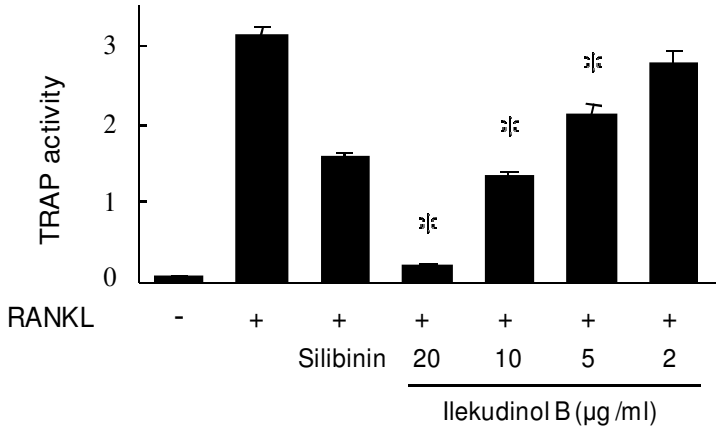
To investigate the effect of Ilekudinol B, we tested whether Ilekudinol B affects the differentiation of osteoblasts. When osteoblasts were cultured for 7 days in the presence of osteogenic agent, the activity of alkaline phosphatase, a marker for osteoblast development, was strongly induced (Figure 4). Alkaline phosphatase activity was not reduced by various concentrations of Ilekudinol B. Together, our results suggested that Ilekudinol B inhibits RANKL-induced osteoclastogenesis from BMNs in a dose-dependent manner, whereas it has no significant effect on osteoblast differentiation.

To investigate the effect of Ilekudinol B on expression of various osteoclastogenesis-associated genes, we performed RT-PCR using BMMs treated with M-CSF and RANKL in the absence or presence of Ilekudinol B. RANKL stimulation increased the expression of NFATc1, which is a key modulator of the late-stage RANKL-induced osteoclastogenesis (Suda et al., 1999; Kim et al., 2005). The induction of NFATc1 gene expression was followed by the expression of TRAP and OSCAR (Kim et al., 2005; Kim et al., 2007). Ilekudinol B inhibited the expression of NFATc1 as well as OSCAR and TRAP, comparable with that of the control (Figure 5). These results suggest that Ilekudinol B can attenuate the key signaling molecules requisite for osteoclast differentiation.

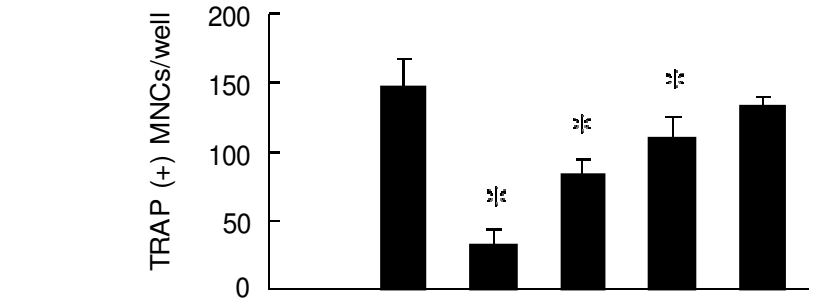
Our results indicate that Ilekudinol B may also act on osteoclast differentiation as an inhibiting agent. This finding suggests that Ilekudinol B may have therapeutic potential for use in the treatment of bone-related disease like postmenopausal osteoporosis and rheumatoid arthritis.

ACKNOWLEDGEMENTS

This study was supported in part by Technology Development Program for Agriculture funded by the Korea Ministry for Food, Agriculture, Forestry and Fisheries (108164-03-2-HD110) and by Basic Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education,Science



A



B

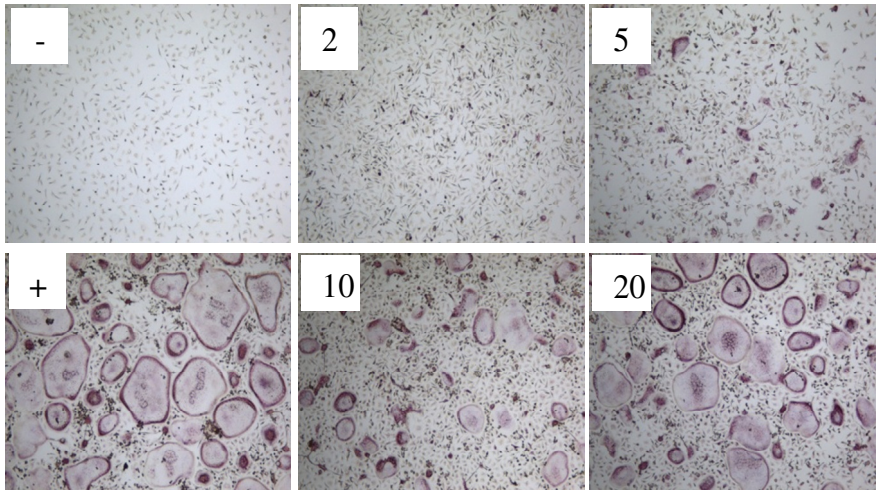


Figure 3. Effect of Ilekudinol B on osteoclast differentiation. (A) BMMs were cultured for 3 days with M-CSF in the absence (-) or presence (+) of RANKL with increasing concentration (2-20 μg/ml) of Ilekudinol B as indicated. Optical density (OD) values for the TRAP activity of osteoclasts were determined at 405 nm. (B) Numbers of TRAP-positive multinucleated cells were counted. Data represent the mean of triplicate samples. * $p < 0.05$. Silibinin was used as a positive control (10 μg/ml) described in "materials and methods".

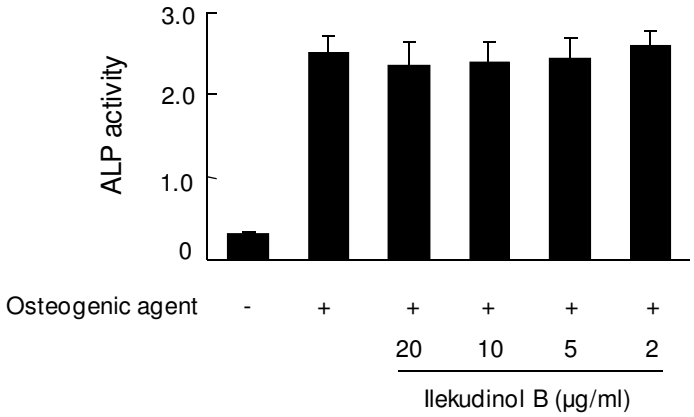


Figure 4. The effects of Ilekudinol B on osteoblast differentiation. Primary calvarial osteoblasts were cultured with osteogenic agent (ascorbic acid and βglycerophosphate) in the presence of various concentration of Ilekudinol B. After 7 days of culture, alkaline phosphatase activity was measured at 570 nm. Data represent the mean of triplicate samples.

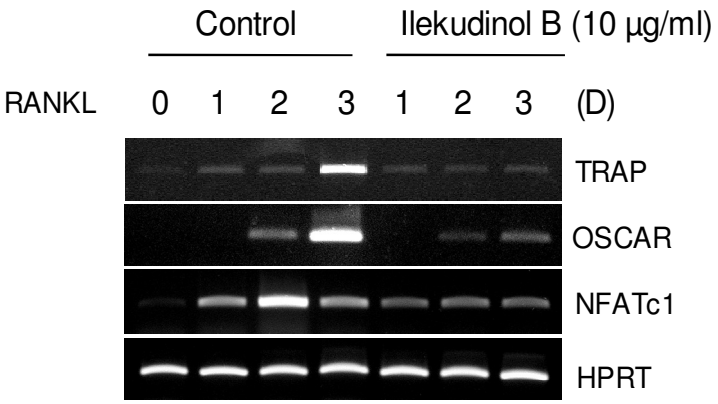


Figure 5. The effects of Ilekudinol B on gene expression. Total RNA was collected from each time point and analyzed by RT-PCR to assess the expression of the indicated genes.

and Technology (2010-0004620).

REFERENCES

Boyle WJ, Simonet WS, Lacey DL (2003). Osteoclast differentiation and activation. Nature, 423: 337-342.

Del, Fattore A, Teti A, Rucci, N (2008). Osteoclast receptors and signaling. Arch. Biochem. Biophys., 473: 147-160.

Han KY, Yang D, Chang EJ, Lee Y, Huang H, Sung SH, Lee ZH, Kim YC, Kim HH (2007). Inhibition of osteoclast differentiation and bone resorption by sauchinone. Biochem Pharmacol., 74: 911-923.

Kim HH, Kim JH, Kwak HB, Huang H, Han SH, Ha H, Lee SW, Woo ER, Lee ZH (2004). Inhibition of osteoclast differentiation and bone resorption by tanshinone IIA isolated from Salvia miltiorrhiza. Biochem Pharmacol., 6: 1647-1656.

Kim JH, Kim K, Jin HM, Song I, Youn BU, Lee J, Kim N (2009). Silibinin inhibits osteoclast differentiation mediated by TNF family members. Mol Cells., 28: 201-207.

Kim K, Kim JH, Lee J, Jin HM, Kook H, Kim KK, Lee SY, Kim N (2007). MafB negatively regulates RANKL-mediated osteoclast differentiation. Blood. 109: 3253-3259.

Kim K, Kim JH, Lee J, Jin HM, Lee SH, Fisher DE, Kook H, Kim KK, Choi Y, Kim N (2005). Nuclear factor of activated T cells c1 induces osteoclast-associated receptor gene expression during tumor necrosis factor-related activation-induced cytokine-mediated osteoclastogenesis. J. Biol. Chem., 280: 35209-53216.

Kwak HB, Lee SW, Lee DG, Hahm KS, Kim KK, Kim HH, Lee ZH (2003). A hybrid peptide derived from cecropin-A and magainin-2 inhibits osteoclast differentiation. Life Sci., 73: 993-1005.

Lee J, Kim K, Kim JH, Jin HM, Choi HK, Lee SH, Kook H, Kim KK, Yokota Y, Lee SY, Choi Y, Kim N (2006). Id helix-loop-helix proteins negatively regulate TRANCE-mediated osteoclast differentiation. Blood, 107: 2686-2693.

Lee JW, Kim JH, Kim K, Jin HM, Lee KB, Chung DJ, Kim N (2007). Ribavirin enhances osteoclast formation through osteoblasts via up-regulation of TRANCE/RANKL. Mol. Cell. Biochem., 296: 17-24.

Lorenzo J, Horowitz M, Choi, Y (2008). Osteoimmunology: interactions of the bone and immune system. Endocr. Rev., 29: 403-440.

Maran A, Gorny G, Oursler MJ, Zhang M, Shogren KL, Yaszemski MJ, Turner RT (2006). 2-Methyoxyestradiol inhibits differentiation and iscytotoxic to osteoclast. J. Cell. Biochem., 99: 425-434.
Roodman GD (2004). Mechanisms of bone metastasis. N. Engl. J. Med., 350: 1655-1664.
Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999). Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr. Rev., 20: 345-357.
Teitelbaum SL (2000). Bone resorption by osteoclasts. Sci., 289: 1504-1508.
Thuong PT, Min BS, Jin WY, Na MK, Lee JP, Seong RS, Lee YM, Song K, Seong Y, Lee HK, Bae K, Kang SS (2006). Anti-complementary activity of ursane-type triterpenoids from *Weigela subsessilis*. Biol. Pharm. Bull., 29: 830-833.
Thuong PT, Na M, Su ND, Seong RS, Lee YM, Sok DE, Bae K (2005). Inhibitory effect of coumarins from *Weigela subsessilis* on low density lipoprotein oxidation. Biol. Pharm. Bull., 28: 1095-1097.