The novel isoflavone derivatives inhibit RANKL-induced osteoclast formation

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ABSTRACT

Isoflavones are compounds structurally similar to the mammalian estrogens and have received considerable attention for their preventive actions on bone loss. Here, we synthesized the novel isoflavone derivatives and examined their activities in bone cells. We found that the novel isoflavone derivatives markedly inhibited the receptor activator of nuclear factor kappa B ligand (RANKL) plus macrophage colony stimulating factor (M-CSF)-induced osteoclastic differentiation from bone marrow stromal cells and RAW264.7 macrophage cells. Treatment of RAW264.7 macrophages with RANKL induced extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) phosphorylation. However, RANKL-induced p38 and JNK but not ERK phosphorylation was attenuated by isoflavone derivatives. Furthermore, RANKL-mediated increase of p65 phosphorylation at Ser^{536}, NF-κB-specific DNA-protein complex formation and κB-luciferase activity was inhibited by isoflavone derivatives. On the other hand, isoflavone derivatives did not affect the cell proliferation and differentiation of human cultured osteoblasts. Our data suggest that the novel isoflavone derivatives inhibit osteoclastogenesis from bone marrow stromal cells and macrophage cells via attenuation of RANKL-induced p38, JNK and NF-κB activation.

Key Words: Isoflavones; Osteoclast; Osteoporosis; RANKL; Chinese Herb

1. INTRODUCTION

Bone undergoes renewal and repair termed "bone remodeling" through the intimate interaction between osteoclasts, which resorb bone, and osteoblasts, which form new bone (Goltzman, 2002). Osteoporosis ensues from an imbalanced bone resorption and bone formation causing a net bone loss that may come from hormonal imbalance, diseases, or medications (e.g. corticosteroids or anti-epileptic agents) (Goltzman, 2002). Current medications for treatment of osteoporosis include bisphosphonates, calcitonin and estrogen. These medications act mainly as anti-resorption agents and maintain bone mass through inhibiting the activities of osteoclasts (Rodan and Martin, 2000).

Osteoclasts are multinucleated cells originating from the fusion of mononuclear progenitors of the monocyte/macrophage family (Teitelbaum, 2000). The maturation of macrophages into osteoclasts in vitro requires the presence of stromal cells or their osteoblast progeny (Udagawa et al., 1990). Extensive research in the last few years has indicated that these cells express M-CSF its receptor for activation of NF-κB (RANK) ligand (RANKL) that are essential for osteoclastogenesis (Yao et al., 2003). Besides M-CSF and RANKL, several inflammatory cytokines and osteotropic agents including tumor necrosis factor (TNF) and interleukin (IL)-1β have also been implicated in the osteoclastogenesis. They act most likely through the osteoblastic modulation of RANKL, its decoy receptor osteoprotegerin, and M-CSF (Yao et al., 1998).

RANKL, a member of the TNF superfamily (Darnay et al., 1999), interacts with the cell surface receptor RANK and in turn recruits TNFR associated factors (TRAF) 1, 2, 3, 5, and 6 (Darnay et al., 1998). Receptor deletion analysis has shown that sequential recruitment of TRAF6 and NF-κB-inducing kinase by RANK leads to the activation of NF-κB, and the recruitment of TRAF2 leads to c-Jun N-terminal kinase (JNK) activation (Darnay and Aggarwal, 1999; Lee et al., 1997). RANKL is also known to activate NF-κB, JNK, p38 and p44/p42 mitogen-activated protein kinase (Darnay and Aggarwal, 1999; Lee et al., 1997), how this cytokine mediates osteoclastogenesis is not fully understood.

Thus agents that can suppress RANKL signaling can suppress osteoclastogenesis-induced bone loss.

Isoflavones are compounds structurally similar to the mammalian estrogens that are found in plant foods, particularly soybeans. They have received considerable attention for their preventive actions on bone loss (Hertrampf et al., 2007). Genistein (4',5,7-trihydroxyisoflavone) is the major isoflavones found in soybeans (Szkudelska and Nogowski, 2007). The potential effects of genistein on bone biology have been extensively studied and it has been found to mainly act as a selective estrogen receptor modulators *in vivo* and *in vitro* to exert beneficial effect on bones (Chen et al., 2003; Uesugi et al., 2001). Here we synthesized the novel isoflavone derivatives and examined their activities in bone cells. We found that the novel isoflavone derivatives decreased the osteoclastogenesis from bone marrow stromal cells and macrophage cells. Additionally, isoflavone derivatives inhibited the RANKL-induced p38, JNK and NF-κB activation in macrophages. In contrast, isoflavone derivatives did not affect the proliferation and differentiation of human osteoblasts. Thus our data suggest that the novel isoflavone derivatives may benefit bone health by decreaseing bone loss through inhibiting the osteoclasts.

2.MATERIALS AND METHODS

2.1 Materials

The isoflavone derivatives (Fig. 1; chemical purity > 95%) were synthesized at the Graduate Institute of Pharmaceutical Chemistry, School of Pharmacy, China Medical University, Taichung, Taiwan (The detail synthesis procedures was provided in Supplemental data). Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for phospho-p38, phospho-ERK, phospho-JNK, p38,

ERK2, JNK and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA, USA). Recombinant human M-CSF, RANKL and BMP-2 were purchased from R&D Systems (Minneapolis, MN, USA). Osteocalcin ELISA kit was purchased from Biosource Technology (Nivelles, Belgium); Osteopontin ELISA kit was purchased from Assay Designs Inc. (Ann Arbor, MI, USA). Alendronate was purchased from Calbiochem (San Diego, CA, USA). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, WI, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cell culture

Bone marrow cells were prepared by flushing out the bone marrow cavity of femurs of 6-8 week-old Sprague-Dawley rats with α-MEM which was supplemented with 20 mM HEPES and 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The non-adherent cells (hematopoietic cells) were collected after 24 h and used as osteoclast precursors. Cells were seeded at a density of 1×10^6 cells/well in 24-well plates in the presence of human recombinant soluble RANKL (50 ng/ml) and M-CSF (20 ng/ml) for 7 days. The culture medium was replaced every 3 days.

Murine RAW264.7 cells (a mouse macrophage cell line obtained from American Type Culture Collection) were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For differentiation of osteoclasts, RAW264.7 cells (2×10^4, in 24-well plate) were cultured in the presence of RANKL (50 ng/ml) and M-CSF (20 ng/ml) for 5 days. The culture medium was replaced every 3 days. Osteoclast formation was measured by quantifying cells that were positively stained by tartrate-resistant acid phosphatase [TRAP (Acid Phosphatase Kit 387-A; Sigma-Aldrich, St. Louis, MO, USA)] according to the manufacture's instructions. Osteoclasts were identified as TRAP-positive stained multinuclear (>3 nuclei) cells using light microscopy. The total

number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted (Tang et al., 2007a).

The human osteoblast-like cell line MG-63 (CRL-1427) was purchased from American Type Culture Collection. Cells were cultured in α-MEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The conditionally immortalized human fetal osteoblastic cell line (hOB; CRL-11372) was purchased from American Type Culture Collection and maintained in a 1:1 mixture of DMEM/Ham's F-12 medium containing 10% FBS supplemented with Geneticin (300 µg/ml) and antibiotics at 37°C (Tang et al., 2009).

2.3 Assay of resorbing activity of osteoclast

Mature osteoclasts were isolated as described previously (Tang et al., 2009). Long bones were isolated from 6-day-old rabbits (body weight, 70–90 g). After removal of muscle and cartilage, the bones were minced in α-MEM. Cells were dissociated from bone fragments by gentle vortexing, then bone fragmentswere allowed to settle under normal gravity. The supernatant was removed and saved, and the mincing and sedimentation were repeated three more times. The supernatants were pooled and centrifuged for 5 min at 60 ×g. The cells were plated in an osteoclast activity assay substrate (OAAS) plate, after overnight incubation, the adherent cells were washed three times with PBS and then treated with trypsin/EDTA for 10 min at 37 °C to remove contaminating cells. Additionally, treatment with 0.1% collagenase for 5 min at room temperature was performed to remove most of the stromal cells. The highly enriched osteoclasts (>90%) were washed three times in PBS.

The mature osteoclasts were plated into a calcium phosphate apatite-coated OAAS plate. After 2-days' cultures, the remaining cells on the plate were lysed using 1 N NaOH. Five images per well were obtained using inverted microscope (200x), and the resorbed area was measured using an image analyzer.

2.4 Quantitative real time PCR

The method of RT-PCR was prepared as described previously (Tang et al., 2007a; Tang et al., 2009). Total RNA was extracted from macrophage using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 µg of total RNA that was reversely transcribed into complementary DNA using oligo(dT) primer. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA, USA). One hundred ng of total cDNA were added per 25-µl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially [β-actin was used as internal control (Applied Biosystems, CA, USA)]. qPCR assays were carried out in triplicate with a StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification in order to calculate the cycle number at which the transcript was detected (denoted C_T).

2.5 Flow cytometry

Differentiated and undifferentiated cells were harvested (1% collagenase for 5 min) and washed twice with FACS washing buffer (1% FCS and 0.1% NaN_3 in PBS), followed by incubation with monoclonal anti-CD51/61-FITC antibody at 4°C for 20 min. After cells were washed three more times with FACS washing buffer, the fluorescence of cells was analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) (Tang et al., 2009; Tang et al., 2007b).

2.6 Western blot analysis

The cellular lysates were prepared as described previously (Tsai et al., 2008).

Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-mouse antibodies against p65, p-p65, p38, p-p38, p-ERK, p-JNK, ERK or JNK (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.7 Transfection and reporter gene assay

Cells were co-transfected with 0.8 μg κB-luciferase plasmid, 0.4 μg β-galactosidase expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. After 24 h transfection, the cells were incubated with the indicated agents. Cell extracts were then prepared, and luciferase and β-galactosidase activities were measured.

2.8 Assay of cell viability and proliferation

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After treatment with isoflavone derivatives for 7 days, cultures were washed with PBS. MTT (0.5 mg/ml) was then added to each well and the mixture was incubated for 2 h at 37°C. Culture medium was then replaced with equal volume of DMSO to dissolve formazan crystals. After shaking at room temperature for 10 min, absorbance of each well was determined at 550 nm using a microplate reader (Bio-Tek, Winooski, VT). Cell proliferation was determined by BrdU assay. Osteoblasts (2×10^4 cells/well) were seeded on 24-well plates. Cells were then incubated in serum-free medium for 24 h before the

addition of isoflavone derivatives. After incubation with isoflavone derivatives for 48 h, BrdU incorporation was assayed according to the protocol of enzyme-linked immunosorbent assay chemiluminescence detection kit (Roche Molecular Biochemicals, New Jersey, USA).

2.9 Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed by using EMSA 'gel shift' kit (Panomics, Redwood City, CA) according to the manufacturer's protocol. Oligonucleotide corresponding to NF-κB-binding sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') was used (Liu et al., 2006). Nuclear extract (3 μg) of cells was incubated with poly d(I-C) at room temperature for 5 min. The nuclear extract was then incubated with biotin-labeled probes at room temperature for 30 min. After electrophoresis on a 6% polyacrylamide gel, the samples on gel were transferred onto a presoaked Immobilon-Nyt membrane (Millipore, Billerica, MA). The membrane was baked at 80°C for 1 h, cross-linked in an oven for 3 min then developed by adding the blocking buffer and streptavidin–horseradish peroxidase conjugate and subjected to western blot analysis

2.10 Measurement of alkaline phosphatase (ALP) activity, collagen content, osteocalcin and osteopontin

Osteoblasts cultured in 24-well plates in the presence or absence of isoflavone derivatives were harvested in 0.2% Nonidet P-40 and the cell suspension was disrupted by sonication. After centrifugation at 1500 × g for 5 min, ALP activity in the supernatant was measured as previously described (Tang et al., 2007a; Tang et al., 2009).

Collagen content was determined by measuring the levels of 4-hydroxyproline in osteoblasts. Briefly, cells cultured in α-MEM for 3 days with or without isoflavone derivatives were hydrolyzed in 6 N HCl for 16 h at 116°C. After lyophilization and

reconstitution of the lysate in distilled water, the amount of 4-hydroxyproline was determined by spectrophotometry at 550 nm.

Cells were treated with various concentrations of isoflavone derivatives for 3 days. The culture medium was then collected for measurement of osteocalcin and osteopontin using osteocalcin and osteopontin ELISA kits according to the manufacture's instruction (Wu et al., 2008).

2.11 Statistics

The values given are means ± S.E.M. The significance of difference between the experimental groups and control was assessed by one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test. The difference was considered to be significant if the P value was <0.05.

3. RESULTS

3.1 The isoflavone derivatives inhibited osteoclastogenesis from bone marrow stromal cells and macrophages

Osteoclasts are specialized monocyte/macrophage family members that differentiate from bone marrow hematopoietic precursors. Osteoclast precursors cultured in the presence of RANKL (50 ng/ml) and M-CSF (20 ng/ml) for 7 days formed of large mature osteoclasts with multi-nuclei characterized by the acquisition of mature phenotypic markers, such as TRAP (Fig 2A; left panel). We next examined whether the novel isoflavone derivatives could reduce RANKL-induced osteoclastogenesis. The novel isoflavone derivatives all inhibited the differentiation of osteoclast in a dose-dependent manner [Alendronate (10 nM) was used for comparison] (Fig. 2A; Table 1). The stimulating effect on osteoclast differentiation was also observed in murine RAW264.7

macrophages, where RANKL (50 ng/ml) and M-CSF (20 ng/ml) were able to induce the osteoclast formation. Cultured for 5 days in RAW264.7 cells, isoflavone derivatives dose-dependently inhibited the formation of TRAP-positive cells (Table 1). Furthermore, compounds 1 and 5 are more effective in reducing osteoclast formation than compound 2, 3, 4 and 6 (Table 1). In addition, treatment of bone marrow stromal cells or RAW264.7 macrophages for 7 days with isoflavone derivatives (3 or 10 μM) did not affect the cell viability, which was assessed by MTT assay [Taxol (100 nM) was used for positive control] (Table 2). As for the functional characterization, we examined whether CD51/61 (vitronectin receptor), TRAP and MMP-9 were expressed in the differentiated osteoclasts. These three molecules have been widely used as specific markers of osteoclasts (Tucker et al., 1999). The undifferentiated RAW264.7 macrophages expressed lower levels of CD51/61 protein (Fig. 2B). On the other hand, incubation of with cells with RANKL and M-CSF for 5 days increased the expression of CD51/61 (Fig. 2B). In the meantime, the mRNA levels of MMP-9 and TRAP, which were not present in undifferentiated RAW264.7 macrophages, were induced by RANKL and M-CSF, as analyzed by RT-PCR (Fig. 2C). Treatment with isoflavone derivatives also inhibited the expression of these markers in RAW264.7 macrophages (Fig. 2B&C). These data suggest that the novel isoflavone derivatives inhibited osteoclastogenesis from bone marrow stromal cells and murine macrophages. To directly evaluate the effect of isoflavone derivatives on the resorption activity of osteoclasts, mature osteoclasts were isolated from long bones of rabbit and cultured on an OAAS plate. Treatment with isoflavone derivatives for 2 days significantly decreased the resorption activity of mature osteoclasts (Fig. 2D).

3.2 Isoflavone derivatives inhibited the RANKL-induced p38 and JNK activation

Three members of the mitogen-activated protein kinase family (MAPK)s, ERK, p38 and JNK have been implicated in the mediation of RANKL-regulated osteoclastogenesis

(Miyazaki et al., 2000). To elucidate the potential signaling pathways of isoflavone derivatives, we examined the activation of MAPKs in RAW264.7 cells treated with RANKL by immunoblotting. Stimulation by RANKL (50 ng/ml) markedly induced the phoshorylation of ERK, p38 and JNK (Fig. 3A). RANKL-induced increase in p38 and JNK but not ERK phosphorylation were inhibited by the pretreatment of cells for 30 min with compound 1 and 5 (Fig. 3B). Therefore, isoflavone derivatives reduced RANKL-induced osteoclast formation through p38 and JNK pathways.

3.3 Isoflavone derivatives inhibited RANKL-induced NF-κB activation

In addition to MAPKs, activation of transcription factor NF-κB is also involved in osteoclast differentiation (Yasuda et al., 1998). Previous studies showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation (Madrid et al., 2001), so the antibody specific against phosphorylated p65 Ser^{536} was used to examine p65 phosphorylation. Treatment of cells with RANKL for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 4A). Pretreatment of cells with compound 1 and 5 attenuated the RANKL-induced p65 phosphorylation (Fig. 4B). Stimulation of RAW264.7 cells with RANKL increased NF-κB DNA binding activity, as revealed by EMSA analysis (Fig. 4C). Similarly, the RANKL-induced increase in NF-κB-specific DNA-protein complex formation was inhibited by compound 1 and 5 (Fig. 4C). To further confirm that the NF-κB activation may be involved in the action of isoflavone derivatives, transient transfection was performed using the κB promoter-luciferase constructs. RAW264.7 cells incubated in the presence of RANKL exhibited increased κB promoter activity (Fig. 4D). The increase of κB promoter activity by RANKL was antagonized by compound 1 and 5 (Fig. 4D). However, compound 1 and 5 did not affect RANKL-induced AP-1 luciferase activity (Fig. 4E). These results suggest that p38, JNK and NF-κB signaling pathways are involved in isoflavone derivatives-reduced osteoclastogenesis from macrophages.

3.4 Isoflavone derivatives did not affect the differentiation and maturation of cultured osteoblasts

We next determined the effect of isoflavone derivatives on the cell viability and proliferation of MG-63 and human osteoblast cells (hFOB) by MTT and BrdU assay. Isoflavone derivatives did not exhibit any significant effect on cell growth at the concentrations used (10 µM) after 7 days of treatment in either cell line (data not shown). Differentiated osteoblasts exhibit and elevated ALP activities that are correlated with high levels of enzyme expression (Tang et al., 2007a). We then investigated the effects of isoflavone derivatives on the ALP activity of osteoblasts. Treatment of osteoblasts with BMP-2 (10 ng/ml) but not isoflavone derivatives (10 µM) for 7 days significantly increased ALP activity (Fig. 5A). The effect of isoflavone derivatives on the terminal differentiation of osteoblast cells was also assessed by determining the synthesis of collagens, osteocalcin and osteopontin. Treatment of MG-63 and hFOB cells with isoflavone derivatives did not affect the expression of collagen, osteocalcin and osteopontin after 7 days (Fig. 5B-D; BMP-2-induced collagen, osteoclacin and osteopontin was used for positive control).

4. DISCUSSION

Phyto-estrogens are polyphenolic compounds found in plants to varying degrees. Soy proteins (Arjmandi et al., 1996; Bahr et al., 2005) and individual isoflavones, such as coumestrol, zearalanol, genistein and daidzein (Draper et al., 1997; Picherit et al., 2000), all attenuate bone loss in ovariectomized rats and mice. Here we synthesized the novel isoflavone derivatives and examined the activities in bone cells. We found that the novel isoflavone derivatives decreased the osteoclastogenesis from bone marrow stromal cells

and macrophage cells. Additionally, isoflavone derivatives inhibited the RANKL-induced p38, JNK and NF-κB activation in macrophages. Therefore, our data suggest that the novel isoflavone derivatives may benefit bone health by decrease bone loss through inhibiting the formation of osteoclasts.

To evaluate the effects of isoflavone derivatives on the formation of osteoclasts, we used a bone marrow culture system from healthy rats and murine macrophages to generate osteoclasts with in vitro RANKL/M-CSF stimulation. To the best of our knowledge, this is the first study to demonstrate that the novel isoflavone derivatives can inhibit the formation of osteoclasts. To simply examine the effect of isoflavone derivatives on resorption activity of osteoclasts, the mature osteoclasts were isolated from long bones of rabbit. Treatment with isoflavone derivatives decreased the resorption activity of mature osteoclasts. Our results suggest that isoflavone derivatives are able to suppress osteoclastic differentiation as well as the resorption activity of mature osteoclasts. We further demonstrated the suppression effects on the expression of MMP-9 and TRAP in differentiated osteoclast. Whether the other signaling pathways, e.g. Cathepsin K (Nishida and Gotoh, 1993), are involved in isoflavone derivatives-inhibited bone resorption needs further investigation. In this study, we found that compound 1 and 5 were more effective in reducing RANKL-induced osteoclastogenesis than other compounds. We presume that the structure of compound 1 and 5 enable them to interfere with the interaction of RANKL and RANK. However, this hypothesis needs further examination.

MAPK family members are proline-directed serine/threonine kinases that play important roles in cell growth, differentiation, and apoptosis (Brown and Sacks, 2009). Some external stimuli activate the phosphorylation of threonine and tyrosine (Berg et al., 2003; Ducy et al., 2000). MAPK family members are classified into the ERK, p38 and JNK groups, and it is widely accepted that peptide growth factors and phorbol esters preferentially activate ERK (Ducy et al., 2000). Furthermore, some studies showed that

RANKL–RANK binding causes the phosphorylation of ERK, p38 and JNK, and that such phosphorylation leads to osteoclast differentiation (Berg et al., 2003; Ducy et al., 2000; Yasuda et al., 1998). In the present study, we used a homogenous clonal population of murine monocytic RAW 264.7 cells to clarify the effects of isoflavone derivatives on the signaling pathways in osteoclast progenitor cells. We found that isoflavone derivatives strongly inhibited the phosphorylation of p38 and JNK but not ERK in RAW 264.7 cells stimulated with RANKL. These findings suggest that the inhibitory effect of isoflavone derivatives on the differentiation of osteoclast into mature osteoclasts may be responsible for the regulation of phosphorylation of p38 and JNK. Further, when mouse bone marrow stromal cells were cultured for 7 days in the presence of both RANKL and M-CSF, isoflavone derivatives significantly reduced osteoclast formation. Based on our findings, we speculated that this phenomenon was caused by the inhibitory effect of isoflavone derivatives on RANK binding to RANKL and subsequent phosphorylation of p38 and JNK signal transduction.

NF-κB plays a critical role in the regulation of the cell cycle, cell adhesion, cytokine production, apoptosis, and other important cellular processes in macrophages. Osteoclast formation and functions are mediated by RANKL-induced NF-κB activation (Soysa and Alles, 2009). Therefore, the inhibition of NF-κB activity might be an effective approach to target osteoclast activity as well as treatment of osteoporosis. In this study, we demonstrated that compound 1 and 5 inhibited RANKL-induced NF-κB activation in RAW 264.7 cells by inhibiting the phosphorylation of p65 and NF-κB-specific DNA-protein complex formation. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that RANKL-increased NF-κB activity was inhibited by compound 1 and 5. These data support the hypothesis that isoflavone derivatives inhibit NF-κB activity in macrophages, resulting in inhibition of osteoclast formation, and resorption activity. It has been reported that AP-1 plays important roles in RANKL-induced osteoclastogenesis (Wagner and Eferl, 2005). However, compound 1

and 5 did not affect the RANKL-induced AP-1 luciferase activity in this study. Therefore, whether and what the other signaling pathways are involved need further investigation.

Bone is a complex tissue composed of several cell types which are continuously undergoing a process of renewal and repair. When resorption and formation of bone are not coordinated and bone breakdown overrides bone building, osteoporosis results (Deal, 2009). Current drugs used to treat osteoporosis include bisphosphonates, calcitonin and estrogen. These are all anti-resorption medications, which maintain bone mass by inhibiting the function of osteoclasts (Goltzman, 2002). Their effects in increasing or recovering bone mass is relatively small, certainly no more than 2% per year (Rodan and Martin, 2000). It is desirable, therefore, to have satisfactory bone-building agents, such as teriparatide, that can stimulate new bone formation and correct the imbalance of trabecular microarchitecture that is characteristic of established osteoporosis (Berg et al., 2003). Since new bone formation is primarily a function of the osteoblast, agents that act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts can enhance bone formation (Berg et al., 2003; Deal, 2009). Here we found that isoflavone derivatives did not affect the proliferation of two osteoblast cell lines. Compared with BMP-2, isoflavone derivatives also did not affect the differentiation marker including ALP, osteocalcin and osteopontin of osteoblasts. These data confirm the anti-resorption effect but not bone formation activity of isoflavone derivatives.

In conclusion, the present study demonstrated that the novel isoflavone derivatives inhibit the osteoclastogenesis from bone marrow stromal cells and macrophages. In addition, the novel isoflavone derivatives attenuation the RANKL-induced p38, JNK and NF-κB activation. However, isoflavone derivatives did not affect the cell proliferation and differentiation of human osteoblast. Therefore, isoflavone derivatives may benefit bone health by reducing osteoclast formation and activity.

ACKNOWLEDGMENTS

FIGURE LEGENDS

Fig. 1   Structures of isoflavone derivatives.

Fig. 2   Inhibition of osteoclast differentiation by isoflavone derivatives.

Osteoclast precursors isolated from long bones of adult male rats were plated on a 24-well plate at a cell density of 1×10^6 cells/well and cultured in the presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml) for 7 days. Following TRAP staining, the cells with more than 3 nuclei were counted (A). Compared with RANKL plus M-CSF, compound 1 and 5 (10 µM) treatment markedly inhibited the differentiation of osteoclast. RAW264.7 cells were treated with M-CSF (20 ng/ml) and RANKL (50 ng/ml) without or with isoflavone derivatives (10 μM) for 5 days. After incubation, cells were treated with anti-CD51/61-FITC and analyzed by flow cytometry (B). RAW264.7 cells were treated with M-CSF (20 ng/ml) and RANKL (50 ng/ml) without or with isoflavone derivatives (10 μM) for 5 days. After incubation, TRAP and MMP-9 mRNAs were detected by qPCR (C). Mature osteoclasts were treated with isoflavone derivatives (10 μM) for 2 days. Resorbed lacunae on the plates were photographed under an inverted microscope. Total resorption area per well was graphed and measured by image analyzer (D). Results are expressed as the mean ± S.E.M. of four independent experiments. *: $P<0.05$ as compared with control group. #: $P<0.05$ as compared with RANKL-treated control group.

Fig. 3   Isoflavone derivatives inhibited the RANKL-induced p38 and JNK phosphorylation.

RAW264.7 cells were incubated with RANKL (50 ng/ml) for indicated time intervals (A), or pretreated with compound 1 and 5 for 30 min followed by stimulation with RANKL (50 ng/ml) for 60 min (B), and the ERK, p38 and JNK

phosphorylation was determined by immunoblotting using phospho-ERK, p38 and JNK specific antibody, respectively. Note that compound 1 and 5 inhibited the RANKL-induced p38 and JNK but not ERK phosphorylation.

Fig. 4   Isoflavone derivatives inhibit RANKL-induced NF-κB activation.
RAW264.7 cells were incubated with RANKL (50 ng/ml) for indicated time intervals (A), or pretreated with isoflavone derivatives for 30 min followed by stimulation with RANKL (50 ng/ml) for 60 min (B). p65 phosphorylation was determined by immunoblotting using phospho-p65 antibody. (C) RAW264.7 cells were pretreated with isoflavone derivatives for 30 min followed by stimulation with RANKL (50 ng/ml) for 60 min, and NF-κB-specific DNA-protein binding activity was measured by EMSA analysis. (D&E) Cells were transfected with κB or AP-1-luciferase expression vector and then pretreated with isoflavone derivatives (10 μM) for 30 min before incubation with RANKL (50 ng/ml) for 24 h. Luciferase activity was then assayed. Results are expressed as the mean ± S.E.M. (n=4). *: $P<0.05$ as compared with control. #: $P<0.05$ as compared with RANKL-treated control group

Fig. 5   Isoflavone derivatives did not affect the differentiation of osteoblasts.
MG-63 or hFOB cells were treated with isoflavone derivatives (10 μM) or BMP-2 (10 ng/ml) for 7 days. ALP activity was assessed using the commercial ALP kit (A). Cells were treated with isoflavone derivatives (10 μM) or BMP-2 (10 ng/ml) for 7 days. Collagen content was determined by measuring the level of 4-hydroxyproline (B). Cells were treated with isoflavone derivatives (10 μM) or BMP-2 (10 ng/ml) for 7 days. The amount of osteocalcin and osteopontin in culture medium was assessed by osteocalcin and osteopontin ELISA kit (C&D). Note that isoflavone derivatives did not affect the differentiation of osteoblast

cells, whereas BMP-2 stimulated the expression of ALP, type I collagen, osteocalcin and osteopontin by MG-63 or hFOB cells. Data are presented as mean ± S.E.M. (*n=4*).

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