

Anti-Osteoclastogenic Activity of Praeruptorin A via Inhibition of p38/Akt-c-Fos-NFATc1 Signaling and PLC γ -Independent Ca²⁺ Oscillation

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Abstract

Background: A decrease of bone mass is a major risk factor for fracture. Several natural products have traditionally been used as herbal medicines to prevent and/or treat bone disorders including osteoporosis. Praeruptorin A is isolated from the dry root extract of *Peucedanum praeruptorum* Dunn and has several biological activities, but its anti-osteoporotic activity has not been studied yet.

Materials and Methods: The effect of praeruptorin A on the differentiation of bone marrow-derived macrophages into osteoclasts was examined by phenotype assay and confirmed by real-time PCR and immunoblotting. The involvement of NFATc1 in the anti-osteoclastogenic action of praeruptorin A was evaluated by its lentiviral ectopic expression. Intracellular Ca²⁺ levels were also measured.

Results: Praeruptorin A inhibited the RANKL-stimulated osteoclast differentiation accompanied by inhibition of p38 and Akt signaling, which could be the reason for praeruptorin A-downregulated expression levels of c-Fos and NFATc1, transcription factors that regulate osteoclast-specific genes, as well as osteoclast fusion-related molecules. The anti-osteoclastogenic effect of praeruptorin A was rescued by overexpression of NFATc1. Praeruptorin A strongly prevented the RANKL-induced Ca²⁺ oscillation without any changes in the phosphorylation of PLC γ .

Conclusion: Praeruptorin A could exhibit its anti-osteoclastogenic activity by inhibiting p38/Akt-c-Fos-NFATc1 signaling and PLC γ -independent Ca²⁺ oscillation.

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Introduction

Bone fracture is a public health problem because it occurs easily in patients with bone-related disorders including osteoporosis. As the elderly population is rapidly increasing, the medical costs of hospitalization caused by fractures have become a serious social issue [1,2].

Bone homeostasis depends on the balance between osteoblastic bone formation and osteoclastic bone resorption, but an imbalance caused by an increased number of osteoclasts or overactivation can lead to impaired bone structure and low bone mass, which are

common characteristics in patients with bone disorders [3,4]. Therefore, a method to pharmaceutically inhibit osteoclast differentiation is one of the therapeutic strategies for preventing and/or treating bone disorders and related fractures [5].

Clinically, osteoclast-targeting bisphosphonates have been widely used to treat patients with osteoporosis and/or prevent osteoporotic fracture. The orally available bisphosphonates principally inhibit the activation of osteoclasts by binding to hydroxyapatite [6], but recently bisphosphonate-related side effects including acute phase response, hypocalcaemia, secondary

hyperparathyroidism, upper gastrointestinal tract problems, musculoskeletal pain, renal toxicity and osteonecrosis of the jaw have been reported [7]. Therefore, there have been several basic and clinical efforts to find anti-osteoporotic phytochemicals in order to minimize adverse side effects [8–10].

Praeruptorin A (Fig. 1A) is isolated from the dry root extract of *Peucedanum praeruptorum* Dunn, which has been used as an herbal medicine with several pharmacological activities [11–14]. Recently, praeruptorin A has been shown to suppress the lipopolysaccharide (LPS)-induced inflammatory response in RAW264.7 cells [15]. Since LPS is a potent stimulator of both inflammation and bone resorption [16,17] and several phytochemicals exhibit dual anti-inflammatory and anti-resorptive activities [18,19], in this study, the anti-osteoclastogenic activity of praeruptorin A was investigated. The functional involvement of praeruptorin A in osteoclast differentiation is not yet clearly understood.

Materials and Methods

Reagents

Praeruptorin A was purchased from Stanford Chemicals (CA). Mouse soluble receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) were purchased from R&D Systems (MN). Penicillin, streptomycin, cell culture medium, and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (NY). The CCK-8 assay kit was from Dojindo Molecular Technologies (ML). Antibodies against c-Fos, NFATc1 and actin were from Santa Cruz Biotechnology (CA). Antibodies against p-p38, p38, p-JNK, JNK, p-ERK, ERK, Akt, p-Akt (Ser473), PLC γ and p-PLC γ were obtained from Cell Signaling Technology (MA).

Osteoclast Differentiation

This study was carried out in strict accordance with the recommendations in the Standard Protocol for Animal Study of Korea Research Institute of Chemical Technology (KRICT;

Permit No. 2012-7D-02-01). The protocol (ID No. 7D-M1) was approved by the Institutional Animal Care and Use Committee of KRICT (IACUC-KRICT). All efforts were made to minimize suffering. In detail, after cervical dislocation, bone marrow cells were obtained from 5-wk-old male ICR mice (Daejeon Science, Daejeon, Korea) by flushing femurs and tibias with α -MEM supplemented with antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin). Bone marrow cells were cultured for 1 day on a culture dish in α -MEM supplemented with 10% FBS and M-CSF (10 ng/ml). Non-adherent bone marrow cells were plated on a Petri dish and cultured for 3 days in the presence of M-CSF (30 ng/ml). After non-adherent cells were washed out, adherent cells were used as bone marrow-derived macrophages (BMMs). For osteoclastogenesis, BMMs (1×10^4 cells/well in a 96-well plate or 3×10^5 cells/well in a 6-well plate) were cultured in the presence of M-CSF (30 ng/ml) and RANKL (10 ng/ml) for 4 days.

Tartrate-resistant Acid Phosphatase (TRAP) Staining and Activity Assay

Cells were fixed with 3.7% formaldehyde for 5 min, permeabilized with 0.1% Triton X-100 for 5 min, and stained with the Leukocyte Acid Phosphatase Kit 387-A (Sigma-Aldrich, MO). TRAP-positive multinuclear cells with three or more nuclei were counted as osteoclasts. To measure TRAP activity, cells were treated with TRAP buffer (100 mM sodium citrate, pH 5.0, 50 mM sodium tartrate) including 3 mM *p*-nitrophenyl phosphate (Sigma-Aldrich) at 37°C for 5min. Reaction mixtures were transferred into a new plate containing an equal volume of 0.1 N NaOH, and optical density values were determined at 405 nm in a Wallac EnVision microplate reader (PerkinElmer, Finland).

Cytotoxicity Assay

BMMs were plated at a density of 1×10^4 cells/well on a 96-well plate in triplicate. After treatment with M-CSF (30 ng/ml) and praeruptorin A, cells were cultured for 3 days. Then, cell

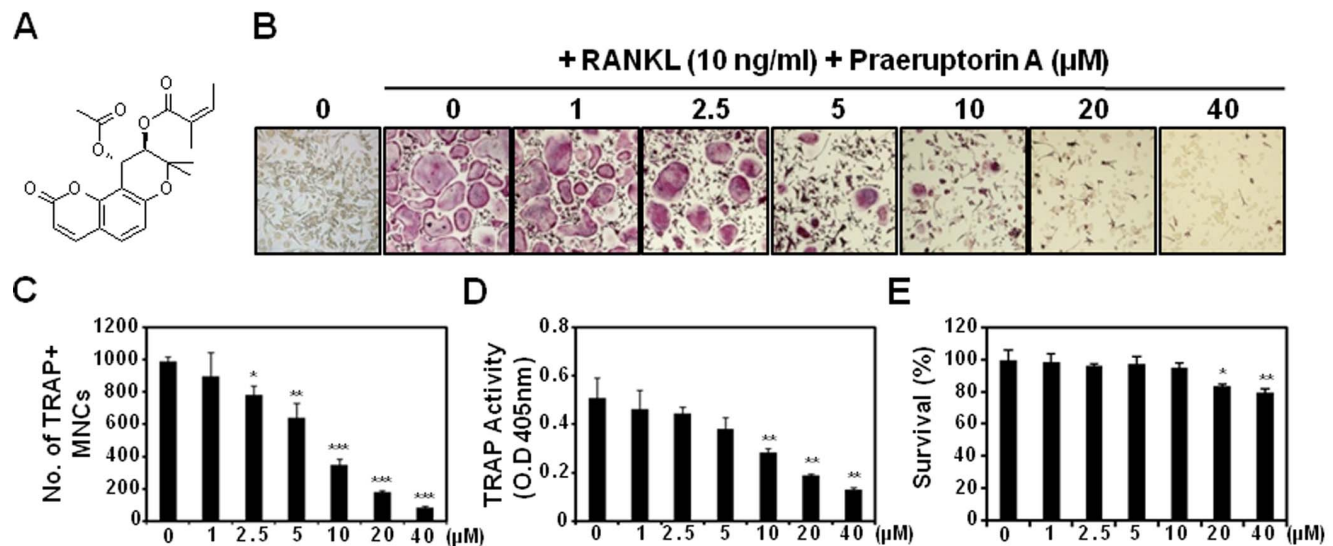


Figure 1. Effect of praeruptorin A on RANKL-induced osteoclast differentiation. (A) Chemical structure of praeruptorin A. (B) BMMs were pretreated with vehicle (0.1% DMSO) or praeruptorin A for 2 h and then incubated with RANKL (10 ng/ml) and M-CSF (30 ng/ml) for 4 days. Multinucleated cells were fixed, permeabilized, and stained with TRAP solution. Mature TRAP-positive multinucleated osteoclasts (MNCs) were photographed under a light microscope. TRAP-positive MNCs (nuclear number >3) were counted (C), and TRAP activity of osteoclasts was measured (D). (E) The effect of praeruptorin A on the viability of BMMs was evaluated by CCK-8 assay. *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$. doi:10.1371/journal.pone.0088974.g001

viability was measured with the CCK-8 kit according to the manufacturer's protocol.

Western Blot Analysis

Western blot analysis was performed as described previously [9,10]. Briefly, cells were washed, lysed, and centrifuged at $10,000 \times g$ for 15 min. After protein quantification of the supernatants by the BCA protein assay (Pierce, IL), proteins were denatured, separated on SDS-PAGE gels, and transferred onto PVDF membranes (Millipore, CA). After incubation with antibody, the membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and visualized with the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan). ImageJ software-based quantification of the detected bands was performed, and the relative, normalized ratio between the density of phosphorylated form and that of the protein itself or actin was presented in each figure.

NFATc1 Luciferase Activity Assay

Luciferase reporter plasmid, NFATc1-luc vector was purchased from Clontech (CA) and pRL-Renilla control vector was purchased from Promega (WI). The full-length human RANK cDNA was amplified from human leukocyte cDNA from Clontech (CA) and cloned into the HindIII-EcoRI site of pcDNA3.1 (Invitrogen). Recombinant human RANKL (hRANKL) was purchased from R&D Systems (CA). For measurement of luciferase activity, human embryonic kidney HEK293T cells were plated at a density of 5×10^4 cells/well on a 48-well plate in triplicate for 1 day. Plasmids containing NFAT-luc (100 ng/well), pcDNA3.1-RANK (100 ng/well), and pRL-Renilla (20 ng/well) were transfected into HEK293T cells with Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's protocol. After 6 h, transfected HEK293T cells were treated with hRANKL (50 ng/mL) and praeruptorin A for 2 days. The luciferase activity was detected in cell extracts with the Dual-Luciferase Reporter Assay System (Promega, WI) and Wallac EnVision microplate reader (PerkinElmer). Luciferase activity assessed in triplicates was normalized to the average renilla luciferase activity.

Real-time PCR

Real-time PCR was performed as described previously [9,10]. Primers were chosen with the online Primer3 design program [20]. The primer sets used in this study are shown in Table 1. Briefly, total RNA was isolated with TRIzol reagent, and the first-strand cDNA was synthesized with the Omniscript RT kit (Qiagen) according to the manufacturer's protocol. SYBR green-based QPCR was performed with the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA). All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta C_T}$ method [21]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard gene. The statistical significance was determined by Student's *t*-test with GAPDH-normalized $2^{-\Delta\Delta C_T}$ values; differences were considered significant at $p < 0.05$.

Retrovirus Preparation and Infection

Retrovirus preparation and infection were conducted as described previously [22]. Briefly, to obtain retroviral particles, pMX-IRES-green fluorescent protein (GFP; the control) or pMX-CA-NFATc1-GFP containing constitutively active (CA)-NFATc1 was transfected into Plat-E cells (Cell Biolabs, Inc., CA) using Lipofectamine 2000 reagent according to the manufacturer's protocol. After viral particles were collected from the culture

Table 1. Primer sequences used in this study.

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
TRAP	GATGACTTTGCCAGTCAGCA	ACATAGCCACACCGTTTCTC
NFATc1	GGGTCAGTGTGACCCGAAGAT	GGAAGTCAGAAGTGGGTGGA
OSCAR	AGGGAAACCTCATCCGTTTG	GAGCCGAAATAAGGCACAG
Cathepsin K	GGCCAACCAAGAAGAAAAC	GTGCTTGCTCCCTTCTGG
DC-STAMP	CCAAGGAGTCGTCATGATT	GGCTGCTTTGATCGTTTCTC
c-Src	CCAGGCTGAGGAGTGGTACT	CAGCTTGCGGATCTTGATG
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

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medium for 48 h, BMMs were incubated with those in the presence of M-CSF (30 ng/ml) and polybrene (10 μ g/mL) for 8 h. For the osteoclast formation assay, BMMs were treated with RANKL (10 ng/ml), M-CSF (30 ng/ml) and praeruptorin A (10 μ M) for 4 days.

Intracellular Ca^{2+} Measurement

Isolated BMMs were seeded at a density of 1×10^5 cells on the cover glass and cultured in the presence of RANKL (10 ng/ml) and M-CSF (30 ng/ml) for 24 h. Cells were incubated with vehicle (DMSO) or praeruptorin A (10 μ M) for 2 h, treated with Fura-2, AM (5 μ M), and then placed in the perfusion chamber to allow continuous perfusion of bath solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4, 310 mOsm) until each experiment was complete. Fluorescent dyes inside the cells were excited at wavelengths of 340 nm and 380 nm sequentially and the emitted fluorescence was passed through 510 nm cut-off filter. Each image was collected with a CCD camera and analyzed with MetaFluor software. All results were digitized to the mean of the ratio (340 nm/380 nm).

Statistical Analysis

All quantitative values are presented as mean \pm SD. Statistical differences were analyzed using Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results

Praeruptorin A Inhibits RANKL-induced Osteoclast Differentiation

The effect of praeruptorin A on RANKL-induced osteoclastogenesis was evaluated in BMMs. Pre-treatment with praeruptorin A before RANKL treatment strongly prevented the RANKL-induced formation of TRAP-positive multinucleated cells in a dose-dependent manner (Fig. 1B and C). Consistent with these results, TRAP activity was also significantly inhibited by praeruptorin A above 10 μ M (Fig. 1D). To clarify the possibility that the anti-osteoclastogenic activity of praeruptorin A could be due to its cytotoxicity in BMMs, we investigated the cytotoxic effect of praeruptorin A in BMMs. As shown in Fig. 1E, praeruptorin A exhibited significant cytotoxicity above 20 μ M, suggesting that anti-osteoclastogenic activity of praeruptorin A without any cytotoxicity could be expected under 10 μ M. Hoechst-stained nuclei supported no difference in cell cytotoxicity or spreading by praeruptorin A at 10 μ M (Fig. S1). Therefore, the cells were treated with 10 μ M of praeruptorin A in subsequent experiments to evaluate its anti-osteoclastogenic mechanism. At 10 μ M,

praeruptorin A also strongly inhibited osteoclast formation by inhibiting cell fusion; when it was added before fusion of preosteoclasts (or treated in the differentiation day 3; Fig. S2), the formation of multinucleated osteoclasts was significantly inhibited.

Praeruptorin A Inhibits RANKL-induced Activation of p38 and Akt

Anti-osteoclastogenic activity of praeruptorin A was confirmed by staining actin ring of mature osteoclasts (Fig. S3). This result was consistent with Fig. 1B. To elucidate the anti-osteoclastogenic mechanism of praeruptorin A, we investigated its effect on the activation of signaling molecules including JNK, p38, ERK, and Akt, which are known to play a role in the early stage of RANKL-induced osteoclast differentiation. After RANKL treatment, all signaling molecules were activated in 5–15 min, but pre-treatment with praeruptorin A 30 min before RANKL treatment attenuated the RANKL-induced phosphorylation of p38 and Akt within 15 min after RANKL treatment (Fig. 2A). The RANKL-induced activation of ERK and JNK were not changed by praeruptorin A.

Praeruptorin A Inhibits RANKL-induced Expression of c-Fos and NFATc1

Additionally, the inhibitory effect of praeruptorin A on the expression of transcription factors such as c-Fos and NFATc1 was evaluated by Western blot analysis. As shown in Fig. 2B, RANKL induced the expression of c-Fos and NFATc1 in the early and late

stages of osteoclastogenesis, respectively. However, those inductions were inhibited by praeruptorin A. In particular, on day 3, the RANKL-induced expression of NFATc1 was strongly blocked by praeruptorin A.

Additionally, the inhibitory effect of praeruptorin A on the activation of NFATc1 was revealed by the NFATc1 luciferase activity assay (Fig. 2C); RANKL significantly induced the transcriptional activity of NFATc1 in HEK293T cells transfected with RANK plasmid and NFATc1 firefly-luciferase reporter plasmid, but the addition of 10 μ M praeruptorin A significantly inhibited the RANKL-induced transcriptional activity of NFATc1.

Praeruptorin A Inhibits RANKL-induced mRNA Expression of Osteoclast-Specific Genes

The anti-osteoclastogenic activity of praeruptorin A was confirmed by evaluating the mRNA expression levels of osteoclast-specific genes. Consistent with the previous data, praeruptorin A significantly attenuated the RANKL-induced mRNA expressions of TRAP and NFATc1 (Fig. 3). Since NFATc1 can regulate the mRNA levels of osteoclast-related molecules including osteoclast-associated receptor (OSCAR), dendrite cell-specific transmembrane protein (DC-STAMP), cathepsin K and c-Src, we further investigated whether praeruptorin A could affect the mRNA expression of these genes during osteoclastogenesis. As expected, the mRNA expressions of these molecules were highly induced by RANKL, but praeruptorin A

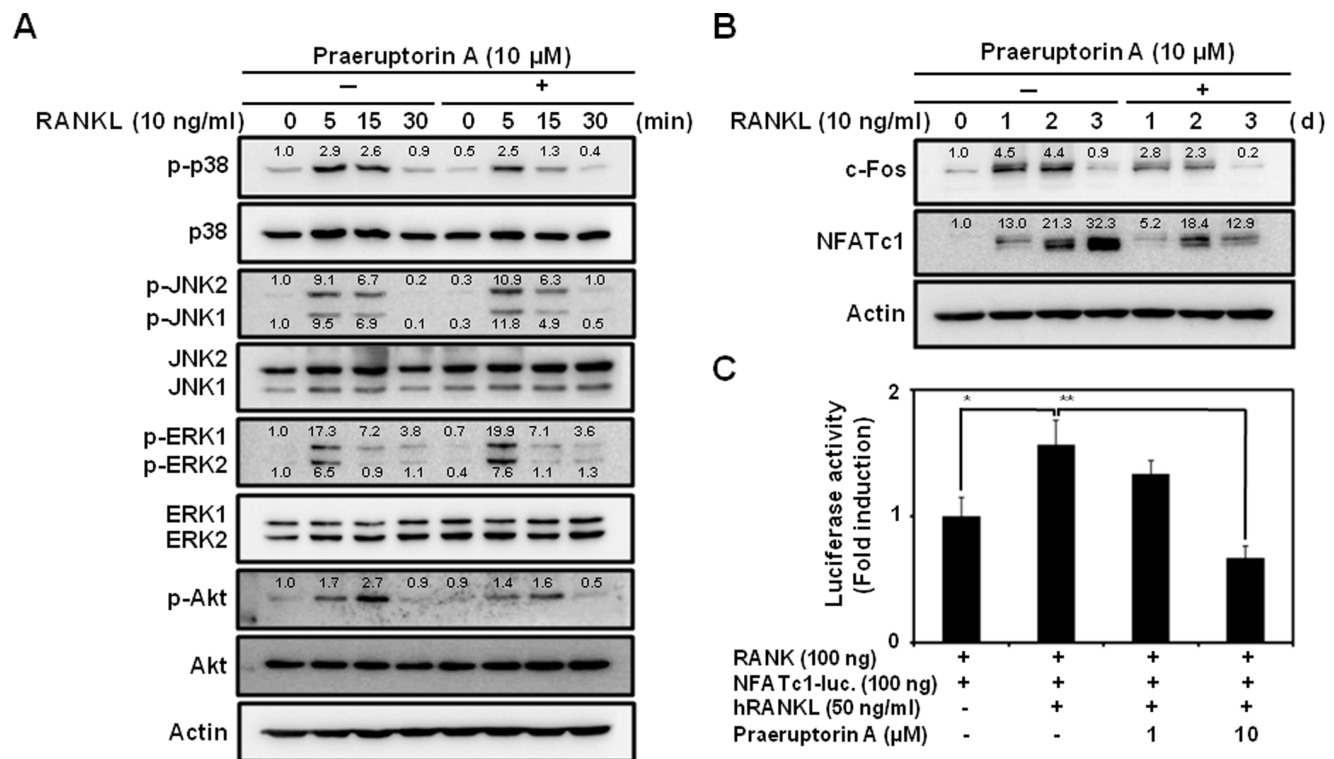


Figure 2. Effect of praeruptorin A on RANKL-induced activation or expression of osteoclast-specific signaling molecules and transcription factors. The effects of praeruptorin A on RANKL-induced phosphorylation of MAP kinases and Akt (A) and expression of transcription factors, c-Fos and NFATc1 (B), were evaluated by Western blot analysis. BMMs were pre-treated with praeruptorin A (10 μ M) 2 h before treatment with RANKL (10 ng/ml) and M-CSF (30 ng/ml). Actin was used as an internal control. Densitometric analysis was performed using ImageJ software and the relative, normalized ratios of p-p38/p38, p-JNKs/JNKs, p-ERKs/ERK, p-Akt/Akt, c-Fos/actin and NFATc1/actin were presented. (C) The effect of praeruptorin A on the transcriptional activity of NFATc1 was evaluated by luciferase activity assay as described in 'Materials and Methods'. *, $P < 0.05$; **, $P < 0.01$.

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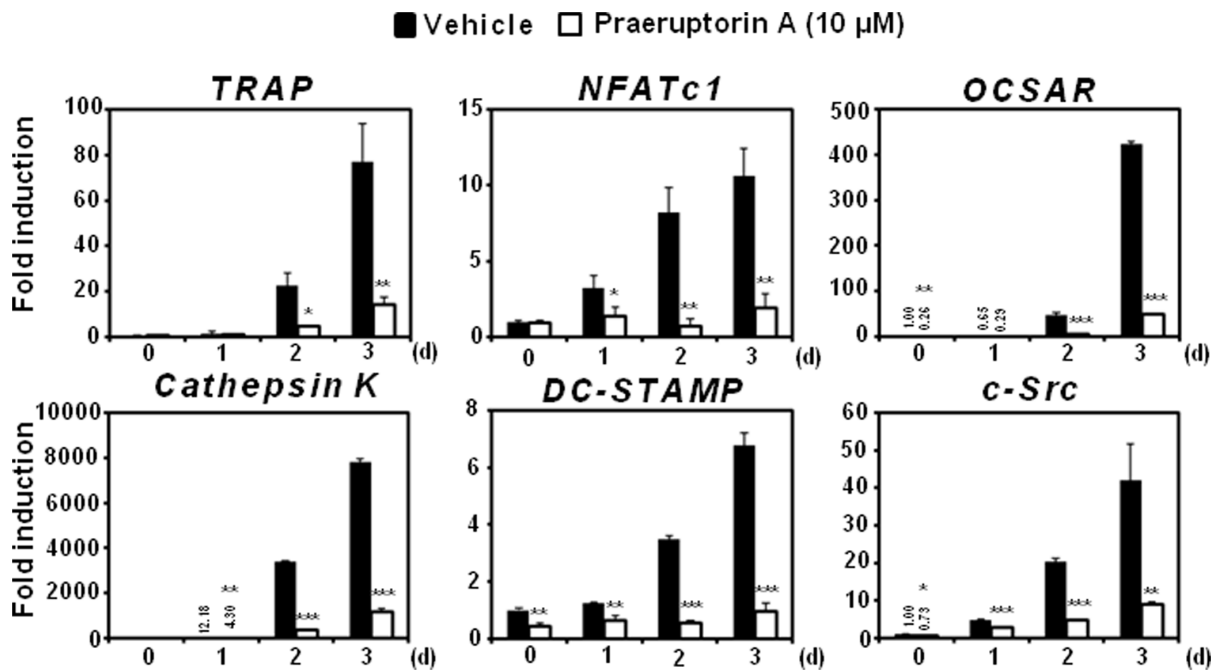


Figure 3. Effect of preruptorin A on RANKL-induced mRNA expressions of osteoclastic-specific genes. BMMs were treated with vehicle (DMSO) or preruptorin A (10 μM) for 2 h and then RANKL (10 ng/ml) was added into cells. The mRNA expression levels of osteoclastic-specific genes were analyzed by real-time PCR. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. doi:10.1371/journal.pone.0088974.g003

dramatically suppressed those inductions in a dose-dependent manner.

Ectopic NFATc1 Rescues Anti-osteoclastogenic Action of Preruptorin A

The anti-osteoclastogenic action of preruptorin A could result from its potential to block p38 and/or Akt signaling pathways that subsequently affect the expression and/or activity of c-Fos and the most distal transcription factor, NFATc1. To clarify this hypothesis, we investigated whether ectopic expression of the constitutively active form of NFATc1 (CA-NFATc1) could rescue the preruptorin A-inhibited formation of TRAP-positive multinucleated osteoclasts. Based on GFP signaling, both the control GFP and CA-NFATc1-GFP plasmid were infected well, and the overexpression of NFATc1 was confirmed by Western blot analysis (Fig. 4D). Consistent with Fig. 1B, the formation of TRAP-positive multinucleated osteoclasts from BMM expressing the control GFP was strongly inhibited by preruptorin A (upper images in Fig. 4A). However, even in the presence of preruptorin A, TRAP-positive multinucleated osteoclasts were derived from BMMs over-expressing NFATc1 (bottom images in Fig. 4A). The ameliorating effect of NFATc1 on the preruptorin A-mediated inhibition of osteoclast differentiation was also confirmed by counting the number of multinucleated osteoclasts, measuring the TRAP activity (Fig. 4B), and evaluating the mRNA expression levels of TRAP, OSCAR, cathepsin K and DC-STAMP (Fig. 4C). Additionally, Western blot analysis revealed that preruptorin A strongly attenuated the Akt activation by the overexpression of activated NFATc1 (Fig. 4D). The overexpression of activated NFATc1 did not induce the phosphorylation of p38.

Preruptorin A Inhibits Ca^{2+} Oscillation without Any Change in PLC γ Phosphorylation

Since several studies have reported the Ca^{2+} channel blocking activity of preruptorin A [23,24], we further evaluated the effect of preruptorin A on the RANKL-induced Ca^{2+} oscillation. As shown in Fig. 5A, when BMMs were treated with M-CSF plus RANKL for 24 h, Ca^{2+} oscillation was triggered, but not with M-CSF alone. However, additional treatment with preruptorin A for 2 h before measuring Ca^{2+} oscillation completely inhibited the RANKL-induced Ca^{2+} oscillation.

We further examined the possibility that preruptorin A could inhibit the RANKL-induced Ca^{2+} oscillation by blocking the RANKL-induced phosphorylation of PLC γ . In BMMs, the RANKL-induced phosphorylation of PLC γ was not changed by preruptorin A.

Discussion

Osteoclasts are functionally essential for sustaining bone health. However, the overactivation of osteoclasts and/or their increased number can lead to diseases characterized to bone loss, which is a risk factor for fracture. In this study, preruptorin A attenuated the RANKL-induced osteoclastogenesis in a dose-dependent manner without any cytotoxicity upto 10 μM. Anti-osteoclastogenic activity of preruptorin A was independent on age, strain and sex of mouse (Fig. S4), and importantly, preruptorin A significantly inhibited the fusion of preosteoclasts (Fig. S2) and the formation of resorptive pits by mature multinucleated osteoclasts (Fig. S5).

RANKL is the most essential cytokine for osteoclast differentiation (or osteoclastogenesis) [25]. The binding of RANKL to RANK, its receptor, triggers the activation of signaling molecules such as MAP kinases, Akt, and phospholipase C γ (PLC γ) that subsequently induce the activation of transcription factors such as

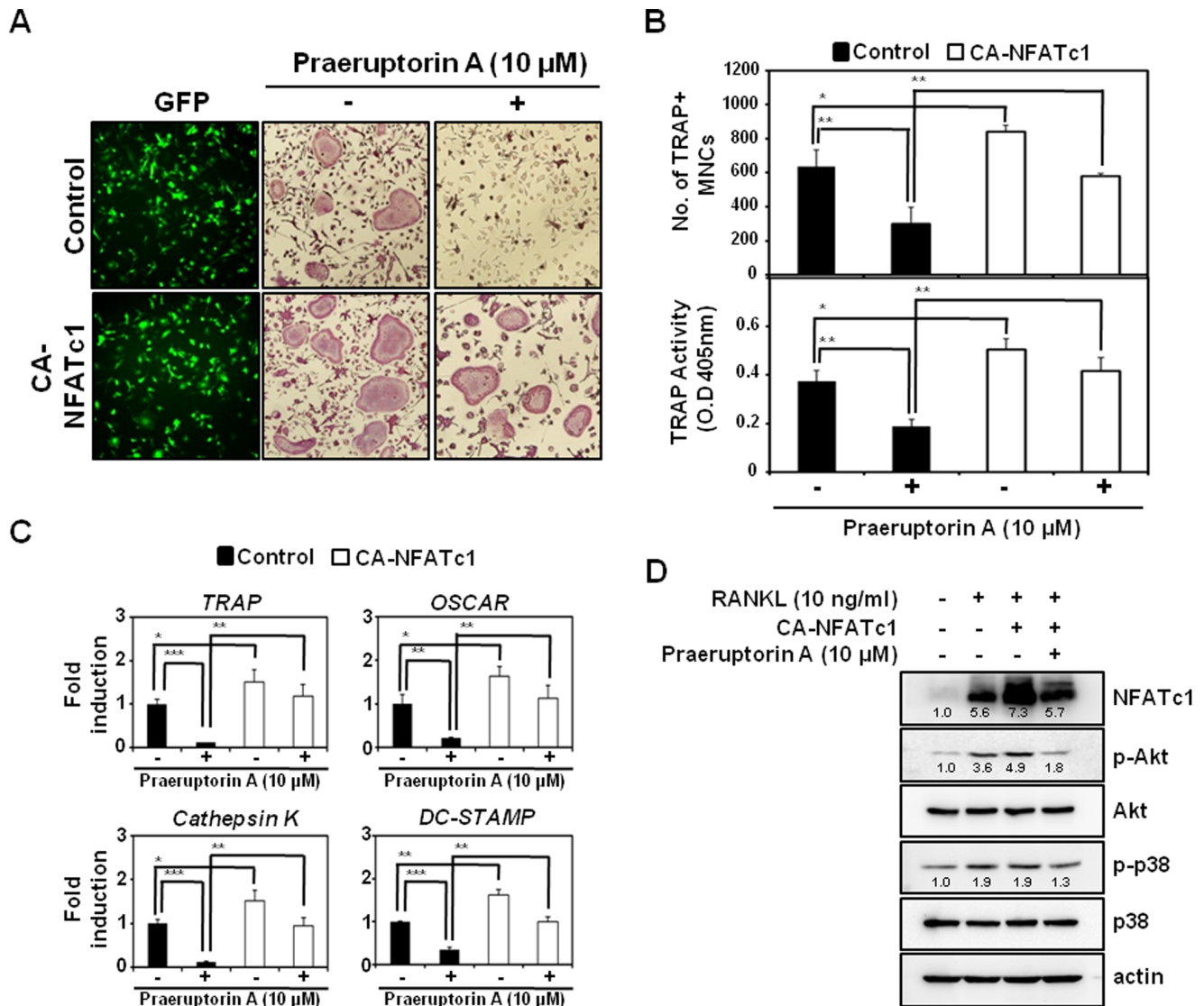


Figure 4. Effect of NFATc1 on anti-osteoclastogenic action of praeruptorin A. (A) BMMs were infected with retroviruses harboring the control GFP or Ca-NFATc1-GFP vectors. Transduced BMMs were cultured with RANKL (10 ng/ml) and M-CSF (30 ng/ml) in the presence of praeruptorin A (10 μ M) or vehicle (DMSO). After incubation for 2 days, GFP expression was visualized under a fluorescence microscope. After 2 additional days, mature TRAP-positive multinucleated osteoclasts were visualized by TRAP staining. (B) TRAP-positive cells (nuclear number >3) were counted as osteoclasts, and TRAP activity was measured at 405 nm. On the differentiation day 2, the mRNA and protein expression levels of osteoclastogenesis-related molecules were analyzed by real-time PCR (C) and Western blot analysis, respectively (D). Densitometric analysis was performed using ImageJ software and the relative, normalized ratios of NFATc1/actin, p-Akt/Akt or p-p38/p38 were presented. *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

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c-Fos and nuclear factor of activated T cells (NFATc1) to regulate the expression of genes required for osteoclast differentiation [26–28]. c-Fos is an essential factor for the induction of NFATc1, which is a master transcription factor that regulates the process of osteoclast differentiation by controlling osteoclast-specific genes [29–31].

Here, praeruptorin A attenuated the RANKL-induced phosphorylation of p38 without affecting JNK and ERK. A pharmacological inhibition experiment using the p38 inhibitor SB203580 revealed direct involvement of p38 in the RANKL-induced osteoclast differentiation [22,32,33]. Furthermore, a study using both p38 inhibitor SB203580 and over-expression of dominant negative MKK3 and MKK6, which are known as upstream kinases of p38, revealed that the p38 signaling pathway could

mediate the induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation [34].

Moreover, praeruptorin A also attenuated the RANKL-induced phosphorylation of Akt. Akt has been known to play a critical role in the survival of osteoclasts rather than in osteoclast differentiation through the phosphoinositide 3-kinase (PI3K) kinase signaling pathway [35,36]. However, a recent study showed the importance of the Akt-NFATc1 signaling axis in osteoclast differentiation [37]; inhibition of Akt phosphorylation by LY294002 resulted in the inhibition of osteoclast differentiation via modulation of RANKL-induced activation of NFATc1.

Thus, the anti-osteoclastogenic action of praeruptorin A could be due to its potential to inhibit both p38 and Akt signaling pathways that consequently downregulate the expression and/or

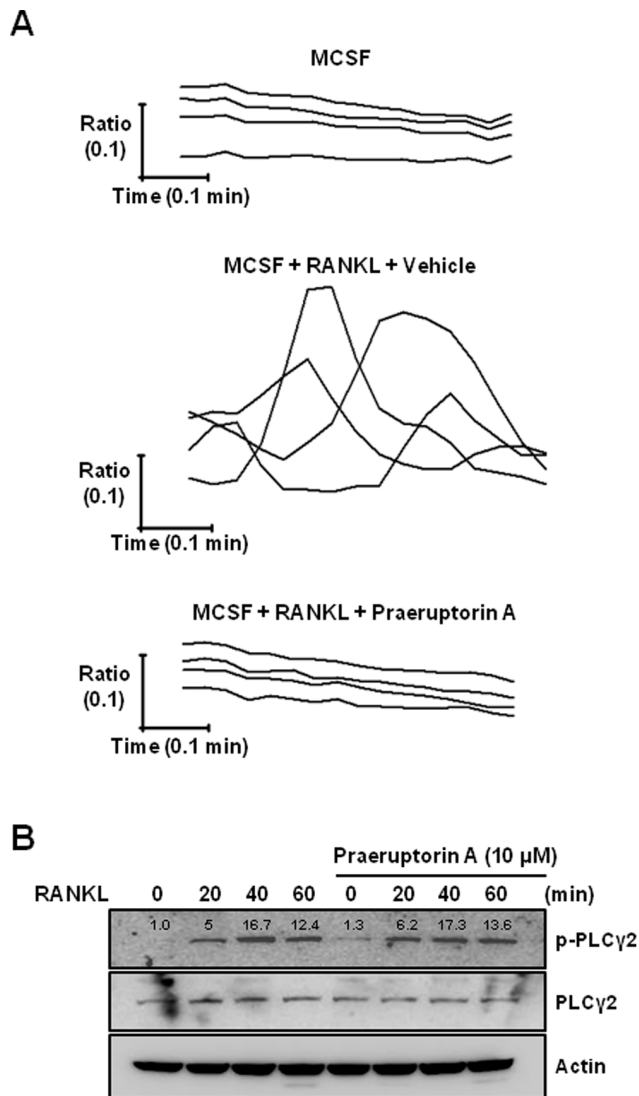


Figure 5. Effect of praeruptorin A on RANKL-induced Ca^{2+} oscillation and PLC γ phosphorylation. (A) The effect of praeruptorin A on the RANKL-induced Ca^{2+} oscillation was evaluated as described in 'Materials and Methods'. Each trace presents intracellular Ca^{2+} mobilization in each cell. (B) The effect of praeruptorin A on the RANKL-induced phosphorylation of PLC γ was evaluated by Western blot analysis. BMMs were pre-treated with praeruptorin A for 2 h before treatment with RANKL. Actin was used as an internal control. Densitometric analysis was performed using ImageJ software and the relative, normalized ratio of p-PLC γ 2/PLC γ 2 was presented. doi:10.1371/journal.pone.0088974.g005

activity of c-Fos and NFATc1. In particular, NFATc1, which is mainly regulated by c-Fos during osteoclastogenesis, plays a role as the most distal transcription factor required for regulating the expression of osteoclast-specific genes including TRAP, OSCAR, DC-STAMP, cathepsin K and c-Src [27,28,38]. TRAP is recognized as a marker of osteoclast differentiation and exhibits bone resorptive activity in the lysosomes [39]. OSCAR is a receptor that controls the PLC γ - Ca^{2+} signaling pathway, which is crucial for the activation of NFATc1 [40]. DC-STAMP and cathepsin K are well-known molecules for fusion and bone resorptive activity, respectively [38,41]. c-Src tyrosine kinase is also required for the maintenance of the osteoclast actin cytoskeleton and the control of bone resorption [42]. In our results,

praeruptorin A significantly inhibited the RANKL-induced expression of c-Fos, NFATc1 and those osteoclast-specific genes. Additionally, praeruptorin A inhibited the RANKL-induced activation of NFATc1.

These results suggested that the downregulation of NFATc1 could be the outcome of the anti-osteoclastogenic action of praeruptorin A by inhibiting p38 and Akt signaling pathways. The hypothesis was proved by the ectopic expression of the constitutively active form of NFATc1; it significantly rescued the anti-osteoclastogenic action of praeruptorin A. The rescue of defected osteoclastogenesis by overexpression of NFATc1 has been reported in several studies [9,43]; for example, NFATc1-deficient embryonic stem cells failed to differentiate into osteoclasts after RANKL treatment, but the ectopic expression of NFATc1 rescued the abrogated osteoclast differentiation [30,44]. Additionally, praeruptorin A strongly attenuated the Akt activation by the overexpression of activated NFATc1, but that did not induce the phosphorylation of p38. These results suggested that the auto-amplification of NFATc1 during osteoclast differentiation could affect the activation of Akt, but not p38, and praeruptorin A has the potential to attenuate the NFATc1-mediated activation of Akt.

Furthermore, the overexpression of c-Fos did not significantly rescue the effect of praeruptorin A on osteoclast differentiation (Fig. S6), but the Western blot analysis revealed the involvement of NF- κ B signaling in anti-osteoclastogenic action of praeruptorin A (Fig. S7). For degradation, I κ B α was phosphorylated 5 min after RANKL treatment, and then free NF- κ B p65 was translocated into nucleus in 15 to 30 min, but this activation of NF- κ B by RANKL was shown to be attenuated by the pretreatment of praeruptorin A. The role of NF- κ B in osteoclast differentiation has been described in several review articles [4,5].

In osteoclast differentiation, RANKL also triggers the activation of PLC γ , which subsequently leads to Ca^{2+} mobilization [45]. As well as activating MAP kinases and Akt, PLC γ -mediated Ca^{2+} mobilization affects the activation of NFATc1 required for regulating osteoclast-specific genes [27]. Importantly, several studies have reported the Ca^{2+} channel blocking activity of praeruptorin A [23,24]. These data clarify the hypothesis that praeruptorin A-mediated inhibition of Ca^{2+} oscillation via PLC γ could also downregulate the activity of NFATc1 during osteoclast differentiation. Interestingly, the RANKL-triggered Ca^{2+} oscillation was inhibited by praeruptorin A, but the RANKL-induced phosphorylation of PLC γ was not changed by praeruptorin A. These data suggest that the anti-osteoclastogenic activity of praeruptorin A involves inhibition of PLC γ -independent Ca^{2+} oscillation.

This is the first report of the anti-osteoclastogenic activity of praeruptorin A and its mode of action; praeruptorin A could inhibit the RANKL-induced osteoclast differentiation by inhibiting p38 and Akt signaling pathways and PLC γ -independent Ca^{2+} oscillation that consequently affect the expression and/or activity of the osteoclast-specific transcription factors, c-Fos and NFATc1. Also, NF- κ B signaling was shown to be partly involved in the anti-osteoclastogenic action of praeruptorin A. In a further study, the binding molecules (or target proteins) of praeruptorin A might be identified, and the mechanism how praeruptorin A inhibits the fusion of preosteoclasts and the pit formation of mature osteoclasts might be elucidated.

Supporting Information

Figure S1 Effect of praeruptorin A on cell spreading during RANKL-induced osteoclast differentiation. BMMs (1×10^4 cells/well) were seeded in a 96-well plate, treated with the

vehicle (0.1% DMSO) or praeruptorin A (10 μ M) for 2 h in the presence of M-CSF (30 ng/ml), and incubated with RANKL (10 ng/ml) for 1 and 3 days. Then, cells were fixed, permeabilized, washed, and incubated with 10 μ g/ml Hoechst 33342. (TIF)

Figure S2 Effect of praeruptorin A on RANKL-induced osteoclast differentiation for the indicated periods. BMMs were cultured with praeruptorin A (10 μ M) for various times periods (indicated the black arrow) in the presence of M-CSF and RANKL. After TRAP staining, TRAP-positive multinuclear cells (MNCs; nuclear number >3) were counted *, $P<0.05$; **, $P<0.01$; *** $P<0.001$. (TIF)

Figure S3 Effect of praeruptorin A on the formation of actin rings during osteoclast differentiation. BMMs (1×10^4 cells/well) were seeded in a 96-well plate, treated with the vehicle (0.1% DMSO) or praeruptorin A (10 μ M) for 2 h in the presence of M-CSF (30 ng/ml), and incubated with RANKL (10 ng/ml) for 4 days. Then, cells were fixed, permeabilized, washed, and stained with Hoechst 33342 and phalloidin-FITC for nucleus and actin rings, respectively. (TIF)

Figure S4 (A) Effect of age, strain or sex on anti-osteoclastogenic action of praeruptorin A. BMMs were isolated from mice (male and female ICR strain, 5-week old; male and female C57BL6/N strain, 5-week old and 8-month old) and cultured with praeruptorin A in the presence of M-CSF and RANKL for 4 days. Osteoclast differentiation was visualized by TRAP staining. (B) Effect of age, strain or sex on anti-osteoclastogenic action of praeruptorin A. BMMs were isolated from mice (male and female ICR strain, 5-week old; male and female C57BL6/N strain, 5-week old and 8-month old) and cultured with praeruptorin A in the presence of M-CSF and RANKL for 4 days. After TRAP staining, TRAP-positive multinuclear cells (MNCs; nuclear number >3) were counted. TRAP activity and cell viability were also evaluated. *, $P<0.05$; **, $P<0.01$; *** $P<0.001$. (TIF)

Figure S5 Anti-resorptive activity of praeruptorin A. (A) After co-culturing BMMs with osteoblasts for 7 days, multinucleated osteoclasts were replated on BioCoat Osteologic MultiTest slides and after 2 h incubation, cells were further incubated with

praeruptorin A and RANKL for 6 h. Then, cells were stained for TRAP (upper images). (B) TRAP-positive multinucleated cells were counted. (C) After removing cells, the resorption pits (indicated by asterisks in bottom images) were observed under a light microscope. The relative resorbing areas were evaluated using the ImageJ program. *** $P<0.001$. (TIF)

Figure S6 Effect of c-Fos on anti-osteoclastogenic action of praeruptorin A. BMMs were infected with retroviruses harboring the control GFP or c-Fos-GFP vectors. Transduced BMMs were cultured with RANKL (10 ng/ml) and M-CSF (30 ng/ml) in the presence of praeruptorin A (10 μ M) or the vehicle (0.1% DMSO). (A) After incubation for 2 days, GFP expression was visualized under a fluorescence microscope (left images). After 2 additional days, mature TRAP-positive multinucleated osteoclasts were visualized by TRAP staining (middle and right images). TRAP-positive cells (nuclear number >3) were counted as osteoclasts (B), and TRAP activity was measured at 405 nm (C). *, $P<0.05$; **, $P<0.01$; *** $P<0.001$. (TIF)

Figure S7 Effect of praeruptorin A on RANKL-induced activation of NF- κ B signaling pathway. BMMs were treated with praeruptorin A (10 μ M) for 30 min, stimulated with RANKL (10 ng/ml) for the indicated time. The expression levels of molecules in cytoplasmic or nuclear protein fractions were evaluated by Western blot analysis. Actin and lamin B1 were used for the loading control of cytosolic and nuclear proteins, respectively. Densitometric analysis was performed using ImageJ software and the relative, normalized ratios of I κ B α /actin, p-I κ B α /actin, cytosolic p65/actin or nuclear p65/lamin B1 were presented. (TIF)

File S1 Materials and Methods. (DOC)

Author Contributions

Conceived and designed the experiments: SHK YJS. Performed the experiments: JTY KJK SWC SHM YSP BJR JMO MSK ME. Analyzed the data: SHK YJS JTY KJK. Contributed reagents/materials/analysis tools: SHK YJS. Wrote the paper: SHK YJS.

References

- Cooper C, Campion G, Melton LJ 3rd (1992) Hip fractures in the elderly: a world-wide projection. *Osteoporos Int* 2: 285–289.
- Lippuner K, von Overbeck J, Perrelet R, Bosshard H, Jaeger P (1997) Incidence and direct medical costs of hospitalizations due to osteoporotic fractures in Switzerland. *Osteoporos Int* 7: 414–425.
- Karsenty G, Wagner EF (2002) Reaching a genetic and molecular understanding of skeletal development. *Dev Cell* 2: 389–406.
- 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.
- Fleisch H (2003) Bisphosphonates in osteoporosis. *Eur Spine J* 12 Suppl 2: S142–146.
- Papapetrou PD (2009) Bisphosphonate-associated adverse events. *Hormones (Athens)* 8: 96–110.
- Morabito N, Crisafulli A, Vergara C, Gaudio A, Lasco A, et al. (2002) Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: a randomized double-blind placebo-controlled study. *J Bone Miner Res* 17: 1904–1912.
- Choi SW, Son YJ, Yun JM, Kim SH (2012) Fisetin Inhibits Osteoclast Differentiation via Downregulation of p38 and c-Fos/NFATc1 Signaling Pathways. *Evid Based Complement Alternat Med* 2012: 810563.
- Choi SW, Moon SH, Yang HJ, Kwon DY, Son YJ, et al. (2013) Antiresorptive activity of bacillus-fermented antler extracts: inhibition of osteoclast differentiation. *Evid Based Complement Alternat Med* 2013: 748687.
- Shen X, Chen G, Zhu G, Fong WF (2006) (+/-)-3'-O, 4'-O-dicinnamoyl-cis-khellactone, a derivative of (+/-)-praeruptorin A, reverses P-glycoprotein mediated multidrug resistance in cancer cells. *Bioorg Med Chem* 14: 7138–7145.
- Wu JY, Fong WF, Zhang JX, Leung CH, Kwong HL, et al. (2003) Reversal of multidrug resistance in cancer cells by pyranocoumarins isolated from *Radix Peucedani*. *Eur J Pharmacol* 473: 9–17.
- Ji Y, Rao MR (1996) [Effects of Peucedanum praeruptorum extractum on blood pressure, left ventricular hypertrophy and hemodynamic changes in left ventricular hypertrophied hypertensive rats]. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 16: 676–678.
- Zhang C, Li W, Xiao YQ (2005) [Comparative studies on pharmacological effects of the main chemical constituents of Peucedanum praeruptorum from He'nan and Jiangxi]. *Zhongguo Zhong Yao Za Zhi* 30: 1356–1358.
- Yu PJ, Ci W, Wang GF, Zhang JY, Wu SY, et al. (2011) Praeruptorin A inhibits lipopolysaccharide-induced inflammatory response in murine macrophages through inhibition of NF- κ B pathway activation. *Phytother Res* 25: 550–556.
- Koide M, Okahashi N, Tanaka R, Kazuno K, Shibasaki K, et al. (1999) Inhibition of experimental bone resorption and osteoclast formation and survival by 2-aminoethanesulphonic acid. *Arch Oral Biol* 44: 711–719.

17. Inada M, Matsumoto C, Uematsu S, Akira S, Miyaura C (2006) Membrane-bound prostaglandin E synthase-1-mediated prostaglandin E2 production by osteoblast plays a critical role in lipopolysaccharide-induced bone loss associated with inflammation. *J Immunol* 177: 1879–1885.
18. Kim MH, Ryu SY, Choi JS, Min YK, Kim SH (2009) Saurolectam inhibits osteoclast differentiation and stimulates apoptosis of mature osteoclasts. *J Cell Physiol* 221: 618–628.
19. Lee SU, Choi YH, Kim YS, Min YK, Rhee M, et al. (2010) Anti-resorptive saurolectam exhibits in vitro anti-inflammatory activity via ERK-NF-kappaB signaling pathway. *Int Immunopharmacol* 10: 298–303.
20. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132: 365–386.
21. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402–408.
22. Yeon JT, Choi SW, Park KI, Choi MK, Kim JJ, et al. (2012) Glutaredoxin2 isoform b (Glx2b) promotes RANKL-induced osteoclastogenesis through activation of the p38-MAPK signaling pathway. *BMB Rep* 45: 171–176.
23. Li JM, Chang TH, Sun XD, Hao LY, Wang YP, et al. (1994) Effect of dl-preruptorin A on calcium current in ventricular cells of guinea pig. *Zhongguo Yao Li Xue Bao* 15: 525–527.
24. Song YL, Jing WH, Zhao HY, Yan R, Li PT, et al. (2012) Stereoselective metabolism of (+/-)-preruptorin A, a calcium channel blocker from *Peucedani Radix*, in pooled liver microsomes of rats and humans. *Xenobiotica* 42: 231–237.
25. Takayanagi H (2005) Inflammatory bone destruction and osteoimmunology. *J Periodontol Res* 40: 287–293.
26. Feng X (2005) RANKing intracellular signaling in osteoclasts. *IUBMB Life* 57: 389–395.
27. Takayanagi H (2007) Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol* 7: 292–304.
28. Takayanagi H (2007) The role of NFAT in osteoclast formation. *Ann N Y Acad Sci* 1116: 227–237.
29. Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, et al. (1994) c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* 266: 443–448.
30. Takayanagi H, Kim S, Koga T, Nishina H, Ishiki M, et al. (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* 3: 889–901.
31. Miyamoto T (2011) Regulators of osteoclast differentiation and cell-cell fusion. *Keio J Med* 60: 101–105.
32. Li X, Udagawa N, Itoh K, Suda K, Murase Y, et al. (2002) p38 MAPK-mediated signals are required for inducing osteoclast differentiation but not for osteoclast function. *Endocrinology* 143: 3105–3113.
33. Matsumoto M, Sudo T, Saito T, Osada H, Tsujimoto M (2000) Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). *J Biol Chem* 275: 31155–31161.
34. Huang H, Chang EJ, Ryu J, Lee ZH, Lee Y, et al. (2006) Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway. *Biochem Biophys Res Commun* 351: 99–105.
35. Gingery A, Bradley E, Shaw A, Oursler MJ (2003) Phosphatidylinositol 3-kinase coordinately activates the MEK/ERK and AKT/NFkappaB pathways to maintain osteoclast survival. *J Cell Biochem* 89: 165–179.
36. Wong BR, Besser D, Kim N, Arron JR, Vologodskaya M, et al. (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol Cell* 4: 1041–1049.
37. Moon JB, Kim JH, Kim K, Youn BU, Ko A, et al. (2012) Akt induces osteoclast differentiation through regulating the GSK3beta/NFATc1 signaling cascade. *J Immunol* 188: 163–169.
38. Kim K, Lee SH, Ha Kim J, Choi Y, Kim N (2008) NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP). *Mol Endocrinol* 22: 176–185.
39. Angel NZ, Walsh N, Forwood MR, Ostrowski MC, Cassady AI, et al. (2000) Transgenic mice overexpressing tartrate-resistant acid phosphatase exhibit an increased rate of bone turnover. *J Bone Miner Res* 15: 103–110.
40. Nemeth K, Schoppet M, Al-Fakhri N, Helas S, Jessberger R, et al. (2011) The role of osteoclast-associated receptor in osteoimmunology. *J Immunol* 186: 13–18.
41. Delaisse JM, Andersen TL, Engsig MT, Henriksen K, Troen T, et al. (2003) Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities. *Microsc Res Tech* 61: 504–513.
42. Miyazaki T, Tanaka S, Sanjay A, Baron R (2006) The role of c-Src kinase in the regulation of osteoclast function. *Mod Rheumatol* 16: 68–74.
43. Lee SH, Kim T, Jeong D, Kim N, Choi Y (2008) The tec family tyrosine kinase Btk Regulates RANKL-induced osteoclast maturation. *J Biol Chem* 283: 11526–11534.
44. Asagiri M, Sato K, Usami T, Ochi S, Nishina H, et al. (2005) Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. *J Exp Med* 202: 1261–1269.
45. Negishi-Koga T, Takayanagi H (2009) Ca2+-NFATc1 signaling is an essential axis of osteoclast differentiation. *Immunol Rev* 231: 241–256.