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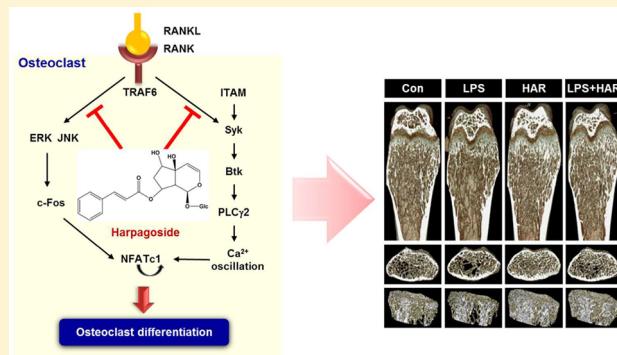
Harpagoside Inhibits RANKL-Induced Osteoclastogenesis via Syk-Btk-PLC γ 2-Ca²⁺ Signaling Pathway and Prevents Inflammation-Mediated Bone Loss

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Supporting Information

ABSTRACT: Harpagoside (HAR) is a natural compound isolated from *Harpagophytum procumbens* (devil's claw) that is reported to have anti-inflammatory effects; however, these effects have not been investigated in the context of bone development. The current study describes for the first time that HAR inhibits receptor activator of nuclear factor κ B ligand (RANKL)-induced osteoclastogenesis *in vitro* and suppresses inflammation-induced bone loss in a mouse model. HAR also inhibited the formation of osteoclasts from mouse bone marrow macrophages (BMMs) in a dose-dependent manner as well as the activity of mature osteoclasts, including filamentous actin (F-actin) ring formation and bone matrix breakdown. This involved a HAR-induced decrease in extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) phosphorylation, leading to the inhibition of Syk-Btk-PLC γ 2-Ca²⁺ in RANKL-dependent early signaling, as well as the activation of c-Fos and nuclear factor of activated T cells cytoplasmic 1 (NFATc1), which resulted in the down-regulation of various target genes. Consistent with these *in vitro* results, HAR blocked lipopolysaccharide (LPS)-induced bone loss in an inflammatory osteoporosis model. However, HAR did not prevent ovariectomy-mediated bone erosion in a postmenopausal osteoporosis model. These results suggest that HAR is a valuable agent against inflammation-related bone disorders but not osteoporosis induced by hormonal abnormalities.



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With the aging of the global population, bone metabolic diseases such as rheumatoid arthritis, osteoporosis, and Paget's disease are increasingly impacting public health as well as the economy.^{1,2} The greatest problem associated with bone disease and first diagnostic indicator especially for osteoporosis is the high risk of fractures, which seriously undermines quality of life and increases the risk of mortality.³

Osteoclasts are the main type of bone-resorbing multi-nucleated giant cell produced by hematopoietic stem cells upon stimulation by the cytokines receptor activator of nuclear factor (NF)- κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), also known as CSF-1. RANKL, a member of the tumor necrosis factor (TNF) family, accelerates cell differentiation and is an osteoclast marker. M-CSF is the main regulator of osteoclast progenitor and macrophage proliferation and survival.^{4–6} The binding of RANKL to the RANK receptor activates multiple downstream signaling pathways including NF- κ B, Akt, and mitogen-activated protein kinases (MAPKs) comprising p38, extracellular signal-regulated kinase (ERK), and c-jun N-terminal kinase (JNK), leading to the activation of the transcription factors c-Fos and nuclear factor of activated T cells cytoplasmic (NFATc)₁ that are required for osteoclast differentiation. The RANKL-RANK axis also stimulates the Ca²⁺ signaling pathway via activation of phospholipase C (PLC) γ . During osteoclastogenesis, activated PLC γ hydrolyzes phosphatidylinositol-4,5-biphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3), causing both the up-regulation of intracellular Ca²⁺ and NFATc1 activation.^{7,8} We recently demonstrated that Ca²⁺ signaling plays a critical role in metabolic bone disorders by showing that vesicle-associated membrane protein B or oleanolic acid acetate regulate RANKL-induced osteoclast differentiation via PLC γ -Ca²⁺-NFAT signaling.^{9,10} Through regulation of this early signaling cascade, NFATc1 induces the expression of various osteoclast-specific genes such as *tartrate-resistant acid phosphatase* (*TRAP*), *osteoclast-associated receptor* (*OSCAR*), β 3-integrin, *dendritic cell-specific transmembrane protein* (*DC-STAMP*), *calcitonin receptor* (*CTR*), and *cathepsin K*.^{11–16}

Various medicinal compounds prepared from crude plant extracts that act on osteoclast differentiation and functions have

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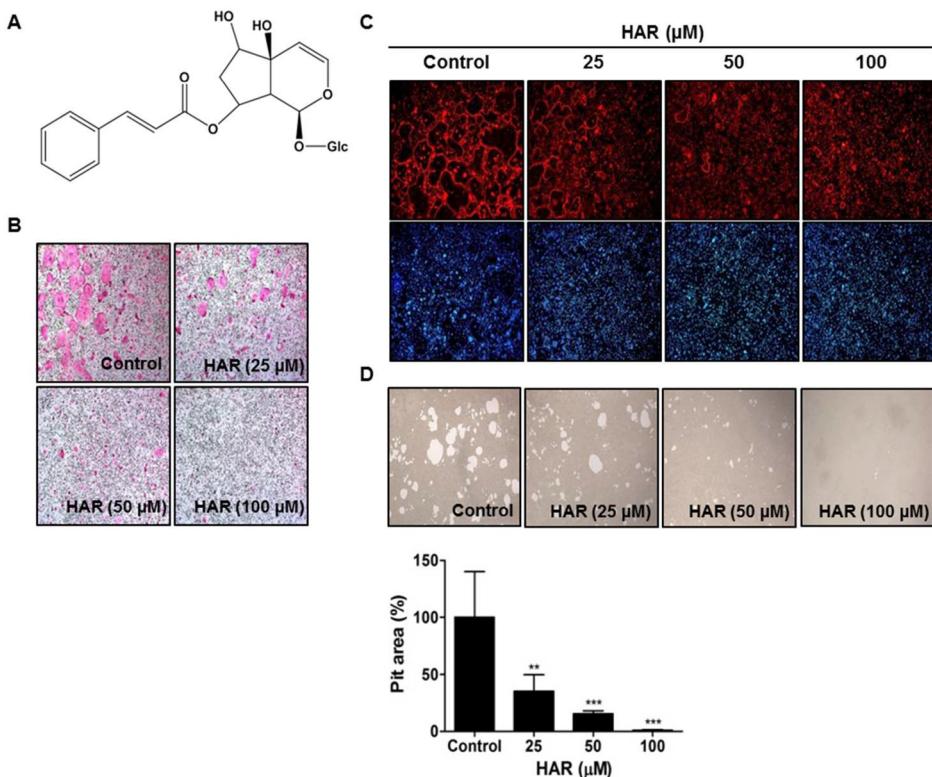


Figure 1. Effect of HAR on osteoclast differentiation and F-actin ring formation, and bone resorption by mature osteoclasts. (A) Chemical structure of HAR. (B) The mouse BMMs were cultured for 3 days with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the absence or presence of HAR. Cells were fixed with 3.7% formalin in PBS, permeabilized with 0.1% Triton X-100 in PBS, and incubated with TRAP reagent. TRAP-positive cells were imaged under a light microscope (*upper*). TRAP-positive multinucleated cells (TRAP⁺ MNCs) were counted as osteoclasts (*lower*). ***P* < 0.01, ****P* < 0.001 vs control group. (C) BMMs were cultured for 4 days with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the absence or presence of HAR. Cells were fixed with 3.7% formalin in PBS, permeabilized with 0.1% Triton X-100 in PBS, and stained with phalloidin and 4',6-diamidino-2'-phenylindole (DAPI). (D) Mature osteoclasts were seeded on hydroxyapatite-coated plates and treated for 24 h with indicated concentrations of HAR. Adherent cells were collected and imaged under a light microscope (*upper*). Pit areas on hydroxyapatite plates were quantified using Image Pro-Plus v.4.5 software (*lower*). ***P* < 0.01, ****P* < 0.001 vs control group.

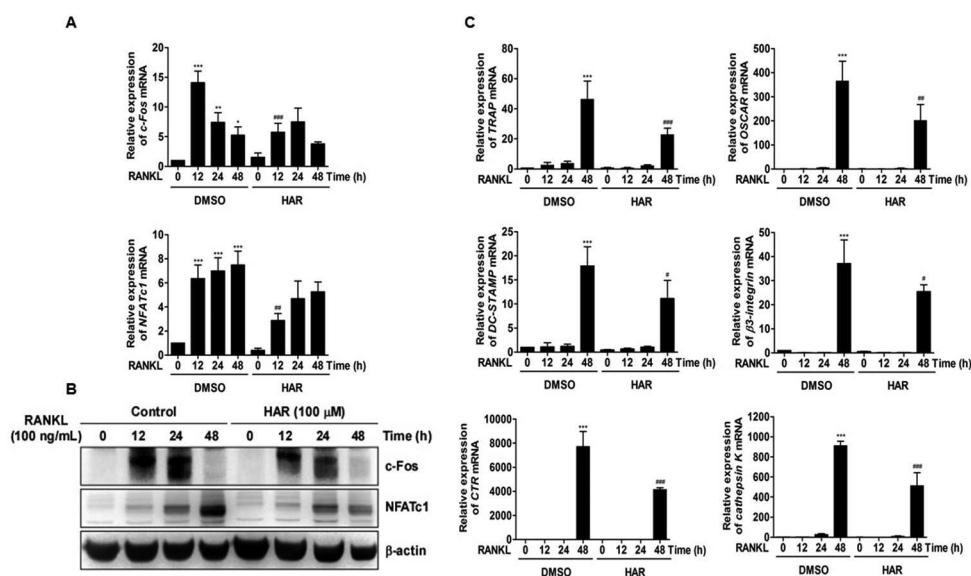


Figure 2. Effect of HAR on RANKL-mediated expression of osteoclast-related genes. BMMs were pretreated with or without HAR (100 μM) for 1 h in the presence of M-CSF (30 ng/mL) and then stimulated with RANKL (100 ng/mL) for the indicated times. (A) Total RNA was isolated from cells and mRNA expression levels were analyzed by quantitative real-time RT-PCR. (B) Effects of HAR on protein expression levels of c-Fos and NFATc1 were evaluated by Western blot analysis, with β-actin used as a loading control. (C) mRNA expression levels were analyzed by quantitative real-time RT-PCR. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs control group and #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs control group at indicated times.

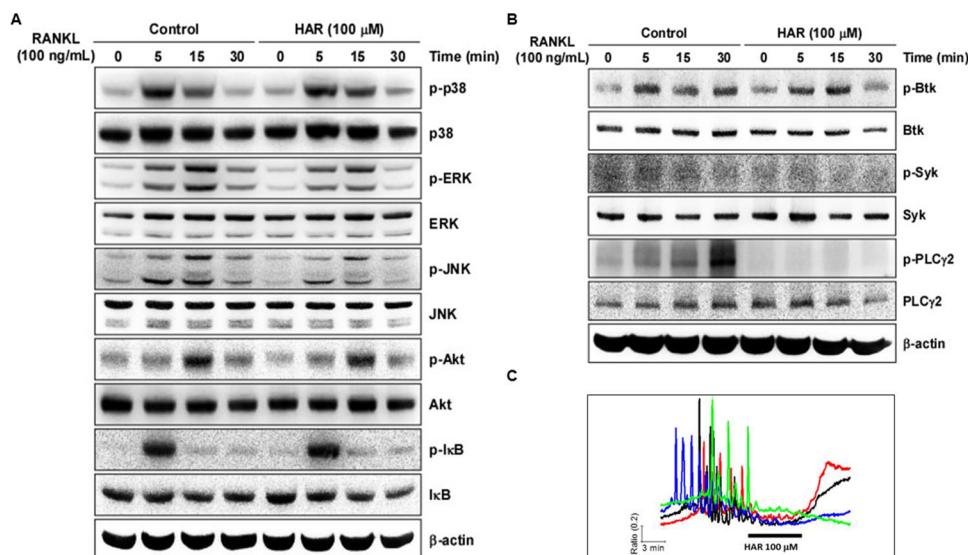


Figure 3. Effect of HAR on RANKL-induced early signaling events. (A) BMMs were pretreated with or without of HAR ($100 \mu\text{M}$) for 1 h in the presence of M-CSF (30 ng/mL) and then stimulated with RANKL (100 ng/mL) for the indicated times. Whole cell lysates were evaluated by Western blot analysis, with β -actin used as a loading control. (B) Whole cell lysates were analyzed by Western blotting. (C) BMMs were seeded on coverslips in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL). After incubation with RANKL for 24 h, HAR ($100 \mu\text{M}$) reduced the amplitude of Ca^{2+} oscillations induced by RANKL. Each value represents the mean \pm SD of three independent experiments with each measurement made in triplicate.

been reported to have therapeutic benefits with few side effects in the treatment or prevention of bone diseases.^{17–22} To identify novel compounds that can function as antiresorptive agents, several natural compounds were screened by analyzing TRAP staining. Harpagoside (HAR, Figure 1A) is an iridoid glycoside isolated from *Harpagophytum procumbens* (devil's claw) that has been used to treat pain, arthritis, fever, ulcers, and boils in Southern Africa²³ and has been described as an anti-inflammatory and analgesic agent.^{24,25} We found that HAR suppressed RANKL-mediated osteoclast differentiation from bone marrow macrophages (BMMs). On the basis of the association between chronic inflammatory and bone diseases,^{26–28} we investigated the pharmacological effects of HAR on RANKL-induced osteoclast differentiation and function *in vitro* and on bone destruction in lipopolysaccharide (LPS)-induced bone loss and ovariectomized (OVX)-induced bone erosion mouse models.

RESULTS AND DISCUSSION

HAR Inhibits RANKL-Induced Osteoclast Differentiation and Function. To evaluate the effect of HAR on osteoclast differentiation, mouse BMMs treated with M-CSF and RANKL were cultured in the presence or absence of HAR. TRAP-positive osteoclasts were observed in higher numbers in control groups, whereas HAR treatment inhibited the formation of TRAP-positive osteoclasts in a concentration-dependent manner (Figure 1B). The XTT assay was carried out to assess cytotoxicity during osteoclast differentiation. HAR had no effect on cell viability at the concentrations tested (Supplementary Figure 1). The formation of filamentous-actin (F-actin) ring structures in osteoclasts is a critical indicator of the bone resorption activity of osteoclasts. We therefore examined whether HAR inhibits osteoclast function and bone resorption. HAR treatment disrupted F-actin ring formation in a dose-dependent manner (Figure 1C). Additionally, in mature osteoclasts seeded on hydroxyapatite-coated

plates, resorption pits were observed in the control group, whereas HAR inhibited resorption in a dose-dependent manner (Figure 1D). These findings indicate that HAR attenuated both the formation and bone resorbing activity of osteoclasts.

HAR Inhibits RANKL-Induced c-Fos and NFATc1 and Osteoclast Marker Gene Expression. To determine whether HAR regulates RANKL-mediated osteoclastogenesis by inhibiting c-Fos and NFATc1 activation, the effects of HAR on RANKL-induced c-Fos and NFATc1 expression were examined. In BMMs treated with RANKL for 12–48 h, c-Fos and NFATc1 protein and mRNA expression increased in the control group, as determined by Western blotting and quantitative real-time PCR, respectively; these were reduced by treatment with HAR (Figure 2A,B). We also examined whether HAR regulates the mRNA expression of the transcription factor genes TRAP, OSCAR, β 3-integrin, DC-STAMP, CTR, and cathepsin K, which are involved in osteoclast formation and function during RANKL-induced osteoclast differentiation. The transcript level of each of these factors was markedly inhibited by HAR treatment (Figure 2C).

It is well-known that c-Fos and NFATc1 are required for the differentiation of osteoclast precursors into bone-resorbing osteoclasts. The c-Fos gene product is a component of the inducible activator protein-1 complex formed by Fos family members c-Fos, FosB, Fos-related antigen (Fra)-1, and Fra-2 in association with the Jun proteins c-Jun, JunB, and JunD,²⁹ which regulates target gene transcription.³⁰ c-Fos contains a C-terminal transactivation domain that is critically involved in oncogenesis and cellular transformation;^{29,31} c-Fos-deficient mice experience osteopetrosis due to a block in osteoclast differentiation, while impaired osteoclastogenesis in mouse BMMs is completely rescued by ectopic c-Fos expression.³² NFATc1, which is strongly induced by RANKL, belongs to the NFAT transcription factor family first identified in T cells.³³ NFATc1-deficient embryonic stem cells do not differentiate into osteoclasts; this can be induced in BMMs by ectopic expression of NFATc1 even in the absence of RANKL.^{33,34}

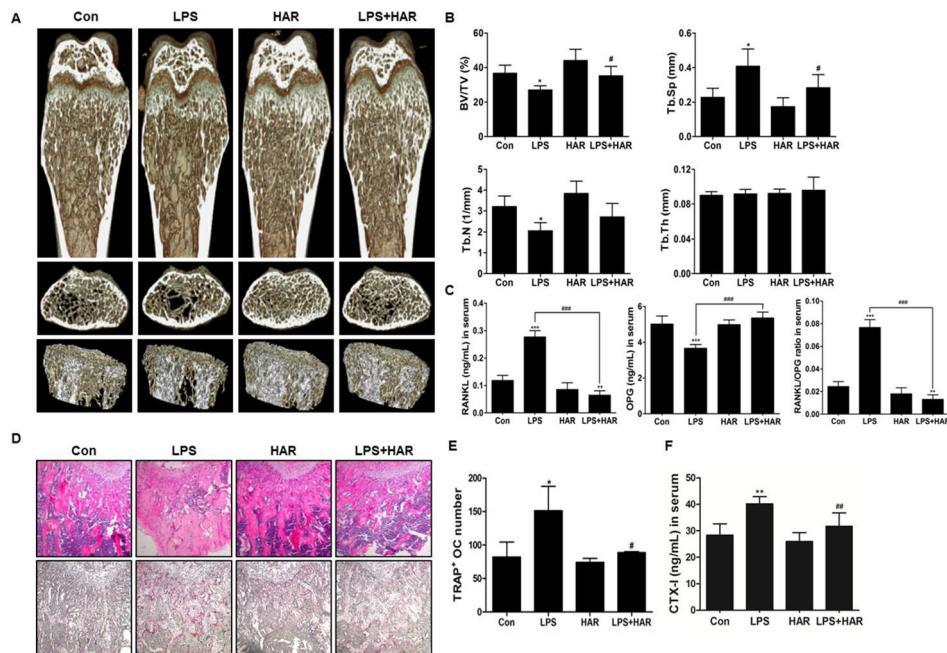


Figure 4. Effect of HAR on LPS-induced bone loss. (A) 5-week-old ICR mice were treated with either PBS (Control), HAR (10 mg/kg), and/or LPS injection (5 mg/kg) and sacrificed on day 10, and radiographs of longitudinal and transverse sections of the proximal femur were obtained by micro-CT. (B) BV/TV, Tb.Sp, Tb.Th, and Tb.N of each femur were determined and analyzed using INFINITT-Xelis software. *P < 0.05 vs control group, #P < 0.05 vs LPS group. (C) Levels of RANKL, OPG, and RANKL/OPG ratio of controls and mice treated with LPS, HAR, or both. ***P < 0.001 vs control group, ***P < 0.001 vs LPS group. (D) Dissected femurs were fixed, decalcified, embedded, and sectioned; sections were stained with hematoxylin and eosin (upper) and treated with TRAP reagent (lower). (E) The number of osteoclasts per field of tissue was analyzed. *P < 0.05 vs control group, #P < 0.05 vs LPS group. (F) Measurement of CTX-I in serum of controls and mice treated with LPS, HAR, or both by ELISA. ***P < 0.001 vs control group, **P < 0.01 vs LPS group.

NFATc1-deficient mice have defects in osteoclastogenesis and show symptoms of osteopetrosis.³⁵ NFATc1 regulation by c-Fos during osteoclastogenesis is required for the expression of osteoclast-specific genes such as *TRAP*, *OSCAR*, β 3-integrin, *DC-STAMP*, *CTR*, and *cathepsin K*.^{12–15} Thus, these results suggest that HAR attenuated the mRNA and protein expression of c-Fos and NFATc1, leading to down-regulation of various transcription factors related with osteoclasts.

HAR Regulates Osteoclastogenesis Via ERK, JNK, and Syk-Btk-PLC γ 2-Ca²⁺ Signaling. To investigate the mechanism underlying the HAR-mediated suppression of osteoclastogenesis, the effects of HAR on RANKL-induced early signaling events were examined. The phosphorylation of ERK and JNK induced by RANKL stimuli was slightly down-regulated by HAR treatment (Figure 3A). Furthermore, the phosphorylation of Syk, Btk, and PLC γ 2 was inhibited by HAR (Figure 3B). On the basis of these observations, the effects of HAR on RANKL-induced Ca²⁺ responses were assessed. HAR treatment decreased the amplitude and frequency of Ca²⁺ oscillations induced by RANKL (Figure 3C).

The upstream events that lead to the induction of c-Fos and NFATc1 involve the RANKL-dependent phosphorylation of several signaling components of the MAPK and PLC γ 2-Ca²⁺ pathways, which are critical for osteoclast activity. The p38 inhibitor SB203580 disrupted osteoclast formation in cocultures of mouse osteoblasts and BMCs by directly targeting osteoclast precursors.³⁶ The JNK inhibitor SP600125 stimulated the apoptotic effect of the RANKL/RANK/tumor necrosis factor receptor-associated factor 6 axis on osteoclasts, whereas the suppression of ERK by overexpression of

dominant-negative Ras induced apoptosis of osteoclast-like cells *in vitro*.^{37,38}

Ca²⁺ signaling is essential for the differentiation of osteoclasts. RANKL triggers PLC γ activation, leading to Ca²⁺ mobilization. The PLC γ family members PLC γ 1 and PLC γ 2 require phosphorylation of their tyrosine residues for catalytic activity, which regulates protein kinase C activation and intracellular Ca²⁺ levels in hematopoietic cells.^{39–41} PLC catalyzes the conversion of PIP2 into IP3, which plays a role in the up-regulation of intracellular Ca²⁺ level and diacylglycerol. The phosphorylation of PLC γ 2 but not PLC γ 1 is required for RANKL-mediated Ca²⁺ signaling in osteoclast differentiation.⁴² PLC γ 2 forms a complex with the scaffold protein GRB-associated binding protein 2—an adaptor molecule that is important for the differentiation of progenitor cells into osteoclasts—leading to its phosphorylation and recruitment.^{42,43} PLC γ 2 phosphorylation is directly linked to Ca²⁺ oscillations and the Ca²⁺-dependent translocation of NFATc1 induced by RANKL.⁴⁴ The PLC γ 2-Ca²⁺-NFATc1 signaling cascade is downstream of the activation of the tyrosine kinases Btk and Tec; these regulate osteoclastogenesis via interactions between RANK and immunoreceptor tyrosine-based activation motif (ITAM) signaling and the phosphorylation of ITAM within the receptor-associated adaptor domain, which leads to the recruitment of Syk tyrosine kinase.^{45,46} In the present study, HAR inhibited Ca²⁺ oscillation via inactivation of Syk, Btk, and PLC γ 2, resulting in the suppression of RANKL-induced osteoclast differentiation (Figure 3). These results demonstrate the involvement of ERK and JNK as well as Syk-Btk-PLC γ 2-Ca²⁺ signaling in the HAR-mediated inhibition of osteoclastogenesis.

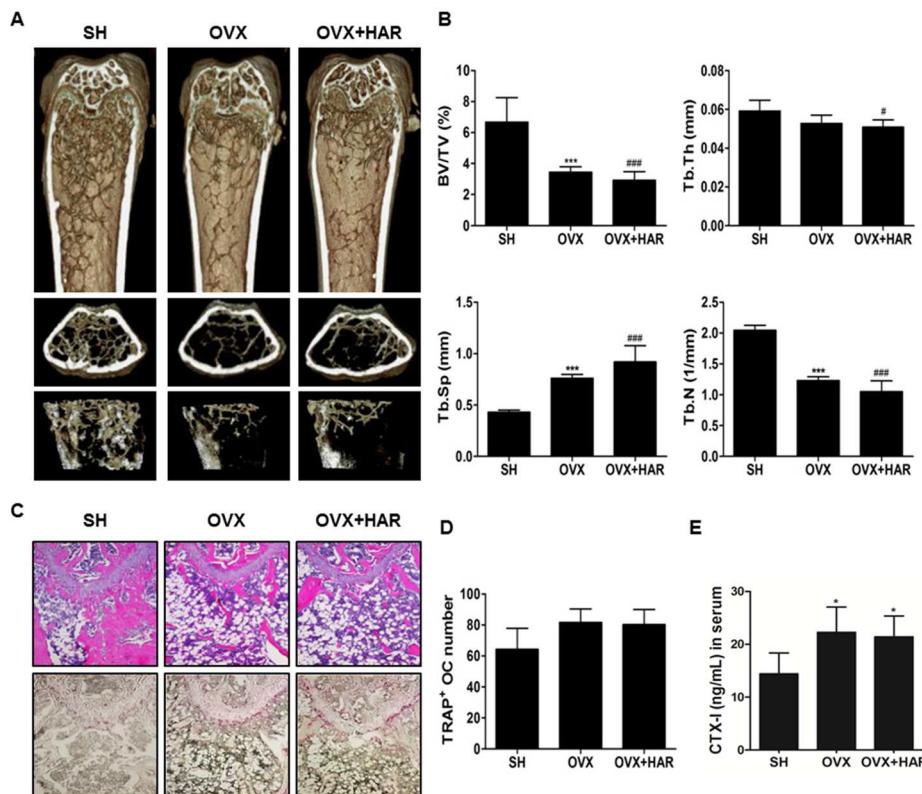


Figure 5. Effect of HAR on OVX-induced bone loss. (A) 8-week-old C57BL/6 mice were ovariectomized at 4-week-old. Four weeks later, HAR (10 mg/kg) or PBS (Control) was administered orally every other day for 28 days, at which time all mice were sacrificed. Radiographs of longitudinal and transverse sections of the proximal femur were obtained by micro-CT. **P < 0.01, ***P < 0.0001 vs OVS group. (B) BV/TV, Tb.Sp, Tb.Th, and Tb.N of each femur were determined and analyzed using INFINITT-Xelis software. ***P < 0.001 vs SH group. (C) Dissected femurs were fixed, decalcified, embedded, and sectioned; sections were stained with hematoxylin and eosin (upper) and treated with TRAP reagent (lower). (D) The number of osteoclasts per field of tissue was analyzed. (E) Measurement of CTX-I in serum of SH, OVX, and OVX mice treated with HAR by ELISA. *P < 0.05 vs SH group.

HAR Suppresses LPS-Induced Bone Loss. It has been proved that LPS—a major cell wall component of Gram-negative bacteria—triggers the release of cytokines, chemokines, metalloproteinases, and other agents that induce bone resorption and are involved in inflammation-mediated bone loss by macrophages.^{47–50} LPS increases the rate of bone resorption by modulating the production of TNF- α , interleukin-1 (IL-1), prostaglandin E₂ (PGE₂), and RANKL derived from osteoblasts and by interacting with preosteoclasts and reducing the expression of RANK and M-CSF receptor.^{51–54} LPS also stimulates osteoclast differentiation and activation via RANKL-induced MAPK signaling.⁵⁵

Therefore, in order to examine the effects of HAR on LPS-induced bone loss *in vivo*, mice were treated with LPS with or without HAR, and 9 days later the femurs were examined by microcomputed tomography (micro-CT). A three-dimensional analysis revealed a loss of trabecular bone following LPS treatment, which was abrogated in the HAR treatment group (Figure 4A): a morphometric analysis revealed that HAR restored the BV/TV, Tb.Sp, and Tb.N, but not the Tb.Th in these mice (Figure 4B). To determine whether the HAR contributes to RANKL and osteoprotegerin (OPG) expression, serum levels of these proteins were measured by enzyme-linked immunosorbent assay (ELISA). HAR treatment resulted in the down-regulation of RANKL and up-regulation of OPG, thereby decreasing the RANKL/OPG ratio in relative to the LPS-only group (Figure 4C). A histological analysis showed that LPS-

induced osteoclast formation and bone loss (Figure 4D), as well as the number of osteoclasts (Figure 4E), were inhibited in the femurs of mice treated with both HAR and LPS. Finally, the serum C-terminal telopeptide of type I collagen (CTX-I) concentration, a bone resorption marker, were substantially higher in the LPS-treated group. Moreover, expressions of CTX-I in serum of LPS+HAR declined effectively than LPS group (Figure 4F).

OVX-Mediated Bone Loss is Unaffected by HAR. The OVX mouse is a preclinical model for the study of osteoporosis and was used here to investigate the effects of HAR on estrogen-induced bone loss. A three-dimensional visualization of the femoral area by micro-CT revealed that HAR did not mitigate the loss of bone mineral density (Figure 5A) nor the decreases in BV/TV, Tb.Sp, and Tb.N (Figure 5B) induced by OVX. A histological examination also showed that osteoclast formation, bone loss, and the number of osteoclasts present in the femur were not restored in HAR-treated OVX mice (Figures 5C,D). Also, in comparison to the Sham (SH) group, serum level of CTX-I was increased in the OVX group. However, the levels were not different between the OVX and OVX mice treated with HAR (Figure 5E). These results demonstrated that HAR restores LPS-induced bone loss, whereas HAR does not have recovery effects on OVX-mediated bone erosion.

Unlike the relationship between LPS and bone environment, estrogen regulates osteoclast formation and activity by

promoting osteoclast apoptosis induced by transforming growth factor $\beta 1$, and estrogen agonists increase OPG production via estrogen receptor- α activation, which suppresses RANKL activity.^{56,57} Estrogen deficiency resulting from OVX induces the up-regulation of osteoclastogenesis by enhancing T cell TNF- α production.⁵⁸ OVX also induces the differentiation of Th17 cells that secrete IL-7, and promotes bone loss by enhancing osteoclast production and inhibiting osteoblast differentiation.⁵⁹ In the present study, micro-CT and histological analyses were carried out on mice treated concurrently with LPS and HAR to evaluate the preventive effect of HAR on bone loss as well as on mice treated with HAR after the development of OVX-induced bone loss to assess the therapeutic effect of HAR. HAR restored bone density in the bone loss model induced by LPS but not OVX, demonstrating a protective effect of HAR against bone erosion. Given that the mechanism of bone loss due to LPS-induced inflammation is dependent on the regulation of PLC $\gamma 2$ -Ca²⁺ signaling, which is not the case for bone erosion resulting from OVX,^{60–62} these findings suggest that HAR exerts its effects by modulating PLC $\gamma 2$ activation. The biggest reason for this result is irrelevance with Syk-Btk-PLC $\gamma 2$ -Ca²⁺ signal pathway in OVX-model. Previously, it was demonstrated that PLC $\gamma 2$ -deficient (PLC $\gamma 2^{-/-}$) mice had considerably higher trabecular bone mass than wild-type mice, whereas OVX-induced bone erosion in PLC $\gamma 2^{-/-}$ mice were similar to wild-type mice.⁶² These results implied the existence of PLC $\gamma 2$ -independent mechanisms provoking estrogen deficiency-induced osteoclast development. Thus, we arrived at the conclusion that OVX-induced bone erosion having PLC $\gamma 2$ -independent mechanisms were not recovered by HAR, which mainly regulates osteoclastogenesis via PLC $\gamma 2$ -dependent signal pathway.

Taken together, the results of this study demonstrate for the first time that HAR suppresses RANKL-induced osteoclast differentiation via ERK, JNK, and Syk-Btk-PLC $\gamma 2$ -Ca²⁺ signaling and downstream activation of NFATc1 and target gene expression. Moreover, HAR restored bone density in an LPS-but not OVX-induced bone loss model. These findings suggest that HAR is a promising therapeutic agent for treating inflammation-associated bone diseases such as inflammatory osteoporosis and rheumatoid arthritis but not postmenopausal osteoporosis. Future studies will address the detailed mechanisms underlying HAR function using *in vivo* models of LPS- and OVX-induced bone loss.

EXPERIMENTAL SECTION

General Experimental Procedures. HAR was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and dissolved in dimethyl sulfoxide (DMSO) followed by dilution. The purity of HAR (>95%) was determined by high-performance liquid chromatography (HPLC) assay. 1,25-Dihydroxyvitamin D₃ (VitD₃), PGE₂, LPS, and monoclonal β -actin antibody were purchased from Sigma-Aldrich as well. Recombinant soluble human RANKL and M-CSF were obtained from PeproTech EC Ltd. (London, U.K.). Penicillin/streptomycin antibiotics, α -minimum essential medium (α -MEM), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Antibodies against c-Fos (sc-7202), NFATc1 (sc-7294), I κ B (sc-371), spleen tyrosine kinase (Syk) (sc-1077), phospho-PLC $\gamma 2$ (sc-101785), and PLC $\gamma 2$ (sc-5283) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies against phospho-p38 MAPK (9211), p38 MAPK (9212), phospho-ERK 1/2 (9101), ERK 1/2 (9102), phospho-JNK (9251), JNK (9252), phospho-Akt (9271), Akt (9272), phospho-inhibitory κ B (I κ B) (2859), Btk (3533), and phospho-Syk (2710) were purchased from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). Antibodies against phospho-

Bruton's tyrosine kinase (Btk) (GTX61791) were purchased from GeneTex (Irvine, CA, U.S.A.). All other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Ethics Statement. Experimental procedures were conducted according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Committee of Wonkwang University (Permit number: WKU-14-45). Surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Animals were monitored daily in order to check health state.

Experimental Animals. Male ICR mice (5 weeks old) or female C57BL/6J mice (10 weeks old) were purchased from Damul Science (Daejeon, Korea) or Central Lab Animal Inc. (Seoul, Korea). Mice were housed under conditions of controlled temperature (22 °C–24 °C) and humidity (55%–60%) on a 12:12 h light/dark cycle.

Mouse BMM Preparation and Osteoclast Differentiation. Bone marrow cells (BMCs) were obtained by flushing the femurs and tibiae of 5-week-old ICR mice and were resuspended in α -MEM supplemented with 10% FBS and 1% penicillin/streptomycin. Nonadherent cells were collected and cultured for 3 days in the presence of M-CSF (30 ng/mL). Floating cells were discarded and cells adhering to the bottom of the culture dish were classified as BMMs. The cells were seeded at 3.5×10^4 /well and cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 days with or without HAR, then fixed in 3.7% formalin for 15 min, permeabilized with 0.1% Triton X-100, and stained for TRAP. TRAP-positive multinucleated cells (MNCs) with greater than five nuclei were counted as osteoclasts.

Cytotoxicity Assay. BMMs were seeded in 96-well plates at a density of 1×10^4 /well. Cells were treated with M-CSF (30 ng/mL) and increasing concentrations of HAR. After 3 days, 50 μ L of sodium 3'-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) (XTT reagent), benzenesulfonic acid hydrate, and N-methyl dibenzopyrazine methyl sulfate were added to each well followed by incubation for 4 h. The optical density at 450 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

F-actin Ring Staining. BMMs were cultured for 3 days with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the presence or absence of HAR. After 3 days, cells were fixed with phosphate-buffered saline (PBS) containing 3.7% formalin for 20 min and permeabilized with PBS containing 0.1% Triton X-100 for 15 min. The cells were blocked with 2.5% bovine serum albumin for 30 min and then stained with phalloidin (Molecular Probes/Life Technologies, Carlsbad, CA, U.S.A.) and 4',6-diamidino-2'-phenylindole (Sigma) at room temperature for 30 min. Images were acquired using a fluorescence microscope (DMLB, Leica, Germany).

Resorption Pit Assay. Primary osteoblasts and BMCs were cocultured on a collagen gel-coated 90 mm dish in the presence of VitD₃ and PGE₂ for 6 days. Cocultured cells were detached by treatment with 0.1% collagenase at 37 °C for 10 min, and then replated on hydroxyapatite-coated plates with or without HAR for 24 h. The cells were then removed and resorption pits were imaged and analyzed using Image Pro-Plus version 4.0 (Media Cybernetics, Rockville, MD, U.S.A.).

Quantitative Real-Time RT-PCR Analysis. Total RNA was isolated using QIAzol reagent (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. RNA (1 μ g) was reverse transcribed using oligo dT primers (10 μ g) and dNTPs (10 mM). The mixture was incubated at 65 °C for 5 min and cDNA was obtained by incubation at 42 °C for 50 min with first strand buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂), 100 mM dithiothreitol, RNase inhibitor, and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Real-time RT-PCR was carried out on an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer Co., Daejeon, Korea) in a 20 μ L reaction mixture containing 10 μ L SYBR Green Premix (Bioneer Co.), 10 pmol each of forward and reverse primers, and 1 μ g cDNA. The amplification conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 1 min, 60 °C for 30 s, and 72 °C for 1 min. Fluorescence resulting from the incorporation of SYBR

Green dye into double-stranded DNA was quantified using the threshold cycle (C_t) value. Relative levels of *c-Fos*, *NFATc1*, TRAP, OSCAR, β 3-integrin, DC-STAMP, CTR, and cathepsin K were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used for real-time RT-PCR are summarized in Table 1.

Table 1. Primer Sequences Used for Real-Time RT-PCR Analysis

gene name		primer sequence (5'→3')
GAPDH	forward	5'-TCA AGA AGG TGG TGA AGC AG-3'
	reverse	5'-AGT GGG AGT TGC TGT TGA AGT-3'
<i>c-Fos</i>	forward	5'-GGT GAA GAC CGT GTC AGG AG-3'
	reverse	5'-TAT TCC GTT CCC TTC GGA TT-3'
<i>NFATc1</i>	forward	5'-GAG TAC ACC TTC CAG CAC CTT-3'
	reverse	5'-TAT GAT GTC GGG GAA AGA GA-3'
TRAP	forward	5'-TCA TGG GTG GTG CTG CT-3'
	reverse	5'-GCC CAC AGC CAC AAA TCT-3'
OSCAR	forward	5'-GGA ATG GTC CTC ATC TCC TT-3'
	reverse	5'-TCC AGG CAG TCT CTT CAG TTT-3'
β 3-integrin	forward	5'-GGA GTG GCT GAT CCA GAT GT-3'
	reverse	5'-TCT GAC CAT CTT CCC TGT CC-3'
DC-STAMP	forward	5'-TCC TCC ATG AAC AAA CAG TTC CA-3'
	reverse	5'-AGA CGT GGT TTA GGA ATG CAG CTC-3'
CTR	forward	5'-TCC AAC AAG GTG CTT GGG AA-3'
	reverse	5'-CTT GAA CTG CGT CCA CTG GC-3'
cathepsin K	forward	5'-CCA GTG GGA GCT ATG GAA GA-3'
	reverse	5'-CTC CAG GTT ATG GGC AGA GA-3'

Western Blot Analysis. Cells were lysed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. Lysates were centrifuged at 14 000g for 20 min, and the protein concentration of the supernatants was determined. Samples were resolved by 8%–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.), which were blocked with 5% skim milk for 1 h and then probed with primary antibodies (1:1000) followed by appropriate secondary antibodies (1:3000, Enzo) conjugated to horseradish peroxidase. Immunoreactivity was visualized using a chemiluminescence detection system (Millipore).

Ca²⁺ Measurements. Isolated BMMs were seeded on 22 × 22 mm coverslips (5 × 10⁴ cells each) and cultured in the presence of M-CSF (30 ng/mL) for 24 h. If necessary, cells were pretreated or directly perfused with RANKL for 24 h along with 100 μ M HAR for the indicated times. Cells were then loaded with 5 μ M Fura-2/AM for 50 min at room temperature, rinsed with bath solution (140 mM NaCl; 5 mM KCl; 1 mM MgCl₂; 10 mM HEPES; 1 mM CaCl₂; 10 mM glucose; and 310 mOsm, pH 7.4) to remove unloaded dye, and then continuously perfused with prewarmed (37 °C) bath solution. To measure the intensity of the Fura-2/AM fluorescence, cells were sequentially excited at 340 and 380 nm, and the fluorescence emitted at 510 nm (ratio = F340/F380) was captured using a charge-coupled device camera. Images were digitized and analyzed with MetaFluor software (Universal Imaging, Bedford Hills, NY, U.S.A.).

LPS-Induced Bone Loss Mouse Model. To examine the effect of HAR on LPS-induced bone destruction, 5-week-old male ICR mice were randomly divided into four groups of five mice each. Mice were treated on day one with PBS (Control), LPS, HAR, or both LPS and HAR (LPS+HAR). HAR (10 mg/kg) and PBS were administered orally 1 day before the first LPS injection (5 mg/kg), and then every other day for 8 days. LPS was injected intraperitoneally on days 2 and 6. All mice were sacrificed on day 10.

OVX-Mediated Bone Loss Mouse Model. To examine the effect of HAR on estrogen deficiency-induced bone loss, 8-week-old female

CS7BL/6 mice were randomly divided into three groups of five mice: sham-operated (SH), OVX control (OVX), and OVX with HAR treatment (OVX+HAR). SH mice were subjected to incision and sutured without removal of the ovaries. Mice of the OVX and OVX +HAR groups were subjected to bilateral ovary removal. After 4 weeks of recovery from surgery, HAR (10 mg/kg) or PBS (control) was administrated orally every other day for 28 days, at which time all mice were sacrificed.

Micro-CT and Histological Analysis. Intact left femur metaphysic regions of each mouse were examined by high-resolution micro-CT analysis (NFR-Polaris-S160; Nanofocus Ray, Iksan, Korea) with a source voltage of 45 kVp, 90 μ A current, and 7 μ m isotropic resolution. Femur scans were performed over a 2 mm distance from the growth plate, with a total of 350 sections per scan. After 3D reconstruction, bone volume per tissue volume (BV/TV), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and trabecular number (Tb.N) were applied to perform quantitative analysis using INFINITT-Xelis software (INFINITT Healthcare, Seoul, Korea). Femurs were fixed in 4% neutral-buffered paraformaldehyde (Sigma) for 1 day, decalcified for 3 weeks in 12% EDTA, and embedded in paraffin. Sections 5 μ m thick were cut using an RM2145 microtome (Leica Microsystems, Bannockburn, IL, U.S.A.). Sections were stained with hematoxylin and eosin for histological examination and other sections were stained for TRAP. Parameters for bone resorption including the number of osteoclasts per field of tissue were quantified using Image Pro-Plus software version 4.0 (Media Cybernetics, Silver Spring, MD, U.S.A.). Nomenclature, symbols, and units used in this study are those recommended by the American Society for Bone Mineral Research (ASBMR) Nomenclature Committee.

Measurements of RANKL, OPG, and CTX-I levels. Serum RANKL and OPG levels were detected using commercial ELISA kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, U.S.A.). Serum CTX-I levels, a specific marker of bone resorption, were determined using a mouse-specific ELISA assay according to the manufacturer's protocol (Nordic Bioscience Diagnostics, Herlev, Denmark).

Statistical Analysis. Each experiment was performed at least three times and all quantitative data are presented as mean \pm SD. Statistical analyses were performed using SPSS (Korean version 14.0; SPSS Inc., Chicago, IL, U.S.A.). Analysis of variance followed by a Tukey posthoc test was used to compare parameters among three groups. $P < 0.05$ was considered statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jnatprod.5b00233](https://doi.org/10.1021/acs.jnatprod.5b00233).

Effect of HAR on cell viability (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Cauley, J. A. *J. Gerontol., Ser. A* **2013**, *68*, 1243–1251.
- (2) Raisz, L. G.; Seeman, E. *J. Bone Miner. Res.* **2001**, *16*, 1948–1952.
- (3) Topini, C.; Topini, D.; Cereria, G.; Nardocci, F.; Topini, G. *Clin. Cases. Miner. Bone. Metab.* **2014**, *11*, 129–131.
- (4) Zaidi, M.; Blair, H. C.; Moonga, B. S.; Abe, E.; Huang, C. L. *J. Bone Miner. Res.* **2003**, *18*, 599–609.
- (5) Tanaka, S.; Nakamura, K.; Takahashi, N.; Suda, T. *Immunol. Rev.* **2005**, *208*, 30–49.
- (6) Arai, F.; Miyamoto, T.; Ohneda, O.; Inada, T.; Sudo, T.; Brasel, K.; Miyata, T.; Anderson, D. M.; Suda, T. *J. Exp. Med.* **1999**, *190*, 1741–1754.
- (7) Carpenter, G.; Ji, Q. *Exp. Cell Res.* **1999**, *253*, 15–24.
- (8) Negishi-Koga, T.; Takayanagi, H. *Immunol. Rev.* **2009**, *231*, 241–256.
- (9) Choi, S. W.; Yeon, J. T.; Park, K. I.; Lee, C. H.; Youn, B. S.; Oh, J.; Lee, M. S. *FEBS Lett.* **2012**, *586*, 263–269.
- (10) Kim, J. Y.; Cheon, Y. H.; Oh, H. M.; Rho, M. C.; Erkhembaatar, M.; Kim, M. S.; Lee, C. H.; Kim, J. J.; Choi, M. K.; Yoon, K. H.; Lee, M. S.; Oh, J. *Bone* **2014**, *60*, 104–111.
- (11) Takayanagi, H. *J. Mol. Med. (Heidelberg, Ger.)* **2005**, *83*, 170–179.
- (12) Lee, Z. H.; Kim, H. H. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 211–214.
- (13) Takeshita, S.; Kaji, K.; Kudo, A. *J. Bone Miner. Res.* **2000**, *15*, 1477–1488.
- (14) Novack, D. V.; Teitelbaum, S. L. *Annu. Rev. Pathol.: Mech. Dis.* **2008**, *3*, 457–484.
- (15) Oursler, M. J. *J. Cell. Biochem.* **2010**, *110*, 1058–1062.
- (16) Kim, J. H.; Kim, K.; Jin, H. M.; Youn, B. U.; Song, I.; Choi, H. S.; Kim, N. *J. Mol. Biol.* **2008**, *383*, 502–511.
- (17) Lee, C. H.; Kwak, S. C.; Kim, J. Y.; Oh, H. M.; Rho, M. C.; Yoon, K. H.; Yoo, W. H.; Lee, M. S.; Oh, J. *J. Pharmacol. Sci.* **2014**, *124*, 344–353.
- (18) Cheon, Y. H.; Song, M. J.; Kim, J. Y.; Kwak, S. C.; Park, J. H.; Lee, C. H.; Kim, J. J.; Kim, J. Y.; Choi, M. K.; Oh, J.; Kim, Y. C.; Yoon, K. H.; Kwak, H. B.; Lee, M. S. *Phytother. Res.* **2014**, *28*, 586–592.
- (19) Kim, J. Y.; Cheon, Y. H.; Yoon, K. H.; Lee, M. S.; Oh, J. *BMB Rep.* **2014**, *47*, 451–456.
- (20) Kwak, S. C.; Lee, C.; Kim, J. Y.; Oh, H. M.; So, H. S.; Lee, M. S.; Rho, M. C.; Oh, J. *Biol. Pharm. Bull.* **2013**, *36*, 1779–1786.
- (21) Kim, J. Y.; Kim, J. Y.; Cheon, Y. H.; Kwak, S. C.; Baek, J. M.; Kim, Y. C.; Yoon, K. H.; Oh, J.; Lee, M. S. *Int. Immunopharmacol.* **2014**, *20*, 213–220.
- (22) Kim, J. Y.; Kim, J. Y.; Kim, J. J.; Oh, J.; Kim, Y. C.; Lee, M. S. *Biol. Pharm. Bull.* **2014**, *37*, 255–261.
- (23) Mncwangi, N.; Chen, W.; Vermaak, I.; Viljoen, A. M.; Gericke, N. *J. Ethnopharmacol.* **2012**, *143*, 755–771.
- (24) Stewart, K. M.; Cole, D. *J. Ethnopharmacol.* **2005**, *100*, 225–236.
- (25) Huang, T. H.; Tran, V. H.; Duke, R. K.; Tan, S.; Chribasik, S.; Roufogalis, B. D.; Duke, C. C. *J. Ethnopharmacol.* **2006**, *104*, 149–155.
- (26) Gokhale, R.; Favus, M. J.; Garrison, T.; Sutton, M. M.; Rich, B.; Kirschner, B. S. *Gastroenterology* **1998**, *114*, 902–911.
- (27) Schoon, E. J.; Blok, B. M.; Geerling, B. J.; Russel, M. G.; Stockbrugger, R. W.; Brummer, R. J. *Gastroenterology* **2000**, *119*, 1203–1208.
- (28) Schett, G.; Kiechl, S.; Weger, S.; Pederiva, A.; Mayr, A.; Petrangeli, M.; Oberholzer, F.; Lorenzini, R.; Redlich, K.; Axmann, R.; Zwerina, J.; Willeit, J. *Arch. Int. Med.* **2006**, *166*, 2495–2501.
- (29) Matsuo, K.; Owens, J. M.; Tonko, M.; Elliott, C.; Chambers, T. J.; Wagner, E. F. *Nat. Genet.* **2000**, *24*, 184–187.
- (30) Milde-Langosch, K.; Roder, H.; Andritzky, B.; Aslan, B.; Hemminger, G.; Brinkmann, A.; Bamberger, C. M.; Loning, T.; Bamberger, A. M. *Breast Cancer Res. Treat.* **2004**, *86*, 139–152.
- (31) Wisdon, R.; Verma, I. M. *Mol. Cell. Biol.* **1993**, *13*, 7429–7438.
- (32) Grigoriadis, A. E.; Wang, Z. Q.; Cecchini, M. G.; Hofstetter, W.; Felix, R.; Fleisch, H. A.; Wagner, E. F. *Science* **1994**, *266*, 443–448.
- (33) Takayanagi, H. *Ann. N. Y. Acad. Sci.* **2007**, *1116*, 227–237.
- (34) Takayanagi, H.; Kim, S.; Koga, T.; Nishina, H.; Isshiki, M.; Yoshida, H.; Saiura, A.; Isobe, M.; Yokochi, T.; Inoue, J.; Wagner, E. F.; Mak, T. W.; Kodama, T.; Taniguchi, T. *Dev. Cell* **2002**, *3*, 889–901.
- (35) Winslow, M. M.; Pan, M.; Starbuck, M.; Gallo, E. M.; Deng, L.; Karsenty, G.; Crabtree, G. R. *Dev. Cell* **2006**, *10*, 771–782.
- (36) Li, X.; Udagawa, N.; Itoh, K.; Suda, K.; Murase, Y.; Nishihara, T.; Suda, T.; Takahashi, N. *Endocrinology* **2002**, *143*, 3105–3113.
- (37) Ikeda, F.; Matsubara, T.; Tsurukai, T.; Hata, K.; Nishimura, R.; Yoneda, T. *J. Bone Miner. Res.* **2008**, *23*, 907–914.
- (38) Miyazaki, T.; Katagiri, H.; Kanegae, Y.; Takayanagi, H.; Sawada, Y.; Yamamoto, A.; Pando, M. P.; Asano, T.; Verma, I. M.; Oda, H.; Nakamura, K.; Tanaka, S. *J. Cell. Biol.* **2000**, *148*, 333–342.
- (39) Wilde, J. I.; Watson, S. P. *Cell. Signalling* **2001**, *13*, 691–701.
- (40) Rebecchi, M. J.; Pentyala, S. N. *Physiol. Rev.* **2000**, *80*, 1291–1335.
- (41) Katan, M. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **1998**, *1436*, 5–17.
- (42) Mao, D.; Epple, H.; Uthgenannt, B.; Novack, D. V.; Faccio, R. J. *Clin. Invest.* **2006**, *116*, 2869–2879.
- (43) Wada, T.; Nakashima, T.; Oliveira-dos-Santos, A. J.; Gasser, J.; Hara, H.; Schett, G.; Penninger, J. M. *Nat. Med.* **2005**, *11*, 394–399.
- (44) Takayanagi, H. *Nat. Rev. Immunol.* **2007**, *7*, 292–304.
- (45) Shinohara, M.; Koga, T.; Okamoto, K.; Sakaguchi, S.; Arai, K.; Yasuda, H.; Takai, T.; Kodama, T.; Morio, T.; Geha, R. S.; Kitamura, D.; Kurosaki, T.; Ellmeier, W.; Takayanagi, H. *Cell* **2008**, *132*, 794–806.
- (46) Mocsai, A.; Humphrey, M. B.; Van Ziffle, J. A.; Hu, Y.; Burghardt, A.; Spusta, S. C.; Majumdar, S.; Lanier, L. L.; Lowell, C. A.; Nakamura, M. C. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 6158–6163.
- (47) Wada, N.; Maeda, H.; Yoshimine, Y.; Akamine, A. *Bone* **2004**, *35*, 629–635.
- (48) Dos Santos, S.; Delattre, A. I.; De Longueville, F.; Bult, H.; Raes, M. *Ann. N. Y. Acad. Sci.* **2007**, *1096*, 70–77.
- (49) Suda, K.; Woo, J. T.; Takami, M.; Sexton, P. M.; Nagai, K. *J. Cell. Physiol.* **2002**, *190*, 101–108.
- (50) Shuto, T.; Jimi, E.; Kukita, T.; Hirata, M.; Koga, T. *Endocrinology* **1994**, *134*, 831–837.
- (51) Agarwal, S.; Piesco, N. P.; Johns, L. P.; Riccelli, A. E. *J. Dent. Res.* **1995**, *74*, 1057–1065.
- (52) Orcel, P.; Feuga, M.; Bielakoff, J.; De Verneuil, M. C. *Am. J. Physiol.* **1993**, *264*, E391–E397.
- (53) Zou, W.; Bar-Shavit, Z. *J. Bone Miner. Res.* **2002**, *17*, 1211–1218.
- (54) Aznar, C.; Fitting, C.; Cavaillon, J. M. *Cytokine+* **1990**, *2*, 259–265.
- (55) Hou, G. Q.; Guo, C.; Song, G. H.; Fang, N.; Fan, W. J.; Chen, X. D.; Yuan, L.; Wang, Z. Q. *Int. J. Mol. Med.* **2013**, *32*, 503–510.
- (56) Hughes, D. E.; Dai, A.; Tiffee, J. C.; Li, H. H.; Mundy, G. R.; Boyce, B. F. *Nat. Med.* **1996**, *2*, 1132–1136.
- (57) Zallone, A. *Ann. N. Y. Acad. Sci.* **2006**, *1068*, 173–179.
- (58) Cenci, S.; Weitzmann, M. N.; Roggia, C.; Namba, N.; Novack, D.; Woodring, J.; Pacifici, R. *J. Clin. Invest.* **2000**, *106*, 1229–1237.
- (59) Tyagi, A. M.; Srivastava, K.; Mansoori, M. N.; Trivedi, R.; Chattopadhyay, N.; Singh, D. *PLoS One* **2012**, *7*, e44552.
- (60) Letari, O.; Nicosia, S.; Chiavaroli, C.; Vacher, P.; Schlegel, W. *J. Immunol.* **1991**, *147*, 980–983.
- (61) Chiang, C. Y.; Veckman, V.; Limmer, K.; David, M. *J. Biol. Chem.* **2012**, *287*, 3704–3709.
- (62) Kertesz, Z.; Gyori, D.; Kormendi, S.; Fekete, T.; Kis-Toth, K.; Jakus, Z.; Schett, G.; Rajnavolgyi, E.; Dobo-Nagy, C.; Mocsai, A. *Eur. J. Clin. Invest.* **2012**, *42*, 49–60.