Full Paper

Genipin Inhibits RANKL-Induced Osteoclast Differentiation Through Proteasome-Mediated Degradation of c-Fos Protein and Suppression of NF- κ B Activation

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Abstract. People over the age of 50 are at risk of osteoporotic fracture, which may lead to increased morbidity and mortality. Osteoclasts are responsible for bone resorption in bone-related disorders. Genipin is a well-known geniposide aglycon derived from *Gardenia jasminoides*, which has long been used in oriental medicine for controlling diverse conditions such as inflammation and infection. We aimed to evaluate the effects of genipin on RANKL-induced osteoclast differentiation and its mechanism of action. Genipin dose-dependently inhibited early stage RANKL-induced osteoclast differentiation in bone marrow macrophages (BMMs) during culture. Genipin inhibited RANKL-induced I κ B degradation and suppressed the mRNA expression of osteoclastic markers such as *NFATc1*, *TRAP*, and *OSCAR* in RANKL-treated BMMs, but did not affect *c-Fos* mRNA expression. Interestingly, genipin markedly inhibited c-Fos protein expression in BMMs, which was reversed in the presence of the proteasome inhibitor MG-132. Furthermore, genipin inhibited RANKL-mediated osteoclast differentiation, which was also rescued by overexpression of *c-Fos* and *NFATc1* in BMMs. Taken together, our findings indicate that genipin down-regulated RANKL-induced osteoclast differentiation through inhibition of c-Fos protein proteolysis as well as inhibition of I κ B degradation. Our findings indicate that genipin could be a useful drug candidate that lacks toxic side effects for the treatment of osteoporosis.

Keywords: genipin, osteoclast, differentiation, c-Fos, NFATc1

Introduction

Osteoporosis is becoming a serious issue in our aging society because it leads to weakened bone structure and fractures in the elderly. In particular, hip fractures are associated with functional impairment, loss of independence, and increased mortality. Thus, careful monitoring for fractures should be provided to postmenopausal

women and those who require steroidal medications to control diseases such as rheumatoid arthritis and chronic obstructive pulmonary disease (1, 2). Under physiological conditions, bone remodeling is maintained by the balanced control of osteoclasts and osteoblasts, where over-activation of osteoclasts is responsible for bone loss. Thus, the mechanisms of most drugs currently used in the treatment of osteoporosis are focused on suppressing osteoclast-mediated bone resorption. Osteoclasts are multinucleated cells that develop from hematopoietic precursors derived from monocyte-macrophage cells, while osteoblasts are derived from mesenchymal stem cells. Direct interaction between receptor activator

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of nuclear factor (NF)- κ B ligand (RANKL), present on the surface of osteoblastic cells, and RANK, present on surface of osteoclasts, is the initial stimulation for osteoclast differentiation (3, 4). RANKL, a member of the tumor necrosis factor (TNF) superfamily, is recognized as the main regulator of osteoclasts and induces key transcription factors, including NF- κ B, c-Fos, and nuclear factor of activated T cells (NFAT) c1, which play a major role in osteoclast differentiation (5). Bisphosphonate has been widely used clinically for the treatment of postmenopausal osteoporosis and steroid-induced osteoporosis. Bisphosphonate exerts its effects by binding to hydroxyapatite in bone tissue, thereby inhibiting osteoclastic activity and inducing apoptosis of osteoclasts (6). In spite of the protective effects of bisphosphonate, its unwanted side effects such as esophagitis and jaw necrosis necessitate the development of new drugs that lack serious side effects and can be used in clinical settings. Genipin, the aglycon of gardenia fruit-derived geniposide, has been traditionally used as a folk medicine in Asia for centuries; and it has recently been reported to have diverse pharmacological functions such as antimicrobial (7), antitumor (8), and anti-inflammatory effects (9, 10). Interestingly, genipin-containing scaffolds have been widely studied for bone tissue engineering due to the ability of this compound to facilitate the proliferation, differentiation, and maturation of osteoblast-like cells (11). However, the effects of genipin on RANKL-induced osteoclast differentiation have not been investigated. Therefore, we examined the effects of genipin on osteoclast differentiation *in vitro*, as well as its signaling pathways.

Materials and Methods

Preparation of geniposide and genipin from Gardenia fructus

The dried fructus of *G. jasminoides* was purchased from an herbal store in Seoul, Korea. Geniposide was isolated by silica gel column chromatography, and genipin was produced by hydrolyzation of geniposide with β -glucosidase, as previously reported (12). The fructus of *G. jasminoides* (18 kg) was extracted with MeOH for 7 d at room temperature. The MeOH extract was concentrated using a rotary evaporator under reduced pressure, resuspended in distilled water, and successively fractionated into hexane, ethyl acetate, and aqueous fractions. Geniposide was purified from the ethyl acetate fraction, which was chromatographed on a silica gel column (silica gel 60; Merck, Darmstadt, Germany) and eluted with a CHCl_3 -MeOH step gradient (100:0, 100:1, 50:1, 30:1, 15:1, 9:1, 5:1, 2:1, 1:1). Fraction F7 was subjected to reverse phase column chromatography

(LiChroPrep RP18; Merck) with step-gradient elution using MeOH/H₂O (5:95, 10:90, 20:80, 40:60, 50:50, 60:40, 80:20, 100:0) to give geniposide. Genipin was produced by treating Geniposide (1.0 g in acetate buffer, pH 5.0) with β -glucosidase (5 mg) for 5 h at 37°C, as previously reported (12). The resulting aglycon was extracted with ether 3 times. The combined extracts were treated with sodium sulfate followed by filtration and concentration *in vacuo*. The concentrates were crystallized in ether to yield genipin. The ¹H-NMR (500 MHz) and ¹³C-NMR (125 MHz) spectra were obtained on a JEOL ECS400 spectrometer (Jeol, Tokyo), with CD₃OD and pyridine-*d*₅ as solvents. The ESI-MS was determined using an Agilent 6430 LC/MS/MS and 1100 LC/MS spectrometer (Agilent, Palo Alto, CA, USA).

Mice and reagents

Five-week-old male ICR mice were purchased from Damul Science (Daejeon, Korea). The mice were maintained at a controlled temperature (22°C – 24°C) and humidity (55% – 60%) range with 12-h light/dark cycles. All experiments in this study were performed in accordance with the animal experiment guidelines of the Institute Committee of Wonkwang University. Recombinant soluble human M-CSF and human RANKL were obtained from PeproTech EC, Ltd. (London, UK). Cycloheximide (CHX) and MG-132 were obtained from Calbiochem (San Diego, CA, USA). Anti-p38, anti-phospho-p38, anti-ERK, anti-phospho-ERK, anti-Akt, anti-phospho-Akt, anti-JNK, anti-phospho-JNK, anti-I κ B, and anti-phospho-I κ B antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-c-Fos and anti-NFATc1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal β -actin antibody was obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), α -minimum essential medium (α -MEM), and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals were of an analytical grade or complied with the standards required for cell culture experiments.

Mouse bone marrow macrophage preparation and osteoclast differentiation

Bone marrow cells (BMCs) were obtained by flushing the femurs and tibiae of 5-week-old ICR mice with α -MEM and suspended in α -MEM supplemented with 10% FBS. Non-adherent cells were collected and cultured for 3 d in the presence of M-CSF (30 ng/ml). Floating cells were discarded and the adherent cells were classified as bone marrow-derived macrophages (BMMs). BMMs were seeded at 3.5×10^4 cells/well in α -MEM/10% FBS and were cultured in the presence

of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 d in the presence or absence of genipin. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) activity, as described below. TRAP-positive multinucleated cells with greater than three nuclei per cell were counted as osteoclasts.

Cytotoxicity assay

BMMs were plated in 96-well plates at a density of 1×10^4 cells/well in triplicate. Cells were treated with M-CSF (30 ng/ml), and increasing concentrations of genipin were added to the mix. After 3 d, XTT reagent (50 μ l) was added to each well. Wells were incubated for 4 h and optical density at 450 nm was determined with an ELISA reader.

Quantitative real-time RT-PCR analysis

Total RNA was isolated using the QIAzol reagent (Qiagen, Valencia, CA, USA) 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 produced by incubating at 42°C for 50 min with the first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 100 mM DTT, RNase inhibitor, and Superscript II reverse transcriptase (Invitrogen). The cDNA was amplified using the following primer sets: *c-Fos*, 5'-GGTGAAGACCGTGTCAGGAG-3' (forward) and 5'-TATTCCGTTCCCTTCGGATT-3' (reverse); *NFATc1*, 5'-GAGTACACCTTCCAGCACCTT-3' (forward) and 5'-TATGATGTCGGGGAAGAGA-3' (reverse); *TRAP*, 5'-TCATGGGTGGTGCTGCT-3' (forward) and 5'-GCCCACAGCCACAAATCT-3' (reverse); *OSCAR*, 5'-GGAATGGTCCTCATCTGCTT-3' (forward) and 5'-GGAATGGTCCTCATCTGCTT-3' (reverse); and *GAPDH*, 5'-TCAAGAAGGTGGTGAAGCAG-3' (forward) and 5'-AGTGGGAGTTGCTGTTGAAGT-3' (reverse). Real-time RT-PCR was conducted using 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 forward primer, 10 pmol reverse primer, and 1 μ g cDNA. The amplification parameters consisted of an initial denaturation at 95°C for 5 min followed by 40 cycles of a 3-step PCR (denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min). The fluorescence resulting from the incorporation of SYBR Green dye into the double-stranded DNA produced during the PCR was quantified using the threshold cycle (C_t) value. Relative levels of *c-Fos*, *NFATc1*, *TRAP*, and *OSCAR* were normalized to that of *GAPDH*.

Western blot analysis

Cells were lysed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. The lysates were centrifuged at $14,000 \times g$ for 20 min and supernatants were collected. Protein concentrations of supernatants were determined and cellular proteins (30 μ g) were resolved by 8%–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Non-specific interactions were blocked with 5% skim milk for 1 h and then probed with the appropriate primary antibodies. Membranes were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase, and immunoreactivity was detected with enhanced chemiluminescence reagents (Millipore). Densitometric values were quantified for each band with the Image Pro-plus program (version 4.0).

Retrovirus preparation and infection

Retroviral vector [pMX-IRES-EGFP, pMX-*c-Fos*-IRES-EGFP, and pMX-constitutively active (*CA*)-*NFATc1*-IRES-EGFP] packaging was performed by transient transfection of the pMX vectors into Plat-E retroviral packaging cells using X-tremeGENE 9 (Roche, Nutley, NJ, USA) according to the manufacturer's protocol. After incubation in fresh medium for 2 d, the culture supernatants of the retrovirus-producing cells were collected. For retroviral infection, nonadherent BMCs were cultured in M-CSF (30 ng/ml) for 2 d. The BMMs were incubated with viral supernatants from pMX-IRES-EGFP, pMX-*c-Fos*-IRES-EGFP, and pMX-*CA-NFATc1*-IRES-EGFP virus-producing Plat-E cells together with polybrene (10 μ g/ml) and M-CSF (30 ng/ml) for 6 h. Retroviral infection efficiency was determined by green fluorescent protein expression and was always > 80%. After infection, the BMMs were induced to differentiate in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 d. The expression of each construct and osteoclast formation was detected using a fluorescence microscope and TRAP staining, respectively.

Bone resorption assay

Mature osteoclasts were prepared from the BMC and primary osteoblast co-culture. BMC (1×10^7 cells) and primary osteoblasts (1×10^6 cells) were seeded on collagen gel-coated culture dishes and cultured for 7 days in the presence of 10^{-8} M 1,25-dihydroxyvitamin D₃ (Sigma) and 10^{-6} M prostaglandin E₂ (PGE₂) (Sigma). The co-cultured cells were detached by 0.1% collagenase

treatment at 37°C for 10 min and were then replated on hydroxyapatite-coated plates (Corning, NY, USA). The cells were incubated on the plates with or without genipin. After 12 h, the cells were removed and the total resorption pits were photographed and analyzed using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistical analyses

Each experiment was performed at least 3 times, and all quantitative data are presented as the mean $\pm$ standard deviation (S.D.). All statistical analyses were performed using SPSS (Korean version 14.0). The Student's *t*-test was used to compare the parameters between 2 groups, while the analysis of variance (ANOVA), followed by the Tukey post-hoc test, was used to compare the parameters among 3 groups. $P < 0.05$ was considered statistically significant.

Results

Genipin inhibits RANKL-mediated osteoclast differentiation in BMMs

To evaluate the effects of genipin on osteoclastogenesis, we treated primary BMMs with genipin in the presence of RANKL and M-CSF. While the BMMs of the control group differentiated into mature TRAP-positive multinucleated osteoclasts, the formation and numbers of TRAP-positive multinucleated cells were dose-dependently reduced by genipin treatment (Fig. 1: B and C). To investigate the differential, time-dependent effects of genipin on RANKL-mediated osteoclast differentiation, BMMs were challenged with genipin at various time points after RANKL treatment. The results showed that the inhibitory effect of genipin on osteoclast differentiation was exerted when BMM cells had been treated with genipin in the early stage after RANKL stimulation. However, the inhibitory effects of genipin on osteoclastogenesis were absent when treatment occurred 3 d after RANKL stimulation (Fig. 2: A and B). Next, we performed the XTT assay to exclude the possibility that the inhibition was due to reduced viability and/or proliferation of the osteoclast precursor cells. The results revealed that genipin did not exert cytotoxic effects at doses that effectively inhibited osteoclast differentiation (Fig. 1D).

Genipin inhibits NF- κ B activation during RANKL-mediated osteoclastogenesis in BMMs

RANKL activates several transducers involved in osteoclastogenesis, including p38, ERK, JNK, Akt, and NF- κ B, which are important transcriptional factors in osteoclast differentiation. To elucidate the mechanism

of inhibitory effects of genipin on RANKL-induced osteoclast differentiation, we investigated the relevant signaling pathways, including p38, ERK, JNK, Akt, and the transcriptional factor NF- κ B. Signaling pathways were observed after osteoclast precursors were pretreated with genipin for 1 h and subsequently stimulated with RANKL at different dose or time point. We found that RANKL-mediated induction of phosphorylation of p38, ERK, JNK, and Akt were not affected by genipin (Fig. 3). In contrast, the degradation of I κ B was significantly and dose dependently inhibited by genipin (Fig. 3A), and phosphorylation of I κ B was suppressed by genipin in a time-dependent manner (Fig. 3B).

Genipin inhibits osteoclast differentiation through c-Fos protein degradation and NFATc1 suppression

c-Fos and NFATc1 play key roles in osteoclast differentiation and regulate the expression of osteoclast-specific genes such as *OSCAR* and *TRAP*. Thus, we explored whether genipin affected the mRNA expression of *c-Fos*, *NFATc1*, *OSCAR*, and *TRAP* during RANKL-induced osteoclastogenesis. Osteoclast precursors were pretreated with genipin and stimulated with RANKL as before. We found that mRNA expression of *NFATc1*, *TRAP*, and *OSCAR* was significantly inhibited by genipin, but not *c-Fos* mRNA expression (Fig. 4A). As expected, *NFATc1* protein levels were increased in response to RANKL and were significantly inhibited by genipin. Interestingly, RANKL-induced c-Fos protein expression was significantly inhibited by genipin (Fig. 4B). Thus, to elucidate the mechanism of genipin inhibition of c-Fos protein levels, despite unchanged c-Fos mRNA expression, we determined whether genipin inhibits the translation of c-Fos mRNA or induces degradation of c-Fos protein by treating with CHX, an inhibitor of new protein biosynthesis, and MG-132, a selective inhibitor of the 26S proteasome. Genipin-induced suppression of c-Fos protein expression was reversed by MG-132 treatment, which suggests that proteasome-mediated degradation is responsible for the effect of genipin on c-Fos protein expression (Fig. 4C).

Induction of c-Fos and NFATc1 expression reverses genipin-induced inhibition of RANKL-mediated osteoclastogenesis

To further examine whether the reduction of c-Fos protein is related to the inhibitory effects of genipin on RANKL-induced osteoclastogenesis, we overexpressed the *c-Fos* gene in BMMs using a retroviral system. The inhibitory effects of genipin were partially reversed by c-Fos overexpression. In addition, we overexpressed the *CA-NFATc1* in BMMs, which also reversed the genipin-mediated inhibition of osteoclast differentiation

Fig. 1. Genipin inhibits RANKL-induced osteoclast differentiation. A) Chemical structure of genipin. B) BMMs were cultured for 4 d with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the absence (control) or presence of varying concentrations of genipin. Cells were fixed with 3.7% formalin, permeabilized with 0.1% Triton X-100, and stained with TRAP solution. Mature TRAP-positive multinucleated osteoclasts (MNCs) were photographed under a light microscope (Magnification $\times 100$). C) TRAP-positive cells were counted as osteoclasts. D) BMMs were cultured for 3 d at the indicated doses of genipin in the presence of M-CSF (30 ng/ml). Cell viability was determined using the XTT assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the control.

Fig. 2. Genipin exerts its inhibitory effects in the early stage of RANKL-induced osteoclast differentiation. A) Genipin treatment was performed at the indicated time point for 1 d with M-CSF (30 ng/ml) and RANKL (100 ng/ml) and TRAP staining performed. Mature TRAP-positive multinucleated osteoclasts (MNCs) were photographed under a light microscope (Magnification $\times 100$). B) TRAP-positive cells were counted as osteoclasts. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the control.

Fig. 3. Genipin inhibits RANKL-mediated NF- κ B activation. BMMs were cultured for 1 d with M-CSF (30 ng/ml). A) BMMs were pretreated with DMSO (control) or genipin (indicated dosage) for 1 h in the presence of M-CSF (30 ng/ml) and subsequently stimulated with RANKL (100 ng/ml) for 5 m. B) BMMs were pretreated with or without genipin (15 μ g/ml) for 1 h prior to RANKL (100 ng/ml) stimulation at the indicated time. Whole-cell lysates were analyzed by western blotting with the indicated antibodies.

(Fig. 5: A, B). These results suggest that genipin suppresses RANKL-mediated osteoclastogenesis in BMMs through the inhibition of c-Fos and NFATc1 expression.

Genipin inhibited the bone-resorbing activity of mature osteoclasts in vitro

We next examined whether genipin inhibits osteoclast function. To investigate whether genipin affects bone-resorbing activity, mature osteoclasts were cultured on hydroxyapatite-coated plates. After 12 h, numerous resorption pits had been generated by mature osteoclasts in the vehicle. Genipin dose-dependently inhibited the area of the resorption pits (Fig. 6), suggesting that genipin inhibits osteoclast differentiation and decreases the bone-resorption activity of mature osteoclasts.

Discussion

Genipin is an aglycon derived from geniposide, which is present in the fruit of *Gardenia jasminoides* Ellis. Genipin is known as a natural cross-linker of proteins, collagen, gelatin, and chitosan. It cross-links with less toxicity than many other commonly used synthetic cross-linking reagents, and its usefulness has been widely evaluated for a large variety of biomedical applications, such as in graft materials (13), corneal implants (14),

and drug delivery (15). Several studies have reported that genipin has anti-inflammatory effects. Genipin inhibits NO production and iNOS expression upon stimulation by lipopolysaccharide/interferon- γ (IFN- γ) in RAW 264.7 cells, a murine macrophage cell line (16), and shows anti-inflammatory effects in a model of LPS-induced acute systemic inflammation (9, 17). We examined the effects of genipin on RANKL-induced osteoclastogenesis to investigate the possibility that it could be a candidate for treating osteoporosis through the regulation of osteoclast differentiation. In the present study, we have shown that genipin significantly inhibited the early stage RANKL-induced osteoclast differentiation without detectable cytotoxicity (Fig. 1). An important initial step in osteoclastogenesis is the interaction between RANKL and its receptor RANK, which leads to activation of various downstream signaling pathways, including p38, ERK, JNK, Akt, and NF- κ B, to induce the expression of osteoclastogenesis-related genes such as *c-Fos*, *NFATc1*, *OSCAR*, and *TRAP* (18). In this study, the major intracellular signaling pathways (JNK, p38 MAPK, and Akt) influencing RANKL-mediated osteoclastogenesis were not affected by genipin. However, genipin inhibited the phosphorylation of I κ B, which leads to NF- κ B (a crucial transcriptional factor for osteoclast differentiation) activation (Fig. 3). Both

Fig. 4. Genipin suppresses RANKL-induced NFATc1 expression in BMMs. **A)** BMMs were stimulated with RANKL (100 ng/ml) and M-CSF (30 ng/ml) in the presence or absence of genipin (15 μ g/ml) for the indicated durations. Total RNA was isolated from cells using QIAzol reagent, and mRNA expression levels were evaluated by real-time RT-PCR. ** $P < 0.01$, *** $P < 0.001$ vs. the control (DMSO); ### $P < 0.001$ vs. the control at 48 h. **B)** Effects of genipin on c-Fos and NFATc1 protein expression levels were evaluated using western blot analysis. β -Actin was used as the internal control. **C)** Plat-E cells were transfected with c-Fos (2 μ g) and subsequently treated with DMSO or genipin (15 μ g/ml). After 48 h of transfection, 2 μ g/ml CHX and 5 μ M MG-132 were added to the cultures 4 h before harvest. c-Fos protein levels were detected by western blot analysis.

p50 and p52 double knockout mice demonstrated osteoclastogenesis defects and severe osteopetrosis, indicating that NF- κ B is crucial factor in osteoclast differentiation (19). I κ B is attached to NF- κ B, preventing it from migrating into the nucleus, and phosphorylation with I κ B kinase (IKK) separates the two proteins. Subsequent ubiquitination and proteasome degradation of I κ B allows the transfer of NF- κ B into the nucleus and transcription of the target gene (20). Our results are consistent with RANKL activation of NF- κ B in osteoclastic precursor cells through IKK activation, and degradation. Genipin inhibited RANKL-induced IKK activation, leading to suppression of NF- κ B activation. Also, it has been reported that inhibitors of NF- κ B activation prevent the osteoclast formation induced directly or indirectly by RANKL in the early stages of osteoclast precursor differentiation, rather than during later stages (21). As shown in Fig. 2, the inhibitory

effects of genipin on early stage osteoclastogenesis may be related to inhibition of the NF- κ B pathway. In particular, two key transcription factors, *c-Fos* and *NFATc1*, are known to play an essential role during RANKL-induced osteoclastogenesis (5, 22). RANKL signaling induces binding of c-Fos to the NFATc1 promoter, which in turn induces the expression of NFATc1. It is well established that c-Fos is a major component of the AP-1 transcription factor complex, which includes members of the Jun family (23). Importantly, *c-Fos*-deficient mice develop osteoporosis with deficiencies in bone remodeling and tooth eruption (24, 25). As shown in Fig. 4, genipin significantly suppressed the RANKL-induced c-Fos and NFATc1 protein expression, without inducing marked changes in c-Fos mRNA expression. It is already known that c-Fos is a short-lived, unstable protein that can be ubiquitinated and targeted to the proteasome-dependent proteolytic

Fig. 5. Overexpression of c-Fos and NFATc1 partially reversed the suppressive effects of genipin. BMMs were infected with retroviruses expressing pMX-IRES-EGFP (pMX), pMX-*c-Fos*-IRES-EGFP, and pMX-*CA-NFATc1*-IRES-EGFP. Infected BMMs were cultured with or without genipin (15 μ g/ml) in the presence of RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 4 d. Thereafter, the cells were fixed and stained for TRAP (top). Mature TRAP-positive multinucleated osteoclasts (MNCs) were photographed under a light microscope (Magnification $\times$ 100). TRAP-positive osteoclasts generated from control BMMs (white column), c-Fos-transduced BMMs (gray column), and NFATc1-transduced BMMs (black column) were counted and measured (bottom). ** P < 0.01, *** P < 0.001.

Fig. 6. Genipin inhibits osteoclastic bone resorption. Mature osteoclasts were seeded on hydroxyapatite-coated plates and treated for 12 h with the indicated concentrations of genipin. Attached cells on the plates were removed and photographed under a light microscope (A). Pit areas were quantified using ImageJ (B). * P < 0.05, *** P < 0.001 vs. the control (DMSO).

pathway (26). Previously, Ito et al. reported that c-Fos protein gradually accumulates during osteoclast differentiation over a span of approximately 2 days post-RANKL stimulation in osteoclast progenitors and that the proteasome pathway is the predominant mechanism for c-Fos degradation in osteoclast progenitors, which plays an important role in the molecular regulatory mechanism of osteoclast differentiation (27). In the present study, we used CHX and MG-132 to confirm that the genipin-mediated inhibition of c-Fos protein expression was not due to translational inhibition of c-Fos, but rather due to a proteasome-mediated degradation. Furthermore, induction of c-Fos or NFATc1 expression partially rescued the genipin-induced suppression of osteoclastogenesis, which indicates that down-regulation of c-Fos is responsible for the inhibitory effects of genipin. Our results suggested that genipin exerts its anti-osteoclastogenic effect by regulating c-Fos protein stability through the induction of proteasomal degradation during osteoclastogenesis. However, further studies are required to determine the mechanism by which genipin acts as an activator of the ubiquitination–proteasome pathway, which leads to proteolytic degradation of c-Fos protein in RANKL-induced osteoclastogenesis.

To infer the *in vivo* effects from *in vitro* activities, knowledge of the metabolism and pharmacokinetics of genipin in animals is essential. Previous studies reported that orally administered genipin (200 mg/kg) resulted in a 78% mortality rate in rats (28) and that the long-term use of genipin/geniposide appears to be associated with mesenteric phlebosclerosis (29). Although our *in vitro* study suggested that cytotoxicity of genipin was not detected, some problems such as toxicity or side effects remain, which may limit its clinical usage. Genipin exerts extensive beneficial effects that offer potential therapeutic benefit to patients with bone-loss disease according to the results from *in vitro* studies. Ongoing studies are being conducted to further explore these properties. Further studies will be focused on both short- and long-term effects of genipin by *in vivo* studies.

Taken together, our results clearly show that genipin has inhibitory effects on RANKL-induced osteoclastogenesis via suppression of NF- κ B activation and proteasome-mediated c-Fos degradation. Hence, we suggest that genipin could be a therapeutic candidate for the treatment of postmenopausal osteoporosis and the inflammatory bone loss observed in conditions such as rheumatoid arthritis and periodontitis.

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Conflicts of Interest

The authors have no financial conflicts of interest.

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