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Corrigendum

Corrigendum to "Effects of gallotannin on osteoclastogenesis and the p38 MAP kinase pathway" [Orthod. Waves 75 (2016) 105–113]

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The authors regret the incorrect spelling of "Gllotannin" in the horizontal axis title of Fig. 3B and vertical axis title of Fig. 3C. The correct spelling is "Gallotannin".

The authors would like to apologise for any inconvenience caused.

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Original article

Effects of gallotannin on osteoclastogenesis and the p38 MAP kinase pathway

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ABSTRACT

Purpose: Osteoclasts are multinucleated giant cells that specialize in bone resorption and work together with bone-forming osteoblasts to maintain bone homeostasis. However, excessive osteoclast activation accounts for bone diseases, such as osteoporosis and periodontitis. In previous studies, natural small-molecule compounds have been shown to regulate osteoclastogenesis and osteoclast functions. Here we demonstrate that gallotannin, a hydrolyzable plant tannin, suppresses osteoclast differentiation.
Methods: We first used an *ex vivo* bone marrow culture system containing both osteoclast precursors and surrounding cells, thereby resembling physiological conditions, to evaluate the suppressive effect of gallotannin. We also used a RANKL-induced osteoclastogenesis assay containing only osteoclast precursors to confirm the suppressive effect of gallotannin in the absence of effects from other cells.
Results: The suppressive effect of gallotannin was associated with the reduced RANKL-mediated induction of NFATc1, a critical transcription factor involved in osteoclast differentiation. We further confirmed that gallotannin reduced the p38 MAPK pathway activation, which is mediated by M-CSF and RANKL. This pathway suppression might underlie the suppression of NFATc1 production and subsequent reduction in osteoclast differentiation.
Conclusion: Our data indicate that the natural small-molecule compound gallotannin might be useful as a novel anti-bone resorptive agent.

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1. Introduction

The normal adult bone undergoes continuous formation and degradation to maintain skeletal homeostasis. Accordingly, an imbalance between osteoblast-mediated bone formation and osteoclast-mediated bone degradation results in bone diseases, such as osteoporosis, rheumatoid arthritis, and periodontitis [1]. The development and activation of osteoclasts, multinucleated giant cells that arise from monocyte-lineage hematopoietic cells, are tightly regulated by the surrounding cells, including osteoblasts, osteocytes, and immune cells [2].

The receptor activator of nuclear factor kappa-B ligand (RANKL) and monocyte colony-stimulating factor (M-CSF) are the key cytokines involved in osteoclast differentiation and function. A deficiency in signaling mediated by either cytokine results in severe osteopetrosis *in vivo* [3,4], and the presence of both cytokines is sufficient to induce the differentiation of osteoclast precursors into osteoclasts *in vitro* [5]. Although M-CSF is constitutively produced by osteoblasts, RANKL is produced by osteoblasts only in response to osteotropic factors [6]. The interaction of RANKL with its cognate receptor, RANK, activates downstream signaling pathways, such as the NF-κB, p38, ERK, JNK, and Akt pathways, thereby inducing the expression of osteoclastogenic transcription factors, such as c-fos, MITF, and NFATc1. These molecules are considered to be key targets in the regulation of osteoclast differentiation and activation [7].

A previous study has demonstrated that natural small-molecule compounds with unique pharmacological activities can provide beneficial effects in the context of human medicine [8]. Ellagitannins and gallotannins, the two subclasses of hydrolyzable tannins, are examples of such compounds that are widely distributed throughout the plant kingdom (*e.g.*, in beans, fruits, vegetables, and nuts) [9]. Ellagitannin and gallotannin are polyesters of glucose with organic acids which are ellagic acid and gallic acid, respectively [9]. Previous study has described the ellagitannin-mediated suppression of RANKL-induced osteoclastogenesis *via* the suppression of p38, JNK, and AP-1 activation [10]. Recently, ellagic acid, the acid component of ellagitannin, was also reported to suppress osteoclast differentiation and function [11]; in other words, both tannins and their acid components might regulate osteoclast differentiation. Gallotannin, the simplest hydrolyzable tannin, exhibits various biological effects, including anti-cancer [12,13] and anti-inflammatory effects [14,15], as well as protective effects against atherosclerosis [16], fatty diet-induced diabetes [17], and diabetic nephropathy [18]. However, the effects of gallotannin on osteoclast differentiation have not yet been characterized.

In the present study, we examined the effects of gallotannin on osteoclast differentiation *in vitro*. We found that gallotannin could suppress osteoclast differentiation in both bone marrow (BM) and bone marrow macrophage (BMM) culture systems. These suppressive effects of gallotannin were associated with decreased RANKL-induced NFATc1 expression in gallotannin-treated osteoclast precursors. Furthermore, we found that gallotannin treatment reduced the activation of p38 MAP kinase in RANKL- and M-CSF-treated osteoclast precursors.

2. Materials and methods

2.1. Ethics

This study was approved by the Institutional Animal Care and Use Committee of Osaka Dental University.

2.2. Cell culture

The ST-2 osteoblastic cell line was obtained from the RIKEN BioResource Center (Tsukuba, Japan) and cultured in α-Modified Eagle's Medium (MEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium).

For BMM preparation, BM was isolated from the tibiae and femora of 6-week-old male ddY mice. Following red blood cell elimination with RBC lysis buffer (BioLegend, San Diego, CA, USA), the BM cells were cultured in complete medium supplemented with 25 ng/mL M-CSF (BioLegend) in a non-tissue culture dish (Sarstedt, Nümbrecht, Germany) for 5–7 days. For further experiments, BMMs were re-plated onto non-tissue culture plates (Sarstedt) prior to flow cytometry or onto tissue culture plates (TPP, Trasadingen, Switzerland) for all other experiments. Cells were counted by hemocytometer in the presence of trypan blue, then calculated the total cell number.

2.3. Osteoclast differentiation

Osteoclast differentiation was assessed by TRAP staining [19]. Briefly, the cells were fixed with 10% formalin and acetone–methanol (1:1), and subsequently incubated with TRAP staining buffer containing 0.1 mg/mL naphthol AS-MX phosphate (Sigma–Aldrich, St. Louis, MO, USA), 0.5% *N*,*N*-dimethylformamide (Wako), and 0.6 mg/mL fast red violet LB salt (Sigma–Aldrich) in 0.1M sodium acetate buffer pH 5.0 with 50 mM sodium tartrate (Wako). TRAP-positive multi nucleated (more than three nuclei) cells were considered osteoclasts.

To induce osteoclast differentiation in a BM culture system, isolated red blood cell-free BM cells were cultured for 7 days in 96-well plates at a density of 6×10^3 cells/well in the presence of 50 nM 1,25 dihydroxyvitamin D_3 (1,25$(OH)_2D_3$; Santa Cruz Biotechnology, Dallas, TX USA). To induce RANKL-mediated osteoclast differentiation, BMMs were stimulated for 3 days in 96-well plates at a density of 1×10^4 cells/well in the presence of 25 ng/mL M-CSF and 50 ng/mL RANKL (Wako). Gallotannin (Santa Cruz Biotechnology) was added to both culture systems at concentrations of 0.1, 1, 10, and 100 nM.

2.4. Flow cytometry (FACS)

In preparation for FACS analysis, adherent cells were washed with PBS, exposed to accutase (Nacalai Tesque, Kyoto, Japan) for 5 min to induce detachment from the culture plate, and immediately washed with FACS buffer (phosphate-buffered saline [PBS] supplemented with 2% calf serum and 0.1% azide).

ST-2 cells were cultured for 3 days in a 6-well plate at a density of 1×10^5 cells/well in the presence of 50 nM 1,25$(OH)_2D_3$ and 50 nM dexamethasone (Wako). Subsequently, the

RANKL expression levels in these cells were assessed using an anti-RANKL antibody (IK22/5, BioLegend). Data were collected on a FACSVerse (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software, version 10.1 (Tree Star, Ashland, OR, USA).

2.5. *Cell proliferation assay*

BMMs cultured in complete medium supplemented with 25 ng/mL M-CSF and 0.1, 1, 10, or 100 nM gallotannin for 24 h were subsequently exposed to 10 μM BrdU (Sigma) for 1 h. The cells were then collected and fixed with pre-chilled 70% ethanol for 20 min at room temperature. Fixed cells were treated with 2 M HCl for 20 min at room temperature to denature DNA, followed by neutralization in 0.1 M $Na_2B_4O_7$ for 2 min. Finally, the cells were incubated with a PE-conjugated anti-BrdU antibody (Bu20a, BioLegend) for 30 min at room temperature. BrdU incorporation was assessed by FACS, and BrdU-positive populations were considered to be proliferating cells.

2.6. *Cell viability assay*

BMMs were cultured in complete medium supplemented with 25 ng/mL M-CSF and 0.1, 1, 10, or 100 nM gallotannin for 24 h. The cells were collected and washed with Annexin V binding buffer (Cayman Chemical, Ann Arbor, MI, USA) and subsequently incubated with APC-conjugated Annexin V (BioLegend) for 15 min. Annexin V binding was assessed by FACS, and Annexin V-negative cells were considered live cells.

2.7. *Western blotting*

BMMs were stimulated with 25 ng/mL M-CSF and 50 ng/mL RANKL for 3 days in a 6-well plate at a density of 1.5×10^5 cells/well prior to the detection of osteoclastogenic transcription factors. To detect the RANKL-induced activation of intracellular signaling molecules, BMMs were cultured overnight in a 6-well plate at a density of 3×10^5 cells/well in the presence of 25 ng/mL M-CSF, followed by stimulation with 50 ng/mL RANKL for the indicated time periods. To detect the M-CSF-induced activation of intracellular signaling molecules, BMMs were cultured for 24 h at a density of 3×10^5 cells/well in the presence of low-dose M-CSF (10 ng/mL) and 50 ng/mL RANKL, followed by stimulation with high-dose M-CSF (50 ng/mL) for the indicated time periods. The cells were lysed directly in sample buffer, sonicated, and boiled for 3 min. Lysates were loaded onto 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (3×10^4 cell equivalents per lane) for protein separation. After transfer of the proteins to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), the membranes were blocked with 5% bovine serum albumin (BSA; Wako) in Tris-buffered saline + Tween-20. Subsequently, the membranes were incubated with anti-NFATc1 (clone: 7A6, Santa Cruz Biotechnology), anti-c-fos (#4384, Cell Signaling Technology [CST], Beverly, MA, USA), anti-β-actin (clone: 2F3, Wako), anti-phospho p38 (#9216, CST), anti-p38 (#9212, CST), anti-phospho ERK1/2 (#9106, CST) and anti-ERK1/2 (#9102, CST) antibodies. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (ECL anti-mouse IgG or anti-rabbit IgG as appropriate; GE Healthcare, Little Chalfont, UK). Finally, the membranes were incubated with a chemiluminescent substrate (SuperSignal West Pico, Thermo, Rockford, IL, USA) and the resulting chemiluminescent signal was detected with a ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

2.8. *Statistical analysis*

All statistical analyses were performed with Prism software 6.0 for Mac OS X (GraphPad, Inc., La Jolla, CA, USA). A one-way analysis of variance (ANOVA) and subsequent Dunnett's multiple comparison tests were used to assess significance.

3. Results

3.1. *Effects of gallotannin on osteoclast differentiation in a BM culture system*

BM cells comprise both hematopoietic and mesenchymal cell populations, which respectively include osteoclast precursors and osteoblast lineage stromal cells. Because osteoblast lineage stromal cells produce RANKL in response to osteotropic factors, BM cell culture in the presence of $1,25(OH)_2D_3$ leads to the generation of osteoclasts [20]. We therefore employed this *ex vivo* BM culture system to investigate the effect of gallotannin on osteoclast differentiation. We observed that the addition of $1,25(OH)_2D_3$ to our BM culture system resulted in the generation of TRAP-positive multinucleic osteoclasts, whereas osteoclastogenesis was suppressed in the presence of gallotannin in a dose-dependent manner (Fig. 1A and B).

Because $1,25(OH)_2D_3$-mediated RANKL expression on osteoblast lineage stromal cells is critical for osteoclast differentiation in a BM culture system [5,21], we examined the effect of gallotannin on RANKL expression on osteoblast lineage cells. For these experiments, we used the ST-2 osteoblastic cell line, which produces RANKL in response to $1,25(OH)_2D_3$ and dexamethasone [22]. $1,25(OH)_2D_3$ and dexamethasone induced the surface expression of RANKL on ST-2 cells, and this expression was not affected by treatment with gallotannin (Fig. 1C). These results suggest that gallotannin inhibits osteoclast differentiation by suppressing the ability of osteoclast precursors to differentiate into osteoclasts.

3.2. *Effects of gallotannin on the RANKL-induced osteoclast differentiation of BMMs*

As gallotannin appeared to directly affect the ability of osteoclast precursors to differentiate into osteoclasts, we employed an *in vitro* RANKL-induced osteoclastogenesis assay, using BMMs as osteoclast precursors [23]. Here, we observed that gallotannin treatment suppressed the RANKL-induced osteoclast differentiation of BMMs (Fig. 2A and B).

According to a previous report, the osteoclast precursor density affects the ability of these cells to differentiate into osteoclasts *in vitro* [24]. Accordingly, we examined the effects of gallotannin on the proliferative activity and viability of BMMs. First, we employed a BrdU incorporation assay to investigate the effects on proliferative activity. Notably,

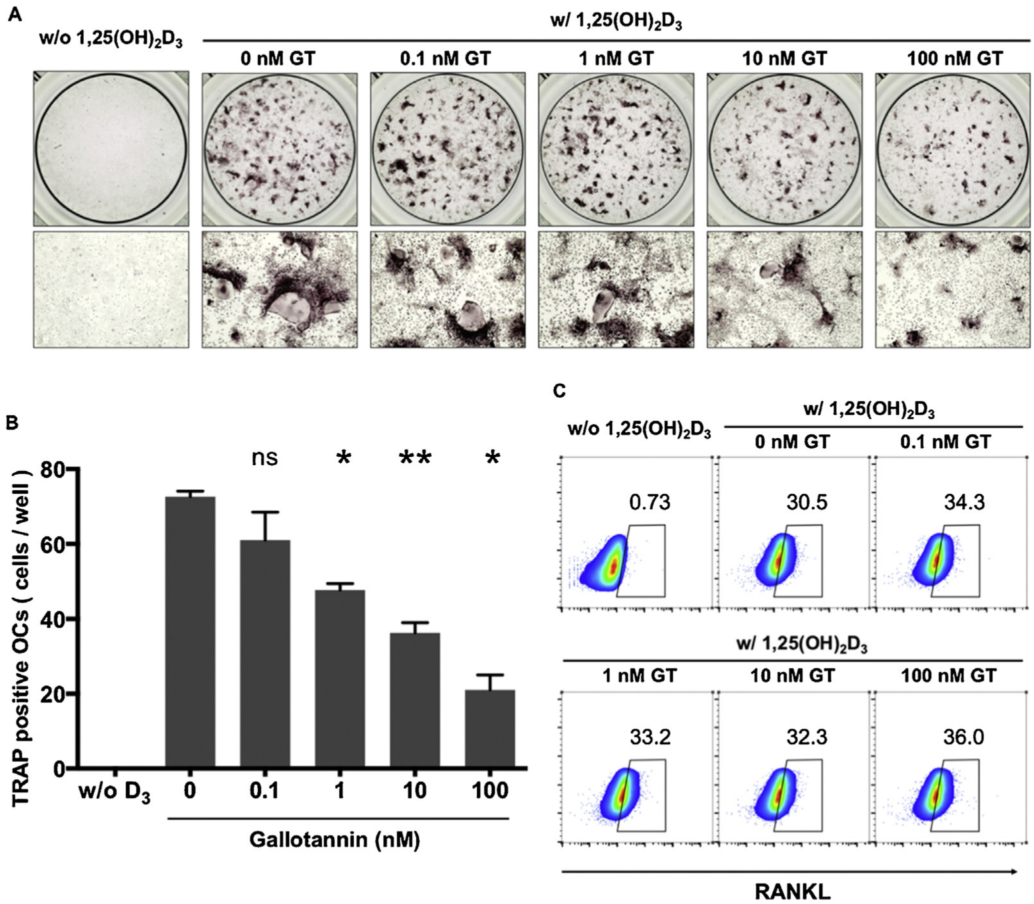

Fig. 1 – Gallotannin suppresses $1,25(OH)_2D_3$-mediated osteoclast differentiation in a bone marrow cell (BM) culture. (A and B) BM cells were cultured for 7 days in the presence of 50 nM $1,25(OH)_2D_3$ and increasing concentrations of gallotannin. Representative images show a TRAP-stained BM culture (A) and the number of TRAP-positive cells containing more than three nuclei. Data were obtained from three independent experiments. Columns and bars represent the means and standard errors of the means; $^{*}p < 0.05$ and $^{**}p < 0.01$, one-way ANOVA followed by Dunnett's multiple comparison test (B). (C) Representative pseudo-color plot of RANKL-positive ST-2 cells cultured for 3 days in the presence of 50 nM $1,25(OH)_2D_3$ and 50 nM dexamethasone with increasing concentrations of gallotannin. GT: gallotannin.

gallotannin concentrations as high as 100 nM did not affect the proliferative activity of BMMs (Fig. 3A). Next, we used Annexin V-APC staining to examine the effects on viability. Similarly, gallotannin concentrations as high as 100 nM did not affect the viability of BMMs (Fig. 3B). Finally, we counted the absolute number of gallotannin-treated and -untreated cells by hemocytomater. Consistent with the results of BrdU incorporation assay and Annexin V staining, gallotannin concentrations as high as 100 nM did not affect the absolute number of BMMs (Fig. 3C). Given that gallotannin could suppress osteoclastogenesis at a concentration of 1 nM (Fig. 2A and B), we concluded that this effect was independent of the proliferative activity and viability of BMMs.

3.3. *Effects of gallotannin on NFATc1 and c-fos expression in RANKL-treated BMMs*

Upon stimulation with RANKL, osteoclast precursors produce and activate the key transcription factor NFATc1, which is indispensable for osteoclast differentiation and considered a hallmark of osteoclast differentiation [25,26]. We observed that NFATc1 expression was induced in response to RANKL treatment, but suppressed in the presence of gallotannin (Fig. 4A). We further assessed the effects of gallotannin on the expression levels of the osteoclastogenic transcription factor c-fos, and found that this expression was blunted in the presence of gallotannin (Fig. 4B). Taken together, gallotannin appears to suppress osteoclast differentiation by inhibiting the expression of osteoclastogenic transcription factors.

3.4. *Effects of gallotannin on RANKL-induced p38 phosphorylation*

In osteoclast precursors, RANKL stimulation leads to the recruitment of TRAF6 and activation of downstream intracellular signaling molecules [7]. In order to examine signal transduction downstream of RANK, BMMs were stimulated with RANKL, with or without gallotannin, for the indicated time periods. Although we observed comparable ERK1/2 phosphorylation kinetics in both gallotannin-treated and

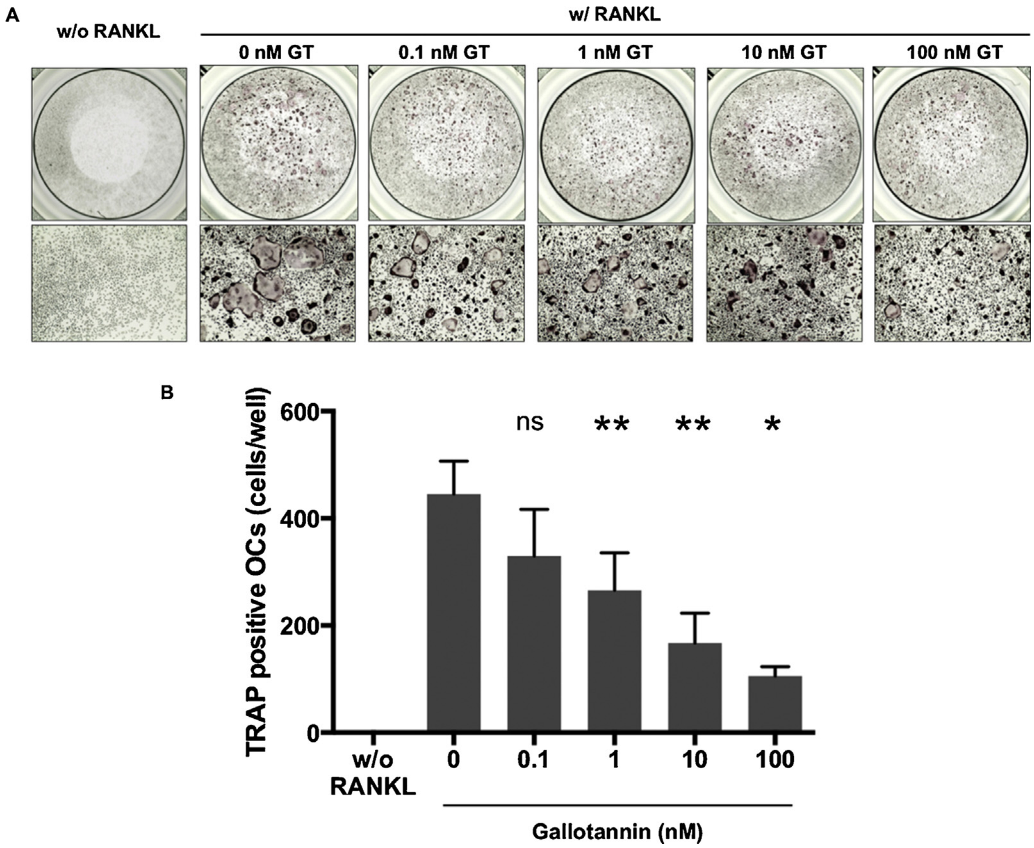

Fig. 2 – Gallotannin suppresses RANKL-induced osteoclast differentiation in a bone marrow macrophage (BMM) culture. BMMs were cultured for 3days in the presence of 25ng/mL M-CSF and 50ng/mL RANKL with increasing concentrations of gallotannin. Representative images show a TRAP-stained BMM culture (A) and the number of TRAP-positive cells containing more than three nuclei (B). Data were obtained from three independent experiments. Columns and bars represent the means and standard errors of the means; *$p < 0.05$ and **$p < 0.01$, one-way ANOVA followed by Dunnett's multiple comparison test.

-untreated BMMs (Fig. 5A), p38 phosphorylation was suppressed in gallotannin-treated BMMs (relative to gallotannin-untreated BMMs; Fig. 5A). These results suggest that gallotannin specifically suppresses RANKL-induced activation of the p38 MAP kinase pathway.

3.5. *Effects of gallotannin on M-CSF-induced p-38 phosphorylation*

Although M-CSF is known to be indispensable to the survival and proliferation of osteoclast precursors, M-CSF signaling has also been described as important for osteoclast differentiation and activation [27,28]. Therefore, we examined whether gallotannin treatment would affect M-CSF-induced intracellular signaling in differentiating osteoclast precursors. BMMs were first treated with RANKL and low-dose M-CSF to induce osteoclast differentiation, followed by stimulation with high-dose M-CSF for the indicated time periods. We observed that high-dose M-CSF induced the phosphorylation of p38 and ERK1/2 in differentiating osteoclast precursors (Fig. 5B). We further observed that although the presence of gallotannin did not affect the ERK1/2 phosphorylation kinetics, gallotannin blunted the phosphorylation of p38 (Fig. 5B). Consistent with the specific inhibitory effect of gallotannin on RANKL-mediated activation of the p38 MAPK pathway, gallotannin also specifically suppressed M-CSF-induced p38 phosphorylation in differentiating osteoclast precursors.

4. Discussion

Previous reports have described the anti-inflammatory effects of gallotannin, particularly with regard to regulatory T cells [29] and macrophages [15,30]. Because monocyte/macrophage lineage cells are considered osteoclast precursors [31], we hypothesized that gallotannin would affect osteoclast differentiation and function. According to our study results, gallotannin suppressed *in vitro* osteoclast differentiation in a BM culture system stimulated with $1{,}25(OH)_2D_3$, as well as a M-CSF/RANKL-treated BMM culture system. Consistently, we found that gallotannin treatment suppressed the expression of NFATc1 in RANKL-treated BMMs. NFATc1 is considered to be a master gene for osteoclast differentiation because the expression and activation of NFATc1 is induced by RANKL stimulation, and inhibition of NFATc1 activity by FK506 results in suppressed RANKL induced osteoclast differentiation [25].

To further elucidate the mechanisms underlying the suppressive effect of gallotannin on osteoclast differentiation,

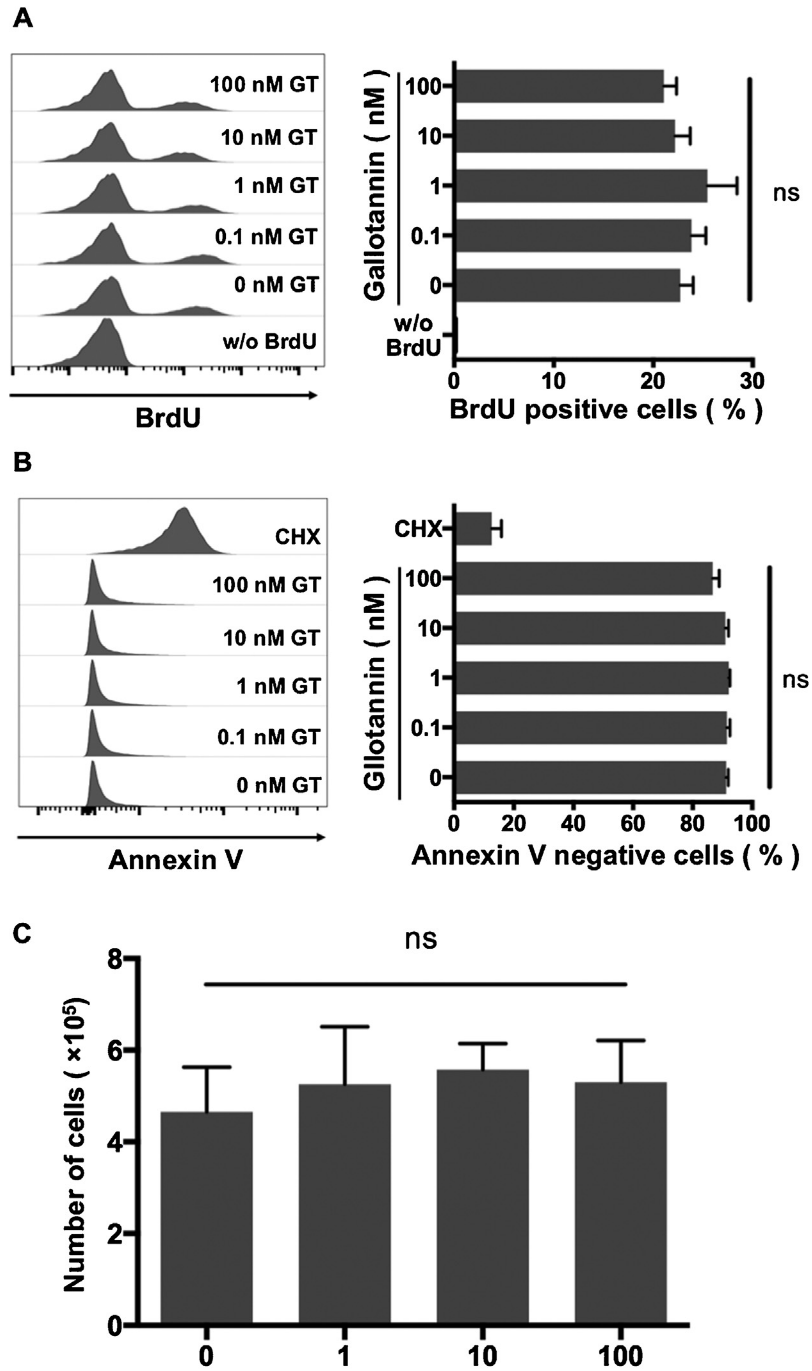

Fig. 3 – Gallotannin did not affect the viability and proliferative activity of bone marrow macrophages (BMMs).
(A) Representative histograms of cells that had incorporated BrdU (left) and the percentage of BrdU-positive cells (right). (B) Representative histograms of BMMs stained with Annexin V (left) and the percentage of Annexin V-negative cells (right). CHX: cycloheximide. (C) Absolute cell number of BMMs cultured in the presence of gallotannin. BMMs were plated at a density of 3×10^5 cells/well in 6-well plates supplemented with 25 ng/mL of M-CSF. Cell number was determined at days 2 by trypan blue exclusion and hemocytometer enumeration.

we examined the effects of gallotannin on RANKL- and M-CSF-mediated intracellular signaling. RANKL activates intracellular signaling cascades through binding to its receptor RANK, through which downstream signaling is transduced and the adapter protein TRAF6 is recruited [7]. We found that although gallotannin did not affect the activation kinetics of ERK1/2, it blunted p38 MAPK activation in RANKL-treated BMMs. We also observed the suppressive effects of gallotannin on M-CSF-

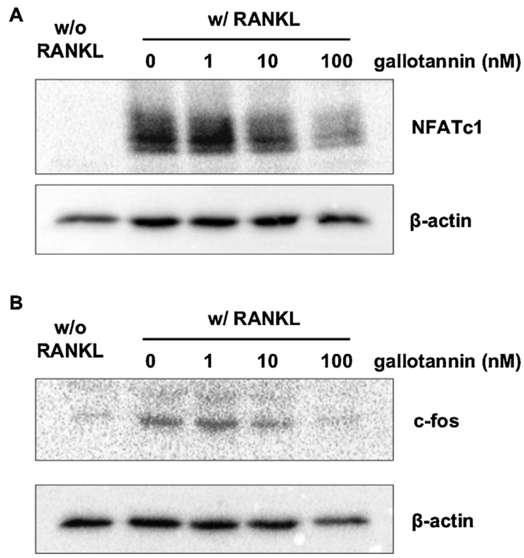

Fig. 4 – Gallotannin suppresses the RANKL-mediated induction of osteoclastogenic transcription factor expression.
Lysates of bone marrow macrophages (BMMs) stimulated for 3 days with 25 ng/mL of M-CSF, 50 ng/mL of RANKL, and increasing concentrations of gallotannin were subjected to a Western blot analysis to evaluate NFATc1 (A) and c-fos (B) expression.

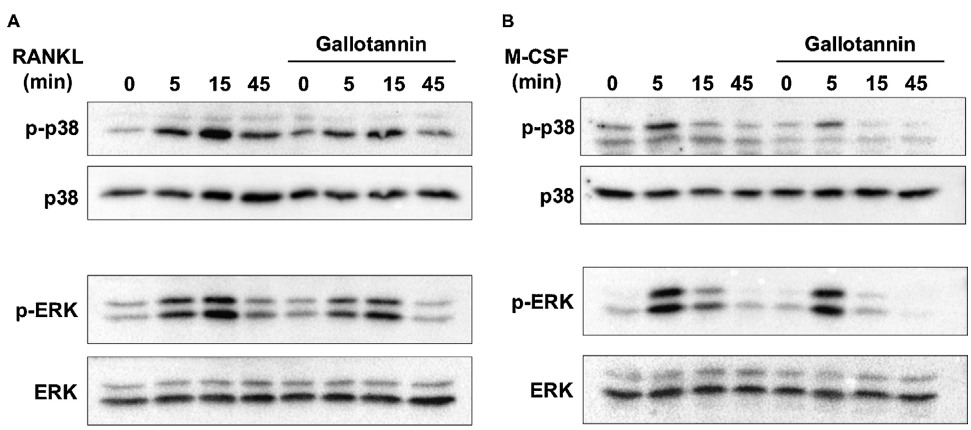

Fig. 5 – Gallotannin suppresses the RANKL- and M-CSF-induced activation of p38 MAP kinase.
Western blot analysis of phospho- and total-p38 and ERK1/2 expression in cell lysates prepared from BMMs stimulated for the indicated periods of time with 50 ng/mL RANKL in the presence or absence of overnight gallotannin pretreatment (A) or cell lysates prepared from osteoclast-differentiating BMMs stimulated for the indicated periods of time with 50 ng/mL M-CSF in the presence or absence of overnight gallotannin pretreatment (B).

induced p38 MAPK activation in BMMs that had been programmed for osteoclast differentiation by pretreatment with RANKL and low-dose M-CSF for 24h. In other words, gallotannin specifically suppressed RANKL- and M-CSF-induced activation of the p-38 MAPK pathway.

We note that while preparing this manuscript, Rantlha et al. reported a similar result in which ellagic acid, the acid component of ellagitannin, inhibited RANKL-induced osteoclastogenesis from a RAW264.7 murine macrophage cell line and human CD14 positive monocytes (osteoclast precursors) [11]. Specifically, ellagic acid suppressed RANKL-induced p38 phosphorylation but had no effects on the activation status of JNK, ERK, and NF-κB [11]. Because ellagic acid is a dimeric derivative of acid component of gallotannin, gallic acid, the two acids might exhibit similar bioactivities with respect to osteoclast precursors. Another point of similarity between our results and the findings of Rantlha et al. was the ability of both compounds to suppress osteoclast differentiation at nanomolar concentrations. Rantlha et al. showed that ellagic acid suppressed osteoclast differentiation at concentrations as low as 100 nM [11]. Similarly, we found that gallotannin suppressed osteoclast differentiation at concentrations ranging from 1 nM to 100 nM. These findings contradict previous reports in which gallotannin [15,16] and ellagic acid [32,33] exhibit its bioactivity against macrophages at micromolar concentrations. The similarities between our report and that of Rantlha et al. lead us to conclude that gallotannin and ellagic acid might share a common osteoclastogenesis suppression mechanism.

In conclusion, we have tested our hypothesis that gallotannin, which is reported to affect the physiological activity of macrophages, might affect osteoclast differentiation and demonstrated that gallotannin suppresses NFATc1 induction and osteoclast differentiation *in vitro*, as well as M-CSF- and RANKL-mediated p38 MAPK phosphorylation. Therefore, gallotannin might exert its anti-osteoclastogenic effects by targeting the p38 MAPK pathway. Because gallotannin is effective at low doses, it could be used clinically to treat bone resorptive diseases.

Conflict of interest

The authors declare no commercial or financial conflicts of interest.

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