# Inhibition of osteoclastogenesis for periprosthetic osteolysis therapy through the suppression of p38 signaling by fraxetin

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**Abstract.** Periprosthetic osteolysis belongs to osteolytic diseases, which often occur due to an imbalance between osteoclast and osteoblast number or activity. Fraxetin, a natural plant extract, inhibits osteoblast apoptosis and has therapeutic potential for treating osteolytic diseases. However, data pertaining to the effects of fraxetin on osteoclasts are limited. In the present study, it was demonstrated that the inhibition of osteoclastogenesis by fraxetin had an important role on the therapy of titanium particle-induced osteolysis *in vivo*. In addition, fraxetin was demonstrated to suppress receptor activator of nuclear factor-κB ligand (RANKL)-mediated osteoclast differentiation and bone resorption *in vitro* in a dose-dependent manner. Fraxetin inhibited osteoclast differentiation and function through the suppression of p38 signaling and subsequently, the suppression of osteoclast-specific gene expression, including tartrate-resistant acid phosphatase, nuclear factor of activated T-cells, cytoplasmic 1, and cathepsin K. In conclusion, fraxetin administration may have potential as a treatment method for periprosthetic osteolysis and other osteolytic diseases.

## Introduction

Bone tissue is constantly breaking-down and building-up to maintain skeleton balance. The bone remodeling process involves bone formation (osteoblast activities) and bone resorption (osteoclast activities) (1). Excess osteoclast activity may lead to adult skeletal osteolytic diseases, including osteoporosis, rheumatoid arthritis, and periodontal disease (2-4). Periprosthetic osteolysis (PO) following total hip arthroplasty (THA) is also a typical osteolytic disease. As a highly successful procedure, THA has become the standard method to address serious joint disease. But aseptic loosening, caused by PO, leads frequently to prosthetic failure. Previous studies have reported that PO is initiated by inflammatory response to wear debris (5,6), then excessive osteoclast formation and bone resorption are stimulated, resulting in osteopenia (4,7).

Osteoclasts are large multinucleated cells, which arise from the hematopoietic monocyte/macrophage lineage (8). Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) are required for osteoclast differentiation and formation (9). RANKL induces osteoclast differentiation by binding to RANK, its cognate receptor (10). Then, downstream signaling pathways are activated, including nuclear factor-κB (NF-κB), c-Jun N-terminal kinase (JNK) 1/2, p38, and extracellular signal-regulated kinase (ERK) 1/2 pathways (10,11). Suppression of these pathways might provide a potential for treating PO through inhibiting osteoclast formation or function.

Fraxetin (7, 8-dihydroxy-6-methoxy coumarin; Fig. 1A), extracted from the Chinese herb *Cortex Fraxini*, has anti-inflammatory, antitumor, antioxidative, antiviral, antihyperglycemic and neuroprotective effects (12,13). In addition, Kuo *et al* (14) reported that fraxetin can inhibit interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and anti-first apoptosis signal (Fas) IgM-mediated apoptosis by suppressing the Fas signal pathway in osteoblastic MG-63 cells. These findings indicate that fraxetin might be a potential therapeutic option to prevent osteolytic diseases. However, the role of fraxetin in osteoclasts has not been reported.

The present study aimed to investigate the therapeutic benefits and mechanism of fraxetin on PO *in vivo*, to assess the effect of fraxetin on osteoclastogenesis and function *in vitro*, and to determine the mechanisms that mediate the effects of fraxetin on osteoclast development and function.

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## Materials and methods

*Reagents*. Mouse macrophage RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS) was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The Cell Counting Kit (CCK)-8 was purchased from Dojindo Molecular Technologies, Inc., (Kumamoto, Japan). Alpha-modified Eagle's medium (α-MEM) was obtained from Gibco; Thermo Fisher Scientific, Inc. Fraxetin, glycine, Tris, sodium dodecyl sulfate (SDS), NaCl, Acid Phosphatase kit and other reagents were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). M-CSF and RANKL were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The SYBR Premix Ex Taq II and Prime Script RT reagent kits were purchased from Takara Bio, Inc. (Otsu, Japan). Antibodies against phosphorylated (p-) JNK1/2 (cat. no. 9251), total JNK1/2 (cat. no. 9252), p-p38 (cat. no. 9211), total p38 (cat. no. 8690), p-ERK1/2 (cat. no. 9101), total ERK1/2 (cat. no. 9102), MMK6 (cat. no. 9264) and β-actin (cat. no. 4967) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) and the working dilution was 1:1,000 for all.

*Animals and experimental design*. This study obtained ethics approval by the Institutional Animal Care And Use Committee (IACUC) of Southern Medical University (SMU; Guangzhou, China), and all experiments involving animals were performed according to the guidelines of SMU IACUC. A mouse calvarial osteolysis model was established to investigate the effects of fraxetin *in vivo* (15). Commercial pure Titanium (Ti) particles were obtained from Johnson Matthey (Royston, UK) (16). The particles were exposed to 180°C for 6 h, and sterilized with 70% ethanol for 48 h to remove adherent endotoxins. The particles with an average diameter of 4.50 mm are similar to wear particles retrieved from periprosthetic tissues (17,18). The concentration of particles in subsequent studies was 0.1 mg/ml. Healthy 8 week-old male C57BL/J6 mice, weighing 23±1 g, were purchased from Southern Medical University Laboratory Animal Center and assigned randomly to three experimental groups (8 mice per group). The sham group was used as a negative control. The vehicle group had calvarial osteolysis mediated by Ti particles. The fraxetin group had calvarial osteolysis and was treated with fraxetin (5 mg/kg/day). For the generation of the calvarial osteolysis model, the Ti particles (30 mg) were embedded on the middle suture of the calvaria (19). Two days later, fraxetin or PBS were locally injected every other day. All animals were housed under conditions of constant temperature and humidity on a 12-h light/dark cycle. Food and water were available *ad libitum*. After two weeks, the mice were sacrificed, and the calvarias were excised, fixed in 4% paraformaldehyde for 15 h and stored at 70% ethanol for micro-computed tomography (μ-CT) analysis.

*μ-CT scanning and histological analysis*. The prepared calvarias were analyzed by high resolution μ-CT. Bones were scanned at an isometric voxel resolution of 8 μm. The X-ray energy parameters were 80 kV and 80 μA. A square region of interest (ROI) was selected to perform the subsequent analyses. Porosity, number of pores, and bone volume/tissue volume (BV/TV) were studied for the ROI (20).

After μ-CT scanning, bones were decalcified in 10% ethylenediaminetetraacetic acid (EDTA; pH 7.4) for 2-3 weeks prior to embedding in paraffin. Sections with thickness of 4-μm were prepared and stained with tartrate-resistant acid phosphatase (TRAP) and hematoxylin and eosin (H&E). Stained sections were observed and photographed on a Leica DM1000 light microscope. At least 3 fields per slide per group were analyzed. For calvarias, the osteolysis area was analyzed using Image J (National Institutes of Health, Bethesda, MD, USA). TRAP-positive osteoclast numbers were determined.

*Osteoclast culture, TRAP staining assay, resorption pit assay and osteoclastogenesis rescue assay*. Primary mouse bone marrow-derived macrophages (BMMs) were obtained from the bone marrow of long bones. BMMs were cultured in α-MEM containing 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 30 ng/ml M-CSF (complete α-MEM). α-MEM containing 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine was used for RAW264.7 cell culture.

BMMs were seeded at a density of 8x10$^3$ cells/well into a 96-well plate with complete α-MEM, RANKL (50 ng/ml), and fraxetin (0, 3, 6, or 12 μM). For the resorption pit assay, bovine bone slices were added into the 96-well plate previously. For the osteoclastogenesis rescue assay, anisomycin (2.5 ng/ml), a potent activator of p38, was added following the fraxetin treatment (21,22). After seven days, the mature osteoclasts were fixed with 4% paraformaldehyde for 30 min and stained using the Acid Phosphatase kit. Osteoclast-like (OCL) cells were characterized as TRAP-positive osteoclasts with >3 nuclei. For the resorption pit assay, mechanical agitation and sonication were used to remove OCL cells from the bone slices. Resorption pits were observed using a scanning electron microscope. For each group, three wells were selected randomly for analysis. Image J software was used to analyze the numbers and the area of OCL cells and the % of resorbed bone surface area.

*Cytotoxicity assay*. The effects of fraxetin on the viability of BMMs were determined by CCK-8 assay. BMMs were plated at a density of 8,000 cells/well in 96-well plates, and cultured in complete α-MEM containing fraxetin (0, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 μM) for 48 h. GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) was used to calculate the half-maximal inhibitory concentration (IC$_{50}$).

*Western blot analysis*. RAW264.7 cells were seeded in 6-well plates at a density of 5x10$^5$ cells/well. First, RAW264.7 cells were treated with placebo or fraxetin for 2 h. Then, the RAW264.7 cells were stimulated with RANKL for 0, 5, 10, 20, 30, or 60 min. Subsequently, cells lysates were collected and western blotting was performed (23). The Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) was used to visualize the antibody reactivity.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay*. BMMs were seeded at a density
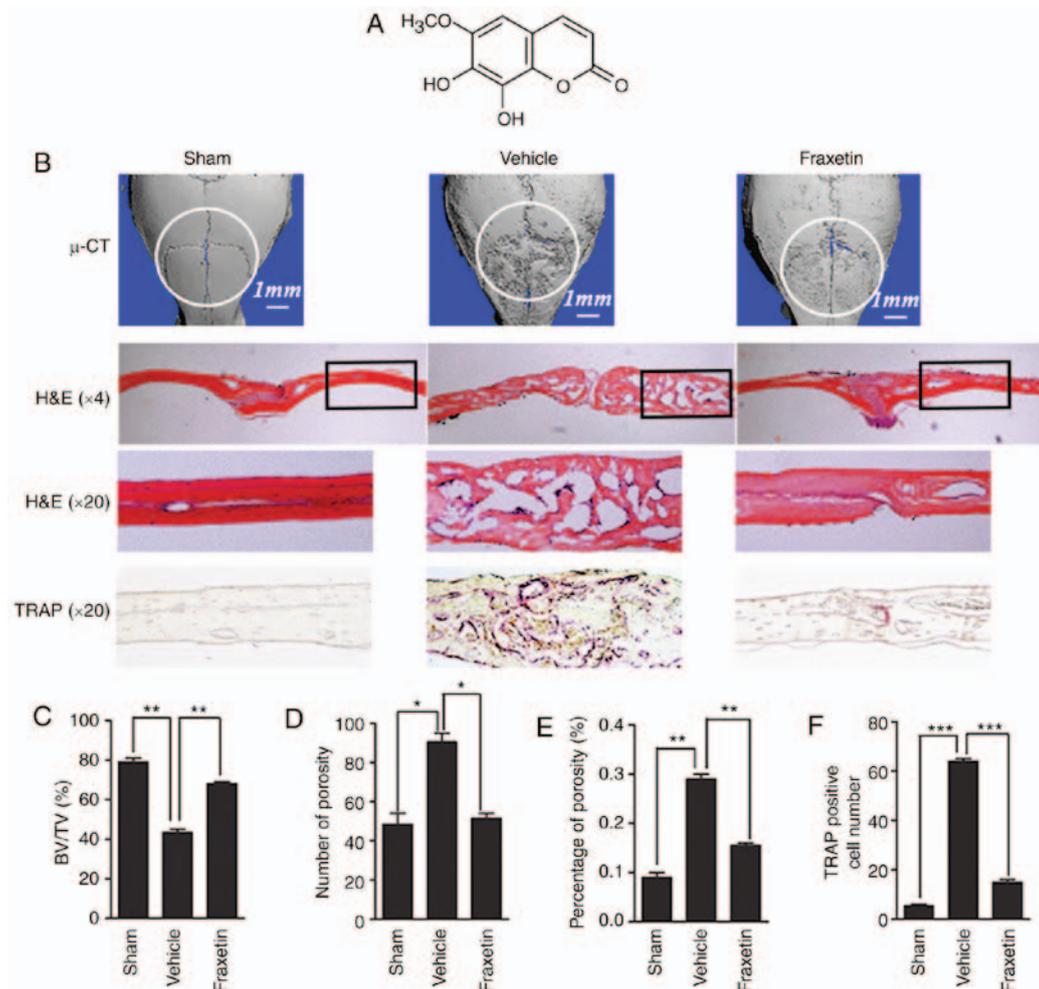
Figure 1. Fraxetin protects against Ti particle-induced osteolysis of mouse calvarias. (A) Chemical structure of fraxetin. (B) Representative images from 3D $\mu$-CT analysis reconstruction (scale bar, 1 mm), H&E staining (magnification, x4 and x20) and TRAP staining (magnification, x20). At least three sections per group were analyzed. (C) Quantified results for BV/TV, (D) number of pores, (E) % of porosity and (F) osteoclast numbers. *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by brackets. $\mu$-CT, micro-computed tomography; H&E, hematoxylin and eosin; TRAP, tartrate-resistant acid phosphatase; BV, bone volume; TV, tissue volume.

of $2x10^5$ cells/well in 6-well plates and cultured in complete $\alpha$-MEM supplemented with 50 ng/ml RANKL. Then, the BMMs were treated with either 0, 3, or 12 $\mu$M fraxetin for 5 days, or 12 $\mu$M fraxetin for 0-5 days. A Qiagen RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) was used to extract total RNA. Reverse transcription was performed with the Prime Script RT kit. qPCR was performed using SYBR Premix Ex Taq II with the following specific primers: TRAP, forward 5'-CTGGAGTGCACGATGCCAGCGACA-3' and reverse 5'-TCCGTGCTCGGCGATGGACCAGA-3'; nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), forward 5'-CCG TTGCTTCCAGAAAATAACA-3' and reverse 5'-TGTGGG ATGTGA ACTCGGAA-3'; cathepsin K (CTSK), forward 5'-CTTCCAATACGTGCAGCAGA-3' and reverse 5'-TCT TCAGGGCTTTCTCGTTC-3'; GAPDH, forward 5'-ACC CAGAAGACTGTGGATGG-3' and reverse 5'-CACATTGGG GGTAGGAACAC-3'. The following cycling conditions were used: 40 cycles of denaturation at 95°C for 5 sec and amplification at 60°C for 24 sec. GAPDH expression was used to normalize transcript levels for the genes under investigation, and all reactions were run in triplicate. The $2^{-\Delta\Delta Cq}$ method was used for quantification (24).

*Statistical analysis.* Results are expressed as the mean ± standard deviation. The significance of differences between groups was analyzed with one-way analysis of variance with a post hoc Bonferroni test, using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

*Fraxetin protects against bone loss induced by wear particles.* A Ti particle-induced calvarial osteolysis model was used to simulate PO. Analysis of parietal bones by $\mu$-CT revealed extensive bone resorption in mice exposed to Ti particles (vehicle group; received Ti injection) compared with the negative controls (sham group; received PBS only injection; Fig. 1B). Bone resorption was lower in mice administered a 5 mg/kg/day concentration of fraxetin compared with the vehicle group (Fig. 1B), demonstrating that fraxetin suppressed osteolysis induced by Ti-particles. Quantification of bone parameters revealed that exposure to fraxetin increased bone volume (Fig. 1C) and decreased porosity (Fig. 1D and E) compared with the vehicle group.
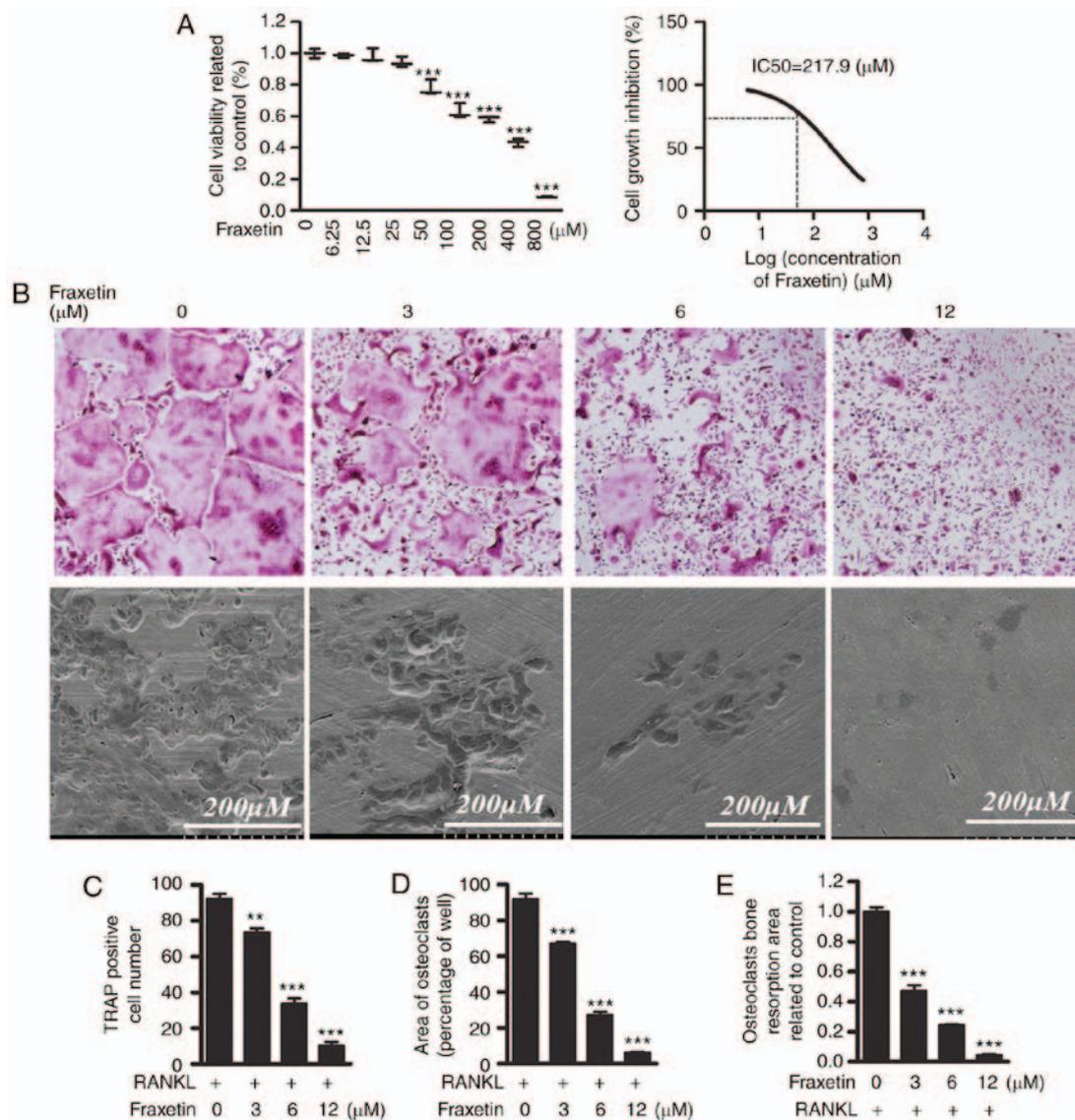
Figure 2. Fraxetin suppresses osteoclast formation and function *in vitro*. (A) Cell cytotoxicity assay. The inhibition rate of BMMs by fraxetin is shown in the left panel, while the $IC_{50}$ curve is shown in the right panel. (B) Fraxetin inhibited RANKL-induced osteoclastogenesis and Fraxetin attenuated bone resorption. (C) Quantification of TRAP-positive cell number, (D) area, and (E) total area of resorption pits following treatment of BMMs with fraxetin compared with untreated cells. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. untreated cells. BMMs, bone marrow-derived macrophages; $IC_{50}$, half-maximal inhibitory concentration; RANKL, receptor activator of nuclear factor-κB ligand.

Histological assessment results demonstrated that fraxetin administration prevented parietal bones from Ti particle-induced osteolysis. H&E staining revealed few osteolytic changes in the sham group mice. Multiple osteolytic changes occurred in the vehicle group, whereas fraxetin administration reversed opposed this effect (Fig. 1B). Accordingly, TRAP staining revealed that the number of OCLs was increased following Ti particle stimulation, while fraxetin administration reversed this effect (Fig. 1B and F). In summary, fraxetin could prevent wear particle-induced bone loss by suppressing osteoclastogenesis.

*Fraxetin inhibits osteoclast formation and function in vitro.* Based on the aforementioned *in vivo* results, it was hypothesized that fraxetin can inhibit osteoclastogenesis *in vitro*. The role and mechanism of fraxetin in normal osteoclast formation and bone resorption function were therefore explored further *in vitro*. First, CCK-8 assays were performed in order to exclude the possibility that a potential cytotoxic effect of fraxetin may be responsible for the decrease in OCL cell numbers. The $IC_{50}$ value of fraxetin was 217.9 µM at 48 h, suggesting that fraxetin might suppress the proliferation of BMM cells at concentrations >217.9 µM (Fig. 2A). Then, differentiated BMMs were induced into TRAP-positive OCL cells (Fig. 2B). Exposure to fraxetin decreased osteoclast formation in a dose dependent manner. The numbers of TRAP-positive OCL cells was suppressed by ~30% in the presence of 3 µM fraxetin (Fig. 2B-D), and was almost completely suppressed by exposure to fraxetin at concentrations >12 µM (Fig. 2 B-D). Since the $IC_{50}$ value of fraxetin was demonstrated to be 217.9 µM, fraxetin could not have affected the proliferation of BMMs at doses 6-12 µM, suggesting that at these latter concentrations of fraxetin impaired osteoclast formation without cell cytotoxicity.
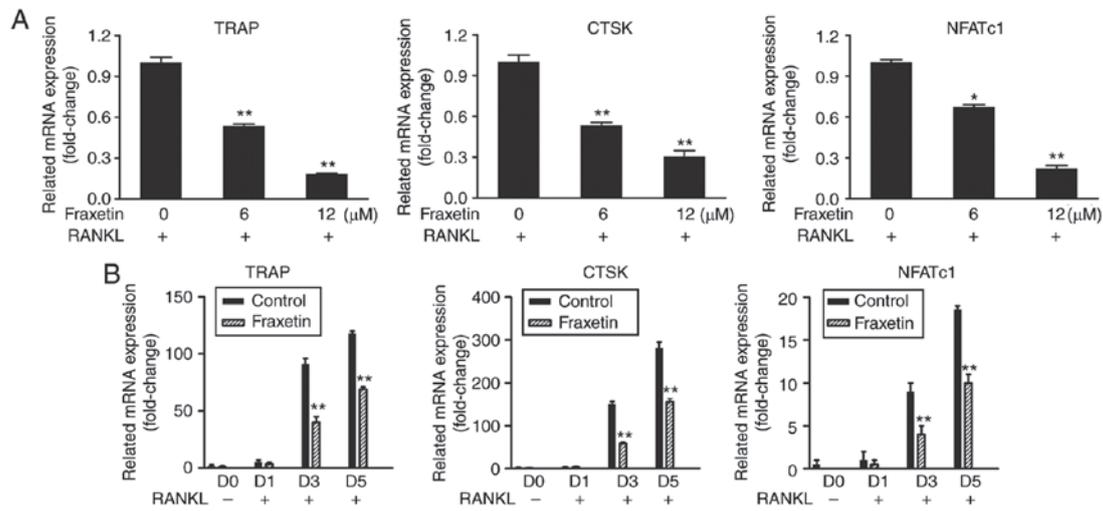
Figure 3. Relative mRNA expression of the osteoclast-specific genes TRAP, CTSK and NFATc1. (A) BMMs were cultured with complete α-MEM, 50 ng/ml RANKL, and the indicated doses of fraxetin for 5 days. (B) BMMs were cultured with complete α-MEM, 50 ng/ml RANKL and 12 μM fraxetin for 0, 1, 3, and 5 days. *P<0.05, **P<0.01 and ***P<0.001 vs. fraxetin free cells. TRAP, tartrate-resistant acid phosphatase; CTSK, cathepsin K; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; BMMs, bone marrow-derived macrophages; RANKL, receptor activator of nuclear factor-κB ligand.
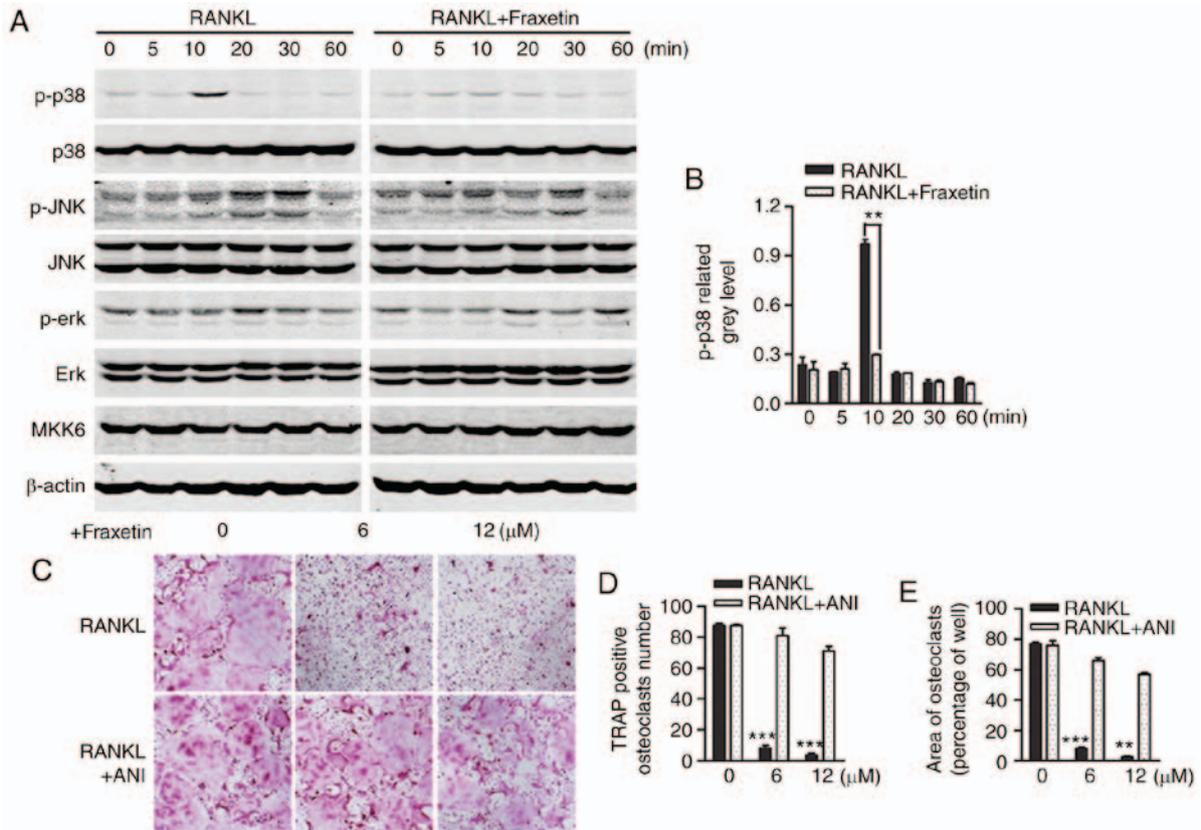


Figure 4. Analysis of the signaling mechanism by which fraxetin inhibits osteoclast differentiation. (A) RAW264.7 cells were treated with or without 12 μM fraxetin for 4 h, and with 50 ng/ml RANKL for the indicated periods. Total protein was separated by gel electrophoresis and levels of protein of interest were determined by western blotting. Representative blots are shown. (B) Phosphorylation of p38 was quantified and normalized to β-actin. **P<0.01 vs. the fraxetin free group. (C) Treatment with ANI neutralized the suppression effect of fraxetin on osteoclast formation. (D) Quantification of the number and (E) area of osteoclasts per treatment group. **P<0.01 and ***P<0.001 vs. RANKL + ANI. RANKL, receptor activator of nuclear factor-κB ligand; ANI, anisomycin; p-, phosphorylated; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MKK6, mitogen-activated protein kinase kinase 6; TRAP, tartrate-resistant acid phosphatase.

Bone resorption is the most important function of osteoclasts. Therefore, bone resorption assays were conducted *in vitro* in order to evaluate the effect of fraxetin on bone

resorption. Scanning electron microscopy analysis revealed osteoclastic bone resorption pits present in BMM cultures in the presence of RANKL. Exposure of BMMs to 3 μM fraxetin

resulted in a reduction in resorptive activity >50%, and at fraxetin concentrations >12 $\mu$M, resorption was completely suppressed (Fig. 2B and E). These results demonstrate that fraxetin administration reduced Ti particle-induced bone resorption in the presence of RANKL *in vitro*.

*Fraxetin suppresses osteoclast activity by downregulating osteoclast-specific gene expression.* RT-qPCR results demonstrated that the mRNA expression levels of TRAP, CTSK and NFATc1 were significantly decreased following fraxetin treatment, compared with untreated control cells, in a time- and dose-dependent manner (Fig. 3). These findings suggest that fraxetin inhibited osteoclast differentiation by suppressing osteoclast-specific gene expression.

*Fraxetin suppresses osteoclast activity by specifically inhibiting the p38 signaling pathway.* Osteoclasts were differentiated from monocytes and macrophages in response to stimulation with M-CSF and RANKL. The RAW264.7 cell line has good stability and can substitute for preosteoclasts *in vitro*. Western blot analysis was conducted in RAW264.7 cells in order to investigate the activity of signaling pathways that determine osteoclast differentiation. Well known, mitogen-activated protein kinase (MAPK) pathways (namely ERK1/2, p38 and JNK1/2) have an important role in osteoclast differentiation downstream of RANK signaling (25). The present western blot results revealed that stimulation of RAW264.7 cells with RANKL induced peak p38 phosphorylation within 10 min, and this effect was suppressed following treatment with 12 $\mu$M fraxetin (Fig. 4A). Densitometry analysis of western blot images confirmed the significant inhibition of p38 phosphorylation by fraxetin (Fig. 4B). To confirm these findings, BMMs exposed to fraxetin were treated with anisomycin, a p38 agonist (21,22). In accordance with previous observations, fraxetin inhibited osteoclast formation, whereas anisomycin precluded this effect (Fig. 4C-E). Conversely, in both the control and fraxetin-treated groups, ERK and JNK phosphorylation were induced, and fraxetin treatment did not oppose this effect (Fig. 4A). Furthermore, MKK6, an upstream regulator of p38, was not affected by RANKL stimulation or fraxetin treatment (Fig. 4A). Taken together, these results demonstrated that fraxetin suppressed osteoclast differentiation by inhibiting p38 signaling, whereas JNK and ERK activity was not affected.

## Discussion

Previous studies have reported that fraxetin is a potential anti-osteolytic agent, based on its properties to inhibit osteoblast apoptosis induced by inflammatory cytokines (14). However, osteoclasts serve an important role in osteolytic diseases, and the effects of fraxetin on these cells have not been characterized. In the present study, fraxetin was demonstrated to suppress PO via inhibition of the stimulatory effects of RANKL-induced p38 signaling on osteoclast formation and bone resorption *in vitro* and *vivo*. Thus, the present findings provide a mechanistic justification for the application of fraxetin to the treatment of PO.

Results from the Ti particle-induced bone loss assay indicated that the inhibition of osteoclastogenesis by fraxetin

*in vivo* may have an important role on the therapy of PO. Additionally, the inhibitory effect of fraxetin on osteoclastogenesis and bone resorption were further investigated *in vitro*. The results demonstrated that fraxetin prevented the stimulatory effects of RANKL through the p38 cascade. Activated p38 signaling is a key procedure in bone destruction, while suppression of p38 activity reduces osteoclast formation and bone resorption (26,27). p38 is involved in the activation of activator protein (AP)-1 components (28), but the molecular mechanisms of their functions are not well understood. A major component of the transcription factor AP-1 is Fos proto-oncogene (also known as c-fos), which also induces NFATc1, a master regulator of osteoclastogenesis, by binding to its promoter region (29,30). The activity of c-fos and NFATc1 has been reported to be regulated by p38 (26,29-32). In addition, the present study demonstrated that MKK6, the kinase upstream of p38 (33), was unaffected by fraxetin administration. It was therefore speculated that fraxetin may prevent the stimulatory effects of RANKL by p38/c-fos/NFATc1 signaling, and subsequently inhibit osteoclastic-specific gene expression (10).

Ultra-high molecular weight polyethylene, Ti, and polymethyl methacrylate (PMMA) are common particles present in tissues around joint prostheses, which are important for today's joint replacement surgeons (34,35). Though metal particles are relatively less frequently used, osteolysis induced by Ti particles is typical (36,37). Thus, Ti particle-induced osteolysis could be representative and suitable for studying the effect of fraxetin on osteolysis caused by wear debris (16,17). The current results demonstrated that fraxetin administration prevented Ti particle-induced osteolysis in a mouse osteolysis model and did not result in any obvious side effects, confirming that fraxetin inhibits osteoclast differentiation and function *in vivo* and suggesting that it may hold potential therapeutic benefits on PO.

The present study has several strengths. It is the first that characterized the suppression effects of fraxetin on osteoclastogenesis. Chinese herbs are less prone to lead to drug resistance and have fewer side effects compared with synthetic drugs. Therefore, investigating the effects of fraxetin further may be a promising approach to develop treatments for PO. The present study also presents a limitation. PO is a complex process that consists of multiple interactions between bone cells and inflammatory reactions *in vivo*. However, the present study only explored the mechanism for osteolytic diseases therapy through the inhibition of osteoclast activities.

Collectively, the present findings demonstrated that fraxetin administration improved PO *in vitro* and *in vivo*, through the suppression of osteoclast development and function via inhibition of the p38 signaling pathway. Therefore, fraxetin may be a potential agent for the treatment of PO and other osteolytic diseases.

## Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

## Authors' contributions

DZC designed the study; JCL, ZXW, and ZPM performed the research; JCL and CZ contributed new reagents or analytical tools; JCL, CZ and ZXW analyzed data; JCL wrote the study.

## Ethics approval and consent to participate

This study obtained ethics approval by the Institutional Animal Care and Use Committee (IACUC) of Southern Medical University (SMU; Guangzhou, China), and all experiments involving animals were performed according to the guidelines of SMU IACUC.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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