CHAPTER FIVE - PSEUROTIN A INHIBITS OSTEOCLASTOGENESIS AND PREVENTS OVARIECTOMIZED-INDUCED BONE LOSS BY SUPPRESSING REACTIVE OXYGEN SPECIES

Publication arising from this chapter:
5.1 Abstract

Rationale: Growing evidence indicates that intracellular reactive oxygen species (ROS) accumulation is a critical factor in the development of osteoporosis by triggering osteoclast formation and function. Pseudoerin A (Pse) is a secondary metabolite isolated from Aspergillus fumigatus with antioxidant properties, recently shown to exhibit a wide range of potential therapeutic applications. However, its effects on osteoporosis remain unknown. This study aimed to explore whether Pse, by suppressing ROS level, is able to inhibit osteoclastogenesis and prevent the bone loss induced by estrogen-deficiency in ovariectomized (OVX) mice.

Methods: The effects of Pse on receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL)-induced osteoclastogenesis and bone resorptive function were examined by tartrate resistant acid phosphatase (TRAcP) staining and hydroxyapatite resorption assay. 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) was used to detect intracellular ROS production in vitro. Western blot assay was used to identify proteins associated with ROS generation and scavenging as well as ROS-mediated signaling cascades including mitogen-activated protein kinases (MAPKs), NF-κB pathways, and nuclear factor of activated T cells 1 (NFATc1) signaling. The expression of osteoclast-specific genes was assessed by qPCR. The in vivo potential of Pse was determined using an OVX mouse model administered with Pse or vehicle for 6 weeks. In vivo ROS production was assessed by intravenous injection of dihydroethidium (DHE) into OVX mice 24h prior to killing. After sacrifice, the bone samples were analyzed using micro-CT and histomorphometry to determine bone volume, osteoclast activity, and ROS level ex vivo.

Results: Pse was demonstrated to inhibit osteoclastogenesis and bone resorptive function in vitro, as well as the downregulation of osteoclast-specific genes including Acp5 (encoding TRAcP), Ctsk (encoding cathepsin K), and Mmp9 (encoding matrix metalloproteinase 9). Mechanistically, Pse suppressed intracellular ROS level by inhibiting RANKL-induced ROS production and enhancing ROS scavenging enzymes, subsequently suppressing MAPK pathway activation (ERK, P38, and JNK) and NF-κB pathways, leading to the inhibition of NFATc1 signaling. Micro-CT and histological data indicated that OVX procedure resulted in a significant bone loss, with dramatically increased the number of osteoclasts on the bone surface as well as increased ROS level in the bone marrow microenvironment; whereas Pse supplementation was capable of effectively preventing these OVX-induced changes.
Conclusion: Pse was demonstrated for the first time as a novel alternative therapy for osteoclast-related bone diseases such as osteoporosis through suppressing ROS level.

5.2 Introduction

The maintenance of bone homeostasis is achieved by the delicate balance between osteoblastic bone formation and osteoclastic bone resorption. Uncoordinated activities of osteoblasts and osteoclasts can result in various perturbations in skeletal structure and function thus leading to bone diseases such as Paget’s disease of bone and osteoporosis (1, 2). Estrogen deficiency-induced bone loss, mainly due to enhanced osteoclast activity, plays an essential role in osteoporosis in postmenopausal women, leading to fragility fractures that are often associated with life-threatening mortality and morbidity, as well as massive economic cost to both individuals and society (3).

Osteoclasts are large multinucleated cells which are derived from hematopoietic progenitors. The formation of mature osteoclasts is dependent on several crucial factors, including macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL). Osteoclast precursor cells undergo survival and proliferation in response to the binding of M-CSF to its receptor c-Fms (4). RANKL is an indispensable and sufficient factor for osteoclastogenesis, via its action on RANK receptor (5). After stimulation with RANKL, recruitment of TNF receptor-associated factor 6 (TRAF6) is induced to activate multiple downstream targets including mitogen-activated protein kinases (MAPKs) and NF-κB pathways, leading to activation of c-Fos and nuclear factor of activated T cells 1 (NFATc1) (6-8). These signaling cascades enable the expression of genes that typify the osteoclast lineage including Acp5 (encoding tartrate-resistant acid phosphatase [TRAcP]), Ctsk (encoding cathepsin K), and Mmp9 (encoding matrix metalloproteinase 9), thus eventually leading to the formation of mature osteoclasts (6).

Growing evidence also indicates that intracellular reactive oxygen species (ROS) play a crucial role during osteoclast formation and bone resorption (9-11). ROS are endogenously produced in osteoclast precursors following stimulation with RANKL, via a signaling cascade involving TRAF6, Rac1, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 (Nox1)(9). Application of oxidant scavenger like N-acetylcysteine (NAC) or Nox inhibitor such as diphenylene iodonium (DPI), was found to inhibit osteoclastogenesis by suppressing RANKL-mediated ROS production (9),
indicating ROS are required for osteoclast differentiation. Cellular protective mechanisms against oxidative stressors also include a variety of cytoprotective or antioxidant enzymes, such as heme oxygenase-1 (HO-1), catalase, glutathione-disulfide reductase (GSR), NAD(P)H: quinone reductase (NQO1), and γ-glutamylcysteine synthetase (GCS) (12, 13). Antioxidants were shown to attenuate osteoclast formation and bone resorption by enhancing expression of the cytoprotective enzymes (14, 15). The downstream targets of ROS in RANKL-mediated signaling still remain unclear; however, a higher level of oxidative stress was suggested to promote osteoclast formation and function through the activation of NF-κB and MAPKs (13, 16). In addition, ROS production is highly involved in bone remodeling and bone homeostasis by promoting bone resorption (16, 17). Estrogen deficiency-induced osteoporosis is associated with a higher level of oxidative stress and can be prevented by increasing antioxidant defenses (18-20). Therefore, these findings might provide a rationale for suppressing ROS as a potential strategy for the treatment of osteoporosis.

Pseurotin A (Pse) is a bioactive secondary metabolite originally isolated from Pseudoeurotium ovalis (21) and later also from several species of Aspergillus (22-24). A single hybrid polyketide synthase/non-ribosomal peptide synthetase (PKS/NRPS) (25) gene and the rtfA gene (26) are associated with the biosynthesis of Pse in Aspergillus fumigatus. The expression of Pse is also induced in response to hypoxia (27). So far, Pse has demonstrated potential therapeutic applications due to its immunosuppressive activity (28), antibacterial activity (29), nematicidal activity (30), antiparasitic as well as anticancer activity (22). Furthermore, Pse was found to have antioxidant and radical-scavenging activity, as demonstrated by its ability to scavenge the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical (31).

Given the significant role of ROS on osteoclast formation and function, the potential antioxidant activity as well as other various potential therapeutic applications of Pse, we hypothesized that Pse might inhibit osteoclasts and thus prevent osteoclast-related osteoporosis. In the present study, we assessed the therapeutic effects of Pse on RANKL-induced osteoclastogenesis in vitro and ovariectomized (OVX)-induced osteoporosis mouse models in vivo, with a focus on determining the antioxidant capacity of Pse as well as elucidating its underlying mechanisms.

5.3 Materials and Methods

5.3.1 Materials and reagents
Pse, obtained from SHANGHAI ZZBIO CO., LTD (Shanghai, China), was dissolved in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Sydney, NSW, Australia) at a concentration of 100 mM and further diluted to working concentrations with culture medium. DMSO of the same dilution was used as a vehicle control. Alpha modified Minimal Essential Medium (α-MEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia). MTS assay kit and luciferase analysis system were purchased from Promega (Sydney, NSW, Australia). Primary antibodies for TRAF6, GSR, IκB-α, β-actin, phospho-ERK, ERK, phospho-P38, P38, NFATc1, Integrin αV, cathepsin K, and V-ATPase-d2 were purchased from Santa Cruz Biotechnology (Dallas, CA, USA). Primary antibodies for HO-1, catalase, phospho-JNK, JNK, and Active Rac1 Detection Kit were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibody for anti-vinculin was purchased from Sigma-Aldrich (Sydney, NSW, Australia). Recombinant M-CSF was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant Glutathione S-transferase (GST)-rRANKL protein was expressed and purified as previously described[32]. Rhodamine Phalloidin, ProLong Gold Antifade Mountant, and 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia) and 4,6-diamidino-2-phenylindole (DAPI) was purchased from Santa Cruz Biotechnology (Santa Cruz, Dallas, CA, USA). Dihydroethidium (DHE) was purchased from ApexBio (Boston, MA, USA).

5.3.2 In vitro osteoclastogenesis assay

Fresh bone marrow macrophages (BMMs) from C57BL/6J mice were isolated using methods approved by the University of Western Australia Animal Ethics Committee (RA/3/100/1244) as described [16]. In brief, bone marrow was flushed from the femur and tibia and then cultured in α-MEM/10% FBS/Penicillin/streptomycin (complete αMEM). To obtain pure BMMs, non-adherent cells were then collected and cultured in complete αMEM containing M-CSF (50 ng/mL). After a further 3 days in culture, the attached cells were used for experimental purposes. BMMs were plated in 96-well plates at a density of 6 × 10³ cell/well overnight. The following day, cells were stimulated with M-CSF and GST-rRANKL (50 ng/mL) in the presence or absence of increasing concentrations of Pse (2.5, 5, 7.5, 10 µM). Medium was replaced every 2 days until osteoclasts formed. The cells were then fixed with 2.5% glutaraldehyde solution for 10 min and stained for tartrate resistant acid phosphatase (TRAcP) enzymatic activity using a leucocyte acid phosphatase staining kit (Sigma-Aldrich, Sydney, NSW, Australia). Cells with more than 3 nuclei were counted as osteoclasts.
5.3.3 Cytotoxicity assay

BMMs were seeded in 96-well plates at a density of $6 \times 10^3$ cells/well and incubated overnight to allow adherence. Cells were then treated with different concentrations of Pse (0, 2.5, 5, 7.5, 10 µM) for 48 h. At the end of the experiment, MTS solution (20 µL/well) was added and the plate was incubated for an additional 2 h. The optical density (OD) was measured by spectrophotometric absorbance at 490 nm using a microplate reader (BMG LABTECH, Ortenberg, Germany).

5.3.4 Staining for podosome belts

To visualize podosome belts, BMMs were seeded onto FBS-coated coverslips (5-mm diameter) in 96-well plates and were induced to form osteoclasts, as described above, in the presence of Pse (0, 5, 10 µM). Osteoclasts were then fixed 4% paraformaldehyde (PFA) for 10 min, permeabilized with 0.1% (v/v) Triton X-100 for 10 min and blocked with 3% BSA in PBS for 1 h. Cells were then incubated with mouse monoclonal anti-vinculin (1:200) at 4 °C overnight, followed by washing in PBS and incubation with a fluorescent secondary anti-mouse antibody (Alexa Fluor 488, green). F-actin was stained with Rhodamine Phalloidin in the dark for 1 h. After that, cells were washed with PBS, stained with DAPI for 10 min, washed again, and mounted in ProLong Gold Antifade Mountant prior to visualization using an NIKON A1Si confocal microscope (Nikon Corporation, Minato, Tokyo, Japan).

5.3.5 Quantitative real-time PCR

BMMs were cultured in 6-well plates ($1 \times 10^5$ cells/well) and stimulated with GST- rRANKL and M-CSF in the presence of Pse (0, 5, 10 µM) until osteoclasts formed. Total RNA was isolated from the cells using Trizol reagent (Life Technologies, Sydney, Australia) and single stranded cDNA was prepared from 1 µg of total RNA template using moloney murine leukemia virus (MMLV) reverse transcriptase with oligo-dT primer (Promega, Sydney, NSW, Australia). For relative quantitative real-time PCR (qPCR), SYBR Green PCR MasterMix (Thermo Fisher Scientific, Scoresby, VIC, Australia) was used. The cycling parameters for PCR were set as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 40 s, followed by an elongation step of 5 min at 72 °C. The specific primers used are as following: Ctsk (forward: 5'-GGGAGAAAAACCTGAAGC-3'; reverse: 5'-ATTCTGGGACTCAGAGC-3'), Acp5 (forward: 5'-TGTGGCCATCTTTATGC-3'; reverse: 5'-GTCATTTCTTGGGGCTT-3'), Atp6v0d2 (Forward: 5ʹ-GTGAGACCTTGGAAAGACCTGAA-3ʹ; Reverse: 5ʹ-GAGAAATGTGCTCAGGGGCT-3ʹ), Mmp9 (Forward: 5ʹ-GTGAGACCTTGGAAAGACCTGAA-3ʹ; Reverse: 5ʹ-GAGAAATGTGCTCAGGGGCT-3ʹ), Nfatc1 (Forward: 5ʹ-CAACGCCCTGACCACCAGATAG-3ʹ; Reverse: 5ʹ-GGCTGCCTTCGCTCCTAGT-3ʹ), C-fos (Forward: 5ʹ-GCGAGCAGAGGACGAGAC-3ʹ; Reverse: 5ʹ-
TTGAAACCGAGAACATC-3'), *Hmbs* (Forward: 5’- AAGGGCTTTTCTGAGGCACC -3’; Reverse: 5’- AGTTGCCCATCTTTTCATCATG-3’), *Hprt1* (Forward: 5’-TCAGTCAACGGGGACATAAA-3’; Reverse: 5’-G GGCTGTACTGCTTAACCAG -3’). The qPCR procedure was performed on a ViiA 7 Real-time PCR machine (Applied Biosystems, Warrington, Cheshire, UK). Target gene expression levels were normalized to average expression of *Hprt1* and *Hmbs*.

5.3.6 Hydroxyapatite resorption assay

To determine whether Pse affects osteoclast function, hydroxyapatite resorption assay was performed as described previously [15, 33, 34]. BMMs were plated in 6-well collagen-coated plates (Corning Inc., Corning, NY, USA) in complete αMEM with M-CSF at a density of 1 x 10⁵ cells/well. The following day cells were stimulated with 50 ng/mL GST-RANKL until osteoclasts began to form. Cells were then gently detached using cell dissociation solution (Sigma-Aldrich, Sydney, NSW, Australia) and an equal number of cells were seeded into each well of hydroxyapatite-coated plate (Corning Inc., Corning, NY, USA). Cells were treated with Pse at increasing concentrations (0, 5, 10 µM) in complete αMEM containing 50 ng/mL GST-RANKL and M-CSF. After 48 h incubation, half of the wells for each group were washed with 10% bleach solution to remove cells and the images of hydroxyapatite resorption areas were captured using a Nikon microscope (Nikon Corporation, Minato, Tokyo, Japan) and quantified using Image J software (NIH, Bethesda, Maryland, USA). The remaining wells were fixed and stained for TRAcP activity as above to count the number of osteoclasts. The resorbed area per well and the percentage of resorbed area per osteoclast were used to quantify the osteoclast activity.

5.3.7 Detection of intracellular ROS production

The intracellular production of ROS was measured as described previously [9]. Briefly, after stimulation with GST-rRANKL (50 ng/mL) in the absence or presence of Pse at stated concentrations (5, 10 µM), BMMs were then incubated in Hank's balanced salt solution containing 5 mM H₂DCFDA for 1 h. Upon oxidation, the nonfluorescent H₂DCFDA will be converted to the highly fluorescent 2’,7’-dichlorofluorescein (DCF). The fluorescence of DCF was measured at an excitation wavelength of 488 nm and an emission wavelength of 515–540 nm using an NIKON A1Si confocal microscope. Cells images were also captured by digital interference contrast (DIC). The mean fluorescence intensity for each cell and the number of ROS-positive cells per field were analyzed using Image J software.
5.3.8 Luciferase reporter assays of NF-κB or NFATc1

RAW264.7 cells (ATCC, Manassas, Virginia, USA), stably transfected with an NF-κB responsive luciferase construct [35] and an NFATc1 responsive luciferase reporter construct [36], were seeded in 48-well plates at a density of $1.5 \times 10^5$ cells/well or $5 \times 10^4$ cells/well overnight, respectively. Cells were then pre-treated with Pse for 1 h and stimulated with GST-rRANKL (50 ng/mL) for 6 h and 24 h; respectively. At the end of the time points, cells were lysed and luciferase activity was measured using the luciferase reporter assay kit (Promega, Sydney, NSW, Australia) and a luminescence reader (BMG LABTECH, Ortenberg, Germany).

5.3.9 Flow cytometric analysis for macrophage marker

Bone marrow was flushed from the long bone (femur and tibia) of mice and then cultured in complete αMEM for 24 h. Non-adherent cells were then collected and cultured in complete αMEM containing M-CSF (50 ng/mL) for 3 days culture. The attached cells were used for flow cytometric analysis. To investigate the purity of bone marrow macrophages (BMMs), cell suspension ($5 \times 10^5$ cells) were incubated with APC conjugated monoclonal antibody for mouse CD11b (Thermo Fisher Scientific, Scoresby, VIC, Australia) or isotype-matched control IgGs for 30 min on ice. After washing 3 times, cells were subjected to flow cytometric analysis using FACSCantoll (BD Biosciences, New Jersey, USA). Data were processed using FlowJo software (FlowJo LLC, Ashland, Oregon, USA).

5.3.10 TUNEL assay

TUNEL assays were performed using an In Situ Cell Death Detection kit (Sigma-Aldrich, Sydney, NSW, Australia). $6 \times 10^3$ BMMs were seeded onto coverslips in 96-well plates and were induced into osteoclasts. To determine whether Pse can induce osteoclast apoptosis, cells were cultured with M-CSF and RANKL for further 2 days after mature osteoclasts formation. Cells were treated with different concentrations of Pse (0, 5, 10 μM). DNase I (3,000 U/mL, 30 min) treated cells were used as positive control. Cells were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 for 15 minutes. Cells were then processed following the manufacturer’s protocol in order to label fragmented DNA. Nuclei were stained with DAPI. The percentage of nuclei was used to evaluate Pse’s effect on apoptosis.

5.3.11 Osteoblast differentiation

To determine whether Pse has effects on osteoblast differentiation, MC3T3-E1 cells (ATCC, Manassas, Virginia, USA) were cultured with α-MEM, 10% FBS, and 1% penicillin-streptomycin at
37°C in a humidified atmosphere of 5% CO₂. To induce osteogenic differentiation, cells (1.0 x 10⁴/well) were seeded in 48-well plates overnight and then changed into osteogenic medium (10 mM β-glycerophosphate and 50 μg/mL ascorbic acid) with different concentrations of Pse (5, 10 μM). BMP-2 (50 ng/mL) treatment was used as positive control; Group without Pse and BMP-2 was negative control. Medium was changed every 3 days. After incubation for 0, 7, and 14 days, cells in each group were fixed in 4% paraformaldehyde for 10 min, washed with PBS for 3 times, and stained by BCIP/NBT liquid substrate system (Sigma-Aldrich, Sydney, NSW, Australia) for alkaline phosphatase (ALP) at 37°C for 30 min.

5.3.11 Western blot Assay

BMM cells were seeded in 6-well plates (1 x 10⁵ cells/well) and stimulated with GST- rRANKL and M-CSF in the presence or absence of Pse (10 μM) for the stated times. Cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer to harvest protein. For GTP-Rac1 detection, cell lysates were collected and incubated with GST-Human PAK1-PBD fusion protein that binds to GTP-bound Rac1 using Active Rac1 Detection Kit (Cell Signaling Technology, Danvers, MA, USA). Protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE Healthcare, Silverwater, Australia). Non-specific binding was blocked by incubation in 5% skim milk powder and the membrane was then incubated with primary antibodies (1:1000) with gentle shaking overnight at 4°C. The next day, membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h. Antibodies were detected with enhanced chemiluminescence substrate (PerkinElmer, Waltham, MA, USA). Images were acquired on an Image-quant LAS 4000 (GE Healthcare, Silverwater, NSW, Australia) and analyzed by ImageJ software.

5.3.12 Ovariectomized (OVX)-induced osteoporosis mouse model

All in vivo experimental procedures were approved by the Institutional Animal Ethics Committee of Sir Run Run Shaw Hospital. Eighteen C57BL/6J mice (females; 18.6 ± 1.4 g, 11 weeks old) were supplied by Animal Experiment Center of Sir Run Run Shaw Hospital (Hangzhou, Zhejiang, China). All mice were randomly divided into three groups: sham group (n=6), OVX group (n=6), and OVX+Pse (5 mg/kg) group (n=6). Bilateral ovariectomy was carried out to induce osteoporosis under chloral hydrate anesthesia for the mice in OVX group and OVX+Pse group, a sham procedure in which the ovaries were only exteriorized but not resected was performed for the mice in sham group. All mice had a 1-week-recovery after operations, then the mice in the OVX +Pse group were administrated
an intraperitoneal injection of Pse at 5 mg/kg every 2 days for 6 weeks. The mice in the sham and OVX groups were intraperitoneally injected with vehicle (1% DMSO in PBS) as a control. ROS production in vivo was determined using DHE following previously described protocols with modifications [37]. In brief, 24 h before killing, each mouse received a 200-μL intravenous injection of DHE at 25 mg/kg.

5.3.13 μCT scanning and analysis
After sacrifice, the femurs were collected and fixed in 10% neutral buffered formalin for 24 h and the excess soft tissue was removed. Left femurs were scanned using a Skyscan 1176 microCT instrument (Bruker microCT, Kontich, Belgium) using the following settings: source voltage, 50 kV; source current, 500 μA; Al 0.5 mm filter; pixel size 9 μm; rotation step, 0.4 degree. The images were then reconstructed with NRecon software (Bruker microCT, Kontich, Belgium) using the following settings: ring artefact correction, 7; smoothing, 2; beam hardening correction, 40%. A refined volume of interest was generated 0.5mm above the growth plate of the distal femur and 1 mm in height. The trabecular bone region of interest (ROI) within this volume was manually defined and bone parameters within this ROI were all determined using a constant threshold (50 ~ 255) for binarization of the trabecular bone. The following parameters, including bone volume per tissue volume (BV/TV), trabecular number (Tb.N), connectivity density (Conn.Dn), and trabecular thickness (Tb.Th), were analyzed by the program CTAn (Bruker microCT, Kontich, Belgium). Cortical bone analysis was performed in the mid shaft (4 mm below the growth plate with a height of 1 mm) and a constant threshold (100~255) for binarization was used.

5.3.14 Bone histomorphometry analysis
Following microCT analysis, femurs were decalcified in 14% EDTA (Sigma-Aldrich, Sydney, NSW, Australia) at 37 °C for 7 days, and then embedded into paraffin for sectioning. Hematoxylin and eosin (H&E) staining and TRAcP activity staining were performed. Section images were acquired using Aperio Scanscope (Mt Waverley, VIC, Australia), and bone histomorphometric analyses were performed using BIOQUANT OSTEO software (Bioquant Image Analysis Corporation, Nashville, TN, USA).

For in vivo ROS fluorescence detection, cryosections of bone tissue were prepared as previously described [38]. Fresh bone tissues were dissected, cleaned and immediately fixed in 4% paraformaldehyde (Sigma-Aldrich, Sydney, NSW, Australia) solution at 4 °C for 4 h. Next,
decalcification was carried out with 0.5 M EDTA overnight at 4 °C under constant rotation, after which the EDTA was exchanged for cryoprotective solution composed of 20% sucrose and 2% polyvinylpyrrolidone (PVP) (Sigma-Aldrich, Sydney, NSW, Australia) for 24 h. Finally, the resultant tissues were embedded and frozen in 8% gelatin in the presence of 20% sucrose and 2% PVP. 5 μm sections were generated and air-dried at room temperature prior to permeabilization for 10 min in 0.3% Triton X-100 (Sigma-Aldrich, Sydney, NSW, Australia). Nuclei were stained with DAPI for 30 min. After washing with PBS, sections were mounted and coverslips were sealed with nail polish. An NIKON A1Si confocal microscope (Nikon Corporation, Minato, Tokyo, Japan) was used for imaging. The fluorescence intensity of 6 random areas of each group were quantified and analyzed using NIS-Elements software (Nikon Corporation, Minato, Tokyo, Japan).

5.3.15 Statistical analysis
Each experiment was repeated at least three times. All quantitative data are presented as mean ± standard deviation (SD). Statistical significance was determined by Student’s t test. A p-value of less than 0.05 was considered to be significant.

5.4 Results

5.4.1 Pse suppresses RANKL-induced osteoclastogenesis in vitro
The purity of isolated precursor BMMs was approximately 99%, as assessed by flow cytometry for CD11b (Figure 5.1). The chemical structure and formula of Pse are shown in Figure 5.2A. To identify the effect of Pse on osteoclastogenesis, BMMs were seeded in 96-well plates and treated with both M-CSF and RANKL in the presence or absence of various concentrations of Pse as indicated. Numerous TRAcP-positive multinucleated osteoclasts formed in the RANKL-induced control group (without Pse), whereas increasing concentrations of Pse exerted a dose-dependent inhibition on osteoclastogenesis (Figure 5.2B and C). To determine any apoptotic effect and potential cytotoxicity of Pse on BMMs, TUNEL assay and MTS assay were performed. Pse was found to have no effect on BMMs apoptosis (Figure 5.3A and B) and proliferation (Figure 5.3C) over the concentrations used in this study. To examine which stage of osteoclastogenesis was affected, cells were treated with 10 μM Pse at indicated time phases (Figure 5.4A). Pse was found to predominantly exert its suppressive effect during mid-late stage (Day 3-6) of osteoclast differentiation, rather than early stage (Day 1-3) (Figure 5.4B).
Figure 5.1 Flow cytometric analysis for bone marrow-derived macrophages. (A) CD11b expression in isotype control stained cells (grey) and APC conjugated CD11b stained cells (red) within single cell population. (B) The percentage of CD11b+ cells were determined in triplicate.

FSC-A, forward scatter area; APC, allophycocyanin.
Figure 5.2 Pse suppresses RANKL-induced osteoclastogenesis in vitro. (A) The chemical structure and formula of Pse. (B) Representative images of TRAcP staining showing that Pse inhibited osteoclastogenesis dose-dependently. BMMs were stimulated with RANKL for 5 days in the absence or presence of indicated concentrations of Pse. (C) Quantification of TRAcP-positive multinucleated cells (nuclei >3) (n=3 per group). All bar graphs are presented as mean ± SD. *p<0.05, **p<0.01 compared with control group (without Pse treatment). Scale bar = 200 µM. BMMs, bone marrow macrophages; Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB ligand; TRAcP, tartrate-resistant acid phosphatase
Figure 5.3 Pse showed no effect on osteoclast apoptosis and viability. (A) After mature osteoclasts induced by RANKL, different concentrations of Pse (0, 5, 10 μM) were used to treat the cells for additional 2 days. As a positive control for apoptosis, DNase I treatment (3,000 U/mL, 30 min) was included. Apoptotic osteoclasts were then assessed by TUNEL assay (green). Nuclei were visualized by DAPI staining. The TUNEL-positive apoptotic nuclei are indicated by white arrows. (B) The quantification of the percentage of TUNEL-positive nuclei in each group (n=5 per group). (C) Effects of Pse on BMM viability after 48 h treatment as measured by MTS assay (n=3 per group). All bar graphs are presented as mean ± SD. **p<0.01 compared with the group without Pse and DNase I treatment. Scale bar=100 μm. DAPI, 4,6-diamidino-2-phenylindole; OD, optical density; Pse, Pseurotin A; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; OC, osteoclast.
Figure 5.4 Pse predominantly suppresses osteoclastogenesis at mid-late stage. (A) Representative images of TRAcP staining showing BMMs treated with Pse for the indicated days during RANKL-induced osteoclastogenesis. (B) Quantification of TRAcP-positive multinucleated cells treated with Pse in different time periods (n=3 per group). All bar graphs are presented as mean ± SD. **p<0.01 compared with control group (without Pse treatment). Scale bar = 200 µM. BMMs, bone marrow macrophages; Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB ligand; TRAcP, tartrate-resistant acid phosphatase.
5.4.2 Pse affects podosome belt formation and inhibits osteoclast-specific genes expression

Cells were co-stained with rhodamine-phalloidin and anti-vinculin to visualize podosome belt formation and morphological changes in cells treated with or without Pse. As shown in Figure 5.5 and Figure 5.6, after RANKL stimulation, well-defined podosome belts with intact nuclei formed in mature osteoclasts. In contrast, smaller osteoclasts with fewer nuclei were observed after Pse treatment (5, 10 µM) (Figure 5.5B and C).

Several osteoclast-specific genes, including c-fos, Nfatc1, Ctsk, Acp5, Atp6v0d2, and Mmp9 are upregulated in BMMs when osteoclast differentiation is induced. We examined these genes at mRNA level using quantitative PCR to observe how Pse affected osteoclast-specific genes expression and found that their expression was inhibited during RANKL-induced osteoclastogenesis following the addition of Pse (Figure 5.7A-F). Collectively, these results further confirmed that Pse suppressed the expression of osteoclast-specific genes and thus osteoclastogenesis in vitro.

5.4.3 Pse inhibits osteoclast resorptive function

We next determined whether Pse has an effect on osteoclast resorptive activity using hydroxyapatite-coated plates. A reduction in the number of osteoclasts and the resorption area were observed at both Pse concentrations (5, 10 µM) (Figure 5.8A and B). In addition, following normalization of resorption area to the number of osteoclasts, a dose-dependent inhibitory effect on osteoclast resorptive function was observed as compared to the control group (Figure 5.8C).

5.4.4 Pse reduces RANKL-induced intracellular ROS level in BMMs

To investigate whether RANKL-induced ROS production during osteoclast differentiation was reduced by Pse, ROS level was visualized using a cell-permeable, oxidation-sensitive dye H₂DCFDA. ROS oxidation of this dye was detected as a fluorescent signal derived from the oxidation product DCF using confocal microscopy. The intensity of DCF fluorescence was significantly decreased in a dose-dependent manner in BMMs when stimulated with M-CSF and RANKL in the presence of Pse (5, 10 µM) (Figure 5.9). Therefore, our results indicated that Pse inhibited osteoclast formation and activity via suppressing ROS.
Figure 5.5 Pse affects podosome belts formation. (A) Representative images showing the impaired podosome belts formation in osteoclasts treated with Pse. Vinculin (green), F-actin (red) and nuclei (blue) staining of osteoclasts on glass coverslip and observed by confocal microscopy. Overlapping staining of vinculin and F-actin is shown in yellow. **(B) Quantification of the nuclei number per osteoclast (n=16 per group). (C) Quantification of the relative area of osteoclast (n=16 per group). All bar graphs are presented as mean ± SD. **p<0.01 compared with the control group (treated with RANKL but without Pse). Scale bar = 200 µM. DAPI: 4,6-diamidino-2-phenylindole; Pse, Pseudo toxin A.
Figure 5.6 Confocal images of podosome belts of osteoclasts. (A) High magnification confocal images showing podosome belts formation in osteoclasts treated with or without Pse. (B) Fluorescence intensity profile through podosome belts (indicated by the white arrow in A). DAPI, 4,6-diamidino-2-phenylindole; Pse, Pseurotin A
Figure 5.7 Pse inhibits osteoclast-specific genes expression. qPCR analysis of osteoclast-specific genes expression of (A) c-fos, (B) Nfatc1, (C) Ctsk, (D) Acp5, (E) Atp6v0d2, and (F) Mmp9 relative to Hprt1 and Hmbs in BMMs stimulated with RANKL for 5 days in the presence of Pse of indicated concentrations (n=3 per group). All bar graphs are presented as mean ± SD. **p<0.01 compared with the control group (treated with RANKL but without Pse). Scale bar = 200 μM. Acp5, acid phosphatase 5, tartrate resistant; Atp6v0d2, ATPase H+ Transporting V0 Subunit D2; BMMs, bone marrow macrophages; c-fos, Proto-oncogene C-Fos; Ctsk, cathepsin K; Mmp9, matrix metalloproteinase 9; Nfatc1, nuclear factor of activated T cells 1; PCR, polymerase chain reaction; Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB ligand; TRAcP, tartrate-resistant acid phosphatase.
Figure 5.8 Pse inhibits osteoclast resorptive function. (A) Representative images showing the osteoclastogenesis and hydroxyapatite resorption in each group. Osteoclasts were seeded in hydroxyapatite-coated plates and treated by RANKL with or without Pse. Half of the wells for each group were stained by TRAcP or washed with 10% bleach solution. (B-C) Quantification of resorbed hydroxyapatite area per well and resorbed area per osteoclast in each group (n=3 per group). All bar graphs are presented as mean ± SD. *p<0.05, compared with control. Scale bar = 200 µM. Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB ligand; TRAcP, tartrate-resistant acid phosphatase.
**Figure 5.9** Pse attenuates RANKL-induced ROS generation in vitro. **(A)** Representative confocal images of RANKL-induced ROS generation in BMMs with or without pre-treatment of Pse. Intracellular ROS generation reacted with the cell permeant, oxidation-sensitive dye H2DCFHDA and was detected in the form of highly fluorescent DCF. The lower panel is a merge of DCF fluorescence and confocal digital interference contrast (DIC) images. **(B)** Quantification of DCF fluorescence intensity averaged on cells of each well (n=3 per group). **(C)** Quantification of the number of ROS-positive cells per field (n=3). All bar graphs are presented as mean ± SD. *p<0.05, compared with control. Scale bar = 200 µM. Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB ligand; ROS, reactive oxygen species.
Figure 5.10 Pse affects ROS-related genes expression in RANKL-stimulated BMMs. The expressions of (A) Nox1, (B) Nrf2, (C) Nrf2/Keap1, (D) HMOX1, (E) CAT, and (F) GCLC were examined by qPCR after 2-day RANKL stimulation in the absence or presence of Pse (5, 10 μM). Gene expression was calibrated using the Hprt1 housekeeping gene. **p<0.01 compared with the group with only RANKL treatment. CAT, catalase; GCLC, gamma-glutamylcysteine synthetase; HMOX1, heme oxygenase 1; Hprt1, hypoxanthine phosphoribosyltransferase 1; Keap1, kelch like ech associated protein 1; Nox1, nicotinamide adenine dinucleotide phosphate oxidase 1; Nrf2, nuclear factor-erythroid 2 related factor 2; Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB (NF-κB) ligand.
5.4.5 Pse suppresses intracellular ROS level by down-regulating the TRAF6/Rac1/Nox1 signaling cascade as well as enhancing expression of antioxidant enzymes

To determine whether Pse affects ROS generation, the activation level of Nox1, known as a major contributing factor in ROS generation (9, 32), was investigated using western blot. BMMs were incubated with RANKL for 2 days in the absence or presence of Pse at different concentrations as indicated. ROS-related genes were determined using qPCR (Figure 5.10). Nox1 expression was significantly up-regulated by RANKL, but dose-dependently inhibited by Pse at the concentrations of 2.5-10 μM (Figure 5.11 C and E). TRAF6 and GTP-Rac1 are required for the Nox1 activation, we therefore examined whether Pse inhibited Nox1 by attenuating TRAF6 and GTP-Rac1. Adaptor protein TRAF6 expression was augmented by RANKL and showed a trend of down-regulation at high concentrations (10 μM), which was not significant (Figure 5.11C and D). GTP-bound Rac1 is a cytosolic component of Nox1 and is responsible for Nox1 activation. As shown in Figure 5.11A and B, GTP-Rac1 activation was significantly enhanced after 5 min and 15 min of RANKL stimulation but was attenuated by Pse treatment in a dose-dependent manner. Next, to examine whether Pse is able to scavenge ROS by up-regulating antioxidant enzymes, the expression of antioxidant enzymes including HO-1, catalase, and GSR were examined. As shown in Figure 5.12A and B, the expression of HO-1 was reduced by RANKL stimulation but was recovered and enhanced dose-dependently by Pse treatment. Similarly, Pse also enhanced the expression levels of catalase (Figure 5.12A and C) and GSR (Figure 5.12A and D). Taken together, these data mechanistically suggest that Pse suppresses RANKL-induced intracellular ROS level via inhibition of ROS generation as well as the enhancement of ROS scavenging.
Figure 5.11 Pse suppresses ROS generation through TRAF6-Rac1-Nox1 signaling cascade. (A) Representative Western Blot images of the effects of Pse on GTP-Rac1. BMMs were pretreated with Pse at indicated concentrations and were then stimulated with RANKL for 0, 5, and 15 min. Cell lysates were collected and incubated with GST-Human PAK1-PBD fusion protein that binds to GTP-bound Rac1. Rac1 activation levels were then detected with anti-Rac1 antibody. (B) Quantification of the ratios of band intensity of GTP-Rac1 relative to Rac1 (n=3 per group). (C) Representative Western Blot images of the effects of Pse on TRAF6 and Nox1 expression. BMMs were stimulated with RANKL (50 ng/mL) in the absence or presence of Pse (2.5, 5, 10 μM) for 2 days before protein collection for Western Blot. (D and E) Quantification of the ratios of band intensity of TRAF6 and Nox1 relative to β-actin (n=3 per group). All bar graphs are presented as mean ± SD. *p<0.01, relative to RANKL-induced control group. BMMs, bone marrow macrophages; GTP, Guanosine-5′-triphosphate; NOX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; Pse, Pseurotin A; Rac1, Ras-related C3 botulinum toxin substrate 1; TRAF6, TNF receptor-associated factor 6.
Figure 5.12 Pse scavenges ROS by enhancing the expression of antioxidant enzymes.

(A) Representative Western Blot images of the effects of Pse on expression of antioxidant enzymes, including HO-1, Catalase, and GSR. BMMs were stimulated with RANKL (50 ng/mL) in the absence or presence of Pse (2.5, 5, 10 μM) for 2 days before protein collection for Western Blot. (B-D) Quantification of the ratios of band intensity of HO-1, Catalase, and GSR relative to β-actin (n=3 per group). All bar graphs are presented as mean ± SD. *p<0.01 relative to RANKL-induced control group. BMMs, bone marrow macrophages; GSR, glutathione-disulfide reductase; HO-1, heme oxygenase-1; Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB ligand; ROS, reactive oxygen species;
5.4.6 Pse interferes with RANKL-induced activation of NF-κB and MAPK pathways

NF-κB and MAPK pathways are regarded as the main signaling pathways activated during osteoclastogenesis (6), and RANKL-induced ROS generation may trigger these signaling events. In order to further explore whether Pse blocked ROS activated osteoclastogenesis via attenuating NF-κB and MAPK signaling, we investigated these two pathways using Western Blot and luciferase assay. For the NF-κB pathway, we analyzed the expression of IκB-α. NF-κB transcription factors are bound to IκB-α and retained in an inactive state; these are activated and released when IκB-α is degraded after stimulation with RANKL (33). Within 60 minutes of stimulation with RANKL, IκB-α degradation was significantly inhibited by Pse (Figure 5.13A and B), thus indicating failure to activate NF-κB signaling. Consistent with inhibition of IκB-α degradation, the results of luciferase assay showed the activation of NF-κB was significantly inhibited by treatment with Pse (Figure 5.13C). For the MAPK signaling pathway, as shown in Figure 5.14, phosphorylation of ERK (Figure 5.14B), p38 (Figure 5.14C), and JNK (Figure 5.14D) relative to total ERK, total p38, and total JNK was suppressed significantly by Pse treatment in BMMs. Collectively, these data suggested Pse had an inhibitory effect on both NF-κB and MAPK activation pathways mediated by RANKL.

5.4.7 Pse attenuates NFATc1 activity and downstream factors

NFATc1 acts as the master transcriptional regulator of osteoclastogenesis (7). In this study, RAW264.7 cells stably expressing an NFATc1-driven luciferase reporter gene were used to measure the NFATc1 transcriptional activity. After stimulation with RANKL, NFATc1 activity was very highly elevated (Figure 5.15A). In contrast, NFATc1 activity was abrogated significantly in a dose-dependent manner after pre-treatment with Pse (2.5, 5, 10 µM) (Figure 5.15A). NFATc1 expression was also found to be suppressed over the course of osteoclast differentiation using Western Blot assay (Figure 5.15B and C). In addition, our results showed that Pse abrogates the elevation of integrin αV (Figure 5.15D), cathepsin K (Figure 5.15E), and V-ATPase-d2 (Figure 5.15F), which are all downstream proteins needed for osteoclast formation and function. Consequently, Pse treatment strongly inhibits NFATc1 activity, thus affecting downstream protein expression.
Figure 5.13 Pse suppresses osteoclast differentiation by inhibiting NF-κB pathway.

(A) Representative Western Blot images of the effects of Pse on IκBα degradation induced by RANKL. BMMs were pretreated with 10 μM Pse for 2 h prior to the addition of RANKL at the indicated time points and the indicated proteins were determined. (B) Quantification of the ratios of band intensity of IκBα relative to β-actin (n=3 per group). (C) Cells were then pre-treated with Pse and then stimulated with GST-rRANKL (50 ng/mL) for 6 h. NF-κB luciferase assay showing that Pse inhibited NF-κB transcriptional activity dose-dependently (n=3 per group). All bar graphs are presented as mean ± SD. *P<0.05, **P<0.01 relative to RANKL-induced control group at the same time point. BMMs, bone marrow macrophages; NF-κB, nuclear factor-κB; Pse, Pseurotin A; RANKL, receptor activator of nuclear factor-κB ligand.
Figure 5.14 Pse suppresses osteoclast differentiation by inhibiting MAPK pathway.

(A) Representative Western Blot images of the effects of Pse on MAPKs pathway, including p-ERK, p-P38, and p-JNK. BMMs were pretreated with 10 μM Pse for 2 h prior to the addition of RANKL at the indicated time points and the indicated proteins were determined. (B-D) Quantification of the ratios of band intensity relative to ERK, P38, and JNK were analyzed (n=3 per group). All bar graphs are presented as mean ± SD. *P<0.05 relative to RANKL-induced control group at the same time point. BMMs, bone marrow macrophages; MAPKs, mitogen-activated protein kinases; Pse, Pseurotin A
Figure 5.15 Pse suppresses osteoclast differentiation by inhibiting NFATc1 signaling.

(A) NFATc1 luciferase assay showing Pse significantly inhibited NFATc1 transcriptional activity (n=3 per group). (B) Representative Western Blot images of the expression levels of NFATc1 and osteoclast-related proteins including Integrin αV, Cathepsin K, and V-ATPase-d2 during osteoclastogenesis. BMMs were stimulated with RANKL in the absence or presence of 10 μM Pse for 0, 1, 3, and 5 days before protein collection for Western Blot. (C-F) Quantification of the ratios of band intensity of NFATc1, Integrin αV, Cathepsin K, and V-ATPase-d2 relative to β-actin (n=3 per group). All bar graphs are presented as mean ± SD. *P<0.05, **P<0.01 relative to RANKL-induced control group at the same time point. BMMs, bone marrow macrophages, NFATc1, nuclear factor of activated T cells 1; Pse, Pseurotin A
5.4.8 Pse prevents OVX-induced bone loss

Our results established that Pse has inhibitory effects on osteoclast formation and function in vitro. The potential of Pse to act as a prophylactic agent to protect against osteoclast-related bone disease in vivo was further explored using an OVX-induced osteoporosis mice model. Mice were either OVX or sham operated and were then intraperitoneally injected with either Pse at a concentration of 5 mg/kg every 2 days, or vehicle only for 6 weeks post-surgery (Figure 5.16A). No adverse events or fatalities were recorded after the OVX procedure and Pse administration. Furthermore, there were no observable effects of Pse on body weight in treated mice relative to non-treated mice (Figure 5.16B). Micro-CT confirmed that Pse prevented the extensive bone loss induced by the OVX procedure in mice femurs (Figure 5.17A). Quantitative analysis of bone parameters also confirmed increased BV/TV, Tb.N and Conn.Dn in Pse treated group relative to the OVX mice without Pse treatment (Figure 5.17B-D). However, Tb.Th remained unchanged in this study (Figure 5.17E). Consistently, histological examination showed that Pse reduced the extent of bone loss induced by OVX (Figure 5.19). Quantification of H&E staining demonstrated that the bone volume and bone surface were both well maintained in Pse treated group compared with non-Pse treated OVX mice (Figure 5.19B and C). Cortical bone related parameters, including cortical thickness (Ct.Th), total cross-sectional area (Tt.Ar), cortical bone area (Ct.Ar), and cortical area fraction (Ct.Ar/Tt.Ar), were also analyzed (Figure 5.18A-E). Pse showed no effect on cortical bone.

5.4.9 Pse reduces osteoclasts and ROS production in the OVX mouse model

Histological staining using TRAcP was performed on femur sections to examine the number of osteoclasts in vivo. As shown in Figure 5.20A, the OVX procedure led to an increased number of TRAcP-positive osteoclasts, whereas Pse treatment prevented the increase of osteoclast number in vivo. This was supported by quantification of osteoclast parameters, demonstrating a reduction in the number and surface area of osteoclasts on the bone surface in Pse treated mice (Figure 5.20B and C). In addition, as Pse exhibited in vitro anti-oxidant activity, in vivo ROS level was also assessed in cryosections of bone tissue using DHE, a probe that detects ROS. Consistent with previous publications (34, 35), ROS fluorescence intensity was highly elevated due to OVX procedure and Pse dramatically reversed the ROS production within the bone marrow microenvironment (Figure 5.21). Taken together, these data indicated that Pse prevented OVX-induced bone loss in vivo by scavenging ROS thus inhibiting osteoclast activity.
Figure 5.16 Experimental design of Pse treatment for ovariectomized (OVX) animal model. 
(A) Schematic illustration of the establishment of OVX mouse model and the experimental design to evaluate Pse’s therapeutic effects. (B) Body weights of all mice recorded at indicated time points. DHE: dihydroethidium; Pse, Pseurotin A; TRAcP: tartrate resistant acid phosphatase; ROS: reactive oxygen species;
Figure 5.17 Pse treatment prevents ovariectomized (OVX)-induced trabecular bone loss in vivo. (A) Representative μCT images showing that the trabecular bone loss was prevented by Pse administration. (B-E) Quantitative analyses of parameters regarding bone microstructure, including BV/TV, Tb.N, Conn.Dn, and Tb.Th (N=6 per group). All bar graphs are presented as mean ± SD. **P<0.01 relative to the OVX group. BV/TV, bone volume per tissue volume; Conn.Dn, connectivity density; NS, non-significant; Pse, Pseurotin A; Tb.N, trabecular number; Tb.Th, trabecular thickness.
Figure 5.18 Cortical bone parameters of all mice in each group. (A) Representative μCT images of femur cortical bone in each group. (B-E) Quantitative analyses of cortical bone related parameters, including cortical thickness (Ct.Th), total cross-sectional area (Tt.Ar), cortical bone area (Ct.Ar), and cortical area fraction (Ct.Ar/Tt.Ar) (N=6 per group). All bar graphs are presented as mean ± SD. *P<0.05, **P<0.01 relative to the OVX group. OVX, ovariectomized; Pse, Pseurotin A; ns, non-significant.
Figure 5.19 Histomorphometry analysis of bone mass. (A) Representative images of HE staining of decalcified bone sections. (B and C) Quantitative analyses of BV/TV and BS in tissue sections (n=4 per group). All bar graphs are presented as mean ± SD. **P<0.01 relative to the OVX group. BM, bone marrow; BS, bone surface; BV/TV, bone volume per tissue volume; HE, hematoxylin and eosin; Pse, Pseurotin A; TB, trabecular bone;
**Figure 5.20 Histomorphometry analysis of osteoclast ex vivo.** (A) Representative images of TRAcP staining of decalcified bone sections. (B-C) Quantitative analyses of N.Oc/BS and Oc.S/BS (n=4 per group). All bar graphs are presented as mean ± SD. **P<0.01 relative to the OVX+Vehicle group. BM, bone marrow; N.Oc/BS, osteoclast number/bone surface; Oc.S/BS, osteoclast surface/bone surface; TB, trabecular bone; TRAcP, tartrate resistant acid phosphatase.**
Figure 5.21 Pse treatment reduces ROS level in OVX mice. (A) Representative images of bone cryosections showing ROS fluorescence in different groups. (B) Quantitative analyses of ROS fluorescence intensity relative to sham group (n=5 per group). All bar graphs are presented as mean ± SD. **P<0.01 relative to the OVX+Vehicle group. BM, bone marrow; DAPI, 4,6-diamidino-2-phenylindole; DHE, dihydroethidium; GP, growth plate; ROS, reactive oxygen species.
5.5 Discussion

Osteoporosis is a skeletal disease which is characterized by low bone mass, leading to reduced bone strength and susceptibility to fracture. It is more frequently diagnosed in the postmenopausal population majorly due to excessive osteoclast activity. Therefore, osteoclasts remain as the major target in dealing with osteoporosis. Current clinically available therapies for osteoporosis such as estrogen replacement, bisphosphonates, and denosumab are effective but have limitations and side-effects including increased risk of breast cancer, osteonecrosis of the jaw, and atypical femur fracture (36, 37). A search for novel alternative drugs is always required for the improved treatment of osteoporosis. In the present study, we elucidated for the first time that Pse inhibited osteoclastogenesis in vitro and prevented the development of OVX-induced osteoporosis in vivo via suppressing ROS level.

Firstly, osteoclastogenesis assay and hydroxyapatite resorption assay in vitro, showed Pse as a potentially novel therapy for osteoclast-related bone disease. Further, we investigated the mechanisms underlying this inhibitory effect on osteoclast formation and function, especially regarding the intracellular ROS level, NF-κB pathway, MAPK pathway, and NFATc1.

Upon stimulation with RANKL, intracellular ROS level was hugely suppressed by Pse treatment, which was accompanied by the reduced activation of NF-κB and MAPKs, leading to the subsequent attenuation of NFATc1. The level of intracellular ROS depends on the balance between the rate of production and the rate of scavenging. ROS are generated during osteoclastogenesis through a signaling cascade including TRAF6, Rac1, and Nox1 (9). Nox1-mediated ROS production was found to regulate RANKL-induced signaling and is required for osteoclastogenesis (9, 32). Therefore, we hypothesized that the down-regulated ROS signaling might be partly due to the inhibition of Nox1. Indeed, Pse effectively attenuated Nox1 expression via the suppression of GTP-Rac1. To scavenge oxidative stress and maintain redox homeostasis, a wide range of antioxidant enzymes are induced. Several ROS scavengers were determined to be upregulated by Pse in this study, such as HO-1, catalase, and GSR. HO-1 induction may act as a defense mechanism to catalyze heme liberated by oxidants (38). Catalase plays a key role in converting hydrogen peroxide into water and oxygen (39). GSR converts glutathione disulphide back to glutathione in an NADPH-consuming process and GSR inhibitor was also found to induce the activation of the NF-κB pathway (40). The mechanisms behind the upregulation of antioxidant enzymes still remain unclear. Nuclear factor-erythroid 2 related
factor 2 (Nrf2) is regarded as an important redox-sensitive transcription factor that positively regulates antioxidant enzymes (12, 13). RANKL stimulation attenuated the gene expression of *Nrf2* to favour ROS signalling, whereas Pse treatment recovered and upregulated *Nrf2* expression dose dependently (*Figure S4*), suggesting that Pse may upregulate antioxidant enzymes at least in part via augmenting *Nrf2*. Taken together, Pse attenuates the ROS level by suppressing ROS production as well as enhancing ROS scavenging.

Accumulating evidence suggests that RANKL-induced ROS signaling regulates MAPK and NF-κB activation (9, 18, 41-43). NF-κB signaling serves a crucial role and appears to be the first event in early osteoclast development from precursors, followed by c-Fos and NFATc1 activation (7). Loss of NF-κB signaling in mice leads to an osteopetrotic bone phenotype due to a defect of osteoclastogenesis (44). Tumor necrosis factor α (TNFα)-induced NF-κB activation is redox-dependently regulated through the dynein light chain LC8 (41). ROS is able to oxidize LC8 to a homodimer linked by a disulphide bond between the Cys² residues of each subunit, which promotes its dissociation from IκBα and thereby allows IκBα’s phosphorylation and degradation by IκB kinase (IKK), thus releasing NF-κB dimers to translocate to nucleus (41). In our study, Pse treatment effectively suppressed RANKL-induced IκB-α degradation, as demonstrated by a higher expression level of IκB-α in the Pse treated group, suggesting the involvement of IκB-dependent inactivation of NF-κB in the inhibitory effect of Pse on osteoclast formation. Furthermore, luciferase assay results consistently supported the inhibitory effect of Pse on NF-κB transcriptional activation.

The MAPK pathways, including three major MAPK family members (ERKs, JNKs, and p38), are also important signaling events involved in osteoclast differentiation stimulated by RANKL (6). ERK is crucial for the survival of osteoclasts (45, 46). Similarly, JNK and p38 are also phosphorylated during osteoclastogenesis in response to RANKL stimulation (46, 47). MAPKs are activated by MAPK kinases through phosphorylation of tyrosine and threonine and inactivated by MAPK phosphatases (MKPs) through dephosphorylation (48). ROS may act as a physiologic second messenger and oxidize tyrosine phosphatases, thus inhibiting MKPs and subsequently activating MAPKs (9, 49). In the present study, the Western Blot results showed that Pse comprehensively attenuated the phosphorylation of ERK, JNK, and p38 thus achieving its inhibitory effects. Thus, ROS is suggested to incorporate into the activation of NF-κB and MAPKs during osteoclastogenesis.
NFATc1 is well-known as a master transcriptional regulator of terminal osteoclast differentiation, and its initiation is regulated in an auto-amplification loop to maintain robust expression by binding to its own promoter (8). NFATc1 modulates the expression of osteoclast downstream gene expression, and NFATc1-deficiency results in inhibition of osteoclastogenesis in vitro and osteopetrosis in vivo (50). Here, our data implicated that the expression level and transcriptional activity of NFATc1 were reduced by Pse following RANKL stimulation. The mechanism behind the reduction of NFATc1 still remains unclear. It could result from the suppression of upstream NF-κB and MAPK signaling or a direct intervention of NFATc1 activation. We further demonstrated that Pse suppressed the expression of osteoclast-specific genes, including Ctsk, Acp5, Atp6v0d2, and Mmp9, which are regulated by NFATc1 directly or indirectly (51, 52), and participate in osteoclast formation and their bone resorptive function. C-Fos, which cooperates with NFATc1 in the context of activator protein 1 (AP-1) and contributes to osteoclast differentiation (53), was also found to be inhibited by Pse treatment at mRNA expression level.

Based on these convincing in vitro results, an OVX mouse model was further used to evaluate whether Pse has therapeutic effects in vivo through its antioxidant activity. It is interesting to note that oxidative stress status has a negative correlation with osteoporotic status. OVX rats were found to have higher oxidative stress in the femurs accompanied by a decreased activity of antioxidant systems compared to sham-operated control rats (34, 54). Furthermore, Altindag et al. found that the oxidative stress index values and total plasma oxidant status in osteoporotic postmenopausal women was higher than that in the healthy group (35). The development of osteoporosis is at least partly due to the imbalance between antioxidant defenses and oxidative stress. This compelling evidence provides a new insight into a potential approach for the treatment of postmenopausal osteoporosis via suppressing oxidative stress.

Our results indicated that Pse exhibited a significant protective effect on OVX-induced bone loss in mice by scavenging ROS. A previous study on ROS detection in bone used direct staining on histological sections to assess ROS level (16). However, the stability of ROS level is likely to be highly variable using this method due to the lengthy tissue fixation and processing times. In this study, a ROS probe was intravenously injected 24h prior to euthanasia, which allowed DHE to be distributed to cells and tissues via the circulation. DHE is a probe that is particularly sensitive to superoxide anion levels and it was previously reported as an in vivo marker for ROS (55). The imaging of bone tissue remains challenging because of its calcified nature. Herein, a protocol of mild fixation,
decalcification, and cryo-sectioning was used to process the bone samples, which was able to effectively preserve cellular morphology and tissue architecture (56). To our knowledge, this is the first time that DHE was used as a ROS probe to detect ROS in vivo using histological sections in an OVX model. Our data showed that ROS level within bone marrow were dramatically suppressed in the Pse treated group, which was accompanied by a reduced number of TRAcP-positive osteoclasts. The prevention of bone loss by Pse was thought to be due to its suppression of oxidative stress and subsequent compromised osteoclast formation and function. However, this probe is not cell or tissue-specific, this data only indicated the total ROS level in bone marrow microenvironment, osteoclast-specific ROS in this study still remains to be further investigated.

In summary, this study has demonstrated for the first time that Pse can suppress intracellular ROS level by inhibiting RANKL-induced ROS production and enhancing expression of antioxidant enzymes, which attenuates the activation of MAPK and NF-κB pathways, subsequently leading to attenuation of NFATc1 as well as its downstream proteins (Figure 5.22). These signaling events contribute to decreased osteoclast formation and bone resorptive function in vitro. Additionally, Pse was also found to prevent estrogen deficiency-induced osteoporosis in vivo via suppressing oxidative stress in the bone marrow microenvironment. Therefore, Pse may serve as a novel candidate or an alternative therapeutic treatment for osteoclast-related bone disease such as osteoporosis.
Figure 5.22 A proposed scheme for the inhibition of Pse on osteoclastogenesis. Upon RANKL binding to RANK, both NF-κB and MAPKs pathways are activated, leading to the amplification of NFATc1. Several osteoclast-specific genes such as Ctsk, Acp5, Atp6v0d2, and Mmp9 are upregulated as a result. These signaling events are mediated by RANKL-induced ROS signaling. Our results demonstrated for the first time that Pse inhibits osteoclastogenesis via suppressing ROS level by inhibiting RANKL-induced ROS production and enhancing expression of antioxidant enzymes. Acp5, acid phosphatase 5, tartrate resistant; AP-1, activator protein 1; Atp6v0d2, ATPase H+ Transporting V0 Subunit D2; NFATc1, nuclear factor of activated T cells 1; c-fos, Proto-oncogene C-Fos; Ctsk, cathepsin K; Mmp9, matrix metallopeptidase 9; NOX, nicotinamide adenine dinucleotide phosphate oxidase; Pse, Pseurotin A; Rac1, Ras-related C3 botulinum toxin substrate 1; RANKL, receptor activator of nuclear factor-κB (NF-κB) ligand; ROS, reactive oxygen species.
Figure 5.23 Pse has no effect on osteoblast differentiation. (A) MC3T3-E1 cells were induced osteogenic differentiation for 0, 7, and 14 days. ALP staining was carried out using BCIP/NBT liquid substrate system. (B) Quantitative analyses of ALP staining area (n=3 per group). (C-F) qPCR analysis of osteoblast genes expression of Alpl, Bglap, Tnfrsf11b, and Sp7/Osx relative to Actb in MC3T3-E1 cells induced in osteogenic medium for 14 days in each group (n=3 per group). All bar graphs are presented as mean ± SD. *p<0.05 **p<0.01 compared with control. Actb, actin beta; Alpl, alkaline phosphatase; Bglap, bone gamma-carboxyglutamate protein (osteocalcin); BMP2, bone morphogenetic protein 2; Sp7/Osx, sp7 transcription factor; Tnfrsf11b, tumor necrosis factor receptor superfamily member 11b.
5.6 Reference


CHAPTER SIX - STEROID-INDUCED OSTEONECROSIS OF THE FEMORAL HEAD REVEAL ENHANCED REACTIVE OXYGEN SPECIES AND HYPERACTIVE OSTEOCLASTS

The manuscript of this chapter is in preparations for submission
6.1 Abstract

Steroid-induced osteonecrosis of the femoral head (ONFH) is a progressive bone disorder which typically results in femoral head collapse and hip joint dysfunction. It is well-accepted that osteoclast hyperactivity contributes to loss of bone structural integrity and subchondral fracture in ONFH. However, the pathophysiologic mechanisms underlying the recruitment and activation of osteoclasts in ONFH remain incompletely understood. Here, we assessed for correlations between reactive oxygen species (ROS) and osteoclasts in steroid-induced osteonecrotic femoral heads from both patients and rat ONFH models. When compared with healthy neighboring bone, the necrotic region of human femoral heads was characterized by robust up-regulated expression of osteoclast-related proteins (cathepsin K and tartrate-resistant acid phosphatase) but pronounced down-regulation of antioxidant enzymes (catalase, γ-glutamylcysteine synthetase [γ-GCSc], and superoxide dismutase 1 [SOD1]). In addition, the ratio of TNFSF11 (encoding RANKL)/TNFRSF11B (encoding OPG) was increased within the necrotic bone. Consistently, in rat ONFH models induced by methylprednisolone (MPSL) and imiquimod (IMI), significant bone loss in the femoral head was observed, attributable to increased numbers of tartrate-resistant acid phosphatase (TRAP) positive osteoclasts. Furthermore, the decreased expression of antioxidant enzymes observed by immunoblotting was accompanied by increased ex-vivo ROS fluorescence signals of dihydroethidium (DHE). Therefore, this study lends support to the rationale that antioxidant agents may be a promising therapeutic avenue to prevent or mitigate the progression of steroid-induced ONFH by inhibiting ROS level and hyperactive osteoclasts.

6.2 Introduction

Osteonecrosis of the femoral head (ONFH) is a progressive degenerative disorder of the hip characterized by subchondral bone microfractures and subsequent collapse of the femoral head, eventually leading to dysfunction of the hip joint [1]. ONFH poses a huge socioeconomic burden, usually affecting young to middle aged individuals, with ~67% of asymptomatic patients rapidly progressing to symptomatic stages and thus requiring total hip arthroplasties (THA) [2]. The exact aetiology of ONFH is unclear, however, steroid administration at the hip joint is an established risk factor, which accounts for ~51% of all reported ONFH cases [3]. For example, in patients who received corticosteroid treatment for systemic lupus erythematosus (SLE), it was reported that an increase of corticosteroids to 10 mg/d correlated with a 3.6% increase in the rate of osteonecrosis,
with daily doses >40 mg predisposing patients to ONFH [4]. Other known contributors include alcohol abuse (31%), tobacco use, coagulation abnormalities, chemotherapy, and genetic factors etc [3, 5].

The pathophysiologic mechanisms underlying steroid-induced ONFH are not fully understood. Recent studies suggest that the onset of ONFH is initiated by impaired microcirculation and necrosis of osteocytes, with magnetic resonance imaging (MRI) detecting pools of edema [1, 6], but without noticeable changes in subchondral bone microstructure, as assessed by computed tomography (CT). Following bone necrosis, a repair process ensues whereby osteoclast-mediated bone resorption dwarfs bone formation by osteoblasts, leading to net loss of subchondral trabecular bone [7]. As the ONFH progresses, the loss of bone and altered microarchitecture compromises the integrity of the femoral head leading to deformity and high vulnerability of collapse [6]. It is widely accepted that excessive osteoclast activity during the regenerative phase, rather than during the necrosis of cells and tissue, directly contributes to the loss of bone integrity and ensuing subchondral bone fracture [6-9]. This contribution is further supported by studies showing that anti-resorptive agents could effectively reduce the risk of femoral head collapse at early-stage ONFH when compared with placebo treatment [10-12].

A better understanding of the activation of osteoclasts during repair process is therefore critical in order to develop effective treatments to preserve the integrity of hip joint prior to femoral head deformation and collapse. Accumulated evidence indicate that reactive oxygen species (ROS) play a crucial role in osteoclast formation and function by modulating receptor activator of NF-κB ligand (RANKL)-induced signalling [13-15]. Our previous work has demonstrated that ROS levels are increased during estrogen deficiency-induced osteoporosis in mice, and that antioxidant agents are effective in inhibiting osteoclast activity and thus preventing bone loss [16]. ROS have also been implicated in the pathogenesis of ONFH, causing damage to blood vessels and osteoblastic cell lineages [17-19]. However, the precise correlation between ROS and osteoclasts in the context of steroid-induced ONFH remains incompletely understood. The aim of this study, therefore, was to correlate the effects of oxidative stress with osteoclast hyperactivity and ONFH, thereby providing a new rationale for the treatment of ONFH.

6.3 Methods

6.3.1 Specimens Collection
Steroid-induced osteonecrotic femoral heads (ARCO stage III-IV) were collected from patients who received total hip arthroplasty (THA) at the First Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China). This study was approved by the Ethical Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (No. Y [2019] 141).

6.3.2 Steroid-induced ONFH rat Model
Male Sprague Dawley (SD) rats at 10-weeks-of-age were provided by Sanyuanli Animal Experiment Center of Guangzhou University of Chinese Medicine (Guangzhou, China). All experimental procedures in this study were approved by the Institutional Animal Ethics Committee of Guangzhou University of Chinese Medicine (NO.20190213001). All rats were housed under a 12-hour light/dark circle and have access to water and food ad libitum. Animals were randomly divided into two groups: control group (n=8) and rat ONFH model group (n=8). Steroid-induced ONFH models were implemented as previously described [20, 21] with modifications. Rats in the ONFH model group were given Imiquimod (IMI, 30 mg/kg) subcutaneously on day 1 and Methylprednisolone (MPSL, 20 mg/kg) intramuscularly on day 2. All injections were repeated on week 4. Rats in the control group were injected with the same amount of saline at the same time point with the model group. Rats were sacrificed and the femurs were collected at 6 weeks following the first injection. Dihydroethidium (DHE, 25 mg/kg) was subcutaneously injected 24 hours prior to sacrifice to visualize the ROS fluorescence signals of DHE [16].

6.3.3 Histomorphometric analysis
Rat femurs or human femoral head regions of interest were collected and the overlying tissues were removed prior to the fixation in 10% neutral buffered formalin (NBF) for 48 hours. Bone tissues were decalcified in EDTA (14%, PH=7.4) (Sigma-Aldrich) at 37 °C for 14 days, in which the EDTA solution was changed every day till the bone became soft for sectioning. Next, the bone samples were placed in the automatic tissue processor for dehydration, followed by paraffin-embedding. Sections of 5-μm thickness were cut using a Leica RM 2035 Biocut Microtome (Leica Microsystems) and collected onto glass slides. Haematoxylin and eosin (HE) staining and tartrate-resistant acid phosphatase (TRAP) staining were implemented to visualise the bone microstructures and osteoclasts. Stained and mounted bone sections were scanned with Uscope MXII-20 (ProSciTech).

6.3.4 In-vivo ROS fluorescence detection
We prepared the cryosections of rat femoral heads as described previously [16] with modifications. Fresh rat femoral heads were fixed using 4% paraformaldehyde (PFA) at 4 °C for 24 hours, which was followed by the decalcification process using EDTA (0.5 M, PH=7.4) for 4 weeks at 4 °C under gentle rocking. The resultant tissues were embedded in Tissue-Tek optimum cutting temperature (O.C.T.) compound (ProSciTech). A thickness of 5-μm sections were obtained at a low speed of cutting and then collected using slides, followed by air-dry at room temperature for 30 minutes. Hoechst solution was used to stain the nuclei prior to mounting using ProLong Gold Antifade Mountant (Thermo Fisher Scientific). The images were observed using a A1Si confocal microscope (NIKON). Appropriate laser settings were chosen based on the excitation/emission wavelengths of the desired fluorochrome (Hoechst, 361nm/497 nm; DHE, 490nm/590 nm) and the regions of interest were captured.

6.3.5 Quantitative real-time polymerase chain reaction (qPCR)

We extracted total RNA from the bone tissue using TRIzol reagent (Life Technologies) and an RNA Extraction Kit (TaKaRa) following the manufacturer’s instructions. The extracted RNA was transcribed in complementary DNA (cDNA) using reverse transcriptase with OligodT primer and moloney murine leukemia virus (MMLV) (Promega). The primers used in this study include TNFSF11, encoding receptor activator of nuclear factor kappa-B ligand (RANKL); TNFRSF11B, encoding osteoprotegerin (OPG); CTSK, encoding cathepsin K; ACP5, encoding TRAP; CAT, encoding catalase; NQO1, encoding NAD(P)H quinone dehydrogenase 1; HMOX1, encoding heme oxygenase 1 (HO1). The primer sequences were listed in Table 6.1. We used SYBRGreen PCR MasterMix (Thermo Fisher Scientific) to perform qPCR in the ViiATM 7 Real-Time PCR System (Applied Biosystems). The qPCR data was extracted using ViiATM 7 software and the expression level of each gene was normalized to the expression of the housekeeping gene - ACTB.
### Table 6.1. Primers sequences

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Sequence (5'-3')</th>
<th>Reverse Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFSF11</td>
<td>CATGTTCGTGGCCCTCCTG</td>
<td>GGATCCATCTGCGCTCTGAA</td>
</tr>
<tr>
<td>TNFRSF11B</td>
<td>GCGCTCCTGTCTTGAGCAT</td>
<td>ACACGGTCTTTCCACTTGCT</td>
</tr>
<tr>
<td>CTSK</td>
<td>GGGGGACATGACAGTGAAG</td>
<td>CAGAGTCTGGGCTCTACCT</td>
</tr>
<tr>
<td>ACP5</td>
<td>GGGAGATCTGTGAGCCAGTG</td>
<td>TTTATCCCTCCCTGCTGC</td>
</tr>
<tr>
<td>CAT</td>
<td>CTCCGGAACAACAGCTTCTC</td>
<td>ATAGAATGCCCCGCACCTGAG</td>
</tr>
<tr>
<td>NQO1</td>
<td>GCTGGTTTGGGCGAGTGTC</td>
<td>CTGCTTTCTACTCCGGAAGG</td>
</tr>
<tr>
<td>HMOX1</td>
<td>CGCTGACCCATGACACCAA</td>
<td>GGGCAGAATCTTGGACCTTGT</td>
</tr>
<tr>
<td>ACTB</td>
<td>ACAGAGCCTCGCTTTGCC</td>
<td>GATATCATCATCCATGGTGAGCTG</td>
</tr>
</tbody>
</table>

### 6.3.6 Western Blot (WB) Assay

The bone tissues of interest were dissected and immerse in liquid nitrogen to snap freeze, which was followed by mechanical grinding using a mortar and pestle. Radioimmunoprecipitation (RIPA) lysis buffer was added and then incubated on ice for 20 minutes. The lysates were then centrifuged at 14,000 rpm for 25 minutes at 4°C. The pellets were discarded, and the supernatants were collected as protein. Protein concentrations were determined using Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime) following the manufacturer’s instructions. The protein extractions were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which was then transferred to nitrocellulose membrane. The membrane was blocked using 5% skim milk (prepared in 1 x TBST) for 1 hour at room temperature (RT) under slow rocking. The primary antibodies diluted in 1% skim milk (in 1 x TBST) were added to the membrane for overnight incubation at 4°C. On the following day, membranes were rinsed with 1XTBST (5 minutes each time for 3 times). Appropriate anti-mouse or rabbit secondary antibodies conjugated with horseradish peroxidase (HRP) were used to incubate the membrane at RT for 1 hour. The proteins were detected using enhanced chemiluminescence substrate (PerkinElmer, Waltham, MA, USA). Images of protein bands were captured using an Image-quant LAS 4000 (GE Healthcare). The intensities of protein bands were analyzed using an ImageJ software (NIH, USA).

### 6.3.7 Micro-CT scanning

Rat femurs were collected, and the excess soft tissues dissected. The femoral heads were scanned in a Skyscan Micro-CT instrument (Bruker) using parameter as the following: source current, 385 μA;
source voltage, 65 kV; pixel size 9 μm; filter, Al 1.0 mm; rotation step, 0.4 degree. The image reconstruction was performed using NRecon software (Bruker) and the data was analysed using the CTAn program (Bruker). The parameters of the femoral head, including bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th) were compared between the control group and ONFH model group.

6.3.8 Statistical Analysis
All numeric data are presented as mean ± standard deviation (SD). Statistical difference was determined using Student’s t test or One-way ANOVA test. The result was considered significantly different when p-value is less than 0.05.

6.4 Results
6.4.1 Imaging of late-stage of human ONFH
Representative anteroposterior and frog-leg lateral radiographs of the pelvis from a 42-year-old female patient with ONFH demonstrated a crescentic lucency in the subchondral area of left femoral head (crescent sign) (Figure 6.1A), suggesting the separation of subchondral bone from the overlying cartilage due to collapse. Joint space narrowing with acetabular involvement was also clearly delineated in Figure 6.1A, based on which the patient was diagnosed as stage IV according to Association Research Circulation Osseous (ARCO) staging system [5]. Furthermore, the magnetic resonance imaging (MRI) indicated the presence of a medium-sized osteonecrotic lesion of the left femoral head, which presents as a low signal intensity area on T1-weighted image and a high signal on T2-weighted image (Figure 6.1B). The sequentially sliced macroscopic sections of the femoral head specimens clearly showed altered femoral head shape and osteonecrotic bone within the subchondral area (Figure 6.1C).

6.4.2 Assessment of osteoclast activity and antioxidant enzymes in the necrotic bone of human femoral head
The osteonecrotic bone tissues were further analysed by histopathological evaluation. H&E staining of decalcified femoral head sections showed the uniform absence of osteocytes within lacunae, indicative of osteonecrosis (Figure 6.2A). TRAP staining revealed that the necrotic bone tissues are widely circumscribed by osteoclasts (Figure 6.2A). To assess for osteoclast activity and explore its relationship with oxidative stress, qPCR was performed to compare the difference of associated
gene expression between healthy bone and necrotic bone (Figure 6.2B). Osteoclast-specific genes, including CTSK and ACP5, were robustly up-regulated in the necrotic bone compared with those in the healthy bone. The ratio of TNFSF11 (encoding RANKL)/TNFRSF11B (encoding OPG) was also increased in the necrotic bone. In contrast, genes encoding anti-oxidant enzymes including CAT (encoding catalase), NQO1 (encoding NAD[P]H quinone dehydrogenase 1), and HMOX1 (encoding heme oxygenase 1 [HO-1]), were notably down-regulated in the necrotic bone. Furthermore, the western blot analyses of the osteoclast-related proteins (RANKL and cathepsin K) and antioxidant enzymes including catalase, γ-glutamylcysteine synthetase (γ-GCSc), and superoxide dismutase 1 (SOD1) further corroborated the mRNA findings, showing similar trends at the protein level (Figure 6.2C). Together, these findings demonstrate that enhanced osteoclast activity is a pathological feature associated with osteonecrosis of the femoral head which may, in part, to the dysregulation of antioxidant enzymes and subsequent high oxidative stress.
Figure 6.1. A representative case of steroid-induced osteonecrosis of femoral head (ONFH, ARCO stage IV). (A) Radiographs delineating the crescent sign (red arrow), flattening of the articular surface of femoral head, and osteoarthritic acetabular changes (red dashed circles). (B) Coronal T1- and T2-weighted magnetic resonance images (MRI) highlighting a medium-sized necrotic lesion of the left femoral head (red dashed circles). (C) Gross appearance of sequentially sliced sections of the femoral head obtained at surgery, showing the deformation of the femoral head (red arrow) and subchondral necrotic bone (black dashed area). ARCO, Association Research Circulation Osseous.
Figure 6.2. Necrotic area of the femoral head has enhanced osteoclast activity and decreased expression of antioxidant enzymes. (A) Representative hematoxylin & Eosin (HE) staining showing the necrotic bone is featured by the presence of empty osteocyte lacunae (green arrows) and tartrate-resistant acid phosphatase (TRAP) staining showing the distributions of osteoclasts on trabecular bone surface (red arrows). (B) qPCR results showing the genes’ expression of osteoclast-specific markers and antioxidant enzymes relative to the ACTB expression. TNFSF11, encoding receptor activator of nuclear factor kappa-B ligand (RANKL); TNFRSF11B, encoding osteoprotegerin (OPG); CTSK, encoding cathepsin K; ACP5, encoding TRAP; CAT, encoding catalase; NQO1, encoding NAD(P)H quinone dehydrogenase 1; HMOX1, encoding heme oxygenase 1 (HO1). (C) Western blot analysis showing the protein expression level of osteoclast-related markers including RANKL and
cathepsin K, as well as anti-oxidant enzymes including catalase, γ-glutamylcysteine synthetase (γ-GCSc), and superoxide dismutase 1 (SOD1). All bar graphs are presented as mean ± SD (n=3 in each group). *P<0.05, **P<0.01 compared with control group (HB). HB, Healthy bone; NB, Necrotic bone.
6.4.3 Histomorphometric analysis of steroid-induced ONFH rat model

To further explore the relationship between osteoclasts and oxidative stress in steroid-induced ONFH we used a rat model to mimics ONFH (Figure 6.3A). The incidence rate of ONFH was 75% (6/8) in the model group with no ONFH observed in the control group. Following sample collection and processing, histomorphometric analyses were performed on decalcified sections of the femoral heads. From the H&E staining (Figure 6.3B), increased bone marrow adiposity and the presence of empty osteocyte lacunae were observed in the subchondral bone tissues, reminiscent of histopathological features observed in patient samples with ONFH. In addition, TRAP staining revealed a morphological increase in osteoclast numbers surrounding the bone samples following steroid administration but were less pronounced in the control group (Figure 6.3B and C).

6.4.4 Micro-CT based analyses of trabecular microstructure of rat femoral heads

To complement the studies, Micro-CT was used to examine the subchondral trabecular architecture and bone structural integrity of the femoral head. As was shown in Figure 6.4A, the bone microstructure in the ONFH model group was notably altered following the use of steroid. Furthermore, the quantification of the parameters including bone volume/tissue volume (BV/TV), trabecular number (Tb.N), and trabecular separation (Tb.Sp) in the ONFH model group were significantly inferior to those of the control group (Figure 6.4B). There was no difference of trabecular thickness (Tb.Th) between these two groups (Figure 6.4B).
Figure 6.3. Steroid-induced osteonecrosis of femoral head (ONFH) of rat and histological evaluation. (A) Schematic illustration of the establishment of rat ONFH model. (B) HE staining of decalcified paraffin-embedding sections showing the increased number of empty lacunae (green arrows) and adipose tissue area (asterisk) in the model group. (B) TRAP staining showing the osteoclasts (red arrows) in the control and model groups. (C) Quantitative analyses of N.Oc/BS and Oc.S/BS (N=5 per group). **P<0.01 compared with control group. N.Oc/BS, osteoclast number/bone surface; Oc.S/BS, osteoclast surface/bone surface.
Figure 6.4. Micro-CT (μCT) analysis of steroid-induced osteonecrosis of femoral head (ONFH) in rat model. (A) Representative μCT scanning images of the femoral head of the control group and model group. (B) Quantification of the bone microstructure parameters of the femoral head, including bone volume/tissue volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th). All bar graphs are presented as mean ± SD (n=6 in each group); *P<0.05 compared with the control group; ns, no significance.
6.4.5 Detection of ROS and osteoclast-related markers in the rat femoral heads

The ex vivo ROS level, as probed by DHE, was detected in the cryosections of femoral head under the confocal microscopy. As was shown in Figure 6.5A, DHE fluorescence of the subchondral area of the femoral head was dramatically enhanced in the model group. The fluorescence intensity profile of the yellow indicated line in the Figure 6.5A showed a clear difference between the control group and model group (Figure 6.5B). Quantification of the DHE fluorescence intensity indicated a higher oxidative stress in the rat femoral head following the steroid administration (Figure 6.5C). Western blot assay of the femoral head tissues further indicated that the expression levels of antioxidant enzymes such as HO-1, catalase, and SOD1 were significantly altered in the model group (Figure 6.5D). In contrast, the expression of the mature osteoclast marker cathepsin K was up-regulated in the ONFH model group (Figure 6.5D). In addition, the expression of RANKL was also increased (Figure 6.5D). Collectively, these results suggest that the use of steroid may lead to high oxidative stress in the femoral head by reducing antioxidant enzymes, which may contribute to the recruitment and/or activation of osteoclasts.

6.5 Discussion

The use of steroids, particularly the prolonged and high-dose administration, is estimated to account for the majority of non-traumatic ONFH cases [4]. ONFH is a progressive bone disorder which typically results in the collapse of the femoral head at end stage. The precise cellular and molecular mechanisms underlying steroid-induced osteonecrosis remain controversial. Recent studies indicate that increased osteoclast number and activity are responsible for the femoral head bone loss and subsequent failure [6, 8, 9]. Given the increasing number of studies illustrating a role for ROS in promoting osteoclast formation and resorption, we sought to elucidate whether alterations of ROS level and osteoclasts correlate with the pathogenesis of ONFH following steroids use. In the present study, ROS-related enzymes and osteoclasts were detected in both patients ONFH specimens and rodent models mimicking ONFH. Specimens of femoral head were obtained from end-stage ONFH patients who received THA and antioxidant enzymes and osteoclasts compared between healthy and necrotic region(s). Steroid-induced ONFH rat models were established to further assess the role of ROS and osteoclasts that played in this bone disorder.
Figure 6.5. Analysis of reactive oxygen species (ROS) level and antioxidant enzymes in the rat femoral head. (A) Cryosections of femoral heads showing ROS level, as probed by dihydroethidium (DHE), in the control group and model group. AC, articular cartilage (white dashed line area); SB, subchondral bone. (B) DHE fluorescence intensity profile through subchondral area of femoral head (indicated by the yellow arrow in A). (C) Quantification of DHE fluorescence intensity relative to the control group (n=5 in each group). (D) Western blot analysis of the protein expression level of osteoclast-related markers including RANKL and cathepsin K, as well as anti-oxidant enzymes including heme oxygenase 1 (HO1), catalase, and superoxide dismutase 1 (SOD1) (n=3 in each group). All bar graphs are presented as mean ± SD. *P<0.05, **P<0.01 compared with control group.
Oxidative stress is featured by an augmented level of ROS which disrupts physiological reduction-oxidation (redox) balance. ROS are known to play a pivotal role in aging and degenerative diseases [22, 23]. The deleterious effects of ROS result from the alternations to the integrity of mitochondrial and nuclear DNA, which can lead to cell apoptosis or necrosis [24]. Accumulating studies have suggested that bone pathophysiology is closely related with redox balance and ROS are key modulators of bone cell functions [25]. High oxidative stress is thought to be associated with the development of various bone disorders, including osteoporosis [26, 27], bone tumours [28], diabetic osteopenia [29], rheumatoid arthritis [30], and ankylosing spondylitis [31]. Recent evidence also indicated ROS are involved in the pathogenesis of steroid-induced ONFH [18, 19, 32] and that approaches inhibiting oxidative stress exerted potential therapeutic effects on ONFH [32-34]. To validate this, a ROS probe (DHE) was administrated on the rats prior to sacrifice, which is distributed via the circulation enabling the direct visualization of ROS in tissues as we previously described [16]. Not surprisingly, the ROS fluorescence intensity in the femoral head was dramatically enhanced following steroid treatment. This is, to the best of our knowledge, the first time that the ROS levels have been directly visualized in the femoral head ex vivo.

Oxidative stress is determined by the imbalance between ROS generation and its scavenging, the latter of which is carried out by well-known antioxidant enzymes such as SOD, HO-1, catalase, and γ-GCS c [35]. The mechanisms underlying the steroid-induced oxidative stress in the femoral head [36] remain unclear, but is thought that the administration of steroid leads to the downregulation of cytoprotective enzymes and subsequently causes redox failure [37, 38]. Indeed, our results confirmed that the antioxidant enzymes (catalase, γ-GCS c, and SOD1) were significantly altered in the necrotic area of patients’ femoral head relative to the healthy area, suggesting the existence of redox failure and the high level of ROS in the necrotic area. SOD plays a major role in cellular redox by reducing superoxide radicals to hydrogen peroxide (H₂O₂) which is less unstable [35]. Catalase then converts H₂O₂ into oxygen and water, thereby protecting the cells from ROS damage [39]. Furthermore, in addition to high ROS signalling in the femoral head, rats who received steroids injection also showed decreased expressions of antioxidant enzymes including HO-1, catalase, and SOD1. These findings collectively suggest that the compromised expressions of antioxidant enzymes are, at least partly, responsible for the oxidative stress due to steroid administration.
The current understanding of the hazards of ROS are that oxidative stress may lead to pathological conditions, including the elevated vascular permeability [40] as well as DNA oxidation injuries [36] which causes cell apoptosis or necrosis [34]. However, whether ROS enhances the osteoclast activity during the pathogenesis of ONFH has not been previously reported. ROS can support the activation of RANKL-induced signalling, which is essential for osteoclast formation and resorptive activity [13]. The exact molecules or proteins that ROS targets remain unclear, but RANKL-induced downstream events such as MAPK [13] and NF-κB signaling [41] appear to be involved. Our results demonstrate that the reduced antioxidant enzymes in the necrotic area are accompanied by the augmented osteoclast-related markers, such as cathepsin K, which were also supported by the TRAP staining indicating that osteoclasts activity is localized to the necrotic area. Consistent with the human specimen, we found that osteoclast numbers were elevated in a rat model of steroid-induced ONFH, which ultimately resulted in decreased femoral trabecular bone volume and thus structural integrity as assessed by μCT. These results indicate that oxidative stress is closely related with the osteoclast activity and thus may coincide with the rapid progression of ONFH. With respect to the difference of the expression of osteoclast signature proteins and antioxidant enzymes between necrotic bone and healthy bone, it remains to be further investigated whether these differences similarly exist in weight-bearing and non-weight bearing bones.

In summary, this study demonstrates that a decline in the expression of antioxidant enzymes correlates with the administration of steroids, which may, in turn, contribute to elevated ROS levels and osteoclast numbers/activity observed during the pathogenesis of ONFH. Oxidative injuries usually present shortly following steroid administration at the very early-stage ONFH [36, 42], and excessive osteoclast activity is also believed to be for a contributor of femoral head collapse. This provides rationale that antioxidant therapy may be a promising alternative to prevent the progression of steroid-induced ONFH by suppressing ROS level and thus inhibiting osteoclasts.

6.6 Reference

42. Lu BB, Li KH. Lipoic acid prevents steroid-induced osteonecrosis in rabbits. Rheumatol Int. 2012; 32: 1679-83.
CHAPTER SEVEN - GENERAL DISCUSSION AND FUTURE DIRECTIONS
7.1 General discussion

Bone is a self-renewal connective tissue which continually undertakes a process of remodelling, including the removal of bone matrix which is carried out by osteoclasts and replacement of new bone matrix which is deposited by osteoblasts. The precise coordination of osteoclastic bone resorption and osteoblastic bone formation is fundamental to maintain bone homeostasis. Under physiological condition, no net change exists in bone mass due to bone remodelling. However, a couple of bone disorders may occur when the balance derails because of various causes, including menopausal estrogen deficiency, aging, physical activities, drugs, and secondary diseases [1]. Osteoclast is considered as the sole type of bone-resorbing cells originated from the monocyte/macrophage lineage. Excessive formation and activity of osteoclasts play a leading role in a variety of bone and joint disorders [2], including osteoporosis, periprosthetic osteolysis after joint arthroplasty, Paget disease of bone, rheumatoid arthritis, subchondral bone destruction in osteonecrosis of femoral head (ONFH). Thus, anti-catabolic strategies targeting aberrant osteoclast formation and function remain promising in the treatment of osteoclast-related bone disorders.

Accumulating evidence demonstrated that ROS play an essential role in osteoclastogenesis by mediating RANKL-induced downstream signaling [3-5]. With the stimulation of RANKL in osteoclast precursors, ROS are endogenously generated via a signaling axis TRAF6/Rac1/ Nox1 (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase 1) [3]. Using oxidant scavengers such as N-acetylcysteine (NAC) or diphenylene iodonium (DPI) is able to suppress osteoclasts formation by attenuating intracellular ROS level [3], suggesting ROS are critical for osteoclastogenesis. The mechanisms underlying ROS-mediated signaling still need to be further elucidated; however, ROS are suggested to promote osteoclast differentiation and bone resorptive function via activating NF-κB and MAPKs pathways [6, 7]. Additionally, ROS was also clearly suggested to participate in NFATc1 induction via regulating calcium signalling [8, 9]. Meanwhile, to protect from the oxidative damage to the cells, a wide range of antioxidant/cytoprotective enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H: quinone reductase (NQO1), catalase, glutathione-disulfide reductase (GSR), and γ-glutamylcysteine synthetase (GCS) [7, 10] are induced to defend against oxidative stress. Hence, these results deliver a rationale for suppressing the level of ROS, by inhibiting ROS production or enhancing antioxidant enzymes, as a potential strategy for inhibiting osteoclasts formation and activity.
In our study, two novel secondary metabolites isolated from *Aspergillus fumigatus*, known as helvolic acid (HA) and pseurotin A (Pse), were identified to have anti-catabolic effects by at least partly inhibiting ROS levels. Our results provided evidence that HA exerted inhibitory effects on osteoclastogenesis via strongly suppressing RANKL-induced activation of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), which is the master transcriptional factor of osteoclast formation. It was further demonstrated that the attenuated NFATc1 expression was due to the inhibitions of multiple upstream signalling cascades, including Ca$^{2+}$ oscillation, intracellular ROS production, and c-Fos signalling. Based on these robust inhibitory effects, we propose that HA could be possibly applied in the development of an alternative drug for osteoclast-related bone disorders.

The anti-catabolic effect of Pse on osteoclast was more extensively examined both *in vitro* and *in vivo*. Pse was demonstrated to inhibit osteoclastogenesis and bone resorptive function *in vitro*, as well as the downregulation of osteoclast-specific genes including *Acp5* (encoding TRAcP), *Ctsk* (encoding cathepsin K), and *Mmp9* (encoding matrix metalloproteinase 9). Mechanistically, Pse suppressed intracellular ROS level by inhibiting RANKL-induced ROS production through TRAF6/Rac1/Nox1 axis and enhancing ROS scavenging enzymes including HO-1, catalase, and GSR, subsequently suppressing MAPK pathway activation (ERK, P38, and JNK) and NF-$\kappa$B pathway, leading to the inhibition of NFATc1 signalling. By establishing the estrogen deficiency-induced osteoporosis mice model, we investigated whether Pse is able to prevent the osteoporosis through its anti-catabolic property. Micro-CT and histological data indicated that OVX procedure resulted in a significant bone loss, with dramatically increased the number of osteoclasts on the bone surface as well as increased ROS level in the bone marrow microenvironment; whereas Pse supplementation was capable of effectively preventing these OVX-induced changes.

By carrying out these studies, two novel small molecules were revealed for the first time to target osteoclasts at least partly through inhibiting ROS production and/or scavenging. Thus, in bone disorders which are associated with hyperactive osteoclasts, these small molecules of anti-catabolic properties may exert therapeutic effects.

Given the inspiring outcome that the treatment of osteoporosis by anti-catabolic interference via ROS suppression, we are interested the role of ROS and osteoclast on the development of ONFH. The current understandings of the hazards of ROS are that oxidative stress may lead to pathological conditions, including the elevated vascular permeability [11] as well as DNA oxidation injuries [12]
which causes cell apoptosis or necrosis [13]. However, whether ROS enhance the osteoclast activity during the pathogenesis of ONFH remains unobserved. It is also accepted that the hyperactive osteoclasts cause the loss of bone structural integrity and subchondral fracture. However, the pathophysiologic mechanisms underlying the activation of osteoclasts in ONFH have not been fully elucidated. These background evidence made us further hypothesize that ROS may enhance the osteoclast activity during the development of ONFH.

Therefore, we explored the correlations between ROS and osteoclasts in both patients’ femoral head and rat ONFH models. The necrotic bone region of human femoral head, compared with healthy bone region, was characterized by dramatically up-regulated expression of osteoclast-related proteins (RANKL and cathepsin K) but down-regulated antioxidant enzymes (catalase, γ-glutamylcysteine synthetase [γ-GCSc], and superoxide dismutase 1 [SOD1]). Next, methylprednisolone (MPSL) and imiquimod (IMI) were used to induce rat ONFH models, in which tartrate-resistant acid phosphatase (TRAP) staining indicated the increased number of osteoclasts, and micro-CT displayed significant bone loss in the femoral head. Furthermore, in the model group, WB assay showed the decreased expression of antioxidant enzymes, which was accompanied by the enhancement of in-vivo ROS fluorescence as probed by dihydroethidium (DHE). These results collectively revealed that the elevation of ROS level and subsequent osteoclast activity during the pathogenesis of ONFH, providing the rationale that anti-oxidant agents may be effective in the prevention and/or treatment of ONFH.

In summary, our studies demonstrated that hyperactive osteoclasts are closely related with the oxidative stress during the pathogenesis of bone disorders such as osteoporosis and ONFH. Thus, anti-catabolic or anti-oxidant agents may achieve good outcomes when encounter these challenging issues. However, there are still some problems remained to be further attempted.

7.2 Future directions

What are the specific targets of the small molecules?

Two small molecules, HA and Pse, have been identified to exert inhibitory effects on osteoclasts. However, current findings are more likely to be phenotype-based results, which lack the evidence of the molecular targets underlying drug therapeutic effects. Thus, our future work will be focusing on discovering the specific binding of these small molecules, which would allow us to gain better
understanding of the molecular actions of these novel potential drugs when they come to bone
diseases.

The primary limitation of traditional affinity-based target identification techniques is that the
structure modifications of the small molecules which affect the bioactivity [14]. To overcome this
limitation, a novel technique, known as Drug affinity responsive target stability (DARTS), was
developed based on the theory that the target protein's structure would be stabilized by the binding
of a small molecule drug [14, 15]. The native small molecules are used without chemical
derivatization for target identification, which allows the drugs to determine their direct binding
targets. In our studies, given these small molecules achieved inhibitory effects at different stages of
osteoclastogenesis (e.g. early-stage on day 1-3, mid-stage on day 3-5, and late-stage on day 5-6),
the cell lysates from corresponding stages can be collected for DARTS assay. By carrying out the
DARTS assays, new significant proteins in osteoclastogenesis may also be revealed and new
therapeutic options may also be proposed.

**Which stage of ONFH should be started with anti-catabolic treatment?**

ONFH is known as a progressive bone disorder, which leads to the catastrophic results of femoral
head and subsequent total hip arthroplasty. Recent evidence also indicated reactive oxygen
species (ROS) are involved in the pathogenesis of steroid-induced ONFH [16-18] and approaches to
inhibiting oxidative stress exerted protective effects on ONFH [13, 18, 19]. The current
understanding of the hazards of ROS is that oxidative stress may lead to pathological conditions,
including the elevated vascular permeability [11] as well as DNA oxidation injuries [12] which
causes cell apoptosis or necrosis [13]. Our studies demonstrated that ROS also enhance the
osteoclast activity in the pathogenesis of ONFH. Oxidative injuries usually present shortly following
steroid administration at the very early-stage ONFH [12, 20] and the hyperactive osteoclasts are
also believed to be responsible for the femoral head collapse. The prevention of femoral head
remains as the core treatment of ONFH. Thus, to maximum the therapeutic effects, whether anti-
oxidant agents should be administrated together with steroids or after remains to be found.

**Effects on other osteoclast-related bone disorders**

Osteoclast plays a leading role in the pathogenesis of bone disorders which includes osteoporosis,
periprosthetic osteolysis after joint arthroplasty, Paget disease of bone, rheumatoid arthritis, ONFH,
and tumour metastases-induced bone destruction. The small molecules identified in our studies
exert inhibitory effects on osteoclast formation and function. Pse was shown to prevent the estrogen deficiency-induced bone loss, HA has not yet been examined in animal models. These anticalcatabolic agents may also be applied to other bone disorders which have not been investigated. Based on the positive effects of Pse and HA on osteoclast, it is of interest to find out whether HA and Pse have therapeutic effect on ONFH.

Off-target effects
The small molecules in these studies have been identified to inhibit osteoclast formation and function without cytotoxicity as assessed by cell proliferation assay. However, when administrated in vivo, whether “off-target” effects exist still remain unknown. Pharmacokinetic and pharmacodynamic analyses are required to be further investigated. Although the mice received the injections of Pseurotin A were monitored on weight, daily activities, and death rate, further detailed information includes the effects on kidney, liver, lung, and heart are still needed to be examined to confirm the safety of use. Also, only one concentration of Pseurotin A has been evaluated. The concentration of injection in vivo should be optimized to maximize the therapeutic effect with minimum adverse effects. In addition, the delivery system which can localized the drug in specific area would also be promising in relieve the side effects.

Reference
APPENDIX
Helvolic acid attenuates osteoclast formation and function via suppressing RANKL-induced NFATc1 activation

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Abstract

Excessive osteoclast formation and function are considered as the main causes of bone lytic disorders such as osteoporosis and osteolysis. Therefore, the osteoclast is a potential therapeutic target for the treatment of osteoporosis or other osteoclast-related diseases. Helvolic acid (HA), a mycotoxin originally isolated from Aspergillus fumigatus, has been discovered as an effective broad-spectrum antibacterial agent and has a wide range of pharmacological properties. Herein, for the first time, HA was demonstrated to be capable of significantly inhibiting receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis and bone resorption in vitro by suppressing nuclear factor of activated T cells 1 (NFATc1) activation. This inhibition was followed by the dramatically decreased expression of NFATc1-targeted genes including Ctr (encoding calcitonin receptor), Acp5 (encoding tartrate-resistant acid phosphatase [TRACP]), Ctsk (encoding cathepsin K), Atp6v0d2 (encoding the vacuolar H+ ATPase V0 subunit d2 [V-ATPase-d2]) and Mmp9 (encoding matrix metallopeptidase 9) which are osteoclast-specific genes required for osteoclast formation and function. Mechanistically, HA was shown to greatly attenuate multiple upstream pathways including extracellular signal-regulated kinase (ERK) phosphorylation, c-Fos signaling, and intracellular Ca\(^{2+}\) oscillation, but had little effect on nuclear factor-κB (NF-κB) activation. In addition, HA also diminished the RANKL-induced generation of intracellular reactive oxygen species. Taken together, our study indicated HA effectively suppressed RANKL-induced osteoclast formation and function. Thus, we propose that HA can be potentially used in the development of a novel drug for osteoclast-related bone diseases.
Pseurotin A Inhibits Osteoclastogenesis and Prevents Ovariectomized-Induced Bone Loss by Suppressing Reactive Oxygen Species

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Abstract

Rationale: Growing evidence indicates that intracellular reactive oxygen species (ROS) accumulation is a critical factor in the development of osteoporosis by triggering osteoclast formation and function. Pseurotin A (Pse) is a secondary metabolite isolated from Aspergillus fumigatus with antioxidant properties, recently shown to exhibit a wide range of potential therapeutic applications. However, its effects on osteoporosis remain unknown. This study aimed to explore whether Pse, by suppressing ROS level, is able to inhibit osteoclastogenesis and prevent the bone loss induced by estrogen-deficiency in ovariectomized (OVX) mice.

Methods: The effects of Pse on receptor activator of nuclear factor-xB (NF-kB) ligand (RANKL)-induced osteoclastogenesis and bone resorptive function were examined by tartrate resistant acid phosphatase (TRACP) staining and hydroxyapatite resorption assay. 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) was used to detect intracellular ROS production in vitro. Western blot assay was used to identify proteins associated with ROS generation and scavenging as well as ROS-mediated signaling cascades including mitogen-activated protein kinases (MAPKs), NF-kB pathways, and nuclear factor of activated T cells 1 (NFATc1) signaling. The expression of osteoclast-specific genes was assessed by qPCR.

Results: Pse was demonstrated to inhibit osteoclastogenesis and bone resorptive function in vitro, as well as the downregulation of osteoclast-specific genes including Acp5 (encoding TRACP), Ctsk (encoding cathepsin K), and Mmp9 (encoding matrix metalloproteinase 9). Mechanistically, Pse suppressed intracellular ROS level by inhibiting RANKL-induced ROS production and enhancing ROS scavenging enzymes, subsequently suppressing MAPK, NF-kB pathways (ERK, JNK, and JNK) and NF-kB pathways, leading to the inhibition of NFATc1 signaling. Micro-CT and histological data indicated that OVX procedure resulted in a significant bone loss, with dramatically increased the number of osteoclasts on the bone surface as well as increased ROS level in the bone marrow microenvironment; whereas Pse supplementation was capable of effectively preventing these OVX-induced changes.

Conclusion: Pse was demonstrated for the first time as a novel alternative therapy for osteoclast-related bone diseases such as osteoporosis through suppressing ROS level.

Key words: Pseurotin A, reactive oxygen species, osteoclast, osteoporosis