



Pristimerin Suppresses RANKL-Induced Osteoclastogenesis and Ameliorates Ovariectomy-Induced Bone Loss

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Specialty section:

This article was submitted to
Experimental Pharmacology and Drug
Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 25 October 2020

Accepted: 07 December 2020

Published: 15 January 2021

Citation:

Qi D, Liu H, Sun X, Luo D, Zhu M, Tao T, Gao C, Zhou C, Zhou W and Xiao J (2021) Pristimerin Suppresses RANKL-Induced Osteoclastogenesis and Ameliorates Ovariectomy-Induced Bone Loss.
Front. Pharmacol. 11:621110.
doi: 10.3389/fphar.2020.621110

Osteoporosis is characterized by bone loss and destruction of trabecular architecture, which greatly increases the burden on the healthcare system. Excessive activation of osteoclasts is an important cause of osteoporosis, and suppression of osteoclastogenesis is helpful for the treatment of osteoporosis. Pristimerin, a natural compound, possesses numerous pharmacological effects via inactivating the NF- κ B and MAPK pathways, which are closely related to osteoclastogenesis process. However, the relationship between Pristimerin and osteoclastogenesis requires further investigation. In this research, we examined the effect of Pristimerin on osteoclastogenesis and investigated the related mechanisms. Our results showed Pristimerin inhibited RANKL-induced osteoclast differentiation and osteoclastic bone resorption *in vitro*, with decreased expression of osteoclastogenesis-related markers including c-Fos, NFATc1, TRAP, Cathepsin K, and MMP-9 at both mRNA and protein levels. Furthermore, Pristimerin suppressed NF- κ B and MAPK signaling pathways, reduced reactive oxygen species (ROS) production and activated the nuclear factor erythroid 2-related factor 2/heme oxygenase 1 (Nrf2/HO-1) signaling during osteoclastogenesis. Our *in vivo* experiments showed that Pristimerin remarkably ameliorated ovariectomy-induced bone loss, reduced serum levels of TNF- α , IL-1 β , IL-6, and RANKL, and increased serum level of osteoprotegerin (OPG). Therefore, our research indicated that Pristimerin is a potential chemical for the treatment of osteoporosis.

Keywords: pristimerin, osteoclastogenesis, MAPK, NF- κ B, Nrf2

INTRODUCTION

Bone, a dynamic organ, is in a constant remodeling to maintain homeostasis. Osteoblast and osteoclast are the two main cells involved in bone remodeling, which are responsible for bone formation and bone resorption, respectively (Siddiqui and Partridge, 2016). A disbalance in bone remodeling causes numerous bone metabolism diseases, such as osteoporosis, osteopetrosis, Paget's disease and rheumatoid arthritis (Roodman and Windle, 2005; Feng and McDonald, 2011; Baum and Gravallesse, 2016).

In recent years, osteoporosis has become one of the most important public health problems, which is characterized by reduced bone density and destruction of trabecular architecture, and can easily lead to fractures and body pain (Ensrud and Crandall, 2017). As bone resorption mainly depends on osteoclasts, excessive activation of osteoclasts is the principal cause of osteoporosis (Charles and Aliprantis, 2014).

Osteoclasts are multinucleated giant cells originated from precursor cells of monocyte/macrophage haematopoietic lineage (Ono and Nakashima, 2018). Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) are the two vital factors for the maturation of osteoclasts (Boyle et al., 2003). The combination of M-CSF and its receptor c-Fms can regulate proliferation, differentiation and survival of osteoclasts and their precursors, as well as induce the expression of RANK on these cells (Boyce, 2013). RANKL associates with RANK on the surface of osteoclast precursors, activates multiple downstream signaling pathways, including NF- κ B, MAPK and activator protein-1 (Nakashima and Takayanagi, 2011), which are most important pathways involved in osteoclastogenesis. Furthermore, pro-inflammatory cytokines, ROS production and Nrf2/HO-1 signals are also strongly associated with osteoclastogenesis (Agidigbi and Kim, 2019; Coury et al., 2019; Sun et al., 2020).

Pristimerin, a natural triterpenoid compound, isolated from the traditional Chinese herbs *Celastraceae* and *Hippocrateaceae*, has shown numerous biological and pharmacological properties, such as anti-inflammatory (Yadav et al., 2010), anti-oxidative (Dos Santos et al., 2010; Hui et al., 2014), anti-tumor (Li et al., 2019), anti-malarial (Figueiredo et al., 1998) and anti-fungal effects (Luo et al., 2005; Gullo et al., 2012). Many previous studies have confirmed that Pristimerin exerts its pharmacological effects through inactivating NF- κ B and MAPK signaling (Deeb et al., 2014; Yousef et al., 2018; Xie et al., 2019), which are similar pathways involved in osteoclastogenesis. However, the role of Pristimerin on osteoclastogenesis need further investigation.

Therefore, we hypothesized that Pristimerin may inhibit osteoclastogenesis through the anti-inflammatory mechanism. To test our hypothesis, we investigated the effect of Pristimerin on osteoclastogenesis both *in vitro* and *in vivo*, and explored the underlying mechanisms.

MATERIALS AND METHODS

Reagents and Antibodies

Pristimerin (HPLC \geq 98%) was bought from Sigma-Aldrich (St.Louis, MO, USA), the compound was dissolved in DMSO, and stored in a refrigerator at -80°C for later use. α -MEM medium and cell counting kit-8 (CCK-8) kit were bought from Boster (Wuhan, China). Specific antibodies against Nrf2, NFATc1, HO-1, MMP-9, NQO-1, c-Fos, tartrate-resistant acid phosphatase (TRAP), GAPDH, and Cathepsin K (CTSK) were obtained from Proteintech Group (Wuhan, China). The Rhodamine-conjugated phalloidin, TRAP staining kit, and DAPI were bought from Sigma-Aldrich

(St.Louis, MO, USA). Specific antibodies against IKK β , p-JNK, JNK, p-ERK, ERK, p-P38, P38, p-I κ B α , I κ B α , p-P65, P65, and p-IKK α/β were bought from Cell Signaling Technology (Beverly, MA, USA). Osteo Assay Surface was bought from Corning Incorporated Life Science (Corning, NY, USA). M-CSF and RANKL were bought from R & D Systems (Minneapolis, MN, USA).

Bone Marrow-Derived Macrophages Culture and Cytotoxicity Assay

We isolated bone marrow-derived macrophages (BMMs) from the femoral and tibial bone marrow of 8-week-old male C57BL/6 mice as showed previously (Guan et al., 2015). BMMs were cultured in α -MEM medium supplemented with 10% fetal bovine serum, streptomycin (100 ug/mL), penicillin (100 U/mL) and M-CSF (30 ng/ml) at 37°C in an incubator. CCK-8 kit was used to test the cytotoxicity of Pristimerin in BMMs. Briefly, 2.4×10^3 BMMs were plated onto 96-well plates each well, and were incubated overnight, then the BMMs were exposed to various concentrations (0, 5, 10, 25, 50, and 75 nM) of Pristimerin for 1, 3, and 5 days. Medium was changed daily. At the end of culture, 10 ul CCK-8 solution was added to each well and incubated at 37°C for 1 h at dark. Finally, the optical density (OD) was measured at 450 nm with a plate reader.

TRAP Staining Assay

We used TRAP staining to assess the effect of Pristimerin on osteoclast formation *in vitro*. Briefly, 1.8×10^4 BMMs were plated onto 96-well plates per well overnight. Then multiple concentrations (0, 5, 10, 25, 50, and 75 nM) of Pristimerin were added to the medium supplemented with RANKL (100 ng/ml) for additional 6–7 days, with medium changed daily. When BMMs were observed to fuse into osteoclasts under a light microscope, the cells were rinsed for 3 times and fixed in 4% paraformaldehyde for 20 min, then the cells were stained using the TRAP kit (Sigma) according to the instructions. Multinucleated (>3 nuclei) TRAP-positive cells were identified as osteoclasts.

Bone Pit and Actin Ring Formation Assay

BMMs were seeded on 0.2% collagen-gel pre-coated 24-well plates and stimulated with RANKL (100 ng/ml) for 6–7 days. Type I collagenase was used to obtain the mature osteoclasts and the cells were plated onto Corning Osteo Assay surface, then the cells were treated with different concentrations (0, 10, 25, 50, and 75 nM) of Pristimerin with or without RANKL (100 ng/ml) supplement for additional 3 days. As for pit formation, the plates were soaked in 5% sodium hypochlorite for 5 min and washed with pure water, then the bone resorption areas were photographed for further quantitative analysis. For actin ring formation, 4% paraformaldehyde was used to fix the cells for 20 min, 0.2% Triton X-100 was used to break the cell membranes for 10 min. Then the Phalloidin and DAPI were applied to stain the cells respectively. Finally, a fluorescence microscope was used to take the actin ring images.

Bone Marrow Stem Cells Isolation and Osteogenic Differentiation Assay

BMSCs were isolated from the femurs and tibias of 5 weeks old SD rats as shown previously (Watanabe et al., 2018), and cultured with DMEM/F12 medium until confluent. Then the BMSCs were digested and seeded on 24-well plates at a density of 1.5×10^4 cells/well. For osteogenic differentiation, the BMSCs were cultured in osteogenic medium (a-MEM medium containing 50 mM Ascorbic acid, 10 nM dexamethasone and 10 mM β -glycerol phosphate) and the medium was replaced every 2 days. ALP staining was performed at day 7 after differentiation using the BCIP/NBT Alkaline phosphatase Color Development Kit (Beyotime, China). Alizarin Red staining was performed at day 14 after differentiation using the Alizarin Red (Cyagen Biosciences) according to the manufacturers' protocols.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA of cultured cells were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 1 μ g RNA was used to synthesize the complementary DNA (cDNA) using the ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan). TB Green Fast qPCR Mix (Takara, Japan) was used to perform RT-PCR. The primer sequences used were as follows (sequences 5'-3', sense and antisense): TRAP: GATGCCAGC GACAAGAGGTT and CATAACAGGGGATGTTGCGAA; MMP-9: CTGGACAGCCAGACACTAAAG and CTCGCG GCAAGTCTTCAGAG; NFATc1: TCTTCCGAGTTCACATCC C and GACAGCACCATCTTCTTCC; CTSK: GAAGAAGAC TCACCAGAAGCAG and TCCAGGTTATGGGCAGAGATT; GAPDH (mouse): AACGACCCCTTCATTGACCTC and CCT TGACTGCCGTTGAAGT; GAPDH (rat): GGTGGACCTCAT GGCCTACA and CTCTCTTGCTCTCAGTATCCTTGCT; ALP: GCACAACATCAAGGACATCG and TCAGTTCTGTTC TTGGGGTACAT; RUNX2: GGGACCGACACAGCCATATA and TCTTAGGGTCTCGGAGGGAA; OCN: GCCCTGACT GCATTCTGCCTCT and TCACCACCTTACTGCCCTCCTG; OPN: CCAGCCAAGGACCAACTACA and GCTGGCAGT GAAGGACTCAT.

Western Blot Analysis

RIPA lysis buffer was used to extract total proteins from cultured BMMs. 15 μ g proteins were separated using 10% SDS-PAGE gel electrophoresis, then the proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA). 5% BSA solutions was used to block the membranes for 1 h at room temperature, then the membranes were incubated with specific primary antibodies for 16 h at 4°C, with a dilution ratio of 1:1,000. Membranes were washed and incubated with the secondary antibodies for another 1 h. Finally, electrochemiluminescence reagents (Thermo Fisher Scientific) were used to acquire the images and the band gray values were analyzed with Image Lab 5.1 software (Bio-Rad, Hercules, CA).

Measurement of Reactive Oxygen Species Expression

DCFH-DA (Beyotime, Shanghai, China) was used to measure the levels of ROS in BMMs. Briefly, BMMs were stimulated with or without RANKL (100 ng/ml), and were exposed to various concentrations (0, 25, 50, and 75 nM) of Pristimerin for 24 h. Then DCFH-DA (10 μ M) was added to each well and incubated at 37°C at dark for 25 min. The cells were washed twice and a fluorescence microscope was used to observe the intracellular ROS level.

Animals and Ovariectomized Mouse Model

An ovariectomized (OVX) osteoporosis model was built to assess the role of Pristimerin on bone loss *in vivo*. All animal experiments were performed on the basis of the recommendations of Animal Experimentation Guidelines, the Ethics Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Thirty 8-week-old female C57BL/6 mice were obtained from the Experimental Animal Center of Tongji Hospital (Wuhan, China) and were randomly divided into three groups (n = 10): The sham + VEH (Sham group treated with vehicle), the OVX + VEH (OVX group treated with vehicle), and the OVX + Pristimerin (OVX group treated with Pristimerin). All mice were anesthetized and the bilateral ovaries were removed to induce osteoporosis except the sham group. Three days after operation, the mice were intraperitoneally injected with Pristimerin (1 mg/kg) or vehicle (DMSO) for 5 days per week. 8 weeks later, all mice were sacrificed, the femurs and sera were collected for following experiments.

μ CT Scanning

After removal of the soft tissues, the femurs were fixed in 4% paraformaldehyde for 3 days. μ CT (Scanco Medical, Bassersdorf, Switzerland) was applied to scan the metaphysical regions of left femurs, the voltage of the source was set to 70 kV, the current of the source was set to 110 μ A, and the isotropic resolution was set to 9 μ m. We used built-in μ CT software to reconstruct three-dimensional images of the femurs and analyzed the bone structural parameters, such as bone volume/tissue volume (BV/TV), trabecular separation (Tb.Sp), trabecular number (Tb.N), and trabecular thickness (Tb.Th).

Histological Analyses

All right femurs were soaked in 10% EDTA for 2 weeks, then the tissues were embedded in paraffin, and were cut at 5 μ m using a microtome. H&E and TRAP staining were performed to observe the bone structure or the osteoclasts.

Measurement of Serum Inflammatory Factors

The sera of all the mice were collected. The levels of TNF- α , IL-1 β , IL-6, OPG, and RANKL in serum were detected using ELISA kits based on the manufacturer's protocols.

Statistical Analyses

The data were showed as means \pm SD, and all experiments were repeated at least three times. Student's t test was used to examine

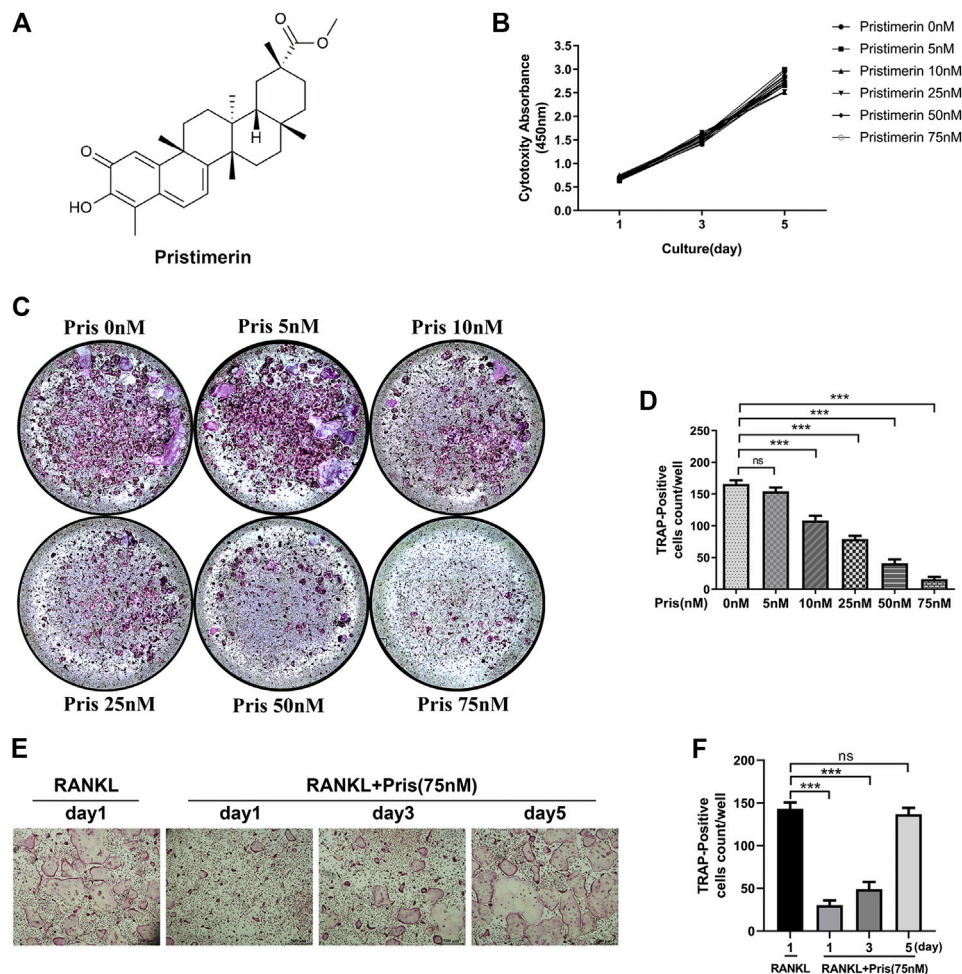


FIGURE 1 | Pristimerin suppresses RANKL-induced osteoclast formation *in vitro* (A) Chemical formula of Pristimerin (B) Pristimerin concentrations lower than 75 nM did not have cytotoxic effect on BMMs. BMMs were treated with various concentrations (0, 5, 10, 25, 50, and 75 nM) of Pristimerin for 1, 3, and 5 days, and the cytotoxicity was evaluated using a CCK-8 kit (C and D) Pristimerin suppressed RANKL-induced osteoclast formation. BMMs were treated with various concentrations (0, 5, 10, 25, 50, and 75 nM) of Pristimerin in the presence of RANKL (100 ng/ml) for 6–7 days. The number of osteoclasts was counted using ImageJ (E and F) Pristimerin suppressed osteoclast formation at early stage (1–3 days), but not late stage (5–7 days). BMMs were treated with Pristimerin (75 nM) at day 1, 3, and 5 after RANKL-induced osteoclastogenesis. The TRAP staining was performed 6–7 days after RANKL stimulation. The data were obtained in three independent experiments and were shown as mean \pm SD ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

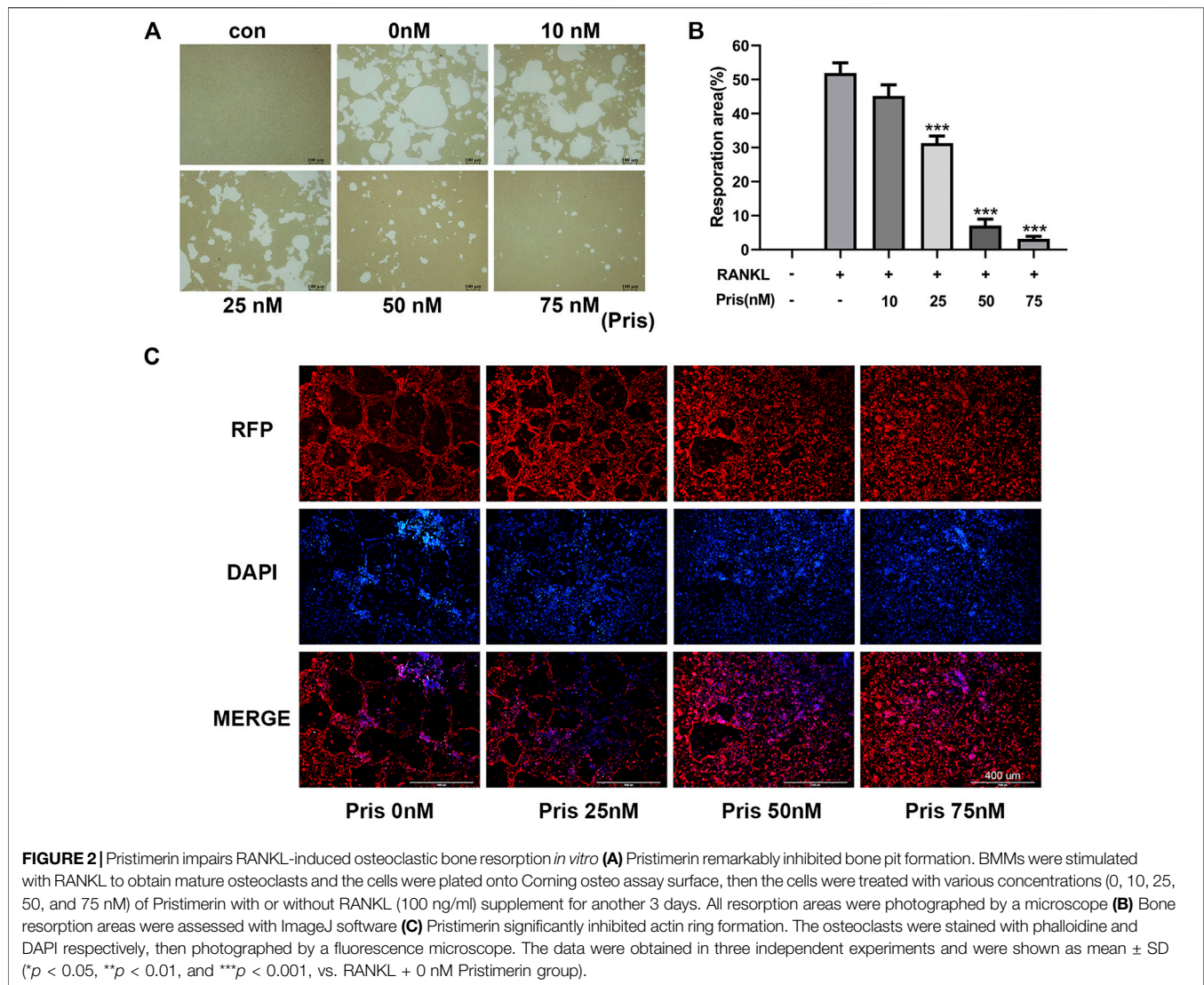
the statistical significance between the two groups, while one-way ANOVA was used for multiple comparisons. A p -value < 0.05 was considered as significant, all statistical analysis was performed with Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Pristimerin Suppresses Receptor Activator of Nuclear Factor- κ B Ligand-Induced Osteoclast Differentiation *in vitro*

Pristimerin (Pris), a natural compound, possesses numerous pharmacological effects, its chemical formula is showed in

Figure 1A. First, we used CCK-8 kit to measure the cytotoxicity of Pristimerin in BMMs. The results demonstrated that there was no obvious effect on the cytotoxicity of BMMs at 1, 3, 5 days after Pristimerin incubation (**Figure 1B**). To test the role of Pristimerin on osteoclasts formation, BMMs were treated with increasing concentrations (0, 5, 10, 25, 50, and 75 nM) of Pristimerin for 6–7 days in the presence of RANKL (100 ng/ml). We found that RANKL-induced osteoclasts formation was obviously suppressed by Pristimerin in a concentration-dependent manner (**Figure 1C,D**). In addition, to identify Pristimerin inhibits osteoclastogenesis at which stage, BMMs were treated with Pristimerin (75 nM) at day 1, 3, and 5 after osteoclastogenesis. The results indicated that Pristimerin inhibited osteoclasts formation at early stage (1–3 days), but not late stage (5–7 days) (**Figure 1E,F**). Therefore, Pristimerin



significantly suppressed RANKL-induced osteoclast formation without cytotoxic effect on BMMs. To explore the role of Pristimerin on osteogenesis in BMSCs, we also performed ALP staining at day 7 and Alizarin Red staining at day 14 after differentiation, the results indicated that there was no obvious effect on osteogenesis in BMSCs after Pristimerin treatment (Supplementary Figure S1A,B).

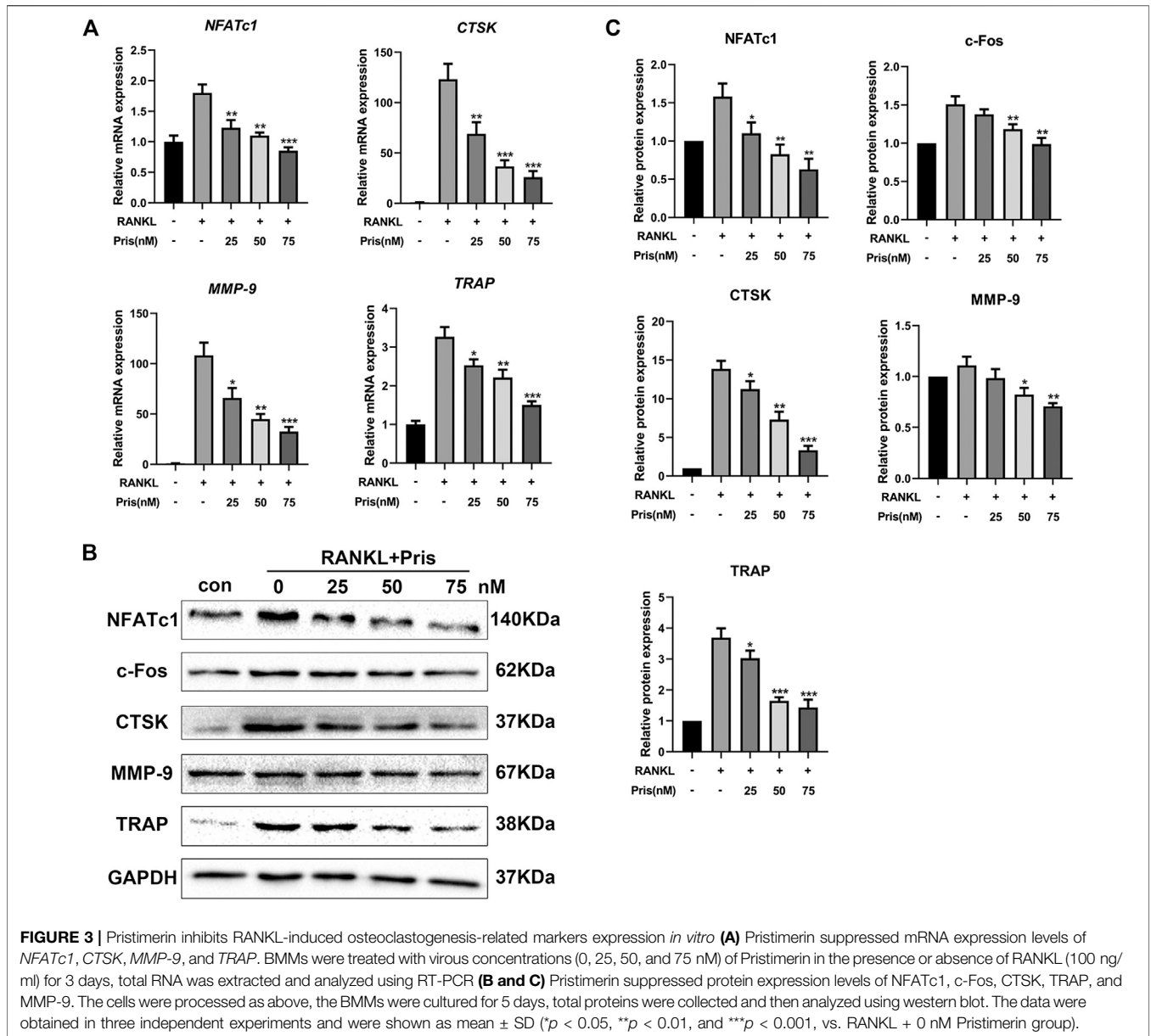
Pristimerin Impairs Osteoclastic Bone Resorption *in vitro*

To explore the effect of Pristimerin on the osteoclast function, pit formation and actin ring formation experiments were performed. The results revealed that the bone resorption area was remarkably reduced by Pristimerin in a dose-dependent manner (Figure 2A,B). Actin ring formation is vital and essential for osteoclast attachment and bone resorption (Wilson et al., 2009). In our study, the actin ring formation was also suppressed after

the treatment of Pristimerin (Figure 2C). Therefore, Pristimerin significantly inhibited osteoclastic bone resorption *in vitro*.

Pristimerin Inhibits Receptor Activator of Nuclear Factor- κ B Ligand-Induced Osteoclastogenesis-Related Markers Expression *in vitro*

To further explore the role of Pristimerin on osteoclastogenesis in BMMs, the mRNA and protein expression levels of osteoclastogenesis-related genes were detected, including *TRAP*, *Cathepsin K*, *NFATc1*, *MMP-9*, and *c-Fos*. These markers play a very important part in osteoclast differentiation and function (Ono and Nakashima, 2018). The results indicated that Pristimerin significantly suppressed the osteoclastogenesis-related markers expression in BMMs in a concentration-dependent manner (Figure 3). To explore the role of Pristimerin on osteogenesis in BMSCs, the mRNA expression levels of osteogenesis-related genes



including *ALP*, *RUNX2*, *OCN*, *OPN* were also detected, the results indicated that there was no obvious effect on osteogenesis after Pristimerin treatment for 7 days (Supplementary Figure 1C).

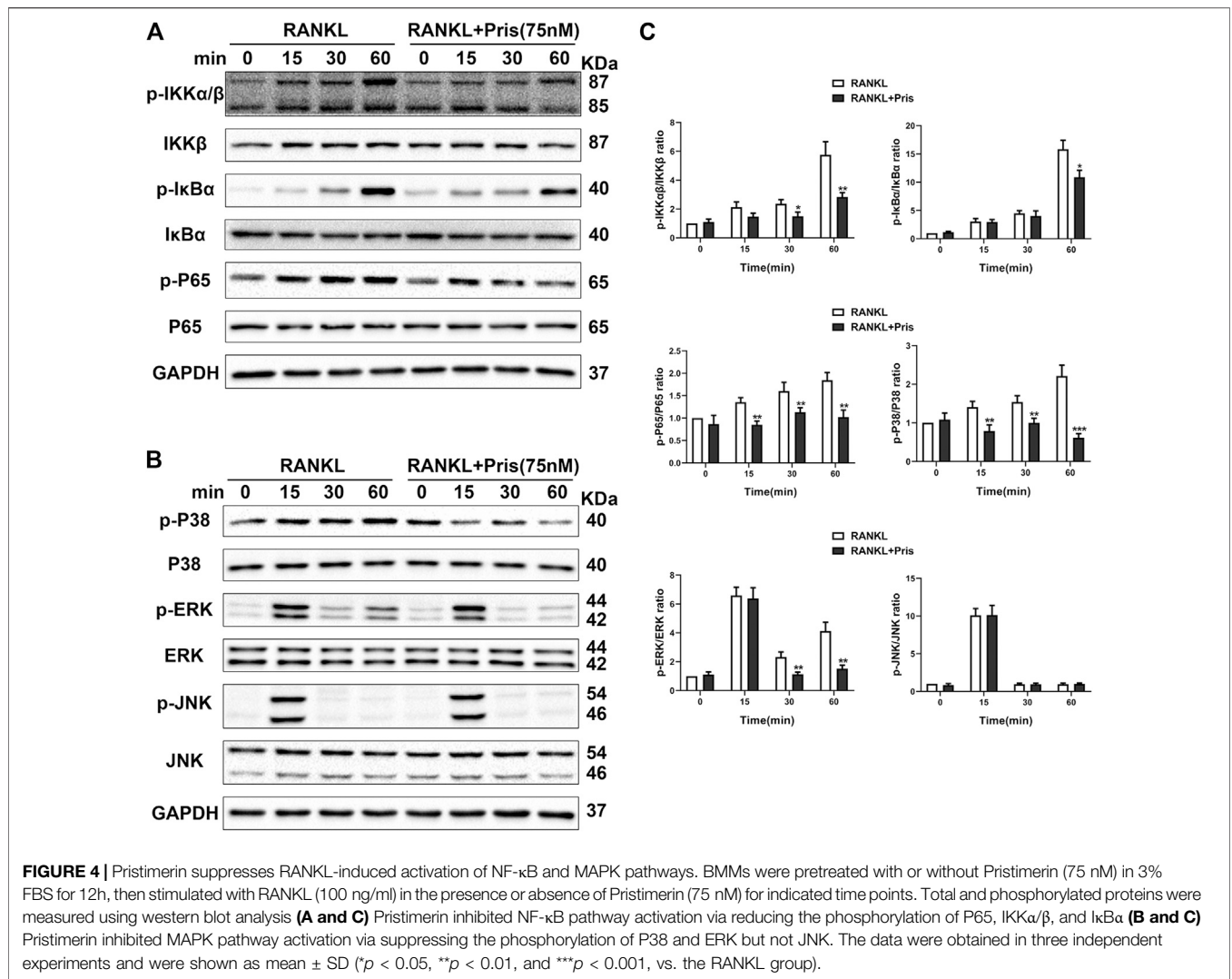
Pristimerin Inhibits NF-κB and MAPK Pathways

To uncover the molecular mechanism underlying the inhibitory effect of Pristimerin on osteoclastogenesis, we examined the expression level of proteins involved in NF-κB and MAPK pathways. Briefly, BMMs were starved with 3% FBS in the presence of Pristimerin (75 nM) or not for 12 h, then RANKL (100 ng/ml) was added to the medium for 0, 15, 30 and 60 min. Western blot results showed Pristimerin decreased the phosphorylation of IκBα, IKKα/β, and P65 (Figure 4A,C), the

phosphorylation of P38 and ERK also decreased, but JNK remained unchanged (Figure 4B,C), indicating both NF-κB and MAPK pathways were suppressed by Pristimerin.

Pristimerin Suppresses Receptor Activator of Nuclear Factor-κB Ligand-Induced Reactive Oxygen Species Production and Activates the Nrf2/HO-1 Signals

Overproduction of ROS promotes osteoclast formation and plays an important part in osteoclastogenesis (Agidigbi and Kim, 2019). The activation of Nrf2/HO-1 signals could induce the expression of various antioxidants and protect cells from oxidative stress (Bellezza et al., 2018). Therefore, the role of Pristimerin on oxidative stress in BMMs was investigated. The



DCFH-DA staining assay showed that Pristimerin treatment significantly reduced the number of ROS-positive cells induced by RANKL stimulation (**Figure 5A,B**). We also tested the effect of Pristimerin on Nrf2/HO-1 signaling. BMMs were treated with different concentrations (0, 25, 50, and 75 nM) of Pristimerin in the presence or absence of RANKL (100 ng/ml) for 3 days, then the total proteins were extracted for western blot. The results showed that the protein levels of Nrf2, HO-1, and NQO-1 decreased after RANKL stimulation, but were significantly increased after various concentrations (0, 25, 50, and 75 nM) of Pristimerin incubation in BMMs (**Figure 5C,D**). Taken together, Pristimerin decreased the ROS production and activated Nrf2/HO-1 signaling.

Pristimerin Ameliorates Ovariectomized-Induced Bone Loss *in vivo*

To test the function of Pristimerin *in vivo*, an OVX-induced osteoporosis model was created to evaluate the potential

protective effect of Pristimerin on osteoporosis. Mice were injected with Pristimerin (1 mg/kg) or vehicle intraperitoneally for 8 weeks. After sacrificing, the left femurs of mice were scanned and analyzed using μCT. The results indicated that the trabecular bone mass of mice in OVX group reduced significantly compared with the sham group, while treatment with Pristimerin dramatically rescued the OVX-induced bone loss (**Figure 6A**), as also confirmed by the bone parameters. Ovariectomy decreased BV/TV, Tb.N, and Tb.Th but increased Tb.Sp in mice, and this was reversed by Pristimerin treatment (**Figure 6B**).

The histological examinations were also performed to identify the effect of Pristimerin on bone loss. H & E staining showed that Pristimerin treatment significantly ameliorated the trabecular bone loss caused by ovariectomy (**Figure 7A**). TRAP staining indicated that Pristimerin application obviously reduced the number of osteoclasts on the trabecular bone surface near distal femur growth plate, compared with the OVX mice (**Figure 7B,C**).

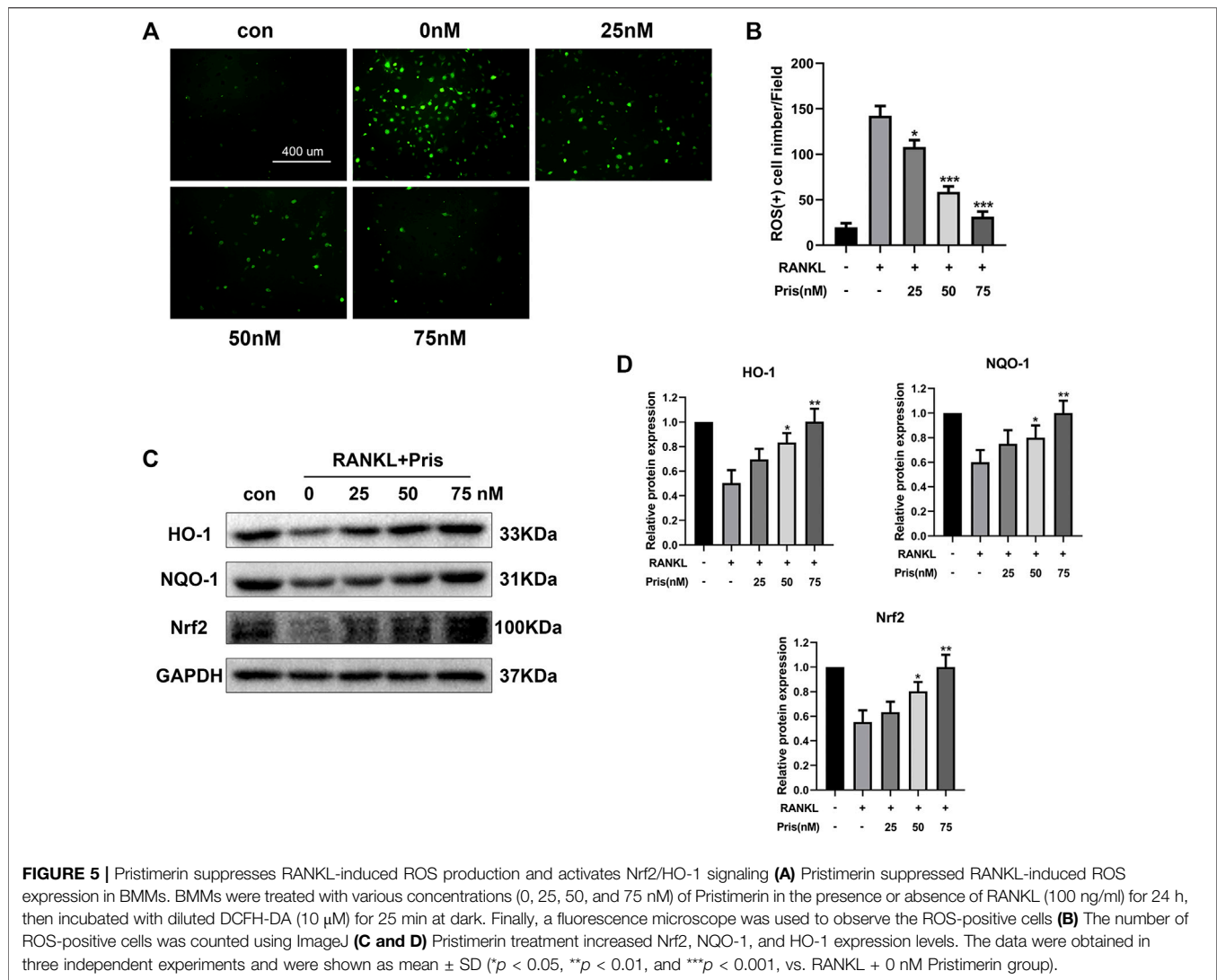


FIGURE 5 | Pristimerin suppresses RANKL-induced ROS production and activates Nrf2/HO-1 signaling **(A)** Pristimerin suppressed RANKL-induced ROS expression in BMMs. BMMs were treated with various concentrations (0, 25, 50, and 75 nM) of Pristimerin in the presence or absence of RANKL (100 ng/ml) for 24 h, then incubated with diluted DCFH-DA (10 μ M) for 25 min at dark. Finally, a fluorescence microscope was used to observe the ROS-positive cells **(B)** The number of ROS-positive cells was counted using ImageJ **(C and D)** Pristimerin treatment increased Nrf2, NQO-1, and HO-1 expression levels. The data were obtained in three independent experiments and were shown as mean \pm SD (* p < 0.05, ** p < 0.01, and *** p < 0.001, vs. RANKL + 0 nM Pristimerin group).

In addition, we tested the levels of TNF- α , IL-1 β , IL-6, OPG, and RANKL in the sera of mice using ELISA kits. We found that compared to the sham group, TNF- α , IL-1 β , IL-6, and RANKL levels increased in the OVX group, and all these inflammatory factors were reversed in OVX treated with Pristimerin group, which confirmed the anti-inflammatory effect of Pristimerin *in vivo*. Moreover, Pristimerin treatment increased OPG level compared to OVX group (Figure 7D).

DISCUSSION

In the current study, the effect of Pristimerin on osteoclastogenesis was revealed *in vitro* and *in vivo*. Our results indicated that Pristimerin inhibited RANKL-induced osteoclast differentiation and function, reduced the expression of osteoclastogenesis-specific markers, including NFATc1, c-Fos, TRAP, CTSK, and MMP-9. The ovariectomized mice were selected for our *in vivo* experiments, which showed that

Pristimerin reduced the levels of inflammatory factors in mice and alleviated the bone loss caused by ovariectomy. We also found Pristimerin inactivated NF- κ B and MAPK pathways and activated Nrf2/HO-1 signaling, which might be the potential molecular mechanisms for its role on osteoclastogenesis (Figure 8). Osteoclastic bone resorption and osteoblastic bone formation maintain bone homeostasis together (Feng and McDonald, 2011), thus some experiments on osteogenesis in BMSCs were performed as a supplement. In the current study, we found there was no obvious effect on osteogenesis after Pristimerin treatment in BMSCs.

Current evidence suggests that RANKL/RANK/OPG axis plays a major part in osteoclastogenesis (Walsh and Choi, 2014). OPG associates with RANKL and functions as a decoy receptor, thus inhibiting the differentiation of osteoclasts (Baud'huin et al., 2007). RANKL binds to RANK, results in the recruitment of TRAF6 in the cytoplasm, then triggers the downstream NF- κ B and MAPK signaling, which subsequently activates transcription factors NFATc1 and c-Fos, thus enhances the expression of

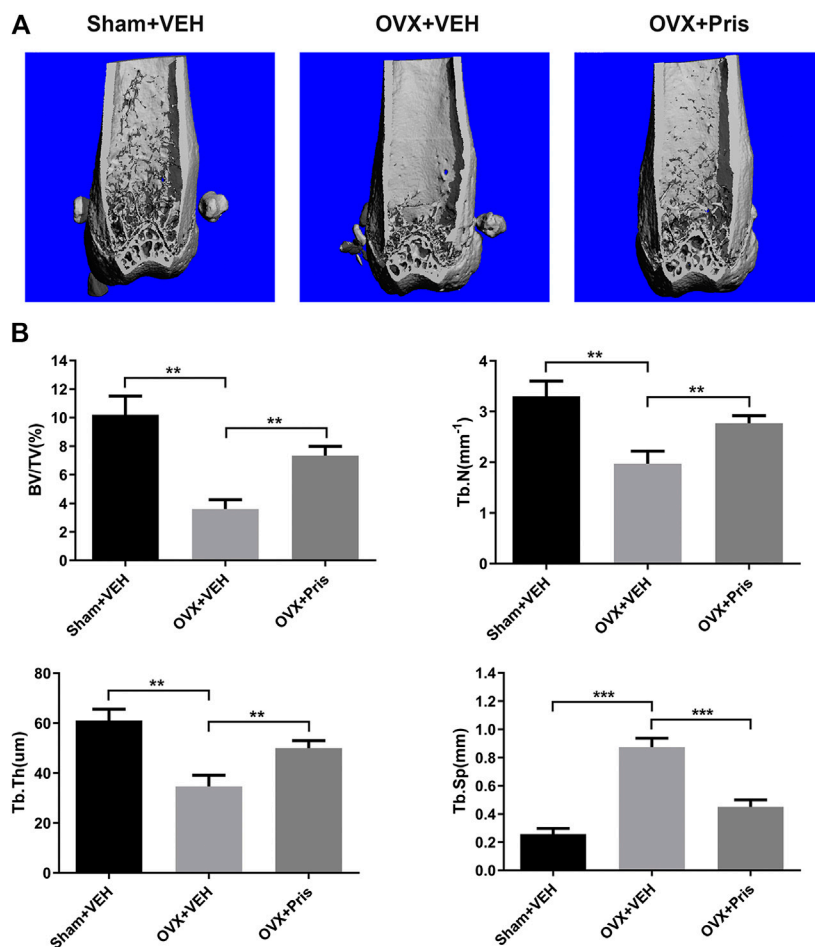


FIGURE 6 | Pristimerin ameliorates OVX-induced bone loss *in vivo* (A) Representative three-dimensional reconstruction images of distal femurs of Sham + VEH (sham group treated with vehicle), OVX + VEH (OVX group treated with vehicle), and OVX + Pristimerin (OVX group treated with Pristimerin). The results showed that Pristimerin treatment could ameliorate OVX-induced bone loss (B) Statistical graphs of bone parameters BV/TV, Tb.Sp, Tb.Th, and Tb.N. Data were described as mean \pm SD (n = 10) (* p < 0.05, ** p < 0.01, and *** p < 0.001, vs. OVX + VEH group).

osteoclastogenesis-related markers, eventually leads to the differentiation and maturation of osteoclasts (Takayanagi et al., 2002; Wada et al., 2006). Recent literatures have clearly demonstrated that Pristimerin could suppress the activation of MAPK and NF- κ B signaling (Tu et al., 2018). One study found that Pristimerin exerted anti-inflammatory effects via blocking the NF- κ B and MAPK signals in murine macrophage RAW264.7 cells (Kim et al., 2013). Another study showed that Pristimerin could suppress LPS-induced neurotoxicity via inhibiting NF- κ B and JNK/AP-1 pathways in BV-2 microglia cells (Hui et al., 2018). In our study, Pristimerin suppressed the phosphorylation of P65, I κ B α , IKK α / β , P38 and ERK, thus blocked the activation of NF- κ B and MAPK, and this might be the molecular basis by which Pristimerin inhibits osteoclastogenesis.

ROS have been shown to mediate the toxicity but also function as signaling molecules (D'Autreaux and Toledano, 2007). The excessive production of ROS may be responsible for various pathological conditions, such as tumors (Prasad et al., 2017),

inflammation (Mittal et al., 2014) and osteoporosis (Agidigbi and Kim, 2019). Nuclear factor erythroid-related factor 2 (Nrf2) is a vital regulator of oxidative stress response, it controls the expression of downstream antioxidant enzymes such as HO-1 and NQO-1 (Tonelli et al., 2018). Nrf2 activation could counteract RANKL-induced ROS production and further suppress osteoclastogenesis (Sun et al., 2015; Liu et al., 2019). One study reported that Pristimerin could protect against DOX-induced cardiotoxicity due to activation of Nrf2 and inhibition of MAPK signaling (El-Agamy et al., 2019). Another study indicated that Pristimerin possessed protective effects against autoimmune hepatitis via the activation of Nrf2/HO-1 pathway, thus exerted its anti-oxidative, anti-inflammatory properties (El-Agamy et al., 2018). In our study, Pristimerin effectively inhibited the generation of ROS induced by RANKL and increased the expression of Nrf2, HO-1, and NQO-1 in BMMs, this could also be one of the mechanisms by which Pristimerin inhibits osteoclastogenesis.

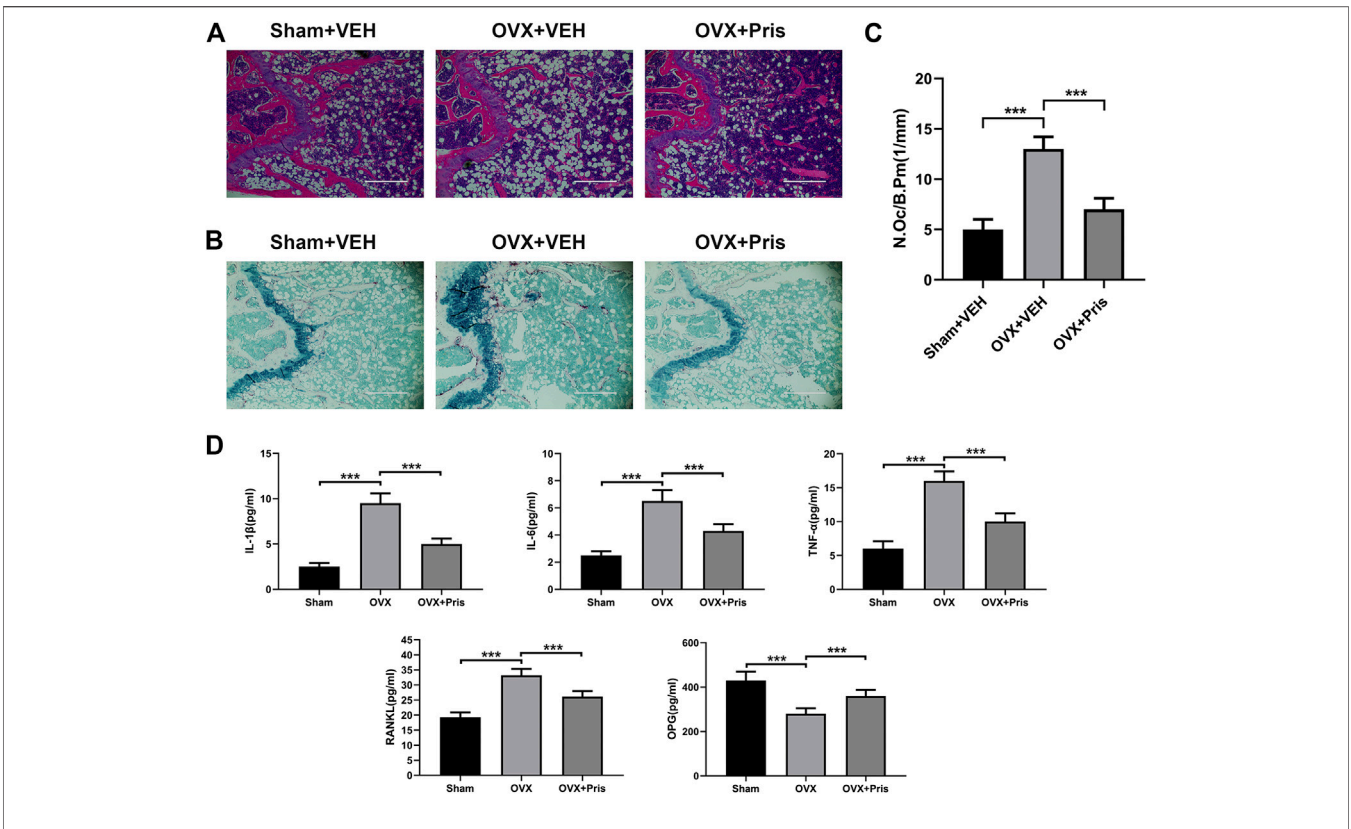
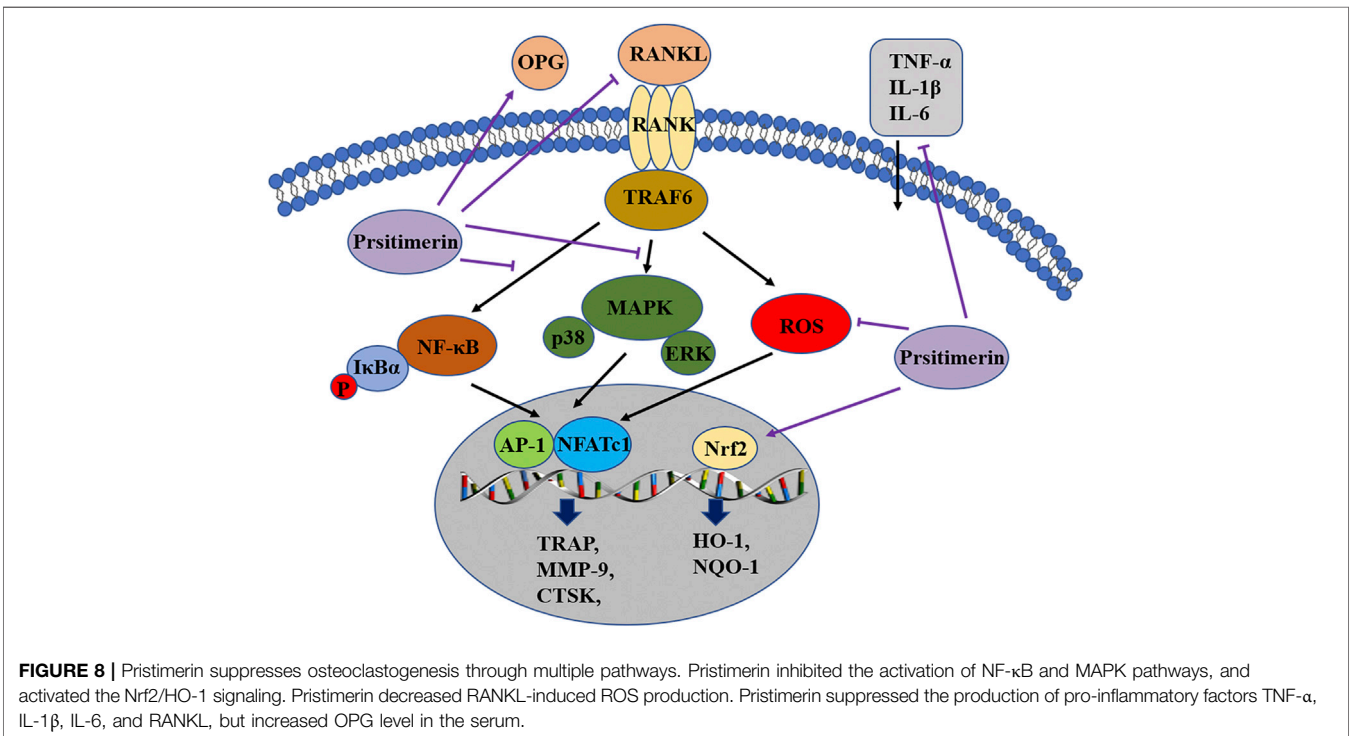


FIGURE 7 | Pristimerin improves histomorphological changes of bone tissue in OVX mice **(A)** Representative H & E staining sections from each group **(B)** Representative TRAP staining sections from each group **(C)** The osteoclasts on the surface of trabecular bone was counted using ImageJ **(D)** Pristimerin increased the serum level of OPG and decreased the serum levels of TNF- α , IL-1 β , IL-6, and RANKL. These cytokines were measured using ELISA kits. Data were described as mean \pm SD (n = 10) (* p < 0.05, ** p < 0.01, and *** p < 0.001, vs. OVX + VEH group).



Inflammation is also closely related to osteoclastogenesis (Adamopoulos, 2018). Studies have implicated that postmenopausal osteoporosis is partially mediated by the release of pro-inflammatory factors due to estrogen withdrawal, such as IL-6, TNF- α , and IL-1 (Mundy, 2007). Overproduction of pro-inflammatory cytokines is also associated with some inflammatory bone destruction diseases, such as periodontitis, rheumatoid arthritis, and multiple myeloma (Zhou et al., 2019). These pro-inflammatory cytokines indirectly mediate osteoclastogenesis via a series of ways, such as increasing RANKL and M-SCF expression, reducing OPG expression, and cross-linking with the RANKL/RANK downstream pathways (Zupan et al., 2013; Rao et al., 2018). Previous studies have confirmed that Pristimerin could decrease the expression levels of pro-inflammatory cytokines in several animal models of tissue damage, such as LPS-induced acute lung injury (Shaaban et al., 2018), ovalbumin-induced allergic airway inflammation (Jin et al., 2016), and adjuvant-induced arthritis (Tong et al., 2014; Deng et al., 2015). In our study, Pristimerin reduced the expression levels of serum inflammatory factors in ovariectomized mice, including TNF- α , IL-1 β , IL-6 and RANKL, which is consistent with other studies.

The limitation of our study is, although we have explored the effect of Pristimerin both on osteoclastogenesis and osteogenesis, but we didn't test the effects of Pristimerin on adipogenesis. As previous studies showed, factors promoting osteogenesis are recognized as inhibitors for adipogenesis and vice versa (Kawai and Rosen, 2010). Thus elevated adipogenesis may impair osteogenesis and bone mass, experiments may take to further elucidate adipose formation after Pristimerin exposure in the next study. In addition, our experiment did not test the toxic effect of Pristimerin on other organs in mice like liver and kidney, which will be needed for the translation of Pristimerin into clinical use, much more work should be done in the future.

Our study indicated that Pristimerin has an obvious inhibitory effect on RANKL-induced osteoclastogenesis and a protective role on ovariectomy-induced bone loss. We believe our study provides persuasive evidence for developing a novel reagent to treat osteoporosis for Pristimerin in the future.

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethics Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

AUTHOR CONTRIBUTIONS

Study design: DQ and JX. Study conduct: DQ, HL, XS, DL, MZ, TT, CZ, and JX. Data collection: CG and WZ. Data analysis: DQ, WZ, and JX. Data interpretation: WZ and JX. Drafted the manuscript: DQ, WZ, and JX. Approved the final version of manuscript: all the authors. WZ and JX take responsibility for the integrity of the data analysis.

FUNDING

This study was supported by National Natural Science Foundation of China (No.81772396; No.81974350).

ACKNOWLEDGMENTS

The authors thank all participants enrolled in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2020.621110/full#supplementary-material>.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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