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Original Article

Tanshinone IIA attenuates osteoclastogenesis in ovariectomized mice by inactivating NF-κB and Akt signaling pathways

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Abstract: Osteoporosis is a common disease associated with age and menopausal status. Postmenopausal osteoporosis is the most common type of primary osteoporosis and is accompanied by increased risk of osteoporotic fracture. Natural and herbal compounds have long been used to prevent and treat many human diseases. Here, we demonstrated that tanshinone IIA prevented ovariectomy-induced bone loss in an *in vivo* mouse model that closely mimics osteoporosis. In addition, we found that tanshinone IIA inhibited the receptor activator of nuclear factor NF-κB ligand (RANKL)-induced osteoclast differentiation and osteoclastogenesis *in vitro*. Tanshinone IIA treatment also abrogated RANKL-induced activation of the NF-κB pathway, PI3-kinase/Akt signaling, and the mitogen-activated protein kinase (MAPK) pathways, including nuclear translocation of NF-κB p65 and phosphorylation of IκB, extracellular signal-regulated kinase (ERK), p38, and Akt. Inactivation of these pathways resulted in deceased expression of osteoclastogenesis-related markers. These results suggest that tanshinone IIA, a natural drug, has the potential to treat and prevent bone loss diseases, including postmenopausal osteoporosis.

Keywords: Tanshinone IIA, postmenopausal osteoporosis, osteoclastogenesis, RANK ligand

Introduction

Osteoporosis is characterized by low bone mineral density and increased bone weakness that is caused by altered bone microstructure [1]. A number of diseases and medications increase the risk for osteoporosis, including alcoholism, kidney disease, surgical removal of the ovaries, chemotherapy, and glucocorticosteroid use. In addition, the risk for osteoporosis increases with age and is more common in women than men. Studies have found that over 50% of postmenopausal Caucasian women will have an osteoporotic-related fracture [1]. A diagnosis of osteoporosis puts an individual at high risk of skeletal fragility and fracture and is the most common reason for a broken bone among the elderly. Although rarely lethal, osteoporotic fractures are associated with reduced quality of life [2].

Osteoporosis occurs when there is an imbalance between osteoclast bone resorption and bone formation by osteoblasts [3]. Osteoblasts are bone-forming cells derived from mesenchymal stem cells (MSCs) through a multistep differentiation pathway. Osteoclasts are large bone-resorbing multinucleated cells that differentiate from mononuclear cells of the monocyte/macrophage lineage. Monocyte/macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) stimulate osteoclast activation and survival [4]. Osteoblasts express both M-CSF and RANKL, both membrane-bound and soluble RANKL, which bind to their receptors, c-Fos and RANK. Following ligand binding, RANK recruits adaptor proteins, TNF receptor-associated factors (TRAFs), to initiate intracellular signaling pathways, including NF-κB, c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK), p38, nuclear factor of activated T cells cytoplasmic 1 (NFATc1), and Akt. These activated pathways then regulate osteoclast formation, function, and survival [3]. RANKL is more highly expressed on the surface

of osteoblasts and lymphocytes from postmenopausal women with osteoporosis compared to premenopausal controls [5], suggesting a role for RANKL in the pathogenesis of postmenopausal osteoporosis. Therefore, we hypothesize that targeting RANKL may be an effective therapeutic avenue to prevent fractures in postmenopausal women with osteoporosis.

Inhibition of bone resorption is an important therapeutic strategy for postmenopausal osteoporosis. In addition to lifestyle changes, a number of medications, such as alendronate, etidronate, risedronate, raloxifene, and strontium ranelate, are recommended as treatment [6]. A monoclonal antibody to RANKL has also recently been developed as a treatment [7]. In addition, traditional Chinese herbs have shown promise in treating osteoporosis. Tanshinone IIA is a diterpene quinone isolated from the danshen root, *Salvia miltiorrhiza* [8], that suppresses bone loss induced by inflammatory disease [9] and suppresses bone loss in animal models of disease [10]. In the past decade, tanshinone IIA has been widely used to protect against cancer, cardiovascular, metabolic, and neurodegenerative diseases [11-14]. Tanshinone IIA has multiple molecular targets [8]. In osteoclast-related bone disease, it targets c-fos, NFATc1, and prostaglandin E2, leading to inhibition of bone loss [9, 15]. However, it is unknown if tanshinone IIA attenuates postmenopausal osteoporosis by targeting RANKL-mediated signaling pathways.

Therefore, in the current study, we evaluated the effects of tanshinone IIA on osteoclast differentiation *in vivo* and *in vitro* in order to evaluate its mechanism of protection. Our results demonstrate that tanshinone IIA has the potential to effectively inhibit postmenopausal osteoporosis through targeting various molecular pathways.

Materials and methods

Reagents

Tanshinone IIA (Figure S1) was purchased from Sigma-Aldrich (#T4952; St. Louis, MO, USA). RAW264.7 cells were obtained from Dr. Hou from the Department of Immunology, Second Military Medical University (Shanghai, China). Recombinant human RANKL was purchased from PeproTech EC (London, England).

MTT assay

RAW264.7 cells were cultured and plated at a density of 10^4 cells/well in a 96-well plate for 24 h. Cells were then treated with various concentrations of tanshinone IIA (0, 2.5, 5, 10, 20, 40, or 80 μg/ml) for 48 h in triplicate. MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml] was added, 10 μl/well, and incubated for an additional 2 h. Absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Hercules, CA, USA). All experiments were repeated three times.

In vitro osteoclastogenesis assay

We sacrificed 8-week-old C57BL/6 mice and isolated bone marrow mononuclear cells (BMMCs) from the femoral bone marrow. RAW264.7 cells were seeded (8×10^3 cells/well) in 24-well plates. Wells were designated as controls (untreated) or as tanshinone IIA-treated (1, 2, or 5 μg/ml). RANKL (50 ng/ml) was also added to the tanshinone IIA-treated cells. After 7 days, cells were stained with a tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma-Aldrich, St. Louis, USA). $TRAP^+$ cells with more than three nuclei were identified as osteoclasts. RAW264.7 cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.1% Triton-X 100/PBS for 5 min and incubated with Cell Navigator F-actin Labeling Kit Green Fluorescence (#22661; AAT Bioquest, Sunnyvale, CA, USA) to visualize F-actin. All experiments were repeated three times.

Pit-formation assays

RAW264.7 cells were seeded (3×10^3 cells/well) on bone biomimetic synthetic surface-coated plates (Corning, St. Lowell, MA, USA) in the absence or presence of RANKL (50 ng/ml) with or without various concentrations of tanshinone IIA, as previously described [16]. Following a 7-day incubation, osteoclast resorbing pits on the bone biomimetic synthetic surface were observed using a light microscope (Olympus-BX53, Tokyo, Japan). Pit areas were quantified using Image-Pro Plus software. All experiments were repeated three times.

NF-κB p65 immunofluorescence

RAW264.7 cells were treated with 5 μg/ml tanshinone IIA for 30 min for immunofluorescent

visualization of NF-κB p65. After treatment, cells were fixed with 4% PFA for 15 min, blocked with 1% bovine serum albumin (BSA) in PBS, and incubated with anti-p65 antibody (Abcam, Cambridge, MA, USA). Cells were then incubated with a biotinylated-goat anti-mouse IgG antibody (Baca) and fluorescein-conjugated streptavidin. Cells were counterstained with propidium iodide. For imaging, three fields of vision were randomly selected and 10 cells were counted per field.

Western blot

Whole-cell lysates of RAW264.7 cells from each treatment group were prepared for western blot analyses using standard blotting procedures. Following electrophoresis, transfer, and blocking, membranes were incubated with primary antibodies to TRAP (1:350), cathepsin K (1:500), TRAF6 (1:250), MMP-9 (1:400), CTR (1:200), p65 (1:350), phosphorylated (P)-p65 (1:500), p50 (1:250), P-p50 (1:400), IκBα (1:350), P-IκBα (1:500), and β-actin (1:1000; loading control) (Santa Cruz, Dallas, TX, USA).

Animals and bone histomorphometric analysis

All procedures were approved by the Animal Ethics Committee of the Second Military Medical University. Eight-week-old female C57BL/6 mice were purchased from the Chinese Academy of Sciences and were ovariectomized (OVX) under anesthesia. Sham-operated mice were used as a control. Mice were randomly divided into three groups: sham group (n=6), OVX mice treated with normal saline (n=6), and OVX mice treated with tanshinone IIA (n=6). Tanshinone IIA (10 mg/kg) was given by intraperitoneal (i.p.) injection daily. After 6 weeks of treatment, all mice were sacrificed and femurs were collected for further examination. Femurs were fixed and decalcified according to standard procedures [17]. Sections (4 μm) were prepared and stained with hematoxylin and eosin (H&E). Bone histomorphometric measurements were performed with an Olympus-BX53 microscope at 40× magnification. All experiments were repeated three times.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (version 16.0; IBM, Armonk, New York, USA). The number of osteoclasts was determined by counting more than three cells following TRAP staining. The F-actin ring was used to determine the number of actin molecules in the pore plate. P65 immunofluorescence in the cell nucleus was used to determine the number of positive cells. The area of bone resorption, Western blot band intensities were measured using the Image-Pro Plus 6 software (Media Cybernetics, Inc., Rockville, MD). All statistical analysis was performed using by GraphPad Prism software (La Jolla, CA, USA). The data with normal distribution were expressed by mean ± SD. The Student's *t*-test was used to compare between the groups. $P<0.05$ indicated a statistically significant difference. Differences with a $P<0.01$ are also indicated.

Results

Tanshinone IIA inhibits osteoclastogenesis and osteoclast function in vitro

To determine the effect of tanshinone IIA on RAW264.7 cell viability and ascertain the appropriate concentration to use for treatments, we first performed an MTT assay. Figure S1B shows that when used at concentrations below 10 μg/ml, tanshinone IIA treatment had no cytotoxic effects. We then evaluated the effects of tanshinone IIA on osteoclastogenesis *in vitro*. RAW264.7 cells were treated with RANKL in the absence and presence of various concentrations of tanshinone IIA. After RANKL treatment, the number of TRAP-positive ($TRAP^+$) cells significantly increased, with no $TRAP^+$ cells observed without RANKL-mediated osteoclastogenesis (**Figure 1A**). However, co-administration of tanshinone IIA with RANKL reduced the number of $TRAP^+$ cells in a dose-dependent manner.

When treated with RANKL, RAW264.7 cells are known to differentiate into mature osteoclasts and form pits on bone biomimetic synthetic surfaces [18]. In our culture system, a number of osteoclast resorbing pits were formed with RANKL treatment. However, the number of pits was significantly reduced when tanshinone IIA was added in a dose-dependent manner (**Figure 1B**), suggesting that tanshinone IIA suppresses osteoclast function.

RANKL also induces RAW264.7 cells to form actin rings, a characteristic feature of mature osteoclasts during osteoclastogenesis [19].

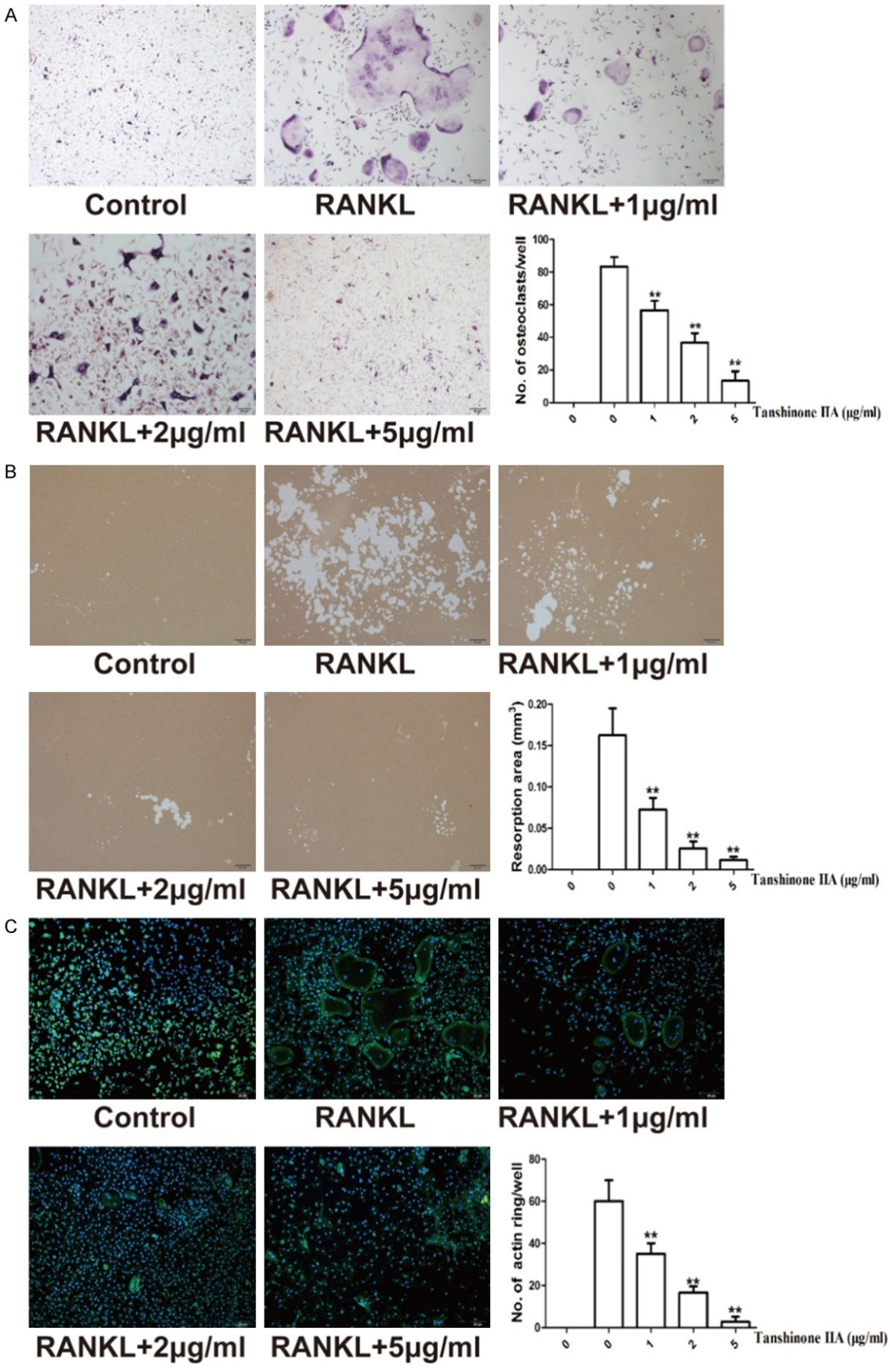
A
Control
RANKL
RANKL+1μg/ml
RANKL+2μg/ml
RANKL+5μg/ml
No. of osteoclasts/well
Tanshinone IIA (μg/ml)
B
Control
RANKL
RANKL+1μg/ml
RANKL+2μg/ml
RANKL+5μg/ml
Resorption area (mm3)
Tanshinone IIA (μg/ml)
C
Control
RANKL
RANKL+1μg/ml
RANKL+2μg/ml
RANKL+5μg/ml
No. of actin ring/well
Tanshinone IIA (μg/ml)

Figure 1. Tanshinone IIA inhibits osteoclastogenesis *in vitro*. A. RAW264.7 cells were treated with RANKL (50 ng/ml) in the absence or presence of various concentrations of tanshinone IIA (1, 2, 5 μg/ml) for 7 days and then stained with TRAP. $TRAP^+$ cells with more than three nuclei were counted as osteoclasts. B. RAW264.7 cells were seeded (3×10^3 cells/well) on bone biomimetic synthetic surface-coated plates in the absence or presence of RANKL (50 ng/ml) with or without tanshinone IIA of various concentrations. After 7 days, osteoclast-resorption pits at the bone biomimetic synthetic surface were observed and pit areas quantified. C. After 7 days of treatment, RAW264.7 cells were fixed, permeabilized, and incubated with Cell Navigator F-actin Labeling Kit Green Fluorescence to visualize F-actin. **P<0.01 compared with the RANKL-only group.

Interestingly, when treated with tanshinone IIA, the size and number of actin ring structures were significantly reduced compared with those without tanshinone IIA treatment (**Figure 1C**). In addition, as the concentration of tanshinone IIA increased, the actin rings decreased. These data suggest that tanshinone IIA suppresses the formation of actin rings in mature osteoclasts. Together, these results demonstrate that tanshinone IIA suppresses osteoclast function.

Tanshinone IIA inhibits RANKL-induced osteoclast differentiation

To determine the effect of tanshinone IIA on RANKL-induced pre-osteoclast differentiation into mature osteoclasts, tanshinone IIA was added to osteoclast differentiation cultures from days 0 to 3. When treatment began on day 0 or 1, no or few $TRAP^+$ cells were observed, indicating that osteoclastogenesis was effectively inhibited by tanshinone IIA administration. However, when treatment began on day 2 or 3, $TRAP^+$ cells were identified, indicating that osteoclastogenesis was not successfully inhibited by tanshinone IIA treatment (**Figure 2A**). Therefore, tanshinone IIA inhibited RANKL-induced osteoclast differentiation at the early stage, when administered prior at the start of differentiation.

Tanshinone IIA suppresses osteoclastogenesis-related gene expression

Next, we determined the effect of tanshinone IIA on the expression of the osteoclastogenesis-related markers TRAP, matrix metalloproteinase 9 (MMP-9), cathepsin K, calcitonin receptor (CTR), and TRAF6. **Figure 2B** (The original diagram of western blots is Figure S2) shows that administration of RANKL increased the expression of all of these proteins. However, when co-treated with tanshinone IIA, TRAP, cathepsin K, TRAF6, MMP-9, and CTR protein expression was significantly inhibited in a dose-dependent manner (P<0.01).

NFATc1 is a well-known master regulator of osteoclastogenesis and function [20]. To determine whether tanshinone IIA regulates the expression of NFATc1, we assessed the effect of tanshinone IIA treatment on *NFATc1* levels by RT-PCR. NFATc1 expression was increased after RANKL induction and then suppressed following tanshinone IIA treatment in a dose-dependent manner (**Figure 2C**).

Tanshinone IIA inhibits RANKL-induced activation of the NF-κB pathway

The NF-κB pathway can be activated by RANKL during osteoclast differentiation [21]. To examine the effect of tanshinone IIA on this pathway, we examined p65 cellular location. RAW264.7 cells had positive p65 staining with and without tanshinone IIA treatment (**Figure 3A**). In the control group, p65 was located in the cytoplasm and was unphosphorylated. After induction with RANKL, p65 translocated to the nucleus. This p65 nuclear translocation was blocked when cells were incubated with 5 μg/ml tanshinone IIA (**Figure 3A**). Our results indicated that RANKL treatment induced p65 activation in RAW264.7 cells (P<0.01) and treatment with tanshinone IIA inhibited p65 translocation (P<0.01) (**Figure 3B**).

We also examined phosphorylation of p65, p50, and IκBα by western blot. Semi-quantitative detection showed that induction with RANKL promoted phosphorylation of p65, p50, and IκBα in RAW264.7 cells. However, treatment with tanshinone IIA remarkably decreased levels of p65, p50, and IκBα phosphorylation (**Figure 3C**, the original diagram of western blots is Figure S3). These data suggest that tanshinone II inhibits activation of the NF-κB pathway that is induced by RANKL during osteoclastogenesis.

Tanshinone IIA inhibits RANKL-induced activation of the MAPK and Akt pathways

Activation of MAPK and Akt pathways also regulates osteoclastogenesis [22]. Therefore,

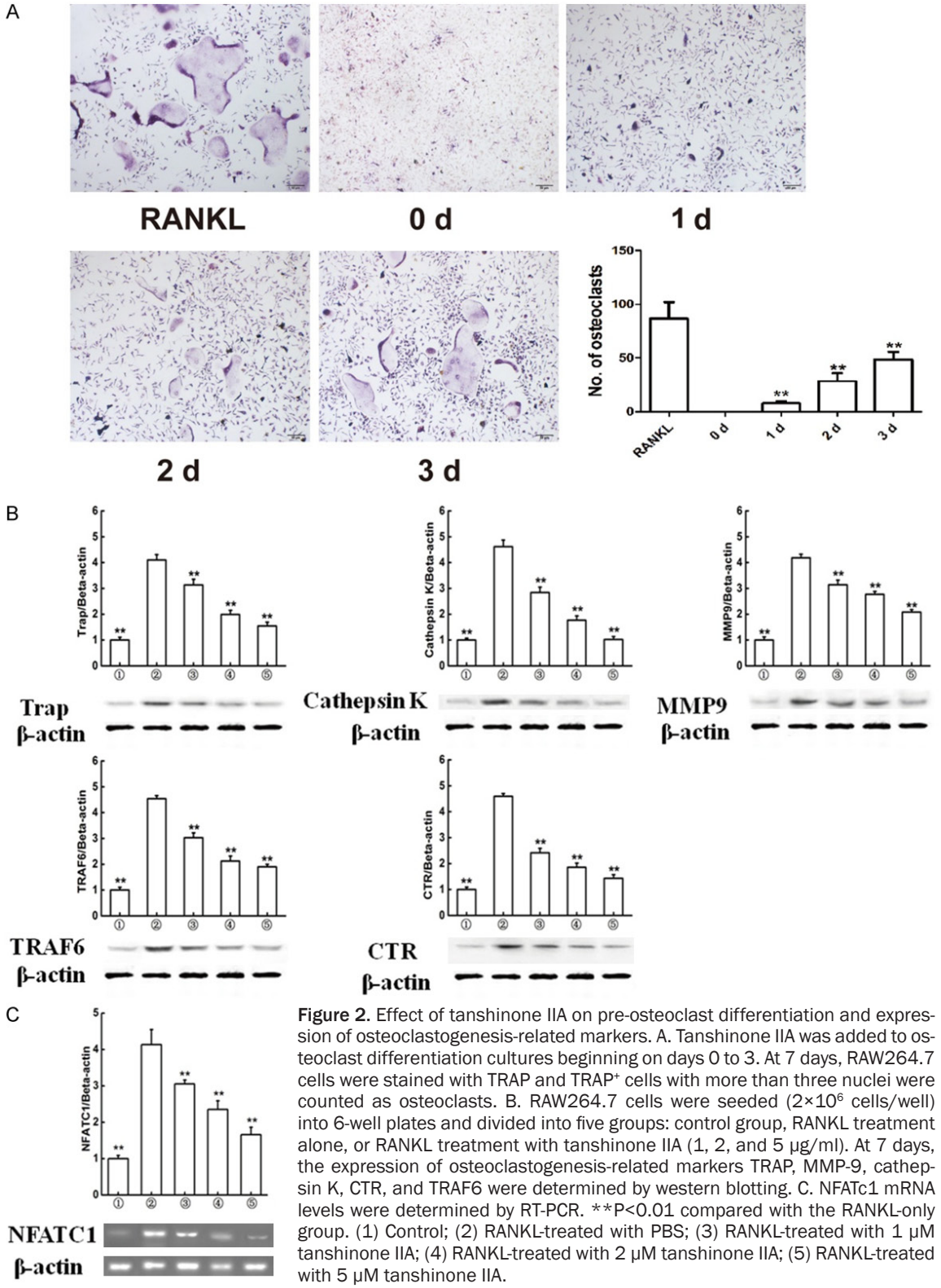

Figure 2. Effect of tanshinone IIA on pre-osteoclast differentiation and expression of osteoclastogenesis-related markers. A. Tanshinone IIA was added to osteoclast differentiation cultures beginning on days 0 to 3. At 7 days, RAW264.7 cells were stained with TRAP and TRAP$^+$ cells with more than three nuclei were counted as osteoclasts. B. RAW264.7 cells were seeded (2×10^6 cells/well) into 6-well plates and divided into five groups: control group, RANKL treatment alone, or RANKL treatment with tanshinone IIA (1, 2, and 5 μg/ml). At 7 days, the expression of osteoclastogenesis-related markers TRAP, MMP-9, cathepsin K, CTR, and TRAF6 were determined by western blotting. C. NFATc1 mRNA levels were determined by RT-PCR. **P<0.01 compared with the RANKL-only group. (1) Control; (2) RANKL-treated with PBS; (3) RANKL-treated with 1 μM tanshinone IIA; (4) RANKL-treated with 2 μM tanshinone IIA; (5) RANKL-treated with 5 μM tanshinone IIA.

we examined the effects of tanshinone IIA on these signaling pathways induced by RANKL. **Figure 4** (The original diagram of western blots is Figure S4) demonstrates that the levels of phosphorylated ERK, JNK, c-fos, and Akt were significantly increased by RANKL treatment. However, this was reversed with tanshinone IIA administration, indicating that tanshinone IIA

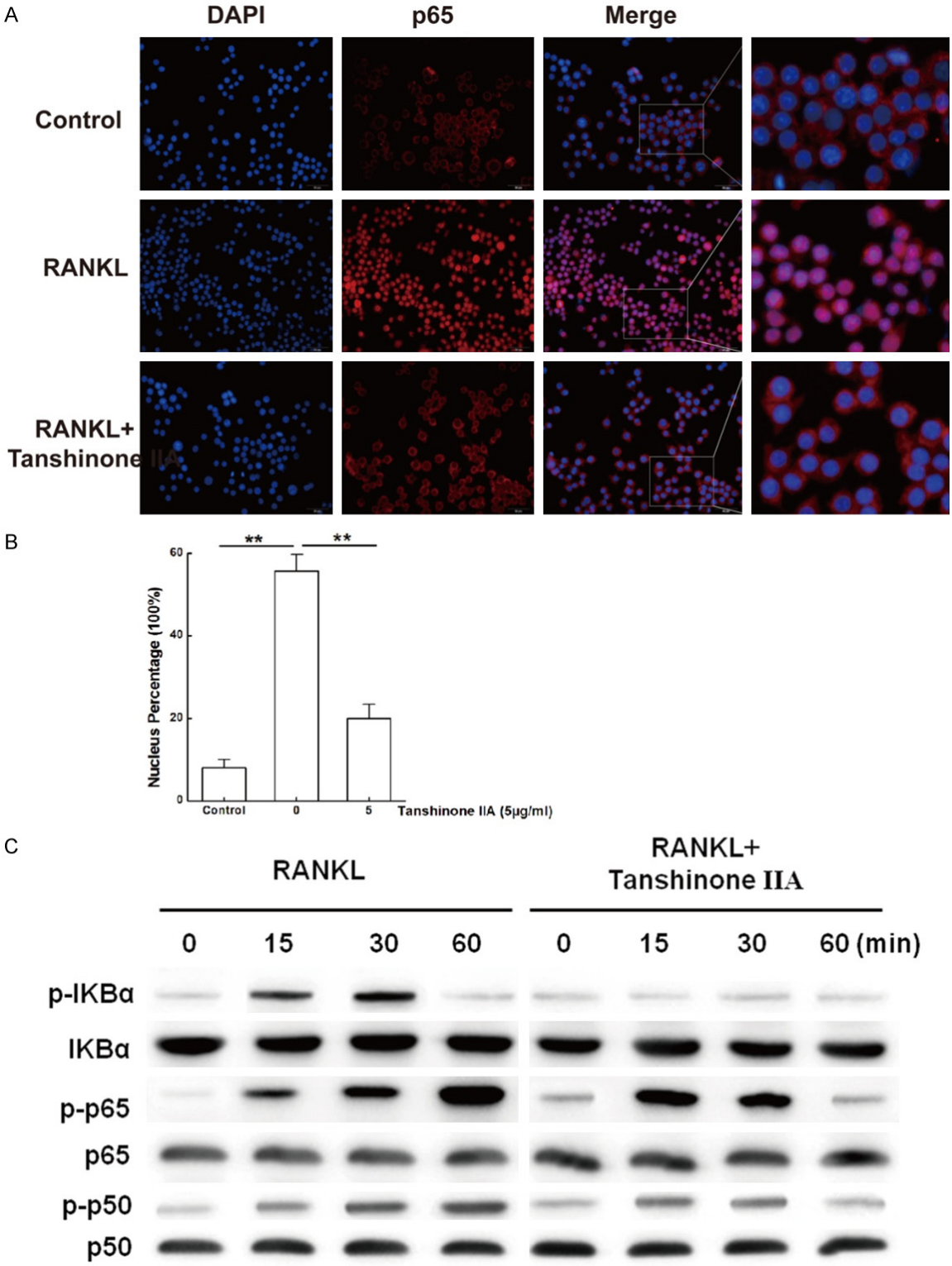

Figure 3. Effect of tanshinone IIA on the NF-κB pathway. A. RAW264.7 cells were treated with RANKL with or without tanshinone IIA for 7 days. Cells were fixed and incubated with an anti-p65 antibody, followed by a biotinylated-goat anti-mouse IgG and fluorescein-conjugated streptavidin. Cells were counterstained with propidium iodide and visualized by microscopy. B. Ratio of the nuclear fluorescence intensity with whole-cell fluorescence intensity. C. Phosphorylation of p65, p50, and IκB proteins was determined by western blotting at the indicated times. **P<0.01 compared with the RANKL-only group.

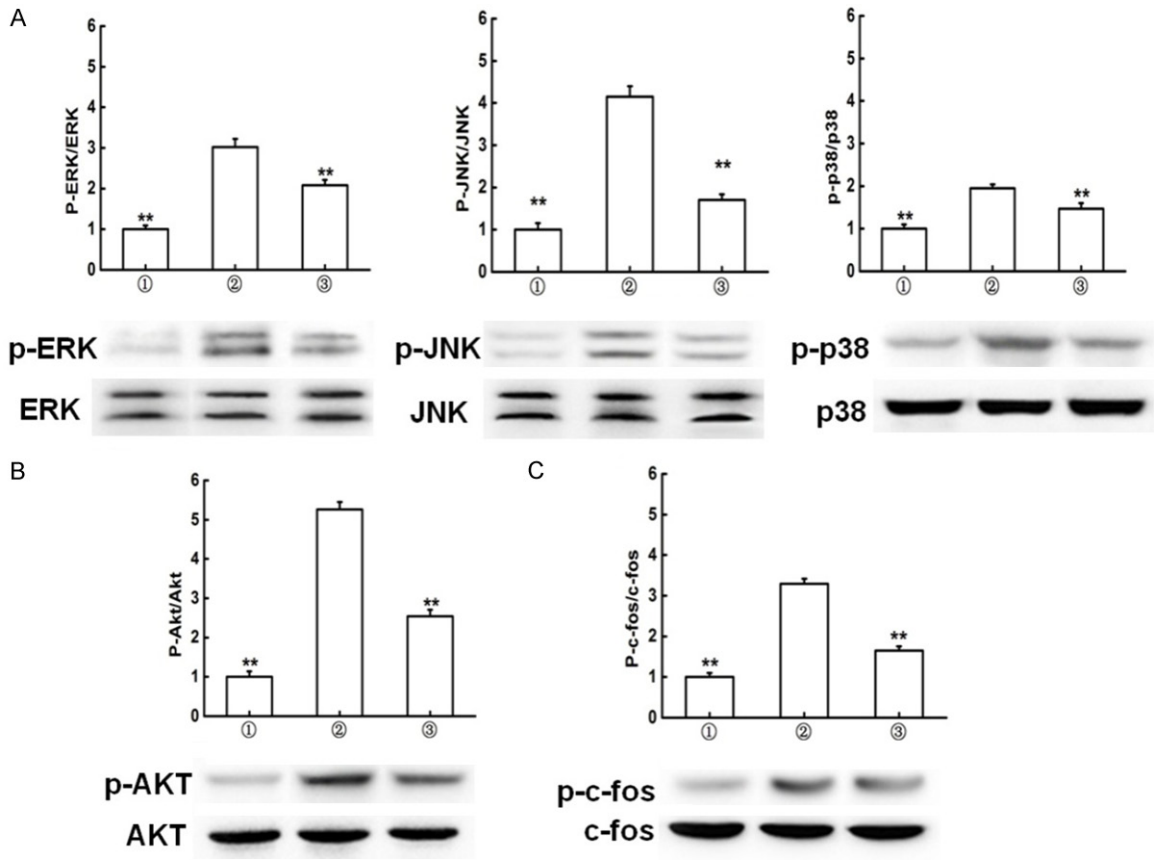

Figure 4. Inhibitory effect of tanshinone IIA on the MAPK and PI3K/Akt pathways. RAW264.7 cells were treated with RANKL alone or with 5 μM tanshinone IIA. Whole-cell lysates were prepared to detect ERK, JNK, and P38 (A), Akt (B), and c-fos (C) phosphorylation by western blotting. (1) Control; (2) RANKL-induced with PBS; (3) RANKL-treated with 5 μM tanshinone IIA. **P<0.01 compared with the RANKL-only group.

interrupts RANKL-induced activation of the MAPK and Akt pathways in osteoclasts.

Effect of tanshinone IIA on bone loss in OVX mice

Finally, we examined if tanshinone IIA treatment was able to prevent OVX-induced bone loss *in vivo*. An OVX mouse model was constructed to mimic menopause-induced bone loss in women. H&E staining revealed that after 6 weeks, OVX mice exhibited a significant loss of trabecular bone when compared with sham-operated mice. However, treatment with tanshinone IIA in OVX mice markedly inhibited trabecular bone loss, as shown by H&E staining, compared with OVX mice treated with normal saline (**Figure 5A**). These results were further corroborated by Micro-CT analyses (**Figure 5B**). The two-dimensional and three-dimensional structures were measured by trabecular BV/TV, BS/TV, Tb. N, Tb.pf, and BMD. The data demonstrated that tanshinone IIA reduced ovariectomy-induced bone loss *in vivo*.

Discussion

In the present study, we investigated the effects of tanshinone IIA on bone loss and osteoclastogenesis *in vivo* and *in vitro*, providing strong evidence that tanshinone IIA delays ovariectomy-induced osteoporosis. Mechanistically, tanshinone IIA treatment inhibited the RANKL-mediated activation of NF-κB, MAPK, and Akt signaling pathways during osteoclastogenesis. These results suggest that tanshinone IIA is a promising therapy for inhibiting bone loss by preventing osteoclast formation.

Osteoporosis results from an imbalance between bone formation and bone resorption. In restoring this balance, it is essential to eliminate risk factors. However, osteoporosis is strongly associated with age and menopause, with postmenopausal osteoporosis (PMOP)

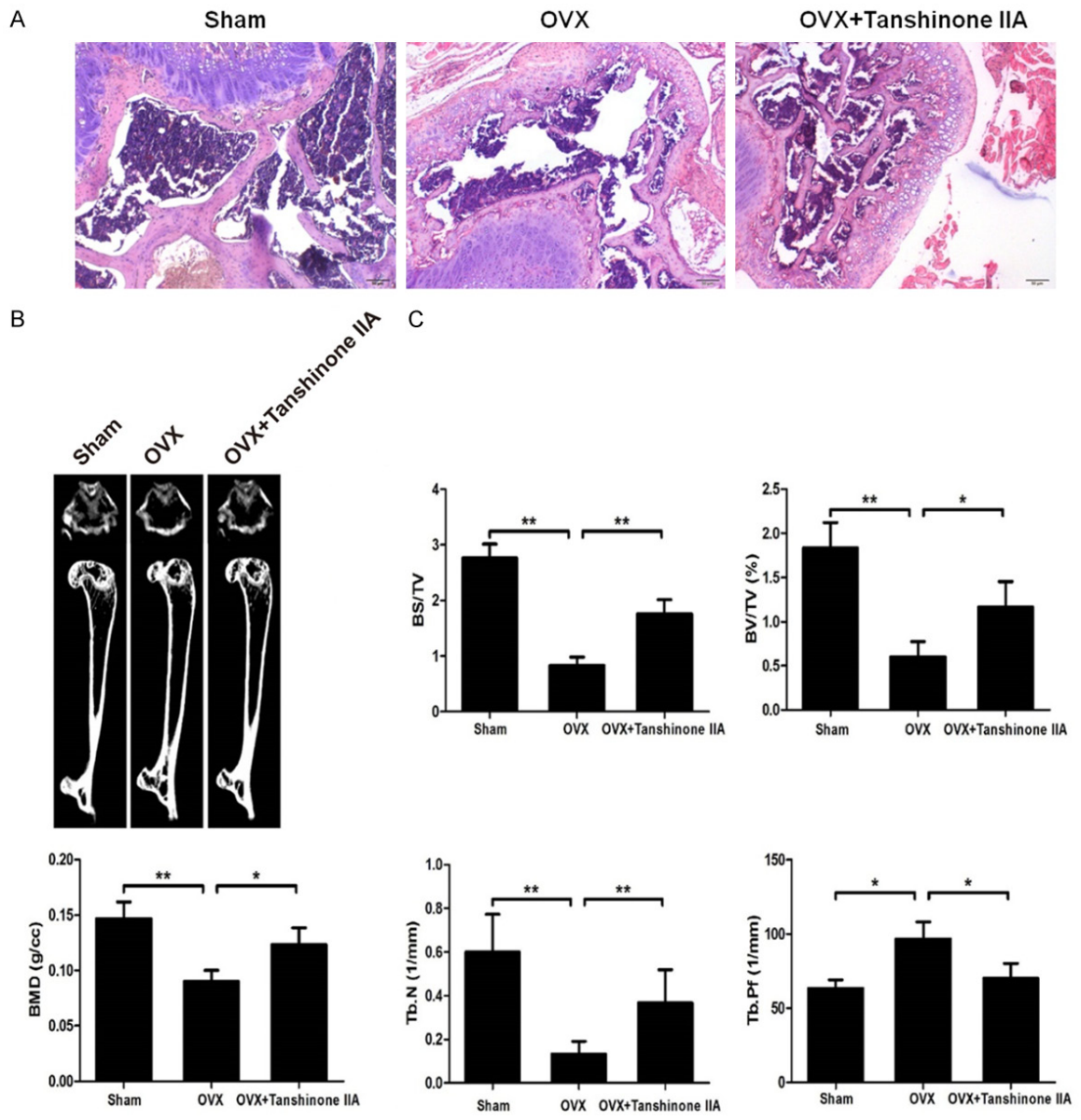

Figure 5. Inhibitory effect of tanshinone IIA on ovariectomy-induced bone loss *in vivo*. A. Representative H&E staining of femoral sections. B. Representative micro-CT sections of femurs from sham-operated, OVX, and OVX + tanshinone IIA-treated mice. C. Bone surface area/total value (BS/TV), bone value/total value (BV/TV), bone mineral density (BMD), trabecular number (Tb.N), and trabecular pattern factor (Tb.pf) were analyzed. *P<0.05, **P<0.01.

increasing the risk of osteoporotic fracture. Various medications for POMP are in development, including inhibitors of bone resorption (odanacatib, bisphosphonates, denosumab, and selective estrogen receptor modulators), stimulators of bone formation (teriparatide), and chemical compounds and monoclonal antibodies that both decrease bone resorption and stimulate bone formation (strontium ranelate, romosozumab, and blosozumab) [23, 24]. During PMOP development, excessive osteoclast formation rather than damage to osteoblast activity is the primary reason for bone loss. Therefore, anti-resorptive drugs are the main treatment for preventing fractures in PMOP patients [25]. However, many of these treatments have negative side effects and therefore they are not indicated for regular use to prevent PMOP when patients do not have a risk of fracture [26].

Herbal medicines have been used to treat various diseases for thousands of years with minimal side effects. Tanshinone IIA is a key bioac-

tive phytochemical of the danshen plant, *S. miltiorrhiza*. Increasing evidence from animal models and patient studies have shown that tanshinone IIA is effective for the treatment of inflammation, atherosclerosis, cardiovascular disease, cancers, and osteoporosis [11, 12, 15, 27] RANKL promotes osteoclast differentiation and is highly expressed in patients with POMP [5]. Therefore, inhibiting RANKL-induced osteoclastogenesis is important to prevent bone disease. Here, we demonstrated that tanshinone IIA significantly inhibited RANKL-mediated differentiation of osteoclast precursors, characterized by decreased numbers of activated osteoclasts, pit-formation, and F-actin rings. This result is in accordance with previous findings [28]. We further explored treatment effects and found that tanshinone IIA only inhibited osteoclastogenesis when RANKL and tanshinone IIA were administrated at nearly the same time. When RANKL-induced osteoclast differentiation was complete, tanshinone IIA treatment had very little effect. *In vivo*, tanshinone IIA significantly prevented bone loss after 6 weeks in OVX mice. In total, this evidence demonstrates that tanshinone IIA, a natural Chinese herb, has the potential to prevent bone loss in patients with POMP when administered at the early stages of osteoporosis.

RANKL binding to RANK on osteoclast precursor cells induces the expression of osteoclastogenesis-related markers, including TRAP, cathepsin K, TRAF6, MMP-9, CTR, and NFATc1. In addition, multiple downstream pathways such as the NF-κB, MAPK, and Akt pathways are activated by RANKL. NF-κB is a major transcription factor that plays an critical role in many diseases [29]. Normally, NF-κB proteins are kept in the cytoplasm in their inactive form, a p50/p65 protein heterodimer that associates with inhibitory IκBα [30]. Upon stimulation with RANKL, IκBα undergoes phosphorylation, unmasking a nuclear localization signal on p65 that allows NF-κB to translocate to the nucleus where it activates gene transcription. These activation targets include NFATc1, a well-known master regulator of osteoclastogenesis. In the present study, we found that p65, p50, and IκBα phosphorylation increased with RANKL-stimulation, which was inhibited by tanshinone IIA pretreatment. We also found that this inhibitory effect was dose-dependent. In addition, RANKL-treated RAW264.7 cells contained elevated levels of nuclear NF-κB p65, while tanshinone IIA treatment reduced the amount of nuclear NF-κB p65. Consistent with a previous study [31], these data confirmed that tanshinone IIA inhibited RANKL-stimulated expression of osteoclastogenesis-related markers in RAW264.7 cells partially through an NF-κB-dependent signaling pathway.

Phosphatidylinositol 3-kinase (PI3-kinase) and MAPKs have been implicated in osteoclast differentiation, at least in part through RANKL signaling [32]. Binding of RANKL to RANK recruits and triggers the activation of cytoplasmic TRAF6 that subsequently induces the activation of PI3-kinase/Akt and MAPKs pathways [33]. These pathways promote differentiation of osteoclast progenitors into mature multinucleated osteoclasts. In the present study, RANKL-stimulation increased the levels of phosphorylated ERK, JNK, c-fos, and Akt, which were then inhibited by tanshinone IIA treatment. Therefore, tanshinone IIA inhibited RANKL-induced activation of Akt and MAPKs, leading to decreased expression the of osteoclastogenesis-related markers TRAP, cathepsin K, TRAF6, MMP-9, CTR, and NFATc1.

Tanshinone IIA has been used clinically, particularly for cardiovascular disease and cancer therapies. However, although increasing evidence indicates the efficacy of tanshinone IIA in preventing bone loss during POMP in animals, its use for the human disease is still unresolved. Further clinical studies are necessary to demonstrate the efficacy and safety of tanshinone IIA treatment in patients with POMP.

In summary, the results of the present investigation demonstrate that tanshinone IIA significantly attenuated RANKL-induced osteoclastogenesis by suppressing the activation of the NF-κB, PI3-kinase/Akt, and MAPK pathways, as well as the transcription factor NFATc1. We found strong evidence supporting tanshinone IIA as a promising and effective therapy for preventing POMP.

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expert in our country. He works in Shanghai Changzheng Hospital. The amount of funding is three hundred thousand yuan. The experiments were performed at Second Military Medical University and Wenzhou Medical University.

Disclosure of conflict of interest

None.

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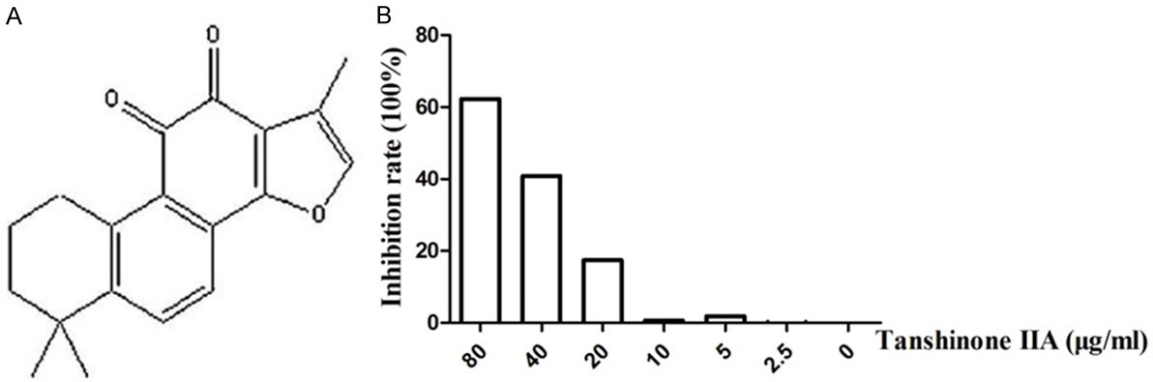

Figure S1. Cytotoxicity of tanshinone IIA *in vitro*. A. Chemical structure of matrine. B. RAW264.7 cells were cultured at a density of 10^4 cells/well in a 96-well plate for 24 h. Cells were then cultured with various concentrations of tanshinone IIA (0, 2.5, 5, 10, 20, 40, and 80 μg/ml) for 48 h. MTT solution was added and incubated for an additional 2 h. Absorbance was measured at 490 nm to measure cell viability.

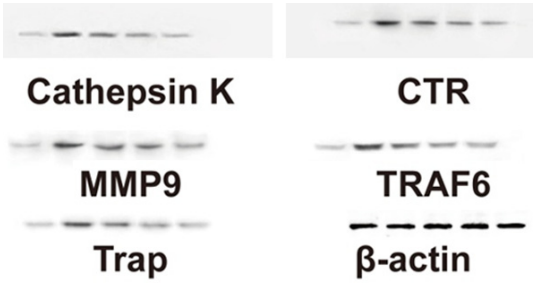

Figure S2. The picture is the original diagram of western blots in **Figure 2**.

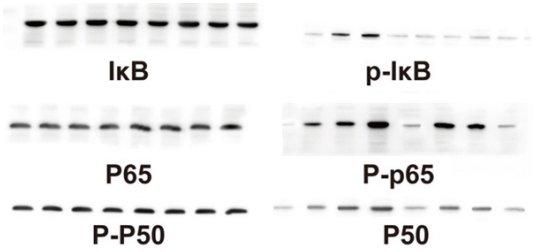

Figure S3. The picture is the original diagram of western blots in **Figure 3**.

Figure S4. The picture is the original diagram of western blots in **Figure 4**.