

# Beneficial effect of pristimerin against the development of osteoporosis in ovariectomy-induced osteoporosis rats by the RANKL/TRAF6/NF- $\kappa$ B pathway

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## Abstract

**Introduction:** Osteoporosis is a major cause of bone fracture in post-menopausal women. We evaluated the effects of pristimerin treatment on ovariectomy-induced osteoporosis, and its possible molecular mechanism.

**Material and methods:** Rats were ovariectomised and biochemical markers of bone formation were determined from serum samples. The microarchitectures of bone tissues were analyzed via micro-CT scans and Western blotting assays. The cytotoxic effects of pristimerin, the differentiation of osteoclasts, and bone reabsorption were evaluated *in vitro* using RAW 264.7 cells.

**Results:** Treatment with pristimerin attenuated changes in markers of bone formation and reabsorption such as creatine kinase (CK), alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), collagen type I fragments (CTX), bone Gla-protein (BGP), and osteocalcin (OC) in the serum of ovariectomised rats. It also appeared to restore the microarchitecture of bone tissue. The expression levels of TNF receptor-associated factor 6 (TRAF-6), nuclear factor  $\kappa$  light chain enhancer of activated B cells (NF- $\kappa$ B p65), and receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) protein were significantly lower, while those of Akt and PI3K were significantly higher, in the bone tissues of the pristimerin-treated group than in negative controls. Pristimerin had no cytotoxic effect on RAW 264.7 cells and reduced the differentiation of osteoclasts, bone reabsorption, and translocation of p65 in RANKL-stimulated RAW 264.7 cells *in vitro*.

**Conclusions:** Pristimerin reduces the effects of osteoporosis by restoring the altered RANKL/TRAF-6/NF- $\kappa$ B pathway in ovariectomised rats.

**Key words:** pristimerin, ovariectomy, RAW 264.7 cells, osteoporosis, osteoclast.

## Introduction

Osteoporosis is a common bone disorder affecting over 2 billion people throughout the world [1]. It is characterised by decreased bone density, increased bone fragility, and changes in bone structure [2]. Post-menopausal osteoporosis also results in an increased incidence of bone fractures [3]. In China, 40.1% of women over the age of 50 years suffer from osteoporosis [4]. Osteoporosis alters bone remodelling by decreasing osteoblast formation and increasing osteoclastic bone reabsorption [5]. In addition, multinucleated osteoclasts form due to the binding of haematopoiet-

ic monocytes to osteoclast precursors, activating the receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) signalling pathway [6]. This enhances the process of osteoclastogenesis [7]. Due to an interaction between RANKL and its receptor RANK, TNF receptor-associated factor 6 (TRAF6) is recruited and upregulates several osteoclast genes, such as DPD, cathepsin, and TRAP [8]. In addition, nuclear factor of activated T cell c1 (NFATc1) is induced by NF- $\kappa$ B and is reported to regulate the activation, fusion, and differentiation of RANKL-induced osteoclasts [9].

Traditional drugs used for the management of postmenopausal osteoporosis have several undesirable side effects including osteonecrosis [10]. During the last few decades, molecules from natural sources have shown beneficial results in the management of chronic disorders. Pristimerin is a triterpenoid isolated from plants in the family Celastraceae [11]. These plants are used in traditional Chinese medicine. Pristimerin has been used for the treatment of inflammatory and autoimmune disorders as well as tumours [12]. It also has the ability to inhibit inducible nitric oxide synthase (iNOS) and reduce NOx [13], which results in anti-inflammatory activity and a hepatoprotective effect [14]. In addition, it inhibits NF- $\kappa$ B and its resulting inflammatory cascade [15], and has a protective effect against arthritis by modulating the RANKL/OPG pathway and reducing inflammatory mediators [12]. We evaluated its protective effects against osteoporosis in ovariectomised rats.

## Material and methods

### Animals

Female Wistar rats were procured from Shanghai Medical College, Shanghai, China. All animals were stored under standard conditions with a 7-day acclimatisation period and free access to a standard chow diet and tap water. All protocols in this study were approved by the Institutional Animal Ethical Committee of Shanghai JiaoTong University, Shanghai, China (SJTU/IAEC/2017/07).

### Chemicals

Pristimerin was provided by Sigma Aldrich Ltd., USA, and alendronate was provided by Hangzhou Moshadong Pharmaceutical Co. Ltd., China. ELISA kits with collagen type I fragments, osteocalcin, cathepsin K, BGP, TNF- $\alpha$ , and IL-1 $\beta$  were purchased from Thermo Fisher Scientific Ltd., USA. Antibodies were procured from Bio-Techne China Ltd., China.

### Procedure

All animals were randomly separated into two different groups. Ovaries were removed from the

ovariectomy group after anaesthetisation with pentobarbital (40 mg/kg, i.p.). The sham group ( $n = 10$ ) underwent surgery without removing the ovaries. After 3 days of recovery, the ovariectomy group was further separated into five groups (each group  $n = 10$ ). Negative controls received saline solution, the standard treatment group received alendronate (2.5 mg/kg), and the three pristimerin groups received 50, 100, and 200 mg/kg over a 4-week period.

### Measurement of biochemical parameters

The plasma concentration of alkaline phosphatase (ALP) was estimated using a colorimetric assay kit. An ELISA kit was used to determine the serum concentration of collagen type I fragments (CTX) and osteocalcin (OC) protein. The levels of cathepsin K, BGP, TNF- $\alpha$ , and IL-1 $\beta$  in serum were estimated using their respective kits. The activity of tartrate-resistant acid phosphatase (TRAP) was estimated using a p-nitrophenyl sodium phosphate assay.

### Measurement of bone microarchitecture

Micro computed tomography (CT) analyses were performed as previously reported [16]. Scans were used to determine the microarchitecture of trabecular bone tissue from the distal right femur. ABA bone analysis software was used to estimate bone morphometric parameters at the growth plate such as trabecular thickness, number of trabeculae, trabecular separation, structure model index, ratio of trabeculae to total bone volume, and bone mineral density.

### Western blotting assay

Protein was extracted from the right tibia and the lysate was centrifuged at 12,000 RPM for 10 min to obtain a supernatant. A bicinchoninic acid quantification assay was used to estimate protein concentration. Proteins were separated via 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was further incubated with primary antibodies including Akt, PI3K, RANKL, NF- $\kappa$ B, p65, TRAF-6, and  $\beta$ -actin. Later, the membrane was washed and the chemiluminescent signals of the blots were imaged.

### Cell culture

RAW 264.7 cells were procured from the Cancer Research Center, Shanghai, China. Dulbecco's modified Eagle medium (DMEM) supplemented with streptomycin (100  $\mu$ g/ml), penicillin (100 U/ml), and FBS (10%) was used to culture the cell line in 5% CO<sub>2</sub> at 37°C.

### MTT assay

The viability of RAW264.7 cells was estimated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Cells ( $3 \times 10^4$  cells/well) were seeded in a 24-well plate and incubated with pristimerin (5, 10, 25, and 50  $\mu\text{M}$ ) overnight. The following day, the cells were incubated with MTT for 4 h. A FlexStation 3 Multi-Mode Microplate Reader was used to detect absorbance at a wavelength of 492 nm.

### Osteoclastogenesis assay

RAW 264.7 cells were seeded into a 24-well plate ( $1 \times 10^6$  cells/well) and cultured with RANKL (50 ng/ml) and pristimerin (5, 10, 25 and 50  $\mu\text{M}$ ). A TRAP staining kit was used to measure the formation of osteoclasts. TRAP-positive, mature osteoclasts were counted using a light microscope.

### Immunofluorescent staining

RAW 264.7 cells were seeded in a 24-well plate ( $1 \times 10^6$  cells/well) and incubated overnight. They were later treated with pristimerin (5, 10, 25, and 50  $\mu\text{M}$ ) for 2 h and further stimulated with RANKL (50 ng/ml) for 1 h. Thereafter, the cells were washed with PBS twice and treated with paraformaldehyde (4%) at room temperature for 15 min to fix the cells. Anti-p65 antibody (1 : 200) was incubated with the cells overnight at 4°C and then Alexa Fluor 488 phalloxin (1 : 500) was used at room temperature for 1 h to label the cells. Cells were washed with PBS and further stained with 4'-6-diamidino-2-phenylindole (DAPI) at room temperature for 3 min. Confocal microscopy was used to visualise the immunohistochemical changes.

### Reabsorption pit assay

RAW 264.7 cells were seeded in a 24-well plate (2000 cells/well) and incubated for 7 days with pristimerin (5, 10, 25, and 50  $\mu\text{M}$ ) and RANKL (50 ng/ml). The medium was changed every 3 days. The cells were treated overnight with ammonium hydroxide (1 M) to remove the attached cells, which then were placed on bone slices. The number of pits that form indicate the level bone reabsorption, which is related to the activity of osteoclasts. Image Pro-Plus software (v. 4.0) was used to count the reabsorption pits.

### Statistical analysis

All data are expressed as mean  $\pm$  SEM ( $n = 10$ ). The statistical analyses were performed using one way ANOVA and Dunnett's post hoc test (GraphPad Prism 6.1., CA, USA). The level of statistical significance was set at  $p < 0.05$ .

## Results

### Effect of pristimerin on bone formation

The effects of pristimerin on markers of bone formation such as CK, ALP, TRAP, CTX, BGP, and OC in the serum of ovariectomised rats are shown in Figure 1. There was a significant increase in the levels of CK, ALP, TRAP, CTX, and BGP and a significant decrease in the level of OC in the serum of negative controls compared to controls. Treatment with pristimerin significantly attenuated the altered level of these biochemical markers.

### Effect of pristimerin on cytokine production

The effects pristimerin on the level of inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  are shown in Figure 2. There was a significant decrease in the level of inflammatory cytokines in the pristimerin and standard treatment group compared to negative controls.

### Effect of pristimerin on bone microarchitecture

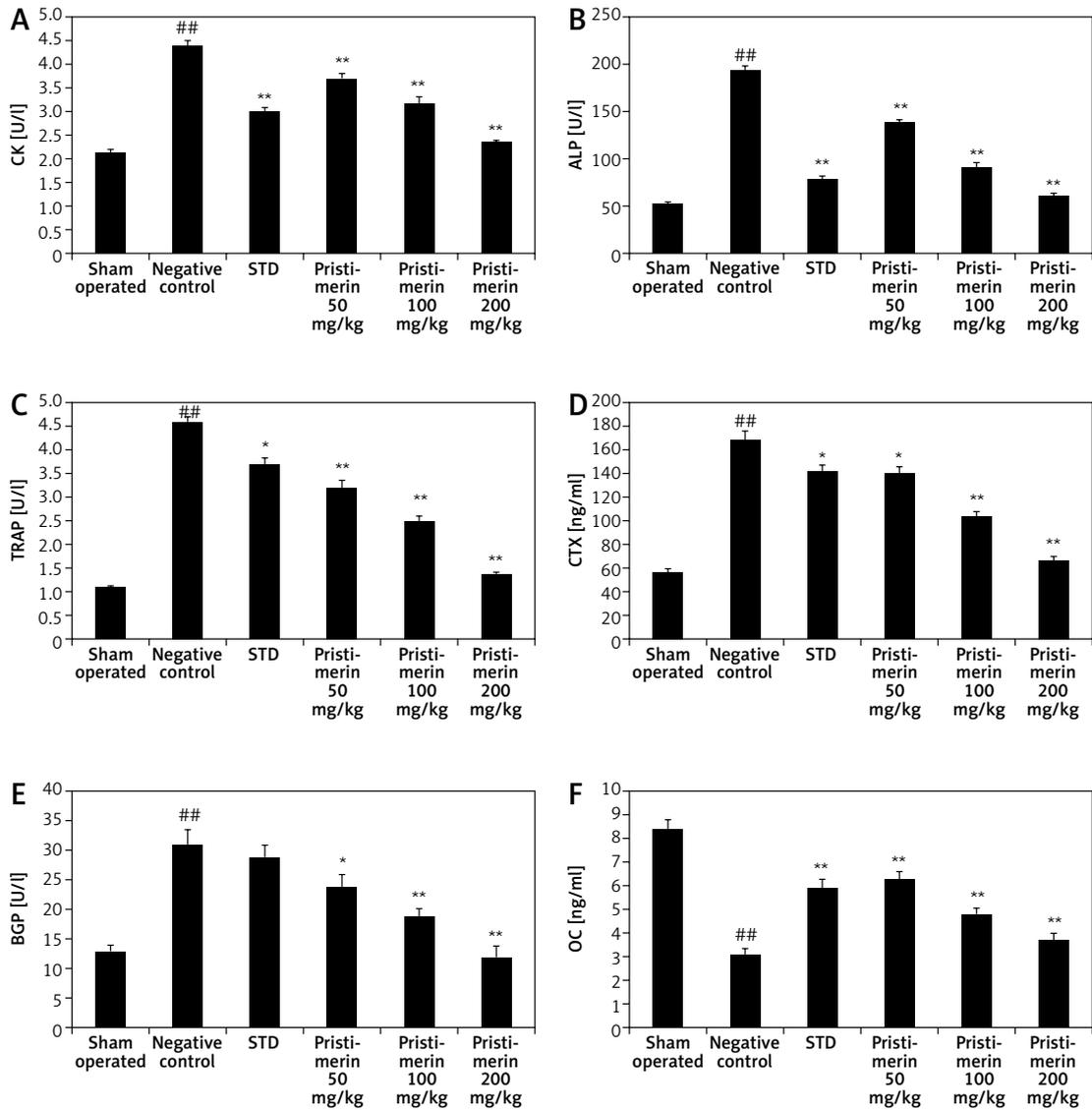
Figure 3 shows the effects of pristimerin on bone microarchitecture. There was a significant ( $p < 0.01$ ) decrease in BMD tubular number, tubular thickness, and BV/TV in the bone tissues of negative controls compared to controls. However, SIM and tubular separation in the bone tissue increased in negative controls compared to controls. Treatment with alendronate and pristimerin attenuated the changes in markers of bone microarchitecture such as BMD, Tb.N. Tb.Th, Tb.Sp, SIM, and BV/TV.

### Effect of pristimerin on the expressions of Akt, PI3K, RANKL, NF- $\kappa$ B p65, and TRAF-6

The effects of pristimerin on the expression levels of Akt, PI3K, RANKL, NF- $\kappa$ B p65, and TRAF-6 in bone tissue are shown in Figure 4. The expression levels of TRAF-6, NF- $\kappa$ B p65, and RANKL were significantly increased ( $p < 0.01$ ), and the expression levels of Akt and PI3K were significantly reduced, in negative controls compared to the sham group. Treatment with pristimerin significantly reversed both trends.

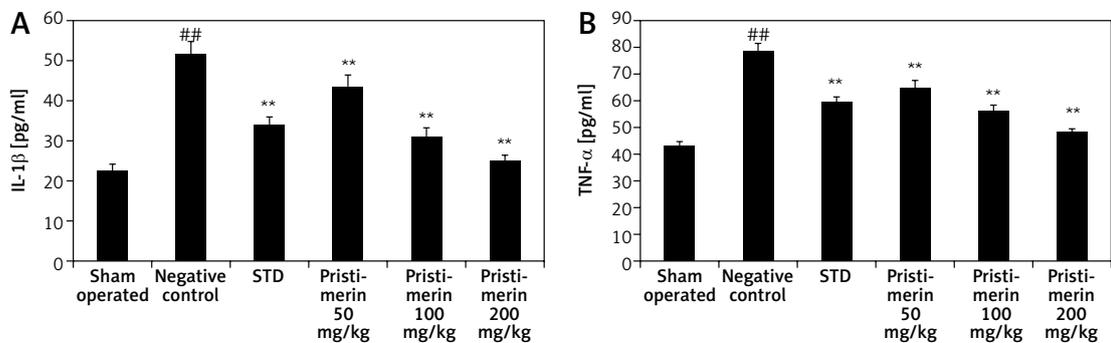
### Effect of pristimerin on viability of cells *in vitro*

Figure 5 shows the results of an MTT assay to assess the viability of RAW 264.7 in the presence of pristimerin. RAW 264.7 cells treated with pristimerin at 5, 10, 25, and 50  $\mu\text{M}$  exhibited 100.1%, 99.4%, 98.39% and 99.0%, viability, respectively. Thus the pristimerin groups show no increase in toxicity compared to the other groups.



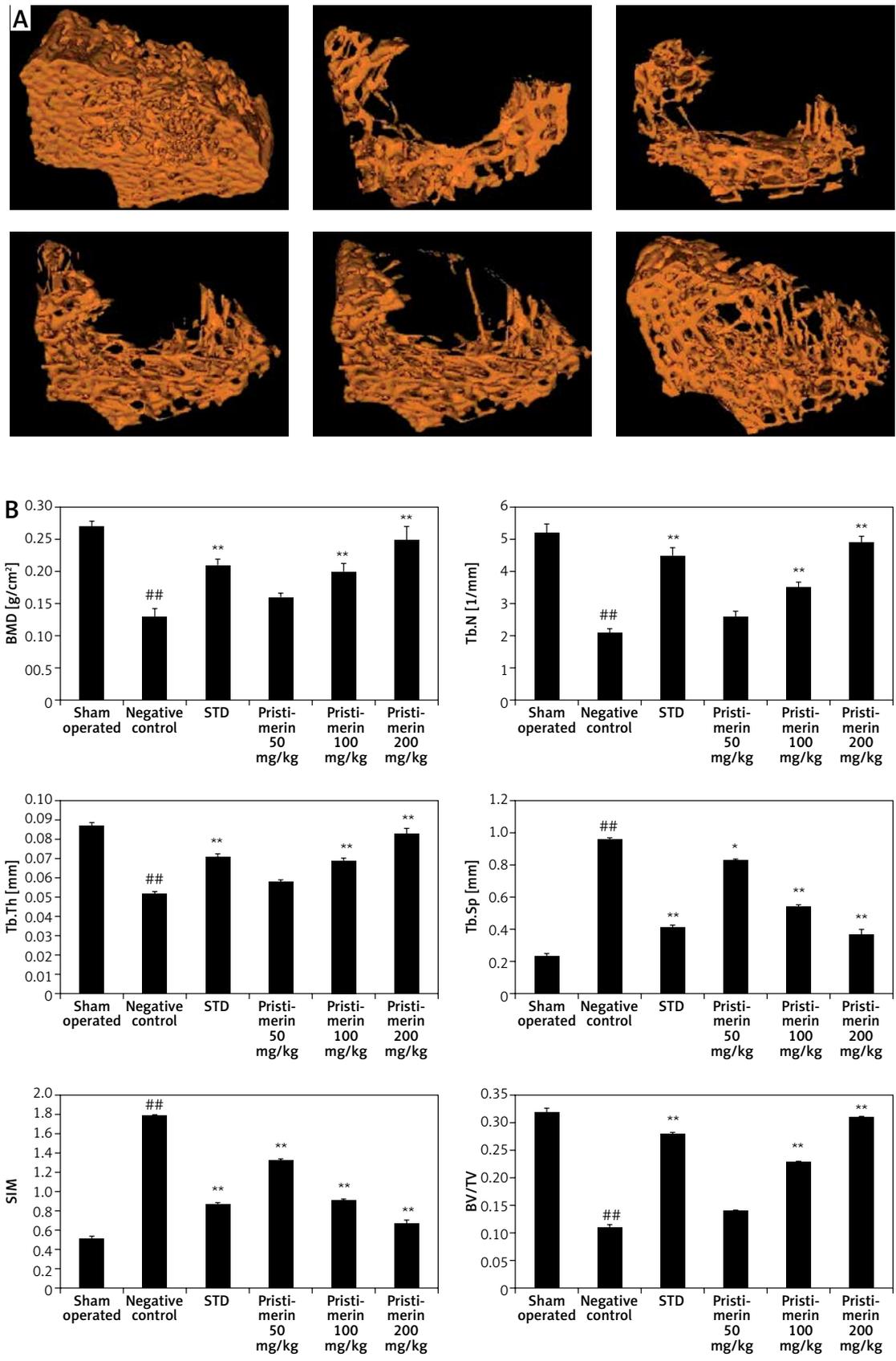
**Figure 1.** Effect of pristimerin on markers of bone formation (CK (A), ALP (B), TRAP (C), CTX (D), BGP (E), and OC (F)) in the serum of ovariectomised rats

Mean  $\pm$  SEM (n = 10), <sup>##</sup>p < 0.01 compared to sham group; \*p < 0.05, <sup>\*\*</sup>p < 0.01 compared to negative controls.



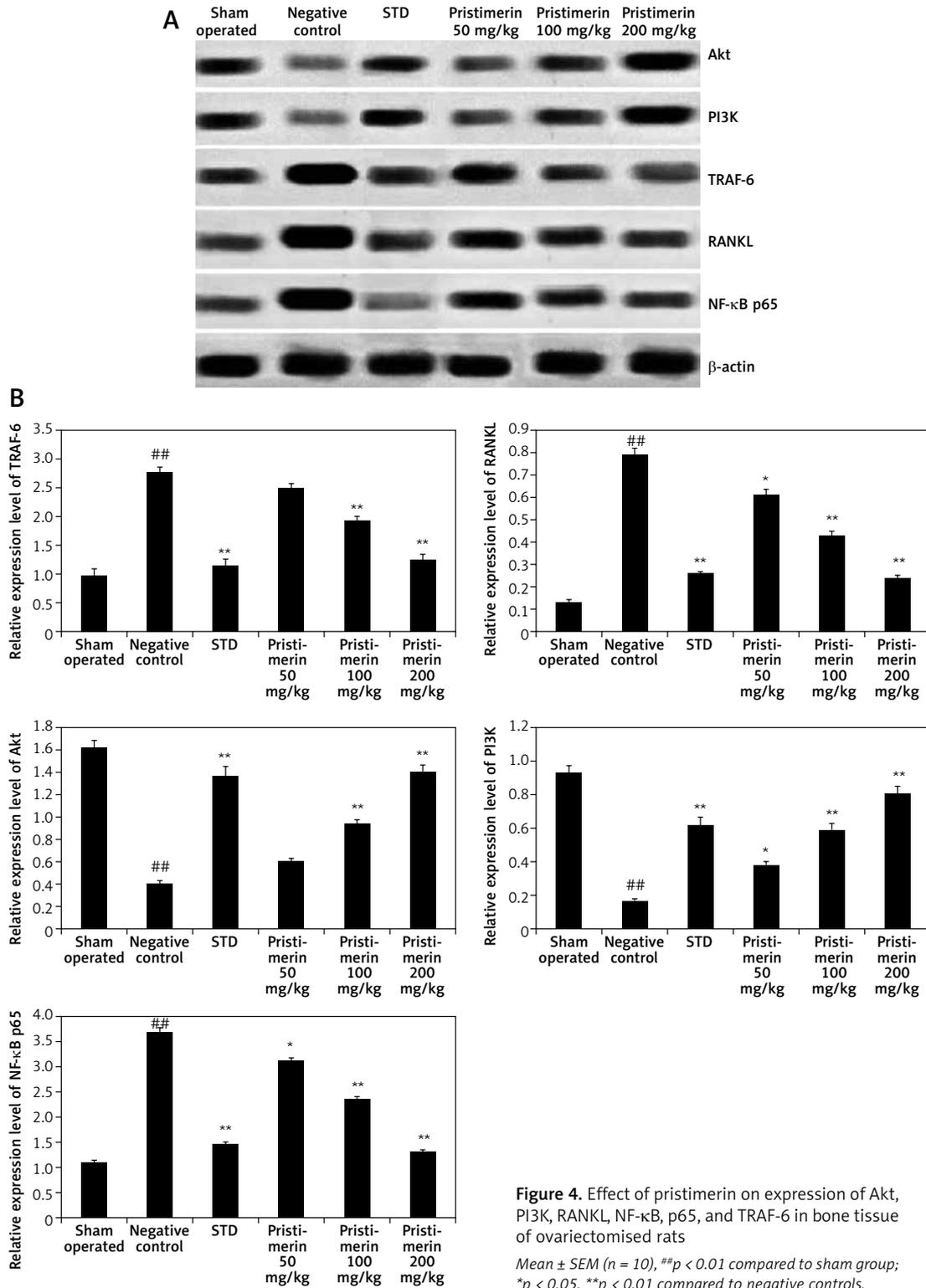
**Figure 2.** Effect of pristimerin on the level of inflammatory cytokines (A – IL-1 $\beta$ , B – TNF- $\alpha$ ) in the serum of ovariectomised rats

Mean  $\pm$  SEM (n = 10), <sup>##</sup>p < 0.01 compared to sham group; \*p < 0.05, <sup>\*\*</sup>p < 0.01 compared to negative controls.



**Figure 3.** Effect of pristimerin on bone microarchitecture in ovariectomised rats. **A** – Computed tomography of trabecula bone tissue. **B** – Microarchitecture of the femur

Mean ± SEM (n = 10), <sup>##</sup>p < 0.01 compared to sham group; <sup>\*</sup>p < 0.05, <sup>\*\*</sup>p < 0.01 compared to negative controls.



**Figure 4.** Effect of pristimerin on expression of Akt, PI3K, RANKL, NF- $\kappa$ B, p65, and TRAF-6 in bone tissue of ovariectomised rats

Mean  $\pm$  SEM (n = 10), <sup>##</sup>p < 0.01 compared to sham group; <sup>\*</sup>p < 0.05, <sup>\*\*</sup>p < 0.01 compared to negative controls.

#### Effect of pristimerin on differentiation of osteoclasts

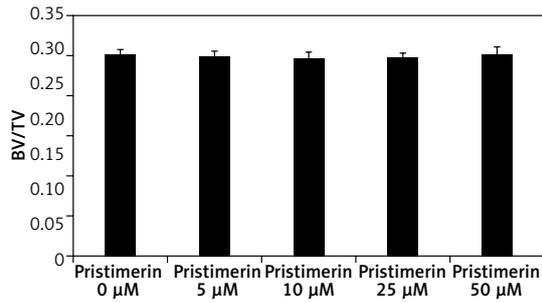
Figure 6 shows the effects of pristimerin on osteoclast differentiation. RANKL increased the formation of osteoclasts in RAW264.7 cells and treatment with pristimerin attenuated this effect.

#### Effect of pristimerin on localisation of p65

Stimulation with RANKL localised p65 to the nucleus and pristimerin blocked this effect (Figure 7).

#### Effect of pristimerin on bone reabsorption

Treatment with pristimerin significantly ( $p < 0.01$ ) reduced the amount of bone reabsorbed by

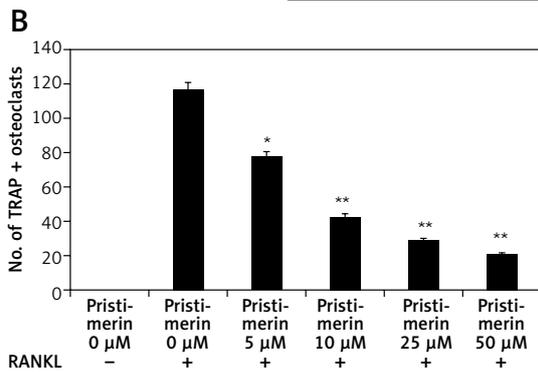
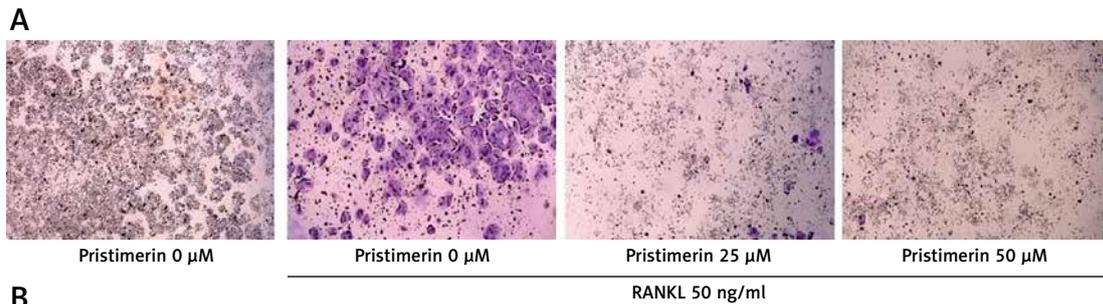


**Figure 5.** Effect of pristimerin on viability of RAW 264.7 cells  
*Mean ± SEM (n = 10).*

RANKL-stimulated RAW264.7 cells compared to the RANKL-only group (Figure 8).

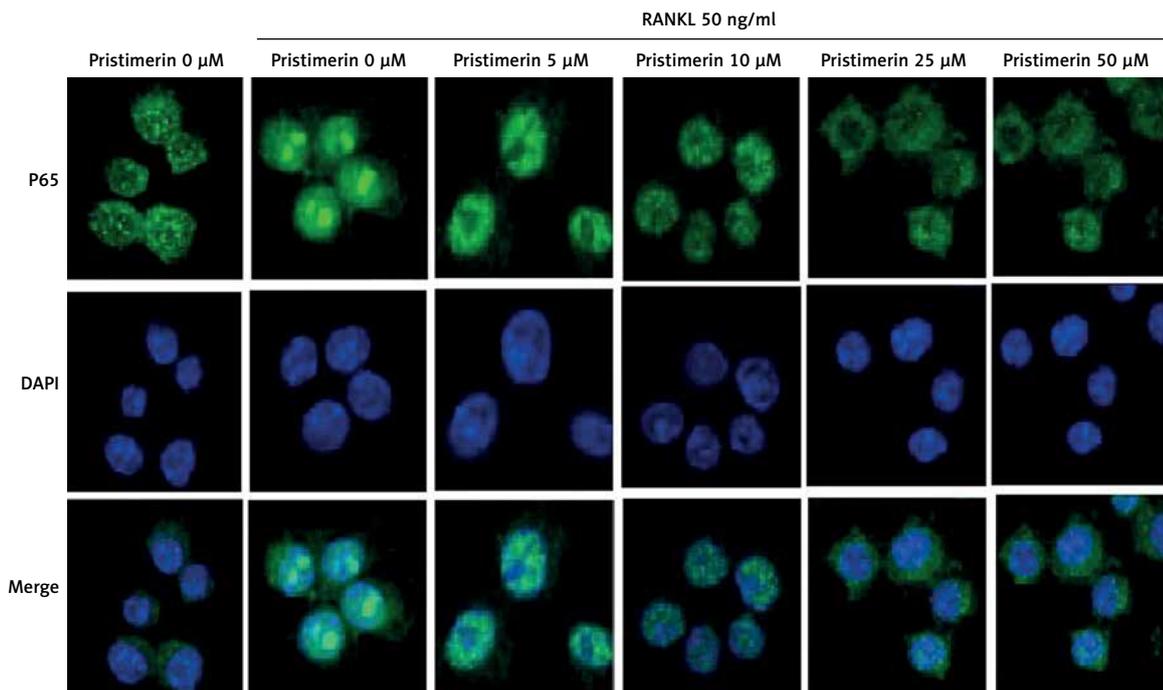
**Discussion**

Osteoporosis is the most common bone disorder affecting post-menopausal women. Current treatment options for osteoporosis have several limitations [2]. There are several markers of bone formation and reabsorption such as ALP, CTX, OC, BGP, and TRAP [17]. Alendronate is the first-line drug for treating osteoporosis and has been shown to restore normal levels of these markers [18]. Our results indicate that treatment with pris-

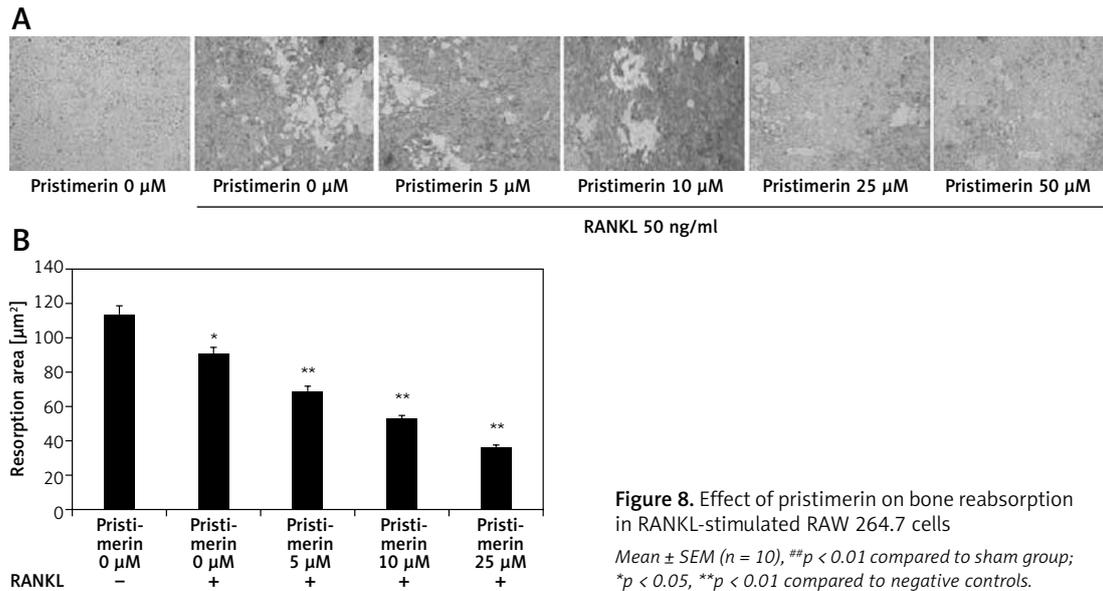


**Figure 6.** Effect of pristimerin on differentiation of osteoclasts. **A** – TRAP-positive multinucleated mature osteoclast cells under a light microscope. **B** – Number of TRAP-positive osteoclast cells

*Mean ± SEM (n = 10), \*\*p < 0.01 compared to sham group; \*p < 0.05, \*\*p < 0.01 compared to negative controls.*



**Figure 7.** Effect of pristimerin on localisation of p65 in RANKL-stimulated RAW 264.7 cells



**Figure 8.** Effect of pristimerin on bone reabsorption in RANKL-stimulated RAW 264.7 cells  
 Mean  $\pm$  SEM (n = 10), \*#p < 0.01 compared to sham group; \*p < 0.05, \*\*p < 0.01 compared to negative controls.

timerin has a similar outcome as treatment with alendronate. Menopause causes a deficiency in oestrogen, which is associated with an increase in the level of inflammatory cytokines [19–21]. Treatment with pristimerin significantly ( $p < 0.01$ ) reduced the level of inflammatory cytokines in oestrogen-deficient mice.

The integrity of the skeletal system is maintained by coordination of the activity between osteoclasts and osteoblasts [22]. RANKL stimulates osteoclastogenesis, increasing bone reabsorption [23]; membrane-bound protein p65, resulting in translocation to the nucleus [24]; and the PI3K and NF- $\kappa$ B pathway, enhancing osteoclastogenic activity [25]. Our data reveal that treatment with pristimerin restores the normal activity of PI3K, NF- $\kappa$ B, and p65 in RANKL-stimulated bone tissue of ovariectomised rats.

TRAF6 is a novel target for the management of osteoporosis [26]. Research has shown that TRAF-6-deficient transgenic mice suffer from osteoporosis and exhibit increased osteoclastogenesis [27]. RANKL is thought to downregulate TRAF-6. Our results suggest that pristimerin treatment restores the normal expression of TRAF-6 in ovariectomised rats.

To further confirm the effects of pristimerin on the RANKL signalling cascade, we performed *in vitro* analyses using RAW 264.7 cells. First, we evaluated the cytotoxic effects of pristimerin on RAW 264.7 cells and found that there were no negative effects on cell viability. Next, we examined TRAP, which is an enzyme released in bone tissue by osteoclastic activity. RANKL increased the number of TRAP-positive cells and treatment with pristimerin significantly reduced their number. In addition, treatment with pristimerin blocked RANKL-stimulated translocation of p65 to the nucleus and

reduced the number of pits in RAW 264.7 cells. This indicates that pristimerin affects the RANKL signalling cascade and bone reabsorption caused by the translocation of p65 in our *in vitro* model.

In conclusion, pristimerin effectively treats osteoporosis by reducing stimulation of the RANKL signalling pathway and restoring normal levels of TRAF-6 and NF- $\kappa$ B, thereby reducing osteoclastic activity in bone tissue.

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### Conflict of interest

The authors declare no conflict of interest.

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