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Article type : Research Article

Pharmacological evaluation of imidazole-derived bisphosphonates on RANKL-induced osteoclast differentiation and function

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Abstract: Bisphosphonates (BPs) have been commonly used in the treatment of osteolytic bone lesions, such as osteoporosis and osteogenesis imperfecta. However, serious side effects can occur

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cbdd.13767

during the therapy. To search for novel potent BPs with lower side effects, a series of imidazole-containing BPs (zoledronic acid (ZOL) ； ZOL derivatives by substitution of the hydrogen at the 2-position on the imidazole ring with a methyl (MIDP), ethyl (EIDP), n-propyl (PIDP) or n-butyl group (BIDP)) were developed and the effects on receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation were investigated using the murine macrophage RAW 264.7 cells at the protein, gene, morphological and functional levels. Influences of these BPs on the cell growth and proliferation of RAW 264.7 were also studied in order to determine cytotoxicity. The results showed that PIDP significantly inhibited the RANKL-induced osteoclast formation in a dose-dependent fashion without inducing cytotoxicity under the concentration of 12.5 μM. It exerted remarkable suppressive effects on the development of actin rings, the bone resorption and the expressions of osteoclastogenesis-related gene and protein markers. The down-regulation of c-Jun N-terminal kinase (JNK), Protein kinase B (Akt) and inhibitor of nuclear factor kappa-B (IκB) phosphorylation in the early signaling event and subsequent inhibition of the expression of c-Fos and nuclear factor of activated T-cells (NFATc1) might be involved in these effects. All these results indicated that PIDP might be a promising drug to treat bone-related disorders.

Keywords: Pharmacology, bisphosphonate, RANKL, osteoclastogenesis, mechanism.

INTRODUCTION

Human bone homeostasis relies on a subtle balance between bone formation and resorption regulated by osteoblasts and osteoclasts, respectively(Boyle et al., 2003). An inappropriate osteoclast activity can result in bone disorders, such as osteopetrosis or excessive bone resorption. The former is caused by the shortage of osteoclast activity, while the latter is the result of an increased osteoclast activity leading to bone loss followed by several skeletal disorders, such as osteoporosis, Paget's disease, hypercalcemia, rheumatoid arthritis, osteoarthritis and lytic bone metastases (Gough et al., 1998; Knowles, 2017; Krzeszinski and Wan, 2015). Thereby, it is crucial to prevent a change in osteoclast activity to maintain bone homeostasis.

Osteoclasts are originated from the monocyte/macrophage lineage of hematopoietic stem

cells(Boyle et al., 2003). After differentiation into multinucleated giant cells, osteoclasts adhere to the bone surface and polarize to form a ruffled border and an actin-rich sealing zone with an isolated resorptive microenvironment. Then, osteoclasts are involved in bone resorption activity by excreting various acids and enzymes(Teitelbaum, 2011). Receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) belongs to the super-family of tumor necrosis factor (TNF), and it is considered as a key cytokine for the regulation of the differentiation and function of human osteoclasts. Binding of RANKL to RANK receptor on osteoclast precursor cells leads to the aggregation of TNF receptor-associated factors 6 (TRAF6), followed by the activation of mitogen-activated protein kinase (MAPK) and transcription factors including NF-κB, c-Fos and nuclear factor of activated T-cells (NFATc1). As a key regulator of osteoclastogenesis, NFATc1 regulates the expression of various genes related with osteoclast including matrix metallopeptidase-9 (MMP-9), tartrate-resistant acid phosphatase (TRAP), cathepsin K and integrin-β3(Fujisaki et al., 2007; Kim and Kim, 2014; Negishi-Koga and Takayanagi, 2009).

Bisphosphonates (BPs) are synthetic analogues of pyrophosphate with a P-C-P backbone. Osteophilic accumulation is observed with BPs, and they possess inhibitory effect on osteoclastic bone resorption(Widler et al., 2002). The third generation BPs, represented by zoledronic acid (ZOL), hold higher anti-resorptive capacity than the previous BPs drugs. They have already been commonly applied to treat osteoporosis, osteogenesis imperfecta, osteitis deformans, tumor-induced osteolytic metastases and other bone fragility related diseases(Baykan et al., 2014; Polascik, 2009; Ruza et al., 2013; Xu et al., 2017). However, ZOL also induces serious side-effects. One of the main adverse effect of ZOL is osteonecrosis of the jaw (ONJ)(Kang et al., 2013; Lee and Suzuki, 2015). In addition, ZOL may also cause bilateral retrobulbar optic neuropathy(Lavado et al., 2017). Therefore, novel BPs drugs with a better pharmacological profile are needed. Although the pathophysiology of ZOL-induced adverse effects, such as ONJ, is still unclear, some studies showed that macrophages play a critical role during the development of ONJ. Reduced number and function of monocytes and macrophages caused by repeated administration of BPs could induce local immune dysfunction, infection and necrosis involved in ONJ(Pazianas, 2011). In addition, as farnesyl pyrophosphate synthase (FPPS) inhibitor, nitrogen-containing BPs

especially ZOL possess worse effects on cell biology than non-nitrogen containing BPs by blocking the mevalonate pathway (Ziebart et al., 2016). Considering that the macrophages RAW 264.7 can differentiate into multinucleated osteoclasts after RANKL stimulation, they are used as model of osteoclast precursors(Odkhuu et al., 2012). Recently, we prepared a series of ZOL derivatives by substitution of the hydrogen at the 2-position on the imidazole ring with a methyl (MIDP), ethyl (EIDP), n-propyl (PIDP) or n-butyl group (BIDP)(Chen et al., 2009; Luo et al., 2005; Niu et al., 2008; Wang et al., 2010) (Scheme 1), and the effects of these imidazole-containing BPs on the proliferation of RAW 264.7 cells and the RANKL-stimulated osteoclastogenesis in RAW 264.7 cells were examined. Due to the relatively low toxicity on osteoclast precursors and high anti-osteoclastogenesis ability, PIDP was selected for further investigations. The effect of PIDP on bone resorption activity, signaling pathways and osteoclastic markers involved in differentiation of osteoclast in RAW 264.7 cells were studied. Our results demonstrated that PIDP might be a promising drug for preventing osteolytic diseases.

MATERIAL AND METHODS

1. Reagents and antibodies

ZOL, MIDP, EIDP, PIDP and BIDP were prepared in our lab with the purity of over 98 % according to our reported methods(Chen et al., 2009; Luo et al., 2005; Niu et al., 2008; Wang et al., 2010). They were dissolved in H_2O to yield 10 mM solutions and stored at -20 °C as the stock solution. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics and polyvinylidene fluoride (PVDF) membrane were obtained from Life Technologies (Grand Island, NY, USA). Soluble recombinant RANKL was purchased from R&D (Lorton, VA, USA). Recombinant mouse FPPS protein was purchased from Sino Biological Inc. (Beijing, P. R. China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isopentenyl pyrophosphate (IPP), geranyl pyrophosphate (GPP), pyrophosphatase, malachite green and phalloidin-fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (St Louis, MO, USA). Tartrate-resistant acid phosphatase (TRAP) activity assay kit, bicinchoninic acid (BCA) protein assay kit, Beyozol, Hoechst 33258, anti-TRAF6 antibody and radioimmunoprecipitation assay

(RIPA) lysis buffer were from Beyotime Institute of Biotechnology (Jiangsu, China). TRAP & ALP (alkaline phosphatase) double-stain kit, Taq polymerase and SYBR-Green were obtained from TaKaRa biotechnology Co. Ltd (Dalian, China). High Capacity cDNA Reverse Transcription Kit was obtained from Applied biosystems (Carlsbad, CA, USA). PCR primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Anti-c-Fos antibody was purchased from Abcam. Western blot antibodies for phospho-p38 mitogen-activated protein kinase (phospho-p38 MAPK), p38 mitogen-activated protein kinase (p38 MAPK), phospho-c-Jun N-terminal kinase (phospho-JNK), c-Jun N-terminal kinase (JNK), phospho-extracellular signal-related kinase (phospho-ERK), extracellular signal-related kinase (ERK), phospho-protein kinase B (phospho-Akt), protein kinase B (Akt) and NF-kappa-B inhibitor alpha (IκB-α) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-NFATc1, phospho-IκB-α, β-actin antibodies, horse radish peroxidase (HRP)-conjugated secondary antibodies and western blot luminol reagent were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Protease and phosphatase inhibitors were obtained from Thermo Fisher Scientific Inc. (Waltham, MA USA). The murine macrophage RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, USA).

2. Cell culture

The cells were cultured in DMEM containing 10 % FBS, 100 mg/mL streptomycin, 100 unit/mL penicillin at 37 °C in a humidified 5 % CO_2 atmosphere.

3. Cell viability assay

Cell viability was determined by the traditional MTT method. RAW 264.7 cells were placed in 96-well plates with a density of 1×10^3 cells/well. After incubation for overnight, the medium was removed and replaced by the indicated concentration of ZOL, MIDP, EIDP, PIDP and BIDP, respectively. The medium was added to the control and blank wells. After 5 days, 20 μL of MTT solution (5 mg/mL) was added and the cells were cultured for another 4 h. The supernatants were then discarded, and cells were dissolved in 150 μL dimethylsulfoxide (DMSO). The optical density (OD) of each well was measured at 490 nm by SpetraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as a percentage of the

control.

4. TRAP staining and activity assay

RAW 264.7 cells (1×10^3 cells/well) were placed in a 96-well plate and further treated for 5 days with different concentrations of ZOL, MIDP, EIDP, PIDP or BIDP in the absence or presence of RANKL (50 ng/mL). TRAP staining assay was used to identify osteoclasts by using TRAP & ALP double-stain kit according to the instructions. TRAP-positive multinucleated cells with 3 or more nuclei were counted as osteoclasts and captured using a microscope.

To measure the suppressive effect of BPs on TRAP activity, RAW 264.7 cells (1×10^4 cells/well) were seeded into a 24-well plate and incubated with different concentrations of PIDP for 5 days in the absence and presence of RANKL (50 ng/mL). TRAP activity was then measured using the TRAP activity assay kit according to the kit instructions and calculated as percent of the control.

5. Actin rings and nucleus staining

RAW 264.7 cells were seeded at 1×10^4 cells/well with complete DMEM medium in 24-well plates. After 24 h, cells were treated with RANKL (50 ng/mL). The medium was changed every 2 days. On the fourth day of differentiation, mature osteoclasts were obtained from RAW 264.7 cells and further incubated with PIDP for 24 h. The cells were then washed twice with phosphate-buffered saline (PBS), fixed with 3.7 % formalin for 10 min, and washed twice again with PBS. Actin rings were stained by using 5 μg/mL phalloidin-FITC for 60 min at 37 °C away from light. The cells were washed twice again with PBS and nuclei were stained with 10 μg/mL Hoechst 33258 for 10 min. Fluorescent staining was examined using the fluorescence microscope (IX51; Olympus).

6. Bone resorption pit assay

RAW 264.7 cells (5×10^3 cells/well) were seeded in Corning Osteo Assay Stripwell plate and incubated overnight. Then different concentrations of PIDP with or without RANKL (50 ng/mL) were added to the medium. The cells were cultured in a humidified atmosphere of 5 % CO_2. The medium was changed every 2 days. Seven days later, cells were washed with 10 % bleach solution. Resorption pits were clearly visualized using a light microscope.

7. FPPS inhibition assay

The inhibitory effect of ZOL and the derivatives on FPPS activity was evaluated using previously described method with appropriate adjustments (Liu et al., 2020). Briefly, the assay was carried out in 96-well plate with 100 μL reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 5 mM GPP, 5 mM IPP, 0.1 U/mL pyrophosphatase and 1.5 μg FPPS in each well. After FPPS and 0.5 μM BPs was pre-incubated in the assay buffer at room temperature for 10 min, GPP and IPP were added. 10 min later, the reaction was terminated by adding 25 μL malachite green work solution containing 10 parts 0.1% malachite green in 3 N H_2SO_4, 2.5 parts 7.5% ammonium molybdate, and 0.2 parts 11% Tween 20. The plate was shaken for 20 min on a plate shaker. The absorbance was measured at 630 nm using SpetraMax M5 microplate reader. Reaction solution without BPs was used as negative control. Reaction solution without FPPS and BPs was set as blank control.

8. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RAW 264.7 cells (4×10^4 cells/well) were seeded in a 6-well plate with complete DMEM medium. After 24 h, different concentrations of PIDP with or without RANKL (50 ng/mL) were added into the medium. Then the cells were cultured at 37 °C in a humidified 5 % CO_2 atmosphere for 5 days and the medium was changed every 2 days. Beyozol was used to extract total RNA according to the manufacturer's instructions. The cDNA was generated from 1.5 μg total RNA by using high capacity cDNA reverse transcription kit according to the instructions. The primers for indicated genes and GAPDH were designed using Primer Premier software and shown in Table S1. The PCR programs were as follows: 25-35 cycles of 1 min at 94 °C (denaturation), 1 min at 54-59 °C (annealing) and a 1 min at 72 °C (extension). The PCR products were analyzed through a 1 % agarose gel, stained with SYBR-Green and analyzed using the Image J software (Scion Corp., Frederick, MD USA).

9. Western blot analysis

RAW 264.7 cells (4×10^4 cells/well) were seeded with complete DMEM medium in a 6-well plate and cultured overnight. Then the cells were treated with different substances. The treated cells were rapidly washed with ice-cold PBS and lysed with RIPA lysis buffer containing protease

and phosphatase inhibitors according to the manufacturer's instructions. The densitometric of the bands were analyzed by using the Image J software.

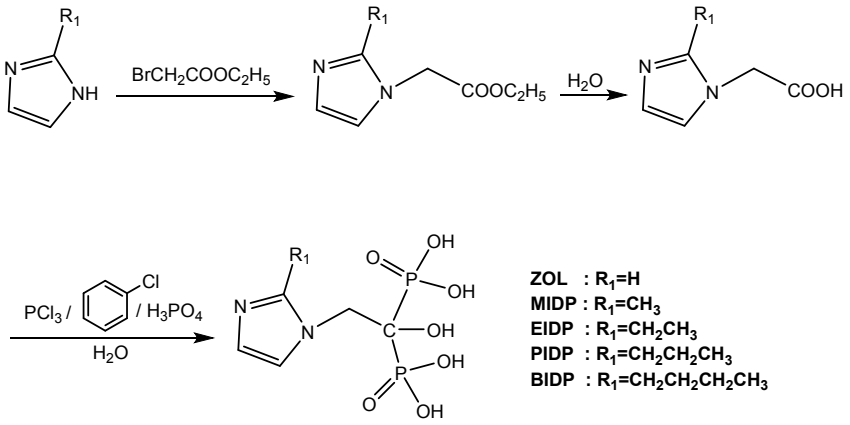
10. Statistical analysis

Data from at least three independent experiments were expressed as mean ± standard deviation (SD). Statistical analysis was performed by GraphPad Prism software (version 5; GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance was identified by the ANOVA test. $p < 0.05$ was regarded to be statistically significant.

RESULTS

1. Syntheses and toxicity of imidazole-containing BPs

All the BPs (ZOL and its derivatives MIDP, EIDP, PIDP and BIDP) were prepared with yield over 35 % according to the strategy described as following (see Scheme 1). The specific synthesis methods and the characteristics of BPs can be checked in supporting material (Fig. S1-10). Cytotoxic effects of ZOL, one representative of the BPs clinical drugs, and its corresponding derivatives (MIDP, EIDP, PIDP and BIDP) on RAW 264.7 cells were tested by MTT assay. Toxicity of these imidazole-containing BPs on cell growth was found to be concentration-dependent (Fig. 1). Among them, ZOL demonstrated the strongest toxicity on RAW 264.7 cells (Fig. S11). It is noteworthy that ZOL, MIDP, EIDP, PIDP and BIDP are nontoxic towards RAW 264.7 cells at concentrations below 3.12, 6.25, 12.5 and 50 μM, respectively. Therefore, a range of concentrations not exceeding these thresholds has been used to estimate the inhibitory effect of these BPs on osteoclasts formation. Thus, we could warrant that the inhibition on osteoclastogenesis could be observed independently from the cytotoxicity on the osteoclast precursors.



Scheme 1. Synthesis strategy of BPs

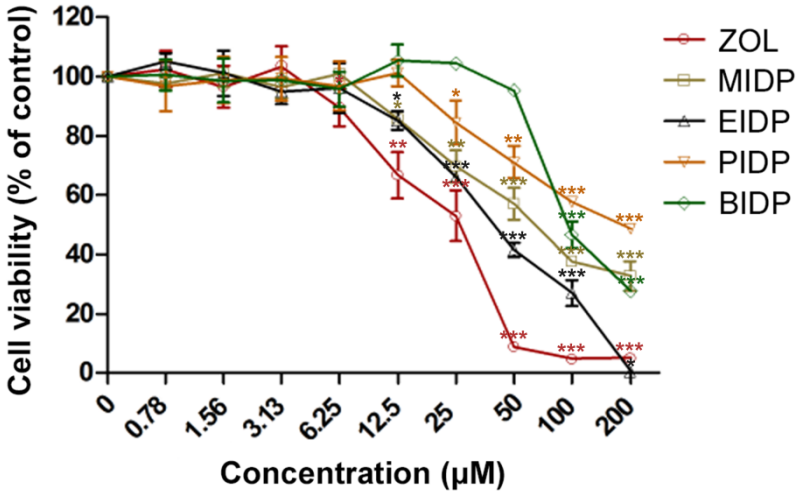


Fig. 1. Inhibition study of BPs on RAW 264.7 cells. Data from four groups of independent experiment were expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the respective control.

2. Effects of BPs on osteoclastogenesis

TRAP is a phenotype marker of osteoclasts, due to its high expression in osteoclasts. To examine the suppressive effects of ZOL, MIDP, EIDP, PIDP and BIDP on RANKL-induced osteoclastogenesis, RANKL-treated RAW 264.7 cells were incubated with different concentrations of these imidazole-containing BPs and then the formation of osteoclasts was determined using TRAP staining assay. As shown in Fig. 2a-c, ZOL, MIDP and EIDP had no

obvious suppression effect on RANKL-stimulated osteoclastogenesis at all tested concentrations. However, PIDP and BIDP showed a concentration dependent inhibition of RANKL-induced osteoclastogenesis. PIDP significantly inhibited osteoclastogenesis at a relatively low concentration compared to BIDP (1.56 μM vs. 6.25 μM) (Fig. 2d-e). PIDP also showed a higher inhibitory effect than BIDP at the same concentrations of 6.25 μM and 12.5 μM (Fig. 2f). These results indicated that PIDP possessed relatively high anti-osteoclastogenesis ability. Accordingly, PIDP was chosen for further study in the following experiments.

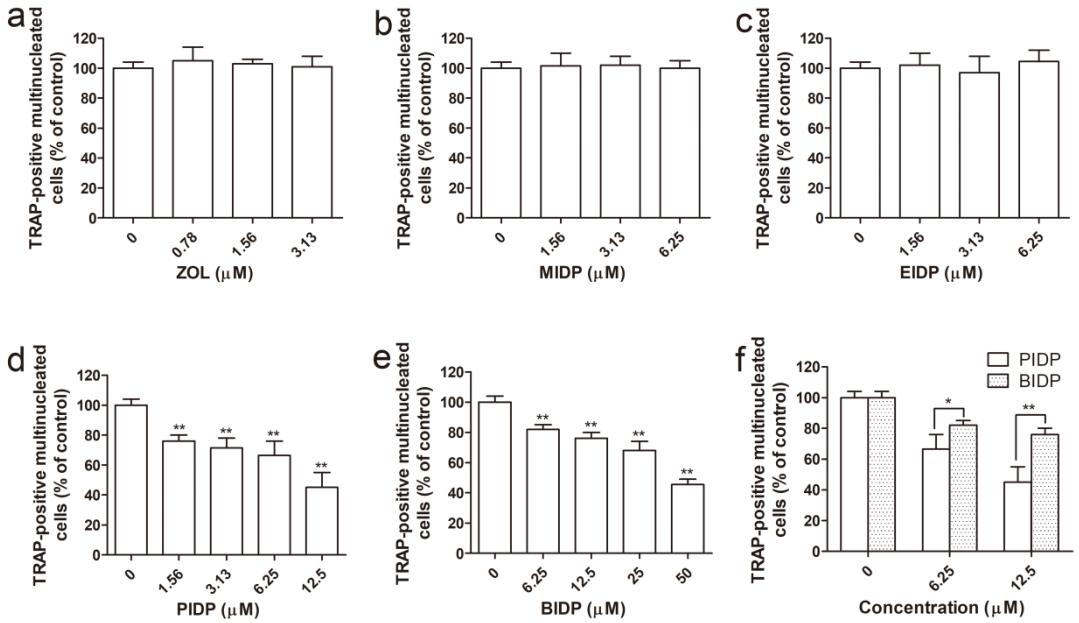


Fig. 2. Effects of ZOL, MIDP, EIDP, PIDP and BIDP on RANKL-induced osteoclastogenesis. (a-e) TRAP-positive multinucleated cells were counted and the results of independent experiments (n = 3) were expressed as mean ± SD. ** $p < 0.01$, compared with the respective control. (f) Comparison of PIDP and BIDP on RANKL-stimulated osteoclastogenesis. * $p < 0.05$, ** $p < 0.01$.

To further examine the inhibition of the formation of RANKL-stimulated osteoclast by PIDP, RANKL-treated RAW 264.7 cells were incubated with varying concentrations of PIDP. Concentrations which could not induce cytotoxicity were chosen in the study, and TRAP staining assay was performed. As clearly exemplified on Fig. 3a, only RAW 264.7 cells cultured with RANKL generated TRAP-positive multinucleated cells (nuclei > 3). However, we observed that

addition of PIDP reduced the number of RANKL-stimulated osteoclasts and inhibited TRAP activity (Fig. 3a-b).

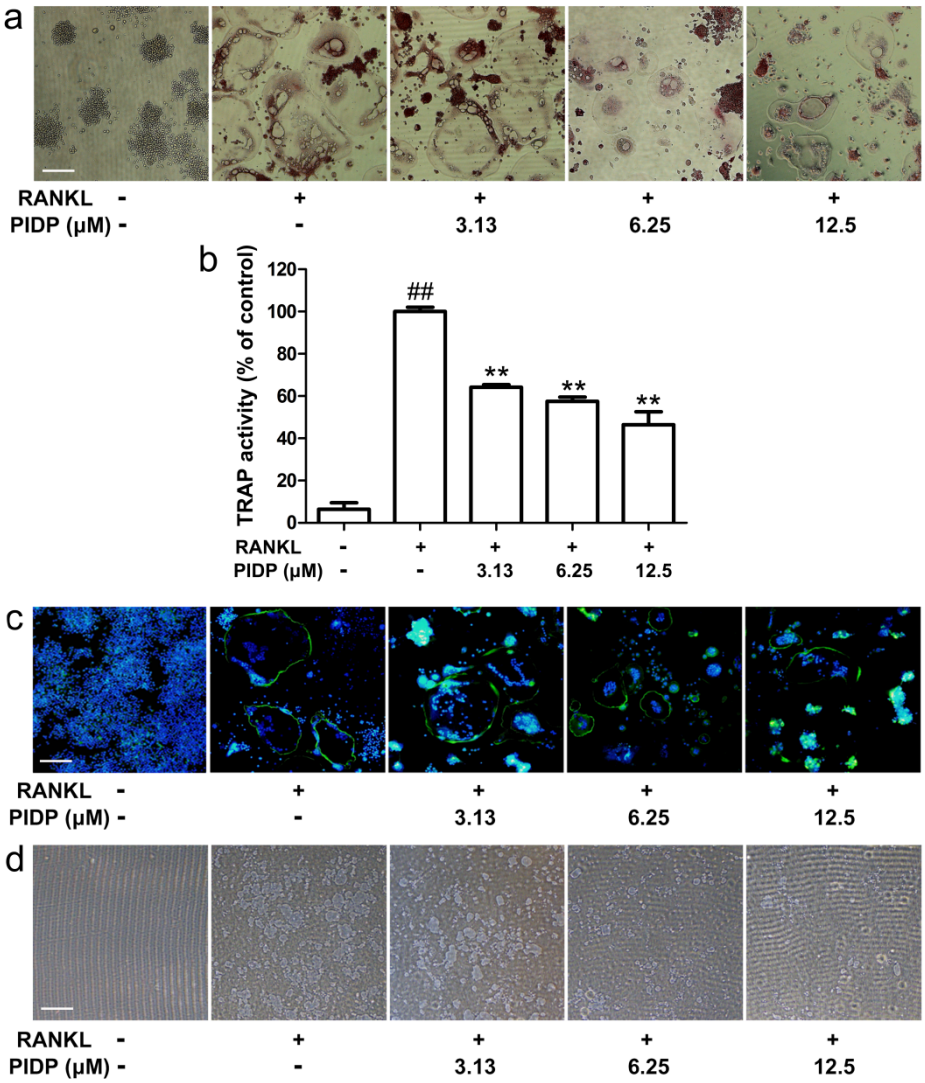


Fig. 3. Investigation of PIDP on RANKL-stimulated osteoclast formation and bone resorption. (a) RAW 264.7 cells were cultured with different concentrations of PIDP with or without 50 ng/mL RANKL. Bar = 80 μm. (b) TRAP activity of RANKL-stimulated osteoclast. ## $p < 0.01$, in comparison with untreated cells. ** $p < 0.01$, in comparison with cells only treated by 50 ng/mL RANKL. (c) Actin filament staining of mature osteoclasts after incubation with varying concentrations of PIDP for 24 h. Bar = 80 μm. (d) Morphology of RAW 264.7 cells cultured for 7 days with different concentrations of PIDP without or with 50 ng/mL RANKL. Bar = 200 μm.

3. Effects of BPs on RANKL-induced bone resorption

By adhesion to the bone surface, the membrane of the multinucleated osteoclasts becomes polarized and an isolated resorptive area surrounded by a tight actin-rich sealing zone is formed. Actin rings are therefore considered as a marker of polarized osteoclasts. When mature osteoclasts were incubated with phalloidin-FITC, actin rings with smooth peripheries were observed. However, addition of PIDP disrupted the actin rings of the mature osteoclasts in a dose-dependent manner, leading to morphological changes, such as irregular or even incomplete cell periphery and a contracted cytoplasm (Fig. 3c).

Osteoclasts are known to excrete hydrogen ions and lytic enzymes to the lacuna, and then the bone matrix is gradually dissolved(Jurdic et al., 2006; Soysa and Alles, 2016). Our results demonstrated that both area and density of bone resorption pits were remarkably reduced by the treatment of PIDP (Fig. 3d).

4. Effect of BPs on the activity of FPPS

Inhibition of FPPS is an important mechanism for the function of the third generation of BPs. So, the inhibitory effect of the BPs on the activity of FPPS was studied. As shown in Fig. 4, the inhibition rate of 0.5 μM ZOL to the enzyme activity of 1.5 μg FPPS was about 87.8%, while the inhibition rates of 0.5 μM MIDP, EIDP, PIDP or BIDP to 1.5 μg FPPS were 47.5%, 39.1%, 36.1% and 26.8%, respectively. The inhibition rates of MIDP, EIDP, PIDP or BIDP were all significantly lower than that of ZOL.

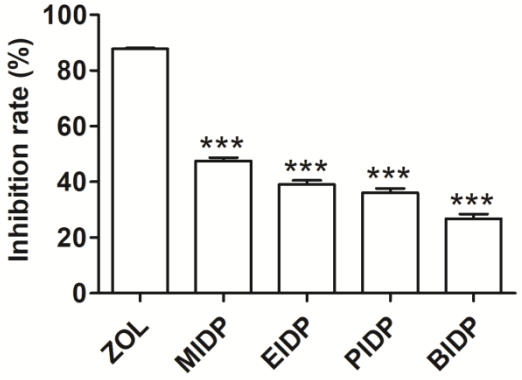


Fig. 4. Effect of BPs on the activity of FPPS. 0.5 μM BPs and 1.5 μg FPPS were pre-incubated for 10 min respectively. The inhibition of the BPs on the activity of FPPS was assayed. *** $p < 0.001$, compared with the ZOL group.

5. Effect of PIDP on RANKL-induced signaling pathways

Distinct RANKL-induced signaling pathways were investigated by western blotting assay after treatment with PIDP. PIDP inhibited the phosphorylation of JNK and strongly reduced the phosphorylation of Akt and IκB (Fig. 5). However, no obvious influence on p38 and ERK activation was observed after treatment of the RAW 264.7 cells with PIDP.

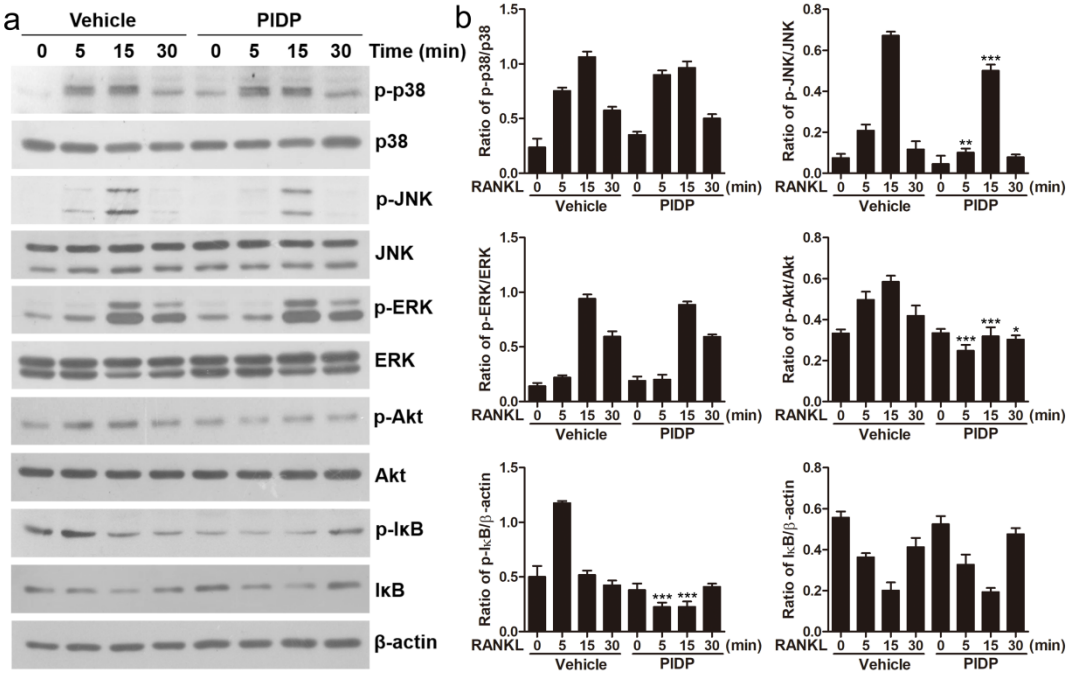


Fig. 5. Effect of PIDP on signal pathways induced by RANKL. (a) RAW 264.7 cells were pretreated without or with 12.5 μM PIDP for 1 h prior to 50 ng/mL RANKL stimulation for a period of time. (b) Statistical analysis of the western blot results. Bar graphs showed the relative ratios of p-p38/p38, p-ERK/ERK, p-JNK/JNK, p-IκB/β-actin, p-Akt/Akt and IκB/β-actin. Data from three groups of independent experiment were expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the respective vehicles.

6. Effect of PIDP on RANKL-induced expressions of TRAF6, NFATc1 and c-Fos

The aggregation of TRAF6 is an early event induced by RANKL/RANK interaction. We determined the effect of PIDP on RANKL-induced expression of TRAF6. As shown in Fig. 6a and c, the expression level of TRAF6 was up-regulated upon exposure to RANKL, but suppressed by PIDP, in a dose-dependent manner.

Transcription factor NFATc1 is involved in osteoclast differentiation and activation induced by RANKL. To further investigate the function of PIDP in osteoclast differentiation, we determined mRNA and protein expression of NFATc1 in RANKL-stimulated RAW 264.7 cells with or without PIDP. The genes related to NFATc1 were also investigated, including MMP-9, cathepsin K, RANK, integrin β3, and TRPV5. mRNA levels of MMP-9, cathepsin K, NFATc1, RANK integrin β3 and TRPV5 were suppressed by PIDP during osteoclastogenesis in a dose-dependent manner (Fig. 6b and d). The expression of NFATc1 protein was up-regulated upon exposure to RANKL, but inhibited by additional treatment with PIDP (Fig. 6a and c). c-Fos is another key transcription factor which is contributing in RANKL-stimulated osteoclastogenesis. Our experiments showed that PIDP exerted inhibitory effects on the mRNA and protein expressions of c-Fos (Fig. 6a-d).

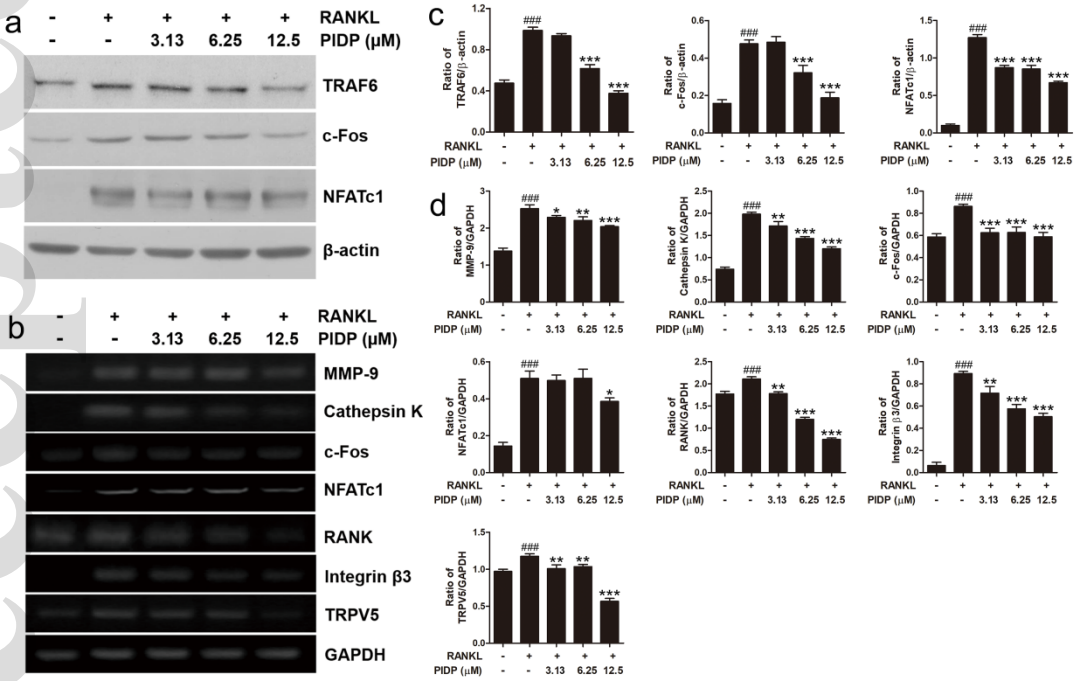


Fig. 6. Effect of PIDP on osteoclastogenesis-related genes and proteins. (a) Suppressive effect of PIDP on RANKL-stimulated TRAF6, c-Fos and NFATc1 protein expressions. (b) Inhibition effect

of PIDP on RANKL-stimulated mRNA expression of MMP-9, cathepsin K, c-Fos, NFATc1, RANK, integrin β3, and transient receptor potential vanilloid 5 (TRPV5). (c) Statistical analysis of the western blot results in Fig. 6a. Bar graphs showed the relative levels of TRAF6, c-Fos and NFATc1. (d) Statistical analysis of the results in Fig. 6b. Bar graphs showed the relative mRNA expression levels of MMP-9, cathepsin K, c-Fos, NFATc1, RANK, integrin β3 and TRPV5. ### $p < 0.001$, in comparison with untreated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, in comparison with cells only treated by 50 ng/mL RANKL.

DISCUSSION

To explore novel BPs drugs with high efficiency and low toxicity, a series of imidazole-containing BPs including MIDP, EIDP, PIDP and BIDP were examined preliminarily in the present study. ZOL was also investigated in parallel and served as the control. Our results suggested that PIDP not only exerted lower cytotoxicity to RAW 264.7 cells than ZOL, but also showed higher anti-bone-resorption capacity on RANKL-induced osteoclastogenesis than other BPs. It was also noted that PIDP suppressed the expression of osteoclastogenesis-associated molecules. These results supported the potential value of PIDP as a promising agent for treatment of bone disorders involving increased osteoclastogenesis. Interestingly, ^{99m}Tc-labeled PIDP was also proved as a potential radio-pharmaceutical for targeted bone imaging(Chen et al., 2009).

In addition to the phosphate groups, BPs also contain two side chains commonly known as R1 and R2. The structure of P-C-P determines the high affinity of BPs to bone mineral. The hydroxyl group at R1 position could enhanced the property of P-C-P structure(van Beek et al., 1998), and the R2 chain was responsible for the properties and potency of each BP(Ruza et al., 2013). It can be seen that the toxicity towards RAW 264.7 cells and the anti-bone-resorptive activity of BPs were mainly related to the R2 side chain. ZOL, MIDP, EIDP, PIDP and BIDP shared the same R1 chain (a hydroxyl group) and the different R2 chain (the group at the 2-position on the imidazole ring was a hydrogen (ZOL), methyl (MIDP), ethyl (EIDP), n-propyl (PIDP) or n-butyl group (BIDP)). For these compounds, the carbon atom increased from 0 to 4 at this specific position, gradually increased the upper limit of non-cytotoxic concentration, which

suggested that the number of carbon atom at the 2-position on the imidazole ring of these BPs was negatively correlated with their toxicity to RAW 264.7 cells. It has been shown that small differences of substituents binding to carbon atom could lead to significant changes in the 3D shape and atomic orientation of BPs, thus affecting its binding affinity with the target (Qiu et al., 2014; Russell et al., 2008). Therefore, the different toxicity of ZOL, MIDP, EIDP, PIDP and BIDP with similar structure may be related to a series of consequences caused by different R2 side chains, including lipophilicity, binding ability to the targets, etc.

Because of the complex mechanism of these BPs, the relationship between the structure and the anti-osteoclast-differentiation bioactivities is also complex. Previous studies have shown that the inhibition of mevalonate pathway by targeting FPPS was related to the anti-osteoclastic activity of BPs (Ebetino et al., 2011). According to our results, the substitution of the hydrogen at the 2-position on the imidazole ring by a methyl, ethyl, n-propyl or n-butyl group reduced the inhibitory activity of the compounds on FPPS and the number of carbon atom at the 2-position on the imidazole ring was negatively correlated with their inhibition to FPPS to some extent. However, PIDP showed more potent ability at inhibiting osteoclast differentiation than ZOL, MIDP, EIDP and BIDP. These indicated that the anti-osteoclastic effects of PIDP may be caused by other more important factors than the inhibition of FPPS.

The pathophysiology of ZOL-induced adverse effects, such as ONJ, remains unclear, but evidences showed that macrophages could play a critical role in the development of ONJ(Pazianas, 2011). It is suggested that repeated administration of BPs could affect the immune system by reducing the number and activity of monocytes and macrophages, and thus be responsible for infection and necrosis(Hoefert et al., 2016). According to our results, no obvious effect on osteoclast formation induced by RANKL was observed with ZOL when RAW 264.7 cells were treated at concentrations below the cytotoxicity threshold. Consequently, inhibition of osteoclast differentiation with ZOL was inevitably associated with toxicity to the precursors of osteoclasts. However, PIDP can effectively inhibit RANKL-stimulated osteoclast formation at concentrations without any cytotoxicity on RAW 264.7 cells. From the above results, we anticipated that PIDP might have a potential effect on inhibiting bone resorption activity of osteoclasts without

producing undesirable side effects. However, the concentration at which toxicity was observed might not be relevant to the *in vivo* situation where the toxicity might actually arise at higher local concentrations which are feasible upon treatment as these BPs presumably accumulate in bone. Therefore, further *in vivo* experiments are still needed.

It is known that the actin rings are essential for bone resorption activity at the late stage of the osteoclast maturation. It has been demonstrated that osteoclasts resulted in bone substrate degradation only within actin ring-defined regions, and consequently the integrity of the actin structure was crucial to osteoclasts-mediated bone resorption(Wagner and Karsenty, 2001). Hence, PIDP was not only inhibiting osteoclast differentiation, but also suppressing bone resorption by altering the morphological structure of osteoclasts.

As mentioned above, resorption lacunae and pit were formed by RANKL stimulated mature osteoclasts(Hsu et al., 1999). Functionally, the pit formation assay is important for identifying the osteoclast phenotype. In the present study, to investigate the effect of PIDP on osteoclastic bone resorption, a commercial osteo assay strip well plate was used to detect the pit formation. The results demonstrated that PIDP had a significant inhibition effect on the function of mature osteoclast and it attenuated the bone resorption activity of osteoclasts.

RANKL/RANK interaction activated a variety of important signal transducing pathways such as MAPKs, Akt and IκB. The MAPKs were related enzymes that selectively phosphorylated threonine and serine residues upon stimulation with extracellular molecules and transmited the stimuli into the nucleus. MAPKs and Akt pathways were involved in the transduction of RANK signal and they were necessary for subsequent differentiation of osteoclast(Gingery et al., 2003; Kong et al., 2015; Matsumoto et al., 2000). Inhibitors of MAPKs (i.e. p38, ERK, JNK) and Akt disturbed osteoclast differentiation induced by RANKL(Cheng et al., 2012; Ikeda et al., 2009; Tao et al., 2011; Yamanaka et al., 2013). The present study demonstrated that PIDP could inhibit the RANKL-stimulated phosphorylation of JNK and Akt, suggesting that PIDP targeted JNK and Akt cascades.

NF-κB is a key transcription factor, which plays a crucial role in osteoclast formation induced by RANKL. Inhibition of NF-κB activation could block the osteoclastogenesis(Jimi et al., 2004).

The binding of IκB with NF-κB prevents NF-κB to migrate to the cell nucleus. Phosphorylation of IκB induces separation of these two proteins, allowing NF-κB to reach the nucleus and activate target genes transcription(Hayden and Ghosh, 2004). Our results suggested that the inhibition effect of PIDP on RANKL-stimulated RAW 264.7 cells might also be related to the reduction of NF-κB.

NFATc1 can up-regulate various genes which are involved in osteoclast function and degradation of bone matrix(Zhao et al., 2010). Osteoclasts cannot be differentiated by embryonic stem cells in which NFATc1 was knocked out, and ectopic expression of NFATc1 resulted in efficient formation of osteoclast from osteoclast precursors without RANKL stimulation(Takayanagi et al., 2002). It is known that ZOL inhibited RANKL-stimulated formation of osteoclast through the down-regulation of NFATc1 gene(Nakagawa et al., 2015). NFATc1 also regulated the expression of many genes in relation with osteoclastogenesis, such as MMP-9, cathepsin K and integrin β3(Crotti et al., 2008; Song et al., 2009). MMP-9 and cathepsin K were found important for the degradation of the organic bone matrix, while integrin β3 contributed to bone resorption through the migration and adhesion of osteoclasts(Broadhead et al., 2011; Costa et al., 2011). In the present study, PIDP decreased both mRNA and protein levels of NFATc1. Additionally, treatment with PIDP suppressed the expression of MMP-9, cathepsin K and integrin β3 genes. Therefore, PIDP inhibited osteoclast formation by down-regulating the expression of these osteoclastogenesis-related genes through the inhibition of NFATc1.

RANKL/RANK interaction induced the aggregation of c-Fos, whereas the interaction of c-Fos with the promoter of NFATc1 led to the NFATc1 expression(Song et al., 2009). Lack of c-Fos in mice resulted in osteopetrosis and defective osteoclast differentiation(Wang et al., 1992). Moreover, it is demonstrated that risedronate, one of the nitrogen-bearing BPs, could also inhibit RANK-stimulated expression of c-Fos and NFATc1(Kwak et al., 2009). Our experiments showed that PIDP exerted inhibitory effects on the mRNA and protein expressions of c-Fos. The results indicated that PIDP might restrain RANKL-induced osteoclastogenesis via the down-regulation of NFATc1 following the suppression of c-Fos. RANK could regulate the expression of c-Fos in osteoclast precursors(Arai et al., 2012). We studied the expression of RANK and showed that

PIDP inhibited the mRNA expression of RANK, and the result demonstrated that PIDP might suppress the expression of c-Fos through its action on the signaling cascades involved in RANKL.

In addition, the effect of PIDP on the expression of TRPV5 (transient receptor potential cation channel subfamily V member 5) was also studied. TRPV5 is known to be involved in RANKL-stimulated Ca^{2+} spike. The inactivation of TRPV5 by siRNA completely inhibited the concentration increase of the intracellular Ca^{2+} induced by RANKL (Chamoux et al., 2010). Moreover, the inactivation of TRPV5 was also necessary to facilitate NF-κB nuclear translocation and NFATc1 activation, and to maintain osteoclast survival and bone-resorption activity(Komarova et al., 2005; Komarova et al., 2003). Our study demonstrated that the mRNA levels of TRPV5 decreased in presence of PIDP, which suggested that the down-regulation of RANKL-induced increase of intracellular Ca^{2+} concentration might be involved in the anti-bone-resorptive effect of PIDP.

CONCLUSIONS

In summary, we demonstrated the effects of a series of imidazole-containing BPs on RANKL-stimulated osteoclast differentiation and activation in RAW 264.7 cells. Therein, PIDP attenuated RANKL-induced osteoclast differentiation and possessed low cytotoxicity on RAW 264.7 cells. Moreover, PIDP exerted suppression effects on the actin rings formation, bone resorption, and the expression of gene and protein associated with osteoclastogenesis. The down-regulation of JNK, Akt and IκB phosphorylation in the early signaling event and subsequent inhibition of c-Fos and NFATc1 expressions might be involved in these effects (Fig. 7). Taken together, our findings supported the potential value of PIDP as a promising agent for the treatment of bone disorders involving increased osteoclastogenesis.

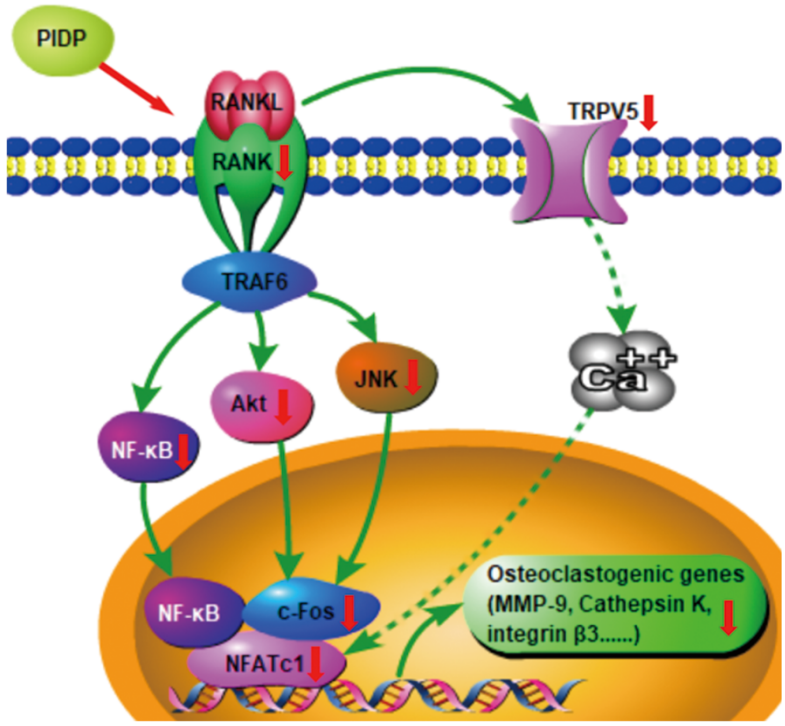


Fig. 7. Potential mechanism of action of PIDP on the regulation of osteoclast differentiation and activation.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

FUNDING

This work was supported by the National Natural Science Foundation of China (81803586), the Natural Science Foundation of Jiangsu Province (BK20181128), the 333 Project of Jiangsu Province (BRA2016518, LGY2018086), the Jiangsu Provincial Medical Youth Talent (QNRC2016626 and QNRC2016629), Innovation Capacity Development Plan of Jiangsu Province (BM2018023-5) and Jiangsu Provincial Key Medical Discipline (ZDXKA2016017).

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