Pharmacological Reports 67 (2015) 1264–1272

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original research article

A pterostilbene derivative suppresses osteoclastogenesis by regulating RANKL-mediated NFκB and MAPK signaling in RAW264.7 cells

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ARTICLE INFO

Article history:
Received 13 December 2014
Received in revised form 18 April 2015
Accepted 14 May 2015
Available online 30 May 2015

Keywords:
PTERC-T
RANKL
Osteoclastogenesis
MAPK
NFκB

ABSTRACT

Background: A dysfunctional osteoclast activity is often the cause of bone destructive diseases, such as osteoporosis, periodontitis, erosive arthritis, and cancer. The NFκB ligand (RANKL) has been identified as a major mediator of bone resorption. Agents that suppress RANKL signaling have the potential to inhibit bone resorption or osteoclastogenesis. The present study aimed to determine the effect of a pterostilbene derivative (PTERC-T) for suppressing RANKL or tumor cells-induced osteoclastogenesis in RAW264.7 murine macrophages.

Methods: Cytotoxicity was measured by MTT assay and inhibitory effect on osteoclastogenesis was analyzed by counting the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells and measuring the expression levels of the osteoclast-specific genes. The reactive oxygen species (ROS) generation was detected by FACS. Further, signaling pathways were analyzed by immunofluorescence and immunoblot analyses.

Results: PTERC-T suppressed the differentiation of monocytes to osteoclasts in a dose and time-dependent manner. The expression of osteoclast marker genes like *TRAP*, cathepsin K (*CTSK*), matrix metalloproteinase 9 (*MMP9*) and transcription factors *c-Fos*, and nuclear factor of activated T cells cytoplasmic 1 (*NFATc1*) were also diminished by PTERC-T. PTERC-T scavenged intracellular ROS generation within osteoclast precursors during RANKL-stimulated osteoclastogenesis. Mechanistically, PTERC-T abrogated the phosphorylation of MAPKs (ERK and JNK) and inhibited RANKL-induced activation of NFκB by suppressing IκBα phosphorylation and preventing NFκB/p65 nuclear translocation.

Conclusions: This study thus identifies PTERC-T as an inhibitor of osteoclast formation and provides evidence for its role in preventing osteoporosis and other bone related disorders. However, further studies are needed to establish its efficacy *in vivo*.

Introduction

The bones undergo constant turnovers and are balanced (homeostasis) by osteoblasts (creating bone) and osteoclasts (destroying bone). Osteoclasts are unique bone-resorbing cells derived from the cells of the monocyte–macrophage lineage. Excessive bone resorption by osteoclasts leads to an imbalance in bone remodeling and causes bone lytic diseases such as osteoporosis, Paget's disease, periodontitis, erosive arthritis, hypercalcemia, and cancer metastasis to the bone [1]. The drugs currently used to treat osteoporosis include bisphosphonates, calcitonin, and estrogen. These drugs are bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts [2]. Since the osteoclasts are responsible for bone resorption, they are one of the main targets for the treatment of osteoporosis.

Many factors are involved in osteoclast differentiation. Receptor activator of nuclear factor (NF)-κB (RANK), its ligand RANKL and the decoy receptor osteoprotegerin (OPG) are essential and central regulators of osteoclast development and functions. RANKL is expressed on the surface of the osteoblastic/stromal cells and also by various cancer cells. It is also directly involved in the differentiation of monocyte–macrophages into osteoclasts. Studies have shown that mice with a disrupted RANKL gene show a lack of osteoclasts, severe osteopetrosis, and defective tooth eruption, indicating that RANKL is essential for osteoclast differentiation [3,4]. In the physiological milieu when RANKL binds to RANK, it

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http://dx.doi.org/10.1016/j.pharep.2015.05.009
1734-1140/

first undergoes trimerization and then binds to an adaptor molecule, i.e., TNF receptor-associated factor 6 (TRAF6). This results in the activation of several downstream signaling cascades, including the NFκB, mitogen-activated protein kinases (MAPK), activating protein 1 (AP-1), and nuclear factor of activated T cells (NFATc1), resulting in the formation of multinucleated bone-resorbing osteoclasts [5,6].

Trans-3,4′,5-trihydroxystilbene or Resveratrol (RESV) is a polyphenol produced by certain plant species, including nuts, berries, and grapes, in response to environmental stress by the action of stilbene synthase enzyme [7]. RESV possesses significant anti-inflammatory and antioxidant properties that may benefit bone health. Studies have showed that RESV increased osteoblast proliferation and differentiation [8,9] and decreased the osteoclast differentiation [9,10]. Given the limited bioavailability of RESV after oral administration, the high concentration required to obtain the desired effects, its potential toxicity and complex mechanisms of actions, RESV analogs may offer advantages over the parent compound.

Pterostilbene (PTER) is a naturally occurring dimethylated analog of RESV that shows pleiotropic health benefits, including anti-oxidant, anti-inflammatory, anti-aging, cardioprotective, and neuroprotective activities [11]. However, after ingestion, the PTER is significantly more bioavailable than RESV [12]. Due to the widespread use and importance of stilbenes, which are small molecules, there is a need for new and active stilbenes. In our recent studies, we have synthesized a PTER derivative, PTER carboxaldehyde thiosemicarbazone (PTERC-T) (which was previously reported as pterostilbene–isothiocyanate conjugate, PTER-ITC) [13–15] with improved activity than PTER and have studied its role in breast [13,14] and prostate [15] cancer prevention (Fig. 1A). In the present study, we investigated the effect of PTERC-T on osteoclast differentiation in RAW264.7 cells and found that PTERC-T acts as a RANKL-mediated osteoclastogenesis inhibitor. This inhibitory effect results due to suppression of reactive oxygen species (ROS) generation and the blockage of RANKL-induced signaling pathways (MAPKs and NFκB), which are required for osteoclast differentiation.

Materials and methods

Reagents

RAW264.7 (murine macrophage) and MDA-MB-468 (human breast adenocarcinoma) cells were obtained from the National Center for Cell Science (NCCS; Pune, India). The cell culture reagents were purchased from GIBCO (Life Technologies, Grand Island, NY). Penicillin, streptomycin, 3-(4,5-dimethyl-2-thiazolyl)2,5diphenyl-2H-tetrazoliumbromide (MTT), cell culture grade

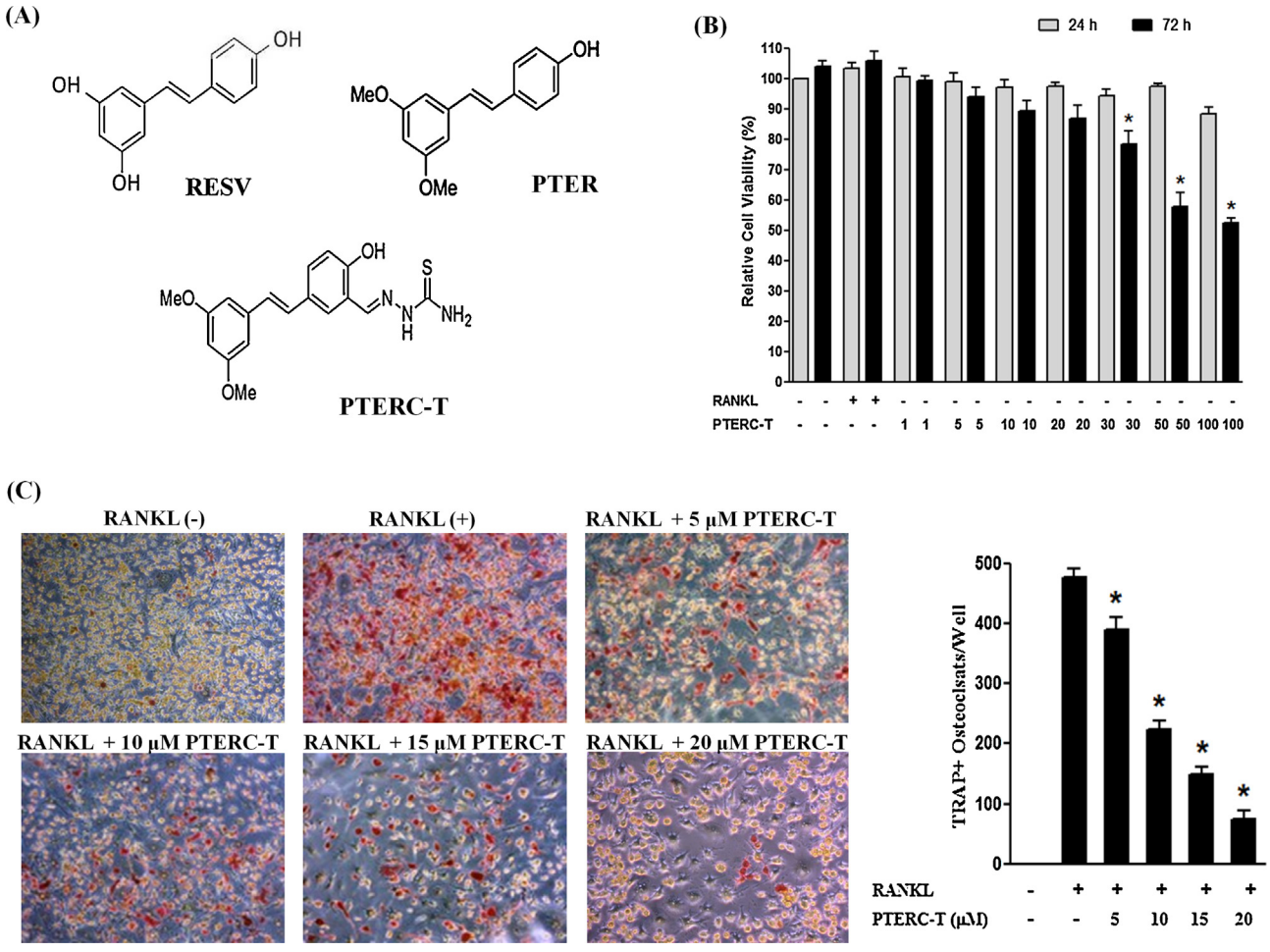

Fig. 1. PTERC-T inhibited RANKL induced osteoclastogenesis of RAW264.7 cells without significant toxicity. (A) Molecular structure of RESV, PTER, and PTERC-T. (B) Effect of PTERC-T on cell viability in RAW264.7 cells. The cell viability was determined by using MTT assay, and the results are expressed as a percentage of surviving cells over the control group. Data are the mean ± SEM of three independent experiments. * Represents statistically significant difference compared to the control group; $p < 0.05$. (C) RAW264.7 cells were cultured for 6 days with 50 ng/ml RANKL and indicated concentrations of PTERC-T. Cells were fixed, and TRAP staining was performed. TRAP-positive cells were photographed (100× magnifications). The histogram on the right panel shows quantification of multinucleated osteoclasts (i.e., those containing two or more nuclei). Values are mean ± SEM from three independent experiments. * Indicate statistically significant differences compared to only RANKL treated groups; $p < 0.05$.

dimethyl sulphoxide (DMSO), agarose and all analytical grade chemicals were obtained from Himedia (Mumbai, India). The reverse transcription-polymerase chain reaction (RT-PCR) kits were from Genei (Bangalore, India). RANKL and BCA protein estimation kits were obtained from Sigma–Aldrich (St. Louis, MO, USA), and 2,7-dichloro dihydro fluorescein diacetate (DCFH-DA) was from Invitrogen (USA). Antibodies for p-ERK, p-JNK, p-P38, p-NFκB p65, p-Ikβ-α, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies were purchased from Genei (Bangalore, India). PTERC-T was synthesized and provided as described previously [13], and its purity was >99%, as identified by the high-performance liquid chromatography (HPLC) analysis.

Cell culture

The RAW264.7 cells were maintained in Dulbecco's modified Eagle's media (DMEM) while the MDA-MB-468 cells were maintained in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (heat inactivated) (both from Invitrogen, Life Technologies) and 1% antibiotic (100 U/ml of penicillin and 100 μg/ml streptomycin) at 37 °C, and 5% CO_2 in a humidified atmosphere.

Cytotoxicity assays

The MTT assay was carried out as described previously [13]. In brief, the RAW264.7 cells were seeded onto 96-well microtiter plates at a density of 5×10^3 cells/well in 200 μl medium. After 16 h, serial dilutions of PTERC-T, initially ranging 0–100 μM in DMSO (dimethyl sulphoxide) in the presence and absence of RANKL (50 ng/ml), were added to the monolayer. The final DMSO concentration for all dilutions was 0.1%, which was used as a vehicle control. The cultures were assayed after 24 h and 72 h by adding 20 μl of 5 mg/ml MTT and incubating for 4 h at 37 °C. MTT-containing medium was then aspirated, and 200 μl of DMSO was added to dissolve the formazone crystal. The optical density (OD) was measured at 570 nm in an ELISA plate reader (Fluostar optima, BMG Labtech, Germany). The absorbance values were expressed as a percentage of control.

Osteoclast differentiation and TRAP staining

Murine RAW264.7 cells were grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. For osteoclasts differentiation, the RAW264.7 cells (2×10^4, in 24-well plate) were cultured in the presence of RANKL (50 ng/ml) and indicated concentration of PTERC-T for 6 days. The culture medium was replaced every alternate days. After the completion of differentiation, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity. For TRAP staining, the cells were first washed with PBS and then fixed with 3.7% paraformaldehyde (pH 7.4) for 15 min at RT. The cells were then permeabilized in 0.1% Triton X-100 for 10 min. The cells were then washed and incubated in TRAP stain (0.3 mg of Fast red violet BB salt per ml of TRAP buffer) till the pink color was developed. Each 100 ml of TRAP buffer contained 50 ml of 0.1 M acetate buffer, 10 ml of 0.3 M sodium tartrate, 1 ml of 10 mg/ml Naphthol AS-MX phosphate and 38.9 ml of milli Q water. The TRAP-positive multinucleated cells containing two or more nuclei were considered to be osteoclast-like cells and were counted under the inverted microscope (Axiovert 25, Zeiss, Germany).

For co-culture experiments with tumor cells, RAW264.7 cells (5×10^3) were incubated in the presence of breast cancer (MDA-MB-468) cells (1×10^3) for 24 h, then exposed to PTERC-T (5, 10 and 20 μM) for 5 days, and finally subjected to TRAP staining.

Semi-quantitative RT-PCR

The total RNA was extracted from the treated cells by using an RNA isolation kit (Genei, Bangalore, India). The samples were then quantified, and equal amounts of the individual treatments were transcribed with the RT-PCR kit according to the manufacturer's instructions (Genei, Bangalore, India). For semi-quantitative RT-PCR, the templates were amplified on an MJ MiniTM Personal Thermo Cycler (Bio-Rad Laboratories). The PCR condition and the primer sequences are provided in the supplementary information. The PCR conditions were optimized to maintain amplification in the linear range to avoid the plateau effect. The PCR products were separated on 1.5% agarose gel and visualized using a gel documentation system (BioRad, USA). The intensity of the bands on agarose gels was analyzed using the ImageJ 1.43 software (NIH, USA) and normalized to β-actin PCR products.

Measurement of intracellular ROS levels

The intracellular ROS production was determined using DCFH-DA, a fluorogenic probe for general ROS such as hydrogen peroxide, peroxynitrite, and hydroxyl radical. In brief, the RAW264.7 cells were grown and treated with different concentrations of PTERC-T (1–20 μM) for 2 h before stimulation with 50 ng/ml of RANKL. Thereafter 1 h of RANKL stimulation (3 h after PTERC-T stimulation) these cells were subsequently incubated with DCFH-DA (at a final concentration of 10 μM) for 30 min at 37 °C. Then the cells were washed with PBS twice, and intracellular ROS accumulation was observed and photographed using a fluorescent microscope (Axiovert 25, Zeiss, Germany).

The quantitative amount of ROS production in RAW264.7 cells was also analyzed by fluorescence-activated cell-sorting (FACS). For this, the RAW264.7 cells were grown and treated as described above. Then the cells were washed in PBS and incubated with DCFH-DA (at a final concentration of 10 μM) for 30 min at 37 °C. The cells were then scraped off, washed in PBS, and the fluorescence intensity was analyzed by FACSverse TM (BD Biosciences, San Jose, CA, USA).

Western blot analysis

For the preparation of cytoplasmic and nuclear fraction, the cells were pelleted by centrifugation at 4 °C at 250 × *g* for 2 min, followed by resuspension in lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 50 mM sodium fluoride, 50 mM glycerol-2-phosphate, 1× protease inhibitor and 0.5% NP40). The samples were then centrifuged for obtaining cytoplasmic fraction. The nuclei pellet was then resuspended in 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM sodium fluoride, 50 mM glycerol-2-phosphate, 1× protease inhibitor, and lysates were generated. Equal amounts of protein lysates (~40 μg) were then analyzed on 12% polyacrylamide gel, followed by immunoblot analysis using a standard protocol. In brief, the proteins were transferred to the nylon membrane, which was blocked with TBS-T buffer (20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% skim milk powder. The blots were first washed with TBS-T buffer and then incubated (overnight, 4 °C) in the same buffer with primary anti-p-NFkB p65, -p-IκBα, -p-ERK, -p-JNK, -p-P38 (1:500) and -β-actin (1:1000) antibodies (all from Santa Cruz Biotechnology). The blots were then washed and incubated with HRP (horseradish peroxidase)-conjugated anti-rabbit or anti-mouse secondary antibody (1:20,000). The color was developed in the dark using the ECL kit (GE Healthcare, Bucks, UK), and the blots were then analyzed by densitometry with ImageJ 1.43 software using β-actin as an internal control.

Immunofluorescence staining

The cells were first washed with PBS, fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 1% bovine serum albumin (BSA) for 30 min at room temperature. The cells were then incubated with rabbit polyclonal p-NFkB p65 antibody (dilution: 1:200) in blocking buffer overnight at 4 °C. The cells were then washed with PBS and incubated with the FITC-labeled anti-rabbit secondary antibody (diluted 1:500 in blocking buffer) for 30 min at room temperature. DAPI (dilution 1:50,000; Sigma) was used to stain the nuclei. All the images were captured using a fluorescence microscope (Axiovert 25, Zeiss, Germany).

Statistical analysis

Data are expressed as mean ± SEM. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test for multiple comparison, using Graph Pad Prism 5.04 (Graph Pad Software, San Diego, CA, USA). A p-value less than 0.05 was considered to be statistically significant.

Results

Effect of PTERC-T on cell viability

The RAW264.7 cells were treated with various concentrations (1–100 μM) of PTERC-T for 24 h and 72 h. The cell viability was determined by using the MTT assay. As shown in Fig. 1B, PTERC-T had no cytotoxic effects on the cells at any of the doses tested after the 24 h. However, when the cells were treated for a longer period (72 h), PTERC-T was found to reduce the cell numbers at a dosage above 30 μM. Hence, in order to exclude PTERC-T-mediated cytotoxicity, non-lethal concentrations (<30 μM) were used in the subsequent experiments.

PTERC-T inhibits RANKL-induced osteoclastogenesis in RAW264.7 cells

In the next phase, we investigate whether PTERC-T can inhibit RANKL-induced osteoclastogenesis. For this, the osteoclast precursor RAW264.7 cells were treated with different concentrations (5, 10, 15 and 20 μM) of PTERC-T in the presence of RANKL and allowed to differentiate into osteoclasts for 6 days. Fig. 1C shows RANKL-induced formation of osteoclasts in the control cells. In contrast, the differentiation into osteoclasts was significantly reduced in the presence of PTERC-T, which was dose-dependent. As little as 5 μM concentration of PTERC-T had a significant effect on RANKL-induced osteoclast formation.

In order to determine whether PTERC-T inhibits osteoclastogenesis in a time-dependent manner, the RAW264.7 cells were incubated with PTERC-T for three to five days and allowed to differentiate into osteoclasts by RANKL. The morphological observations clearly demonstrated that the RAW264.7 cells differentiated into osteoclasts after RANKL addition and that PTERC-T inhibited this differentiation (Fig. 2A). The extent of suppression was measured by counting the number of TRAP-positive osteoclasts per well (Fig. 2A lower panel). The study showed that RANKL induced osteoclast differentiation in a time-dependent manner; a maximum of TRAP-positive osteoclasts (per well) was observed at day 5 (Fig. 2A). On the other hand, PTERC-T decreased the number of TRAP-positive osteoclasts in a dose-dependent manner, with a strong inhibition at 20 μM at all days examined (Fig. 2A) ($p < 0.05$).

PTERC-T inhibits osteoclastogenesis induced by tumor cells

Osteoclastogenesis is commonly linked with various cancers, including breast cancer, through the activation of RANK-RANKL signaling pathway [16]. Breast cancer cell lines, including MDA-MB-468 cells, are known to constitutively express NFκB and RANKL. Hence, in the next part of the study, we investigated whether PTERC-T inhibits tumor cell-induced osteoclastogenesis of RAW264.7 cells. Our result indicated that incubating monocytes with MDA-MB-468 cells (Fig. 2B) induced osteoclast differentiation and that PTERC-T suppressed this differentiation in a dose-dependent manner (as determined by reduction in the number of TRAP positive cell numbers). These results indicated that osteoclastogenesis induced by tumor cells was significantly suppressed in the presence of PTERC-T.

Effects of PTERC-T on expression of osteoclastic marker gene in RANKL stimulated RAW264.7 cells

In order to further evaluate the osteoclastic changes, we examined the effect of PTERC-T on mRNA expression levels of osteoclast specific genes using semi-quantitative RT-PCR. The osteoclastic markers including TRAP, CTSK, and MMP9 were significantly up-regulated upon treatment with RANKL. However, the up-regulation of osteoclastic marker genes was attenuated by the addition of PTERC-T in a dose dependent manner (Fig. 3A).

PTERC-T inhibits RANKL-induced osteoclast specific transcription factors

c-Fos and NFATc1 are considered as the two most important osteoclast specific transcription factors. The NFATc1 gene has been identified as the most strongly induced transcription factor after RANKL stimulation. It is also regarded as the master regulator of osteoclastogenesis. In order to elucidate the molecular mechanisms of the effect of PTERC-T on osteoclastogenesis, we examined whether PTERC-T modulated these transcription factors by RT-PCR in RANKL-stimulated RAW264.7 cells. In accordance with the previous results, treatment with RANKL significantly up-regulated both c-Fos and NFATc1 mRNA levels (Fig. 3B) while pre-treatment with PTERC-T significantly down-regulated RANKL-induced mRNA expressions of both these genes.

Effects on ROS generation stimulated by RANKL

Since it is known that intracellular ROS production is correlated with RANKL stimulated osteoclastogenesis [17,18], we investigated whether PTERC-T inhibits ROS production during RANKL-mediated osteoclast differentiation using a cell-permeable, oxidation-sensitive dye, DCFH-DA. As shown in Fig. 3C, the intracellular ROS concentration was increased by stimulation with RANKL and this increase was dose-dependently inhibited by PTERC-T. To further confirm this result, we used flow cytometry to quantitatively determine the intracellular ROS in PTERC-T treated cells. The results showed that stimulation of RAW254.7 cells with RANKL alone substantially increased intracellular ROS levels which however was prevented in cells pretreated with increasing dose of PTERC-T, which are consistent with the fluorescent microscopy results (Fig. 3D).

PTERC-T inhibits early RANKL signaling pathway in RAW264.7 cells

RANKL induces the activation of three well-known MAPKs and NFκB in osteoclast precursors that are required for early osteoclast differentiation. In order to understand the probable mechanisms by which PTERC-T inhibits osteoclastogenesis, we investigated the

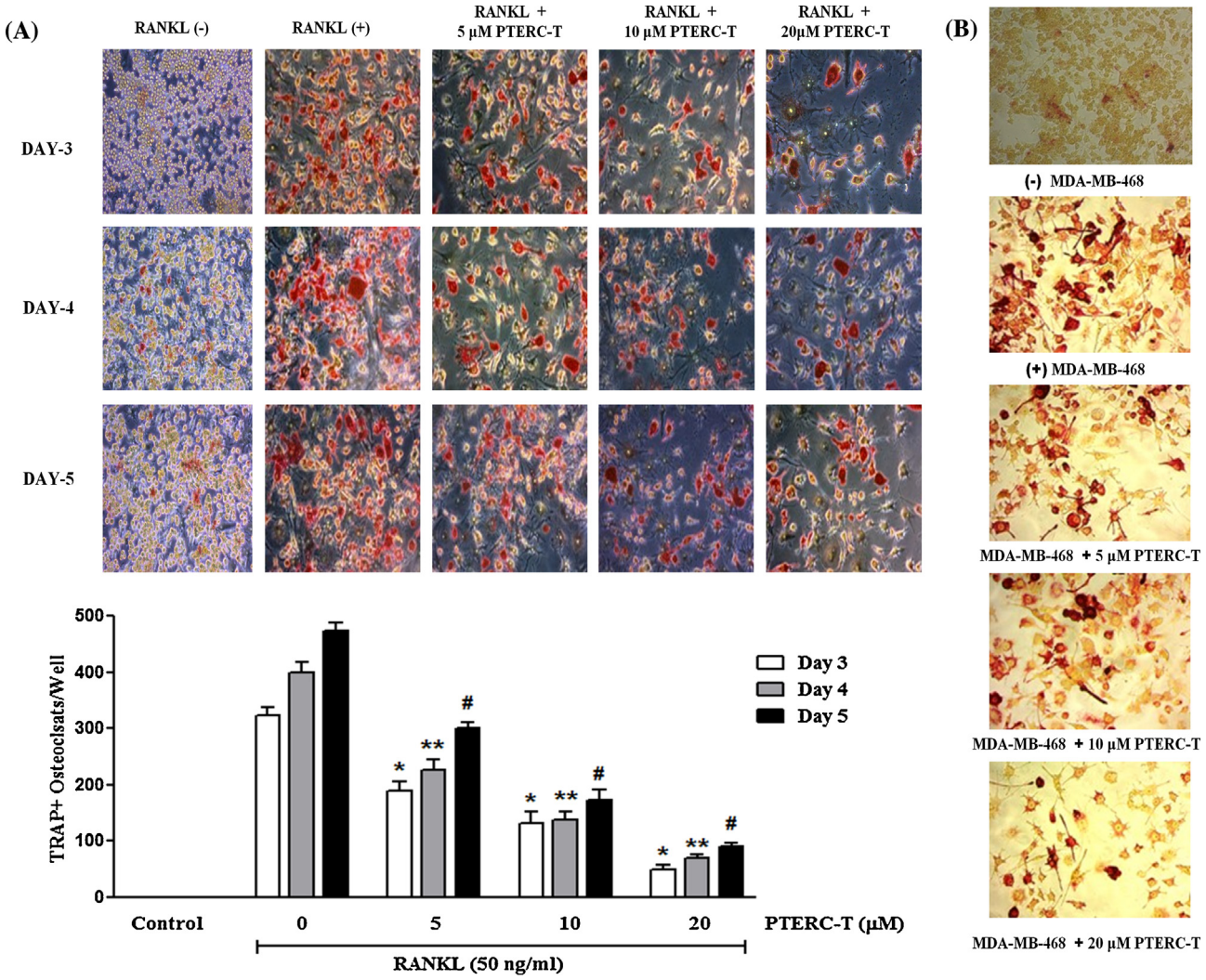

Fig. 2. PTERC-T inhibits RANKL and tumor cell-induced osteoclastogenesis. RAW264.7 cells were incubated with RANKL alone (50 ng/ml) or with RANKL and PTERC-T (5, 10 and 20 μM) for 3, 4 or 5 days and then stained for TRAP expression. (A) TRAP-positive cells were photographed (100× magnifications). The histogram on the lower panel shows quantification of multinucleated osteoclasts (i.e., those containing two or more nuclei). Values are mean ± SEM from three independent experiments. *, ** and # indicate statistically significant differences compared to only RANKL treated groups at day 3, 4 and 5 respectively, $p < 0.05$. (B) PTERC-T suppressed osteoclastogenesis induced by tumor cells. RAW264.7 cells were co-incubated in the presence of MDA-MB-468 cells and exposed to PTERC-T for 5 days, and finally stained for TRAP expression. Figures show one representative experiment of three performed.

effect of PTERC-T on MAPKs and NFκB activation in macrophages. For this, the RAW264.7 cells were pre-treated with 5, 10 and 20 μM of PTERC-T for 2 h and then stimulated with 50 ng/ml RANKL for 30 min. Our results showed that RANKL strongly activated p38 MAPK, ERK 1/2, and JNK phosphorylation in RAW264.7 cells, whereas RANKL-induced phosphorylation was inhibited significantly by PTERC-T: most effectively for JNK followed by ERK 1/2 and non-significant for p38 MAPK (Fig. 4A) ($p < 0.05$).

RANKL-induced NFκB activation is essential for initiating osteoclast differentiation. We investigated NFκB activation in RAW264.7 cells by employing two different approaches to determine whether PTERC-T inhibited NFκB mediated osteoclastogenesis. First, using the immunoblot analysis, we showed that PTERC-T reduced RANKL-induced phosphorylation and degradation of IκBα (Fig. 4B, upper panel) and the subsequent nuclear translocation of NFκB subunit (Fig. 4B, lower panel). Secondly, we performed immunofluorescence staining of p65 with or without PTERC-T treatment. As shown in Fig. 5, in the absence of RANKL, most of the p65 sub-units were located in the cytoplasm. Upon RANKL stimulation, almost all p65 sub-units were located in the nucleus after 30 min of incubation. However, the RANKL-induced p65 nuclear translocation was blocked when pre-treated with PTERC-T. These results indicated that PTERC-T can inhibit RANKL-mediated activation of the NFκB pathway.

Discussion

The bone is constantly regenerated through the continuous formation by osteoblasts and resorption by osteoclasts, which is termed as "remodeling". Excessive bone resorption plays a central role in the pathological conditions responsible for bone-related disorders [1]. Thus, there is a requirement of safe, efficacious, and affordable compounds that can inhibit bone loss. To date, various drugs have been developed for the treatment of bone loss related diseases. One such compound is RESV, a polyphenolic phytoestrogen with osteogenic and osteoinductive properties [8–10]. Despite its promising properties, RESV's rapid metabolism and low bioavailability have precluded its advancement in clinical use [19]. Structural modifications of RESV may lead to analogs with

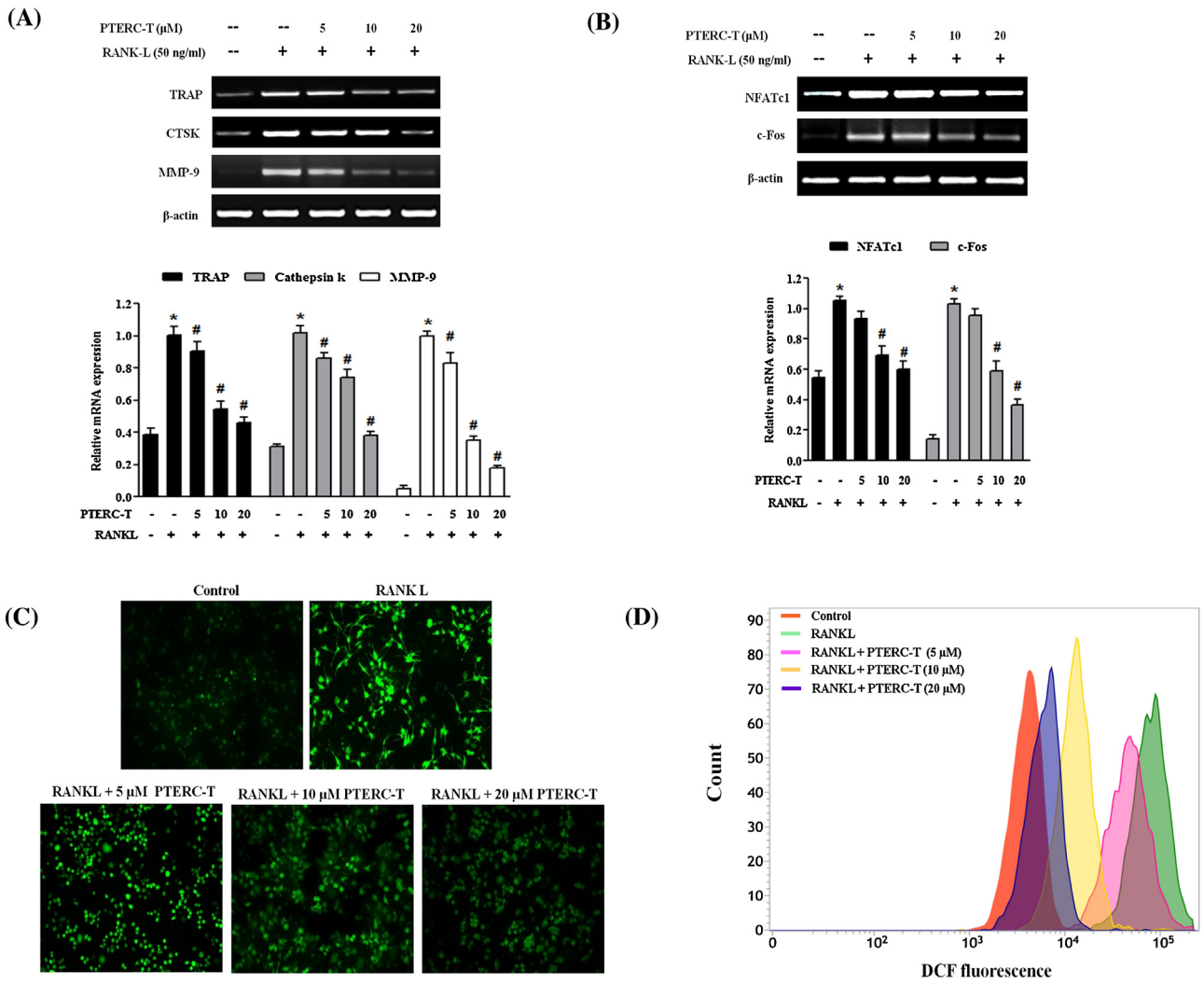

Fig. 3. PTERC-T inhibited RANKL induced mRNA expression levels of osteoclasts related genes and transcription factors. (A) RAW264.7 cells were cultured with the indicated concentration of PTERC-T in the presence of RANKL (50 ng/ml). After 5 days of culture, the mRNA expression levels of osteoclasts related genes were determined by RT-PCR. (B) Inhibition of NFATc1 and c-Fos expression by PTERC-T. RAW264.7 cells were preincubated with vehicle or indicated concentrations of PTERC-T for 2 h and then treated with RANKL (50 ng/ml) for another 48 h. Total RNA was isolated, and RT-PCR was performed to detect NFATc1 and c-Fos mRNA levels with β-actin mRNA as an endogenous control. The histogram on the lower panel of each figure shows relative band intensities normalized to the corresponding β-actin level. Bar shows mean ± SEM of three independent experiments. * and # indicate statistically significant differences with respect to vehicle treated and only RANKL treated groups respectively, $p < 0.05$. (C) ROS accumulation within cells in response to different doses of PTERC-T treatments as estimated by H2-DCF-DA staining. Magnification, 100×. (D) FACS analysis of ROS generation using DCFH-DA in cells exposed to RANKL (50 ng/ml) with or without PTERC-T pretreatment. The figure was obtained from three independent experiments with similar patterns.

improved potency and specificity compared with the parental agent, thereby becoming more safe and tolerable. In this study, we used a synthetic derivative of PTER (an analog of resveratrol) and tested its effect on osteoclasts differentiation *in vitro*. For this study, we used a homogeneous, clonal population of murine monocytic cell, RAW264.7 cells, and stimulated it with RANKL to differentiate it into osteoclasts in the presence of PTERC-T. This system does not contain any osteoblast/bone marrow stromal cells or cytokine-like macrophage-colony-stimulating factor and helps to focus on RANKL signaling in preosteoclast cells. Our results showed that PTERC-T inhibits RANKL-induced osteoclast differentiation without causing any significant decrease in the viability of RAW264.7 cells.

In bone regulation, RANKL-induced intracellular ROS production serves to regulate the RANKL signaling pathways required for osteoclast differentiation and acts as an upstream component of signaling pathways that mediate osteoclast activation and survival [17,18]. Here, ROS was generated within the RAW264.7 cells during RANKL simulated osteoclast differentiation. This ROS production decreased distinctly when the RAW264.7 cells were pre-treated with PTERC-T. From these findings, we hypothesized that PTERC-T may suppress osteoclast differentiation by scavenging the generated intracellular ROS.

Studies have suggested several transcription factors for mediating the induction of the genes implicated in osteoclastic differentiation in response to RANKL. The transcription factors like, NFκB, c-Fos, and NFATc1 functions downstream of RANKL signaling for osteoclasts differentiation. The c-Fos/c-Jun/NFATc1 pathway is important for the development of osteoclast, and thus, the lack of any of these three components can arrest osteoclastogenesis [20]. In this study, the RANKL-induced expression of c-Fos and NFATc1 was dramatically down-regulated by pre-treatment with PTERC-T. NFATc1 can regulate the expression of many genes associated with osteoclast differentiation and function, such as TRAP, latent transforming growth factor beta binding protein 3 (LTBP3), chloride channel (ClC7), MMP9, CTR, Cathepsin K and c-Src. In this study, the NFATc1-regulated gene expression, such as TRAP, MMP9, and CTSK, was examined. Our

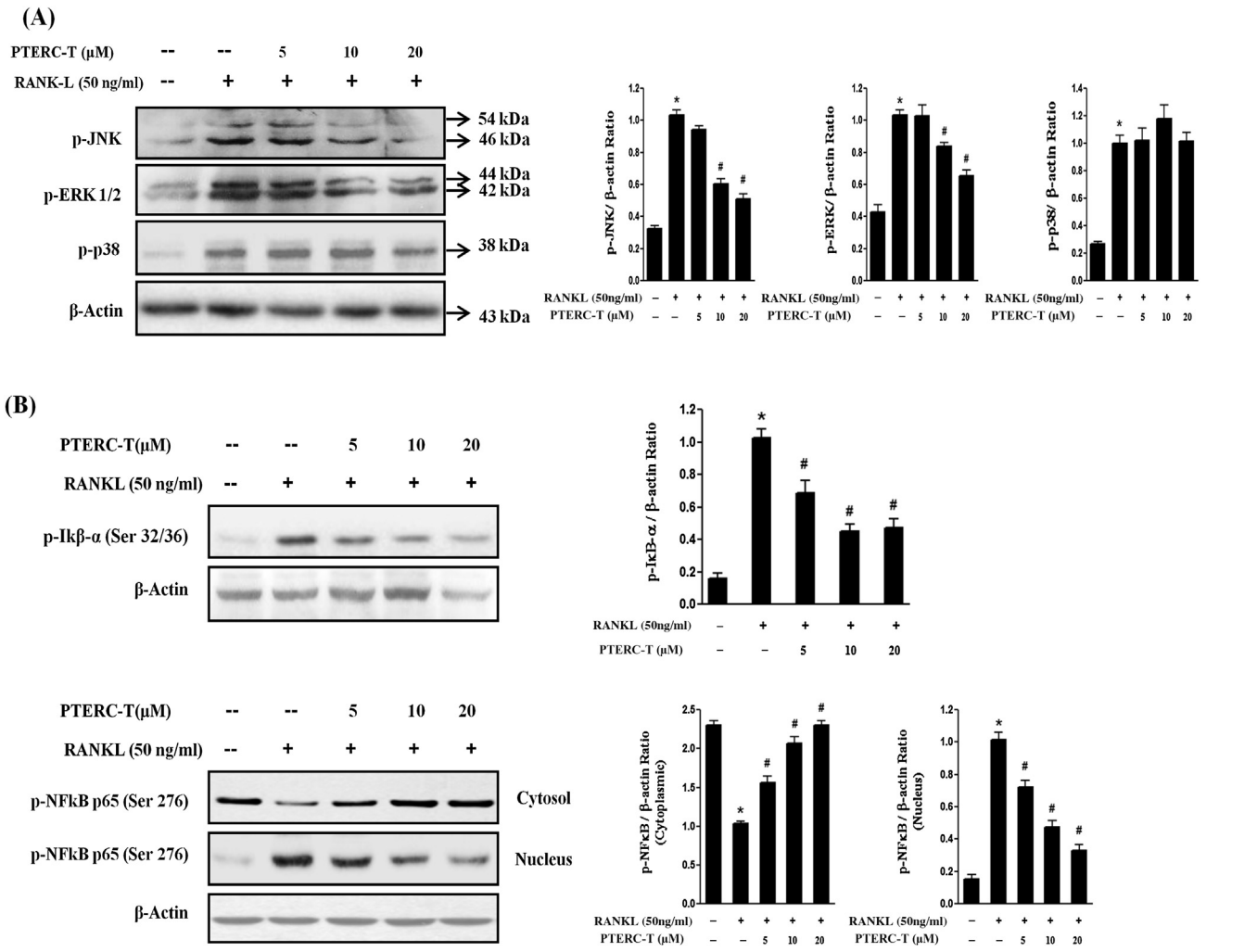

Fig. 4. PTERC-T inhibits RANKL induced MAPK and NFκB/IκB signaling pathways. RAW264.7 cells were preincubated with the indicated concentrations of PTERC-T for 2 h, and then treated with 50 ng/ml of RANKL for 30 min. Thereafter cellular proteins (total, cytoplasmic or nuclear) were extracted and subjected to western blot analysis for (A) MAPK and (B) NFκB dependent pathways. The histogram in the right panel of each figure shows relative band intensities normalized to the corresponding β-actin level. Bars show mean ± SEM of three independent experiments. * and # indicate significant differences with respect to vehicle treated and only RANKL-treated groups, respectively; $p < 0.05$.

results indicated that the expression of all these genes was decreased by PTERC-T, suggesting that PTERC-T affects not only the expression of NFATc1 but also its regulated downstream gene expressions.

The importance of NFκB in RANKL/RANK pathways for osteoclastogenesis is well understood by the findings from genetic and pharmacological studies. The classical NFκB signaling pathway involves activation of the IκB kinase (IKK) complex, which phosphorylates IκBα and targets it for ubiquitin-dependent degradation [21,22]. Our results showed that PTERC-T inhibited cytoplasmic degradation of IκBα and the nuclear translocation of p50 and p65 proteins, resulting in reduced levels of NFκB transactivation. The results indicated that inhibition of the NFκB-dependent pathway is one of the mechanisms involved in the anti-osteoclastogenic effect of PTERC-T. Besides the NFκB pathway, RANKL stimulation has also been shown to activate three major subfamilies of MAPKs (p38 MAPK, ERK 1/2 and JNK). These MAPKs also play pivotal roles in the development of osteoclasts and are thus key molecular targets for therapeutic application in inflammatory bone disease [23]. In this study, we demonstrated that PTERC-T inhibited RANKL-induced phosphorylation of JNK and ERK in RAW264.7 cell, suggesting that it can suppress MAPK cascades.

In addition to these *in vitro* data, *in vivo* studies are further warranted to verify the osteoprotective role of PTERC-T. Previously, *in vivo* efficacy of PTERC-T associated with various pharmacological actions has been elucidated [13]. Oral administration of PTERC-T demonstrated prominent antitumor effect at 100 mg/kg body weight [13] and anti-inflammatory activity at 50 mg/kg body weight [24] without any side effects, such as toxicity. Presumably, consistent with the *in vitro* results, in reference to *in vivo* treatment dose of PTERC-T, oral administration of PTERC-T in the range of 50–100 mg/kg body weight could suppress excessive bone resorption through down regulating RANKL expression of osteoblasts as well as interfering with molecular signaling of osteoclastogenesis. Since RESV increases osteoblast's proliferation and differentiation [8,25,26] similarly, PTERC-T also could increase bone formation through enhancing osteoblastogenesis and expression of osteoblast specific marker genes. Accordingly, *in vivo* treatment with PTERC-T is likely to regulate abnormally high bone turnover rate, leading to improvement of pathologic condition of osteoporosis. However, further *in vivo* experiments are needed to conclusively elucidate the bioavailability status of this compound.

From the above findings, it can be concluded that PTERC-T inhibits RANKL and breast cancer cell-induced osteoclastogenesis *in*

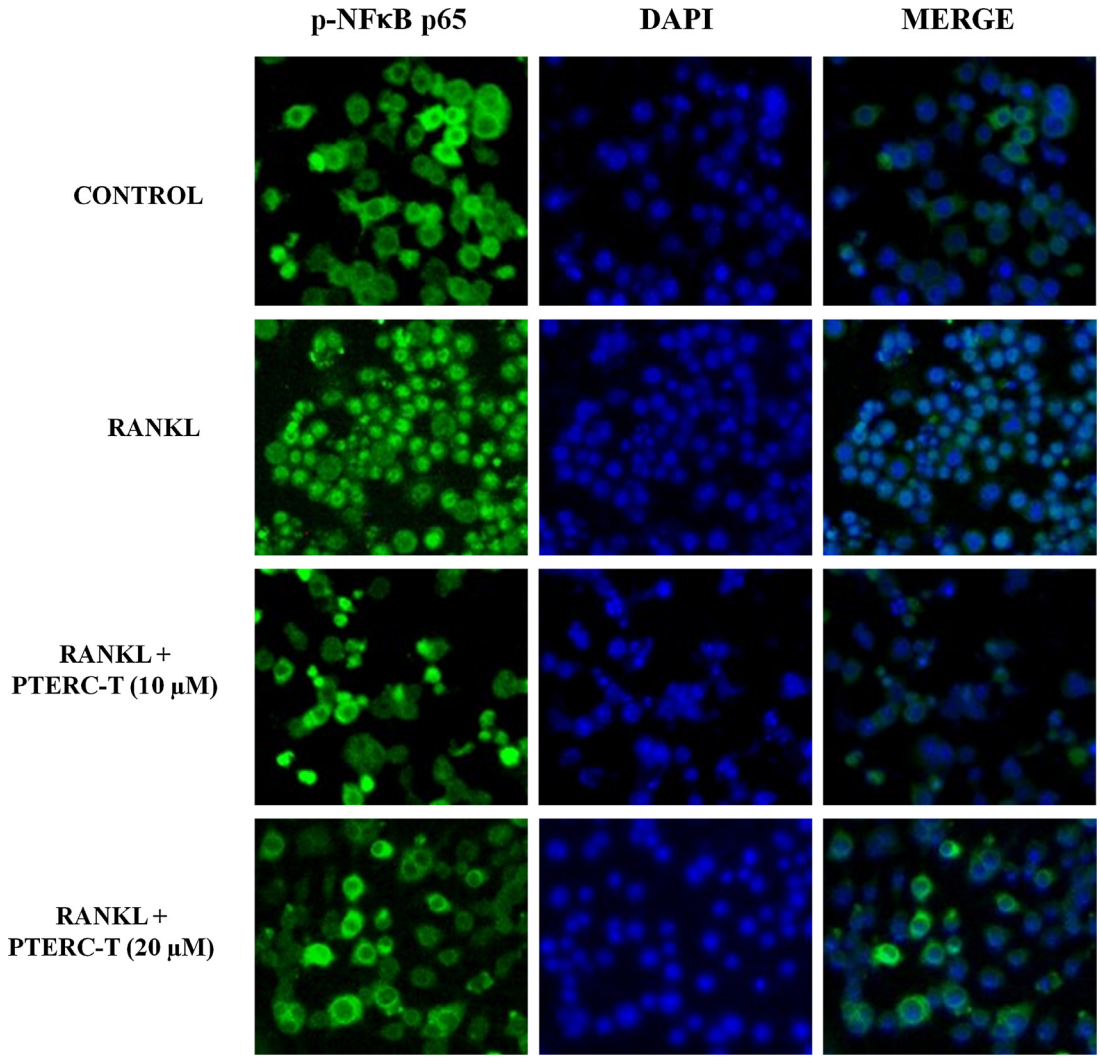

Fig. 5. PTERC-T inhibits RANKL induced NFκB nuclear translocation. Nuclear translocation of p-NFκB p65 was detected by immunofluorescence analysis in cultured RAW264.7 cells. RAW264.7 cells were incubated with 10 and 20 μM PTERC-T for 2 h, and then treated with 50 ng/ml RANKL for 30 min. Immunofluorescence staining was performed to locate p-NFκB p65 (green). Nuclear counter-staining was performed using DAPI (blue). Magnification, 200×. (For interpretation of the references to color in this legend, the reader is referred to the web version of the article.)

vitro. PTERC-T prevents RANKL-induced activation of MAPKs and transcription factors such as NFκB, c-Fos and NFATc1. Although additional experiments are needed to confirm the efficacy of PTERC-T in treating disease conditions *in vivo*, our results indicate that PTERC-T may be a therapy for disorders associated with bone loss after further validations based on the current data.

Conflict of interest

The authors have no conflict of interest to declare.

Funding

This work was supported by research grants from Council for Scientific and Industrial Research and Ministry of Human Resources and Development (MHRD), Government of India as research fellowships to KN and project assistantship to PR, respectively.

Acknowledgements

The authors would like to convey their sincere thanks to Srinivasa Rao Palla and Dr. Rama Krishna Peddinti of Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee, India for providing synthetic pterostilbene carboxaldehyde thiosemicarbazone (PTERC-T) for this study. The authors would also like to thank Reseapro Scientific Services (P) Ltd., India, for English editing of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pharep.2015.05.009.

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