Pharmacological Reports xxx (2015) xxx–xxx

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 xxx

*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

Q2 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 nuclear factor-kB (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 antioxidant, 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

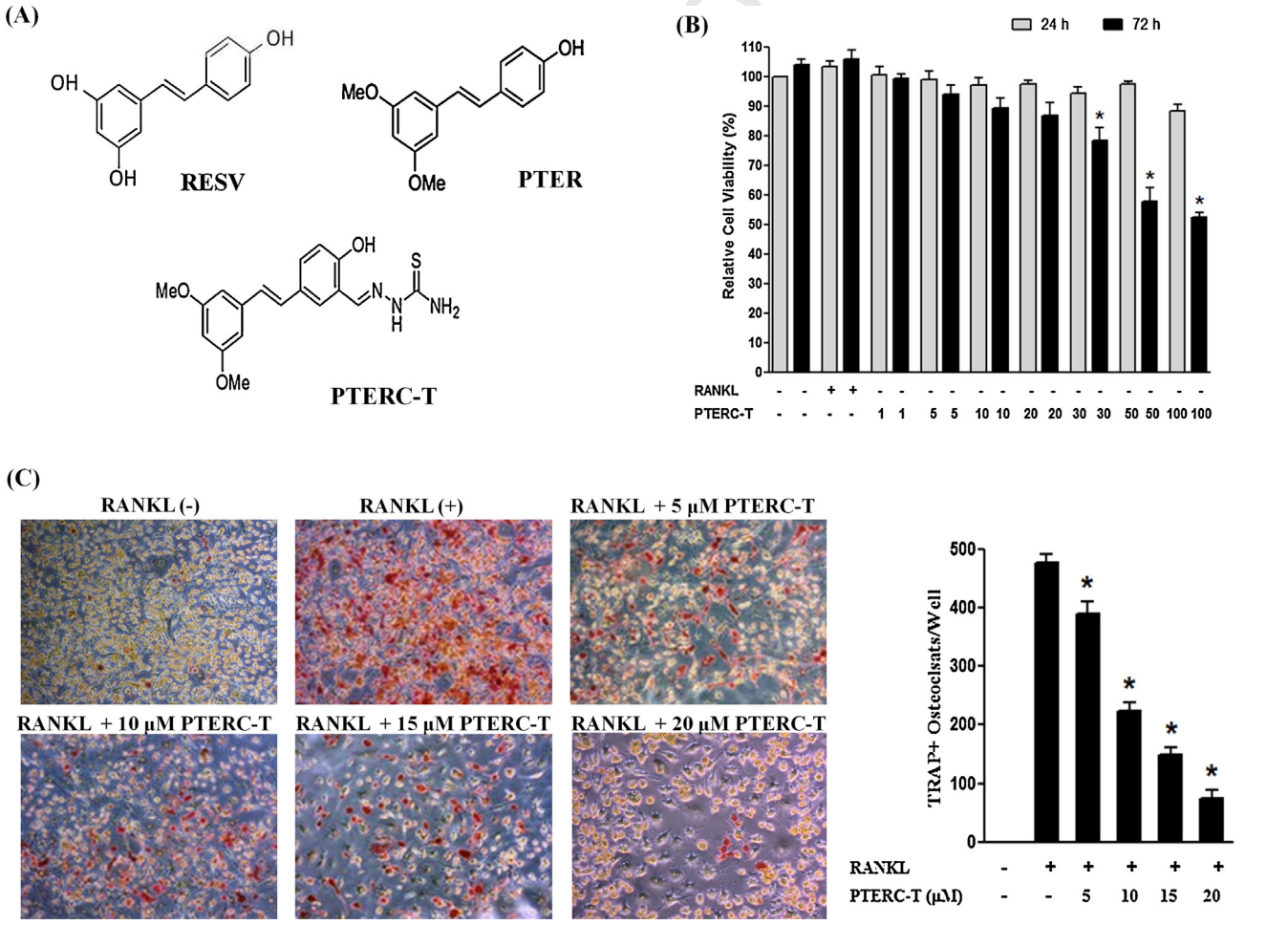


**Fig. 1.** PTERC-T inhibited RANKL induced osteoclastogenesis of RAW264.7 cells without significant toxicity. (A) Molecular structure of RESV, PTER, and PTERC-T. (B) Effect of PTERC-T on cell viability in RAW264.7 cells. The cell viability was determined by using MTT assay, and the results are expressed 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; $p < 0.05$.

Please cite this article in press as: Nikhil K, et al. A pterostilbene derivative suppresses osteoclastogenesis by regulating RANKL-mediated NFκB and MAPK signaling in RAW264.7 cells. Pharmacol Rep (2015), http://dx.doi.org/10.1016/j.pharep.2015.05.009