

## Inhibition of bone resorption by Tanshinone VI isolated from *Salvia miltiorrhiza* Bunge

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### Abstract

During the last decade, a more detailed knowledge of molecular mechanisms involved in osteoclastogenesis has driven research efforts in the development and screening of compound libraries of several small molecules that specifically inhibit the pathway involved in the commitment of the osteoclast precursor cells. Natural compounds that suppress osteoclast differentiation may have therapeutic value in treating osteoporosis and other bone erosive diseases such as rheumatoid arthritis or metastasis associated with bone loss. In ongoing investigation into anti-osteoporotic compounds from natural products we have analyzed the effect of Tanshinone VI on osteoclasts differentiation, using a physiologic three-dimensional osteoblast/bone marrow model of cell co-culture. Tanshinone VI is an abietane diterpene extracted from the root of *Salvia miltiorrhiza* Bunge (Labiatae), a Chinese traditional crude drug, "Tan-Shen". Tanshinone has been widely used in clinical practice for the prevention of cardiac diseases, arthritis and other inflammation-related disorders based on its pharmacological actions in multiple tissues. Although Tanshinone VI A has been used as a medicinal agent in the treatment of many diseases, its role in osteoclast-related bone diseases remains unknown. We showed previously that Tanshinone VI greatly inhibits osteoclast differentiation and suppresses bone resorption through disruption of the actin ring; subsequently, we intended to examine the precise inhibitory mechanism of Tanshinone VI on osteoclast differentiating factor. This study shows, for the first time, that Tanshinone VI prevents osteoclast differentiation by inhibiting RANKL expression and NF $\kappa$ B induction.

The strength and integrity of the human skeleton depends on a delicate equilibrium between bone resorption and formation. Bone resorption is an elementary cellular activity in the modelling of our skeleton during growth

and development. Later in life a most important physiological process in the skeleton is bone remodelling, which is locally initiated by resorption. During remodelling bone resorption is coupled to new bone formation that ensures renewal of bone with only minor local and temporary bone loss. Cells responsible for bone resorption and subsequent bone formation are osteoclasts and osteoblasts, respectively.

The osteoclast is derived from the pluripotent hematopoietic stem cell, which gives rise to a myeloid stem cell that can further differentiate into megakaryocytes, granulocytes, monocytes/macrophages and osteoclasts. The respective bone resorbing and forming actions of osteoclasts and osteoblasts are finely coupled, so that in a healthy adult the bone mass remains remarkably stable. Imbalances between osteoclast and osteoblast activities can arise from a wide variety of hormonal changes or perturbations of inflammatory and growth factors, resulting in postmenopausal osteoporosis, Paget's disease, lytic bone metastases, or rheumatoid arthritis, leading to increased bone resorption and crippling bone damage.<sup>1</sup> Remarkable progress has been made in recent years on elucidating the molecular mechanisms of osteoclast differentiation and activation, especially the discovery of an osteoclast differentiation factor, i.e. receptor activator for nuclear factor  $\kappa$ B ligand (RANKL).<sup>2</sup>

RANKL, a novel member of tumour necrosis factor (TNF) family of cytokines, is produced in various tissues and is especially abundantly expressed in bone and in lymphoid tissues.<sup>3</sup> RANK/RANKL interaction on the surface of osteoclasts and their precursor cells triggers signalling through several enzymatic pathways, leading to activation of some adaptor proteins such as TRAF-6,<sup>4</sup> TAB-2, IRAK 1-3 and Src, that activate Akt, AP-1 and NF- $\kappa$ B.<sup>5</sup> Within these transcription factors, RANKL specifically induces expression of the NFAT family member NFATc1, which is the master regulator of OC differentiation. This induction is dependent on NF- $\kappa$ B pathway via RANKL-RANK stimulation.<sup>6</sup> The NF- $\kappa$ B signal transduction pathway has long been recognized as critical for osteoclast development and function<sup>7,8</sup> and double knock-out of p50 and p52 NF- $\kappa$ B subunits leads to osteoporosis, due to a severe defect in osteoclast differentiation in these mice.<sup>7</sup> Consequently, this pathway regulates many osteoclast specific genes, such as tartrate-resistant acid phosphatase (TRAP), which results in the activation of enzymes that mediate the secretion of various substances, thus leading to the final step of matrix and mineral dissolution.<sup>9</sup> During the last decade, a more detailed knowledge of these molecular mechanisms involved in

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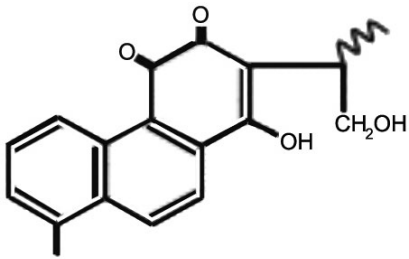
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osteoclastogenesis has driven research efforts in the development and screening of compound libraries of several small molecules that specifically inhibit these pathways. Natural compounds suppressing osteoclast differentiation may have therapeutic value in treating osteoporosis and other bone erosive diseases, such as rheumatoid arthritis associated with bone loss.<sup>10</sup> In ongoing investigation into anti bone resorption compounds from natural products, we have analyzed the effect of Tanshinone VI, an abietane diterpene extracted from the root of *Salvia miltiorrhiza* Bunge (Labiatae), a Chinese traditional crude drug, "Tan-Shen". Tanshinone has been widely used in clinical practice for the prevention of cardiac diseases, arthritis and other inflammation-related disorders based on its pharmacological actions in multiple tissues. *Salvia miltiorrhiza* extract contains several diterpene derivatives, such as Tanshinone IIA<sup>11-15</sup> and Tanshinone VI (Tan) (Figure 1) that have some pharmacological activities on cardiac fibroblasts, cardiomyocytes etc.; however, their effects on osteoclast differentiation and function are unknown.

The crude Tanshinone VI used in the present investigation was obtained by extraction with ethanol-n-hexane (1:1, v/v) from *S. miltiorrhiza* Bunge. Preparative HSCCC with the two-phase solvent systems A composed of n-hexane-ethanol-water (10:5.5:4.5, v/v) and B composed of n-hexane-ethanol-water (10:7:3, v/v) was successfully performed in a stepwise elution yielding six relatively pure diterpenoids from 300 mg of the crude extract in a single run.

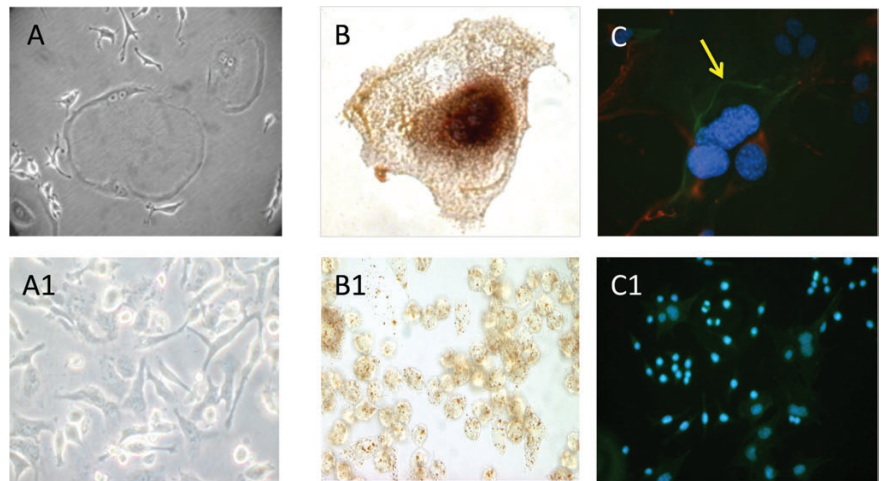
To test the effect of Tanshinone VI on osteoclast differentiation we used a physiologic three-dimensional osteoblast/bone marrow model of cell co-culture.<sup>16</sup> Osteoclasts were



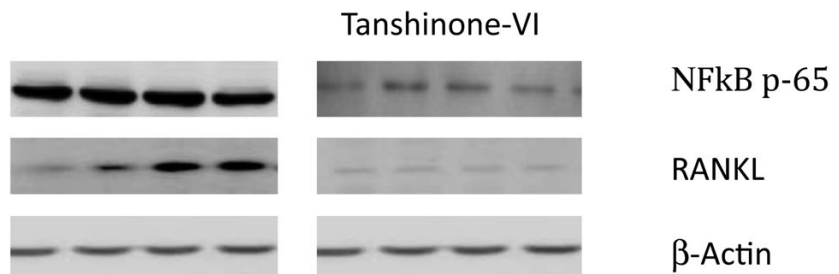
**Figure 1.** Chemical structure of Tanshinone VI extracted from Danshen (*Salvia miltiorhiza*).

identified in the mixed population derived from a murine co-culture model, developed using monocyte-macrophage cell line RAW 264.7 and osteoblast cell line type CRL 12257, that form mature osteoclasts within 4 days in absence of exogenous cytokines.<sup>16</sup> In order to examine viabilities of cells involved in co-culture model in response to Tanshinone VI, cells were treated with 10 ng/mL for 4 days with Tanshinone VI, according with previous works.<sup>14,15</sup> The effect of Tanshinone VI revealed a significant inhibitory effects on osteoclast differentiation (Figure 2 A-A1), demonstrated as an inhibitory effect on formation of TRAP-positive multinucleated osteoclasts (Figure 2, B-B1). In order to exclude this indirect effect, we used the culture condition under which osteoclasts are differentiated from bone marrow-derived macrophages in the presence of M-CSF plus RANKL without the support of osteoblast cells (*data not shown*). In this culture, the inhibitory effect of Tanshinone VI on osteoclastogenesis was also observed. With the aim to investigate the effect of Tanshinone VI on functionality of osteoclast, we have analyzed by immunohistochemistry the formation of the actin ring that is a pre-requisite for osteoclasts bone resorption.<sup>16-18</sup> Staining F-actin with phalloidin showed a ring structure in mature osteoclasts purified from control co-culture characterized by clear margin (Figure 2 C, yellow arrow). On the opposite, Tanshinone VI treatment affects the actin ring structure, resulting as a loose and fuzzy structure (Figure 2, C1).

Looking at the expression of RANKL and Nf  $\kappa$ B, it was found that both significantly decreased in co-culture treated with Tanshinone VI in respect of vehicles (Figure, 3). These results suggest that Tanshinone VI may affect RANKL-induced signalling involved in osteoclastogenesis. Based on these data, we hypothesize that pharmacological suppression of NF- $\kappa$ B and RANKL induced by Tanshinone VI *in vivo* may be an effective approach to improve bone loss suppressing osteoclastic bone resorption.



**Figure 2.** Effects of Tanshinone VI (10 ng/mL) on osteoclast differentiation and maturation. A-A1. Phase contrast observation of cell untreated characterized by the presence of multinucleated cell (A) *vs* cells treated with Tanshinone VI that are characterized by undifferentiated mononuclear cells (A1). B-B1. Immunoenzymatic assay for TRAP reaction revealed that cell untreated (B) evidenced a consistent positivity to TRAP reaction in contrast to cell treated with Tanshinone VI (B1). C-C1. Immunocytochemical analysis of actin ring. Immunocytochemistry demonstrated that untreated cells are characterized by a functional actin ring (high functionality-yellow arrow) in contrast to its total absence on cells treated with Tanshinone VI. Scale bar 50  $\mu$ m.



**Figure 3.** Immunoblotting assay. Cells were pre-treated with Tanshinone VI (10ng/mL) for 4 days. After treatment protein was extracted and RANKL and NFKB proteins were determined by western blotting. The analysis revealed that Tanshinone VI reduced the Nf  $\kappa$ B induction and Expression of RANKL over all time periods (day 1, day 2, day 3, day 4) that are key factors in the osteoclastogenesis differentiation and maturation.

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