The Therapeutic Effect and Mechanism of Actions of Natural Compounds in Osteoporosis

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DOCTOR OF PHILOSOPHY

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DECLARATION

This is to certify that all the work contained herein was performed by myself, except where indicated otherwise.

__________________
Lin Zhou

__________________
W/Prof. Jiake Xu

__________________
Dr Jennifer Tickner
Bone is a dynamic organ, which is continuously remodeling and changing, and thus maintaining an internal homeostasis. This homeostasis involves the balancing of a variety of bone cells, such as osteoclasts, osteoblasts, osteocytes and chondrocytes. Among these cells, osteoclasts and osteoblasts are most important. The imbalance of bone resorption by osteoclasts and bone formation by osteoblasts leads to a series of disease, such as osteoporosis, osteopetrosis and Paget’s disease.

Osteoclasts are large-sized multinucleated cells which originate from hemopoietic stem cells. The over-activation of osteoclasts is the main pathogenic factor of osteoporosis. The interaction of receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) and RANK results in the activation of a series of transcription factors, such as NF-κB, Mitogen Activated Protein kinase (MAPK) and Nuclear Factor of Activated T-cell cytoplasmic 1 (NFATc1), ultimately leading to the formation of mature osteoclasts.

In order to identify the new potential treatments against bone loss, we screened hundreds of natural compounds isolated from both native Australian plants and from a Chinese natural chemical library. This study has identified several natural compounds which have inhibitory effects on osteoclasts in vitro and/or in vivo. Among these effective compounds, the present study mainly focuses on four natural compounds, including berberine sulfate, dihydroartemisinin, fangchinoline and cumambrin A. The screening results also contributed to some papers published by my colleagues. These related compounds include SC514, triptolide, andrographolide and eriodictyol, which are listed in the appendix.

Osteoclastogenesis assay and luciferase reporter gene assay suggested that berberine sulfate, dihydroartemisinin, fangchinoline and cumambrin A could inhibit formation and differentiation of osteoclasts in a dose dependent manner. In addition, the four compounds could both inhibit NF-κB and NFAT activity through luciferase reporter gene assay. Consistently, the four compounds could significantly suppress the level of osteoclast marker genes expression, such as calcitonin receptor (CTR), cathepsin K (CTSK), tartrate-resistant acid phosphatase (TRAcP), Matrix Metalloproteinase 9
(MMP-9), NFATc1 and V-ATPase d2. The effects of the compounds on the function of osteoclasts were determined by bone resorption assay. The results suggested that the resorption activity of osteoclasts were significantly suppressed by these compounds.

Next, the effects of the above compounds on RANKL-induced signal pathways were further examined by western blot assay. The results showed that berberine sulfate and fangchinoline inhibited the phosphorylation of MAPKs, and the protein expression level of V-ATPase d2 and NFAT, while dihydroartemisinin and cumambrin A also suppressed the degradation of IκBα.

In order to further evaluate the effect of compounds on protecting against bone loss, an ovariectomized (OVX) mouse model was applied to study dihydroartemisinin, fangchinoline and cumambrin A. Consistent with in vitro study, these compounds protected against OVX-induced bone loss with a significant increase of BV/TV and trabecular number, and a significant decrease of trabecular separation. Thus, these compounds inhibited osteoclast formation and differentiation in vitro and prevented OVX-induced bone loss in vivo.

Previous studies have found that these natural compounds have anti-tumor, anti-inflammatory, anti-hypertensive and anti-leukemic activities. The present study has showed the anti-osteoclast effects of these compounds. Taken together, this study may help to find new potential treatments against bone disease caused by over-activated osteoclasts, such as osteoporosis.
ACKNOWLEDGEMENTS

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I also wish to thank UWA China Scholarships for support me my living and tuition fees during these years. It is very fortunate for me to get this financial support and make my live much easier.

It is also important for me to acknowledge my colleagues. Mingli Yang worked with me side by side during these four years. Jinbo Yuan helped me with MicroCT scan. Dian Teguh, Benjamin Ng, Vincent Kuek, Baysie Lim, gave me a lot of advice and support. I would also like to extend my heartfelt gratitude to colleagues and fridends, such as Fangming Song, Gaurav Jadhav, Jacob Kenny, Chao Wang, Heng Qiu. Thank you all for your friendships and support to my work. It is also a funny time to work with all of you over these years.

I can’t presume my dream without love and support from my family. I feel very fortunate to have such open minded parents. Thanks for your unconditional love and endless support for me. Without that, none of the work is possible. My deepest thanks
are reserved for my beloved husband, Baishen Wei. Your love and support have made this stressful and hard time bearable.

Last but not least, I would like to thank all those who helped me directly or indirectly to complete my PhD study.
**PUBLISHED PAPERS**


PUBLISHED ABSTRACTS


Zhou Lin, Yang Mingli, Tickner Jennifer, Xu Jiake. (2015) “Cumambrin A prevents osteoclastogenesis and bone resorption via the Regulation of
RANKL-induced Signalling Pathways” Australian and New Zealand Orthopaedic Research Society 21st annual meeting, Hobart, Tasmania. (Oral presentation)
# ABBREVIATION

The following abbreviations are used throughout this thesis:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>18S</td>
<td>18s ribosomal RNA</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Alpha modified eagles medium</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMM(s)</td>
<td>Bone marrow macrophage(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BV</td>
<td>Bone volume</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CTR</td>
<td>Calcitonin receptor</td>
</tr>
<tr>
<td>CTSK</td>
<td>Cathepsin K</td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>Dendritic cell-specific transmembrane protein</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DHA</td>
<td>Dihydroartemisin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAse</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotheritol</td>
</tr>
<tr>
<td>D2</td>
<td>V-ATPase d2</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HSCs</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>IκBα</td>
<td>Inhibitor kappaB α</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R-associated kinases</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>JNK</td>
<td>C-jun N-terminal kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>micro-CT</td>
<td>Micro computed tomography</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>Na₃VO₄</td>
<td>Sodium vanadate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NFATc1</td>
<td>Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OB</td>
<td>Osteoblast</td>
</tr>
<tr>
<td>OC</td>
<td>Osteoclast</td>
</tr>
<tr>
<td>OCL</td>
<td>Osteoclast-like cells</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Oligo</td>
<td>Oligonucleotide</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>OSCAR</td>
<td>osteoclast-associated receptor</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomized</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>p-ERK</td>
<td>Phosphorylated-ERK</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKCδ</td>
<td>protein kinase Cδ</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>p-Src</td>
<td>phosphorylation of c-Src</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor κB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RNAsin</td>
<td>Ribonuclease inhibitor</td>
</tr>
<tr>
<td>ROI</td>
<td>Reactive oxygen intermediate</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>Runx</td>
<td>Runt-related transcription factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SERMs</td>
<td>Selective estrogen receptor modulators</td>
</tr>
<tr>
<td>SLs</td>
<td>sesquiterpene lactones</td>
</tr>
<tr>
<td>TAB</td>
<td>TGF beta-activated protein kinase 1-binding protein</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF beta-activated kinase</td>
</tr>
<tr>
<td>Tb.N</td>
<td>Trabecular number</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Tb.Sp</td>
<td>Trabecular space</td>
</tr>
<tr>
<td>Tb.Th</td>
<td>Trabecular thick</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-beta activated kinase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>Topo</td>
<td>Topoisomerase</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>TRAcP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TV</td>
<td>Tissue volume</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar-H⁺ ATPase</td>
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<th>Title</th>
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Chapter 1
Osteoclast biology
### 1.1 Introduction

Bone is a dynamic tissue, which provides a stable frame for our body. The role of bone is for movement, protection and support of our body. It is also a hemopoietic organ and storage place for minerals, mainly calcium and phosphate. Organics and inorganics including calcium and phosphate make the bone have characteristics of both toughness and hardness. Bone remodelling continues through lifetime, allowing our body to grow and endure mechanical damage (Raisz L. G. 1999). The process involves several types of cells, including osteoclasts, osteoblasts, osteocytes and chondrocytes, but the most important actions are bone resorption by osteoclasts and bone formation by osteoblasts (Raggatt L. J. and N. C. Partridge 2010). Osteoblasts also affect osteoclast regulated bone resorption by secreting Receptor activator of nuclear factor kappa-B ligand (RANKL) to stimulate the formation and differentiation of osteoclasts (Tanaka Y. et al. 2005). The imbalance of bone resorption and bone formation causes bone diseases, including osteoporosis, Paget’s disease of bone, osteopetrosis and rickets (Feng X. and J. M. McDonald 2011).

Among these bone remodelling disorder related diseases, osteoporosis is most common. According to Australia Health Survey by self-reported diagnosis of osteoporosis, people over 50-year old have a percentage of 15% of women and 3% of men who got osteoporosis. Another recent survey carried out by measuring bone density reported that people over 50-year old have a percentage of 23% of women and 6% of men (Australian Institute of Health and Welfare . 2014). Ethnicity also affects osteoporosis; a 1-year cohort study which surveyed nearly $200 000 postmenopausal American women reported that black American women had highest bone mineral density (BMD) and had reduced risk of fracture (Barrett-Connor E. et al. 2005). Osteoporosis is a huge burden to society. An Osteoporosis Australia study reported that the estimated total costs of osteoporosis is AUD $2,754 million in 2012 all over Australia (Watts J.J. et al. 2013).

Osteoblasts are the single nucleated cells that produce new bone. Osteoblasts are usually on the surface of bone. The active osteoblasts are fat and cuboidal; while the inactive
osteoblasts are flattened. Osteoblasts embedded in bone matrix are osteocytes. Osteoblasts arise from mesenchymal stem cells through two different ways: intramembranous ossification and endochondral ossification (Olsen B. R. et al. 2000). The function of osteoblasts is to deposit unmineralised osteoid and subsequent mineralization of this organic matrix to form bone. Osteoblasts orchestrate bone formation through secreting several extracellular proteins, such as osteocalcin, alkaline phosphatase, type I collagen, proteoglycan, bone sialoprotein and osteopontin (OPN) (Rattner A. et al. 2000; Aubin J. E. 2001; Hall B. K. 2005). The overactivity of osteoblasts results in forming too much bone tissue, which is a hallmark of several diseases including Paget’s disease of bone and osteopetrosis; while the lack of osteoblasts lead to damage of bone formation occurs in osteoporosis and osteolytic diseases (Raisz L. G. 1999; Feng X. and J. M. McDonald 2011).

Osteoclasts originate from hematopoietic stem cells and are characterized by having a large cell size and more than one nucleus. Osteoclasts also have a ruffled membrane and sealing zone (Teti A. et al. 1991), and express tartrate-resistant acid phosphatase (TRAcP). The function of osteoclasts is to remove old or injured bone, and thus clear out space for osteoblasts to form new healthy bone (Pierce A. M. et al. 1991; Miyamoto T. and T. Suda 2003). However, excessive bone resorption causes many bone diseases, of which main one is osteoporosis (Woo J. T. et al. 2008). Thus, this study focused on osteoclasts as a target for treating osteoporosis. This chapter will elaborate the formation, differentiation and function of osteoclasts, and related operating signalling pathways.
1.2 Origin of osteoclasts

The term “osteoclast” is the combination of osteo- (from Greek word osteon, meaning bone) with –clast (from Greek word klastos, meaning broken). Since osteoclasts were first described in 1873, the origin of osteoclasts has been debated. After a century of controversy, it was gradually acknowledged that osteoclasts arise from the monocyte phagocytic system (Nijweide P. J. et al. 1986). It has now been further understood that osteoclasts are the fusion of macrophages and are derived from the hematopoietic stem cells (HSCs) in the bone marrow (Hayase Y. et al. 1997; Teitelbaum S. L. 2000).

1.3 Osteoclast morphology

Osteoclasts can be easily separated from other cells by their large size and multiple nuclei. The size of osteoclasts is often from 30 µm to 100 µm, but can be up to 150 µm; the number of nuclei is usually from 3-10, but can be range from 2-100 (Basle M. F. et al. 1988; Jain N. and R. S. Weinstein 2009; Vincent C. et al. 2009). The large size of osteoclasts allow osteoclasts to contain a great amount of cell organelles, such as endoplasmic reticulum, mitochondria, Golgi apparatus as well as lysosomes, vesicles, vacuoles, which endow osteoclasts with the function of energy production, protein synthesis and secretion (especially for lysosomal enzyme), and vesicular transport abilities. Vacuoles containing lysosomes are filled with TRAcP, making osteoclasts available to be visualized by staining for this enzyme (Holtrop M. E. and G. J. King 1977; Vaananen H. K. et al. 2000).

The active osteoclasts which are resorbing bone on the bone surface have two arresting features, which are the ruffled border and clear sealing zones (Suda T. et al. 1992; Stenbeck G. 2002) (Figure 1.1). The ruffled border is a domain on the cell membrane of osteoclasts which is sealed by a ring-like structure. The ruffled border sits at the bone surface and is situated on the resorbed cavity (Baron R. 1989; Salo J. et al. 1997). The ruffled border can be very extensive and secretes acid and transports materials into the bone resorption compartment. The formation and expansion of the ruffled border require an important molecule, αvβ3 integrin, which facilitates osteoclasts adhesion to
the bone surface and regulates the bone resorption function of osteoclasts (Nakamura I. et al. 2003). Mice lacking β3 showed irregular ruffled border and an osteosclerotic phenotype (McHugh K. P. et al. 2000). Vacuolar-H+ ATPase (V-ATPase), which is situated in the ruffled border, provides protons across the ruffled membrane to dissolve minerals of bone (Gay C. V. 1996). Lysosomal acid phosphatase (LAP)/TRAcP and matrix metalloproteinases (MMPs) are also located in the ruffled border and surrounding area (Delaisse J. M. et al. 2000; Suter A. et al. 2001).

The clear zone got its name by presenting a vacant area which is absent of organelles. The clear zone, which presents a ring-like structure, surrounds the ruffled border and seals the ruffled border from the area outside osteoclasts. Thus, the clear zone is also called the sealing zone (Holtrop M. E. and G. J. King 1977). αvβ3 integrin, a cell attachment molecule, is located in the clear zone. MMPs are also located in the clear zone, contributing to the degradation of bone matrix (Irie K. et al. 2001).
Figure 1.1 The morphology of osteoclasts. Ruffled border and clear/sealing zone are two obvious features of osteoclast structure.
1.4 Osteoclasts function

The major function of osteoclasts is to resorb bone by dissolution and removal organic and inorganic components. Active osteoclasts are located in Howship's lacunae, which is the cavity formed by osteoclasts resorbing bone. Upon stimulation, osteoclasts attach closely to bone surface to facilitate itself become polarization and form the specific structure of ruffled border and sealing zone. Osteoclasts degrade inorganic mineral, mainly calcium and phosphate, through secreting acid from the ruffled border, and also dissolve organic matrix of bone, primarily type I collagen, by producing cathepsin K and MMPs (Teitelbaum S. L. 2000). In addition, osteoclasts play a role in medullary hematopoiesis. Osteoclasts not only provide cavity for hypertrophic chondrocytes to form blood vessel at the presence of angiogenic factors and for the settlement of hematopoietic stem cells (HSCs), but also produce signalling molecules to regulate HSC niches (Blin-Wakkach C. et al. 2014). The reduction of osteoclasts and osteopetrosis together leads to extramedullary hematopoiesis (Takami M. 2011).

1.5 Formation of osteoclasts

The formation and differentiation of mature osteoclasts from bone marrow macrophages (BMM) requires the critical cytokines RANKL and Macrophage colony-stimulating factor (M-CSF) which are secreted by osteoblasts and stromal cells (Udagawa N. et al. 1999). These two cytokines both have critical roles to promote osteoclastogenesis, but with some distinctions. M-CSF is necessary for the survival, proliferation and differentiation of early progenitors (Feng X. 2005; Hodge J. M. et al. 2007); while RANKL contributes to the differentiation of later progenitors (osteoclast precursor) to mature osteoclasts. Osteoblasts also produce osteoprotegerin (OPG), which suppresses the differentiation of osteoclasts (Boyce B. F. and L. Xing 2007; Wright H. L. et al. 2009). Through RANKL/RANK/OPG, osteoblasts regulate the survival and differentiation of osteoclasts.

1.5.1 M-CSF
M-CSF, also known as colony stimulating factor 1 (CSF1), is an important cytokine to stimulate HSCs into monocyte/macrophage-lineage cells (Stanley E. R. et al. 1997). The physiological function of M-CSF is very extensive, including regulating bone metabolism, improving immune system, clearing lipoproteins, and supporting fertility and pregnancy (Hume D. A. and K. P. MacDonald 2012). M-CSF is secreted from osteoblasts, which is stimulated by parathyroid hormone. Stromal cells also release M-CSF, induced by inflammatory cytokines, such as TNF-α and IL-1 (Ross F. P. 2006). The role of M-CSF on osteoclastogenesis involves the survival, proliferation and differentiation of early progenitors into mature phagocytes. The enhancement role of M-CSF on osteoclasts is indicated by op/op mice, in which no effective M-CSF is produced. These mice show an osteopetrotic phenotype owing to the severe shortage of osteoclasts (Wiktor-Jedrzejczak W. et al. 1990; Kodama H. et al. 1991). Furthermore, the rescue study by which M-CSF was injected into op/op mice showed that osteopetrosis in these mutant mice were reversed (Wiktor-Jedrzejczak W. et al. 1991).

Colony-stimulating factor-1 receptor (CSF-1R, also named c-Fms), which is encoded by c-Fms proto-oncogene, is the only receptor for M-CSF on osteoclasts. This receptor is one of the growth factor family members, which have an intrinsic tyrosine-specific protein kinase function. The other well-known members of this family include insulin, and insulin-like growth factor-I (IGF-1), the receptors for epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) (Sherr C. J. et al. 1988). The role of c-Fms was confirmed by a study which reported that the mice lacking c-Fms oncogene presented an osteopetrotic phenotype similar to the op/op mice (Dai X. M. et al. 2002).

The interaction of M-CSF with its sole receptor c-Fms, results in a number of signalling cascades that are necessary for the formation of osteoclasts, including ERK1/2, PI3K, and c-Cbl. In addition, phospholipase C gamma (PLCγ) may also be involved in M-CSF induced osteoclastogenesis through releasing intracellular calcium (Newton A. C. 2001; Nakamura I. et al. 2003).

1.5.2 RANKL
RANKL, a type II transmembrane protein, also known as osteoprotegerin ligand (OPGL), TNF-related activation-induced cytokine (TRANCE), and osteoclast differentiation factor (ODF), is a critical factor for the formation and differentiation of osteoclasts. RANKL was first found to be a potential factor for osteoclastogenesis by two different groups almost at the same time in 1998 (Lacey D. L. et al. 1998; Yasuda H. et al. 1998). The effect of RANKL on osteoclastogenesis was then confirmed by a study which reported that mice lacking the RANKL encoding gene (Tnflsfl1) showed an osteopetrotic phenotype, due to the deficiency of mature osteoclasts (Kong Y. Y. et al. 1999). RANKL is produced from osteoblasts/bone marrow stromal cells, endothelial cells and T cells (Kular J. et al. 2012). RANKL could be detected in many organs and tissues, especially lung, thymus and lymph nodes. The other organs and tissues which could also express RANKL include bone marrow, stomach, and peripheral blood, (Wada T. et al. 2006). Although the expression of RANKL in bone marrow is relatively low, it exerts a pivotal role in bone metabolism.

RANKL consists of three parts, which are a membrane-anchoring domain, connecting stalk, and a receptor-binding ectodomain. RANKL structure shows a similar functional structure to other TNF cytokines. Each module of the trimeric structure contains two relatively conserved β sheets and super variable loops linking them, forming a specific β-sandwich jellyroll topologic structure. The role of first β sheet, which including strands A”, A, H, C, F, is to form the inner trimeric structure; while the role of the second β sheet, which including strands B’, B, G, D, E, is to constitute the outside surface structure. The loops formed by linking strands contribute to the attachment of the receptor (Liu C. et al. 2010).

RANK, a type I transmembrane protein, is the receptor of RANKL. RANK consists of 616 amino acids, of which 184 amino acids constitute the extracellular domain, 21 amino acids constitute the transmembrane domain, the remain amino acids constitute cytoplasmic domain (Schramek D. and J. M. Penninger 2011). The role of RANK in osteoclastogenesis is supported by a study reporting that mice lacking the RANK gene (Tnfrsf11a) are osteopetrotic (Li J. et al. 2000).
The interaction of RANKL with RANK activates a series of signalling pathways including JNK, ERK, p38, NFATc1, AKT, and NF-κB, through the recruitment of TNFR-associated factors (TRAFs), which are adaptor proteins, to RANK. The details of these signalling pathways will be discussed in the later part of this chapter.

In addition to inducing osteoclastogenesis, RANKL also has diverse roles in other tissues and organs. One study found that female mice lacking RANKL showed defective mammary gland development during pregnancy, resulting in the death of neonate babies (Fata J. E. et al. 2000). Further study showed that the RANKL-RANK signalling pathway could increase proliferation and decrease differentiation of mammary epithelial cells (Gonzalez-Suarez E. et al. 2007). These studies indicated that RANKL plays a role in breast development. Recent studies found that RANKL is also involved in regulating body temperature (Hanada R. et al. 2009), activating the immune response (Akiyama T. et al. 2012), and suppressing chronic Colitis (Totsuka T. et al. 2009). In addition, primary and metastatic bone tumours release RANKL, which in turn leads to further bone destruction, resulting in a viscous circle in tumour related osteolysis (Schramek D. and J. M. Penninger 2011).

1.5.3 OPG

Osteoprotegerin (OPG), which is encoded by the TNFRSF11B gene, is a cytokine of the TNF super family. OPG is also termed osteoclastogenesis inhibitory factor (OCIF). In addition to osteoblasts, OPG is also expressed by vascular smooth muscle cells (VSMCs) and endothelial cells (ECs). OPG is a polypeptide of 401 amino acids and analogous to secreted glycoprotein. OPG is a decoy receptor of RANKL, competitively antagonizing RANKL-RANK binding during osteoclastogenesis. The inhibitory function of OPG on osteoclastogenesis was indicated in a study which reported that transgenic mice overexpressing OPG present with an osteopetrotic phenotype (Simonet W. S. et al. 1997). Consistently, another study reported that the coculture of osteoblasts and BMM of OPG defective mice resulted in the formation of osteoclasts without the presence of other osteoclastogenic cytokines. Using this RANKL un-antagonistic model, this study also confirmed that RANKL functions as a membrane associated
protein as determined by the fact that osteoclasts only form in the presence of osteoblasts (Udagawa N. et al. 2000).

In addition, a high level of OPG was detected in the serum of patients with cardiovascular disease, suggesting the role of OPG as a peripheral biomarker of heart disease (Venuraju S. M. et al. 2010). OPG may also link with diabetic diseases, as female diabetics have higher serum OPG levels compared to healthy controls (Browner W. S. et al. 2001). In addition, increased quantities of OPG congregate in aorta of patients of diabetes (Olesen P. et al. 2005). These studies indicate the correlation between bone disease and heart disease, and bone disease with diabetes (Blazquez-Medela A. M. et al. 2011).
Fig.1.2 The formation of mature osteoclasts from osteoclast precursors. Osteoblasts/stromal cells release M-CSF and RANKL to induce the maturation of osteoclasts. Osteoblasts also regulate osteoclasts through secreting OPG as a decoy receptor of RANKL to inhibit osteoclast formation.
1.6 RANKL induced signalling pathways

Through the recruitment of the adaptor molecule TRAF6, the combination of RANKL and RANK induced many signalling pathways to promote osteoclast formation and functions. These signalling pathways include NF-κB, MAPKs (ERK, JNK, p38), NFATc1 and Akt (Wada T. et al. 2006). NF-κB is critical for RANKL/RANK induced signalling pathways not only in mice but also in humans. MAPKs, another well-known pathway of RANKL induced signalling molecules during osteoclastogenesis, is a big family which contains ERK1/2, JNK1/2 and p38. The downstream effector of MAPKs is the activator protein-1 (AP-1) transcription factor complex, of which the components c-Jun, JunB, c-Fos and Fra are important cytokines in promoting osteoclast formation and function. NFAT is the downstream of MAPKs and NF-κB for regulating osteoclastogenesis. Interestingly, MAPKs can also amplify themselves by recruiting transcription factors. Anti-apoptotic serine/threonine kinase (Akt/PKB,) which is regulated by Src kinase is a recently discovered pathway. Akt is an important molecule for the survival of osteoclasts (Wada T. et al. 2006; Akiyama T. et al. 2012).
Figure 1.3 RANKL/RANK induced signalling pathways. The main signalling pathways include NF-κB, MAPKs and Akt pathways.
1.6.1 RANKL-induced NF-κB pathway

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a pleiotropic protein which is involved in multiple physiological functions including immune regulation, inflammatory responses, the differentiation and survival of cells, and the growth and invasion of tumours (Oechinghaus A. and S. Ghosh 2009). The immune response to infection by NF-κB is operated through kappa-light-chain, which is an immunoglobulin like structure. Much later, NF-κB was also found to support synaptic plasticity and memory (Albensi B. C. and M. P. Mattson 2000).

The five subunits from the NF-κB family were classified into two groups, which are the NF-κB subfamily (which includes p50 and p52) and the Rel subfamily (which includes RelA [p65], RelB and cRel). All the family members share a common Rel homology domain (RHD), which is highly conserved at the N-terminus. The Rel subfamily proteins have C-terminal transactivation domains to activate transcription in very extensive species. The NF-κB subfamily are expressed as the precursor proteins NF-κB1 (p105) and 2 (p100), the C-termini of these proteins, which contain multiple copies of ankyrin repeats, undergo degradation to release the mature subunits p50 and p52 (Gilmore T. D. 2006).

The activation of NF-κB can be divided into two pathways, termed the canonical and non-canonical pathways. The canonical pathway is induced by RANKL, TNF and IL-1β, followed by the activation of IKK2 and IKKγ/NEMO, which results in the phosphorylation and subsequent proteolytic processing of IκB (p105) to release the mature subunit NF-κB1 (p50), and the activation of RelA (p65) and cRel. The non-canonical NF-κB pathway can also be induced by RANKL, as well as lymphotoxin-α and BAFF. Receptor activation is followed by the activation of IKK1 and NF-κB-inducing kinase (NIK), leading to the phosphorylation and subsequent proteolytic processing of NF-κB2 precursor (p100) to release the mature subunit NF-κB2 (p52), which then dimerizes with RelB (Otero J. E. et al. 2012).
The role of p50 and p52 in osteoclastogenesis were confirmed in a study in which the mice lacking p50 and p52 present osteopetrosis. In addition, the single knockout mice showed that p50 plays a major role in the differentiation of osteoclasts (Iotsova V. et al. 1997). When activated they bind to Rel proteins to form heterodimers, which then translocate to the nucleus to activate the transcription of a series of osteoclast downstream genes, including NFATc1, Cathepsin K, Calcitonin Receptor, V-ATPase d2, and MMP-9. These marker genes in turn regulate osteoclast formation and function.
1.6.2 RANKL-induced ERK pathway

Extracellular-signal-regulated kinase (ERK) is a member of the MAPK family. ERK is an important protein which connects the signals from receptors on the surface to gene transcription.

After external stimulation, the Ras-Raf-MEK cascade is activated, which then phosphorylates ERK1 and ERK2, which are related serine/threonine-specific protein kinases. MEK1/2 catalyzes the phosphorylation of ERK1 at Tyr204 followed by Thr202, and also catalyzes the phosphorylation of ERK2 at Tyr187 followed by Tyr185. The phosphorylation of ERK1/2 results in phosphorylation and activation of a series of cytoplasmic and nuclear factors to implement various physiological functions, such as gene transcription and expression, cell adhesion, mitosis, survival, proliferation and differentiation (Roskoski R., Jr. 2012).

One study reported that PD98059, a MEK1 inhibitor, enhanced apoptosis of osteoclasts and contributed to the loss of ruffled borders. This study reveals the role of ERK in the survival and formation of osteoclasts (Nakamura H. et al. 2003). The role of ERK on osteoclast formation and function were elaborated in a study which reported that ERK1/2 promoted osteoclastogenesis and bone resorption by using ERK1 defective mice and ERK2 defective mice. These mice have increased amount of bone mass compared to wild type mice. In addition, the role of ERK1 on osteoclast formation and function was much greater than ERK2 (He Y. et al. 2011).

1.6.3 RANKL-induced JNK pathway

c-Jun N-terminal kinase (JNK), also named as stress-activated protein kinase, is another member of the MAPK family. JNK was firstly recognized by its functions to phosphorylate c-Jun at Ser-63 and Ser-73 and its responses to external stress.

In the Ras-Raf-MEK cascade, MKK 4 and MKK7 are involved in the phosphorylation of JNK, resulting in the phosphorylation of c-Jun. There are three JNK genes which encode up to ten JNK isoforms. Both JNK1 and JNK2 are widely expressed in various
types of cells, and each has four isoforms. JNK3 has only two isoforms, and is only expressed in the central nervous system (CNS) neurons, cardiac smooth muscle, and testis (Waetzig V. and T. Herdegen 2005).

The aberrant activation of JNK is related to various diseases, such as neurological disorders (including Alzheimer’s disease and Parkinson’s disease), type 2 diabetes, obesity, and fatty liver disease (Cui J. et al. 2007).

JNK activated c-Jun phosphorylation exerts an important role in osteoclastogenesis (Shevde N. K. et al. 2000; Takayanagi H. et al. 2000). It was reported that osteoclast formation and activation were impaired in the BMM derived from JNK1 defective but not JNK2 defective mice through c-Jun and phosphorylation of c-Jun, suggesting that JNK1 is a critical protein in osteoclast formation and function (David J. P. et al. 2002).

1.6.4 RANKL-induced p38 pathway

p38, also termed as RK (CDC2-related protein kinase) or CSBP (Cytokinin Specific Binding Protein), is another member of the MAPK family. Similar to JNK, p38 is also activated by cytokines and environmental stress to exert multiple functions such as cytokine production and activation, cell survival, proliferation, differentiation and apoptosis.

There are four isoforms of p38, p38α (encoded by MAPK14), p38β (encoded by MAPK11), p38γ (encoded by MAPK12 / ERK6), and p38δ (encoded by MAPK13 / SAPK4). Notably, p38α and p38β, which are expressed in a variety of cells, share some common and distinct physiological actions, while p38γ and p38δ are only expressed in limited cell types and tissue, but have specialized roles (Zarubin T. and J. Han 2005; Cuadrado A. and A. R. Nebreda 2010).

Among the four isoforms, p38α is the only isoform which is expressed in osteoclasts, suggesting the possible role of p38α on osteoclasts. After the stimulation of TNFα and RANKL, p38α is activated by M KK3/6 through TRAF2 and TRAF6 respectively. Then, the activated p38α phosphorylates and activates p65, NFATc1, STAT1 and MITF to participate in the multiple cellular functions (Thouverey C. and J. Caverzasio 2015).
1.6.5 RANKL-induced NFAT pathway

Nuclear factor of activated T-cells (NFAT) is a transcription factor family that is important for immune responses. This family contains five members, which are NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), NFATc4 (NFAT3), and NFAT5 (Crabtree G. R. and E. N. Olson 2002).

Among these members, NFATc1 is highly induced by RANKL (Takayanagi H. et al. 2002). A study using gene expression analysis reported NFATc1 plays an important role in osteoclastogenesis. In this study, the gene expression of NFATc1 was closely related with the maturation of osteoclasts. In addition, the differentiation of osteoclasts was greatly impaired when NFATc1 was inhibited by introducing antisense NFATc1 (Ishida N. et al. 2002). In addition, the overexpression of NFATc1 in RAW cells enhances osteoclastogenesis, and the effects could be blocked by overexpression of dominant-negative c-Jun, suggesting c-Jun is critical in the RANKL-induced NFAT pathway (Ikeda F. et al. 2004).

NFATc1 is a downstream transcription factor of RANKL-induced signalling pathways. After the combination of RANKL and RANK, the recruited TRAF6 activates NF-κB and MAPK. MAPK then triggers the induction of AP-1 which consists of c-Fos, c-Jun, ATF and MAF protein families (Karin M. 1995). In addition, c-Fos can also be induced by RANKL (Takayanagi H. et al. 2002) and play a critical role in osteoclastogenesis since c-Fos knockout mice presented osteopetrosis due to the deficiency of mature osteoclasts (Wang Z. Q. et al. 1992). NF-κB and AP-1 then result in the induction of NFATc1.

In addition, calcium pathway is another major pathway for the induction of NFATc1. The binding of RANKL and RANK activates costimulatory receptors, which include osteoclast-associated receptor (OSCAR), triggering receptor expressed in myeloid cells-2 (TREM-2), paired immunoglobulin-like receptor-A (PIR-A), and signal-regulatory protein β1 (SIRPβ1). The ligands for the costimulatory receptors are unclear. Costimulatory receptors then associate with DAP12 and FcRγ, which are ITAM-harboring adaptor. The significant role of DAP12 and FcRγ on osteoclast
differentiation is indicated in a study, which showed that DAP12 and FcRγ double deficient mice developed severe osteopetrosis (Koga T. et al. 2004). The activated ITAM leads to the recruitment of SYK, resulting in the phosphorylation of PLCγ. The activated PLCγ causes the generation of IP3, which then binds to its receptor and subsequently trigger the Ca^{2+} release from the endoplasmic reticulum (Hwang S. Y. and J. W. Putney, Jr. 2011). The transient increased intracellular calcium causes calcium oscillation. Calcium oscillation then activates CAMKIV and calcineurin and leads to resultant induction of NFATc1. It is notable that calcium oscillation also contributes to the amplification of NFATc1 via recruitment of other transcription factors (NF-κB, NFATc2 and c-Fos). After activation, NFATc1 is translocated to the nucleus and then regulates the transcription of osteoclast specific genes, such as CTSK, V-ATPase d2 and TRAcP (Takayanagi H. 2007).
Figure 1.4 RANKL-induced NFATc1 pathway. This schematic describes the RANKL-induce NFATc1 signalling pathway in osteoclastogenesis.
1.6.6 RANKL-induced Akt pathway

Akt pathway or PI3K/Akt pathway plays an important role in cell survival and formation. This pathway is activated by both RANKL and MCSF, and has been proposed to enhance osteoclastogenesis (Stern P. H. 2007).

The activation of RANKL-induced Akt pathway is associated with TRAF6–Src–PI3-kinase interaction (Wong B. R. et al. 1999). Phosphatidylinositol 3-kinase (PI3K) family contains three types of members, which are class I, class II, and class III. Among these members, only class I is involved in lipid phosphorylation stimulated by extracellular stimulation. After activation, class I PI3K (which consists of regulatory subunit p85 and a catalytic subunit p110) catalyzes the addition of phosphate to the inositol ring of phosphoinositides, finally forming PI 3,4,5-triphosphate (PI (3,4,5) P3), which has a high affinity to Akt. After the binding of PI (3,4,5) P3 and Akt, the Akt was partially activated by phosphorylation at Thr308 through phosphatidylinositol-dependent kinase 1 (PDK1), or fully activated by phosphorylation at Ser473 through phosphatidylinositol-dependent kinase 2 (PDK2) (Cantley L. C. 2002).

Inhibition of PDK and Akt were found to effectively suppress osteoclastogenesis. In addition, the partially activated Akt, which are phosphorylated at Thr308 but not Ser473, participate in this RANKL-induced pathway during osteoclastogenesis (Kawasaki T. et al. 2012). A recent study showed similar results and also reported that the overexpression of Akt promotes the level of phosphorylated GSK3β and induction of NFATc1, thus providing evidence for the role of the PI3K/Akt/GSK3β/NFATc1 signalling axis in RANKL-induced osteoclastogenesis (Moon J. B. et al. 2012).

The six RANKL-induced pathways in osteoclastogenesis which has been discussed in chapter 1.6 could be summarized in Figure 1.5 (Leibbrandt A. and J. M. Penninger 2008).
Figure 1.5 RANKL-induced pathways promoting osteoclastogenesis. The combination of RANKL (membrane bound RANKL or soluble cleaved RANKL) and RANK induces the recruitment of TRAFs, and then activates NF-κB, Akt and MAPK pathways. NF-κB can be activated through canonical or non-canonical routes. MAPK family includes three members, which are JNK, p38 and ERK. All of them are important in osteoclastogenesis. Akt, which could be activated by RANKL and MCSF, is critical in survival of osteoclasts. Calcium signalling was activated by PLCγ, then triggers the induction of NFATc1 through calcineurin (Leibbrandt A. and J. M. Penninger 2008).
Chapter 2

Potential natural compounds for inhibiting osteoclasts formation and function
2.1 Introduction

Osteoporosis is a common geriatric disease affecting people all over the world. It is a huge burden to government and individuals. The costs of osteoporosis are, in many aspects, the social and personal costs for treating the disease and the costs for related fractures, the loss of labour force, as well as costs for patients care and residence support. In 2012, the total amount of these direct and indirect costs in Australia was estimated to be AUD $2,754 million (Watts J.J. et al. 2013).

The agents used for the treatment of osteoporosis can be divided into two categories: anti-resorptive drugs (bisphosphonates, estrogen and raloxifene) and anabolic drugs (parathyroid hormone, PTH). Although these drugs have significant effects on osteoporosis, the obvious side effects restrict their wide usage. Bisphosphonates can effectively increase BMD and reduce the risk of fracture (Eriksen E. F. et al. 2014). However, bisphosphonates are also associated with an increased relative risk of osteonecrosis of the jaw and atypical subtrochanteric femoral fractures (Shane E. 2010; Chamizo Carmona E. et al. 2013). Estrogen replacement is a common treatment for osteoporosis in post-menopausal women. The serious potential risks of estrogen include breast and uterine cancer, stroke, heart attacks and blood clots (Rossouw J. E. et al. 2002). Raloxifene is one of the selective estrogen receptor modulators (SERMs). It is effective in the treatment of osteoporosis, and reduces breast cancer which is one of the side effects of estrogen treatment (Chen J. H. et al. 2011). However, raloxifene increases the risks of stroke and heart disease (Taylor H. S. 2009). PTH comes in two forms: intact PTH (PTH1-84) and teriparatide (PTH1-34; TPTD). Although intact PTH and teriparatide have significant therapeutic effects in patients with severe osteoporosis, the duration of PTH therapy is restricted to 48 months due to the possible risk of osteosarcoma (Hodsman A. B. et al. 2005; Lippuner K. 2012).

RANKL-targeted therapy has become a new therapeutic method in the treatment of osteoporosis. Denosumab, a fully human anti-RANKL IgG2 monoclonal antibody, has been used for the treatment of osteoporosis and tumor-related osteolytic diseases (Lewiecki E. M. and J. P. Bilezikian 2012). Denosumab is now considered the optimal
treatment for osteoporosis because it is effective, specific, stable in the blood and relatively well tolerated (Josse R. et al. 2013; Yasuda H. 2013). The injection of OPG-Fc fusion protein (the recombinant form of mature OPG and an immunoglobulin Fc fragment of IgG1) was effective in preventing bone loss (Bekker P. J. et al. 2001; Body J. J. et al. 2003). However, the clinical use of OPG-Fc was limited due to the need for high doses and unwanted side effects on the immune system; this has led to it being replaced by denosumab (Boyce B. F. and L. Xing 2008).

Although denosumab has replaced bisphosphonates as the current frontline treatment for osteoporosis, its side effects such as the possible hypocalcaemia (Gupta A. and L. March) warrant the development of new safe and effective optional treatments. Natural compounds may provide alternative treatments for osteoporosis, since numerous natural compounds were found to be inhibitors of bone resorption and to protect against bone loss in OVX mice (Putnam S. E. et al. 2007). We screened a large number of natural compounds and identified some natural compounds that have potential ability for treating osteoporosis. The effects of the studied natural compounds on osteoclasts number were showed in Appendix.
2.2 Berberine sulfate

Berberine is extracted from the stems, bark, roots and rhizomes of berberis plants, which have been used as anti-microbial medicine in ancient China for treating dysentery and gastroenteritis (Pitea M. et al. 1972; Sturm S. and H. Stuppner 1998). Berberine significantly inhibits osteoclast formation and function in vivo and in vitro (Li H. et al. 1999; Hu J. P. et al. 2008; Wei P. et al. 2009). However, the absorption of berberine via the intestinal wall is very poor. Its apparent permeability co-efficient (Papp) across the intestinal tissue is in the order of only $10^{-7}$ cm/S (Baird Alan W. et al. 1997). After oral administration, berberine is typically undetectable in blood (Yu C. P. et al. 2012). The commonly used salt forms of berberine include berberine hydrochloride and berberine sulfate. The chemical structures of berberin, berberin hyfrochloride and berberine sulfates are shown in Figure 2.1. Berberine sulfate has better bioavailability than berberine since berberine sulfate is both stable and clearly detected in the blood after oral administration (Qiu F. et al. 2008). The solubility of berberine sulfate is greater than berberine hydrochloride (Luo Xiang et al. 2013). The solubility of drugs is one of the most crucial factors of bioavailability (Savjani K. T. et al. 2012). Thus, berberine sulfate has an advantage over other salt forms of berberine. Berberine sulfate is also effective in treating other diseases. For example, berberine sulfate was found to have obvious anti-bacterial effects (Sun D. et al. 1988; Sun D. et al. 1988), hence can be used in the treatment of diarrhea (Rabbani G. H. et al. 1987). Berberine sulfate was also found to inhibit LPS-stimulated myocardial TNF-α production, and hence is beneficial in the treatment of inflammation caused cardiac dysfunction (Yang J. et al. 2006), and also to increase survival of LPS-induced endotoxemia (Li F. et al. 2006). Berberine sulfate was also reported to sustain hemostatic systems by promoting blood coagulation (Ziablitskii V. M. et al. 1996) and has an anti-heparin effect (Sabir M. and N. K. Bhide 1971).
Figure 2.1 Chemical structures of the selected natural compounds and their analogues.
2.3 Dihydroartemisinin

Artemisinin, a powerful drug for treating malaria, is a sesquiterpene lactone which is produced from the leafy portions of *Artemisia annua*. The plant has been used to treat fever and malaria in ancient China for more than two thousand years (Klayman D. L. 1985). Dihydroartemisinin (DHA), a water-soluble semi-synthetic derivative of artemisinin (Lin A. J. et al. 1987; Tu Y. 1999), is combined with piperaquine for clinical usage (Ashley E. A. et al. 2005). DHA has been reported to be an effective anti-malaria drug (Ashley E. A. et al. 2005). The chemical structures of DHA and artemisinin are shown in Figure 2.1.

In addition to anti-malaria, DHA has been found to be an inhibitor of cancer cells, such as breast cancer cells (Singh N. P. and H. Lai 2001; Ericsson T. et al. 2014) and lung cancer cells (Liu L. K. et al. 2014; Zuo Z. J. et al. 2014). It can also suppress tumor angiogenesis (Jung M. et al. 2006). The inhibitory effects of DHA on different types of cancer cells are through regulating NF-κB, MAPK and AKT pathways (Chen H. et al. 2010; Hwang Y. P. et al. 2010; Ji Y. et al. 2011; Lee J. et al. 2012). Considering the known importance of these pathways in osteoclast formation and function, it is interesting to examine the effect of DHA on osteoclastogenesis.

2.4 Fangchinoline

Fangchinoline, an alkaloid, is produced from the tuberous root of *Stephania tetrandra S. Moore* and *Cyclea peltata Diels*, which are from *Menispermaceae* family (Huang K. C. 1993). The difference between Fangchinoline and its analogue tetrandrine is that fangchinoline has a hydroxyl group in one of the side chains of the isoquinoline ring, whereas tetrandrine has a methoxy group (Choi H. S. et al. 2000). This slight distinction gives the two compounds different medicinal effects. The chemical structures of fangchinoline and tetrandrine are shown in Figure 2.1. Fangchinoline and tetrandrine have been used in ancient China for treating inflammation for centuries.

Fangchinoline has been found to have various pharmacological properties in treating diseases. For example, fangchinoline presents anti-inflammatory effects through
inhibiting cyclooxygenase, and human interleukin-6 (Choi H. S. et al. 2000). Fangchinoline also inhibits histamine release (Nakamura K. et al. 1992), acts as an anti-oxidant (Gulcin I. et al. 2010) and provides neural protection through inhibition of glutamate release (Lin T. Y. et al. 2009). In addition, fangchinoline was reported to have anti-tumour activity in various cancer cells, such as breast cancer cells (Wang C. D. et al. 2014), lung cancer cells (Luo X. et al. 2016) and prostate cancer cells (Wang C. D. et al. 2010). Moreover, fangchinoline was also identified to be an inhibitor of HIV virus (Wan Z. et al. 2012). Inflammation promotes osteoclast formation and activity, thus, we tested the effects of fangchinoline on osteoclastogenesis.

2.5 Cumambrin A

Cumambrin A, isolated from Handelia trichophylla, is a member of the sesquiterpene lactones (SLs) family (Tarasov V. A. et al.). Sesquiterpene lactones (SLs) are characterized by a terpenes structure that consists of three isoprene units (sesquiterpene), and a lactone ring. SLs were used in our study as they have previously been shown to have anti-inflammatory activity through their ability to inhibit the NF-κB pathway (Rungeler P. et al. 1999). It was reported that parthenolide, a member of SLs family, suppresses osteoclastogenesis both in vitro and in vivo via inhibiting NF-κB activity (Yip K. H. et al. 2004). The structures of cumambrin A and parthenolide are shown in Figure 2.1.

Cumambrin A has not been deeply studied yet. It significantly suppressed NF-κB activity in our NF-κB luciferase reporter assay. Thus, it is interesting to study the effect of cumambrin A on osteoclastogenesis in our established system.

2.6 Other natural compounds

SC-514 is a synthetic cell-permeable (thienothienyl) amino-acetamide compound and has anti-inflammatory effects (Kishore N. et al. 2003). The chemical structure of SC-514 is shown in Figure 2.1. SC-514 is a selective inhibitor of IKKβ and does not affect other isoforms of IKK. IKKβ is one of the subunits of IKK which leads to the activation of NF-κB. It was reported that IKKβ is necessary for osteoclasts formation.
both in vitro and in vivo (Ruocco M. G. et al. 2005). The inhibition of IKKβ has been found to have anti-tumor and anti-inflammatory effects in many studies (Buss H. et al. 2004; Lee K. H. et al. 2013; Johnson J. et al. 2014). Thus, the effects of SC-514 on osteoclastogenesis should be studied.

Eriodictyol is a flavonoid derived from plants such as Citrus limon, and Eriodictyon californicum. The chemical structure of eriodictyol is shown in Figure 2.1. Eriodictyol has been demonstrated to have widespread pharmacological properties, such as anti-oxidant (Lee K. H. et al. 2013), anti-tumor (Habtemariam S. 1997) and anti-inflammatory activities (Lee J. K. 2011; Lee E. et al. 2013). Using our luciferase gene reporter assay, we found that eriodictyol significantly inhibited NF-κB activity. Thus, we studied the effect of eriodictyol on osteoclastogenesis.

Andrographolide is a bicyclic diterpenoid lactone which is derived from the stem and leaves of Andrographis paniculata. The chemical structure of andrographolide is shown in Figure 2.1. A. paniculata has been used in ancient China for centuries in the treatment of various diseases, such as diarrhea, dysentery, pharyngolaryngitis, cough with thick sputum, carbuncle, sores, cholecystitis, hypertension, scald, epistaxis and snake bites (Akbar S. 2011). The pharmacological functions of andrographolide include anti-asthmatic (Nguyen V. S. et al. 2015), anti-inflammatory (Abu-Ghefreh A. A. et al. 2009), anti-tumor (Zhang Q. Q. et al. 2014), and neuroprotective effects (Al Batran R. et al. 2013). In addition, andrographolide has been shown to prevent LPS-induced bone loss (Zhai Z. J. et al. 2014) and breast cancer-induced bone loss (Zhai Z. J. et al. 2014). However, estrogen deficiency-induced bone loss, which reflects postmenopausal osteoporosis, has not been studied yet.

Triptolide, a highly oxygenated diterpene triepoxide, is extracted from root and stem of Tripterygium wilfordii Hook f. This plant has strong anti-inflammatory and immunosuppressive properties and has been used to treat inflammatory lesions of leprosy, and patients with rheumatoid arthritis, but it is also quite toxic (Tao X. and P. E. Lipsky 2000). The chemical structure of triptolide is shown in Figure 2.1. Its pharmacological functions include anti-tumor (Kiviharju T. M. et al. 2002),
anti-leukemic (Chen J. et al. 2015), anti-inflammatory and neuroprotective effects (Bai S. et al. 2016). The effect of triptolide on osteoclastogenesis has not been studied yet, thus it is interesting to examine whether triptolide inhibits osteoclast formation and function in vitro and in vivo.

2.7 Summary of screened natural compounds

My preliminary data and previous studies suggested that natural compounds may be effective inhibitors of osteoclasts formation and function. Hence, the impacts of four compounds on osteoclasts formation and functions were studied in this thesis.
Chapter 3
Hypothesis and aims
Despite current available treatments, osteoporosis-related fractures still result in significant morbidity and mortality. The development of novel therapies is vital for improving therapeutic strategies against osteolytic bone diseases. We have identified several potential natural compound candidates for inhibiting osteoclast number using osteoclastogenesis assay. In this study, we aim to examine the effect of these natural compounds on osteoclast formation and the possible mechanisms by which natural compounds regulate osteoclastogenesis, as well as their therapeutic potential in osteolytic diseases in vivo.

In this study, we hypothesized that:

1. Natural compounds inhibit osteoclast formation in vitro.
2. Natural compounds inhibit osteoclast function in vitro.
3. Natural compounds suppress bone loss in vivo.

The aims of our study are:

1. Investigate the effects of natural compounds on the formation of osteoclasts by in vitro osteoclastogenesis assay.
2. Investigate the effects of natural compounds on the activity of NF-κB and NFAT by luciferase reporter gene assay.
3. Investigate the effects of natural compounds on the proliferation of BMM by MTS assay.
5. Determine the effects of natural compounds on osteoclast marker gene expression by reverse transcription (RT)-PCR.
6. Investigate the effects of natural compounds on RANKL-induced signaling pathways in osteoclasts by western blot analysis.

7. Investigate the potential therapeutic effects of natural compounds \textit{in vivo}. 
Chapter 4
Materials and methods
### 4.1 Materials

#### 4.1.1 General reagents and chemical reagents

<table>
<thead>
<tr>
<th>Reagents</th>
<th>MANUFACTURER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α,25-dihydroxyvitamin D3</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>β-glycerophosphate</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>30% Acrylamide/Bis solution</td>
<td>Bio-Rad Laboratories, Hercules, CA, USA</td>
</tr>
<tr>
<td>Acetone</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Acrylamide 99.9%</td>
<td>Bio-Rad Laboratories, USA</td>
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<tr>
<td>Agar Powder</td>
<td>BDH Laboratory Supplies, UK</td>
</tr>
<tr>
<td>Agarose Powder</td>
<td>Promega Corp, Madison, Wi, USA</td>
</tr>
<tr>
<td>Alcian Blue 8GX</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Alizarin Red S Monohydrate</td>
<td>MP Biochemicals Inc., USA</td>
</tr>
<tr>
<td>Aluminium Sulfate</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
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<tr>
<td>Ampicillin</td>
<td>Sigma Chemical Co., USA</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Baxter water (PCR grade water)</td>
<td>Baxter Healthcare Pty. Ltd, Australia</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Promega Corp., USA</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Citric acid (C₆H₈O₇)</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Supplier</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>ClearMount™ Mounting solution</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>DePeX mounting media</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Dimethyl sulphoxide (DMSO)</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Di-Sodium Phosphate (Na₂HPO₄)</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>DPX</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Eosin Y disodium salt</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Ethanol (100%)</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
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<tr>
<td>Ethylene tetra-acetic acid (EDTA) Disodium</td>
<td>Boehringer Ingleheim Corp, Indpl, IN, USA</td>
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<tr>
<td>Glacial acetic acid</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Glycerol</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Glycine</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Guanosine diphosphate</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Haematoxylin</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Supplier Information</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
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<tr>
<td>Leukocyte Alkaline Phosphatase Kit</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Lysozyme (Chicken egg white)</td>
<td>Sigma-Aldrich, USA</td>
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<tr>
<td>Methanol</td>
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</tr>
<tr>
<td>Multicore Buffer (10×)</td>
<td>Promega Corp, Madison, Wi, USA</td>
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<tr>
<td>Oligo dT</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Phenol red</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Phenylmethanesulfonyl fluoride (PMSF)</td>
<td>Boehringer Ingleheim Corp, Indpl, IN, USA</td>
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<tr>
<td>Phosphoric acid</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Prolong Gold Antifade Reagent</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td>Ringer solution</td>
<td>Baxter Healthcare Pty. Ltd, Australia</td>
</tr>
<tr>
<td>RNA sample loading buffer</td>
<td>Promega Corp, Madison, Wi, USA</td>
</tr>
<tr>
<td>RNAsin (ribonuclease inhibitor)</td>
<td>Promega Corp, Madison, Wi, USA</td>
</tr>
<tr>
<td>Skim Milk Powder</td>
<td>Standard supermarket brand</td>
</tr>
<tr>
<td>Sodium acetate trihydrate</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Chemical</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>BDH Laboratory Supplies, Poole, Dorset, England</td>
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<tr>
<td>Sodium orthovanadate (Na3VO4)</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Sodium phosphate, Dibasic, Anhydrous (Na2HPO4)</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Sodium phosphate, Monobasic, Anhydrous (NaH2PO4)</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
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<tr>
<td>Sodium tartrate dihydrate</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
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<tr>
<td>Streptavidin-horseradish peroxidase</td>
<td>Dako, Victoria, Australia</td>
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<tr>
<td>Tartaric acid</td>
<td>Sigma-Aldrich, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED)</td>
<td>Bio-Rad, USA</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma-Aldrich, USA</td>
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<tr>
<td>Trizma Base, Tris</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>TRIzol® Reagent</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Xylene</td>
<td>AnalaR, UK</td>
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<tr>
<td>Zinc Chloride</td>
<td>Sigma-Aldrich, USA</td>
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4.1.2 Cell line

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<tr>
<th>CELL LINE</th>
<th>DESCRIPTION</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW264.7</td>
<td>Murine monocyte cell line</td>
<td>The American Type Culture Collection (Rockville, MD, USA)</td>
</tr>
<tr>
<td>P3K</td>
<td>Stable RAW264.7 C4 cells transfected with a luciferase reporter gene 3κB-Luc-SV40 which contain three κB sites from the interferon gene.</td>
<td>They were generated as previously described (Wang C. et al. 2003).</td>
</tr>
</tbody>
</table>

4.1.3 Tissue culture regents

<table>
<thead>
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<th>DESCRIPTION</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-Modification of Eagles Medium (α-MEM)</td>
<td>Thermo, Electron, Sydney, Australia</td>
</tr>
<tr>
<td>Cell Dissociation Buffer</td>
<td>Sigma Chemical Co, St Louis, Mo, USA</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium (DMEM)</td>
<td>Thermo, Electron, Sydney, Australia</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>Invitrogen, New Zealand</td>
</tr>
<tr>
<td>GlutaMAX™</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>Hanks’ Balanced Salt Solution (HBSS)</td>
<td>Invitrogen, New Zealand</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>Gibco BRL, Life Technologies, Vic, Australia</td>
</tr>
<tr>
<td>Product</td>
<td>Supplier</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>Gibco BRL, Life Technologies, Vic, Australia</td>
</tr>
<tr>
<td>Puromycin</td>
<td>Sigma Chemical Co., USA</td>
</tr>
<tr>
<td>TrypLE™ Express</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>Trypsin-EDTA (pH 7.0)</td>
<td>Gibco BRL, Life Technologies, Vic, Australia</td>
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### 4.1.4 Vectors

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<th>SUPPLIER</th>
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<tr>
<td>P3κB-Luc</td>
<td>Promega Corp., USA</td>
</tr>
<tr>
<td>pNFATc1-TA-Luc</td>
<td>BD Bioscience, MA, USA</td>
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### 4.1.5 Cytokines

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<th>SUPPLIER</th>
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<tbody>
<tr>
<td>M-CSF</td>
<td>Produced in the Cell Signalling Group, School of Pathology and Laboratory Medicine, UWA, using conditioned media from CMG14-12 cells.</td>
</tr>
<tr>
<td>RANKL-GST</td>
<td>Generated as previously described (Xu J. et al. 2000).</td>
</tr>
</tbody>
</table>

### 4.1.6 Pharmacological compounds

<table>
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<tr>
<th>DESCRIPTION</th>
<th>SUPPLIER</th>
<th>STOCK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine sulfate</td>
<td>Mansite, Chengdu, China</td>
<td>100 mM (Diluted in DMSO)</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>Mansite, Chengdu, China</td>
<td>100 mM (Diluted in DMSO)</td>
</tr>
<tr>
<td>Compound</td>
<td>Location</td>
<td>Concentration</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Fangchinoline</td>
<td>Mansite, Chengdu, China</td>
<td>100 mM</td>
</tr>
<tr>
<td></td>
<td>(Diluted in DMSO)</td>
<td></td>
</tr>
<tr>
<td>Cumambrin A</td>
<td>Provided by Dr. Qiong Gu from School of Pharmaceutical Sciences, Sun Yat-Sen University.</td>
<td>100 mM</td>
</tr>
<tr>
<td></td>
<td>(Diluted in DMSO)</td>
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### 4.1.7 Molecular Products

<table>
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<tr>
<th>Molecular Product</th>
<th>Manufacturer and Country of Manufacture</th>
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<tr>
<td>1 kb DNA ladder</td>
<td>Promega Corporation, USA</td>
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<tr>
<td>100 bp DNA ladder</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>dNTPs (dATP, dGTP, dTTP, dCTP)</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>GoTaq® Green Master Mix</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>GoTaq® qPCR Master Mix</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT)</td>
<td>Promega Corporation, USA</td>
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<tr>
<td>M-MLV RT Buffer (5x)</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>Precision Plus Protein™ Standards</td>
<td>Bio-Rad, USA</td>
</tr>
<tr>
<td>Proteinase K (20 mg/ml)</td>
<td>Thermo Fisher Scientific, USA</td>
</tr>
<tr>
<td>OligodT</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>SYBR® Safe DNA Gel Stain</td>
<td>Thermo Fisher Scientific, USA</td>
</tr>
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### 4.1.8. Primers for PCR Reactions

<table>
<thead>
<tr>
<th>PRIMERS</th>
<th>SEQUENCE</th>
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| 18sRNA | F: 5' ACCATAAACGATGCGACT  
R: 5' TGTCATCCTGTCGTC |
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer sequence</th>
<th>Reverse Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcitonin Receptor</td>
<td>5'TGGTTGAGGTTGTGCCCA3'</td>
<td>5'CTCGTGGGTTTGCTCCATC3'</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>5'GGGAGAAAAAACCTGAAGC3'</td>
<td>5'ATTCTGGGGACTCAGAGC3'</td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>5'CTTGCAACCTAAGGGCAAAG3'</td>
<td>5'TCAACAGCTCTGTCTGACC3'</td>
</tr>
<tr>
<td>MMP9</td>
<td>5'CGTGTCCTGGAGATTGACTTGA3'</td>
<td>5'TTGGAAAACCTACACGCAGA3'</td>
</tr>
<tr>
<td>NFATc1</td>
<td>5'CAACGCCCTGACCCGATAG3'</td>
<td>5'GGCTGCCTTCCGTCTCATAG3'</td>
</tr>
<tr>
<td>TRAP</td>
<td>5'TGTGGCCATCTTTATGCT3'</td>
<td>5'GTCATTTCTTTGGGGCTT3'</td>
</tr>
<tr>
<td>V-ATPase d2</td>
<td>5'GGATCCGAATTCATGCTTCAG3'</td>
<td>5'GGTCTAGATTATAAAATTGGAATGTAGCT3'</td>
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### 4.1.9 Antibodies

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>SUPPLIER</th>
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</thead>
<tbody>
<tr>
<td>Anti-Beta-actin (JLA20)</td>
<td>Developmental Studies Hybridoma Bank, USA</td>
</tr>
<tr>
<td>Anti-c-FOS</td>
<td>Cell Signaling Technology, USA</td>
</tr>
<tr>
<td>Anti-ERK 1/2</td>
<td>Promega Corp, Madison, Wi, USA</td>
</tr>
<tr>
<td>Antibody Description</td>
<td>Supplier</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>Anti-phospho-ERK 1/2</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>Anti-IκBα (C21)</td>
<td>Santa Cruz Biotechnology CA, USA</td>
</tr>
<tr>
<td>Anti-mouse IgG (Fab specific) Peroxidase conjugate</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Anti-NFATc1 (7A6)</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>Anti-P38</td>
<td>Cell Signaling, USA</td>
</tr>
<tr>
<td>Anti-phospho-P38</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>Anti-rabbit IgG (whole molecule) Peroxidase conjugate</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Anti-SAPK/JNK antibody</td>
<td>Cell Signaling, USA</td>
</tr>
<tr>
<td>Anti-phospho-SAPK/JNK antibody</td>
<td>Cell Signaling, USA</td>
</tr>
<tr>
<td>Anti-V-ATPase d2 subunit</td>
<td>Produced within the laboratory(Feng H. et al. 2009)</td>
</tr>
</tbody>
</table>
### 4.1.10 Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition, Preparation and Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Phosphatase (ALP) assay substrate</td>
<td>5 mg tablet of p-NPP was diluted in 1ml of ALP assay reagent buffer so that the final concentration of substrate solution was 70 mM. (Freshly prepared just before use )</td>
</tr>
<tr>
<td>ALP assay reagent buffer</td>
<td>420mM 2-amino-2-methyl-1-propanol (AMP) dissolved in 200 ml Milli-Q water. Adjusted to PH 10.3 with concentrated NaOH. 1mM MgCl₂ and 1mM ZnCl₂ solutions were then added. The solution could be stored at room temperature for 6 months.</td>
</tr>
<tr>
<td>APS</td>
<td>10% (w/v) ammonium persulphate dissolved in ddH₂O. Stored at 4°C.</td>
</tr>
<tr>
<td>BSA-PBS</td>
<td>0.2% (w/v) BSA dissolved in 1x PBS. Filter sterilized and stored at 4°C.</td>
</tr>
<tr>
<td>Collagenase Type-II solution</td>
<td>2 mg/ml Collagenase Type-II in serum free DMEM. Filter sterilized.</td>
</tr>
<tr>
<td>EDTA</td>
<td>14% (w/v) EDTA diluted in Milli-Q water. Adjusted to pH 8 to dissolve EDTA and then adjusted to pH 7.4. Stored at room temperature.</td>
</tr>
<tr>
<td>Luciferase Lysis Buffer</td>
<td>25 mM Tris-HCl, pH 7.8, 2 mM EDTA, 10% Glycerol, 1% Triton-X 100. Stored at 4°C.</td>
</tr>
<tr>
<td>Buffer</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PBS</td>
<td>10x PBS stock solution: 70 mM Na₂HPO₄, 30 mM NaH₂PO₄, 1.3 M NaCl dissolved in Milli-Q water. 1x PBS: 10x PBS stock solution diluted 1:10 in ddH₂O and adjusted to pH 7.4. Stored at room temperature.</td>
</tr>
<tr>
<td>PMSF</td>
<td>Prepared as stock 17.4 mg/ml in ethanol. Aliquots stored at -20°C.</td>
</tr>
<tr>
<td>RIPA Buffer</td>
<td>50 mM Tris-HCl, 150 mM Sodium Chloride, 1% Nonident P-40, 0.5% sodium deoxycholate, 0.1% SDS. Adjusted to pH 7.5 and stored at 4°C.</td>
</tr>
<tr>
<td>RIPA Cell Lysis Buffer</td>
<td>100 µg/ml PMSF, 1x protease inhibitor cocktail, 1 mM sodium orthovanadate and 500 µg/ml DNaseI mixed in RIPA Buffer. Kept on ice and used immediately.</td>
</tr>
<tr>
<td>SDS-PAGE Loading Buffer</td>
<td>4x SDS-PAGE Loading Buffer: 240 nM Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% glycerol, 0.04% bromophenol blue, 5% β-mercaptoethanol in Milli-Q water. Stored at 4°C.</td>
</tr>
<tr>
<td>SDS-PAGE Running Buffer</td>
<td>10x Stock solution: 25 mM Trizma base, 1.92 M Glycine and 1% (w/v) SDS. 1x Solution: 10x stock solution diluted 1:10 in Milli-Q water. Stored at 4°C.</td>
</tr>
<tr>
<td>SDS-PAGE Separating Gel Buffer</td>
<td>1.5 M Trizma base in Milli-Q water. Adjusted to pH 8.8. Stored at room temperature.</td>
</tr>
<tr>
<td><strong>SDS-PAGE Stacking Gel Buffer</strong></td>
<td>1 M Trizma base in Milli-Q water. Adjusted to pH 6.8. Stored at room temperature.</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5 M stock solution in ddH2O. Stored at room temperature.</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>5 M stock solution in ddH2O. Stored at room temperature.</td>
</tr>
<tr>
<td>Sodium Vanadate (Na₃VO₄)</td>
<td>0.25 M stock solution from Na₃VO₄ powder in ddH2O. The solution was activated through depolymerization at pH 10.0 with a pH-boiling cycle until the solution was clear and colorless, and the pH stabilized at 10. Aliquots were stored at -20°C.</td>
</tr>
<tr>
<td>TAE</td>
<td>50x TAE stock solution: 2 M Trizma base, 5.71% (v/v) glacial acetic acid and 50 mM EDTA.</td>
</tr>
<tr>
<td>Tris-Buffered Saline (TBS)</td>
<td>10x TBS stock solution: 0.5 M Trizma base and 1.5 M NaCl dissolved in Milli-Q water and adjusted to pH 7.4. 1x TBS solution: 10x TBS stock solution diluted 1:10 in Milli-Q water. Stored at room temperature.</td>
</tr>
<tr>
<td>TBS-Tween (0.1%)</td>
<td>1x TBS solution and 0.1% (v/v) Tween20. Stored at room temperature.</td>
</tr>
<tr>
<td>TRAcP Stain Solution</td>
<td>TRAcP Stain Solution A: 100 mM sodium acetate trihydrate, 50 mM sodium tartrate dehydrate and 0.22% glacial acetic acid.</td>
</tr>
<tr>
<td><strong>TRAcP Stain Solution</strong></td>
<td>5 mg Naphthol AS-MX dissolved in 250 µl 2ethoxyethanol and 30 mg Fast Red-Violet LB salt dissolved in 50 ml of TRAcP Stain Solution A.</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Adjusted to pH 5. Aliquot stored at -20°C. Filtered before use.</td>
</tr>
<tr>
<td><strong>Triton X-100/PBS</strong></td>
<td>0.1% (v/v) Triton X-100 in 1x PBS. Filtered and stored at 4°C.</td>
</tr>
<tr>
<td><strong>Western Blot Membrane Stripping Buffer</strong></td>
<td>62.5 mM Trizma base and 2% (w/v) SDS dissolved in Milli-Q water and adjusted to pH 6.7. 100 mM 2-mercaptoethnaol added. Stored at room temperature. Heated to 55°C before use.</td>
</tr>
<tr>
<td><strong>Western Blot Transfer Buffer</strong></td>
<td>25 mM Trizma base, 192 mM Glycine and 10% (v/v) Methanol prepared in in MilliQ water. Stored at 4°C.</td>
</tr>
</tbody>
</table>
### 4.1.11 Other reagents and materials

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amersham Protran Supported 0.45 μm Nitrocellulose Membrane</td>
<td>GE Healthcare, USA</td>
</tr>
<tr>
<td>BCA Protein Assay Kit</td>
<td>Pierce Biotechnology Inc., USA</td>
</tr>
<tr>
<td>Bovine bone slices</td>
<td>Weir’s butcher, WA, Australia</td>
</tr>
<tr>
<td>Carbon dioxide (CO$_2$) gas</td>
<td>BOC Gases, Australia</td>
</tr>
<tr>
<td>Cell culture flasks: T75, T25</td>
<td>Nunc, Denmark</td>
</tr>
<tr>
<td>Cell culture plates: 6, 12, 24, 48 and 96 wells</td>
<td>Nunc, Denmark</td>
</tr>
<tr>
<td>Cell scraper</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)</td>
<td>Promega Corporation, USA</td>
</tr>
<tr>
<td>Centrifuge tubes: 15, 50 ml</td>
<td>Corning, USA</td>
</tr>
<tr>
<td>Chloral Hydrate Solution</td>
<td>Sigma, China</td>
</tr>
<tr>
<td>Cling film</td>
<td>Glad, Australia</td>
</tr>
<tr>
<td>Collagen coated 6-well cell culture plate</td>
<td>Corning, USA</td>
</tr>
<tr>
<td>Cover slips (22mm×22mm)</td>
<td>Knittel Glaser, Germany</td>
</tr>
<tr>
<td>Crypure tube 1.6 ml</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Eppendorf centrifuge Tubes: 0.5 ml</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Eppendorf centrifuge Tubes: 1.5, 2 ml</td>
<td>Axygen, USA</td>
</tr>
<tr>
<td>Falcon Cell strainer 100 μm Nylon</td>
<td>BD Biosciences, USA</td>
</tr>
<tr>
<td>Item</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Filter paper</td>
<td>Whatmans International, UK</td>
</tr>
<tr>
<td>Filtropur S 0.2 syringe filter</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Glass slides</td>
<td>Knittel Glaser, Germany</td>
</tr>
<tr>
<td>Leukocyte Alkaline Phosphatase Kit</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Liquid nitrogen gas</td>
<td>BOC gases, Australia</td>
</tr>
<tr>
<td>Non-sterile examination gloves</td>
<td>Ansell Limited, Australia</td>
</tr>
<tr>
<td>Osteo assay strip well</td>
<td>Corning, NY, USA</td>
</tr>
<tr>
<td>PCR 0.2 ml tubes</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Precision Plus Protein™ Standards</td>
<td>Bio-Rad, Hercules, CA, USA</td>
</tr>
<tr>
<td>Precision Glida™ Needle (0.5mm × 25mm)</td>
<td>BD, Temse, Belgium</td>
</tr>
<tr>
<td>Reservoirs Autoclavable: 75 ml</td>
<td>Thermo Scientific, USA</td>
</tr>
<tr>
<td>Serological pipettes: 10, 25 ml</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Surgical blades sterile</td>
<td>Swann-Morton, England</td>
</tr>
<tr>
<td>SYBR® Green PCR Master Mix</td>
<td>Imgenex, USA</td>
</tr>
<tr>
<td>Syringe Filters: 0.2, 0.45 and 0.8 μm</td>
<td>Pall Corporation, USA; Crown Scientific, Australia</td>
</tr>
<tr>
<td>Syringe: 5 ml, 20 ml</td>
<td>BD, Temse, Belgium</td>
</tr>
<tr>
<td>Transfer pipette 1ml, 3.5 ml</td>
<td>Sarstedt, Germany</td>
</tr>
<tr>
<td>Western Lightning Ultra</td>
<td>PerkinElmer, USA</td>
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### 4.1.12 Equipment and software

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Biosystems ViiA™ 7 Real-Time PCR System</td>
<td>Thermo Scientific, USA</td>
</tr>
<tr>
<td>Adobe® Photoshop® version 5.0</td>
<td>Adobe System Inc, USA</td>
</tr>
<tr>
<td>Agarose gel assembly</td>
<td>Bio-Rad, Hercules, CA, USA</td>
</tr>
<tr>
<td>Auto vortex mixer, CSV90</td>
<td>Crown Scientific Pty Ltd, WA, Australia</td>
</tr>
<tr>
<td>Biofuge A microcentrifuge</td>
<td>Heraeus Sepatech, Germany</td>
</tr>
<tr>
<td>Centr-M2 centrifuge</td>
<td>International equipment company, USA</td>
</tr>
<tr>
<td>Clayson Oven</td>
<td>Quantum Scientific Pty Ltd, Australia</td>
</tr>
<tr>
<td>Clemco fume hood</td>
<td>Oliphant Pty Ltd, Sydney, Australia</td>
</tr>
<tr>
<td>Cryo 1°C Freezing container</td>
<td>Nalgene Labware, Selby Biolab, Australia</td>
</tr>
<tr>
<td>Eppendorf Biophotometer plus</td>
<td>Fisher scientific, USA</td>
</tr>
<tr>
<td>Eppendorf centrifuge 5810R, 5430R</td>
<td>Fisher scientific, USA</td>
</tr>
<tr>
<td>Eppendorf Thermomixer comfort</td>
<td>Fisher scientific, USA</td>
</tr>
<tr>
<td>FujiFilm LAS-3000 Gel Doc. System</td>
<td>FujiFilm, Fuji Photo Co. Ltd, Japan</td>
</tr>
<tr>
<td>Gene Amp PCR system (2400)</td>
<td>Perkins-Elmer, Norwalk, USA</td>
</tr>
<tr>
<td>Grant W-14 water bath</td>
<td>Selby Scientific and Medical, USA</td>
</tr>
<tr>
<td>ImageQuant LAS 4000</td>
<td>GE Healthcare, USA</td>
</tr>
<tr>
<td>Laboratory Refrigerator 4°C</td>
<td>Labec, Australia</td>
</tr>
<tr>
<td>Equipment Description</td>
<td>Manufacturer/Supplier</td>
</tr>
<tr>
<td>------------------------------------------------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Laboratory Refrigerator -20°C</td>
<td>Hisense, Australia</td>
</tr>
<tr>
<td>Laboratory Refrigerator -80°C</td>
<td>Thermo Scientific, USA</td>
</tr>
<tr>
<td>Magnetic Stirrer/Heat Block, model 209-1</td>
<td>IEC Australia Industrial Equipment &amp; control Pty Ltd, Australia</td>
</tr>
<tr>
<td>Medium Reciprocating Shaker, model RM2</td>
<td>Ratek Instruments Pty Ltd, Australia</td>
</tr>
<tr>
<td>Micro-CT 1176 compact</td>
<td>Skyscan, Belgium</td>
</tr>
<tr>
<td>MiniSpin® and MiniSpin® Plus microcentrifuge</td>
<td>Eppendorf AG, Germany</td>
</tr>
<tr>
<td>Model 680 Microplate Reader</td>
<td>Bio-Rad, USA</td>
</tr>
<tr>
<td>Nikon Digital Sight DS-Fi1</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Nikon Digital Sight DS-QiMc</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Nikon Digital Sight DS-U3</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Nikon Eclipse 50i</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Nikon Eclipse Ti-U</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Nikon Intensilight C-HGFIE</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Orbital mixing incubator</td>
<td>RATEK Instruments, Australia</td>
</tr>
<tr>
<td>Parafilm laboratory film</td>
<td>American National Can™, USA</td>
</tr>
<tr>
<td>PC-960C Cooled Thermal cycler</td>
<td>Corbette Research, Australia</td>
</tr>
<tr>
<td>pH211 microprocessor pH meter</td>
<td>Hanna Instruments, Australia</td>
</tr>
<tr>
<td>POLARstar multifunctional microplate reader</td>
<td>POLARstar OPTIMA, BMG, Germany</td>
</tr>
<tr>
<td>Equipment Description</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Rocking platform mixer</td>
<td>RATEX Instrument, Australia</td>
</tr>
<tr>
<td>Scanning Electron Microscope</td>
<td>FEI Cooperation, Oregon, USA</td>
</tr>
<tr>
<td>Tissue Processor TP 1020</td>
<td>Leica Microsystems, Wetzlar, Germany</td>
</tr>
<tr>
<td>Tissue-Tek tissue embedding workstation (Cryo/Thermal/Dispensing consoles)</td>
<td>Miles Scientific, USA</td>
</tr>
<tr>
<td>Tuttnauer® 3870E Tabletop Digital Autoclave</td>
<td>Heidolph, USA</td>
</tr>
<tr>
<td>UV Transilluminator (UVT)</td>
<td>Fujifilm Medical Systems USA Inc, USA</td>
</tr>
<tr>
<td>Water jacketed CO₂ incubator</td>
<td>Forma Scientific, Ohio, USA</td>
</tr>
</tbody>
</table>
4.2 Methods

4.2.1 Osteoclastogenesis assay

Freshly isolated BMM cells from 6-week old C57BL/6 mice were seeded in T75 flasks and cultured in complete α-MEM medium (α-MEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin) with M-CSF (50 ng/ml). Medium was changed every two days. After cells reached confluence, they were transferred to 96-well plates (6×10³/well). After overnight incubation in CO₂ incubator at 37°C to allow attachment, cells were stimulated with RANKL (50 ng/ml) and incubated with natural compounds. Medium was changed every two days and culture maintained until osteoclasts formed.

4.2.2 Cell fixation

Cells were fixed for staining by washing with 1 × PBS and then adding 2.5% glutaraldehyde to cells. After incubation at room temperature for 10 min, the cells were gently washed with 1 × PBS three times.

4.2.3 TRAcP staining

Osteoclast cultures were stained for TRAcP activity after glutaraldehyde fixation. Aliquots of TRAcP were thawed in 37°C, then added to the cells at 50 – 100 μl per well. The cell plates were then incubated at 37°C and protected from light. After 30 min or when the colour changed to red, the cells were washed three times with ddH₂O. Staining was visualised by light microscopy using a Nikon TiU inverted microscope. Images were taken using Nikon Basic Research software and cells manually counted using ImageJ software. Plates were then dried for long term storage at 4°C.

4.2.4 MTS assay

Freshly isolated BMM cells were plated in T75 flasks and then seeded in 96-well plates at 6 × 10³/well when cells were confluent. The culture medium is complete α-MEM
with M-CSF. After overnight incubation, the cells were incubated with different concentrations of natural compounds. Then, 20 μl MTS solution (Promega) was added to the plates. After 2 h, the plates were read by a micro-plate reader at the absorbance of 490 nm.

4.2.5 Luciferase assay

Raw cells transfected with NF-κB or NFAT luciferase reporter genes were seeded in T75 flasks and cultured with complete α-MEM. After cells reached 80% confluence, the cells were seeded into 48-well plates at a density of 1.5 × 10^{5} for NF-κB luciferase assay or 5 × 10^{4} for NFAT luciferase assay. After overnight incubation to allow attachment of the cells, the cells were first pre-treated with natural compounds for 1 h, then stimulated with RANKL for 6 h (NF-κB luciferase assay) or 24 h (NFAT luciferase assay).

Cells were washed with 1 × PBS once, and then lysed with luciferase assay solution (100 µl each well). The cells can be stored at −20 °C until later processing. On the next day, the cell lysates were thawed and collected by scraping cell plates. Then 50 µl cell lysates were added into each well of a white solid plate very gently to avoid any bubbles. The white solid plate was then read on a BMG Polar Star Optima luminescence machine (BMG, Germany). The value of each well was read as soon as 50 µl luciferase substrate was added to each well automatically by the machine.

4.2.6 Polymerase Chain Reaction (PCR)

Freshly isolated BMM cells from 6–week old C57BL/6 mice were plated in T75 flasks and cultured by complete α-MEM medium with M-CSF. When the cells are almost confluent, they were seeded into 6-well plates at the density of 1 × 10^{5}/well. After overnight incubation, the cells were stimulated with RANKL (50 ng/ml) and incubated with different concentrations of natural compounds. The medium was changed every two days for five days or until mature osteoclasts were formed. Then cells were washed once with 1 × PBS, then harvested using TRIzol (1ml/well). The cell lysates could be stored at −80 °C for later use.
The next day, the cells lysates were thawed and then put at room temperature for 5 min. After that, 200 µl of chloroform was added to each sample, which was then shaken vigorously and kept at room temperature for 3 min. Samples were then centrifuged at 12000g for 15 min. The supernatant was collected carefully without disturbing the middle interphase layer. Then 750 µl of isopropyl alcohol was added to supernatant. The mixture was then incubated for 10 min and centrifuged at 12000g for another 10 min. After that, the supernatant was discarded, the remaining precipitate was washed with 1 ml 75% ethanol, then air dried for 10 mins. The remaining RNA pellet was resuspended with 20 µl of nuclease-free water and then incubated at 55 °C for 10 min. RNA samples can be stored in -80 °C for later use.

The concentrations of RNA samples were measured by NanoDrop™. cDNA was generated for each template RNA sample through reverse transcriptase as follows; 1 µg of template RNA was dissolved in 15 µl nuclease free water and then mixed with 0.25 µl Oligo–dT and 2.5 µl ddH2O. Then, the mixture was incubated on PCR machine at 70° C for 5 min. After that, 5 µl of 5 × buffer, 0.5 µl MMLV-RT, 0.5 µl RNAsin and 1.25 µl dNTP were added to the mixture, which was then incubated on PCR machine at 42° C 60 min, 92° C 10 min, then held at 4 °C.

cDNA was then diluted 1:5 with nuclease-free water. For each sample, 1 µl diluted cDNA was mixed with 3 µl nuclease-free water, 5 µl 2 × GoTaq, 0.5 µl 20 µM forward primer and 0.5 µl 20 µM reverse primer. The mixture (10 µl) was incubated at 94 °C 5min; 30 circles of 94° C 40 s, 60° C 40 s, 72°C 40 s; then 72°C 10 min, 4°C ∞.

At the same time, a 1.5% (w/v) agarose gel was prepared by adding 1.5 g agarose to 100 ml 1 × TAE buffer. Agarose was dissolved by heating for 5 min in microwave and 5 µl of DNA gel stain was added into the solution. A 20 tooth comb was inserted into the gel. The PCR products were added into the well (8 µl each well). Then, the gel was run at 90 V for 30 min. After that, the gel was read on UV transilluminator.

Alternatively, each diluted cDNA sample was added to a 384-well plate at 5.4 µl per well and mixed with 6 µl SYBR Green and 0.6 µl primer (0.3 µl forward primer plus 0.3 µl reverse primer). Then, the plate was incubated on ViiA™ 7 Real-time PCR
machine which was set at 95 °C for 5 minutes; 40 cycles of 95 °C for 15 seconds; 58 °C for 20 seconds; 72 °C for 20 seconds; and 72 °C for 1 minute then 4 °C ∞. The obtained value was normalized to house-keeping gene 18S, and expressed as fold change relative to the control sample.

4.2.7 Hydroxyapatite resorption assay

BMM were seeded on 6-well collagen-coated plates at the density of 1×10^5 cells/well. The next day, BMM were stimulated with 50 ng/ml RANKL for three days or when osteoclasts began to form. Then, the medium was removed from the plates. The cells were then washed once with 1 × PBS, then very gently harvested using cell disassociation solution (300 µl each well). The harvested multinucleated cells were seeded on 96-well hydroxyapatite-coated plates. Then, the cells were incubated with natural compounds and 50 ng/ml of RANKL.

After 2 days, cells were fixed and stained with TRAcP staining buffer for osteoclast counts as described above, or lysed with 10% bleach. The bleached wells were dried and the resorbed area was photographed by Nikon microscope and analysed by Image J software.

4.2.8 Western blot assay

BMM seeded in 6-well plates were stimulated with RANKL on day 1, 3 and 5 in the presence of natural compounds. Then cells were harvested and lysed by RIPA lysis buffer. The cell lysates were centrifuged at 448 × g for 15 min at 4 °C. The supernatant was collected and store at −20°C for later use.

The separating gel and stacking gel were prepared as follows:

<table>
<thead>
<tr>
<th>Separating gel</th>
<th>10%</th>
<th>12.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ H₂O</td>
<td>2.97 ml</td>
<td>2.345 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH=8.8</td>
<td>1.875 ml</td>
<td>1.875 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>10% SDS</td>
<td>75 μl</td>
<td>75 μl</td>
</tr>
<tr>
<td>30% Acrylamide/Bis solution</td>
<td>2.5 ml</td>
<td>3.125 ml</td>
</tr>
<tr>
<td>10% Ammonium persulfate (APS)</td>
<td>75 μl</td>
<td>75 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 μl</td>
<td>6 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>7.5 ml</strong></td>
<td><strong>7.5 ml</strong></td>
</tr>
</tbody>
</table>

**stacking gel (for one gel)**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ H₂O</td>
<td>2.062 ml</td>
</tr>
<tr>
<td>1 M Tris-HCl, pH=6.8</td>
<td>375 μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>30 μl</td>
</tr>
<tr>
<td>30% Acrylamide/Bis solution</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>10% Ammonium persulfate (APS)</td>
<td>30 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>3 μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>3 ml</strong></td>
</tr>
</tbody>
</table>

Then, protein samples were mixed with 4x SDS-PAGE loading buffer and were boiled at 99 °C for 5 min. After that, they were loaded into the SDS-PAGE gels. The gels were run at 100 V for 2 h. Protein was then transferred to nitrocellulose membranes and the membrane was cut into smaller pieces according to the expected size of the protein. The cut membrane was then blocked with 5% skimmed milk in 1 × TBST for at least 1 h. After washing with 1 × TBST solution, the membrane was incubated with primary antibody overnight at 4 °C. Then, the membrane was washed with 1 × TBST three times, and then incubated with secondary antibody for 1 h at room temperature. After that, the membrane was washed with 1 × TBST twice and 1 × TBS twice, and then visualised by FujiFilm LAS-3000 imaging system.
4.2.9 Ovariectomy (OVX) animal model

For animal experiments, all the mice were housed in plastic cages with hay shavings in a constant temperature (22°C) environment and a 12 h light and 12h dark alternate lighting condition. Food and water were provided *ad libitum* to the mice. The animal experiments were carried out in accordance to the protocols approved by the University of Western Australia Animal Ethics Committee and the First Affiliated Hospital of Guangxi Medical University Research Ethical committee (SCXK - (JUN) 2012-0004, Beijing, China). When the mice were 7-weeks old, ovariectomy or sham operations were performed. All the mice were injected with 10% chloral hydrate solution for general anaesthesia. For ovariectomy operation, each ovary and its capsule as well as part of the oviduct was resected from small dorsal incisions. The ovaries and oviducts were positioned by a dissecting microscope. After that, operative incisions were stitched up by a synthetic absorbable suture thread (5-0). For sham group, the dorsal incisions were closed without removing ovaries and related organs. One week later, natural compounds or 10% DMSO in sterile water were injected intraperitoneally into mice every two days. The mice were divided into four groups: OVX group, sham group, OVX + drug low–dose group and OVX + drug high–dose group. After 6 weeks, all the mice were euthanized and sacrificed to collect their tibias for analysis.

The tissue samples were fixed in 4% paraformaldehyde (PFA) for around 24 h, and then washed three times with 1 × PBS. After that, the tissue samples were stored in 70% ethanol for later use.

4.2.10 Micro-CT analysis and bone histomorphometric analysis

Samples were then scanned by a Skyscan 1176 micro-CT instrument (Bruker micoCT, Belgium) with the resolution of 9 µm. The parameters of the instrument were set as follows: voltage 50 kV, current 800 µA, filter 0.5 mm aluminium. The scanned images were reconstructed by NRecon software and then analysed by CTAn software. The region of interest is set at the distance of 1 mm, from 0.5 to 1.5 mm below the bottom of the growth plate. The trabecular bone volume was manually drawn to exclude the
cortical bone. The trabecular regions were then binarised and analysed by CTAn software.

For bone histomorphometric analysis, tibia samples were washed once with $1 \times$ PBS, and then decalcified by 14% EDTA for 7 days. EDTA solution was changed every two days. Samples were handled as follows: dehydrating through 70% ethanol twice for 1.5 h each time, 80% ethanol for 1.5 h, 95% ethanol for 1.5 h, 100% ethanol twice for 1.5 h each time; clearing through xylene twice for 2 h each time; immersing through paraffin wax three times for 2.5 h each time at 60°C. Then, samples were embedded in paraffin blocks, and cut into 5 μm thick sections, which were then stained with H&E or TRAcP solutions.

The procedures of H&E staining and TRAcP staining were as follow:

<table>
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<th>H&amp;E staining</th>
<th>Reagents</th>
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<td>9</td>
<td>Tap water</td>
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<td>Haematoxylin</td>
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**TRAcP staining**

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<td>13</td>
<td>Tap water</td>
<td>Rinse</td>
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The slides were mounted in DPX mounting medium and scanned by the Scanscope XT machine (Aperio) at 20 × objective dry lenses on the next day or later. The results were analysed by BIOQUANT OSTEO software (Nashville, USA).

4.2.11 Statistical analysis

All the values presented in this thesis represent the mean of at least three independent experiments. The data is shown as the mean ± standard error of the mean (SEM). Statistical analyses used in this thesis are unpaired Student’s t-test which is for a direct comparison, or analysis of variance (ANOVA) which is for multiple comparisons. Statistical significance was defined as $P < 0.05$. 
Chapter 5

Berberine sulfate attenuates osteoclast differentiation through RANKL induced NF-κB and NFAT pathways
Statements of permission from co-authors

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Signature: ___________________________ Date: __________
Statements of authorship

Berberine sulfate attenuates osteoclast differentiation through RANKL induced NF-κB and NFAT pathways

Lin Zhou, Fangming Song, Qian Liu, Mingli Yang, Jinmin Zhao, Renxiang Tan, Jun Xu, Ge Zhang, Julian M. W. Quinn, Jennifer Tickner and Jiake Xu

Lin Zhou

Contributed to cell culture, PCR and signalling studies. Major contribution to writing of the manuscript.

Fangming Song

Contributed to cell culture, PCR and signalling studies. Also contributed to writing of the manuscript.

Mingli Yang

Contributed to MTS assay and data analysis.

Jinmin Zhao

Contributed to supply of natural compounds and data analysis.

Renxiang Tan

Contributed to the analysis and interpretation of research
Jun Xu
Contributed to supply of natural compounds and data analysis.

Ge Zhang
Contributed to screening design and data analysis.

Julian M. W. Quinn
Contributed to data analysis and revise the manuscript.

Jennifer Tickner
Contributed to design and supervise the overall study.

Jiake Xu
Contributed to the design, supervise the overall study, revise the manuscript and interpretation of research. Acted as corresponding author.
Berberine Sulfate Attenuates Osteoclast Differentiation through RANKL Induced NF-κB and NFAT Pathways

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† These authors contributed equally to this work.

Abstract: Osteoporosis, a metabolic bone disease, is characterized by an excessive formation and activation of osteoclasts. Anti-catabolic treatment using natural compounds has been proposed as a potential therapeutic strategy against the osteoclast related osteolytic diseases. In this study, the activity of berberine sulfate (an orally available form of berberine) on osteoclast differentiation and its underlying molecular mechanisms of action were investigated. Using bone marrow macrophages (BMMs) derived osteoclast culture system, we showed that berberine sulfate at the dose of 0.25, 0.5 and 1 μM significantly inhibited the formation of osteoclasts. Notably, berberine sulfate at these doses did not affect the BMM viability. In addition, we observed that berberine sulfate inhibited the expression of osteoclast marker genes, including cathepsin K (Csk), nuclear factor of activated T cells cytoplasmic 1 (NFATc1), tartrate resistant acid phosphatase (TRACP, Acp5) and Vacuolar-type H+-ATPase V0 subunit D2 (V-ATPase d2). Luciferase reporter gene assay and Western blot analysis further revealed that berberine sulfate inhibits receptor for activation of nuclear factor ligand (RANKL)-induced NF-κB and NFAT activity. Taken together, our results suggest that berberine sulfate is a natural compound potentially useful for the treatment of osteoporosis.

Keywords: berberine sulfate; osteoclast; RANKL; NF-κB and NFAT pathway

1. Introduction

Bone remodelling is a continuous and dynamic process regulated by osteoclastic bone resorption and osteoblastic bone formation [1]. Overproduction and/or excessive activation of osteoclasts can
lead to osteolytic bone diseases, such as postmenopausal osteoporosis and Paget's disease of the bones [2].

Osteoclasts are multinuclear giant cells derived from the monocyte/macrophage lineage of hematopoietic stem cells. There are two key osteoclastogenic cytokines: macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor κB (NF-κB) (RANK) ligand (RANKL). M-CSF causes the proliferation of early macrophage/osteoclast precursors, while RANKL acting via its receptor RANK on these precursors causes them to differentiate into mature osteoclasts. RANKL-RANK interaction activates a cascade of intracellular signaling pathways including nuclear factor (NF)-κB, Fli-3 kinase/AKT, nuclear factor of activated T cells cytoplasmic 1 (NFATc1), and MAPKs. Among these pathways, NF-κB plays a pivotal role early in the initiation of osteoclastogenesis [3]. Recent studies have also identified NFATc1 as a key RANKL-mediated transcription factor. NFATc1 is capable of inducing transcription of diverse osteoclast-associated genes, such as those coding for tartrate-resistant acid phosphatase (TRACP, Acp5), cathepsin K (Ctsk), and calcitonin receptor [4]. Therefore, inhibition of RANKL-mediated signalling molecules may contribute to the treatment and prevention of osteolytic bone diseases.

Berberine, an isoquinoline derivative alkaloid, is isolated from several natural herbs, such as Cortex phellodendri (Huangbai) and Rhizoma coptidis (Huanglian). Berberine has multiple pharmacological effects, including anti-bacterial [5], anti-tumour [6], and apoptosis-induction actions [7–9] but is poorly absorbed into the bloodstream when taken orally. Previous studies also suggested that berberine inhibits osteoclast formation and bone resorption [10], bone loss in ovariectomized (OVX) rats [11], and NF-κB and Akt pathways in osteoclasts [12].

In search for natural compounds that can inhibit osteolysis, we have conducted drug screening using combined osteoclastogenesis assays, and NF-κB and NFAT luciferase reporter gene assays. To this end, we have identified novel compounds that inhibit osteoclast formation and osteolysis [13,14]. In this study, we found that berberine sulfate, a derivative formula of unsulfated berberine that is easily absorbed, inhibits osteoclast differentiation. In addition, we determined the inhibitory effects of berberine sulfate on RANKL-induced osteoclast marker genes expression and NF-κB and NFAT pathways. Our results suggest a potential role for berberine sulfate in the treatment of osteoporosis.

2. Results

2.1. Berberine Sulfate Inhibits RANKL-Induced Osteoclastogenesis

To examine the effect of berberine sulfate on the formation of osteoclasts, osteoclastogenesis assay was performed. Bone marrow macrophages (BMMs) were stimulated by RANKL for five days at the presence of varying concentrations of berberine sulfate, then fixed and stained for TRACP activity. Our results showed that the number of osteoclasts was significantly reduced in a dose-dependent manner, with an IC_{50} of 0.25 μM (Figure 1B–D). In addition, the size of osteoclasts was also decreased (Figure 1C). Cell proliferation assay (MTS) results showed that berberine sulfate has no effect on cell viability at doses up to 10 μM (Figure 1E). Thus, the inhibitory effect of berberine sulfate on osteoclast formation was not due to the toxicity of berberine sulfate.

2.2. Berberine Sulfate Suppresses RANKL-Induced Osteoclast Function

To study the effect of berberine sulfate on mature osteoclast resorptive function, mature osteoclasts were seeded on hydroxyapatite-coated plates and then treated with berberine sulfate for 48 h. Our results showed that the percentage of area resorbed per osteoclast was significantly reduced in the presence of berberine sulfate at the concentration of 0.5 μM and resorption was almost completely absent in the 1 μM group. A small reduction in osteoclast number was also observed at the dose of 1 μM (Figure 2). These results suggested that berberine sulfate suppresses mature osteoclast resorptive function.
Figure 1. Berberine sulfate inhibits RANKL-induced osteoclastogenesis in BMM cells. (A) Chemical structure of berberine sulfate. The molecular weight of berberine sulfate is 433.43; (B) The 96 well-plate showing the effects of different concentration of berberine sulfate on the BMMs derived osteoclast-like cell formation. BMM cells (6 x 10^3 cell/well) were cultured in the presence of M-CSF and GST-rRANKL (50 ng/mL) with or without different concentration of berberine sulfate for five days. Then, the cells were stained for TRAcP. “—” means RANKL untreated; and “+” means RANKL treated; (C) Enlarged images of B (scale bar: 100 μm); (D) Osteoclast cell counts showing TRAcP-positive multinucleated cells. (n = 3); (E) Effect of berberine sulfate on the viability of BMM cells as measured by MTS assay. (n = 3). *** p < 0.001 (versus RANKL-treated control).

Figure 2. Berberine sulfate suppresses osteoclast function. (A) Representative images of osteoclastic resorption and TRAcP staining on hydroxyapatite coated surfaces (Scale bars, 500 μm); (B) The effect of berberine sulfate on the number of TRAcP positive multinucleated cells (Nuclei ≥ 3, counted as osteoclasts); (C) Percentage of the area of hydroxyapatite surface resorbed per osteoclast. * p < 0.05, *** p < 0.001 relative to untreated controls.
2.3. Berberine Sulfate Suppresses RANKL-Induced Osteoclast-Associated Gene Expression

To further investigate the effects of berberine sulfate on osteoclast formation, we examined the effect of berberine sulfate on osteoclast marker mRNA expression. Real time PCR analysis was performed on osteoclast culture. BMMs were stimulated with RANKL for five days in the presence of varying concentrations of berberine sulfate. Total RNA were extracted, and followed by real time PCR analysis. Our results showed that berberine sulfate reduces mRNA levels of cathepsin K (Ctsk), NFATc1, TRACP and Vacuolar-type H+ ATPase V0 subunit D2 (V-ATPase d2), (Figure 3) consistent with the inhibitory effect of berberine sulfate on osteoclastogenesis.

![Figure 3](image)

**Figure 3.** Effect of berberine sulfate on mRNA levels of osteoclast-associated genes. Real time-PCR analysis was performed to examine osteoclast-specific gene expression (Ctsk, V-ATPase d2, NFATc1, and TRAcP), and results were normalized to the expression of GAPDH. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with untreated control.

2.4. Berberine Sulfate Inhibits RANKL-Induced NF-κB and NFAT Activity

To explore the molecular mechanism of action by which berberine sulfate inhibits osteoclast formation, the effects of berberine sulfate on RANKL-induced NF-κB and NFAT activities were tested using luciferase reporter gene assays. RAW264.7 cells stably transfected with a NF-κB luciferase reporter construct were seeded in 48 wells at 1.5 × 10⁵ cells/well and RAW264.7 cells stably transfected with an NFAT luciferase reporter construct were seeded at a density of 5 × 10⁴ cells/well and left overnight to attach. The next day, the cells were pre-treated with berberine sulfate for one hour, and then stimulated with RANKL for six hours for NF-κB luciferase assay and 24 h for NFAT luciferase assay, respectively. Our results showed that berberine sulfate significantly inhibited NF-κB (Figure 4A) and NFAT (Figure 5A) activities from the dose of 1 μM, with an IC50 of approximately 5 μM.

![Figure 4](image)

**Figure 4.** Cont.
Figure 4. Berberine sulfate attenuated RANKL-stimulated NF-κB activity. (A) RAW264.7 cells stably transfected with an NF-κB transcriptional luciferase reporter construct, were pretreated with indicated concentration of berberine sulfate for 1 h and subsequently stimulated with RANKL (50 ng/mL) for an additional 6 h. The cells were harvested, and the luciferase activity was assayed. \( n = 6 \). \( \beta \)-RANKL untreated; “+” means RANKL treated. * \( p < 0.05 \), ** \( p < 0.01 \) versus untreated control; (B) BMM cells were pretreated with serum-free culture medium for 3 h, and berberine sulfate (1 μM) for 1 h and then were incubated with RANKL (100 ng/mL) for indicated times. The levels of IκB-α were evaluated by Western blot analysis.

2.5. Berberine Sulfate Inhibits RANKL-Induced IκBα Protein Degradation and NFATc1 Protein Expression

To further study the role of berberine sulfate in RANKL-induced NF-κB and NFAT signal pathways, Western blot analysis was performed to test the effects of berberine sulfate on IκBα pro degradation and NFATc1 protein expression. For detecting IκBα protein degradation, BMMs were stimulated by RANKL at 5, 10, 20, 30 and 60 min in the presence or absence of berberine sulfate. Results showed that IκBα degradation was inhibited by berberine sulfate at the concentration of 1 μM (Figure 4B). The inhibition was especially clear at 20 and 30 min of RANKL stimulation. For detecting NFATc1 protein expression, BMMs were stimulated with RANKL at Day 1, 3 and 5 in the presence or absence of berberine sulfate. The expression of NFATc1 was also reduced by berberine sulfate at the concentration of 1 μM (Figure 5B). These results further attested the inhibitory effect of berberine sulfate on RANKL-induced NF-κB and NFAT signal pathways.

Figure 5. Berberine sulfate suppressed RANKL-induced NFAT activity. (A) RAW264.7 cells were stably transfected with an NFAT-dependent transcriptional luciferase reporter construct and then pretreated with indicated concentration of berberine sulfate for 1 h, prior to the addition of RANKL (50 ng/mL) for 24 h. The cells were harvested, and the luciferase activity was measured (\( n = 6 \)). “-” means RANKL untreated; “+” means RANKL treated. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) compared with untreated control; (B) BMM cells were pretreated with berberine sulfate for 1 h, followed by RANKL (100 ng/mL) stimulation for indicated times. The cell lysates were probed for NFATc1 protein levels using Western blot analysis.
3. Discussion

Postmenopausal osteoporosis is a disease featured by an imbalance between the activities of osteoclasts and osteoblasts, with excessive osteoclastic bone resorption predominating over osteoblast bone formation. Therefore, osteoclasts are prime targets of commonly used anti-catabolic drugs such as bisphosphonates (BPs), and Denosumab, a fully human anti-RANKL IgG2 monoclonal antibody. In this study, through screening of natural compounds that exhibit inhibitory effects on osteoclastogenesis and RANKL signalling, we found that berberine sulfate is capable of inhibiting osteoclast formation, osteoclast marker genes expression, and RANKL-induced NF-κB and NFAT activities, suggesting that berberine sulfate is a potential anti-catabolic agent for the treatment of osteoporosis.

Berberine was previously reported to have a wide range of pharmacological effects [15]. However, absorption of berberine via the intestinal wall is very poor [16]. Indeed, after oral administration, berberine is typically undetectable in blood [17]. The metabolites of berberine after absorption when detectable are mainly sulfate or glucuronide conjugates [18]. For these reasons, berberine sulfate is a commonly used salt form of berberine as this form is both stable and clearly detected in the blood after oral administration. While berberine itself has been examined for its effects on osteoclast formation, its sulfated derivative has not. In this study, we found that berberine sulfate strongly inhibits osteoclastogenesis with an IC_{50} of approximately 0.25 μM.

RANKL, a member of the tumour necrosis factor (TNF) super family, was identified as a critical cytokine for osteoclastogenesis [19]. The binding of RANKL and RANK activates several downstream molecules which are important for regulating osteoclast differentiation, function and survival, including NF-κB, AP-1, TRAFs, NFATc1 and ionized calcium [3,20]. NF-κB pathway is the most important among RANKL-induced early signaling pathways, as without this signal no osteoclast formation can occur. RANKL binding causes the binding of its receptor RANK to TRAF6 which interacts with TAB2, forming a complex to activate the downstream TAK1, inducing the phosphorylation of IKKα and β. The activated IKKα and β induce the phosphorylation of IκB [21,22]. The phosphorylated IκB is then degraded via proteasome pathway, allowing the release of NF-κB to the nucleus [23–25]. In our study, we found that NF-κB activity and IκBα protein degradation were inhibited by berberine sulfate. However, the phosphorylation of ERK was not inhibited by berberine sulfate (Figure S1). These results are consistent with a previous study, which suggested that berberine prevented the IκBα degradation but not the phosphorylation of ERK and P38 [12].

NFATc1, a RANKL-induced transcription factor that is dependent upon prior NF-κB activation, plays a key regulatory role in osteoclast differentiation and indeed can drive osteoclast formation when it is overexpressed in osteoclastic progenitor cells [4]. Its stability and nuclear translocation is also dependent on Ca^{2+} dependent calcineurin activity [4], making it essential to use activity assays (such as the luciferase based system employed here) to investigate the effects of an anti-osteoclastic factor such as berberine sulfate. Together with NF-κB, AP-1 and other transcription factors NFATc1 elicits the expression of osteoclast-associated genes such as TRAcP (Acp5), MMP9, calcitonin receptor, Ctsk, V-ATPase d2, c-Src and other genes that are required for osteoclast function. In this study, we found that berberine sulfate suppressed RANKL-induced NFATc1 transcriptional activity and NFATc1 protein levels. It is not clear whether berberine sulfate acts directly on NFATc1 expression or stability, since it is possible that its primary action is to suppress NF-κB and only indirectly NFATc1. However, consistent with reduced NFATc1 protein levels, berberine sulfate was observed to reduce mRNA expression of osteoclast associated proteins Ctsk, V-ATPase d2, and TRAcP (Acp5), downstream targeted genes of NFATc1 activity, which is consistent with the inhibitory effects of berberine sulfate on NFATc1 transcriptional activity. However, the exact molecular targets of berberine in osteoclasts are not currently known. Further work is required to determine if berberine sulfate is internalized to inactivate RANKL-dependent signaling pathways intracellularly, or whether it interferes with cell surface interactions such as RANKL binding to RANK, or binding to other specific transmembrane receptors.
4. Materials and Methods

4.1. Media and Reagents

Berberine sulfate (Purity > 98%) was purchased from Mansite (Chengdu, China), and dissolved in Dimethyl sulfoxide (DMSO). α Modification of Minimal Essential Medium (α-MEM) and fetal bovine serum (FBS) was purchased from TRAC (Sydney, Australia). Recombinant GST-rRANKL protein was expressed and purified as previously described [26] and recombinant macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Minneapolis, MN, USA). MTS reagent and luciferase analysis reagents were obtained from Promega (Sydney, Australia). Antibodies against NFATc1, IkBα, ERK, phosphorylated ERK, and β-actin were obtained from Santa Cruz Biotechnology (Dallas, CA, USA). Tartrate resistant acid phosphatase (TRACP) enzymatic activity was detected using the Leukocyte acid phosphatase staining kit (Sigma, St. Louis, MO, USA).

4.2. Cell Culture

RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). RAW264.7 cells and freshly isolated bone marrow macrophages (BMM) from C57BL/6 mice were grown in α-MEM supplemented with 10% heat inactivated FBS, 2 mM 1-glutamine and 100 U/mL penicillin/streptomycin (complete α-MEM). Primary BMM were grown in complete α-MEM with the addition of macrophage-colony stimulating factor (M-CSF). All cell cultures were maintained in 5% CO₂ at 37 °C.

4.3. In Vitro Osteoclastogenesis Assay

BMM cells (at a density of 6 × 10³ cell/well in 96-well plate) were seeded and cultured in the presence of complete α-MEM medium containing M-CSF and GST-rRANKL (50 ng/mL) with or without different concentration of berberine sulfate. The medium as above described was changed every 2 days. After 5 days, cultured cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Fixed cells were then histochemically stained as per manufacturer’s instructions using a TRACP staining kit (Sigma, St. Louis, MO, USA). The osteoclast-like cells (OCL) were quantified as the total number of TRACP-positive multinucleated cells (more than 3 nuclei).

4.4. Cytotoxicity Assays

BMMs were plated onto 96-well plate (6 × 10³ cell/well) overnight, and then treated with complete α-MEM medium with M-CSF (25 ng/mL) and different concentration of berberine sulfate for 48 h. Next, 20 μL of MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium), was added and incubated for 2 h. Cell viability assay was measured by optical density at 490 nm using a microplate reader (ThermoFisher, Waltham, MA, USA).

4.5. Hydroxyapatite Resorption Assay

For the experiment, 1 × 10⁵ cells/well BMMs were seeded onto 6-well collagen-coated culture plates overnight at 37 °C. Then, the cells were stimulated with 50 ng/mL GST-rRANKL and M-CSF until osteoclasts were formed. The cells were gently harvested using cell dissociation buffer, and equal numbers of multinucleated cells were seeded on hydroxyapatite-coated plates (Corning, New York, NY, USA). The cells were then treated with different concentrations of berberine sulfate in the presence of 50 ng/mL GST-rRANKL and M-CSF. After 48 h, half of the wells were fixed and stained for TRACP activity for osteoclast counting and the remainder of the wells were bleached and dried for hydroxyapatite resorption visualisation using a Nikon microscope (Nikon Corporation, Tokyo, Japan) and analysed using Image J software (National Institutes of Health, Bethesda, MD, USA).

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4.6. RNA Isolation and Analysis

BMMs were cultured with berberine sulfate at various concentration in the presence of M-CSF (25 ng/mL) and GST-rRANKL (50 ng/mL) for 5 days on 6 well plate (1 × 10^5 cell/well). Then, total RNA from cells were isolated using Trizol regent (Life Technologies, Mulgrave, Australia). cDNA was synthesized from 1 μg of RNA using reverse transcriptase with oligo-dT primer. All PCR reactions used specific primers following the mouse sequences: Cathepsin K (Ctsk) (forward: 5'-GGG AGA AAA ACC TGA AGC-3'; reverse: 5'-ATT CTG GGG ACT CAG AGC-3'), TRAcP (Acp5) (forward: 5'-TGT GCC CAT CTT TAT GCT-3'; reverse: 5'-GTC ATT TCT TTG GGG CTT-3'), NFATc1 (forward: 5'-CAA CCC CCT GAC CAC CGA TAG-3'; reverse: 5'-GGC TGG CTT CCG TCT CAT AGT-3'), V-ATPase-d2 (forward: 5'-GTG AGA CCT TGG AAG ACC TGA A-3'; reverse: 5'-GAG AAA TGT GCT CAG GGG CT-3'), GAPDH (forward: 5'-ACC ACA GTC CAT GCC ATG AC-3'; reverse: 5'-TCC ACC CTG TGG CTG TA-3'), qPCR reactions were performed on a ViiA™ 7 Real-time PCR machine (Applied Biosystems, Paisley, UK). The comparative 2^-ΔΔCt method was used to calculate the relative expression of each target gene. The mean Ct value of target genes in the experimental group was normalized to the Ct value of GAPDH to give a ΔCt value, which was further normalized to control samples to obtain ΔΔCt. Three independent cultures were carried out, and all experiments were performed in triplicate.

4.7. NF-κB and NFAT Luciferase Reporter Gene Assays

NF-κB and NFAT activation were measured by luciferase reporter gene assays. Briefly, RAW264.7 cells stably transfected with an NF-κB luciferase construct (κB-Luc-SV40) [27] and with an NFATc1 luciferase reporter construct [28]; respectively. The transfected cells were plated in 48-well plates (1.5 × 10^5 and 5 × 10^4 cells/well; respectively). Then, cells were pre-treated with different concentration of berberine sulfate for 1 h, followed by GST-rRANKL (50 ng/mL) stimulation for 6 h (NF-κB luciferase report gene assay) or 24 h (NFAT luciferase reporter gene assay). Analysis of luciferase activity was performed in accordance with the manufacturer’s instructions (Promega, Sydney, Australia).

4.8. Western Blot Assays

Protein was extracted from cells in culture by scraping the cells into radioimmunoprecipitation assay (RIPA) buffer. Samples were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes; these were then blocked with 5% skim milk for 1 h at room temperature and probed with primary antibodies overnight at 4 °C. After three washes, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h. Protein bands were then visualized using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Sydney, Australia).

4.9. Statistical Analysis

All data were shown as the mean ± SEM with at least 3 independent experiments. Statistical analysis used the student’s t test to assess statistical differences, p values less than 0.05 were considered to be significant.

5. Conclusions

We have identified that berberine sulfate, a commonly used salt form of berberine, is capable of inhibiting osteoclast formation and the expression of osteoclast marker genes through the suppression of RANKL-induced NF-κB and NFAT activation. Our study suggests that berberine sulfate is a potential therapeutic agent for the treatment of osteoporosis.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/16/11/25998/s1.
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Author Contributions: Lin Zhou, Fangming Song, and Qian Liu performed cell culture, PCR and signalling studies; Mingli Yang performed the cell proliferation assay (MTS) analysis and data analysis; Lin Zhou and Fangming Song wrote the manuscript cooperatively; Jinmin Zhao, Renxiang Tan, Jun Xu, Ge Zhang, Julian M. W. Quinn contributed to the supply of natural compounds, screening design and data analysis; Jiakai Xu and Jennifer Tickner designed and supervised the overall study. Jiakai Xu and Julian M. W. Quinn revised the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References


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Chapter 6
Dihydroartemisinin, an anti-malaria drug, suppresses estrogen deficiency-induced osteoporosis, osteoclast formation, and RANKL-induced signaling pathways
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Dihydroartemisinin, an anti-malaria drug, suppresses estrogen deficiency-induced osteoporosis, osteoclast formation, and RANKL-induced signaling pathways

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Lin Zhou
Contributed to cell culture, PCR, signaling and animal studies. Major contribution to writing of the manuscript.

Qian Liu
Contributed to providing natural compounds and animal studies.

Mingli Yang
Contributed to MTS assay.

Tao Wang
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Jinbo Yuan
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Jinmin Zhao
Contributed to data analysis and revised the manuscript.

Jennifer Tickner
Contributed to design and supervise the overall study.

Jiake Xu
Contributed to the design, supervise the overall study, revise the manuscript and interpretation of research. Acted as corresponding author.
Dihydroartemisinin, an Anti-Malaria Drug, Suppresses Estrogen Deficiency-Induced Osteoporosis, Osteoclast Formation, and RANKL-Induced Signaling Pathways

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ABSTRACT
Osteoporosis is an osteolytic disease that features enhanced osteoclast formation and bone resorption. Identification of agents that can inhibit osteoclast formation and function is important for the treatment of osteoporosis. Dihydroartemisinin is a natural compound used to treat malaria but its role in osteoporosis is not known. Here, we found that dihydroartemisinin can suppress RANKL-induced osteoclastogenesis and bone resorption in a dose-dependent manner. Dihydroartemisinin inhibited the expression of osteoclast marker genes such as cathepsin K, calcitonin receptor, and tartrate-resistant acid phosphatase (TRACP). Furthermore, dihydroartemisinin inhibited RANKL-induced NF-κB and NFAT activity. In addition, using an in vivo ovariectomized mouse model, we show that dihydroartemisinin is able to reverse the bone loss caused by ovariectomy. Together, this study shows that dihydroartemisinin attenuates bone loss in ovariectomized mice through inhibiting RANKL-induced osteoclast formation and function. This indicates that dihydroartemisinin, the first physiology or medicine nobel prize discovery of China, is a potential treatment option against osteolytic bone disease. © 2015 American Society for Bone and Mineral Research.

KEY WORDS: DIHYDROARTEMISININ; OSTEOCLAST; RANKL; BONE RESORPTION; OSTEOLYSIS

Introduction
Bone is a continuously renewing tissue that is formed by mineralization of an organic matrix. Bone formation by osteoblasts and bone resorption by osteoclasts contributes to the balance of bone remodeling. An imbalance between osteoclast and osteoblast formation and function causes many osteopathic diseases, such as osteoporosis and Paget’s disease.1,2 Osteoporosis is a common age-related degenerative disease that often affects postmenopausal women due to estrogen deficiency. According to the 2004 U.S. Surgeon General’s report, it was estimated that women over 50 years old have a 50% risk of fragility fracture during the remainder of their lifetime, whereas men of all age have a 20% risk of fragility fracture throughout the remainder of their lifetime.3 Fractures caused by osteoporosis are associated with significant morbidity and mortality, and place a large economic burden on society.4,5

The functional role of the osteoclast is to resorb bone by secreting acid and proteases to dissolve the organic and mineral components of bone. Receptor activator of nuclear factor kappa-B ligand (RANKL), also called osteoprotegerin ligand (OPGL), TNF-related activation-induced cytokine (TRANCE), and osteoclast differentiation factor (ODF), is a critical cytokine for the formation and activation of osteoclasts.6,7 The interaction between RANKL and its receptor RANK activates several transcription factors, such as NF-κB, AKT, activator protein 1 (AP-1), mitogen-activated protein kinase (MAPK), and nuclear factor of activated T-cell cytoplasmic 1 (NFATc1). Activation of these downstream factors triggers the expression of genes governing osteoclast differentiation and function, including tartrate-resistant acid phosphatase (TRACP), cathepsin K, matrix metalloproteinase 9 (MMP-9), and calcitonin receptor (CTR), ultimately resulting in the production of mature multinucleated osteoclasts.8-10 RANKL is now recognized as an attractive target for the treatment of osteoporosis, because inhibition of RANKL using antibodies, peptides, and natural compounds could prevent osteoclast formation and function. This study aims to determine the effect of natural compounds on inhibiting RANKL-induced osteoclast formation and function.11 Artemisinin is an effective drug for treating malaria. It belongs to the family of sesquiterpene lactones, which are produced by the Artemisia annua plant.12-14 Dihydroartemisinin (DHA) is a water-soluble semisynthetic derivative of artemisinin15,16 and it is commercially combined with piperaquine as an effective therapy for malaria17 with few side effects.18 DHA has also
been found to have inhibitory effects on cancer cells, mainly through modulating the NF-κB pathway. However, the effect of DHA on osteoclast formation and osteoporosis is not known.

In this study, we examine the role of DHA in osteoporosis in vivo using an ovariectomized (OVX) mouse model, and elucidate its cellular and molecular mechanism of action. We show that DHA has a protective effect in an estrogen deficiency-induced osteoporosis mouse model. Furthermore, we found that DHA inhibits the formation and function of osteoclasts via inhibition of the NF-κB and NFAT pathways. Because DHA is already used for treatment of malaria and has antitumor activity, our study further indicates that DHA could be beneficial for the treatment of osteolytic bone conditions, such as cancer or inflammation-induced osteolysis.

Materials and Methods

Materials and reagents

DHA was purchased from Chengdu Must Bio-Technology Co., Ltd (Chengdu, Sichuan Province, China) (Fig. 1A), and dissolved in dimethyl sulfoxide (DMSO). Alpha-MoM Medium (α-MEM) and fetal bovine serum (FBS) were obtained from TRACSE (Sydney, Australia). Anti-P-ERK and anti-iκBα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-NFATC1 antibody was purchased from BD Biosciences (San Jose, CA, USA) and Vacular-type H+ -ATPase V0 subunit d2 (V-ATPase d2) was generated as described.206 Mouse anti-β-Actin (catalog number: JLA20) was purchased from Developmental Studies Hybridoma Bank (DSHB) (Iowa City, IA, USA). The luciferase assay system and MTS assay kit were purchased from Promega (Sydney, Australia). Recombinant GST-rRANKL protein was produced according to a previous article and recombinant macrophage colony stimulating factor (M-CSF) was used as described.

In vitro osteoclastogenesis assay

Freshly isolated bone marrow macrophages (BMMs) from C57BL/6 mice were cultured in T75 flasks in α-MEM containing M-CSF. When confluent, BMMs were seeded into 96-well plates at 1 × 10^5 cells per well with α-MEM and M-CSF overnight. The next day, BMMs were stimulated with 50 ng/mL RANKL in the presence of DHA for 2 days until osteoclasts formed. The plate was then fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for 10 min, and stained for TRAP. TRAP-positive multinucleated cells with three or more nuclei were scored as osteoclast-like (OCL) cells.

In vitro osteoblast differentiation and mineralization

For osteoblastogenesis assays, osteoblast precursors from adult calvaria or long bones were obtained as outgrowth from collagenase-treated bone pieces as described.225 The cells were plated into culture dishes at a cell density of 1 × 10^4 cells/mL in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (complete DMEM). When confluent, osteogenic media (complete DMEM, 10 mM dexamethasone, 10 mM ß-glycerophosphate, and 50 µg/mL ascorbate) was added and cells cultured for 7 days (alkaline phosphatase assay) or 21 days (mineralization). Alkaline phosphatase staining was performed using the Leukocyte alkaline phosphatase staining kit (Sigma, Castle Hill, Australia). Mineralization was observed following fixation and staining with 1% alizarin red (image) software (NIH, Bethesda, MD, USA) was used to measure the mineralized area.

MTS assay for cell proliferation and viability

MTS assay was used to determine the effects of compounds on BMNs using a commercially available MTS assay kit (Promega, Sydney, Australia). BMNs were seeded on 96-well plates at 6 × 10^3 cells per well and incubated overnight. Varying concentrations of DHA were then added to BMNs and the cells were incubated with the compounds for 48 hours. A MTS/phenazine methosulfate (PMS) mixture was then added to each well for 2 hours according to the manufacturer’s instructions. The absorbance of MTS was measured by spectrophotometric absorbance at 490 nm using an ELISA plate reader (BMG LABTECH GMBH, Ortenberg, Germany).

Hydroxyapatite resorption assay

BMNs were seeded on six-well collagen-coated culture plates at a density of 1 × 10^3 cells per well. Cells were allowed to adhere overnight at 37°C and cells were then stimulated with 50 ng/mL RANKL and M-CSF until osteoclasts began to form. The cells were gently harvested using cell dissociation solution, counted, and equal numbers of multinucleated cells were seeded onto hydroxyapatite-coated plates (Corning, Inc., Corning, NY, USA). DHA was added to the cells after they were seeded onto the hydroxyapatite plate. After 48 hours, one-half of the wells were fixed and stained for TRAP activity for osteoclast counting and the remainder of the wells were bleached and dried for hydroxyapatite resorption visualization using a Nikon microscope and analyzed using Image J software.

RNA extraction and analysis

Freshly isolated BMNs from C57BL/6 mice were cultured in T75 flasks then seeded in six-well plates at a density of 1 × 10^4 cells per well. Cells were then stimulated with RANKL and M-CSF in the presence of DHA for 5 days. After osteoclasts were formed, cells were lysed, and total RNA was extracted using Trizol (Life Technologies, Mulgrave, Australia) in accordance with the manufacturer’s protocol. For reverse transcription (RT)-PCR, single-stranded cDNA was reverse transcribed from 1 µg total RNA using reverse transcriptase with an oligo-dT primer. For relative quantitative real-time PCR (qPCR), SYBR Green PCR MasterMix was used. The cycling parameters for PCR were set as follows: 94°C for 5 min, followed by 30 cycles of 94°C (40 s), 60°C (40 s), 72°C (40 s), followed by an elongation step of 5 min at 72°C. PCR reactions used specific primers for detecting and quantifying the following genes: cathepsin K (forward: 5’-GGGAGAAA-AACCTGAAAGC-3’; reverse: 5’-ATCTCGGGGACTGACGGC-3’), calcitonin receptor (forward: 5’-TGGTTGGTTGGTGGCAG-3’; reverse: 5’-CTGTTGGTTGGTGGTCAT-3’), TRAP (forward: 5’-TGTGGCCACCTTTATGCT-3’; reverse: 5’-GTCTTGGTGGTGGTCAT-3’), and 18S RNA (forward: 5’-ACCATAGATGGCCGACT-3’; reverse: 5’-TGTCAATCCTGTCGTGGTGC-3’). qPCR reaction results were read on a Viia 7Real-time PCR machine (Applied Biosystems, Warrington, UK). The comparative 2^-ΔΔCt method was used to calculate the relative expression of each target gene. The mean Ct value of target genes in the experimental group was normalized to the Ct value of 18S to give a ΔCt value, which was further normalized to control samples to obtain ΔΔCt.
independent cultures were carried out, and all experiments were performed in triplicate.

NF-κB and NFAT luciferase assay

For NF-κB and NFAT luciferase assays, RAW264.7 cells stably transfected with an NF-κB luciferase reporter construct (3xκB-Luc-SV40) or an NFAT luciferase reporter construct were seeded onto a 48-well plate at the density of 1.5 × 10^5 or 5 × 10^4 cells per well. The following day, the cells were treated with DHA only for 1 hour, followed by stimulation with RANKL for 6 hours (NF-κB luciferase reporter gene assays) or 24 hours (NFAT luciferase reporter gene assays) in the presence of DHA. Cells were then lysed and centrifuged for 20 min at 14 000 g at 4°C. Luciferase activity was then measured using the Promega luciferase kit and a BMG Polar Star Optima luminometer reader (BMG LABTECH GMBH, Ortenberg, Germany).

Western blot assays

Freshly isolated BMMS from C57BL/6 mice were seeded onto six-well plates, and stimulated with RANKL and M-CSF in the presence of DHA. After 5 days, osteoclasts had formed and cells were lysed with radioligand precipitation assay (RIPA) buffer for protein extraction. Protein was separated by SDS-PAGE electrophoresis, and then transferred to a nitrocellulose membrane. The membrane was blocked in 5% skim milk for 1 hour then probed with specific antibodies, including P-ERK, ERK, IκBα, NFATc1, V-ATPase-d2, and β-actin antibodies. After washing three times with TBST, membranes were incubated with appropriate HRP-conjugated secondary antibodies. Finally, the membranes were washed using enhanced chemiluminiscence (ECL) reagents (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions and imaged using an Image quant LAS 4000 (GE Healthcare, Silverwater, Australia).

O VX mouse model

The animal experiments were approved by the Institutional Animal Ethics Committee of Guangxi Medical University (SCXX-2012-0004, Beijing, China). Eighteen specific pathogen-free C57BL/6J mice age 7 weeks were randomly divided into three groups containing six mice: sham group, O VX group, and O VX-DHA (1 mg/kg) group. Mice were housed in individually ventilated cages (IVC) with standard chow and water ad libitum, with a 12-hour light/dark cycle. The O VX group and O VX-DHA group were given an ovariolectomy operation, whereas the sham group were given a sham operation as a control. The mice were anesthetized with 10% chloral hydrate solution. Small dorsal incisions were made, and each ovary, together with its capsule and part of the oviduct, was removed. A dissecting microscope was used to locate these organs. Each incision was closed by one stitch performed with a 5-0 synthetic absorbable suture. After the procedure all mice had 1 week for postoperative recovery. Then the procedure or both groups were given an intraperitoneal injection of DHA at 1 mg/kg every 2 days. The mice in the sham and O VX groups were intraperitoneally injected with 1% DMSO as a control. After 6 weeks, all the mice were euthanized. Their tibiae were collected and fixed in 10% neutral buffered formalin. Excess soft tissue was removed and the cleaned tibiae were prepared for micro-computed tomography (μCT) analyses by wrapping in tissue soaked in PBS and loading into a 2-ml Eppendorf tube to maintain position, hydration, and to prevent movement artifacts. Tubes were then immobilized using foam inserts in the bed of a SkyScan 1176 μCT instrument (Bruker microCT, Kontich, Belgium). Each tibia was then imaged with the following instrument settings: 50 kV, 500 μA, 0.5 mm AI filter, 952.2 ms exposure, pixel size 8.89 μm, 2 frame averaging, 0.4-degree rotation step through 180 degrees. Images were then reconstructed using NRecon software (Bruker microCT, Kontich, Belgium) with a constant threshold value. A volume of interest containing the proximal head of the tibia was generated and this volume was loaded into the analyses program CTAn (Bruker microCT, Kontich, Belgium). A refined volume of interest was then generated 0.5 mm below the growth plate and 1 mm in height. The trabecular bone region of interest (ROI) within this volume was manually defined and bone parameters within this ROI were determined using a constant threshold for binarization of the trabecular bone. Following μCT analyses bones were decalcified and embedded into paraffin blocks for sectioning and staining. Sequential 5-μm-thick sections were prepared and stained using hematoxylin and eosin, or for TRACP activity. Sections were scanned using Aperio Scanscope, and bone histomorphometric analyses were performed using BIOQUANT OSTEOST software (Biocut Image Analysis Corporation, Nashville, TN, USA).

Results

DHA inhibits RANKL-induced osteoclastogenesis

In order to determine the effect of DHA on the formation of osteoclasts, osteoclastogenesis assays were performed. BMMS were seeded in 96-well plates at 6 × 10^4 per well. After overnight incubation, DHA was added to the plate every 2 days until osteoclasts were formed. The results showed that DHA inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner. DHA significantly inhibited osteoclast formation at doses greater than 0.5 μM (Fig. 1B, C). To determine any cytotoxic effects of DHA on BMMS, a cell survival assay was performed. Our results showed that concentrations of DHA which were effective at inhibiting osteoclastogenesis (0.5 μM and 1 μM) did not have any cytotoxic effects on BMMS (Fig. 1D). Similar results were also found when BMMS were treated with artemisinin; however, the effect of artemisinin was weaker than that of DHA. The minimum dose of artemisinin that significantly inhibited osteoclastogenesis was 1 μM (Supplementary Fig. 1). We also assessed the effect of DHA on osteoblast formation and mineralization using in vitro osteoclastogenesis assays. We found no effect of DHA on osteoblast alkaline phosphatase staining, and no changes in mineralization in osteoblast cultures (Supplementary Fig. 2).

Thus, for the subsequent studies, DHA was chosen as a prototype to further examine its mechanism of action in osteoclastogenesis and therapeutic potential in vivo.

DHA suppresses RANKL-induced osteoclast function

Hydroxyapatite-coated plates were used to test the effect of 48-hour treatment with DHA on mature osteoclast resorptive function. The percentage of resorbed area per osteoclast was
Reduced in the presence of DHA. The number of osteoclasts was also reduced by DHA but to a lesser degree relative to resorbing area (Fig. 2A, B, C). These results suggested that DHA restrains the bone-resorbing activity of osteoclasts.

**DHA inhibits RANKL-induced gene expression**

Real-time PCR was used to assess the effect of DHA on RANKL-induced gene expression levels during osteoclastogenesis. The results showed that the expression of the osteoclast marker genes cathepsin K, calcitonin receptor, and TRACP were significantly and dose-dependently inhibited by DHA at day 5 of culture. These results were consistent with osteoclast formation and activity assays (Fig. 3).

**DHA suppresses RANKL-induced NF-κB activation and ERK phosphorylation**

In order to further explore the mechanism of inhibition by DHA on osteoclast differentiation and activity, NF-κB luciferase reporter assay and IκBα degradation analyses were performed. RAW264.7 cells stably transduced with an NF-κB luciferase reporter construct were pretreated with different concentrations of DHA up to 5 μM for 1 hour, then stimulated with 50 ng/mL RANKL for another 6 hours. For Western blot assay, BMMs pretreated for 1 hour with 1 μM DHA were stimulated with RANKL for 0, 5, 10, 20, 30, and 60 min. The results showed that DHA significantly inhibited NF-κB activation in a dose-dependent manner from the concentration of 0.5 μM (Fig. 4A). The results of Western blot analyses showed that DHA at 1 μM significantly inhibited IκBα degradation relative to no treatment at 10 min poststimulation (Fig. 4B, C), and also inhibited the RANKL-induced phosphorylation of ERK relative to total ERK (Fig. 4B, C).

**DHA suppresses RANKL-induced NFAT activation**

To examine the effect of DHA on NFAT activation, NFAT luciferase reporter assay was performed. Similar to the NF-κB luciferase reporter assay, RAW264.7 cells stably transfected with an NFAT luciferase reporter construct were first pretreated with different concentrations of DHA up to 5 μM for 1 hour, followed by RANKL (50 ng/ml) stimulation for another 24 hours. Cells were then harvested for luciferase activity measurement. The results showed that NFAT activation was significantly reduced by DHA at concentrations greater than 0.5 μM. DHA produced a greater than 10-fold reduction in NFAT activation at the concentrations of 2.5 and 5 μM (Fig. 5A).

NFATc1 is a key transcription factor regulating the differentiation of osteoclasts, and autoamplifies its own transcription.
Fig. 2. DHA suppresses hydroxyapatite resorption. (A) Representative images of TRACP staining and osteoclastic resorption on hydroxyapatite-coated surfaces (scale bars = 500 μm). (B) Quantitative analyses of the number of TRACP-positive multinucleated cells. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001 relative to DHA-untreated controls. (C) Quantitative analyses of the percentage of the area of hydroxyapatite surface resorbed per osteoclast. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001 relative to DHA-untreated controls. MNCs = multinucleated cells.

Fig. 3. DHA suppresses RANKL-induced gene expression during osteoclastogenesis. Real-time PCR analyses was performed on RNA extracted from cells stimulated for 5 days with RANKL and varying concentrations of DHA. Gene expression of osteoclast marker genes cathepsin K, calcitonin receptor, and TRACP was normalized to 18S RNA and then compared to RANKL-only control samples to obtain the relative fold change. n = 3; *p < 0.05, **p < 0.01, ***p < 0.001 relative to RANKL-treated, DHA-untreated controls.
DHA inhibits NFATc1 protein levels. Western blot assay was performed with BMMs stimulated by RANKL (50 ng/ml) for 0, 1, 3, and 5 days in the presence or absence of DHA. Protein levels of NFATc1 and V-ATPase-d2 were significantly reduced by DHA treatment consistent with the observed reduction in osteoclast formation (Fig. S8, C). The maximum reduction of V-ATPase-d2 was at 5 days whereas the maximum reduction of NFATc1 was at 3 days (Fig. S8, C).

DHA inhibits ovariectomy-induced bone loss

To determine the effect of DHA on ovariectomy-induced bone loss, mice were either sham operated or ovariectomized (OVX) and were then treated with either DHA at a concentration of 1 mg/kg every 2 days, or DMSO only control, by intraperitoneal injection for 6 weeks post-surgery. There were no adverse events recorded after the OVX procedure or the subsequent treatment with DHA. The tibias were then collected for μCT analysis. Our results showed that DHA treatment protected the mice from bone loss associated with ovariectomy, as shown by increased BV/TV in treated mice relative to the OVX mice without treatment (Fig. 6A, B). Furthermore, trabecular separation (Tb. Sp) was significantly reduced in the OVX + DHA group when compared with the OVX control group (Fig. 6A, B). These results indicated that DHA protected against ovariectomy-induced bone loss.

To further confirm the effect of DHA on ovariectomy-induced bone loss, histomorphometric analyses were also performed. Consistent with the μCT results, there was an increase in BV/TV in the OVX + DHA group when compared with the OVX group (Fig. 7A, B). In addition, osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone surface (N.Oc/BS) were decreased in DHA-treated OVX mice as compared with the OVX group. Consistent with our in vitro results the osteoblast number/bone surface (N.Ob/BS) was not significantly changed after DHA treatment versus the OVX group (Fig. 7A, B). This result...
suggested that DHA protected against ovariectomy-induced bone loss by inhibiting osteoclast number and activity.

**Discussion**

Artemisinin (Qinghaosu) is a natural compound extracted from *Artemisia annua*, which has been used to treat malaria in China for more than 2000 years. DHA is a semisynthetic derivative of artemisinin and is used in combination with piperaquine for the treatment of malaria patients, which is an effective remedy for malaria. Recently, DHA was also found to inhibit cancer cells, such as breast cancer cells and lung cancer cells. Furthermore, DHA was able to inhibit tumor angiogenesis. DHA was found to exert these inhibitory effects on cancer cells through regulating the NF-κB pathway. In this study, we explored the effect of DHA on osteoclast formation and activity, and in an O VX-induced bone loss mouse model, and we found that DHA is more effective than artemisinin in inhibiting osteoclast formation.

Our results showed that DHA inhibited RANKL-induced osteoclastogenesis at concentrations of 0.5 μM and higher without affecting the viability of BMMs; however, mature osteoclast survival and function were affected at these doses, resulting in significant reductions in osteoclast activity. We also found that osteoblast formation and function were not affected by DHA treatment. These findings suggest that DHA might represent an efficient drug prototype for the treatment of osteoclast-mediated osteolysis.
In concordance with its inhibitory effect on osteoclastogenesis, DHA was found to suppress NF-κB in a luciferase reporter gene assay and significantly inhibit osteoclast marker gene expression. The RANKL-induced NF-κB pathway is the major signal pathway activated during osteoclastogenesis. The interaction between RANK and RANKL results in the rapid degradation of IκB by the proteasome and subsequent release of NF-κB, which then translocates from the cytoplasm to the nucleus. It then initiates transcription of osteoclast-specific genes necessary for osteoclast differentiation and function. Our results have demonstrated an inhibitory effect of DHA on NF-κB activation through modulating IκB protein, degradation, and downstream gene expression, indicating that DHA inhibits the NF-κB signaling pathway similar to its known mechanism of action in other cell types.

DHA was also able to reduce NFAT activity in a luciferase reporter gene assay, and the expression of NFAT by Western blot assay. RANKL-induced NFATc1 is another important signaling pathway leading to osteoclast formation. The NFAT signaling cascade activates phospholipase Cγ (PLCγ) and leads to the release of intracellular Ca²⁺, resulting in the activation of calcineurin and transcription and auto amplification of NFATc1. Our studies are the first to identify NFATc1 regulation by DHA, although it is interesting to note that the mechanism of DHA action in Plasmodium falciparum is to disrupt sarcodoplasmic reticulum (SR) calcium transport ATPase (SERCA) pumps resulting in Ca²⁺ disturbances. Although it has been reported that artesimisin does not inhibit mammalian SERCA isoforms, DHA has not been assessed. The importance of Ca²⁺ signaling in osteoclast NFAT activation may explain the sensitivity of osteoclasts to DHA. Similar inhibitory effects have been noted on osteoclast survival and differentiation using other SERCA inhibitors such as thapsigargin.

NFAT signaling is also downstream of NF-κB, thus the suppression of NFATc1 by DHA could potentially result from the suppression of NF-κB signaling. Thus, the inhibitory effect on NFATc1 by DHA could be due to indirect inhibition of NFAT through NF-κB. Our results showed that downstream targets of NFAT activation, including V-ATPase-d2 that is important for osteoclast fusion, are inhibited by DHA, suggesting that suppression of NFATc1 is an important component of the mechanism of DHA inhibition of RANKL-induced osteoclastogenesis.
In addition, our Western blot results showed that DHA inhibits RANKL-induced ERK phosphorylation. The binding of RANKL to RANK also activates ERK, which is a MAPK pathway that is downstream from tumor necrosis factor receptor-associated factor 6 (TRAF6). Inhibition of ERK phosphorylation has been observed in human umbilical vein endothelial cells (HUVECs) treated with DHA, and ERK is crucial for the survival of osteoclasts. Hence the observed reduction in ERK phosphorylation in the presence of DHA may account for the reduced survival that we observed in mature osteoclast cultures. Thus, the effect of DHA on osteoclasts is multifactorial via the inhibition of multiple signaling pathways, including NF-κB, ERK, and NFATc1 signaling pathways.

Based on the in vitro results, an OVX animal model was used for the evaluation of the function of DHA in osteoporosis. Our results showed that OVX mice were protected from bone loss by DHA treatment via inhibiting osteoclast formation and function, with no toxicity in mice. These preclinical experimental results are consistent with the in vitro results, and suggest that in addition to its antimarial and anticancer effects, DHA has a new potential therapeutic effect against osteoporosis and other lytic bone diseases.

Disclosures

All authors state that they have no conflicts of interest.

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Authors’ roles: Lin Zhou and Qin Liu performed cell culture, PCR and signalling studies; Mingli Yang, Jun Yao and Jianwen Cheng performed the MTT analysis and data analysis; Lin Zhou, Tao Wang, Jibing Yuan and Xie Liu performed animal experiments. Lin Zhou wrote the manuscript; Jiaku Xu, Jennifer Tickner and Jinmin Zhao designed and supervised the overall study and revised the manuscript.

References

Chapter 7
Fangchinoline inhibits osteoclast formation and function through RANKL-induced NFAT activity (manuscript in preparation)
Fangchinoline inhibits osteoclast formation and function through RANKL-induced NFAT activity

**ABSTRACT** Osteoporosis is a disease characterized by abnormally increased formation and function of osteoclasts. Anti-RANKL treatment using natural medicine is a potential therapy for osteoporosis. Here, we studied the effect of fangchinoline, which is extracted from the root of *Stephania tetrandra* S. Moore, on osteoclast formation and function. We found that fangchinoline inhibited osteoclastogenesis at doses of 0.5 and 1 µM. In addition, we also examined the mechanism of the inhibitory effect of fangchinoline on osteoclasts. We found that fangchinoline down regulated NFATc1 activity and expression. However, fangchinoline did not affect IκBα degradation and MAPK pathways. In addition, we also found that fangchinoline could protect against bone loss in OVX mice. Taken together, fangchinoline may be a potential compound for osteoporosis.

**KEY WORDS:** fangchinoline, RANKL, osteoclast, NF-κB, NFATc1, MAPK

**INTRODUCTION**

Bone is a hard tissue that forms the endoskeleton of vertebrates. It is a dynamic tissue that goes through a continuous procedure of remodelling and renewing to maintain internal homeostasis throughout life (McHugh K. P. et al. 2000). Osteoclast-induced bone resorption and osteoblast-induced bone formation are two critical components for this process. Increased numbers and overactivity of osteoclasts is a key cause of osteoporosis, which is the most common bone disorder in elderly people. Osteoporosis is a disease of low bone density and poor bone architecture, which increases the risk of fracture in low impact falls. Osteoclasts, giant multinucleated cells, are derived from hematopoietic stem cells. The formation and differentiation of osteoclasts requires two key cytokines, receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). The combination of RANKL and its receptor RANK activates a series of signalling pathways including NF-κB, MAPKs and
NFAT pathways (Wada T. et al. 2006). RANKL-targeted treatment has become a new promising option for osteoclast-related bone diseases.

Fangchinoline is a bisbenzylisoquinoline alkaloid derived from the root of *Menispermaceae* family such as *Stephania tetrandra* S. Moore and *Cyclea peltata* Diels (Huang K. C. 1993). Fangchinoline has numerous pharmacological properties such as anti-inflammatory, anti-oxidant, and neural protection effects (Choi H. S. et al. 2000; Abu-Ghefreh A. A. et al. 2009; Gulcin I. et al. 2010). In addition, tetrandrine, the analogue of fangchinoline, was found to inhibit osteoclastogenesis as well as osteoclast function *in vivo* and *in vitro* (Takahashi T. et al. 2012). Thus, we examined the anti-resorptive effect of fangchinoline on osteoclasts.

**MATERIALS AND METHODS**

**Materials**

Fangchinoline with a purity ≥98% was purchased from Mansite (Chengdu, China). Alpha modified Minimal Essential Medium (α-MEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Scoresby, Australia). Penicillin-Streptomycin and GlutaMAX were purchased from Thermo Fisher Scientific (Scoresby, Australia). The production and purification of recombinant RANKL were described in a previous study (Xu J. et al. 2000). Antibodies to IκBα (C21), phosphorylated ERK, phosphorylated P38, NFATc1 (7A6) and β-actin were obtained from Santa Cruz Biotechnology (Paso Robles, CA, USA). Antibodies to P38, phosphorylated JNK and JNK were ordered from Cell signaling (Danvers, MA, USA). Antibody to ERK, MTS and luciferase assay kits were purchased from Promega (Madison, WI, USA). Antibody to v-ATPase-d2 was produced as reported previously (Feng H. et al. 2009).

**Osteoclastogenesis assay**

Freshly isolated BMM from C57BL/6 mice were plated in T75 flasks and cultured in α-MEM supplemented with M-CSF (50 ng/ml). When cells were confluent, BMM were seeded in 96-well plates at the concentration of 6 × 10³ cells per well. After overnight incubation to allow attachment, cells were differentiated with RANKL (50 ng/ml) and
incubated with different concentrations of fangchinoline. Medium was changed every two days for 5 days or until mature osteoclasts were formed. For investigating which stage of osteoclastogenesis is mostly affected by fangchinoline, BMM were treated with RANKL (50ng/ml) for 5 days, while 1μM fangchinoline was added to BMM at either day 1, 3 or 5. Then, the cells were fixed in paraformaldehyde for 10 min, followed by three washes with 1 × PBS. After that, the cells were stained with TRAcP staining buffer for counting multinucleated cells and image acquired using a light microscope.

**MTS assay**

BMM were seeded into 96-well plates at the density of 6 × 10³ cells per well and cultured in α-MEM with M-CSF for overnight incubation. BMM were then incubated with different concentrations of fangchinoline for 48 h. After that, the cells were treated with 20 µl of MTS solution (Promega, Madison, WI, USA) for 2 h, and then absorbance read with a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm.

**Hydroxyapatite resorption assay**

The effect of fangchinoline on activity of osteoclasts was tested by hydroxyapatite resorption assay. BMM were seeded in 6-well collagen-coated plates (1 × 10⁵/well) for overnight incubation. Then, the cells were stimulated with RANKL (50ng/ml) for every two days until osteoclasts began to form. After that, the cells were harvested with cell dissociation solution and cultured in hydroxyapatite-coated 96 well plates (Corning, Sullivan Park, NY, USA). Cells were treated with RANKL and fangchinoline for another 48 h. Then, half of the wells were fixed with 2.5 % glutaraldehyde and stained with TRAcP staining buffer for counting the number of multinucleated cells. The remaining wells were bleached with 10% bleaching solution for 10 min and then the images were taken for calculating resorbed area by Image J software.

**RNA isolation and analysis**

BMM were seeded in 6-well plates at the density of 1 × 10⁵ per well and stimulated with RANKL (50ng/ml) and treated with fangchinoline in various concentrations for 5 days. Then total RNA was extracted by TRIzol Reagent according to the manufacturer’s
instructions (Life Technologies, Mulgrave, Australia). Single-stranded cDNA was synthesized from 1μg of RNA using reverse transcriptase with oligo-dT primer. The specific primers used in qPCR reactions were designed as follows: mouse cathepsin K (Ctsk) (forward: 5’-GGG AGA AAA ACC TGA AGC-3’; reverse:5’-ATT CTG GGG ACT CAG AGC-3’), mouse calcitonin receptor (forward: 5’-TGG TTG AGG TTG TGC CCA-3’; reverse: 5’-CTC GTG GGT TTG CCT CAT C-3’), V-ATPase-d2 (forward: 5’-GTG AGA CCT TGG AAG ACC TGA A-3’; reverse:5’-GAG AAA TGT GCT CAG GGG CT-3’), mouse MMP9 (forward: 5’-CGT GTC TGG AGA TTC GAC TTG A-3’; reverse: 5’-TTG GAA ACT CAC ACG CCA GA-3’), mouse NFATc1 (forward: 5’-CAA CGC CCT GAC CAC CGA TAG-3’; reverse: 5’-GGC TGC CTT CCG TCT CAT AGT-3’), mouse TRAcP (Acp5) (forward: 5’-TGG GGC CAT CTT TAT GCT G-3’; reverse: 5’-GTC ATT TCT TTG GGG CCTT-3’), and mouse GAPDH (forward: 5’-ACC ACA GTC CAT GCC ATC AC-3’; reverse: 5’-TCC ACC ACC CTG TTG CTG TA-3’).

qPCR reactions were performed though ViiA™ 7 Real-time PCR system (Applied Biosystems, Paisley, United Kingdom). All the qPCR reactions were run in triplicates, and normalized by housekeeping gene GAPDH and further normalized by control samples.

**NF-κB and NFAT luciferase reporter gene assay**

RAW264.7 cells stably transfected with an NF-κB luciferase reporter construct (3kB-Luc-SV40) (Wang C. et al. 2003) or with an NFATc1 luciferase reporter construct (van der Kraan A. G. et al. 2013) were used in this experiment to determine the effect of fangchinoline on NF-κB and NFAT activation. Transfected cells were seeded in 48-well plates at the density of 1.5×10⁵ cells/well (NF-κB luciferase reporter gene assay) or 5×10⁴ cells/well (NFAT luciferase reporter gene assay). After overnight incubation, cells were pre-treated with fangchinoline for 1 h, and then incubated with RANKL (50ng/ml) for 6 h (NF-κB luciferase reporter gene assay) or 24 h (NFAT luciferase reporter gene assay); respectively. Then, the cells were harvested and lysed for measuring luciferase activity using the luciferase assay system (Promega, Sydney, Australia) following the manufacturer’s instruction.
Western blot assays

BMM cells were seeded in 6-well plates overnight at the density of $1 \times 10^5$ cells per well. After 3 h serum starvation, cells were pre-treated with fangchinoline for 1h, then stimulated with RANKL for 0, 10, 20, 30 and 60 min. For long time course western blot assay, cells were cultured in 6-well plates at $1 \times 10^6$ cells per well. Fangchinoline was added to the cells on the next day. Then, the cells were stimulated by RANKL at day 1, 3 and 5. Cells were harvested and lysed by RIPA lysis buffer on ice. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk for at least 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. After three times washing with 1× PBS, membranes were incubated with HRP-conjugated secondary antibodies for 1 h. Proteins on the membranes were visualized by the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Sydney, Australia).

Ovariectomy (OVX) animal model

For OVX experiments, C57BL/6 mice were used in this study. The in vivo experiments were conducted according to the protocols proposed by The Guangxi Medical University Ethics Committee [SCXK - (JUN) 2012-0004, Beijing, China] and the University of Western Australia Animal Ethics Committee. All the mice were raised in standard cages, with the temperature set at 22°C and the lighting condition set at 12 h light and 12 h dark cycle. Mice aged 7-weeks were anesthetized with chloral hydrate and subjected to ovariectomy or sham operation. After one week to allow recovery from the surgery, ovariectomized mice received intraperitoneal injection of fangchinoline at the concentration of 1 mg/kg and 5 mg/kg every two days. In the meantime, mice from OVX control group and sham operation group were injected with 10% DMSO for comparison. After six-weeks of treatment, all the mice were sacrificed and their femurs were removed for analyzing bone parameters by micro-CT.

Micro-CT analysis
The collected femurs were fixed with 4% paraformaldehyde (PFA) for 24 h, followed by three washes with 1 × PBS. Then, samples were scanned by a Skyscan 1176 micro-CT instrument (Bruker microCT, Kartuizersweg, Belgium), using 500 μA source current, 50kV voltage and 0.5mm aluminium filter. Samples were reconstructed using standardized parameters (Hwang Y. P. et al. 2010) with NRecon software (Bruker microCT, Kartuizersweg, Belgium), and analyzed using CTAn software (Bruker microCT, Kartuizersweg, Belgium). The regions of interest were set from 0.5 to 1.5 mm below the bottom of the growth plate. The following trabecular bone parameters were measured: bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp).

Statistical analysis

All data demonstrated in this study are representative of one of three or more independent experiments. The data was expressed as mean ± SEM. Statistical significance was determined by paired or unpaired Student’s t-tests using Microsoft Excel 2010. P value <0.05 was considered to be statistically significant.

RESULTS

Fangchinoline inhibits osteoclastogenesis

To investigate the effect of fangchinoline treatment (Figure 1 A) on osteoclast formation, BMM cells were incubated with different concentrations of fangchinoline (0.125, 0.25, 0.5, 1 μM) and RANKL (50ng/ml) for 5 days, or 1 μM fangchinoline was added to BMM for 1, 3 or 5 days in the continuous presence of RANKL (50 ng/ml). The results suggested that fangchinoline inhibited osteoclastogenesis at 0.5 and 1 μM in a dose-dependent manner (Figure 1 B, C). In addition, fangchinoline significantly inhibited osteoclastogenesis only when added during the early stages of culture (Figure 1 D, E), suggesting that fangchinoline influenced early stages of the osteoclast formation process. MTS results showed that fangchinoline did not impact the viability of BMM (Figure 1 F), which implied that the inhibitory effect of fangchinoline on osteoclasts was not caused by cytotoxicity.
Fangchinoline reduces osteoclastic resorption

To examine the effect of fangchinoline on osteoclasts function, hydroxyapatite-coated 96-well plates were used in our study. Mature osteoclasts were incubated with fangchinoline (0.5 and 1 μM) on hydroxyapatite-coated 96-well plates for 48 h, and the area resorbed per osteoclast was determined. From light microscopy, it was visible that resorption area was reduced by fangchinoline (Figure 2 A). Consistently, the resorption area per osteoclast analysed by Image J was reduced by fangchinoline, while the osteoclast number was not significantly affected (Figure 2 B, C).

Fangchinoline suppresses the expression of osteoclast marker genes

To investigate fangchinoline on RANKL-induced gene expression in osteoclasts, qPCR was applied in this study. As shown in Figure 3, fangchinoline dose dependently reduced RANKL-induced osteoclast markers genes, including cathepsin K, calcitonin receptor, V-ATPase-d2, MMP9, NFAT and TRAcP. This result is consistent with inhibitory effect of fangchinoline on the osteoclast formation and function.

Fangchinoline inhibits NF-κB activity, but not IκBα degradation and the MAPK pathway

To further study the molecular mechanism by which fangchinoline inhibits osteoclastogenesis, luciferase and western blot assays were used. Luciferase assay showed that fangchinoline inhibits NF-κB activity at the dose of 1 μM (Figure 4 A). However, short-time course western blot results showed that IκBα degradation was not affected by fangchinoline, indicating NF-κB activity was suppressed downstream of IκBα (Figure 4 B C). In addition, fangchinoline did not inhibit the phosphorylation of ERK1/2, P38 and JNK1/2 (Figure 5).

Fangchinoline inhibits NFAT activity, and protein expression of NFATc1 and V-ATPase-d2

The effect of fangchinoline on NFAT transcriptional activity was tested by NFAT luciferase assay. RAW264.7 cells transfected with NFAT luciferase gene reporter construct were pre-treated with fangchinoline for 1h, and then incubated with RANKL
(50 ng/ml) for 24h. The result showed that RANKL-induced NFAT activity was inhibited by fangchinoline from 0.5 μM in a dose-dependent manner (Figure 6 A). Consistently, the expression of NFATc1 was also inhibited by fangchinoline as shown by western blot analysis (Figure 6 B). In addition, V-ATPase-d2, which is induced by NFATc1, was also inhibited by fangchinoline at 1 μM (Figure 6 B).

**Fangchinoline protects against OVX-induced bone loss**

To determine the effect of fangchinoline on osteoporosis-induced bone loss, OVX mice which imitate postmenopausal osteoporosis were used in this study. The mice were divided into four groups: sham group, OVX group, OVX + fangchinoline (1mg/kg) group and OVX + fangchinoline (5mg/kg) group. Fangchinoline-treated mice had significantly increased bone mass from the 3D images (Figure 7 A) and the bone parameters (Figure 7 B) as determined by micro-CT. As shown in Figure 7 B, fangchinoline-treated OVX mice had a dose dependent increase in BV/TV and Tb.Th, and decreased Tb.Sp. These results indicated that fangchinoline has therapeutic effect to inhibit OVX-induced bone loss.

**DISCUSSION**

Osteoporosis is a common disease in elderly populations and may reduce life expectancy by causing fracture, resulting in death (Pinkerton J. V. et al. 2013). The development of osteoporosis is through the overproduction and overactivity of osteoclasts (Boyle W. J. et al. 2003). Current treatments against osteoporosis have notable side effects, such as bisphosphonates induced osteonecrosis of jaw and estrogen-induced breast cancer (Rachner T. D. et al. 2011; Lippuner K. 2012). Natural compounds may provide an optional treatment to osteoporosis since natural compounds arising from Chinese medicine have been considered to be relatively safer than pharmaceutical chemicals (He Y. et al. 2011). Numerous natural compounds have been found to attenuate osteoporosis based on cell culture and animal experiments (Bai S. et al. 2016). In this study, we explored the therapeutic effect of fangchinoline on osteoclastogenesis and OVX-induce bone loss.
Modern studies have reported that fangchinoline has extensive pharmacological properties including anti-inflammatory (Choi H. S. et al. 2000), anti-oxidant (Gulcin I. et al. 2010), anti-tumour (Wang C. D. et al. 2014; Luo X. et al. 2016), anti-HIV (Wan Z. et al. 2012) and neural protection effects (Lin T. Y. et al. 2009). In addition, tetrandrine, which has similar structure to fangchinoline, suppressed RANKL-induced osteoclast differentiation and bone loss in sciatic-neurectomized mice (Takahashi T. et al. 2012). In this study, we found fangchinoline suppressed RANKL-induced osteoclastogenesis and ameliorated bone loss in OVX mice.

Osteoclasts, arising from the monocyte-macrophage lineage, are the specific cells which contribute to bone resorption (Yavropoulou M. P. and J. G. Yovos 2008). RANKL and M-CSF are two critical cytokines for the formation and differentiation of osteoclasts. In this study, osteoclastogenesis was dose-dependently inhibited by fangchinoline in the presence of RANKL and M-CSF. MTS results suggested the fangchinoline exerted the inhibitory effect of osteoclastogenesis not through cytotoxicity. Cytotoxic drugs are harmful to human body (Kiron SS. and M. Saritha 2009). The test of cytotoxicity could reflect the toxic effect in vivo (Riss T. L. et al. 2011). Thus, the effective dose of fangchinoline may be safe in clinical trial. Consistently, fangchinoline also down regulated the expression of a series of osteoclasts marker genes, including cathepsin K, calcitonin receptor, V-ATPase-d2, MMP9, NFAT and TRAcP.

The inhibitory effect of fangchinoline on osteoclasts function was studied in vitro using hydroxyapatite-coated plates, which mimics in vivo bone surface to study drug-affected cell functional activity. The hydroxyapatite resorption assay is a simple assay to quantitatively measure the osteoclastic resorbed area. Our results showed that fangchinoline significantly inhibited mature osteoclasts resorption at the concentration of 0.5 and 1 μM, without affecting cell number. Bone resorption is a process that osteoclasts degrade bone and subsequently release calcium to blood (Teitelbaum S. L. 2000). The increased bone resorption has critical involvement in the development of osteolytic diseases. Thus, the inhibiting effect of fangchinoline on bone resorption is essential for the potential role of treating osteoporosis. The active osteoclasts degrade inorganic mineral, mainly calcium and phosphate, through secreting acid from the
ruffled border, and also dissolve organic matrix of bone, primarily type I collagen, by producing cathepsin K and MMPs (Teitelbaum S. L. 2000), consistent with our results that fangchinoline down regulates the gene expression of cathepsin K and MMP9.

The therapeutic effect of fangchinoline on in vivo bone loss was investigated by using OVX mice. The OVX animal model, which mimics estrogen withdrawal in postmenopausal women, is the most common experimental method for assessing bone microarchitectural structure of postmenopausal osteoporosis in response to drugs. The process of bone resorption at first surpasses bone formation after ovariectomy, causing the decline of bone mass. Before long, bone resorption and formation reach a new balance (Lelovas P. P. et al. 2008). In our study, fangchinoline protected the decrease of bone mass reflected by following parameters: the increased BV/TV and Tb.N, and decrease Tb.Sp in OVX mice which were given intraperitoneal injection of fangchinoline. Thus, fangchinoline protects OVX-induced bone loss. However, whether the protective of fangchinoline is through inhibiting the osteoclasts activity needs the conduction of bone histochemistry to analyse the relative parameter such as osteoclast number/ bone surface (N.Oc/BS) and osteoclast surface/ bone surface (Oc.S/BS).

The combination of RANKL and RANK recruits TRAF6, which is an adaptor protein from the TNF receptor-associated factor (TRAF) protein family, initiating a series of chemical reactions. The involved molecules include NF-κB and NFATc1 transcription factors and MAPK families (ERK, P38, JNK) (Jimi E. et al. 1999). NF-κB has been a well-known cytokine for survival and differentiation of osteoclasts (Franzoso G. et al. 1997). In our study, NF-κB activity was significantly inhibited by fangchinoline, suggesting the inhibitory effect of NF-κB activity contribute to part of the underlining mechanisms through which fangchinoline regulates osteoclastogenesis. However, fangchinoline had little effect on IκBα degradation, implying that fangchinoline inhibits NF-κB activity through downstream of IκBα degradation or other unknown regulatory events. In addition, the inhibitory effect of fangchinoline on osteoclasts is not through MAPK signalling since fangchinoline did not affect the phosphorylation of ERK, P38 and JNK. NFATc1, a master transcription factor for osteoclastogenesis, could be induced by RANKL and auto-amplifies its own transcription. NFATc1 transcription is
regulated by calcineurin which is a calcium and calmodulin dependent serine and threonine protein phosphatase (Takayanagi H. 2007). Our results showed that fangchinoline inhibited NFAT activity and also down regulated the expression of NFATc1 and V-ATPase-d2. V-ATPase-d2 is the downstream of NFATc1 (Feng H. et al. 2009), and contributes to the fusion of osteoclasts (Lee S. H. et al. 2006).

In conclusion, fangchinoline attenuated osteoclastogenesis and ameliorated bone loss in OVX mice through suppressing NF-κB and NFAT activity. Our results may offer a novel candidate drug for the therapy of osteoporosis and other osteoclast-mediated bone diseases. However, it requires further investigation to explore the precise molecular targets of fangchinoline. In addition, we will conduct bone histochemistry to analyse the effect of fangchinoline on osteoclast activity in vivo.
REFERENCE


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Figure legends

Figure 1. Osteoclastogenesis and MTS assay. (A) Chemical structure of fangchinoline. (B) RANKL-induced osteoclastogenesis was suppressed by fangchinoline in a dose dependent manner, which was visualised by light microscope. (scale bars= 100 μm) (C) Consistently, the number of multi-nucleated cells (≥3 nuclei) was significantly decreased from 0.5 μM by counting under optical microscopy. (D) Osteoclast images showed that the treating of fangchinoline at day 1 inhibited osteoclastogenesis. (scale bars= 100 μm) (E) Multi-nucleated cells (≥3 nuclei) counting also showed that fangchinoline inhibited osteoclastogenesis mainly at day 1. (F) MTS results indicated that fangchinoline didn’t affect cell viability. ** < 0.01, *** < 0.001.

Figure 2. Resorption assay. (A) Visualized TRAcP positive multinucleated cells (left side) and resorption pits (right side) on hydroxyapatite-coated plates (scale bars, 500 μm). (B) Fangchinoline didn’t affect osteoclast (≥3 nuclei) number. (C) Pits area of per osteoclast was significantly decreased upon the treatment of fangchinoline in a dose-dependent manner on hydroxyapatite-coated plates. * < 0.05, ** < 0.01, *** < 0.001.

Figure 3. qPCR assay for osteoclast marker genes. BMMs were cultured with fangchinoline and RANKL for 5 days. Then, total RNA was extracted for cDNA transcription and qPCR was performed for determining the expression of osteoclast marker genes. The results showed that fangchinoline significantly inhibited the gene levels of cathepsin K, calcitonin receptor, v-ATPase-d2, MMP9, NFATc1 and TRAcP. * < 0.05, ** < 0.01, *** < 0.001.

Figure 4. Fangchinoline suppresses RANKL-induced NF-κB activity, but not IκB degradation. (A) NF-κB luciferase assay results suggested that fangchinoline reduced NF-κB activity at 1 μM. (B) BMM cells were pre-treated with fangchinoline for 1 h, then stimulated by RANKL at different time points (0, 5, 10, 20, 30 60 min), followed by harvested for WB assay. (C) The ratios of the density of IκB-α bands relative to
β-actin bands were determined using Image J. n=3. The results showed that IκBα degradation was not affected by fangchinoline. ** < 0.01.

Figure 5. Fangchinoline doesn't affect RANKL-induced MAPK pathway. (A) Cells for detecting the effect of fangchinoline on MAPK pathway by WB assay were treated and harvested same as WB assay for assessing IκBα degradation. (B) The ratios of the density of P-ERK1/P-ERK2 bands relative to ERK1/ ERK2, P-P38 bands relative to P38 bands and P-JNK1/P-JNK2 bands relative to JNK1/JNK2 bands were determined using Image J. n=3. The results showed that fangchinoline had no effect on the phosphorylation of ERK1/2, P38 and JNK1/2, indicating the inhibitory fangchinoline on osteoclastogenesis was not through MAPK pathway. The results showed that IκBα degradation was not affected by fangchinoline.

Figure 6. Fangchinoline inhibits NFAT activity, as well as the expression of NFATc1 and v-ATPase-d2. (A) NFAT luciferase results showed that fangchinoline inhibited NFAT activity from 0.5 μM in a dose dependent manner. (B) BMM were treated with fangchinoline for 5 days and stimulated with RANKL at day 1, 3 and 5. Then, WB assay was performed on the cell lysates. (C) The ratios of the density of NFATc1 bands relative to β-actin bands and v-ATPase-d2 relative to β-actin bands were determined using Image J. n=3. It was shown from results that the protein levels of NFATc1 and v-ATPase-d2 were significantly inhibited by fangchinoline at day 3 and 5. * < 0.05, ** < 0.01, *** < 0.001.

Figure 7. OVX mice experiment. (A) Constructed 3D images of proximal femur from sham, OVX, OVX mice injected with low doses and high doses of fangchinoline. (B) The microstructure of bone mass was analysed by following parameters: BV/TV, Tb.Th, Tb.Sp and Tb.N (* P < 0.05, ** P < 0.01). * < 0.05, ** < 0.01, *** < 0.001.
Fig. 2
Fig. 3
Fig 4

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A

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P-ERK1/2
ERK1/2
P-P38
P38
P-JNK1/2
JNK1/2

B

Fig. 5
Chapter 8
Cumambrin A prevents osteoclastogenesis and bone resorption via the regulation of RANKL-induced signaling pathways (manuscript in preparation)
Cumambrin A prevents osteoclastogenesis and bone resorption via the regulation of RANKL-induced signaling pathways

ABSTRACT As the principal cells responsible for bone resorption and pathological bone loss, osteoclasts have become the main targets for anti-resorptive treatment. In this study, we found that cumambrin A can significantly inhibit RANKL-induced osteoclast formation and bone resorption through the suppression of NF-κB and NFAT activity. In addition, cumambrin A can also inhibit RANKL-induced ERK phosphorylation. Treatment with cumambrin A at 5 μM did not affect the viability of osteoclast precursor cells. Further, cumambrin A inhibits the expression of osteoclast marker genes, including cathepsin K, calcitonin receptor, and v-ATPase d2. Using an in vivo OVX mouse model we showed that cumambrin A protected against estrogen withdrawal-induced bone loss. Collectively, our results reveal that cumambrin A can suppress RANKL-induced osteoclast formation and bone resorption, suggesting cumambrin A may be a potential treatment for bone destruction related disease.

KEY WORDS: Cumambrin A, RANKL, NF-κB, NFATc1, ERK

INTRODUCTION

Bone is a dynamic tissue that is continuously reshaping, repairing and renewing to maintain bone integrity. These events are collectively termed bone remodelling, which is the predominant metabolic process regulating bone structure and function during life time (Lewellen T. K. et al. 1977; Teitelbaum S. L. 2000; Boyle W. J. et al. 2003). The bone remodelling process consists of bone resorption by osteoclasts and bone synthesis by osteoblasts (Tomoyasu A. et al. 1998). The imbalance of bone remodelling results in the development of bone diseases such as osteoporosis and osteosclerosis.

Osteoclasts arise from hematopoietic stem cells and differentiate through monocyte/macrophage precursors to mature osteoclasts marked by a clear zone and ruffled border (Boyle W. J. et al. 2003). The receptor activator of nuclear factor-kappa B ligand (RANKL), which is a type II transmembrane protein from the super family of TNF (Blair H. C. and N. A. Athanasou 2004), is essential for osteoclast formation and
activation (Lacey D. L. et al. 1998; Tomoyasu A. et al. 1998; Kong Y. Y. et al. 1999). Binding of RANKL to RANK results in the activation of a series of key transcription factors, such as NF-κB, Activator Protein 1 (AP-1), and Nuclear Factor of Activated T-cell cytoplasmic 1 (NFATc1), regulating the expression of osteoclast related genes, such as TRAcP, Cathepsin K, Matrix Metalloproteinase 9 (MMP-9), and subsequently leading to the formation and activation of mature multinucleated osteoclast (Zhu L. L. et al. 2005; Takayanagi H. 2009; Zou W. and S. L. Teitelbaum 2010).

Sesquiterpene lactones (SLs) are a large class of compounds which share the similar chemical structure consisting of sequiterpene and a lactone ring. Most SLs are derived from plants in the Asteraceae family (Schmidt T.J. 1999). Numerous species of this family are used in traditional medicine for the treatment of inflammation, and SLs have been identified as their active constituents. Multiple studies have suggested that SLs exert their anti-inflammatory activity through inhibiting NF-κB (Hehner S. P. et al. 1998; Lyss G. et al. 1998; Mazor R. L. et al. 2000; Garcia-Pinieres A. J. et al. 2001).

In this study, several natural compounds containing SL structure were screened for their effects on osteoclasts by NF-κB luciferase assay. Among the screened compounds, we have found that cumambrin A was one of the most effective compounds which suppressed NF-κB activity. Thus, we further investigated the inhibitory effects of cumambrin A on osteoclasts and the underlying molecular mechanism for this inhibitory effects.

MATERIALS AND METHODS

Media and reagents

Cumambrin A (>95% purity) was provided by Dr. Qiong Gu from the School of Pharmaceutical Sciences, Sun Yatsun University. Alpha modified Minimal Essential Medium (α-MEM) and fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Scoresby, Australia). Anti-NFATc1 antibody was obtained from BD Bioscience (Sydney, Australia). Anti-ÎºBα, anti p-ERK1/2 and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology Santa Cruz Biotechnology (Paso Robles, CA, USA). Antibodies to total ERK1/2 were purchased from Promega (Madison, WI, USA) and v-ATPase-d2 was produced as the description in a previously published paper (Feng H. et al. 2009). Luciferase assay system was obtained from
Promega (Sydney, Australia). Recombinant GST-rRANKL protein was generated as previously published (Xu J. et al. 2000).

**In vitro osteoclastogenesis assay**

BMM were cultured in complete α-MEM with M-CSF. Upon reaching confluence, BMM were then seeded on to 96-well plates (6x10³) and cultured with cumambrin A in the presence of RANKL (50 ng/ml). Medium and cumambrin A were replaced every two days. After five days cells were fixed with 2.5% glutaraldehyde (Fisher Scientific, USA) and stained with TRAcP. Images were taken and the number of osteoclasts (defined as TRAcP positive cells with three or more nuclei) was counted under light microscopy.

**Cell proliferation assay (MTS)**

Cell proliferation was assessed using MTS assay according to the manufacturer’s instructions (Promega, Sydney, Australia). Briefly, BMM cells were seeded onto a 96-well plate at the density of 6x10³ and cultured with complete α-MEM and M-CSF (25 ng/ml). After cells were cultured overnight to attach to the plate, cells were then incubated with cumambrin A for 2 days. MTS/PMS mixture was added to the cells and incubated for 2 hours. Then each well was read by an ELISA plate reader (Bio-Rad, Hercules, CA, USA) to obtain the absorbance value and compared to untreated controls.

**Hydroxyapatite resorption assay**

BMM were cultured in a 6 well collagen-coated plate at a cell density of 1 x 10⁵ cells/well and incubated overnight. Cells were then stimulated with 50ng/ml RANKL every two days until small osteoclasts were formed. Osteoclasts were then dissociated from the collagen plate and seeded into a hydroxyapatite-coated plates at consistent cell density. Cells were treated with RANKL to keep cell viability in the presence or absence of Cumambrin A. After 48 hours the plate was fixed with 2.5% glutaraldehyde (Fisher Scientific, USA) and stained with TRAcP for visualizing and counting osteoclasts; or bleached with 10% bleach solution to remove cells and measuring the resorbed area through Image J software.

**Luciferase assay**
RAW cells stably transfected with NF-κB luciferase reporter gene (Wang C. et al. 2003) or NFAT luciferase reporter gene (van der Kraan A. G. et al. 2013) were seeded onto a 48-well plate at the density of 1.5x10^5 and 5 x10^4; respectively. On the next day cumambrin A was added to the cells at different concentrations. After 1h, cells were treated with RANKL for 6 h (for NF-κB luciferase reporter gene) and 24 h (for NFAT luciferase reporter gene); respectively. At the endpoint, cells were lysed and harvested. Cell lysates were added to a white solid 96-well plate for measuring luciferase activity by BMG Polar Star Optima luminescence reader.

**RNA isolation and analysis**

For RNA isolation, BMM were seeded on to 6-well plates (1x10^5) and differentiated with RANKL in the presence or absence of cumambin A. Cells were harvested and total RNA was extracted with TRIzol® Reagent (Life Technologies™). For RT-PCR, single stranded cDNA was obtained by reverse transcribing 1μg of total RNA using reverse transcriptase with oligo-dT primers according to manufacturer’s instructions (Promega, Sydney, Australia). QPCR was then performed using a ViiA™ 7 Real-time PCR system (Applied Biosystems, Paisley, United Kingdom), at the following conditions: 94°C, 5 minutes; 30 cycles of 94°C, 60°C, and 72°C at 40 seconds; and then 72°C, 10 minutes. The following mouse primers were used: TRAcP (Forward, 5’-CAGCAGCCAAGGAGGACTAC -3’; Reverse, 5’- ACATAGCCCACACC GTTCTC -3’), calcitonin receptor (Forward: 5’- TGGTTGAGGTTGTGCCCA-3’; reverse: 5’-CTCGTGGGGTTTCCTC -3’), cathepsin K (Forward, 5’-CCAGTGGGAGCTATGGAAGA -3’; Reverse, 5’- AAGTGGTTTCTGGCCAGTTC -3’), 18S (Forward, 5’- TGTCAATCCTGTCCGTGTC -3’; Reverse, 5’-ACCATAACGATGCCGACT -3’). The obtained value was normalized to house-keeping gene 18S, and expressed as fold change relative to the control sample.

**Western blot analysis**

BMM cells were seeded into 6-well plates as described above. For short time course experiments, the cells underwent serum starvation for 3 h prior to 1 h pretreatment with cumambrin A. RANKL was then added to the cells for 0, 5, 10, 20, 30 and 60 min. For
long time course, the cells were stimulated with RANKL in the presence or absence of cumambrin A on Day 1, Day 3 and Day 5. Cells were lysed with RIPA buffer on ice for protein extraction. SDS-PAGE electrophoresis was performed to separate proteins, which were then transferred onto nitrocellulose membranes. Membranes were blocked in a 5% skim milk with TBS-Tween solution for one hour, then probed with the following antibodies: anti-ERK1/2, anti-pERK1/2, anti-IκBα, anti-v-ATPase-d2, anti-NFAT and β-actin overnight. After washing with TBS-Tween corresponding HRP-conjugated secondary antibodies were then added for 1h to detect bound antibody. The membranes were developed using Enhanced Chemiluminescence (ECL) reagents and imaged on FujiFilm LAS-3000 gel documentation system.

**Ovariectomized (OVX) mouse model**

The OVX mouse model was used to test the effect of cumambrin A on hormone withdrawal induced bone loss. This *in vivo* experiment was approved by the Institutional Animal Ethics Committee of Guangxi medical university. A total of 24 C57BL/6 mice were randomly divided into 4 groups; sham group, OVX group, OVX + cumambrin A (1mg/kg) group and OVX + cumambrin A (5mg/kg) group (6 mice in each group). After acclimatization for one week, mice aged 7 weeks old were anesthetized by intraperitoneal injection with chloral hydrate. Sham control mice were given a sham operation, while other three groups were given an ovariectomy operation. Animals had one week postoperative recovery after the surgery. For the next six weeks, the OVX + cumambrin A groups were intraperitoneally injected with Cumambrin A at 1mg/kg and 5 mg/kg respectively. The OVX and sham control groups were intraperitoneally injected with 4% DMSO. After 6 weeks of treatment mice were euthanized and their femurs were collected for micro-CT analysis (Skyscan 1174; Skyscan, Aartselaar, Belgium). The femurs of the mice were scanned at avoxel resolution of 9 µm. The scan settings and reconstruction parameters were as described previously (Wang T. et al. 2015).

**Statistical analysis**
Data presented are representative results obtained from a triplicate set of three independent experiments or the mean ± SEM of those experiments. A student’s t-test was used to test statistical significance, with P-values of < 0.05 considered statistically significant.

RESULTS

Cumambrin A inhibits RANKL-induced osteoclastogenesis

To examine the effects of cumambrin A on RANKL-induced osteoclast growth, BMM cells were incubated with varying concentrations of cumambrin A (0, 0.5, 1, 2, 5 µM). After five days, osteoclasts were formed, then stained with TRAP, fixed and counted. Only osteoclasts with greater than 3 nuclei were included in the count. Cumambrin A treatment resulted in reduced size and number of osteoclasts (Fig. 1B). Wells containing the higher doses of cumambrin A had a darker color by scanning (Fig. 1C). The minimum concentration which can significantly reduce the number of osteoclasts is 0.5 µM (Fig. 1D). As a result, this data suggests that cumambrin A inhibits osteoclast growth in a dose-dependent manner. In addition, MTS results showed that the inhibition of osteoclastogenesis by cumambrin A at doses up to 5 µM was not due to the suppression of BMM cell viability.

Cumambrin A impairs osteoclastic resorption

Hydroxyapatite resorption assay was performed to determine the effect of cumambrin A on the function of osteoclasts. In our study, mature osteoclast numbers were not reduced by cumambrin A at the concentration of 1 and 2 µM, but resorptive area was significantly reduced by cumambrin A (at 1 and 2µM) (Fig. 2 A B). These results revealed that cumambrin A inhibits bone resorption without affecting the apoptosis of osteoclasts.

Cumambrin A suppresses RANKL-induced gene expression

To further examine the role of cumambrin A in osteoclast differentiation, qPCR was performed to examine the effect of cumambrin A on osteoclast marker gene expression. Consistently, cumambrin A inhibited the expression of osteoclastic marker genes.
cathepsin K, calcitonin receptor, and TRAcP during RANKL induced osteoclastogenesis from 0.5 μM in a dose-dependent manner (Fig. 3).

**Cumambrin A suppresses RANKL-induced NF-κB activation as well as IκBα degradation and phosphorylation of ERK**

To explore the effect of cumambrin A on RANKL-induced signalling transduction pathways, NF-κB luciferase and WB assay was performed. Our results showed that Cumambrin A can significantly inhibit NF-κB activation from the concentration of 5 μM (Fig. 4 A). In addition, cumambrin A at this concentration could also inhibit IκBα degradation revealing the importance of the NF-κB pathway in cumambrin A mediated inhibition of osteoclastogenesis. Furthermore, the same concentration of cumambrin A also decreased the phosphorylation of MAPK family member ERK, another important signaling pathway regulating the survival and activation of osteoclasts (Fig. 4 B).

**Cumambrin A inhibits RANKL-induced NFAT activation as well as the expression of NFATc1 and v-ATPase-d2**

RANKL-induced NFAT pathway is another critical pathway for osteoclastogenesis. Luciferase reported assay showed that cumambrin A can significantly inhibit NFAT activity from 0.5 μM (Fig. 5 A). To study the role of cumambrin A on the expression of NFATc1 and v-ATPase-d2, we also performed western blotting on long time course cell culture protein lysates. Our results reveal that both RANKL-induced v-ATPase-d2 and RANKL-induced NFATc1 protein expression were significantly suppressed by cumambrin A. From our study, v-ATPase-d2 was reduced at day 3 and more significantly at day 5, while NFATc1 was more significantly reduced at day 3 rather than day 5 (Fig. 5 B).

**Cumambrin A prevents ovariectomy-induced bone loss**

The OVX mice were injected with cumambrin A every two days for 6 weeks. Then the femurs were collected for micro-CT analysis. From our results, the BV/TV (bone volume/tissue volume) was increased in the OVX mice which were injected with cumambrin A (5 mg/kg) when compared with OVX controls. In addition, trabecular number was also increased in the OVX mice which were injected with cumambrin A (5
mg/kg) compared with OVX controls. These results suggested that higher doses of cumambrin A (5 mg/kg) were able to prevent or reverse the bone loss induced by ovariectomy (Fig 6. A B).

DISSCUSION

Osteoporosis is a common and degenerative bone disease. The key feature of this disease is the decrease in bone mass and density. Osteoclasts are the main cells leading to the loss of bone mass and density. The binding of RANK and RANKL results in activation and formation of osteoclasts through NF-κB, NFATc1 and mitogen activated protein kinase (MAPK) pathways (Wada T. et al. 2006; Boyce B. F. and L. Xing 2008). Natural compounds are now interested for their safety when compared with pharmaceutical chemicals (Jiang M. et al. 2011). Natural compounds may provide alternative treatments for osteoporosis since numerous natural compounds were found to be inhibitors of bone resorption and protect bone loss in OVX mice (Putnam S. E. et al. 2007). Sesquiterpene lactones (SLs) are chemical compounds characterized by containing sesquiterpene structure consisting of three isoprene units and a lactone ring. Recently, interest in the anti-inflammatory activity of SLs has led to the identification of their ability to inhibit the NF-κB pathway. Cumambrin A is a member of the SL family, and is a natural compound isolated from Chrysanthemum indicum. In this study, we studied the effect of cumambrin A on osteoclast formation and function.

Osteoclasts are the fusion of macrophages and are derived from the hematopoietic stem cells in the bone marrow (Teitelbaum S. L. 2000). Our results showed that cumambrin A inhibited osteoclastogenesis in a dose-dependent manner. The results are consistent to PCR results which revealed that cumambrin A reduced the gene expression of osteoclast marker genes such as CTR, cathepsin K and TRAcP during RANKL-induced osteoclastogenesis. Osteoclasts function was tested by hydroxyapatite resorption assay. Consistently, cumambrin A inhibited osteoclastic resorption.

RANKL, a type II transmembrane protein, is a critical factor for the formation and differentiation of osteoclasts. The combination of RANKL and RANK induced many
signalling pathways to promote osteoclast formation and functions via the recruitment of the adaptor molecule TRAF6 (Soysa N. S. et al. 2012). The related pathways include NF-κB, MAPKs (ERK, JNK, p38), NFATc1. To explore the potential mechanisms by which cumambrin A inhibited osteoclastogenesis and osteoclastic resorption, luciferase assay and western blot analysis were performed.

Luciferase results showed that cumambrin A inhibited RANKL-induced NF-κB activity in a dose dependent manner. Short-time course western blot also showed cumambrin A inhibited RANKL-induced IκB-α degradation. The importance of NF-κB was indicated in double knock out of p50 and p52 subunits studies, which found that p50/- and p52/- mice developed osteopetrosis due to the markedly reduced osteoclasts (Iotsova V. et al. 1997). NF-κB is released through the degradation of IκBα and then transferred from cytoplasm to nucleus and subsequently plays a positive role on osteoclast differentiation and function (Mercurio F. and A. M. Manning 1999; Mercurio F. and A. M. Manning 1999; Dixit V. and T. W. Mak 2002; Brown K. D. et al. 2008). Thus, our luciferase assay and western blot analysis together revealed that Cumambrin A inhibits RANKL-induced NF-κB signaling pathway.

NFATc1 upregulates the expression of osteoclast marker genes, such as TRAcP, calcitonin receptor and cathepsin K, and subsequently promotes osteoclast function and differentiation (Asagiri M. et al. 2005). NFATc1 is a downstream master transcription factor of RANKL-induced signalling pathways including NF-κB and AP-1 (Leibbrandt A. and J. M. Penninger 2008; Leibbrandt Andreas and Josef M. Penninger 2012). In addition, the induction of NFATc1 is also dependent on calcium signaling pathway. Furthermore, the transcription of NFATc1 could also be auto-amplified by NFATc1 itself during calcium signaling (Takayanagi H. 2007). Results of NFAT luciferase assay showed that cumambrin A inhibited RANKL-induced NFAT activity in a dose-dependent manner. The suppression was more significant compared with NF-κB. The long-time course western blot analysis result was also consistent with this result. NFATc1 expression was significantly suppressed on day 3 by cumambrin A. So, the luciferase assay and western blot together confirm that cumambrin A inhibited RANKL-induced NFAT pathway. In addition, our western blot results showed that
cumambrin A inhibit ERK phosphorylation in short time course and v-ATPase-d2 expression in long time course. In addition to RANKL, ERK could be also activated by M-CSF (Kikuta J. and M. Ishii 2013). ERK is critical for the survival of osteoclasts (Miyazaki T. et al. 2000; Nakamura H. et al. 2003) while v-ATPase-d2 promotes the fusion of osteoclasts (Lee S. H. et al. 2006). In summary, cumambrin A inhibits osteoclastogenesis and bone resorption. This effect is possibly exerted by affecting some very important signaling pathways that include NF-κB, ERK and NFATc1 signaling pathway.

OVX mouse model is an animal model which mimics the physiological characteristics in postmenopausal women. The bone loss features of OVX model are similar to postmenopausal bone changes in human (Lee W. S. and W. Yao 2001). In consistent to the in vitro experiments, our in vivo experiments suggested that cumambrin A protects ovariectomy-induced bone loss by increasing BV/TV and trabecular number. The protecting effect was showed in the dose of 5 mg/kg.

Over all, the present study revealed that cumambrin A may provide a potential treatment to osteoporosis. However, it requires the conduction of bone histochemistry to analyze the effect of cumambrin A on osteoclast activity in vivo.


Figure legend

Fig 1. Cumambrin A prevents RANKL-induced osteoclastogenesis. (A) The molecular structure of cumambrin A. (B) Inhibitory effect of cumambrin on osteoclastogenesis assay. BMM were stimulated with RANKL and cultured with different concentrations of cumambrin A. After 5 days, the cells were fixed and stained with TRAcP. The images of cells were taken by light microscope at 100X magnification (C) The photo of the plate were taken to reveal the dose dependent effect of cumambrin A on osteoclastogenesis. (D) Quantification of the effect of cumambrin A at increasing doses by counting TRAcP+ multinucleated cells (Nuclei ≥3) as osteoclasts. (E) The effect of cumambrin A on cell viability. BMM cells were incubated with cumambrin A at different concentrations for 48 hours and then incubated with MTS/PMS mixture for 2 h, then absorbance at 490nm was measured. *p <0.05, **p<0.01, ***p<0.001 relative to cumambrin A -untreated controls.

Fig 2. Cumambrin A inhibits hydroxyapatite resorption. (A) Representative images of resorbed area and TRAP-positive multinucleated cells on hydroxyapatite coated surfaces (scale bars, 500 µm). (B) The effect of cumambrin A on the number of TRAP+ multinucleated cells (Nuclei ≥3, counted as osteoclasts). (C) Percentage of the area of hydroxyapatite surface resorbed per osteoclast. *p<0.05, **p<0.01 relative to cumambrin A -untreated controls.

Fig 3. Cumambrin A down-regulates RANKL-induced gene expression during osteoclastogenesis. BMM were stimulated with RANKL and cultured with different concentrations of cumambrin A. After 5 days, RNA was extracted, followed by quantitative PCR analysis. The comparative 2-ΔΔCT method was used to calculate the relative expression of each target gene. The mean Ct value of target genes in the experimental group was normalized to the Ct value of 18S to give a ΔCt value, which was further normalized to RANKL-treated control samples to obtain ΔΔCt. *p<0.05, **p<0.01, ***p<0.001 relative to RANKL-treated, cumambrin A -untreated controls.

Fig 4. Cumambrin A inhibits RANKL-stimulated NF-κB activity, IκBα degradation and ERK phosphorylation. (A) The bar graph depicts the NF-κB luciferase activity of
RAW264.7 cells stably transfected with an NF-κB luciferase reporter construct. Cells were treated by different concentrations of cumambrin A and stimulated with RANKL for 6 h. (B) BMM cells were pretreated with cumambrin A for 1 h, then stimulated with RANKL for 0, 5, 10, 20, 30 and 60 min. The cells were harvested for protein extraction. The protein were separated and transferred to PVDF membranes, and then probed for IκB-α, p-ERK, ERK and β-actin antibodies. *p<0.05 relative to RANKL-treated, Cumambrin A-untreated controls.

Fig 5. Cumambrin A inhibits RANKL-stimulated NFAT activity, and also suppressed the expression of NFATc1 and v-ATPase-d2. (A) The bar graph depicts the NFAT luciferase activity of RAW264.7 cells stably transfected with an NFAT luciferase reporter construct. Cells were treated by different concentrations of cumambrin A and stimulated with RANKL for 24 h. (B) BMM cells were with RANKL at day 1, 3 and 5 in the presence of cumambrin A. The cells were harvested for protein extraction. The protein were separated and transferred to PVDF membranes, and then probed for NFATc1, v-ATPase-d2 and β-actin antibodies. *p<0.05, **p<0.01, ***p<0.001 relative to RANKL-treated, cumambrin A-untreated controls.

Fig 6. Cumambrin A ameliorates OVX-induced bone loss. (A) The 3D images of proximal femurs from sham, OVX, OVX + cumambrin A (1 mg/ml), OVX + cumambrin A (5 mg/ml). (B) The micro-CT analysis results of the above four groups using the following parameters: BV/TV, Trabecular thickness, Trabecular separation, Trabecular number. *p<0.05, relative to OVX controls.
Figure 1

A

Chemical structure of cumambrin A.

B

Images showing the effects of RANKL and cumambrin A on osteoclast activity.

C

Table showing the concentration of cumambrin A (µM) and its effect on TRAP+ multinucleated cells.

D

Graph showing the number of TRAP+ multinucleated cells (TRAP+ MNCs) for different concentrations of cumambrin A (µM).

E

Bar graph showing cell viability for different concentrations of cumambrin A (µM).

Figure 1
Figure 2
Figure 3

Normalize to 18S (relative ratio to control)

Fold change

Cathepsin K

Calcitonin receptor

TRACP

Cumambrin A(μM) 0 0.5 1 2

Fold change

**

Figure 3
Figure 5
A

Sham  OVX  OVX + cumambrin A (1mg/ml)  OVX + cumambrin A (5mg/ml)

Figure 6
Chapter 9

General discussion and future directions
9.1 General discussion

Osteoporosis is a silent disease, which increases the risk of fragility fractures with associated morbidity and mortality. According to Australia Health Survey by self-reported diagnosis of osteoporosis, people over 50-year old have a percentage of 15% of women and 3% of men who got osteoporosis in 2011 to 2012 (Australian Institute of Health and Welfare . 2014). Osteoporotic fracture is quite common and affected around 9 million people in the world annually (Johnell O. and J. A. Kanis 2006). The risk of death was increased around 20% in the patients with hip and spine fractures (Ioannidis G. et al. 2009). Thus osteoporosis is a devastating disease.

Recent therapies targeting osteoporosis are split into two main classes: anti-resorptive drugs and bone-formation drugs. Anti-resorptive drugs include bisphosphonates, SERMs, and estrogen replacement therapy. Bone-formation drugs include PTH and strontium ranelate. Compared with anti-resorptive drugs, bone-formation drugs are relatively new and undergoing development. However, most of these drugs have severe side effects. For example, the major adverse effect of bisphosphonates is osteonecrosis of the jaw (Shane E. 2010; Chamizo Carmona E. et al. 2013). Estrogen supplementation has been associated with increased incidence of breast cancer, stroke and heart disease. In addition, it is not suitable for males and pre-menopausal females (Rossouw J. E. et al. 2002). Raloxifene (one of the SERMs) could greatly reduce the risk of breast cancer, but not the risk of stroke and heart disease (Taylor H. S. 2009). PTH has significant benefits to treat osteoporosis, but should be stopped after 2 years due to the possible risk of osteosarcoma (Hodsman A. B. et al. 2005; Lippuner K. 2012). Strontium ranelate is a very effective novel drug for osteoporosis (Cianferotti L. et al. 2013). However, it increases the risk of venous thromboembolism and cardiovascular disease (MHRA 2014).

RANKL has become a hot target for anti-resorptive treatment. For example, denosumab, a fully human anti-RANKL IgG2 monoclonal antibody, is available in clinics. Accumulating studies showed that denosumab is very efficient and well tolerated (Josse R. et al. 2013; Yasuda H. 2013). Thus, it has become the first-line
medicine for osteoporosis. In our study, natural compounds were studied as anti-RANKL or anti-resorptive treatments for osteoporosis. Natural compounds were derived from fruits, leaves, stems, bark, rhizomes, and roots of the plants. Most of these plants have been used in traditional Chinese medicine for centuries. When compared with pharmaceutical chemicals natural compounds are gaining much interest for their safety profiles (Jiang M. et al. 2011).

In this study, we screened hundreds of natural compounds by osteoclastogenesis. Among these compounds, four compounds (berberine sulfate, dihydroartemisinin, fangchinoline, and cumambrin A) were taken into further study. The study results of berberine sulfate and dihydroartemisin (DHA) have been accepted for publication. The results of fangchinoline and cumambrin A have contributed to two further manuscripts which will be submitted to journals soon. Other effective compounds from my screening list were collaboratively studied with my lab colleagues and the published papers of these compounds are attached in the appendix.

Most compounds used for this study were extracted from common Chinese medicinal herbs. For example, berberine sulfate is extracted from the stems, bark, roots and rhizomes of berberis plants, which have been used as anti-microbial medicine in ancient China for treating dysentery and gastroenteritis. DHA is derived from the leafy portions of *Artemisia annua* plants, which has been used as anti-malaria medicine for a long time in traditional China. Fangchinoline is isolated from the tuberous root of Menispermaceae family plants such as *Stephania tetrandra* S. Moore and *Cyclea peltata* Diels. These plants were used for treating inflammatory diseases in China for centuries. Cumambrin A is produced from *Handelia trichophylla*, which also has a long medicinal history in China.

Although the four compounds have different chemical structure and are extracted from different herbs, they do have similar pharmacological effects, especially anti-inflammatory effect. For example, berberine sulfate is the salt form of berberine, which is a kind of isoquinoline alkaloid. Berberine sulfate has notable anti-bacterial effects, thus was used in the treatment of diarrhea (Rabbani G. H. et al. 1987; Sun D. et
DHA, a semi-synthetic derivative of artemisinin, is a kind of sesquiterpene lactone. DHA has strong anti-malarial activity (Ashley E. A. et al. 2005). In addition, DHA also has anti-inflammatory (Ashley E. A. et al. 2005) and anti-tumor effects (Crespo-Ortiz M. P. and M. Q. Wei 2012; Jia L. and X. Li 2013). Fangchinoline is a bisbenzylisoquinoline alkaloid and has multiple pharmacological effects including anti-inflammatory (Choi H. S. et al. 2000), anti-oxidant (Gulcin I. et al. 2010), anti-tumor (Wang C. D. et al. 2010; Wang C. D. et al. 2014; Luo X. et al. 2016), anti-HIV (Wan Z. et al. 2012) and neural protection (Lin T. Y. et al. 2009). Cumambrin is a member of sesquiterpene lactones (SLs) family which has obvious anti-NF-κB effects (Rungele P. et al. 1999). So far, the pharmacological effect of cumambrin A has not been deeply studied.

These studied compounds have been shown to inhibit osteoclastogenesis in a dose-dependent manner. Consistently, they also suppressed osteoclast gene markers, such as cathepsin K, calcitonin receptor, and v-ATPase d2. The impacts of the natural compounds on osteoclasts function were assessed in vitro using hydroxyapatite resorption assay. The results showed all the studied natural compounds inhibited osteoclastic resorption on hydroxyapatite-coated plates. In order to test whether the effective doses of the natural compounds on osteoclasts were not due to the cytotoxicity, MTS assay was applied. In our study, the highest working concentrations of the natural compounds did not affect cell proliferation. Thus, the natural compounds inhibited RANKL-induced osteoclastogenesis not through cytotoxicity.

To explore the intrinsic molecular mechanisms by which natural compounds inhibited osteoclasts, luciferase and western blot assays were applied. The luciferase results showed that all of these four compounds inhibit NF-κB and NFAT activities. DHA and cumambrin A inhibited IκBα degradation, consistent with the NF-κB luciferase results. However, fangchinoline and berberine sulfate didn’t affect IκBα degradation. The importance of NF-κB was indicated in double knock out of p50 and p52 subunits studies, which found that p50/- and p52/- mice developed osteopetrosis due to the markedly reduced osteoclasts (Franzoso G. et al. 1997; Iotsova V. et al. 1997). Before activation, NF-κB is bound to IκBα. When simulated, IκBα is phosphorylated and
degraded to release transcription factor, NF-κB, which then translocates to nucleus to function in osteoclasts (Karin M. and Y. Ben-Neriah 2000). Since fangchinoline and berberine sulfate didn’t inhibit IκBα degradation, they may regulate NF-κB through downstream pathway of IκBα or other unknown cytokines.

RANKL-induced MAPK pathways are also critical in osteoclastogenesis. The major members of MAPK family are ERK1/2, JNK1/2 and p38 (Soysa N. S. et al. 2012). In our study, cumambrin A inhibited the phosphorylation of ERK1/2 through western blot assay. ERK plays an important role in the survival and formation of osteoclasts (Nakamura H. et al. 2003).

NFATc1 is a downstream transcription factor of RANKL-induced signalling pathways. The induction of NFATc1 could be through NF-κB and AP1. Additionally, calcium pathway is another major pathway for the induction of NFATc1. It is interesting that NFATc1 also amplifies itself by calcium signalling and the subsequent recruitment of other transcription factors (NF-κB, NFATc2 and c-Fos) (Takayanagi H. 2007). v-ATPase d2 plays a positive role in osteoclast fusion, since v-ATPase d2 knockout mice presented defective osteoclast fusion and enhanced bone formation (Lee S. H. et al. 2006). In our study, all of the four compounds inhibited NFATc1 and v-ATPase d2, which may be because NFATc1 is the master transcription factor regulating osteoclast marker genes and v-ATPase d2 is critical in the maturation of osteoclasts.

Three compounds (dihydroartemisinin, fangchinoline, cumambrin A) were tested in the in vivo experiments. The microstructure of bone was assessed by the following parameters: bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp). Consistently, these compounds ameliorated estrogen deficiency-induced bone loss.

Thus, our study might provide novel therapeutic agents targeting osteoclast induced bone disease.

**9.2 Future directions**
Although we have found a few molecular mechanisms by which the studied natural compounds functioned in osteoclasts, the more accurate and direct targets still need to be explored. In addition, other kinds of animal models could be applied to explore the effects of the natural compounds on other pathophysiological factors induced bone loss. The animal models that could be studied in the future include LPS-induced bone loss, breast cancer-induced bone loss, titanium-particle-induced bone loss, aging-induced bone loss and sciatic-neurectomized-induced bone loss.

In addition to osteoclasts, osteoblasts also play an important role in the maintaining of normal bone mass. They exert this effect through forming new bones and regulating the formation and function of osteoclasts (Caetano-Lopes J. et al. 2007). Thus, it is important to examine the effect of the natural compounds on osteoblast differentiation and function.

Furthermore, bone histochemistry is essential for assessing the effect of natural compounds on the microstructure in the bones of the ovariectomized mice, such osteoclasts and osteoblasts activity. Thus, bone histochemistry is important for us to understand the mechanisms of the anti-osteoporotic effect of the natural compounds. Due to the time limitation, bone histochemistry has not been performed in the study of fangchinoline and cumambrin A.

Lastly, the clinical application of the natural compounds will be evaluated in the future. The drug safety and efficacy will undergo clinical trials in patients with osteoporosis and other bone destruction related diseases. This plan will be very essential to examine the actual curative effects of the studied natural compounds.

The future directions could be summarized as follows:

1) Exploring the accurate molecular targets for the natural compounds.

2) Applying more animal models for studying the effects of natural compounds on osteoclasts related disease.

3) Conducting osteoblast culture and histomorphometric analysis for natural
compounds.

4) Evaluation of the therapeutic roles of natural compounds by clinical trials.
Chapter 10
References


Jia L. and Li X. (2013). "[The role and mechanism of anticancer of dihydroartemisinin]." Lin Chung Er Bi Yan Hou Jing Wai Ke Za Zhi 27(18): 1033-1036.


osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL." Proc Natl Acad Sci U S A 95(7): 3597-3602.


Appendix

The screening of natural compounds and other published effective natural compounds
The effects of natural compounds on inhibiting RANKL-induced osteoclastogenesis

<table>
<thead>
<tr>
<th>Compounds name</th>
<th>Origins</th>
<th>Inhibitory effect on RANKL-induced Osteoclast number (IC50)</th>
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<td>Triptolide</td>
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</table>
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Statements of authorship

Triptolide inhibits osteoclast formation, bone resorption, RANKL-mediated NF-κB activation and titanium particle-induced osteolysis in a mouse model

Jianbin Huang, Lin Zhou, Huafei Wu, Nathan Pavlos, Shek Man Chim, Qian Liu, Jinmin Zhao, Wei Xue, Ren Xiang Tan, Jiming Ye, Jun Xu, Estabelle S. Ang, Haotian Feng, Jennifer Tickner, Jiakex Xu, Yue Ding

Jianbin Huang
Contributed to the execution of cell culture, animal experiments and data analysis. Major contribution to writing of the manuscript.

Lin Zhou
Contributed to initial screening of natural compounds, the execution of RT-PCR and analysis of the results.

Huafei Wu
Contributed to the execution of bone resorption assay and animal experiment.

Nathan Pavlos
Contributed to the analysis of the results and interpretation of research.

Shek Man Chim
Contributed to the analysis of the results and interpretation of research.

Qian Liu
Contributed to the execution of cell culture.

Jinmin Zhao
Contributed to the analysis of the results.
Wei Xue
Contributed to the analysis of the results.

Ming H. Zheng (Co-supervisor)
Contributed to the analysis and interpretation of research.

Ren Xiang Tan
Contributed to the analysis and interpretation of research.

Jiming Ye
Contributed to the interpretation of research.

Jun Xu
Contributed to providing of the natural compounds.

Estabelle S. Ang
Contributed to the planning of the research.

Haotian Feng
Contributed to the planning of the research.

Jennifer Tickner
Contributed to the planning and analysis of the research.

Jiake Xu
Contributed to the planning, analysis of the research. Also contributed to revising of the manuscript. Acted as corresponding author.

Yue Ding
Contributed to the planning and analysis of the research. Also contributed to revising of
the manuscript. Acted as corresponding author.
Triptolide inhibits osteoclast formation, bone resorption, RANKL-mediated NF-κB activation and titanium particle-induced osteolysis in a mouse model

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The RANKL-induced NF-κB signaling pathway is required for osteoclast formation and function. By screening for compounds that inhibit RANKL-induced NF-κB activation using a luciferase reporter gene assay in RAW264.7 cells, we identified triptolide (PC-109), as a candidate compound targeting osteoclast differentiation and osteoclast-mediated osteolysis. Triptolide (PC2490) is an active compound of the medicinal herb Tripterygium wilfordii Hook F (TWHF) or Lei Gong Teng with known anti-inflammatory properties. We found that triptolide inhibited osteoclastogenesis and bone resorption, as well as RANKL-induced NF-κB activities as monitored by luciferase reporter gene assays and the nuclear translocation of p65. In vivo studies showed that triptolide attenuates titanium-induced osteolysis and osteoclast formation in a mouse calvarial model. Considering that drugs which protect against localized bone loss are critically needed for the effective treatment of particle-induced osteolysis, our data suggest that triptolide might have therapeutic potential for the treatment of bone lytic diseases caused by prosthetic wear particles.

1. Introduction

Osteoclasts are multinucleated, terminally differentiated cells that are responsible for the degradation of mineralized bone matrix (Teitelbaum, 2000). Osteoclasts differentiate from mononuclear cells of the monocyte and macrophage lineage through a process collectively known as osteoclastogenesis (Teitelbaum, 2000). Pivotal to this process is the transcription factor nuclear factor kappa B (NF-κB), which plays an important role in both osteoclast differentiation and survival (Boyce et al., 2010; Novack et al., 2003; Russo et al., 2005; Xing et al., 2002; Xu et al., 2005). Likewise, the receptor activator of NF-κB ligand (RANKL) is a key cytokine for osteoclast differentiation, survival and function (Kong et al., 1999; Yamaura et al., 1998). Binding of RANKL to its cognate receptor RANK is essential for osteoclastogenesis, with a disruption in either RANK or RANK manifesting in severe osteoporosis due to a lack of osteoclast formation (Brage et al., 2004; Jones et al., 2002; Kong et al., 1999; Yoshitake et al., 2008). Thus, RANKL and its associated signaling cascades serve as potential therapeutic targets for the treatment of osteoclast-related diseases.

Prosthetic wear particles, such as polymethylmethacrylate and titanium particles are involved in the initiation and development
of periarticular osteolysis that leads to irreversible aseptic loosening of prostheses (Goodman et al., 2006). These particles can stimulate osteoclast-mediated bone resorption (Goodman et al., 2006), an action that can promote periarticular osteolysis in the bone microenvironment. In addition, direct and indirect mechanisms of particle-induced osteolysis may involve exacerbated inflammation with the production of pro-inflammatory cytokines, impaired periarticular bone formation, and compromised bone regeneration (Wang et al., 2004). To date, effective therapy against particle-induced osteolysis is limited to surgical revision. Identification of drugs that can inhibit particle-induced osteolysis therefore remains a major challenge in the treatment of particle-induced osteolysis.

Triptolide (C28H36O10) is a highly oxygenated diterpene triepoxide and contains 9,11-epoxide and the 14β-oriented-hydroxyl system, which are necessary for anti-leukemic and immunosuppressive activities (Tao et al., 2001). Triptolide is involved in antitumor activity against prostate cells (Kwiharju et al., 2002), possesses significant therapeutic benefit for immunosuppression (Kusunoki et al., 2004), has anti-inflammatory and anti-leukemic activity (Tao and Lipsky, 2000; Tao et al., 2002), and blocks the induction of NF-κB activity by TNF-α, through functioning as an NF-κB inhibitor (Füller et al., 2003). Previous studies have shown that triptolide inhibits NF-κB transcriptional activation triggered by phorbol 12-myristate 13-acetate (PMA), PMA/Jonu and TNF-α stimuli via specific binding of the p50–p65 heterodimer to DNA (Qiu et al., 1999). However, no data have been available in regard to whether triptolide exerts a therapeutic effect on particle-induced osteolysis, as well as on RANKL-mediated osteoclastogenesis and NF-κB activity.

Through the biological screening for compounds that inhibit RANKL-induced NF-κB activation by a luciferase reporter gene assay in RAW264.7 cells, we identified Triptolide (PC490), as a candidate compound targeting osteoclasts and osteoclast-mediated osteolysis. Our results demonstrate that triptolide suppresses osteoclastogenesis, bone resorption and RANKL-mediated NF-κB activation, and attenuates particle-induced osteolysis in vivo, suggesting that triptolide possesses therapeutic potential for the treatment of osteolysis.

2. Materials and methods

2.1. Media and reagents

RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD), α-Modification of Eagle’s Medium (α-MEM) and fetal bovine serum (FBS) were purchased from TRACE (Sydney, NSW, Australia). L-glutamine, penicillin and streptomycin were purchased from Gibco BRL (Melbourne, Australia). Triptolide was purchased from Astra Saffhire Bioscience Pty. Ltd. (AU-Redfern, NSW, Australia). Triptolide powder was dissolved in dimethyl sulfoxide (DMSO) and then diluted in phosphate buffered saline (PBS). Natural compounds (with a purity ≥98%) were obtained from Professor Jun Xu of San Yat-sen University, Professor Ren Xiang Tan of Nanjing University, Professor Jinming Ye RMIT University, Melbourne, or Chengfu MUST Bio-technology Co., LTD (China). All compounds were dissolved in dimethyl sulfoxide (DMSO) purchased from BDH laboratory supplies (Poole, Dorset, England). Recombinant GST-RANKL, was prepared as previously described (Xu et al., 2000).

2.2. Drug screening assay and NF-κB activity in RAW264.7 cells

Drug biological screening assays were conducted to evaluate RANKL-mediated NF-κB activity using RAW264.7 cells stably transfected with an NF-κB-driven luciferase reporter gene construct (3xB-Luc-SV40) (Wang et al., 2003) with natural and synthetic compounds at concentrations of 1–10 μM. To investigate the effect of triptolide on RANKL-induced NF-κB activity, RAW264.7 cells stably transfected with an NF-κB-driven luciferase reporter gene construct (3xB-Luc-SV40 (Wang et al., 2003) were treated with various doses of triptolide for 1 hour prior to incubation with 100 ng/ml RANKL for 8 hours, Luciferase activities were measured using a POLARstar Optima luminescence detection system (BMG Labtech) (Wang et al., 2003).

2.3. Osteoclastogenesis assay

Primary bone marrow macrophages (BMM) isolated from C57BL/6 mice were seeded (1 × 10⁶ cells/well) in a 96-well cell culture plate and incubated overnight with 10 ng/ml of M-CSF before stimulation with RANKL (100 ng/ml) in various concentrations of triptolide. Culture medium was replenished every second day. After 5 days incubation, cells were fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) activity. TRAP-positive multinucleated cells with >3 nuclei were scored as osteoclasts (Ang et al., 2009).

2.4. Bone resorption pit assay

To examine the effect of triptolide on osteoclast-bone resorption, BMM derived osteoclasts cultured in collagen-coated plates were cultured in α-MEM medium supplemented with 10% FBS and 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 mg/ml amphotericin and 50 IU/ml procollagenase at 37 °C in a water saturated atmosphere with 5% CO₂ and fed by medium replacement every 2–3 days for a total of 5–7 days. Osteoclasts cultured in collagen-coated plates were then washed once with PBS and treated for 20 minutes with 250 μl of cell dissociation buffer (Sigma) per 25-mm-diameter well. The detached cells were pelleted and resuspended in 150 μl of α-MEM, aliquoted onto 150 μm thick bone slices in wells of a 96 well plate, and incubated for 2 hours to ensure proper attachment of cells onto the bone substrate before the addition of triptolide. After cell attachment, the bone slices were treated either with Triptolide (5 nM) or left untreated. After 48 hours at 37 °C incubation, the bone slices were fixed with 4% paraformaldehyde, osteoclasts were TRAP stained and counted, prior to treatment with 2 M NaOH. Cells were removed by gentle brushing and sonication. The resorption pits were visualized by a Philips XL30 scanning electron microscope (SEM). The percentage of resorbed area on bone surface normalized to osteoclast number was quantified using Scion Image software (Scion Cooperation, National Institute of Health, USA) (Pavlos et al., 2011; Yip et al., 2006).

2.5. p65 immunohistochemistry

BMM were seeded (2 × 10⁶/well) in 100 μl into 96-well plates and incubated overnight. Cells were then pre-incubated with triptolide for 1 hour before stimulation with RANKL (100 ng/ml) for up to 30 min. Immunohistochemistry was performed with anti-p65 antibody (Santa Cruz Biotechnology, Inc.) diluted 1:200 in 0.1% BSA–PBS, and with anti-mouse IgG (Santa Cruz Biotechnology, Inc.) as a negative control as previously described (Ang et al., 2009).

2.6. Preparation of titanium particles

Commercial pure titanium particles, with a density of 4.507 g/cm³, were purchased from Alfa Aesar (Ward Hill, MA, USA). These particles were diluted with pure water and filtered by Millipore filter membranes (Billericia, MA, USA) of a series of sizes (pore diameter: 0.2, 1.2, and 10 μm). Three ranges of particle size, 0.2–1.2 μm
(Ti-0.2), 12–10 μm (Ti-1.2) and >10 μm (Ti-10) were obtained after filtration. All particles were washed with 70% ethanol for 24 h at room temperature and then dried in a biological drying oven. The dried particles were sterilized with ethylene oxide. According to particle weight and density, the concentration of the particles suspended in PBS was adjusted to 4 × 10^9 mm^3/ml. The level of endotoxin in particle solutions was measured using the limulus amebocyte lysate assay (QL-1000; Bio Whittaker, Walkersville, MD, USA), and the results showed that the endotoxin level was under the detection level of 0.01 EU/ml. An image analysis system (Nikon, Tokyo, Japan) was used to confirm the exact size of the particles, which showed that there were no significant differences in size between different particles (data not shown).

2.7. Animal surgery and experimental protocol

Twenty 11-week-old C57BL/6 male mice were purchased from the Animal Laboratory of Sun Yat-sen University (Guangzhou, Guangdong, China). All mice were handled in accordance with Chinese animal protection guidelines and the use of laboratory animals. Animals were housed in quarantine under local vivarium conditions (24 °C, 12 h/12 h light/dark cycle) for 1 week. The surgical procedures have been described previously (Viedemeyer et al., 2007). Briefly, mice were operated under general anesthesia via intraperitoneal injection of chloral hydrate. All mice were 12 weeks of age at surgery. A 0.5 × 0.5 cm^2 area of periosteum was exposed by making a 10-mm midline sagittal incision over the calvaria. These mice were divided into 3 groups randomly. Each of five mice received local calvaria injection of 100 μl Ti-0.2 particles; five mice received 100 μl Ti-0.2 particles and 50 μl triptolide suspension (equivalent to 1 mg/kg); five mice sham-operated with 100 μl PBS constituted the sham control. The incision was closed using nonabsorbable suture (Yangzhou Guo Tai Co., Ltd, China). The mice were returned to their cages after they were able to right themselves. Water and food were given ad libitum. No mice died during the experiments, and no wound complications were observed. Fourteen days postoperatively, the mice were euthanized, and the calvaria harvested for CT scan analysis and micro-CT imaging.

2.8. Micro-computed tomography

The skulls were analyzed with a high-resolution micro-CT (μCT 80; Scanco Medical AG, Bruttisellen, Switzerland) to perform qualitative analyses of the calvarial bone. Micro-CT analysis focused on the bony properties in the area of the sagittal suture of the skull. The radiographic projections (n = 500) were acquired at 55 kV and 145 μA with a fixed exposure time of 300 ms and all projection frames were recorded 6 times and then averaged. 3D images were reconstructed with a voxel average size of 12 μm, using the manufacturer's reconstruction software (Scanco Medical AG).

2.9. Histology

Paraffin embedded sections (4 μm) were taken in the frontal plane centered over the area of particle-induced osteolysis and stained with Goldner dye. The Goldner-stained sections were analyzed by transmission light microscopy (Axioshot, Zeiss, Germany). The existence and dimension of granulomatous foreign body reaction and bone resorption were evaluated using a standard high-quality light microscope. Histomorphometric analysis was made by image analysis software (UTHSCSA Image Tool, IT version 3.0; University of Texas, San Antonio, TX) as described previously (Viedemeyer et al., 2007).

2.10. Statistical analyses

Data were presented as mean ± SEM. Student’s t-test was used to determine statistical significance between groups. A p-value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Triptolide inhibits osteoclastogenesis in vitro

Using a high-throughput luciferase reporter gene assay to screen a library of 120 compounds, consisting of natural compounds and their derivatives, for potential inhibitors of RANKL-induced activation of NF-κB, we identified triptolide (Fig. 1), as a candidate compound that potently inhibited RANKL-induced NF-κB activation (Table 1). Given that NF-κB activity is crucial to osteoclast formation and function, we first investigated the cellular and molecular basis of the inhibitory effects of triptolide on osteoclastogenesis in vitro using an established primary bone marrow macrophage (BMM) osteoclastogenic culture system. As shown in Fig. 2, mouse BMMs cells cultured in M-CSF and RANKL (100 ng/ml) alone formed multinucleated osteoclast-like cells showing typical TRAP-positive activity (pink reaction product). On the other hand, BMM cells treated with triptolide demonstrated a dose-dependent reduction of RANKL-induced osteoclast formation both in numbers and in size (Fig. 2). In the presence of triptolide, RANKL treatment is still able to induce TRAP expression with a smaller size of osteoclast, suggesting that triptolide might affect both the early stage of osteoclast differentiation and the cell fusion at the later stage.

3.2. Triptolide inhibits osteoclastic bone resorption

Next, to examine the inhibitory effect of triptolide on osteoclastic bone resorption, a bone resorption pit assay was performed using BM/M-derived osteoclasts. As shown in Fig. 3A, triptolide (5 nM) suppressed osteoclastic bone resorption activity, as evidenced by the visible reduction in bone resorbive pits by SEM. Quantitative analysis of the total resorption area revealed the area of bone resorption normalized with osteoclast numbers was significantly reduced in osteoclasts treated with triptolide (Fig. 3B) compared with controls treated with vehicle (DMSO) only. These results indicate that triptolide impairs osteoclastic resorptive function in vitro.

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<th>Table 1</th>
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<td>Compounds name</td>
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3.3. Triptolide suppresses RANKL-induced activation of NF-κB in osteoclast-like cells

As aforementioned, the NF-κB pathway plays a crucial role in RANKL-induced osteoclastogenesis. Therefore, we next investigated the effect of triptolide on NF-κB activity using RAW264.7 cells stably transfected with an NF-κB-driven luciferase reporter gene construct (3xLuc-NFκB). As shown in Fig. 4, RANKL induced a significant increase in NF-κB activation compared to unstimulated RAW264.7 cells which served as a basal control. Treatment with triptolide showed dose-dependent suppression of RANKL-stimulated NF-κB activation in RAW264.7 cells at concentrations of 0.62 nM, 1.25 nM and 2.5 nM (p<0.001).

3.4. Triptolide suppresses RANKL-induced p65 nuclear translocation

To further explore the effect of triptolide on NF-κB activity, we evaluated the effect of triptolide on RANKL-mediated p65 nuclear translocation in BMMs. Fig. 5 shows that treatment of BMMs with triptolide decreases the RANKL-induced cytosol-nuclear translocation of p65, as compared to vehicle control cells which possessed a higher percentage of cells displaying clear nuclear translocation of p65. Anti-mouse IgG, which served as a negative control, showed negligible staining (Fig. 5).

3.5. Triptolide treatment reduced titanium particle-induced osteolysis of the calvarial bone

Next, we examined the effect of triptolide on titanium particle-induced osteolysis in vivo using our previously published procedures (Wedemeyer et al., 2007). During the treatment, we did not observe weight loss and abnormal behavior of triptolide-treated mice. Micro-CT analysis showed that administration of titanium particles to the surface of mouse calvarial bone elicited a localized inflammatory and osteolytic response which significantly widened the cranial suture width. By comparison, Sham-operated mice show no obvious signs of osteolytic activity. Importantly, treatment with triptolide significantly decreased the cranial suture width induced by titanium particles (Fig. 6).

To enable a more detailed examination of the effects of triptolide on sagittal suture resorption area and osteoclast formation in vivo, we performed a histological assessment of the calvaria. As shown in Fig. 6C, titanium particles caused a significant increase in sagittal suture resorption area, whereas co-injection with triptolide significantly reduced the titanium particle-induced sagittal suture resorption area. Taken

Fig. 1. Overview of the chemical structure of natural compounds and their derivatives used for initial screening and identification of potential NF-κB modulators. YC4-2 is a tetracyclic terpenoid derivative of oleanolic acid. LH8, 13, 16, 22, 32 and 45 are derivatives of berberine. HuM8 and 13 are derivatives of matrine.
Fig. 2. Triptolide inhibits RANKL-induced osteoclastogenesis. (A) Chemical structure of triptolide. (B) BM mesenchymal cells cultured in a 96-well plate in the presence of RANKL (100 ng/ml) and M-CSF (10 ng/ml) with or without triptolide for 5 days were fixed with 4% paraformaldehyde and stained for TRAP activity. (C) Representative light microscope images showing the effect of triptolide on RANKL-induced osteoclast formation with morphological changes in comparison with BM mesenchymal cells cultured in the absence of RANKL. (D) Quantitative analysis shows the mean number of TRAP-positive multinucleated cells (MNC). **p < 0.01, ***p < 0.001 compared to RANKL-treated control, n = 3.

Together, these findings indicate that triptolide attenuates osteoclast-mediated osteolysis.

4. Discussion

In the present study, we addressed the therapeutic potential of triptolide as a candidate inhibitor of osteoclastogenesis, bone resorption, RANKL-induced NF-κB signaling pathways and particle-induced osteolysis. Previous studies have shown that triptolide
specifically targets NF-κB p65 to block NF-κB signaling via inhibiting NF-κB transcriptional activation through the C-terminal portion of NF-κB p65 in T-cells (Qiu et al., 1999). Dai et al. also revealed that triptolide inhibited the binding of NF-κB p65/p50 in COX-2 promoters in lipopolysaccharide (LPS)-stimulated human A172 cells (Dai et al., 2005). Similarly, Zhu et al. reported that triptolide inhibits NF-κB transcriptional activity via blocking the association of p65 with CREB-binding protein (CBP)/p300 and via decreasing the p65 protein expression (Zhu et al., 2009). Consistent with these observations, our studies provide further evidence that triptolide efficiently impairs NF-κB activity and p65 nuclear translocation during RANKL-induced osteoclastogenesis.

Prosthetic wear particles play a pathological role in the initiation and development of periprosthetic osteolysis, leading to irreversible aseptic loosening of prostheses (Goodman et al., 2006). Polymethylmethacrylate and titanium particles have been commonly associated with clinical aseptic joint loosening and over-activation of osteoclasts (Goodman et al., 2006). Many orthopedic implants fail due to particle-stimulated aseptic loosening. To date, therapy against particle-induced osteolysis is limited to surgical revision. Thus, identification of drugs that can suppress particle-induced osteolysis will improve treatment options for particle-stimulated aseptic loosening. In this study, we demonstrate that triptolide effectively suppressed titanium particle-induced osteolysis. Osteolysis induced by

Fig. 5. Triptolide inhibits RANKL-induced p65 nuclear translocation. (A,B) Treatment with RANKL induces p65 translocation from the cytoplasm to the nucleus in BMM cells. RANKL-induced nuclear translocation was inhibited by pre-treatment with triptolide. (C) Negative control cells treated as in (B), and stained with anti-mouse IgG antibody. Black arrow indicates the localization of p65 in the cytoplasm, and white arrow specifies the presence of p65 in the nucleus. (E) The percentage of cells showing nuclear translocation of p65 was reduced in cells treated with RANKL+ triptolide (5 nM) compared to RANKL only controls. (***p < 0.001, n=3).
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Eriodictyol Inhibits RANKL-Induced Osteoclast Formation and Function Via Inhibition of NFATc1 Activity

Fangming Song, Lin Zhou, Jinmin Zhao, Qian Liu, Mingli Yang, Renxiang Tan, Jun Xu, Ge Zhang, Julian M.W. Quinn, Jennifer Tickner, Yuanjiao Huang and Jiake Xu

Fangming Song
Contributed to the execution of cell culture, PCR and data analysis. Major contribution to writing of the manuscript.

Lin Zhou
Contributed to initial screening of natural compounds, the execution of cell culture and analysis of the results.

Jinmin Zhao
Contributed to analysis of the results.

Qian Liu
Contributed to the providing of the natural compounds.

Mingli Yang
Contributed to the execution of MTS assay.

Renxiang Tan
Contributed to the interpretation of the research.

Jun Xu
Contributed to the analysis of the results.
Ge Zhang
Contributed to the analysis of the results.

Julian M.W. Quinn
Contributed to the analysis of the results and revising of the manuscript.

Jennifer Tickner
Contributed to the planning and analysis of the research.

Yuanjiao Huang
Contributed to the planning and analysis of the research. Acted as corresponding author.

Jiake Xu
Contributed to the planning, analysis of the research. Also contributed to revising of the manuscript. Acted as corresponding author.
Eriodictyol Inhibits RANKL-Induced Osteoclast Formation and Function Via Inhibition of NFATc1 Activity

FANGMING SONG,1,2,3 LIN ZHOU,2 JINMIN ZHAO,1,4 QIAN LIU,1,4 MINGLI YANG,2 RENXIANG TAN,2 JUN XU,6 GE ZHANG,7 JULIAN M.W. QUINN,8 JENNIFER TICKNER,2 YUANJIAO HUANG1,9* AND JIAKE XU1,2,10

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7Institute for Advancing Translational Medicine in Bone and Joint Diseases, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China
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Receptor activator of nuclear factor κB ligand (RANKL) induces differentiation and function of osteoclasts through triggering multiple signaling cascades, including NF-κB, MAPK, and Ca2+-dependent signals, which induce and activate critical transcription factor NFATc1. Targeting these signaling cascades may serve as an effective therapy against osteoclast-related diseases. Here, by screening a panel of natural plant extracts with known anti-inflammatory, anti-tumor, or anti-oxidant properties for possible anti-osteoclastogenic activities we identified Eriodictyol. This flavonoid potently suppressed RANKL-induced osteoclastogenesis and bone resorption in a dose-dependent manner without detectable cytotoxicity, suppressing RANKL-induced NF-κB, MAPK, and Ca2+ signaling pathways. Eriodictyol also strongly inhibited RANKL-induced c-Fos levels (a critical component of AP-1 transcription factor required by osteoclasts) and subsequent activation of NFATc1, concomitant with reduced expression of osteoclast-specific genes including cathepsin K (Ctsk), V-ATPase-a3 subunit, and tartrate resistant acid phosphatase (TRACP/Arp5). Taken together, these data provide evidence that Eriodictyol could be useful for the prevention and treatment of osteolytic disorders associated with abnormally increased osteoclast formation and function.


Bone is a dynamic and living organ, which continuously undergoes the process of repair and renewal throughout life (Teitelbaum, 2000). This process involves osteoclast activity (bone formation) and osteoclast activity (bone resorption).

However, bone disorders, such as osteoporosis and Paget’s disease of bone, are characterized by bone loss owing to the excessive formation and production of osteoclasts (Manolagas and Jilka, 1995; Kular et al, 2012).
Osteoclasts are multinucleated cells that are derived from the differentiation of monocyte/macrophage lineage hematopoietic cells. The process of macrophage differentiation into osteoclasts depends on two essential cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) (Boyle et al., 2003). RANKL is a key cytokine for osteoclast differentiation, function, and survival, and exerts its biological effects through interacting with its receptor, Receptor activator of NF-κB (RANK). The binding of RANKL to RANK results in activation of MAPK, NF-κB, and Ca²⁺ signaling transduction pathways followed by stimulation of several transcription factors, such as c-Fos and NFATc1, which are indispensable for osteoclastogenesis (Baud’huin et al., 2007). Osteoclast-specific gene expression is initiated by NFATc1 in response to RANKL stimulation (Negishi-Koga and Talayanoj, 2009). M-CSF is a key factor responsible for inducing survival and proliferation of osteoclast precursors. M-CSF can also modulate the levels of RANK in bone marrow macrophage cells (BMMs) sensitizing them to RANKL stimulation (Takayanagi, 2007). Thus, impairment of RANKL-induced molecular and signaling pathways can be useful for the treatment of osteoclast-related disease.

Eriodictyol, a flavonoid extracted from plants such as Citrus limon, has a wide range of biomedical and pharmacological effects, which include antioxidant (Lee et al., 2015) and anti-inflammatory activities (Lee, 2011; Lee et al., 2013). However, the cellular and molecular actions of Eriodictyol on RANKL-induced osteoclast formation and function are not clear. In order to assess the impact of Eriodictyol on RANKL-mediated osteoclastogenesis, we investigated the effects and mechanism of action of Eriodictyol in regulating formation and function of osteoclasts. Our results demonstrate that Eriodictyol is capable of inhibiting RANKL-induced osteoclastogenesis and bone resorption through suppression of NFATc1 activation via NF-κB, MAPK, and Ca²⁺ signaling pathways. Collectively, these data suggest that Eriodictyol is a novel and potential candidate for treatment of osteolytic bone diseases.

Materials and Methods

Materials

Alpha modified Minimal Essential Medium (α-MEM) and fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Scoresby, Australia). Eriodictyol with a purity >95% was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China) and prepared at a concentration of 100 μM in Dimethyl sulfoxide (DMSO). DMSO was used as vehicle in these assays and shows no effect. Antibodies specific for NFATc1, IκBα, ERK, JNK, p38, phosphorylated (p) IκBα, p-ERK, p-p38, p-JNK, and p-actin were obtained from Santa Cruz Biotechnology (San Jose, CA). Vascular-type H~1~ATPase V0 subunit d2 (V-ATPase d2) was obtained as previously described (Feng et al., 2009). The MTS and luciferase assay system were obtained from Promega (Sydney, Australia). Recombinant macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Minneapolis, MN). L-ascorbic acid 2-phosphate staining kits were obtained from Sigma–Aldrich (Sydney, Australia). Recombinant GST-RANKL protein was expressed and purified as previously described (Xu et al., 2000).

Cell culture

RAW264.7 cells (mouse macrophage cells) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in α-MEM supplemented with 10% FBS, 2 mM l-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (complete medium). Bone marrow macrophage cells (BMMs) were isolated from 6-week-old C57BL/6j mice by flushing the marrow from the femur and tibia, and then culture in complete medium in the presence of M-CSF (50 ng/mL).

Drug screening assay and osteoclastogenesis assay

Drug screening assays were conducted using BMMs isolated as described above to evaluate RANKL-induced osteoclastogenesis. BMMs were plated into 96-well culture plates at a density of 6 × 10⁵ cells/well, and treated with complete medium containing M-CSF and GST-RANKL (50ng/mL) in the presence or absence of natural compounds (screening assay—10μM) or varying concentrations of Eriodictyol. The cell culture medium was changed every 2 days. After 5 days, cells were fixed with 4% paraformaldehyde for 10 min, washed three times with PBS, and then stained for tartrate resistant acid phosphatase (TRACP) enzymatic activity using a leucyce acid phosphate staining kit (Sigma–Aldrich, Sydney, Australia), following the manufacturer’s procedures. TRACP-positive multinucleated cells (>3 nuclei) were counted as osteoclasts.

Cytotoxicity assays

BMMs were seeded into a 96-well plate at 6 × 10⁵ cells/well and left overnight to adhere. The following day, the cells were incubated with varying concentrations of Eriodictyol for 48 h. At the end of the experiment, MTS solution (20 μL/well) was added and incubated with cells for 2 h. The absorbance at 490 nm was read with a microplate reader (Thermo Labsystem Multiscan Spectrum, Thermo labsystem, Chantilly, VA).

Hydroxyapatite resorption assay

To measure osteoclast activity, osteoclasts were first generated from BMMs (1 × 10⁶ cells per well) cultured onto 6-well collagen-coated plates and stimulated with 50 ng/mL GST-RANKL and M-CSF until mature osteoclasts were generated. Cells were gently detached from the plate using cell dissociation solution (Sigma–Aldrich, Sydney, Australia) and mature osteoclasts were seeded into individual wells in hydroxyapatite-coated 96-well plates (Corning, Inc., Corning, NY). Starting osteoclast number was the same in each well. Mature osteoclasts were incubated in medium containing GST-RANKL and M-CSF with or without Eriodictyol at the indicated concentration. After 48 h, half of the wells were histochemically stained for TRACP activity as above to assess the number of multinucleated cells per well. The remaining wells were bleached for 10 min to remove the cells and allow measurement of the resorbed areas, which were photographed under standard laboratory conditions, and ImageJ software (NIH, Bethesda, MD) to quantify the percentage area of hydroxyapatite surface resorbed by the osteoclasts.

Luciferase reporter assays

To investigate NF-κB and NFATc1 transcripational activation, RAW264.7 cells were stably transfected with either an NF-κB-responsive luciferase construct (Wang et al., 2003) or an NFATc1-responsive luciferase reporter construct (van der Kraan et al., 2013). Transfected cells were cultured in 48-well plates at a density of 1.5 × 10⁵ cells/well (NF-κB luciferase reporter gene assay) or 5 × 10⁵ cells/well (NFATc1 luciferase reporter gene assay), respectively, and pretreated with various concentrations of Eriodictyol for 1 h. Following pre-treatment cells were subsequently stimulated with GST-RANKL (50ng/mL) for 6 h (NF-κB luciferase reporter gene assay) or 24h (NFATc1 luciferase reporter gene assay) and luciferase activity was detected using the
**Table 1.** The effect of natural compounds on RANKL-induced osteoclastogenesis

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Origin</th>
<th>IC50 (nM)</th>
<th>Osteoclast number (IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eriodictyol</td>
<td>Natural</td>
<td>22.3 ± 2.2</td>
<td>22.3 ± 2.2</td>
</tr>
<tr>
<td>Sinapigenine</td>
<td>Natural</td>
<td>55.4 ± 4.4</td>
<td>55.4 ± 4.4</td>
</tr>
<tr>
<td>Threoxyanate</td>
<td>Natural</td>
<td>27.6 ± 2.6</td>
<td>27.6 ± 2.6</td>
</tr>
<tr>
<td>Jervine</td>
<td>Natural</td>
<td>14.8 ± 3.8</td>
<td>14.8 ± 3.8</td>
</tr>
<tr>
<td>Venosamine</td>
<td>Natural</td>
<td>29.4 ± 3.9</td>
<td>29.4 ± 3.9</td>
</tr>
<tr>
<td>Olapridin</td>
<td>Natural</td>
<td>27.5 ± 3.6</td>
<td>27.5 ± 3.6</td>
</tr>
<tr>
<td>Pedunculolide</td>
<td>Natural</td>
<td>26.4 ± 3.7</td>
<td>26.4 ± 3.7</td>
</tr>
<tr>
<td>Bartalin</td>
<td>Natural</td>
<td>23.6 ± 3.2</td>
<td>23.6 ± 3.2</td>
</tr>
<tr>
<td>Oncogluconoid</td>
<td>Natural</td>
<td>21.7 ± 2.9</td>
<td>21.7 ± 2.9</td>
</tr>
<tr>
<td>Beclina</td>
<td>Natural</td>
<td>20.8 ± 2.8</td>
<td>20.8 ± 2.8</td>
</tr>
<tr>
<td>Germacrone</td>
<td>Natural</td>
<td>19.9 ± 2.7</td>
<td>19.9 ± 2.7</td>
</tr>
<tr>
<td>Paeciniflorin</td>
<td>Natural</td>
<td>19.2 ± 2.6</td>
<td>19.2 ± 2.6</td>
</tr>
<tr>
<td>Albinin</td>
<td>Natural</td>
<td>18.5 ± 2.5</td>
<td>18.5 ± 2.5</td>
</tr>
<tr>
<td>Alpinin</td>
<td>Natural</td>
<td>17.8 ± 2.4</td>
<td>17.8 ± 2.4</td>
</tr>
<tr>
<td>Calycosin</td>
<td>Natural</td>
<td>17.1 ± 2.3</td>
<td>17.1 ± 2.3</td>
</tr>
<tr>
<td>Tectorigenin</td>
<td>Natural</td>
<td>16.4 ± 2.2</td>
<td>16.4 ± 2.2</td>
</tr>
<tr>
<td>Martine</td>
<td>Natural</td>
<td>15.7 ± 2.1</td>
<td>15.7 ± 2.1</td>
</tr>
<tr>
<td>Artabien</td>
<td>Natural</td>
<td>15.0 ± 2.0</td>
<td>15.0 ± 2.0</td>
</tr>
<tr>
<td>Hyodeoxycholic acid</td>
<td>Natural</td>
<td>14.3 ± 1.9</td>
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</tr>
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<td>Ethyl ferulate</td>
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<td>13.6 ± 1.8</td>
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<td>Natural</td>
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<td>12.2 ± 1.6</td>
</tr>
<tr>
<td>Hydrochloride</td>
<td>Natural</td>
<td>11.5 ± 1.5</td>
<td>11.5 ± 1.5</td>
</tr>
</tbody>
</table>

Luciferase reporter assay system according to the manufacturer’s protocol (Promega, Sydney, Australia).

**Reverse transcription (RT)-PCR analysis of gene expression**

Total RNA was isolated from cells using Trizol reagent according to the manufacturer’s protocol (ThermoFisher Scientific, Scoresby Australia). The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase with 1 μg of RNA template and oligo-dT primers. Polymerase chain reaction amplification of specific sequences was performed using the following cycle: 94°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 60°C for 40 sec, and 72°C for 40 sec, and a final extension step of 5 min at 72°C. Reaction products (2 μL) were separated using agarose gel electrophoresis and visualized on an Image-quant LAS 4000 (GE Healthcare, Silverwater, Australia) and analyzed by ImageJ software.

The following specific primers (based on the mouse sequences) were used:

- **Cathespin K (Catk)** (Forward: 5'-GGGAGAABAACTGAAGC-3'; Reverse: 5'-ATTCTGACCAGATGACGC-3'), TRACP (Acp5) (Forward: 5'-TGTGCCGACATCTTATGCT-3'; Reverse: 5'-GTCATTCTTGGGCTT-3').

---

**Fig. 1.** Eriodictyol suppresses RANKL-induced osteoclastogenesis. A: Structure of Eriodictyol. B: Representative images of RANKL-induced osteoclastogenesis in the presence of indicated concentrations of Eriodictyol (Magnification 20×; scale bar, 100 μm). C: Low power image of TRACP staining showing the dose dependent effect of Eriodictyol treatment. D: Quantification of the effect of Eriodictyol treatment on the number of TRACP-positive multinucleated cells (nuclei ~3) (n = 3). E: Survival of BMMs in the presence of Eriodictyol as assessed by MTS cell viability assay (n = 3). **p** means RANKL untreated; ***p** means RANKL treated. **p** < 0.05, ***p** < 0.01, ****p** < 0.001 relative to RANKL-stimulated controls.
Fig. 2. ERTH inhibits RANKL-induced hydroxyapatite resorption. A: Representative images of hydroxyapatite resorption (left) with corresponding TRAP stained osteoclasts (right). White arrow indicated TRAP-positive multinucleated cells, black arrow indicated mononuclear cells (Mag = 4x; scale bar, 100 μm). B: Quantification of the number of TRAP-positive cells (nuclei > 3) (n = 3). C: The percentage area of hydroxyapatite resorption per osteoclast (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 relative to RANKL-stimulated controls.

V-ATPase-2d (Forward: 5'-TGG AGA CCT TGG AAG ACC TGA A-3'; Reverse: 5'-GAG AAA TGT GCT CAGGGGCT-3'), GAPDH (Forward: 5'-ACCACAGTCCATGGCATCAC-3'; Reverse: 5'-TCCACACCCTTGTTGCTGA-3').

**Western blotting**

BMM cells were cultured in 6-well plates and stimulated with 100 ng/mL GST-RANKL for the stated times. Cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer, and proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Silverwater, Australia). The membranes were blocked in 5% skim milk for 1 h, and then probed with various specific primary antibodies with gentle shaking overnight. Membranes were washed and subsequently incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies. Antibody reactivity was then detected with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscataway, NJ), visualized on an Imagequant LAS 4000 (GE Healthcare, Silverwater, Australia) and analyzed by ImageJ software.

**Measurement of intracellular Ca²⁺ oscillation**

Ca²⁺ oscillations were investigated using the calcium binding dye Fluo-4-AM according to the manufacturer's method (Molecular Probes, Thermo Fisher Scientific, Scoresby, Australia). Briefly, BMMs were seeded into a 48-well plate at a concentration of 1 x 10⁶ cells/well. The following day, the medium was replaced with complete medium containing 10 μM ERTH supplemented with GST-RANKL and M-CSF for 24 h. Cells were then washed in Assay buffer (HANKS balanced salt solution supplemented with 1 mM probenecid and 1% FBS) and then incubated with 4 μM Fluo-4 AM solution (Fluo-4-AM dissolved in 20% pluronic-F127 (w/v)) in DMSO diluted in Assay buffer, for 45 min at 37°C. Cells were washed once in Assay buffer and incubated at room temperature for 20 min followed by a further two washes in Assay buffer and visualization of fluorescence using an inverted fluorescent microscope (Nikon, Tokyo, Japan) at an excitation wavelength of 488 nm. Images were captured every 2 sec for 3 min and calcium flux analyzed using Nikon Basic Research Software. Cells that showed more than one calcium peak within the observed time frame were considered to be oscillating, with the oscillation intensity calculated as the difference between the maximum and minimum of fluorescence intensities within the oscillating cell area.

**Statistical analysis**

All experiments were repeated as the mean ± SEM and statistical significance determined by Student’s t-test. All experiments were repeated at least three times with results presented as the average of triplicate experiments or the data from a representative experiment that was repeated at least three times. A P-value < 0.05 was considered statistically significant.

**Results**

ERTH inhibits osteoclastogenesis

To identify compounds that have the potential to inhibit osteoclastogenesis, our preliminary screen assessed 20 natural compounds at a standardized concentration of 10 μM for their ability to inhibit RANKL-stimulated osteoclast formation from mouse BMM (Table 1 and Supplementary Fig. S1). From this screen we identified ERTH (Fig. 1A) exerted a strong inhibitory effect on RANKL-induced osteoclast formation. We further investigated the dose dependency of the effect of
**ERIODICTYOL INHIBITS RANKL-INDUCED OSTEOCLAST**

![Diagram showing NF-κB luciferase activity and protein levels over time with RANKL concentration]

**Fig. 3.** Eriodictyol attenuates RANKL induced NF-κB activity, but has little effect on IkB-α. A: Luciferase activity in RANKL stimulated RAW264.7 cells transfected with an NF-κB luciferase construct. Transfected cells were pre-treated with indicated concentrations of Eriodictyol and subsequently stimulated with GST-RANKL (50 ng/mL) (n = 3). "*" means RANKL treated; "**" means RIKL untreated; "***" means RANKL treated. *P < 0.05, **P < 0.01, ***P < 0.001 relative to RANKL-stimulated controls. B: Protein lysates from BMMs pre-treated with Eriodictyol (10 μM), followed by stimulation with GST-RANKL (50 ng/mL) for indicated times. Western-blot was probed for IkB-α and β-actin, the amount of IkB-α was normalized to β-actin and expressed as a ratio.

Eriodictyol on osteoclast formation using BMMs cultures stimulated with RANKL and M-CSF. Increasing doses of Eriodictyol exhibited a dose-dependent inhibition on the number of TRAcP positive osteoclasts formed (Fig. 1B–D). Both the size and number of osteoclasts were significantly suppressed as the concentration of Eriodictyol increased from 2.5 to 10 μM. The inhibitory concentration (IC50) of Eriodictyol for osteoclastogenesis was approximately 5 μM (Fig. 1D). We next performed a cell viability assay to determine whether the inhibitory effect of Eriodictyol on osteoclast formation was due to cytotoxic effects. Our results showed that Eriodictyol concentrations ranging from 0.25 to 10 μM were not cytotoxic to BMMs (Fig. 1E). Collectively, these results indicate that Eriodictyol inhibits osteoclastogenesis in a dose-dependent manner without causing cell cytotoxicity.

**Eriodictyol inhibits osteoclastic resorption**

To investigate the effect of Eriodictyol on resorption by mature osteoclasts, a hydroxyapatite resorption assay was used. We observed a large amount of hydroxyapatite resorption in the presence of RANKL alone; however, treatment with Eriodictyol caused a visible reduction in resorption area (Fig. 2A). Consistent with the lack of cytotoxicity observed in BMM, mature osteoclast number was not affected by treatment with Eriodictyol (Fig. 2B). However, resorption area per osteoclast was significantly reduced at 5 or 10 μM Eriodictyol (Fig. 2C), with almost complete inhibition of hydroxyapatite resorption in cultures treated with 10 μM Eriodictyol. Taken together, these data show that Eriodictyol suppresses osteoclastic resorption without affecting the survival of mature osteoclasts.

**Eriodictyol suppresses RANKL-induced NF-κB and MAPK pathways**

To further elucidate the molecular mechanism by which treatment with Eriodictyol results in suppression of osteoclastogenesis, we investigated the effect of Eriodictyol on the RANKL-induced signaling pathways, NF-κB, and MAPK. Activation of NF-κB, as assessed by luciferase assay, was greatly reduced, with a significant reduction in NF-κB activity at concentrations greater than 2.5 μM (Fig. 3A). To determine how Eriodictyol was impacting NF-κB activity we examined the protein levels of inhibitor of NF-κB (IkB-α) using western-blot. BMM were pre-treated with 10 μM Eriodictyol and then stimulated with RANKL and RANKL induced IkB-α degradation was analysed. As shown in Figure 3B, pretreatment with Eriodictyol failed to inhibit the degradation of IkB-α.
Interestingly, we found that the phosphorylation of ERK1/2, JNK1/2, and p38 were strongly inhibited by pre-treatment with Eriodictyol, compared to untreated RANKL-induced only cells (Fig. 4A). Taken together, these findings indicate that Eriodictyol exerts an inhibitory effect on RANKL-induced activation of NF-κB and MAPK signaling pathways.

**Eriodictyol inhibits RANKL-induced Ca^{2+} oscillations**

RANKL stimulation results in the activation of Ca^{2+} signal transduction pathways, which initiate Ca^{2+} oscillations that result in stabilization and nuclear translocation of NFATc1 transcription factor. To determine the effect of Eriodictyol on RANKL-induced Ca^{2+} signaling, intracellular Ca^{2+} oscillations were observed in RANKL-stimulated BMMs. As expected, treatment with RANKL induced Ca^{2+} oscillations whereas no Ca^{2+} flux was observed in the RANKL-unstimulated group (Fig. 5A, B). Treatment with Eriodictyol significantly attenuated the observed RANKL induced Ca^{2+} oscillations (Fig. 5C, D). These results suggest that Eriodictyol abrogates the Ca^{2+} signaling pathway mediated by RANKL.

**Eriodictyol represses NFATc1 and c-fos activation, and associated downstream protein expression**

As Ca^{2+} oscillations are critical for activation of NFATc1, we utilized an NFATc1 luciferase reporter gene construct in RAW264.7 cells to investigate the effect of Eriodictyol on RANKL-induced NFATc1 activity. Pre-treatment with Eriodictyol resulted in a dose-dependent impairment of NFATc1 activation (Fig. 6A). Consistent with the inhibition of NFATc1 activation observed in the RAW264.7 cells, the increase in protein levels of NFATc1 observed in RANKL-stimulated BMM was notably blocked by Eriodictyol treatment on days 3 and 5 of culture (Fig. 6B). In confirmation of the observed reductions in NFATc1 activity, expression of the NFATc1 regulated V-ATPase subunit d2 was also suppressed by Eriodictyol (Fig. 6B).

RANKL binding to cognate receptor RANK also results in activation of the transcription factor AP-1, via induction of the protein expression of critical component c-Fos. Western blot analysis of c-Fos protein expression showed that c-Fos was maximally induced on days 3 and 5 following RANKL stimulation of BMM (Fig. 6B). Treatment with Eriodictyol strongly blocked the expression of c-Fos relative to the RANKL-treated group. Collectively, these data demonstrate that Eriodictyol blocks the activation of NFATc1 and its downstream target V-ATPase-d2 via regulation of RANKL-induced NF-κB, MAPK, and Ca^{2+} pathways.

**Eriodictyol inhibits RANKL-induced gene expression**

Activation of NF-κB and NFATc1 results in the up-regulation of osteoclast marker gene expression. Based on the inhibitory effect of Eriodictyol on these pathways we investigated the effect of Eriodictyol (5 and 10 μM) on osteoclast gene expression. As shown in Figure 7, the expression of osteoclast marker genes (Ctsk, TRACP, and V-ATPase-d2) was reduced dose-dependently following treatment with Eriodictyol, suggesting that Eriodictyol attenuates osteoclast-specific gene expression.

**Discussion**

In adult skeleton bone remodeling is constantly active through coordinated balance between osteoclast-induced bone resorption and osteoblast-induced bone formation. When the balance is perturbed bone diseases such as osteoporosis are developed; these osteolytic diseases are associated with excessive osteoclastic activity (Boyle et al., 2003).
ERIODICTYOL INHIBITS RANKL-INDUCED OSTEOCLAST

![Graphs A, B, C, D showing calcium oscillation](image)

Fig. 5. Eriodictyol blocks RANKL-induced Ca^{2+} oscillation. BMMS were stimulated with GST-RANKL (50 ng/mL) for 24 h and then calcium flux was assessed using Fluo4 calcium indicator. A: Representative calcium fluctuations within three cells treated with RANKL only. B: Representative calcium fluctuations within three cells treated with M-CSF only (negative control). C: Representative calcium fluctuations within three cells treated with RANKL and Eriodictyol (10 μM). D: Average change in intensity per cell. Calcium fluctuations were analysed across multiple cells for each condition and maximum peak intensity minus baseline intensity was calculated (n > 16 individual cells/well, three wells/treatment) (n = 3). **“−” means RANKL untreated; “−−” means RANKL treated. **P < 0.01 relative to RANKL-stimulated controls.

Osteoclasts are formed by differentiation and fusion of macrophage precursors cells (Shinohara and Takayanagi, 2007). There are several cytokines that are critical for osteoclast differentiation, such as M-CSF, interleukin-1, transforming growth factor-β, and RANKL. Of these factors, RANKL, in concert with its receptor RANK, has been identified as a key factor regulating both osteoclast formation in the presence of M-CSF, and inducing osteoclast activity (Lacey et al., 1998; Kong et al., 1999). As a critical regulator of both osteoclast formation and function, RANKL is an attractive target for treatment of osteolytic diseases.

Current therapies focus on inhibiting the resorptive function of osteoclasts. Several treatments are clinically available targeting bone disorders, including estrogen replacement therapy, bisphosphonates, and denosumab. However, these treatments have some concerning side effects, including increased risk of breast cancer, gastrointestinal distress, and thromboembolism (Rodan and Martin, 2000; Rachner et al., 2011; Lippuner, 2012). Considering these concerns, it is important to search for alternative approaches for the treatment of excessive osteoclastic activity. Interestingly, due to the triggers of inflammation, cancer, and oxidative stress in the risk of bone loss, remarkable progress has been made in developing potent osteoclast inhibitor to inhibit those triggers (Abu-Amer, 2009; Wauquier et al., 2009). Of note, natural products exhibit a promising alternative treatment for bone diseases (Putnam et al., 2007). Recently, natural compounds like mangiferin, phloretin, and naringin, extracted from natural plants, have been demonstrated to possess inhibitory effects on osteoclast activity and may serve as potential therapies to treat skeletal diseases (Ang et al., 2011a, b; Kim et al., 2012). Therefore, we selected 20 compounds from commercial libraries for further study, based on their anti-inflammatory, anti-tumor or anti-oxidant benefits. For instance, germacrone inhibits human hepatoma cells growth via regulation of G2/M cell cycle and apoptosis (Liu et al., 2013); alpinetin exerts suppression effects on production of NO and PGE2 in lipopolysaccharides induced RAW 264.7 cells (Lee et al., 2012); maraine prevents oxidative damage associated with the translocation of Nrf2 (Zhang et al., 2012). Through screening the effect of natural compounds on osteoclast formation, we observed that Eriodictyol significantly suppressed RANKL-induced osteoclastogenesis in a dose-dependent manner, with further work detailed in this manuscript showing the key mechanisms by which Eriodictyol exerts this action.

Mature osteoclasts express a variety of specialized proteins that play essential roles in the degradation of bone matrix, in particular, osteoclasts deficient in V-ATPase-d2 or Ctsk, exhibit dysfunctional bone resorption resulting in osteoporosis (Gowen et al., 1999; Li et al., 1999). In the present study, we found that Eriodictyol abrogated the resorption of a hydroxyapatite substrate by mature osteoclasts as well as the expression of V-ATPase-d2 and Ctsk genes.

Dissecting the mechanism of action of an osteoclast inhibitor such Eriodictyol offers valuable information about the molecular targets of its actions. Eriodictyol suppresses many of
Fig. 6. Eriodictyol abrogates RANKL-induced c-Fos and NFATc1 protein expression. A: Luciferase activity in RANKL stimulated RAW264.7 cells transfected with an NFAT luciferase construct. Transfected cells were pre-treated with indicated concentrations of Eriodictyol, and subsequently stimulated with GST-rRANKL (50 ng/mL) (n = 3). “-” means RANKL untreated; “+” means RANKL treated. * P < 0.05, ** P < 0.01, *** P < 0.001 relative to RANKL-stimulated controls. B: Cell lysates from BMMs pre-treated with Eriodictyol (10 μM) and then stimulated with GST-rRANKL (50 ng/mL) for the indicated times. Western-blot was probed with c-Fos, NFATc1, V-ATPase-d2, and β-actin specific antibodies. Protein levels are expressed as the ratio relative to β-actin.

The critical pathways in osteoclast precursors, which explains its potency, but it is not clear at this stage what its main molecular target is in osteoclast precursors. RANK engagement by RANKL leads to recruitment of TRAF6, resulting in subsequent recruitment of IKK, which phosphorylates and degrades IκB-α to release the activated form of NF-κB. This is followed by translocation of NF-κB to the nucleus and subsequent activation of osteoclastogenesis gene transcription either directly or by inducing production of other transcription factors required for osteoclast formation and function (D’Donato et al., 1997). Deletion of p50 and p52 subunits of NF-κB in mice results in osteoporosis, demonstrating that NF-κB acts as a significant regulator in the generation of osteoclasts (Boyce et al., 1999) and osteocytic bone conditions (Xu et al., 2009). In this study, Eriodictyol suppressed the activity of NF-κB, although Eriodictyol failed to prevent the degradation of IκB-α, perhaps indicating effects on NF-κB downstream of IκB. Since, several other pathways investigated here, including AP-1 and NFATc1 depend upon NF-κB activity, this may indicate that blockade of NF-κB is a primary action of Eriodictyol. This compound has previously been found to suppress inflammatory responses (e.g., lipopolysaccharide-elicited responses) that depend on NF-κB (Lee, 2011). It is important to note; however, that RANKL signaling also activates supporting signaling pathways that are also required for successful osteoclast formation, notably MAPK and Ca²⁺ dependent pathways, which are not NF-κB dependent. This might indicate multiple molecular targets of Eriodictyol or effects upstream of NF-κB and MAPK, perhaps involving TRAF6 or RANK.

The MAPK pathways play a crucial role in the modulation of osteoclast differentiation. Three MAPK pathways are relevant to osteoclast formation, namely ERK1/2, JNK, and p38, which are all activated by the binding of RANKL to RANK (Kobayashi et al., 2001). Inhibition of ERK activity affects both osteoclast survival and formation (Hooten et al., 2002; Nakamura et al., 2003). Dominant-negative JNK abrogates RANKL-stimulated osteoclastogenesis, while activation of p38 exerts many effects on osteoclast differentiation but not on osteoclast function (Matsumoto et al., 2000; Li et al., 2002; Ikeda et al., 2004).
study, Eriodictyol inhibited RANKL-induced phosphorylation of ERK, JNK, and p38, implying that all of these pathways are affected and suggesting several ways that osteoclast formation is reduced by Eriodictyol.

RANKL treatment of BMMs also activates c-Fos, a component of the AP-1 transcription factor complex involved in osteoclast and macrophage differentiation. Mice with mutations in c-Fos exhibit severe osteopetrosis diseases, due to reduced numbers of mature osteoclasts (Grigoriadis et al., 1994). In this study, we found that Eriodictyol strongly reduced the protein level of c-Fos, consistent with its inhibition of MAPK, and NF-kB pathways.
Along with inhibition of NF-κB, MAPKs, and c-Fos by ERioduct, we further examined its impact on NFATc1, a transcription factor that is indispensable for osteoclast formation. NFATc1 is a member of the NFAT family of proteins (NFATc1, NFATc2, NFATc3, NFATc4, and NFATc-5). Importantly, NFATc1 is a master regulator of osteoclastogenesis, and can be spatio-temporally induced and activated by RANKL-stimulated signaling pathways (Shinohara and Takayanagi, 2007; Nakashima et al., 2012). NFATc1 can auto-regulate itself through binding motifs in promoter regions of its own gene and that of other NFAT family members (which bind a similar motif). It is notable that the NFATc1 promoter region can also recruit other transcription factors (NF-κB, NFATc2, and c-Fos) to amplify NFATc1 expression and downstream transcriptional processes (Asagiri et al., 2005; Alpargatis et al., 2008). Interestingly, ERK/MAPK, JNK/MAPK, and p38/MAPK also trigger induction of c-Fos and NFATc1 (Takayanagi et al., 2002; Ikeda et al., 2004; Monje et al., 2005; Huang et al., 2006). After activation of NFATc1, downstream osteoclastic genes are elicited, such as Csk, V-AKTase-δ2, and TRAPδ (Ballan et al., 2009; Feng et al., 2009). Our study demonstrated that Erioduct inhibited the protein level of NFATc1 with associated down-regulation of osteoclast-specific genes. From the combined suppression of osteoclast genes (Csk, V-AKTase-δ2, and TRAPδ) and suppression of NF-κB activity, MAPKs, and c-Fos level by Erioduct, it was inferred that Erioduct attenuated the process of RANKL-induced osteoclastogenesis. Moreover, c-Fos down-regulation by Erioduct (mediated by suppression of the induction of c-Fos, finally, down-regulation of NFATc1. In addition to the above observations with NF-κB and MAPK, NFATc1 is also sensitive to the release of intracellular Ca2+ during osteoclast differentiation. RANKL-induced Ca2+ oscillation activates the auto-amplification loop of NFATc1 required for osteoclastogenesis (Takayanagi et al., 2002; Negishi-Koga and Takayanagi, 2009). We found that Erioduct could inhibit the intensity of Ca2+ oscillation in response to RANKL, congruent with the pivotal role of Calcium signaling-induced suppression of NFATc1 (Takayanagi et al., 2002; Kogu et al., 2004).

In summary, we observed that Erioduct prevented RANKL-induced osteoclast formation and function through inhibiting NFATc1 activity and RANKL-induced NF-κB, MAPK, and Ca2+ signaling. Our study highlighted that the regulation of the transcription factor NFATc1 is a master switch in the course of RANKL-induced osteoclastogenesis. Our findings thus provide a cellular and molecular mechanism for the inhibitory effect of Erioduct on RANKL-induced osteoclastogenesis.

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Literature Cited


Lee YS, Yang H. 2015. Inflammation and RANKL-induced osteoclast formation in vivo/vo to confirm its suitability as a potential treatment against osteolytic bone diseases. Thus, Erioduct could offer a promising therapeutic strategy against osteoclastogenesis and other osteoclast-related diseases.

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Statements of authorship

Andrographolide inhibits ovariectomy-induced bone loss via the suppression of RANKL signaling Pathways

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Contributed to the execution of cell culture, animal experiments and data analysis. Major contribution to writing of the manuscript.

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Contributed to the planning, analysis of the research. Also contributed to revising of the manuscript. Acted as corresponding author.
Andrographolide Inhibits Ovariectomy-Induced Bone Loss via the Suppression of RANKL Signaling Pathways

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Abstract: Osteoporosis is a debilitating skeletal disorder with an increased risk of low-energy fracture, which commonly occurs among postmenopausal women. Andrographolide (AP), a natural product isolated from Andrographis paniculata, has been found to have anti-inflammatory, anti-cancer, anti-asthmatic, and neuro-protective properties. However, its therapeutic effect on osteoporosis is unknown. In this study, an ovariectomy (OVX) mouse model was used to evaluate the therapeutic effects of AP on post-menopausal osteoporosis by using micro-computed tomography (micro-CT). Bone marrow-derived osteoclast culture was used to examine the inhibitory effect of AP on osteoclastogenesis. Real time PCR was employed to examine the effect of AP on the expression of osteoclast marker genes. The activities of transcriptional factors NF-κB and Nfatc1 were evaluated using a luciferase reporter assay, and the IkBα protein level was analyzed by Western blot. We found that OVX mice treated with AP have greater bone volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) compared to vehicle-treated OVX mice. AP inhibited RANKL-induced osteoclastogenesis, the expression of osteoclast marker genes including cathepsin K (Ctsk), TRACP (Acp5), and Nfatc1, as well as the transcriptional activities of NF-κB and Nfatc1. In conclusion, our results suggest that AP inhibits estrogen deficiency-induced bone loss in mice via the suppression of RANKL-induced osteoclastogenesis and NF-κB and Nfatc1 activities and, thus, might have therapeutic potential for osteoporosis.

Keywords: andrographolide; osteoclastogenesis; RANKL; OVX; bone loss

1. Introduction

The maintenance of bone homeostasis involves two coordinated actions; namely bone formation (by osteoblasts) and bone resorption (by osteoclasts). The balance between the activities of osteoblasts and osteoclasts is critical to ensure normal bone structure. When this balance is disrupted, an increase or a decrease in bone mass will occur [1]. Enhanced osteoclast formation and/or activation are known mechanisms underlying osteolytic diseases such as osteoporosis, Paget’s disease of bone, metastatic
bone disease, and erosive arthritis [2]. Thus, osteoclasts have become key targets in the treatment of osteoporosis and other osteoclast-related osteolytic diseases [3].

Osteoclasts are specialized giant polykaryons, which are formed by the fusion of mononuclear progenitors of monocyte/macrophage lineage, and are responsible for bone resorption. Osteoclast formation, survival, and activation are regulated by several key cytokines. Although macrophage colony-stimulating factor-1 (MCSF-1) is necessary for the proliferation and survival of osteoclast progenitor cells [4], the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) is a critical cytokine for osteoclast differentiation [5]. Upon binding to its receptor RANK on osteoclast progenitor cells, RANKL recruits an adaptor molecule, tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). This in turn results in a cascade of intracellular signaling, which includes IκB kinase (IKK), RANKL-Induced Nuclear Factor-κB (NF-κB), extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK (p38 mitogen-activated protein kinases), Akt, Proto-oncogene tyrosine-protein kinase Src (c-Src), activator protein 1 (AP-1) and nuclear factor and activator of transcription (NFATc1) [6,7]. Among these signaling molecules, NF-κB plays an indispensable role in osteoclastogenesis. Following the phosphorylation of IκBα by the activated IκB kinase, NF-κB is released from the NF-κB/IκBα complex, and translocated to the nucleus, where it binds to its DNA target sequences, and triggers the expression of specific genes important for the formation of osteoclasts [8]. Based on the current understanding of this molecular mechanism, the inhibition of RANKL signaling by anti-RANKL antibodies or anti-NF-κB compounds is of prime focus in developing anti-catabolic therapies for osteoporosis.

In recent years, the use of natural herbal products to inhibit osteoclast formation and osteoclast-related diseases has garnered attention. Andrographolide (AP), a bicyclic diterpenoid lactone, is a naturally occurring product isolated from the leaves of a traditional Chinese herb, Andrographis paniculata, and is used for the treatment of respiratory infections, inflammation, fever, and diarrhea [9]. Modern pharmacological studies suggest that AP exerts anti-cancer [10], anti-inflammatory [11,12], anti-asthmatic [13], and neuro-protective effects [14] partly by regulating NF-κB. Furthermore, AP has the potential to prevent inflammatory osteolysis [15,16] and human breast cancer-induced bone loss [17]. However, the therapeutic effect of AP on post-menopausal osteoporosis is unknown. Thus, in the present study, we investigated the direct effects of AP on postmenopausal osteoporosis using an ovariectomy (OVX) mouse model, and explored its molecular mechanisms of action.

2. Results

2.1. Andrographolide (AP) Inhibits Ovariectomy-Induced Bone Loss in Vivo

To examine the effects of AP on bone loss in vivo, OVX mice were used to mimic postmenopausal osteoporosis. Tibias from OVX mice treated with AP or vehicle were analyzed using micro-CT. The trabecular bone mass in OVX mice was markedly reduced compared to sham-operated mice at seven weeks post-ovariectomy, which was confirmed by 3D reconstruction (Figure 1A) and quantitative bone parameters (Figure 1B). AP treated OVX group showed a dose-dependent therapeutic effect (at dose of 5 mg/kg/2 days) in preventing bone loss induced by OVX, with significant increases in bone volume per tissue volume (BV/TV) and trabecular number (Tb.N), and a decrease in trabecular separation (Tb.Sp) as compared to vehicle treated OVX group (Figure 1). AP had no significant effect on cortex thickness (Cor.Th) (Figure 1B).

![Figure 1. Cont.](image-url)
Figure 1. Andrographolide inhibits OVX-induced bone loss. A week after ovariectomy, mice were injected with vehicle (1% DMSO) or AP (1 or 5 mg/kg) every two days for a six-week period. (A) representative 3D images showing tibias of mice scanned with micro-CT; and (B) analyses of microstructural indices including bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and cortex thickness (Cor.Th) calculated by micro-CT. n = 6, ** p < 0.005, *** p < 0.001.

2.2. AP Inhibits Receptor Activator of Nuclear Factor-κB Ligand (RANKL)-Induced Osteoclastogenesis

To examine the effect of AP on osteoclastogenesis, we first tested the effect of AP on bone marrow macrophages (BMM) cell viability using a MTS assay. As shown in Figure 2B, AP had no cytotoxic effects within the dose range of up to 20 μM. To determine the dose dependent effect of AP on RANKL-induced osteoclastogenesis, BMMs were cultured in the presence of RANKL with varying concentrations of AP, and stained for tartrate-resistant acid phosphatase (TRACP) activity. After five days of RANKL stimulation, the BMMs were fully differentiated into osteoclasts (Figure 2C). However, when AP was added to BMMs during the course of osteoclastogenesis, osteoclast formation was inhibited in a dose-dependent manner, as demonstrated by the significantly decreased total number of osteoclasts. Addition of AP at the dose of one, five, and 10 μM resulted in a huge reduction in osteoclast number (Figure 2D). Taken together, these data showed a dose-dependent inhibitory effect of AP on RANKL-induced osteoclastogenesis.

Figure 2. Cont.
Figure 2. Effect of andrographolide on RANKL-induced osteoclast formation. BMMs were cultured in the presence of RANKL with different doses of AP for five days. (A) Chemical structure of AP; (B) cell viability measured using a MTS assay, in which BMMs were treated with varying concentrations of AP for 48 h; (C) Light microscopy images showing the effect of AP (one, five, and 10 μM) on RANKL-induced osteoclast formation (Mag = 20×); and (D) cell count of osteoclast like cells with TRACP-positive multinucleated cells. *** p < 0.001.

2.3. AP Suppresses Osteoclast-Related Gene Expression

To further investigate the effect of AP on osteoclast differentiation, the expression of osteoclast-related genes, which are up-regulated in response to RANKL, were examined by real-time PCR. As demonstrated in Figure 3, the expressions of osteoclasts marker genes cathepsin K (Ctsk), ATP6v0d2, NFATc1, and TRACP (Acp5), were induced by RANKL. Treatment with AP at the concentration of 10 μM, decreased the RANKL-induced mRNA expression of these genes in a dose-dependent manner. This result is consistent with its inhibitory effect on osteoclast formation as shown in Figure 2.

Figure 3. Andrographolide attenuates RANKL-induced gene expression. mRNA analysis of Ctsk (A); ATP6v0d2 (B); NFATc1(C) and TRACP (D) genes from BMMs that were treated with M-CSF (25 ng/mL), RANKL (50 ng/mL) and varying doses of AP (1, 5 and 10 μM) for five days. mRNA level were determined by real-time PCR and normalized to gene expression of GAPDH. All experiments were run in triplicate. ** p < 0.005; *** p < 0.001.
2.4. AP Suppresses RANKL-Induced Nuclear Factor-κB (NF-κB) Activation

The NF-κB pathway has been shown to play an essential role in RANKL-induced osteoclastogenesis [18]. To explore the possible mechanism underlying the inhibitory effect of AP on osteoclastogenesis, we investigated the effect of AP on IκBα degradation and NF-κB activation. As shown in Figure 4A, IκBα degradation was observed at 10 min after RANKL stimulation, and then IκBα was re-synthesised at 30 min (Figure 4B). AP (10 μM) treatment delayed the RANKL-induced IκBα degradation, which was most evident at 60 min. The effect of AP on modulating NF-κB transcriptional activity was also tested using RAW 264.7 cells stably transfected with an NF-κB luciferase reporter construct (3xκB-Luc-SV40). As shown in Figure 4B, NF-κB transcriptional activation increased significantly after RANKL stimulation compared to the control group. However, treatment with AP showed a dose-dependent suppression of the NF-κB activation at concentrations of one and 10 μM.

![Figure 4](image_url)

Figure 4. Andrographolide inhibits RANKL-induced NF-κB and NFATc1 activities. (A) BMM were seeded at a density of 5 × 10^5 per well. After attachment overnight, cells were pre-treated with AP for 1 h and then stimulated with M-CSF (25 ng/mL) and RANKL (50 ng/mL) for indicated times. Lysate of cells were collected by using RIPA Lysis Buffer, and separated with 10% SDS-PAGE following by transferring onto nitrocellulose membrane. The membrane was blocked and probed with antibodies to IκBα and β-actin. Signal intensities of bands were detected, and shown as a ratio of IκBα/β-actin; (B) RAW 264.7 cells, which transfected with 3xκB-Luc-SV40 reporter gene, were pre-treated with one or 10 μM AP for 1 h followed by RANKL stimulation for 6 h. Cell lysates were collected and examined for luciferase activity; (C) BMMs were treated with AP (10 μM) and M-CSF and RANKL for indicated times. Cell lysates were collected for Western blot analysis using antibodies to NFATc1 and β-actin; (D) RAW 264.7 cells stably transfected with an NFATc1 luciferase reporter construct were cultured with AP at one or 10 μM in the presence of RANKL. Luciferase activity was measured after 24 h; and (E) BMMs were treated with AP (10 μM), and stimulated with RANKL (50 ng/mL). Cell lysates were analyzed by Western blot with antibodies to ERK and p-ERK. All experiments were run in triplicate. Student’s t-test was used for statistical analysis. *p < 0.05; **p < 0.005; ***p < 0.001.
2.5. AP Suppresses RANKL-Induced Nuclear Factor of Activated T-Cells (NFATc1) Activation

NFATc1, a transcription factor belonging to the NFAT family, regulates the expression of osteoclast specific genes, such as Ctsk, TRACP (Acp5), calcitonin receptor (Ctr), and osteoclast-associated receptor (Oscar) [19,20]. As stated above, AP inhibited RANKL-induced gene expression of Ctsk, TRACP (Acp5), and NFATc1. Therefore, we further investigated its regulatory effect on the protein level of NFATc1. Western blot analysis showed that the protein level of NFATc1 was increased at day three after RANKL stimulation, and was more pronounced at day five (Figure 4C). Notably, when AP (10 μM) was added to the BMM culture, the protein level of NFATc1 was suppressed. The transcriptional activity of NFATc1 was also analyzed by using a luciferase reporter gene assay. The transcriptional activity of NFAT was dramatically up-regulated by RANKL, but this effect was strongly inhibited by AP in a dose dependent manner (Figure 4D).

2.6. AP Does not Affect RANKL-Induced Extracellular Signal-Regulated Kinases (ERK) Phosphorylation

ERK is one of the three subfamilies of MAPKs, which also play an important role in RANKL-induced osteoclastogenesis [21,22]. Therefore, we explored whether this signaling was intervened by AP during RANKL stimulation. The results showed that AP has no obvious effect on the ERK phosphorylation induced by RANKL (Figure 4E).

3. Discussion

Osteoporosis is the most common bone disorder, caused by excessive bone resorption by osteoclasts without adequate bone formation by osteoblasts [23]. Therefore, osteoclasts become the key targets of commonly-used drugs in the treatment of osteoporosis and other osteoclast-related osteolytic isease [24]. Many plant-derived polyphenols, such as mangiferin [25], curcumin [26], and naringin [27] have been shown to attenuate osteoporosis through negative regulation of osteoclastogenesis. In this study, we demonstrated that AP effectively inhibits bone loss induced by O VX. We also found that AP was capable of attenuating osteoclastogenesis via the inhibition of RANKL-induced NF-κB and NFATc1 activities. Understanding the therapeutic effect of AP on bone loss induced by estrogen deficiency and the underlining mechanisms might provide vital information for developing potential therapies against post-menopausal osteoporosis and osteoclast-related diseases.

Increased bone resorption, trabecular thinning, and decreased connections between the remaining trabeculae are the predominant features of post-menopausal estrogen withdrawal [28], and ovariectomy could mimic this phenomenon in animals. In this study, we evaluated whether AP can prevent bone loss induced by estrogen deficiency in C57BL/6 mice. The data showed that OVX mice lead to damaged bone microstructure and decreased trabecular bone compared to sham-operated mice. Intrapitoneal injection of AP significantly suppressed the bone loss induced by OVX, accompanied by increased BV/TV and Tb.N, and decreased Tb.Sp (Figure 1). Our results also suggested that AP had no significant effect on Cor.Th. However, the direct effects of AP on osteoblasts and bone formation require further investigation.

Osteoclasts, originated from monocyte-macrophage lineage, are unique cells in body responsible for bone resorption [29]. Excessive osteoclast formation and activity cause bone disorders, such as osteoporosis, metastatic bone disease, and inflammatory arthritis, where bone resorption exceeds bone formation [30]. In this study, we demonstrated that AP inhibits osteoclast formation in a dose-dependent manner. In order to choose an optimal dose of AP for BMMs culture, cell viability was examined at varying concentrations of AP. The result showed that AP has no cytotoxic effect on BMMs within the dose range of up to 20 μM. In the experiment of cell culture, AP at the dose of five or 10 μM, inhibited the formation of osteoclasts and the expression of osteoclast maker genes, including Ctsk, ATP6v0d2, NFATc1, and TRACP. These results are consistent with the therapeutic effect of AP on bone loss in vivo.
Upon RANKL binding, RANK recruits an adaptor protein TRAF6, which then induces a cascade of intracellular events. Among the molecules involved, NF-κB and NFATc1 are two well-documented transcription factors that are important for osteoclastogenesis [31,32]. The stimulatory signal from TRAF6 triggers phosphorylation and subsequent degradation of IκBα, leading to the activation of NF-κB. It is well known that the activation of NF-κB is critical for osteoclast differentiation, resorption, and survival [33]. Notably, NF-κB p50<sup>−/−</sup> and p52<sup>−/−</sup> double-knockout mice exhibit severe osteopetrosis, since they fail to generate mature osteoclasts [31].

Previous reports have identified the inhibitory ability of AP on NF-κB activity. For example, in 293 cells, AP covalently modified NF-κB and abrogated the TNF-α-induced NF-κB binding to its DNA target sequence [34]. In the permanent cerebral ischemia rat model, AP exerted neuro-protective effects by suppressing NF-κB activation [14]. The attenuation of RANKL-induced NF-κB activity by AP in osteoclasts is consistent with these results and might be part of the underlining mechanisms for the inhibitory effect of AP on osteoclastogenesis in vitro and bone loss in vivo.

NFATc1, a well-known master transcription factor, regulates the expression of osteoclast-specific genes such as the Ctsk, TRACP (Acp5), MMP9, Ctr, ATP6V0d2, and e-Src that are associated with osteoclast differentiation and function [19,35]. In addition, this function is regulated by NF-κB activity [32]. Conditional knockout of NFATc1 in mice resulted in a severe osteopetrotic phenotype and inhibition of osteoclastogenesis [32,36]. Although the exact details of the mechanism are unclear, here, we demonstrated that AP down-regulates RANKL-induced NFAT transcription in a dose-dependent manner. The protein level of RANKL-induced NFATc1 in osteoclasts was also suppressed significantly by AP. These results indicate that AP exerts the inhibitory effect on osteoclastogenesis partly by suppressing the NFATc1-mediated pathway. Therefore, AP not only affects the activation of NF-κB and NFATc1, but also the expression of their downstream genes, which contributes to its inhibitory effect on osteoclast formation and function.

Estrogen suppresses osteoclast formation by inhibiting osteoclastogenic cytokines such as IL-1, IL-6, IL-11, and TNF-α [37,38]. There is evidence that AP could also alleviate lipopolysaccharide (LPS)-induced osteolysis in mice [16]. Given that AP has strong anti-inflammatory effects and that inflammatory cytokine production positively influences osteoclastic bone resorption, it is possible that the therapeutic effect of AP on OVX mice bone mass is partly due to its anti-inflammatory property. In addition, there is a possibility of an estrogen-like effect of andrographolide that might contribute to its protective effect of OVX-induced bone loss. However, to our knowledge, there is no report that AP has an estrogen-like effect to date.

Taken together, our results indicate that AP inhibits osteoclastogenesis in vitro and bone loss in the OVX mouse model. Furthermore, our data demonstrate that the inhibitory effect of AP on osteoclasts is due to, at least in part, the suppression of RANKL-induced NF-κB and NFATc1 signaling pathways. Thus, AP might serve as a potential therapeutic agent for the treatment of osteoporosis and other osteoclast-related diseases.

4. Materials and Methods

4.1. Animals

Seven-week-old female C57BL/6 mice were purchased from the Academy of Military Medical Sciences, and housed in the SPF experimental center of Guangxi Medical University. The experimental procedures and animal facility were approved by the Animal Care and Welfare Committee of Guangxi Medical University (SYXK2009-0004). After a week of acclimatization, all animals were anaesthetized with 10% chloral hydrate and bilateral ovariectomy was performed, except for mice in the sham operated group (mice in the sham operated group were anaesthetized, and the ovaries were exposed but not removed). After post-surgery recovery for one week, all OVX mice were randomly divided into three groups (n = 6): OVX control group, AP 1 mg/kg group, and AP 5 mg/kg group. The AP groups were intraperitoneally injected with AP at corresponding
concentrations in 1% DMSO in physiological saline, while the OVX control group received 1% DMSO as vehicles. All injections were administered every two days for a period of six weeks. At the end of treatment, all mice were sacrificed for the extraction of tibias, which were fixed in 4% paraformaldehyde (PFA) at room temperature (25 °C) for 24 h followed by three rinses with 1× PBS. The bone microarchitecture of proximal tibias was measured using a micro-tomography scanner (Skyscan, Aartselaar, Belgium) with the setting as following: 50 kV, 500 μA, constant exposure, 0.5 mm Al filter, voxel size 9 nm, 0.4° angular rotation. 3D structures were reconstructed using NRecon software (Skyscan). A region 0.5 mm from the growth plate and 1 mm in height within the proximal tibia was analyzed by CTAn (Skyscan) software to evaluated trabecular structure parameters, including bone volume per tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). Cortex thickness (Cor.Th) was evaluated through the same way but using a region 5 mm from the growth plate.

4.2. Reagents and Antibodies

AP (99.50%, assay by HPLC) was purchased from Mansite (Chengdu, Sichuan, China). For in vitro study, AP was dissolved in DMSO and the final concentration of DMSO in the culture was less than 0.02%. Alpha Modified Eagle’s Medium (αMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). Primary antibodies for β-actin, IκBα and NFATc1 were purchased from Cell Signaling Technology (Danvers, MA, USA). Recombinant GST-RANKL proteins were expressed and purified as previously described [39].

4.3. In Vitro Osteoclastogenesis Assay

To evaluate the effect of AP on osteoclast differentiation, in vitro osteoclastogenesis assay was performed. BMMs were obtained from six-week-old C57BL/6 mice by flushing femurs and tibias with αMEM and were cultured in a T-75 flask with a complete medium containing αMEM and 25 ng/mL macrophage-colony stimulating factor (M-CSF). The medium was changed every two days. After 5–7 days of proliferation and attaining confluence, BMMs were harvested by washing with phosphate-buffered saline (PBS) and trypsinization for 30 min. To generate osteoclasts, BMMs were plated in 96-well plates at a density of 6 × 10³ cells per well, incubated overnight, and stimulated with M-CSF (25 ng/mL) and RANKL (50 ng/mL). Simultaneously, the cells were treated with or without AP. The culture medium was replaced every 2–3 days. After five days, the cells were fixed with 4% paraformaldehyde in PBS) for 10 min at room temperature, rinsed with 1× PBS, and stained with tartrate-resistant acid phosphatase (TRACP) staining buffer. TRACP-positive multinucleated cells with more than three nuclei were counted as osteoclast-like (OCL) cells.

4.4. Cytotoxicity Assay

BMMs were plated in 96-well plates (6 × 10³ cells per well) and cultured overnight at 37 °C. The cells were then treated with M-CSF (25 ng/mL) and varying concentrations (0, 0.5, 1, 1.25, 5, 10, 20 μM) of AP. After culturing for two days, 50 μL MTS was added to each well and incubated for 4 h. The optical density (OD) was measured at 490 nm wavelength by using a BMG plate reader (BMG, Offenburg, Germany).

4.5. Real Time Polymerase Chain Reaction (Real-Time PCR)

For real-time PCR, BMMs were seeded into 6-well plate at a density of 1 × 10⁵ cells per well and cultured with M-CSF (25 ng/mL) and RANKL (50 ng/mL) for five days in the presence of varying AP concentration (1, 5 and 10 μM). Total RNA was isolated using the RNAeasy Mini Kit (Qiagen, Valencia, CA, USA) and reverse-transcription was conducted using 1 μg of total RNA with oligo-dT primers at 42 °C for 1 h. All PCRs were performed using 2 μg of respective cDNA. Quantification of mRNA was performed using ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Tag kit (TaKaRa Biotechnology, Dalian, Liaoning, China), and
normalized to housekeeping gene GAPDH. The following cycling parameters were used: 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s. The mouse primers used in this study are presented in Table 1.

<table>
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<tr>
<th>mRNA</th>
<th>Primer</th>
<th>Sequences (5'–3')</th>
<th>Product (bp)</th>
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</thead>
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<td>Csk</td>
<td>Forward</td>
<td>GGAGAAACACCTGAGGC</td>
<td>350</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>ATTTCTGGGACCCGAGC</td>
<td></td>
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<tr>
<td>ATP6v0d2</td>
<td>Forward</td>
<td>GTGAGACCTTGGAGACCTGAA</td>
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<td></td>
<td>Reverse</td>
<td>GAGAAATGTGCTCAGGGGGCT</td>
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<tr>
<td>NFATc1</td>
<td>Forward</td>
<td>CAACGCCCTGACCCACCGATAG</td>
<td>392</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCTGCTTTCCGCTCTCATAGG</td>
<td></td>
</tr>
<tr>
<td>TRACP (Acp5)</td>
<td>Forward</td>
<td>TGGGACATCATTTATGCCT</td>
<td>462</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTACTTCTTTTGCCGCTT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>ACCACAGTCATGACCACACCTACAC</td>
<td>452</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>TCCACACACTGTGGTTGCTGTA</td>
<td></td>
</tr>
</tbody>
</table>

4.6. Western Blot Assay

BMBS were seeded into six-well plates at a density of 5 x 10^5 cells per well and incubated overnight. Following pre-treatment with AP for 1 h, the cells were stimulated with RANKL (50 ng/mL) for the indicated times. The cells were then lysed with RIPA Lysis Buffer (Millipore, Billerica, MA, USA) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Proteins were separated by 10% SDS-PAGE, and then transferred onto a nitrocellulose membrane (Bio-Rad, Gladesville, NSW, Australia). Non-specific interactions were blocked using 5% skim milk powder (SMP) in 1 x TBS-Tween (TBST) for one hour. After washing with 1 x TBST, membrane was immunoblotted with antibodies against ERK, p-ERK, IκBα, NFATc1 and β-actin, visualized using enhanced chemiluminescence (ECL) reagents under a FujiFilm LAS-3000 system to detect the immunoreactivity. Signal intensities were quantified using NIH ImageJ software.

4.7. Luciferase Reporter Gene Assay for NF-κB and NFAT

To test the effect of AP on RANKL-induced NF-κB activation, RAW 264.7 cells stably transfected with a NF-κB luciferase construct (3xB-Luc-SV40) [40] were used in this assay. Cells were plated in 48-well plates and pre-treated with AP for 1 h, followed by RANKL (50 ng/mL) stimulation for 6 h. Cells were harvested and lysates isolated for luciferase assay. Luciferase activity was measured using a Promega Luciferase Assay system (Promega, Madison, WI, USA) according to manufacturer’s instructions. NFAT activity was test through the same way, but using RAW 264.7 cells which stably transfected with an NFATc1 luciferase reporter construct [41], and stimulated with RANKL for 24 h.

4.8. Statistical Analysis

All data are represented as mean ± standard deviation (SD) from three donors. Two-tailed Student’s t-test was used for statistical analysis. * p < 0.05, ** p < 0.01 or *** p < 0.001 were considered statistically significant.

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Author Contributions: Tao Wang and Qian Liu performed cell culture; Lin Zhou performed the MTS analysis; Xi Xi Lin, Rong Zeng, and Xiaoman Liang partly contributed to ovariectomy (O VX) mice experiment; Jin Bo Yuan performed Micro-CT scanning; Tao Wang and Qian Liu analyzed data and wrote this manuscript cooperatively; Jiak Xue and Jinmin Zhao designed and supervised the study, and revised the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References


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Statements of authorship

SC-514, a selective inhibitor of IKKβ attenuates RANKL-induced osteoclastogenesis and NF-kB activation

Qian Liu, Huafei Wu, Shek Man Chim, Lin Zhou, Jinmin Zhao, Haotian Feng, Qingli Wei, Qing Wang, Ming H. Zheng, Ren Xiang Tan, Qiong Gu, Jun Xu, Nathan Pavlos, Jennifer Tickner, Jiake Xu,

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Contributed to the execution of cell culture, PCR, western blot assay and data analysis. Major contribution to writing of the manuscript.

Huafei Wu
Contributed to the execution of cell culture and data analysis.

Shek Man Chim
Contributed to the execution of western blot assay and data analysis.

Lin Zhou
Contributed to the screening of the natural compounds.

Jinmin Zhao
Contributed to the execution of micro-CT scanning.

Haotian Feng
Contributed to the execution of animal experiment.

Qingli Wei
Contributed to the execution of animal experiment.

Qing Wang
Contributed to the analysis of the results.

**Ming H. Zheng**
Contributed to revising of the manuscript.

**Ren Xiang Tan**
Contributed to the analysis of the results.

**Qiong Gu**
Contributed to providing of the natural compounds.

**Jun Xu**
Contributed to providing of the natural compounds.

**Nathan Pavlos**
Contributed to the analysis of the results.

**Jennifer Tickner**
Contributed to the revising of the manuscript.

**Jiake Xu**
Contributed to the planning, analysis of the research. Also contributed to revising of the manuscript. Acted as corresponding author.
SC-514, a selective inhibitor of IKKβ attenuates RANKL-induced osteoclastogenesis and NF-κB activation

Qian Liu, Huafei Wu, Shen Man Chim, Lin Zhou, Jinmin Zhao, Haotian Feng, Qingli Wei, Qing Wang, Ming H. Zheng, Ren Xiang Tan, Qiong Gu, Jun Xu, Nathan Pavlos, Jennifer Tickner, Jiale Xu

SC-514, a selective inhibitor of IKKβ attenuates RANKL-induced osteoclastogenesis and NF-κB activation

ABSTRACT

The RANKL-induced NF-κB signaling pathway is essential for osteoclastogenesis. This study aims to identify specific inhibitors targeting NF-κB signaling pathway, which might serve as useful small molecule inhibitors for the treatment and alleviation of osteoclast-mediated bone lytic diseases. By screening for compounds that selectively inhibit RANKL-induced NF-κB activation in RAW264.7 cells as monitored by luciferase reporter gene assay, we identified SC-514, a specific inhibitor of IKKβ, as a candidate compound targeting osteoclastogenesis. SC-514 dose-dependently inhibits RANKL-induced osteoclastogenesis with an IC50 of <5 μM. At high concentrations, SC-514 (≥12.5 μM) induced apoptosis and caspase 3 activation in RAW264.7 cells. Moreover, SC-514 specifically suppressed NF-κB activity owing to delayed RANKL-induced degradation of IκBα and inhibition of p65 nuclear translocation. Taken together, our results indicate that SC-514 impairs RANKL-induced osteoclastogenesis and NF-κB activation. Thus, targeting IKKβ by SC-514 presents as a potential treatment for osteoclast-related disorders such as osteoporosis and cancer-induced bone loss.

1. Introduction

Osteoclasts are multinucleated bone-resorbing cells derived from precursors of the monocyte-macrophage lineage [1]. Over-production and activation of osteoclasts is a pathological feature of many osteolytic bone disorders such as osteoporosis, periodontitis, and aseptic loosening of orthopedic implants [1]. The receptor activator of NF-κB ligand (RANKL), a member of the tumor necrosis factor (TNF) receptor–ligand family, is a crucial factor for the differentiation of osteoclasts in vitro and ex vivo [2-4]. Targeted ablation of RANK in mice results in a lack of osteoclasts, severe osteoporosis, and a defect in tooth eruption [5]. During osteoclastogenesis, RANKL signaling is initiated by binding to its receptor, RANK, which results in the recruitment of TNF receptor associated factor (TRAF) adapter proteins and the activation of NF-κB, leading to the induced-expression of specific genes that have DNA-binding sites unique for NF-κB. NF-κB is an essential transcription factor that is vital to both osteoclast differentiation and survival [6-11]. An essential step in the NF-κB activation cascade is the phosphorylation of inhibitor κB (IκB) by the IκB kinase (IKK) complex which consists of IκK alpha (IKKα), IκK beta (IKKβ), and IκK gamma (NEMO) with catalytic domain or regulatory domain [12]. Interestingly, IKKβ, but not IκKα, has been shown to be a critical mediator of osteoclast survival and is required for inflammation-induced bone loss [9]. Using conditional KO mouse models, it has been shown that IκKα is required for osteoclast formation in vitro, but it is dispensable in vivo. On the other hand, IκKβ is required for RANKL-induced osteoclastogenesis both in vitro and in vivo [9]. In addition, IκK downstream targets p50/p2 and p65 are also important for osteoclastogenesis [13,14].

SC-514, a selective inhibitor of IKKβ is a cell-permeable (thienothiophenyl)aminooacetamide compound that displays anti-inflammatory properties [15]. As a potent, reversibly,
ATP-competitive, and highly selective inhibitor, SC-514 inhibits various forms of recombinant IKKβ with IC50 values of 3–12 μM. SC-514 has selectivity on IKKβ and little effect on other members of the IKK family, including other IKK isoforms or serine-threonine and tyrosine kinases in vitro. SC-514 inhibits expression of NF-kB-dependent cytokines such as IL-6 and IL-8, through the inhibition of IKKβ-mediated phosphorylation of IκBα [15]. SC-514 is also effective in an acute inflammation model by blocking TNF-α production in LPS-challenged rats [15]. Previous studies have also reported the inhibitory effects of SC-514 on IKKβ in the treatment of tumors and inflammation [16–26]. However, the effect of SC-514 on osteoclastogenesis and RANKL-induced NF-κB signaling pathways is hitherto unknown. In the present study, we investigated the effects of SC-514 on in vitro osteoclast formation and NF-κB signaling. Our results demonstrate that SC-514 is a potent inhibitor of osteoclast formation and NF-κB activity and thus might serve as a useful treatment for osteoclast-related disorders such as osteoporosis.

2. Methods

2.1. Media and reagents

RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). Alpha Modification of Eagle’s Medium (α-MEM) and fetal bovine serum (FBS) were purchased from TRAC (Sydney, NSW, Australia). l-Glutamine, penicillin and streptomycin were purchased from Gibco BRL (Melbourne, Australia). SC-514 (BML-EI343) was purchased from Enzo Life Sciences (Sapphire Bioscience Pty. Ltd., Australia). Natural compounds (with a purity >98%) and synthetic compounds were obtained from Professor Jun Xu of San Yat-sen University, Professor Ren Xiang Tan of Nanjing University, or Chengdu MUST Bio-technology Co., Ltd. (China). All compounds were dissolved in dimethyl sulfoxide (DMSO) purchased from BDH laboratory supplies (Poole, Dorset, England). GST-RANKL was purified and used as previously described [3].

2.2. NF-κB activity in RAW264.7 cells and drug screening assay

Drug screening assays were conducted using RAW264.7 cells stably transfected with an NF-κB-driven luciferase reporter gene construct (Seb-Luc-SV40) [27] with natural and synthetic compounds at concentrations of 1–10 μM. To investigate the effect of SC-514 on NF-κB activity, RAW264.7 cells stably transfected with an NF-κB-driven luciferase reporter gene were pretreated with various doses of SC-514 (0–1.25 μM) for 1 h followed by 100 ng/mL RANKL for 8 h. Luciferase activities were measured using a POLARStar OPTIMA plate reader (BMG Labtech, Germany).

2.3. Dose-dependent osteoclastogenesis assay

For primary cell cultures, bone marrow cells isolated from C57BL/6 mice were cultured for 3 days in complete medium containing α-MEM supplemented with 10% FBS, 2 mM l-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin with the addition of 10 ng/mL macrophage-colony stimulating factor (M-CSF; R&D Systems, NSW, Australia). Cells were kept in a humidified 37 °C water-jacketed incubator (Forma Scientific) at 5% CO2 and 95% air. Bone marrow derived macrophages (BMM) were then seeded (8 × 106 cells/well) into a 96-well cell culture plate and incubated overnight. Then cells were stimulated with M-CSF; RANKL (250 pg/mL) and treated with various concentrations of SC-514 (1.25–5 μM). Culture medium was replenished every second day. After 5–7 days incubation, cells were fixed with 4% paraformaldehyde (Sigma Aldrich, NSW, Australia) and stained for tartrate-resistant acid phosphatase (TRAP) activity to identify osteoclasts using TRACP staining kit (Sigma Aldrich, NSW, Australia). TRAP-positive multinucleated cells with >3 nuclei were scored as osteoclasts. Photographs of individual wells were taken using Nikon Coolpix 995.

2.4. Apoptosis assay

Apoptosis assays were performed as previously described [28]. In brief, 1 × 106 RAW264.7 cells were seeded in 2 mL of complete media in a 6-well cell culture plate and then incubated in a humidified atmosphere of 5% CO2 and 95% air at 37 °C overnight. Media was removed after overnight incubation. SC-514 with various concentrations of 0 μM, 3.1 μM, 6.2 μM, 12.5 μM, 25 μM, or 50 μM was added to the wells, and cells were incubated for 24 h. Cells were collected and resuspended in 0.5 mL of 1 × Binding Buffer (BD-Pharmingen, NSW, Australia). Aliquots of cell suspensions (100 μL) were then stained with Annexin V–PE (5 μL; BD-Pharmingen, NSW, Australia) and/or 7-aminoactinomycin D (7-AAD) (5 μL; BD-Pharmingen, NSW, Australia). Cells were incubated in the dark at room temperature for 15 min, followed by addition of 400 μL of 1 × Binding Buffer and analysis by flow cytometry (FACSCalibur, Becton Dickinson, NSW, Australia). The percentage of apoptotic cells in the population was obtained.

2.5. Confocal microscopy

RAW264.7 cells (1 × 104 cells/well) were seeded onto sterilized cover-slips in 24-well plates. Cells were stimulated with 100 ng/mL of RANKL and incubated in a humidified atmosphere of 5% CO2 at 37 °C for 5 days. Complete media with RANKL (100 ng/mL) was replaced every other day. After osteoclast formation was observed, cells were treated for 24 h with or without SC-514 (10 μM and 50 μM). Cells were washed twice with 1 × PBS (pH 7.4), and then fixed for 30 min with 0.5% 4% paraformaldehyde. Fixed cells were washed 3 times with 1 × PBS and then permeabilized with 0.5% of 0.1% Triton X-100 (Sigma Aldrich, NSW, Australia) in 1 × PBS for 5 min at room temperature. Following permeabilization, cells were washed twice with 0.2% BSA (Sigma Aldrich, NSW, Australia) in 1 × PBS (0.25% BSA-PBS) and incubated in the dark for 2 h with Rhodamine-Conjugated Phalloidin F-actin stain (1:100; 1:150; Life Technologies, Victoria, Australia) to visualize filamentsous actin. The cells were washed 4 times with 0.2% BSA-PBS and a further 4 times with 1 × PBS and counter-stained in the dark at room temperature for 3 min with the Hoechst 33258 DNA nuclei stain (1:10000 in PBS; Life Technologies, Victoria, Australia) for nuclear DNA staining. After washing the cells were mounted onto slides with anti-fade mounting media for confocal microscopy using a confocal laser scanning microscope (MRC-1000 Bio-Rad) equipped with a Krypton-Argon laser to an epifluorescence Nikon Diaphot 300 inverted microscope. The slides were viewed under oil immersion objective lens (Nikon, NA = 0.7) and the images (512 × 512 pixel) were collected as Bio-Rad PIC files and viewed using Confocal Assistant™ version 4.0.2. For microtubule staining, a monoclonal anti-α-tubulin antibody (Sigma, St. Louis, USA) (1:500 diluted in 0.25% BSA-PBS) was used with Alexa Fluor 488 secondary antibodies (Molecular Probes Inc., Eugene, USA) at 1:500 dilution and the fluorescent images were collected as described above.

2.6. Caspase-3 assay

RAW264.7 cells were seeded (2 × 105 cells/well) into 24-well plates, cultured overnight, and then treated with various doses of...
SC-514 for 24 h. The activity of caspase-3 was measured as previously described [29]. The treated cells were harvested by trypsinization and vigorous pipetting. The cells were pelleted, washed once in PBS and then lysed by three freeze thaw cycles in 20 μL of lysis buffer containing 50 mM HEPEs (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/mL pepstatin A and 10 μg/mL leupeptin. The chemicals in lysis buffer were purchased from Sigma Aldrich, NSW, Australia. The protein content of the lysate was determined by a Bradford assay (BioRad, NSW, Australia). The activity of caspase-3 in lysates was determined using a kinetic assay, in buffer containing 50 mM HEPEs (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA and 10% glycerol (Sigma Aldrich, NSW, Australia), by monitoring the cleavage of acetyl-DEVD-APC in the presence or absence of the caspase-3 inhibitor Ac-DEVD-CHO (1 μM) (Promega, Sydney, Australia). The changes in fluorescence of AFC were measured at 510 nm after excitation at 400 nm in a multifunctional microplate reader (POLAstar OPTIMA, BMG, Germany).

2.7. Western blot analysis of iNκBα

Bone marrow cells isolated from C57Bl/6 mice were cultured for 4 days in complete medium in an incubator as described above. BMM cells (1 x 10⁶ cell/well) were seeded in 6-well plates and incubated overnight. Cells were pretreated with SC-514 (5 μM) or left untreated for 4 h followed by stimulation with RANKL (100 ng/mL) for 0, 10, 20, and 60 min. Cells were lysed in lysis buffer.
buffer and proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad, NSW, Australia). Membranes were blocked with 5% (w/v) skim milk powder (SMP) in 1× TBS-Tween (TBST) and probed with primary antibodies for β-actin (1:5000: Santa Cruz Biotechnology, Inc., CA, USA) in 1% (w/v) SMP in TBST. After three washes with TBST, membranes were incubated with secondary antibodies diluted 1:5000 in 1% (w/v) SMP in TBST as previously described [30]. The membranes were then visualized using FujiFilm LAS-3000. Signal intensities were quantified by NIH imagej software.

2.8. p65 immunochemistry

BMM were seeded (2 × 10⁴/well in 100 µL) into 96-well plates and incubated overnight. Cells were then pre-incubated with SC-514 (5 µM) for 1 h before stimulation with RANKL (100 ng/mL) for up to 30 min. After washing with 1× PBS, cells were fixed with 4% paraformaldehyde for 20 min, washed three more times with 1× PBS and then permeabilized for 5 min with 0.1% Triton X-100 in PBS. Following two washes with 0.1% BSA-PBS, cells were incubated at 37°C for 45 min with 50 µL of anti-p65 antibody (Santa Cruz Biotechnology, Inc., CA, USA) diluted 1:200 in 0.1% BSA-PBS. Anti-IgG was used as a negative control (Sigma Aldrich, NSW, Australia). The cells were then washed four times with 0.1% BSA-PBS, four times with 1× PBS, once with 0.1% BSA-PBS before the addition of streptavidin-hors eradish peroxidase (Dako, Victoria, Australia). After 20 min incubation at room temperature and washing (four times with 0.1% BSA-PBS, four times with PBS, once with 0.1% BSA-PBS), Dako Liquid DAB (20 mL: Dako,

![Fig. 2. SC-514 causes morphological changes in OCL cells. (A) RAW264.7 cells seeded on 6-well plates were treated with various doses of SC-514 (0–50 µM). Representative light microscope images showing the appearance of RAW264.7 cells treated with SC-514. (B) Representative confocal images of osteoclast like cells (OCL) treated with SC-514 (0, 10 and 50 µM). The changes in morphological characteristics of OCL cells include the loss of F-actin (red), microtubule array (green), and fragmentation of DNA nuclei (blue) at high doses of SC-514 (50 µM). Representative results from three independent experiments are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
Victoria, Australia) was added for 30 min or until brown coloration appeared [31].

2.9. Statistical analyses

All data shown represent one of at least three independent experiments. Data were presented as mean ± SEM. Student’s t-test was used to determine statistical significance of differences between groups. A p-value of <0.05 was considered to be statistically significant.

3. Results

3.1. SC-514 inhibits RANKL-induced osteoclastogenesis

Using a high-throughput luciferase reporter gene assay to screen a compound library (total number of 95 compounds) for potential inhibitors of RANKL-induced activation of NF-κB, we identified SC-514 (Fig. 3A), a selective inhibitor of IKKβ, as a candidate compound found to attenuate RANKL-induced activation of NF-κB (Table 1). Given that NF-κB activity is crucial to osteoclast differentiation and function we first investigated the effects of SC-514 on RANKL-induced osteoclast formation using an established BMM osteoclastogenic culture system. As shown in Fig. 1B, BMM cells cultured in the presence of RANKL and M-CSF form multilaminated TRAP positive osteoclast-like (OCL) cells (Fig. 1B and C). By comparison, BMM cells treated with increasing doses of SC-514 demonstrated a dose-dependent inhibition of RANKL-induced osteoclastogenesis, with treatment with ≥5 μM SC-514 resulting in a significant attenuation of RANKL-induced OCL formation (Fig. 1B and C).

3.2. SC-514 induces apoptotic cell death and activation of caspase 3 in OCL cells

Next, we assessed the effect(s) of SC-514 on both precursor and mature OCL cells at higher concentrations. RAW264.7 cells not stimulated with RANKL and in the absence of SC-514 remained well-attached and retained their characteristic ovoid cell morphology. With increasing concentrations of SC-514 (>12.5 μM), both the morphology and density of RAW264.7 cells was significantly altered. In this instance, RAW264.7 cells became more rounded with cytoplasmic condensation and increasing amounts of detached cell debris observed within the culture media, indicative of cell death (Fig. 2A).

To further investigate the effects of SC-514 on the morphology of mature OCL cells, RAW264.7 cells stimulated with RANKL to become OCL cells were challenged with differing concentrations of SC-514 for 24 h and then triple stained for F-actin, α-tubulin and Hoechst 33258 to visualize the cytoskeleton and nuclei, respectively (Fig. 2B). Untreated OCL cells typically displayed intact nuclei (blue) and well-defined F-actin rich podosomal belts (red), and a spatially organized microtubule array (green). Little disturbance in podosome belt or microtubule organization were observed in OCL cells treated with SC-514 at concentrations of <10 μM. On the other hand, an almost complete loss of podosome microtubule integrity was observed in OCL cells treated with high concentrations of SC-514 (50 μM), probably reflecting cytotoxicity and/or apoptosis. Consistently, these cytoskeletal changes were frequently accompanied by nuclear fragmentation, a hallmark of apoptosis.

To better determine whether the morphological aberrations observed following SC-514 treatment were a direct consequence of cellular apoptosis and not simply drug induced cytotoxicity we next performed apoptosis assays on SC-514 treated RAW264.7 cells using flow cytometry. In this instance, cells stained positive for Annexin V-PE and negative for 7-AAD accounted for early apoptosis, whereas cells positive for both Annexin V-PE and 7-AAD were considered to be undergoing late apoptosis/necrosis. Rates of apoptosis were low in RAW264.7 cells either untreated or treated with SC-514 (≤6.2 μM) (Fig. 3A). When treated with SC-514 at concentration ≥6.2 μM, the percentage of apoptotic events was substantially increased with >45% of total cells deemed as early apoptotic (Fig. 3A).

Caspase 3 activation plays a key role in the initiation of cellular events during the apoptotic process. Therefore, to complement the Annexin V-PE assay we monitored caspase 3 activity before and after SC-514 treatment by ELISA. In keeping with the flow cytometric data, caspase 3 activity of RAW264.7 cells was not significantly changed when cells were exposed to SC-514 at low concentrations (≤6.2 μM) (Fig. 3B). By comparison caspase 3 activities were elicited significantly following treatments with SC-514 at doses ≥12.5 μM (Fig. 3B).

3.3. SC-514 suppresses RANKL-induced activation of NF-κB

To confirm the inhibitory effects of SC-514 on NF-κB signaling we employed RAW264.7 cells stably transfected
with a NF-κB-driven luciferase reporter gene construct (3x-B- luc-SV40). As expected, RANKL-induced a ~5-fold increase in NF-κB-mediated luciferase gene expression as compared with RANKL-ununtreated cells which served as a control (Fig. 4A). By comparison, with increasing concentrations of SC-514 (≥0.62 μM), RANKL-induced transcriptional NF-κB activity was significantly suppressed (p < 0.001) (Fig. 4A). Treatment of SC-514 alone showed a trend of reduction of the basal level of NF-κB activity in RAW264.7 cells at 1.25 μM (Fig. 4A).

As aforementioned, during RANKL mediated NF-κB signaling, IKK is the convergence point that leads to the inducible phosphorylation and degradation of IκB, which is required for NF-κB activation. Given that SC-514 is a selective inhibitor of IKKβ we next examined its capacity to block RANKL-induced IκBα degradation. As shown in Fig. 4, BMMs treated with RANKL alone showed maximal degradation of IκBα after 20 min, and resynthesis of IκBα at 60 min. In comparison, preincubation of BMMs with SC-514 (5 μM) partially delayed the RANKL-induced

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Fig. 4. The NF-κB pathway in RAW264.7 cells is affected by SC-514 treatment. (A) SC-514 suppresses RANKL-induced NF-κB-dependent transcription. RAW264.7 cells stably transfected with the NF-κB-Luc/SV40 reporter gene were pretreated with various doses of SC-514 (0–1.25 μM) for 1 h followed by 100 ng/ml RANKL for 8 h. Luciferase activities were measured as described in Section 2 and are expressed as relative light units. n = 3 wells. (**p < 0.001 compared to RANKL-stimulated control). (B) Representative Western blot image showing SC-514 delays IκBα degradation. Cell extracts were prepared from BMM cells that were pretreated with SC-514 (5 μM) or left untreated for 4 h followed by stimulation with RANKL (100 ng/ml) for 0, 10, 20, and 60 min. Proteins extracted were subject to Western blot analysis using primary antibodies for IκBα or β-actin. (C) Average ratio of IκBα relative to β-actin. Western blot signal intensities were quantified by ImageJ software. IκBα/β-actin ratios were normalized to 0 min. n = 3 independent experiments. (*p < 0.05, **p < 0.01, N.S.: not significant).
degradation/resynthesis ratio of IκBα, most evident after 60 min (Fig. 4B and C).

There was a slight reduction of IκBα level in SC-514 treated BMMs at 0 min. This observation was consistent with a previous study demonstrating that DMSO slightly activated NF-κB activities in comparison to unstimulated cells [32].

Finally, to further explore the influence of SC-514 on NF-κB activity, we also evaluated the effect of SC-514 on RANKL-mediated p65 nuclear translocation in BMMs. As shown in Fig. 5, pre-treatment of BMMs with 5 μM SC-514 significantly decreases the nuclear translocation of p65 upon RANKL stimulation, as compared to vehicle pre-treated control cells. Anti-IgG antibody was used as a negative control.

4. Discussion

In this study, we document, for the first time, the inhibitory effects of SC-514 on RANKL-induced activation of NF-κB and osteoclastogenesis. Additionally, we have shown that SC-514 delays IκBα degradation and decreases the nuclear translocation of p65 upon RANKL stimulation. At higher concentration, SC-514 disrupts cytoskeletal organization and induces cellular apoptosis, an effect that may negatively impact RANKL-stimulated p65 nuclear translocation, which in turn drives the NF-κB signaling pathway.

In recent years, significant progress has been made in the search for small molecules that specifically and selectively inhibit NF-κB
activation. For instance, non-steroidal anti-inflammatory drugs including aspirin and salicylates, sulindac, and its analogs, and salicylates that are known as immunomodulatory drugs, also have inhibitory effects on NF-κB activation. Other major efforts have aimed to develop novel small molecule inhibitors that exhibit selectivity for IKK or NF-κB [44]. These studies have provided a solid framework to support the use of NF-κB inhibitors in the therapies for the treatment of various diseases including cancer, inflammation and autoimmune disorders [44]. The findings presented in the current study further support and extend the notion that selective inhibitors to IKK, like SC-514 might also serve as small molecule inhibitors for the treatment and alleviation of osteoclast-mediated bone lytic diseases. With our growing understanding of the molecular contribution of NF-κB in regulating osteoclastogenesis and osteolytic bone disease such as Paget’s disease of bone [11,31,45], effective and selective inhibitors of NF-κB might be developed by specific inhibition of IKK activity.

Interestingly, in addition to attenuating multiple facets of the NF-κB activation pathway (including IkBα degradation and p65 nuclear translocation), we found that SC-514 induces apoptosis of osteoclast like cells. This observation was important because osteoclast apoptosis directly influences the life span of osteoclasts, which in turn regulates the rate of osteoclastic bone resorption. NF-κB is commonly involved in suppressing apoptosis by inducing the expression of antiapoptotic genes, which include inhibitor of apoptosis proteins (IAPs), TRAF2, Bcl-2 family, A20, and c-FLIP [46,47]. Given that SC-514 is a selective inhibitor of IKKβ [15], the observed suppression of NF-κB activity via IKK and elevation of caspase-3 activity likely account for the induction of osteoclast apoptosis.

Recent studies have suggested the potential therapeutic effects of SC-514 in the inhibition of inflammation. For instance, treatment of rat astrocytes with high concentrations of SC-514 (100 μM) inhibited chemokine production stimulated with either IL-1β or TNF-α [26]. Similarly, treatment of mouse skin cells with SC-514 (1 μM) attenuates TPA-induced activation of Akt and NF-κB in vitro [19]. SC-514 has also been reported to strongly reduce IL-8 levels in HepG2 cells during an inflammatory response [24], the level of phospho-p65/p65Ser-536 in C81 cells [21], and microglial TLR2 expression in response to TNF-α treatment [25]. Given that inflammation often results in the activation of NF-κB, together with the fact that the production of pro-inflammatory cytokines has been linked to osteoclastic conditions, SC-514 could therefore be potentially beneficial in the treatment of inflammation-induced osteolytic diseases via the inhibition of IKK complexes.

In summary, this study unveils SC-514 as a potent inhibitor of RANKL-induced NF-κB activation and osteoclastogenesis. These findings indicate that SC-514 may be useful for the prevention or treatment of osteolytic disorders such as osteoporosis, tumor metastasis to bone, and/or inflammation elicited bone loss.

Conflicts of interest

All authors state that they have no conflicts of interest.

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