สารออกฤทธิ์ชีวภาพยับยั้งการแปรสภาพของออสตีโอคลาสต์

นางสาวชนม์นรี วิสุทธิ์สิทธิวงศ์

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BIOACTIVE COMPOUNDS SUPPRESSING DIFFERENTIATION OF OSTEOCLASTS

Miss Chonnaree Wisutsitthiwong

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Industrial Microbiology

Department of Microbiology

Faculty of Science

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Thesis Title	BIOACTIVE COMPOUNDS SUPPRESSING DIFFERENTIATION OF OSTEOCLASTS
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ชนม์นรี วิสุทธิ์สิทธิวงศ์:สารออกฤทธิ์ชีวภาพยับยั้งการแปรสภาพของออสตีโอคลาสต์. (Bioactive compounds suppressing differentiation of osteoclasts) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ธนาภัทร ปาลกะ, 75 หน้า.

การสร้างและการสลายกระดูกเป็นกระบวนการที่เกี่ยวข้องกับการรักษาภาวะสมดุลกระดูก ความสมดุลของการทำงานของเซลล์ออสตีโอบลาสต์และเซลล์ออสตีโอคลาสต์จึงเป็นตัวควบคุมในกระบวนการสร้างกระดูกใหม่ ความบกพร่องที่เกิดขึ้นในกระบวนการเพื่อคงสภาพของกระดูกนี้ก่อให้เกิดโรคต่าง ๆ ที่เกี่ยวข้องกับกระดูก รวมไปถึงโรคกระดูกพรุน ดังนั้น เซลล์ออสตีโอคลาสต์จึงเป็นเป้าหมายหนึ่งในการป้องกันและรักษาโรคกระดูกพรุน งานวิจัยนี้มีวัตถุประสงค์เพื่อค้นพบสารออกฤทธิ์ชีวภาพจากธรรมชาติที่มีสมบัติยับยั้งการสลายกระดูก โดยคัดกรองสารสกัดธรรมชาติที่ได้จากพืชซึ่งมีสมบัติยับยั้งกระบวนการเปลี่ยนสภาพของเซลล์ออสตีโอคลาสต์ สารสกัดธรรมชาติที่นำมาคัดกรอง คือ สารสกัดจากพืช *Kaempferia parviflora* จำนวน 10 ชนิด และ สารประกอบลิโมนอยด์ (limonoid) จากพืช *Xylocarpus moluccensis* จำนวน 6 ชนิด พบว่า 7-oxo-deacetoxygedunin (7-OG) ซึ่งเป็นสารประกอบลิโมนอยด์จากพืช *Xylocarpus moluccensis* สามารถยับยั้งกระบวนการเปลี่ยนสภาพของเซลล์ออสตีโอคลาสต์ที่ชักนำโดย RANKL ได้ โดยไปลดกิจกรรมของเอนไซม์ tatrate resistant acid phosphatase (TRAP) ของเซลล์พรีออสตีโอคลาสต์ที่ถูกกระตุ้นด้วยไซโตไคน์ Receptor Activator of Nuclear Factor-κB ligand (RANKL) ในเซลล์ไลน์ RAW264.7 โดยมีค่า IC50 ของความเป็นพิษต่อเซลล์มากกว่า 22.80 ไมโครโมลาร์ และค่า IC50 ของการกดการเปลี่ยนสภาพของเซลล์ออสตีโอคลาสต์เท่ากับ 2.50 ไมโครโมลาร์ จากการวิเคราะห์ผลของ 7-OG ต่อการแสดงออกของยีน *NFATc1* และ *Cathepsin K* ซึ่งเป็นยีนที่เกี่ยวข้องกับการเปลี่ยนสภาพและการทำงานของเซลล์ออสตีโอคลาสต์ตามลำดับ พบว่า 7-OG สามารถกดการแสดงออกของทั้งสองยีนนี้อย่างมีนัยสำคัญ ($p<0.05$) เมื่อศึกษาผลของ 7-OG ต่อการส่งสัญญาณภายในเซลล์ พบว่า 7-OG สามารถกดการกระตุ้นวิถีสัญญาณ NF-κB และ MAPK ที่เหนี่ยวนำโดย RANKL ผลการทดลองเหล่านี้แสดงให้เห็นว่า 7-OG สามารถกดการเปลี่ยนสภาพของเซลล์ออสตีโอคลาสต์ที่ถูกกระตุ้นด้วยไซโตไคน์ RANKL ได้โดยผ่านวิถีสัญญาณ NF-κB และ MAPK และมีความเป็นไปได้ที่จะนำไปพัฒนาเป็นยาในการรักษาโรคกระดูกพรุนต่อไป

ภาควิชาจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชาจุลชีววิทยาทางอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2554 .....

5272268323: MAJOR INDUSTRIAL MICROBIOLOGY
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CHONNAREE WISUTSITTHIWONG: BIOACTIVE COMPOUNDS SUPPRESSING DIFFERENTIATION OF OSTEOCLASTS.
ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., 75 pp.

Bone formation and bone resorption are the processes which maintain bone homeostasis. The balance in functioning of osteoblasts and osteoclasts regulates bone remodeling. Dysregulated bone homeostasis results in bone diseases including osteoporosis. Therefore, osteoclasts are one of the target cells for anti-resorptive therapies to treat osteoporosis. To discover new anti-resorptive agents, we screened natural compounds from plants that inhibit osteoclastogenesis. In this study, we screened ten compounds from *Kaempferia parviflora* and six limonoid compounds from *Xylocarpus moluccensis*. We found that 7-oxo-deacetoxygedunin (7-OG), a limonoid compound from *Xylocarpus moluccensis* markedly inhibited the differentiation of osteoclasts induced by receptor activator of nuclear factor-κB ligand (RANKL). Treatment with 7-OG decreased the tartrate resistant acid phosphatase (TRAP) activity in pre-osteoclasts which was induced by RANKL in RAW264.7 cell line. The IC50 for cytotoxicity and anti-osteoclastogenic activity was more than 22.80 μM and 2.50 μM, respectively. We investigated the effects of 7-OG on the expression of osteoclast-related genes, *NFATc1* and *Cathepsin K*, and found that treatment with 7-OG significantly suppressed the expression of both genes ($p<0.05$). Detailed analysis uncovered that 7-OG treatment suppressed the activation of NF-κB and MAPK signaling pathways. These results suggested that 7-OG inhibits osteoclastogenesis induced by RANKL by inferring with NF-κB and MAPK pathways and has potential to be a therapeutic agent for osteoporosis.

Department : Microbiology Student's Signature .....
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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µm	Micrometer
µM	Micromolar
/	Per
:	Ratio
7-OG	7-oxo-deacetoxygedunin
Ab	Anitibody
bp	Base pair
BMD	Bone mineral density
BSA	Bovine serum albumin
cDNA	Complementary DNA
CO_2	Carbon dioxide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	dATP, dCTP, dGTP and dTTP
ER	Estrogen receptor
FBS	Fetal bovine serum
g (centrifugation speed)	Gravity
hr	Hour
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IC50	50% of inhibitory concentration

IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
kDa	Kilo Dalton
LPS	Lipopolysaccharide
m	murine
mA	Milliampere
M-CSF	macrophage colony-stimulating factor
mg	Milligram
min	minute
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N (concentration)	Normal
NED	*N*-(1-naphthyl)-ethylenediamine dihydrochloride
nm	Nanometer
ng	Nanogram
NO	Nitric oxide
OPG	Osteoprotegerin
OD	Optical density
OVX	Ovariectomy
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline – Tween20
PCR	Polymerase chain reaction
psi	Pound per square inch

PVDF	Polyvinylidine fluoride
qPCR	Quantitative polymerase chain reaction
RANKL	Receptor activator of NF-κB ligand
RNA	Ribonucleic acid
rpm	Round per minute
rRANKL	Recombinant RANKL
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
sec	Second
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase
U	Unit
v	volume
w	weight
α	Alpha
β	Beta
δ	Delta
γ	Gamma
κ	Kappa

CHAPTER I

BACKGROUND

Osteoporosis is the common bone disease that occurs in elderly female after menopause (40-80 years of age) because of estrogen deficiency. An imbalance between osteoblast-bone forming cells and osteoclast-bone resorbing cells enhances osteoclastogenesis causing increase bone resorption, low bone mass and high risk of fracture (Pongchaiyakul et al., 2008).

Osteoclasts are multinucleated giant cells, derived from monocyte/macrophage lineage. Mature osteoclasts adhere to bone matrix and secrete acids and lytic enzymes to degrade the bone resulting in bone loss (Boyle et al., 2003). Receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are the key cytokines for osteoclast development (Suda et al., 1992).

Two major types of anti-resorptive agents, bisphosphonates and calcitonin are the most prescribed treatments for osteoporosis. Bisphosphonates inhibit lifespan and functions of osteoclasts but they have several side effects such as heartburn, abdominal pain, pain in muscles and joints, difficulty swallowing etc. and calcitonin inhibits functions of osteoclasts without affecting their survival. Although calcitonin transiently inhibits bone resorption but continuous treatment with calcitonin decreases its inhibitory effects, because of the down-regulation of calcitonin receptors on osteoclasts (Woo et al., 2006).

Several natural compounds from plants or microorganisms can inhibit bone resorption suggesting that they can be used for therapeutic treatment of bone diseases. In recent studies, several types of compounds with anti-osteoclastogenic activity have

been reported such as flavonoid from onion (Woo et al., 2004), isoflavone from soybean (Tang et al., 2010), FK-506 from *Streptomyces* sp. (Igarashi et al., 2004). These compounds markedly inhibit differentiation of osteoclast from bone marrow derived monocytes/macrophages or RAW264.7 cell line.

Therefore, in this study, we aimed at screening compounds from plants for anti-osteoclastogenic activity and studying the molecular mechanisms of such activities. The compounds which were identified in this study have potentials in becoming lead compounds for anti-bone resorption drug to prevent and treat bone diseases.

In order to identify new natural agent for osteoporosis treatment, we identified the limonoid compound 7-oxo-deacetoxygedunin (7-OG) from seeds of *Xylocarpus moluccensis* (Family Meliaceae), which was collected from the southern area of Thailand (Ravangpai et al., 2011). This compound has strong anti-osteoclastogenic activity but low toxicity. Moreover, this compound has been reported that it has anti-inflammatory activity because it can suppress nitric oxide production in RAW264.7 cell line treated with lipopolysaccharide (LPS) (Ravangpai et al., 2011).

Objective

To screen compounds from plants for anti-osteoclastic activity and study its molecular mechanisms

CHAPTER II

LITERATURE REVIEWS

2.1 Bone remodeling cycle

Bone is a dynamic tissue that is regulated by osteoblast-bone forming cells and osteoclast-bone resorbing cells to maintain mineral homeostasis (Kearns and Kallmes, 2008). Bone formation is mediated by mature osteoblasts to synthesize the bone matrix and mineralization and bone resorption is mediated by mature osteoclasts to remove mineralized tissues by secreting acids and lytic enzymes. Bone remodeling cycle occurs by a mult-istep process : a) a resting period before the beginning of new remodeling cycle; b) mature osteoclasts create the cavity to degrade bone matrix; c) osteoblasts precursors prepare the bone surface to begin bone formation; d) mature osteoblasts form the matrix with new bone. This process in human occurs in approximately 4 months and is regulated by various cytokines and hormones such as estrogen (Figure 2.1).

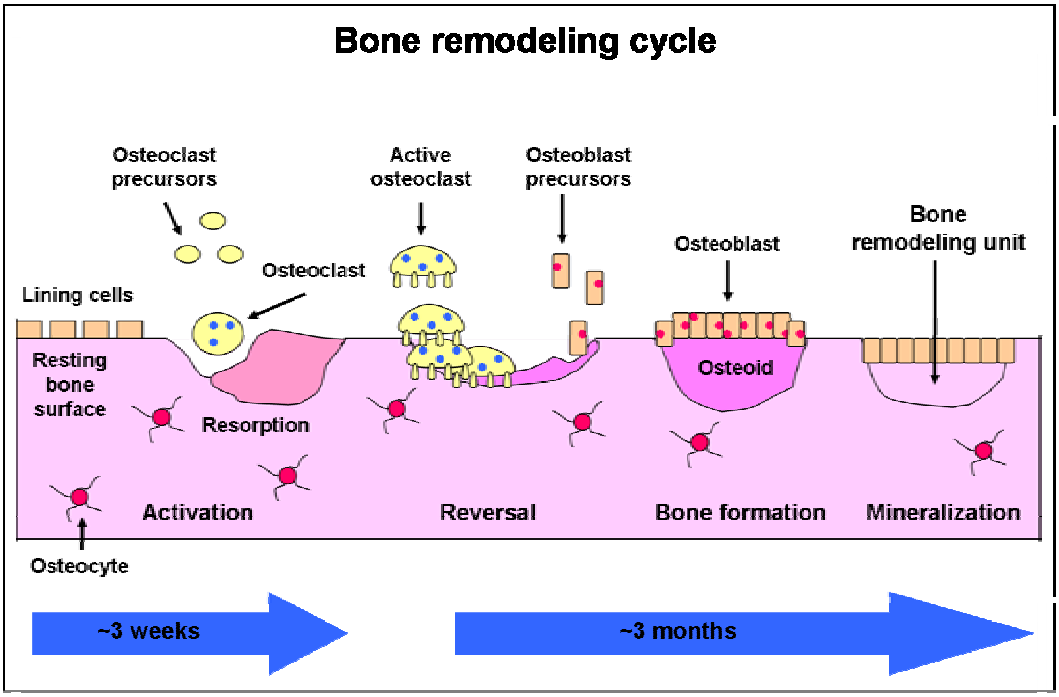


Fig 2.1 Bone remodeling cycle. The action of osteoclasts to degrade old bone and osteoblasts to form new bone is depicted (Bringhurst et al., 2011).

2.1.1 Osteoporosis

Osteoporosis is one of public health that is associated with aging disorders. It is characterized by decrease in bone mass and bone mineral density (Sipos et al., 2009). Low bone mass increases high risk of fractures of wrist, tibia and hip (Kessenich, 1997). The rate of bone loss in both women and men age 40-50 years is 0.3-0.5 percent per year and this rate increases 10-fold in postmenopausal women or after castration in men (Chin and Sarno, 1995). An imbalance between bone formation and bone resorption in bone remodeling cycle by increasing in bone resorption comparing to bone formation result in this condition. Osteoporosis occurs in postmenopausal women mainly because of the deficiency in estrogen production. Estrogen directly involves in the signaling cascade during bone remodeling. Therefore, the low level of estrogen results in bone loss and osteoporosis in women. Age-related osteoporosis may occur because the activity of osteoblasts in forming new bone is impaired while the resorption is normal or increases (Kessenich, 1997). Various risk factors for osteoporosis have been reported

such as alcohol abuse, cigarette smoking, endocrine diseases (e.g., thyrotoxicosis, primary hyperparathyroidism) and rheumatoid arthritis (Eastell, 1998). Furthermore, parathyroid hormone, calcium and vitamin D regulate immune system and function in the pathogenesis of osteoporosis (Clowes et al., 2005).

2.1.2 Diagnosis and Treatment of osteoporosis

In general, bone mineral density (BMD) is measured for diagnosis of osteoporosis. There are several techniques for measuring bone mineral density. The most useful technique is dual-energy x-ray absorptiometry (DEXA) (Eastell, 1998). Measurement of biochemical markers of bone remodeling in serum or urine is another useful method. For example, the measurement of serum alkaline phosphatase or serum osteocalcin which are the biochemical markers for bone formation can be used to detect the condition of bone-related disorders. The measurement of serum tartrate-resistant acid phosphatase or urinary excretion of pyridinium cross-link of collagen are used as the biochemical markers of bone resorption. These techniques are used for predicting fracture risk and monitoring clinical treatment (Eastell, 1998).

Several therapies are available for clinical treatment of osteoporosis. Most aim at reducing the risk of fractures by increasing bone mineral density. The current therapies are listed below

Bisphosphonates

Alendronate, etidronate and risedronate are the drugs in the bisphosphonate group. The mode of action of bisphophonates is shortening the lifespan of mature osteoclasts so they can prevent the bone fractures. Bisphosphonates have several side effects such as heartburn, abdominal pain, pain in muscles and joints, difficulty in swallowing.

Calcitonin

Calcitonin is produced by thyroid C cell. It can inhibit bone resorption by binding to the calcitonin receptor on osteoclasts. They increase the bone mineral density but it is expensive and its side effects are nausea and diarrhea (Eastell, 1998). Moreover, the patients may become resistant to calcitonin after a long term use because of down-regulation of calcitonin receptors on osteoclasts.

Estrogen-replacement therapy

Estrogen can prevent bone loss and relieve menopausal symptoms in postmenopausal women. There are, however, many side effects associated with this therapy. For example, it can increase the risk of breast carcinoma and endometrial carcinoma or the return of menstrual bleeding (Eastell, 1998).

2.2 Osteoclastogenesis

Osteoclasts are large multinucleated cells which are differentiated from monocyte/macrophage progenitors (Wilson et al., 2009). The formation of mature osteoclast occurs by the binding of macrophage colony-stimulating factor (M-CSF) to c-fms receptor on osteoclast precursor and RANK receptors is engaged by its ligand, receptor activator of NF-κB ligand (RANKL) which is produced by osteoblast in the membrane-bond form and the soluble form. Both cytokines play a crucial role in osteoclast differentiation and activation. M-CSF is essential for the proliferation and survival of osteoclast precursors and RANKL regulates the formation of mature osteoclast (Lorenzo et al., 2008). Osteoprotegerin (OPG) which is produced by osteoblasts blocks osteoclast differentiation by binding to RANKL. OPG negatively regulates the binding of RANKL to RANK receptor to balance the bone formation and resorption processes *in vivo* (Shinohara and Takayanagi, 2007) (Figure 2.2).

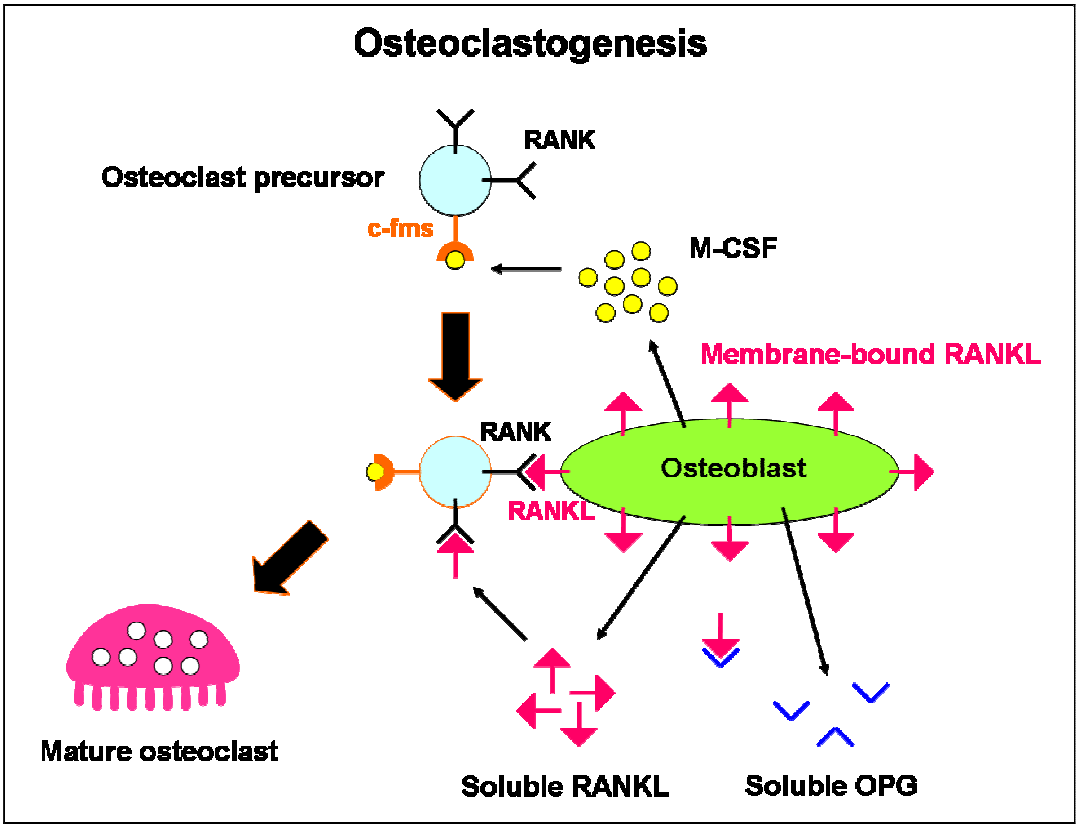


Figure 2.2 Molecules in osteoclastogenesis. Osteoclast precursor is induced by M-CSF and RANKL via binding to their specific receptors, c-fms and RANK receptor, respectively. These cytokines and OPG are expressed by osteoblasts. RANKL induces the differentiation and activation of mature osteoclasts while OPG suppresses this process. (Lorenzo et al., 2008)

2.3 Signaling pathways in osteoclast differentiation

Because millions of people worldwide are at risk or affected by osteoporosis and other bone diseases, the understanding of the molecular mechanisms in osteoclast differentiation is important for developing novel drugs for clinical treatment. Several factors and signaling pathways that are involved in osteoclast differentiation and functions have been reported and they are reviewed below (Figure 2.3).

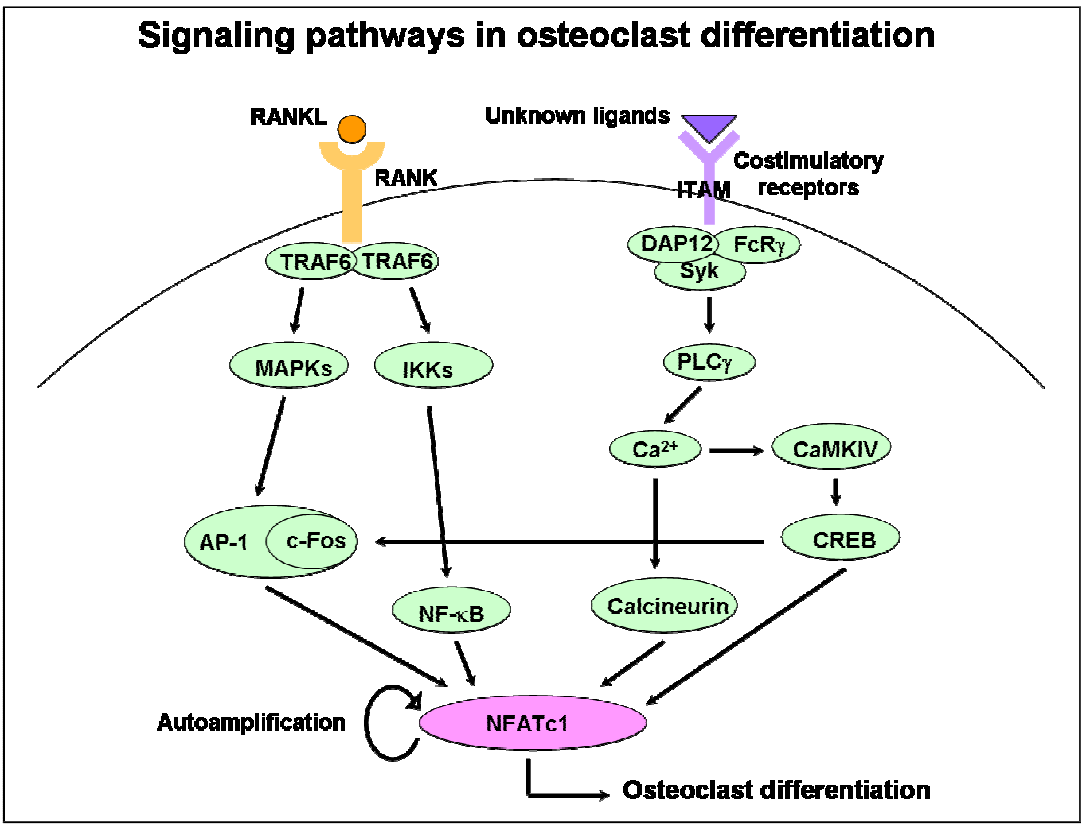


Figure 2.3 Signaling pathways in osteoclast differentiation. The signaling pathways are activated by RANKL and ITAM. Stimulation by RANKL results in the activation of MAPK and NF-ΚB pathway which induce NFATc1 expression via TRAF6. The phosphorylation of ITAM upon stimulation by an unknown ligand results in the activation of calcium signaling which activates CaMK/CREB pathway and calcineurin pathway. These pathways are required for the optimal induction of NFATc1 expression and its autoamplification (Shinohara and Takayanagi, 2007).

TRAF (TNF receptor-associated factor) 6

TRAF6 is an adaptor protein that mediates signaling pathways of several cytokine receptors such as Toll/IL-1R family and TNF receptor superfamily. TRAF6 is a major adaptor molecule associated with RANK receptor (Tanaka et al., 2003). It is essential for the activation of MAPK and NF-ΚB pathways which is involved in osteoclast differentiation and their functions.

MAPKs (Mitogen activated protein kinase)

MAPK family members consist of p38-MAPK (p38α, β, γ and δ), c-Jun N-terminal kinase (JNK1, 2 and 3), extracellular signal-regulated kinase (ERK1 and 2) (Wada et al., 2006) (Figure 2.4). The phosphorylated forms of these MAPKs which are activated form play a crucial role in osteoclastogenesis. Previous studies reported that p38 is involved in the induction of *Cathepsin K* mRNA expression (Matsumoto et al., 2004). Moreover, inhibition of ERK by MEK inhibitor did not affect the differentiation of osteoclast but ERK is essential for the survival of osteoclasts (Asagiri and Takayanagi, 2007). On the other hand, the induction of RANKL, IL-1, TNFα and LPS induced the phosphorylation of p38-MAPK in osteoclast precursors but not in osteoclasts. Taken together, these data suggested that p38-MAPK is essential for osteoclast differentiation but not for osteoclast functions (Li et al., 2002).

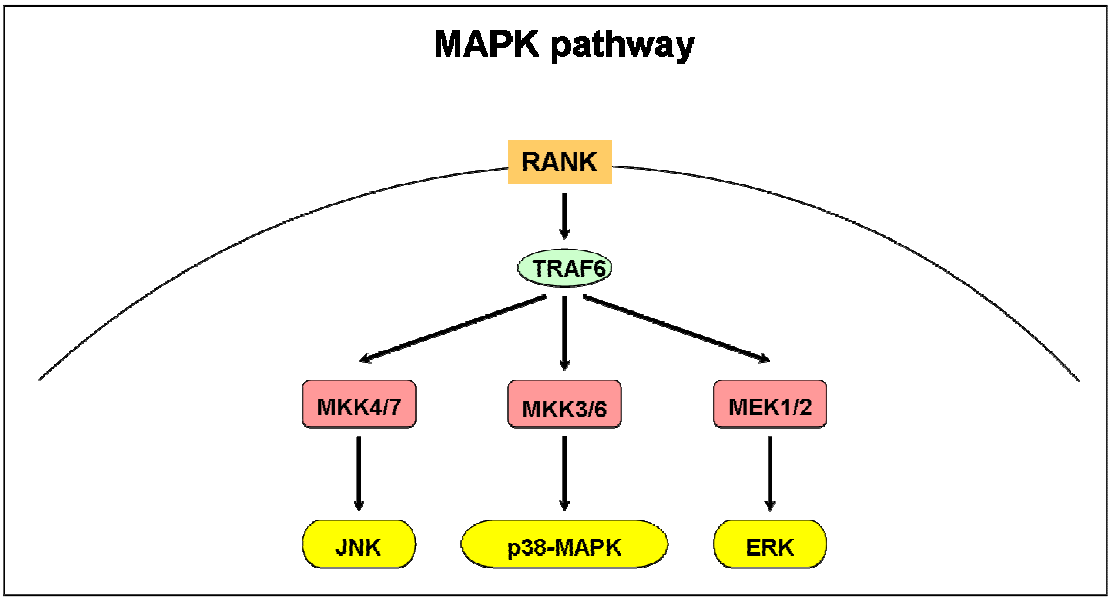


Figure 2.4 Activation of MAPK pathway. MAPK pathway is activated via TRAF6. MKK4/7, MKK3/6 and MEK1/2 are downstream targets of TRAF6 and can be activated by phosphorylation. JNK, p38-MAPK and ERK are phosphorylated by MKK4/7, MKK3/6 and MEK1/2, respectively, resulting in the induction of transcription of specific target genes (Wada et al., 2006).

c-Fos and AP-1 proteins

Activator protein-1 (AP-1) is a dimeric complex consisting of the Fos (c-Fos, Fos-B, Fra-1 and Fra-2), Jun (c-Jun, JunB and JunD) and ATF (ATFa, ATF2, ATF3, ATF4 and B-ATF) proteins (Asagiri and Takayanagi, 2007). C-Fos forms heterodimer with AP1 which play an important role in the osteoclast differentiation. Osteoclast formation cannot occur in the absence of these transcription factors (Boyce et al., 2005). Previous studies reported that Fra-1 was controlled by JNK because the JNK inhibitor also inhibited the expression of Fra-1 (Chang et al., 2009). These data suggested that the activation of AP-1 components is regulated by MAPK pathway

NF-κB (nuclear factor kappa B)

The family of NF-κB transcription factor consists of p65/RelA, p50, RelB, p52, IκBα, IκBβ, IκBε, NF-κB1/p105 and NF-κB2/p100. The activation of NF-κB is essential for immune responses, inflammation and bone formation (Abu-Amer et al., 2008). There are two major signaling transduction pathway of this protein family, the classical pathway (canonical pathway) and the alternative pathway (non-canonical pathway) (Figure 2.5). Both pathways are activated via RANK/RANKL and is mediated by TRAF1, 2, 3, 5 and 6 in osteoclastogenesis (Sun, 2011).

Furthermore, previous study reported that the inhibition of IKK/NF-κB in osteoblasts prevented bone loss by maintaining bone formation in mice with ovariectomy (OVX). And they found the expression of Fra-1, the essential transcription factor for bone matrix formation, increased after the inhibition of IKK/NF-κB. These data suggested that IKK/NF-κB is the target for treatment of osteoporosis (Chang et al., 2009).

NF-κB signaling pathway is activated via the stimulation of TRAF6 which is associated with RANK receptor in osteoclasts. Previous studies reported that the subunits of IKK multisubunit enzyme, IKKγ (NEMO) and IKKβ, are essential for osteoclastogenesis in the NF-κB classical pathway both *in vitro* and *in vivo* but another

subunit, IKKα, is essential only *in vitro*. These results indicated that the NF-κB classical pathway is important in osteoclast differentiation and functions (Ruocco et al., 2005).

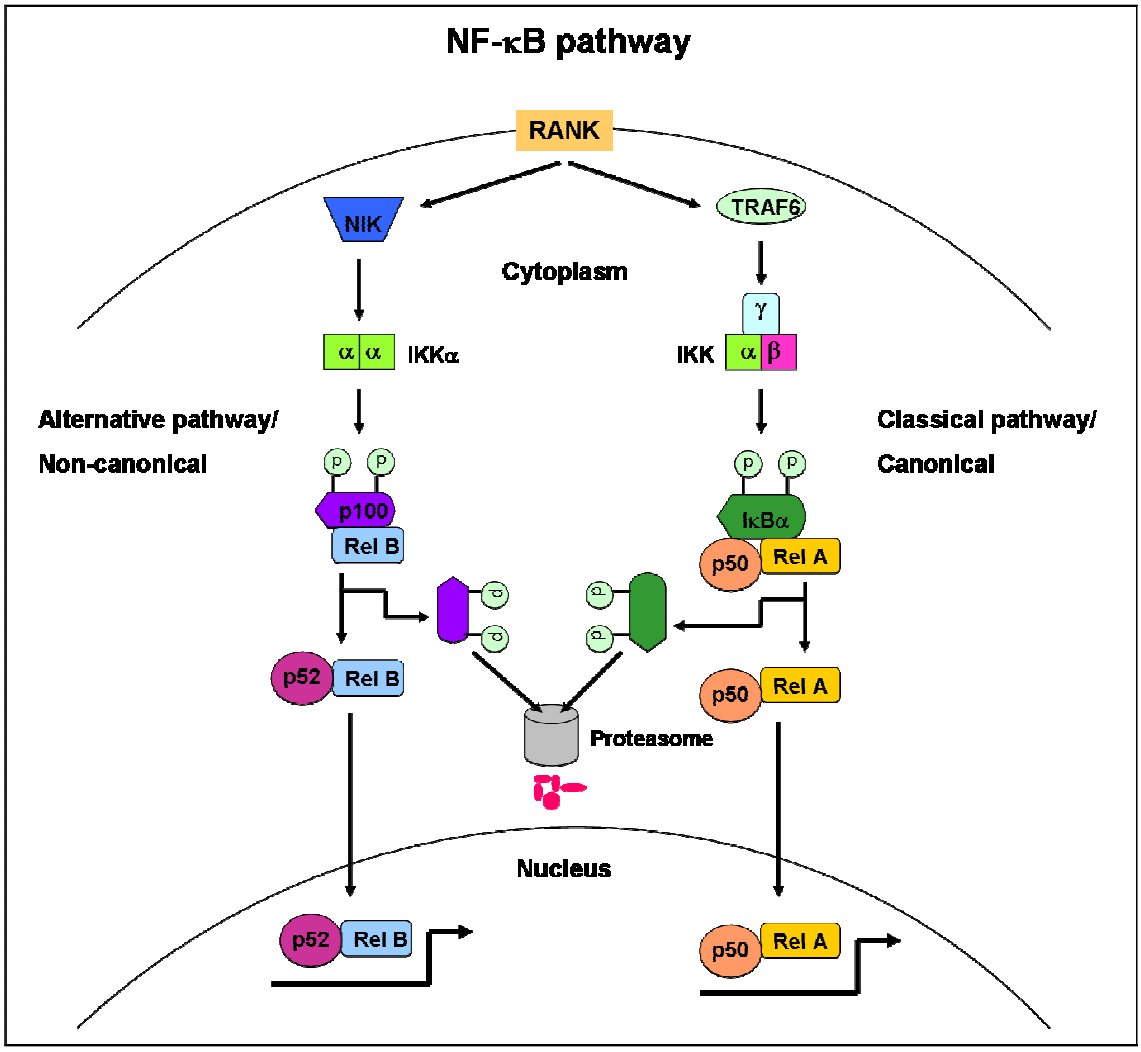


Figure 2.5 Activation of NF-κB pathway. In the classical pathway, NF-κB is activated via TRAF6. IKK complex which consists of IKKα, IKKβ and IKKγ (NEMO) subunits are activated which results in the phosphorylation of IκBα. The phosphorylated form of IκBα is targeted for degradation by the proteasomes which generates the complex of p50-Rel A (p65) in an active form and these complex translocate to the nucleus (Soysa and Alles, 2009). Alternative pathway or non-canonical pathway is activated via NF-κB-inducing kinase (NIK). The homodimers of IKKα is activated resulting in the

phosphorylation of p100. The phosphorylated form of p100 is degraded by proteasomes which generates Rel-B in an active form. Rel-B forms a complex with p52 and translocate to the nucleus.

NFAT (Nuclear factor of activated T cell) c1

NFAT is a transcription factor which was originally discovered in T cells. NFAT families consist of five members including NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), NFATc4 (NFAT3), and NFAT5. NFATc1 is the master transcription factor in osteoclastogenesis. Previous studies reported that NFATc1-deficient mice exhibit severe osteopetrosis, a condition of excessive bone formation. These data suggested that NFATc1 play a critical role in the differentiation and functions of osteoclasts (Takayanagi, 2007). Furthermore, it has been reported that calcium signaling is essential for the autoamplification of NFATc1 during osteoclast formation. The activation of calcium signaling and NFATc1 leads to autoregulates *NFATc1* promoter during osteoclastogenesis (Asagiri et al., 2005).

There are several target genes of NFATc1 which are involved in osteoclast functions including Cathepsin K (degradation of bone matrix), TRAP (tartrat-resistant acid phosphatase production), β3 integin (adherence of osteoclast to bone matrix), dendritic cell-specific transmembrane protein or DC-STAMP (cell-cell fusion of osteoclast precursors), osteoclast-associated receptor or OSCAR (cell-cell interaction between osteoclast and osteoblast) and calcitonin receptor. These osteoclast-related genes are directly regulate by NFATc1 (Negishi-Koga and Takayanagi, 2009).

Cathepsin K (CTSK) is a member of cysteine protease family which is expressed in mature osteoclast. Cathepsin K cleaves the helical and telopeptide of collagen Type I which is found more than 97 percent in bone matrix (Figure 2.6). The action of cathepsin K is important in the degradation of bone matrix during bone resorption (Zaidi et al., 2001). Metrix metalloproteinases (MMPs), another protease which is synthesized by mature osteoclasts consist of MMP-9, MMP-10, MMP-12 and MMP-14. MMP-9 and

MMP-14, they are the key enzymes involved in the migration of osteoclasts through collagen (Delaisse et al., 2003).

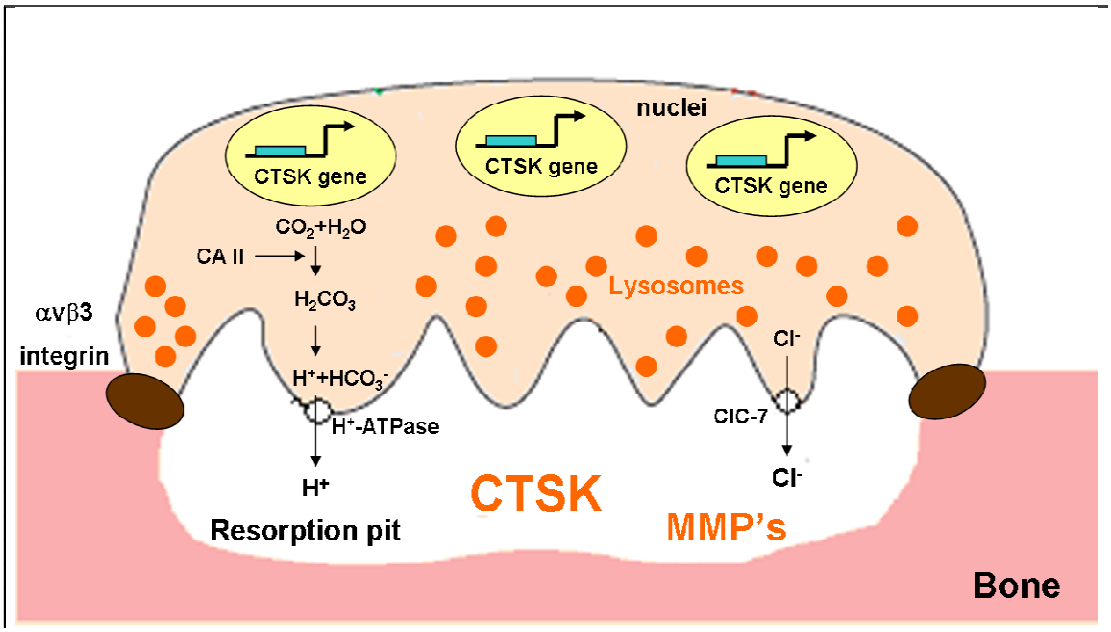


Figure 2.6 Regulations of Cathepsin K and its role during bone resorption by osteoclast. The osteoclast adheres to bone by the binding of αvβ3 integrin to bone matrix. The H^+-ATPase proton pump transports H^+ ions to the lacuna causing acidic condition. The electrical balance in the cell is maintained by Cl^- ions which are released by chloride channel (CIC-7). Cathepsin K (CTSK) and matrix metalloproteinases (MMPs) are transported from endoplasmic reticulum in lysosomes into the resorption pit (Troen, 2006).

Interferon regulatory factor-8 (IRF8) is a member of IRF familes. IRF8 was reported to be expressed in B lymphocytes and activated T lymphocytes, monocytes and macrophages (Zhao et al., 2009). Previous studies reported that IRF8 was a negative regulator of osteoclastogenesis because the levels of IRF8 were reduced during the initial step of osteoclastogenesis stimulated by RANKL. The activation of NF-κB and AP-1 occurr in osteoclast precursors which are induced by RANKL, resulting in the positive regulation of *NFATc1* promoter. At the same time, stimulation with RANKL

downregulates the levels of IRF8 results in releasing NFATc1 to autoamplify its own expression (Zhao et al., 2009) (Figure 2.7).

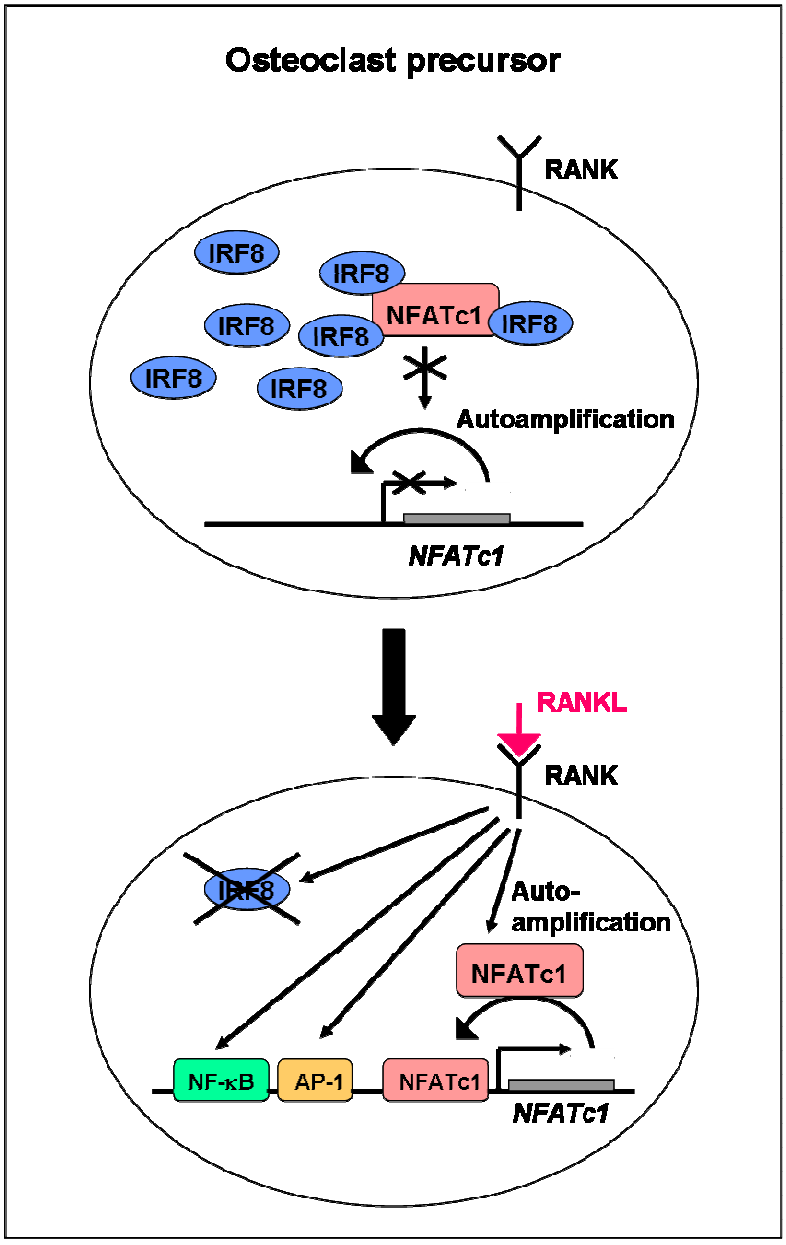


Figure 2.7 The negative roles of IRF8 in osteoclastogenesis. IRF8 is present in abundance in osteoclast precursors. In the absence of RANKL, IRF8 suppresses the activity of NFATc1 by binding to NFATc1 causing the transcription of the target genes to

be suppressed. In the presence of RANKL, the levels of IRF8 are reduced and NFATc1 can be freed to stimulate its own promoter (Zhao et al., 2009).

2.4 7-oxo-deacetoxygedunin (7-OG)

Limonoids are triterpene derivatives found only in plant of family Meliaceae such as mahogony and family Rutaceae such as citrus fruits (Manners, 2007). Limonoids are derived from limonin and first characterized in 1949 as the bitterness agents from navel orange juice. The chemical composition of limonin is $C_{26}H_{30}O_8$ (Figure 2.8) and its molecular weight is 470 (Roy and Saraf, 2006). Limonoids are moderately polar, insoluble in water but soluble in ketones, alcohols and hydrocarbons (Pudhom et al., 2010). Previous studies reported the bioactivities of limonoid compounds such as anti-colon cancer activity of limonoids from *Xylocarpus granatum* (Uddin et al., 2007), anti-malarial activity of limonoids from *Khaya grandifoliola* of the combination between these limonoids and chloroquine exhibits additive effect for anti-malarial activity (Kayser et al., 2003). The anti-HIV activity of limonoids from *Clausena excavate* was also reported (Sunthitikawinsakul et al., 2003).

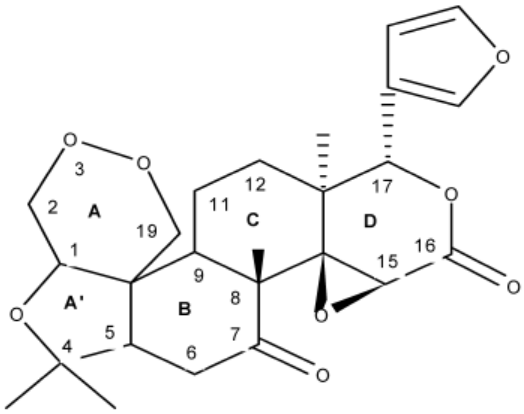


Figure 2.8 The structure of limonin from citrus (Roy and Saraf, 2006)

7-oxo-deacetoxygedunin (7-OG) is a limonoid compound from seeds of *Xylocarpus moluccensis* or cedar mangrove which collected from Phuket, in southern Thailand. It has an inhibitory effect against nitric oxide production in LPS-stimulated

macrophages (Ravangpai et al., 2011). Moreover, 7-OG obtained from *Gaurea grandiflora* (Family Meliaceae) inhibits ATP synthesis and phospholyrating electron flows in thylakoids of spinach (Achnine et al., 1999)

CHAPTER III

MATERIALS AND METHODS

3.1 Cell line and media

A macrophage like cell line, RAW264.7 (ATCC TIB-71) was used in this study. RAW264.7 was cultured in DMEM (Hyclone, UK) and supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, UK), 1% 10 mM sodium pyruvate (Hyclone, UK), 1% HEPES free acid (Hyclone, UK), 100 U/ml penicillin G (General Drugs House, Thailand) and 0.4 mg/ml streptomycin (M & H Manufacturing, Thailand) and incubated at 37 °C in a humidified atmosphere of 5% in the CO_2 incubator (Thermo Electron Corporation, USA).

3.1.1 Cell preservations

RAW264.7 was collected from culture dishes and centrifuged at 1000 rpm for 5 min. Cell pellets were resuspended in 1 ml of freezing media (10%(v/v) DMSO (Sigma Aldrich, USA) in DMEM complete media) and transferred to cryogenic vialx (Corning Incorporation, USA). Cells were immediately stored at -80 °C and moved to store in Liquid Nitrogen Tank model 34 HC Taylor Wharton Cryogenic (Harsco Corporation, USA) after at least 24 hr at -80°C for long term storage.

3.2 Cell preparation

After removing culture media, RAW264.7 was collected from culture dishes (Hycon, Germany) by using cold PBS and centrifuged at 1000 rpm for 5 min (Hettich, Germany). The supernatant was discarded and the cell pellets were resuspended in DMEM complete media. Viable cells were diluted in trypan blue dye and counted by using a hemacytometer. The cell numbers were calculated by using the following formula:

Cell number (cells/ml) = number of counted cells in 16-large square x dilution factor x 10^4

3.3 MTT assay

RAW264.7 was cultured at density of 1 x 10^4 cells/well in 96-well plates (Corning Incorporation, USA) and treated with 7-OG dissolved in DMSO (a kind gift from Dr. Khanitha Pudhom, Department of Chemistry, Faculty of Science, Chulalongkorn University) at various concentrations for 24 hrs. Ten microliter of 5 mg/ml MTT solution was added at 20 hr after treatment and cells were incubated further for 4 hrs at 37° C in a humidified atmosphere with 5% CO_2. At the end of treatment, 0.04 N HCl in isopropanol (200 µl) was added to dissolve the purple formazan and the absorbance was measured at 540 nm by microplate reader (Biochrom Anthos 2010, Cambridge, UK). DMSO was used as a vehicle control. Percent of cell viability was calculated by using the following formula:

% cell viability = (Abs test – Abs blank) x 100 / Abs control – Abs blank

3.4 TRAP activity assay and TRAP staining

RAW264.7 (1x10^4 cells/well, in total volume of 500 µl or 5x10^3 cells/well in total volume of 250 µl) was cultured with 100 ng/ml rRANKL (PeproTech) in the presence of 7-OG and using DMSO as the vehicle control in 24 or 48-well plate for 5 days at 37°C and 5% CO_2. During this period, 100 ng/ml of rRANKL, 7-OG or vehicle control DMSO and fresh media were changed every 2 days. After 5 days in culture, cells were washed with PBS and fixed with 10% formaldehyde for 10 min. After fixation, cells were treated with 95% ethanol for 1 min and washed with PBS. To measure TRAP activity, 500 µl of 50 mM citrate buffer (pH4.6) containing 10 mM sodium tartrate and 10 mM *p*-nitrophenylphosphate (Sigma Aldrich, USA) was added to the fixed cells and incubated for 30 min. The solutions were transferred to microcentrifuge tubes (Axygen Scientific,

USA) containing 500 µl of 0.1 N NaOH. The absorbance was measured at 410 nm by microplate reader.

For TRAP staining, cells were fixed and permeabilized as described above. After washing with PBS, 500 µl of 50 mM acetate buffer (pH 5.0) containing 50 mM sodium tartrate and 0.1 mg/ml naphthol AS-MX phos phate and 0.6 mg/ml Fast red violet LB salt (Sigma Aldrich, USA) was added to the fixed cells and incubated for 30 min. Cells were washed with PBS and the osteoclasts (TRAP-positive multinucleated cells (3 or more nuclei) were counted under a light microscope.

3.5 RNA extraction

RAW264.7 ($8x10^4$ cells/well in total volume 500 µl) was plated in 24-well plate overnight to allow for adhesion. Cells were treated with 100 ng/ml rRANKL in the presence or the absence of 7-OG at indicated concentration for 0, 6, 12, 24, 48 and 72 hrs. DMSO was used as vehicle control. During this period, rRANKL, 7-OG or DMSO and fresh media were changed every 2 days. Total RNA was isolated by using TriZol reagent (1 ml/well) (Invitrogen, UK) and transferred to microcentrifuge tubes. The samples were extracted with 200 µl of chloroform (Lab-Scan, Ireland) and vigorously mixed for 15 sec. After incubation for 2-3 min, the samples were centrifuged at 12000x*g* for 15 min at 4 °C using refrigerated centrifuge (model 1920 (Kubota, Japan)). The aqueous phase was carefully collected and gently mixed with 400 µl of isopropanol (Merck, Germany). The samples were incubated for 10 min at room temperature to precipitate RNA and centrifuged at 12000x*g* for 10 min at 4 °C. The supernatants were removed and RNA pellets were washed with 1 ml of cold 75% ethanol. After mixing by Vortex mixer (model G560E (Scientific Industries, USA)), the samples were centrifuged at 7500x*g* for 5 min at 4 °C. The resulting RNA pellets were air dried for 10-15 min. RNA pellets were resuspended in 10-15 µl of 0.01% diethylpyrocarbonate (DEPC) water and incubated for 10 min at 55-60 °C. RNA samples were stored at -80 °C until use.

3.5.1 Quantitation of RNA using Quan iT Assays

Total RNA was measured by using Quan-iT (Invitrogen, UK) according to the manufacturer's instruction. Quan-iT reagent and buffer were prepared as working solution. To prepared standard RNA, 10 µl of 0 ng/ml and 10 ng/ml RNA were mixed with 190 µl of working solution. RNA samples were diluted 1:10 in Hypure® water PCR grade (Hyclone, England). One hundred ninety eight µl of working solution was mixed with 2 µl of diluted RNA sample. The standard RNA were used to calibrate and measured RNA concentrations. The concentrations of RNA were calculated by using the following formula:

RNA (µg/ml) = measured concentration x dilution factor

3.6 cDNA synthesis by reverse transcription

Total RNA (0.2 µg) was used to synthesized cDNA. Total RNA was mixed with 0.2 µg of random hexamer (Qiagen, Germany) and adjusted the volume to 12.5 µl by DEPC water. The mixtures were heated at 65 °C for 5 min. The PCR master mix was prepared by mixing 1x Reverse transcriptase buffer (Fermentas, Canada), 1 mM dNTP mix (Fermentas, Canada) and 20 U of RNase inhibitor (Fermentas, Canada). After heating the RNA mixtures, the master mix was added to the mixtures and finally 200 U of reverse transcriptase per reaction was added to the mixtures. The reactions were performed by Bioer Life Express (Bioer Technology, China) at 25 °C for 10 min, 42°C for 60 min and 70 °C for 10 min. The cDNA was stored at -20°C until use.

3.7 Quantitative polymerase chain reaction (qPCR)

qPCR was performed by using Maxima™ SYBR Green qPCR Master Mix (Fermentas). Two µl of cDNA, 0.3 µM of forward and reverse primer and nuclease free water were prepared. The specific primers used to amplify each target genes were as follows (Table 3.1). The reactions were performed in MJ Mini personal Thermal cycler (BioRad, USA) by conditions as follows; 95°C for 10 min, 95°C for 5 min, annealing

temperature of each gene indicated in Table 3.1 for 30 sec and 72°C for 1 min and 72°C for 10 min, 40 cycles. The relative expression of mRNA levels were calculated and analyzed by $2^{-\Delta\Delta_{CT}}$.

Gene	Primer sequences (5′→3′)	Annealing temp. (°C)	Product size (bp)	Ref.
NFATc1	Forward: GGTAACTCTGTCTTTCTAA CCTTAAGCTC Reverse: GTGATGACCCCAGCATGC ACCAGTCACAG	62	240	(Nishikawa et al., 2010)
Cathepsin K	Forward: GGCCAACTCAAGAAGAAA Reverse: GTACCCTCTGCATTTAGC	58	225	(Yasuda et al., 2004)
IRF8	Forward: GGAAAGCCTTACCTGCTG AC Reverse: AAGGTCACCGTGGTCCTT	55	112	(Nishikawa et al., 2010)
β-actin	Forward: ACCAACTGGGACGACATG GAGAA Reverse: GTGGTGGTGAAGCTGTAG CC	55	380	(Kuroda et al., 2002)

Table 3.1 Primer sequences and conditions used in qPCR for osteoclast-related genes.

3.8 Western blot

3.8.1 Protein extraction and quantitation

RAW264.7 ($8x10^4$ cells/well, in total volume 500 µl) was plated in 24-well plate overnight to allow for adhesion. Cells were pretreated with 9 µM 7-OG or vehicle control DMSO for 30 min and stimulated with 100 ng/ml rRANKL for 0, 15, 30 and 60 min. After removing culture supernatant, cells were washed with 1 ml of cold PBS and added with 35 µl of cold RIPA buffer composed of 1% (v/v) phosphatase inhibitor (Sigma Aldrich, USA). After mixing a few times by micropipetted (Gilson, France) for cell lysis, cell lysates were collected and centrifuged at 5000 rpm for 5 min at room temperature. The supernatants were kept at -80 °C.

Protein concentrations were measured by using BCA (bicinchoninic acid) ™ protein assay (PIERCE, USA) according to the manufacturer's instruction. Reagent A and reagent B were prepared by mixing at ratio 50:1 as the working reagent. 1 mg/ml Bovine Serum Albumin (BSA) was used as protein standard. BSA was prepared in 96-well plate at concentrations 0, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml by diluting in sterile deionized water. The samples were diluted at 1:10 in sterile deionized water and added with 200 µl of working reagent. After incubation at 37 °C, the absorbance was measured at 540 nm by microplate reader and calculated the protein concentrations.

3.8.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Twenty micrograms of proteins were mixed with 2x Laemmli buffer by an equal volume of cell lysates. The protein samples were heated at 100 °C for 5 min on Thermomixer Compact (Microcentrifuge tubes, Germany). The samples and molecular weight marker (BioRad, USA) were loaded on 10% separating gel and electrophoresis was carried out at 100 volts for 105 min using Protein III system (BioRad, USA).

The stacking gel was removed after separation and the separating gel was equilibrared in the transfer buffer (Appendix). Polyvinylidene fluoride (PVDF) membrane (GE Healthcare, USA) was prepared by soaking in an absolute methanol (Merck,

Germany) and washed twice in deionized water. PVDF membrane and 6 pieces of Whatman filter paper were immersed in transfer buffer. PVDF membrane, filter paper and gel were placed on a semi-dry transfer Trans-Blot® SD (BioRad, USA). The protein samples were transferred at 90 mA for 90 min.

3.8.3 Antibody probing

The PVDF membrane was blocked twice with 3% non-fat dried milk in PBS and 0.05% Tween-20 (blocking solution) for 5 min at room temperature on Labnet Rocker 25 (Labnet International Inc, USA). The membrane was probed with primary antibody preparing in blocking solution with the working dilution as follows: mouse anti-IκBα at 1:2000 dilution, rabbit anti-phospho-p65 at 1:4000 dilution, rabbit anti-p65 at 1:4000 dilution, rabbit anti-phospho-p38 at 1:4000 dilution, rabbit anti-p38 at 1:4000 dilution, rabbit anti phospho-p44/42 at 1:2000 dilution, rabbit anti-p44/42 at 1:2000 dilution (all antibodies were from Cell Signaling Technology, MA, USA) and mouse anti-β-actin at 1:5000 dilution (Chemicon International, USA) at 4 °C overnight. The membrane was washed with PBST for 5 min twice and for 15 min twice after removing the primary antibody. The secondary antibody, donkey anti-rabbit IgG or sheep anti-mouse IgG conjugated with horse-raddish peroxidase (HRP) (Amersham Bioscience, UK) at 1:2000 or 1:4000 or 1:5000 dilutions were added and incubated for 1 hr at room temperature with rocking.

3.8.4 Signal detection by chemiluminescence

Solution A and solution B were prepared as chemiluminescent substrates. The membrane was immersed in the mixture of solution A and solution B and incubated for 1 min by rocking. After incubation, the membrane was wrapped with plastic and placed on Hypercassette (Amersham Bioscience, UK). The wrapped membrane was exposed to High Performance Chemiluminescence X-ray Film in the dark (Amersham Bioscience, UK). Exposured time of IκBα, p-p65, p65, p-p38, p38, p-p44/42, p44/42 and β-actin were 15 sec, 8 min, 5 min, 8 min, 5 min, 30 sec, 15 sec and 5 sec, respectively. The film

was immersed in developer solution (J-Nasen, Thailand) for 5-10 sec and washed with water. Subsequently, the film was immersed in fixer solution (J-Nasen, Thailand) for 1-2 min and washed with water.

3.9 Immunofluorescent staining

RAW264.7 cells were pretreated with 9 μM 7-OG or DMSO for 30 min and stimulated with 100 ng/ml rRANKL for 0, 15, 30 and 60 min. Cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton-X 100 for 2 min and blocked with 10% FBS in PBS for 10 min and 10% 2.4G2 (anti-Fcγ R) in 10% FBS for 10 min. After blocking, rabbit anti-p65 antibody (Cell Signaling Technology, USA) at 1:100 dilution in 1.5% FBS were added and incubated for 1 h. Cells were washed with PBS and added secondary antibody, anti-rabbit IgG (H + L, (Fab′)2 fragment) Alexa Fluor® 555 (Cell Signaling Technology, USA), was added and incubated for 1 h in the dark. The cells were visualized under fluorescent microscopy (Olympus, Japan).

3.10 Nitric oxide assay

RAW264.7 ($5x10^3$ cells/well in total volume 250 μl) were cultured with 100 ng/ml rRANKL in the presence of 7-OG at indicated concentration or DMSO as the vehicle control in 24 or 48-well plate for 5 days at 37°C and 5% CO_2. During this period, rRANKL, culture supernatants were collected and rRANKL, 7-OG, DMSO and fresh media were changed every 2 days. To determine nitric oxide production, 999 μl of DMEM complete media containing 1 μl of 0.1 M sodium nitrite was prepared as nitrite standard at concentrations 0, 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 μM in 96-well plate. Fifty μl of culture supernatants were added in each well. Subsequently, sulfanilamide was added 50 μl/well and incubated for 10 min at room temperature in the dark. *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED) was added 50 μl/well and incubated for 10 min at room temperature in the dark. The absorbance at 540 nm was measured by using a microplate reader (Anthos, UK).

3.11 Statistical analysis

The data were analyzed by an independent Student's t-test using SPSS software (SPSS15). p value < 0.05 was considered to be statistically significant.

CHAPTER IV

RESULTS

4.1 Screening of bioactive compounds for anti-osteoclastogenic activity

To screen bioactive compounds with anti-osteoclastogenic activity, ten bioactive compounds from *Kaempferia parviflora* and six limonoid compounds from *Xylocarpus moluccensis* (kind gifts from Dr. Pattara Swasdi and Dr. Kanitha Pudhom, Department of Chemistry, Faculty of Science, Chulalongkorn University) were first evaluated for the cytotoxicity by MTT assay. The bioactive compounds which maintained more than 80% of cell viability were further analyzed for the anti-osteoclastogenic activity. The results were summarized in Table 4.1.

Bioactive compounds	IC50 of cytotoxicity (μM)	Anti-osteoclastogenic activity (%inhibition)
Kaempferia parviflora		
KP1	22.88	No activity
KP2	57.19	No activity
KP3	28.93	No activity
KP4	6.30	No activity
KP5	204.69	No activity
KP6	48.36	No activity
KP7	61.62	No activity
KP8	128.38	No activity
KP9	71.29	No activity
KP10	12.86	No activity
Xylocarpus moluccensis		
XMPH1	-	No activity

XMPH2	-	No activity
XMPH3 (7-OG)	26.29	94.94
XMPH4	16.66	81.23
XMPH5	-	No activity
XMPH6	-	No activity

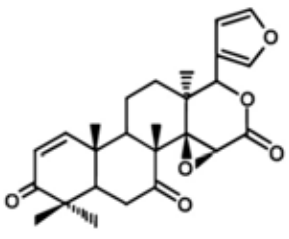
Table 4.1 IC50 of cytotoxicity and anti-osteoclastogenic activity of bioactive compounds from *Kaempferia parviflora* and limonoid compounds from *Xylocarpus moluccensis*

XMPH3 (7-OG) with low cytotoxicity and highest anti-osteoclastogenic activity was chosen for further investigation of its molecular mechanism.

4.2 Effect of 7-OG on osteoclast differentiation in RAW264.7 cell line

4.2.1 The cytotoxicity of 7-OG

To examine the cytotoxicity of 7-OG (Figure 4.1), treatment with various concentrations of 7-OG did not affect cell viability which were more than 80% of cell viability even at 10 μM (Figure 4.2). IC50 for cell viability of 7-OG was more than 22.80 μM. In this study, we used 7-OG at 9 μM for further experiments because it did not affect the cell viability of RAW264.7 cell line.



7-oxo-deacetoxygedunin

Figure 4.1 The structure of 7-oxo-deacetoxygedunin (7-OG)

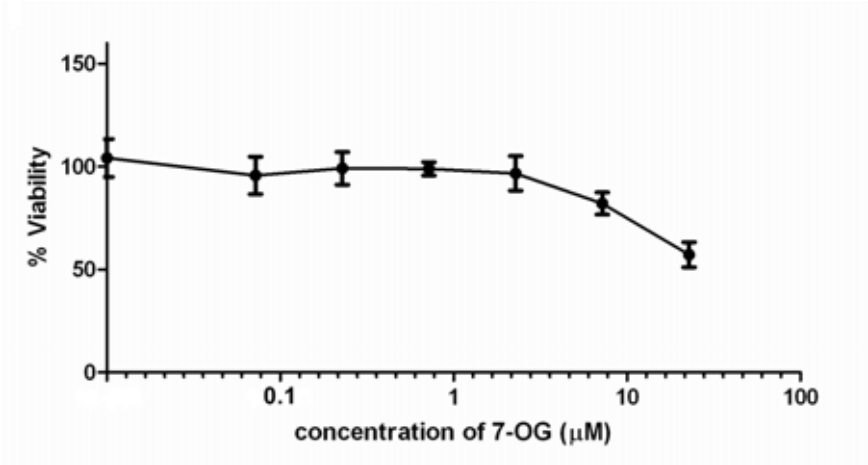


Figure 4.2 7-oxo-deacetoxygedunin (7-OG) and its cytotoxicity

RAW264.7 was treated with 7-OG at various concentrations for 24 hrs. Cell viability was evaluated by MTT assay. Data were shown as mean±SD.

4.2.2 Suppressing of TRAP activity and the appearance of TRAP positive multinucleated osteoclasts by treatment with 7-OG

To determine the anti-osteoclastogenic activity of 7-OG, TRAP staining was carried out after treatment of RAW264.7 cell line with 7-OG at various concentrations. As shown in Figure 4.3, the IC50 for anti-osteoclast activity determined by non-linear regression analysis was 2.50 μM. TRAP activity of RAW264.7 cell line treated with 7-OG at 9 μM significantly decreased compare to the DMSO control treatment (Figure 4.4) and inhibited the appearance of TRAP positive multinucleated osteoclasts compare to the DMSO control treatment (Figure 4.5). FK506 at 10 μM was used as positive control but it did not strongly inhibit the appearance of TRAP positive multinucleated osteoclasts. These results suggested that 7-OG effectively suppressed osteoclast differentiation with no toxicity at the effective dose.

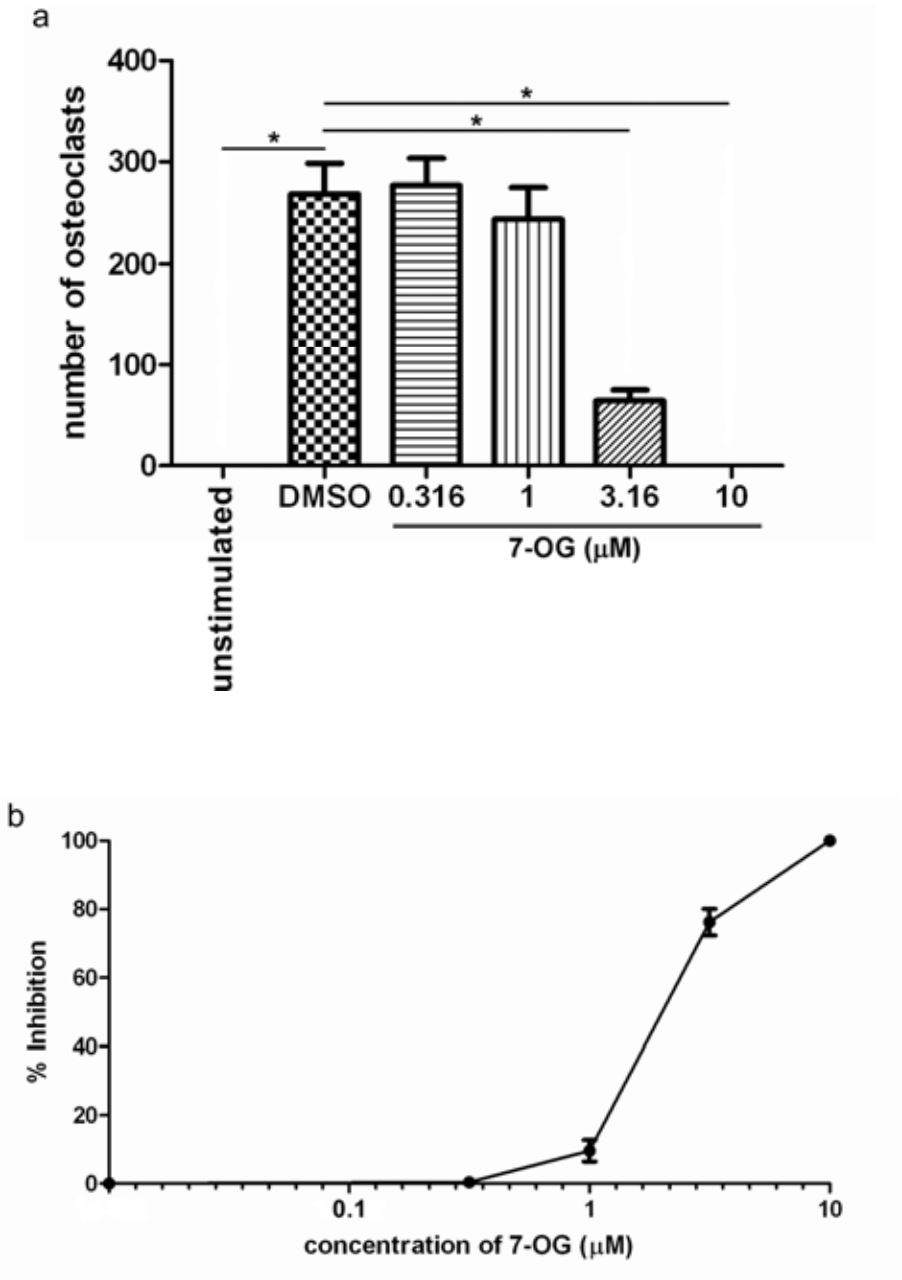


Figure 4.3 IC50 of anti-osteoclastogenic activity of 7-OG

RAW264.7 cells were cultured with 100 ng/ml rRANKL in the presence of 7-OG at various concentrations or vehicle control DMSO for 5 days. TRAP positive multinucleated cells were counted under the light microscope (a) and the percent of inhibition (b) was calculated. An IC50 of 7-OG was 4.14 μM. Data were shown as mean±SD and represented two independent experiments ($p < 0.05$), in triplicate.

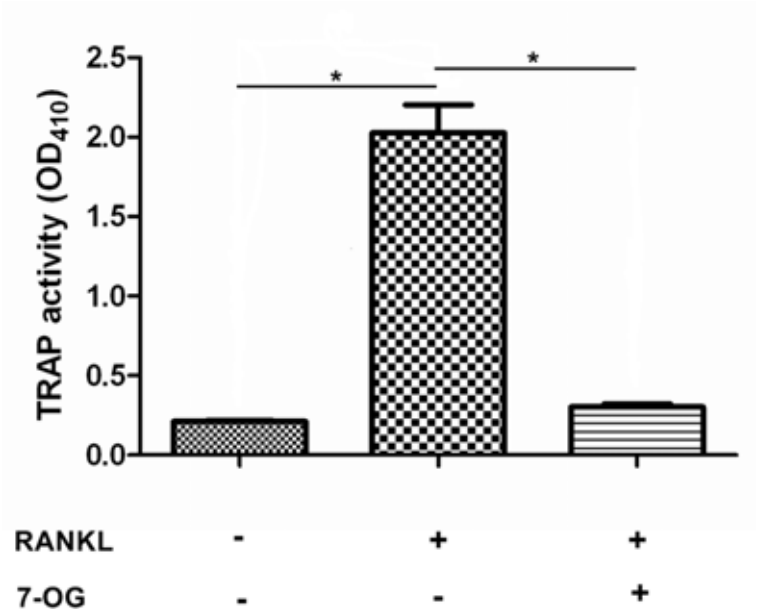


Figure 4.4 The effect of 7-OG on TRAP activity induced by RANKL

RAW264.7 was cultured with 100 ng/ml rRANKL in the presence of 7-OG (9 μM) or vehicle control DMSO for 5 days. After TRAP activity assay, the absorbance was measured at 410 nm by a microplate reader. Data were shown as mean±SD and represented two independent experiments ($p < 0.05$), in triplicate.

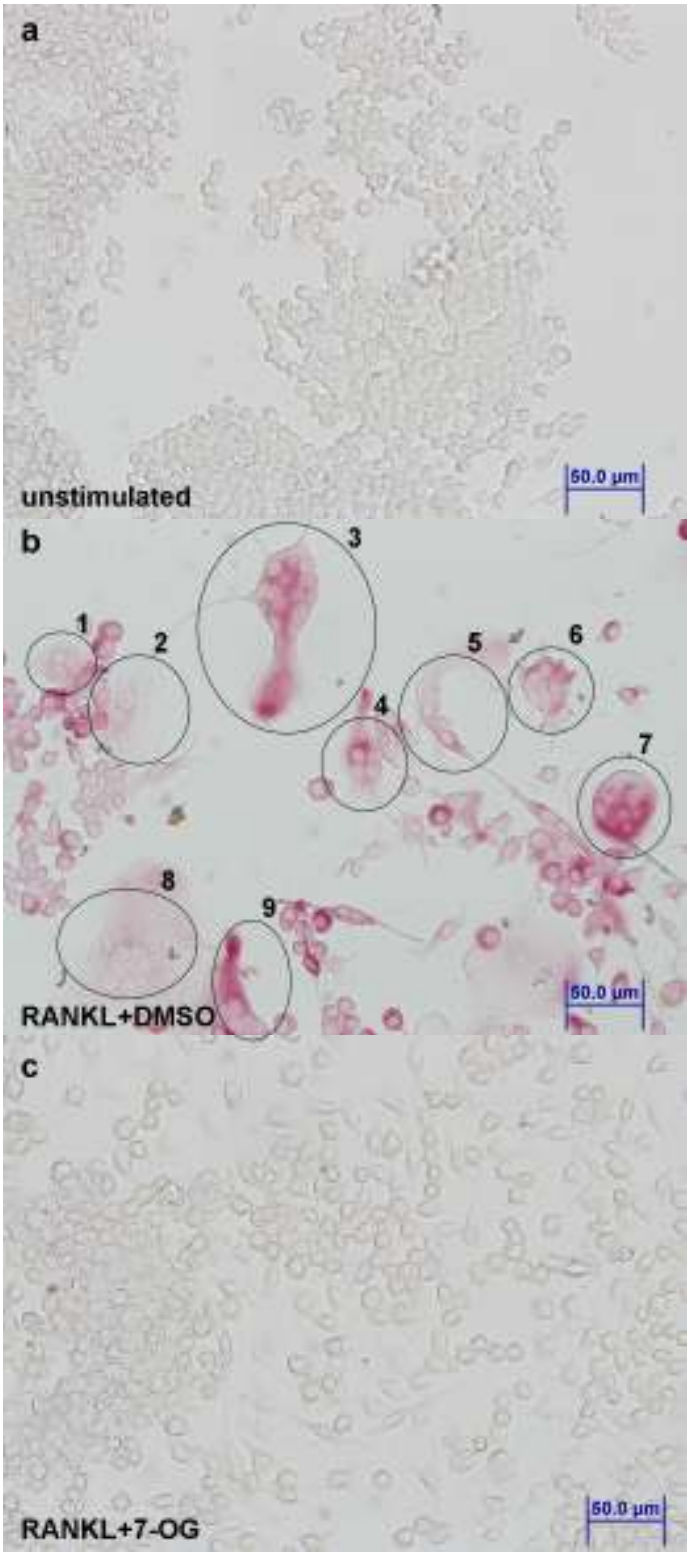
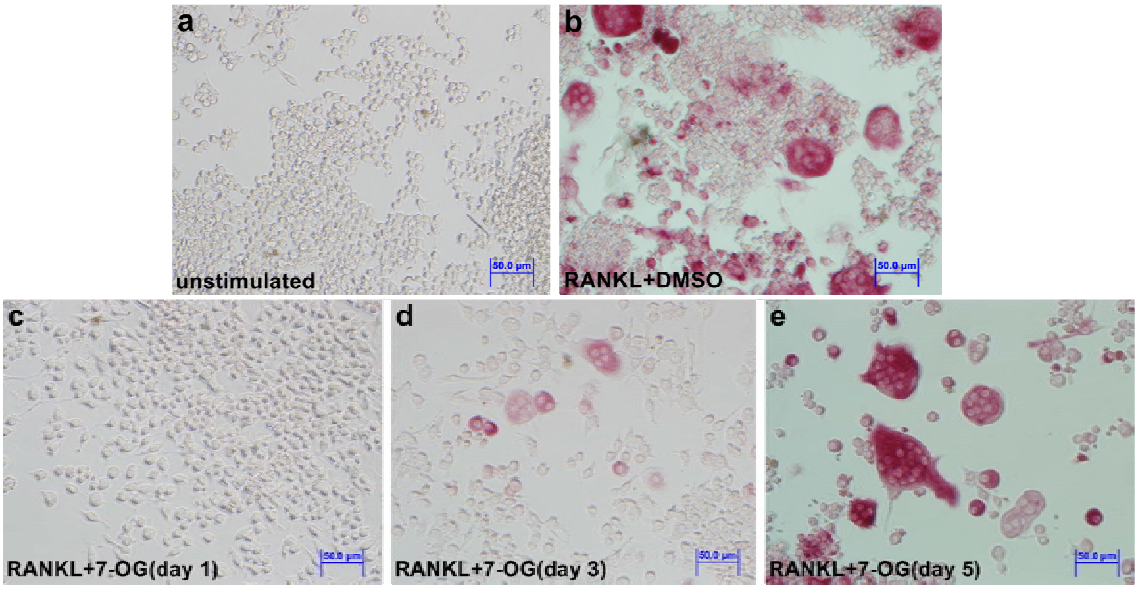


Figure 4.5 The effect of 7-OG on TRAP staining induced by RANKL in RAW264.7

RAW264.7 was cultured with 100 ng/ml rRANKL in the presence of 7-OG at 9 μM or vehicle control DMSO for 5 days. TRAP positive multinucleated cells were visualized under a light microscope (a-c).

4.2.3 Effects of 7-OG treatment at early step of osteoclast differentiation

To examine the steps that 7-OG suppresses osteoclast differentiation, treatment with 7-OG (9 μM) at the beginning of the culture significantly inhibited TRAP positive multinucleated osteoclasts (Figure 4.6 c) but 7-OG partially inhibited TRAP positive multinucleated osteoclasts when 7-OG was added on day 3 after culturing with rRANKL. There are multinucleated cells (approximately 30 cells/well compared to when 7-OG was added to the beginning of the culture) and they were not multi-nucleated cells compared to the control DMSO (Figure 4.6 d). Furthermore, 7-OG could not inhibit the formation of TRAP positive multinucleated osteoclasts when 7-OG was added at day 5 after culture in the presence of rRANKL. There are multinucleated cells approximately 300 cells/well, which was similar to the control DMSO treated cells (Figure 4.6 e). These results suggested that 7-OG suppressed osteoclast differentiation at early step and there was no effect on mature osteoclasts.



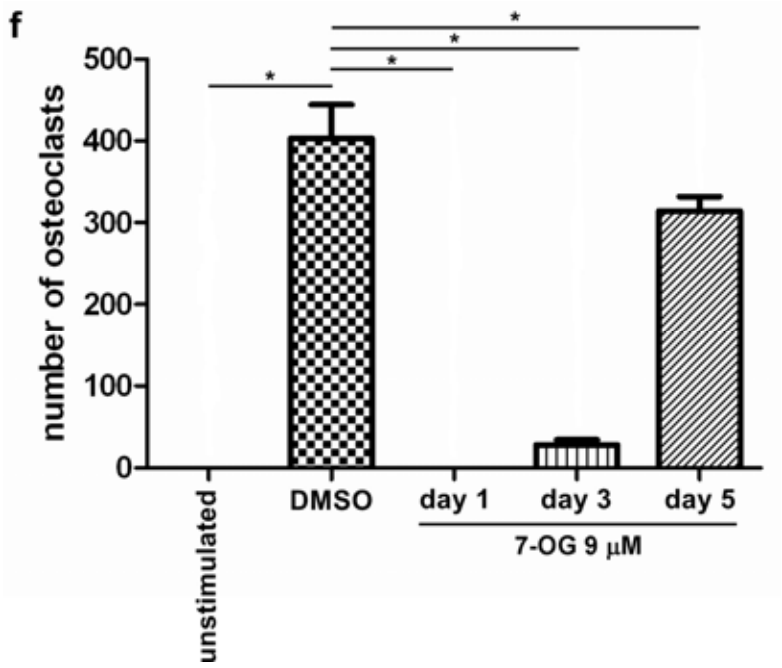


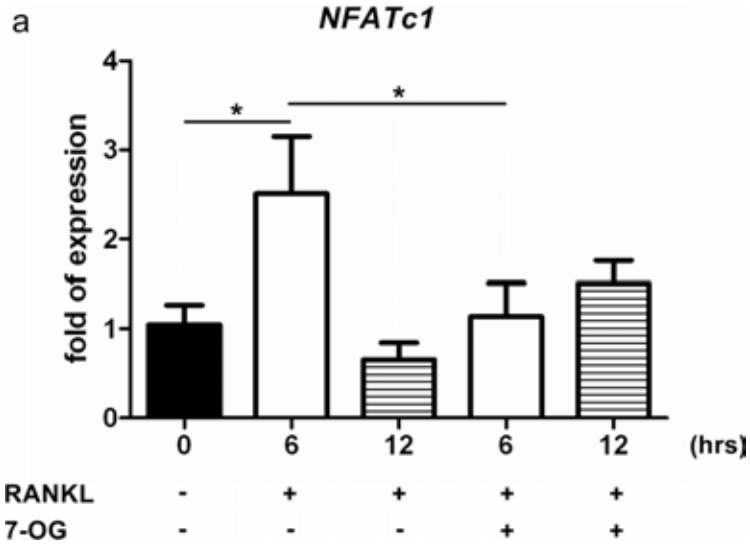
Figure 4.6 Effect of 7-OG on early step of osteoclast differentiation

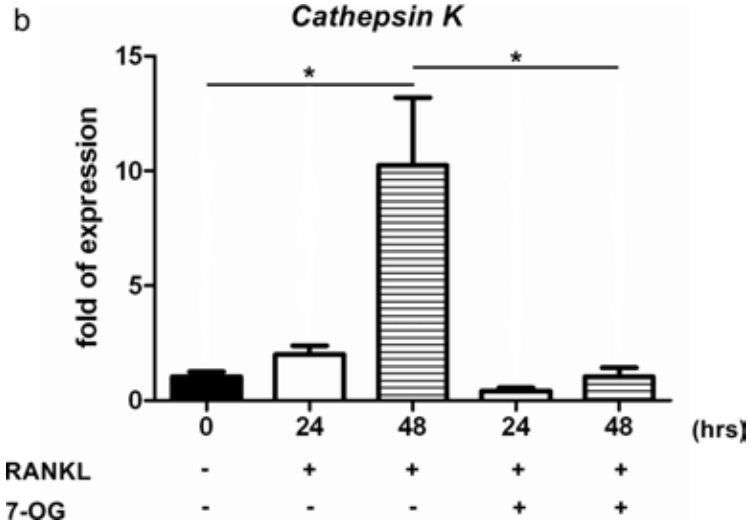
RAW264.7 was cultured with 100 ng/ml rRANKL. 7-OG was added to culture on day 1, day 3 and day 5. DMSO was used as a control. TRAP positive multinucleated cells were visualized under a light microscope (a-e), and the number of osteoclasts were counted (f). Data were shown as mean±SD and represented two independent experiments ($p < 0.05$), in triplicate.

4.3 Effect of 7-OG on osteoclast-related gene expression

To investigate the effect of 7-OG on osteoclast-related gene expression, the expression of *NFATc1*, *Cathepsin K* and *IRF8* were investigated by qPCR. Treatment with 7-OG at 9 μM effectively suppressed the expression of *NFATc1* at 6 hrs compared to control DMSO treatment (Figure 4.7a). The expression of *Cathepsin K*, the expression of which is regulated by NFATc1, was also suppressed by 7-OG treatment at 48 hrs (Figure 4.7b). The down regulation of *IRF8* mRNA, the negative regulator of *NFATc1*, was delayed at 6 hrs but the expression at 12 hrs was not different compare to the control (Figure 4.7c). The expression of *Cathepsin K* was not increased in RAW264.7 treated with 7-OG indicated that osteoclast formation did not occur because the

expression of *Cathepsin K* normally increases in mature osteoclasts. The downregulation of *IRF8* was delayed when RAW264.7 was treated with 7-OG, indicating that 7-OG indirectly suppresses the functions of NFATc1 by maintaining the level of IRF8.





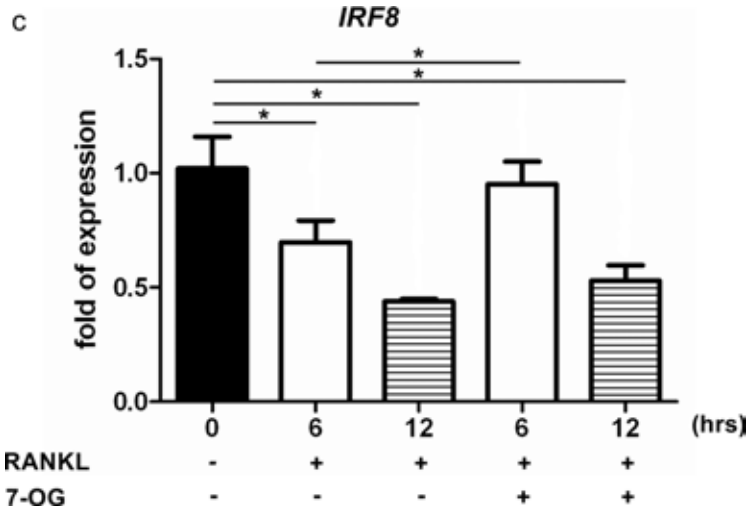


Figure 4.7 Effect of 7-OG on osteoclast-related gene expression

RAW264.7 was treated with 100 ng/ml rRANKL in the presence of 7-OG (9 μM) or vehicle control DMSO for indicated time. The expression of *NFATc1* (a), *Cathepsin K* (b) and *IRF8* (c) were determined by qPCR. Data were shown as mean±SD and represented two independent experiments ($p < 0.05$), in triplicate.

4.4 Effect of 7-OG on the activation of NF-κB and MAPK signaling pathways

To investigate the effect of 7-OG on the signaling pathways involved in the differentiation of osteoclasts, the activation of NF-κB and MAPK was evaluated. After stimulation with RANKL, the level of IκBα was not different in RAW264.7 treated with DMSO or 7-OG (Figure 4.8). The level of phophorylated form of one of the subunits of NF-κB, phospho-p65 decreased at 30 and 60 min after treatment with 7-OG (Figure 4.8). To confirm this result, the immunofluorescent staining of p65 was performed. The results showed that majority of p65 was translocated to the nucleus at 15 and 30 min after stimulation with RANKL and this translocation recovered at 60 min (Figure 4.9). In contrast, p65 was not found in the nuclei at any time points in RAW264.7 treated with 7-OG (Figure 4.9 e-h). Taken together, these results suggest that 7-OG interferes with activation of NF-κB pathway upon stimulation with RANKL.

For MAPK pathway, the activations of p38 and ERK were evaluated. The levels of both phospho-p38 and phospho-p44/42 increased at 15 min and 60 min upon RANKL

treatment. In contrast, both were decreased in RAW264.7 treated with 7-OG (Figure 4.8). These results suggest that 7-OG also suppresses the activation of MAPK signaling pathways.

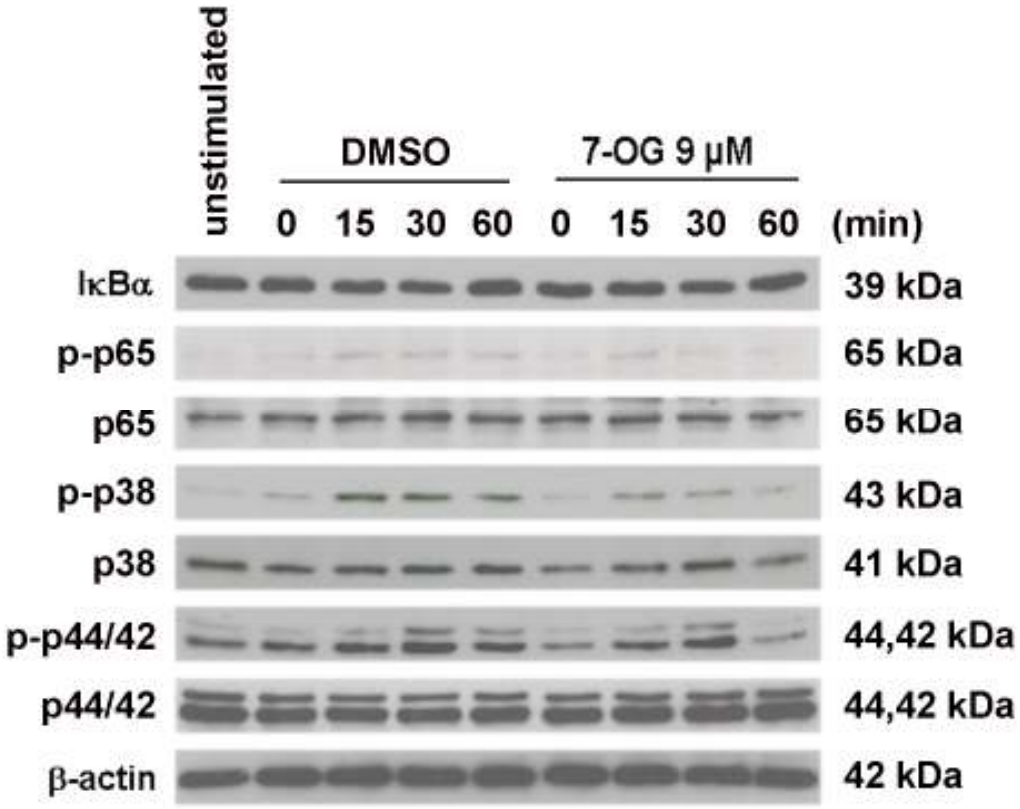


Figure 4.8 Effect of 7-OG on the activation of NF-**κ**B and MAPK signaling pathways RAW264.7 was pretreated with 7-OG (9 μM) or vehicle control DMSO for 30 min and stimulated with 100 ng/ml rRANKL for indicated time. Cell lysates were analyzed by Western blot and β-actin was used as loading control.

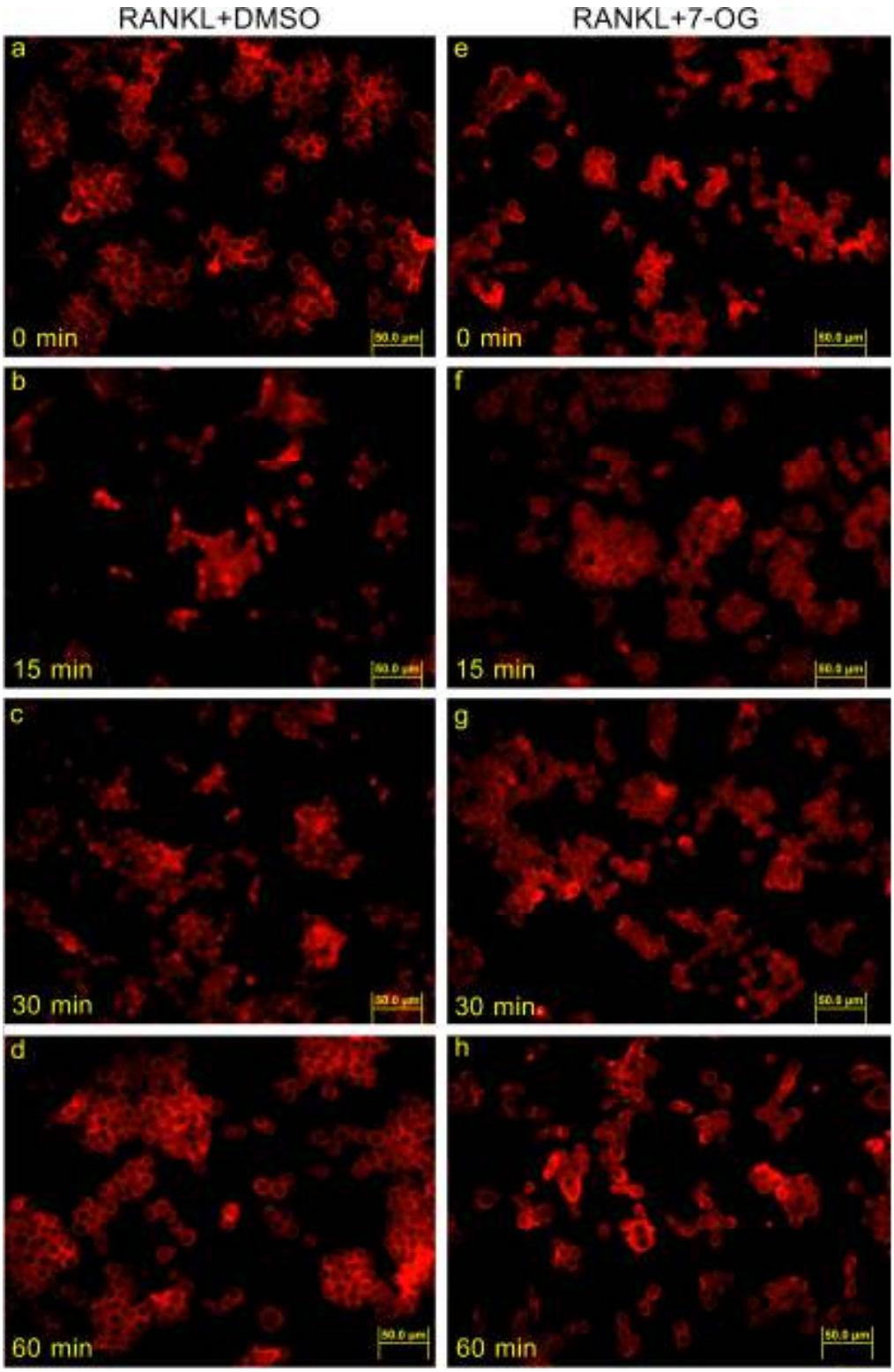


Figure 4.9 Effect of 7-OG on nuclear localization of NF-ΚB p65

RAW264.7 was pretreated with 7-OG (9 μM) or vehicle control DMSO for 30 min and stimulated with 100 ng/ml rRANKL for indicated time. The cells were subjected to immunofluorescent staining for NF-κB p65 and visualized under a fluorescent microscope.

4.5 Effect of 7-OG on nitric oxide production

To determine the effect of 7-OG on nitric oxide production which can induce apoptosis of osteoclast, the culture supernatant was collected from unstimulated, control DMSO and 7-OG treated cells at various time points. The range of amount nitric oxide produced was 1 to 3 μM (Figure 4.10). The apoptosis assay was not carry out but the trace amount of nitric oxide production has no effect on apoptosis of osteoclast. On the other hand, from the result of 4.1.3, it showed that 7-OG suppressed the early step of osteoclast differentiation so there is no mature osteoclast in the treatment of 7-OG at 9 μM. This result suggests that apoptosis of mature osteoclast is not induced in 7-OG treatment.

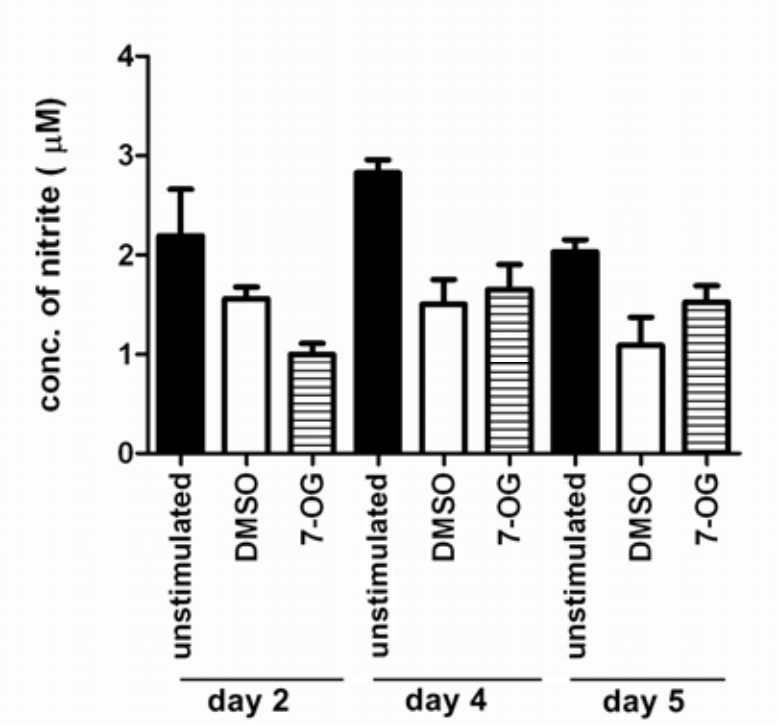


Figure 4.10 Effect of 7-OG on nitric oxide production

RAW264.7 cells were cultured with 100 ng/ml rRANKL in the presence of 7-OG (9 μM) or vehicle control DMSO for 5 days. Culture supernatant was collected every 2 days and before TRAP staining to determine nitric oxide production. Data were shown as mean±SD, in triplicate.

CHAPTER V

DISCUSSION

Ten bioactive compounds from *Kaempferia parviflora* exhibited no activity for anti-osteoclastogenic activity, but among six limonoid compounds from *Xylocarpus moluccensis*, there were two limonoid compounds which exhibited strong anti-osteoclastogenic activity, XMPH3 (7-oxo-deacetoxygedunin or 7-OG) and XMPH4 (7-deacetylgedunin). The percent inhibition of anti-osteoclastogenic activity of 7-OG and 7-deacetylgedunin were 94.94% at 9 μM and 81.23% at 4.5 μM, respectively. Because the cytotoxicity of 7-acetylgedunin was higher than 7-OG, 7-OG was chosen for further study the molecular mechanisms in suppressing osteoclast differentiation.

In this study, 7-OG has a strong anti-osteoclastogenic activity because the TRAP activity of RAW264.7 treated with DMSO is significantly higher than that treated with 7-OG approximately 10 fold in the presence of 100 ng/ml RANKL. In agreement with the TRAP activity, TRAP staining assay showed that there were TRAP-positive multinucleated cells almost 300 cells/well in cells treated with RANKL and DMSO but there was almost no TRAP-positive multinucleated cells when treated with RANKL and 7-OG. These results suggest that 7-OG at 9 μM effectively inhibit the osteoclast formation. Furthermore, adding 7-OG to the culture at different time points after RANKL stimulation identified the effect of 7-OG at early step because 7-OG did not affect the preosteoclast and mature osteoclast even though when added on day 5 compared to the control DMSO.

To confirm that 7-OG suppresses the osteoclast differentiation, expressions of osteoclast-related genes, *NFATc1*, *Cathepsin K* and *IRF8* were investigated. The

expression of *NFATc1* was not induced at any time points in RAW264.7 treated with 7-OG compared to the vehicle control DMSO treated cells. This result was consistent with the results of TRAP staining because 7-OG suppresses the differentiation of osteoclasts at an early step and *NFATc1* is one of the genes induced at an early stage of osteoclast differentiation. Next, the expression of *Cathepsin K* was suppressed in RAW264.7 treated with 7-OG at all time points tested. In addition, the delayed in downregulation of *IRF8* was also detected in RAW264.7 treated with 7-OG. IRF8 is a negative regulator of NFATc1 and the binding of IRF8 to NFATc1 in osteoclast precursor leads to the suppression of NFATc1 activity such as autoamplification. The levels of IRF8 were reported to be reduced in the presence of RANKL (Zhao et al., 2009). This result suggests that 7-OG may suppress the activity of NFATc1 directly by modulating *NFATc1* expression and indirectly by modulating the reduction of IRF8 levels.

To investigate the effect of 7-OG on the signaling pathways involved in osteoclast differentiation, the activation of NF-κB and MAPK signaling pathway were focused. The effect of 7-OG on the level of IκBα was not different but its effect on the level of phosphorylated p65 could be clearly detected. Immunofluorescent staining revealed that there were few cells that exhibited p65 in the nuclei of RAW264.7 treated with 7-OG. These results suggest that treatment with 7-OG clearly interferes with activation of NF-κB p65. In the MAPK pathway, treatment with 7-OG decreased the levels of phosphorylated p38 and p44/42 (ERK1/2). These results showed that 7-OG can suppress the activation of both NF-κB and MAPK pathways which leads to suppression of osteoclast differentiation.

To investigate the effect of 7-OG on apoptosis of osteoclast, the amount of nitric oxide was determined. The amount of nitric oxide in all of the timepoints tested was less than 3 μM. This result suggested that the trace amount of nitric oxide production may not be sufficient to induce apoptosis in osteoclast. The previous experiments showed that there was no mature osteoclast by treatment with 7-OG because 7-OG suppressed

this process at early step of osteoclast differentiation. Previous study reported that stimulation of bone marrow cells with osteoblasts co-cultured with IL-1β, IFN-γ and TNF-α enhanced nitric oxide production and the nitric oxide in turn induces apoptosis of osteoclast progenitors (van't Hof and Ralston, 1997). Moreover, high concentration of nitric oxide production can suppress osteoclast formation and its activity by inhibiting the cathepsin K activity (van't Hof and Ralston, 2001).

There are several bioactive compounds which have been reported that have anti-osteoclastogenic activities such as FK506 and symbioimine which suppress the differentiation of osteoclast precursor to preosteoclast, concananycin B and prodigiosin 25-C which suppress the activation of multinucleated osteoclast to mature osteoclast and reveromycin A and cyclosporin A which induce apoptosis of mature osteoclast. These compounds obtained from microorganisms such as bacteria or fungi (Woo et al., 2008). Furthermore, bioactive compounds which obtained from plants such as root of *Achyranthes bidentata* (chinese herb) (He et al., 2010), leaf of *Ficus carica* (fig) (Park et al., 2009) and stem bark of *Erythrina variegata* (legume) (Zhang et al., 2007) can inhibit the differentiation and formation of osteoclasts.

The derivatives of 7-OG, 7-deacetylgedunin, was found to have anti-osteoclastogenic activity similar to 7-OG. The percent of inhibition was approximately 81.23% at 4.5 μM but it exhibited higher cytotoxicity. The structure of 7-deacetylgedunin is different from 7-OG at C-atom position 7. The function group at this position of 7-deacetylgedunin is -OH whereas 7-OG is -O. Therefore, the functional group at this position may be critical for the biological activities of 7-OG. 7-OG has potentials in becoming lead compounds for anti-bone resorption drug to prevent and treat bone disorders because it has a strong anti-osteoclastogenic activity.

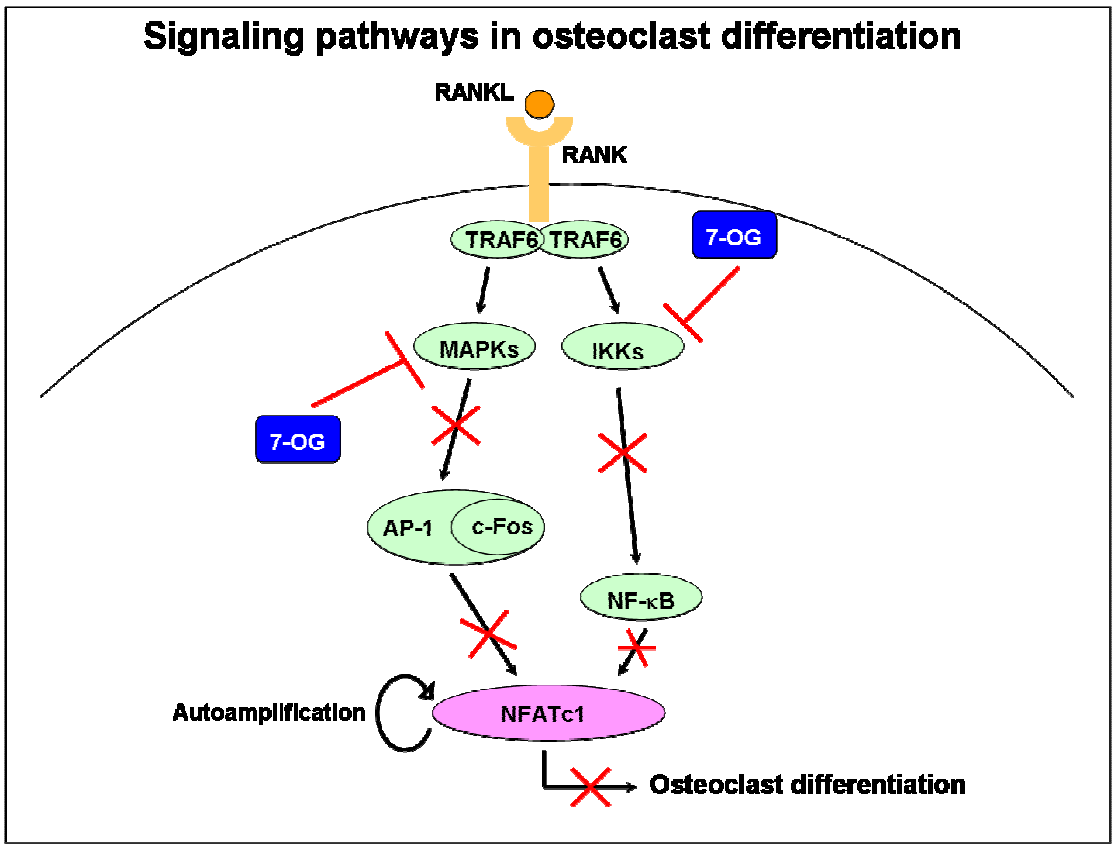


Figure 5.1 The mechanism of 7-OG in suppressing osteoclast differentiation

CHAPTER VI

CONCLUSION

(1) 7-oxo-deacetoxygedunin (7-OG), a limonoid compound obtained from *Xylocarpus moluccensis* has a strong anti-osteoclastogenic activity by suppressing the formation of TRAP positive multinucleated cells and TRAP activity which induced by RANKL.

(2) 7-OG has an effect on osteoclast-related gene expression by suppressing the expression of *NFATc1* and *Cathepsin K* and delayed in downregulating of *IRF8*.

(3) 7-OG inhibited the translocation of NF-κB p65 to the nucleus but it had no effect on the level of IκBα in NF-κB pathway and 7-OG decreased the levels of phosphorylated p38 and p44/42 (ERK 1/2) in MAPK pathway.

(4) 7-OG had no effect on nitric oxide production.

Suggestion for the future work

Study the effect of 7-OG on the molecular mechanisms which involve osteoclast differentiation and osteoblast functions in animal model such as mice with ovariectomy (OVX) should be conducted for further evaluating its potential as anti-osteoporosis drug.

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APPENDIX

APPENDIX

1. DMEM Complete media 100 ml

DMEM	90 ml
FBS	10 ml
100 mM Sodium pyruvate	1 ml
HEPES free acid	1 ml
Penicillin G 10^6 U/ml	10 µl
Streptomycin 500 mg/ml	50 µl

2. DMEM freezing media

DMEM complete media	9 ml
DMSO	1 ml

3. FBS inactivation

FBS must be inactivated at 56°C for 30 min using water bath before use.

4. Penicillin G and streptomycin solution

Penicillin G and streptomycin were prepared at final concentration 10^6 U/ml and 500 mg/ml in sterile deionized water, respectively. The solutions were sterilized by using 0.45 µM filter and kept in aliquots at -20°C

5. DEPC water for dissolving RNA 100 ml

10 µl of DEPC (0.01% v/v) was added to 100 ml of HPLC water and incubated overnight at room temperature. The DEPC water was autoclaved at 121°C and pressure 15 psi for 15 min.

6. 2x Laemmli buffer (SDS-dye) 10 ml

1 M Tris-HCl pH 6.8	1 ml
10% SDS	4 ml

99.5% glycerol	2.01 ml
HPLC water	2.989 ml
Bromphenol blue	0.001 g

7. SDS-polyacrylamide gel preparation

7.1 5% stacking gel 2 ml

Sterile water	1.204 ml
40% Acrylamide and Bis-acrylamide solution	0.25 ml
1 M Tris-HCl pH6.8	0.504 ml
10% SDS	0.02 ml
10% APS	0.02 ml
TEMED	0.002 ml

7.2 10% separating gel

Sterile water	3.836 ml
40% Acrylamide and Bis-acrylamide solution	2 ml
1 M Tris-HCl pH6.8	2 ml
10% SDS	0.08 ml
10% APS	0.08 ml
TEMED	0.004 ml

8. RIPA buffer for protein extraction

1 M Tris-HCl pH 7.4	0.5 ml
0.5 M NaCl	3 ml
Nonidet p-40	0.5 ml
10% sodium deoxycholate	0.5 ml
20% SDS	0.05 ml
Protease inhibitor	1 tablet
Deionized water	5.45

9. 1x PBS pH 7.4

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Deionized water	1000 ml

Adjusted pH to 7.4 with 10 N NaOH and autoclaved at 121°C and pressure 15 psi for 15 min

10. PBST (washing buffer)

1x PBS	1000 ml
Tween20	500 µl

11. Blocking solution

PBST	100 ml
Non-fat dry milk	3 g

12. 5x running buffer

Trisma base	15.1 g
Glycine	94 g
SDS	5 g
Deionized water	1000 ml

13. Transfer buffer

Trisma base	5.08 g
Glycine	2.9 g
SDS	0.37 g
Deionized water	800 ml
Absolute methanol	200 ml

14. ECL substrate

14.1 90 mM coumaric acid

Coumaric acid	74 mg
DMSO	5 ml

The solution was kept in aliquots at -20°C

14.2 250 mM luminol

Luminol	222 mg
DMSO	5 ml

The solution was kept in aliquots at -20°C

14.4 Solution A

100 mM Tris-HCl pH8.8	2.5 ml
90 mM coumaric acid	11 µl
250 mM luminol	25 µl

14.5 Solution B

100 mM Tris-HCl pH8.8	2.5 ml
30% H_2O_2	1.5 µl

15. Film developer and fixer

Film developer and fixer were diluted in tap water at dilution 1:4 in total volume 50 ml

16. TRAP solution for TRAP staining and TRAP activity assay

16.1 10% formaldehyde 10 ml

40% formaldehyde	2.5 ml
Deionized water	7.5 ml

16.2 95% ethanol 10 ml

Absolute ethanol	9.5 ml
Deionized water	0.5 ml

16.3 50 mM sodium acetate with 50 mM sodium tartrate

Glacial acetic acid	11.025 ml
Sodium acetate trihydrate	6.8 g
Sodium tartrate dihydrate	11.5 g
Deionized water	1000 ml

Adjusted pH to 5.0 with 10 N NaOH and autoclaved at 121°C and pressure 15 psi for 15 min

16.4 10mg/ml naphtol AS-MX phosphate

Naphtol AS-MX phosphate	10 mg
N,N-dimethlformamide (DMF)	1 ml

16.5 TRAP solution for TRAP staining assay

Fast red violet LB salt	3 mg
10mg/ml naphtol AS-MX phosphate	50 µl
50 mM sodium acetate with 50 mM sodium tartrate	5 ml

16.6 50 mM citrate buffer with 10 mM sodium tartrate

Citric acid	10.51 g
Sodium citrate dihydrate	14.71 g
Sodium tartrate dihydrate	2.3 g

Adjusted pH to 4.6 with 10 N NaOH and autoclaved at 121°C and pressure 15 psi for 15 min

16.7 TRAP solution for TRAP activity assay

p-nitrophenylphosphate	18.375 g

50 mM citrate buffer with 10 mM sodium tartrate 5 ml

17. 5 mg/ml MTT solution

MTT	50 mg
Sterile 1x PBS	10 ml

The solution was sterilized by using 0.45 µM filter and kept in aliquots at $4^{\circ}C$

18. 0.04 N HCL in isopropanol

Conc. HCl	0.331 ml
Isopropanol	80 ml

Adjusted volume to 100 ml by using isopropanol in volume metric flask

19. Sulfanilamide solution (1% w/v in 5% phosphoric acid)

Sulfanilamide	1 g
85% phosphoric acid	5.88 ml
Deionized water	94.12 ml

20. *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED) solution (0.1% w/v)

NED	0.1 g
Deionized water	100 ml

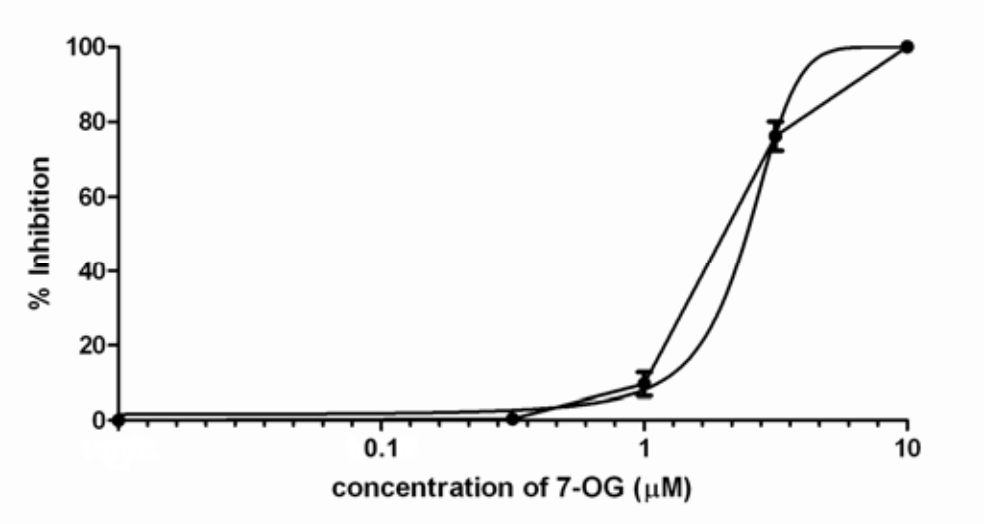
21. 4% paraformaldehyde

4 g of paraformaldehyde was dissolved in 100 ml of 1x PBS and added a few drop of 1 N NaOH. The solution was heated at $65^{\circ}C$ in water bath and cooled to room temperature. The solution was adjusted pH to 7.4.

22. 0.2% triton-X 100

Triton-X 100	20 µl
Deionized water	10 ml

23. Determination of IC50 for anti-osteoclast activity



Determination of IC50 for anti-osteoclast activity was determined by non-linear regression analysis using GraphPad Prism software.

Biography

Chonnaree Wisutsitthiwong was born in Prachuap khiri khan, Thailand on April 16, 1987. After graduation with the Bachelor's degree of Science from the Department of Microbiology, Faculty of Science at Kasetsart University in 2008, she subsequently enrolled in the Master Program of Science in Industrial Microbiology at Chulalongkorn University in 2009.

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