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Original Paper

Psoralen and Bakuchiol Ameliorate M-CSF Plus RANKL-Induced Osteoclast Differentiation and Bone Resorption Via Inhibition of AKT and AP-1 Pathways in Vitro

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Key Words
Psoralen • Bakuchiol • Osteoclast • Differentiation • Bone resorption

Abstract
Background/Aims: Psoralen and bakuchiol are the main active compounds found in the traditional Chinese medicine *Psoralea corylifolia L.*, and have been used to treat osteoporosis. This study aims to investigate the anti-osteoporosis effects of these two compounds using osteoclasts precursor differentiation and bone absorption assays *in vitro*. ***Methods:*** Primary mouse osteoclasts precursor cells were induced by M-CSF (macrophage colony stimulating factor) plus RANKL (receptor activator of nuclear factor kappa-B ligand) *in vitro*. TRACP (tartrate-resistant acid phosphatase) enzyme activity and toluidine blue staining were used to observe the effects of psoralen and bakuchiol on osteoclast differentiation and bone resorption, respectively. Gelatin zymography was used to assess MMP (matrix metalloproteinase) activity, and ELISA was performed to measure cathepsin K activity. Western blotting analysis for expression of phosphorylated AKT, ERK, NF-kB, and c-jun; and immunofluorescence analysis for c-jun and p65 nuclear translocation in induced osteoclasts were then used to determine the mechanism of anti-bone resorption of psoralen and bakuchiol. ***Results:*** Mature osteoclasts were induced by M-CSF plus RANKL from primary bone marrow macrophages *in vitro*. Both psoralen and bakuchiol significantly inhibited TRACP enzyme activity and slightly decreased the number of $TRACP^+$ multinuclear osteoclasts induced by M-CSF plus RANKL. Bakuchiol significantly decreased bone lacunae area and attenuated MMP-2 activity induced by M-CSF

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plus RANKL in osteoclasts. Both psoralen and bakuchiol significantly decreased the expression and nuclear translocation of phosphorylated c-jun stimulated by M-CSF plus RANKL, but no significant effect on p65 translocation was observed in osteoclasts. Additionally, bakuchiol significantly attenuated the increased of M-CSF plus RANKL-induced phosphorylation of AKT in osteoclasts. ***Conclusions:*** Psoralen and bakuchiol ameliorated M-CSF plus RANKL-induced osteoclast differentiation and bone resorption via inhibition of AKT and AP-1 pathways activation *in vitro*.

Introduction

Osteoporosis is an age-related health problem for both women and men. It is the most common metabolic bone disease, characterized by decreased bone strength and increased risk of bone fracture, especially in elderly women. The most frequent cause of osteoporosis in women is the decreased estrogen level that occurs during menopause [1, 2]. Osteoporosis in men above the age of 70 is also recognized as an important public health issue [3, 4]. Emerging data demonstrate that serum estradiol level is an important prediction factor in men, establishing a threshold estradiol level of 18pg/ml , below which bone loss and fracture risk increase remarkably [5].

Bone is constantly remodeled during via osteoblast formation and osteoclast resorption. The imbalance between osteoclast and osteoblast formation and function leads to various osteopathic disease, particularly those involving bone loss caused by excessive bone resorption, such as osteoporosis and rheumatoid arthritis. Osteoclasts are multinucleated cells specialized for bone resorption. Osteoclasts show present a “foamy” appearance due to their high concentration of vesicles and vacuoles. These vacuoles include lysosomes filled with TRACP and cathepsin K. During bone resorption, TRACP, cathepsin K and MMPs are released to digest the organic components of the bone matrix.

Differentiation of osteoclast precursor cells termed osteoclastogenesis and bone resorption by mature osteoclasts requires RANKL and M-CSF to induce the expression of RANK, a receptor for RANK ligand (RANKL) [6]. Interaction between RANK and RANKL then recruit tumor necrosis factor (TNF) -receptor-associated factors and activates downstream signaling pathways, including AKT (protein kinase B), ERK (extracellular signal-regulated kinase), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), JNKs (c-Jun N-terminal kinases), and p38 mitogen-activated protein kinases [7, 8]. Immediately after these signaling molecules are activated, NFATC1 (nuclear factor of activated T cells) is elicited as a master factor for osteoclast differentiation. NFATC1 plays a predominant role in the regulation of gene expression for osteoclast maturation, including TRACP, cathepsin K and MMPs, which are required for the bone resorption processes mediated by mature osteoclasts [9, 10].

Psoralea corylifolia L., a well-known traditional Chinese medicine, is a kidney tonifying plant generally used in formulas that are prescribed for the treatment of fractures and bone and joint diseases in traditional Chinese medicine. It is reported to improve BMD (bone mineral density) promote new bone formation, promote osteoblast cell proliferation [11] and differentiation [12, 13]. Bakuchiol is reported to increase uterine weight, reduce bone loss, and show strong estrogen activity in ovariectomized Sprague-Dawley rats [14]. Although many studies have reported the effects of *Psoralea corylifolia L.* in osteoporosis, for example improved BMD [12-17], the specific effects and mechanisms of its active ingredients psoralen and bakuchiol on the inhibition of osteoclast differentiation and bone resorption *in vitro* have not yet been studied . In this study, we investigated the inhibitory effects of psoralen and bakuchiol on osteoclast differentiation function using primary osteoclast cells induced by M-CSF plus RANKL and potential underlying mechanisms.

Materials and Methods

Drugs and reagents

Psoralen and bakuchiol were obtained from the Food and Drug Verification Research Institute of China (Beijing, China). α-MEM was purchased from Hyclone (Hyclone Logan, UT, USA). FBS (Fetal Bovine Serum) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). M-CSF and RANKL were purchased from R&D Systems Inc. (Minneapolis, Minnesota, USA). TRACP and Cathepsin K Activity assay kits were obtained from Sigma (St. Louis, MO, USA). p-ERK, p-AKT, p-jun, p-p65, ERK, AKT, Jun, p65, rabbit IgG (Alexa Fluor 555-conjugate), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody, DAPI (4, 6-diamidino-2-phenylindole), and ProLong® Gold Anti-Fade Reagent were purchased from Cell Signaling Technology (Danvers, MA, USA). PNPP-NA2 was purchased from Amresco (Cochran Road Solon, OH, USA). L(+)-tartaric acid was purchased from Solarbio (Beijing, China). SDS-PAGE (12%) was purchased from Invitrogen (Waltham, MA USA). Bone slices were purchased from Nordic Bioscience (Herlev, Denmark). ECL kit was purchased from GE Healthcare Life Sciences (Marlborough, USA). Psoralen and bakuchiol were weighed accurately and dissolved in DMSO to make a stock solution at a concentration of 0.05mM or 0.1mM (stored at -20°C). When the components were added to cells, the stock solution was diluted by MEM.

Isolation of osteoclast precursor cells

Kunming mice (4–6-weeks-old) were purchased from the Institute of Hygiene and Environmental Medicine, Academy of Military Medical Sciences (SCXK 2009-003). Bone marrow cells were obtained from mouse tibias as described previously [18]. Briefly, bone marrow was flushed from tibias with culture medium (α-MEM, 15% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin) (referred to as MEM in following text) using a 26-gauge needle. After washing with MEM, marrow cells were cultured in 75 cm^2 culture flasks for 24h at 37°C in 5% CO_2, then non-adherent cells were collected and centrifuged at 1, 000 rpm, 5 min,25°C, and suspended in MEM at 5×10^6 cells/ ml and plated in 96-well plates. After 48 h incubation at 37°C, cells were washed twice with D-Hanks. Isolated BMMs (Bone Marrow-Derived Macrophages) attached to the bottom of the plate were maintained in MEM. For differentiation tests, the cells were plated in 6-well plates or 12-well plates on glass cover slips or other plates.

Measurement of TRACP enzyme activity

The bone marrow cells were planted in 96 well plates at 5×10^6 cells/ ml. After 48h incubation at 37°C, osteoclast precursor BMMs attached to the bottom of the culture plate were kept and cultured in MEM containing different concentrations of M-CSF and RANKL. The cells were harvested on day 8 for the measurement of TRACP enzyme activity as described previously [19]. Briefly, the cells were washed twice with PBS and 30 μL 0.1% Triton X-100 was added for 10 min to lyse the cells. Then 100 μL of PNPP substrate solution (2 g/L p-nitro-disodium phenylphosphate, 7.6 g/L sodium L-Tartrate, pH 5.2) was added and incubated at 37°C for 30 min. 100μL 1M NaOH was added to stop the reaction. Absorbance was measured at 405 nm on a micro-plate reader (Victor™X5, PerkinElmer).

Differentiation of osteoclast precursor cell assay

The bone marrow cells were plated in 96-well plates at 5×10^6 cells/ ml. After 48h incubation at 37°C, osteoclast precursor, BMMs attached to the bottom of the culture plate were kept and cultured in MEM containing different concentrations of M-CSF and RANKL. Media was changed every 3 days over a 15-day period. At the end of the assay, cells (day 15) were stained for TRACP using a leukocyte acid phosphatase kit (Sigma) according to the manufacturer's instructions [20]. Briefly, cells were fixed in fixative solution (25 ml citrate solution, 65 ml acetone and 8 ml 37% formaldehyde) for 30s, and then stained according to the procedure outlined in the kit. $TRACP^+$ multinuclear cells containing three or more nuclei were considered to be osteoclasts and were evaluated using a reflected light microscope.

Observation and assay of pit formation

The bone marrow cells were plated at a density of 5×10^6 cells/ml in 96-well plates inside which bovine bone slices were placed. After 48 h incubation at 37°C, the osteoclast precursor, BMMs attached to the bottom of the culture plate were kept and cultured in MEM containing different concentrations of M-CSF and RANKL. At the same time, psoralen or bakuchiol were added to the culture media. Media was changed every

3 days over a 15-day period. At the end of the assay, the bone slices in 96-well plate were fixed with 2.5% glutaraldehyde for 7min, and attached cells were removed ultrasonically in 0.25M NH_4OH solution. The bone slices were then dehydrated through a gradient of alcohol (100–30%) and stained with 1% toluidine blue to visualize the bone lacuna. Lacuna number and area were analyzed using an Eclipse TS100 (Nikon) microscope with NIS-Elements F system [18].

Measurement of calcium concentration

Calcium concentration in the culture medium was used to indirectly reflect the progress of bone lacuna in the bone slices. In brief, the osteoclast precursor, BMMs were cultured in MEM with or without M-CSF plus RANKL for 15 days in 96-well plates into which bone slices were added. Half the medium was changed every two days. During a 15-day period incubation, culture media was collected and diluted with 1% GR nitric acid by 20 times. The calcium concentration of diluted media was then measured by atomic spectrophotometry (PE Company, 2100 Atom Absorption Spectra instrument) [21].

Measurement of MMPs activity

MMPs activity was detected using the gelatin zymography assay [22]. Briefly, the osteoclast precursor, BMMs were seeded into 96-well plates and treated with psoralen or bakuchiol in the presence or absence of M-CSF plus RANKL for 15 days. The supernatant was collected to measure MMP activity. The samples were separated by electrophoresis on 7.5% SDS acrylamide gel containing 1% gelatin. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 to remove the SDS, then incubated in 1% Triton X-100 followed by 5 mM $CaCl_2$ at 37°C for 36 h. The gels were then stained with 0.1% Coomassie brilliant blue R-250 and de-stained in 10% acetic acid in H_2O. MMP activity was identified as transparent bands against the blue background of the Coomassie blue stain and signals were detected using a GENE Genius Bio Imaging System (SynGene, Cambridge, United Kingdom).

Measurement of cathepsin K activity by ELISA

The bone marrow cells were seeded at a density of 5×10^6 cells/ ml into 96-well plates. After 48 h incubation at 37°C, the osteoclast precursor BMMs attached to the bottom of the culture plate were kept and cultured in MEM and treated with psoralen or bakuchiol in the presence or absence of M-CSF plus RANKL for 15 days. Cells were then collected and proteins extracted using lysis buffer (1ml 1×TBS containing 20μL Triton X-100, 20μL glycerol) for 10 min on ice after washing twice with D-Hanks. A commercial cathepsin K activity assay kit (Sigma) was used to measure cathepsin K activity according to the manufacturer's instructions [23].

Western blotting analysis to identify cell signaling pathways

To identify cell signaling pathways, western blotting analysis was performed as we have previous described [24]. The bone marrow cells were seeded at a density of 5×10^6 cells/ml into 6-well plates. After 48 h incubation at 37°C, the attached osteoclast precursor cells were treated with psoralen or bakuchiol in the presence or absence of M-CSF plus RANKL for 15 days. After 15 days of culture in MEM in 96-well plates, osteoclasts were lysed by the addition of cold RIPA lysis buffer (Beyotime, China) and equal amounts of protein (30 μg) were subjected to SDS-PAGE gel electrophoresis. The proteins were then transferred onto PVDF membranes. After 1h blocking at room temperature in 5% skimmed-milk, the membranes were incubated with anti-p-ERK, anti-p-AKT, anti-p-p65, anti-p-IκB, and anti-p-c-jun primary antibodies overnight, followed by incubation with an appropriate secondary antibody conjugated to horseradish peroxidase (HRP). The blots were visualized by enhanced chemiluminescence using an ECL kit. For normalization purposes, the same membranes were then stripped and re-probed with identical primary antibodies. Signals were detected using a VersaDoc™ Imaging System (BioRAD). The band gray densities were quantified by Image J software and normalized to the corresponding total ERK, AKT, p65, IκB, c-jun.

Immunocytochemistry analysis for p65 and c-jun nuclear translocation

The bone marrow cells were seeded at a density of 5×10^6 cells/ ml into 24-well plates in which slips were placed. After 48 h incubation at 37°C, cells were washed twice with D-Hanks. Isolated BMMs attached to the bottom of the plate were then maintained in MEM with psoralen or bakuchiol in the presence or absence of M-CSF plus RANKL for 15 days. MEM was aspirated and the cells were fixed for 15 min at room

temperature in 4% formaldehyde. The cells were then washed three times in 1×PBSand blocked for 60 min with blocking buffer (PBS/0.3% Triton X-100/5% normal serum: from the same species as the secondary antibody). Primary antibodies for anti-p65 and anti-c-jun diluted in antibody dilution buffer (PBS/0.3% Triton X-100/1% BSA) were then added and incubated overnight at 4°C and the wells were washed three times in PBS. Fluorochrome-conjugated secondary antibody was then incubated for 1h at room temperature in the dark. The secondary antibody was then aspirated and the wells washed in PBS. Slides were then covered with ProLong® Gold Anti-Fade reagent (CST). Translocation was visualized using a Nikon E400 microscope under a fluorescence microscope (Nikon, Japan).

Statistical analysis

Data were expressed as mean±SD. One-way ANOVA was used to determine significant differences among all groups followed by Dunnett's post hoc test. *P* values less than 0.05 were considered to be significant.

Results

Development of TRACP⁺ osteoclasts containing three or more nuclei from primary bone marrow macrophages

Mature osteoclasts containing three or more nuclei were produced from primary bone marrow macrophages cultured in the presence of 5 ng/ml M-CSF and 15 ng/ml RANKL for 8 days (Fig. 1A), with TRACP enzyme activity reaching a maximum level at approximately day 5 (Fig. 1B). 10 ng/ml M-CSF (plus15 ng/ml or 30 ng/ml RANKL) treatment significantly increased the mean number of osteoclast compared with 5 ng/ml M-CSF (plus 15 ng/ml or 30 ng/ml RANKL) on day 8 (Fig. 1C). Concomitantly, 10 ng/ml M-CSF plus 30 ng/ml RANKL significantly increased the number of mature osteoclasts containing three or more nuclei in 15 days (Fig. 1D). Therefore, M-CSF at 15 ng/ml and RANKL at 30 ng/ml were selected to induce bone marrow macrophage differentiation into mature osteoclasts, and day eight was chosen as the time point to measure TRACP enzyme activity.

Psoralen and bakuchiol inhibited osteoclast differentiation (TRACP enzyme activity)

After 8 days of treatment, osteoclast differentiation was determined by the quantification of TRACP enzyme activity. As shown in Fig. 2A and 2B, 10 ng/ml M-CSF plus 30 ng/ml RANKL significantly increased TRACP enzyme activity in osteoclasts. Compared with the M-CSF plus RANKL-induced group, 0.05 and 0.1μM psoralen and 0.1μM bakuchiol significantly inhibited TRACP enzyme activity (Fig. 2A, 2B). These data suggested that psoralen and

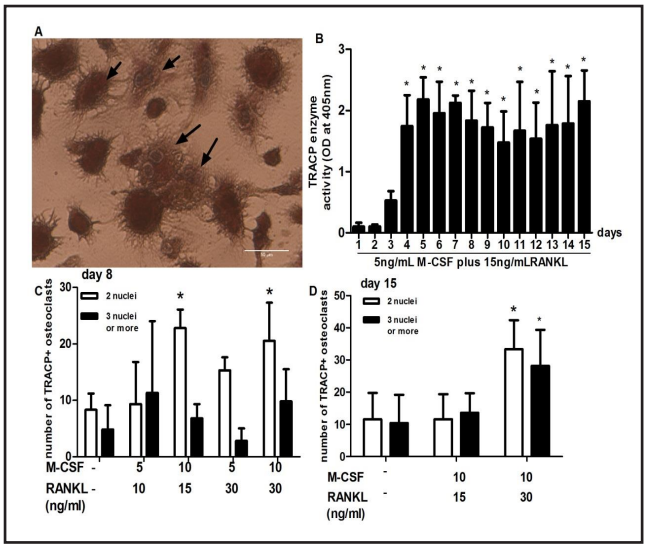

Fig. 1. The differentiation of osteoclast precursors induced by different concentrations of M-CSF plus RANKL. (A) Morphology of osteoclasts induced by 5 ng/ml M-CSF and 15 ng/ml RANKL for 8 days. Arrow: TRACP⁺ osteoclasts with two or more nuclei. (B) The activity of TRACP enzyme in the presence of 5 ng/ml M-CSF and 15 ng/ml RANKL for 15 days. (C, D) The number of osteoclasts induced by mouse M-CSF (5 or 10 ng/ml) plus RANKL (15 or 30 ng/ml) for 8 days or 15 days. Results show means ± SD of three independent experiments, n = 6. * P<0.05 vs. M-CSF and RANKL 0 ng/ml group (Control group).

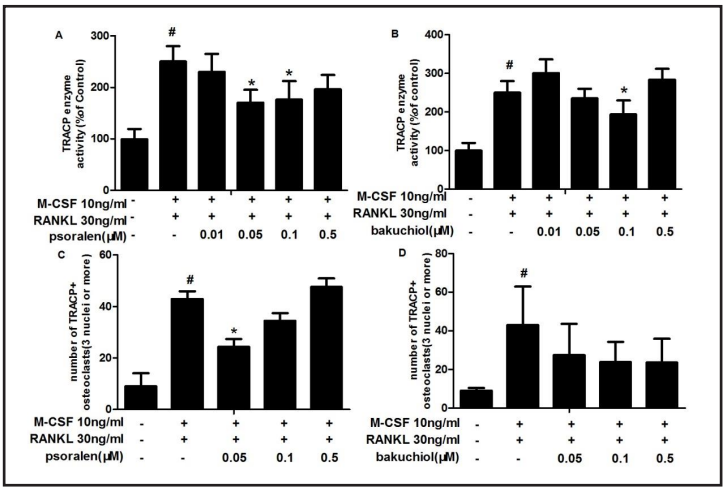

Fig. 2. The effects of psoralen or bakuchiol on TRACP enzyme activity and mature osteoclast formation induced by M-CSF plus RANKL. (A, B) The analysis of TRACP enzyme activity shows inhibition by psoralen or bakuchiol in M-CSF plus RANKL for 8 days. n=27; (C, D) The number of mature osteoclasts was counted in osteoclast precursors induced by M-CSF plus RANKL for 15 days. Results show means ± SD of three independent experiments. n = 6, #P<0.05 vs. Non-induced group. *P<0.05 vs. M-CSF plus RANKL-induced group.

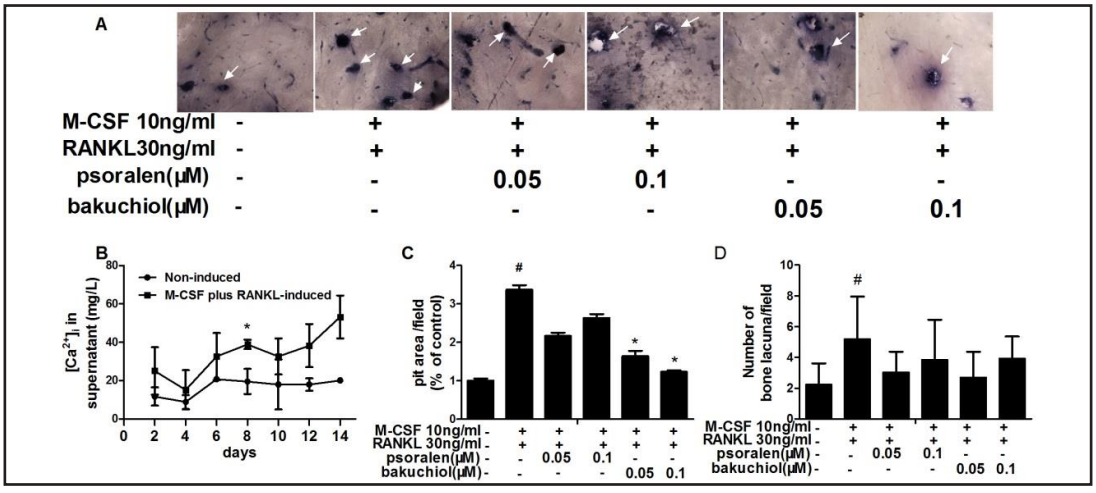

Fig. 3. Psoralen and bakuchiol inhibit bone lacunae formation in bone slices. (A) Toluidine blue staining to visualize bone lacunae in bone slice. Arrow: the bone lacunae. (B) $[Ca^{2+}]_i$ in the osteoclast culture medium released from bone slices was measured every 2 days. (C, D) Number and area of bone lacunae for A were analyzed using an Eclipse TS100 (Nikon) microscope with NIS-Elements F system. Results show means ± SD of three independent experiments, n = 6, #P<0.05 vs. Non-induced group. *P<0.05 vs. M-CSF plus RANKL-induced group. Bakuchiol attenuated MMP-2 activity induced by M-CSF plus RANKL in osteoclasts.

bakuchiol inhibited osteoclast differentiation. However, except for the 0.05 μM psoralen group, psoralen and bakuchiol only slightly decreased the number of $TRACP^+$ multinuclear osteoclasts, compared with M-CSF plus RANKL induced group after 15 days, with no significant difference occured (Fig. 2C, 2D). Together, these data showed that psoralen and bakuchiol inhibited osteoclast differentiation, but had no effects on the formation of $TRACP^+$ mature multinuclear osteoclasts.

Psoralen and bakuchiol decreased bone resorption

During treatment of osteoclasts with M-CSF plus RANKL, the calcium released from the bone slices increased within days, which suggested that bone lacunae were forming on bone slices absorbed by osteoclasts (Fig. 3B). Day 16 was selected to observe the area and number of bone lacuna.

10 ng/ml M-CSF plus 30 ng/ml RANKL significantly increased bone lacunae area and the number of bone lacunae (Fig. 3A, 3C and 3D). Psoralen slightly decreased the bone

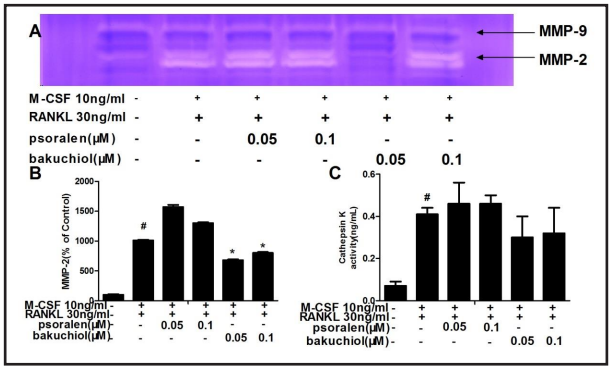

Fig. 4. Psoralen or bakuchiol inhibit MMPs and cathepsin K activity in M-CSF and RANKL-induced osteoclasts. (A, B) MMP2/9 activity in culture media was measured by gelatin zymography on day16, Blot (A) and band density data (B) is representative of three independent experiments. (C) Cathepsin K activity was detected by an ELISA cathepsin K assay kit. Results show means ± SD, n = 6, #P<0.05 vs. non-induced group. *P<0.05 vs. M-CSF plus RANKL-induced group.

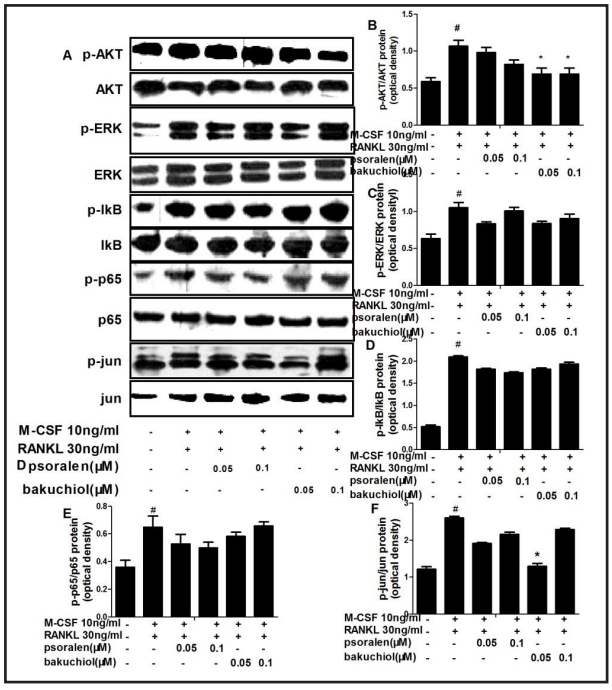

Fig. 5. Effects of psoralen and bakuchiol on p-AKT, p-ERK, p- I-kB, p-p65, and p-c-jun expression in M-CSF and RANKL-induced osteoclasts. (A) Representative image of p-AKT, p-ERK, p-I-kB, p-p65, and p-c-jun expression detected by Western blots. (B, C, D, E, F) Representative Western blots and corresponding band intensities to p-AKT, p-ERK, p-I-kB, p-p65 and p-c-jun were quantified. The images shown are representative of three independent experiments. Results show means ± SD of three independent experiments, n = 6, #P<0.05 vs. non-induced group. *P<0.05 vs. M-CSF plus RANKL-induced group.

lacunae area, while 0.05 and 0.1μM bakuchiol significantly decreased the bone lacunae area compared with the vehicle group (Fig. 3C). However, both psoralen and bakuchiol did not decrease the number of bone lacunae compared with the M-CSF plus RANKL-induced group (Fig. 3D).

Osteoclasts exert their effects of bone matrix degradation using a series of enzymes including TRACP, MMPs and cathepsin K.

Gelatin zymography and ELISA were used to detect MMP2/9 and cathepsin K activities, respectively. 10 ng/ml M-CSF plus 30 ng/ml RANKL significantly increased MMP-2 activity in osteoclasts Compared to the M-CSF plus RANKL-induced group, bakuchiol, but not psoralen, significantly attenuated MMP-2 activity induced by M-CSF plus RANKL (Fig. 4A, 4B and 4C). No significant differences were observed between groups for MMP-9 expression (results not shown). Neither psoralen nor bakuchiol restored the increased cathepsin K activity induced by M-CSF plus RANKL in osteoclasts (Fig. 4C) to baseline levels.

Psoralen and bakuchiol decreased the phosphorylation of c-jun induced by M-CSF plus RANKL in osteoclasts

To elucidate the mechanism of psoralen or bakuchiol on inhibition of M-CSF plus RANKL-induced osteoclastogenesis, we investigated the phosphorylation of downstream signaling pathways for osteoclast proliferation and differentiation including AKT, ERK, transcription

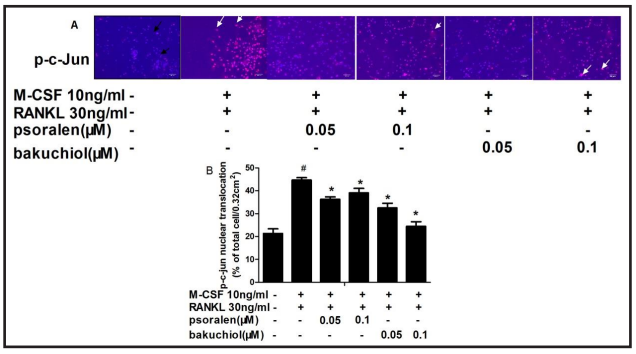

Fig. 6. Effects of psoralen or bakuchiol on c-jun translocation into the nucleus in osteoclasts induced by M-CSF and RANKL for 8 days. (A) Representative image of c-jun translocation into the nucleus. Black arrows show the nucleus (blue) without p-c-jun translocated into nucleus, white arrows represented the nucleus (blue) with p-c-jun (bright orange dye) translocated into nucleus. Translocation was visualized in five fields of view/bone slice. Images and data are representative of three independent experiments. n = 6, #P<0.05 vs. Non-induced group. *P<0.05 vs. M-CSF plus RANKL-induced group.

factor AP-1 (including c-jun subunit), NF-KB (including p65), and I-KB, which together constitute the main RANKL signaling cascades for osteoclast proliferation and differentiation [25, 26]. Osteoclasts were incubated with M-CSF plus RANKL in the presence or absence of psoralen or bakuchiol for eight days. M-CSF plus RANKL- induced group significantly increased the phosphorylation of AKT, ERK, I-KB, p65 and c-jun signaling factors in the cells compared to the non-induced group (Fig. 5A, B, C, D, E and F). Psoralen did not affect the phosphorylation of AKT, ERK, I-KB, p65 or c-jun in M-CSF plus RANKL induced osteoclasts, (Fig. 5A, B, C, D, E and F). However, bakuchiol significantly attenuated the increase of M-CSF plus RANKL-induced phosphorylation of AKT and c-jun compared with the M-CSF plus RANKL-induced group (Fig. 5A, B and F).

Psoralen and bakuchiol inhibited M-CSF plus RANKL-induced nuclear translocation of c-jun in osteoclasts

Osteoclast precursor cells induced by M-CSF plus RANKL were treated with different concentrations of psoralen or bakuchiol for eight days. Low levels of p65 (data not shown) and high levels of c-jun were translocated to the nucleus in the presence of M-CSF plus RANKL (Fig. 6A, 6B), and both psoralen and bakuchiol significantly decreased nuclear translocation of c-jun stimulated by M-CSF plus RANKL (Fig. 6A, 6B).

Discussion

Experimental results from this study show that both psoralen and bakuchiol significantly inhibited TRACP activity and slightly decreased the number of $TRACP^+$ multiclear osteoclasts induced by M-CSF plus RANKL. Bakuchiol significantly decreased bone lacunae area and attenuated MMP-2 expression induced by M-CSF plus RANKL in osteoclasts. However, neither psoralen or bakuchiol decreased the number of bone lacunae or restored the increased cathepsin K activity induced by M-CSF plus RANKL. Psoralen and bakuchiol ameliorated M-CSF plus RANKL-induced osteoclast differentiation and bone resorption *in vitro* via the inhibition of AKT and AP-1 pathway activation.

We first tested the effects of psoralen and bakuchiol on TRACP enzyme activity in M-CSF plus RANKL-induced osteoclasts. In primary osteoclast cell cultures, we found that TRACP enzyme activity was induced by M-CSF plus RANKL, which is consistent with previous studies [19]. Bone resorption is a process of bone remodeling that is mediated by osteoclasts and result in bone loss. Excessive bone resorption is related to bone loss diseases such as osteoporosis and rheumatoid arthritis. We hypothesized that suppression of RANKL-induced osteoclastogenesis and its inhibition by psoralen or bakuchiol may be associated with the inhibition of TRACP, MMPs and cathepsin K enzyme activities.

Our findings suggest that psoralen had a direct effect on TRACP enzyme activity, but no effect on the formation of mature osteoclasts, bone resorption ability, or the activity of

osteoclastic MMPs and cathepsin K. Conversely, bakuchiol significantly inhibited M-CSF plus RANKL-induced TRACP enzyme and MMP-2 activities, and decreased cathepsin K expression. We also demonstrated an inhibitory effect of bakuchiol on osteoclasts resorption properties *in vitro*. Two adaptor molecules were found to be involved in the signal pathways, which are AKT and c-jun (AP-1 subunit) signals. Additionally, nuclear translocation of c-jun was also inhibited by bakuchiol and psoralen.

RANKL-induced osteoclastogenesis is mediated by M-CSF and RANKL. RANKL predominantly activates AKT, ERK, NF-kB and AP-1 signaling pathways [27-29]. MAPK-NFATc1 constitutes the main downstream signaling cascade of RANKL during early-stage osteoclastogenesis (within 24h from RANKL-stimulation), whilst TRACP and cathepsin K are required for bone resorption during late-stage osteoclastogenesis (>48h from RANKL-stimulation). p38, JNK and ERK are significantly activated by RANKL during osteoclastogenesis, but when tetracycline antibiotics were added to the cultures, these signaling pathways were not suppressed in osteoclasts [27]. Similarly, previous studies reported that harmine failed to suppress ERK, p38, and I-κB signaling pathways during osteoclastogenesis [29], which was confirmed in our results. AKT signaling plays an important role in osteoclast formation [30]. Kim and Park reported that gene knockout of OC-STAMP (osteoclast stimulatory transmembrane protein) reduces osteoclast precursor cell differentiation and maturation, coinciding with decreased expression of c-jun, c-Fms, TRACP, cathepsin K, and MMP-9[31]. C-jun plays an important role in osteoclast differentiation by estrogen [32]. Recent research reported that zedoary turmeric oil inhibited the formation of osteoclasts via c-jun N-terminal kinase (JNK) induction by RANKL/AP-1 signaling [33]. Consistent with these previous studies, our results showed that AKT phosphorylation was enhanced in osteoclasts induced by M-CSF plus RANKL, and bakuchiol significantly inhibited phosphorylation of AKT. We also found that psoralen and bakuchiol reduced c-jun activation and decreased its translocation into the nucleus. Furthermore, bakuchiol reduced the activity of TRACP enzyme, cathepsin K and MMP-2 in osteoclasts.

A number of previous studies have been conducted on the effect of *Psoralea corylifolia L.* on osteoporosis. One study showed that *Psoralea corylifolia L.* extract leads to a significant increase in bone volume/tissue volume ratio, increased trabecular thickness , and BMD in ovariectomized rats [34]. Additionally, water extracts of *Psoralea corylifolia L.* have been shown to increase bone density, serum concentration of 25-hydroxyvitation D, and osteocalcin, and inhibit bone absorption in osteoporosis rats [35]. Psoralidin was shown to inhibited lipopolysaccharide-induced bone resorption by suppressing inflammatory cytokines [36]. Meanwhile, bakuchiol reduced postmenopausal bone loss by increasing alkaline phosphatase, Ca^{2+} concentration, serum E2 concentration, and BMD, and decreased the levels of inorganic P[14]. It has been reported that bavachalcone extracted from *Psoralea corylifolia L.* inhibited osteoclasts differentiation through suppression of NFATC1 induction by RANKL [15]. Similarly, we found that bakuchiol inhibited osteoclast TRACP enzyme activity, and lowered MMP-2 and cathepsin K activity, thus reducing the area and number of bone lacunae to prevent osteoporotic bone resorption. Taking our data together with the previous work detailed above, there is clear evidence that *Psoralea corylifolia L.* may be a potential drug candidate for the treatment of osteoporosis.

However, there are some limitations to this study. First, we only measured the effects of two components, psoralen and bakuchiol, on osteoclast differentiation and bone resorption *in vitro.* Whether administration of psoralen and bakuchiol at clinical dosage can exert anti-osteoporosis effects in animals and humans needs further investigation. Second, phosphorylation and translocation of the classical NF-κB subunit p65 was not observed on day eight during bone resorption. More time points are therefore needed to determine the mechanism of bone resorption for psoralen and bakuchiol.

In conclusion, this study shows that psoralen and bakuchiol ameliorate M-CSF plus RANKL-induced osteoclasts differentiation and bone resorption via inhibition of AKT and AP-1 pathways activation *in vitro*. Therefore, *Psorlea corylifolil L.* represents a promising

candidate herb for the development of a therapeutic approach to address bone resorptive diseases caused by soluble RANKL and M-CSF. Further studies to test and develop this therapeutic approach for resorptive diseases may be warranted.

Disclosure Statement

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