3-(4-Methoxyl)-1-(2-(4-coumarin)prop)-2-en-1-one inhibits the differentiation of osteoclasts

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Abstract

Background: Osteoclasts are large terminal-differentiated cells with multiple nuclei and are the only cells with bone resorption activity in the body. The abnormal migration and differentiation of osteoclasts may accelerate bone absorption, a crucial process in the occurrence and development of osteoporosis. Regulating the differentiation and activation of osteoclasts is a breakthrough point for the prevention and treatment of osteoporosis. Coumarin derivative of 3-(4-methoxyl)-1-(2-(4-coumarin) prop)-2-en-1-one (MCPEO) was selected in this study. We aimed to investigate the effects of (MCPEO) on osteoclast differentiation.

Methods: Bone marrow mononuclear cells (BM-MNCs) were collected from 6-week-old ICR mice and inoculated in vitro. BM-MNC and RAW264.7 cells were induced by the receptor activator of nuclear factor κ B ligand (RANKL) to differentiate them into osteoclasts. The cells were then added with 0.01, 0.05, 0.1, 0.5, and 1 μM MCPEO. The cell viability of differentiated osteoclasts was analyzed by methylthiazolyldiphenyl-tetrazolium bromide assay. The differentiated osteoclasts were detected by tartrate-resistant acid phosphatase (TRAP) staining. Miuse osteoclast activation was investigated by absorptive activity analysis. Filamentous actin (F-actin) staining was employed to identify the formation of F-actin rings in the differentiated mouse osteoclasts. The change level of critical transcription factors related to osteoclast differentiation was determined by Western blot analysis.

Results: Data show that MCPEO affected the cell viability of differentiated osteoclasts, inhibited the formation of TRAP-positive polynuclear cells, and decreased the absorption activity and the formation of F-actin rings in the differentiated osteoclasts. Furthermore, MCPEO influenced the change level of crucial transcription factors related to osteoclast differentiation.

Conclusions: MCPEO inhibited the differentiation of RANKL-induced BM-MNC and RAW264.7 cells into osteoclasts.

Keywords: 3-(4-methoxyl)-1-(2-(4-coumarin)prop)-2-en-1-one, bone marrow cell,

RAW264.7 cell, osteoclast, differentiation

Background

Bones are not quiescent tissues but persistent metabolic connective tissues. Old bone matrix is constantly replaced by newly formed bone matrix through a continuous process known as bone remodeling, which is critical to maintain bone mass and strength [1]. Osteoclasts are derived from hematopoietic stem cells and are responsible for the absorption of old bones. These cells are derived from mesenchymal stem cells by secreting and mineralizing the bone matrix and can reconstruct the resorbed bone mass. The synergy between these two types of cells maintains bone metabolism balance [2,3]. Disparity in bone metabolism may lead to various skeletal related diseases, such as osteoporosis, rheumatoid arthritis, multiple myeloma, and aseptic prosthesis, which may be caused by the pathological increase or hyperfunction of osteoclasts [4]. Bone marrow stem cells, monocytes, macrophages, and mononuclear macrophages in various normal and abnormal (infection and tumor) tissues in the hematopoietic mononuclear-macrophage system can form osteoclasts and be stimulated by the parathyroid hormone, macrophage colony-stimulating factor, tumor necrosis factor-alpha, lipopolysaccharide, and interleukin. Osteoclasts are large terminal-differentiated cells with multiple nuclei and are the only cells with bone resorption activity in the body [5]. The abnormal migration and differentiation of osteoclasts may accelerate bone absorption, a crucial process in the occurrence and development of osteoporosis. Regulating the differentiation and activation of osteoclasts is a breakthrough point for the prevention and treatment of osteoporosis [6].

Coumarin is commonly found in higher plants, animals, and microbial metabolites and exhibits the advantages of low molecular weight, relatively simple synthesis, and high bioavailability [7]. Coumarins, flavonoids, lignans, and stilbenes are classified as phytoestrogens because their structures and functions are similar to those of animal estrogens. The main biological activities of coumarins include anti-menopausal, anti-osteoporotic, anti-tumor, anti-cardiovascular diseases, anti-dementia, anti-inflammatory, and anti-coagulation activities [8]. However, most

of the bioactive mechanisms of coumarins and their derivatives remain unclear and unevaluated. For example, the mechanism of coumarins and their derivatives in inhibiting osteoporosis remains unknown [9]. Hence, scholars must continue to explore the biological activities of the effective ingredients in coumarins and their derivatives and synthesize and screen those with low toxicity for the treatment of several diseases.

In this study, the coumarin derivative of 3-(4-methoxyl)-1-(2-(4-coumarin) prop)-2-en-1-one (MCPEO) was selected. The effects of MCPEO on osteoclast differentiation were analyzed. Results provide a reference for the screening of therapeutic drugs for skeletal metabolic disorders.

Methods

Animals and cells

Six-week-old ICR mice were obtained from Qinglong Mountain Animal Breeding Farm, Jiangning District, Nanjing, Jiangsu Province, China (License number: SCXK 2017-0001) and were executed through cervical dislocation. The experiment was approved by the Ethics Committee Experimental Animal Ethics Branch of Bengbu Medical College. RAW264.7 cell (TCM13) was bought from the cell resource center of Chinese Academy of Sciences, Shanghai Life Sciences Institute (China).

Reagents

Minimum essential medium alpha (α-MEM) and fetal bovine serum (FBS) were obtained from GE Healthcare (Utah, USA). The receptor activator of nuclear factor κ B ligand (RANKL) was acquired from Peprotech Inc. (New Jersey, USA). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Solarbio Life Sciences (Beijing, China). Acid phosphatase kit 387-A (tartrate-resistant acid phosphatase [TRAP] staining kit) was provided by Sigma–Aldrich (Missouri, USA). Corning Osteo Assay Surface (COAS) was bought from Corning Inc. (New York,

USA). TRITC-conjugated phalloidin was supplied by Invitrogen Inc. (California, USA). NFATc1, c-Fos, c-Jun, p-c-Fos, and p-c-Jun primary antibodies were purchased from Cell Signaling Technology Inc. (Danvers, USA). The primary and secondary antibodies of β-actin were obtained from Santa Cruz Biotechnology (Texas, USA). Radio immunoprecipitation assay lysis buffer, bicinchoninic acid protein assay kit, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel superquick preparation kit, and nitrocellulose membrane were obtained from Beyotime Biotechnology (Shanghai, China).

Bone marrow mononuclear cell (BM-MNC) isolation, osteoclast induction, and MCPEO treatment

Six-week-old ICR mice were sacrificed through cervical dislocation and placed in 75% ethanol to instantaneously sterilize the body surface. The long bones of the limbs were separated, and the attached soft tissues were thoroughly removed. The bones were cleaned twice with aseptic PBS and placed in flat dishes. The joints of both ends of the medullary cavity were cut off, and the marrow cavity was washed repeatedly with serum-free α-MEM medium by using a 10 mL sterile syringe. Cell suspension was collected and centrifuged at 1000 rpm/min for 5 min. The cells were resuspended in a-MEM medium containing 15% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin, inoculated into six-well culture plates, and cultured overnight at 37 °C and 5% CO_2 under saturated humidity. The cell suspension was regarded as BM-MNCs. BM-MNC density was adjusted to 1×10^6 cells/mL and resuspended in α-MEM medium containing 100 ng/mL RANKL, which contained 15% IU/mL penicillin and 100 μg/mL streptomycin. BM-MNCs were seeded into 96-well culture plates (0.2 mL/well) for MTT and TRAP staining and into COAS (0.2 mL/well) for osteoclast absorption activity detection. BM-MNCs were seeded into 12-well culture plates (1 mL/well) for filamentous actin (F-actin) staining. After 7 days of cultivation, the cells in all groups were added with 0.01, 0.05, 0.1, 0.5, and 1 μM MCPEO and DMSO (solvent control, MCPEO was dissolved in DMSO) and cultured for another day.

RANKL induced osteoclast formation and MCPEO treatment

RAW264.7 cells were re-suspended in α-MEM medium containing 10% FBS, 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL/mL streptomycin. The cells were inoculated into 96-well culture plate (200 μL / well) for TRAP staining and MTT assay, and the cells were inoculated into six-well culture plate (2mL/ well) for total protein extraction and follow-up Western blot. At 4 h of incubation, RANKL was added into the culture. After another 3 days of induction, the cells in all groups were added with 0.01, 0.05, 0.1, 0.5, and 1 μM MCPEO and DMSO (solvent control, MCPEO was dissolved in DMSO) and cultured for another day.

Cell viability detection (MTT assay)

BM-MNC and RAW264.7 cells were induced by RANKL and treated with various MCPEO concentrations. After incubation, the culture medium was discarded, and the cells were washed three times with PBS. Serum-free DMEM containing 0.05 mg/mL MTT was added into each well, and the samples were incubated at 37 °C for 4 h. The liquid was removed, and 150 μL of DMSO was added into each well. After 10 s of shaking, the optical density was recorded at 595 nm by using an enzyme-labeled meter.

Detection of osteoclast formation by TRAP staining

BM-MNC and RAW264.7 cells were induced by RANKL and treated with various MCPEO concentrations. At the end of incubation, the culture medium was discarded, and the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. TRAP staining was performed in accordance with the manufacturer's instructions. Under the inverted microscope, five visual fields were randomly selected from each well to count more than three nuclei TRAP-positive cells labeled as differentiated osteoclasts.

Analysis of the absorption activity of differentiated osteoclasts

The BM-MNCs were induced by RANKL and treated with various MCPEO concentrations. After incubation, the medium was removed, the COAS culture plates

were washed with double-distilled water. The cells were cleaned using an ultrasonic cleaning instrument for 3 min. Hypochloric acid (10%) was added into each well of the plate, which was incubated at room temperature for 5 min. The COAS culture plates were washed with double-distilled water twice, air dried at room temperature for 3–5 h, and observed under an inverted microscope. Five visual fields were randomly selected for each well and photographed. The areas of absorption pits were calculated by image analysis software (version 1.0, JEDA).

F-actin staining for differentiated osteoclasts

The BM-MNCs were induced by RANKL and treated with various MCPEO concentrations. At the end of incubation, the medium was removed. The cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. The samples were washed three times with PBS, and 0.1% Triton X-100 was added for 5 min for cell membrane penetration. The samples were washed three times with PBS and blocked using 1% BSA for 30 min. F-actin staining was conducted according to the manufacturer's instructions. The cells were added with 50 μg/mL phalloidin-TRITC staining solution, incubated at room temperature in the dark for 20 min, washed three times with PBS, observed under an inverted fluorescence microscope, and photographed with red channel.

Western blotting assay

RAW264.7 cells were induced by RANKL and treated with various MCPEO concentrations. At the end of culture, the cells were collected, the total protein was extracted, and the concentration was measured and adjusted. The protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% skimmed milk at room temperature for 60 min. c-Fos, p-c-Fos, c-Jun, p-c-Jun, NFATc1, and β-actin primary antibodies were added into the nitrocellulose membrane and incubated overnight on ice. The nitrocellulose membrane was then washed with TBST, and the second antibody was added and incubated at room temperature. Enhanced chemiluminescence was

performed.

Statistical analysis

All experiments were repeated in triplicate, and data were presented as the mean ± standard error of the mean (SEM). The statistical differences between groups were evaluated by ANOVA, followed by Tukey's post-hoc test using SPSS v.17.0 software. Difference at $P < 0.05$ was considered statistically significant.

Results

MCPEO affected the viability of differentiated osteoclasts

MCPEO affected the cell viability of RANKL-induced BM-MNCs (differentiated osteoclasts). Compared with that in the control groups (RANKL + DMSO groups) (100% ± 27.92%), the cell viability of differentiated osteoclasts was significantly restrained in the 0.05 μM (66.48% ± 21.08%) ($P < 0.05$), 0.5 (41.24% ± 28.06%), and 1 μM (51.32% ± 22.49%) MCPEO groups ($P < 0.01$) (Fig. 1A).

MCPEO influenced the cell viability of differentiated osteoclasts from RAW264.7 cells. Compared with that in the control groups (RANKL + DMSO groups) (102.33% ± 14.36%), the cell viability of differentiated osteoclasts showed extremely significant depression in the 0.1 (82.80% ± 10.38%), 0.5 μM (82.48% ± 5.34%) ($P < 0.01$), and 1 μM (84.61% ± 12.46%) MCPEO groups ($P < 0.05$) (Fig. 1B).

MCPEO restricted the differentiation of osteoclasts

RANKL could induce the differentiation of BM-MNC and RAW264.7 cells into TRAP-positive cells (osteoclasts); however, the process was inhibited by MCPEO (Fig. 2A). For BM-MNC-induced osteoclast part, the number of TRAP-positive cells in the control groups (RANKL + DMSO groups) was 30.60 ± 6.50. Compared with the control groups, the 0.01 (29.40 ± 2.75) and 0.05 μM (19.47 ± 2.54) MCPEO groups did not exhibit a significantly decreased number of TRAP-positive cells.

Meanwhile, the number of TRAP-positive cells decreased markedly in the 0.1 (10.07 ± 1.10) and 0.5 μM (5.6 ± 0.72) MCPEO groups ($P < 0.05$) and in 1 μM (6.33 ± 1.50) MCPEO groups ($P < 0.01$) (Fig. 2B). For the RAW264.7 cell-induced osteoclasts, compared with the control groups (RANKL + DMSO groups) (21.47 ± 2.00), the 0.01 μM (21.53 ± 2.83) MCPEO groups did not considerably reduced the number of TRAP-positive cells. Nevertheless, the number of TRAP-positive cells decreased markedly ($P < 0.05$ or $P < 0.01$) in the 0.05 (15.80 ± 1.31), 0.1 (8.80 ± 0.35), 0.5 (5.00 ± 0.53), and 1 μM (5.40 ± 1.59) MCPEO groups (Fig. 2C).

MCPEO influenced the resorption activity of differentiated osteoclasts

RANKL could induce the differentiation of BM-MNCs into mature osteoclasts with absorptive activity, and the differentiated osteoclasts could erode the COAS and generate absorption pits at its bottom (Fig. 3A). The absorption pit area statistics shows that MCPEO inhibited the absorption activity of osteoclasts. Compared with the RANKL + DMSO groups (control groups) (143,960.66 ± 26,956.42 μm^2), the RANKL groups (184,386.66 ± 106,56.15 μm^2) did not remarkably affect the absorption activity of differentiated osteoclasts, indicating that DMSO had no effect on osteoclast activation. Compared with the control groups, the 0.01 μM (122,979.03 ± 477,20.22 μm^2) MCPEO groups did not notably decrease the absorption activity of osteoclasts. The absorption activity of osteoclasts in 0.05 (86993.32 ± 9277.47 μm^2), and 0.1 μM (90600.38 ± 10518.70 μm^2) MCPEO groups were significantly lower than that those in the control groups ($P < 0$ 05). Compared with the control group, the 0.5 (39846.56 ± 10193.40 μm^2) and 1 μM (18220.00 ± 9733.15 μm^2) MCPEO groups showed significant decrease ($P < 0.$ 05) in osteoclast absorption activity (Fig. 3B).

MCPEO affected the F-actin morphology in differentiated osteoclasts

The results of Phalloidin-TRITC staining showed that after 8 days of induction, the intact and regular F-actin rings appeared in the RANKL-stimulated BM-MNCs and in the RANKL + DMSO groups. MCPEO could inhibit the formation of F-actin rings in the differentiated osteoclasts. At specific concentrations (0.5 and 1 μM), this

derivative could inhibit osteoclast differentiation and reduce F-actin ring formation (Fig. 4).

Effects of MCPEO on the change level of critical transcription factors related to osteoclast differentiation

During the differentiation of RAW264.7 cells into osteoclasts induced by RANKL, the expression levels of NFATc1, p-c-Jun, p-c-Fos, and c-Fos, which are the key transcription factors of osteoclast differentiation, were upregulated. However, MCPEO could inhibit their expression, and the inhibitory effects became increasingly evident with the increased MCPEO concentrations (Fig.5).

Discussion

In this study, MCPEO could inhibit the differentiation of osteoclasts from RANKL-induced BM-MNC and RAW264.7 cells, prevent the formation of TRAP-positive multinucleated cells, reduce the absorption activity of differentiated osteoclasts, and hinder the formation of F-actin rings in differentiated osteoclasts. Furthermore, MCPEO influenced the change level of critical transcription factors related to osteoclast differentiation.

Patients with osteoporosis are characterized with decreased bone density and increased fracture risk. Current treatments to promote bone synthesis include the use of estrogen receptor analogues and bisphosphonates as anti-bone resorption agents and specific drugs such as parathyroid hormones. However, given the side effects, some defects occur in the two schemes above [10]. Therefore, developing new anti-osteoporosis drugs is urgent.

Coumarin derivatives can influence bone metabolism by regulating the differentiation of osteoblasts or osteoclasts, which show anti-osteoporosis effects. For example, osthole, a coumarin derivative from Chinese herbal medicine, can stimulate the differentiation of rat skull osteoblasts through bone morphogenetic protein (BMP)-2/ p38MAPK/Runx-2/osterix pathway [11]. In addition, osthole considerably promotes the formation of new bones on the surface of mouse skull through the

β-catenin-BMP signaling pathway. Bone loss occurs in ovariectomized rats, and osthole can solve this problem by improving bone microstructure, tissue morphological parameters, and biomechanical properties [12]. Imperatorin and bergapten are coumarin derivatives that can increase the expression level of BMP-2 and promote bone formation through the p38 and ERK-dependent signaling pathways [13]. Coumarin–dihydropyridine hybrids can promote the formation of osteoblast in vitro and inhibit bone resorption in ovariectomized animal models. These hybrids can increase bone mineral density and volume, promote the expression of osteogenic gene, and increase bone formation and mineral adhesion rates [14]. Psoralen is a coumarin derivative that is extracted from Chinese herbal medicine, promotes the differentiation of osteoblasts in mouse skull by activating BMP signaling pathways, increases the expression level of osteoblast-specific genes, and enhances the activity of alkaline phosphatase [12]. Coumarin compounds, such as aesculin [15], wedelolactone [16], osthole [17,18], and cinnamoyloxy-mammeisin [19], can inhibit the differentiation and activation of osteoclasts and potentially treat osteoporosis.

The anti-osteoporosis biological activities of coumarin derivatives provide additional options for the treatment of bone metabolic disorders. MCPEO was synthesized in our laboratory, and its biological activity was preliminarily explored. To provide the bases for the treatments of skeletal metabolic disorders, we determined the effects of MCPEO on osteoclast differentiation.

Osteoclasts have high activity of TRAP enzyme, which is involved in the bone resorption of these cells and can be used as a marker of their activity. The serum TRAP enzyme level is considerably increased due to the decreased estrogen level. Bone resorption activity is stronger than bone formation in menopausal women. In addition, serum TRAP enzyme level is remarkably increased in patients with primary osteoporosis caused by aging and is negatively correlated with bone density. TRAP enzyme level can be used as a monitoring index for primary osteoporosis. TRAP staining is the simplest direct method to identify osteoclast differentiation. For TRAP staining, the cytoplasm of the osteoclasts is wine red, and the characteristics of multiple nuclei are evident. MCPEO can inhibit the differentiation of BM-MNCs into

TRAP positive cells (osteoclasts). The results were consistent with the inhibitory effects of other coumarin derivatives on osteoclast differentiation [17,19].

Mature osteoclast plays a unique role in bone degradation, a physiological process that regulates bone tissue development and bone remodeling. Osteoclast is absorbed and migrates on the bone surface until apoptosis [20]. Sealing zones are first assembled in the osteoclast, which consists of dense podosomes, to form the F-actin rings and immobilize the cell on the surface of mineralized matrix. Podosomes and F-actin rings are rich and evident in normal osteoclasts. In the sealing zone, the plasma membrane of osteoclasts that is in contact with the bone is rearranged into a ruffled border. Proton and chloride ions are secreted from the cells, acidify the extracellular media, and dissolve the hydroxyapatite (inorganic components) in the bone. Acid proteases, such as cathepsin K and MMP9, and phosphatase, such as TRAP, are also produced by osteoclast and further degrade bone matrix proteins (organic components). Osteoclasts form new adhesion structures on the bone surface that are adjacent to the existing resorption lacunae to continuously exert its absorption activity and are repolarized to complete their bone resorption function [21,22]. MCPEO can suppress the bone resorption activity by inhibiting the formation of F-actin rings in osteoclasts. The results were consistent with the inhibitory effects of coumarin derivatives, such as wedelolactone and osthole, on osteoclast activation [16,18].

RANKL binds to the receptor activator of nuclear factor kappa B, which is located on the surface of osteoclast precursor. The intracellular domain of RANK subsequently recruits the TNF receptor-activating factor (TRAF) family, such as TRAF2/6. Activated TRAF stimulates the key transcription factors related to osteoclast differentiation and functions, such as AP-1 family (Fos/Jun) and activated T nuclear factor (NFATc1). These transcription factors induce the expression of osteoclast-related genes. NFATc1 is the master transcription factor in osteoclast differentiation. NFATc1 with AP-1 (Fos/Jun), PU.1, and microphthalmia-associated transcription factor form specific transcription complexes, which then act in the promoter region of genes, such as TRAP, CTR, cathepsin K, β 3 integrin, and OSCAR,

which are related to the osteoclast attachment, migration, acidification, and degradation of bone matrix [23,24]. This study indicated that MCPEO affected the expression of NFATc1 and AP1 family proteins during osteoclast differentiation.

Conclusions

MCPEO can inhibit osteoclast differentiation specifically by preventing the formation of TRAP-positive cells, reducing the absorptive capacity of osteoclasts, inhibiting the formation of F-actin rings, and affecting the change level of key transcription factors related to osteoclast differentiation.

Abbreviations

RANKL: receptor activator for nuclear factor-κ B ligand; MCPEO: 3-(4-methoxyl)-1-(2-(4-coumarin)prop)-2-en-1-one; BM-MNCs: bone marrow mononuclear cells; TRAP: tartrate-resistant acid phosphatase; COAS: corning osteo assay surface; TRAF: TNF receptor-activating factor; NFATc1: activated T nuclear factor1.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Authors' contributions

FYX, WYH, NDQ and XW cultured the cells, helped to design the study, analysed and interpreted the results, and was a major contributor in the writing of the manuscript. TBD, MYJ, HZY and GZ conducted the MTT assay, tartrate-resistant acid phosphatase staining, and osteoclast absorption activity assay. MQ, YTT and WXD performed the (F)-actin staining. SJH, LYH, and ZYN collected the total protein and conducted the western blot analysis. WX and FJX cultured the cells. HYJ provided experimental ideas, analysed and interpreted the results, and was a major contributor in the writing of the manuscript. All authors read and approved the final manuscript.

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References

1. Zhao B. TNF and Bone Remodeling. Curr Osteoporos Rep, 2017; 15: 126-134.
2. Teitelbaum, SL. Bone resorption by osteoclasts. Science, 2000; 289: 1504-1508.

3. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature, 2003; 423: 349-355.
4. Katsimbri P. The biology of normal bone remodelling. Eur J Cancer Care (Engl), 2017; 26: 1-5.
5. Boyle WJ, Simonet WS, Lacey, D.L. Osteoclast differentiation and activation. Nature, 2003; 423: 337-342.
6. Rachner TD, Khosla S, Hofbauer LC. Osteoporosis: now and the future. Lancet, 2011; 377: 1276-1287.
7. Perumalsamy H, Sankarapandian K, Veerappan K, Natarajan S, Kandaswamy N, Thangavelu L, et al. In silico and in vitro analysis of coumarin derivative induced anticancer effects by undergoing intrinsic pathway mediated apoptosis in human stomach cancer. Phytomedicine, 2018; 46: 119-130.
8. van Duursen MBM. Modulation of estrogen synthesis and metabolism by phytoestrogens in vitro and the implications for women's health. Toxicol Res (Camb), 2017; 6: 772-794.
9. Ibrar A, Shehzadi SA, Saeed F, Khan I. Developing hybrid molecule therapeutics for diverse enzyme inhibitory action: Active role of coumarin-based structural leads in drug discovery. Bioorg Med Chem, 2018; 26: 3731-3762.
10. Haas, A.V. & LeBoff, M.S. Osteoanabolic Agents for Osteoporosis. J Endocr Soc, 2018; 2: 922-932.
11. Ming LG, Zhou J, Cheng GZ, Ma HP, Chen KM. Osthol, a coumarin isolated from common cnidium fruit, enhances the differentiation and maturation of osteoblasts in vitro. Pharmacology, 2011; 88: 33-43.
12. Tang DZ, Hou W, Zhou Q, Zhang M, Holz J, Sheu TJ, et al. Osthole stimulates osteoblast differentiation and bone formation by activation of beta-catenin-BMP signaling. J Bone Miner Res, 2010; 25: 1234-1245.
13. Tang CH, Yang RS, Chien MY, Chen CC, Fu WM. Enhancement of bone morphogenetic protein-2 expression and bone formation by coumarin derivatives via p38 and ERK-dependent pathway in osteoblasts. Eur J Pharmacol, 2008; 579: 40-49.

14. Sashidhara KV, Kumar M, Khedgikar V, Kushwaha P, Modukuri RK, Kumar A, et al. Discovery of coumarin-dihydropyridine hybrids as bone anabolic agents. J Med Chem, 2013; 56: 109-122.
15. Zhao XL, Chen LF, Wang Z. Aesculin modulates bone metabolism by suppressing receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis and transduction signals. Biochem Biophys Res Commun, 2017; 488: 15-21.
16. Hsieh CJ, Kuo PL, Hou MF, Hung JY, Chang FR, Hsu YC, et al. Wedelolactone inhibits breast cancer-induced osteoclastogenesis by decreasing Akt/mTOR signaling. Int J Oncol, 2015; 46: 555-562.
17. Kuo PL, Hsu YL, Chang CH, Chang JK. Osthole-mediated cell differentiation through bone morphogenetic protein-2/p38 and extracellular signal-regulated kinase 1/2 pathway in human osteoblast cells. J Pharmacol Exp Ther, 2005; 314: 1290-1299.
18. Li X, Xue C, Wang L, Tang D, Huang J, Zhao Y, et al. Osteoprotective effects of osthole in a mouse model of 5/6 nephrectomy through inhibiting osteoclast formation. Mol Med Rep, 2016; 14: 3769-3776.
19. da Cunha MG, Ramos-Junior ES, Franchin M, Taira TM, Beutler JA, Franco GCN, et al. Effects of Cinnamoyloxy-mammeisin from Geopropolis on Osteoclast Differentiation and Porphyromonas gingivalis-Induced Periodontitis. J Nat Prod, 2017; 80: 1893-1899.
20. Saxena R, Pan G, McDonald JM. Osteoblast and osteoclast differentiation in modeled microgravity. Ann N Y Acad Sci, 2007; 1116: 494-498.
21. Georgess D, Machuca-Gayet I, Blangy A, Jurdic P. Podosome organization drives osteoclast-mediated bone resorption. Cell Adh Migr, 2014; 8: 191-204.
22. Touaitahuata H, Blangy A, Vives V. Modulation of osteoclast differentiation and bone resorption by Rho GTPases. Small GTPases, 2014; 5: e28119.
23. Metzger CE, Narayanan SA. The Role of Osteocytes in Inflammatory Bone Loss. Front Endocrinol (Lausanne), 2019; 10: 285.
24. Tanaka Y. Clinical immunity in bone and joints. J Bone Miner Metab, 2019; 37: 2-8.

Figure legends

Fig. 1 Influence of MCPEO on the cell viability of RANKL-induced BM-MNC and RAW264.7 cells.

(A, B) BM-MNCs were resuspended in α-MEM medium with RANKL and seeded into 96-well culture plates for 7 days. RAW264.7 cells were resuspended in α-MEM medium with RANKL and seeded into 96-well culture plates for 3 days. Various MCPEO concentrations were added into each group and incubated overnight. After cultivation, the samples were treated, the OD value of each group was measured, and the relative cell viability was calculated. The results indicated that MCPEO inhibited the cell viability of RANKL-induced BM-MNC and RAW264.7 cells (differentiated osteoclasts). The results are expressed as mean ± S.E.M. $^{**}P < 0.01$, $^{*}P < 0.05$ versus control groups (con).

Fig. 2 Effects of MCPEO on the osteoclast differentiation from RANKL-stimulated BM-MNC and RAW264.7 cells.

(A) BM-MNCs were resuspended in α-MEM medium with RANKL and seeded into 96-well culture plates for 7 days. RAW264.7 cells were resuspended in α-MEM medium with RANKL and seeded into 96-well culture plates for 3 days. Various MCPEO concentrations were added into each group and incubated overnight. After incubation, TRAP staining was conducted. TRAP-positive multinucleated cells (TRAP+) were photographed. (B) TRAP+ cells in RANKL-stimulated BM-MNCs in various groups were counted and compared. (C) TRAP+ cells in RANKL-stimulated RAW264.7 cells in various groups were counted and compared. The results are expressed as mean ± SEM $^{**}P<0.01$, $^{*}P < 0.05$ versus RANKL + DMSO (con). Original magnification: 100×.

Fig. 3 MCPEO influenced the resorption activity of RANKL-stimulated BM-MNCs (differentiated osteoclasts).

(A) BM-MNCs were seeded into COAS plates, and various MCPEO

concentrations were added into the cells after 7 days of RANKL pretreatment. After cultivation, cells were cleaned from the COAS, and pit formations were observed and photographed. (B) Resorption lacunae area was calculated and compared. The results are expressed as mean ± SEM $^{**}P < 0.01$, $^{*}P < 0.05$ versus RANKL + DMSO (con). Original magnification: 100×.

Fig. 4 MCPEO influenced the F-actin ring formation in RANKL-stimulated BM-MNCs (differentiated osteoclasts).

BM-MNCs were seeded into 12-well plates, and various MCPEO concentrations were added into the cells after 7 days of RANKL pretreatment. After cultivation, TRITC-conjugated phalloidin staining was performed. F-actin rings were observed and photographed. Original magnification: 100×.

Fig. 5 MCPEO affected the change level of the crucial transcription factors during the osteoclastogenesis.

RAW264.7 cells were resuspended in α-MEM medium with RANKL and seeded into six-well culture plates for 3 days. Various MCPEO concentrations were added into each group and incubated overnight. At the end of cultivation, the total protein was collected, and the change level of the crucial transcription factors was detected by Western blot analysis.

Fig.1

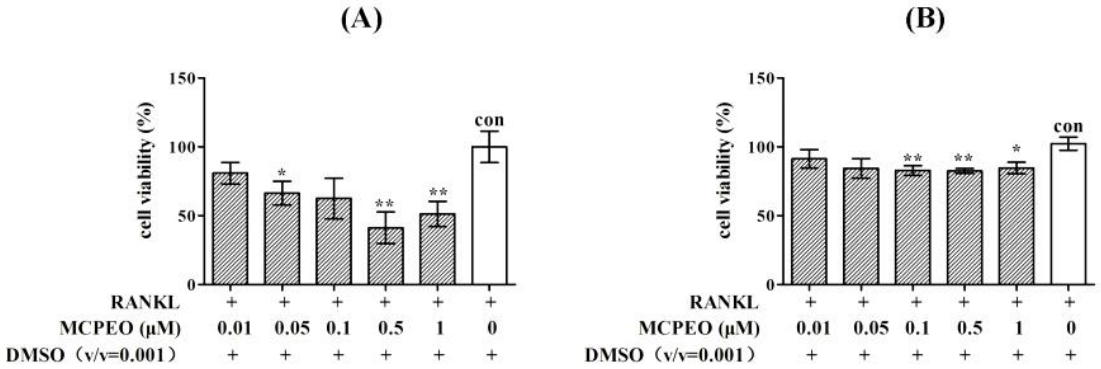

(A)
(B)
cell viability (%)
150
100
50
0
con
*
**
RANKL + + + + + +
MCPEO (μM) 0.01 0.05 0.1 0.5 1 0
DMSO（v/v=0.001） + + + + + +

Fig.2

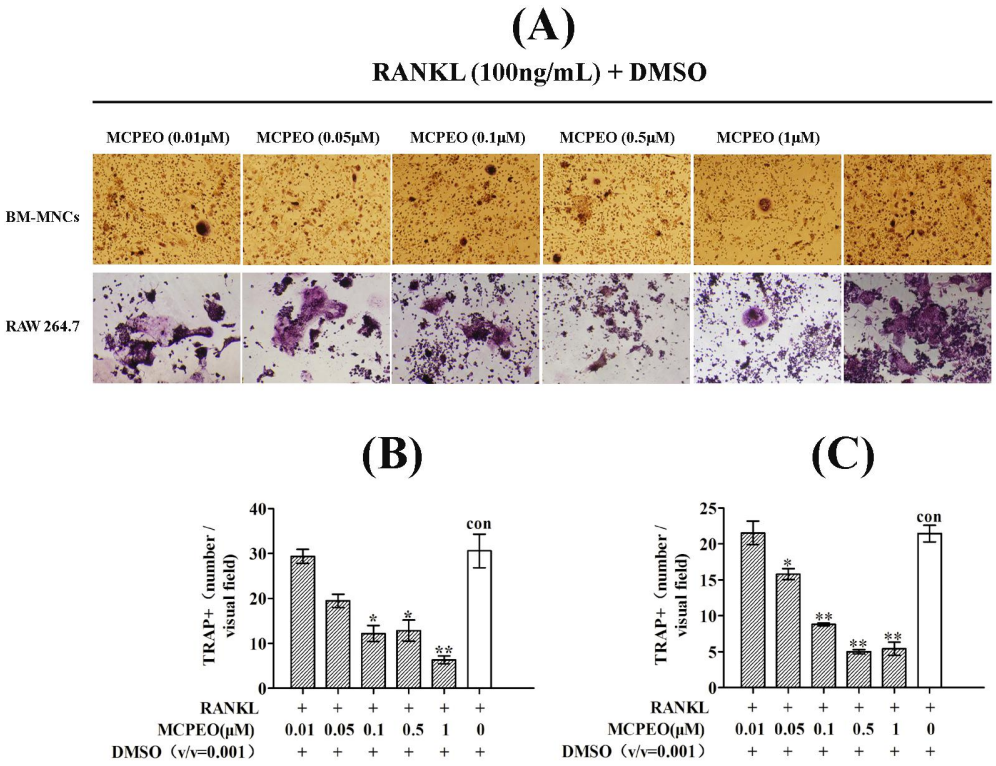

(A)
RANKL (100ng/mL) + DMSO
MCPEO (0.01μM)
MCPEO (0.05μM)
MCPEO (0.1μM)
MCPEO (0.5μM)
MCPEO (1μM)
BM-MNCs
RAW 264.7
(B)
TRAP+（number / visual field)
con
RANKL + + + + + +
MCPEO(μM) 0.01 0.05 0.1 0.5 1 0
DMSO（v/v=0.001） + + + + + +
(C)
TRAP+（number / visual field)
con
RANKL + + + + + +
MCPEO(μM) 0.01 0.05 0.1 0.5 1 0
DMSO（v/v=0.001） + + + + + +

Fig.3

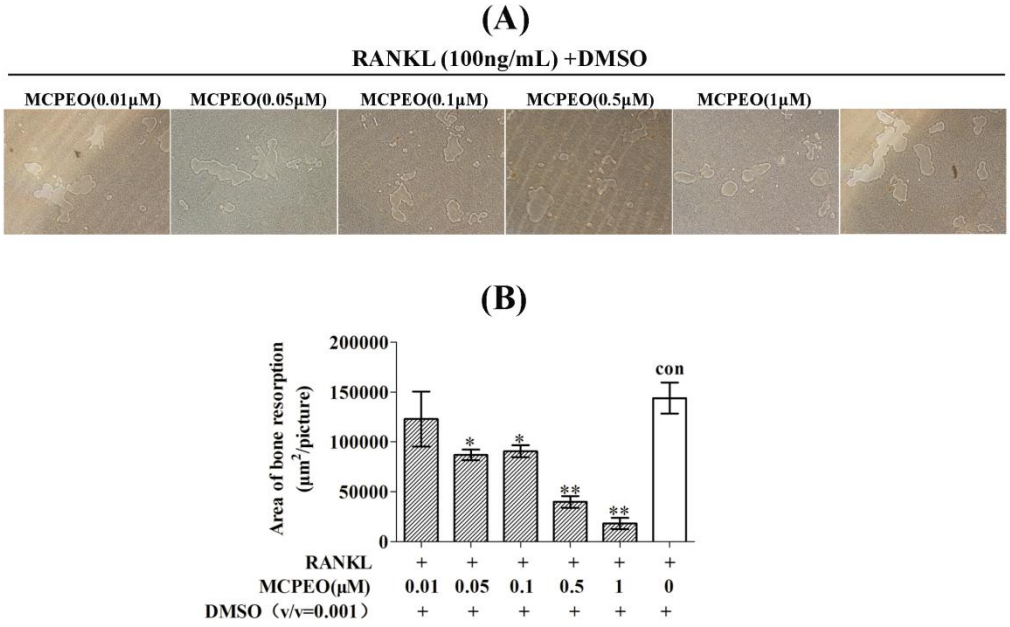

(A)
RANKL (100ng/mL) +DMSO
MCPEO(0.01μM)
MCPEO(0.05μM)
MCPEO(0.1μM)
MCPEO(0.5μM)
MCPEO(1μM)
(B)
Area of bone resorption (μm²/picture)
200000
150000
100000
50000
0
con
*
*
**
**
RANKL + + + + + +
MCPEO(μM) 0.01 0.05 0.1 0.5 1 0
DMSO（v/v=0.001） + + + + + +

Fig.4

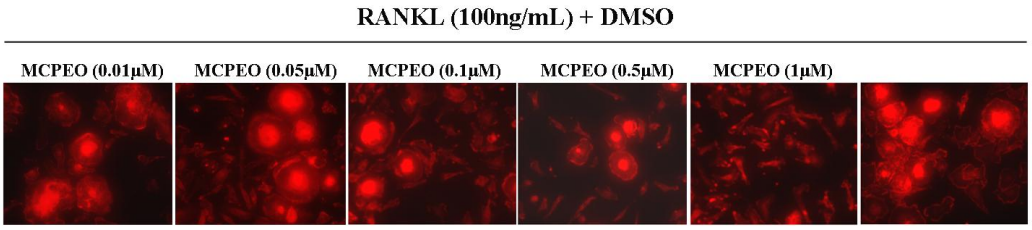
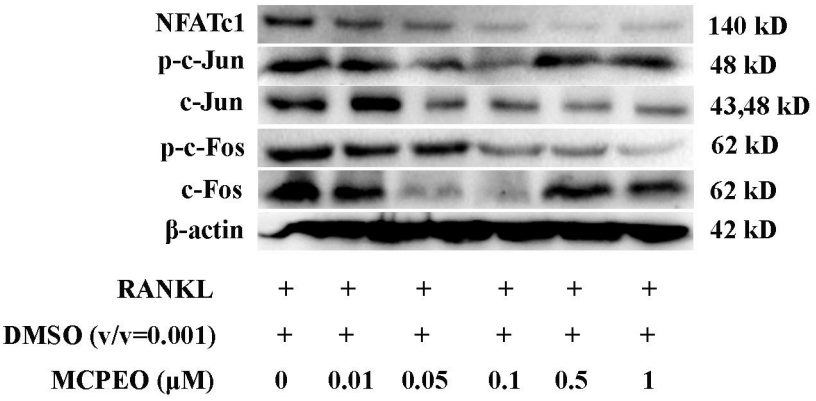

RANKL (100ng/mL) + DMSO
MCPEO (0.01μM)
MCPEO (0.05μM)
MCPEO (0.1μM)
MCPEO (0.5μM)
MCPEO (1μM)

Fig.5


NFATc1
p-c-Jun
c-Jun
p-c-Fos
c-Fos
β-actin
140 kD
48 kD
43,48 kD
62 kD
62 kD
42 kD
RANKL + + + + + +
DMSO (v/v=0.001) + + + + + +
MCPEO (μM) 0 0.01 0.05 0.1 0.5 1

Figures

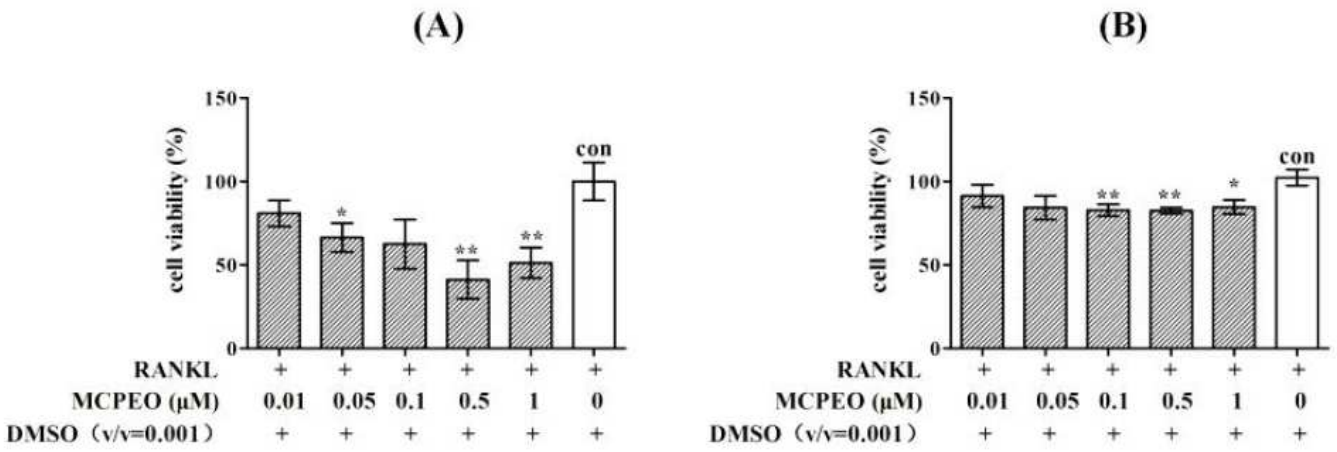


Figure 1

Influence of MCPEO on the cell viability of RANKL-induced BM-MNC and RAW264.7 cells. (A, B) BM-MNCs were resuspended in α-MEM medium with RANKL and seeded into 96-well culture plates for 7 days. RAW264.7 cells were resuspended in α-MEM medium with RANKL and seeded into 96-well culture plates for 3 days. Various MCPEO concentrations were added into each group and incubated overnight. After cultivation, the samples were treated, the OD value of each group was measured, and the relative cell viability was calculated. The results indicated that MCPEO inhibited the cell viability of RANKL-induced BM-MNC and RAW264.7 cells (differentiated osteoclasts). The results are expressed as mean ± S.E.M. **P < 0.01, *P < 0.05 versus control groups (con).

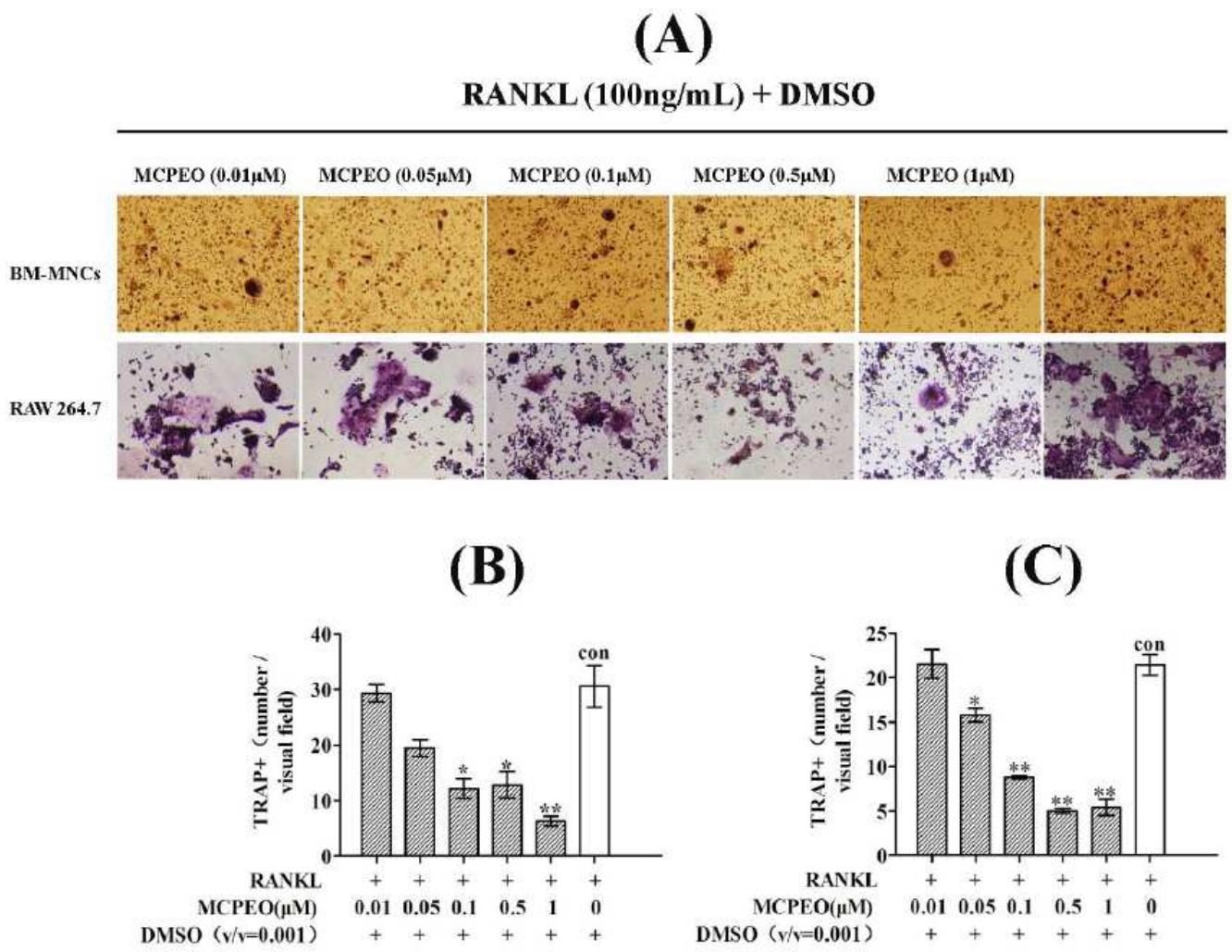


Figure 2

Effects of MCPEO on the osteoclast differentiation from RANKL-stimulated BM-MNC and RAW264.7 cells. (A) BM-MNCs were resuspended in α-MEM medium with RANKL and seeded into 96-well culture plates for 7 days. RAW264.7 cells were resuspended in α-MEM medium with RANKL and seeded into 96-well culture plates for 3 days. Various MCPEO concentrations were added into each group and incubated overnight. After incubation, TRAP staining was conducted. TRAP-positive multinucleated cells (TRAP+) were photographed. (B) TRAP+ cells in RANKL-stimulated BM-MNCs in various groups were counted and compared. (C) TRAP+ cells in RANKL-stimulated RAW264.7 cells in various groups were counted and compared. The results are expressed as mean ± SEM **P<0.01, *P < 0.05 versus RANKL + DMSO (con). Original magnification: 100×.

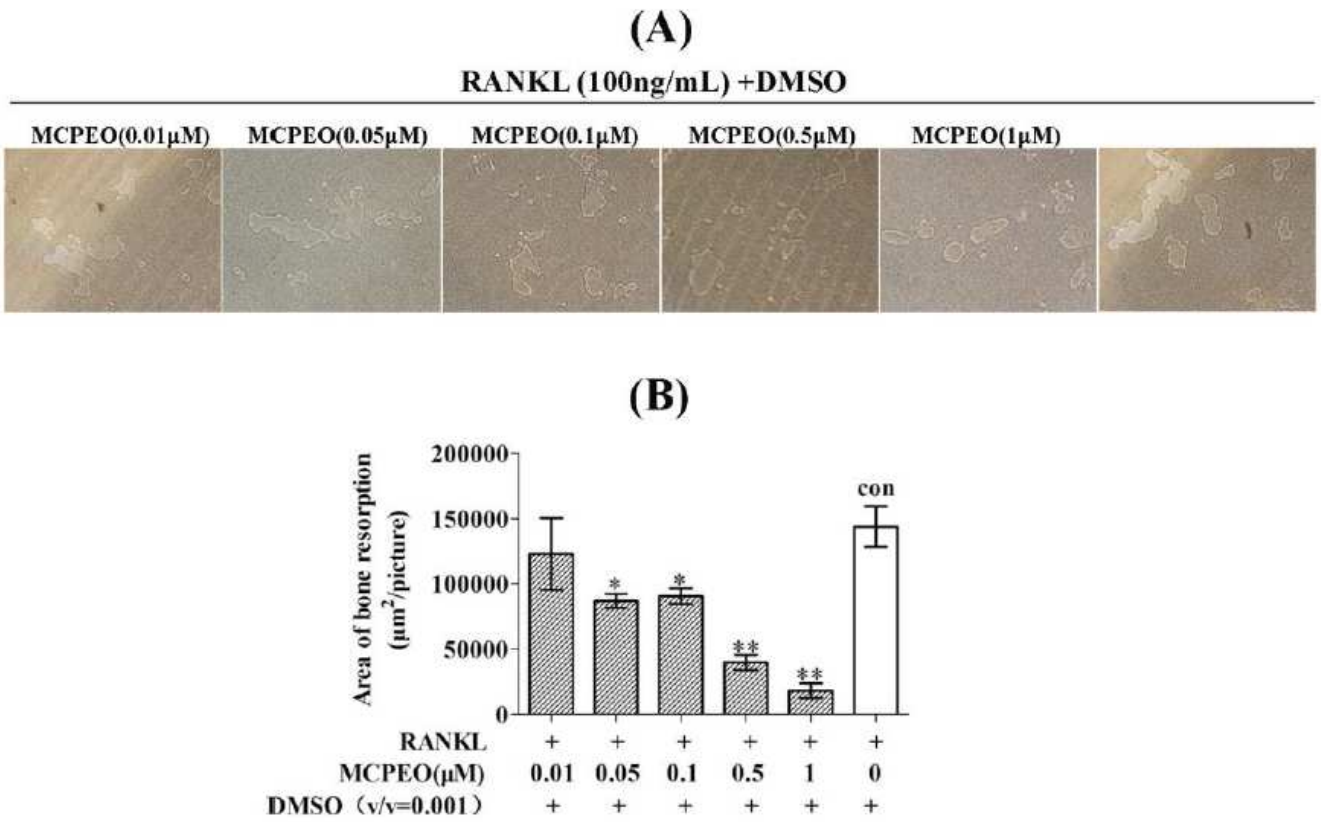


Figure 3

MCPEO influenced the resorption activity of RANKL-stimulated BM-MNCs (differentiated osteoclasts). (A) BM-MNCs were seeded into COAS plates, and various MCPEO concentrations were added into the cells after 7 days of RANKL pretreatment. After cultivation, cells were cleaned from the COAS, and pit formations were observed and photographed. (B) Resorption lacunae area was calculated and compared. The results are expressed as mean ± SEM **P < 0.01, *P < 0.05 versus RANKL + DMSO (con). Original magnification: 100×.

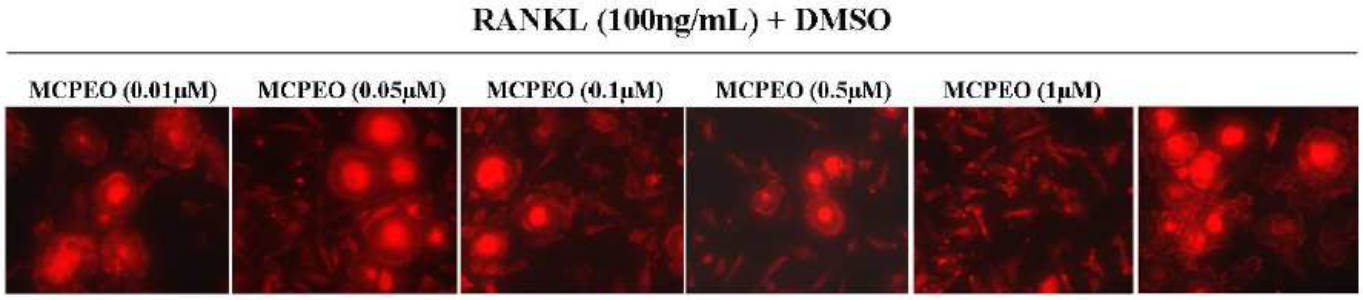


Figure 4

MCPEO influenced the F-actin ring formation in RANKL-stimulated BM-MNCs (differentiated osteoclasts). BM-MNCs were seeded into 12-well plates, and various MCPEO concentrations were added into the cells after 7 days of RANKL pretreatment. After cultivation, TRITC-conjugated phalloidin staining was performed. F-actin rings were observed and photographed. Original magnification: 100×.

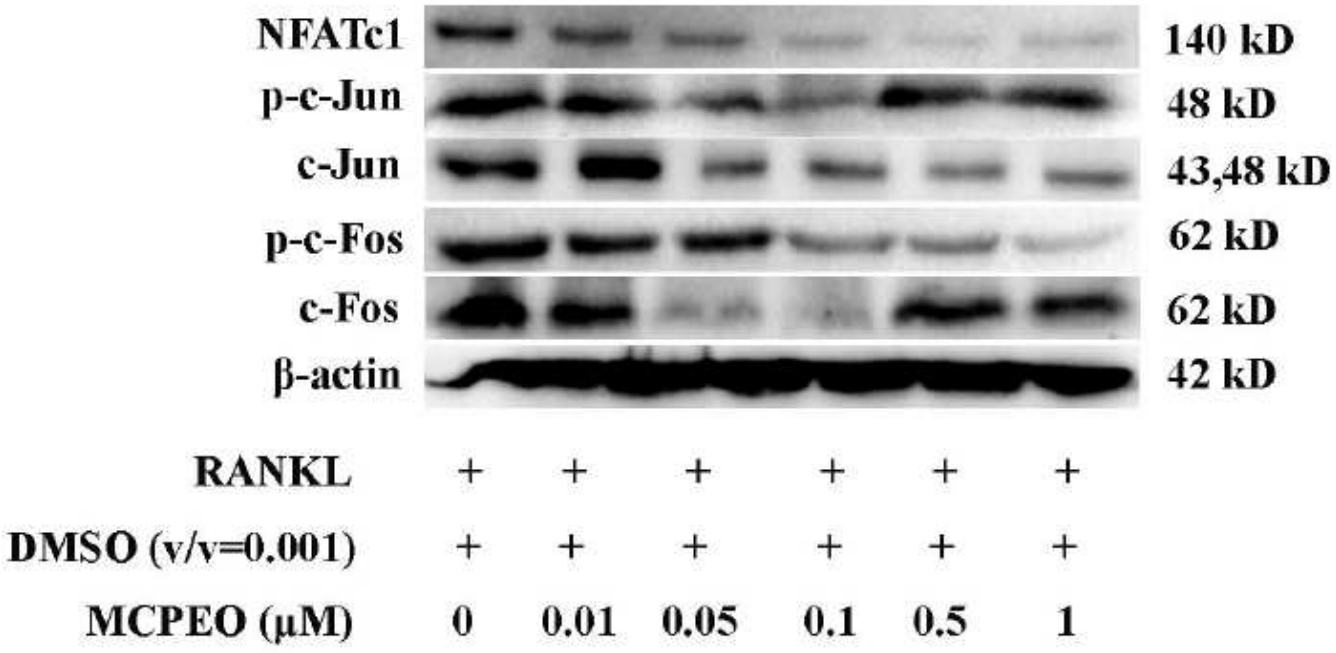


Figure 5

MCPEO affected the change level of the crucial transcription factors during the osteoclastogenesis. RAW264.7 cells were resuspended in α-MEM medium with RANKL and seeded into six-well culture plates for 3 days. Various MCPEO concentrations were added into each group and incubated overnight. At the end of cultivation, the total protein was collected, and the change level of the crucial transcription factors was detected by Western blot analysis.