Original Paper

Monocrotaline Suppresses RANKL-Induced Osteoclastogenesis *In Vitro* and Prevents LPS-Induced Bone Loss *In Vivo*

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Key Words

Monocrotaline • Osteolysis • Osteoclast • MAPK signaling pathways

Abstract

Background/Aims: Extensive osteoclast formation plays a critical role in bone diseases, including rheumatoid arthritis, periodontitis and the aseptic loosening of orthopedic implants. Thus, identification of agents that can suppress osteoclast formation and bone resorption is important for the treatment of these diseases. Monocrotaline (Mon), the major bioactive component of *crotalaria sessiliflora* has been investigated for its anti-cancer activities. However, the effect of Mon on osteoclast formation and osteolysis is not known. **Methods:** The bone marrow macrophages (BMMs) were cultured with M-CSF and RANKL followed by Mon treatment. Then the effects of Mon on osteoclast differentiation were evaluated by counting TRAP (+) multinucleated cells. Moreover, effects of Mon on hydroxyapatite resorption activity of mature osteoclast were studied through resorption areas measurement. The involved potential signaling pathways were analyzed by performed Western blotting and quantitative real-time PCR examination. Further, we established a mouse calvarial osteolysis model to measure the osteolysis suppressing effect of Mon *in vivo*. **Results:** In this study, we show that Mon can inhibit RANKL-induced osteoclast formation and function in a dose-dependent manner. Mon inhibits the expression of osteoclast marker genes such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K. Furthermore, Mon inhibits RANKL-induced the activation of p38 and JNK. Consistent with *in vitro* results, Mon exhibits protective effects in an *in vivo* mouse model of LPS-induced calvarial osteolysis. **Conclusion:** Taken together our data demonstrate that Mon may be a potential prophylactic anti-osteoclastic agent for the treatment of osteolytic diseases caused by excessive osteoclast formation and function.

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Introduction

Bone homeostasis is tightly regulated by the bone formation of osteoblast (OB) and bone resorption of osteoclast (OC) [1-4]. In this process, extensive osteoclast formation plays a critical role in bone diseases such as periodontitis and [5, 6], rheumatoid arthritis [7] and the aseptic loosening of orthopedic implants [8, 9]. Thus, the agents which inhibit excessive OC formation and bone resorption are candidates for the prevention and treatment of pathological bone loss.

OCs are multinucleated giant cells and differentiated from cells of hematopoietic monocyte/macrophage lineage under the presence of two critical factors: the receptor activator of NF- κ B ligand (RANKL) and the macrophage/monocyte colony-stimulating factor (M-CSF) [10]. RANKL is a member of the tumor necrosis factor (TNF) family [11]. Binding of RANKL to RANK results in the recruitment of the TNF receptor-associated factor 6 (TRAF6). Then, TRAF6 activates several downstream targets, including nuclear factor- κ B (NF- κ B), p38 mitogen-activated protein kinase (p38), c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). Activation of these downstream targets triggers the expression of genes regulating OC differentiation and function, including cathepsin K, calcitonin receptor (CTR), tartrate-resistant acid phosphatase (TRAP), and nuclear factor of activated T cells c1 (NFATc1) [12-14].

RANKL is now considered as an attractive target for the treatment of pathological bone lysis, because suppression of RANKL using anti-bodies, peptides, and natural compounds could inhibit OC formation and function. The present study aims to research the effect of natural compounds on suppressing RANKL-induced OC formation and function. Many plant-derived natural compounds have been used in traditional medicine for the treatment of numerous diseases. Monocrotaline (Mon), the main bioactive component isolated from the plant *Crotalaria sessiliflora*, was found to have a therapeutic effect on cancers, such as skin cancer and cervical cancer [15]. Mon is considered to be an alkylating agent, which plays an anticancer effect by alkylation of DNA [16]. In addition, high dose of Mon is used to induce hypertension in animal models [17]. However, its roles in OC formation and lipopolysaccharide (LPS)-induced osteolysis are not yet known.

In the present study, we reported that Mon could attenuate RANKL-induced OC formation (*in vitro*) and LPS-induced bone loss models (*in vivo*). In addition, *in vitro* studies indicated that Mon suppresses RANKL-induced OC differentiation by inhibiting the p38 and ERK signaling pathways. From the *in vivo* data obtained by micro-CT and histology analysis, we further confirmed that Mon significantly prevented LPS-induced severe bone loss by inhibiting OC number.

Materials and Methods

Reagents, cells and anti-bodies

Mon was purchased from Solarbio (Beijing, China). Fetal bovine serum (FBS) and cell culture medium were obtained from Gibco-BRL (Sydney, Australia). Recombinant mouse M-CSF and RANKL were purchased from R&D Systems (Minneapolis, MN, USA). TRAP staining kit was obtained from Sigma (St. Louis, MO, USA). TRIZOL reagent was bought from Invitrogen. PrimeScript™ RT reagent Kit was obtained from TaKaRa Biotechnology. RAW264.7 cells were purchased from Cell Bank (Chinese Academy of Sciences, Shanghai, China). The inhibitors of protease and phosphatase were purchased from Solarbio (Beijing, China). Antibodies for p-p38, p38, p-JNK, JNK, p-ERK, ERK, p-NF- κ B p65, NF- κ B p65, I κ B α and β -actin were obtained from Cell Signaling Technology (MAPK Family Antibody Sampler Kit #9926T, Phospho-MAPK Family Antibody Sampler Kit #9910T, and NF- κ B Family Antibody Sampler Kit #9936).

MTS assay

The cell viability effect of Mon was determined by the MTS cytotoxicity assay (Thermo Fisher Scientific, USA). BMMs were seeded in 96-well plates at a density of 4000 cells/well and incubated with M-CSF (30

ng/mL) in different concentrations of Mon for another 48 and 96 hours. BMSCs were cultured in 96-well plates in complete DMEM at a density of 3000 cells/well with increasing concentrations of Mon for 48 and 96 hours. At the end of the experiment, MTS solution (10 μ L) was added to each well and incubated at 37 °C for 2 hours, the absorbance was measured by a 96-well microplate reader (Bio-tek Instruments Inc., USA).

Osteoclast differentiation and TRAP staining assay in vitro

Bone marrow macrophages (BMMs) were obtained by flushing the tibiae and femurs of 6-week-old C57/BL6 mice with α -MEM. The bone marrow cells were suspended in α -MEM containing 10% (v/v) FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin and incubated for 3 days in the presence of 30 ng/mL M-CSF. Adherent cells were used as BMMs. In order to generate OCs, BMMs were cultured in α -MEM supplemented with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the absence or presence of Mon (1, 2 or 4 μ M) for 4 days. The cells were fixed in 4 % paraformaldehyde for 15 minutes, and the osteoclast formation was investigated by TRAP staining kit according to the manufacturer's instruction. TRAP (+) cells were observed under a light microscope (Olympus, Tokyo, Japan), and multinucleated (≥ 3 nuclei) cells were counted as OC cells.

Hydroxyapatite resorption assay

To perform the hydroxyapatite resorption assay, BMMs were cultured on to six-well collagen-coated culture plates at a density of 1.5×10^5 cells/well. Cells were allowed to adhere overnight and then stimulated with 100 ng/mL RANKL and 30 ng/mL M-CSF until osteoclasts began to form. The cells were gently harvested using cell dissociation solution and equal numbers of multinucleated cells were seeded onto hydroxyapatite-coated 48-well plate (COSMO BIO CO., LTD. TOKYO, JAPAN). Multinucleated cells were treated with M-CSF (30 ng/mL), RANKL (100 ng/mL) and indicated concentrations of Mon until generate multinucleated osteoclasts. Then, the cells were sequentially cultured for another 24 h. Adherent cells were removed by incubating with 5% sodium hypochlorite for 5 min. The resorption areas were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Osteoblastic cell culture and assays

For OB differentiation analysis, the calvariae of neonatal mice were digested with 0.1% collagenase and 0.2% dispase, and the isolated cells were combined and cultured. To examine the effect of Mon on OB formation, the primary OBs were cultured with 50 μ g/mL ascorbic acid, 10 mM β -glycerophosphate and 10^{-7} mM dexamethasone (DXM) on the 24-well chambers, and the medium was added in 0, 1, 2 or 4 μ M Mon. ALP and Alizarin Red staining was performed using a staining kit (Beyotime Biotechnology Co. Ltd., Shanghai, China) on days 7 and 21 of culture according to the manufacturer's instructions.

RNA extraction and quantitative PCR assay

Total RNA was extracted from the cells using TRIzol® reagent according to the manufacturer's protocol. Equal amounts of total RNA were reverse-transcribed to cDNA using reverse transcriptase (Promega). Real-time PCR assays were carried out with Faststart Universal SYBR Green Master (ROX) in an ABI 7300 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA). The detector was programmed with the following PCR conditions: 40 cycles of 5 sec denaturation at 95 °C and 34 sec amplification at 60 °C. All reactions were normalized to the housekeeping genes, including GAPDH and β -actin. The Primers for osteoclastogenic genes used in this study were as follows: c-Fos (forward, 5' -ATGATCTTCTCGGGTTTCAACG-3'; reverse, 5' -CAGTCTGCTGCATAGAAGGAACCG-3'), NFATc1 (forward, 5'-TGCTCCTCCTGCTGCTC-3'; reverse, 5'-GCAGAAGGTGGAGGTGCAGC-3'), CTSK (forward, 5'-CTTCCAATACGTGCAGCAGA-3'; reverse, 5'-TCTTCAGGGCTTCTCGTTC-3'), TRAP (forward, 5'-CTTCCAATACGTGCAGCAGA-3'; reverse, 5'-CCCCAGAGACATGATGAAGTCA -3'), CTR (forward, 5'-TGCAGACAACCTTTGGTTGG-3'; reverse, 5'-TCGGTTTCTTCTCCTCTGGA-3'), GAPDH (forward, 5'-CACCATGGGAGAAGGCCGGG-3'; reverse, 3'-GACGGACACATTGGGGGTAG-5'), β -actin (forward, 5'-TCTGCTGGAAGGTGGACAGT-3'; reverse, 3'-CCTCTATGCCAACACAGTGC-5').

Western-blotting analysis

RAW264.7 cells were treated with vehicle or Mon for 4 h, and then the cells were stimulated with RANKL (100 ng/mL) for indicated time. After that, cells were lysed with RIPA buffer plus protease and

phosphatase inhibitor cocktail. Collected cell lysates and centrifuged at 12,000 rpm for 10 min. Protein concentrations were assessed using BCA protein assay reagent (Beyotime, China), and 30 µg of whole cell lysates was then subjected to 10 % SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% BSA in TBST containing 0.1% Tween-20 for 1 h, and then incubated with primary antibodies (1:1000) overnight at 4 °C. The membranes were washed in 1 × TBST and then incubated secondary anti-bodies (1:5000) for 2 hours at room temperature. Immunoreactive bands were detected by exposing in an Odyssey infrared imaging system (LI-COR).

LPS-induced calvarial osteolysis mice model

We established a mouse calvarial osteolysis model to measure the osteolysis suppressing effect of Mon *in vivo*. Briefly, 32 healthy 8-week-old C57/BL6 male mice were randomly assigned to four groups: PBS control (sham), LPS in PBS (vehicle), and LPS together with low (2 mg/kg) and high (5 mg/kg) concentrations of Mon (Low and High dose, respectively). All treatments were administered under light anesthesia through subcutaneous injection over the sagittal midline suture of the calvarium. PBS and Mon were injected 1 day before the injection of LPS (5mg/kg, prophylactic treatment). PBS and Mon were then injected every other day over a 7-day period. At the end of the experiment, all mice were sacrificed on day 7. Calvarias were separated and fixed in 4% paraformaldehyde for micro-CT and histological analysis. In addition, the pathological examination of liver, kidney and spleen was performed.

Statistical analysis

All data was expressed as the mean ± S.D and analyzed by one-way ANOVA tests using SPSS 19.0 software (SPSS Inc., USA). Homogeneity of variance test was used to test and confirm that the data were normally distributed. Then, Student-Newman-Keuls post hoc tests, were used to determine the significance of difference between results, with *P<0.05, **P<0.01 being regarded as significant.

Results

Mon inhibited osteoclast differentiation without toxicity to BMMs

In order to determine the effect of Mon on the differentiation of OCs, osteoclastogenesis assay was performed. BMMs were seeded in 96-well plates at 5000 per well. After overnight incubation, various concentrations of Mon were added to the plate until OCs formation. As shown in Fig. 1B, Mon inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner. Mon significantly inhibited OC differentiation at doses greater than 0.5 µM (Fig. 1 C, D). To determine whether the inhibition of osteoclast differentiation by Mon is associated with cellular toxicity or not, we further analyzed the cell viability by MTS assay. The results indicated that the anti-osteoclastogenic effect of Mon was not associated with cellular toxicity (Fig. 1 E, F). To explore the inhibitory effect of Mon on osteoclast differentiation in detail, BMMs were treated with RANKL, M-CSF and Mon (4 µM) at four different time points (Fig. 1 G and H). After exposure to Mon for 24 hours, the culture medium containing Mon was washed out and changed to Mon-free culture medium. The results showed that Mon (4 µM) completely inhibited osteoclast differentiation when the cells were exposed on day 0-1 after stimulating by RANKL. A similar inhibitory effect was observed when Mon was added on day 1-2. However, the inhibitory effect was diminished when Mon was added on day 2-3 or day 3-4. These data suggest that Mon suppresses the early cellular events of osteoclastogenesis.

Mon inhibited the expression levels of osteoclastic marker genes in vitro

Osteoclast differentiation is associated with the upregulation of marker genes in response to RANKL [4]. We further analyzed the inhibitory effects of Mon on OC differentiation by evaluating the RANKL-induced mRNA expression levels of osteoclastic marker genes, such as c-Fos, NFATc1, TRAP, CTSK, V-ATPase-a3 and V-ATPase-d2. As demonstrated by quantitative real-time PCR, RANKL markedly induced the expression of all evaluated genes. In contrast, Mon substantially suppressed osteoclastic marker gene expression (Fig. 2 A-F). Therefore, these results indicated that Mon suppressed osteoclastic marker genes expression *in vitro*.

Mon inhibited hydroxyapatite resorption *in vitro*

Since Mon inhibited osteoclast differentiation and formation, we next investigated whether Mon could impair osteoclastic hydroxyapatite resorption *in vitro*. Thus, multinucleated cells were seeded on a hydroxyapatite-coated plate and treated with M-CSF (30 ng/mL), RANKL (100 ng/mL) and indicated concentrations of Mon. The total resorption areas were measured and analysed by Image J. As shown in Fig. 3 A, Mon treatment substantially reduced hydroxyapatite resorption area. The hydroxyapatite resorption area of wells treated with 1 μ M Mon was 35% less than the control group. However, hydroxyapatite resorption was partially abolished after treatment with 4 μ M Mon (Fig. 3 B). Together, these data suggested that Mon inhibited osteoclastic hydroxyapatite resorption *in vitro*.

Mon inhibited the phosphorylation of p38 and JNK during RANKL-induced osteoclastogenesis *in vitro*

To gain further insights into the molecular mechanism by which Mon exerts its inhibitory action on the differentiation of

Fig. 1. Mon inhibited osteoclast differentiation without toxicity to BMMs. (A) Chemical structure of monocrotaline (Mon). (B) BMMs were cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) with various concentrations of Mon for 4 days. Then, the cells were fixed with 4% paraformaldehyde and stained for TRAP. Scale bars, 100 μ m. (C, D, H) The number and areas of osteoclast cells were determined as described in the Methods section. (E, F) BMMs were treated with various concentrations of Mon, M-CSF (30 ng/mL) for 48 h and 96 h, respectively. Cell viability was determined by MTS assay. (G) BMMs were cultured as in A, except the cells were treated with 390 nM Art on the indicated days. The data are presented as the mean $\pm$ SD (** p <0.01), n = 6.

Fig. 2. Mon inhibited the expression levels of osteoclastic marker genes *in vitro*. (A-F) BMMs were incubated for 5 days with M-CSF and RANKL in the presence or absence of Mon. The mRNA expression of c-Fos, NFATc1, TRAP, CTSK, V-ATPase-d2 and V-ATPase-a3 was analyzed by real-time RT-PCR. The mRNA levels of the target gene were normalized relative to the mRNA levels of GAPDH and β -actin. The results are presented as the mean $\pm$ SD (** p <0.01), n = 6.

BMM into osteoclasts, we next observed the influence of Mon (4 μ M) on the RANKL-induced NF- κ B and MAPK signaling pathways. NF- κ B is one of the key signal transducers, involved in the early phase of RANKL-induced osteoclast differentiation [12, 18]. Upon stimulation with RANKL, its receptor RANK binds the signalling adaptor molecule TRAF6. TRAF6 promotes the phosphorylation and degradation of I κ B α , leading to the phosphorylation and nuclear translocation of NF- κ B p65 subunit and activation of NF- κ B signaling [19-21]. However, Mon had no effect on RANKL-induced I κ B α degradation or p65 activation (Fig. 4 A), indicating that it does not influence the NF- κ B signaling pathway.

Previous studies have showed that three major subfamilies of MAPKs, p38, JNK and ERK, play key roles in the development of osteoclasts [22]. Therefore, we further investigated whether these signalling pathways were involved in the inhibition of osteoclastogenesis by Mon. Interestingly, within 15 minutes of RANKL stimulation of RAW264.7 cells, three MAPK family members (p38, JNK and ERK) show increased phosphorylation that persists for up to 30 minutes. By comparison, co-treatment of RAW264.7 cells with RANKL and Mon (4 μ M) partially suppressed RANKL-induced phosphorylation of p38 and JNK at 15 and 30 minutes (Fig. 4 B-D).

Mon did not affect osteoblast differentiation in vitro

Bone homeostasis is a continuous process of bone resorption by OC and bone formation by OB, so the effect of Mon on primary OBs was analyzed [23, 24]. Alkaline phosphatase (ALP) is an early differentiation marker of osteoblasts, and the expression of ALP was assayed. The results showed that the expression of ALP was not significant difference between control and Mon-treated groups (Fig. 5 A). Alizarin red staining was used to analyze the calcium content of the constructs after 21 days of OBs culture. However, the total mineralized area of each dish was similar between the control group and Mon-treated group (Fig. 5 B). Next, the effect of Mon on primary OBs viability was analyzed. As shown in Fig. 5 C and D, the cell viability was not affected by Mon. These data suggested that Mon did not affect OB differentiation and mineralization.

Mon suppressed LPS-induced osteolysis in murine calvaria

Activation of OCs plays a significant role in destructive bone diseases. To further analyze the potential protective effects of Mon under pathological conditions, we used an

Fig. 3. Mon inhibited hydroxyapatite resorption in vitro. (A) BMMs were plated onto hydroxyapatite-coated plates and treated with the indicated concentrations of Mon. Attached cells on the plates were removed by 5% sodium hypochlorite and photographed under a light microscope. (B) The total resorption areas were measured using Image J and are shown in this graph. The data are presented as the mean $\pm$ SD (** p <0.01), n = 6.

LPS-induced inflammatory osteolysis in mouse calvaria model. 8-week-old C57BL/6 mice received local subcutaneous injections of PBS, LPS, LPS with Low dose or LPS with High dose onto the sagittal suture of calvarium for 7 days. One day before LPS injection (prophylactic treatment), the LPS with Low dose, LPS with High dose and PBS groups were injected with Mon or PBS, respectively. Seven days after LPS injection, micro-CT 3D reconstruction showed that extensive bone resorption had occurred in the midline suture of calvaria in the LPS groups when compared with the PBS-treated controls. Quantitative analyses confirmed the marked reduction in BV/TV, and significant increases in both the number and percentage of porosity in LPS-treated groups. By comparison, Mon treatment clearly reduced the bone loss induced by LPS in this model (Fig. 6 A-D). Histological examination confirmed the protective effects of Mon on LPS-induced bone erosion. As shown in Fig. 6 E, LPS injection led to inflammatory bone loss and increased number of TRAP (+) OCs. Interestingly, inflammatory bone erosion was not observed in Mon-treated mouse calvaria tissue sections, an observation consistent with the diminished number of TRAP (+) OCs and OC per bone surface (OC/BS) (Fig. 7 A-D). These data indicated that Mon prevented the inflammation induced bone loss.

Fig. 4. Mon inhibited RANKL-induced activation of p38 and JNK signalling pathways. (A, B) RAW264.7 cells were incubated in serum-free medium and pretreated with or without Mon (4 μ M) for 4 h. Then the cells were stimulated by RANKL (100 ng/mL) for the indicated times. Cells were lysed for western blotting with specific anti-bodies against p-NF- κ B P65, NF- κ B P65, I κ B α , β -actin, p-p38, p38, p-JNK, JNK, p-ERK and ERK. (C, D) The relative expressions were determined by densitometric analysis and phosphorylated p38 and JNK band were expressed as a percentage of its total protein counterpart. All experiments are presented as the mean $\pm$ SD (** p <0.01), n = 5.

Fig. 5. Mon does not affect osteoblast in vitro. (A, B) Primary osteoblasts were treated with indicated concentrations of Mon in the presence of ascorbic acid (A.A), β -glycerophosphate (β -gly) and dexamethasone (DXM) for 7, 21 days during osteoblastic differentiation. The ALP (Alkaline phosphatase) and Alizarin Red Staining demonstrated no significant difference between control and Mon treated group. (C, D) Primary osteoblasts were inoculated in 96-well plates at a density of 3000 cells/well and cultured with different concentrations of Mon, and cell viability was determined by MTS assay (top). The data are presented as the mean $\pm$ SD (** p <0.01), n = 6.

Discussion

Imbalances in bone homeostasis favouring an increased activity of OCs lead to excessive bone destruction, and are characteristic of many lytic bone diseases [25], including periodontitis [26], rheumatoid arthritis [27, 28] and the aseptic loosening of orthopedic implants [29]. The major modalities currently used in these diseases treatment, including bisphosphonates [30], selective estrogen receptor modulators (SERM) [31], and denosumab (a RANKL Inhibitor) [32]. However, these therapies are associated with side effects, including renal toxicity and osteonecrosis [33], endometrial cancer risk [34], and osteonecrosis of the jaws [35]. Recently, Cathepsin-k inhibitors came in to light due to the significant role of this protein in bone resorption and the promising results were showed in clinical trials. Unfortunately, Cathepsin-k inhibitors can cause serious adverse effects such as atrial fibrillation, stroke and atypical fractures [36]. Thus we still need to develop new drugs for the treatment of bone loss diseases.

Natural compounds have been a successful source of therapeutic agents and drug leads. The previous studies have been proven that many plants, such as artesunate [37] and picrasidine I [38] are helpful for lytic bone disease treatment. Mon is a alkaloid derived from the seeds of the *crotalaria spectabilis* plant, has a therapeutic effect on cancers, such as skin cancer and cervical cancer [15]. Mon is an alkylating agent, which plays an anticancer effect by alkylation of DNA [16]. In addition, high dose of Mon is used to

Fig. 6. Mon suppressed LPS-induced osteolysis in murine calvaria. (A) Representative three-dimensional micro-CT reconstructed images of the mice calvaria from each treatment group. (B-D) Bone volume to tissue volume (BV/TV, %), number of porosity and the percentage of total porosity of each sample were measured and analyzed. The data are presented as the mean $\pm$ SD (** $p < 0.01$).

Fig. 7. Mon protected LPS-induced calvaria histological damage. (A) Histological assessment of the protective effect of Mon. Calvarial bone sections were stained with H&E and TRAP. The LPS group exhibited an obvious osteolytic lesion with abundant TRAP (+) OCs present in the lesion, whereas the Mon treated group revealed reduced TRAP (+) OC number and osteolysis. Scale bars, 100 μ m. (B-D) Histomorphometric analysis of bone volume against tissue volume (BV/TV, %), TRAP (+) OC numbers, and TRAP (+) OC numbers against bone surface. The values are expressed as mean $\pm$ SD (** $p < 0.01$).

induce hypertension in animal models through receiving an intraperitoneal injection [17]. In this study, we investigated the effect of Mon on OC formation and function *in vitro* and *in vivo*. Our data showed that Mon effectively inhibited RANKL-induced OC formation and osteoclastic marker genes in dose-dependent. We also found that Mon prevented the ability of hydroxyapatite resorption *in vitro*. In addition, we demonstrated that Mon suppressed RANKL-induced osteoclastogenesis via p38 and JNK signaling pathways in OCs. Finally, *in vivo* data indicated that Mon attenuated LPS-induced bone loss by suppressing OC activity.

OCs are differentiated from cells of hematopoietic monocyte/macrophage lineage under the presence of two key factors: M-CSF and RANKL [10]. Previous studies have showed that M-CSF induces the proliferation of OC precursor cells (BMMs), and RANKL induces subsequent differentiation of OC precursors into OCs [39]. In our results, Mon had no effect on OC precursor proliferation by MTS assay, whereas it inhibited differentiation of OC precursors into OCs induced by RANKL, suggesting that Mon inhibited osteoclastogenesis at the step of OC precursor differentiation into OCs.

RANKL/RANK is a key signaling event, which commits OC precursors to the OC lineage, regulates mRNA expression levels of OC marker genes, initiates bone resorption and maintains OCs survival [40]. Binding of RANKL to RANK, leads to the early activation of NF- κ B and MAPK (p38, JNK and ERK) signaling pathways. The activation of NF- κ B signaling cascade is an important and indispensable early RANKL-induced signaling event in OC differentiation and inhibiting its activation result in failed osteoclastogenesis *in vitro* and osteopetrosis *in vivo* [41]. The prerequisite for NF- κ B activation requires phosphorylation and degradation of I κ B, which is an inhibitory protein, attached to NF- κ B blocking it from migrating into the nucleus. Subsequent ubiquitination and proteasome degradation of I κ B releases homo and hetero NF- κ B dimers such as NF- κ B p65/RelA, which translocate to the nucleus and transcription of target genes [25]. Unfortunately, our data showed that Mon did not affect the degradation of I κ B and the phosphorylation of NF- κ B p65/RelA. The MAPK signaling hub (p38, JNK and ERK) is another important event required for osteoclastogenesis [22] and the activation of P38 and JNK, in particular, is required for the formation of OCs [42]. This study showed that Mon inhibited the phosphorylation of p38 and JNK during RANKL-induced osteoclastogenesis.

The inhibitory effect of Mon on osteoclastogenesis was also affirmed by evaluating the RANKL-induced mRNA expression levels of OC marker genes. Activation of the MAPK and NF- κ B signaling pathways induce the formation and function of OCs by upregulating the osteoclastogenic transcription factors such as c-Fos and NFATc1. The c-Fos/NFATc1 pathway plays a crucial role in OC development, and the lack of these two factors arrest OC differentiation [42]. For example, the importance of c-Fos in osteoclastogenesis has been confirmed in c-Fos deficient mice which exhibit a severe osteopetrotic phenotype due to impaired OC formation [43]. In our study, we clearly demonstrated that Mon had an anti-osteoclastogenic potential by attenuating RANKL-induced activation of c-Fos and NFATc1 during osteoclastogenesis. NFATc1, a master transcription regulator in OC formation and function [44], regulates the expression of osteoclastic special genes [45], including TRAP, CTSK, V-ATPase-d2 and V-ATPase-a3 [24]. Our results showed that mRNA levels of TRAP, CTSK, V-ATPase-d2 and V-ATPase-a3 were markedly decreased by Mon treatment. These data clearly showed that Mon not only inhibited the c-Fos and NFATc1 but it also suppressed the downstream effectors such as TRAP, CTSK, V-ATPase-d2 and V-ATPase-a3.

Furthermore, bone homeostasis is a continuous process of bone resorption by OC and bone formation by OB [24, 46]. Thus, the effect of Mon on OBs was also analyzed. However, the cell viability, ALP expression and total mineralized area of each dish were not significant difference between control and Mon-treated groups. Our data suggested that Mon did not affect OBs differentiation and mineralization.

Owing to the anti-osteoclastogenic and anti-resorptive effects *in vitro*, the administration of Mon protected against inflammatory calvarial bone loss induced by LPS *in vivo*. Lipopolysaccharide (LPS) is a Gram-negative bacteria-derived cell wall product, which has been recognized as a critical factor implicated in the development of osteolysis

[47]. In addition, LPS plays a significant role in bone resorption by activating OC differentiation and function [48, 49]. To further determine the potential protective effects of Mon under pathological conditions, we used an LPS-induced inflammatory osteolysis in mouse calvaria model. The results showed that Mon greatly reduced bone resorption and the number of TRAP (+) OCs. Thus, the suppression of LPS-stimulated bone destruction could be attributed to Mon-induced inhibition of osteoclastogenesis. Unfortunately, high dose (60 mg/kg) of Mon can induce hypertension in animals via receiving an intraperitoneal injection after 21 days [17].

However, Mon was merely injected over the local calvarium with 2 mg/kg or 5mg/kg every two days for 7 days in this study, according to previous report [37]. Importantly, to detect the potential side effects of Mon in our study, the pathological examination of liver, kidney and spleen have been done, and the results showed that Mon had no effect on them (Fig. 8). In addition, chronic toxicity test will be done in a series studies of Mon-prevented osteolysis.

In summary, we first time demonstrated that Mon can suppress osteoclast differentiation and mediate osteoclast activity *in vitro* and *in vivo*. Furthermore, we clarified that these inhibitory effects of Mon occur through suppressing RANKL-induced activation of p38 and JNK. These data suggest that Mon could be a potential agent in the prevention or treatment of lytic bone diseases.

Disclosure Statement

The authors declare no conflicts of interests, financial or otherwise.

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Fig. 8. Mon had no toxic effect on liver, spleen and kidney. (A) To detect Mon potential side effects in this study, the pathological examination of liver, kidney and spleen have been done. Interestingly, H&E staining were revealed that Mon had no toxic effect on liver, spleen and kidney.

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