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Taxifolin Inhibits Receptor Activator of NF-κB Ligand-Induced Osteoclastogenesis of Human Bone Marrow-Derived Macrophages in vitro and Prevents Lipopolysaccharide-Induced Bone Loss in vivo

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Keywords
Taxifolin · Osteoclast formation · MSC · NF-κB · Osteolysis

Abstract
It has been reported that taxifolin inhibit osteoclastogenesis in RAW264.7 cells. In our research, the inhibition effects of taxifolin on the osteoclastogenesis of human bone marrow-derived macrophages (BMMs) induced by receptor activator of NF-κB ligand (RANKL) as well as the protection effects in lipopolysaccharide-induced bone lysis mouse model have been demonstrated. In vitro, taxifolin inhibited RANKL-induced osteoclast differentiation of human BMMs without cytotoxicity. Moreover, taxifolin significantly suppressed RANKL-induced gene expression, including tartrate-resistant acid phosphatase, matrix metalloproteinase-9 nuclear factor of activated T cells 1 and cathepsin K, and F-actin ring formation. Further studies showed that taxifolin inhibit osteoclastogenesis via the suppression of the NF-κB signaling pathway. In vivo, taxifolin prevented bone loss in mouse calvarial osteolysis model. In conclusion, the results suggested that taxifolin has a therapeutic potential for osteoclastogenesis-related diseases such as osteoporosis, osteolysis, and rheumatoid arthritis.

Introduction

Bone is a continuously remodeling organ that the resorption of old bone and the formation of new bone are mediated by osteoclasts and osteoblasts [1]. Osteoclasts are multinucleated cells that are differentiated from monocyte/macrophage hematopoietic lineage cells [2], which play an important role in bone homeostasis [3]. Abnormal activation of osteoclasts resulted in bone loss-associated diseases, including osteoporosis [4], rheumatoid arthritis [5], inflammatory osteolysis

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[6] and Paget's disease [7]. Therefore, reduction of bone resorption by suppression of osteoclast formation has been proposed as a key way for the treatment of these diseases.

The cytokines macrophage-colony stimulating factor (M-CSF) and the receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) ligand (RANKL), a tumor necrosis factor family member, stimulate the differentiation of bone marrow (BM) precursors to osteoclasts [8]. M-CSF induces survival and proliferation as well as mediates the expression of RANKL receptor (RANK) in BM cells. Binding of RANKL to RANK activates several downstream signaling pathways such as NF-κB and mitogen-activated protein kinases (MAPKs) that regulate the production of essential proteins, including nuclear factor of activated T cells cytoplasmic 1 (NFATc1) and tartrate-resistant acid phosphatase (TRAP) for osteoclast differentiation [9, 10]. When mature osteoclasts adhere to bone matrix, acid and osteolytic enzymes were secreted to degrade bone tissue [11]. Therefore, blocking of signaling pathways that involved in osteoclast differentiation is a promising therapeutic approach for preventing diseases of bone loss.

Flavonoids comprise a large number of polyphenolic compounds that extensively exist in natural plants [12, 13]. It is reported that some of flavonoids have several beneficial effects, including oxidation-resisting, antibacterial and inflammation suppression properties [14, 15]. Several studies revealed that some of flavonoids inhibit resorption of bone [16]. Icariin, the active ingredient extracted from *Epimedium pubescens*, suppress lipopolysaccharide (LPS)-induced osteoclasts differentiation through the inhibition of the p38 and JNK pathway activation [17, 18]. Naringenin, a naturally occurring flavanone in grapefruit and tomatoes, was shown to inhibit osteoclastogenesis by suppressing the p38 signaling pathway and protect against titanium particle-induced bone loss [19]. Taxifolin, a catechol-type flavonoid with oxidation-resisting property founded in green tea, has been described to suppress osteoclastogenesis in RAW264.7 cells [20]. However, whether taxifolin affects the differentiation of human BM cells to osteoclast and the specific pathway related remains unknown.

In the present study, osteoclast formation from human BM cells (BMCs) under taxifolin treatment as well as the in vivo effect of taxifolin in an LPS-induced mouse calvarial osteolysis model was investigated to elucidate the underlying molecular mechanism of taxifolin in osteoclastogenesis.

Materials and Methods

Reagents

Taxifolin, LPS and Acid Phosphatase kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CCK-8 kit was purchased from Dojindo Molecular Technology (Kumamoto, Japan). Recombinant human M-CSF and human RANKL were purchased from Peprotech (Rocky Hill, NJ, USA). Antibodies against NF-κB p65 (ab16502) and beta-actin (ab8227) were purchased from Abcam (Cambridge, MA, USA). Antibodies against p-S536 p65 (3033S), IκBα (4814S) and p-Ser32 IκBα were purchased from Cell Signaling Technology (Beverly, MA, USA).

BM Derived Macrophage Preparation and Osteoclast Culture

BM samples were obtained by aspiration from donors at Xiangya Hospital (Changsha, Hunan, China) and primary human BMCs were separated from BM. As described in our previous research [22], BMCs collected by density gradient centrifugation from BM were cultured in α-MEM supplemented with 10% FBS. Floating cells were collected and further incubated with M-CSF (30 ng/mL) for 3 days. The attached cells were classified as BM derived macrophages (BMMs). The effect of taxifolin on osteoclastogenesis were evaluated by treating BMM with RANKL (200 ng/mL) and various concentrations of taxifolin for 6 days. The degree of osteoclast differentiation was evaluated by TRAP staining. The RAW 264.7 cells (Cyagen, Guangzhou, China) were incubated in DMEM medium with 10% FBS at incubator.

Cytotoxicity Assay

Cell viability was evaluated by CCK-8 assay. BMMs (5.0×10^3 cells/well) were seeded in 96-well plates with different density of taxifolin (25, 50, 100, 150, 200, 400, and 600 μmol/L) for 48 h. Next, 10 μL of CCK-8 reagent was added to each well for 4 h at 37 °C. Absorbance was measured and found to be 450 nm and cell viability was also calculated.

TRAP Staining

BMMs were treated in the presence or absence of different concentration of taxifolin with RANKL (200 ng/mL) for 6 days. The cells were fixed with 4% (w/v) paraformaldehyde and stained with the Acid Phosphatase kit (Sigma Chemical, St. Louis, MO, USA) following the manufacturer's protocol. After incubation at 37 °C for 1 h, cells were washed and TRAP-positive cells were counted using the light microscopy.

Real-Time Quantitative PCR

Total cellular RNA was extracted from cultured cells by TRIzol (Invitrogen, Carlsbad, CA, USA) method. cDNAs were synthesized and Real-time PCR was performed using SYBR Green Master Mix with rhodamine X (ROX; Takara, Kusatsu, Shiga, Japan) following the manufacturer's protocol. The sequences of primers used for detection included the following: TRAP, AAGGCGAGAGATTCTTTCCCTG (forward), and ACTGGGGACAATTCACTAGAGC (reverse); β-actin, ACTCTTCCAGCCTTCCTTCC (forward), and GTACTTGCGCTCAGGAGGAG (reverse); NFATc1, ACCACCTTTCCGCAACCA (forward), and TTCCGTTTCCCGTTGCA (reverse); matrix metalloproteinase-9 (MMP-9), CTGGACAGCCAGACACTAAAG (forward), and

CTCGCGGCAAGTCTTCAGAG (reverse); cathepsin K (CTK), CACTGCTCTCTTCAGGGCTT (forward), and ACGGAGGCATTGACTCTGAA (reverse).

F-Actin Ring Immunofluorescence

Taxifolin-treated osteoclasts were fixed with 4% (w/v) paraformaldehyde and permeabilized with 0.1% (v/v) Triton X-100, then incubated with rhodamine-conjugated phalloidin (1:100, Yeasen, Shanghai, China) for 30 min. After rinsing with phosphate-buffered saline for 3 times, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, MO, USA). Fluorescent staining was filmed using a laser-scanning microscope (FV10-ASW 1.7; Olympus, Tokyo, Japan).

Luciferase Reporter Gene Assay, Western Blot

Luciferase reporter gene assay and western blot were performed as described previously [22].

In vivo Osteolysis Model

Mouse calvarial osteolysis model induced by LPS was used. Twenty-male 8-week-old C57/BL6 mice were randomly assigned into 4 groups and received daily injections of LPS alone (5 mg/kg body weight), LPS with low taxifolin (1 mg/kg body weight; low), LPS with high taxifolin (10 mg/kg body weight; high) and phosphate-buffered saline as control. After 7 days of injection, the mice were euthanized and the calvaria were dissected. After 48 h fixation with 4% paraformaldehyde, three-dimensional reconstructions images of mouse calvarias were obtained using a high-resolution micro-computed tomography (μCT) scanner (Skyscan 1176; Skyscan; Aartselaar, Belgium). After reconstruction, a square region of interest around the midline suture was selected for further analysis, the quantitative analysis was performed by Skyscan CT analyzer software (CTAn version 1.7, Skyscan, Antwerpen, Belgium). The parameters measured were bone volume/tissue volume (BV/TV, %) and total porosity (%). After μCT scanning the calvaria were decalcified, dehydrated and sectioned for hematoxylin and eosin staining and TRAP staining. The specimens were examined and photographed and TRAP-positive multinucleated osteoclasts were counted.

Statistical Analysis

Experiments were conducted more than 3 times separately. Data were presented as the mean ± SD. Statistical analyses were performed using SPSS 11.0 software (SPSS, Chicago, IL, USA). The data were assessed using Student *t* tests or analysis of variance, and *p* values <0.05 were considered to be significant.

Results

Taxifolin Inhibited RANKL-Induced Osteoclast Differentiation of Human BMMs

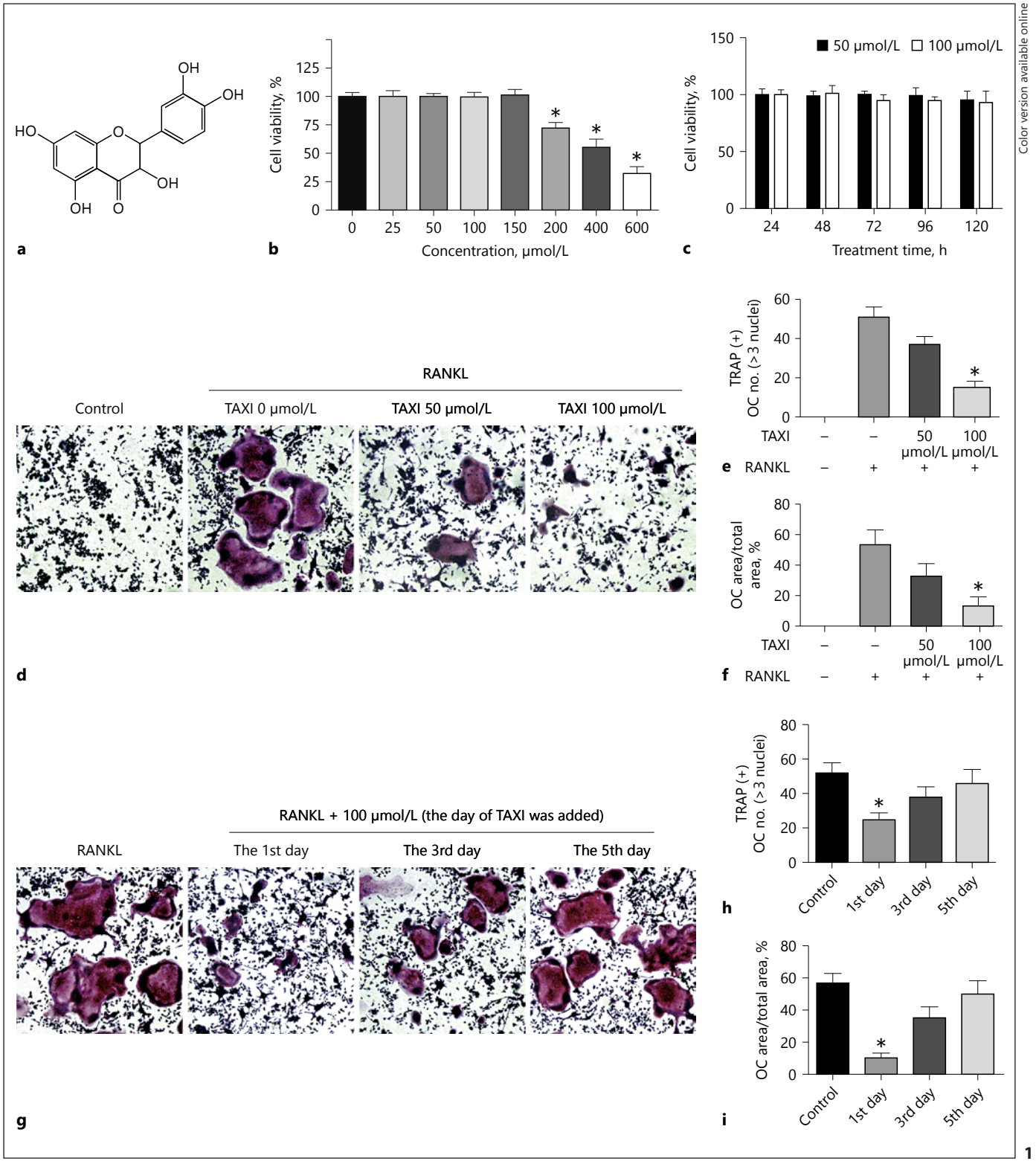
To verify the potential cytotoxic effects of taxifolin, CCK-8 assay were performed to evaluate the taxifolin's effect on proliferation of human BMMs. The results showed that the concentration up to 150 μmol/L have no cytotoxic effect on the human BMMs. Additionally, 200 μmol/L taxifolin treatment for 48 h inhibited cell proliferation (Fig. 1b). In addition, the cytotoxic effect of 50 and 100 μmol/L taxifolin on the human BMMs for different times were determined, the result verify that low concentration taxifolin has no effect on the viability of human BMMs (Fig. 1c). To examine the effect of taxifolin on RANKL-induced osteoclastogenesis, BMMs were incubated with 50/100 μmol/L taxifolin under RANKL stimulation for 6 days. The development of TRAP-positive multinuclear osteoclasts was evaluated. As shown in Figure 1d–f, taxifolin suppressed osteoclast formation and the area of osteoclasts in a dose-dependent manner. Moreover, the effect of taxifolin on osteoclasts formation exerted on which stage was determined by early, middle and late stage supplement with taxifolin. When taxifolin was supplied on the first day during RANKL treatment, the remarkable inhibition effect was observed (Fig. 1g–i).

Taxifolin Inhibited Osteoclast-Related-Gene Expression and F-Actin Ring Formation

To further analyze the inhibitory effect of taxifolin, the mRNA expression of several osteoclast-related genes was examined, including TRAP, NFATc1, MMP-9, and CTK. BMMs were incubated with 50/100 μmol/L taxifolin in the presence of 20 ng/mL M-CSF and 200 ng/mL RANKL for 6 days. Real-time-PCR was performed to assess the mRNA expression level. The results demonstrated that the mRNA expression level was blocked by taxifolin in a concentration-dependent manner (Fig. 2a–d). Furthermore, the F-actin ring formation, a precondition for osteoclastic bone resorption, was examined by confocal microscopy. Taxifolin dose-dependently disrupted the development and the area of osteoclast F-actin ring and cytoskeletal structure (Fig. 2e, f). These data confirmed that taxifolin impaired osteoclastogenesis and the osteoclast-specific genes expression.

Taxifolin Inhibited RANKL-Mediated NF-κB Signaling Pathway Activation

NF-κB is a main transcription factor for RANKL-activated osteoclastogenesis. To test whether NF-κB mediated taxifolin-induced inhibitory effect on osteoclastogenesis of BMM, NF-κB-luciferase reporter plasmid (NF-κB-Luc) were transfected into the RAW264.7 cells and luciferase activity was detected. The result showed that taxifolin treatment attenuates NF-κB-Luc activity in a dose-dependent manner (Fig. 3a). Furthermore, the phosphorylation of key members in NF-κB signaling pathway, like IκBα and P65, were detected. As shown in Figure 3b, stimulation of BMMs with RANKL for 1 h sig-



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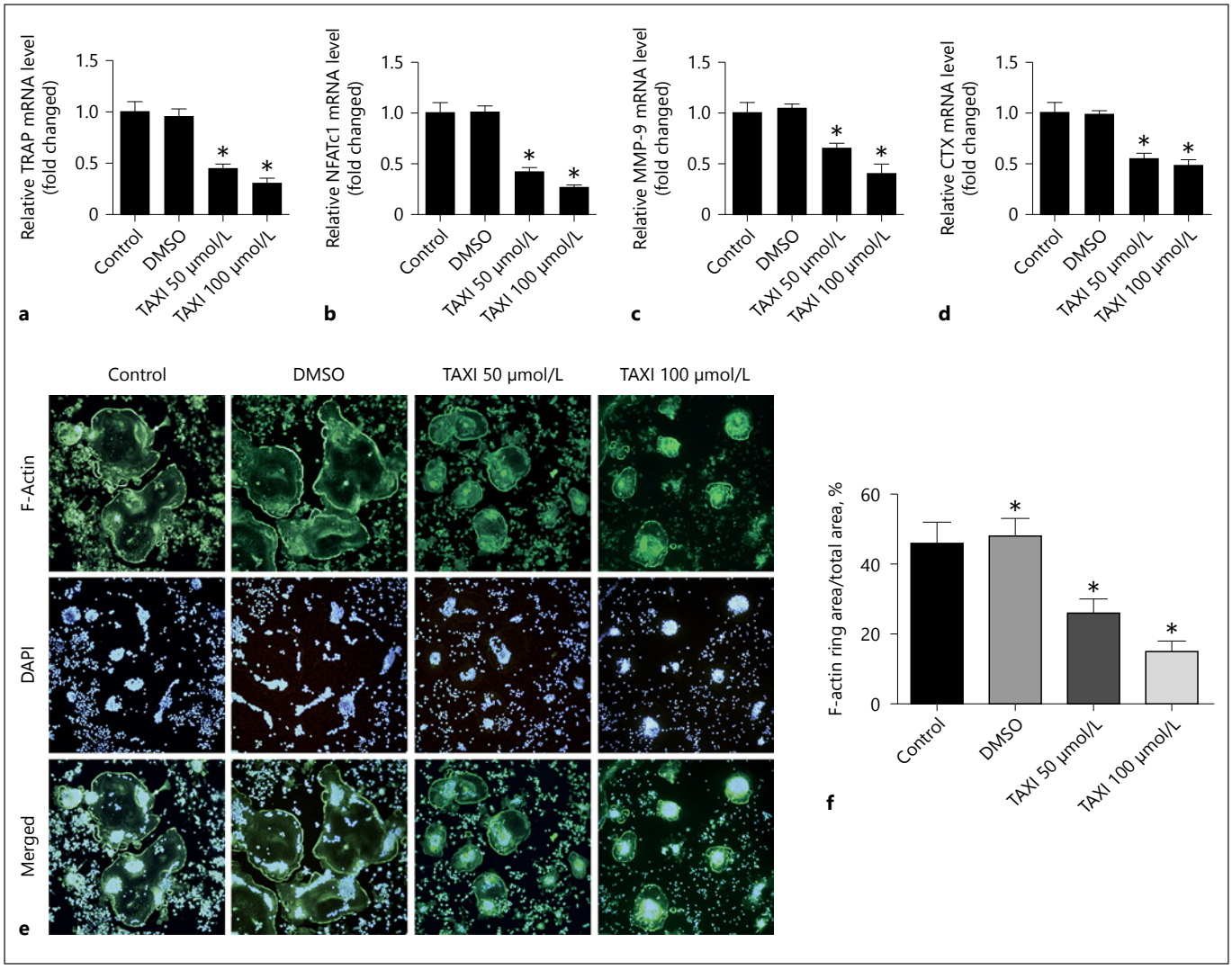

Fig. 2. Taxifolin suppressed osteoclast marker genes expression and the development of F-actin ring. BMM cells were incubated with M-CSF (20 ng/mL) and RANKL (200 ng/mL) with or without 50 or 100 μmol/L TAXI for 6 days. **a**–**d** Quantitative real-time PCR analysis was used to determine the mRNA levels of osteoclast marker genes (TRAP, NFATc1, MMP-9, and CTK). The control group was set to 1.0. * $p < 0.05$ versus the control group ($n = 3$). **e** and **f** The cells were fixed and stained with Rhodamine phalloidin and DAPI. Fluorescence images were shown. The relative areas of F-actin ring were quantified. * $p < 0.05$ versus the control group ($n = 3$). TRAP, tartrate-resistant acid phosphatase; TAXI, taxifolin; NFATc1, nuclear factor of activated T cells 1; MMP-9, matrix metalloproteinase-9.

Fig. 1. Taxifolin prevented RANKL-induced osteoclast differentiation of human BMMs. **a** Chemical structure of TAXI. **b** BMMs were incubated with various concentration (25, 50, 100, 150, 200, 400, and 600 μmol/L) of TAXI for 48 h. CCK-8 assays were performed to determine the cytotoxic effects of TAXI. * $p < 0.05$ versus the 0 μmol/L group ($n = 3$). **c** BMMs were incubated with 50 and 100 μmol/L TAXI for different times (24, 48, 72, 96, and 120 h). CCK-8 assays were performed to determine the cytotoxic effects of TAXI. * $p < 0.05$ versus the 24 h group ($n = 3$). **d**–**f** Human BMMs were incubated with M-CSF (20 ng/mL) and RANKL (200 ng/mL) with or without TAXI (50, 100 μmol/L) for 6 days. Osteoclastogenesis was determined by TRAP staining. The numbers of TRAP-positive osteoclasts were counted and the relative areas of osteoclasts were measured. * $p < 0.05$ versus the RANKL group ($n = 3$). **g**–**i** The effect of different stages of addition of TAXI (100 μmol/L) on osteoclastogenesis of BMMs. BMM cells were incubated with RANKL (200 ng/mL) and M-CSF (20 ng/mL) for 6 days with TAXI for 1, 3, or 5 days (TAXI was added in day 5, 3, and 1). Osteoclastogenesis was determined by TRAP staining. The TRAP-positive osteoclasts were counted and the relative areas of osteoclasts were measured. * $p < 0.05$ versus the RANKL group ($n = 3$). RANKL, receptor activator of NF-κB ligand; TAXI, taxifolin; TRAP, tartrate-resistant acid phosphatase.

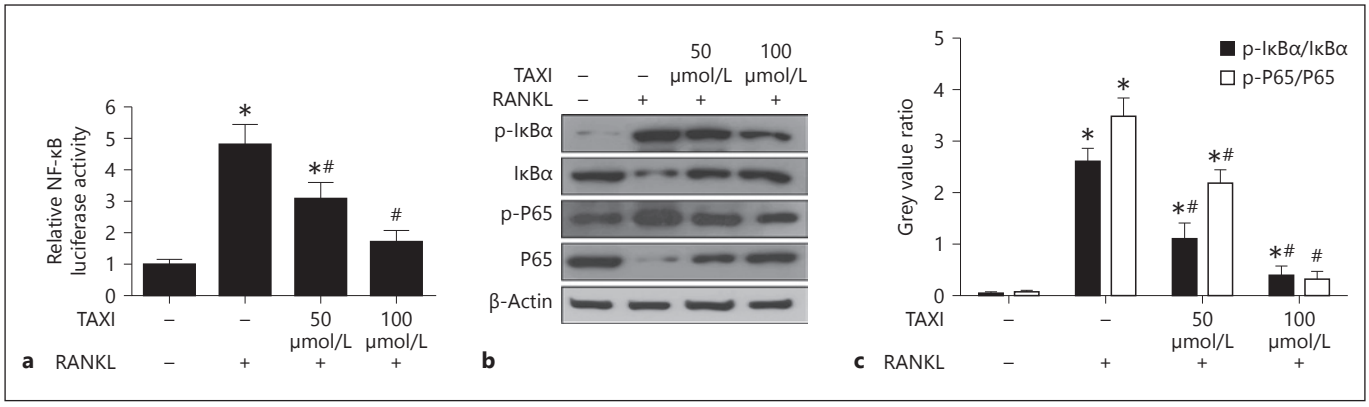

Fig. 3. Taxifolin suppressed RANKL-induced activation of NF-κB signaling pathway. **a** The NF-κB-Luc was transiently transfected into RAW264.7 cells for 24 h. The cells were treated with 50 or 100 μmol/L TAXI in the presence or absence of RANKL (100 ng/mL) overnight after which luciferase activity was determined. **b** BMM cells cultured with 50 or 100 μmol/L TAXI for 4 h prior to stimulation with 100 ng/mL RANKL for 1 h. The key member levels of NF-κB signaling, including p-IκBα, IκBα, p-P65 and P65, were determined by western blotting. β-actin was used as an internal reference. **c** The value of p-IκBα/IκBα and p-P65/P65 was shown in the graph. * $p < 0.05$ versus the control group, # $p < 0.05$ versus the RANKL-treated group ($n = 3$). RANKL, receptor activator of NF-κB ligand.

nificantly increased the phosphorylation degree of IκBα and P65. By comparison, treat BMMs with taxifolin prior to RANKL attenuated RANKL-mediated increased phosphorylation level, indicating that taxifolin influences RANKL-induced osteoclastogenesis partially via inhibition of NF-κB signaling pathway.

Taxifolin Prevented LPS-Induced Bone Loss in vivo

At last, in order to address the effect of taxifolin on in vivo bone pathological lysis, calvarial osteolytic mouse model induced by LPS was used. Mice received local LPS implantation with or without taxifolin onto the calvarium for 7 days. The bone loss was assessed by μCT examination. Massive bone loss following LPS injection was revealed when compared with the sham group (Fig. 4a), suggesting that calvarial osteolysis had occurred in the LPS treatment group. Quantitative analysis confirmed the pronounced decrease in the BV/TV and increase in bone porosity rate in LPS injection group (Fig. 4b, c). However, LPS-induced bone loss was dampened by high concentration taxifolin treatment and the reduction in BV/TV, increase in porosity rate was also rescued (Fig. 4). The histological analysis confirmed that the taxifolin treatment attenuate the LPS-induced bone loss. The numbers of TRAP-positive osteoclasts were reduced in mice received taxifolin injection with LPS implantation (Fig. 4d, e). All in all, our data suggested that taxifolin protects against LPS-induced osteolytsis in vivo.

Discussion

Flavonoids, which consist of a large group of polyphenlic compounds, were ubiquitously existed in fruit and vegetables [12]. The latest research has found flavonoid intakes influence bone metabolism [21]. Flavonoids may prevent bone loss by stimulate bone formation and inhibit bone resorption. Taxifolin, a flavonol with strong anti-oxidative effect, has been proven to promote osteogenic differentiation of human BMSCs in our previous research [22]. It has been proved that several other flavonoids and active components isolated from Chinese herb, such as scutellarin [23], schisantherin [24], tenuigenin [25], and gastrodin [26], inhibit osteoclast formation via RANKL or NFATc1 signaling pathways. In this investigation, the suppression effects of taxifolin on the RANKL-induced osteoclastogenesis of human BMMs as well as the protection effects in LPS-induced bone lysis mouse model have been demonstrated.

Previous study has indicated that taxifolin inhibits osteoclastogenesis of RAW264.7 cells [20]. Since osteoclasts were considered to be formed by the fusion and differentiation of the monocyte/macrophage lineage cells [8], human BMMs were chosen in the current study to verify the inhibition effects of taxifolin on osteoclastogenesis of primary cells. Before the metabolic actions of taxifolin on osteoclasts differentiation were studied, the possible cytotoxic effects of taxifolin were examined. The CCK-8 assays showed that the concentration of taxifolin

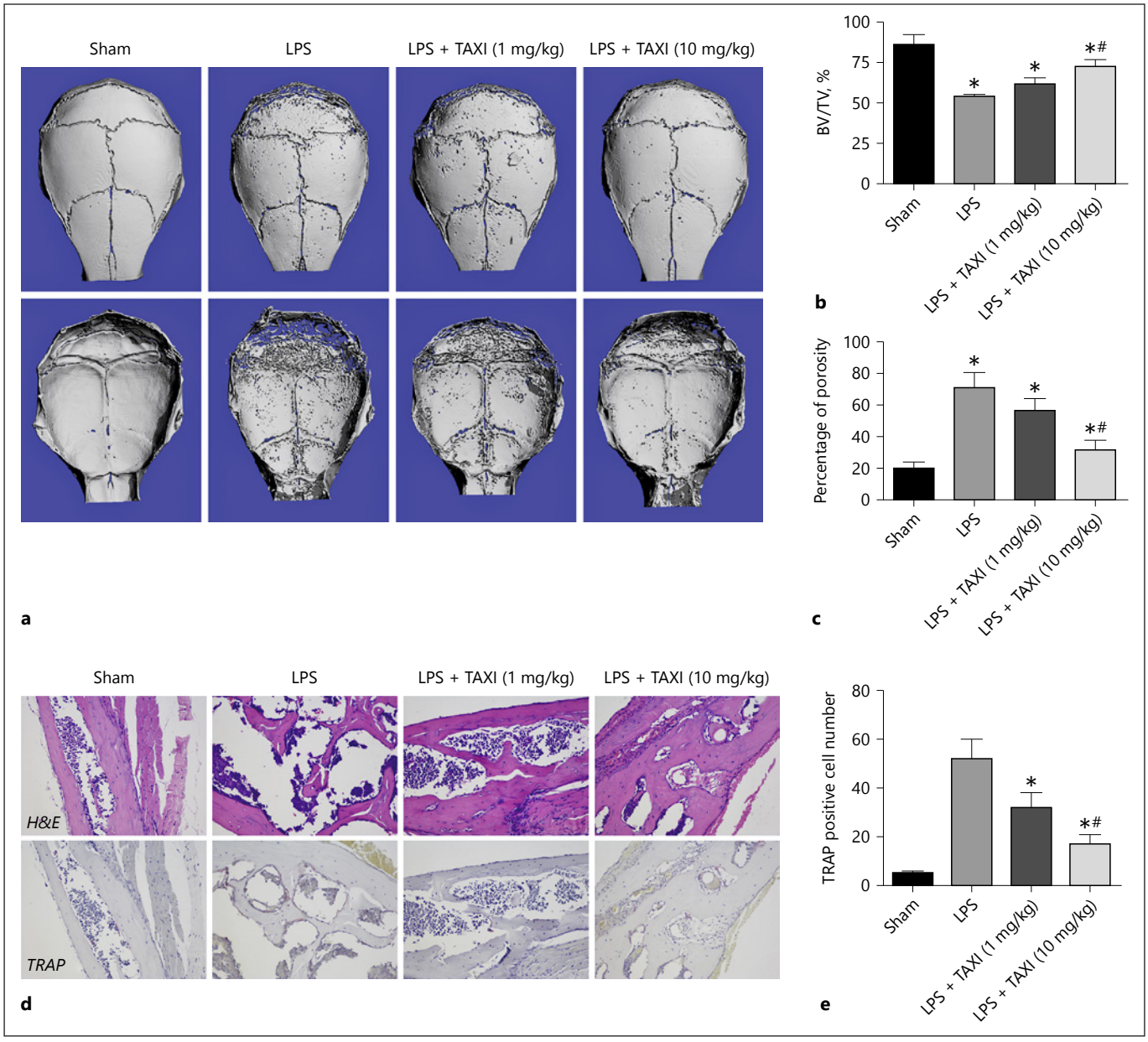

Fig. 4. Taxifolin prevented LPS-induced osteolysis in mouse calvaria. **a** The mice received local LPS implantation with or without taxifolin (1 or 10 mg/kg body weight) subcutaneous injection for 7 days. Representative μCT images of calvarias from each group were shown. **b** and **c** BV/TV, %, the percentage of total porosity were evaluated and analyzed. Values were expressed as mean ± SD, * $p < 0.05$ compared to the sham group, # $p < 0.05$ compared to LPS-treated group ($n = 3$). **d** and **e** Calvarias sections were stained with H&E (20×) and TRAP (20×). The numbers of TRAP-positive osteoclasts were counted. * $p < 0.05$ versus the sham group ($n = 3$), # $p < 0.05$ versus the LPS-treated group ($n = 3$). LPS, lipopolysaccharide; TAXI, taxifolin; BV/TV, Bone volume/tissue volume; H&E, hematoxylin and eosin; TRAP, tartrate-resistant acid phosphatase.

below 150 μmol/L were non-toxicity for human BMMs. Thus, 50 and 100 μmol/L were chosen for subsequent research and had no cytotoxicity for as long as 120 h treatment. The results demonstrated that taxifolin inhibits RANKL-induced osteoclastogenesis of human BMMs in a dose-dependent manner. During the initial process of BMMs differentiate into osteoclasts, the binding of RANKL to RANKL receptor played a vital role in triggering various signaling pathways, such as ERK, JNK, MAPK, p38, and NF-κB signaling pathways [9, 10, 27,

28]. The activation of these pathways induced the specific genes expression such as TRAP, NFATc1, MMP-9 and CTK, which related to osteoclasts differentiation and function. In the current study, the expression of TRAP, NFATc1, MMP-9, and CTK were also downregulated after taxifolin treatment. NFATc1, the master regulator in osteoclastogenesis, was a key downstream gene of NF-κB at the initial stage of osteoclastogenesis [29]. After RANKL-induced NF-kB translocation, NF-kB subunits p50 and p65 are recruited to the NFATc1 promoter induce the expression of NFATc1 during osteoclast formation [30]. The induction of NFATc1 upregulates the osteoclast-specific genes expression, such as TRAP, MMP-9, and CTK. Previous studies have shown, taxifolin exerted its biological effect via modulating NF-κB signaling [22, 31]. In the present research, we observed that the taxifolin treatment inhibits the RANKL-induced NF-κB pathway activation, which was accomplished by the suppression of IκBα and NF-κB p65 phosphorylation. Thus, the result implied that the suppression of NF-κB signaling may partly responsible for the inhibitory effect of taxifolin on NFATc1 expression.

Mature osteoclasts adhered to bone matrix and secreted proton ions and lysosomal enzymes into the sealing zone to degrade the bone matrix. The formation of sealing zone is essential for osteoclastic bone resorption [32]. F-actin, a major component of sealing zone, formed an organized ring, which required the signal of integrin receptors such as integrin αvβ3. In this study, the formation of F-actin ring was impaired by taxifolin treatment. Considering taxifolin can decrease NFATc1 activity and integrin αvβ3 is one of the target genes of NFATc1 [33], it is reasonable to suppose that the inhibition of NFATc1 by taxifolin disrupt the F-actin ring formation, while more studies are needed to validate this. In addition, in accordance with the results in vitro, taxifolin treatment protected against LPS-induced osteolysis in the murine calvaria. LPS induced cytokines releasing from macrophages and monocytes via the NF-κB and MAPK signaling pathways that induce the formation and activation of osteoclasts [34]. The protective effect of taxifolin on LPS-stimulated bone loss could be partially attributed to its suppression on NF-κB signaling pathway. However, further researches were needed to confirm this hypothesis.

However, the current study had some limitations. In the first place, in our previous study, taxifolin promotes osteogenic differentiation of human BM mesenchymal stem cells (MSCs). The results obtained in this study demonstrated that taxifolin treatment inhibited LPS-induced bone loss maybe via the inhibition of osteoclast differentiation and function. It remains unclear which effect of taxifolin play the leading role in this protective effect. However, this issue is presently one of the ongoing researches in our laboratory. Second, although NF-κB pathway was crucial for osteoclast differentiation, there were many other pivotal pathways involved in osteoclastogenesis. Further investigations were needed to discover the regulation of taxifolin on other potential signaling pathways.

Collectively, taxifolin inhibited the osteoclast differentiation and function of human BMMs in a dose-response manner. Moreover, the NF-κB signaling pathway might play a vital role in this phenomenon. It indicated that taxifolin could be a potential therapeutic chemical for osteoclastogenesis-related diseases such as osteoporosis, osteolysis, and rheumatoid arthritis.

Acknowledgments

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Statement of Ethics

This research was approved by the Ethics Committee of Xiangya Hospital of Central South University (No. 201703359 and No. 201703358). Informed consent was signed by each BM donor prior to inclusion in the study. All animal experiments and procedures were approved by Department of Laboratory Animals of Central South University.

Disclosure Statement

The authors declare no competing financial interests.

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