Original Paper

Shikimic Acid Inhibits Osteoclastogenesis in Vivo and in Vitro by Blocking RANK/ TRAF6 Association and Suppressing NF- κ B and MAPK Signaling Pathways

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Key Words

Shikimic acid • Postmenopausal osteoporosis • Osteoclasts • NFATc1

Abstract

Background/Aims: Bone homeostasis is associated with the balance between bone-resorbing osteoclasts and bone-forming osteoblasts. Unbalanced bone homeostasis as a result of reduced osteogenesis or excessive osteoclastogenesis can lead to disorders such as osteoporosis, Paget's disease, and rheumatoid arthritis. Shikimic acid is a cyclohexanecarboxylic acid, reported to exhibit pharmacological properties including anti-inflammatory and antioxidant activities. However, its effects on bone homeostasis remain unknown. **Methods:** First, the *in vitro* MTT cell viability assay was performed. Tartrate-resistant acid phosphatase (TRAP) and actin ring formation assays, as well as immunofluorescence staining were then performed to evaluate osteoclastogenesis. Potential signaling pathways were characterized by western blotting and verified in overexpression experiments. Related factors were examined by western blotting, reverse transcription polymerase chain reaction, electrophoretic mobility shift assay, and co-immunoprecipitation. Ovariectomized mice were used for the *in vivo* study. **Results:** TRAP staining showed that shikimic acid significantly inhibited osteoclastogenesis and pit resorption in bone marrow monocytes and RAW264.7 cells, and actin ring formation assays showed that shikimic acid suppressed the bone resorption function of osteoclasts. Furthermore, shikimic acid inhibited the receptor activator of nuclear factor- κ B RANK/tumor necrosis factor receptor-associated factor 6 (TRAF6) association, suppressed nuclear factor- κ B and mitogen-activated protein kinase signaling pathways, and downregulated nuclear factor of activated T-cell cytoplasmic 1. The expression of osteoclastogenesis biomarkers, including TRAF6, calcitonin receptor, TRAP, cathepsin K, and matrix metalloproteinase-9, was inhibited.

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In vivo, shikimic acid also significantly ameliorated bone loss and prevented osteoclastogenesis in ovariectomized mice. **Conclusion:** Shikimic acid inhibited osteoclastogenesis and osteoclast function by blocking RANK ligand-induced recruitment of TRAF6, as well as downstream signaling pathways *in vitro*. Shikimic acid also reduced ovariectomy-induced osteoclastogenesis and bone loss *in vivo*.

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Introduction

Bone homeostasis is associated with the balance between bone-resorbing osteoclasts and bone-forming osteoblasts. Impaired osteogenesis by osteoblasts and excessive osteoclastogenesis by osteoclasts can cause disorders such as osteoporosis, Paget's disease, and rheumatoid arthritis (RA) [1]. Osteoclasts are differentiated from bone marrow monocytes (BMMs) following stimulation by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) [2]. The binding of M-CSF and c-Fms activates Akt and extracellular signal-regulated kinase (ERK) 1/2, and maintains proliferation of osteoclast precursor cells. In addition, the binding of RANKL to its receptor activator of nuclear factor- κ B (RANK) receptor recruits tumor necrosis factor receptor-associated factors (TRAFs). Subsequently, RANK-TRAF (especially RANK-TRAF6) complexes activate multiple downstream signaling pathways [3]. Nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) is the central transcriptional factor in osteoclastogenesis and is responsible for the transcription of osteoclast-related genes, including matrix metalloproteinase (MMP)-9, cathepsin K, latent transforming growth factor beta binding protein 3, tartrate-resistant acid phosphatase (TRAP), and c-Src [4]. Finally, intracellular Ca^{2+} is released, and NFATc1 is activated and osteoclastogenesis is promoted [5]. Previous studies demonstrated that overactive osteoclast function could also be induced by a deficiency of estrogen accompanied by increased levels of proinflammatory cytokines and RANKL after menopause [6, 7].

Shikimic acid is a compound found in many plants and is extracted from *Illicium verum* Hook.f [8]. This compound has attracted much interest because of its pharmacological safety and biological activities, and has been demonstrated to have several pharmacological effects, including anti-inflammatory and antioxidant activities [9-11]. However, the effects of shikimic acid on osteoclastogenesis have not been reported.

In our previous study, we found that low-dose shikimic acid inhibited the proliferation and differentiation of osteoclasts. Therefore, we hypothesized that it could regulate osteoclastogenesis and serve as an alternative option for the treatment of bone-related diseases such as postmenopausal osteoporosis. Thus, the aims of the present study were to characterize the effects of shikimic acid on osteoclastogenesis and to investigate the underlying molecular mechanisms of this process.

Materials and Methods

Reagents and antibodies

Shikimic acid (Fig. 1A) was provided by Professor Hu (Second Military Medical University, Shanghai, China). RAW264.7 cells were provided by Professor Hou (Second Military Medical University). Penicillin-streptomycin, α -modified essential medium, and fetal bovine serum were obtained from Puhe Biotechnology (Wuxi, China).

Cytotoxicity assay

BMMs were cultured for 48 h with different concentrations (0, 21.25, 42.5, 85, 170, 340, and 680 $\mu\text{g}/\text{mL}$) of shikimic acid, an MTT solution was added to the plates, and the cells were treated for 2 h. An enzyme-linked immunosorbent assay plate reader was used to measure the absorbance at 490 nm.

In vitro osteoclastogenesis assay

BMMs were extracted from the femurs of C57BL/6 mice [28]. Then, 20 ng/mL M-CSF and 50 ng/mL RANKL were used to induce cells, with or without various concentrations of shikimic acid (21.25, 42.5, and 85 µg/mL). After 7 days, TRAP staining (Sigma-Aldrich, St. Louis, MO) was performed according to the manufacturer's instructions. TRAP-positive cells with more than three nuclei were considered as osteoclasts.

F-actin ring formation and pit formation assays

Cells were fixed with 4% paraformaldehyde for 15 min following induction with 20 ng/mL M-CSF and 50 ng/mL RANKL for 7 days. Cells were then washed at least three times using phosphate-buffered saline and treated with FITC-phalloidin for 1 h, followed by 4',6-diamidino-2-phenylindole staining for 10 min. The pit formation assay was performed as previously described [7].

Immunofluorescence staining

We examined the effects of shikimic acid (85 µg/mL) on the nuclear translocation of p65 in RAW264.7 cells. Immunofluorescence was performed as previously described [29]. Cells were fixed for 15 min using 4% paraformaldehyde, washed for 10 min with 0.2% Triton X-100 solution, and blocked with 1% bovine serum albumin. Anti-p65 antibody, anti-mouse IgG antibody, and fluorescein-conjugated streptavidin were then sequentially added. Propidium iodide was used to counterstain the cells, and 10 cells were selected from a random region for imaging.

Western blotting

RAW264.7 cells (2×10^6 cells/well) were incubated in six-well plates and treated with RANKL or shikimic acid (85 µg/mL) for 0, 15, 30, and 60 min, and phosphorylation of IκB, p65, p50, ERK, p38, and JNK was evaluated by western blotting. Three groups of RAW264.7 cells (treated with M-CSF, with RANKL and M-CSF, or with RANKL, M-CSF, and shikimic acid at 21.25, 42.5, or 85 µg/mL, respectively) were cultured in six-well plates for 7 days. The levels of TRAP, CTR, TRAF6, MMP-9, and cathepsin K were then determined. Mouse anti-β-actin was used as the primary antibody. The secondary antibodies were donkey anti-goat horseradish peroxidase (Abcam, Cambridge, UK) and anti-glyceraldehyde 3-phosphate dehydrogenase (Abcam).

Fig. 1. Shikimic acid inhibits osteoclastogenesis *in vitro*. (A) Chemical structure of shikimic acid. (B) MTT analysis of the cytotoxic effects of shikimic acid in RAW264.7 cells. (C) TRAP-staining of mouse RANKL-induced BMMs. (D) TRAP-staining of mouse RANKL-induced RAW 264.7 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Animal model

We used the ovariectomized mouse model in a pathogen-free animal laboratory as previously described [30]. Six-week-old female C57BL/6 mice were obtained from Slack (Shanghai, China). Mice were divided into three groups: a sham group, ovariectomy group, and treatment group. For treatment, 10 mg/kg shikimic acid was injected intraperitoneally every day. Femur and arterial blood samples were collected 6 weeks later. The blood was centrifuged for 5 min at 3,000 rpm, and the supernatant was stored at -80°C .

Histological analyses

Femur bone samples were fixed for 4 days using 4% paraformaldehyde and decalcified for 3 weeks with 10% tetracycline-EDTA. Each distal femur was sliced into 4- μm sections for H&E and TRAP staining. For calcein labeling, mice were subcutaneously injected with calcein (20 mg/kg) on days 1 and 7. The mice were sacrificed on day 10, and the femurs were cut into 50- μm -thick sections. Double calcein labeling was captured using a fluorescence microscope. We calculated the trabecular bone area, number of osteoclasts, and osteogenic rate within the selected areas using ImageJ software (National Institutes of Health, Bethesda, MD).

Micro CT analysis

We analyzed 100 section planes from the growth plate in each bone (Skyscan1172, Antwerp, Belgium). The metaphyseal region and trabecular bone were detected by built-in software. BMD, BV/TV, BS/TV, and Tb.N were calculated. We reconstructed the bone structure image slices in two- and three-dimensional images.

EMSA

Nuclear proteins were extracted and the transcription factors bound to specific DNA sequences were examined by EMSA as previously described [31]. Nuclei were extracted from RANKL-induced RAW264.7 cells with or without shikimic acid treatment for 0 and 30 min, then incubated for 30 min at 37°C with ^{32}P -end-labeled 45-mer double-stranded NF- κB oligonucleotide from the HIV long terminal repeat. The DNA-protein complex was separated from oligonucleotides using 6.6% native polyacrylamide gels, and the gels were quantitated using ImageJ software.

RT-PCR

RNA was extracted from cells and the NFATc1 transcripts in the extracts were quantified using SYBR Green dye and normalized with β -actin. The following primer sets were used: mouse NFATc1: forward, 5'-TGGAGAAGCAGACACAGAC-3' and reverse, 5'-GCGGAAAGGTGGTATCTCAA-3'; mouse β -actin: forward, 5'-GTACGCCAACACAGTGCTG-3' and reverse, 5'-CGTCATACTCCTGCTTGCTG-3'.

Lentivirus infection of RAW264.7 cells

RAW264.7 cells were treated with trypsin to prepare a cell suspension, which was seeded into six-well plates. The cells were infected with Lv-NC, Lv-shRNA-NFATc-1, or Lv-NFATc-1 overnight. Following infection, the cells were incubated with shikimic acid and cultured for 3 days. Finally, the cells were subjected to TRAP staining as previously described.

Statistical analysis

The experiments were performed in triplicate and results are presented as the mean $\pm$ standard deviation. Student's *t*-test was used to determine statistical differences; values of $P < 0.05$ were considered significant.

Results

Shikimic acid had limited effect on differentiation of bone marrow stromal cells to osteoblasts and adipocytes

Bone remodeling is modulated by the activity of osteoclasts and osteoblasts. We first evaluated the effects of shikimic acid on osteogenesis and adipogenesis of bone marrow stromal cells (BMSCs) [12]. Alizarin Red staining and alkaline phosphatase results indicate

that shikimic acid (85 µg/mL) had limited effect on the formation of calcium nodules (Supplemental Figure S1). Oil Red O staining also showed that shikimic acid had limited effect on the *in vitro* formation of fat granules (Supplemental Figure S1 - for all supplemental material see www.karger.com/10.1159/000496039/). We therefore concluded that shikimic acid had limited effects on osteogenesis or adipogenesis.

Shikimic acid suppressed osteoclastogenesis in BMMs and RAW264.7 cells

The MTT cell viability assay was performed before the *in vitro* studies. The results indicated that shikimic acid had no obvious cytotoxic effects at concentrations below 85 µg/mL (Fig. 1B). We then examined the effects of shikimic acid on osteoclastogenesis using RAW264.7 cells and BMMs, both of which are standard models of osteoclastogenesis. Cells were treated with 21.25, 42.5, and 85 µg/mL shikimic acid. Compared with the control group, the number of TRAP-positive cells was significantly increased following RANKL induction, and shikimic acid significantly decreased the number of TRAP-positive cells in a dose-dependent manner (Fig. 1C and D). Together, the results show that shikimic acid suppressed osteoclastogenesis in BMMs and RAW264.7 cells.

Shikimic acid inhibited osteoclast function in vitro

The actin ring formation assay was used to characterize the role of shikimic acid in cytoskeleton formation, which is one of the most important processes of osteoclastogenesis. Fluorescein isothiocyanate (FITC)-phalloidin staining showed that BMMs differentiated into mature osteoclasts and formed actin rings following induction by RANKL. However, when cells were incubated with 21.25, 42.5, and 85 µg/mL shikimic acid, the size and number of cells decreased in a dose-dependent manner (Fig. 2A), indicating that shikimic acid inhibited actin ring formation of osteoclasts.

To identify the role of shikimic acid in osteoclast function, we performed bone resorption assays using RAW264.7 cells. The results show that cells differentiated into mature osteoclasts and formed clear pits on the bone biomimetic synthetic surface induced by RANKL/M-CSF. However, the administration of shikimic acid significantly reduced the resorbed area, suggesting that shikimic acid suppressed osteoclast function (Fig. 2B).

Shikimic acid had limited effect on BMM proliferation and differentiation induced by M-CSF

To identify the osteoclastogenesis stage affected by shikimic acid, its effects of on M-CSF-induced BMM proliferation were examined. RANK and c-fms levels were determined using reverse transcription polymerase chain reaction (RT-PCR). The expression levels of RANK and c-fms in RAW264.7 cells did not change significantly following incubation with shikimic acid, indicating that shikimic acid had limited effect on BMM proliferation induced by M-CSF (Supplemental Figure S2).

Shikimic acid suppressed osteoclastogenesis at an early stage

To identify the stage of osteoclastogenesis affected by shikimic acid, BMMs were treated on days 1, 3, and 5 and RAW264.7 cells on days 1, 2, and 3. The results show that shikimic acid mainly inhibited osteoclast differentiation on day 1 and was no longer effective at later stages (day 5 for BMMs and day 3 for RAW264.7 cells; Fig. 3A and B). Together, the results indicate that shikimic acid did not affect mature osteoclast formation but inhibited the differentiation of osteoclast precursors induced by RANKL at an early stage.

Shikimic acid suppressed the nuclear factor-κB pathway in osteoclastogenesis

Immunofluorescence staining was performed to localize p65 with or without shikimic acid treatment of RAW264.7 cells (Fig. 4A). p65 translocated into the nucleus following induction with RANKL in RAW264.7 cells. However, the nuclear translocation of p65 was blocked by shikimic acid. The ratio of fluorescence intensities, which reflected the fluorescence intensity at the nuclear site relative to the whole-cell fluorescence intensity, showed that

Fig. 2. Shikimic acid inhibits osteoclast function. The resorbed area was quantified by ImageJ software. (A) F-actin staining of mouse RANKL-induced BMMs. (B) The resorption area on the bone biomimetic synthetic surface. Data are presented as the mean $\pm$ standard error of the mean (SEM) and analyzed by one-way analysis of variance. BMMs were treated with different concentrations of shikimic acid for F-actin ring staining ($n = 10$); BMMs were treated with different concentrations of shikimic acid for bone resorption assay ($n = 10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 3. Shikimic acid inhibits osteoclastogenesis at an early stage. (A) Effect of shikimic acid on RANKL-induced BMM differentiation on different days. (B) Effect of shikimic acid on RANKL-induced RAW264.7 cell differentiation on different days. Data are presented as the mean $\pm$ SEM. BMMs treated with different concentrations of shikimic acid for osteoclastogenesis ($n = 10$); RAW264.7 cells treated with different concentrations of shikimic acid for osteoclastogenesis ($n = 10$).

p65 in RAW264.7 cells was activated when induced by M-CSF and RANKL. Following shikimic acid treatment of RAW264.7 cells, nuclear translocation of p65 was significantly inhibited (Fig. 4B). Western blotting results show that phosphorylation of p50, p65, and I κ Ba was increased, reaching peak levels at 30, 30–60, and 60 min, respectively, following induction with M-CSF and RANKL in RAW264.7 cells, indicating that their activation sequence involved the nuclear factor (NF)- κ B pathway. The phosphorylation levels of these factors in RAW264.7 cells were significantly inhibited following incubation with shikimic acid (Fig. 4C). Next the DNA-binding activity of p65 was characterized using an electrophoretic mobility shift assay (EMSA), which showed that DNA-binding activity was significantly inhibited following the addition of shikimic acid to RAW264.7 cells (Fig. 4D). Taken together, the results show that shikimic acid inhibited RANKL-induced NF- κ B pathway activation.

Fig. 4. Shikimic acid suppresses the NF- κ B pathway in osteoclastogenesis. The ratio of the fluorescence intensity was quantified by ImageJ software. (A) The nuclear translocation of p65 induced by RANKL. (B) Ratio of fluorescence intensity. (C) Western blot of phosphorylation of I κ B, p65, and p50 (D) Electrophoretic mobility gel shift assay of phosphorylation of I κ B, p65, and p50.

Shikimic acid suppressed the mitogen-activated protein kinase signaling pathway in osteoclastogenesis

The mitogen-activated protein kinase (MAPK) signaling pathway has an important role in osteoclastogenesis [13]. The phosphorylation of ERK, p38, and c-Jun N-terminal kinase (JNK), the major intermediates of the MAPK pathway, were examined by western blotting in RAW264.7 cell extracts. The semi-quantitative results show that the phosphorylation levels of these intermediates significantly increased following incubation with M-CSF and RANKL in RAW264.7 cells, reaching peak levels within 30 min. Upon addition of shikimic acid to these cells, phosphorylation of the intermediates was inhibited (Fig. 5A). Phosphorylation of ERK and p38 was significantly inhibited 15 min after induction of RANKL in RAW264.7 cells, while the inhibitory effects of phosphorylated JNK reached a peak at 30–60 min (Fig. 5B). Overall, the results indicate that shikimic acid inhibited osteoclastogenesis via the MAPK signaling pathway.

Shikimic acid blocked the association between RANK and TRAF6 induced by RANKL and suppressed markers of osteoclastogenesis-related gene expression

NFATc1 is a downstream regulator in osteoclastogenesis that mediates the expression of various osteoclastogenesis-related genes and regulates osteoclast function and differentiation [14]. RAW264.7 cells were induced with RANKL after NFATc1 overexpression. TRAP-staining

Fig. 5. Shikimic acid suppresses the MAPK pathway in osteoclastogenesis and markers of osteoclastogenesis-related gene expression. (A) Western blot of phosphorylation of ERK, P38, and JNK, which were associated with the MAPK signaling pathway. (B) Quantification of phosphorylation of ERK, p38, and JNK. (C) TRAP staining of RAW264.7 cells with or without overexpression of NFATc1. (D) Co-IP of interaction between TRAF6 and RANK. (E) NFATc1, Cathepsin K, CTR, TRAF6, MMP-9, and TRAP levels were assessed in five different groups: 1 RAW264.7 cells; 2 RAW264.7 cells incubated with M-CSF and RANKL; 3 RAW264.7 cells incubated with M-CSF, RANKL, and 21.25 μg/mL shikimic acid; 4 RAW264.7 cells incubated with M-CSF, RANKL, and 42.5 μg/mL shikimic acid; and 5 RAW264.7 cells incubated with M-CSF, RANKL, and 85 μg/mL shikimic acid. *P<0.05, **P<0.01, ***P<0.001.

Fig. 6. Shikimic acid reduces ovariectomy-induced bone loss in vivo. (A) H&E staining of distal femur sections and quantification of trabecular area from each group. (B) Micro CT analysis of the distal femur from each group. (C) Calculation of BMD, Tb.N, BS/TV, and BV/TV. (D) Representative TRAP-stained histologic distal femur sections. (E) Calcein labeling of distal femur sections. (F) Serum levels of CTX-1, TRAcP5B, TNF-α, and IL-6 were examined. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

showed that the number of TRAP-positive cells increased significantly in cells overexpressing NFATc1 and, importantly, the inhibitory effects of shikimic acid on osteoclastogenesis were reversed by overexpression in RAW264.7 cells (Fig. 5C), suggesting that the effect of shikimic acid is likely upstream of osteoclastogenesis. Co-immunoprecipitation (co-IP) was performed to examine the effects of shikimic acid on the RANK/TRAF6 interaction. RAW264.7 cells were treated in the absence and presence of shikimic acid and induced with or without RANKL. The cell lysates were then immunoprecipitated with anti-TRAF6 antibody and treated with anti-RANK antibody. The results show that RANK recruited TRAF6 induced by RANKL. However, shikimic acid significantly suppressed this interaction (Fig. 5D). Together, the results demonstrated the inhibitory effects of shikimic acid on the RANKL-induced association between RANK and TRAF6.

Western blotting showed that the expression levels of osteoclastogenesis-related genes, including cathepsin K, TRAP, calcitonin receptor (CTR), and MMP-9, were significantly upregulated when induced by M-CSF and RANKL, but downregulated in a dose-dependent manner when RAW264.7 cells were treated with different concentrations of shikimic acid (Fig. 5E). RT-PCR showed that NFATc1 expression in RAW264.7 cells increased following induction with M-CSF and RANKL, whereas it decreased significantly in a dose-dependent manner following the addition of shikimic acid (Fig. 5E).

Shikimic acid prevented ovariectomy-induced bone loss in mice by suppressing osteoclastogenesis

Bone homeostasis is a balanced process mediated by osteoblasts and osteoclasts. The over-activation of osteoclasts leads to an imbalance in bone resorption, which results in osteoclast-related diseases such as postmenopausal osteoporosis [15, 16]. We therefore examined whether shikimic acid affected ovariectomy-induced osteoporosis in mice. After 6 weeks, hematoxylin and eosin (H&E) staining showed that, compared with sham-operated mice, the ovariectomized mice had significant trabecular bone loss, and this was abrogated in mice treated with shikimic acid (Fig. 6A). Micro computed tomography (CT) analysis, including bone volume/total volume (BV/TV), bone surface area/total volume (BS/TV), bone mineral density (BMD), and trabecular number (Tb.N), showed similar results. The two- and three-dimensional structures are shown in Fig. 6B and 6C. As shown by TRAP staining, the number of osteoclasts was significantly increased in ovariectomized mice, and shikimic acid administration significantly reduced the number of osteoclasts (Fig. 6D). Calcein labeling of distal femurs further showed that the bone formation rate declined after ovariectomy in mice, and this effect was reversed following shikimic acid treatment (Fig. 6E).

To characterize the effects of shikimic acid on ovariectomy-induced proinflammatory cytokine secretion and osteoclastogenesis serum markers, we also compared the serum levels of tumor necrosis factor (TNF)- α , C-telopeptide of type I collagen (CTX-1), tartrate-resistant acid phosphatase (TRAcP) 5B, and interleukin (IL)-6 among the three groups. Compared with the sham-operated group, the levels of these serum factors were significantly higher in ovariectomized mice. However, for the ovariectomized mice treated with shikimic acid, the levels were significantly decreased, which demonstrated that shikimic acid treatment suppressed ovariectomy-induced proinflammatory cytokine secretion and osteoclast activity (Fig. 6F).

Discussion

In this study, using a series of *in vivo* and *in vitro* experiments, we showed that shikimic acid played an important role in bone-related pathology. To our knowledge, this is the first report of the inhibitory effects of shikimic acid on osteoclastogenesis. We characterized the

Fig. 7. Shikimic acid suppresses the expression of RANKL-induced proteins involved in osteoclastogenesis. Schematic diagram of the role of shikimic acid in inhibiting osteoclastogenesis. The NF- κ B and MAPK signaling pathways are activated by RANKL-RANK signaling, inducing the expression of NFATc1 and the regulation of osteoclastogenesis-related expression of

genes, including Cathepsin K, CTR, TRAF6, MMP-9, and TRAP. Following shikimic acid administration, the expression of RANK and the association between RANK and TRAF6 are blocked, and the NF- κ B and MAPK signaling pathways are then suppressed. As a result, NFATc1 expression is decreased and osteoclast differentiation is inhibited. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

molecular mechanisms involved, and found that shikimic acid inhibited the transcription of NFATc1 by blocking the association between RANK and TRAF6, thus suppressing RANKL-activated NF- κ B and MAPK pathways. Furthermore, we demonstrated that shikimic acid reversed the bone loss caused by ovariectomy in mice (Fig. 7).

Bone homeostasis is mediated by the balance between bone-resorbing osteoclasts and bone-forming osteoblasts [17]. Disruption of osteogenesis by osteoblasts and excessive osteoclastogenesis by osteoclasts can lead to diseases such as osteoporosis, Paget's disease, and RA [1]. Studies of alternative agents for the treatment of bone-related diseases are therefore of considerable scientific and public interest [18].

Shikimic acid, a compound found in many plants, has been shown to exhibit potential pharmacological properties both in animal and human models, especially with regard to its anti-inflammatory and antioxidant activities [10, 11, 19]. It has been shown to be effective in treating ischemic injury, by stimulating the microcirculation [20]. However, its pharmacological mechanisms remain unknown, and the effects and probable molecular signaling pathways involved in osteoclastogenesis have not been reported.

We performed MTT assays before *in vitro* studies to exclude the possibility of cytotoxic effects of this compound. The subsequent experiments showed that shikimic acid suppressed the formation of mature osteoclasts. However, there was a limited effect on BMM proliferation and differentiation. We then investigated the stage of osteoclastogenesis affected by shikimic acid. The results show that shikimic acid inhibited osteoclastogenesis at an early stage during the process of differentiation of pre-osteoclasts into mature osteoclasts.

NFATc1, an important regulator in osteoclastogenesis, mediates the expression of osteoclastogenesis-related genes and regulates osteoclast function [21]. In this study, we found that the inhibitory effects of shikimic acid were reversed by overexpression of NFATc1 in RAW264.7 cells, which suggested that shikimic acid affected an upstream target of osteoclastogenesis.

The RANKL signaling pathway induces osteoclastogenesis and bone loss, and blockage of this pathway has been shown to decrease bone loss [22]. When RANKL is bound to its RANK receptor, it recruits TRAFs (TRAF1, 2, 3, 5, and 6), thereby activating downstream signaling pathways [23, 24]. The TRAF6 pathway induces phosphorylation of MAPKs, followed by ERK, p38, and JNK [25]. We therefore conducted co-IP assays and showed that shikimic acid

blocked the RANKL-induced interaction between RANK and TRAF6, and suppressed the phosphorylation of MAPKs and NF- κ B. The expression levels of osteoclastogenesis-related markers, including MMP-9, TRAP, CTR, TRAF6, and cathepsin K, were then partially reduced. Finally, the process of osteoclastogenesis was blocked. Overall, the results show that shikimic acid inhibited osteoclastogenesis by modulating the association between RANK and TRAF6, suppressed MAPK and NF- κ B pathways, and finally downregulated the expression of NFATc1.

Osteoclasts are known to be the only cells that undergo bone resorption during bone homeostasis [26]. We confirmed our *in vitro* results using ovariectomized mice. Histomorphometric staining and micro CT analyses showed that shikimic acid suppressed osteoclastogenesis and osteoclast activities to ameliorate bone loss *in vivo*. The serum levels of IL-6, TNF- α , CTX-1, and TRAcP were also reduced by shikimic acid, which was consistent with the TRAP staining of the distal femur. These results demonstrated that shikimic acid affected osteoclastogenesis, at least partially, in the setting of postmenopausal osteoporosis.

The effects of shikimic acid were similar to those of bisphosphonates, which are widely used for the treatment for osteoporosis. However, the effects of shikimic acid on osteogenesis and bone formation remain unclear. No significant difference was found in BMSC osteogenesis and adipogenesis among the different groups in our preliminary experiments. As a result, we concluded that shikimic acid had limited effects on osteoblast differentiation, and that it mainly affected osteoclastogenesis. Further studies are needed to characterize the role of shikimic acid in other osteoclastogenesis-related diseases. In addition, the side effects of shikimic acid are important and require further study as some osteoporosis drugs can cause damage to the intestinal tract [27].

Conclusion

Our findings demonstrated that shikimic acid inhibited osteoclastogenesis by blocking the association between RANK and TRAF6, inhibiting NF- κ B and MAPK pathways *in vitro*, and further ameliorating ovariectomy-induced bone loss *in vivo*. Our results suggested that shikimic acid could provide a novel, alternative, protective therapeutic agent for bone-related diseases such as postmenopausal osteoporosis.

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Disclosure Statement

All the authors declare no conflicts of interest.

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