Iguratimod Suppresses Osteoclastogenesis and Prevents Ovariectomy-Induced Bone Loss through the Inhibition of RANKL Signaling Pathway

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Abstract: Iguratimod is a novel disease-modifying antirheumatic drug which is widely used in China. Recent evidence showed that iguratimod attenuated Walker 256 rat mammary gland carcinoma cells induced bone destruction and osteoclasts formation in vivo. Thus, we presumed that iguratimod may suppress osteoclastogenesis and prevent osteoclast-related diseases including postmenopausal osteoporosis. In this study, ovariectomized (OVX) mice were used to detect the effects of iguratimod in vivo. Bone marrow mononuclear cells (BMMCs) were cultured to detect the effects of iguratimod on receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL)-induced osteoclastogenesis in vitro and the molecular mechanisms involved. The results demonstrated that iguratimod prevented ovariectomy-induced bone loss by suppressing osteoclast activity. Consistently, iguratimod suppressed RANKL-induced osteoclastogenesis and bone resorption. Furthermore, iguratimod inhibited RANKL-stimulated tumor necrosis factor receptor-associated factor 6 (TRAF6) - transforming growth factor β-activated kinase-1 (TAK1) complex formation, followed by suppressing the activation of downstream mitogen-activated protein kinase (MAPK), NF-κB and c-fos. Iguratimod subsequently decreased the expression of osteoclast-specific genes via targeting nuclear factor of activated T cells c1 (NFATc1). In conclusion, our results clearly show that iguratimod can inhibit OVX-induced bone loss and osteoclastogenesis through modulating RANKL signaling. Therefore, iguratimod may be used as a novel therapy to mitigate postmenopausal osteoporosis.

Keywords: iguratimod; postmenopausal osteoporosis; ovariectomy; osteoclast; RANKL; MAPK pathway; NF-κB pathway; TRAF6; TAK1

1. Introduction

Bone homeostasis is maintained by repeated cycles of bone formation and bone resorption in a process called bone remodeling [1]. Balance of the bone remodeling process can be broken by many bone metabolism diseases. Postmenopausal osteoporosis is one such systemic skeletal disease characterized by low bone density and micro architectural deterioration that lead to increased bone fragility thus making the bone susceptible to fracture [2]. Moreover, lack of functional ovaries leads to a decline in estrogen levels that increases bone formation and, to a much higher extent, increases bone resorption, leading to net bone loss [3]. Estrogen deficiency may also result in the over
expression of RANKL by B lymphocytes [4], which may in part explain the excessive osteoclast formation and trabecular bone loss in postmenopausal osteoporosis patients.

Osteoclasts are multinucleated giant cells differentiated from monocyte-macrophage lineage precursor cells, and possess a unique ability to resorb bones. Osteoclast differentiation is primarily governed by 2 key cytokines, macrophage colony-stimulating factor (M-CSF) and RANKL. M-CSF supports cell survival and proliferation while RANKL serves as a signal for osteoclastogenesis [5-7]. The binding of RANKL and its receptor RANK on osteoclast precursor cells induces the recruitment and accumulation of TRAF6, followed by the association of TRAF6 to TAK1 [8]. TRAF6-TAK1 complex subsequently activates downstream signaling pathways including NF-κB [9,10], MAPKs and activator protein 1 (AP-1) [9,11,12]. Up-regulated AP-1 increases the expression of nuclear factor of activated T cells c1 (NFATc1) [13], which is a master factor that activates the expression of osteoclast-specific genes and subsequently results in enhanced differentiation and function of osteoclasts [14-17]. Interfering with these steps in the pathways may help prevent pathological bone loss.

Iguratimod (N-[3-(formylamino)-4-oxo-6-phenoxy-4H-chromen-7-yl]methanesulfonamide) (T-614), a novel anti-rheumatoid arthritis drug, is reported to exert its therapeutic effect by reducing the production of inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α [18,19]. These anti-inflammatory effects of iguratimod are due to its ability to suppress NF-κB activation, which is indispensable for osteoclastogenesis [20]. Iguratimod also suppresses TNF-α and IL-17 induced MAPK activation in fibroblast-like synoviocytes [21]. Together, these reports indicate a likely relationship between iguratimod and osteoclastogenesis. Moreover, in type II collagen-induced arthritis and spontaneous arthritis models, iguratimod can reduce joint destruction and bone resorption [22]. These studies suggest that iguratimod may be a latent inhibitor of osteoclasts activation. However, whether iguratimod can be a therapeutic agent to treat postmenopausal osteoporosis is unknown. In this study, we investigated the effects of iguratimod on bone loss and osteoclasts formation in vivo and in vitro models, and elucidated the underlying molecular mechanisms.

2. Results

2.1. Effect of iguratimod on OVX-induced bone loss

C57/BL6 mice were randomized into 3 groups: SHAM group (sham-operated, vehicle-treated mice), OVX group (ovariectomized, vehicle-treated mice) and OVX+T-614 group (ovariectomized, iguratimod-treated mice). Six weeks after operation, mice were sacrificed. Then, we used micro-computed tomography (μ-CT) scanning to analyze the trabecular bone changes in distal femoral metaphyses of mice. The results demonstrated significant decrease in trabecular bone volume/tissue volume (BV/TV), trabecular bone number (Tb.N) and increase in structure model index (SMI), trabecular separation (Tb. Sp) in the OVX group when compared with the SHAM group. Compared with the OVX group, treatment of OVX mice with iguratimod (OVX+T-614 group) significantly attenuated trabecular bone loss revealed by changes in histomorphometric parameters (Figure 1A, B). In addition, mice in the OVX and OVX+T-614 groups exhibited markedly decreased wet weight of uterus (Figure 1C) compared with the SHAM group, suggesting the success of ovariectomy.
Figure 1. Iguratimod alleviates bone loss in ovariectomized mice. Mice were sacrificed 6 weeks after the operation. (A) μ-CT images of distal femurs from representative specimens from the SHAM, OVX and OVX+T-614 groups. (B) Histograms represent the 3D trabecular structural parameters of the distal femur: trabecular bone volume/tissue volume (BV/TV), structure model index (SMI), trabecular number (Tb. N) and trabecular separation (Tb. Sp). (C) Mice uterus was isolated and weighed. Scale bar, 1 mm. Data are presented as means ± SD. n=10. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 versus SHAM. # p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001 versus OVX.

Hematoxylin and eosin (H&E) staining of the femoral sections were then used to further corroborate these results. Trabeculae in the OVX group were rare and thin in regions proximal and distal to the growth plate. Treatment with iguratimod significantly increased trabecular density and thickness when compared to the OVX group (Figure 2A).

We then stained femoral sections with tartrate-resistant acid phosphatase (TRAP) to investigate the effects of iguratimod on osteoclasts differentiation. Mice in the OVX+T-614 group had reduced numbers of TRAP-positive multinucleated cells, analyzed by the number of osteoclasts per version, compared with mice in the OVX group (Figure 2B, C).

Moreover, compared with the OVX group, mice in the OVX+T-614 group also displayed decreased serum levels of type 1 collagen cross-linked C-terminal telopeptide (CTX-I), which is a biomarker of bone resorption (Figure 2D). These results together suggest that treatment with iguratimod can attenuate OVX-induced bone loss by inhibiting the differentiation of osteoclasts.
2.2. Effect of iguratimod on RANKL-mediated osteoclastogenesis and osteoclasts function in vitro

To further explore the impact of iguratimod on osteoclastogenesis, we tested its effect on bone marrow mononuclear cells (BMMCs). We first detected the potential cytotoxicity of iguratimod using the Cell Counting Kit-8 (CCK-8) and 4′, 6-diamidino-2-phenylindole (DAPI) staining. As shown in Figure 3, even at 30 μg/mL concentration, iguratimod did not influence the viability and proliferation of BMMCs.

We then treated BMMCs with different concentrations of iguratimod (from 0.3 μg/mL to 30 μg/mL) in the presence of RANKL (50 ng/mL) and M-CSF (30 ng/mL) for 5 days. As shown in Figure 4A, C, iguratimod strongly inhibited RANKL-mediated osteoclastogenesis in a dose-dependent manner. At the concentration of 3 μg/mL iguratimod, there were no visible TRAP-positive multinucleated cells.

To evaluate the effect of iguratimod on the bone resorption function of osteoclasts, an Osteo Assay Surface plate was used. After seeding onto a bone slice, BMMCs were cultured with RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 7 days, and then additional 5 days in the presence of different concentrations of iguratimod. As shown in Figure 4B, D, iguratimod significantly suppressed the bone resorption function of osteoclasts.
Figure 3. Influence of iguratimod on BMMCs viability and proliferation. (A) BMMCs were cultured with 0 (vehicle, dimethyl sulfoxide, DMSO), 0.3, 3 or 30 μg/ml iguratimod in the presence of M-CSF (30 ng/mL) for 1, 3 or 5 days. Cell viability was assessed using a CCK-8 assay and calculated using the formula: Cell viability = X/ctrl × 100%. Data from 3 independent experiments are presented as means ± SD. (B) After 1, 3 or 5 days in culture, BMMCs were stained with DAPI, and images of cell nuclei were obtained. Scale bar, 70 μm.
Figure 4. Iguratimod suppresses osteoclasts differentiation and function in vitro. (A, C) BMMCs were incubated with 0 (vehicle), 0.3, 3 or 30 μg/ml iguratimod in the presence of RANKL (50 ng/mL) and M-CSF (30 ng/mL) for 5 days and then stained with TRAP (A). TRAP-positive multinucleated cells in each well were counted (C). (B, D) BMMCs seeded onto a Corning Osteo Assay Surface were incubated with RANKL (100 ng/mL) and M-CSF (30 ng/mL) for 7 days and then treated with 0 (vehicle), 0.3, 3 or 30 μg/ml iguratimod for an additional 5 days. Subsequently images of bone resorption were obtained (B) and quantified by Image J (D). Scale bar, 200 μm. All data are from 3 independent experiments and are presented as means ± SD. **** p<0.0001 versus vehicle-treated controls (T-614 0 μg/mL).

2.3. Effect of iguratimod on RANKL-induced c-Fos, NFATc1 and osteoclast marker gene expression

Osteoclast formation and function are affected by osteoclast marker genes such as TRAP, cathepsin K and matrix metalloproteinase (MMP)-9. All these are target genes of a master transcription factor, NFATc1, which is in part regulated by its upstream activator, c-Fos. We then examined the effect of iguratimod on these pathways. As shown in Figure 5A, quantitative real-time polymerase chain reaction (qRT-PCR) showed that incubation with RANKL significantly increased the mRNA expression of all these genes in BMMCs, especially on day 5. Treatment with iguratimod (3 μg/mL) drastically suppressed the RANKL-induced up-regulation of all these genes. The effects of iguratimod on the expression of c-Fos and NFATc1 were corroborated by immunoblotting (Figure 5B). These data suggest that iguratimod is a potent suppressor of c-Fos, NFATc1, TRAP, cathepsin K and MMP-9 expression in both early- and late- stage osteoclastogenesis.

Figure 5. Iguratimod inhibits the expression of c-Fos, NFATc1 and osteoclast marker genes. BMMCs were treated with vehicle or 3 μg/ml iguratimod in the presence of RANKL (50 ng/mL) and M-CSF (30 ng/mL). (A, B) After 2 days (A) and 5 days (B) of incubation, total RNA was extracted from the cells and used for qRT-PCR. Data are presented as means ± SD. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. (ctrl, M-CSF treated; R+M, RANKL+M-CSF; R+M+T, RANKL+M-CSF+T-614) (C) Proteins were extracted at indicated times and protein expression levels of c-Fos and NFATc1 were detected by Western blotting. The experiments were repeated 3 times independently.
2.4. Effect of iguratimod on MAPKs and NF-κB activation

Activation of MAPKs and NF-κB is essential for RANKL-mediated osteoclastogenesis. Therefore, we explored the effect of iguratimod on these pathways using immunoblotting. As shown in Figure 6A, iguratimod suppressed RANKL-induced phosphorylation of JNK, p38 and ERK. As for NF-κB, Western blotting showed that iguratimod significantly inhibited the phosphorylation of NF-κB p65. Moreover, data from the electrophoretic mobility shift assay (EMSA) demonstrated that iguratimod also suppressed the DNA binding activity of p65 by 23%. These results indicate that iguratimod can suppress RANKL-mediated activation of MAPK and NF-κB signaling pathways in BMMCs.

Figure 6. Iguratimod inhibits RANKL-induced activation of MAPKs and NF-κB. (A) BMMCs were starved with 0.5% FBS in α-MEM for 12 h and pretreated with vehicle (DMSO) or 3 μg/ml iguratimod for 1 h followed by treatment with RANKL (100 ng/mL) for indicated times. The cell lysates were extracted for immunoblotting. Protein expression levels of p-JNK, JNK, p-p38, p38, p-ERK, ERK, p-p65 and p65 were detected using Western blotting. (B) BMMCs were starved with 0.5% FBS in α-MEM for 12 h and pretreated with vehicle (DMSO) or 3 μg/ml iguratimod for 1 h and followed by treatment with RANKL (100 ng/mL) for 30 min. Nuclear proteins were extracted from the cells and subjected to EMSA. The experiments were repeated 3 times independently.

2.5. Effect of iguratimod on TRAF6-TAK1 complex

The formation of TRAF6-TAK1 complex mediated by RANKL is a vital step prior to the activation of MAPKs and NF-κB. As iguratimod could suppress the activation of MAPKs and NF-κB, we next examined whether iguratimod can suppress TRAF6-TAK1 complex formation. When treated with RANKL (100 ng/mL), co-immunoprecipitation of TRAF6 and TAK1 increased. However, treatment with iguratimod significantly suppressed RANKL-induced co-immunoprecipitation of TRAF6 and TAK1 (Figure 7). These data indicate that iguratimod can block RANKL-induced osteoclastogenesis via suppressing the formation of TRAF6-TAK1 complex and activation of downstream pathways.
Figure 7. Influence of iguratimod on the formation of TRAF6 and TAK1 complex. BMMCs were starved with 0.5% FBS in α-MEM for 12 h and pretreated with vehicle (DMSO) or 3 μg/ml iguratimod for 1 h, followed by stimulation with RANKL (100 ng/mL) for 5 min. (A, B) Cell extracts were immunoprecipitated (IP) with anti-TRAF6 or anti-TAK1 antibody. Bound proteins were immunoblotted with anti-TAK1 or anti-TRAF6 antibody, respectively. The experiments were independently repeated 3 times.

3. Discussion

In a previous study we observed that iguratimod reduced osteoclasts formation and bone destruction in the Walker 256 rat mammary gland carcinoma cells induced bone cancer pain model [23]. Other studies showed that mammary carcinoma cells are not bone-resorbing cells, but they stimulate osteocytes to express RANKL [24,25]. Over expression of RANKL results in pathologically enhanced osteoclasts formation and bone destruction. Considering the similar roles osteoclasts play in malignant and benign bone resorption, we hypothesized that iguratimod may also have therapeutic effects in benign bone metabolism diseases such as postmenopause osteoporosis.

Dysfuction of ovaries induces estrogen deficiency and subsequently leads to the over expression of RANKL, which contributes to excessive osteoclastogenesis and trabecular bone loss [4]. Our rationale to use ovariectomized (OVX) mice as animal models in this study is based on reports that all major characteristics of bone loss associated with estrogen deficiency in humans can be mimicked in ovariectomized mice [3,26,27]. Our data showed that ovariectomy in mice led to trabecular bone loss, increased osteoclasts formation and increased serum levels of CTX-I, whereas these osteoporotic effects were significantly alleviated by treatment with iguratimod. Taking into account the vital role of RANKL in postmenopausal bone loss, we presume that targeting RANKL-induced osteoclastogenesis may be a reasonable explanation for the therapeutic effects of iguratimod in OVX mice.

Stimulation of RANKL facilitates NF-κB and MAPKs activation. NF-κB is a vital regulator of osteoclastogenesis [20,28]. Phosphorylated p65 was transferred into nucleus and evoked the initial induction of NFATc1, which subsequently binds to its own promoter and switches to
auto-amplification [29]. The activation of AP-1 complex (c-Fos/c-Jun dimer), which is triggered by MAPKs, also contributes to the induction and further auto-amplification of NFATc1 [13]. A previous report showed that stimulation of RANKL failed to increase NFATc1 levels in c-Fos deficient cells [30]. Finally, NFATc1 activates osteoclast marker genes and promotes the formation and function of osteoclasts [31-33]. Our results revealed that iguratimod suppressed RANKL-mediated osteoclastogenesis and bone resorption in a dose-dependent manner. It is reported that in rheumatoid arthritis patients treated with the recommended dose of iguratimod, the serum levels of iguratimod were about 3 μg/ml [18]. Thus we chose 3 μg/ml as an effective concentration to explore the influences of iguratimod on osteoclastogenesis signaling. Our data showed that iguratimod drastically suppressed the enhanced expression of NFATc1 induced by RANKL. The activation of NFATc1 regulates a number of osteoclastogenesis marker genes, including MMP-9 and cathepsin K, which can directly degrade collagens in demineralized hard tissues [17,34]. In addition, phosphorylation of JNK, ERK and p38 triggered by RANKL were significantly suppressed by iguratimod. Moreover, iguratimod also had a suppressive effect on the phosphorylation of p65. NF-κB binding to DNA was suppressed by 23% through iguratimod treatment. This result is consistent with previous findings [35]. Taken together, the prevention of RANKL-mediated bone resorption by iguratimod may attribute to the suppression of NFATc1 expression via decreasing MAPKs-mediated AP-1 activation and blocking the NF-κB pathway.

As a consequence of RANKL binding, the cytoplasmic domain of RANK recruits a number of TRAFs among which TRAF6 is crucial for osteoclastogenesis [36,37]. Impaired osteoclast function was observed in TRAF6 knockout mice leading to severe osteopetrosis [36]. Recruitment of TRAF6 leads to the formation of TRAF6-TAK1 complex resulting in the activation of TAK1 [38]. As a serine/threonine kinase in the MAPK kinase kinase (MAPKKK) family, TAK1 can activate MEK1/2, MKK3/6 and MKK4/7, which then phosphorylate JNK, ERK and p38 [9,11,12]. On the other hand, TAK1 can also activate the NF-κB pathway via phosphorylation of inhibitor of NF-κB (IκB) and IκB kinase (IKK) [9,10]. Our immunoprecipitation assay revealed that RANKL stimulation rapidly evoked the accumulation of TRAF6-TAK1 complexes, while iguratimod significantly suppressed this effect. These data suggest that iguratimod can disrupt intracellular signaling cascade in osteoclastogenesis, at least in part, by targeting TRAF6-TAK1 complex formation.

Limitations in this study should be noted. First, our in vivo study showed that iguratimod significantly alleviated ovariectomy-induced bone loss via suppressing osteoclastogenesis. However, we did not study whether iguratimod can influence other pathways that are related to ovariectomy-induced bone loss. A previous study showed that iguratimod stimulated bone morphogenetic protein-2-induced bone formation in vivo and osteoblastic differentiation in vitro [39]. Therefore, further studies are needed to validate the influence of iguratimod on osteoblastogenesis in OVX mice. Moreover, inflammatory cytokines, including TNF-α, IL-1α and IL-6, were reported to promote osteoclastogenesis through multiple mechanisms [40,41]. Therefore, whether the inhibitory effects of iguratimod on bone loss attribute to its well-known anti-inflammatory activities needs further investigation. Second, although our data indicate that iguratimod interfered with TRAF6-TAK1 complex formation, the exact targets of iguratimod remain unknown. Studies have shown that TRAF6 can catalyze the synthesis of non-degradative, lysine-63-linked polyubiquitin chains [38,42]. This TRAF6 auto-ubiquitinylation is a prerequisite to recruit and activate TAK1 [42]. Whether iguratimod interferes with the ubiquitinylation of TRAF6 or whether there are other targets will be topics of further studies.

In conclusion, our data demonstrates that iguratimod prevented ovariectomy-induced bone loss in vivo and RANKL-induced osteoclastogenesis in vitro. Considering the lifelong need to treat osteoporosis and that iguratimod is well tolerated in long-term use [43-45], the anti-osteoclastogenic activity of iguratimod under clinical settings should be addressed in future.
4. Materials and Methods

4.1. Reagents and Antibodies

Iguratimod was provided by Simcere Pharmaceutical (Nanjing, China). The drug was suspended in 0.5 % methylcellulose solution for in vivo use, and dissolved in dimethyl sulphoxide (DMSO) for in vitro use. Recombinant soluble mouse M-CSF and RANKL were obtained from PeproTech (Rocky Hill, NJ, USA). Rabbit antibodies against phosphorylated and total MAPKs, p-NF-κB p65, NF-κB p65, NFATc1 and TAK1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit antibody against c-Fos was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibody against TRAF6 was purchased from Abcam (Cambridge, MA, USA). Anti-GAPDH was purchased from Boster (Wuhan, China).

4.2. Animals

Twelve-week-old C57/BL6 female mice (28±1g) (Experimental Animal Center of Tongji Hospital, Wuhan, China) were maintained at a constant temperature of 25 °C under a 12-h light/12-h dark cycle with free access to food and water. This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) at Tongji Hospital.

4.3. Experimental Groups

Animals were divided randomly into 3 groups (n=10 mice/group). Group 1 (SHAM) animals were sham-operated and treated with 0.5% methylcellulose solution (vehicle); Group 2 (OVX) animals underwent bilateral ovariectomies and were treated with vehicle, and Group 3 (OVX+T-614) animals underwent bilateral ovariectomies and were treated with iguratimod (30 mg/kg/day)[21]. All treatments began on day 1 after operation and were administered orally. After 6 weeks, mice were sacrificed to isolate femurs and uteri for use in the following experiments.

4.4. Bone structure analysis

The distal femoral bone structure was analysed with a μ-CT system (μ-CT50 Scanco Medical, Bassersdorf, Switzerland). Scans were obtained at 100 kV and 98 μA; the resolution was set to 10.5 μm. 3D reconstruction were analyzed using the build-in software in the μ-CT system. Trabecular structural parameters including bone volume/tissue volume (BV/TV), structure model indexes (SMI), trabecular number (Tb.N.) and trabecular separation (Tb. Sp.) were also evaluated.

4.5. Histological analysis

For histological analysis, femur samples were fixed in 4% paraformaldehyde for 24 h, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution for 3 weeks and embedded in paraffin wax. H&E staining was performed to observe the trabecular structure. TRAP (Sigma-Aldrich, St. Louis, MO, USA) staining was performed following standard protocols and the numbers of osteoclasts near femoral metaphysis were counted. Images were obtained using Leica Microsystems (Wetzlar, Germany).

4.6. Serum Biochemistry

For serum biochemistry analysis, a retro-orbital puncture was performed immediately prior to sacrifice to collect blood from each mouse. Sera were then extracted and serum levels of CTX-I were measured with ELISA kits (IDS Nordic, Herlev, Denmark) according to the manufacturer’s protocol.
4.7. Cell cultures

Bone marrow mononuclear cells (BMMCs) were obtained from 6-week-old C57BL/6 mice as described previously [46,47]. Briefly, marrow cavity of isolated femurs and tibias were exposed and flushed with Alpha Minimum Essential Medium ($\alpha$-MEM). Cells were then collected and cultured in $\alpha$-MEM with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and M-CSF (30 ng/ml). After 24 h, nonadherent cells were collected and supplemented with M-CSF (30 ng/ml). After 3 days, adherent cells were seeded in 6-well plates or 96-well plates and cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) to induce osteoclast formation.

4.8. In vitro osteoclastogenesis assay

BMMCs were plated in 96-well plates at a density of 10000 cells/well and cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the presence of vehicle or iguratimod. The culture medium was replaced every 2 days. After 5 days, TRAP staining was performed according to the manufacturer’s protocol using the TRAP staining kit (SigmaAldrich, St. Louis, USA). Images were obtained and TRAP-positive multinucleated (>3 nuclei) cells were counted as osteoclasts.

4.9. Bone pit formation by osteoclasts

BMMCs were seeded at a density of 20000 cells/well in a Corning Osteo Assay Surface plate (Corning Incorporated Life Science, New York, USA). Cells were cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 7 days, then treated with vehicle or various concentrations of iguratimod for additional 5 days. Then, the plate was washed with 5% sodium hypochlorite for 5 min. Images of bone resorption were captured by Leica Microsystems (Wetzlar, Germany) and quantified by Image J.

4.10. Cell proliferation assay and DAPI staining

Cell viability was assessed using CCK-8 assay (Boster, Wuhan, China). BMMCs were seeded at a density of 5000 cells/well in 96-well plates. After 24 h, BMMCs were treated with 0 (vehicle), 0.3, 3 or 30 μg/ml iguratimod in the presence of M-CSF (30 ng/mL). After 1, 3 and 5 days, medium containing 10% CCK-8 was added to each well and then incubated in darkness at 37°C for 1 h. The absorbance was measured on an ELX800 absorbance microplate reader (Bio-Tek, Vermont, USA) at a wavelength of 450 nm. For DAPI staining, after treatment with different concentrations of iguratimod for 1, 3 and 5 days, cells were washed 3 times with phosphate buffer saline (PBS) followed by staining with DAPI (Boster, Wuhan, China) for 5 min.

4.11. RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA was extracted from osteoclasts using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as described [47]. First-strand cDNA was synthesized using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) to perform qRT-PCR using the THUNKERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and a Bio-Rad Q5 instrument (Bio-Rad Laboratories, Hercules, CA, USA). All reactions were performed according to the manufacturer’s instructions, and target gene expression was normalized to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used for qRT-PCR: 5’-CACGCGCCGTGACACCGATAG-3’ (forward) and 5’-GGGAAAGTCAGAAGTGGGGA-3’ (reverse) for NFATc1, 5’-AACGCCGACCAGTGCTGGA-3’ (forward) and 5’-TACCTGTGTGGACATGACC-3’ (reverse) for TRAP, 5’-TGTATAACGCCACGGCAAA-3’ (forward) and 5’-GGTTCACATTATCGTCACA-3’ (reverse) for cathepsin K, 5’-TCCAGTACCAAGACAGCCCT-3’ (forward) and 5’-TTGCACGTGACGGTGGA-3’ (reverse) for MMP-9, 5’-GGTGAAGACCGTGTCAGGAG-3’ (forward) and 5’-TATTCCGTTTCCTCAGGATT-3’ (reverse) for c-Fos, and...
5’-CTCCCACTCTTCCACCTTCG-3’ (forward) and 5’-TTGCTGTAGCCGTATTCCATT-3’ (reverse) for GAPDH.

4.12. Western blot analysis

Cell lysates were prepared with the RIPA Lysis Buffer (Boster, Wuhan, China) containing 1mM phenylmethanesulfonyl fluoride (PMSF). Western blot analysis was then performed as described [47,48]. In brief, total cell proteins were separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Subsequently, membranes were blocked with 5% bovine serum albumin and immunoblotted with primary antibodies against p-p65, p65, p-JNK, JNK, p-ERK, ERK, p-p38, p38 MAPK, c-Fos or NFATc1 overnight at 4°C. Then, the membranes were incubated with horseradish peroxidase (HRP)-labelled secondary antibodies (Boster, Wuhan, China) for 1 h at room temperature. Immunoreactivity was detected with enhanced chemiluminescence (Boster, Wuhan, China) and images were taken by ChemiDoc™ XRS+System with Image Lab™ Software (BIO-RAD Laboratories Inc., Munich, Germany).

4.13. Electrophoretic mobility shift assay

BMMCs were pretreated with or without 3 μg/ml iguratimod for 1 h and stimulated with 100 ng/ml RANKL for 30 min. Extraction of nuclear proteins was performed using a nuclear and cytoplasmic protein extraction kit according to the manufacturer’s instructions (Boster, Wuhan, China). The DNA-binding activity of NF-κB was detected using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, MA, USA) [47,48]. Briefly, equal amounts of nuclear extracts were incubated with biotin end labeled probe and electrophoresed on a 6% polyacrylamide gel. Then, protein-DNA complexes were transferred onto a positively charged nylon membrane (Millipore, MA, USA) for chemiluminescence band-detection. The NF-κB probe (Beyotime Institute of Biotechnology, Jiangsu, China) used for EMSA was 5’-AGTTGAGGGGACTTTCCCAGGC-3’.


Cell lysates were extracted with the Immunoprecipitation RIPA buffer (Byotime, Wuhan, China) followed by immunoprecipitation using SureBeads (Bio-Rad, CA, USA) according to the manufacturer’s protocol. Briefly, SureBeads were incubated with anti-TRAF6 and anti-TAK1 antibody for 60min, followed by incubation with different groups of cell lysates for 90 min. After washing with PBS-T (PBS with 0.1% tween-20), immune complexes were analyzed by Western blotting.

4.15. Statistical analysis

All quantitative data are expressed as means ± SD. Statistical analysis between 2 groups was performed using Student’s t-test. Statistical comparison of more than 2 groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey test. Statistical significance was defined as P<0.05 in all tests.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: The image of isolated uteri. Figure S2: Body weight of mice.

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Sample Availability: **Samples of iguratimod are available from the authors.**

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