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Iguratimod (T-614) suppresses RANKL-induced osteoclast differentiation and migration in RAW264.7 cells via NF-κB and MAPK pathways

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Introduction: Iguratimod (T-614) has been confirmed as a highly efficacious and safe novel disease-modifying anti-rheumatic drug (DMARD) for rheumatoid arthritis therapy in China and Japan due to its potent anti-inflammation effect. Here, we investigate the effects of Iguratimod on osteoclast differentiation, migration and function.
Methods: The effect of Iguratimod on osteoclastogenesis, migration and bone resorption were assessed by TRAP staining, transwell migration assay and osteologic discs, respectively. Relative expressions of osteoclastic related genes, chemokines and transcription factors were assessed by reverse transcription polymerase chain reaction (RT-PCR) and signaling pathways were analyzed by western blotting.
Results: Iguratimod significantly inhibits osteoclast differentiation, migration and bone resorption in RANKL-induced RAW264.7 cell in a dose-dependent manner. The expressions of osteoclastic related genes including TRAP, CTSK and CTR were increased in RAW264.7 cell upon RANKL stimulation but were obviously suppressed in the presence of Iguratimod. RANKL induced the expression of chemokines including CCL7, CCL4 and CCL12 and osteoclastic related transcription factors of c-Fos, c-Jun and NFATc1 could be significantly inhibited by Iguratimod in a dose dependent manner. Western blotting indicated Iguratimod could suppress the activation of MAPKs and NF-κB pathway in RANKL induced osteoclastogenesis in RAW264.7.
Conclusions: These findings revealed a directly inhibitory role of Iguratimod on osteoclast formation and function, which is distinct from previous report, suggesting Iguratimod provide a unique therapeutic strategy for RA and especially in light of preventing bone destruction.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial inflammation, cartilage and bone destruction [1]. Despite intensive effort in the development of new therapies for preventing disease progress in RA, a proportion of patients still failed to current classical synthetic disease-modifying anti-rheumatic drugs (sDMARDs) or biological (bDMARDs) [2]. Thus, to seek novel therapeutic agents for RA is still imperative.

About 80% bone erosion of RA patients occurs within 1 year after diagnosis [3], which results in severe structural damage and poor functional outcome in RA. Though the precise etiology of bone erosion in RA still remains elusive, osteoclast, formed by fusion of mononuclear precursors of the monocyte/macrophage, is the cell ultimately responsible for bone destruction in RA. The substantial evidence demonstrates that enhanced activation of osteoclast precursors and mature osteoclasts are abundant at sites of arthritic bone erosions in RA patients and collagen-induced murine arthritis, suggesting osteoclast is an attractive therapeutic target for RA [4,5].

The accelerated osteoclast activation is the result of the cumulative burden of inflammation in RA [6]. Proinflammatory cytokines, such as IL1β, TNFα, IL-6 and IL-17, accumulated in inflammatory joint and circulation of RA, have been suggested play a central role in osteoclastogenesis and bone resorption in RA via receptor activator of nuclear factor κB ligand (RANKL) signaling pathway [7]. Cytokine inhibiter such as TNF blockers (infliximab, etanercept, adalimumab, certolizumab-pegol and golimumab) and IL-6 receptor (IL-6R) blockade (tocilizumab) displayed a potent anti-inflammatory effect and consequently markedly ameliorate joint destruction in RA [8], highlighting the crucial role of inflammatory

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mediated bone erosion in RA. Notably, a proportion of RA patients with low level of disease activity and even those with clinical remission after biologic therapy still experience a continued progression of bone erosion [9,10]. Mice deficiency in osteoclast could develop inflammation but are protected from bone erosion in a serum transfer model of arthritis [11]. Moreover, blockade of osteoclastogenesis by Denosumab, a fully human monoclonal antibody to RANKL activity, could significantly prevent joint damage regardless of inflammation [12], implying the processes of inflammation and osteoclast medicated bone erosion might be dissociated in some patients with RA. Conventional antirheumatic drugs for RA, such as methotrexate and leflunomide, seem to have bone-protective effects mainly based on their ability to effectively suppressing immune reaction and inflammation. Thus, an idea therapeutic strategy for RA should target at both inflammation and osteoclast.

Iguratimod (T-614, N-[7-methanesulfonamido-4-oxo-6-(phenoxy)-chromen-3-yl]formamide) is a member of the family of methanesulfonanilide, which has been confirmed as a highly efficacious and safe novel DMARD for rheumatoid arthritis therapy in China and Japan [13–15]. The antiarthritic mechanism of Iguratimod was mainly attributed to its anti-inflammation effect. The accumulated evidences indicated that Iguratimod could suppress inflammatory cytokines of interleukin-1β(IL-1β), IL-6, IL-8, IL-17, tumor necrosis factor-α and nuclear factor-kappaB production in RA synovial cells and human monocytic THP-1 cells [13,16–19]. In CIA models, Iguratimod could significantly inhibit joints inflammation and bone erosion [20]. Moreover, recently study showed Iguratimod suppress IL-17 signaling in fibroblast-like synoviocytes, highlighting the potent anti-inflammatory role in RA. Interestingly, in addition to inhibit IL-17 expression, we recently found Iguratimod is capable of suppression RANKL/OPG in RASFs [21], which prompt us to study effects of Iguratimod on osteoclast differentiation, migration and function. Our data revealed a direct role of Iguratimod on osteoclast, which is linked to the effective treatment for RA, especially for bone erosion.

2. Methods

2.1. Chemicals

Iguratimod was kindly provided by Simcere Pharmaceutical (Nanjing, China). Human recombinant RANKL and M-CSF proteins were purchased from PeproTech and R&D Systems, respectively. A rabbit polyclonal antibody (Ab) against β-actin was purchased from Abcam. Rabbit polyclonal Ab against phospho-c-Jun N-terminal kinases (JNK), phospho-extracellular signal-regulated kinases (ERK), and phospho-p38 MAPK was purchased from Cell Signaling Technology. Rabbit polyclonal Abs against transcription factors c-Fos, c-Jun and NFATc1 were purchased from Santa Cruz Biotechnology.

2.2. Cell viability assay

The RAW264.7 cells (ATCC) were plated into 96-well plates at a density of 1×10^3 cells/well and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate, plus various concentrations of Iguratimod, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Cell viability in different concentration of Iguratimod treated RAW264.7 cells was measured by the CCK-8 assay (CCK-8; Dojindo China Co., Ltd., Shanghai), in which cellular dehydrogenase activity in the living cells was detected. Cell viability was expressed as a percentage of the value in the untreated control culture. All experiments were performed in triplicate on 3 separate occasions.

2.3. Osteoclast differentiation and TRAP staining

To assess the effect of Iguratimod on osteoclastogenesis, RAW264.7 cells were plated at a density of 2×10^4 cells/well in a 12-well culture plate and cultured under following three conditions: 1) culture medium (CM) only; 2) CM plus 100 ng/ml RANKL and 20 ng/ml M-CSF; and 3) CM with RANKL & M-CSF, plus various concentrations of Iguratimod. After 3–5 days (d), cells were washed twice with 1× PBS, fixed with 4% paraformaldehyde for 10 minutes (min), and stained for TRAP using an Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma) according to the manufacturer's instructions. TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts under a light microscope.

2.4. Bone resorption assay

To assess the effect of Iguratimod on RANKL-induced bone resorption, RAW264.7 cells were seeded on 24-well osteologic discs (BD Biosciences) at a density of 0.5×10^3 cells per well and treated with 100 ng/ml RANKL and 20 ng/ml M-CSF for 10 days. After 10 days of culture, cells were removed and resorption pits were examined by light microscope and photographed. Percentage of resorbed area to the total area was determined using Image-J software at 100-fold magnification.

2.5. Transwell migration assay

RAW264.7 (1.5×10^4) were added to transwell inserts (8 Am pores, 6.5 mm polycarbonate membranes, Costar, Corning, NY) and then were placed into a 24-well dish containing αMEM and 10% FCS with or without RANKL (50 ng/ml) and Iguratimod. Cells were incubated for 24 h at 37 °C (5% CO_2) and then the upper wells containing unmigrated cells were removed and the migrated cells on the lower wells were counted by light microscopy.

2.6. Real time RT-PCR analysis

Total RNA was isolated with TRIzol reagent (Invitrogen Inc., USA) following the manufacturer's instructions. A SuperScript TM III Platinum® SYBR® Green one-step quantitative real-time polymerase chain reaction (qRT-PCR) kit (Invitrogen, Carlsbad, CA, USA) was used. Relative expressions of osteoclastic markers including TRAP, CTSK, CTR and MMP-9, and transcriptional factors of c-Fos, c-Jun and NFATc1 were normalized using the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated by the $2^{-\Delta\Delta_{Ct}}$ method. The following primers were used: CCL4, 5′-TTCCTGCTGTTTCTCTTACA CCT-3′ (sense) and 5′-CTGTCTGCCTCTTTTGGTCAG-3′ (anti-sense); CCL7, 5′-GCTGCTTTCAGCATCCAAGTG-3′ (sense) and 5′-GCTGCTTTCA GCATCCAAGTG-3′ (anti-sense); CCL12, 5′-ATTTCCACACTTCTATGCCT CCT-3′ (sense) and 5′-ATCCAGTATGGTCCTGAAGATCA-3′ (anti-sense); TRAP, 5′-CCAATGCCAAAGAGATCGCC-3′ (sense) and 5′-TCTGTGCAGAGA CGTTGCCAAG-3′(anti-sense); CTSK,5′-GACGCAGCGATGCTAACTAA-3′ (sense) and 5′-CCAGCACAGAGT-CCACAACT-3′ (anti-sense); CTR 5′-TCAGGAACCACGGAATCCTC-3′ (sense) and 5′-ACATTC-AAGCGGATGCGT CT-3′ (anti-sense); MMP-9, 5′-CTGGACAGCCAGACACTAAAG-3′ (sense) and 3′-CTCGCGGCAAGTCTTCAGAG-3′(anti-sense); c-Fos, 5′-ATGATGTT CTCGGGTTTCAA-CG-3′ (sense) and 5′-CAGTCTGCTGCATAGAAGGAAC CG-3′ (anti-sense); c-Jun, 5′-ACTCGGA-CCTTCTCACGTCG-3′ (sense) and 5′-TAGACCGGAGGCTCACTGTG-3′ (anti-sense); NFATc1, 5′-CCGTTGCTTC CAGAAAATAACA-3′(sense) and 5′-TGTGGGATGTGAACTCGGAA-3′ (anti-sense); glyceraldehyde-3-phosphate dehydrogenase(GAPDH), 5′-GCAC AGTCAAGGCCGAG-AAT-3′(sense) and 5′-GCCTTCTCCATGGTGGTGAA-3′ (antisense).

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