Biochemical Pharmacology 86 (2013) 1775–1783

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

SC-514, a selective inhibitor of IKKβ attenuates RANKL-induced osteoclastogenesis and NF-κB activation

Qian Liu [a,b,c,1], Huafei Wu [b,c,1], Shek Man Chim [b], Lin Zhou [b], Jinmin Zhao [a], Haotian Feng [d], Qingli Wei [e], Qing Wang [f], Ming H. Zheng [c], Ren Xiang Tan [g], Qiong Gu [h], Jun Xu [h], Nathan Pavlos [c], Jennifer Tickner [b], Jiake Xu [b,*]

[a] Department of Orthopaedic Surgery, The First Affiliated Hospital of Guangxi Medical University, Guangxi 530021, China
[b] School of Pathology and Laboratory Medicine, The University of Western Australia, Crawley 6009, Western Australia, Australia
[c] Centre for Orthopaedic Research, School of Surgery, The University of Western Australia, Crawley 6009, Western Australia, Australia
[d] Program of Nutrition and Bone & Joint Health, Nestlé R&D (China) Ltd. Building 5, No. 5 Dijin Road, Haidian District, Beijing 100095, China
[e] College of Chemistry and Molecular Engineering, Qingdao University of Science & Technology, Qingdao 266042, China
[f] Institute of Medical Information, School of Biomedical Engineering, Southern Medical University, Guangzhou 510515, China
[g] Institute of Functional Biomolecules, Medical School, Nanjing University, Nanjing 210093, China
[h] Research Center for Drug Discovery (RCDD), School of Pharmaceutical Sciences, Sun Yat-Sen University, 132 East Circle at University City, Guangzhou 510006, China

ARTICLE INFO

Article history:
Received 30 August 2013
Accepted 19 September 2013
Available online 30 September 2013

Keywords:
SC-514
Bone marrow cells
Osteoclastogenesis
RANKL
NF-κB

ABSTRACT

The RANKL-induced NF-κB signaling pathway is essential for osteoclastogenesis. This study aims to identify specific inhibitors targeting NF-κB signaling pathway, which might serve as useful small molecule inhibitors for the treatment and alleviation of osteoclast-mediated bone lytic diseases. By screening for compounds that selectively inhibit RANKL-induced NF-κB activation in RAW264.7 cells as monitored by luciferase reporter gene assay, we identified SC-514, a specific inhibitor of IKKβ, as a candidate compound targeting osteoclastogenesis. SC-514 dose-dependently inhibits RANKL-induced osteoclastogenesis with an IC50 of <5 μM. At high concentrations, SC-514 (≥12.5 μM) induced apoptosis and caspase 3 activation in RAW264.7 cells. Moreover, SC-514 specifically suppressed NF-κB activity owing to delayed RANKL-induced degradation of IκBα and inhibition of p65 nuclear translocation. Taken together, our results indicate that SC-514 impairs RANKL-induced osteoclastogenesis and NF-κB activation. Thus, targeting IKKβ by SC-514 presents as a potential treatment for osteoclast-related disorders such as osteoporosis and cancer-induced bone loss.

1. Introduction

Osteoclasts are multinucleated bone-resorbing cells derived from precursors of the monocyte-macrophage lineage [1]. Overproduction and activation of osteoclasts is a pathological feature of many osteolytic bone disorders such as osteoporosis, periodontitis, and aseptic loosening of orthopedic implants [1]. The receptor activator of NF-κB ligand (RANKL), a member of the tumor necrosis factor (TNF) receptor–ligand family, is a crucial factor for the differentiation of osteoclasts *in vivo* and *ex vivo* [2–4]. Targeted ablation of RANKL in mice results in a lack of osteoclasts, severe osteopetrosis, and a defect in tooth eruption [5]. During osteoclastogenesis RANKL signaling is initiated by binding to its receptor, RANK, which results in the recruitment of TNF receptor associated factor (TRAF) adapter proteins and the activation of NF-κB, leading to the induced-expression of specific genes that have DNA-binding sites unique for NF-κB. NF-κB is an essential transcription factor that is vital to both osteoclast differentiation and survival [6–11]. An essential step in the NF-κB activation cascade is the phosphorylation of Inhibitor κBs (IκB) by the IκB kinase (IKK) complex which consists of IKK alpha (IKKα), IKK beta (IKKβ), and IKK gamma/NEMO with catalytic domain or regulatory domain [12]. Interestingly, IKKβ, but not IKKα, has been shown to be a critical mediator of osteoclast survival and is required for inflammation-induced bone loss [9]. Using conditional KO mouse models, it has been shown that IKKα is required for osteoclast formation *in vitro*, but it is dispensable *in vivo*. On the other hand, IKKβ is required for RANKL-induced osteoclastogenesis both *in vitro* and *in vivo* [9]. In addition, IKK downstream targets p50/52 and p65 are also important for osteoclastogenesis [13,14].

SC-514, a selective inhibitor of IKKβ is a cell-permeable (thienothienyl)amino-acetamide compound that displays anti-inflammatory properties [15]. As a potent, reversible,

* Corresponding author.
E-mail address: jiake.xu@uwa.edu.au (J. Xu).
[1] Equal contributors of the work.

0006-2952/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2013.09.017

ATP-competitive, and highly selective inhibitor, SC-514 inhibits various forms of recombinant IKKβ with IC50 values of 3–12 μM. SC-514 has selectivity on IKKβ and little effect on other members of the IKK family, including other IKK isoforms or serine-threonine and tyrosine kinases *in vitro*. SC-514 inhibits expression of NF-κB-dependent cytokines such as IL-6 and IL-8, through the inhibition of IKKβ-mediated phosphorylation of IκBα [15]. SC-514 is also effective in an acute inflammation model by blocking TNF-α production in LPS-challenged rats [15]. Previous studies have also reported the inhibitory effects of SC-514 on IKKβ in the treatment of tumors and inflammation [16–26]. However, the effect of SC-514 on osteoclastogenesis and RANKL-induced NF-κB signaling pathways is hitherto unknown. In the present study, we investigated the effects of SC-514 on *in vitro* osteoclast formation and NF-κB signaling. Our results demonstrate that SC-514 is a potent inhibitor of osteoclast formation and NF-κB activity and thus might serve as a useful treatment for osteoclast-related disorders such as osteoporosis.

2. Methods

2.1. Media and reagents

RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). Alpha Modification of Eagle's Medium (α-MEM) and fetal bovine serum (FBS) were purchased from TRACE (Sydney, NSW, Australia). L-Glutamine, penicillin and streptomycin were purchased from Gibco BRL (Melbourne, Australia). SC-514 (BML-EI343) was purchased from Enzo Life Sciences (Sapphire Bioscience Pty. Ltd. Australia). Natural compounds (with a purity ≥98%) and synthetic compounds were obtained from Professor Jun Xu of San Yat-sen University, Professor Ren Xiang Tan of Nanjing University, or Chengdu MUST Biotechnology Co., Ltd. (China). All compounds were dissolved in dimethyl sulfoxide (DMSO) purchased from BDH laboratory supplies (Poole, Dorset, England). GST-RANKL was purified and used as previously described [3].

2.2. NF-κB activity in RAW264.7 cells and drug screening assay

Drug screening assays were conducted using RAW264.7 cells stably transfected with an NF-κB-driven luciferase reporter gene construct (3κB-Luc-SV40) [27] with natural and synthetic compounds at concentrations of 1–10 μM. To investigate the effect of SC-514 on NF-κB activity, RAW264.7 cells stably transfected with an NF-κB-driven luciferase reporter gene were pretreated with various doses of SC-514 (0–1.25 μM) for 1 h followed by 100 ng/mL RANKL for 8 h. Luciferase activities were measured using a POLARStar OPTIMA plate reader (BMG Labtech, Germany).

2.3. Dose-dependent osteoclastogenesis assay

For primary cell cultures, bone marrow cells isolated from C57BL/6 mice were cultured for 3 days in complete medium containing α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin with the addition of 10 ng/mL macrophage-colony stimulating factor (M-CSF; R&D Systems, NSW, Australia). Cells were kept in a humidified 37 °C water-jacketed incubator (Forma Scientific) at 5% CO_2 and 95% air. Bone marrow derived macrophages (BMM) were then seeded (8×10^3 cells/well) into a 96-well cell culture plate and incubated overnight. Then cells were stimulated with M-CSF/RANKL (25/100 ng/mL) and treated with various concentrations of SC-514 (1.25–5 μM). Culture medium was replenished every second day. After 5–7 days incubation, cells were fixed with 4% paraformaldehyde (Sigma Aldrich, NSW, Australia) and stained for tartrate-resistant acid phosphatase (TRAP) activity to identify osteoclasts using TRACP staining kit (Sigma Aldrich, NSW, Australia). TRAP-positive multinucleated cells with >3 nuclei were scored as osteoclasts. Photographs of individual wells were taken using Nikon Coolpix 995.

2.4. Apoptosis assay

Apoptosis assays were performed as previously described [28]. In brief, 1×10^6 RAW264.7 cells were seeded in 2 mL of complete media in a 6-well cell culture plate and then incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C overnight. Media was removed after overnight incubation. SC-514 with various concentrations of 0 μM, 3.1 μM, 6.2 μM, 12.5 μM, 25 μM, or 50 μM was added to the wells, and cells were incubated for 24 h. Cells were collected and resuspended in 0.5 mL of 1 × Binding Buffer (BD-Pharmingen, NSW, Australia). Aliquots of cell suspensions (100 μL) were then stained with Annexin V-PE (5 μL: BD-Pharmingen, NSW, Australia) and/or 7-aminoactinomycin D (7-AAD) (5 μL: BD-Pharmingen, NSW, Australia). Cells were incubated in the dark at room temperature for 15 min, followed by addition of 400 μL of 1 × Binding Buffer and analysis by flow cytometry (FACSCalibur, Becton Dickinson, NSW, Australia). The percentage of apoptotic cells in the population was obtained.

2.5. Confocal microscopy

RAW264.7 cells (1×10^4 cells/well) were seeded onto sterilized cover-slips in 24-well plates. Cell were stimulated with 100 ng/mL of RANKL and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 5 days. Complete media with RANKL (100 ng/mL) was replaced every other day. After osteoclast formation was observed, cells were treated for 24 h with or without SC-514 (10 μM and 50 μM). Cells were washed twice with 1 × PBS (pH 7.4), and then fixed for 30 min with 0.5 mL 4% paraformaldehyde. Fixed cells were washed 3 times with 1 × PBS and then permeabilized with 0.5 mL of 0.1% Triton X-100 (Sigma Aldrich, NSW, Australia) in 1 × PBS for 5 min at room temperature. Following permeabilisation, cells were washed twice with 0.2% BSA (Sigma Aldrich, NSW, Australia) in 1 × PBS (0.2% BSA-PBS) and incubated in the dark for 2 h with Rhodamine-Conjugated Phalloidin F-actin stain (1:100–1:150; Life Technologies, Victoria, Australia) to visualize filamentous actin. The cells were washed 4 times with 0.2% BSA-PBS and a further 4 times with 1 × PBS and counter-stained in the dark at room temperature for 3 min with the Hoechst 33258 DNA nuclei stain (1:10,000 in PBS; Life Technologies, Victoria, Australia) for nuclear DNA staining. After washing the cells were mounted onto slides with anti-fade mounting media for confocal microscopy using a confocal laser scanning microscope (MRC-1000 Bio-Rad) equipped with a Krypton-Argon laser to an epifluorescent Nikon Diaphoto 300 inverted microscope. The slides were viewed under oil immersion objective lens (Nikon, NA = 0.7) and the images (512 × 512 pixel) were collected as Bio-Rad PIC files and viewed using Confocal Assistant™ version 4.02. For microtubule staining, a monoclonal anti-α-tubulin antibody (Sigma, St. Louis, USA) (1:500 diluted in 0.2% BSA-PBS) was used with Alexa Fluor 488 secondary antibodies (Molecular Probes Inc., Eugene, USA) at 1:500 dilution and the fluorescent images were collected as described above.

2.6. Caspase-3 assay

RAW264.7 cells were seeded (2×10^6 cells/well) into 24-well plates, cultured overnight, and then treated with various doses of