International Immunopharmacology 34 (2016) 212–219

Contents lists available at ScienceDirect

International Immunopharmacology

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

Tatarinan O, a lignin-like compound from the roots of *Acorus tatarinowii Schott* inhibits osteoclast differentiation through suppressing the expression of c-Fos and NFATc1

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ARTICLE INFO

Article history:
Received 24 December 2015
Received in revised form 28 February 2016
Accepted 1 March 2016
Available online 9 March 2016

Keywords:
Tatarinan O
Osteoclast
Bone resorption
NFATc1
c-Fos
RANKL

ABSTRACT

Osteoclasts (OC) are large multinucleated cells derived from monocyte/macrophage precursors. Suppressing osteoclastogenesis is considered as an effective therapeutic approach to erosive bone disease. The root of *Acorus tatarinowii Schott*, a well-known traditional Chinese medicine was used to treat rheumatosis and other inflammatory disease. However, the effects of tatarinan O (TO), one of the lignin-like compounds isolated from the roots of *Acorus tatarinowii Schott* during bone development are still unclear. In the present study, we explored the effect of TO on RANKL-induced osteoclastogenesis in vitro. TO was found to suppress osteoclast differentiation from RANKL-stimulated mouse bone marrow macrophages (BMMs) without significant cytotoxicity. TO also dose-dependently suppressed bone resorption activity of mature osteoclasts. Additionally, TO apparently inhibited the expression of osteoclastic marker genes, such as MMP-9, Cts K and TRAP. Furthermore, our results showed that TO decreased RANKL-induced expression of c-Fos and NFATc1 without influencing NF-κB activation and MAPK phosphorylation. Hence, for the first time we revealed that TO dose-dependently inhibited osteoclastogenesis from RANKL-stimulated mouse BMMs via decreasing the expression of NFATc1 and c-Fos.

1. Introduction

Bone is a dynamic organ that is continuously molded by an extremely dynamic process which depends on the balance between the bone-resorption and formation mediated by osteoclasts and osteoblasts, respectively [1]. Osteoclasts are large multinucleated cells (MNCs) differentiated from the monocyte/macrophage precursors and responsible for bone resorption [2]. Excessive osteoclastogenesis leads to many bone-loss related diseases, including rheumatoid and osteoarthritis, osteoporosis, malignant bone diseases, periodontal diseases, bacteria-induced osteolysis and Paget's disease of bone [2–4].

It has been verified that two primary cytokines, macrophage colony stimulation factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are essential factors for osteoclastogenesis [2,5]. RANKL binds to the cell surface RANK receptor, then RANKL/RANK/TNF receptor-associated factor 6 (TRAF 6) complexes activate nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) including p38, ERK and JNK [5–8]. In addition, RANKL also up-regulates the expression of key transcription factors during osteoclastogenesis, including c-Fos and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) [7–10]. The activation of above-mentioned signaling pathways controls the expression of osteoclastogenesis-related marker genes directly, including matrix-metalloproteinases 9 (MMP-9), cathepsin K (Cts K) and tartrate-resistant acid phosphatase (TRAP), which lead to bone resorption pit formation during osteoclastogenesis [11]. Thus, RANKL signaling is regarded as the primary target of anti-resorptive agents that inhibit osteoclastogenesis and bone loss.

The roots of *Acorus tatarinowii Schott* is a well-known traditional Chinese medicine used to treat dementia, rheumatosis and other inflammatory disease [12,13]. Previous investigations on the title plant led to the characterization of sesquiterpenoids [14,15], alkaloids [14,16], and lignans [17]. Many natural components have been reported to restrain osteoclastogenesis and bone resorption [10,18–20]. To identify novel compounds that can function as anti-resorptive agents, several natural compounds were evaluated by analyzing TRAP staining. Tatarinan O (TO), one of the lignin-like compounds isolated from the roots of *Acorus*

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http://dx.doi.org/10.1016/j.intimp.2016.03.001
1567-5769/

tatarinowii Schott [21] was found to repress RANKL-mediated osteoclast differentiation from bone marrow macrophages (BMMs).

The aim of this study was to investigate the inhibitory effect of TO on osteoclast formation in RANKL-stimulated BMMs and further clarify the mechanism underlying the suppressive effect of TO on RANKL-induced osteoclastogenesis.

2. Materials and methods

2.1. Chemical analyses

The NMR spectra were run on a Bruker AM-400 spectrometer with TMS as internal standard. HR-ESI-MS spectra were carried out on a Bruker Apex IV FT-MS spectrometer. Column chromatographic separations were carried out on silica gel H-60 (Qingdao Haiyang Chemical Group Corporation, Qingdao, China), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), and LiChroprep RP-18 (40–63 μm, Merckmillipore, Billerica, MA, USA). TLC was carried out on Silica gel HSGF254 plates (Yantai Chemical Industrial Institute, Yantai, China), and spots were visualized by spraying with concentrated sulfuric acid-vanillin solution followed by heating.

2.2. Plant material and reagents

The roots of *Acorus tatarinowii Schott*, in dried form, were purchased from Kangqiao Pharmaceutical Co., Ltd., Shanghai, China, in 2010. A reference sample was deposited in the Tongji Herbarium. Phosphate-buffered saline (PBS), α-MEM, penicillin, streptomycin and fetal bovine serum (FBS) were obtained from Thermo Scientific (Rockford, IL, USA). Phosphatase inhibitor cocktail, protease inhibitor cocktail and MTT were purchased from Sigma (St. Louis, MO, USA). Primary antibodies including anti-c-Fos, anti-JNK, anti-p-JNK, anti-IκB-α, anti-p65, anti-p-p65, anti-ERK1/2, anti-p-ERK1/2, anti-NFATc1, anti-p38, anti-p-p38 and anti-β-actin were from Cell Signaling Technology (Beverly, MA, USA). Soluble mouse recombinant M-CSF was obtained from Perprotech (Rocky Hill, NJ, USA). Soluble mouse recombinant RANKL was purchased from Prospec (Israel). All other chemicals were of reagent grade or complied with the standards required for cell culture.

2.3. Extraction and isolation

The air-dried roots of *Acorus tatarinowii Schott* (5.0 kg) were powdered and then extracted three times with 95% ethanol at room temperature. The combined ethanol extracts were concentrated under reduced pressure to give a residue (700 g), which was then partitioned successively with $CHCl_3$ and *n*-BuOH. The $CHCl_3$ extract (200 g) was subjected to silica gel column chromatography using a gradient solvent system of petroleum ether-acetone (50:1, 25:1, 15:1, 10:1, 5:1, 2:1, 1:1, v/v) to afford 6 fractions (Fr. 1–Fr. 6). Fr. 2 was subjected to silica gel column chromatography repeatedly and absorbed materials were eluted with petroleum ether-EtOAc (15:1, 10:1, 5:1, 2:1, 1:1, v/v) to afford 6 fractions (Fr. 2a–Fr.2f). Fr. 2a was subjected to repeated silica gel column chromatography with gradient elution with petroleum ether-acetone (20:1, 10:1, 5:1, 2:1, 1:1, v/v) to yield tatarinan O (12.0 mg, purity ≥99%).

2.4. Cell culture

Bone marrow cells were isolated from 6-week-old male C57BL/6 mice as previously described with some modifications [22,23]. In brief, bone marrow cells isolated from mice tibiae and femora were incubated overnight in α-MEM medium with 10% FBS, PS (100 U/mL penicillin and 100 μg/mL streptomycin) to obtain a single-cell suspension. Nonadherent cells were collected and seeded at an appropriate density in both 48-well plates and Corning OsteoAssay Surface 24-well plates, then incubated in α-MEM with 10% FBS, PS and M-CSF (20 ng/mL) for 2 days. The adherent cells were used as bone marrow derived macrophages (BMMs).

Table 1
Primer sequences used in quantitative real-time PCR.

Primers	Gene sequence
Cts K sense	5′-AATACCTCCCTCTCGATCCTACA-3′
Cts K antisense	5′-TGGTTCTTGACTGGAGTAACGTA-3′
MMP-9 sense	5′-CTGGACAGCCAGACACTAAAG-3′
MMP-9 antisense	5′-CTCGCGGCAAGTCTTCAGAG-3′
TRAP sense	5′-CACTCCCACCCTGAGATTTGT-3′
TRAP antisense	5′-CATCGTCTGCACGGTTCTG-3′
NFATc1 sense	5′-GGAGAGTCCGAGAATCGAGAT-3′
NFATc1 antisense	5′-TTGCAGCTAGGAAGTACGTCT-3′
c-Fos sense	5′-CGGGTTTCAACGCCGACTA-3′
c-Fos antisense	5′-TTGGCACTAGAGACGGACAGA-3′
β-actin sense	5′-GGCTGTATTCCCCTCCATCG-3′
β-actin antisense	5′-CCAGTTGGTAACAATGCCATGT-3′

2.5. Cell viability assessment

BMMs (4×10^5 cells) were plated in 96-well plates and cultured in α-MEM medium supplemented with 10% FBS, PS, M-CSF (20 ng/mL), RANKL (20 ng/mL) and various concentrations of TO up to 6 days. At the end of the culture, MTT solution was added to each well. After for 4 h incubation, the medium was removed, and then DMSO was added to dissolve the formazan dye. The plates were read at 570 nm using a microplate reader (Tecan, San Jose, CA, USA).

2.6. Osteoclast differentiation and TRAP staining assay

BMMs (6×10^4 cells) were cultured in 48-well plates in α-MEM medium supplemented with 10% FBS, PS, M-CSF (20 ng/mL), RANKL (20 ng/mL) and various concentrations of TO for 3 days. After fixing for 30 s, the cells were stained using TRAP staining kit (Sigma, St. Louis, MO, USA) as the manufacturer's protocols indicated. The cells were incubated at 37 °C in dark environment for 1 h, followed by 3 times wash with distilled water. TRAP-positive multinucleated cells with more than three nuclei were counted as osteoclasts.

2.7. Bone resorption assay and modified Von Kossa staining

BMMs (1×10^5 cells) were seeded in Corning OsteoAssay Surface 24-well plates in α-MEM medium supplemented with 10% FBS, PS, M-CSF (20 ng/mL), RANKL (20 ng/mL) and various concentrations of TO for 6 days. Media and remedy were changed every 3 days during the 6 days culture period. The plates were incubated with 5% sodium hypochlorite for 5 min to remove cells, then stained by modified Von Kossa method as described [23,24]. The areas of total resorption pits were quantified using Image J. Two wells were assessed per treatment in three different experiments.

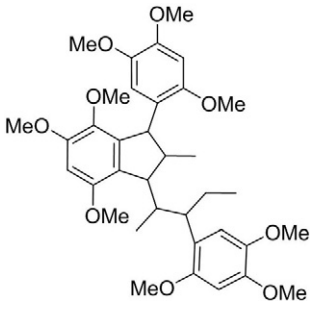

Fig. 1. Chemical structure of TO.