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Inhibitory effect of brazilin on osteoclast differentiation and its mechanism of action

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ABSTRACT

Brazilin isolated from *Caesalpinia sappan* has long been known as a natural red pigment. Our study evaluated the inhibitory effect of brazilin on osteoclast differentiation and investigated its mechanism of action. Our results demonstrated that brazilin inhibited receptor activator of nuclear factor kappa-B ligand (RANKL)-mediated osteoclast differentiation in RAW264.7 cells in a dose-dependent manner, without any evidence of cytotoxicity. The mRNA expression of tartrate-resistant acid phosphatase (TRAP), nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), matrix metalloproteinase 9 (MMP-9), and cathepsin K in RANKL-treated RAW264.7 cells was inhibited by brazilin treatment. Brazilin also decreased RANKL-induced expression of inflammatory mediator genes such as inducible nitric oxide synthase, iNOS; cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 and inhibited extracellular signal-regulated kinases (ERK) and nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) p65 phosphorylation in RANKL-stimulated RAW264.7 cells. A lipopolysaccharide (LPS)-induced osteoporosis study was also performed to assess the effects of brazilin in vivo. Micro-computed tomography (CT) analysis of the femurs showed that LPS treatment causes bone loss in mice, but it was significantly attenuated after co-treatment with brazilin (100 mg/kg). Therefore, brazilin may have therapeutic potential in preventing bone loss.

1. Introduction

Bone metabolism comprises osteoclastic bone resorption and osteoblastic bone formation. The balance between the function of these cells is responsible for maintaining bone homeostasis [1,2]. Osteoporosis may be caused by either decreased or increased osteoclast activities, both of which decrease bone density. Major factors that increase the risk of developing osteoporosis are menopause, thyroid hyperactivity, diabetes, stress, smoking, lack of exercise, physical aging, and glucocorticoid use [3,4].

The differentiation of osteoclasts is guided by various differentiations inducing factors produced by precursor cells of the macrophage family [2]. Receptor activator of nuclear factor kappa-B ligand (RANKL), which is secreted from osteoblasts, combines with RANK and presents in osteoclast precursor cells and on the surface of osteoclasts, facilitating the differentiation of osteoclast precursor cells into osteoclasts [5,6]. Differentiated osteoclasts promote the expression of various osteoclast-related proteins such as transcription factors (nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; c-fos, c-jun, activator protein 1, AP-1; nuclear factor of activated T-cells, cytoplasmic 1, NFATc1), mitogen activated protein kinase (MAPK), extracellular signal related kinase (ERK), c-jun N terminal kinase (JNK), activation process comprising the p38, and activation of another kinase such as Src or Akt, tartarate resistant acid phosphatase (TRAP), cathepsin K, and calcitonin receptor [7].

Caesalpinia sappan L. is a species of flowering tree in the legume family, *Fabaceae* [8]. As one of the main components of *C. sappan*, brazilin (Fig. 1A) is a colorless flavonoid dye [9]. The physiological activities of brazilin include anti-pain [9,10], hematopoietic [11], anti-inflammatory [12,13], immunoregulatory [14], and anti-microbial [15], and hypoglycemic [16] effects.

The aim of this study is to evaluate the effect of the factors involved in osteoclast differentiation and function, and to determine whether brazilin isolated from *C. sappan* is an effective inhibitor of bone resorption in osteoporosis. We measured the generation capacity of TRAP in a RANKL-stimulated mouse macrophage RAW264.7 cell, as well as the relevant gene expressions of TRAP, matrix metalloproteinase 9 (MMP-9), cathepsin K, NFATc1, microphthalmia-associated transcription factor (MITF), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, and interleukin (IL)-6. In addition, we evaluated the effect of phosphorylated JNK and p38, to assess the effects of ERK cell signaling on osteoclasts.

2. Materials and methods

2.1. Chemicals and reagents

Brazilin was isolated from *C. sappan* as described previously [14]. The TRAP staining kit and 3-(4,5-dimethylthiazol-2-yl)-2,5-

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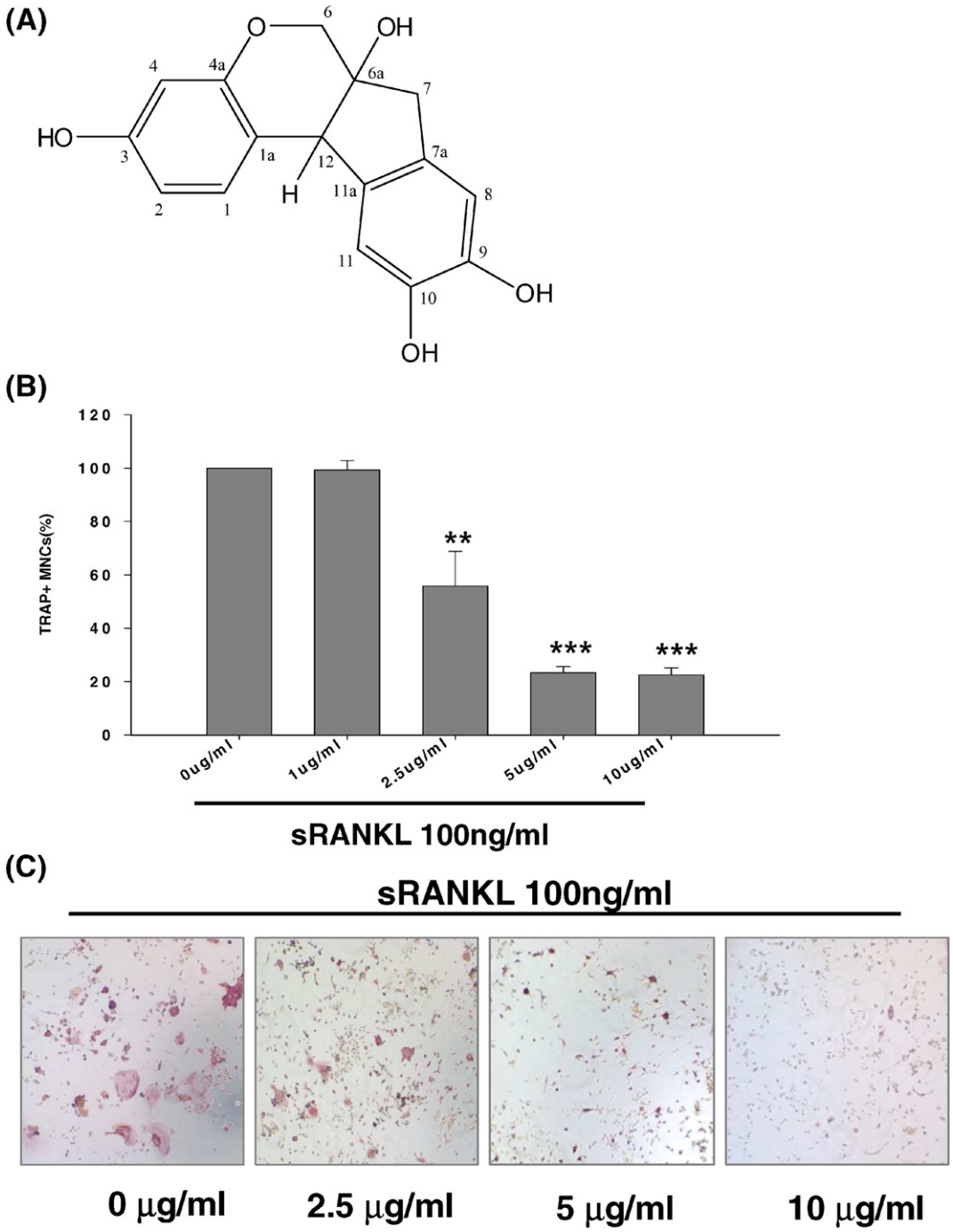

Fig. 1. Brazilin significantly inhibited receptor activator of nuclear factor kappa-B ligand (RANKL)-induced multinucleated osteoclast formation in a dose-dependent manner. (A) Chemical structure of brazilin. (B) Tartrate-resistant acid phosphatase (TRAP+) multinucleated cells were counted. (B) Representative pictures for TRAP (+) multinucleated osteoclasts. The results are expressed as a percent of the control group. ***$p < 0.001$, **$p < 0.01$ as compared with the control group.

diphenyltetrazolium bromide (MTT) assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant soluble RANKL (rsRANKL) was purchased from Peprotech (USA). Minimum essential medium α (α-MEM) was purchased from Gibco (Grand Island, NY, USA). RNAisol and all polymerase chain reaction (PCR) reagents were obtained from TAKARA (Japan). Anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-ERK, anti-phospho-JNK, anti-phospho-NF-κB, anti-p38-MAPK, anti-ERK, anti-JNK, and anti-β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Imprinting Control Region (ICR) mice were purchased from Orient Bio (Seongnam, Republic of Korea).

2.2. RAW 264.7 cell culture and osteoclast differentiation

Mouse monocyte/macrophage RAW 264.7 cells were purchased from ATCC (CRL-TBI-71) and maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed every 3 days. In order to differentiate the RAW 264.7 cells into osteoclasts, cells were seeded into 96-well plates in α-minimal essential media (α-MEM) supplemented with 10% FBS and 100 ng/ml sRANKL at a density of 5×10^3 cells/well. Multinucleated osteoclasts were observed from the differentiation day 4.