Bavachin counteracts receptor activator of nuclear factor- κ B-induced osteoclastogenesis though the suppression of nuclear factor- κ B signaling pathway in RAW264.7 cells

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The aim of this study was to evaluate the biological effects and cellular signaling pathways associated with the anti-osteoclastogenesis effects of bavachin, a phytoestrogen, in the receptor activator of nuclear factor- κ B ligand (RANKL)-treated RAW264.7 cells. The cell viability of RAW264.7 cells was not affected upon treatment with 5–20 μ M bavachin. Furthermore, osteoclastogenesis was suppressed by bavachin in a dose-dependent manner in RAW264.7 cells treated with RANKL. Tartrate-resistant acid phosphatase, matrix metalloproteinase-9, and cathepsin K, which are closely associated with osteoclastogenesis, were significantly downregulated by bavachin in the presence of RANKL. Additionally, bavachin decreased inflammatory molecules, such as nitric oxide, inducible nitric oxide synthase, cyclooxygenase-2, and prostaglandin E2 in RAW264.7 cells treated with RANKL. Bavachin suppressed the RANKL-induced phosphorylation of nuclear factor- κ B and subsequently inhibited the translocation of nuclear factor- κ B from the cytosol to the nucleus. Taken together, the obtained data suggest that bavachin may prevent the osteoclast-mediated bone destructive disorders.

Key Words: Bavachin, Phytoestrogen, Osteoclast, Receptor activator of nuclear factor- κ B ligand, Osteoclastogenesis

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INTRODUCTION

The homeostasis between bone resorption and bone formation is precisely regulated by osteoclasts and osteoblasts, respectively [1]. Generally, bone resorption is closely associated with osteoclast formation and tartrate-resistant acid phosphatase (TRAP) activity, whereas bone formation is related to osteoblast proliferation, alkaline phosphatase activity, and mineralization [2]. Hence, excessively increased osteoclasts, which are

bone-resorbing multinucleated cells differentiated from mononuclear precursors, such as monocytes and macrophages [3], may lead to various bone-related diseases, such as osteopenia and osteoporosis [4]. Furthermore, the inhibition of osteoclast differentiation from monocytes and macrophages is closely associated with the prevention of osteopenic diseases. However, the osteoclast differentiation of monocytes and macrophages is initiated by the receptor activator of nuclear factor- κ B ligand (RANKL), which is synthesized by osteoblasts and induced by reactive oxygen

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Fig. 1. Chemical structure and cellular cytotoxicity of bavachin. (A) Chemical structure of bavachin (7-hydroxy-2-(4-hydroxyphenyl)-6-(3-methylbut-2-enyl)-2,3-dihydrochromen-4-one). (B) Bavachin did not increase cell cytotoxicity in RAW264.7 cells. CAS, chemical abstracts service.

species, pro-inflammatory cytokines, and growth factors [5].

As shown in Fig. 1A, bavachin is a phytoestrogen derived from the herbal plant *Psoralea corylifolia* [6]. Recent studies showed that bavachin has various physiological properties, such as anti-inflammation [6], anti-oxidant [7] effects and anti-cancer activities [8]. Therefore, the aim of the present study was to evaluate the biological effects and cellular signaling pathways of bavachin on osteoclast differentiation in murine RAW264.7 macrophage cells treated with RANKL.

MATERIALS AND METHODS

Cell culture

The murine RAW264.7 macrophage cell line was obtained from Korean Cell Line Bank (Seoul, Korea) and were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL of penicillin, and 100 μ g/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Cytotoxicity assay

To determine the cytotoxicity of bavachin, an MTT assay was performed in RAW264.7 cells. Briefly, RAW264.7 cells were seeded at a density of 2×10^5 cells/ml in 96-well plates and allowed to attach to the wells overnight. After incubation, the cultured cells were treated with 5, 10, and 20 μ M bavachin in triplicate and incubated at 37°C in a 5% humidified CO_2 incubator for 24 hours. MTT solution was added to each well and the cells were further incubated for 4 hours at 37°C. Thereafter, the cells were resuspended in 200 μ L dimethyl sulfoxide and the optical density of the solution was determined using a spectrometer (Epoch Spectrophotometer, BioTek, Winooski, VT, USA) at a wavelength of 570 nm.

TRAP staining and activity assay

To perform the TRAP staining and TRAP activity assay, RAW264.7 cells were seeded at a density of 2×10^5 cells/well in an 8-well chamber slide and allowed to attach to the well overnight. Thereafter, RAW264.7 cells were treated with 20 μ M bavachin in the presence or absence of 100 ng/mL RANKL (Sigma-Aldrich, St. Louis, MO, USA) for 5 days. Next, the cells were fixed in 10% formalin for 10 minutes for TRAP staining. Sequentially, TRAP-positive multinucleated cells were imaged and counted by

microscopy (Eclipse TE2000; Nikon Instruments, Melville, NY, USA) to evaluate osteoclast differentiation.

For the TRAP activity assay, RAW264.7 cells treated with 20 μ M bavachin in the presence or absence of 100 ng/mL RANKL for 5 days were fixed in 10% formalin for 10 minutes and ethanol/acetone (1:1) for 1 minute. Subsequently, the cells were incubated in 50 mM citrate buffer (pH 4.6) containing 10 mM tartrate and 5 mM p-nitrophenylphosphate for 1 hour. The reaction mixtures were transferred to new well plates containing an equal volume of 0.1 N NaOH to stop the reaction, and the absorbance of each well was measured at 410 nm using a spectrophotometer (Epoch Spectrophotometer).

Gelatin zymography assay

Matrix metalloproteinase (MMP) activity was detected using the gelatin zymography assay. RAW264.7 cells were seeded into 12-well culture plates and allowed to attach to the wells overnight. Thereafter, RAW264.7 cells were stimulated with bavachin (10 and 20 μ M) in the presence or absence of 100 ng/mL RANKL for 24 hours. Subsequently, the same volumes of the culture supernatants were used for the gelatin zymography assays to evaluate MMP activity. The samples were electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide gels copolymerized with 1% gelatin. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 and incubated in 1% Triton X-100, 5 mM CaCl_2 , and 5 μ M ZnCl_2 (pH 7.5) at 37°C for 72 hours. The gel was stained with 0.1% Coomassie brilliant blue R250 and destained in 10% methanol and 10% glacial acetic acid in H_2O . MMPs were detected as transparent bands on the blue background of a Coomassie blue-stained gel.

Western blotting

RAW264.7 cells were seeded into 12-well culture plates and allowed to attach to the wells overnight. Thereafter, RAW264.7 cells were stimulated with bavachin (10 μ M and 20 μ M) in the presence or absence of 100 ng/mL RANKL for 5 days. Subsequently, the cells were harvested, lysed. Lysates were then centrifuged at 14,000 $\times$ g for 10 minutes at 4°C. The protein concentration of cell lysates

was determined using the bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL, USA). Protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Thereafter, to verify the alteration of osteoclastogenic markers, such as cathepsin K, MMP-9, and NFATc1, the membranes were incubated with primary antibodies for overnight at 4°C and then incubated with horseradish peroxidase-conjugated secondary antibodies. The immunoreactive bands were visualized using the ECL System (Amersham Biosciences, Amersham, UK) and were exposed to radiographic film.

Measurement of total NO production

RAW264.7 cells were seeded into 12-well culture plates and allowed to attach to the wells overnight. Thereafter, RAW264.7 cells were stimulated with bavachin (10 μ M and 20 μ M) in the presence or absence of 100 ng/mL RANKL for 24 hours. Subsequently, the same volumes of these culture supernatants were used for the NO assays to evaluate NO production. Briefly, 50 μ L of culture supernatants were allowed to react with 50 μ L of sulfanilamide and 50 μ L of N-1-naphthylethylenediamine dihydrochloride. Absorbance was measured at 540 nm using a spectrophotometer (Epoch Spectrophotometer).

Prostaglandin E_2 (PGE_2) assay

To measure PGE_2 , RAW264.7 cells were seeded into 12-well culture plates and allowed to attach to the wells overnight. Thereafter, RAW264.7 cells were stimulated with bavachin (10 μ M and 20 μ M) in the presence or absence of 100 ng/mL RANKL for 24 hours. The concentration of PGE_2 was then measured using the PGE_2 Parameter Assay Kit (R&D Systems, Inc. Minneapolis, MN, USA) according to the manufacturer's instructions.

Nuclear translocation

To observe the nuclear translocation of nuclear factor (NF)- κ B, RAW264.7 cells were pre-treated with 20 μ M bavachin for 30 minutes before treatment with 100 ng/mL

RANKL. After 30 minutes, RAW264.7 cells were fixed with 1% paraformaldehyde, permeabilized in 0.2% Triton X-100, and extensively washed with phosphate-buffered saline. Non-specific signals were blocked with normal goat serum. After multiple washes, RAW264.7 cells were incubated overnight at 4°C with a rabbit anti-total-NF- κ B antibody (SC-10175; Santa Cruz Biotechnology Inc., Dallas, TX, USA) followed by incubation with FITC-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Rockford, IL, USA). In addition, as a nuclear counterstain, all cells were stained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). Nuclear images were visualized by Confocal Laser Scanning Microscope (TCS SP5; Leica Microsystems, Buffalo Grove, IL, USA).

Statistical analysis

Analysis of variance was performed using StatView 5.0 software (SAS Institute, Cary, NC, USA), with *p*-values less than 0.05 considered to be statistically significant in each test.

RESULTS

Bavachin does not affect the viability of RAW264.7 cells

To verify the cytotoxicity of bavachin in RAW264.7 cells, an MTT assay was performed in RAW264.7 cells treated with various concentration of bavachin (5, 10, and 20 μ M) for 24 hours. As shown in Fig. 1B, the relative cell viabilities of RAW264.7 cells compared with non-treated control (100% $\pm$ 6%) were 105% $\pm$ 7%, 111% $\pm$ 3%, and 108% $\pm$ 8% in the presence of 5, 10, and 20 μ M bavachin, respectively. The data indicate that these concentrations of bavachin did not alter the viability of RAW264.7 cells. These data demonstrate the biological safety of bavachin in RAW264.7 cells.

Bavachin inhibits RANKL-induced osteoclastogenesis in RAW264.7 cells

TRAP staining was performed to determine whether

RANKL-induced osteoclastogenesis occurred in RAW264.7 cells treated with 20 μ M bavachin in the presence or absence of 100 ng/mL RANKL for 5 days. As shown in Fig. 2A, TRAP-positive multinucleated cells were not observed in RAW264.7 cells treated with 20 μ M bavachin as well as in non-treated controls. Moreover, TRAP activity in RAW264.7 cells treated 100 ng/mL RANKL was significantly increased by approximately 2.9 $\pm$ 0.25-fold compared with non-treated controls. The upregulated TRAP activities in RAW264.7 cells treated 100 ng/mL RANKL were dose-dependently downregulated by approximately 1.1 $\pm$ 0.03-fold by 20 μ M bavachin, respectively, as shown in Fig. 2B. These data consistently indicated that bavachin inhibits RANKL-induced osteoclastogenesis in RAW264.7 cells.

Bavachin suppresses the expression of biomarkers associated with osteoclastogenesis in RAW264.7 cells

To verify the anti-osteoclastogenic effects of bavachin in RAW264.7 cells, the alteration of biomarkers associated with osteoclastogenesis were observed by western blot. As shown in Fig. 2C, the expression of biomarkers associated with osteoclastogenesis, such as cathepsin K, NFATc1, and c-Fos were significantly upregulated in RAW264.7 cells treated with 100 ng/mL RANKL. In contrast, bavachin suppressed the expression of cathepsin K, NFATc1, and c-Fos in RAW264.7 cells compared with in non-treated controls. Furthermore, the expression and activation of MMP-9 in RAW264.7 cells treated with bavachin in the presence or absence of RANKL was observed by gelatin zymography as shown in Fig. 2D. The upregulated MMP-9 in RAW264.7 cells treated with 100 ng/mL RANKL were dose-dependently suppressed by 10 and 20 μ M bavachin. Moreover, RANKL-induced activation of MMPs was dose-dependently decreased by 10 and 20 μ M bavachin in RAW264.7 cells. These data indicate that bavachin counteracts RANKL-induced osteoclastogenesis by downregulating biomarkers associated with osteoclastogenesis in RAW264.7 cells.

Fig. 2. Bavachin suppresses receptor activator of nuclear factor- κ B ligand (RANKL)-induced osteoclast differentiation in RAW264.7 cells. (A) Bavachin suppressed the tartrate-resistant acid phosphatase (TRAP)-positive RAW264.7 cells treated with RANKL. (B) Bavachin suppressed TRAP activity in RAW264.7 cells treated with RANKL. (C) Bavachin suppressed dose-dependently the expression of osteoclastogenic biomarkers such as cathepsin K, NFATc1, and c-Fos in RAW264.7 cells treated with RANKL. (D) Bavachin inhibited the activation and expression of matrix metalloproteinases-9 in RAW264.7 cells treated with RANKL.

Anti-osteoclastogenesis effects of bavachin are mediated by the suppression of inducible nitric oxide synthase (iNOS), nitric oxide (NO), cyclooxygenase-2 (COX-2), and PGE₂ in RAW264.7 cells

The alterations in iNOS, NO production, COX-2, and PGE₂, which are factors involved in oxidative stress, in the RAW264.7 cells treated with bavachin in the presence or absence of RANKL were observed as shown in Fig. 3. NO production was significantly increased by approximately $212\% \pm 13\%$ in RAW264.7 cells treated with 100 ng/mL RANKL compared with non-treated controls ($100\% \pm 3\%$). NO production was significantly downregulated by approximately $58\% \pm 2\%$ ($p < 0.01$) and $46\% \pm 2.5\%$ ($p < 0.01$) in RAW264.7 cells treated with 10 and 20 μ M bavachin, respectively, compared with non-treated controls. However, RANKL-induced NO upregulation was dramatically downregulated

by approximately $63\% \pm 2\%$ ($p < 0.01$) and $55\% \pm 4\%$ ($p < 0.01$) by 10 and 20 μ M bavachin, respectively, as shown in Fig. 3A. Moreover, although the expression of iNOS and COX-2 were significantly upregulated in RAW264.7 cells treated with 100 ng/mL RANKL, expression was dramatically downregulated by bavachin treatment as shown in Fig. 3B. Subsequently, RANKL-induced PGE₂ upregulation was significantly suppressed in RAW264.7 cells treated with 20 μ M bavachin as shown in Fig. 3C. These data indicate that the anti-osteoclastogenesis effects of bavachin are mediated by the suppression of iNOS, NO, COX-2, and PGE₂ in RAW264.7 cells.

RANKL-induced NF- κ B phosphorylation is inhibited by bavachin in RAW264.7 cells

To determine whether bavachin suppresses the phos-

Fig. 3. Bavachin suppresses inflammation mediators associated with osteoclast differentiation in RAW264.7 cells treated with receptor activator of nuclear factor- κ B ligand (RANKL). (A) Bavachin suppressed nitric oxide (NO) production in RAW264.7 cells treated with RANKL. (B) Bavachin inhibited the expression of inducible nitric oxide synthase (iNOS) and COX-2, which are upstream molecules of NO and prostaglandin E₂ (PGE₂), respectively, in RAW264.7 cells treated with RANKL. (C) Bavachin suppressed PGE₂ production in RAW264.7 cells treated with RANKL.

phorylation of NF- κ B in the presence of RANKL, western blotting was performed as shown in Fig. 4A. The phosphorylation of NF- κ B was significantly induced by 100 ng/mL RANKL in RAW264.7 cells. However, the phosphorylation of NF- κ B was downregulated in RAW264.7 cells treated

with 10 and 20 μ M bavachin compared with non-treated controls. The phosphorylation of NF- κ B after treatment of RANKL was significantly suppressed by 10 and 20 μ M bavachin. Subsequently, the phosphorylation of NF- κ B initiated the translocation of cytosolic NF- κ B to the nucleus. Therefore, the translocation of cytosolic NF- κ B to the nucleus was observed in RAW264.7 cells after bavachin treatment with or without RANKL as shown in Fig. 4B. The phosphorylated NF- κ B was significantly accumulated in the nucleus of RAW264.7 cells treated with 100 ng/mL RANKL. Phosphorylation of NF- κ B was not observed in RAW264.7 cells treated with 20 μ M bavachin. However, RANKL-induced NF- κ B accumulation in the nucleus was significantly inhibited by 20 μ M bavachin treatment, as shown in Fig. 4B. These data indicate that the anti-osteoclastogenesis effects of bavachin are mediated by the inhibition of RANKL-induced NF- κ B phosphorylation in RAW264.7 cells.

DISCUSSION

Bone as a representative connective tissue in the body and undergoes turnover and remodeling. Therefore, bone homeostasis is precisely regulated by two core processes, including osteoblast-mediated bone regeneration and osteoclast-mediated bone resorption [9]. However, osteoclasts are closely associated with bone resorption. Therefore, increased osteoclast differentiation from monocytes and macrophages may result in osteoporosis as bone resorption is accelerated by the release of proteolytic enzymes to digest connective tissues and acid [10]. Furthermore, recent studies reported that estrogen deficiency accelerates bone resorption by inducing osteoclast differentiation [11]. These studies suggest that estrogen analogs with long-term biological safety and physiological functions similar to estrogen may protect against osteoporosis by inhibiting osteoclast differentiation from monocytes and macrophages.

Bavachin is a phytoestrogen-derived molecule present in herbal plants, such as *Psoralea corylifolia* and *Piper longum* [8,12]. Recent studies reported that bavachin has antioxidant activity [7], shows anti-catabolic effects in articular cartilage [13], is physiologically similar to

Fig. 4. Bavachin inhibits receptor activator of nuclear factor- κ B ligand (RANKL)-induced osteoclast differentiation by inhibiting the nuclear factor (NF)- κ B cellular signaling pathway. (A) Bavachin inhibited the phosphorylation of NF- κ B in RAW264.7 cells treated with RANKL. (B) Bavachin suppressed the nuclear translocation of cytosolic NF- κ B in RAW264.7 cells treated with RANKL.

estrogen [12], and is involved in osteoblastic proliferation [14]. Therefore, we hypothesized that bavachin inhibits osteoclast differentiation from macrophages treated with RANKL.

We first evaluated the biological safety of bavachin in RAW264.7 cells. As shown in Fig. 1, 5–20 μ M bavachin did not affect the viabilities of RAW264.7 cells. Recently, Lee et al. [13] reported that cytotoxicity was not observed in human normal oral keratinocytes treated with 0.5–10 μ M bavachin for 24 hours. Moreover, the cell survival rate was maintained at over 80% in primary rat chondrocytes exposed to 0.5–1 μ M bavachin for 21 days compared with non-treated controls [13]. These data indicate that bavachin is a biologically safe phytoestrogen that can be used in long-term treatment.

RANKL is a major inducer of osteoclast differentiation through the binding with its receptor RANK in macrophages [15,16]. Therefore, to determine whether bavachin inhibits RANKL-induced osteoclast differentiation, RAW264.7 cells were treated with RANKL in the presence or absence of bavachin. Thereafter, TRAP staining was performed to verify osteoclast differentiation. As shown in Fig. 2A and 2B, bavachin suppressed osteoclast differentiation from RAW264.7 cells. Although previous studies reported that some phytoestrogens, such as genistein, formononetin, daidzein, and resveratrol suppressed osteoclast differentiation [17–20], we demonstrated the anti-osteoclastogenesis potential of bavachin in present study.

The degradation of organic bone matrix is related to the process of osteoclastic bone resorption and is mediated by proteolytic enzymes such as TRAP, cathepsin K, MMP-9, NFATc1, and c-Fos, which are secreted from osteoclasts [21]. Hence, to confirm the inhibition of RANKL-induced osteoclast differentiation in RAW264.7 cells treated with bavachin, the alteration of biomarkers associated with osteoclastogenesis were observed as shown in Fig. 2C and 2D. According to our results, MMP activation was significantly suppressed by bavachin. Subsequently, upregulated cathepsin K, MMP-9, NFATc1, and c-Fos in RAW264.7 cells treated with RANKL were significantly downregulated by bavachin. These data confirm that bavachin suppresses RANKL-induced osteoclast differentiation in RAW264.7 cells. Furthermore, bavachin may

suppress bone resorption by inhibiting the expression of osteoclastogenic biomarkers such as TRAP, cathepsin K, MMP-9, NFATc1, and c-Fos secreted from osteoclasts.

Recent studies have shown that inflammatory cytokines are closely associated with osteoclast differentiation [22,23]. Sequentially, NO produced by iNOS in response to inflammatory cytokines is important for promoting osteoclast differentiation and functions [24]. Moreover, the release of inflammatory cytokines triggers the upregulation of COX-2 and its downstream target molecule PGE₂, which participate in osteoclast formation and bone resorption. Therefore, the inhibition of NO, iNOS, COX-2, and PGE₂ may inhibit RANKL-induced osteoclast differentiation. As shown in Fig. 3, bavachin significantly suppressed NO production, iNOS, COX-2, and PGE₂ in the RAW264.7 cells treated with RANKL. These data indicate that bavachin inhibits RANKL-induced osteoclast differentiation by suppressing inflammation mediators such as NO, iNOS, COX-2, and PGE₂ in RAW264.7 cells.

However, the binding of RANKL with its receptor RANK leads to the activation of the NF- κ B and mitogen-activated protein kinase cellular signaling pathways during osteoclast differentiation [25]. Particularly, NF- κ B signaling is closely associated with osteoclastogenesis by promoting the initial induction of transcriptional genes related to inflammatory mediators [25]. Moreover, Jimi et al. [26] reported that inhibition of the NF- κ B cellular signaling pathway blocked osteoclastogenesis and prevented inflammatory bone destruction. Therefore, we hypothesized that bavachin inhibits the phosphorylation of NF- κ B in the RAW264.7 cells treated with RANKL to suppress osteoclast differentiation. As shown in Fig. 4, bavachin significantly suppressed the phosphorylation and nuclear translocation of NF- κ B in RAW264.7 cells treated with RANKL. These data indicate that bavachin suppresses the RANKL-induced osteoclast differentiation of RAW264.7 cells by suppressing the NF- κ B cellular signaling pathway. In conclusion, this is the first study to report that bavachin inhibits RANKL-induced osteoclast differentiation in macrophages. These results suggest that bavachin with biologically safe may have a potentially preventive effects for bone destructive disorders as an estrogen analog.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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