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Bavachalcone inhibits osteoclast differentiation through suppression of NFATc1 induction by RANKL

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ABSTRACT

Osteoclasts are cells that have a specialized role for bone resorption and are responsible for many bone diseases such as osteoporosis. As herbal products are invaluable sources in discovery of compounds for new therapies, we sought to identify compounds efficacious in suppressing osteoclastogenesis from medicinal plants that have been implicated for treatment of osteoporotic conditions. Bavachalcone was isolated from *Psoralea corylifolia*, and its effects on osteoclast differentiation were evaluated with primary cultures of osteoclast precursor cells. In addition, the molecular mechanism of action was investigated. Bavachalcone inhibited osteoclast formation from precursor cells with the IC_{50} of ~1.5 μg ml^{-1}. The activation of MEK, ERK, and Akt by receptor activator of nuclear factor kappaB ligand (RANKL), the osteoclast differentiation factor, was prominently reduced in the presence of bavachalcone. The induction of c-Fos and NFATc1, key transcription factors for osteoclastogenesis, by RANKL was also suppressed by bavachalcone. In conclusion, bavachalcone inhibits osteoclastogenesis by interfering with the ERK and Akt signaling pathways and the induction of c-Fos and NFATc1 during differentiation. Our results suggest that bavachalcone may be useful as a therapeutic drug for bone resorption-associated diseases.

1. Introduction

Psoralea corylifolia has long been used for prevention and treatment of several disorders including osteoporosis as folk medicine in Asian countries. However, efforts to evaluate the efficacy in experimental settings and to identify principles responsible for its effect have been scarce. In a few previous studies related to bone-associated effects, crude fractions of *P. corylifolia* were shown to promote bone calcification in vivo and two flavonoid compounds isolated from the plant fruit were reported to increase oteoblastic proliferation of UMR106 cell line in culture [1,2]. However, the molecular mechanisms for the bone anabolic effect of *P. corylifolia* remain unrevealed. We newly isolated a single compound, bavachalcone, from *P. corylifolia* (Fig. 1) and evaluated its effect on osteoclasts, the cells responsible for bone resorption.

The osteoclast-mediated bone resorption and the osteoblast-induced bone formation are the critical activities that determine the bone mass of adults. A disruption in the balance leads to various bone-related disorders such as osteoporosis, osteomalacia, and osteopetrosis [3,4]. Osteoclasts are derived from monocyte/macrophage lineage precursor cells through a differentiation process primarily governed by two key cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL). RANKL provides an essential signal for osteoclastic differ-

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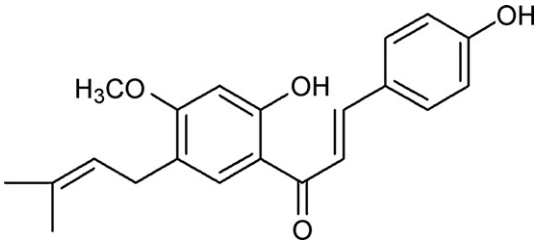

Fig. 1 – Chemical structure of bavachalcone.

entiation while M-CSF supports cell proliferation and survival during osteoclastogenesis. Osteoblasts, which are originated from mesenchymal stem cells, produce these cytokines in response to several stimuli like prostaglandin E_2 (PGE_2), vitamin D_3 (VtD_3), and IL-1 to support osteoclatogenesis [5–7].

RANKL signal in osteoclast precursor cells evokes the activation of the three major mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 [8]. The phosphoinositide kinase-3/Akt pathway is also stimulated by RANKL [9]. These signaling pathways ultimately lead to induction and activation of the transcription factors involved in expression of genes that characterize osteoclasts. These transcription factors include nuclear factor of activated T cells c1 (NFATc1), activator protein 1 (AP-1), and nuclear factor kappa B (NF-κB) [8,10,11]. In line with the importance of these transcription factors for osteoclast differentiation, genetic studies showed that deficiency in c-Fos, a component of the AP-1, or NFATc1 results in blockade of osteoclastogenesis [11–13]. NF-κB is also an important transcription factor for osteoclastogenesis by RANKL. The double knockout mice, lacking both p50 and p52, exhibit osteopetrotic phenotype with a prominent decrease in the number of osteoclasts [14].

In this study, we tested the effect of bavachalcone on RANKL-induced osteoclastogenesis using primary osteoclast precursors. Bavachalcone significantly suppressed osteoclast differentiation by RANKL. The anti-osteoclastogenic mechanism of bavachalcone included the inhibition of RANKL-induced activation of MAPKs and expression of c-Fos and NFATc1. Our data suggest that bavachalcone may be useful as a therapeutic agent for bone erosive disorders such as osteoporosis.

2. Materials and methods

2.1. Isolation of bavachalcone

Bavachalcone was isolated from dried seeds of *P. corylifolia*. The isolation method and spectral data were previously described [15]. The chemical structure of bavachalcone is shown in Fig. 1.

2.2. Reagents

RANKL was purchased from PeproTech (Rocky Hill, NJ). M-CSF was from R&D Systems (Minneapolis, MN). Specific antibodies against phospho-JNK (#9251), JNK (#9252), phospho-ERK (#9101), ERK (#9102), phospho-p38 (#9211), p38 (#9212), phosho-Akt (#9271), Akt (# 9272), phospho-IκB (#9241), IκB (#9242), phospho-MEK (#9121), and MEK (#9122) were purchased from Cell Signaling (Beverly, MA). Specific antibodies against NFATc1 (sc-7294) and β-actin (sc-1616) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and anti-c-Fos antibody was obtained from Upstate Biotechnology, Inc. (Waltham, MA). Other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

2.3. Preparation of bone marrow-derived macrophages (BMMs) and in vitro osteoclast generation

Total bone marrow cells were collected from tibia and femur of 5-week-old ICR mouse by flushing the marrow space with α-MEM (WelGENE Inc., Daegu, Korea). After removing the red blood cells (RBCs) with ACK buffer (0.01 mM EDTA, 0.011 M $KHCO_3$, and 0.155 M NH_4Cl, pH 7.3), cells were cultured for 1 day in α-MEM containing 10% fetal bovine serum (FBS). Nonadherent cells were collected and further cultured with 30 ng ml^{-1} M-CSF in α-MEM containing 10% FBS. After 3 days, culture medium was removed and adherent cells (BMMs) were used for osteoclastogenesis. BMMs were cultured for 3 days in medium containing 30 ng ml^{-1} M-CSF and 200 ng ml^{-1} RANKL. TRAP-positive multinucleated cells (MNCs) with more than three nuclei were counted as osteoclast cells.

2.4. Co-culture

Osteoblast cells were prepared from the calvaria of 1-day-old mouse as described previously [16]. Primary osteoblasts were seeded on 96-well plate (1×10^4 per well). After 1 day, RBC-free bone marrow cells prepared as above were added at 1×10^5 cells per well to the osteoblasts and cultured for 5 days in the presence of vitamin D_3 (VtD_3) (10^{-6} M) and prostagrandin E_2 (PGE_2) (10^{-8} M).

2.5. Tartrate resistant acid phosphatase (TRAP) staining

To confirm the generation of osteoclast cells, cells were fixed with 3.7% formalin for 10 min. Fixed cells were permeablized in 0.1% Triton X-100 for 1 min and washed with distilled water. Cells were stained for tartrate resistant acid phosphatase (TRAP) with the Leukocyte Acid Phosphatase Kit (Sigma, Cat. No. 387A-1KT) in 37 °C incubator for 15 min. Cells were washed three times with distilled water and photographed under a light microscope.

2.6. Reverse transcription-polymerase chain reaction analysis (RT-PCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized by using the Superscript II (Invitrogen). PCR was performed with mouse specific primers: c-Fos, 5′-CTGGTGCAGCCCACTCTGGTC-3′ (forward) and 5′-CTTTCAGCAGATTGGCAATCTC-3′ (reverse); NFATc1, 5′-CAACGCCCTGACCACCGATA-3′ (forward) and 5′-GGCTG-CCTTCCGTCTCATAG-3′ (reverse); and β-actin, 5′-AACCC-TAAGGCCAACCGTGA-3′ (forward) and 5′-ATGGATGCCACAG-GATTCCA-3′ (reverse). The sequences of reaction products were confirmed and the reactions in the absence of reverse transcriptase did not generate any product.

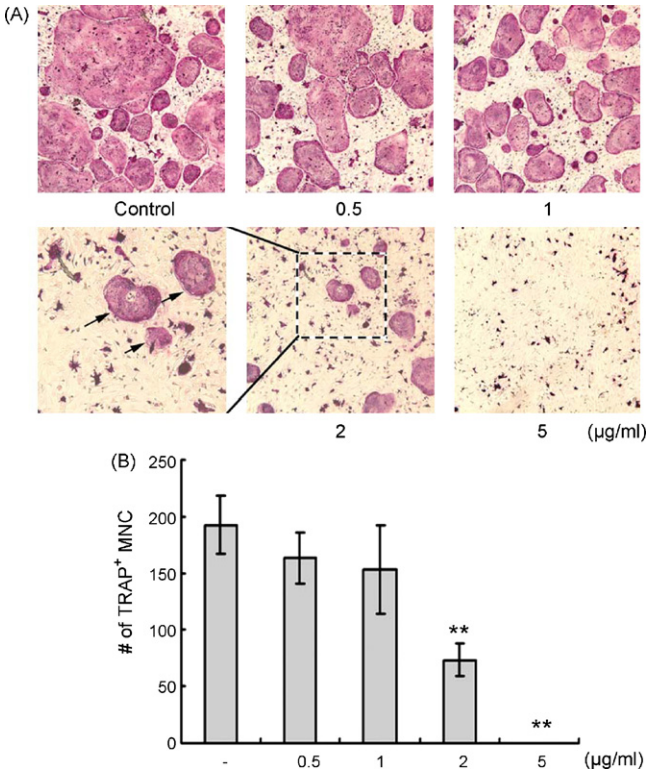

Fig. 2 – Bavachalcone inhibits osteoclastogenesis in co-culture. (A) Whole bone marrow cells and calvarial osteoblasts were cultured with indicated dose of bavachalcone in the presence of VtD_3 (10^{-6} M) and PGE_2 (10^{-8} M). After 5 days, cells were fixed and stained for TRAP. (B) TRAP positive multinucleated cells (≥three nuclei) were counted. BC, bavachalcone. Arrows indicate TRAP positive multinucleated cells. $^{**}p < 0.01$ versus DMSO-treated cells.

2.7. Immunoblotting

After washing with phosphate buffered saline (PBS), total cell lysates were prepared by lysing the cells in cold lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 1 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin, and 1% Triton X-100). Total cell lysates were incubated for 20 min and centrifuged at 10,000 × *g* for 20 min at 4 °C. Harvested proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, NJ). The membrane was probed with specific antibodies and immuno-reactivity was detected by using enhanced chemi-luminescence reagents.

2.8. Cytotoxicity assay

BMMs (1×10^4 per well) were seeded on 96-well plates. Cells were cultured with the indicated dose of bavachalcone in the presence of M-CSF (30 ng ml^{-1}) and RANKL (200 ng ml^{-1}). After 16 h incubation, media were changed with the α-MEM containing 10% solution of Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) and cells were incubated for 2 h at 37 °C. The absorbance in each well was measured using the Benchmark microplate reader (Bio-Rad, Hercules, CA) at 450 nm with a reference at 655 nm.

2.9. Resorption pit assay

BMMs (3×10^4 per well) were seeded on calcium phosphate coated-plates (48-well plates). Cells were cultured for 5 days in medium containing 30 ng ml^{-1} M-CSF and 200 ng ml^{-1} RANKL in the presence or absence of bavachalcone (5 μg ml^{-1}). Cells were removed by washing with distilled water. The resorbed areas were measured using the Image J program.

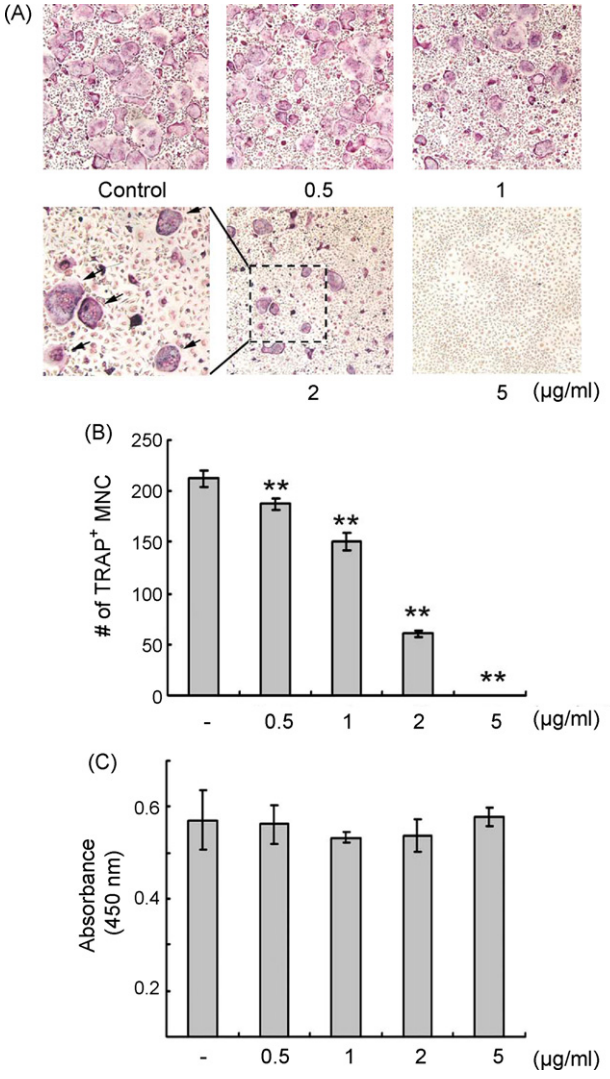

Fig. 3 – Bavachalcone inhibits RANKL-induced osteoclastogenesis. (A) BMMs were cultured with the indicated dose of bavachalcone in the presence of M-CSF (30 ng ml^{-1}) and RANKL (200 ng ml^{-1}). After 3 days, cells were fixed and stained for TRAP. (B) TRAP positive multinucleated cells (≥three nuclei) were counted. (C) Cells viability was measured with CCK reagents as described in Section 2. Arrows indicate TRAP positive multinucleated cells. $^{**}p < 0.01$ versus DMSO-treated cells.

2.10. Statistical analysis

All experiments were performed three or more times. Data are presented as the means ± S.D. The Student's paired *t*-test was used to determine the significance of differences between indicated samples.

3. Results

3.1. Bavachalcone suppresses osteoclastogenesis in vitro

It has been known that osteoblasts support osteoclast formation by expressing the cytokines essential for osteoclast differentiation, M-CSF and RANKL, in response to stimuli like PGE_2 and VtD_3 [5,6]. To investigate whether bavachalcone regulates osteoclastogenesis, we used a co-culture system in which osteoblasts and bone marrow cells that contain osteoclast precursor cells were cultured together in the presence of PGE_2 and VtD_3. Bavachalcone inhibited osteoclast differentiation in the co-culture system (Fig. 2A and B).

To test whether bavachalcone can directly affect osteoclast precursor cells, we next performed a single culture system in which bone marrow-derived macrophages (BMMs) are cultured directly with M-CSF and RANKL in the absence of osteoblasts (Fig. 3A and B). Consistent with the anti-osteoclastogenic effects of bavachalcone in the co-culture system, bavachalcone dose-dependently blocked osteoclast differentiation in this culture system. Bavachalcone showed complete inhibitory effect at 5 $\mu g\,ml^{-1}$. No morphological abnormality was observed in bavachalcone treated-cells. The inhibitory pattern of bavachalcone in the co-culture and the highly purified BMM culture systems were similar. These results indicate that bavachalcone would mainly affect osteoclast precursors rather than osteoblasts. This inhibitory activity of bavachalcone could have been due to its effect on viability of the osteoclast precursor cells. Therefore, we tested cytotoxicity of bavachalcone. Bavachalcone did not exhibit any cytotoxicity at the concentrations used (Fig. 3C). Together, these results suggest that bavachalcone exerts a direct anti-osteoclastogenic effect on the bone marrow-derived precursor cells without interfering with cell viability.

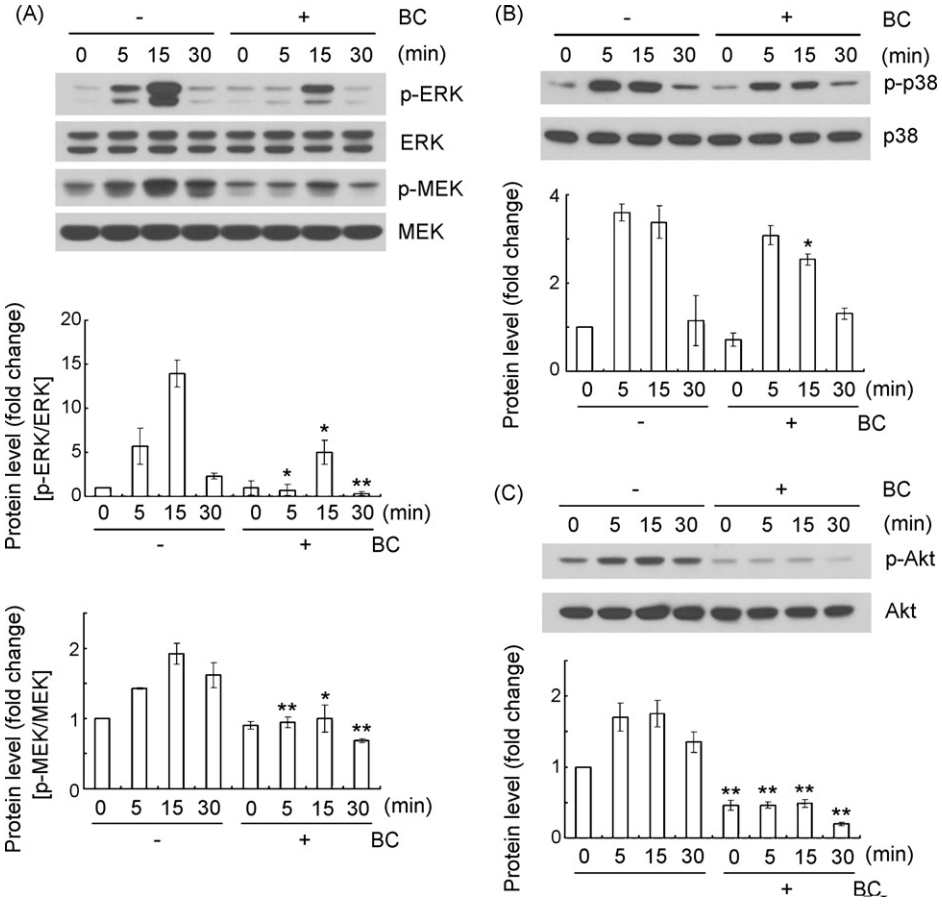

Fig. 4 – Bavachalcone decreases the activation of ERK and Akt by RANKL. (A) BMMs were serum-starved for 4 h, pretreated with bavachalcone (5 $\mu g\,ml^{-1}$) or vehicle (DMSO) for 1 h, and stimulated with RANKL (500 ng ml^{-1}) for the indicated time. Total protein extracts were analyzed by Western blotting with phospho-ERK and phospho-MEK antibodies. Blots were stripped and reprobed with ERK and MEK antibodies. (B) BMMs were treated as in (A) and cell lysates were immunoblotted with an antibody specific for phospho-p38. The blot was stripped and reprobed with anti-p38. (C) BMMs were treated as in (A) and cell lysates were immunoblotted with an antibody specific for phospho-Akt. The blot was stripped and reprobed with an anti-Akt antibody. $^{**}p < 0.01$, $^{*}p < 0.05$ versus DMSO-treated BMMs.

3.2. *Bavachalcone suppresses the RANKL-stimulated activation of ERK and Akt*

The ERK, JNK, and p38 MAPK signaling pathways and the PI3K/Akt signaling pathways mediate various cellular responses including differentiation. In fact, RANKL has been shown to stimulate the activation of MAPKs and Akt in osteoclast precursors [8]. So, we investigated whether MAPKs and Akt are related to the negative function of bavachalcone on osteoclastogenesis. The RANKL-induced ERK phosphorylation was significantly reduced by bavachalcone treatment (Fig. 4A). Similarly, the phosphorylation of MEK, the upstream kinase of ERK, was down-regulated by bavachalcone (Fig. 4A). Also, bavachalcone weakly inhibited RANKL-induced phosphorylation of p38 (Fig. 4B). Although bavachalcone slightly delayed the phosphorylation of JNK by RANKL, it did not reduce the extent of JNK phosphorylation (data not shown). The effect of bavachalcone on Akt phosphorylation induced by RANKL was strongly suppressive (Fig. 4C). These results suggest that the anti-osteoclastogenic effect of bavachalcone was mainly caused by disturbing ERK and Akt signaling rather than JNK and p38 signaling.

3.3. *Bavachalcone inhibits RANKL-induced c-Fos expression*

We next investigated effects of bavachalcone on the RANKL-induced c-Fos expression. c-Fos, a transcription factor required for osteoclast differentiation, is activated by phosphorylated MAPKs and have a role for induction of NFATc1, another transcription factor critical to osteoclastogenesis [11,12,17]. RANKL stimulation clearly up-regulated the mRNA level of c-Fos in DMSO-treated BMMs (Fig. 5A). Conversely, RANKL failed to elevate c-Fos mRNA level in the presence of bavachalcone (Fig. 5A). When the protein level of c-Fos was accessed by Western blotting, RANKL significantly increased the c-Fos protein expression from an early time point (12 h) (Fig. 5B). However, the extent of c-Fos protein up-regulated by RANKL was markedly lower in the presence of bavachalcone (Fig. 5B).

3.4. *Bavachalcone downregulates the induction of NFATc1 by RANKL*

Since bavachalcone suppressed RANKL-induced c-Fos expression, we investigated potential role of bavachalcone in the NFATc1 induction by RANKL. NFATc1 is one of the key transcription factors involved in osteoclast differentiation by RANKL [11,13]. As shown in Fig. 6A, the mRNA expression of NFATc1 increased time-dependently in DMSO-treated BMMs upon treatment with RANKL (Fig. 6A). In contrast, addition of bavachalcone almost completely blocked the induction of NFATc1 mRNA by RANKL (Fig. 6A). Similarly, bavachalcone abrogated the elevation of NFATc1 protein level by RANKL (Fig. 6B).

3.5. *The effect of bavachalcone on resorption pit formation*

Functional osteoclasts have ability to generate resorption pits on bone or calcium phosphate coated matrix. To examine whether the effect of bavachalcone on osteoclastogenesis could be reflected on the osteoclastic activity, we performed the in vitro resorption pit assay using calcium phosphate coated-plates. Bavachalcone itself did not cause any change to calcium phosphate coated-plates (Fig. 7A and B). Many resorption pits were generated in wells with RANKL-treated cells (Fig. 7C). In contrast, the treatment of bavachalcone strongly inhibited formation of resorption pits by the RANKL-treated cells (Fig. 7D). The measurement of resorbed areas revealed more than 90% inhibition by bavachalcone (Fig. 7E).

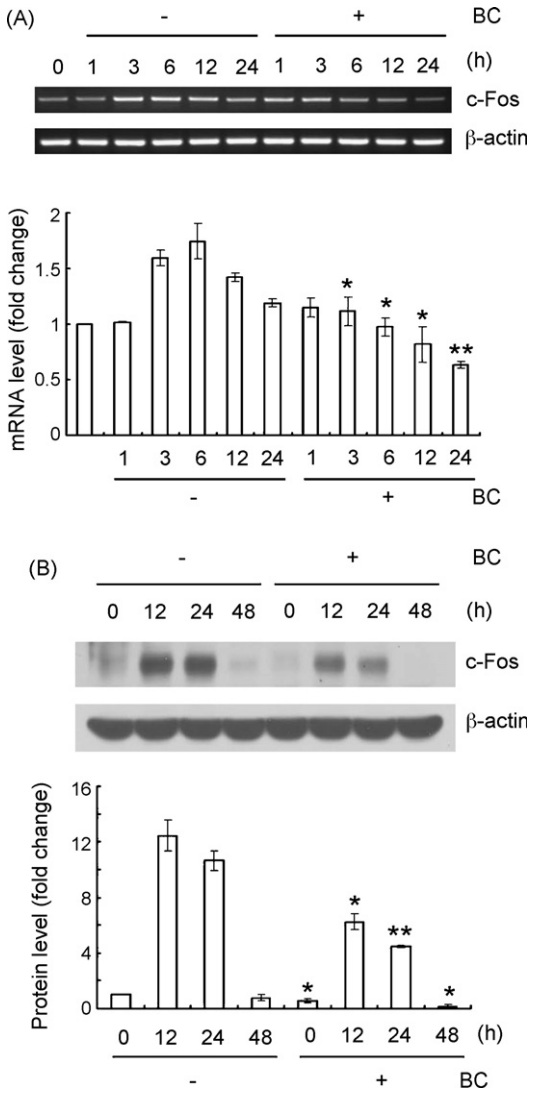

Fig. 5 – Bavachalcone suppresses c-Fos induction by RANKL. (A) BMMs were cultured in medium containing 30 ng ml^{-1} M-CSF plus 200 ng ml^{-1} RANKL for the indicated time with or without bavachalcone (5 μg ml^{-1}). Total RNA was prepared as described in Section 2 and analyzed by RT-PCR. (B) BMMs were cultured with M-CSF and RANKL for the indicated time with or without bavachalcone (5 μg ml^{-1}). Whole cell lysates were prepared and analyzed by western blotting with a c-Fos specific antibody. The blot was reprobed with an anti-β-actin antibody. $^{**}p < 0.01$, $^{*}p < 0.05$ versus DMSO-treated BMMs.

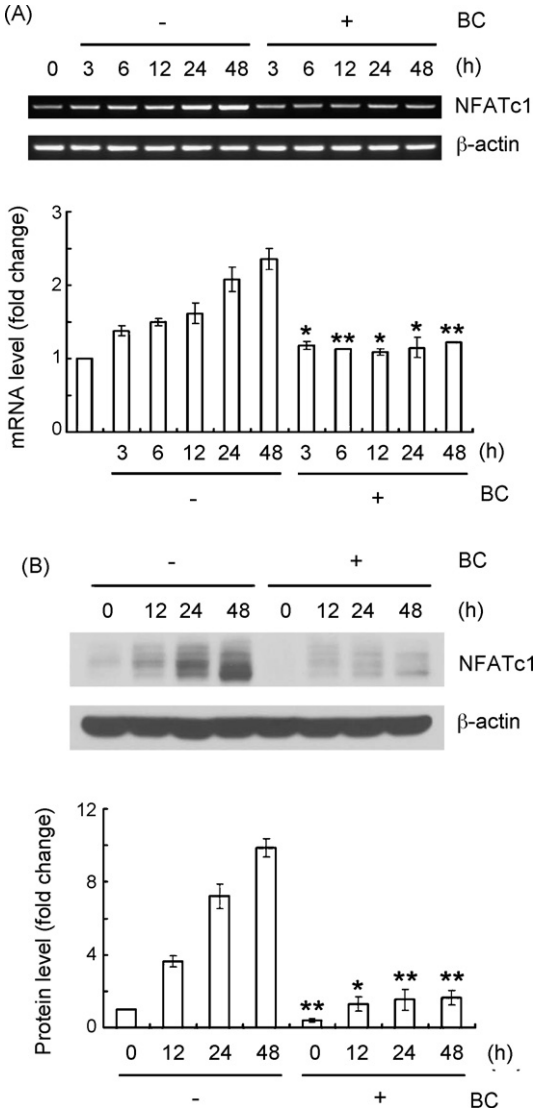

Fig. 6 – RANKL-induced NFATc1 expression was down-regulated by bavachalcone. (A) BMMs were cultured with 30 ng ml^{-1} M-CSF and 200 ng ml^{-1} RANKL for the indicated time in presence or absence of bavachalcone (5 μg ml^{-1}). Total RNA was prepared and accessed by RT-PCR to examine the NFATc1 mRNA level. (B) BMMs were cultured in medium containing 30 ng ml^{-1} M-CSF plus 200 ng ml^{-1} RANKL for the indicated time with or without bavachalcone (5 μg ml^{-1}). Whole cell lysates were prepared and analyzed by immunoblotting with NFATc1 and β-actin specific antibodies. $^{**}p < 0.01$, $^{*}p < 0.05$ versus DMSO treated BMMs.

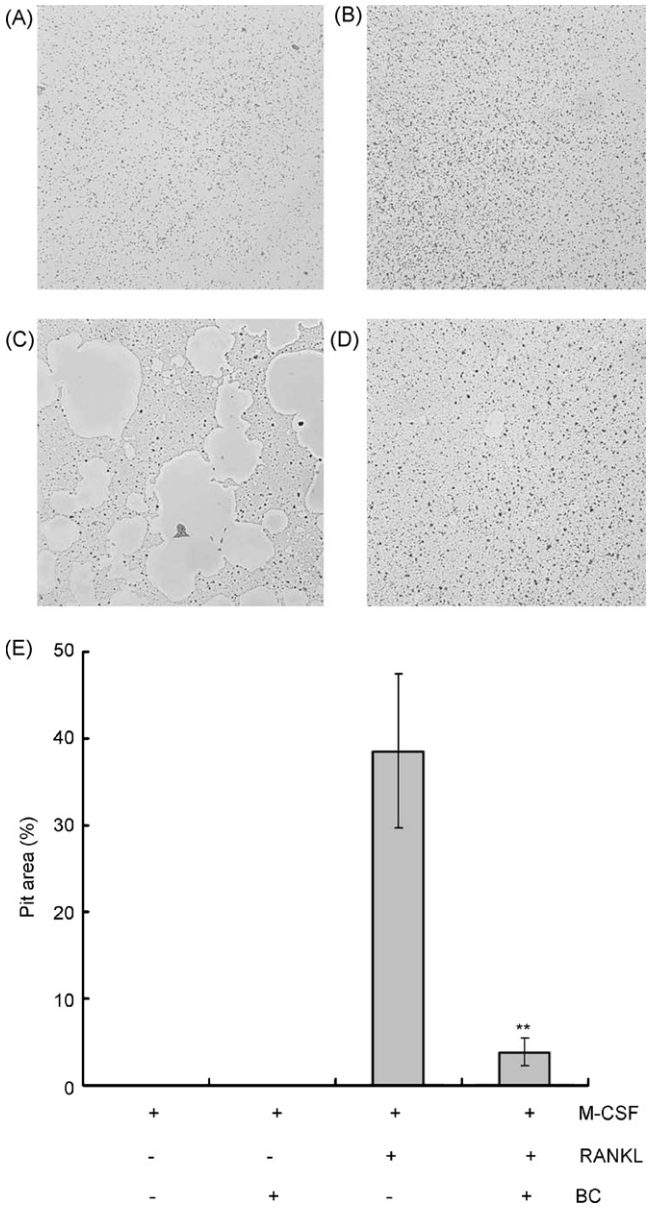

Fig. 7 – Bavachalcone inhibits generation of resorption pits. BMMs were cultured for 5 days with 30 ng ml^{-1} M-CSF in presence (A) or absence (B) of bavachalcone (5 μg ml^{-1}) or 30 ng ml^{-1} M-CSF and 200 ng ml^{-1} RANKL in presence (C) or absence (D) of bavachalcone (5 μg ml^{-1}) on calcium phosphate coated-plates. Cells were removed by washing with distilled water and resorbed areas were measured. $^{**}p < 0.01$ versus RANKL-treated BMMs without bavachalcone.

4. Discussion

In previous reports, the expression of RANKL has been shown to be enhanced in the bone marrow stromal cells or osteoblasts by several factors, such as VtD_3, PGE_2, and IL-1 [5–7]. RANKL induces osteoclast differentiation through binding with its receptor, RANK, which is present in the plasma membrane of BMMs. So, we carried out the co-culture, in which calvarial osteoblast cells and bone marrow cells are cultured in the presence of VtD_3 and PGE_2. In this system, bavachalcone dose-dependently inhibited osteoclastogenesis. Bavachalcone also attenuated RANKL-induced osteoclastogenesis in purified BMM culture system. In these two culture systems, bavachalcone exhibited similar inhibitory effects at same doses, indicating that anti-osteoclastogenic function of bavachalcone is mainly caused by direct effect on BMMs rather than on osteoblasts.

c-Fos and NFATc1 are crucial transcription factors in RANKL-induced osteoclastogenesis [11–13]. The role of c-Fos transcription factor in osteoclastogenesis has been revealed by knockout experiments [12]. c-Fos knockout mice exhibited serious osteopetrotic phenotype, due to the failure of osteoclast formation. The importance of NFATc1 on osteoclastogenesis was supported by the in vitro experiments where NFATc1 knockout mice-derived stem cells failed to differentiate into osteoclast and that BMMs, when forced to express NFATc1, differentiated to osteoclasts in the absence of RANKL [18,19]. In our study, bavachalcone significantly suppressed not only c-Fos induction but also NFATc1 up-regulation by RANKL. We suggest that the down-regulation of c-Fos by bavachalcone may be the major factor of suppression of NFATc1 expression by RANKL.

MAPKs have been implicated in RANK signaling. The AP-1 transcription factor plays an important role in osteoclastogenesis. Also, MAPKs regulate the activation of AP-1, which is a heterodimeric complex of combined c-Fos and c-Jun transcription factors. The importance of p38 on osteoclastogenesis was demonstrated by experiment using p38 specific inhibitors [20]. In our study, bavachalcone significantly inhibited the phosphorylation of ERK as well as MEK by RANKL. Bavachalcone slightly inhibited phosphorylation of p38, whereas it did not show significant effect on the activation of JNK. These results indicate that the suppression of c-Fos expression by bavachalcone may be mainly due to down-regulation of ERK activation by RANKL.

It has been shown that NF-κB is involved in RANK signaling pathway [21]. Importance of NF-κB for osteoclastogenesis was confirmed by genetic studies. p50/p52-deficient mice exhibited osteopetrotic phenotype, due to a defect in osteoclast formation [14]. In the classical NF-κB signaling pathway, I-κB is degraded in ubiquitin-dependent manner upon phosphorylation and NF-κB released from I-κB inhibition translocates to the nucleus to exert transcriptional activity. In our study, bavachalcone did not affect RANKL-induced phosphorylation of I-κB (Supplementary Fig. 1), suggesting that the effect of bavachalcone on osteoclast differentiation is likely to be independent on the classical NF-κB activation pathway.

Previous studies reported that *P. corylifolia* extracts had effects on bone calcification and osteoblast proliferation, suggesting potential use for improvement of osteoporotic disorders [1,2]. Those studies were related to osteoblasts, and were limited to experiments using the crude extract. In our study, bavachalcone isolated from *P. corylifolia* negatively regulated osteoclast differentiation by RANKL in primary BMMs. In addition, we revealed the molecular mechanism for the inhibitory action of bavachalcone on osteoclastogenesis. Although additional experiments are needed to confirm its efficacy in disease conditions in vivo and to improve its potency perhaps via structural modifications, we propose a possibility that bavachalcone can be used in development of a therapeutic drug for osteoporosis.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.03.007.

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