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# Alisol-B, a novel phyto-steroid, suppresses the RANKL-induced osteoclast formation and prevents bone loss in mice

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ABSTRACT

Osteoclasts, bone-resorbing multinucleated cells, are differentiated from hemopoietic progenitors of the monocyte/macrophage lineage. Bone resorption by osteoclasts is considered a potential therapeutic target to the treatment of erosive bone diseases, including osteoporosis, rheumatoid arthritis, and periodontitis. In the present study, we found that alisol-B, a phyto-steroid from *Alisma orientale* Juzepczuk, exhibited inhibitory effects on osteoclastogenesis both *in vitro* and *in vivo*. Although RT-PCR analysis showed that alisol-B did not affect the $1\alpha,25(OH)_2D_3$-induced expressions of RANKL, OPG and M-CSF mRNAs in osteoblasts, addition of alisol-B to co-cultures of mouse bone marrow cells and primary osteoblasts with $10^{-8}$ M $1\alpha,25(OH)_2D_3$ caused significant inhibition of osteoclastogenesis. We further examined the direct effects of alisol-B on osteoclast precursors. Alisol-B strongly inhibited RANKL-induced osteoclast formation when added during the early stage of cultures, suggesting that alisol-B acts on osteoclast precursors to inhibit RANKL/RANK signaling. Among the RANK signaling pathways, alisol-B inhibited the phosphorylation of JNK, which are upregulated in response to RANKL in bone marrow macrophages, alisol-B also inhibited RANKL-induced expression of NFATc1 and c-Fos, which are key transcription factors for osteoclastogenesis. In addition, alisol-B suppressed the pit-forming activity and disrupted the actin ring formation of mature osteoclasts. In a hypercalcemic mouse model induced by 2-methylene-19-nor-(20*S*)-$1\alpha,25(OH)_2D_3$ (2MD), an analog of $1\alpha,25(OH)_2D_3$, administration of alisol-B significantly suppressed 2MD-induced hypercalcemia as resulting from the inhibition of osteoclastogenesis. Taken together, these findings suggest that alisol-B may be a potential novel therapeutic molecule for bone disorders by targeting the differentiation of osteoclasts as well as their functions.


## 1. Introduction

Bone, a mineralized connective tissue, is continuously remodeled by two coupled processes: bone resorption by osteoclasts and bone formation by osteoblasts. This remodeling is responsible for normal skeletal formation, skeletal functions and mineral homeostasis. Therefore, regulations of the recruitment, proliferation and differentiation of osteoclasts and osteoblasts are essential for the maintenance of bone mass. Deregulation in one of these processes can lead to bone diseases such as osteoporosis.

Osteoclasts, multinucleated cells, are differentiated from hematopoietic precursor cells of the monocyte/macrophage lineage under the tight regulation of osteoblasts [1]. Osteoblasts express two cytokines essential for osteoclastogenesis, namely receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [2–5]. M-CSF is constitutively expressed by osteoblasts, whereas RANKL is inducibly expressed by osteoblasts in response to osteotropic hormones and factors including $1\alpha$,25-dihydroxy-vitamin $D_3$ [$1\alpha,25(OH)_2D_3$] and prostaglandin $E_2$. Osteoblasts also produce osteoprotegrin (OPG), a soluble decoy receptor for RANKL, which inhibits osteoclastogenesis by blocking RANKL/RANK interactions.

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Osteoclast precursors, such as bone marrow-derived macrophages (BMMs), express RANK and c-fms, the receptors for RANKL and M-CSF, respectively, and differentiate into mature osteoclasts in the presence of these ligands [6,7].

RANKL, a member of the tumor necrosis factor (TNF) family cytokines [8] that interacts with the receptor RANK, which in turn recruits tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) [9,10]. TRAF1, 2, 3 and 5 bind to the carboxyl-terminal end of RANK trimers, whereas TRAF6 binds more closely to the membrane. The downstream intracellular signaling mediated by RANK in osteoclast progenitor cells includes TRAF6-dependent activation of nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPKs) and AP-1, as well as activation of c-Src and the phosphatidylinositol 3-kinase/Akt pathway [11–13]. In addition, immunoreceptor tyrosine-based activation motif-mediated costimulatory signals have been shown to be required for the expression of nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), a master transcription factor for osteoclast differentiation [14,15].

To discover new types of anti-resorptive agents, we screened for natural compounds that regulate osteoclast differentiation. In our screening, we have found that alisol-B has strong inhibitory effects on osteoclast formation *in vitro*. Alisol-B (Fig. 1A), which is derived from *Alisma orientale* Juzepczuk and is a famous traditional Chinese medicine that has been widely used for diuretic, hypolipidemic, anti-inflammatory and anti-diabetic purposes in China for more than a thousand years [16,17]. Many reports have identified and described specific activities of alisol-B, such as induction of apoptosis in cancer cells and vascular smooth muscle cells [18–20], anti-inhibitory effects on lipopolysaccharide [21], anti-complementary activities [22] and anti-allergic activities [23]. However, its beneficial effects on bone metabolism have not previously been evaluated. In the present study, we investigated the effects of alisol-B on osteoclast differentiation and function,

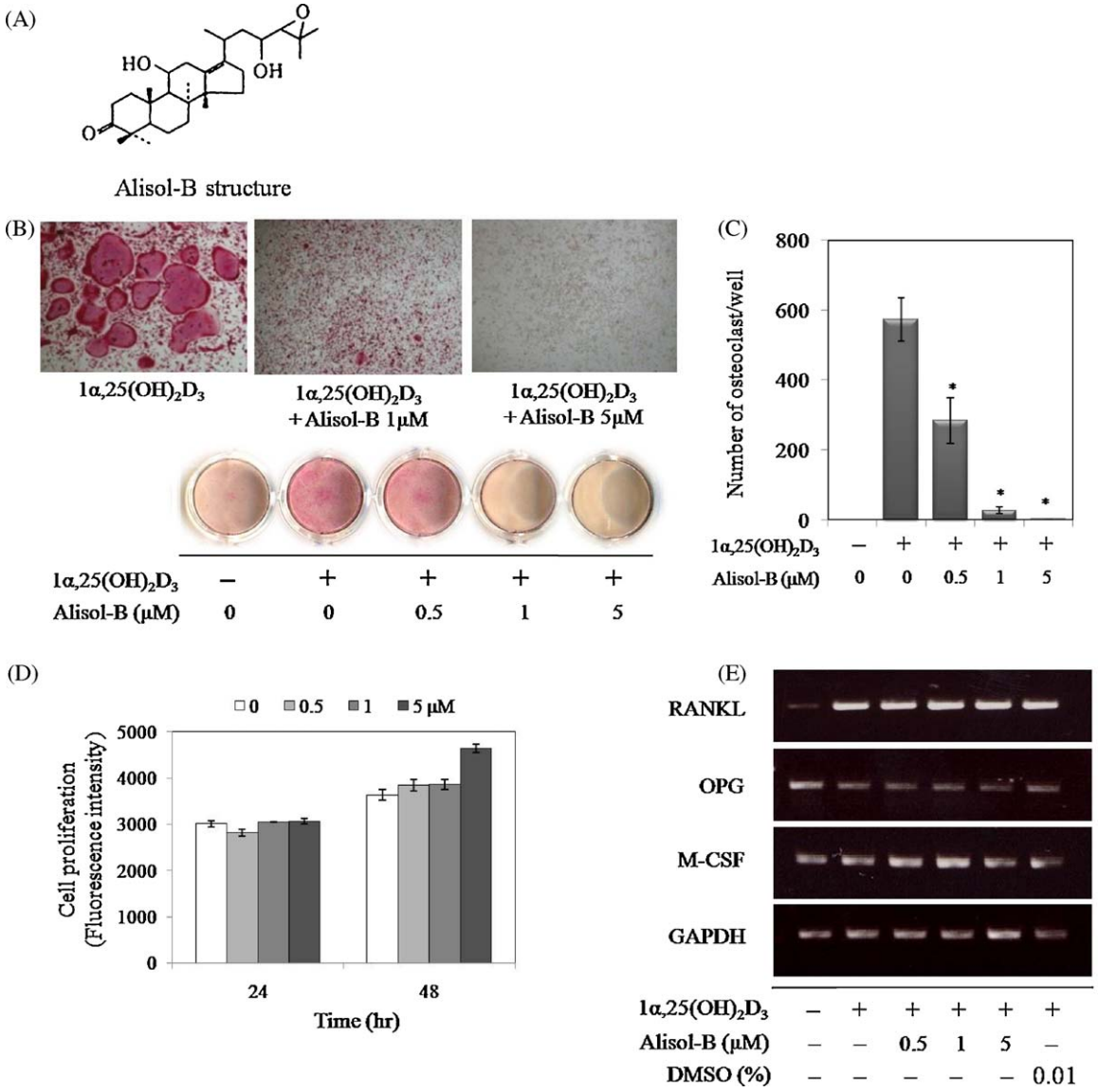


**Fig. 1.** Effects of alisol-B on $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis in mouse co-cultures. (A) Structure of alisol-B. (B) Primary osteoblasts and BMCs were co-cultured in 24-well plates for 6 days in the presence of $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M). After the 6-day co-culture, the cells were fixed and stained for TRAP. (C) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. (D) Effects of alisol-B on cell viability in mouse calvarial osteoblasts. Mouse calvarial osteoblasts were cultured with alisol-B at 0.5, 1 or 5 μM for 2 days. After the indicated times, Alamar blue reagent was added to each well. The fluorescence was measured with an excitation wavelength of 560 nm and an emission wavelength of 590 nm, and the cell viability was calculated. (E) Mouse osteoblasts were cultured for 24 h in the presence of $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) and alisol-B (0.5, 1 or 5 μM). Total RNA was then extracted from osteoblasts, and the expression levels of RANKL, OPG and M-CSF mRNAs were analyzed by RT-PCR. The results are expressed as means ± SD of three cultures. $^{*}p < 0.05$.

RANKL-induced signaling pathways, and bone resorption in an *in vivo* model.

## 2. Materials and methods

### 2.1. Mice and reagents

Newborn and 5-week-old ddY mice and 7- to 8-week-old C57BL/6J (B6) (wild-type) mice were purchased from Japan Shizuoka Laboratories Animal Center (Shizuoka, Japan). All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Matsumoto Dental University. Alisol-B, 1α,25-dihydroxyvitamin $D_3$ [1α,25$(OH)_2D_3$] and prostaglandin $E_2$ ($PGE_2$) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Recombinant human M-CSF (Leukoprol) was purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan). Recombinant soluble RANKL (sRANKL) was purchased from PeproTech EC Ltd. (London, UK). Anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK, anti-extracellular signal-regulated kinase (ERK) 1/2, anti-phospho-ERK1/2, anti-p38 MAPK, anti-phospho-p38 MAPK and anti-inhibitory κB (IκB) rabbit polyclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). An anti-NFATc1 mouse polyclonal antibody was purchased from Affinity Bio Reagents Co. Ltd. (Rockford, IL). An anti-c-Fos rabbit polyclonal antibody was purchased from Calbiochem (San Diego, CA). An anti-β-actin mouse polyclonal antibody and ICI-182870 were purchased from Sigma Chemical Co. (St. Louis, MO). Specific PCR primers for mouse RANKL, osteoprotegerin (OPG), M-CSF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Life Technologies Inc. (Tokyo, Japan). 2-Methylene-19-nor-(20*S*)-1α,25$(OH)_2D_3$ (2MD) compound was kindly provided by Dr. Shimizu Masato from Tokyo Medical and Dental University. All other chemicals and reagents were of analytical grade.

### 2.2. Cell proliferation assay

Cell proliferation was determined by the Alamar blue assay (Biosource International, Camarillo, CA) according to the manufacturer's instructions. Mouse bone marrow cells (BMCs; $5 \times 10^4$ cells/well) were cultured in 96-well plates with various concentrations of alisol-B for a total of 3 days. At various time points, the Alamar blue reagent was added to individual wells. The fluorescence was measured with an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

### 2.3. Murine BMMs and co-cultures

Primary calvarial osteoblasts were obtained from the calvariae of neonatal ddY mice using collagenase (Wako) and dispase (Godo Shusei Co., Tokyo) [24]. Mice are anesthetized with ether, and sacrificed by decapitation. The heads were placed in a petri dish containing PBS(−). The calvariae were collected and the attached skin and muscle was carefully removed. The calvariae were placed in a 50-ml centrifuge tube and washed twice with α-MEM containing 0.1% bacterial collagenase and 0.2% dispase for 10 min at 37 °C in a shaking water bath (120 cycles/min). The collagenase solution was recovered, and fresh collagenase solution (10 ml) is added. Calvariae were further incubated for another 10 min at 37 °C in a shaking water bath (120 cycles/min). This step was repeated five times. The primary osteoblasts isolated in fractions 2–5 were collected by centrifugation, and cultured for 3 days in α-MEM containing 10% FBS in 10-cm culture dishes. To confirm the cells were osteoblasts, alkaline phosphatase staining and 1α,25$(OH)_2D_3$-induced osteoclast formation were performed. BMCs were obtained from the long bones of 4- to 6-week-old ddY male mice. To obtain BMMs, BMCs were cultured for 3 days in α-MEM containing fetal bovine serum (FBS; 10%) and M-CSF (50 ng/ml) in 100-mm culture dishes. After culture for 1 day, cells that had not attached to the culture plates were collected and used as BMMs. In the BMM culture system, cells were cultured on 96-well plates in the presence of M-CSF (50 ng/ml) for 3 days and then treated with RANKL (100 ng/ml) for a further 3 days. In the co-culture system, BMCs were cultured on 24-well plates with mouse primary osteoblasts in the presence of 1α,25-$(OH)_2D_3$ ($10^{-8}$ M) for 6 days. After the cultures, some of the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme for osteoclasts. To obtain mature osteoclasts, BMCs ($1 \times 10^7$ cells) and osteoblasts ($1 \times 10^6$ cells) were co-cultured for 6 days in α-MEM containing 10% FBS in collagen gel-coated 100-mm plates, and supplemented with $10^{-8}$ M 1α,25$(OH)_2D_3$ and $10^{-6}$ M $PGE_2$. The plates were treated with collagenase and whole cells were harvested for use in subsequent experiments.

### 2.4. Pit formation and actin ring formation assays

For resorption pit assays, aliquots of the crude mature osteoclast preparation described above were placed on dentin slices in 96-well plates [25]. After preincubation for 2 h, the dentin slices were transferred to 48-well plates (1 dentin slice/well) containing 0.3 ml of α-MEM supplemented with 10% FBS and cultured with or without alisol-B for 48 h. Dentin slices incubated with calcitonin ($10^{-8}$ M) for the same period were regarded as positive controls. Resorption pits on the dentin slices were visualized by staining with Mayer's hematoxylin solution. The number of resorption pits on each slice was counted. Another dentin slices were assessed for actin ring formation. Briefly, after fixation of the cells with 4% paraformaldehyde, F-actin was stained with rhodamine-conjugated phalloidin for assessment of ring formation by fluorescence microscopy as previously described [26].

### 2.5. Western blot analysis

Cells were lysed with RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM β-glycerophosphate, 1% NP-40, 1 mM $Na_3VO_4$ and 1× protease inhibitor cocktail). The extracted proteins were separated by SDS-PAGE and electrotransferred onto PVDF membranes. Proteins were detected by incubation with primary antibodies against c-JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, ERK1/2, p38 MAPK, phospho-p38 MAPK, IκB, NFATc1, c-Fos and β-actin, followed by secondary antibodies conjugated with horseradish peroxidase. Immuno-complexes were visualized by a chemiluminescence reaction using ECL reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK).

### 2.6. PCR amplification of reverse-transcribed mRNA

For RT-PCR analysis, mouse osteoblasts were cultured in α-MEM containing 10% FBS and $10^{-8}$ M 1α,25$(OH)_2D_3$ with or without alisol-B on 60-mm culture dishes. After culture for 24 h, total cellular RNA was extracted from the cells using TRIzol solution (Life Technologies Inc.). First-strand cDNA was synthesized from the total RNA extracts with random primers and subjected to PCR amplification with Ex *Taq* polymerase (Takara Biochemicals, Shiga, Japan) using specific PCR primers: mouse RANKL, 5′-CGC TCT GTT CCT GTA CTT TCG AGC G-3′ (forward) and 5′-TCG TGC TCC CTC CTT TCA TCA GGT T-3′ (reverse); mouse OPG, 5′-TGG AGA TCG AAT TCT GCT TG-3′ (forward) and 5′-TCA AGT GCT TGA GGG CAT AC-3′ (reverse); mouse M-CSF, 5′-GAG AAG ACT GAT GGT ACA TCC-3′ (forward) and 5′-CTA TAC TGG CAG TTC CAC C-3′ (reverse); mouse GAPDH, 5′-ACC ACA GTC CAT GCC ATC AC-3′

(forward) and 5′-TCC ACC ACC CTG TTG CTG TA-3′ (reverse). The PCR products were separated by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining and UV light illumination. The sizes of the PCR products for mouse RANKL, OPG, M-CSF and GAPDH were 587, 720, 516 and 452 bp, respectively.

### 2.7. Treatment with 2MD in mice and histological analysis of bone

A stock solution of 2MD compound dissolved in ethanol was diluted in propylene glycol at 1:9. Next, 30 pmol of 2MD and 100 μM alisol-B (0.1 ml/day) were intraperitoneally injected into C57BL/6J mice ($n = 6$) every other day and every day for 5 days, respectively. Blood samples from the mice with or without 2MD and alisol-B treatment were collected from the tail (days 0–4) or by heart puncture (day 5) under anesthesia with diethyl ether. The femora, tibiae, spleen, liver and kidney were also removed from each mouse. For TRAP staining, the tibiae were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h at 4 °C. The specimens were then decalcified with 16% EDTA (pH 7.3) for 2 weeks at 4 °C, dehydrated through a series of graded ethanol solutions, embedded in paraffin and cut into 4-μm-thick sections. Double staining with hematoxylin and TRAP was performed on the specimens and TRAP-positive osteoclasts were detected under a light microscope as described previously [27]. The serum concentration of calcium was measured using a calcium E kit (Wako, Osaka, Japan). The serum level of RANKL was measured using a sensitive ELISA kit (R&D Systems Inc., Minneapolis, MN) as described previously [28] and the serum level of TRAP5b was measured using a sensitive ELISA kit (IDS Ltd., Tyne and Wear, UK).

### 2.8. Statistical analysis

The data were expressed as means ± SD. Statistical analyses were performed by an unpaired two-tailed Student's *t*-test assuming unequal variances. Values of $p < 0.05$ were considered to indicate statistical significance.

## 3. Results

### 3.1. Alisol-B inhibits osteoclast formation in the mouse co-culture system without affecting osteoblast activity

To clarify effects of alisol-B on osteoclast formation, BMCs were co-cultured with osteoblasts derived from mouse calvariae in the presence of $10^{-8}$ M $1\alpha,25(OH)_2D_3$. Many TRAP-positive osteoclasts were formed in the co-cultures within 6 days in response to $1\alpha,25(OH)_2D_3$ (Fig. 1B and C). We found that treatment with alisol-B dose-dependently inhibited osteoclast formation on the co-cultures. Complete inhibition of osteoclast formation was observed in co-cultures treated with alisol-B at 5 μM. Prior to examined the effect of mouse calvaria osteoblast activity, we confirmed the typical phenotype of osteoblasts with ALP staining and $1\alpha,25(OH)_2D_3$-induced osteoclast formation on co-culture method. To clarify the effects of alisol-B on mouse calvaria osteoblasts, we examined its effects on the proliferation of osteoblasts. Primary osteoblasts were cultured with the increasing concentration of alisol-B and cell growth of osteoblasts was monitored by Alamar blue assay. The cytotoxicity of alisol-B was not observed in osteoblast cultures at the time points measured (Fig. 1D). We next examined the effects of alisol-B on the expression of RANKL, OPG (a decoy receptor for RANKL) and M-CSF mRNAs in osteoblasts treated with $1\alpha,25(OH)_2D_3$. Treatment of osteoblasts with $1\alpha,25(OH)_2D_3$ enhanced the expression of RANKL, suppressed the expression of OPG and sustained the expression of M-CSF (Fig. 1E). Alisol-B showed no effects on the upregulation of RANKL mRNA expression and also had no effects on the OPG and M-CSF mRNA expression levels in osteoblasts. These results suggest that alisol-B may act directly on osteoclast precursors but not on osteoblasts for the inhibition of osteoclast formation.

### 3.2. Alisol-B targets the early stage of osteoclast differentiation stimulated by sRANKL

We next examined whether alisol-B directly acts on osteoclast precursor cells to inhibit RANKL-induced osteoclastogenesis. To clarify effects of alisol-B on cell growth of osteoclast precursors, BMMs were cultured in the presence of M-CSF together with or without the increasing concentration of alisol-B (Fig. 2A). The growth of BMMs was not affected by alisol-B at 5 μM. We examined whether alisol-B inhibits RANKL-induced osteoclast formation in BMM cultures (Fig. 2B). Mouse BMMs were cultured in the presence of RANKL and M-CSF together with or without various concentrations of alisol-B. BMMs were formed from BMCs in culture within 3 days in response to M-CSF. As shown in Fig. 2B, alisol-B inhibited osteoclast formation in a dose-dependent manner when added throughout the entire culture period. To explore the inhibitory effect of alisol-B on osteoclast formation in detail, we added alisol-B into BMM cultures treated with RANKL and M-CSF at three different time points (Fig. 2C). After exposure to alisol-B for 24 h, the culture media containing alisol-B were washed out and changed into alisol-B free culture media. The results revealed that even 5 μM alisol-B was able to completely inhibit osteoclast formation when the cells were exposed at day 0–1 after sRANKL treatment (Fig. 2D). The similar inhibitory effect on osteoclast formation was observed when alisol-B was added at days 1–2 (data not shown). However, the inhibitory effect was decreased when alisol-B was added at days 2–3. These findings suggest that alisol-B inhibits early cellular events for osteoclastogenesis. We also examined the effect of ICI-182780, a known estrogen receptor antagonist, on the inhibitory effect of alisol-B on osteoclast formation (data not shown). In fact, this inhibitory effect of alisol-B was not affected by ICI-182780. Therefore, these results suggest that the inhibitory effect of alisol-B was not due to estrogen-like activity.

### 3.3. Alisol-B inhibits osteoclast differentiation via suppression of RANKL-mediated JNK activation

A key signaling event induced by the binding of RANKL to RANK is the activation of MAPKs, NF-κB and Akt signaling. To elucidate the inhibitory mechanism and pathway influenced by alisol-B, BMMs were treated with RANKL together with or without alisol-B for 0–120 min. The phosphorylation of MAPKs reached a maximal level within 10 min and then returned to the basal level in response to RANKL (data not shown). As shown in Fig. 3A, RANKL-induced phosphorylation of ERK, p38, Akt and degradation of IκB was not affected by the treatment with alisol-B. In contrast, phosphorylation of JNK was decreased by treatment with alisol-B. The NFATc1 and c-Fos pathways play critical and fundamental roles in osteoclast development, and a lack of either of these two transcription factors arrests osteoclastogenesis [29]. We then examined the effect of alisol-B on expression and activation of NFATc1 and c-Fos (Fig. 3B). Alisol-B impaired the RANKL-stimulated active form (dephosphorylation) of NFATc1. RANKL stimulated expression of c-Fos, which was strongly inhibited by adding alisol-B. Taken together, these findings demonstrate that alisol-B inhibited osteoclast formation involved from down-regulation of JNK phosphorylation.

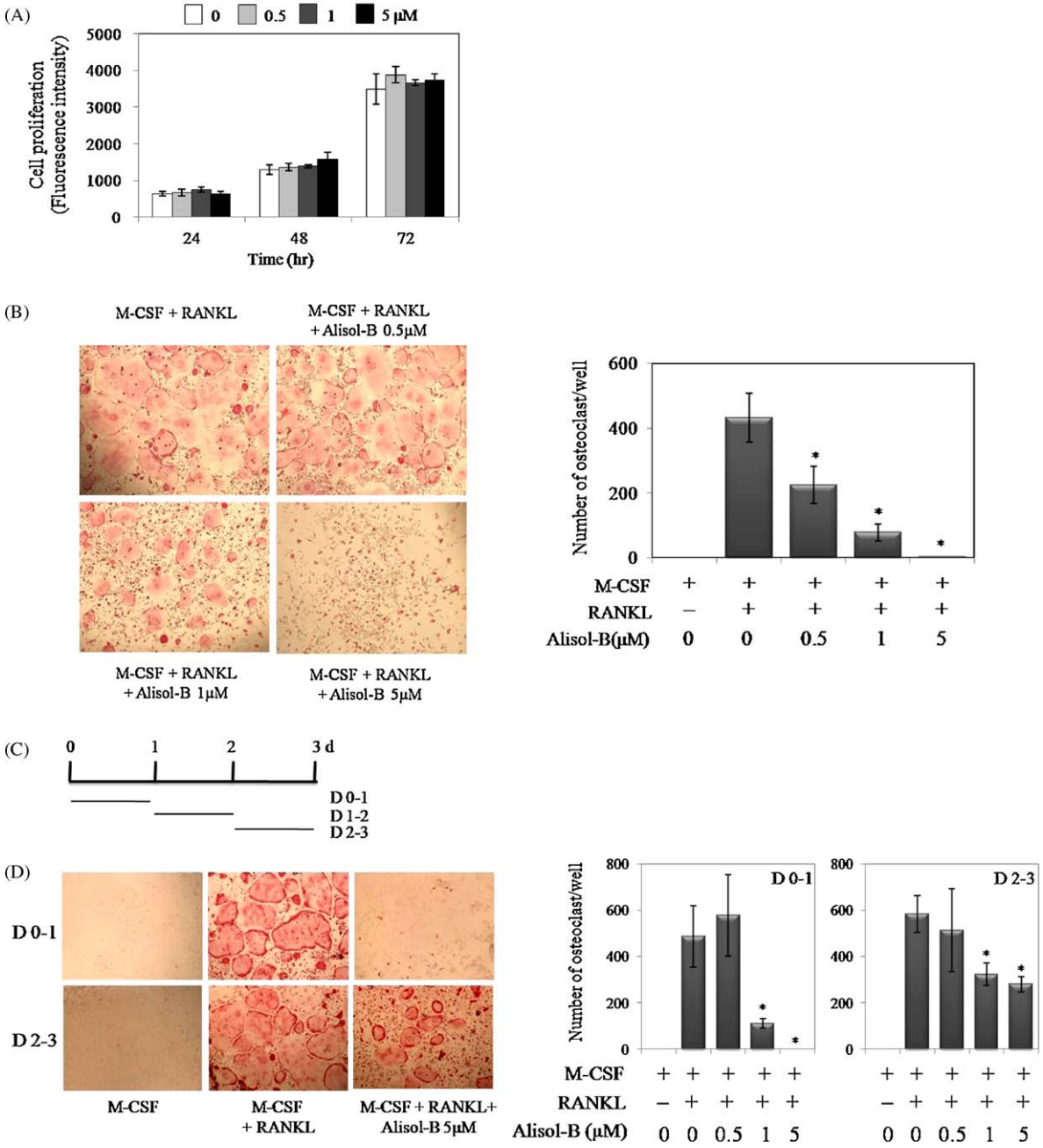


**Fig. 2.** Effects of alisol-B on RANKL-induced osteoclast formation in mouse BMMs. (A) BMCs were cultured with alisol-B for 72 h in 96-well plates. Cell viability was determined by Alamar blue assays. The fluorescence was measured with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. (B) Mouse BMMs were cultured with RANKL (100 ng/ml) and M-CSF (50 ng/ml). After culture for 3 days, the cells were fixed and stained for TRAP. TRAP-positive multinucleated cells containing more than five nuclei were counted as osteoclasts. TRAP staining of BMMs in 96-well plates is also shown. (C) Durations of exposure to alisol-B in days. (D) TRAP staining of BMMs in 96-well plates is also shown. TRAP-positive multinucleated cells counted as osteoclasts. The results are expressed as means ± SD of three cultures. $^{*}p < 0.05$.

### 3.4. Alisol-B inhibits osteoclast function in vitro

We next examined whether alisol-B inhibits osteoclast function. When osteoclasts obtained from co-cultures of BMCs with osteoblasts in the presence of $1\alpha,25(OH)_2D_3$ were inoculated on dentin slices and cultured for 48 h, many resorption pits were formed on the slices (Fig. 4A, upper panels). Alisol-B significantly inhibited the pit-forming activity of osteoclasts. Although the number of pits on the slices was markedly decreased in the presence of alisol-B (Fig. 4B, left panel), the number of TRAP-positive cells observed on the slices treated with alisol-B, even at 5 μM, was not affected compared with the control group (data not shown). Actin ring formation in osteoclasts is essential for the expression of their bone resorption function. When osteoclasts with actin rings were treated with alisol-B for 48 h, their size was constricted and the ring formation was disrupted (Fig. 4A down panels). These data suggest that the inhibition of the pit-forming activity of mature osteoclasts by alisol-B was caused mainly by disruption of the actin ring formation.

### 3.5. Administration of alisol-B prevents bone resorption in 2MD-mediated hypercalcemia in mice

2MD, an analog $1\alpha,25(OH)_2D_3$, was synthesized by the introduction of a methylene group at carbon 2, removal of a methylene group at carbon 10 [30] and alteration of the

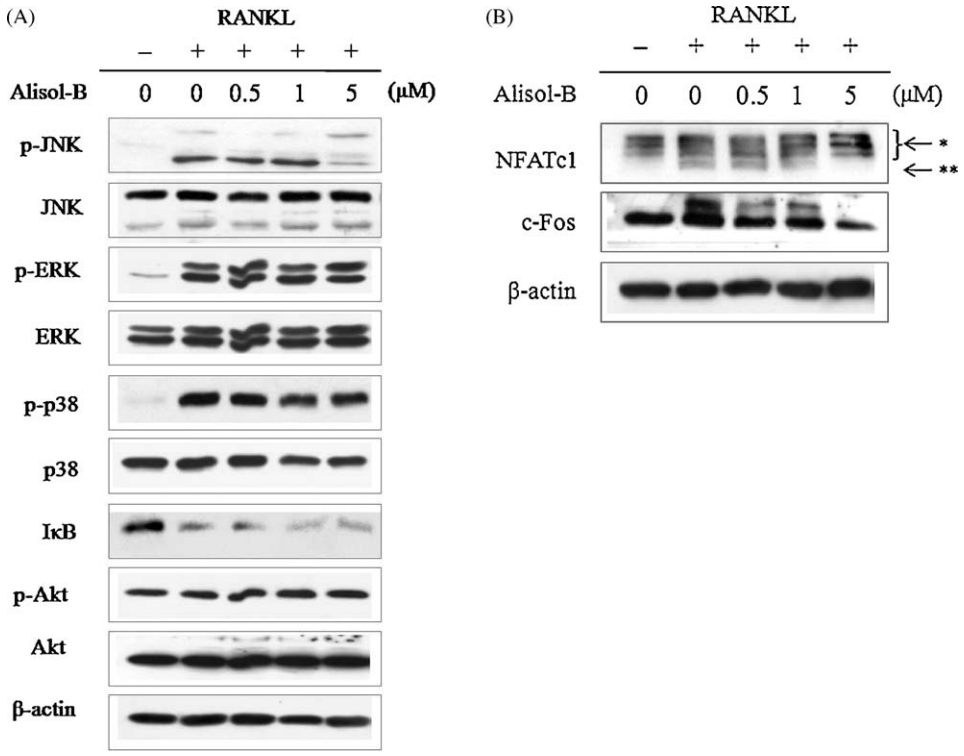


**Fig. 3.** Effects of alisol-B on RANKL-induced signaling pathways. Mouse BMMs were prepared from bone marrow cultures treated with M-CSF. (A) BMMs were treated with alisol-B and RANKL for 10 min and assessed for the phosphorylation of JNK, ERK, p38 MAPK and Akt as well as the degradation of IκBα. The immunoblots were then stripped and reprobed for total JNK, ERK, p38 MAPK and Akt. (B) Effects of alisol-B on the expression levels of NFATc1 and c-Fos during osteoclastogenesis. The cells were incubated alone or in the presence of alisol-B and stimulated with RANKL (100 ng/ml) for 24 h. Cytoplasmic extracts were prepared, separated by SDS-PAGE, transferred onto nitrocellulose membranes and immunoblotted with specific antibodies. β-Actin was used as an internal control. *Non-activated form and **activated form.

stereochemistry of carbon 20 to yield the 20*S* derivative (Fig. 5A). This compound more strongly stimulated RANKL expression in osteoblasts and osteoclast progenitor differentiation into osteoclasts than $1\alpha,25(OH)_2D_3$. After 2MD administration, bone resorption was reported to be strongly accelerated and positive hypercalcemic actions were concomitantly detected in mice [31]. There were no abnormal symptoms such as convulsion, stroke or unusual body temperature after intraperitoneal injection with alisol-B. After the animals were sacrificed, the solid organs, including the kidneys, liver and intestine appeared normal under the naked eye. To ascertain its biological potency *in vivo*, we first examined the effects of alisol-B on bone resorption by histological analyses of the tibial epiphyses. The number of TRAP-positive osteoclasts was significantly increased in the 2MD-treated group compared with the control (Fig. 5B). In the group treated with both alisol-B and 2MD, the number of TRAP-positive osteoclasts was strongly reduced (Fig. 5B). Regarding the serum concentrations of Ca, TRAP5b and RANKL, the 2MD-treated mice exhibited highly upregulated Ca, TRAP5b and RANKL levels (Fig. 5C). Administration of alisol-B to the 2MD-treated mice decreased serum levels of Ca and TRAP5b to the normal conditions, but had no effect on the serum level of RANKL. These results suggest that alisol-B has potent inhibitory effects on bone resorption by suppressing osteoclast differentiation *in vivo*.

## 4. Discussion

Plants used in traditional medicines have been recognized as one of the main sources for drug discovery and development. Since natural products of plant origin still form a major part of traditional medicine systems, a resurgence of interest in herbal medicines has occurred in Western countries as an alternative source of drugs, often for intractable diseases such as rheumatoid arthritis [32].

In the present study, we have shown that alisol-B effectively inhibited osteoclast formation in a mouse co-culture system. Furthermore, RANKL-induced osteoclast formation in mouse BMM cultures was also inhibited by alisol-B at 5 μM. However, RT-PCR analysis showed that alisol-B did not affect the expression levels of RANKL, OPG and M-CSF mRNAs in osteoblasts with or without $1\alpha,25(OH)_2D_3$ treatment. Taken together, these findings suggest that alisol-B act directly on osteoclast precursors and inhibit osteoclast formation, but not on osteoblasts. Furthermore, alisol-B strongly inhibited RANKL-induced osteoclast formation when added during at the early stage of the culture. In the terminal stage of the culture, the inhibitory effect of alisol-B on osteoclast formation was attenuated suggesting that alisol-B acts more strongly on osteoclast precursors.

Osteoclast differentiation is a multistep process that involves cell proliferation, commitment, fusion and activation [33,34]. Under normal conditions, the RANKL–RANK axis appears to be essential for osteoclastogenesis [35,36] and costimulatory immunoreceptors lead to robust induction of NFATc1, which is a necessary and sufficient factor for osteoclast differentiation [37–41]. In the present study, we examined the inhibitory mechanism of alisol-B on RANKL/RANK signaling and found that alisol-B decreased the phosphorylation of JNK. The expressions of NFATc1 and c-Fos also concomitantly inhibited the response to alisol-B. NFATc1 is a key transcription factor for the expression of TRAP and other osteoclastogenesis-associated genes [42–45]. Furthermore, introduction of an NFATc1 siRNA converts TRAP-positive cells into TRAP-negative cells [46]. In turn, c-Fos is one of the key factors required for NFATc1 induction by RANKL [40,47]. Takayanagi [40] showed that c-Fos is required for NFATc1-induced TRAP promoter activity. Matsuo et al. [47] reported that NFATc1 is a transcriptional target of c-Fos, and that NFATc1 transfection can rescue osteoclastogenesis in c-Fos-deficient macrophages in the presence

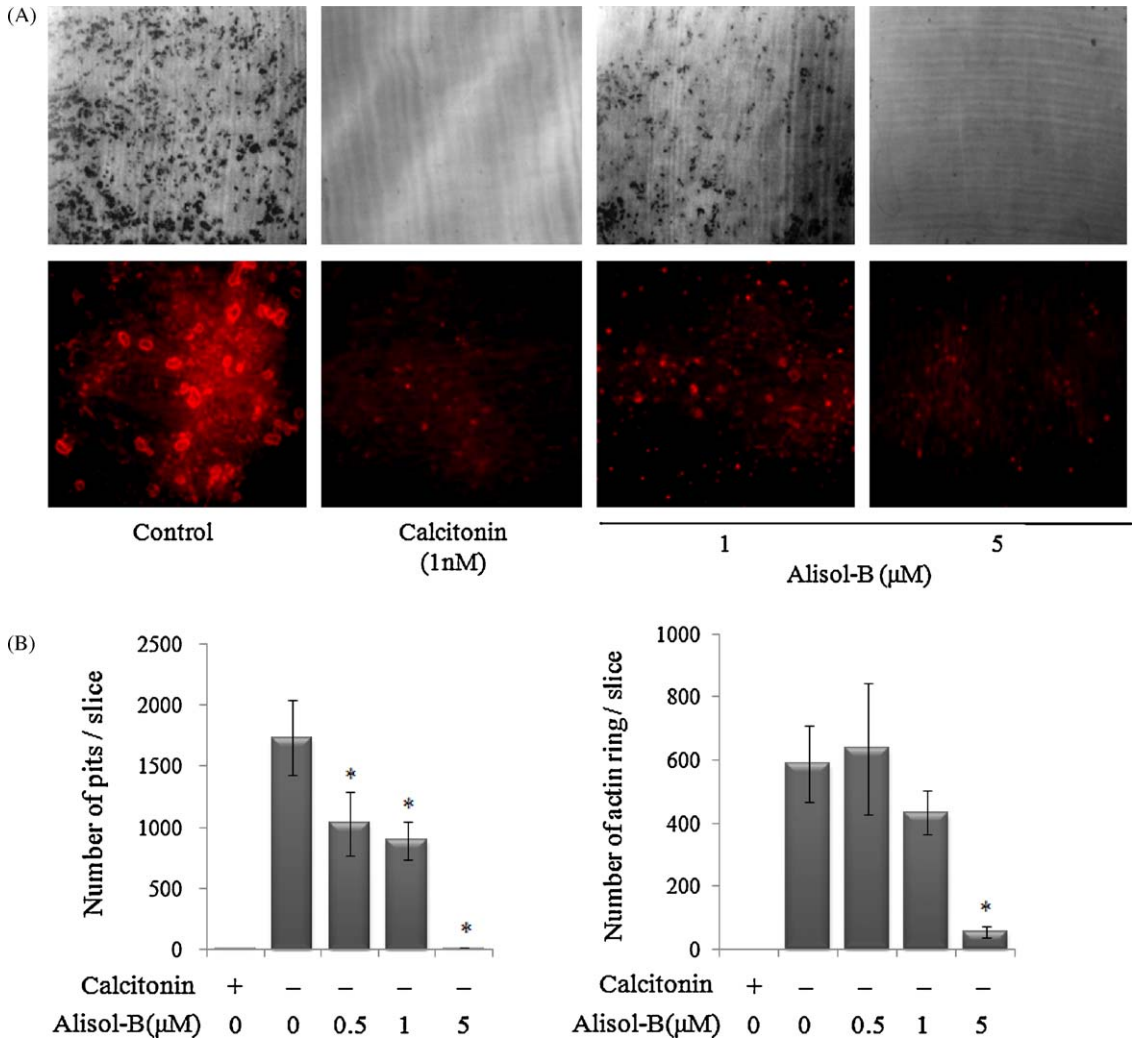


**Fig. 4.** Effects of alisol-B on bone resorption and actin ring formation. The mature osteoclasts were cultured on dentin slices with increasing concentrations of alisol-B. (A) After culture for 48 h, cells were removed and the dentin slices were stained with Mayer's hematoxylin to identify resorption pits (upper panels). After culture for 48 h, the cells were fixed with paraformaldehyde, permeabilized with Triton-X100, and incubated with rhodamine-conjugated phalloidin. F-actin was detected using a fluorescence microscope (lower panels). (B) The numbers of resorption pits (left panel) and actin rings (right panel) were counted. The results are expressed as means ± SD of three cultures. $^{*}p < 0.05$.

of RANKL. Therefore, the inhibitory mechanism of alisol-B implies to relate these axes in osteoclast precursor signaling.

To resorb bone, osteoclasts form actin rings, and this ring formation is essential for bone resorption by osteoclasts [48]. This unique cytoskeletal organization is thought to be a functional marker for activated osteoclasts [49,50]. Therefore, the identification of drugs that can disturb the integrity of these actin rings could be a useful approach for therapies aimed at slowing bone resorption. Alisol-B significantly disrupted actin rings in osteoclasts. Moreover, we examined whether the effects of alisol-B on the actin ring formation by mature osteoclasts were reversible. Once an actin ring was destroyed by treatment with alisol-B, it could be partially recovered within 24 h after the removal of alisol-B (data not shown). These results indicated that the alisol-B-treated non-functional osteoclasts were still alive. The regulation of osteoclast survival is an important mechanism in the regulation of the mature osteoclast bone-resorbing activity. Chen et al. [19] reported that alisol-B (10–100 μM) induces apoptosis in vascular smooth muscle cells and lymphocytes, accompanied by significant reductions in the mitochondrial membrane potential. In particular, alisol-B (10–100 μM) was found to increase the c-myc and bax mRNA and protein levels. Furthermore, alisol-B shows significant cytotoxicity toward various cancer cells, such as SK-OV3, B16-F10 and HT1080 cells [51,52]. We cannot exclude the possibility that the inhibitory effect of alisol-B on bone resorption is partially due to apoptosis of mature osteoclasts. However, in our experiments, alisol-B (5 μM) showed no inhibitory effects on the growth of calvarial osteoblasts and BMMs in culture. In addition, the number of osteoclasts on dentin slices was not significantly affected by alisol-B added to the pit formation assay. Therefore, these results suggest that the alisol-B-induced apoptosis is not the main pathway for the inhibitory action of alisol-B on osteoclastic bone resorption.

The active form of vitamin $D_3$, $1\alpha,25(OH)_2D_3$, is a principal regulator of calcium and phosphorus homeostasis through its actions on the intestine, kidney and bone. Vitamin D deficiency can lead to rickets and osteomalacia through a failure in bone formation caused by a defect of mineralization [53,54]. $1\alpha,25(OH)_2D_3$ is a secosteroid hormone that functions through a nuclear receptor, the vitamin D receptor (VDR). VDR is a ligand-dependent transcription factor and is believed to act by binding as a retinoid X receptor (RXR)/VDR heterodimer to vitamin D-responsive elements (VDREs) found in the promoter of its target genes [55]. 2MD, an analog of $1\alpha,25(OH)_2D_3$, was synthesized by introducing a methylene group at carbon 2, removal of a methylene group at carbon 10, and alteration of the

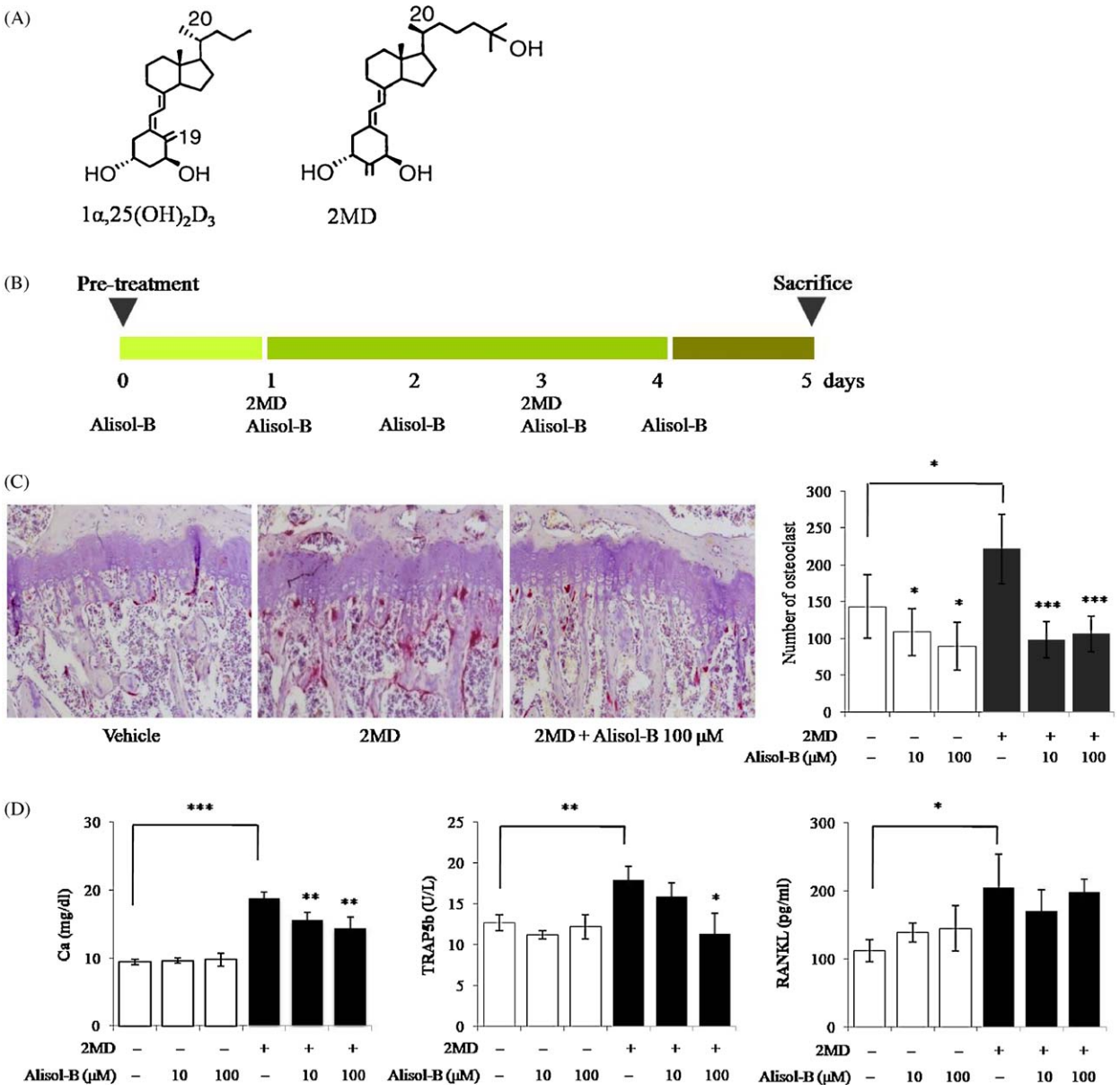


**Fig. 5.** Effects of alisol-B evaluated by histological analysis and the body constitution of 2MD-treated mice. Mice (7–8 weeks of age) were intraperitoneally injected with alisol-B and 2MD. (A) Structure of $1\alpha,25(OH)_2D_3$ and 2MD. (B) Schedule for the *in vivo* experiments and structures of $1\alpha,25(OH)_2D_3$ and 2MD. (C) Histological evaluation (double staining of TRAP and hematoxylin) of tibial bones. The osteoclast numbers in the tibial sections were counted (right panel). (D) Results of serum biochemical analyses. The serum Ca, TRAP5b and RANKL levels were determined. Data were expressed as means ± SD ($n$ = 5–6/group). $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$.

stereochemistry of carbon 20 to yield the 20*S* derivative [56]. Yamamoto et al. [57] reported that the increased potency of 2MD is likely due to enhanced binding of the VDR to the active promoters. In addition, 2MD is more potent in promoting interactions with the RXR and co-activators such as SRC-1 (steroid receptor co-activator 1) and DRIP205 (VDR-interacting protein 205) [57]. 2MD was 100 times as potent as $1\alpha,25(OH)_2D_3$ in stimulating the osteoclast formation *in vitro* and in inducing the expression of receptor activator of NF-κB ligand (RANKL) and 25-hydroxyvitamin D3-24 hydroxylase mRNAs in osteoblasts. They also showed the hypercalcemic effect of 2MD in $OPG^{-/-}$ mouse. The elevations in the serum concentrations of RANKL and Ca were much greater in 2MD analog-treated $OPG^{-/-}$ mice than in $1\alpha,25(OH)_2D_3$-treated mice [31]. Consistently, we found that 2MD treatment markedly increased the osteoclast numbers in bone tissues in mice and also highly upregulated the serum concentrations of Ca, TRAP5b and RANKL. Serum TRAP5b activity is reported to be a good indicator of osteoclastic bone resorption in mice [58]. In our results, both the hypercalcemic action and the enhanced osteoclast formation in mice treated with 2MD were significantly suppressed by the administration of alisol-B, but the serum level of RANKL was not decreased. We showed the inhibitory effects of alisol-B on osteoclast differentiation and function in *in vitro* assays. However, alisol-B exhibited no inhibitory effect on RANKL expression in osteoblasts in culture. In agreement with those *in vitro* activities, these *in vivo* results further support the notion that the inhibition of bone resorption by alisol-B occurs as a direct effect on osteoclast precursors, with no effect on RANKL expression on osteoblasts.

Alisol-B inhibited osteoclast differentiation and suppressed pit formation by osteoclast on dentine slices *in vitro*, Furthermore, alisol-B decreased the number of TRAP-positive osteoclasts in histological analyses of the tibial epiphyses, and the serum levels of

Ca and TRAP5b increased by 2MD *in vivo*. Bisphosphonates (BPPs), which are used to treat osteoporosis, accumulate in bone minerals and are incorporated into osteoclasts, and then induce apoptosis of osteoclasts [59,60]. Because BPPs accumulate in bone minerals, they ultimately decrease the overall bone turnover by continuous, rather than transient, inhibition of bone resorption *in vivo* [59,60]. Recent studies showed that the bisphosphonate alendronate impaired the anabolic effects of PTH to increase bone mineral density in women and men with osteoporosis [61,62]. Thus, specific anti-resorptive drugs should transiently inhibit bone resorption. Alisol-B does not accumulate in bone minerals. Therefore, alisol-B might prevent osteoporosis when administered intermittently with the anabolic drug PTH.

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