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Biselyngbyaside, Isolated From Marine Cyanobacteria, Inhibits Osteoclastogenesis and Induces Apoptosis in Mature Osteoclasts

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ABSTRACT

The mass and function of bones depend on the maintenance of a complicated balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. An inhibitor of osteoclast differentiation and/or function is expected to be useful for treatment of bone lytic diseases such as osteoporosis, rheumatoid arthritis, and tumor metastasis into bone. Biselyngbyaside is a recently isolated macrolide compound from marine cyanobacteria *Lyngbya* sp. that shows wide-spectrum cytotoxicity toward human tumor cell lines. In this study, we investigated the effects of biselyngbyaside on osteoclast differentiation and function. Biselyngbyaside inhibited receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis in mouse monocytic RAW264 cells and primary bone marrow-derived macrophages at a low concentration. Similarly, biselyngbyaside suppressed osteoblastic cell-mediated osteoclast differentiation in cocultures. In the RANKL-induced signaling pathway, biselyngbyaside inhibited the expression of c-Fos and NFATc1, which are important transcription factors in osteoclast differentiation. In mature osteoclasts, biselyngbyaside decreased resorption-pit formation. Biselyngbyaside also induced apoptosis accompanied by the induction of caspase-3 activation and nuclear condensation, and these effects were negated by the pancaspase inhibitor z-VAD-FMK. Taken together, the present findings indicate that biselyngbyaside suppresses bone resorption via inhibition of osteoclastogenesis and induction of apoptosis. Thus, biselyngbyaside may be useful for the prevention of bone lytic diseases. J. Cell. Biochem. 113: 440–448, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: BISELYNGBYASIDE; OSTEOCLAST; DIFFERENTIATION; BONE RESORPTION; APOPTOSIS; MACROLIDE

Bone remodeling occurs continuously to maintain the bone mass during life, and is comprised of opposing processes involving bone resorption by osteoclasts and bone formation by osteoblasts. Osteoclasts are differentiated from hematopoietic cells of the monocyte/macrophage lineage, and the bone lytic function of osteoclasts is involved in many bone-destructive diseases, such as osteoporosis, hypercalcemia, rheumatoid arthritis, tumor metastasis into bone, periodontitis and Paget's disease [Rodan and Martin,

T. Yonezawa and N. Mase contributed equally to this work.

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2000]. Therefore, osteoclastogenesis is a major therapeutic target for these diseases. Receptor activator of nuclear factor-κB (RANK) ligand (RANKL), a member of the tumor necrosis factor family, is expressed on osteoblasts and plays crucial roles in osteoclast differentiation and activation. Binding of RANKL to its receptor RANK on osteoclast precursors causes activation of many signaling pathways, including those involving mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK), and transcription factor nuclear factor-κB (NF-κB) via tumor necrosis factor receptor-associated factor 6 (TRAF6) [Boyle et al., 2003; Feng, 2005]. Activation of these RANKL-induced signaling pathways induces the expression and activation of c-Fos and nuclear factor of activated T cells (NFAT) c1, which are known to be critical regulators of osteoclast differentiation [Boyle et al., 2003; Feng, 2005].

Mature osteoclasts are large multinucleated cells formed through the cell fusion of immature mononuclear osteoclasts and show characteristic ring-like structures, which have an actin-rich constitution that is important for bone resorption accompanied by attachment to bone, formation of a sealing zone and secretion of protons and proteases to resorb bone. Abrogation of the survival or function of mature osteoclasts is assumed to suppress bone resorption.

Recently, we identified a novel 18-membered macrolide glycoside, biselyngbyaside, in marine cyanobacteria *Lyngbya* sp. and found that it caused growth inhibition in a variety of human tumor cell lines [Teruya et al., 2009]. In the present study, we examined the effects of biselyngbyaside on osteoclast differentiation and function.

MATERIALS AND METHODS

REAGENTS

Biselyngbyaside was isolated from marine cyanobacteria *Lyngbya* sp. [Teruya et al., 2009]. The chemical structure of biselyngbyaside is shown in Figure 1A. Fast red-violet LB salt and naphthol AS-MX phosphate were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Recombinant murine macrophage colony-stimulating factor (M-CSF) and recombinant human RANKL were obtained from PeproTech EC Ltd (London, England). PD98059 was obtained from Calbiochem (La Jolla, CA). α-Minimum essential medium (α-MEM) was purchased from Gibco (Grand Island, NY). Anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-ERK, anti-phospho-JNK (Thr183/Tyr185), anti-phospho-inhibitor of NF-κB (IκB), anti-p38 MAPK, anti-ERK, anti-JNK and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Cell Signaling Technology (Danvers, MA). An anti-IκB antibody was purchased from Calbiochem. An anti-c-Fos-antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-NFATc1 antibody was purchased from BD Biosciences (San Jose, CA). 4-6-Diamidino-2-phenylindole (DAPI) was purchased from Dojindo (Kumamoto, Japan). Horseradish peroxidase-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG were obtained from GE Healthcare (Piscataway, NJ). All other chemical reagents were purchased from Wako Pure Chemical Industries Ltd (Tokyo, Japan) or Sigma-Aldrich Fine Chemicals unless otherwise stated.

ANIMALS

This experimental animal study was approved by the Animal Experiment Committee of Chubu University. The mice were

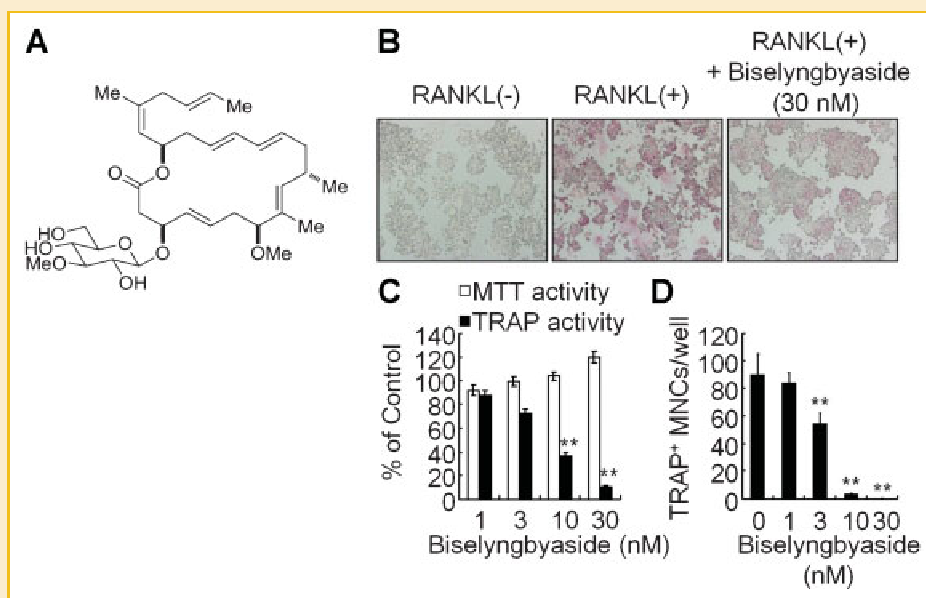

Fig. 1. Effects of biselyngbyaside on osteoclast differentiation of RAW264 cells. A: Structure of biselyngbyaside. B–D: RAW264 cells were cultured in the presence of RANKL (50 ng/ml) with or without biselyngbyaside for 3 days. B: The cells were fixed and stained for TRAP. C: The cell viability was determined by the MTT assay and the TRAP activity was measured. D: $TRAP^+$ MNCs containing more than three nuclei were counted. Data are expressed as the mean ± SD of at least three cultures. $^{**}P < 0.005$ versus control cells.

maintained in accordance with the guidelines of the Animal Experiment Committee of Chubu University.

OSTEOCLAST DIFFERENTIATION

RAW264 cells, a murine macrophage cell line supplied by the RIKEN Cell Bank (Tsukuba, Japan), were grown in α-MEM containing 10% fetal bovine serum (FBS) and passaged every 3 days. The cells were seeded at 4×10^3 cells/well in 96-well plates. After 24 h, RANKL (50 ng/ml) was added and cultured for various times. For differentiation into mature osteoclasts possessing osteoclast specific ring-like cytoskeletal structures, RAW264 cells (6.5×10^3 cells/well in 96-well plates) were cultured for 4 days in the presence of RANKL (50 ng/ml) and PD98059 (20 μM), a MAPK inhibitor that accelerates osteoclastogenesis [Hotokezaka et al., 2002].

Bone marrow cells (BMCs) were isolated from the tibiae and femora of 4- to 6-week-old ddY mice (SLC, Inc., Shizuoka, Japan) and incubated for 3 days in α-MEM containing 10% inactivated FBS with M-CSF (30 ng/ml) at a density of 2×10^7 cells/100-mm dish. After the incubation, the adherent cells were collected as bone marrow-derived macrophages (BMMs). The BMMs were seeded at 2×10^4 cells/well in 96-well plates and cultured in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 days.

To determine osteoblast-mediated osteoclast differentiation, BMCs (2×10^5 cells/well) were cocultured with stromal/osteoblastic cell line UAMS-32 cells [O'Brien et al., 1999] (1×10^4 cells/well) in 96-well plates in the presence of 1α,25-dihydroxyvitamin D_3 (10 nM) and prostaglandin E_2 (1 μM) for 5 days.

Osteoclast formation was evaluated by measuring the tartrate-resistant acid phosphatase (TRAP) activity as an early differentiation marker and counting the number of TRAP-positive multinucleated cells with more than three nuclei after TRAP staining as described previously [Woo et al., 2004; Yonezawa et al., 2011].

VIABILITY ASSAY

Cell viability was evaluated using an MTT or XTT assay kit (Roche Diagnostics, Mannheim, Germany). The absorbances of MTT and XTT were measured at 570 and 450 nm, respectively, using a microplate reader.

WESTERN BLOTTING

RAW264 cells (1×10^6 cells/well in 6-well plates) were pretreated with biselyngbyaside and then stimulated with RANKL for various times. The cells were washed with phosphate-buffered saline (PBS) and lysed with ice-cold lysis buffer comprising 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 50 mM NaF, 0.27 M sucrose, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mg/ml Pefabloc SC (Roche Diagnostics). The lysates were centrifuged at 12,000 rpm for 10 min at 4°C and the supernatants were collected as protein samples. The concentrations of the protein samples were determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were added to 5× Laemmli sample loading buffer and boiled for 5 min. Next, the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare) by electroblotting. The membranes were blocked with 3% skim milk in 50 mM Tris–HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20 for 30 min at room temperature, and then incubated with a primary antibody overnight at 4°C. After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Antigen–antibody complexes on the membranes were visualized using an ECL Western Blot Detection Kit (GE Healthcare) according to the manufacturer's instructions. The membranes were then stripped with a stripping buffer (Restore™ Western Blot Stripping Buffer; Pierce, Rockford, IL) and reprobed with another primary antibody. Quantitative measurements were performed using Image J software (National Institutes of Health freeware).

PIT-FORMATION ASSAY

BMCs (2×10^7 cells) and UAMS32 cells (1×10^6 cells) were cocultured on collagen-coated 100-mm diameter culture dishes in the presence of 1α,25-dihydroxyvitamin D_3 (10 nM) and prostaglandin E_2 (1 μM) for 6 days. To obtain bone-resorbing mature osteoclasts, the cocultured cells were detached by treatment with 0.2% collagenase at 37°C for 10 min, and then cultured on dentin slices in 96-well plates with or without biselyngbyaside for 24 h. After the incubation, the cells were removed and the resorption pits were stained with 1% toluidine blue solution. Images of the resorbed pits were obtained under a light microscope (Nikon, Tokyo, Japan).

CASPASE-3 ACTIVITY

RAW264 cells or mature osteoclasts derived from RAW264 cells were cultured with or without biselyngbyaside or cycloheximide for 6 h. After the culture, the cells were washed with PBS(−) twice and lysed with ice-cold lysis buffer comprising 100 mM Tris–HCl pH 7.5, 1 mM DTT, 1% Triton X-100, and 1 mM PMSF. The lysates were centrifuged at 12,000 rpm for 10 min at 4°C and the supernatants were collected as protein samples. Supernatants (30 μg protein) were incubated in lysis buffer containing 10 μM Ac-DEVD-AMC (Peptide Institute, Osaka, Japan), a fluorogenic caspase-3 substrate, for 1 h at 37°C. The fluorescence (excitation at 355 nm; emission at 460 nm) was measured with a fluorometer (PerkinElmer, Foster City, CA).

DAPI STAINING

Mature osteoclasts derived from RAW264 cells were cultured with or without biselyngbyaside or cycloheximide for 6 h. After the culture, the cells were fixed with 10% formalin in PBS for 15 min at room temperature, and then incubated with 1 μg/ml DAPI in PBS for 30 min for nuclear staining. The distribution of DAPI was visualized under a fluorescence microscope (Nikon).

ACRIDINE ORANGE STAINING

While acridine orange is known to bind to DNA and exhibit green fluorescence, it also accumulates into cell organelles and shows red or orange fluorescence in acidic organelles. Mature osteoclasts derived from RAW264 cells were cultured with or without biselyngbyaside or concanamycin A for 6 h and then further cultured with acridine orange (10 μg/ml) for 30 min to determine the acidification of vacuolar organelles. Fluorescence images were

taken under a fluorescence microscope (Olympus, Tokyo, Japan) with excitation of 490 nm and emission at 530–640 nm.

STATISTICAL ANALYSIS

For each series of experiments, the data were expressed as means ± SD. The data were analyzed by Student's *t*-test or ANOVA followed by a post hoc Dunnett's test. All statistical analyses were performed using SPSS software ver. 17.0 (SPSS, Inc., Chicago, IL).

RESULTS

BISELYNGBYASIDE INHIBITS OSTEOCLAST DIFFERENTIATION IN RAW264 CELLS, BMMS, AND COCULTURES

Mouse RAW264 macrophage cells retain the capacity to differentiate into osteoclast-like cells during culture in the presence of RANKL. Accordingly, treatment of the cells with RANKL increased the activity of TRAP, an osteoclast-specific enzyme, and induced TRAP-stained osteoclasts (Fig. 1B). We then examined the effects of biselyngbyaside on osteoclastogenesis in RAW264 cells, and found that biselyngbyaside inhibited RANKL-induced osteoclast formation at 30 nM (Fig. 1B). Biselyngbyaside dose-dependently inhibited RANKL-induced TRAP activity without affecting the cell viability (Fig. 1C). The IC_{50} value for the inhibitory effect of biselyngbyaside on the increase in TRAP activity was approximately 6 nM. Biselyngbyaside also reduced the number of TRAP-positive multinuclear osteoclasts ($TRAP^+$ MNCs) (Fig. 1D).

To confirm the effects of biselyngbyaside, we examined primary cultures of BMMs. Biselyngbyaside inhibited M-CSF and RANKL-induced TRAP activity in BMMs but had no effect on the cell viability (Fig. 2A,B), similar to the findings in RAW264 cells. The M-CSF and RANKL-induced formation of $TRAP^+$ MNCs was also attenuated by biselyngbyaside (Fig. 2A,C). Under physiological conditions, osteoclastogenesis is evoked by the interaction of osteoclast precursors with RANKL-expressing osteoblasts. Similar inhibitory effects of biselyngbyaside on osteoblast-mediated osteoclast formation were observed in cocultures of BMCs and osteoblastic UAMS-32 cells (Fig. 2D–F). To reveal whether this suppression of osteoclastogenesis was related to the effect on osteoblasts, the mRNA expressions of RANKL and its decoy receptor osteoprotegerin (OPG) were determined by real-time PCR. However, biselyngbyaside did not influence the mRNA expressions of RANKL and OPG (data not shown). These findings indicate that biselyngbyaside has the ability to inhibit osteoclastogenesis through direct actions on osteoclast progenitors, but not on osteoblasts.

BISELYNGBYASIDE INHIBITS BOTH EARLY-STAGE AND LATE-STAGE OSTEOCLAST DIFFERENTIATION IN RAW264 CELLS

Osteoclastogenesis proceeds through multiple differentiation steps including formation of mononuclear osteoclasts, formation of multinucleated osteoclasts by cell fusion and osteoclast polarization with actin rearrangement. To examine which stages of osteoclast differentiation are affected by biselyngbyaside, RAW264 cells were cultured with RANKL and biselyngbyaside

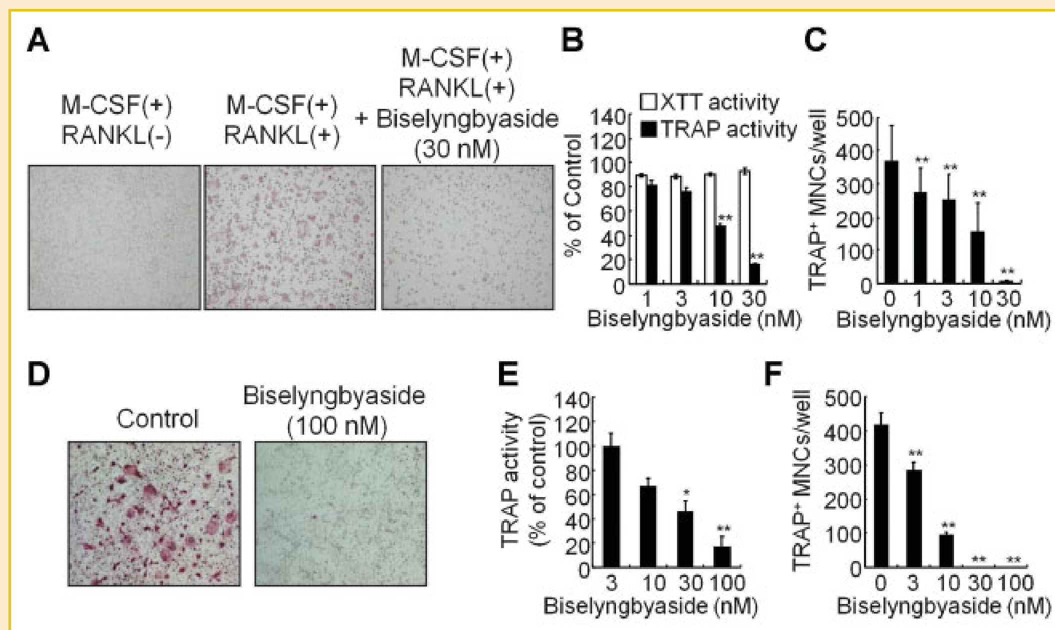

Fig. 2. Effects of biselyngbyaside on osteoclast differentiation in BMMs and cocultures of BMCs and UAMS-32 cells. A–C: BMCs were cultured with M-CSF (30 ng/ml) for 3 days to induce BMMs, and the BMMs were further cultured in the presence of RANKL (100 ng/ml) and M-CSF (30 ng/ml) with or without biselyngbyaside for 3 days. A: The cells were fixed and stained for TRAP. B: The cell viability was determined by the XTT assay and the TRAP activity was measured. C: $TRAP^+$ MNCs containing more than three nuclei were counted. D–F: BMCs and osteoblastic UAMS-32 cells were cultured in the presence of 1α,25 dihydroxyvitamin D_3 (10 nM) and prostaglandin E_2 (1 μM) with or without biselyngbyaside for 5 days. D: The cells were fixed and stained for TRAP. E: The TRAP activity was measured. F: $TRAP^+$ MNCs containing more than three nuclei were counted. Data are expressed as the mean ± SD of at least three cultures. $^*P < 0.05$, $^{**}P < 0.005$, versus control cells.

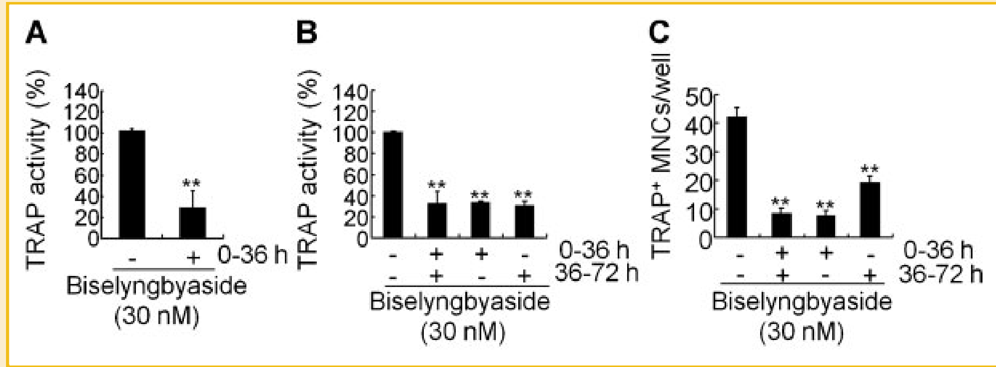

Fig. 3. Effects of biselyngbyaside on early-stage and late-stage osteoclast differentiation in RAW264 cells. A–C: RAW264 cells were cultured in the presence of RANKL (50 ng/ml) for 36 h (A) or 72 h (B,C). The periods of biselyngbyaside treatment are indicated. A,B: The TRAP activity was measured. C: $TRAP^+$ MNCs containing more than three nuclei were counted. Data are expressed as the mean ± SD of at least three cultures. $^*P < 0.05$, $^{**}P < 0.005$, versus control cells.

for 36 h, which is sufficient time for the differentiation of precursor cells into mononuclear osteoclasts, but not for the fusion of mononuclear osteoclasts into multinucleated osteoclasts. Biselyngbyaside suppressed the RANKL-induced TRAP activity (Fig. 3A), indicating that biselyngbyaside inhibits mononuclear osteoclast formation in the early stage of osteoclastogenesis. Next, the treatment times with biselyngbyaside were divided into three periods, 0–36, 36–72, and 0–72 h. The TRAP activity was reduced by biselyngbyaside at 0–36 and 36–72 h, as well as at 0–72 h (Fig. 3B). Furthermore, the formation of $TRAP^+$ MNCs was decreased in the presence of biselyngbyaside at all three periods, although the effect of the treatment at 36–72 h was slightly weaker than those at 0–36 and 0–72 h (Fig. 3C). These findings suggest that biselyngbyaside affects not only early mononuclear osteoclast formation, but also late multinuclear osteoclast formation by cell fusion.

BISELYNGBYASIDE SUPPRESSES RANKL-INDUCED EXPRESSION OF C-FOS AND NFATC1

To investigate the mode of action of biselyngbyaside on osteoclastogenesis, we investigated the effects of biselyngbyaside on the activation of key signaling molecules activated by RANKL/RANK signaling. Biselyngbyaside did not affect the phosphorylation of MAPKs (JNK, p38 MAPK, and ERK) and IκB, which were induced at the early stage of RANKL exposure (Fig. 4A). However, biselyngbyaside did inhibit the RANKL-induced expression of c-Fos and NFATc1, which are important transcription factors for osteoclast differentiation (Fig. 4B). These findings indicate that the suppression of osteoclastogenesis by biselyngbyaside is mediated by attenuated expression of c-Fos and NFATc1.

BISELYNGBYASIDE INHIBITS RESORPTION-PIT FORMATION BY MATURE OSTEOCLASTS

To evaluate the effects of biselyngbyaside on the bone-resorbing activity of differentiated mature osteoclasts, mature osteoclasts induced by coculture were cultured on dentin slices in the presence of biselyngbyaside, and the resorption-pit formation was assessed. Biselyngbyaside markedly suppressed the resorption-pit formation and the number of resorption pits was dramatically decreased (Fig. 5A,B). The attachment onto bone slices was not affected by the presence of biselyngbyaside (data not shown). These findings indicate that biselyngbyaside inhibits the bone-resorbing activity of mature osteoclasts.

BISELYNGBYASIDE DECREASES CELL SURVIVAL SPECIFICALLY IN MATURE OSTEOCLASTS

Bone-resorbing osteoclasts have ring-like cytoskeletal structures that are crucial for their bone-lytic activity and are considered to be a marker for mature osteoclasts. Mature osteoclasts with ring-like cytoskeletal structures were derived from RAW264 cells by culture with RANKL and PD98059 [Yonezawa et al., 2007] and treated with biselyngbyaside for 24 h. Biselyngbyaside decreased the number of mature osteoclasts with ring-like structures (Fig. 6A,B). MTT assays showed that biselyngbyaside decreased the cell viability of mature osteoclasts, but had no effect on the viability of undifferentiated RAW264 cells (Fig. 6C). These findings indicate that biselyngbyaside specifically suppresses cell survival in mature osteoclasts, but not in osteoclast precursor cells.

BISELYNGBYASIDE INDUCES APOPTOSIS SPECIFICALLY IN MATURE OSTEOCLASTS

Next, we measured the caspase-3 activity to determine whether the destruction of the ring-like structures and the decrease in cell viability of mature osteoclasts were accompanied by apoptosis. The results revealed that biselyngbyaside dose-dependently increased the caspase-3 activity in a similar manner to the apoptosis inducer cycloheximide (Fig. 7A). The biselyngbyaside-induced activation of caspase-3 was inhibited by the pancaspase inhibitor z-VAD-FMK (Fig. 7A). Although cycloheximide also increased the caspase-3 activity in preosteoclastic RAW264 cells, biselyngbyaside had no effect on the caspase-3 activity in RAW264 cells (data not shown). Furthermore, nuclear shrinkage and fragmentation were observed in biselyngbyaside-treated mature osteoclasts (Fig. 7B). These findings suggest that biselyngbyaside induces apoptosis specifically in mature osteoclasts.

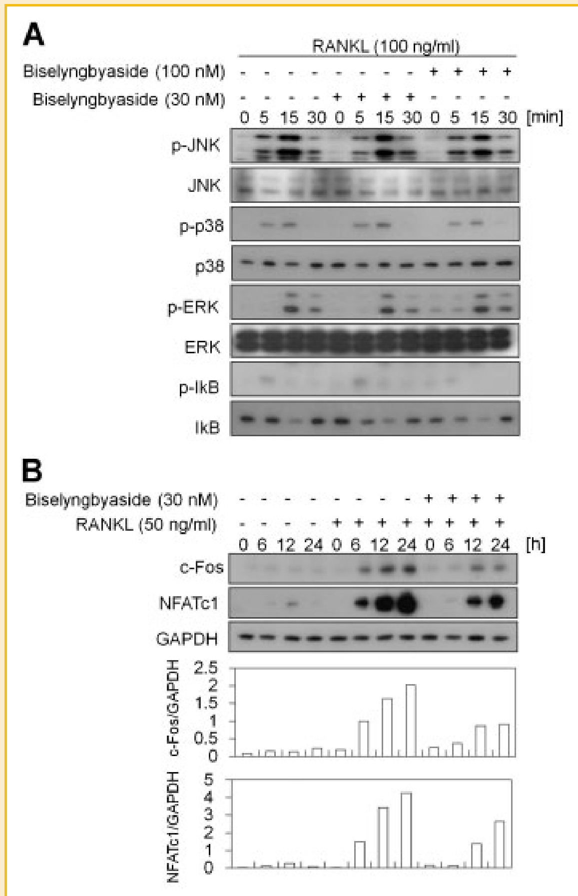

Fig. 4. Effects of biselyngbyaside on RANKL-induced phosphorylation of MAPKs (p38 MAPK, ERK, and JNK) and IκB and expression of c-Fos and NFATc1. A: RAW264 cells were preincubated in the presence of biselyngbyaside for 1 h and then treated with RANKL (100 ng/ml) for the indicated times. Cell lysates were collected and separated by 10% SDS-PAGE. The levels of phosphorylated (p) and non-phosphorylated p38 MAPK, ERK, JNK, and IκB were determined by Western blotting. B: RAW264 cells were incubated in the presence of biselyngbyaside and RANKL (50 ng/ml) for the indicated times. The expression levels of c-Fos, NFATc1, and GAPDH were determined by Western blotting. The relative expression levels of c-Fos and NFATc1 normalized by the GAPDH expression levels were determined using Image J software. The results shown are representative of more than three independent experiments.

BISELYNGBYASIDE DOES NOT AFFECT THE V-ATPASE ACTIVITY IN MATURE OSTEOCLASTS

Vacuolar type H^+-ATPase (V-ATPase) is very important molecule in proton secretion for the degradation of bone matrix. Concanamycin, an 18-membered macrolide like biselyngbyaside, is a specific inhibitor of V-ATPase, and was reported to induce apoptosis in osteoclasts and inhibit osteoclastic bone resorption [Nishihara et al., 1995; Woo et al., 1996; Okahashi et al., 1997]. Therefore, the effects of biselyngbyaside on the acidification of vacuolar organelles were assessed using acridine orange, which shows a fluorescence shift from green to orange in acidic organelles. Osteoclasts without any treatment showed orange fluorescence, indicating vacuole acidification (Fig. 8). The orange fluorescence was quenched by treatment with concanamycin A (Fig. 8). Although biselyngbyaside caused

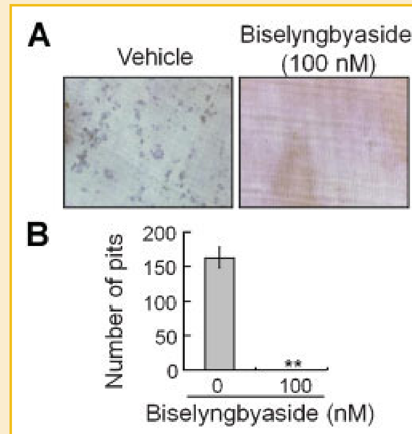

Fig. 5. Effects of biselyngbyaside on osteoclastic resorption-pit formation. A,B: Mature osteoclasts from cocultures of BMCs and UAMS-32 cells were cultured on dentin slices with or without 100 nM biselyngbyaside for 24 h. A: The cells were removed and the resorption pits were stained with 1% toluidine blue solution. B: The number of resorption pits was counted. The data are representative of more three independent experiments, and are expressed as the mean ± SD of at least three cultures. $^{*}P < 0.05$, $^{**}P < 0.005$, versus control cells.

morphological changes, orange fluorescence was still observed in the presence of biselyngbyaside (Fig. 8). These findings suggest that biselyngbyaside does not possess inhibitory activity toward V-ATPase and that V-ATPase is not involved in the biselyngbyaside-induced apoptosis in osteoclasts.

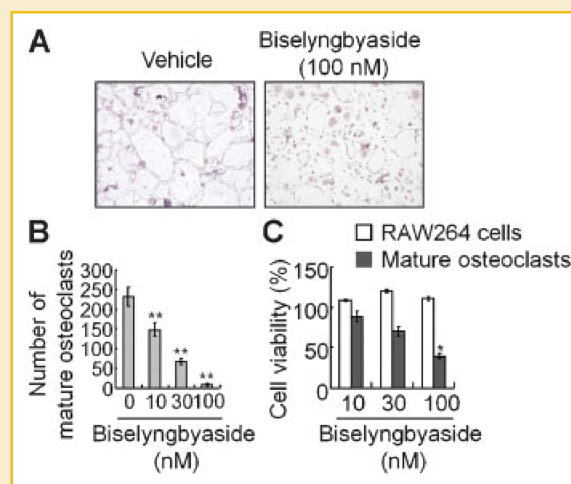

Fig. 6. Effects of biselyngbyaside on the survival of mature osteoclasts and preosteoclastic RAW264 cells. A–C: Mature osteoclasts derived from RAW264 cells were cultured with or without biselyngbyaside for 24 h. A: The cells were stained for TRAP and photographed. B: The TRAP-positive multinuclear cells with ring-like structures were counted as mature osteoclasts. C: The cell viability of RAW264 cells and mature osteoclasts was determined by the MTT assay. The data are expressed as the mean ± SD of at least three cultures. $^{*}P < 0.05$, $^{**}P < 0.005$, versus control cells.

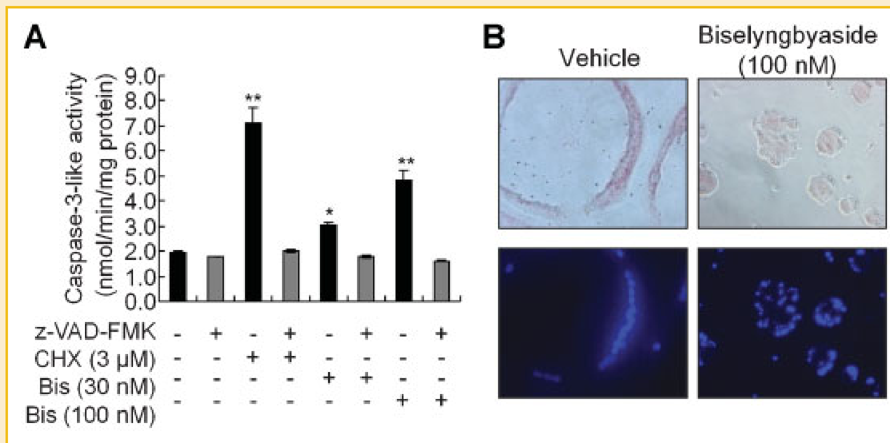

Fig. 7. Effects of biselyngbyaside on apoptosis of mature osteoclasts. A: Mature osteoclasts derived from RAW264 cells were cultured in the presence of biselyngbyaside (Bis) or cycloheximide (CHX) with or without zVAD-FMK (20 μM) for 6 h. Cell lysates were collected and the caspase-3-like activity was measured using Ac-DVED-AMC as a substrate. The data are expressed as the mean ± SD of at least three cultures. $^{*}P < 0.05$, $^{**}P < 0.005$, versus control cells. B: After a similar culture, the cells were fixed and stained for TRAP. The nuclei were stained with DAPI to determine the morphological changes associated with apoptosis. The data are representative of more three independent experiments.

DISCUSSION

Macrolides are characterized by a large macrocyclic lactone ring structure and widely used clinically as antibiotics [Driggers et al., 2008]. Many macrolides have been identified from various bacteria, particularly some strains of actinomycetes. Oceans are a good source of new active compounds. Many marine natural products have been reported and some of these compounds are now being developed as clinical drugs [Molinski et al., 2009; Blunt et al., 2010]. We recently reported a new 18-membered macrolide glycoside, biselyngbyaside, derived from marine cyanobacteria *Lyngbya* sp. as a wide-spectrum growth inhibitor of tumor cells [Teruya et al., 2009]. In the present study, we found that biselyngbyaside inhibits the RANKL-induced increase in TRAP activity and formation of multinucleated osteoclasts in mouse monocytic cell line RAW264 cells. We further confirmed that biselyngbyaside attenuates osteoclastogenesis in primary BMMs and cocultures of primary BMCs and osteoblastic UAMS32 cells. These inhibitory effects of biselyngbyaside were highly specific to the differentiation of osteoclasts and more effective than the growth inhibition of cancer cell lines, since IC_{50} values for osteoclastogenesis in RAW264 cells, BMMs, and cocultures were 3.3, 6.4, and 4.6 nM, respectively, whereas the

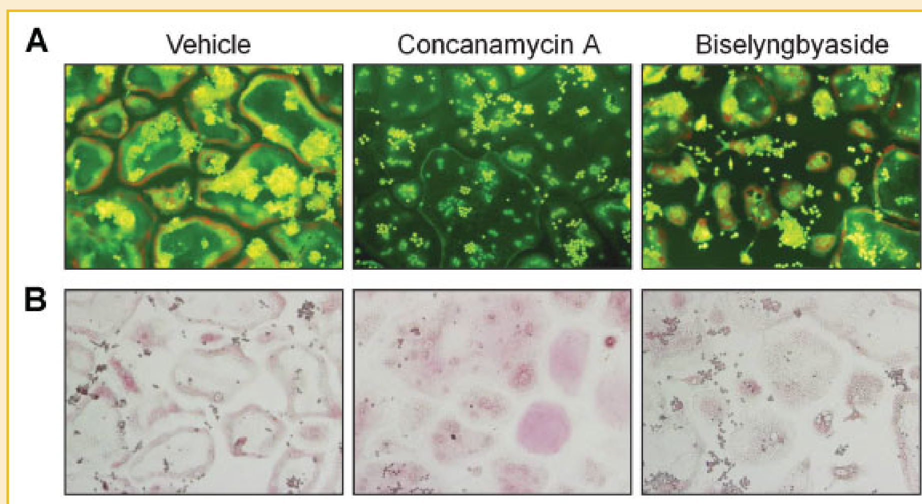

Fig. 8. Effects of biselyngbyaside on the V-ATPase activity in mature osteoclasts. A,B: Mature osteoclasts derived from RAW264 cells were cultured with or without biselyngbyaside (30 nM) or concanamycin A (30 nM) for 6 h. The cells were then stained with acridine orange (A) and stained for TRAP (B). Similar results were obtained in two other independent experiments.

average IC_{50} values for growth inhibition against 39 human cancer cell lines was 0.60 μM [Teruya et al., 2009].

RANKL–RANK binding rapidly leads to the activation of many signaling molecules, including MAPKs (ERK, JNK, and p38 MAPK) and NF-κB, through the adaptor protein TRAF6 and subsequently induces the expression of the transcription factors c-Fos and NFATc1, which play key roles in osteoclastogenesis [Boyle et al., 2003; Feng, 2005]. Our mechanistic experiments revealed that biselyngbyaside inhibits the RANKL-induced expression of the master transcription factors c-Fos and NFATc1, without affecting early signaling events such as the phosphorylation of MAPKs and IκB. Similar to RANKL-induced signaling, lipopolysaccharide (LPS) activates MAPKs and NF-κB signaling via TRAF6 and act as an inducer of bone resorption. Biselyngbyaside did not inhibit LPS-induced nitric oxide production (unpublished data), which is involved in MAPK and NF-κB signaling [Xie et al., 1994; Ajizian et al., 1999; Feng et al., 1999]. This observation is consistent with the findings that biselyngbyaside had no effect on RANKL-induced activation of early signaling molecules such as MAPKs and NF-κB. Thus, biselyngbyaside may modulate RANKL-specific signaling pathways that involve c-Fos and NFATc1 and differ from those activated by LPS. Tacrolimus (FK506), a 23-membered macrolide, is an inhibitor of NFAT signaling and was reported to inhibit late-stage osteoclastogenesis with less marked effects on early-stage osteoclastogenesis [Hirotani et al., 2004; Miyazaki et al., 2007]. However, biselyngbyaside suppressed both early-stage and late-stage osteoclast differentiation, indicating that it affects osteoclastogenesis through other pathways that are distinct from those of tacrolimus. Recently, the participation of costimulatory signals for RANK in osteoclastogenesis has been reported [Asagiri and Takayanagi, 2007]. An immunoreceptor tyrosine-based activation motif (ITAM) is critical for the activation of calcium signaling in immune cells. Fc receptor common γ subunit (FcRγ) and DNAX-activating protein 12 (DAP12), which are ITAM-harboring adaptor proteins, were reported to be involved in the formation of osteoclasts through activation of the Ca^{2+}-NFAT pathway, even though it is not fully understood how RANKL can specifically activate ITAM signaling in osteoclastogenesis [Asagiri and Takayanagi, 2007]. Further studies on the effects of biselyngbyaside on this pathway as well as other pathways may reveal the details of its mechanism of action on osteoclast differentiation.

In the present study, we also found that biselyngbyaside specifically decreases cell survival in differentiated osteoclasts, accompanied by caspase-3 activation and nuclear condensation, and attenuates osteoclastic resorption-pit formation. These findings suggest that biselyngbyaside induces osteoclast apoptosis and inhibits bone resorption. The formation of an acidic extracellular microenvironment is essential for osteoclastic bone resorption and V-ATPase plays a crucial role in this process. Some macrolide compounds including concanamycin A, which is an 18-membered macrolide glycoside like biselyngbyaside, have been reported to induce osteoclast cell death and suppress bone resorption through the inhibition of V-ATPase activity [Nishihara et al., 1995; Woo et al., 1996; Okahashi et al., 1997]. However, biselyngbyaside did not attenuate the acidification of vacuolar organelles, suggesting that biselyngbyaside induces apoptosis in osteoclasts through a novel pathway that differs from those of the other macrolides.

In conclusion, we found that biselyngbyaside inhibits osteoclastogenesis by suppressing c-Fos and NFATc1 expression and induces apoptosis of osteoclasts via distinct pathways from other macrolides that suppress osteoclastogenesis or induce osteoclast apoptosis. Thus, biselyngbyaside may become a new class of agents for bone lytic disorders mediated by osteoclastic bone resorption, including osteoporosis, periodontitis, and tumor metastasis into bone.

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