Halenaquinone inhibits RANKL-induced osteoclastogenesis

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ABSTRACT

Halenaquinone was isolated from the marine sponge *Petrosia alfiani* as an inhibitor of osteoclastogenic differentiation of murine RAW264 cells. It inhibited the RANKL (receptor activator of nuclear factor-κB ligand)-induced upregulation of TRAP (tartrate-resistant acid phosphatase) activity as well as the formation of multinuclear osteoclasts. In addition, halenaquinone substantially suppressed RANKL-induced IκB degradation and Akt phosphorylation. Thus, these results suggest that halenaquinone inhibits RANKL-induced osteoclastogenesis at least by suppressing the NF-κB and Akt signaling pathways.

Bone homeostasis is regulated by the balance between bone formation by osteoblasts and bone resorption by osteoclasts.[1] Osteoclasts were previously shown to differentiate from a monocyte/macrophage lineage when stimulated with receptor activator of nuclear factor-κB ligand (RANKL).[1-3] RANKL stimuli are known to activate several downstream signaling pathways such as the NF-κB and MAPK signaling pathways, which upregulates the expression of osteoclast-specific genes including those encoding tartrate-resistant acid phosphatase (TRAP) and enzymes involved in cell fusion. Since the deregulation of osteoclast functions has been associated with several diseases including osteoporosis and bone-metastasis, considerable and widespread attention has been focused on compounds that affect osteoclastogenesis and the functions of osteoclasts for the treatment of osteoclast-related diseases.[4,5] In the present study, we isolated halenaquinone[6] (**1**) (Fig. 1) from the marine sponge *Petrosia alfiani* as an inhibitor of osteoclastogenic differentiation of murine RAW264 cells.

Screening was performed by measuring the RANKL-induced upregulation of TRAP activity in RAW264 cells.[7] EtOAc- and water-soluble fractions derived from the EtOH extracts of 250 marine sponges and marine-derived fungi were subjected to screening. One of the hits was obtained from the EtOAc-soluble fraction prepared from the EtOH

extract of the marine sponge *P. alfiani*,[8] which was collected in Indonesia in December 2006. This sponge (400 g, wet weight) was soaked in EtOH immediately after being collected. The extract was concentrated and the residual aqueous layer was extracted with EtOAc and then *n*-BuOH. Purification of an inhibitor of osteoclastogenesis from the EtOAc- and *n*-BuOH-soluble fractions by SiO_2 column chromatography afforded **1** (35.6 mg).[8]

Halenaquinone (**1**) completely inhibited the RANKL-induced upregulation of TRAP activity in RAW264 cells at a concentration of 20 μM (Fig. 2A).[7] The IC_{50} value of **1** was 2 μM. We next determined whether **1** suppressed the formation of multinuclear osteoclasts.[9] The RANKL stimulation induced the differentiation of RAW264 cells and formation of TRAP-positive multinuclear osteoclasts (Fig. 2B (upper panel) and C), whereas the presence of **1** at a concentration of 20 μM clearly decreased the formation of these cells (Fig. 2B (lower panel) and C). Based on these results, we concluded that **1** inhibited RANKL-induced osteoclastogenesis.

Since its discovery,[6] **1** has been reported to exhibit inhibitory activities against several enzymes, such as a virus protein tyrosine kinase (v-Src),[10] phosphoinositide 3-kinase (PI3K),[11] Cdc25B phosphatase,[12] RAD51 (homologous-paring activity),[13] phospholipase A_2,[14] and farnesyltransferase,[14] and is also cytotoxic.[15] We have been

searching for proteasome inhibitors from marine sources[16] and demonstrated that **1** inhibited the chymotrypsin-like activity of the proteasome[17] with an IC_{50} value of 0.6 μM.[18]

Since recent studies reported that several proteasome inhibitors inhibited osteoclastogenesis,[19-22] we next determined whether **1** could inhibit the RANKL-induced degradation of IκB in the NF-κB signaling pathway, which induces osteoclastogenesis. IκB has been shown to sequestrate NF-κB in the cytoplasm and, upon the activation of upstream signaling regulators, IκB is degraded by the proteasome, leading to the nuclear entrance of NF-κB and its involvement in the activation of transcription.[23] When RAW264 cells were pretreated with 20 μM **1** for 2 h followed by the stimulation with RANKL, the degradation of IκB[24] 15 min after the RANKL treatment was suppressed by **1** (Fig. 3A). At a higher concentration, **1** also slightly inhibited the RANKL-induced degradation of IκB (Fig. 3B). On the other hand, the finding that PI3K and its downstream Akt kinase are involved in osteoclastogenesis,[25] together with the above study on the inhibition of PI3K by **1**,[11] led us to assume that **1** may affect osteoclastogenesis by inhibiting the PI3K-Akt signaling pathway. Akt phosphorylation[24] was also shown to be suppressed to some extent by **1** (Fig. 3A). Taken together, these findings indicated that **1** may have an inhibitory effect on

RANKL-induced osteoclastogenesis at least via its suppression of the NF-κB and Akt signaling pathways. Since the inhibitory activities of **1** against the degradation of IκB and phosphorylation of Akt appear to be weak, it can be inferred that **1** inhibits other proteins as targets. Therefore, further studies are needed to identify these proteins. In conclusion, this is the first study to describe the inhibitory effects of **1** on osteoclastogenesis.

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References and notes

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7. The murine RAW264 cell line was obtained from the RIKEN Cell Bank (Tsukuba) and maintained in MEMα medium containing 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) and 1 x penicillin/streptomycin (Wako) under a humidified atmosphere containing 5% CO_2 at 37°C. In the TRAP assay, RAW264 cells were seeded on a 96-well plate (1,000 cells/well), cultured for 1 d, and were then treated with sRANKL (a soluble form of RANKL; Oriental Yeast; 50 ng/mL) and samples (10 μg/mL; DMSO, control) for 4 d. The cells were then washed with phosphate-buffered saline (PBS) and lysed with TRAP buffer (50 mM sodium tartrate, 50 mM sodium acetate, 150 mM KCl, 0.1% TritonX-100, 1 mM sodium ascorbate, and 0.1 mM $FeCl_3$, pH 5.7; 100 μL/well) on ice for 10 min. The resulting cell extract (20 μL) was added to 100 μL of TRAP buffer containing 2.5 mM *p*-nitrophenyl phosphate (Thermo Fisher Scientific) and incubated at 37°C for 4 h. To stop the reaction, 50 μL of 0.9 M NaOH was added to the reaction mixture and the reaction product (*p*-nitrophenolate) was measured as the absorbance at 405 nm.

8. A voucher specimen (RMNH POR 8525) of *Petrosia alfiani* has been deposited in the Naturalis Biodiversity Center, the Netherlands. The EtOAc-soluble (3.9 g) and *n*-BuOH-soluble (1.5 g) fractions were separately subjected to SiO_2 column chromatography (MeOH/$CHCl_3$). The fraction (1.5 g) eluted with 5% MeOH/$CHCl_3$ from the EtOAc-soluble fraction and the fraction (32 mg) eluted with 5% MeOH/$CHCl_3$ from the *n*-BuOH-soluble fraction were combined and purified by SiO_2 column chromatography with *n*-hexane/EtOAc (6:4) to afford **1** (35.6 mg), which was identified by comparison of its spectral data with those in the literature (reference 6).

9. RAW264 cells seeded on a 96-well plate (1,000 cells/well) or 12-well plate (6,000 cells/well) were treated with sRANKL (50 ng/mL) in the presence of **1** (DMSO, control) and allowed to differentiate for 4 d. The differentiated cells were washed with PBS and treated with 4% paraformaldehyde solution for 10 min at room temperature. After washing with PBS, the cells were stained with TRAP-staining solution, which consisted of 50 mM sodium tartrate, 45 mM sodium acetate, 0.1 mg/mL naphthol AS-MX phosphate (Sigma-Aldrich), and 0.6 mg/mL fast red violet LB salt (Sigma-Aldrich), pH 5.2, for 1 h or longer at room temperature. TRAP-positive cells that stained red and contained 3 or more nuclei were

determined to be multinuclear osteoclasts.

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17. Briefly, the chymotrypsin-like activity of the proteasome was measured with the proteasome prepared from rat livers using the fluorogenic substrate, Suc-Leu-Leu-Val-Tyr-MCA (Peptide Institute). Details of this assay have been described in reference 16.

18. Proteasome inhibitory activities of halenaquinone and its several analogs are under investigation in our laboratory and will be published elsewhere.

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24. RAW264 cells were seeded on 35-mm cell culture dishes (400,000 cells/dish). After 1 d, the cells were pretreated with 20 μM **1** (DMSO, control) for 2 h and were then stimulated with sRANKL (250 ng/mL). The stimulated cells were washed with PBS and lysed with sample buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (50 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 0.01% bromophenol blue, and 2% 2-mercaptoethanol) and sonication. After boiling and centrifugation, the supernatants were collected and subjected to SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes using the iBlot Gel Transfer Device (Life Technologies). Immunoblotting was performed on an iBind Western Device (Life Technologies) according to the manufacturer's instructions, using an anti-IκBα mouse monoclonal antibody, anti-phospho-Akt (Ser473) rabbit monoclonal antibody, anti-Akt (pan) rabbit monoclonal antibody, and anti-α-tubulin mouse monoclonal antibody (Cell Signaling Technology). The blots were visualized with Luminata

Crescendo (Merck Millipore) and signals were detected with ChemiDoc XRS$^+$ (Bio-Rad).

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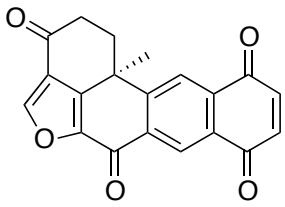
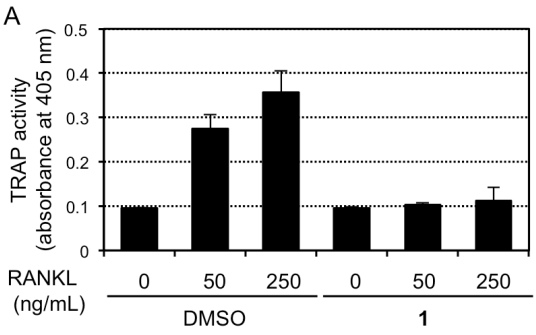
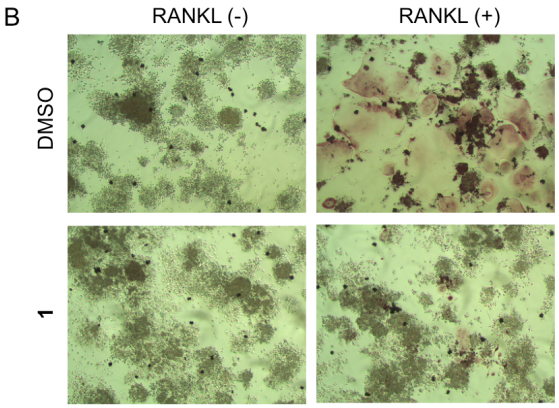


Figure 1. Structure of **1**.





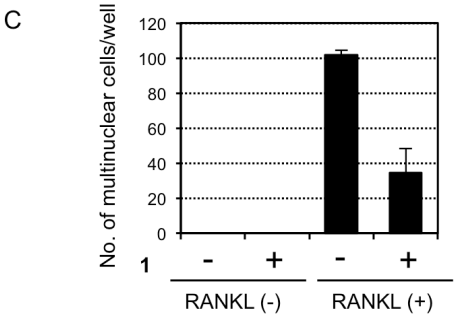


Figure 2. Inhibitory effects of **1** on RANKL-induced osteoclastogenesis.
(A) RAW264 cells were treated with RANKL at the indicated concentrations in the presence or absence of 20 μM **1** and allowed to differentiate for 4 d. TRAP activity was measured as the absorbance at 405 nm. (B) RAW264 cells were allowed to differentiate by the treatment with RANKL (50 ng/mL) in the presence or absence of 20 μM **1** for 4 d and were then stained with TRAP-staining solution. TRAP-positive cells stained red. (C) The TRAP-positive multinuclear cells (nuclei ≥ 3) in (B) were counted.

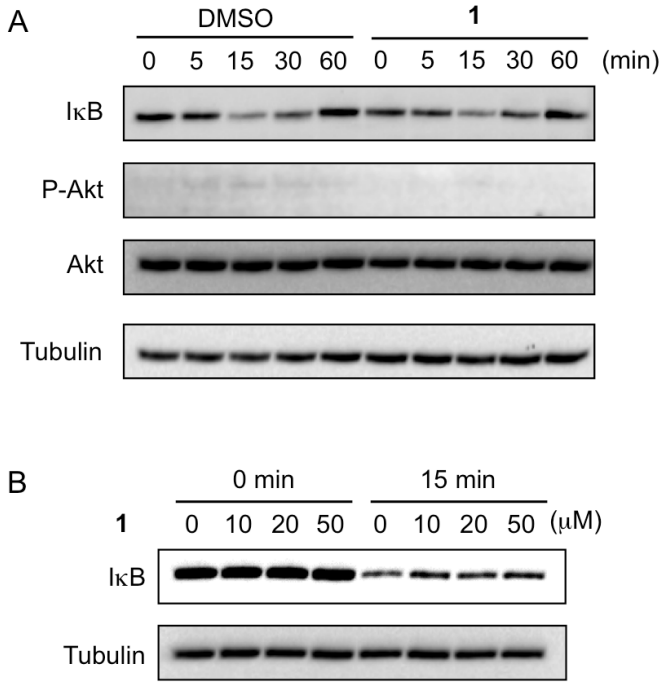


Figure 3. Inhibitory effects of **1** on RANKL-induced IκB degradation and Akt phosphorylation.
(A) RAW264 cells were pretreated with or without 20 μM **1** for 2 h and were then stimulated with RANKL (250 ng/mL). Cell extracts were prepared at the indicated times after the RANKL treatment and subjected to western blotting with the indicated antibodies (tubulin, control). P-Akt, phosphorylated Akt. (B) The **1**-mediated inhibition of the degradation of IκB at various concentrations was measured 15 min after the RANKL treatment by western blotting.