Supporting Information

In Vitro and *In Vivo* Osteogenic Activity of Largazole

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Experimental Section

Cell culture. Mouse bi-potent mesenchymal precursor C2C12 cells were purchased from American Type Culture Collection (VA). C2C12 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) containing 10 % FBS, 100 U/ml of penicillin, and 100 mg/ml streptomycin. The medium was changed every 3 days.

Cell growth assay. Cells were plated in a 96-well plate at 1×10^3 cells/well. After 24 h, cells were incubated in serially diluted compound for 1 or 3 days. Cell growth was evaluated in triplicate using Cell Counting Kit-8 (Dojindo Molecular Technologies, ML) according to the manufacturer's protocol; absorbance was measured at 450 nm using the Wallac EnVision microplate reader (PerkinElmer, Finland). Absorbance was converted to cell number using a standard curve. Significance was determined by Student's t-test and differences were considered significant when $P < 0.05$.

Alkaline phosphatase staining and activity assay. C2C12 cells were plated at 5×10^3 cells/well in a 96-well plate and incubated for 1 day and then the medium containing 5% FBS and largazole was changed. The medium was changed every 3 days and on the differentiation day 6, cells were washed twice with PBS, fixed with 10% formalin in PBS for 30 sec, rinsed with deionized water, and stained using the Alkaline Phosphatase (ALP) Kit (Sigma) under protection from direct light. Images of stained cells were captured under a microscope equipped with a DP70 digital camera (Olympus Optical, Japan). To measure ALP activity, cells were washed twice with PBS and sonicated in lysis buffer (10 mM of Tris-HCl, pH 7.5, 0.5 mM of $MgCl_2$, and 0.1% Triton X-100). After centrifugation at 10,000 × g for 20 min at 4°C, ALP activity in the supernatant was measured in triplicate using the LabAssay ALP Kit (Wako Pure Chemicals Industries). Protein concentration was measured using the BCA Protein Assay kit (Pierce). Significance was determined by Student's *t*-test and differences were considered significant when $P < 0.05$.

Evaluation of mRNA expression level. Primers were designed using an on-line primer design program (Table S1).[1] Total RNA was isolated using TRIzol reagent (Life Technologies, MD) according to manufacturer's protocol. The concentration and purity of total RNA were calculated by measuring absorbance at 260 and 280 nm. First strand cDNA was synthesized using 2 μg of total RNA and 1 μM of oligo-dT_{18} primer and Omniscript Reverse Transcriptase (Qiagen, CA). SYBR green-based quantitative PCR was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA) with 3 μl of first-strand cDNA diluted 1:50 and 20 pmole of primers, according to the manufacturer's protocols. The PCR reaction consisted of three segments. The first segment (95°C for 10 min) activated the polymerase; the second segment included 3-step cycling (40 cycles) at 94°C for 40 sec (denaturation), 60°C for 40 sec (annealing), and 72°C for 1 min (extension); the third segment was performed to generate PCR product temperature dissociation curves ('melting curves') at 95°C for 1 min, 55°C for 30 sec, 95°C for 30 sec. All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta CT}$ method.[2] GAPDH was used as the control gene. Significance was determined by Student's t-test with GAPDH-normalized $2^{-\Delta\Delta CT}$ values. Differences were considered significant when $P < 0.05$.

Western blot analysis. Cells were homogenized in buffer consisting of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20, 1 mM PMSF, and one protease inhibitor cocktail tablet (Roche, Germany) at 4 °C and then centrifuged at 10,000 × g for 15 min. The supernatant was used as the cytoplasmic protein fraction and nuclear proteins were extracted using NucBuster Protein Extraction kit (Novagen, Germany). Protein concentrations were determined using the BCA protein assay kit (Pierce, IL). Samples (20 μg) were mixed with sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 15 min, and loaded onto 10% polyacrylamide gels. Electrophoresis was performed using the Mini Protean 3 Cell (Bio-Rad, CA). The resolved proteins were transferred to a nitrocellulose membrane (Scheicher & Schnell BioScience, Germany). To ascertain the amount of protein loaded and the transfer efficiency, the membranes were stained with Ponceau S staining solution. For immunoanalysis, the membranes were

washed and incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 3% nonfat dry milk) and then incubated with diluted primary antibodies (1:1000) for 2 h at room temperature. Antibodies used in this study were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Following the primary antibody reactions, the membranes were washed with blocking buffer three times (15 min each) and then probed with diluted secondary antibodies (1:2000) for 1 h. The membranes were washed three times (15 min each) and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) using the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan).

HDAC activity assay. HDAC activity was measured by using HDAC Fluorescent Activity Assay kit (BIOMOL, Plymouth Meeting, PA) with a fluorogenic histone deacetylase lysyl substrate and developer. The assay was performed exactly as recommended by the manufacturer. The increase in fluorescence intensity at 460 nm (emission) after excitation at 360 nm was monitored using Wallac EnVision microplate reader (PerkinElmer, Finland).

Runx2 luciferase reporter assay. To measure Runx2 activity, p6×osteoblast-specific cis-acting element (OSE) 2-luc reporter vector was transiently transfected into C2C12 cells that were seeded in a 96-well plate at 5×10^3 cells/well as described previously (Kim et al., 2004). After 24 h, medium was replaced with DMEM containing 5% FBS with or without tanshinone IIA and/or BMP-2. After 24 h, the cells were lysed, and luciferase activity was measured using luciferase reporter assay system (Promega) and the Wallac EnVision microplate reader.

***In vivo* murine calvarial bone formation assay.** *In vivo* bone-forming activity of largazole was evaluated using lyophilized collagen sponges as described previously.[3] Briefly, collagen sponges loaded with 5 μl of vehicle or largazole were implanted over the calvarial bones of mice (n = 5 per group). Three weeks after drug implantation, the calvarias were harvested, fixed in 4% paraformaldehyde, decalcified in 12% EDTA, embedded in paraffin, and sectioned. Sections were deparaffinized through graded xylene

washes, dehydrated in a graded series of ethanol washes, and stained with hematoxylin and eosin (H&E).

Bone regeneration assay. All rabbits were anesthetized with an intramuscular dose of 0.1 ml/kg Zoletil (Virbac, France). The head was shaved, and the cutaneous surface was disinfected with povidone iod solution prior to the operation. The calvaria bone was exposed through a skin incision of ~4 cm in length. Four similar circular bicortical defects (3-mm diameter) were made in the parietal bone using a trephine on a slow-speed electric handpiece, applying 0.9% physiologic saline irrigation. Defects were filled with 10 mg of bone graft substitute, MBCP (Biomatlante, France) soaked with 50 μl of PBS or largazole. Closure of periosteum and subcutaneous tissues was done with lactomer (Syneture), while the skin was relocated with nylon:polyamide (Syneture). Postoperative antibiotics were administered Gentamicin. Rabbits with defects (n = 4 for each test group) were sacrificed at 4 weeks after surgery.

Induction of multinucleated osteoclasts. RAW264.7 cells were purchased from the ATCC and maintained in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml streptomycin with a change of medium every 3 days in humidified atmosphere of 5% CO_2 in air at 37°C. For the osteoclast differentiation, RAW264.7 cells were suspended in α-minimal essential medium (α-MEM) supplemented with 10% FBS and 100 ng/ml RANKL (R&D Systems Inc., MN) and plated in a 96-well plate at 1×10^3 cells/well. The next day, cells were treated with largazole and then after an additional 3 days, multinucleated osteoclasts were observed.

Tartrate-resistant acid phosphatase (TRAP) staining and activity assay. Multinucleated osteoclasts were fixed with 10% formalin for 10 min and ethanol/acetone (1:1) for 1 min, and then stained by Leukocyte Acid Phosphatase Kit 387-A (Sigma, MO). The images of TRAP-positive multinucleated cells were captured under a microscope with DP Controller (Olympus Optical, Japan). For measuring TRAP activity, multinucleated osteoclasts were fixed with 10% formalin for 10 min and 95% ethanol for 1 min, and then 100 μl of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium

tartrate and 5 mM *p*-nitrophenylphosphate (Sigma) was added to the dried cells. After incubation for 1 h, the enzyme reaction mixtures in the wells were transferred into new plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 410 nm, and TRAP activity was presented as % of control. The experiment was performed in triplicate.

References

[1] Rozen, S.; Skaletsky, H. J. *Methods Mol. Biol.* **2000**, *132*, 365–386.

[2] Livak, K. J.; Schmittgen, T. D. *Methods* **2001**, *25*, 402–408.

[3] Ha, H.; Lee, J. H.; Kim, H. N.; Kim, H. M.; Kwak, H. B.; Lee, S.; Kim, H. H.; Lee, Z. H. *J. Immunol.* **2006**, *176*, 111–117.

Figure S1. Effect of largazole on the cell viability. C2C12 cells (4×10^3 cells/well) were cultured in a 96-well plate for 1 day and treated with largazole. Cell growth was evaluated after 1 and 3 days.

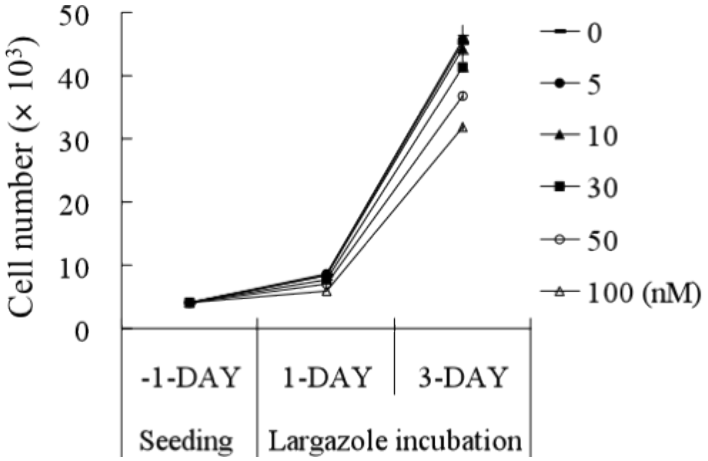

Figure S2. Effect of largazole on the expression level of HDACs in C2C12 cells. Cells (2 × 10^6 cells/plate) were cultured in a 100-mm plate for 24 h and treated with largazole. After 24 h, protein extracts were prepared and western blot analysis was performed. Actin was used as a loading control.

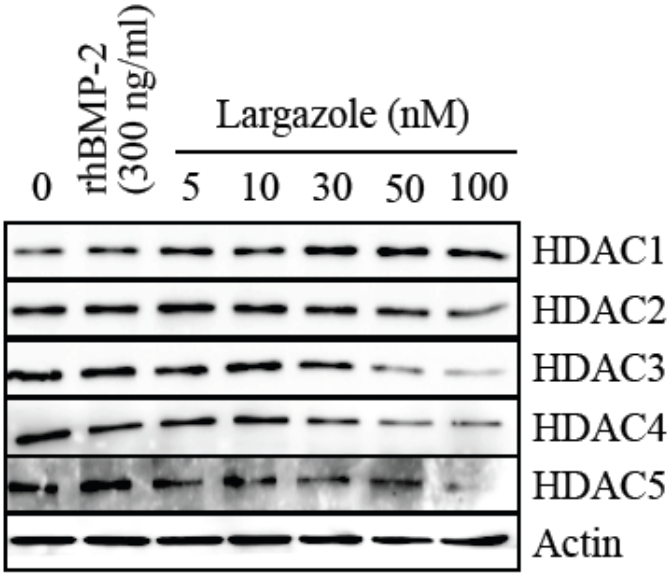

Figure S3. Effect of largazole on osteoclastogenesis. (A) Effect of largazole on the RANKL-induced TRAP activity. (B) Effect of largazole on the RANKL-induced formation of TRAP-positive multinucleated osteoclasts. The effect of largazole on osteoclast differentiation was evaluated in RAW264.7 cells. RAW264.7 cells were plated in 96-well plates at the density 1×10^3, treated with serially diluted largazole, and incubated until multinucleated osteoclasts were observed under a microscope before TRAP activity and its staining were performed.

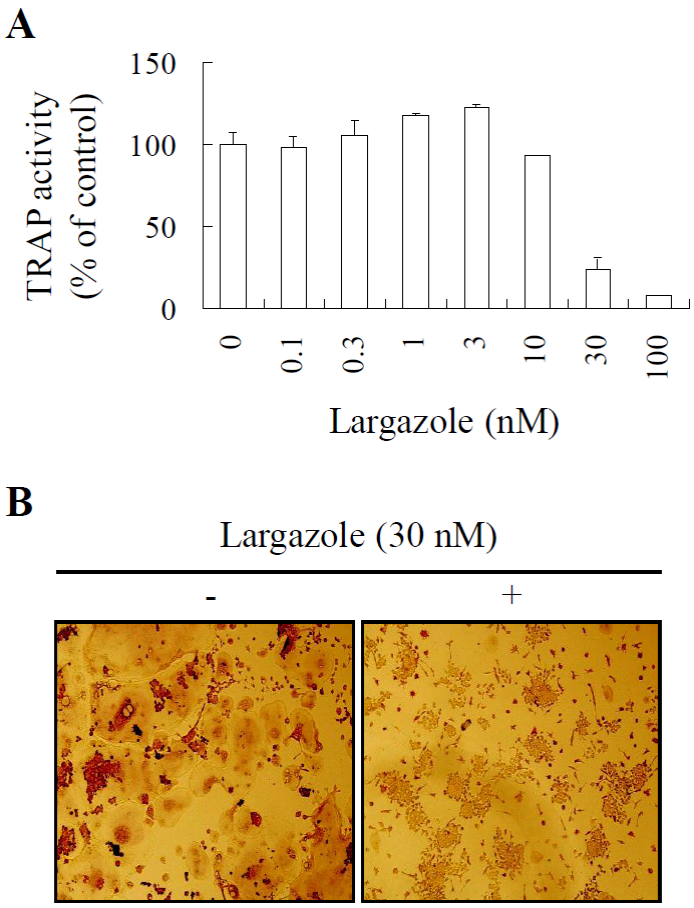

Table S1. RT-PCR Primers.

Target Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
ALP	GCTGATCATTCCCACGTTTT	CTGGGCCTGGTAGTTGTTGT
OPN	CGATGATGATGACGATGGAG	TGGCATCAGGATACTGTTCATC
RUNX2	GCCGGGAATGATGAGAACTA	GGACCGTCCACTGTCACTT
BMP-2	GCTCCACAAACGAGAAAAGC	AGCAAGGGGAAAAGGACACT
BMP-4	CCTGGTAACCGAATGCTGAT	AGCCGGTAAAGATCCCTCAT
BMP-6	TTCTTCAAGGTGAGCGAGGT	TAGTTGGCAGCGTAGCCTTT
BMP-7	CGATACCACCATCGGGAGTTC	AAGGTCTCGTTGTCAAATCGC
BMP-9	CAGAACTGGGAACAAGCATCC	GCCGCTGAGGTTTAGGCTG
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Table S2. Effect of largazole on the mRNA induction of osteoblastogenesis markers in C2C12 cells. The mRNA levels were evaluated by quantitative real-time PCR in cells (1×10^6 cells/60-mm plate) treated with largazole for 6 days. Fold changes relative to the control are presented as mean ± standard deviation. rhBMP-2 (300 ng/ml) was used as the reference of osteoblastogenesis inducer.

Largazole (nM)	Target Genes	
	ALP	*OPN*
0	1.00 ± 0.13	1.00 ± 0.04
1	1.85 ± 0.06[a]	1.33 ± 0.19
50	324.04 ± 1.59[c]	2.28 ± 0.12[b]
100	263.33 ± 11.61[b]	0.83 ± 0.04[a]
rhBMP-2	11.12 ± 0.00[c]	2.35 ± 0.41[a]

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.