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In Vitro and *In Vivo* Osteogenic Activity of Largazole

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Abstract

Due to their capability of modifying chromatin structure and thereby regulating gene transcription, histone deacetylases (HDACs) have been reported to play important roles in osteogenesis and considered a promising potential therapeutic target for bone diseases, including osteoporosis. We showed that the novel marine-derived HDAC inhibitor largazole exhibits *in vitro* and *in vivo* osteogenic activity. Largazole significantly induced the expression of ALP and OPN. The osteogenic activity of largazole was mediated through the increased expression of Runx2 and BMPs. Importantly, largazole showed *in vivo* bone-forming efficacy in the mouse calvarial bone formation assay and the rabbit calvarial bone fracture healing model. The dual action of largazole to stimulate bone formation and inhibit bone resorption would be a useful feature in drug development for bone-related disorders.

Keywords

largazole; osteogenic activity; histone deacetylases; Runx2; bone morphogenetic protein

The cyclic depsipeptide largazole (Figure 1A), isolated from a cyanobacterium of the genus *Symploca* by Luesch and co-workers, is a marine natural product with novel chemical scaffold.[1] Largazole possesses highly differential growth-inhibitory activity, preferentially targeting transformed over non-transformed cells. The intriguing structure and biological activity of largazole have attracted strong interest from the synthetic chemistry community to establish synthetic routes to largazole and to investigate its potential as a cancer therapeutic. Luesch and Hong reported the first total synthesis of largazole, identified histone deacetylases (HDACs) as the molecular targets, and demonstrated antitumor activity in a xenograft mouse model.[2,3] Following our initial report, additional total and formal syntheses, further studies on its HDAC inhibition, and structure–activity relationship studies have appeared in the literature.[4–17]

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Supporting Information Available. General experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

Osteogenesis is a complex process associated with dramatic changes in gene expression.[18] Due to their capability of modifying chromatin structure and thereby modulating gene transcription, HDACs have been reported to play important roles in osteogenesis and considered a promising potential therapeutic target for bone diseases, including osteoporosis.[19] Several HDAC inhibitors, including trichostatin A,[20] sodium butyrate,[20] and valproic acid[21] have been tested for their osteogenic activity, but none have been shown effective in *in vivo* models for further development as potential therapeutics for bone-related diseases. Moreover, it has been reported that long-term treatment with valproic acid increases the incidence of osteoporosis and bone fracture.[22,23] Herein, we report the *in vitro* and *in vivo* osteogenic activity and the significant potential of largazole in bone formation, repair and regeneration.

Murine pluripotent mesenchymal precursor C2C12 cells were used as an *in vitro* model of osteogenesis. The C2C12 cells have been known to be committed into osteoblasts with the induction of several osteogenic bone morphogenetic protein (BMP) isoforms such as BMP-2, 4, 6, 7, and 9.[24] Before evaluating the osteogenic activity of largazole, we examined the effect of largazole on viability of C2C12 cells and found that largazole did not exhibit a significant level of cytotoxicity up to 100 nM (see the Supporting Information for details).

Next, we examined the effects of largazole on the inhibition of HDAC activity and the accumulation of acetylated histones in C2C12 cells to confirm its HDAC inhibitory activity. Largazole decreased the total HDAC activity in C2C12 cells (Figure 1B)[25] and significantly increased the levels of acetylated histones in a dose-dependent manner (Figure 1C).

The effect of largazole on the commitment of C2C12 cells into osteoblasts was determined by the expression level of alkaline phosphatase (ALP), an early phase marker of osteoblast differentiation. Up to 50 nM, largazole increased the expression of ALP in a dose-dependent manner (Figure 1D). In addition, largazole significantly induced the expression of ALP and osteopontin (OPN) at the transcript level (see the Supporting Information for details).

Since Runx2 has been shown to be critical in osteogenesis and regulates the expression of bone-specific genes such as ALP and OPN,[26] we examined the effect of largazole on the activation and mRNA expression of Runx2 using the p6×osteoblast-specific *cis*-acting element 2-luciferase reporter[27] and real-time PCR, respectively (Figure 2). Relative to the DMSO control, largazole at 50 nM significantly increased the activity and mRNA level of Runx2 by 29-fold and 3-fold, respectively (Figure 2A and 2B). Since HDACs have been identified as co-repressor proteins to affect Runx2 activity,[28] these results suggested that inhibition of HDACs by largazole increases Runx2 activation, potentiates osteoblast differentiation, and increases bone formation.

From the data showing that the increased expression of ALP induced by largazole was inhibited by noggin, a specific inhibitor of BMPs (see Figure 1D), we hypothesized that the osteogenic activity of largazole could result from its potential to increase the expression of BMPs. To test this hypothesis, we evaluated the effect of largazole on the mRNA expression levels of BMPs (Figure 2B). Largazole significantly induced the expressions of BMP-2, 4, 6, 7, and 9, supporting our hypothesis that the osteogenic activity of largazole is mediated through the increase of the expression and/or activity of Runx2 and BMPs. Recently, BMP-2 and BMP-7 with strong osteogenic activity have been approved for clinical applications including spinal fusion, fracture healing, and dental tissue engineering.[29,30] Therefore, anabolic agents such as largazole that stimulate BMP expression or the BMP-signaling pathway would be useful for the treatment of osteoblast-related diseases by bone formation or regeneration.[31,32] In addition, we found that largazole inhibits the formation of multinucleated osteoclasts (see the Supporting Information for details), suggesting that

largazole might elicit the dual action to stimulate bone formation and suppress bone resorption.

Next, we examined *in vivo* osteogenic activity of largazole using two independent *in vivo* models, the mouse calvarial bone formation assay and the rabbit calvarial bone fracture healing model.

In the mouse calvarial bone formation assay, when collagen sponges soaked with largazole were implanted in calvarial bones, largazole induced woven bone formation over the periosteum of the calvarial bones (Figure 3). The woven bone formation at the lower concentration (10 μM, Figure 3B) was more significant than that at the higher concentration (50 μM, Figure 3C). This data suggested that largazole may show the biphasic effect which has been observed with other osteogenic compounds.[33–35] Further studies are required to determine the optimal dose for the highest *in vivo* efficacy.

We further evaluated *in vivo* bone-forming activity of largazole in the rabbit calvarial bone fracture healing model (Figure 4). In this study, macroporous biphasic calcium phosphate (MBCP) was used as a basic scaffold to induce bone formation. While an incomplete bone formation was observed with MBCPs alone (Figure 4A), newly-formed bones in direct contact with MBCPs mixed with largazole were observed (Figure 4B and 4C). Since there is a significantly unmet clinical need for small molecules to improve the biological properties of the scaffold and its osteoconductivity, this data clearly shows the great potential of largazole for improvement of the property of bone graft substitutes in bone defect reconstruction.

In summary, we showed that the HDAC inhibitor largazole exhibits *in vitro* and *in vivo* osteogenic activity. Largazole significantly induced the expression of ALP and OPN. The osteogenic activity of largazole was mediated through the increased expression of Runx2 and BMPs. More importantly, largazole showed *in vivo* bone-forming efficacy in the mouse calvarial bone formation assay and the rabbit calvarial bone fracture healing model. The dual action of largazole to stimulate bone formation and inhibit bone resorption would be a useful feature in drug development for bone-related disorders. Taken together, largazole shows the great clinical potential as a novel treatment for bone-related diseases in addition to its already proven potential as an antitumor agent.[3]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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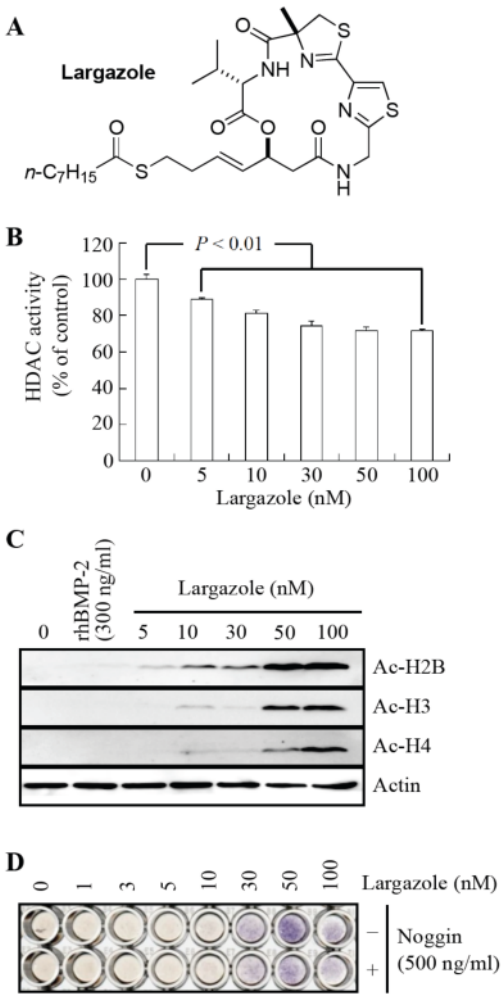

Figure 1.

Largazole exhibited HDAC inhibitory and osteogenic activities in C2C12 cells. (A) Structure of largazole. (B) Effect of largazole on the total HDAC activity in C2C12 cells. Cells (4×10^3 cells/well) were cultured in a 96-well plate for 24 h and pre-treated with largazole for 1 h before incubation with deacetylase Lysyl substrate of the HDAC Fluorescent Activity Assay kit. HDAC activity was measured according to the manufacturer's protocol. (C) Effect of largazole on the level of hyperacetylated histones 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. (D) Effect of largazole on osteoblast differentiation in C2C12 cells. The effect of largazole on osteoblast differentiation was evaluated by ALP staining. Cells (4×10^3 cells/well) were cultured in a 96-well plate for 24 h and treated with largazole for 6 days. Medium was changed every 3 days. Noggin (a specific BMP inhibitor) was co-treated with largazole.

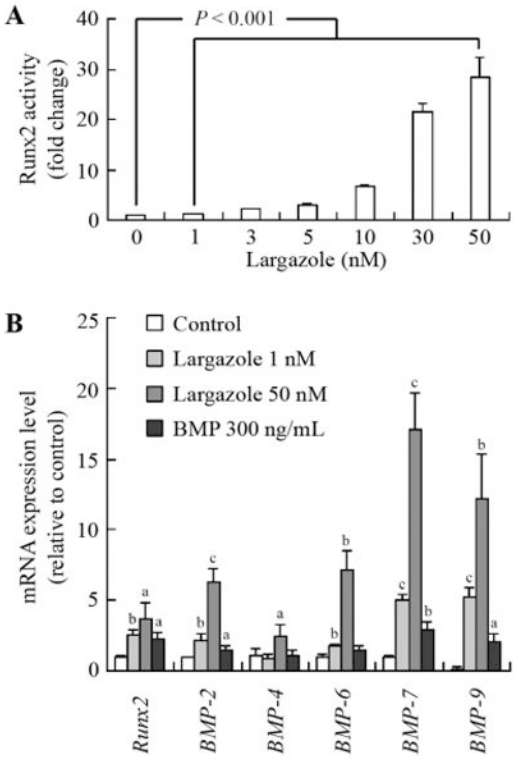

Figure 2.

Effect of largazole on the Runx2 activation and mRNA induction of Runx2 and BMPs in C2C12 cells. (A) Runx2 activation was measured by luciferase reporter assay using 6×OSE2-luc plasmid-transfected C2C12 cells. The cells (4×10^3 cells/well) were cultured in a 96-well plate for 1 day and incubated with DMEM containing 5% FBS in the presence or absence of largazole for 1 day before luciferase activity assay. (B) 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. rhBMP-2 (300 ng/ml) was used as the reference of osteoblastogenesis inducer ($^a P < 0.05$; $^b P < 0.01$; $^c P < 0.001$).

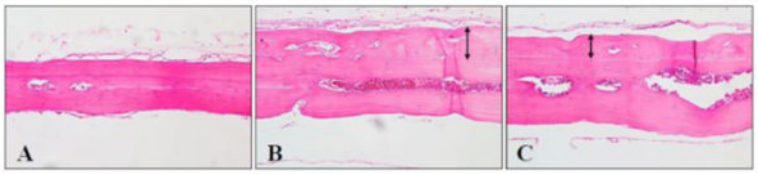

Figure 3.

Largazole exhibited bone-forming activity in mouse calvaria. Collagen sponges containing 5 μl of PBS vehicle (A) or largazole (B, 10 μM; C, 50 μM) were placed onto mouse calvarial bones. After a 3- week implantation, the mice were sacrificied. Calvarial bones were removed, fixed, decalcified, embedded in paraffin, and sectioned. Sections were stained with H&E and photographed at 200× magnification. Arrows indicated the region of newly-forming woven bone.

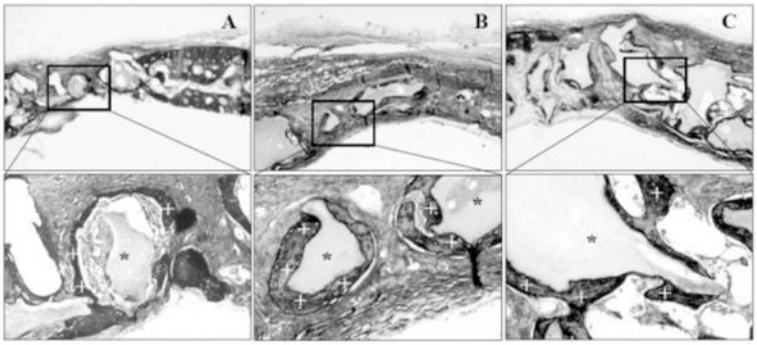

Figure 4.

Largazole exhibited bone-regenerating activity in rabbit calvaria. Bone graft substitute (10 mg), MBCP (indicated by a black asterisk (*); Biomatlante, France) was mixed with 50 μl of PBS (A) or largazole (B, 100 nM; C, 250 nM), and defects were filled with these mixtures. Rabbits were sacrificed at 4 weeks after surgery. The bone (indicated by ‘white +’) grows into and around bone graft substitute. Box images in the top were magnified in the bottom.