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Berberine derivative, Q8, stimulates osteogenic differentiation

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ABSTRACT

Berberine has been implicated to be involved in maintaining bone health due to its anti-oxidative and osteogenic properties. However, low potency and low bioavailability limit the clinical development of the drug. To overcome these obstacles, we previously synthesized a compound, Q8, which is a structural homolog of berberine. The present study examined the pharmacological functions of Q8 to evaluate its potential use in bone regeneration with respect to osteoblast differentiation. Here, we report that Q8 enhanced BMP4-induced alkaline phosphatase (ALP) activity and transcription from the ALP promoter. In addition, Q8 suppressed the expression and activity of PPARγ (a known negative regulator of osteogenesis due to its stimulatory effects on adipogenesis and its role as an adipogenic transcription factor), which in turn increases β-catenin expression in the nucleus, and ultimately promotes osteoblast differentiation. Meanwhile, Q8 reversed the inhibitory effects of the PPARγ agonist, rosiglitazone, on osteoblast differentiation. This study demonstrated that Q8 promotes osteoblast differentiation via inhibition of PPARγ and the enhancement of osteoblast function by Q8 may contribute to the prevention for osteoporosis.

1. Introduction

Osteoporosis is a serious public health issue, arising more often in older people and in women after menopause, in which the density and quality of bone are reduced [1]. Among the several risk factors associated with osteoporosis, bone remodeling is most critical in terms of bone formation and homeostasis [2]. Bone remodeling is tightly regulated by osteoclasts and osteoblasts involved in the process of resorption followed by replacement of bone; this process occurs throughout a person's life [2,3]. Therefore, homeostatic balance between osteoclastic bone resorption and osteoblastic bone formation is vital for the prevention of osteoporosis.

For osteoblast maturation, there are three major stages of cell processes: proliferation, differentiation, and mineralization [4]. Osteoblast differentiation is a regulatory mechanism that affects new bone formation by directly inducing or regulating various signaling pathways and osteogenic factors [5]. Multiple signaling pathways, such as BMP (bone morphogenetic protein), Wnt, and Notch signaling pathways, regulate osteogenic target genes and mediate osteoblast differentiation [6]. Transcription factors, including runt-related transcription factor 2 (Runx-2) and osterix (Osx), increase the activity and expression of various osteogenic markers, subsequently enhancing osteoblast differentiation [7].

Berberine is an active compound found in *Rhizoma coptidis* and has been commonly used as an ingredient of many medicines in China for centuries [8]. Modern research has found that berberine exhibits anti-inflammatory effects by inhibiting reactive oxygen species production and mitogen-activated protein kinase signal and also shows anti-tumor effects by promoting apoptosis and suppressing tumor metastasis [9]. In addition, associated with bone forming effects, berberine promotes osteogenic differentiation via activation of Runx2 and p38 MAPK [10]. Oral administration of berberine increases bone mineral density in senile osteoporosis mice model [11]. In spite of these pharmacological effects, drug development of berberine has been impeded, since berberine also has several side effects including induction of high bilirubin levels, low blood pressure, and drug interactions with cyclosporine and other medications metabolized by the liver cytochrome P450 3A4 enzyme [12–15]. Therefore, our group has designed and synthesized a new mimic drug, Q8, based on berberine structure. The quaternary amine group of berberine was substituted with an uncharged tertiary amine and the position of methoxy group was

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changed in Q8 [16] (Fig. 1A). In the present study, we evaluated the osteoblastogenic effects of Q8.

2. Materials and methods

2.1. Antibodies, reagents, and plasmids

Antibody against PPARγ (MAB3872) was purchased from Chemicon International Inc.; antibodies against lamin B (sc-6216) and glycogen synthase kinase-3α/β (GSK3α/β, sc-7291) were obtained from Santa Cruz Biotechnology; antibody against phospho-GSK3α/β (#9331) was obtained from Cell Signaling Technologies; antibodies against β-catenin (C2206) and α-tubulin (B-5-1-2) were obtained from Sigma-Aldrich. Recombinant human bone morphogenetic protein 4 (BMP4) was purchased from R&D Systems, and berberine chloride (B3251) was purchased from Sigma-Aldrich and was dissolved in dimethyl sulfoxide (DMSO). Q8 compound was synthesized and analyzed as previous described [16]. For preparing plasmid constructs expressing PPARγ, full length PPARγ was subcloned into pCS4 vector. A construct for expressing β-catenin was based on the pEGFP-C1 vector. PPRE-Luc contains the consensus PPAR responsive element (PPRE). TOP flash containing native LEF/TCF binding sites was used as a positive control. FOP flash containing four mutated LEF/TCF binding sites was used as a negative control counterpart. The constructs for ALP-Luc (−900 bp) [17], BSP-Luc (−938 bp) [18], and OC-Luc (−1.1 kbp) [18] were prepared as described previously.

2.2. Cell culture and transient transfection

The C2C12 mouse myoblasts cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic. The DMEM, FBS, and the antibiotics were purchased from Life Technologies (Grand Island, NY, USA). The transient transfection was performed by using a polyethyleneimine (PEI, Polysciences, Warrington, PA, USA) -mediated method. The total amount of transfected plasmid in each group was equalized by adding the empty vector.

2.3. Luciferase reporter assay

The C2C12 cells were plated in 24-well plates one day before transfection at a density of 2×10^4 cells/well. The cells were transfected with a CMV promoter-driven β-galactosidase reporter (pCMV-β-gal), luciferase reporter, and the indicated combinations of the expression plasmids, and 24 h after transfection, the cells were treated with the indicated reagents. The Luciferase Reporter Assay kit (Promega) was used to measure the luciferase activity, and all experiments were performed in triplicate.

2.4. Immunoblotting

The C2C12 cells were lysed in ice-cold lysis buffer [25 mM HEPES (pH 7.5), 150 mM sodium chloride (NaCl), 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM sodium fluoride (NaF), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium vanadate (Na_3VO_4), 250 M HEPES (pH 10, l g/mL leupeptin, and l g/mL aprotinin)]. The cellular lysates were cleared by centrifugation at 13,200 rpm at 4 °C, and the supernatants were subjected to immunoblotting. All the proteins samples were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis

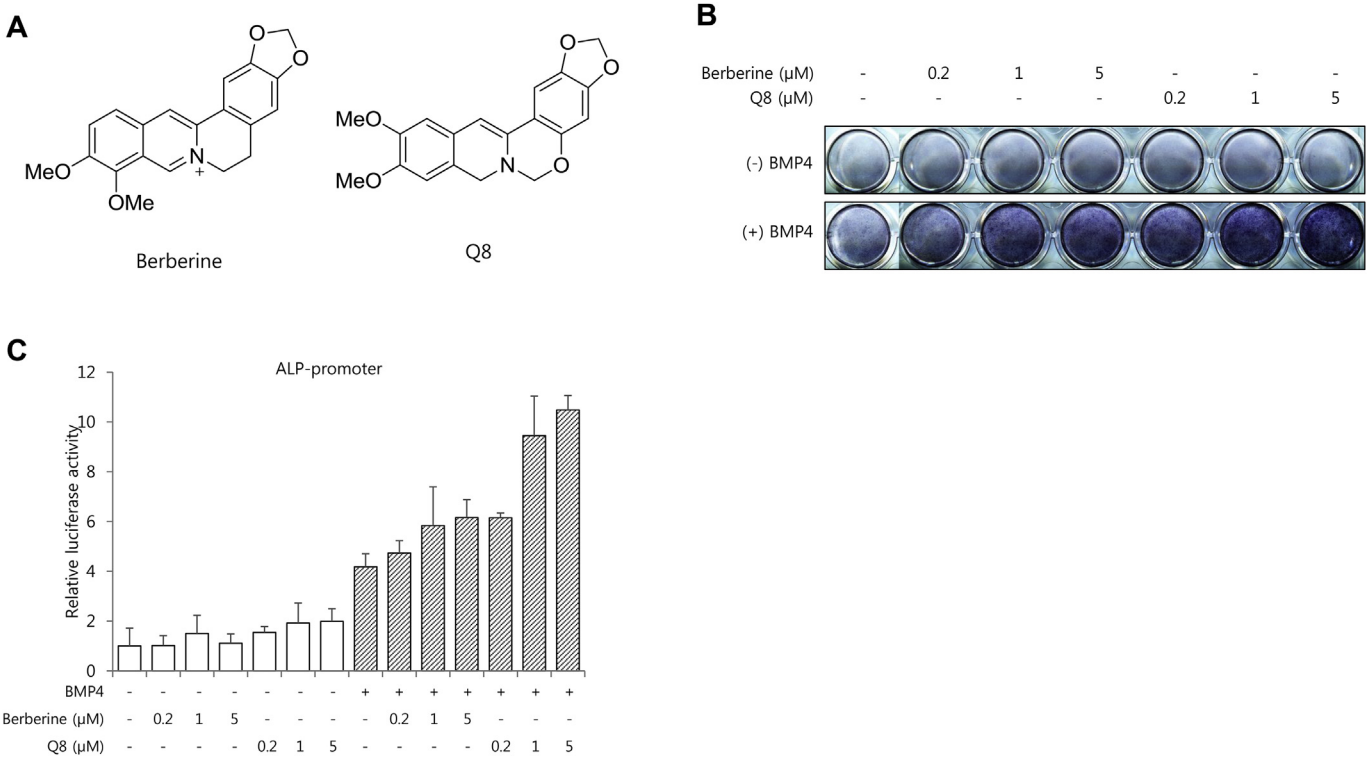

Fig. 1. Q8 increases bone morphogenetic protein 4 (BMP4)-induced osteogenic effects (**A**) Structures of berberine and Q8. (**B**) C2C12 cells were treated with BMP4 (30 ng/mL) and berberine or Q8 (0.2, 1, or 5 μM) for 3 days. Osteoblast differentiation was examined using alkaline phosphatase (ALP) staining. (**C**) C2C12 cells were transfected with pCMV-β-gal (0.1 μg), ALP-luciferase reporters (0.3 μg), with increasing concentrations of berberine or Q8 (0.2, 1, or 5 μM). Luciferase activities were measured. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with BMP4-treated group; ANOVA–Bonferroni *post hoc* test, $n = 3$.