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Berberine derivative WJCPR11 enhances osteoblast differentiation

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Berberine is a phytocompound from plants viz. *Phellodendri cortex* and *Coptis rhizome*, used to treat a variety of diseases. It is effective in preventing osteoporosis, but it is less effective than drugs currently used in clinical practice. In this study, we used a novel berberine derivative, WJCPR11, to promote osteoblast differentiation and to investigate its use in the prevention and treatment of osteoporosis. WJCPR11 at a safe concentration without toxicity increased alkaline phosphatase (ALP) activity induced by bone morphogenetic protein 2 (BMP2) dose-dependently. The mRNA expression of ALP, osteocalcin (OC), runt-related transcription factor 2 (Runx2), and osterix was increased, with the ALP level increasing the most. In addition, the protein abundance of bone sialoprotein (BSP), collagen, type I, alpha 1, Runx2, and osterix were also increased. Moreover, the transcriptional activity of ALP, BSP, and OC was increased by WJCPR11, with OC showing the most significant increase. The results indicate that osteoblast differentiation is promoted by WJCPR11, and it could play a role in the prevention of osteoporosis.

Keywords: Alkaline phosphatase, Bone morphogenetic protein 2, Bone sialoprotein, Osterix, Osteoporosis, Runx2

Bone is a special connective tissue in which the remodeling process occurs even after development and growth[1]. Bone homeostasis is the balance of bone formation by osteoblasts and bone resorption by osteoclasts[2]. However, after menopause, the rapid decrease in secretion of estrogen, aging, and decreased intake of vitamin D can cause bone resorption to become dominant over bone formation so that the metabolic steady-state in the bone is disrupted[3]. This imbalance causes excessive bone absorption and can result in osteoporosis[4]. Hence, various substances that promote bone formation by osteoblast differentiation or inhibit the activity of osteoclasts to prevent a decrease in bone mass have been studied for treatment of osteoporosis[5].

Osteoblast differentiation is important in maintaining bone homeostasis[6]. Osteoblasts are generally maintained by growth hormones, cytokines, and various transcription factors[7]. Bone morphogenetic proteins (BMP) are cytokines that play an important role in regulating osteoblast differentiation and bone formation[8]. In osteoblast differentiation, runt-related transcription factor 2 (Runx2) is key[9]. Runx2 induces osteoblast differentiation by binding to promoters of osteoblast differentiation marker genes such as osterix, osteocalcin (OC), alkaline phosphatase (ALP), and collagen type I alpha 1 (Col1α1) to increase the expression of the genes[10]. Osterix is expressed at the later stage of differentiation into osteoblasts that produce bone[11]. OC is expressed when differentiation is complete, and ALP is expressed in the early stages of osteoblast differentiation[12].

Berberine, an isoquinoline alkaloid is a main component of plants such as *Phellodendri cortex* and *Coptis rhizoma*[13]. This substance is known to exhibit anti-microbial activity, anti-inflammatory activity, anti-tumor, anti-motility, and hemostatic activity[14]. Berberine has been used clinically for many years to treat various diseases and is known to be effective in improving osteoporosis by promoting osteoblast differentiation via the p38 MAPK-Runx2 pathway[15]. However, compared to previously developed bisphosphonate-based osteoporosis treatment,

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#Equal contribution

berberine is less effective and has not been used as a clinical treatment[16]. Therefore, in this study, we assessed the expression of osteogenic genes by observing the effect of WJCPR11, one of the previously synthesized berberine derivative (Fig. 1) on osteoblast differentiation.

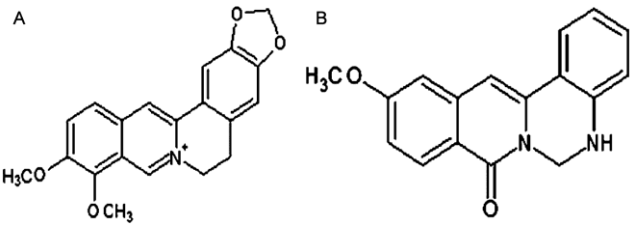

Fig. 1 — Structures of (A) berberine; and (B) WJCPR11.

Materials and Methods

Cell culture and osteoblast differentiation induction

C2C12 is a pre-myoblastic cell line derived from mice. We cultured cells in Dulbecco's modified Eagle's Medium (DMEM, Gibco, Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics (Thermo Fisher Scientific, Waltham, MA, USA) in an environment of 5% CO_2 maintained at 37°C. To induce osteoblast differentiation, we added BMP2 (500 ng/mL) and cultured cells for 24-48 h until greater than 80% confluence.

Reagents and antibodies

BMP2 was purchased from R&D Systems (Minneapolis, MN, USA). Berberine derivative compounds were synthesized as reported previously[17]. For preparation of WJCPR11, dimethyl sulfoxide (DMSO) was used as a solvent, and 0.1% DMSO was used for all controls. Anti-Runx2, anti-bone sialoprotein (BSP), and anti-α-tubulin were purchased from Cell Signaling (Beverly, MA, USA). Anti-Osterix and anti-Col1α1 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

Alkaline phosphatase staining

Differentiated C2C12 cells were fixed with 4% formaldehyde at 20ºC for 15 min. Then, BCIP/NBT color development substrate (Sigma-Aldrich, St. Louis, MO, USA) was added, and the activity of ALP was observed at 15 min intervals. For analysis, a sample was dissolved in DMSO, and the absorbance was measured at a wavelength of 480 nm and analyzed quantitatively.

Cytotoxicity assay

The effect of concentration of WJCPR11 on cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Thermo Fisher Scientific) assay. C2C12 cells were grown in a 24-well plate, treated with different concentrations of WJCPR11, and cultured for 48 h. MTT (5 μg/mL) solution was added to each well and incubated for 1 hour. Then, the supernatant was removed, and isopropanol (500 μL) was added to dissolve the MTT crystals. Finally, 150 μL of dissolved crystal was added to each well in a 96-well plate, and the absorbance was measured and quantitatively analyzed at a wavelength of 490 nm.

RNA extraction and real-time polymerase chain reaction

RNA was extracted by the phenol-chloroform method using TRIzol solution. TRIzol (1 mL) was placed in each well of cultured cells in a 6-well plate, treated with 200 μL of chloroform, and centrifuged at 13,200 rpm for 15 min at 4°C. Next, after separating the supernatant, the same volume of isopropanol was added, mixed, and centrifuged at 13,200 rpm for 15 min at 4°C. After removing the supernatant, the resulting pellet was washed with 80% EtOH and centrifuged at 13,200 rpm for 15 s; the final pellet was dissolved in DEPC water to quantify the concentration. cDNA was synthesized using an oligo (dT) primer and reverse transcriptase (Takara, Nojihigashi, Japan). RT-PCR was conducted in a StepOne Real-Time PCR System (Molecular Devices, San Jose, CA, USA) using TOPreal qPCR 2X PreMIX (SYBR Green with low ROX). All samples were subjected to an initial reaction at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s to obtain CT values. The expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to standardize the expression level of mRNA, and ΔCt was determined by subtracting the Ct value of GAPDH from the target value. The expression level of each gene was calculated by 2-ΔΔCt.

Protein extraction, electrophoresis, and western blotting

After washing the cells in a 6-well plate, they were resuspended in phosphate-buffered saline for protein extraction and maintained on ice. The cells were dissociated using RIPA lysis buffer (Thermo Fisher Scientific) for 20 min. The cell extract in the supernatant was obtained by centrifugation at 13,200 rpm for 30 min at 4°C. The concentration of the extracted protein was quantified using BCA protein assay kit (Thermo Fisher Scientific), and then separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then

transferred to a polyvinylidene fluoride membrane (GE Healthcare, Buckinghamshire, UK). The membrane was blocked with 5% milk for 1 h and washed with Tris-buffered saline. Then, the primary antibody of each protein was added and reacted at 21°C, followed by incubation with a suitable secondary antibody for about 1 hour. After the reaction was complete, an enhanced chemi-luminescence (GE Healthcare) solution was added, and the protein expression was analyzed by an LAS-4000 (Fujifilm, Tokyo, Japan).

Luciferase reporter assay

Transfection was performed using polyethyleneimine (Polysciences, Warrington, PA, USA). ALP, BSP, and OC were used as reporter genes, and β-gal was used as an internal control. At 24 h after transfection, berberine was added to the cells; 24 h later, the activity was measured using a luciferase reporter assay kit as previously described[18].

Statistical analysis

Statistics were analyzed using GraphPad Prism 5.03 software (La Jolla, CA, USA). Experimental results are expressed as mean ± standard deviation. A p-value <0.05 was judged to be statistically significant.

Results

WJCPR11 increases ALP activity during osteoblast differentiation

The level of ALP activity detected by staining plays an important role as an indicator of osteoblast differentiation. We observed the differentiation of cells according to concentration of WJCPR11. As a control group, we used cells that had not been treated with BMP2. For experimental samples, BMP2 was added to C2C12 cells, and the effect was assessed by treating cells with WJCPR11 dose-dependently. When the concentration of the WJCPR11 was 5 or 25 μM, ALP showed relatively high activity. Measured quantitatively, the activity of ALP increased from low to high concentrations of WJCPR11 (Fig. 2 A and B).

WJCPR11 is non-toxic at effective concentrations

An MTT assay was used to determine the toxicity of WJCPR11 on cells. The concentration of WJCPR11 added to cells was 0.04, 0.2, 1, 5, 25, 125, and 200 μM. The viability was observed after an incubation time of 48 h. As the concentration of the WJCPR11 increased, the survival rate was similar to that of the control group until 25 μM, after which cytotoxicity was observed (Fig. 3).

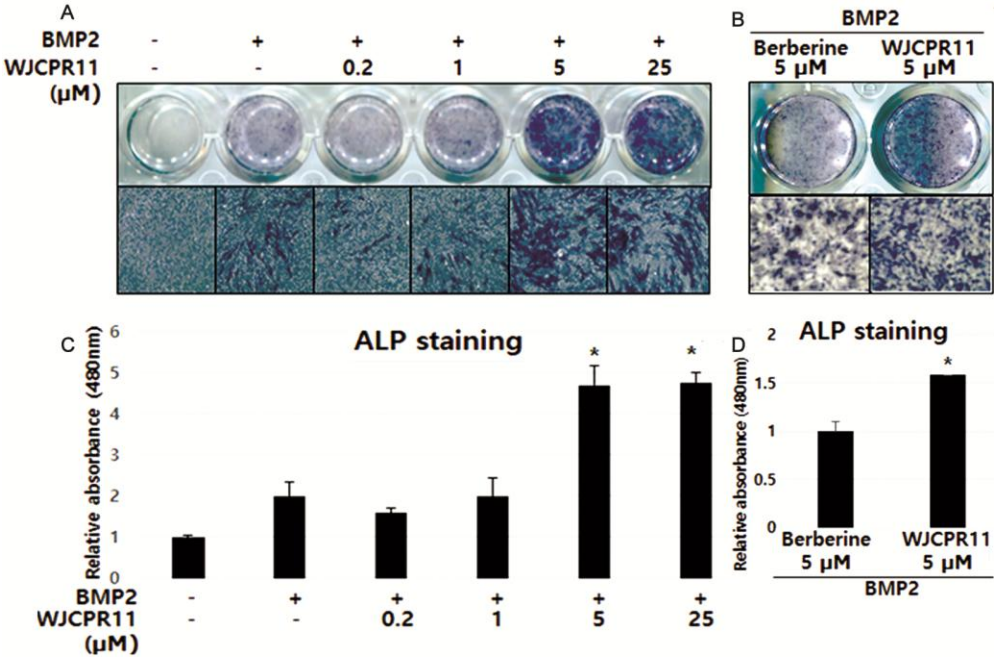

Fig. 2 — Alkaline phosphatase (ALP) staining of WJCPR11 during osteoblast differentiation. [(A) C2C12 cells were treated with BMP2 (500 ng/mL) and WJCPR11 (0.2, 1, 5, 25 μM) for 3 days. Osteoblast differentiation was measured using ALP staining; (B) The relative absorption was normalized to that of the control group. **P* <0.05 compared with the BMP2-treated group; (C) C2C12 cells were treated with BMP2 (500 ng/mL) and berberine or WJCPR11 (5 μM) for 3 days. Osteoblast differentiation was measured using ALP staining; and (D) The relative absorption was normalized to that of the control group. **P* <0.05 compared with BMP2- and berberine-treated group].

WJCPR11 enhances osteogenic gene expression during osteoblast differentiation

Osteoblasts increase the expression of certain genes during bone formation. In the C2C12 differentiation process, we added 1 and 5 μM concentrations of WJCPR11 and then performed real-time RT-PCR to observe mRNA expression. The mRNA expression of ALP, a representative osteoblast differentiation indicator, was higher than that of the control group. The mRNA expression of OC, Runx2, and osterix was also increased (Fig. 4).

WJCPR11 increases osteogenic protein levels during osteoblast differentiation

Next, to discover how WJCPR11 regulates genes related to osteoblast differentiation, we conducted western blot analysis using Runx2, osterix, Col1α1, and BSP antibodies. As a control, we used an α-tubulin antibody. The protein levels of BSP and Col1α1, an osteoblast differentiation indicator, were increased by treatment with WJCPR11 compared to when BMP2 was used alone. In addition, the protein levels of the transcription factors Runx2 and osterix were also increased with WJCPR11 treatment (Fig. 5).

WJCPR11 regulates the transcription activity of osteoblastic genes

ALP-Luc, BSP-Luc, and OC-Luc were introduced into C2C12 cells to analyze the effects of WJCPR11 on transcriptional activity. OC-Luc transcriptional activity was increased by BMP2, while that of ALP-Luc and BSP-Luc was not. ALP-Luc and OC-Luc increased dose-dependently from 0.2 to 5 μM and decreased slightly at 25 μM. Among these, the gene expression of OC-Luc was highest at 5 μM. The gene expression of BSP-Luc increased dose-dependently (Fig. 6).

Discussion

Of late, osteoporosis has gained increased attention from researchers, and various approaches to treat osteoporosis have been developed. Treatment of osteoporosis is divided into bone resorption inhibitors and bone growth stimulators including parathyroid

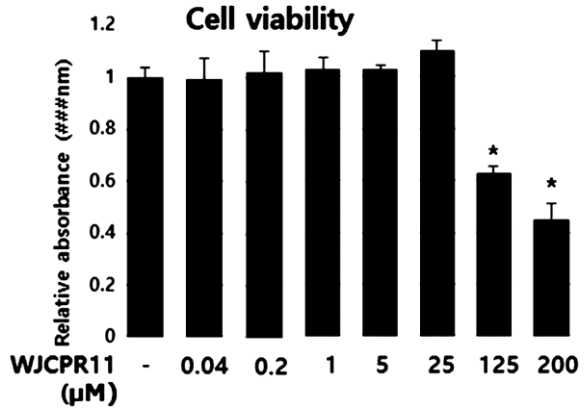

Fig. 3 — Cytotoxicity assessment according to WJCPR11 concentration. [C2C12 cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. C2C12 cells were treated with WJCPR11 at various concentrations for 48 h. *P <0.05 compared with the control group].

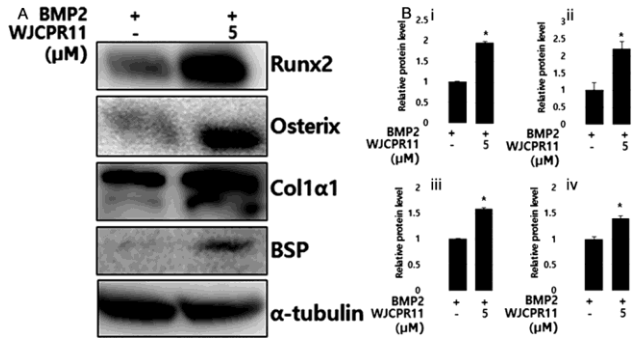

Fig. 5 — Effects of WJCPR11 on the protein levels of osteogenic genes. [C2C12 cells were treated with BMP2 (500 ng/mL) and 5 μM of WJCPR11 for 3 days. Western blotting analysis was performed using antibodies against runt-related transcription factor 2 (Runx2), osterix, Col1α1, and bone sialoprotein (BSP). α-Tubulin was used as a negative control. *P <0.05 compared with the BMP2-treated group].

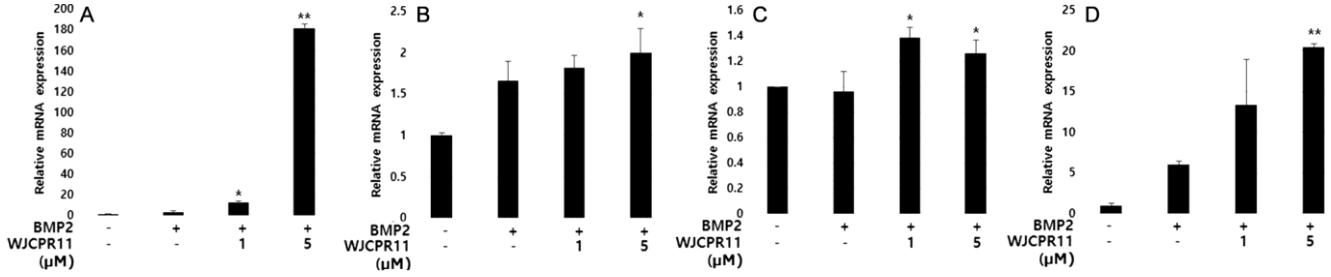

Fig. 4 — Effects of WJCPR11 on the mRNA expression of osteogenic genes. Real-time PCR was performed to confirm the levels of osteoblast differentiation-specific genes. C2C12 cells were incubated with BMP2 (500 ng/mL) and WJCPR11 (1, 5 μM) for 3 days. We used (A) alkaline phosphatase (ALP); (B) osteocalcin; (C) runt-related transcription factor 2 (Runx2); and (D) osterix as target primers. [Glyceraldehyde 3-phosphate dehydrogenase was used as a negative control. *P <0.05, **P <0.01 compared with BMP2-treated group].

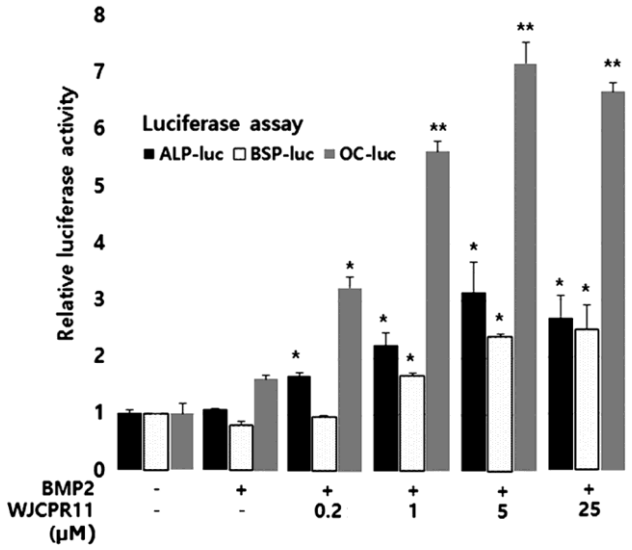

Fig. 6 — Effects of WJCPR11 on the transcriptional activity of osteogenic genes. [C2C12 cells were transfected with luciferase reporters (ALP-Luc, BSP-Luc, or OC-Luc). β-gal was used as a reporter assay and internal control. After 24 h, the cells were treated with WJCPR11 at the indicated concentration. After 24 h, luciferase activities were measured. **P* <0.05, ***P* <0.01 compared with the BMP2-treated group].

hormone preparations, denosumab, and bisphosphonates[19-21]. Among these, bisphosphonates, which are bone resorption inhibitors, account for 70-80% of the drugs used in the treatment of osteoporosis and are representative of the treatment at present[22,23]. However, current used bisphosphonates containing alendronate, ibandronate, and risedronate can cause several side effects such as bone, joint or muscle pain, irritation of the esophagus and gastric ulcer[24]. Above all, these drugs have the limitation of the absence of anabolic activity that forms new bones[25]. Therefore, to overcome the shortcomings of the therapy currently in use, new treatment and routes of administration are being developed. Research shows that osteoporosis is treated by promoting differentiation of osteoblasts with osteogenic agents[26]. Parathyroid hormone (PTH), which stimulates bone formation, is known to promote osteoblast differentiation by activating the PKR-like endoplasmic reticulum kinase (PERK)-eukaryotic initiation factor 2α (EIF2α)-activated transcription factor 4 (ATF4) signaling pathway[27]. In addition, Abaloparatide, a PTH-related analog, can decrease the rate of vertebral fractures and increase BMD, showing potential as a therapeutic agent for osteoporosis[28]. However, Abaloparatide has side effects such as hypercalciuria, dizziness, and nausea, and administration to patients for more than 2 years is not recommended[29]. Osteoporosis drugs are often taken for long periods and often in older age groups; thus, many studies are needed to ensure their safety. Therefore, to improve side effects, a study was conducted on berberine derivatives, a new substance capable of promoting bone formation.

In previous several studies, it has indicated that natural substances can promote osteoblast differentiation[30-32]. For example, an herbal extract (ExMH-PGR) containing gilgyeong root (Platycodon grandiflorum root) is effective in preventing and treating osteoporosis by inhibiting TRAP activity and actin ring formation to promote osteoblast activity[33]. In addition, the ethanol extract of *Leonurus sibiricus* is expected to be effective in improving osteoporosis by promoting the activity of the basic phosphatase involved in osteoblast differentiation and reducing the number of multinuclear osteoclasts and TRAP activity[34]. However, research on the related genes, mechanisms of action, purification, separation of active ingredients, etc. is insufficient, and the drug has limited efficacy.

Berberine is effective in preventing osteoporosis by promoting osteoblast differentiation[35]. Research has shown that Q8, a structurally similar compound to berberine, can prevent osteoporosis by improving the function of osteoblasts through inhibition of PPARγ[36]. In addition, a study to investigate the mechanism of action of berberine in osteoporosis-induced mice found that the levels of OC, ALP, MDA, and RANKL were reduced by the antioxidant effect of berberine[37]. However, there is a disadvantage in that the effect is not higher than that of bisphosphonate, which is the osteoporosis treatment currently used[38,39]. In addition, it has not been verified in clinical trials to be developed as a new drug[40].

The present study involved promotion of bone formation with a berberine derivative. The WJCPR11 used in the experiment showed no toxicity at a high concentration and showed efficacy and high ALP activity at this concentration. ALP is a bone metabolizing enzyme that specifically acts on bone and is related to the cellular function of osteoblasts. Its increase was observed clearly at 5 and 25 μM concentrations of WJCPR11, which indicates that WJCPR11 promotes osteoblast differentiation dose-dependently. In addition, mRNA expression of ALP was highest, and that of OC, Runx2, and osterix also was increased, indicating that WJCPR11 induces the expression of genes related to osteoblast

differentiation. In addition, the protein levels of BSP, Col1α1, Runx2, and osterix were increased. Particularly, Runx2, which is involved in the early stage of osteoblast differentiation and is essential for bone formation and osteoblast differentiation, and osterix, a key factor expressed in the later development of osteoblasts, were increased. Therefore, based on the increased expression of two genes important for bone formation, a luciferase assay was performed using RNA and protein transcription factors to discover the effect of WJCPR11. As a result, the transcriptional activity of ALP, BSP, and OC was increased by WJCPR11, with OC showing the most significant increase. These results suggest that WJCPR11 plays an important role in the later stage of osteoblast differentiation.

Conclusion

In this study, we synthesized berberine derivative WJCPR11 as a novel osteogenic compound, and have demonstrated its effeciency in preventing osteoporosis by promoting osteoblast differentiation of pre-osteoblast C2C12 cells without affecting the cell viability. The key markers for osteoblast differentiation including Runx2 and osterix were enhanced, and transcriptional activity of OC was markedly increased in BMP2-signaling dependent mechanism. It indicates possible use of WJCPR11 in regulating anabolic effects during bone remodeling process and potential replacement for the current treatment of osteoporosis. However, further studies on the mechanism of action, animal experiments, and clinical trials are necessary to validate this study.

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Conflict of interest

Authors declare no competings interests.

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