Original Research

A quinoxaline-based compound ameliorates bone loss in ovariectomized mice

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Impact statement

Glucagon-like peptide 1 (GLP-1) is an intestinal hormone with the activity of promoting insulin release. Several GLP-1 receptor peptide agonists are approved for the treatment of type 2 diabetes. Many studies have shown that GLP-1 plays a role in regulating bone homeostasis. However, these peptide molecules have the disadvantages of difficulty in preparation, instability, high preparation cost, and need for injection, which limit their clinical application. The small molecule compound 6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline (DMB) can directly activate GLP-1R and increase the affinity of natural GLP-1 to GLP-1R. We evaluated the effectiveness of DMB in the treatment of menopausal osteoporosis in ovariectomized (OVX) mice. We found that DMB can effectively prevent the loss of bone mineral density and improve the microstructure of trabeculae and the biomechanical properties of bone. These results indicate that the quinoxaline-based GLP-1R small molecule agonist DMB may be a potential drug candidate for the treatment of postmenopausal osteoporosis.

Abstract

DMB (6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline) is a quinoxaline-based compound that has been investigated as a glucagon-like peptide-1 receptor (GLP-1R) agonist. To clarify anti-osteoporosis effect of DMB, an osteoporotic mice model was established by ovariectomy (OVX) operation. The OVX mice were given intraperitoneally DMB, exendin-4 (EX-4), or 17β-estradiol (E_2) for two months. Then bone mass and structure, and bone morphometric parameters were examined by micro-CT. Weight gain and food consumption, bone turnover markers, and biomechanical strength of the femur were tested, and bone histomorphometry was analyzed. The food intake and weight gain was obviously reduced by E_2 or EX-4, but not DMB. However, DMB or EX-4 treatment obviously inhibited skeletal deterioration and enhanced bone strength. The improvement involved in the increased osteoblast number and level of bone formation markers, and reduced osteoclasts number and level of bone resorption markers. In addition, DMB was found to stimulate osteoblastogenesis-related marker gene expression. These results demonstrated that DMB ameliorated bone loss mainly via induction of bone formation, which suggests that the small molecule compound might be applied to the management of postmenopausal osteoporosis.

Keywords: Osteoporosis, glucagon-like peptide 1 receptor, small molecule agonist, 6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline, bone metabolism

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Introduction

Osteoporosis is a senile skeletal disease accompanied by a rapid and continuous decline in bone mineral density.[1] About half of women after menopause would have fractures caused by osteoporosis, so new treatments are required to deal with postmenopausal bone loss.[2–4] It is more evidence that glucagon-like peptide 1 receptor agonists (GLP-1RAs) play essential roles in regulating bone

homeostasis.[5–9] A GLP-1RA exendin-4 (EX-4) could facilitate senescent primary rat osteoblasts proliferation.[6] Another GLP-1RA, liraglutide, was also found to regulate murine pre-osteoblast MC3T3-E1 cell differentiation.[10] GLP-1 or its agonists could promote bone formation and improve bone strength in animal models of osteoporosis.[5,7,11–13]

In general, most experimental studies support the view that GLP-1RA has a beneficial effect on bone health, although divergent effects of GLP-1RAs on skeletal health have been found in humans [10,14,15] and more clinical studies are needed. However, GLP-1 and its derivatives, as peptide molecular, are difficult to prepare, have poor storage stability and high cost, and must be administered by subcutaneous or intravenous injection, which limits their clinical application.[16–19] While small molecule GLP-1RA can overcome these shortcomings.[17,20]

In recent years several small molecules GLP-1RA have been successfully developed, containing a class of quinoxalines and cyclobutane derivatives.[21–23] A quinoxaline compound, 6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline (DMB), could directly activate GLP-1R and enhance the binding affinity of GLP-1 for GLP-1R. *In vitro*, DMB could induce GLP-1R-mediated cAMP formation and promote insulin release in both mouse pancreatic islets and BRIN-BD11 beta cells.[24,25] *In vivo*, administration of DMB to mice in combination with glucose could significantly minimize the overall glycemic excursion.[24,25] However, it is not clear whether DMB has similar effects on bone metabolism. Hence, we examine the impacts of DMB on bone micro-architecture and metabolism in a menopausal OVX mouse model.

Materials and methods

Experimental animal model

Seven-week-old female C57BL/6J mice (16–18 g) were sourced from the Animal Center of the Fourth Military Medical University (Xi'an, China). All 50 animals were divided into two groups with SHAM group (control, ovary intact, $n = 10$) and OVX group (ovariectomy, $n = 40$). For OVX operation,[26] the mice were anesthetized with pentobarbital sodium. The back skin was shaved, and a cut was made to expose the muscles. Then both ovaries were separated, bound with sterile suture, and removed. The sham-operated mice were handled similarly, but the ovaries were not removed. Four weeks after surgery, the 40 OVX mice then were randomly subdivided into four groups (each $n = 10$): OVX group (olive oil), OVX+E_2 group (17β-estradiol, 10 μg/kg), OVX+DMB group (DMB, 1 mg/kg), and OVX+EX-4 group (EX-4, 4.2 μg/kg). DMB were purchased from Beijing Yisiyan Technology Development Center (Beijing, China). EX-4 was purchased from GL Biochem Ltd. (Shanghai, China). The mice were injected intraperitoneally for eight weeks. The dose of DMB was determined based on our previous results. Weight gain and food consumption were tested every four weeks.

After eight weeks of treatment, the mice were sacrificed, and blood samples were collected for biochemical analysis. The day before the experiment, the animals were fasted in metabolic cages (Hatteras Instruments, Cary, NC, USA) for 24 h, and urine samples were collected and acidified with 2 mL 1 M HCl. The femurs and vertebrae were dissected and stored for histomorphometric analysis and polymerase chain reaction analysis.

All experiments and animal welfares were carried out in accordance with regulations approved by the Animal Ethics Committees of the Xi'an Medical University (Xi'an, China).

Tomography analysis

After eight weeks of treatment, mice were anesthetized and the femurs were scanned with a micro-computed tomography (Micro-CT) system (SIEMENS, Germany). All scanning data were analyzed and three-dimensional (3D) reconstruction of the femoral head were performed basing on images. The bone mineral content (BMC), bone mineral density (BMD), and structural parameters bone volume/tissue volume ratio (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were calculated based on the 3D reconstruction results.

Three-point bending test

A material testing machine (MTS 858 Mini Bionix II, MN, USA) was used to perform three-point-ben test which was done as described previously.[27] The femur were thawed and subjected to increasing load at the loading rate of 1.2 mm/min until fracture. The load–deformation curve was generated to analyze the biomechanical properties of femur. According to the curve, the following biomechanical parameters were obtained: the maximum load (N), stiffness (N/mm), ultimate stress (MPa), and Young's modulus (MPa).

ELISA

N-terminal propeptide of type 1 procollagen (P1NP), osteocalcin (OC), and C-terminal cross-linked telopeptides of type 1 collagen (CTXI) were quantified in serum by ELISA (Cusabio Biotech Co., Wuhan, China). Urine deoxypyridinoline (DPD) level was analyzed by a commercial kit (Quidel Corporation, CA, USA). Creatinine (CRE) was quantified using a commercial picric acid colorimetric assay (R&D Systems, USA).

Quantitative RT-PCR assays

Vertebrae were frozen rapidly in liquid nitrogen and then homogenized with a ceramic grinder. RNeasy Mini Kit (Qiagen, Shanghai, China) and Prime Script RT reagent Kit (Takara, Japan) were used for RNA extraction and reverse transcription. Primer sequences described by Gao *et al*.[28] are used in this study (Supplementary Table 1). Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed by Mx3000P QPCR system with SYBR Premix EX Taq (TaKaRa). The relative gene expression of gene was calculated using $2^{-\Delta\Delta Ct}$ method. All RNA expressions were normalized to GAPDH.

Histological evaluation

The femurs were fixed, decalcified, and embedded in paraffin. Five micron paraffin sections were stained with hematoxylin and eosin (HE) or tartrate-resistant acid phosphatase (TRAP). Osteoblast numbers per tissue area (N. Ob/T.Ar) and osteoclast numbers per tissue area (N.Oc/T. Ar) were quantified under microscope.[29]

Statistical analysis

The data were expressed as means ± standard deviation (SD) and were analyzed using Prism Version 5 software (GraphPad Software Inc., USA). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a *post hoc* multiple comparison using Student–Newman–Keuls *t* test (normal distribution of data was checked by Kolmogorov–Smirnov test). $P < 0.05$ were interpreted as statistically significant.

Results

Effects of DMB on weight gain and food intake

OVX operation could lead to higher weight gain and food intake in mice compared with sham operation (Table 1). While treatment with E_2 or EX-4 but not DMB could abrogate OVX-induced the increase of weight gain and food intake.

DMB improved bone mass and strength of OVX mice

A marked bone loss and significant deterioration of bone architecture were found in OVX mice. After treatment with DMB, EX-4 or E_2, femoral BMC, BMD, and the trabecular microstructure were significantly improved (Figure 1). Consistently, administration of DMB, EX-4, or E_2 for two months also led to a significant increase in BV/TV, Tb.N, and Tb.Th, and a remarkable reduction in Tb.Sp compared to OVX mice (Figure 2).

Biomechanical analysis revealed that the maximum load, stiffness, Young's modulus, and ultimate stress of femur in OVX group were significantly lowered compared to SHAM group. However, the change of these biomechanical parameters were obviously attenuated in DMB, EX-4, or E_2 treatment group (Table 2).

DMB improved biochemical parameters of OVX mice

To determine the effects of DMB on bone turnover, bone formation biomarkers (OC and P1NP) and resorption biomarkers (DPD/CRE ratio and CTXI) were analyzed. Urinary DPD/CRE and serum CTXI, OC, and P1NP levels were increased much more in OVX mice (Figure 3). DMB or EX-4 treatment significantly decreased DPD/CRE ratio and serum CTXI and elevated serum P1NP level in OVX mice (Figure 3). E_2 remarkably lowered OC level of OVX mice (Figure 3(c)). However, DMB or EX-4 did not alter serum OC level of OVX mice.

DMB affected the expression bone metabolism-related genes

ALP is a marker for osteoblast activity and OC is produced by osteoblasts during bone formation. Runx2 is pivotal for bone formation and osteoblast differentiation. Col1 is an early marker in bone formation, and it is produced when mesenchymal stromal cells differentiate into osteoblasts.[30] Therefore, the effects of DMB on Runx2, ALP, Col1, and OC messenger RNA (mRNA) expression levels were measured. Compared with the OVX group, DMB markedly increased the four genes expression level, respectively (Figure 4). In addition, EX-4 also increased the mRNA levels of Runx2 and ALP, but did not change the Col1 and OC level (Figure 4).

DMB increased osteoblast numbers and reduced osteoclast numbers

The impacts of DMB on osteoclast or osteoblast numbers were analyzed by staining femoral sections with TRAP or HE, respectively. TRAP positive cells (dark purple staining) with at least three nuclei were deemed osteoclasts. Osteoblasts are generally cuboidal and arranged along the edge of the trabecular bone in a thin layer (Figure 5). Compared to the SHAM group, OVX caused a marked increase in the number of TRAP-positive osteoclasts and a marked decrease in the number of osteoblasts (Figure 5). However, DMB or EX-4 treatment abrogated the effect on the number of osteoclasts or osteoblasts. E_2 also markedly reduced osteoclast numbers, while slightly elevated osteoblast numbers.

Table 1. Effects of DMB treatment on body weight and food intake.

Time	SHAM	OVX	OVX + E_2	OVX + EX-4	OVX + DMB
Body weight (g)					
Before treatment	20.14 ± 0.81[a]	21.35 ± 0.95	21.15 ± 0.79	21.21 ± 1.00	21.41 ± 0.98
4 weeks after treatment	21.54 ± 1.74[b]	23.54 ± 0.91	21.98 ± 1.16[b]	22.48 ± 0.85	23.18 ± 1.59
8 weeks after treatment	22.92 ± 1.20[b]	25.5 ± 0.87	23.45 ± 1.78[b]	24.15 ± 0.87[a]	25.12 ± 1.50
Food intake (g/day)					
Before treatment	5.67 ± 1.12	5.16 ± 1.14	5.38 ± 1.05	5.75 ± 1.20	5.59 ± 0.83
0–4weeks after treatment	6.34 ± 1.08[a]	7.97 ± 1.28	6.69 ± 1.23[a]	7.33 ± 1.11	7.83 ± 0.94
5–8 weeks after treatment	6.47 ± 1.14[b]	8.19 ± 1.04	7.01 ± 1.11[a]	7.11 ± 0.92[a]	7.99 ± 1.06

All measurements are expressed as the mean ± SD (n =10); sham operation was performed by exposing the ovaries without isolation (SHAM); OVX treated with vehicle (OVX); OVX treated with 17β-estradiol (OVX + E_2, 10 μg/kg/d); OVX treated with DMB (OVX + DMB, 1 mg/kg/d); and OVX treated with exendin-4 (OVX + EX-4, 4.2 μg/kg/d).

[a] $P < 0.05$.

[b] $P < 0.01$ compared with OVX.

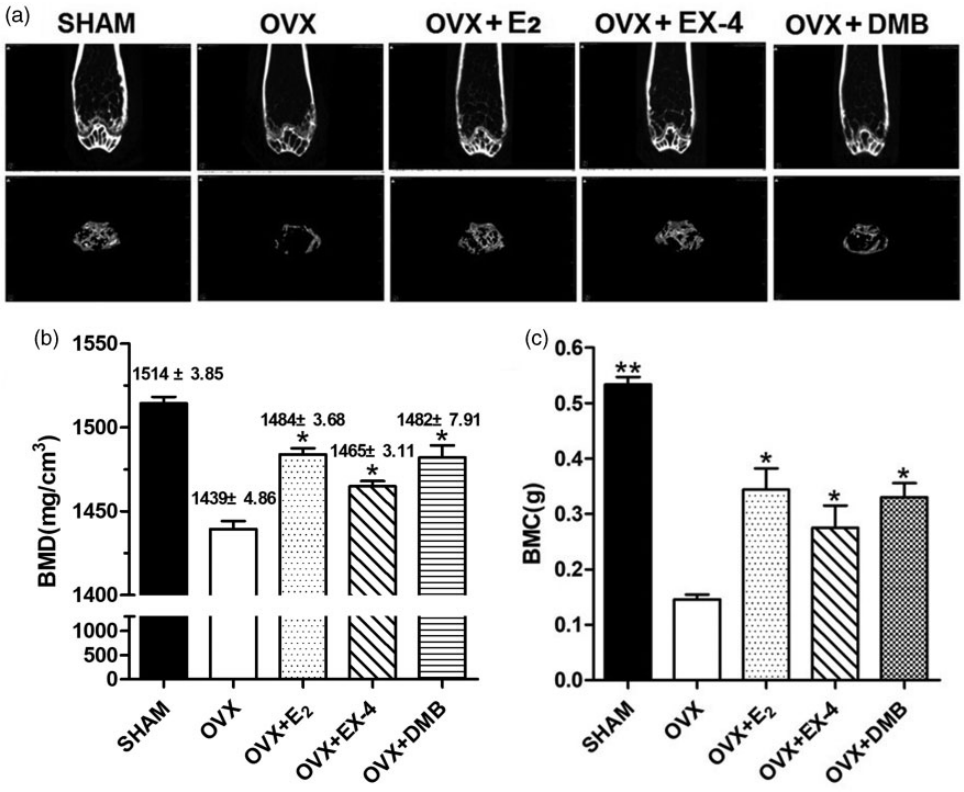

Figure 1. Effects of DMB on (a) bone trabecular architecture, (b) BMD, and (c) BMC in femur. ($n = 10$); *$P < 0.05$, **$P < 0.01$ vs. OVX group. BMD: bone mineral density; BMC: bone mineral content; DMB: (6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline); OVX: ovariectomy; E_2: 17β-estradiol; EX-4: exendin-4.

Discussion

In recent years, GLP-1 and its peptide GLP-1RA have attracted increased research interest, because activation GLP-1R can not only regulate glucose metabolism but also protect neuron from various damaged responses [31,32] and involve in the bone metabolism pathways, including their effects and possible relationship with osteoporosis.[33] To evaluate the anti-osteoporosis impacts of DMB on bone remodeling, we established a mouse model of osteoporosis. OVX-induced bone loss in female mice is regarded as a good model for menopause-related osteoporosis. The estrogen deficiency that occurs after ovariectomy would lead to a significant increase in food consumption, body weight, and bone loss in mice.[34,35] E_2, as a positive control, reverses these changes, and decreases food intake, promotes weight loss and improves bone status in OVX mice.

GLP-1 produces a variety of biological activities related to the wide distribution of its receptor.[36] GLP-1Rs are also distributed in the brain tissue, including the areas implicated in the regulation of feed intake and energy expenditure. Although the blood–brain barrier (BBB) blocks most substances from taking in the brain, it is reported that EX-4 and liraglutide could enter the brain by crossing BBB and produce the receptor-mediated effects such as inhibiting feed consumption and weight gain in rodents.[37] Our result is consistent with the previous results. However, DMB treatment has no impact on feed consumption and weight gain in OVX mice. *In vitro*, the quinoxaline compound DMB could directly bind and activate GLP-1R. It is not yet clear whether DMB could pass through the BBB and activate GLP-1R in brain tissue. Further investigations will be required to describe tissue distribution of DMB in mice. This may explain why DMB has no effects on food intake and weight gain. In addition, small molecule agonists are different from peptide agonists in molecular weight and spatial structure, so small molecules may not completely mimicking the action of larger peptide agonists.[23,38] This may also be the reason why DMB and EX-4 have different effects on food intake and body weight of OVX mice.

BMD variation was considered as an indicator of osteoporosis.[13] Reduction of BMD and the destruction of bone structure can lower mechanical strength and elevate bone fragility. Our results revealed that DMB could increase bone density, improve the bone microstructure, and enhance mechanical strength in OVX mice. Consistent with previous reports, we found that EX-4 also exhibited beneficial effects on trabecular bone of osteoporotic mice. To investigate potential mechanisms, biochemical marker detection and bone histological examination were carried out. In this study, the improvement of bone mass in DMB-treated OVX mice might be associated with the increase in osteoblast number and serum level of bone formation biomarkers (P1NP), with a parallel decrease in the osteoclast number and level of bone resorption markers (serum CTXI and urine DPD/CRE ratio). The results indicated that DMB improved bone metabolism in OVX mice.

Runx2 is believed to be involved in the osteoblast differentiation. In addition, ALP and Col1 are specific bone formation markers. The qRT-PCR results demonstrated that

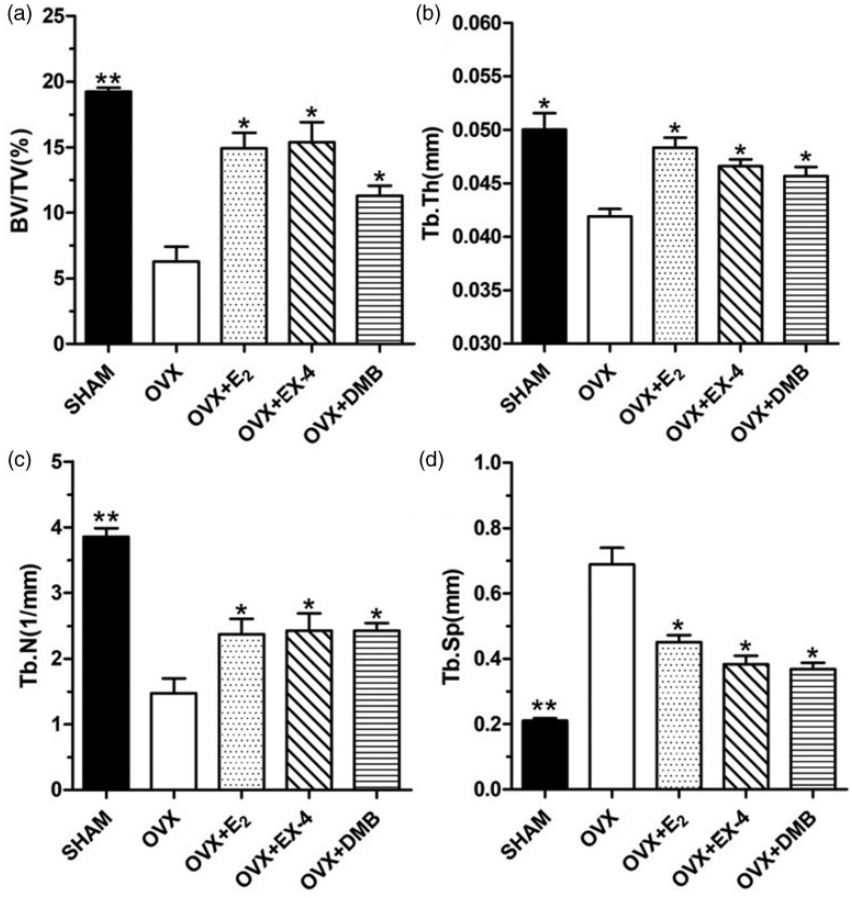

Figure 2. Analysis of bone morphometric parameters of femur. (a) BV/TV; (b) Tb.Th; (c) Tb.N; (d) Tb.Sp. ($n = 10$). *$P < 0.05$, **$P < 0.01$, compared to OVX group. DMB: (6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline); OVX: ovariectomy; E_2: 17β-estradiol; EX-4: exendin-4; BV/TV: bone volume/tissue volume ratio; Tb.Th: trabecular thickness; Tb.N: trabecular number; Tb.Sp: trabecular separation.

Table 2. Effects of DMB on biomechanical properties in femoral diaphysis evaluated by three point bending test in OVX mice.

Group	Maximum load (N)	Stiffness (N/mm)	Stress (Mpa)	Young's modulus (Mpa)
SHAM	16.79 ± 0.40^{b}	71.63 ± 3.16^{b}	31.44 ± 1.69^{c}	8.57 ± 0.59^{c}
OVX	10.98 ± 0.23	58.95 ± 2.64	13.80 ± 1.07	2.91 ± 0.23
OVX + E_2	14.63 ± 0.33^{a}	82.37 ± 3.13^{a}	19.31 ± 0.83^{b}	4.06 ± 0.31^{a}
OVX + EX-4	12.62 ± 0.26^{a}	71.59 ± 2.53^{a}	16.66 ± 0.98^{a}	3.01 ± 0.26^{a}
OVX + DMB	12.66 ± 0.30^{a}	68.94 ± 1.61^{a}	20.44 ± 1.04^{b}	5.23 ± 0.32^{b}

All measurements are expressed as the mean ± SD (n =10); sham operation was performed by exposing the ovaries without isolation (SHAM); OVX treated with vehicle (OVX); OVX treated with 17β-estradiol (OVX+E_2, 10 μg/kg/d); OVX treated with DMB (OVX+DMB, 1 mg/kg/d) and OVX treated with exendin-4 (OVX+EX-4, 4.2 μg/kg/d).
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$ compared with OVX.

DMB markedly enhanced the mRNA expression levels of Runx2, ALP, and Col1, indicating it may regulate the osteogenesis. It is reported that high serum OC level was found in postmenopausal osteoporosis patients, which is consistent with high bone turnover status.[39–42] In this study, the serum OC level in OVX group was obviously higher than that in SHAM group. While a marked reduction in the serum OC levels and urine DPD/CRE ratio were found in E_2-treated OVX mice, indicating an inhibition of bone turnover, which are consistent with a previous study.[13] Interestingly, compared to the OVX group, DMB treatment only changed the expression level of OC mRNA in bone, but did not affect serum OC. According to reports, there are two sources of OC in serum. Part of OC comes from the synthesis and release of osteoblasts during bone formation. The other part of OC comes from the degradation of osteoclastic bone matrix during bone resorption.[42] That means that OC mRNA level only reflects the rate of bone formation. Therefore, our results suggested that DMB may prevent bone loss mainly by promoting bone formation in OVX mice.

The demonstration that EX-4 and DMB ameliorate the symptoms of osteoporosis in OVX mice confirms

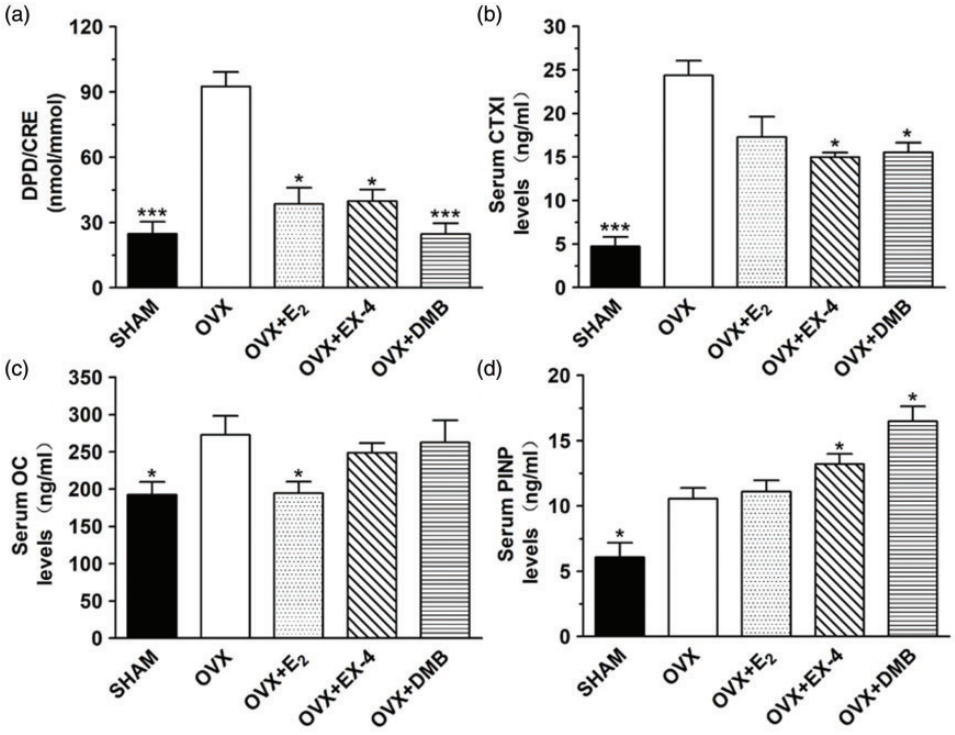

Figure 3. Effects of DMB on the biochemical markers of bone turnover in OVX mice. (a) Urinary DPD/CRE ratio, and the levels of (b) serum CTXI, (c) OC, and (d) P1NP were analyzed by ELISA ($n = 10$); $^{*}P < 0.05$, $^{***}P < 0.001$ vs. OVX group. OC: osteocalcin; DPD: deoxypyridinoline; CRE: creatinine; P1NP: N-terminal propeptide of type 1 procollagen; CTXI: C-terminal cross-linked telopeptides of type 1 collagen; DMB: (6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline); OVX: ovariectomy; E_2: 17β-estradiol; EX-4: exendin-4.

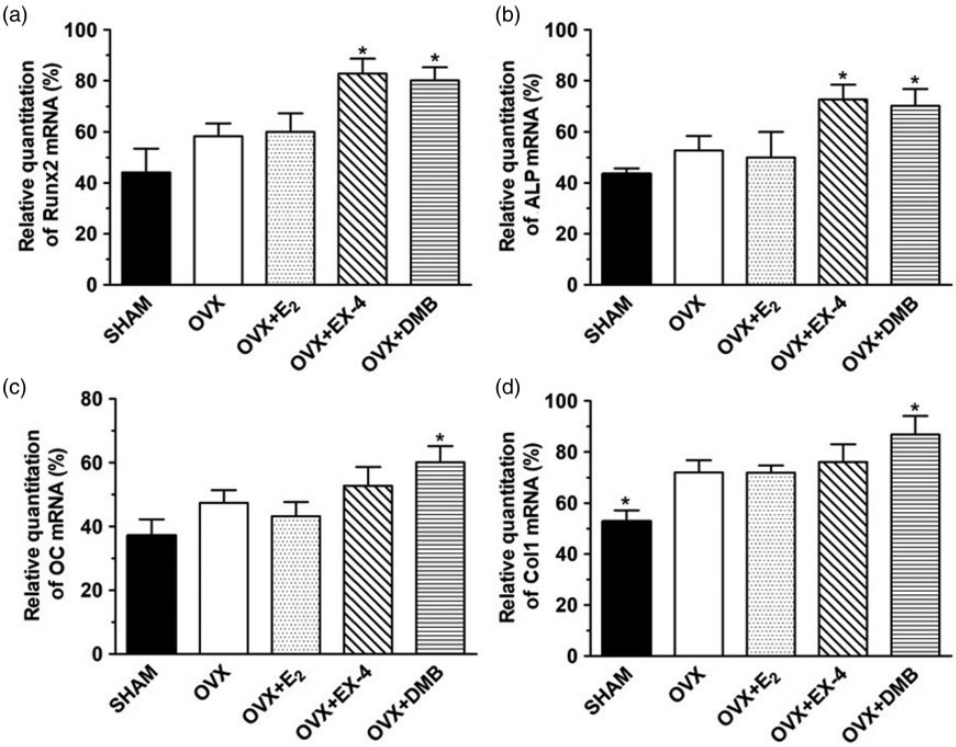

Figure 4. Effects of DMB on mRNA expressions of (a) Runx2, (b) ALP, (c) OC, and (d) Col1 in OVX mice ($n = 10$), $^{*}P < 0.05$ vs. OVX group. DMB: (6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline); OVX: ovariectomy; E_2: 17β-estradiol; EX-4: exendin-4; mRNA: messenger RNA.

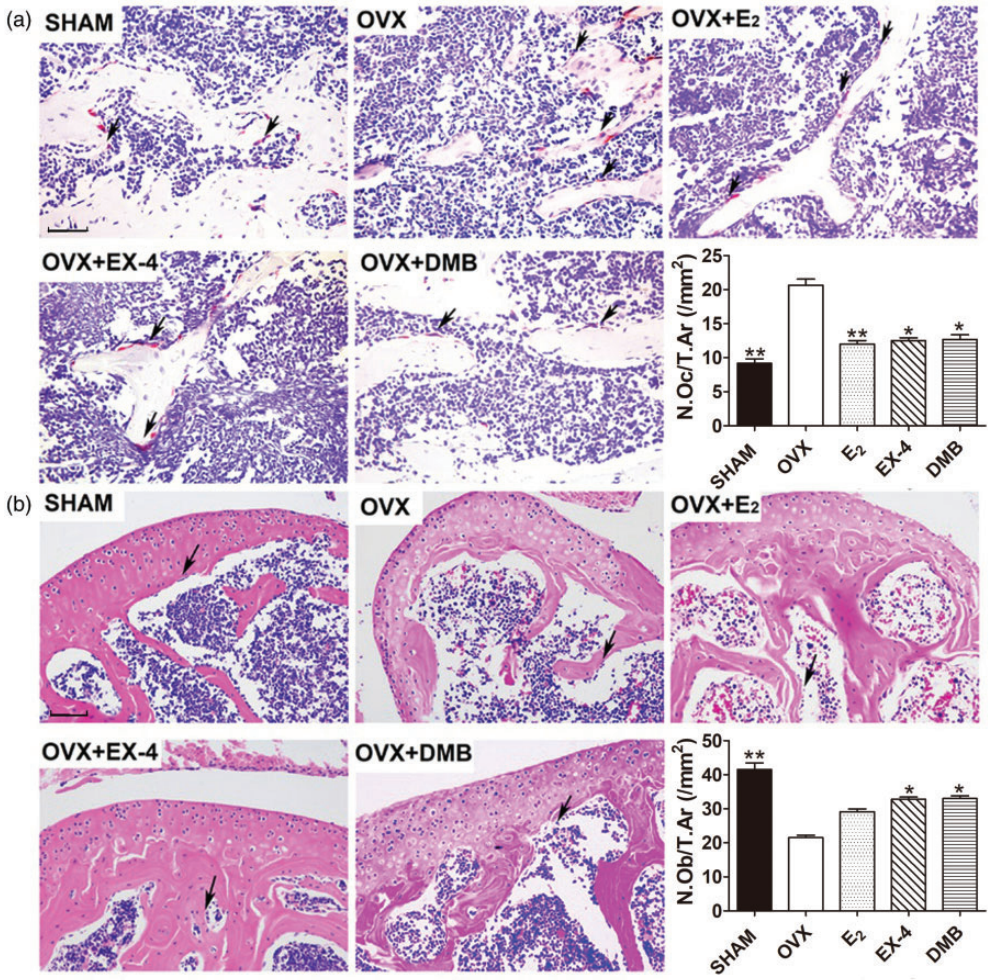

Figure 5. The effect of DMB on the number of osteoclasts and osteoblasts. (a) Osteoclasts or (b) osteoblasts were visualized using TRAP staining or HE staining on femoral sections. Basing on histomorphometric analysis the number of osteoclasts per tissue area (N.Oc/T. Ar/mm^2), or the numbers of osteoblasts per tissue area (N. Ob/T.Ar/mm^2) was obtained ($n = 10$); *$P < 0.05$, **$P < 0.01$ vs. OVX group. The bar length represents 100 μm. DMB: (6,7-dichloro-2-methylsulfonyl-3-Ntert-butylaminoquinoxaline); OVX: ovariectomy; E_2: 17β-estradiol; EX-4: exendin-4; N.Ob/T.Ar: osteoblast numbers per tissue area; N.Oc/T. Ar/mm^2: osteoclast numbers per tissue area. (A color version of this figure is available in the online journal.)

previously reported osteogenic effects of GLP-1 in animal models.[13,25,43] However, the effects DMB on the expression of Col1 and OC were different form EX-4, suggesting that their antiosteoporotic mechanisms might be somewhat different. GLP-1R activation by peptide agonists requires binding predominantly to the large N-terminal domain of the receptor.[23,38] Due to the difference in molecular size and spatial structure of peptide molecules and small molecule compounds, they may have different activation mechanism for GLP-1R. Moreover, bone metabolism relies on complex signaling pathways and control mechanisms to achieve proper rates of growth and differentiation. It is reported that GLP-1R is involved in some bone metabolism signaling pathways including Wnt signal,[44] the receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin (OPG) system,[45] sclerostin,[46] DKK expression[5] and so on. Although the small molecular compound DMB can improve bone density and help to prevent osteoporosis. However, the role of DMB on GLP-1R-mediated or non-GLP-1R-mediated signaling pathways, and the anti-osteoporosis effect in other animal models or in humans need further research in the future. To conclude, basing on the actions of DMB in promoting bone formation, clinical use of the small molecule GLP-1RA or anago-allosteric modulator might be an advantageous option to treat postmenopausal osteoporosis patients.

AUTHORS' CONTRIBUTIONS

YZ, XYX, NW, and ZC performed the experiments. YYG, FL, and ZH performed data analyses. YW, YZ, and HL designed and wrote the manuscript. All authors edited and approved the final version of the manuscript. YZ, XYX, and YYG contributed equally to this paper.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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