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A Novel Vitamin D Receptor Agonist, VS-105, Improves Bone Mineral Density without Affecting Serum Calcium in a Postmenopausal Osteoporosis Rat Model

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Abstract

Background and objectives: VS-105, a novel vitamin D receptor agonist with significantly less hypercalcemic side effects than calcitriol, is a useful tool to investigate whether or not a vitamin D receptor agonist at non-hypercalcemic doses could improve bone mineral density (BMD).

Methods: VS-105 and calcitriol were evaluated in an ovariectomized (OVX) osteoporosis rat model and in calvariae bone organ culture.

Results: Treatment of OVX rats by VS-105 (0.1, 0.2 or 0.5 µg/kg, intraperitoneal, 3×/week, for 90 days) significantly improved BMD in the L3 lumbar vertebra in a dose-dependent manner (sham vs. OVX/vehicle: 324 ± 14 vs. 279 ± 10 mg/cm²; VS-105 at 0.1, 0.2 and 0.5 µg/kg: 306 ± 9 , 329 ± 12 , and 327 ± 10 mg/cm², respectively) without affecting serum calcium (Ca). Calcitriol at 0.1 µg/kg significantly increased BMD but it also increased serum Ca. VS-105 and calcitriol at the test doses significantly suppressed serum parathyroid hormone and promoted tibia bone growth. With respect to biomarkers of bone remodeling, calcitriol and VS-105 both significantly elevated serum osteocalcin. In the calvariae bone organ culture, net Ca release was significantly less in VS-105-treated groups (vs. calcitriol).

Conclusions: VS-105 is efficacious in improving BMD in a dose range that does not affect serum Ca in OVX rats; the improvement in BMD by VS-105 is attributable to increased osteoblastic activity and reduced osteoclastic bone resorption.

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Author contributions

The present study was completed by the collaborative efforts of all authors. The manuscript was written by Wu-Wong and reviewed and approved by other authors.

Conflict of interest

Chen YW, Chen T, Wessale JL and Wu-Wong JR work for Vidasym.

Keywords

Bone mineral density; Osteoporosis; PTH; Vitamin D receptor; Vitamin D analog

Introduction

It is well documented that vitamin D is essential for bone health.¹ Vitamin D₃, synthesized in the skin, is not active and needs to be converted to 25-hydroxyvitamin D₃ (25(OH)D₃) and then further hydroxylated by 1-alpha-hydroxylase CYP27B1 to form the active hormone, calcitriol (1,25(OH)₂D₃). Calcitriol, the active metabolite of vitamin D, is a secosteroid hormone that, by activating the vitamin D receptor (VDR), regulates a variety of physiological processes and functions in various cells and tissues,^{2,3} including bone formation, intestinal calcium (Ca) transport, and synthesis of parathyroid hormone (PTH).⁴⁻⁹

Nutritional vitamin D (the inactive precursor of calcitriol) supplementation for the prevention and/or treatment of osteoporosis is advocated by some proponents but the subject remains an area of considerable controversy. However, vitamin D receptor agonists (VDRAs), such as calcitriol, 1 α -hydroxyvitamin D₃ (alfacalcidol), and eldcalcitol (ED-71, Ediolol®), have been used as therapeutic agents for osteoporosis for many years in a number of countries, albeit not in the USA.¹⁰⁻¹² These drugs increase bone mineral density (BMD) and reduce the incidence of bone fracture in patients with osteoporosis.^{10,11}

Despite encouraging clinical experience with VDRAs' benefits for the bone, in the USA, they are mainly indicated for managing secondary hyperparathyroidism in chronic kidney disease.^{13,14} The fact that VDRAs are not more widely used for treating osteoporosis is in part due to the hypercalcemic side effects of the current VDRAs; calcitriol, alfacalcidol and also eldcalcitol are known to induce hypercalcemia at therapeutic doses.¹⁵⁻¹⁷ Data exist to support that calcitriol, alfacalcidol and eldcalcitol exert direct effects on the bone.^{18,19} At the same time, these compounds, given at therapeutic doses, raise serum Ca, which plays an important role in the bone remodeling process. Thus, it is important to investigate whether or not VDRAs could exert beneficial effects on BMD, independent of a change in serum Ca.

We have previously reported that VS-105, a novel VDRA, exhibits a significantly wider therapeutic index than calcitriol, alfacalcitol and paricalcitol, when comparing their efficacies on reducing serum PTH vs. hypercalcemic side effects in the 5/6 nephrectomized uremic rats.²⁰ The mechanism(s) of action for the less hypercalcemic side effect of VS-105 is attributable to its reduced effect on stimulating intestinal Ca absorption and on releasing Ca from the bone.²¹ VS-105 has completed a Phase 1 clinical study involving healthy subjects ([Clinicaltrials.gov #NCT03043482](https://clinicaltrials.gov/ct2/show/study/NCT03043482)); the data show that VS-105 is well tolerated with no drug-related adverse events or other issues. In this report, to investigate whether or not a VDRA can improve BMD independently of raising serum Ca, we compared calcitriol and VS-105 in an OVX rat model of osteoporosis and also in the calvariae bone organ culture. The results suggest that VS-105, in a dose range that does not induce hypercalcemia, is efficacious in stimulating bone formation with reduced bone resorption, leading to increased BMD.

Materials and methods

Materials

VS-105 ((1R,3R)-5-((E)-2-((3 α S,7 α S)-1-((R)-1-((S)-3-hydroxy-2,3-dimethylbutoxy)ethyl)-7 α -methyldihydro-1H-inden-4(2H,5H,6H,7H,7 α H)-ylidene)ethylidene)-2-methylenecyclohexane-1,3-diol) and calcitriol (1,25-dihydroxyvitamin D₃) were synthesized by Vidasym (Chicago, IL, USA). The synthesis scheme of VS-105 was published previously.²² All other reagents used were of analytical grade.

OVX rats

Female Sprague-Dawley rats at 8 months of age underwent a bilateral ovariectomy. Two weeks after the surgery, animals were administered vehicle (5% ethanol + 95% propylene glycol, 0.4 ml/kg), calcitriol, or VS-105 (doses as indicated), 3 \times /week, intraperitoneally, for 90 days ($n = 8-12$ per group). The dose range for VS-105 was chosen based on previous studies comparing its efficacy on suppressing serum PTH and its effect on affecting serum Ca.^{20,21} Untreated, age-matched sham rats served as controls. Blood samples were collected on day 0 (24 h before the first dose) and also on day 91 (24 h after the last dosing), and assayed for serum PTH, phosphate (Pi) and total Ca. At the end of the study, L3 lumbar vertebra and tibia bone samples were collected for further analyses (an approach similar to that used by Fu *et al.*,²³ Uchiyama *et al.*²⁴ and Wang *et al.*²⁵ for testing the effects of an agent on BMD). All animal studies were conducted under the auspice of the Office of Animal Care and Institutional Biosafety, University of Illinois at Chicago, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Per the NIH guidelines, the number of animals used in this study was according to the approved protocol to achieve a statistically significant group difference. The standard photoperiod for rodent rooms is 14 h of light and 10 h of darkness.

Details of animal grouping

Due to the difficulty in processing the bone samples from these rats, the study was staged in batches such that each batch had <30 rats and each batch always included sham and OVX-vehicle (as control) animals. All drug treatment groups were also represented in each batch. At the end of the studies, the data were compiled. The treatment groups and number of rats per group at the end of the study (a total of 69 rats) were: sham, $n = 11$; OVX-vehicle, $n = 12$; VS-105 at 0.1 $\mu\text{g}/\text{kg}$, $n = 9$; VS-105 at 0.2 $\mu\text{g}/\text{kg}$, $n = 9$; VS-105 at 0.5 $\mu\text{g}/\text{kg}$, $n = 10$; calcitriol at 0.02 $\mu\text{g}/\text{kg}$, $n = 9$; and calcitriol at 0.1 $\mu\text{g}/\text{kg}$, $n = 9$.

Measurements of physiological parameters

Serum PTH was measured using a rat intact PTH ELISA kit, obtained from Immotopics (San Clemente, CA, USA). Serum Ca was measured using the Stanbio LiquiColor Ca Assay Kit (Boerne, TX, USA). The serum Pi was determined using a Pi colorimetric assay (Catalog #K410-500; BioVision, Milpitas, CA, USA).

Three-dimensional computed microtomography analyses

Lumbar vertebra (L3) samples were fixed in 10% formalin for 3 days and then transferred to 70% alcohol. Three-dimensional computed microtomographic analyses of the L3 samples were performed with a 40 micro-CT (SCANCO Medical AG, Bassersdorf, Switzerland). The x-ray source voltage was 55 kVp, the source current was 145 μ A, and the integration time was 300 ms. The scanning resolution was set at a 10-micron voxel size. The Scanco 40 micro-CT was calibrated using a method reported by Mashiatulla *et al.*²⁶ A reconstruction of the bitmap dataset was used to build the 3-dimensional images. BMD from micro-CT was mean density of all voxels within the volume of interest. The analysis was conducted in a blinded manner, independently by a micro-CT technician who was not involved in the animal studies.

Bone growth assessment

Bone was fixed in 10% formalin for 3 days and then transferred to 70% alcohol. The tibia was isolated, cleaned and demineralized by treating with decalcifying solution (Thermo Scientific, Kalamazoo, MI, USA) and then transferred back to 70% alcohol. The tibia was embedded in paraffin and cut into 5- μ m sections. For the measurement of growth plate thickness, slides with longitudinal sections of tibia were stained with hematoxylin-eosin and the images were scanned with an Aperio ScanScope slide scanner (Leica Biosystems, Buffalo Grove, IL, USA), and then analyzed in a blinded manner. In blood samples collected from OVX and sham rats, and serum osteocalcin (as a marker for bone growth and osteoblastic activity)²⁷ was measured using an ELISA kit obtained from Immotopics.

Bone resorption assessment by *ex vivo* calvariae culture

The approach was as described in our previous publication.²⁸ Briefly, 1 week-old mice (CD-1/ICR mice) were sacrificed and the hemi-calvariae were removed and prepared for organ culture. Calvariae were incubated in DMEM containing 1 μ M indomethacin, 15% heat-inactivated horse serum and 10 μ g/mL heparin (resorption medium) overnight, and then transferred to fresh resorption medium without indomethacin and incubated with test agents (at 10^{-10} – 10^{-7} M) for 4 days. The amount of Ca released into the medium was determined. Due to the difficulty in handling these samples, the *ex vivo* bone culture study was staged such that each batch had <20 samples and each batch always included the negative control (C, no addition of drug) and the positive control (calcitriol at 10^{-8} M). All drug treatment groups were also represented in each batch. At the end of these experiments, the data were compiled. The treatment groups and the number of samples per group at the end of the study were: control (C, no addition of drug), $n = 20$; VS-105 at 10^{-10} M, $n = 4$; VS-105 at 10^{-9} M, $n = 4$; VS-105 at 10^{-8} M, $n = 8$; VS-105 at 10^{-7} M, $n = 4$; calcitriol at 10^{-10} M, $n = 4$; calcitriol at 10^{-9} M, $n = 4$; calcitriol at 10^{-8} M, $n = 10$; calcitriol at 10^{-7} M, $n = 4$.

Statistical analysis

Differences between sham and OVX rats with different treatments were assessed using a one-way ANOVA followed by a Dunnett's post-hoc test. A *t*-test with 95% confidence intervals of difference was used to assess differences between two groups. Statistical

significance was defined as $p < 0.05$, with $p < 0.001$ indicating highly statistically significant.

Results

Serum PTH, Pi and Ca in OVX rats

The chemical structures of calcitriol and VS-105 are shown in Figure 1. Figure 2a shows that calcitriol at 0.02 $\mu\text{g}/\text{kg}$ had no effect on serum Ca, but calcitriol at 0.1 $\mu\text{g}/\text{kg}$ significantly raised the serum Ca level. As a comparison, VS-105 at the test doses did not have significant effects on serum Ca. Serum Ca trended slightly higher on day 91 for the VS-105 0.5 $\mu\text{g}/\text{kg}$ group, but the difference (vs. pre-dosing) did not reach statistical significance. VS-105 and calcitriol produced significant suppression of serum PTH at all test doses (Fig. 2b). Both compounds exhibited no significant effect on serum Pi at all test doses (Fig. 2c).

Micro-CT scanning of L3 vertebrae from OVX rats

Figure 3a shows representative 3-D micro-CT scans; the quantitative results are summarized in Figure 3b–d. Compared to sham rats, BMD, bone volume/tissue volume and trabecular thickness were significantly reduced in the OVX rats treated with vehicle. Calcitriol at 0.02 $\mu\text{g}/\text{kg}$ exhibited a modest effect but the hypercalcemic dose at 0.1 $\mu\text{g}/\text{kg}$ produced a significant elevation in the three parameters above the sham level. In comparison, VS-105 improved the three parameters in a dose-dependent manner. When compared with the vehicle group, statistically significant improvement was observed for the VS-105 groups at doses of 0.2 and 0.5 $\mu\text{g}/\text{kg}$. When compared with the sham group, there were no significant differences observed between sham and VS-105 at all three doses.

Tibia growth plate in OVX rats

Figure 4a shows representative hematoxylin-eosin stained tibia. The quantitative results are summarized in Figure 4b. Compared to sham rats, the growth plate was significantly smaller in the OVX rats. Treatment with either VS-105 or calcitriol alone at all test doses resulted in significant restoration of the growth plate to the sham level. Tibia samples from the 0.2 $\mu\text{g}/\text{kg}$ VS-105 group were collected but not processed since the results are unequivocal that VS-105 at 0.1 $\mu\text{g}/\text{kg}$, similar to the VS-105 0.5 $\mu\text{g}/\text{kg}$ dose, already restored the growth plate to the sham level.

Serum osteocalcin in OVX rats

As shown in Figure 5, calcitriol at 0.1 $\mu\text{g}/\text{kg}$ significantly increased the serum osteocalcin level but the 0.02 $\mu\text{g}/\text{kg}$ dose of calcitriol produced no effect. Serum osteocalcin was significantly elevated by VS-105 in a dose-dependent manner.

Bone resorption in ex vivo calvariae culture

In the *ex vivo* calvariae culture (Fig. 6), the effect of calcitriol on Ca release reached a plateau at 1 nM, while VS-105 stimulated Ca release from the bone in a dose-dependent manner (vs. control – no drug). When comparing VS-105 and calcitriol at the same dose

such as 0.1 and 1 nM, it is evident that calcitriol induced significantly more Ca release. These data suggest that there was less bone resorption in the VS-105-treated samples.

Discussion

Calcitriol, alfacalcidol and eldecacitol have been used as therapeutic agents for osteoporosis in several countries (albeit not in the USA) for many years. These drugs exert direct effects on the bone.^{18,19} For example, eldecacitol has been shown to possess a strong inhibitory effect on bone resorption.^{18,19} At the same time, these drugs also raise serum Ca^{24,29,30}; Ca is known to impact various factors (e.g., PTH and Pi) involved in the bone remodeling process via a complex yet tightly regulated system.^{31–33} Thus, to delineate the direct vs. indirect (via raising Ca) effect of a VDRA on the bone, VS-105, with its significantly wider therapeutic window than calcitriol, was chosen as a tool to investigate whether or not a VDRA can exert beneficial effects on BMD without raising serum Ca.

In the OVX rat model, our data show that serum Pi was not significantly altered either in vehicle- or drug-treated groups. Interestingly, calcitriol at the two test doses suppressed PTH to a similar level, yet the low calcitriol dose of 0.02 µg/kg exhibited no significant effect on BMD. In comparison, the three test doses of VS-105 suppressed PTH to a similar level, and VS-105 improved bone parameters in a dose-dependent manner. These results suggest that the efficacy of VDRA on the bone is likely independent of their effects on serum phosphate and/or PTH in this OVX rat model.

Regarding BMD and serum Ca, calcitriol induced hypercalcemia at 0.1 µg/kg but not at 0.02 µg/kg. Meanwhile, calcitriol showed no significant effect on BMD at 0.02 µg/kg, yet it increased BMD at 0.1 µg/kg to a level significantly higher than that observed in the sham rats. In comparison, VS-105 increased BMD in a dose-dependent manner to a level similar to that in the sham rats without affecting serum Ca. These data suggest that different VDRA may exhibit differential effects on BMD and serum Ca. Seemingly, there is a correlation between serum Ca and BMD for calcitriol, but such a correlation is not observed for VS-105.

A word of caution should be added based on previous experiences with VDRA. While VDRA currently in clinical use for treating osteoporosis demonstrate consistent data when comparing animal studies and human trials, a lack of correlation between preclinical and clinical studies exists at least for one VDRA: 2MD. This compound restores various bone parameters, including BMD, to the sham level at non-hypercalcemic doses, and significantly raises BMD above the sham level at the hypercalcemic doses of 2.5–10 ng/kg/day in the OVX rat model.³⁴ However, in the clinical studies, the drug failed to increase BMD in postmenopausal women with osteopenia.³⁵ It is suggested that the lack of effect on BMD in the clinical studies is attributable to the dual activity of 2MD on stimulating bone formation and bone resorption.³⁵

In this study, attempts were made to investigate how VS-105 affects bone resorption vs. bone formation. Osteocalcin (or bone gamma-carboxyglutamic acid-containing protein) is secreted by osteoblasts during the bone formation phase of the remodeling process.^{36,37} It is

worth noting that, for serum osteocalcin in the OVX rat model, no significant difference was observed between sham and the OVX rat treated with vehicle; although, serum osteocalcin trended higher in the OVX rats, which is consistent with previous reports such as that by Ma *et al.*³⁸ However, Uchiyama *et al.*²⁴ reported that serum osteocalcin was significantly increased in the OVX rat, and further increased by eldecalcitol (ED-71) at a hypercalcemic dose (0.2 µg/kg) but not at a non-hypercalcemic dose. Our data show that in the OVX rat, VS-105 increased the serum osteocalcin level in a dose-dependent manner without affecting serum Ca. In comparison, calcitriol significantly increased the serum osteocalcin level only at 0.1 µg/kg but had no effect at 0.02 µg/kg. In the *ex vivo* calvariae culture used to investigate bone resorption, significantly less Ca release was observed in the VS-105-treated groups.

Previously, we reported that calcitriol induces more Ca release from the bone than paricalcitol in the *ex vivo* calvariae culture.²⁸ The data from the current study suggest that there is less Ca release and thus less bone resorption in the VS-105-treated samples. Consistently, the tibia growth plate was restored back to the sham level by VS-105. Although more studies are needed to further investigate the differential effects of VS-105 on osteoblasts and osteoclasts, the data obtained so far suggest that the efficacy of VS-105 on increasing BMD is likely due to its reduced effect on bone resorption while it effectively stimulates osteoblastic activity. Our data further highlight the possibility that VDRAs' effects on bone formation and resorption may be unique to each VDRA.

Future directions

Various vitamin D analogs with similar structural and biological characteristics have been shown to increase BMD and to improve bone strength in the OVX rat model of osteoporosis by their direct effects on osteoblasts and osteoclasts. However, in the clinical setting, different vitamin D analogs seem to perform differently, with at least one vitamin D analog not exhibiting efficacy in improving BMD in postmenopausal women with osteopenia after 1 year of treatment, albeit this compound was efficacious in the OVX rats. Thus, whether VS-105 is useful for the treatment of osteoporosis awaits the results from future clinical trials evaluating VS-105 in postmenopausal women with osteoporosis.

Conclusions

In summary, in this report, we demonstrate that VDR activation by VS-105 improves bone parameters, including BMD, without causing hypercalcemia in the OVX rat model of osteoporosis. The improvement of BMD by VS-105 is attributable to increased osteoblastic activity and reduced osteoclastic bone resorption.

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Data sharing statement

All data used to support the findings of this study are included within the article.

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Abbreviations:

Ca	calcium
OVX	ovariectomized
Pi	phosphate
PTH	parathyroid hormone
VDR	vitamin D receptor
VDRAs	VDR agonists

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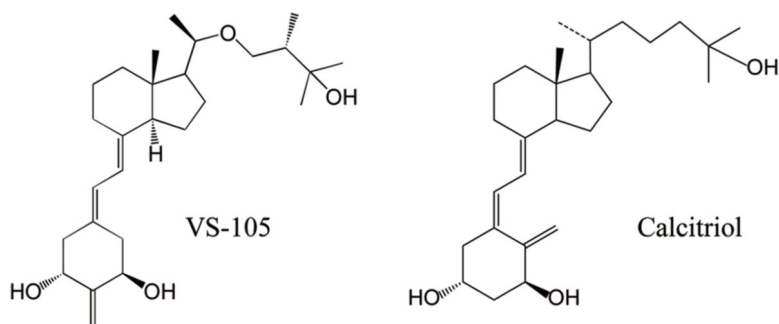


Fig. 1.
Structures of VS-105 and calcitriol.

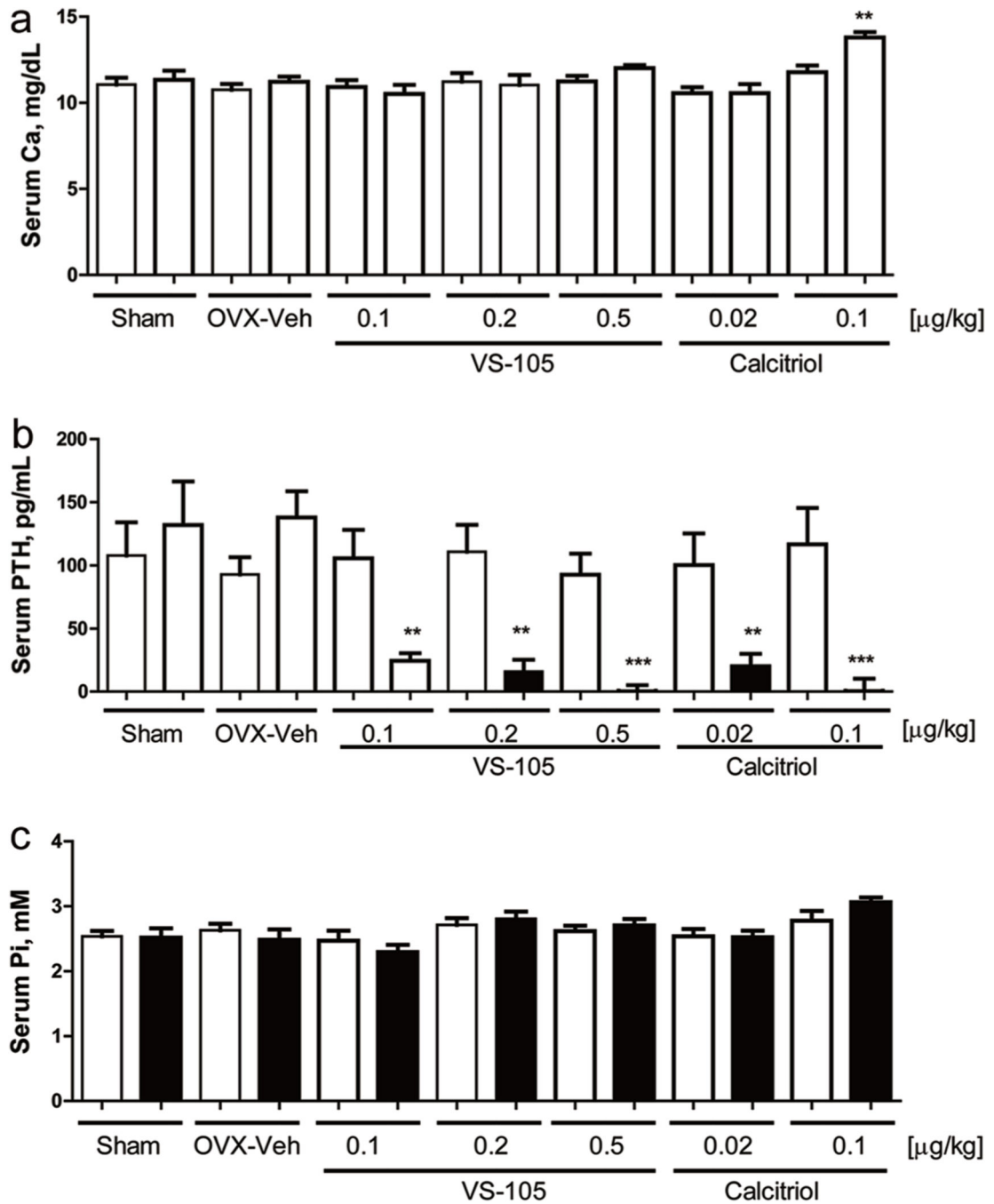


Fig. 2. Effects of VS-105 and calcitriol on serum Ca, Pi, and PTH levels in OVX rats. Rats were treated as described in the “Materials and Methods” (VS-105 at 0.1, 0.2, 0.5 $\mu\text{g}/\text{kg}$; calcitriol at 0.02, 0.1 $\mu\text{g}/\text{kg}$). Blood samples were collected for the measurement of serum Ca (a), PTH (b), and Pi (c) levels. Mean \pm standard error of the mean was calculated for each group. Unpaired *t*-test with 95% confidence intervals of difference was performed to assess differences between baseline day 0 (before treatment: white bar) and day 91 (after treatment: black bar). ** $p < 0.01$, *** $p < 0.001$ vs. pre-dosing, same group. Ca, calcium; OVX, ovariectomized; Pi, phosphate; PTH, parathyroid hormone; Veh, vehicle.

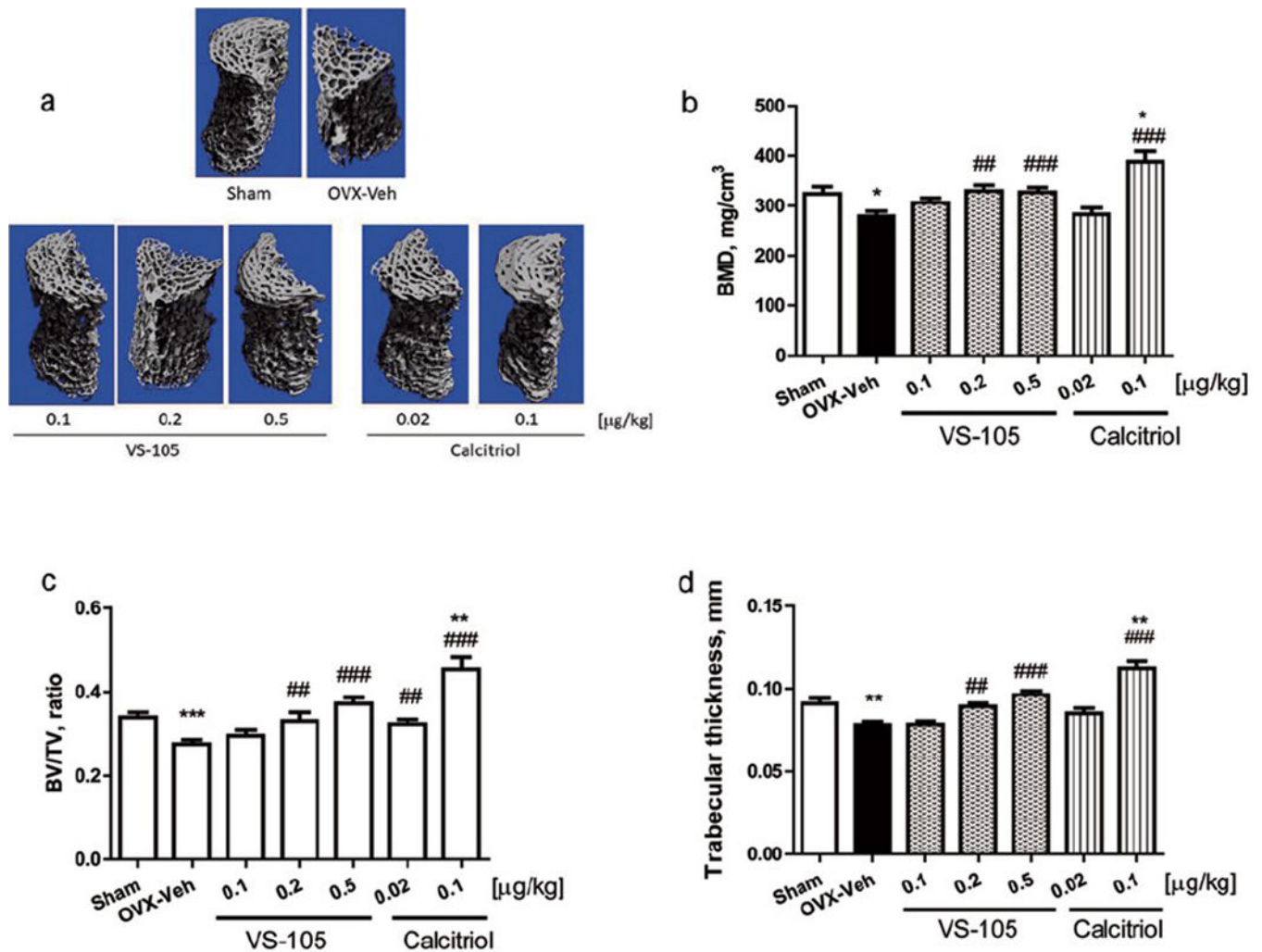


Fig. 3. Effects of VS-105 and calcitriol on L3 lumbar vertebra parameters in OVX rats. Rats were treated as described in the “Materials and Methods” (VS-105 at 0.1, 0.2, 0.5 μg/kg; calcitriol at 0.02, 0.1 μg/kg). Lumbar vertebra (L3) samples were fixed and three-dimensional computed microtomography analysis was conducted in a blinded manner. (a) Representative 3-D micro-CT scans of whole vertebra (L3) from each group in similar orientation. (b) BMD. (c) BV/TV. (d) Trabecular thickness. Differences among different treatments were assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test. ## $p < 0.01$, ### $p < 0.001$ vs. vehicle (OVX-Veh). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham. BMD, bone mineral density; BV/TV, bone volume/tissue volume; OVX, ovariectomized; Veh, vehicle.

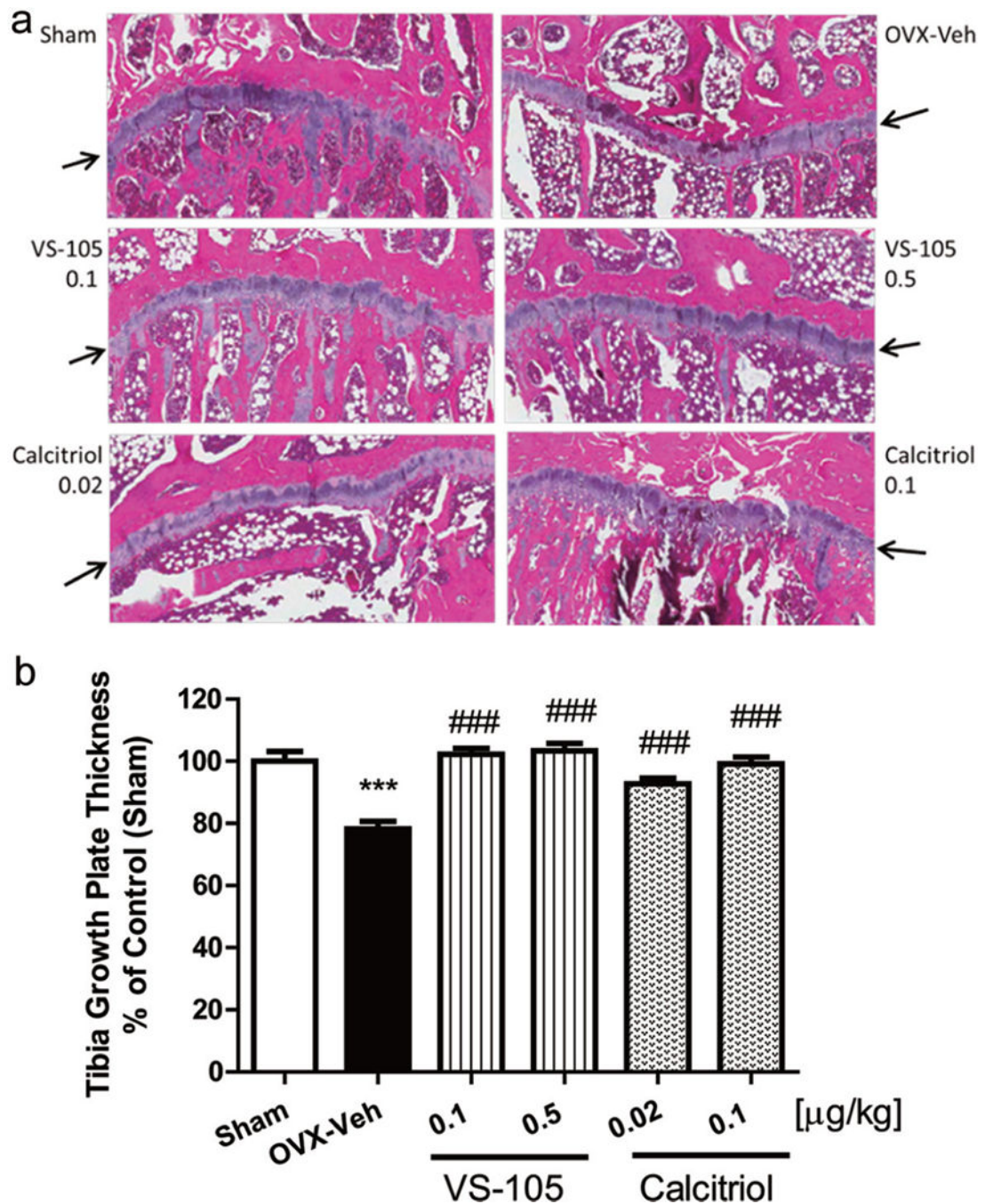


Fig. 4. Effects of VS-105 and calcitriol on tibia growth plate thickness in OVX rats. Rats were treated and tibia sections were stained as described in the “Materials and Methods”. (a) Representative (hematoxylin-eosin-stained) tibia with the growth plate region (black arrow). (b) Analyzed data for tibia growth plate thickness (expressed as % of sham). Differences among different treatments were assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test. ### $p < 0.001$ vs. vehicle (OVX-Veh). *** $p < 0.001$ vs. sham. OVX, ovariectomized.

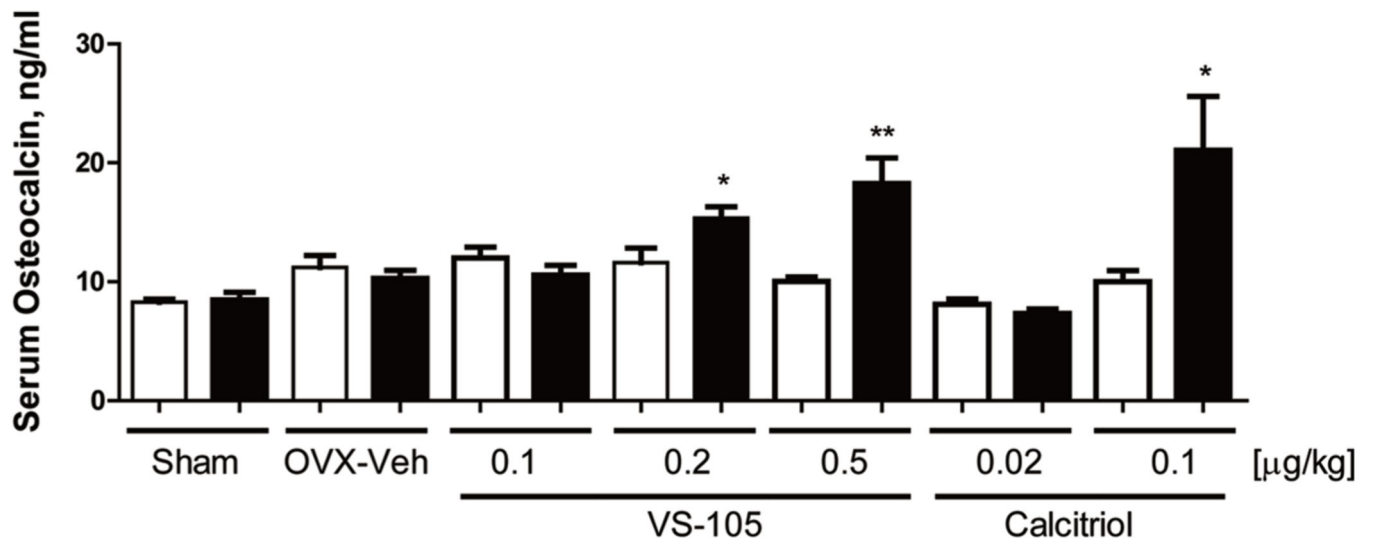


Fig. 5. Effects of VS-105 and calcitriol on serum osteocalcin in OVX rats.

Rats were treated as described in the “Materials and Methods”. Blood samples were collected for the measurement of serum osteocalcin. Unpaired *t*-test with 95% confidence intervals of difference was performed to assess differences between baseline day 0 (before treatment: white bar) and day 91 (after treatment: black bar). **p* < 0.05, ***p* < 0.01 vs. pre-dosing, same group. OVX, ovariectomized; Veh, vehicle.

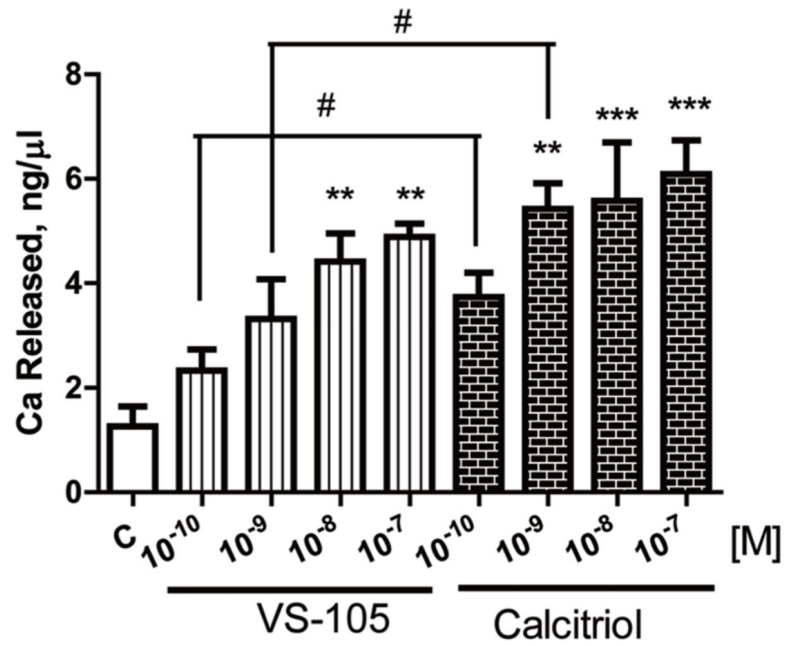


Fig. 6. Effects of VS-105 and calcitriol on bone resorption in *ex vivo* calvariae culture. Hemi-calvariae were prepared from 1 week-old mice as described in the “Materials and Methods”, and treated with test agents for 4 days (doses as indicated). The amount of calcium released into the medium was determined. C: Control, vehicle only. Differences among different treatments were assessed using a one-way ANOVA followed by a Dunnett’s post-hoc test. ** $p < 0.01$, *** $p < 0.001$ vs. control (vehicle). Unpaired t -test with 95% confidence intervals of difference was performed to assess differences between VS-105 and calcitriol at the same dose. # $p < 0.05$.