WY 1048, a 17-methyl 19-nor D-ring analog of vitamin D_3, in combination with risedronate restores bone mass in a mouse model of postmenopausal osteoporosis.

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Abstract

Bisphosphonates like risedronate inhibit osteoclast-mediated bone resorption and are therefore used in the prevention and treatment of osteoporosis. Also vitamin D_3 and calcium supplementation is commonly used in the prevention or treatment of osteoporosis. Combined therapy of risedronate with $1,25(OH)_2D_3$, the active metabolite of vitamin D_3, may be advantageous over the use of either monotherapy, but bears a risk of causing hypercalcemia thereby decreasing the therapeutic window for osteoporosis treatment. In this study, we evaluated the effect on bone mass of the combination of risedronate with the 17-methyl 19-nor five-membered D-ring vitamin D_3 analog WY 1048 in a mouse ovariectomy model for postmenopausal osteoporosis. Ovariectomy-induced bone loss was restored by administration of risedronate or a combination of risedronate with $1,25(OH)_2D_3$. However, the combination of WY 1048 with risedronate induced an even higher increase on total body and spine bone mineral density and on trabecular and cortical bone mass. Our data indicate that combination therapy of risedronate with WY 1048 was superior in restoring and improving bone mass over a combination of risedronate with $1,25(OH)_2D_3$ with minimal calcemic side effects.

Key words: vitamin D, risedronate, vitamin D analog, ovariectomy, osteoporosis

Vitamin D receptor (VDR), Ovariectomy (OVX), Bone mineral density (BMD)

1 Introduction

Osteoporosis is a metabolic bone disease characterized by a decreased bone mass and impaired bone architecture, both contributing to reduced bone strength and increased fracture risk. Although osteoporosis is prevalent in both sexes, more women are affected mainly because of the loss of the bone-protective effects of estrogens during menopause [1]. Among the first-line treatments for the prevention and treatment of osteoporosis in post-menopausal women are the bisphosphonates such as risedronate. Risedronate binds to the bone matrix where it leads to an increased osteoclast apoptosis rate. Already after a few days, this increase in osteoclast apoptosis leads to decreased bone resorption and a net gain in bone mass [2, 3].

Also the use of vitamin D_3 and calcium supplementation has been implicated in the management of osteoporosis since with ageing, the capacity of the kidney to produce active vitamin D_3 declines with negative consequences for calcium uptake [4]. Indeed, vitamin D_3 and, more specifically, its hormonally active form, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], is a major regulator of calcium homeostasis by stimulating intestinal calcium absorption and renal calcium re-absorption [5]. Moreover, in preclinical models of postmenopausal osteoporosis active vitamin D_3 metabolites can fully prevent estrogen-induced loss of trabecular and cortical bone by suppressing bone resorption [6–9]. Nevertheless, the use of active vitamin D_3 metabolites in the treatment of osteoporosis remains paradoxical as they, especially at higher doses, excessively stimulate intestinal calcium absorption resulting in calcemic side effects [10]. In addition, during a negative calcium balance vitamin D_3 metabolites aim to maintain normocalcemia by transferring calcium from the bone to the serum, which adversely impacts bone mass and strength [11].

Interestingly, a number of recent studies have shown that the vitamin D_3 analog eldecalcitol (ED-71) is a potent compound in the restoration of estrogen-induced bone loss by suppression of osteoclastic bone resorption and its use for the treatment of postmenopausal osteoporosis is approved in Japan [12, 13]. In our laboratories, the vitamin D_3 analog WY 1048, a 17-methyl 19-nor five-membered D-ring analog of 1,25$(OH)_2D_3$, was developed (**Figure 1**). This compound has a lower affinity (50%) for the vitamin D receptor (VDR) and a weaker binding (7%) to the vitamin D binding protein compared with 1,25$(OH)_2D_3$. Despite this lower VDR affinity, 10-fold lower concentrations of WY 1048, compared with 1,25$(OH)_2D_3$, were sufficient to reach half-maximal inhibition of MCF-7 breast cancer cell proliferation and 3-fold lower concentrations were enough to obtain half-maximal differentiation of HL60 promyelocytic cells [14, 15]. Evaluation of its calcemic effects revealed that a short-term treatment with 0.2 µg/kg/d WY 1048 induced a small gain in femoral calcium content without affecting serum and urinary calcium concentrations. Treatment with the same dose of 0.2 µg/kg/d 1,25$(OH)_2D_3$ led to overt hypercalcemia and hypercalciuria without increasing femoral calcium content (Supplementary Figure 1). This initial finding led us to evaluate the capacity of WY 1048 to restore bone loss in a preclinical mouse model of estrogen deficiency-induced osteoporosis either alone or in combination with risedronate.

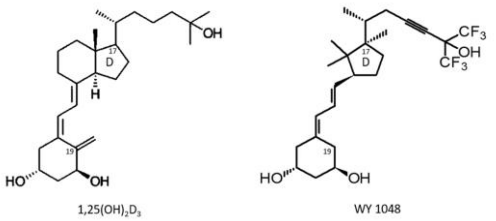


Figure 1. The chemical structure of 1,25(OH)$_2$D$_3$ and of WY 1048*, a 17-methyl-19-nor-five-membered D-ring analog of 1,25(OH)$_2$D$_3$. Modified from "Analogs of calcitriol"* [15].

2 Materials and methods

2.1 Substances

WY 1048 (17-methyl-19-nor-five-membered D-ring-1α,25-dihydroxyvitamin D_3) was originally synthesized by Yusheng Wu, P. De Clercq and the late M. Vandewalle from the University of Ghent (Belgium). Risedronate and calcitriol were purchased from Sigma Aldrich.

2.2 Animal procedures

For the ovariectomy (OVX) experiment, 12 week old, sham-operated (n=10) and ovariectomized mice (n=100) C3H mice (Charles River Laboratories) were used. Starting from 4 weeks after the surgical procedures, sham-operated mice received arachis oil as vehicle control (group 1). OVX mice were randomized into 10 different treatment groups (10 mice/group) as shown in **Table 1** and received daily doses of vehicle (arachis oil, group 2), risedronate (3 µg/kg/d, group 3), $1,25(OH)_2D_3$ (0.05 µg/kg/d or 0.125 µg/kg/d, groups 4 and 5) or a combination of risedronate and calcitriol (groups 6 and 7), WY 1048 (0.2 µg/kg/d or 0.5 µg/kg/d, groups 8 and 9) or a combination of risedronate and WY 1048 (groups 10 and 11). All compounds were administered orally via gavage on a daily basis over a period of 8 weeks. All animals were killed by an overdose of pentobarbital anesthesia. To confirm success of ovariectomy and estrogen depletion in OVX mice, the uterus was isolated and checked for atrophy. Mice were housed in a conventional animal facility with 12 h dark/light cycles and constant temperature with food and water supplied ad libitum. All animal experiments were approved by the ethical committee of the KU Leuven.

Table 1. Animal procedures, treatment groups.

	Group (n=10)										
	1	2	3	4	5	6	7	8	9	10	11
Risedronate (µg/kg/d)			3			3	3			3	3
$1,25(OH)_2D_3$ (µg/kg/d)				0.05	0.125	0.05	0.125				
WY 1048 (µg/kg/d)								0.2	0.5	0.2	0.5
surgical procedure:	sham	OVX									

2.3 Serum, urine, and bone biochemistry

For the OVX experiment, mice were transferred to metabolic cages (5 mice per cage) after 4 and 8 weeks of treatment to obtain 24 h urine collections. Twelve weeks after surgery (i.e. after 8 weeks of treatment) mice were euthanized and blood serum was obtained via cardiac puncture.
Calcium concentrations were determined in the individual serum samples and in the collective urine samples by a colorimetric assay (Synchron CX4 system, Beckman Instruments).

2.4 Micro-computed tomography (µCT)

Distal femur µCT images were taken on the high resolution Skyscan 1172 system (Bruker, Kontich, Belgium). The scanner X-ray source was set at a voltage of 50 kV and a current of 200 µA and a 0.5 mm aluminum filter was applied. Scans were taken with a pixel size of 5 µm, the scanning angular rotation was 180° with an angular increment of 0.4° and with a frame averaging of 2. Serial tomographs were reconstructed from raw data with the cone-beam reconstruction software (NRecon software, Skyscan, Bruker) based on the Feldkamp algorithm [16]. 3D morphometric analysis of reconstructed datasets was performed using the CT Analyzer software (CTAn, Bruker). Cortical and trabecular volumes of interest were manually selected [2 – 2.5 mm (cortical) and 0.5 – 1.5 mm (trabecular) under the selected reference point (point were trabecular bone is fully separated into four compartments on a transverse plane)]. Cortical and trabecular regions of interest were selected using the “automated trabecular and cortical bone selection method” (Method note, Bruker). Binary images for analysis were generated using a global thresholding of 80-255 (cortical) or 75-255 (trabecular). 3D models were constructed with the CTvox software (Bruker) using different selections [2.35 – 2.5 mm (cortical) and 0.25 – 2.75 mm (trabecular) under the same reference point].

2.5 Whole body dual energy X-ray absorptiometry (DEXA)

Areal total body and spine (lumbar vertebrae L1-L5) bone mineral density (BMD) and bone mineral content (BMC) were analyzed *in vivo* by dual-energy X-ray absorptiometry (DEXA, PIXImus densitometer; Lunar Corp., Madison, WI, USA) using ultra high resolution (0.18 x 0.18 pixels, resolution of 1.6 line pairs/mm) and software version 1.45.

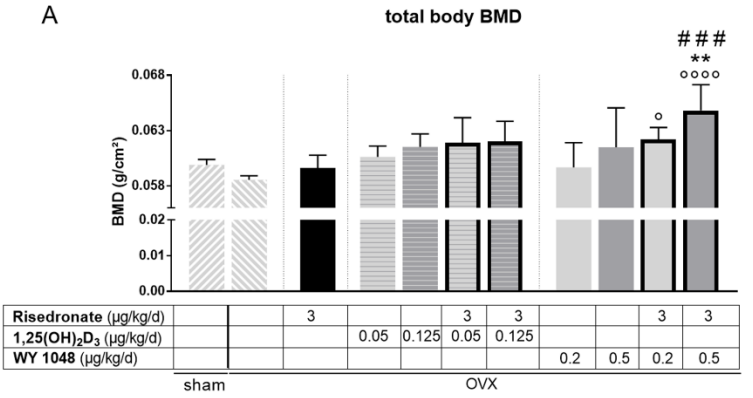
2.6 Statistical analysis

All statistical analyses were performed using Prism 7.04 (Graphpad Software, La Jolla, CA, USA). Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was carried out to detect overall differences followed by Sidak multiple comparison test to calculate intergroup differences. Differences were considered significant at $p<0.05$.

3 Results

3.1 Total body and spine BMD

Total body and spine BMD were decreased upon ovariectomy. However, for total body, statistical significance was lost after correction for multiple comparisons (**Figure 2**). Treatment with the antiresorptive agent risedronate fully restored total body and spine BMD. In addition, also single treatments with 1,25(OH)$_2$D$_3$ or the vitamin D analog WY 1048 led to a dose-dependent restoration of ovariectomy-induced bone loss, specifically at the level of the lumbar vertebrae L1-L5. The combination of 1,25(OH)$_2$D$_3$ with risedronate did not improve BMD above the levels observed after treatment with either agent alone. In contrast, the combination of WY 1048 with risedronate improved spine BMD levels significantly above the effects obtained by treatment with the single compounds. Especially, the combination of the highest dose of WY 1048 with risedronate in OVX mice resulted in a significant increase of BMD well above the levels observed in risedronate-treated OVX mice and even above the total body BMD of sham-operated, vehicle-treated mice. Similar observations were made for total body and spine BMC (data not shown).



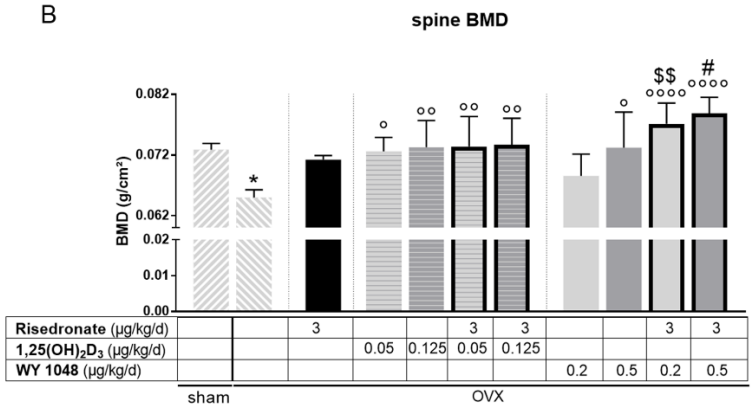
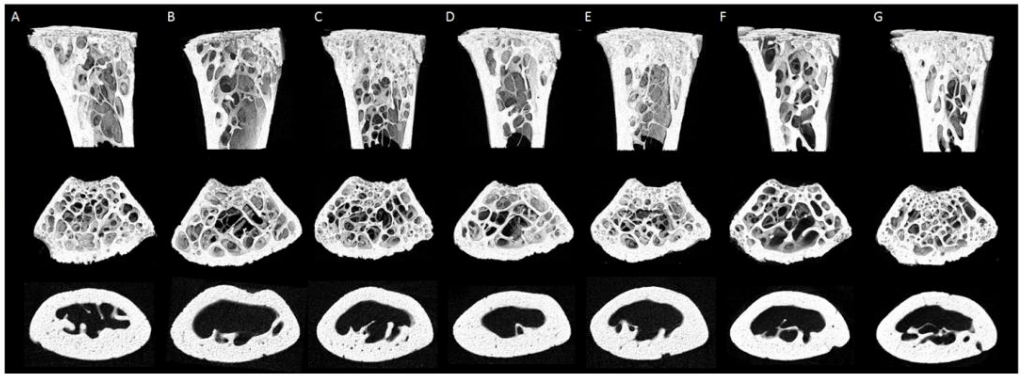


Figure 2. Combined therapy of risedronate and WY 1048 significantly improves both total body and spine BMD. *BMD was measured by DEXA for total body (A) and spine at the lumbar vertebrae L1-L5 (B) at 12 weeks post-surgery. Data are represented as mean ± SD values (n=6/group). *p<0.05 vs. SHAM vehicle; °p<0.05 vs. OVX vehicle; #p<0.05 vs. risedronate; $p<0.05 vs. dose respective monotherapy.*

3.2 µCT trabecular and cortical bone

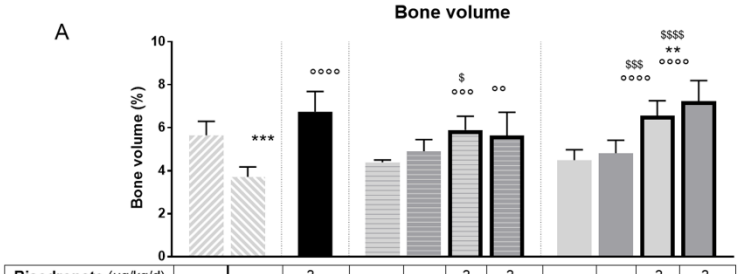
µCT analysis of the distal femur confirmed that ovariectomy induced significant trabecular bone loss, which was accompanied by a decrease in trabecular number and especially by a reduction of trabecular thickness (**Figure 3**, **Figure 4**). Treatment with risedronate efficiently restored OVX-induced bone mass (**Figure 4A)**, mainly by enhancing trabecular number (**Figure 4B**), whereas trabecular thickness was not affected (**Figure 4C**). Single treatment with either $1,25(OH)_2D_3$ or WY 1048 dose-dependently enhanced trabecular bone mass in ovariectomized mice but was not as effective as risedronate. In contrast to risedronate, both $1,25(OH)_2D_3$ and its analog WY 1048 had little effect on trabecular number whereas they increased trabecular thickness, and this was especially clear for the higher dose of WY 1048.

Addition of risedronate to treatment with $1,25(OH)_2D_3$ or WY 1048 led to a significant rise in trabecular bone mass, but only the combination with the higher dose of WY 1048 (0.5 µg/kg/d) surpassed the effect of risedronate alone. Interestingly, this combination treatment resulted in a trabecular bone mass that even significantly exceeded trabecular bone mass of sham-operated, vehicle-treated mice by increasing both trabecular number and thickness (**Figure 4**).



Risedronate (µg/kg/d)		3		3			3
1,25(OH)$_2$D$_3$ (µg/kg/d)			0.125	0.125			
WY 1048 (µg/kg/d)					0.5	0.5	
	sham	OVX					

Figure 3. Representative 3D models *of trabecular bone (sagittal and transverse plane) and cortical bone (transverse plane) for the indicated groups. (A) SHAM vehicle, (B) OVX vehicle, (C) OVX risedronate, (D) OVX 1,25(OH)$_2$D$_3$, (E) OVX 1,25(OH)$_2$D$_3$ + risedronate, (F) OVX WY 1048, (G) OVX WY 1048 + risedronate.*



Risedronate (µg/kg/d)		3			3	3			3	3
1,25(OH)$_2$D$_3$ (µg/kg/d)			0.05	0.125	0.05	0.125				
WY 1048 (µg/kg/d)							0.2	0.5	0.2	0.5
	sham	OVX								

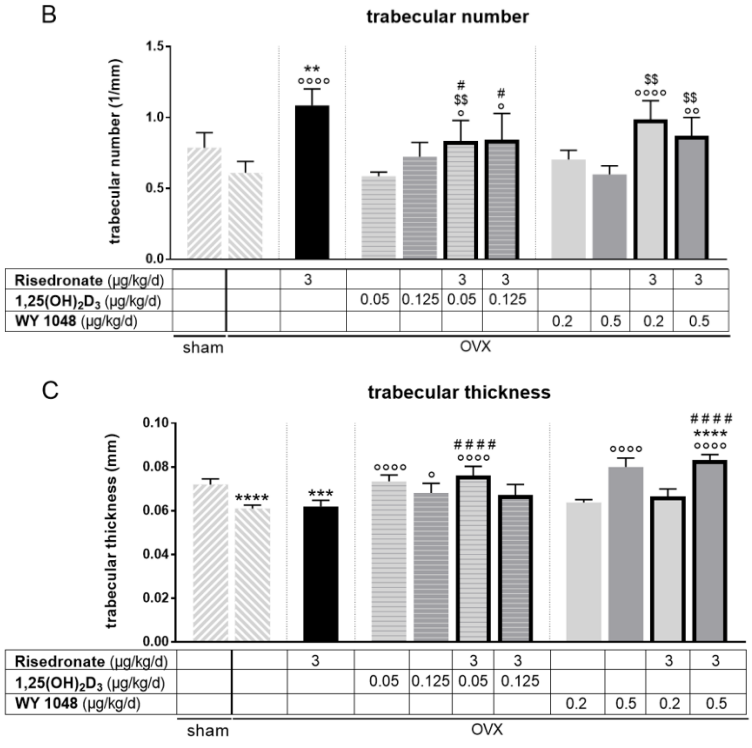


Figure 4. Combination of risedronate with the highest dose of WY 1048 significantly increases bone volume compared to all other treatments. *Microcomputed tomography data on bone volume (A), trabecular number (B) and trabecular thickness (C) of the selected trabecular region of the distal femur in sham and OVX mice treated with the indicated drugs and doses. Values represent mean ± SD values (n=6/group). *p<0.05 vs. SHAM vehicle; °p<0.05 vs. OVX vehicle; $p<0.05 vs. dose respective monotherapy.*

In this mouse model, ovariectomy did not result in cortical changes of the femur (**Figure 5**). Indeed, cortical thickness nor cortical porosity was significantly altered in OVX vehicle-treated mice. In addition, treatment with risedronate did not affect any of the cortical parameters. In contrast, $1,25(OH)_2D_3$ at the lower dose of 0.05 µg/kg/d and WY 1048 at the higher dose of 0.5 µg/kg/d both significantly enhanced cortical thickness and reduced cortical porosity. Again, the combination of risedronate with the higher dose of WY 1048 resulted in a cortical thickness that exceeded that of sham-operated, vehicle-treated mice.

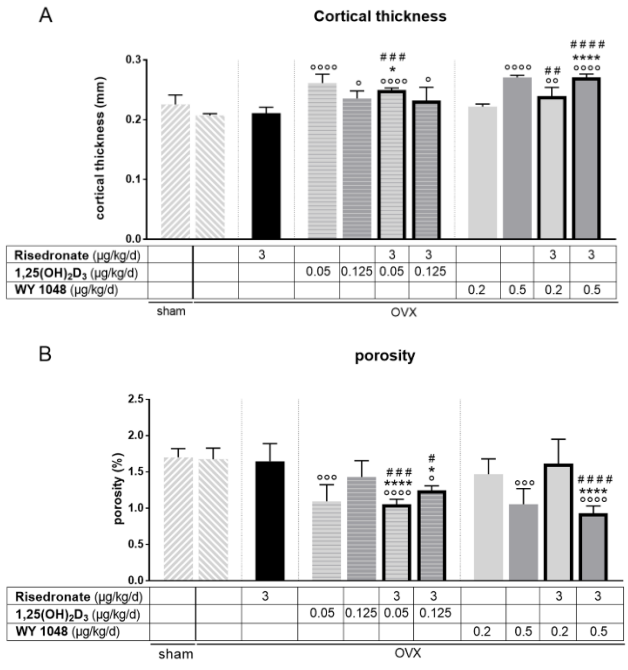
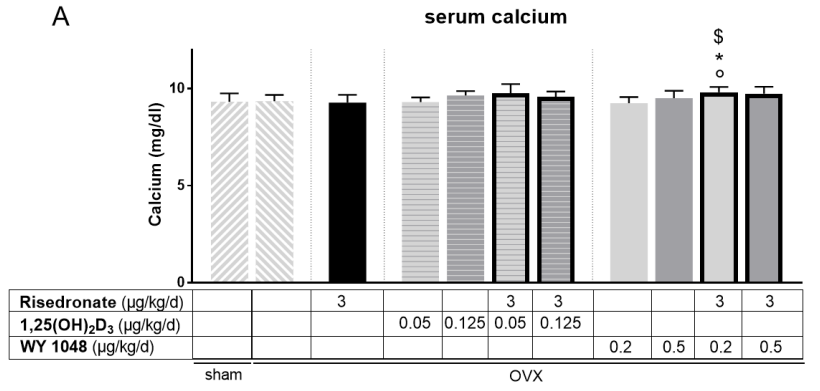


Figure 5. Combination of risedronate with the highest dose of WY 1048 significantly improves cortical thickness and porosity. *Microcomputed tomography data on cortical thickness (A) and porosity (B) of the selected cortical region of the distal femur in sham and OVX mice treated with the indicated drugs and doses. Values represent mean ± SD values (n=6/group). *p<0.05 vs. SHAM vehicle; °p<0.05 vs. OVX vehicle; #p<0.05 vs. risedronate.*

3.3 Serum biochemistry

Interestingly, none of the three drugs caused overt hypercalcemia in the doses used in the current study (**Figure 6A**). Even 4 weeks after treatments were initiated, all urinary calcium levels were stable but, after 8 weeks, small dose-dependent increases in urinary calcium levels were observed in the groups that were treated with $1,25(OH)_2D_3$ or with WY 1048 (**Figure 6B**). Interestingly, addition of risedronate to treatment with $1,25(OH)_2D_3$ seemed to reduce urinary calcium clearance.



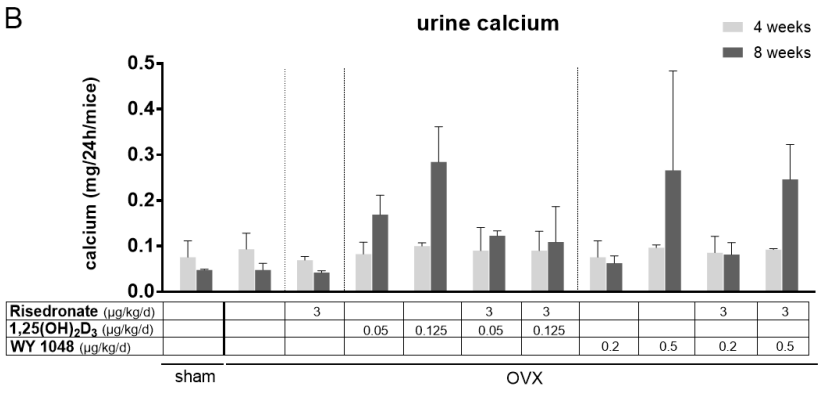


Figure 6. Treatment with $1,25(OH)_2D_3$ or with WY 1048 resulted in minimal calcemic side effects*. Serum calcium (A) and urinary calcium (B) levels were measured at 4 weeks and 8 weeks after treatment initiation with the indicated doses of risedronate, WY 1048, calcitriol or their combinations (n=10). Values of serum and urinary calcium represent mean ± SD. *p<0.05 vs. SHAM vehicle; °p<0.05 vs. OVX vehicle; $p<0.05 vs. dose respective monotherapy.*

4 Discussion

Bisphosphonates like risedronate have been used in the prevention and treatment of postmenopausal osteoporosis for over two decades because of their ability to reduce fracture risk by ~50% [2, 16]. Risedronate is often combined with vitamin D_3 because they collaborate by acting on different pathways [18]. Risedronate binds to hydroxyapatite molecules in the bone matrix where it is taken up by osteoclasts. Within the osteoclast, risedronate inhibits a key enzyme in the mevalonate pathway that is essential to osteoclast function, ultimately leading to increased osteoclast apoptosis rates [1, 3]. This decrease in osteoclast activity and number can result in the formation of new trabeculae, which may explain the increase in trabecular number that we observe. Vitamin D_3 on the other hand improves the calcium balance and its effects on bone seem to depend on the mineral supply. Certainly during a positive calcium balance, $1,25(OH)_2D_3$ may promote mineral deposition in bone matrix and although still controversial, some effects might be direct via mature osteoblasts through activation of the Wnt signaling pathway [13, 17]. Interestingly, combining risedronate, especially at higher doses, with vitamin D_3

metabolites tends to suppress the calcemic side effects of $1,25(OH)_2D_3$, possibly by preventing the drop in PTH levels after treatment with vitamin D_3 metabolites [17, 18]. Although a combination of risedronate with vitamin D_3 metabolites seems to be the ideal treatment strategy for postmenopausal osteoporosis, caution has to be taken because excess dosing of $1,25(OH)_2D_3$ results in increased bone resorption and decreased bone mineralization, limiting the therapeutic potential of $1,25(OH)_2D_3$ [18–20]. Based on our observation that, for most measured bone parameters, $1,25(OH)_2D_3$ at the higher dose (0.125 µg/kg/d) is less effective in restoring bone mass than the lower dose (0.05 µg/kg/d) suggests that the higher dose might exceed the optimal dosing. Hence, new vitamin D_3 analogs with less calcemic side effects and reduced catabolic effects on bone are needed to improve treatment strategies. In this study, we evaluated the capacity of our vitamin D_3 analog WY 1048 to restore bone loss and reduce calcemic side effects in an OVX mouse model of postmenopausal osteoporosis, alone or in combination with risedronate. Because low BMD is one of the most important predictors of a future fracture [23], we first evaluated whether the combination of risedronate with WY 1048 affected BMD. Our DEXA and additional pQCT data (supplementary information) indeed showed that a combined treatment with risedronate and WY 1048 successfully enhanced both trabecular and cortical BMD and BMC. While treatment with risedronate increased bone mass and density by increasing trabecular number, WY 1048 increases trabecular thickness possibly by supplying extra calcium to the bone and thereby enlarging the existing mineral matrix. This apparent difference in mechanism of action may explain that the most prominent increase in trabecular bone was observed when risedronate and WY 1048 were combined. Knowing that the anti-fracture efficacy of a treatment is largely related to its ability to decrease cortical porosity, we also evaluated cortical porosity [22, 23]. Risedronate has been shown to slow down or partly reverse cortical deterioration [17]. However, we did not observe an effect on cortical parameters after treatment with risedronate alone. Cortical bone has a low bone matrix volume compared to its surface area as opposed to trabecular bone that has a high bone matrix volume compared to its surface area [26]. Therefore, trabecular bone is more readily infiltrated by risedronate compared to cortical bone and may explain why we observe a protective effect of risedronate on trabecular bone and not on cortical bone. Also other studies reported that changes in cortical bone occur slower than changes in trabecular bone [27]. It is therefore possible that cortical effects would have been observed when treatment with risedronate was pursued for a longer time. Unexpectedly, the combination of $1,25(OH)_2D_3$ and risedronate seemed to reverse the calcemic effects of $1,25(OH)_2D_3$ whereas this beneficial effect was not observed upon a combined treatment of risedronate with WY 1048. Nevertheless, serum and urinary increases in calcium levels remained minimal. Today, risedronate is often prescribed in combination with vitamin D_3 to reduce the risk of fractures or to improve and speed up recovery of an existing fracture. However the potential of new potent analogs deserve further studying especially because the $1,25(OH)_2D_3$ analog, ED-71 has been shown to increase bone mass to a greater extent than the vitamin D_3 analog alfacalcidol [13]. In this study, we show that a combination therapy with our vitamin D_3 analog WY 1048 and risedronate represents a promising anti-osteoporotic drug in a preclinical model of ovariectomy-induced bone loss. Future experiments are however needed to elucidate the underlying mechanism of action.

5 Acknowledgements

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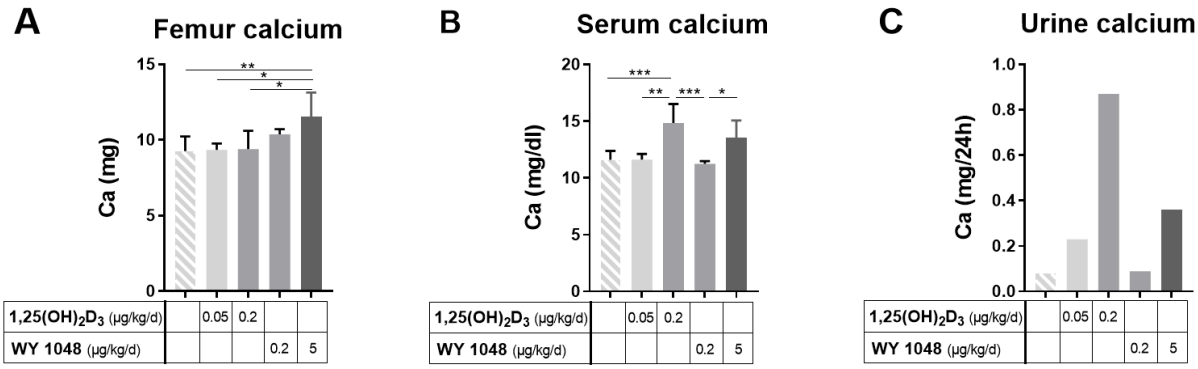
Supplementary information

Toxicity test

Materials and methods

Eight week old female NMRI mice (Charles River Laboratories) were intraperitoneally injected for 7 consecutive days with either vehicle (arachis oil, n=6), 1,25(OH)$_2$D$_3$ (0.05 µg/kg/d or 0.2 µg/kg/d, n=6), or WY 1048 (0.2 µg/kg/d or 5 µg/kg/d, n=5). Mice were then transferred to metabolic cages (5-6 mice per cage, resulting in 1 urine sample per experimental group) to obtain 24 h urine collections. Thereafter, mice were euthanized and blood was collected via cardiac puncture. Femurs were dissected, snap-frozen in liquid N_2 and stored at -80°C for the assessment of calcium content.
For the assessment of femoral calcium content, femurs were dried overnight at 100°C and ashed for 8 h at 500°C. Femoral ashes were dissolved overnight in 1 ml 1N HCl and diluted 1/50 in milliQ water for the measurement of calcium concentration.

figures



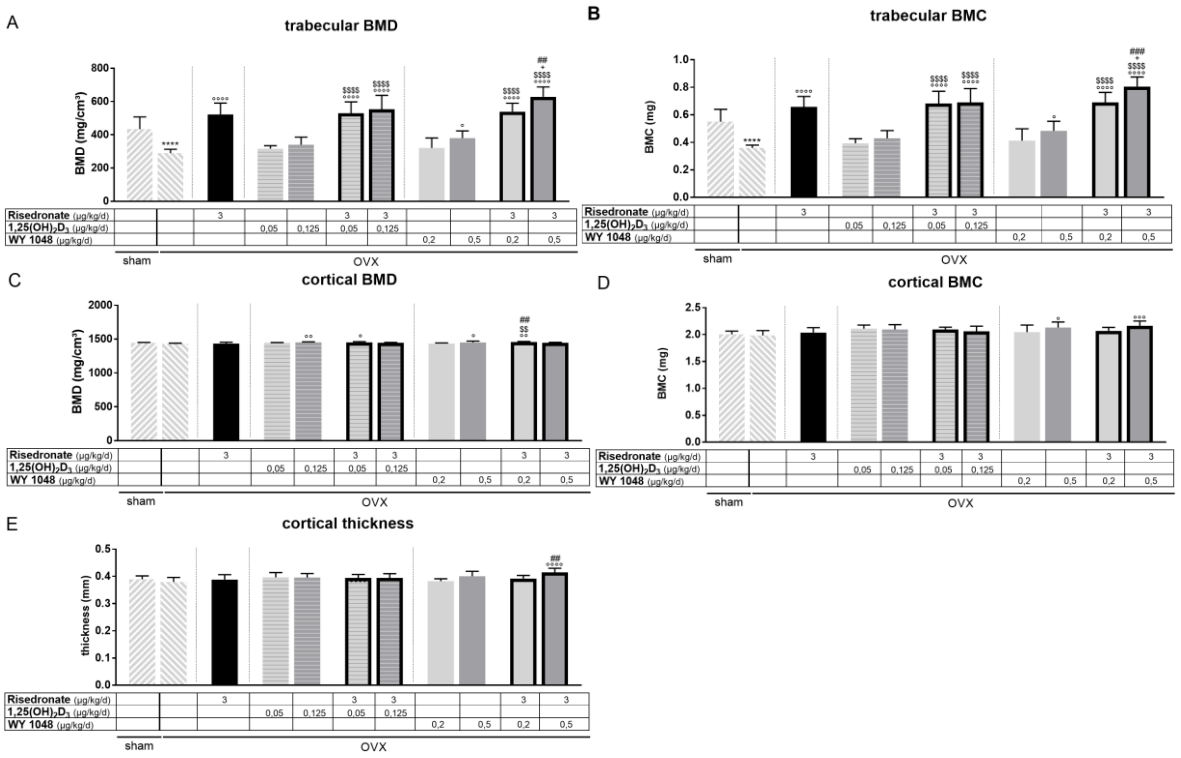
Supplementary figure 1. Toxicity evaluation of WY1048 revealed that this compound resulted in a gain in femoral calcium content with limited effects on serum or urinary calcium levels. *Mice (n=5-6/group) were treated with the indicated drugs and doses during 7 consecutive days. Femoral (A) and serum calcium (B) data are represented as mean ± SD values. Urine calcium (C) data is represented as mean. ***p<0.001, ****p<0.0001 between the indicated groups.*

Peripheral quantitative computed tomography (pQCT)

Materials and methods

Ex vivo bone densitometry of the femur was determined by pQCT. Trabecular bone parameters and cortical bone parameters were assessed using the Stratec XCT Research M+ densitometer (Norland Medical Systems, Fort Atkinson,WI, USA). Slices of 0.2 mm thickness were scanned using a voxel size of 0.070 mm. Three scans were taken from the distal end of the femur *ex vivo* (at 2.5 +/- 0.25 mm), using contmode 1, peelmode 20, and a density threshold of 280 mg/cm^3. The trabecular bone region was defined by setting an inner threshold corresponding to 30% of the total cross-sectional area. These three metaphyseal scans were performed to measure the average trabecular volumetric BMD and BMC. A second scan was taken 7 mm from the distal end of the femur using separation mode 1 and a density threshold of 710 mg/cm^3. These mild-diaphyseal scans were performed to determine cortical volumetric BMD and BMC and cortical thickness.

Figures



Supplementary figure 2. Combined therapy of risedronate and WY 1048 significantly improves both trabecular and cortical BMD and BMC and additionally increases cortical thickness*. Bones were measured by pQCT to obtain trabecular BMD (A) and BMC (B), cortical BMD (C) and BMC (D) and cortical thickness (E). Data is represented as mean ± SD values (n=6/group). *p<0.05 vs. SHAM vehicle; °p<0.05 vs. OVX vehicle; #p<0.05 vs. risedronate; +p<0.05 vs. dose respective calcitriol combination and $p<0.05 vs. dose respective monotherapy.*