

Materials and Methods

Materials

Lyophilized ONO-4819 was prepared at Minase Research Institute, Ono Pharmaceutical Co. Ltd., Osaka, Japan, and was dissolved in saline and stored at 4°C. Risedronate sodium was obtained from LKT Laboratories Inc. (St. Paul, Minnesota USA) , and diluted with saline on the day of use.

Experimental protocol

The following experiments were performed in accordance with the Guideline for Animal Experiments established by the Research Headquarters, Ono Pharmaceutical Co., Ltd. Female Sprague-Dawley rats 32 weeks of age and weighing 350-590 g (purchased from CLEA Japan Inc. Tokyo, Japan) were used. The rats were housed individually in metal cages at 24°C with a 12 h/12 h light-dark cycle, and fed standard rodent chow (CRF-1, Oriental Yeast Co., Ltd; Tokyo, Japan) and tap water *ad libitum* until used in experiments. At 33 weeks of age, the rats were randomized into 9 groups with 10 animals in each, and were subjected to sham operation (n=10) or ovariectomy (OVX, n=80). After a week of recovery period, the animals were given the following treatments for 11 weeks: Three OVX groups were injected s.c. with ONO-4819 at 1, 3 or 10 $\mu\text{g}/\text{kg}$ body weight (BW) twice a day; and 2 OVX groups, s.c. with risedronate at 1 or 5 $\mu\text{g}/\text{kg}$ BW twice a week. Two OVX groups were treated with ONO-4819 (3 $\mu\text{g}/\text{kg}$ BW) plus risedronate at 1 or 5 $\mu\text{g}/\text{kg}$ BW. Sham and OVX control groups were treated with vehicle solution twice a day. The doses of ONO-4819 were determined

based on our previous observations that doses between 3 and 10 $\mu\text{g}/\text{kg}$ were sufficient to protect against bone loss induced by ovariectomy (8). The doses of risedronate were employed according to a previous study in mature rats (3). For histomorphometric analysis, the rats were injected with tetracycline and calcein on days 14 and 4, respectively, before killing. All rats were sacrificed by exsanguination from the abdominal aorta, and their tibiae were then harvested for further analysis. All measurements and analyses were performed blinded to treatment.

Bone Mineral Density Measurements

Bone mineral density (BMD) was determined by peripheral quantitative computed tomography (pQCT). Tomographic scans were performed *ex vivo* on excised tibiae by using a Stratec XCT Research SA+ device (Norland Corp., Fort Atkinson, WI, USA). The machine was adapted for measurements in small animals and was calibrated with hydroxyapatite embedded in acrylic plastic as a standard. The precision error in the measurements of trabecular BMD was 3.64%.

The bones were kept at 4°C in saline throughout the course of the scan. A voxel size of 0.12 mm was chosen for scanning. Transverse images (0.46-mm thickness) of the tibiae were scanned at 4.0 mm from the proximal plateau. Analyses were performed by using Stratec software (Version 5.40B). For the analysis of trabecular bone, contour mode 2 and Peel mode 20 with a threshold setting of 169 mg/cm^3 was used.

Cortical density was calculated based on CT images obtained by scanning with a micro-CT scanner ($\mu\text{CT}40$; Basserdorf, Switzerland) (9,10). Twenty slices of

cortical axial sections in the proximal diaphysis (1.0 cm from the growth plate) were scanned at 8 microns. The average linear attenuation of whole cortical bone was calibrated by linear attenuation of a hydroxyapatite phantom. The precision error in the measurements of cortical density was 0.25%.

Microstructure analysis

Trabecular microarchitecture at the proximal tibiae was analyzed by micro CT. The process was piloted by a Compaq Alpha DS10 workstation (Compaq Computer Corporation, Delaware, DE), and an open VMS system in a cluster configuration was used to perform three-dimensional (3D) analysis.

The proximal tibia was positioned to be scanned cranio-caudally, using 320 slices with 16-micron increments at 55 kVp and 72 μ A. To obtain original 3D images of the proximal tibia from 1 mm below the growth plate (11), we used a threshold value of 275 to binarize the spongiosa and cortex in this analysis system (12). The threshold value of 275 was selected based on our investigation to adequately separate bone and other components by using discrimination analysis.

On the original 3D images, the following morphometric indices were directly determined from the binarized volume of interest (VOI): The trabecular bone volume fraction (BV/TV) was calculated from the bone volume (BV) and tissue volume (TV). Mean trabecular thickness (Tb.Th) was determined from local thickness by using the distance transformation method. Trabecular separation (Tb.Sp) was calculated by applying the same technique used for the direct thickness calculation to the non-bone

parts of the 3D image. Trabecular number (Tb.N) was calculated by taking the inverse of the mean distance between the middle axes of the structure (the ridge number density, 13). . The Structure Model Index (SMI) was used as a parameter to quantify the characteristic form of a three-dimensionally described structure in terms of the amount of plates and rods composing the structure (14). The degree of anisotropy (DA), which defines the direction and magnitude of the preferred orientation of trabeculae, was determined as the ratio between the maximal and minimal radii of the mean intercept length (MIL) ellipsoid (15). Connectivity density (mm^{-3}), which is a topological parameter that estimates the number of trabecular connections per cubic millimeter, was also determined. The perforation of plates without the breaking of trabecular connections is known to artificially increase the connectivity density (16,17). The software of 3D microstructure analysis was provided by SCANCO Medical (Basserdorf, Switzerland). The precision errors in the measurement of microstructural parameters of the rat proximal metaphysis were 2.2 ± 0.8 % (sham) and 3.5 ± 1.2 % (OVX) in connectivity density at the highest, and 0.6 ± 0.2 % (sham) and 0.8 ± 0.3 % (OVX) in Tb.Th at the lowest.

Histomorphometry

Right tibiae were fixed in 70% ethanol, stained with Villanueva bone stain, dehydrated with sequential changes of ethanol (70%, 95%, 100%), and then infiltrated by and embedded in methylmethacrylate without decalcification. A longitudinal section of the right tibia was cut at a thickness of $5\mu\text{m}$ with a RM2155 microtome

(Leica Inc., Nussloch, Germany).

Histomorphometric analysis was performed with a semiautomated digitizing image analyzer. The system consisted of a light or epifluorescence microscope/Olympus, BH-2 (Olympus America Inc., Melville, NY) and a digitizing pad/Numonics 2206 (Numonics Corp., Montomerville) coupled to a computer with histomorphometric software (OsteoMetrics, Atlanta, GA). The following parameters were measured: total tissue area (T.Ar, mm^2), total cancellous bone area (B.Ar, mm^2), total cancellous bone perimeter (B.Pm, mm), single-labeled bone perimeter (sL.Pm, mm), double-labeled bone perimeter (dL.Pm, mm), and interlabeled width (Ir.L.Wi, μm). The following parameters were then calculated: cancellous bone volume (BV/TV, %), single-labeled surface (sLS/BS, %), double-labeled surface (dLS/BS, %), mineralizing surface (MS/BS, %) ($= \text{dLS/BS} + \text{sLS/BS} \times 1/2$), bone formation rate (BFR/BS, $\text{mm}^3/\text{mm}^2/\text{y}$) and the ratio of labeled to eroded perimeters. Osteoclast surfaces (OcS/BS) and osteoblast surfaces (ObS/BS) were measured on sections stained for tartrate-resistant acid phosphatase (TRAP) activity.

Finite element analysis (FEA)

The segmented cubic bone volume was directly incorporated into an FE model to determine the elastic properties of the trabecular network by using the voxel-based FEA program. The 3D-binary image was used to generate an FE model by converting the pixels of the solid phase to correspondingly shaped 8-node brick elements (18). By use of a special-purpose FE-solver, compression and shear tests were simulated in 3

orthogonal directions. For these simulations, the tissue elastic properties were set as linear elastic and isotropic with a Young's modulus of 10 GPa and a Poisson's ratio of 0.3. von Mises stress (N/mm^2) was calculated by using the FEA program (19,20). The number of elements in each datum set ranged from 150,000 elements for low bone mass to more than 20 million elements for high bone mass.

Statistics

Data were expressed as the means \pm standard error (SE). Statistical analysis was carried out by analysis of variance (ANOVA), using StatLight (Yukms, Co., Ltd. Tokyo, Japan). Nonparametric analysis was used to compensate for lack of a normal distribution of the data. Differences between the groups were tested by using the Kruskal-Wallis test. p -values were calculated by use of the Steel test for multiple group comparison. Wilcoxon's rank sum test was used when differences between only 2 groups were tested. A p -value of < 0.05 was considered significant.

The interactions of ONO-4819 and risedronate treatments were investigated by using a two-way ANOVA model. The additive effect was defined when the interaction was not significant ($p > 0.10$).

Results

Effects of ONO-4819 vs. risedronate on trabecular and cortical bone density

Table 1 summarizes the results for BMD at the proximal tibiae of OVX rats treated with ONO-4819 or risedronate alone or in combination. Eleven weeks after OVX, the BMD decreased significantly compared with that of the sham group. Treatment of OVX rats with either ONO-4819 or risedronate alone for 11 weeks increased the BMD in a dose-dependent manner, compared with that for the OVX vehicle treatment group. The highest dose of ONO-4819 exerted a greater effect than that of risedronate, and increased the BMD above the sham level. Adding risedronate at 5 $\mu\text{g}/\text{kg}$ to ONO-4819 at 3 $\mu\text{g}/\text{kg}$ caused a further increase in the BMD, compared with that found for ONO-4819 (3 $\mu\text{g}/\text{kg}$) alone. Treatment with ONO-4819, either alone or in combination with risedronate, did not result in any changes in body weight, serum calcium or phosphate concentrations (data not shown).

The effects of these drugs on the cortical density at the tibial diaphysis were analyzed by using micro-CT. As shown in Table 1, the cortical density decreased substantially following OVX, and administration of ONO-4819 to OVX rats increased it significantly and dose-dependently. Risedronate, too, increased the cortical density, although to a lesser extent than ONO-4819. Adding risedronate to ONO-4819 tended to cause a further increase in the cortical density, compared with that obtained with ONO-4819 alone; however the difference did not reach statistical significance. Thus, ONO-4819 was capable of increasing the bone mineral density not only in the trabecular but also in the cortical compartment.

Effects of ONO-4819 vs. risedronate on trabecular microstructure and biomechanical properties

Table 2 summarizes the effects of ONO-4819 and risedronate, alone or in combination, on the microstructural parameters of the trabecular bone of the proximal tibiae of OVX rats, as determined by micro CT. Trabeculae of the estrogen-deficient aging rats were characterized by substantial decreases in bone volume fraction (BV/TV), connectivity density (ConnD), trabecular number as well as thickness, and by an increase in the structure model index (SMI). Monotherapy of OVX rats with either ONO-4819 or risedronate restored the 3D-bone volume in a dose-dependent manner, with ONO-4819 showing the greater potency. Treatment with risedronate, an anti-resorptive drug, was associated with a robust increase in connectivity density (Table 2). ONO-4819, a new anabolic compound, was also effective in restoring connectivity; however, its effects were more striking in keeping SMI at the sham level, implying that ONO-4819 is peculiar in its ability to maintain plate-like structure even in the estrogen-deficient state. Combined treatment with ONO-4819 and risedronate had an additive effect in increasing the 3D-bone volume. This combination therapy was also very effective in improving the microarchitectural deterioration caused by the estrogen deficiency, since it caused a further increase in connectivity and a decrease in SMI (Table 2). Thus, the characteristic effects of each drug on trabecular microstructure were preserved in the combination treatment.

Biomechanical strength was assessed by using FEA. As shown in Figure 1, von

Mises stress decreased significantly following OVX, confirming the mechanically incompetent property of bone in estrogen deficiency. Treatment of OVX rats with ONO-4819 increased von Mises stress dose-dependently, with the highest dose showing a robust increase far above the sham level. Risedronate, too, increased the biomechanical strength at the higher dose, but only to the sham level. Adding risedronate at 5 $\mu\text{g}/\text{kg}$ to ONO-4819 at 3 $\mu\text{g}/\text{kg}$ caused a further increase in von Mises stress, compared with that recorded for ONO-4819 (3 $\mu\text{g}/\text{kg}$) alone (Figure 1).

Histomorphometric analysis

The mechanisms of action of ONO-4819 vis-à-vis risedronate at the tissue and cell levels were further examined by using histomorphometry at the proximal tibiae of drug-treated OVX rats, the results of which are illustrated in Figure 2.

As expected, the bone metabolism of rats with estrogen deficiency was characterized by high turnover, i.e., marked increases in bone resorption parameters with concomitant increases in bone formation indices. As a net result of bone resorption activity exceeding bone formation ability, a significant reduction in the bone volume fraction ensued at the tissue level, as already described above. Treatment of OVX rats with ONO-4819 caused a robust and dose-dependent increase in the rate of bone formation, which was associated with increases in both osteoblast surface and mineralizing surface, suggesting that this EP4 agonist stimulated the differentiation/recruitment of osteoblasts as well as their mineralizing function. ONO-4819 did not stimulate bone resorption, but rather reduced osteoclast surface area at 3 $\mu\text{g}/\text{kg}$ BW, suggesting that ONO-4819

exerted an anti-resorptive function within a certain dose range.

The administration of risedronate to the OVX rats caused an almost complete suppression of bone resorption parameters, such as osteoclast surface, to sham levels (Figure 2). Concomitantly, risedronate markedly suppressed the rate of bone formation, causing a decrease in the mineralizing surface. Adding risedronate at 5 $\mu\text{g}/\text{kg}$ to ONO-4819 at 3 $\mu\text{g}/\text{kg}$ did not affect the osteoblast surface but dampened the mineralizing activity of ONO-4819, although the mineralizing surface remained still elevated, between sham and OVX levels. Adding ONO-4819 to risedronate did not interfere with the anti-resorptive function of risedronate, and the osteoclast surface area remained suppressed almost to the sham level (Figure 2).

In order to estimate the net effects on bone remodeling, formation/resorption ratio was calculated as labeled to eroded surface. As shown in Figure 3, OVX had very little effect on this ratio, whereas ONO-4819 caused a significant elevation, again illustrating its robust activity in stimulating bone formation. The effect of ONO-4819 was marked at 3 $\mu\text{g}/\text{kg}$, at which dose it rather suppressed bone resorption while stimulating bone formation relative to bone resorption. Risedronate alone reduced the labeled to eroded perimeter even below the OVX level, whereas the combined treatment with ONO-4819 and risedronate was effective in maintaining the ratio at a significantly heightened level. These results suggest that ONO-4819 is a potent bone anabolic agent, with also an anti-resorptive activity within a certain dose range, and that its combination with risedronate can suppress bone resorption without losing its anabolic function.

Drug interaction between ONO-4819 and risedronate

In order to further examine whether the effects of ONO-4819 and risedronate on BMD, microstructural parameters, and biomechanical strength were independent and additive, we assessed the interactions of these 2 drugs by two-way ANOVA. As shown in Table 3, the interaction was not significant for most parameters, and therefore it was judged that treatment with ONO-4819 and risedronate had independent and additive effects on BMD, microstructural parameters, cortical density and bone strength.

Discussion

In the present study, we employed estrogen-deficient mature rats, in which accelerated bone resorption occurs with relatively insufficient bone formation, thereby leading to bone loss and microstructural deterioration. By detailed histomorphometry, we have shown that a selective EP4 agonist, ONO-4819, potently stimulated bone formation by increasing osteoblast differentiation as well as mineralizing activity by individual osteoblasts. These effects on bone cell activity were translated into microstructural changes in the trabeculae following treatment with ONO-4819, represented by almost normalization of SMI with a substantial increase in trabecular thickness. Thus, our data suggest that ONO-4819 was capable of preventing thinning of trabeculae and converting their rod-like structure following OVX to a plate-like one. Another interesting and important finding is that ONO-4819 increased the cortical density of tibial diaphysis significantly. In this respect, ONO-4819 may be dissimilar to the only anabolic drug now available, PTH, which is known to induce intracortical porosity (21).

Interestingly, we found that ONO-4819 at 3 $\mu\text{g}/\text{kg}$ BW significantly inhibited bone resorption, suggesting that the compound exerted an anti-resorptive effect depending on its dosage. This may be reflected in the observed increase in connectivity density of trabeculae caused by ONO-4819, although this agonist was not as potent as risedronate in this respect. PGE_2 is known to stimulate osteoclast formation through activation of EP4 (7) and EP2 (22) receptors and subsequent induction of RANKL expression, an essential cytokine for osteoclastogenesis(23). In contrast, biphasic

effects of PGE₂ (24) and even inhibitory effects on osteoclast formation (25) as well as bone resorption activity (26) have recently been reported. These are all based on the results obtained from *ex vivo* cultures; whereas our current findings represent the first, to our knowledge, showing that an EP4 agonist can exert an inhibitory effect on bone resorption *in vivo*.

Risedronate, by almost completely inhibiting bone resorption with concomitant suppression of bone formation, was very effective in restoring connectivity with only a modest effect on trabecular thickness and SMI. These results are consistent with those of previous preclinical studies reporting the effects of typical anti-resorptive drugs such as risedronate and alendronate on the trabecular microstructure (27). Although ONO-4819 and risedronate are both effective in improving biomechanical strength, it is conceivable that the underlying mechanisms are quite different in view of their distinct effects on bone remodeling and trabecular microarchitecture. In fact, ONO-4819 was more potent than risedronate in increasing mechanical strength, which is presumably due mainly to the anabolic nature of its action.

The combined use of anti-resorptive drugs with anabolic agents is a logical strategy for the treatment of osteoporosis, which is caused by uncoupling of formation to resorption; and many attempts have been made using it in animal experiments as well as in clinical studies (28 - 31). In the combined therapy of ovariectomized rats with PTH and 17- β -estradiol, the bone resorption that decreased in association with bone formation activity was significantly lower than that in the group receiving PTH alone (32). In the study of old female sheep, the anabolic effect of PTH on bone was not

maintained when PTH was co-administered with the bisphosphonate tiludronate (33). The combined treatment of ovariectomized rats with prostaglandin and alendronate showed that the anabolic action of PGE₂ was not blocked by inhibition of bone resorption by alendronate and that the combined treatment increased the mechanical strength of the femoral shaft, even above that found for the non ovariectomized animals (34). In the current study, the combination of ONO-4819 and risedronate caused additive effects on BV/TV, SMI and connectivity density, compared with either drug alone; and the respective effects of both agents on microstructure were also preserved. Although ONO-4819 alone at the highest dose exhibited a robust anabolic effect in the present study without any notable side effects, high doses of the EP4-selective agonist could cause diarrhea, hypotension and abnormalities of intestinal epithelium (8). Thus, for future clinical application, the additive effect by combination of a lower dose of ONO-4819 and risedronate still offers a therapeutic merit.

In conclusion, the present study, which focused on the microarchitecture of trabecular bone, demonstrates that the representative anti-resorptive drug risedronate maintained trabecular connectivity, whereas ONO-4819 was effective in preventing the development of fragile rod-like trabeculae and maintaining or even increasing the plate-like structure of healthy bone. In addition, ONO-4819 is one of the few drugs that are capable of increasing trabecular thickness, owing to its potent anabolic action. When the 2 drugs were combined, the beneficial effects of each on the structural components were maintained, resulting in their additive effects on bone strength. Further studies are required to test the combined benefits of bisphosphonates and EP4

agonists in clinical settings as well as in primate models of osteoporosis.

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Figure legends

Figure 1 Effects of ONO-4819 vs. risedronate on biomechanical strength

Thirty-three-week-old OVX rats were treated with ONO-4819, risedronate or their combination at the indicated doses (in $\mu\text{g}/\text{kg}$) for 11 weeks, and biomechanical strength was determined at the proximal tibiae by finite element analysis. Data are expressed as the mean \pm SEM (n=10 each). * $p < 0.05$ vs. Sham group by Wilcoxon's rank sum test, # $p < 0.05$ vs. OVX group by Steel's test, § $p < 0.05$ vs. ONO-4819 (3 $\mu\text{g}/\text{kg}$) alone by the Steel test

Figure 2 Results of histomorphometric analysis

Thirty-three-week-old OVX rats were treated with ONO-4819, risedronate or their combination at the indicated doses (in $\mu\text{g}/\text{kg}$) for 11 weeks, and histomorphometric analysis was performed at the secondary spongiosa of the proximal tibial metaphysis. BFR: bone formation rate, Ob.S: osteoblast surface, MS: mineralizing surface, Oc.S: osteoclast surface. Data were corrected for bone surface (BS) and expressed as the mean \pm SEM (n=10 each). * $p < 0.05$ vs. Sham group by Wilcoxon's rank sum test, # $p < 0.05$ vs. OVX group (by Steel's test for comparison with ONO-4819-treated group and by Wilcoxon's rank sum test for comparison between risedronate-treated and combined treatment groups.), § $p < 0.05$ vs. ONO-4819 (3 $\mu\text{g}/\text{kg}$) alone by Wilcoxon's rank sum test.

Figure 3 Effects of ONO-4819 vs. risedronate on formation/resorption ratio

Thirty-three-week-old OVX rats were treated with ONO-4819, risedronate or their combination at the indicated doses (in $\mu\text{g}/\text{kg}$) for 11 weeks, and histomorphometric analysis was performed at the secondary spongiosa of the proximal tibial metaphysis. As an index of net bone formation, the ratio of labeled surface to eroded surface was calculated. Data are expressed as the mean \pm SEM (n=10 each). [#]p<0.05 vs. OVX group (Steel's test was used for comparison with ONO-4819-treated groups; and Wilcoxon's rank sum test, for that between risedronate-treated group and combined treatment group.).

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