Treatment with the combination of ibandronate plus eldecalcitol has a synergistic effect on inhibition of bone resorption without suppressing bone formation in ovariectomized rats

Sadaoki Sakai a, Satoshi Takeda a, Masanori Sugimoto b, Masaru Shimizu c, Yasushi Shimonaka a, Kenji Yogo a, Junko Hashimoto d, Frieder Bauss e, Koichi Endo a,*

a Product Research Department, Fuji-Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513 Japan
b Pharmacology 3, Pharmacology Laboratories, Research Headquarters, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Kita-ku, Saitama 331-9530 Japan
c Discovery Pharmacology Dept. 1, Fuji-Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513 Japan
d Primary Lifecycle Management Dept., Chugai Pharmaceutical Co., Ltd., 2-1-1 Nihombashi Muromachi, Chuo-ku, Tokyo 103-8324 Japan
e Roche Pharmaceutical Research and Early Development, Discovery Oncology, Roche Innovation Center Penzberg, Nonnenwald 2, D-82377 Penzberg, Germany

Abstract

Bisphosphonates are widely used in the treatment of osteoporosis and contribute to the reduction of bone fractures. Ibandronate (IBN) is a highly potent, nitrogen-containing bisphosphonate, which is administered orally or intravenously at extended dosing intervals. Vitamin D or active vitamin D3 derivatives are also used in the treatment of osteoporosis, and are often used in combination with other drugs. In this study, we investigated the effect of treatment with the combination of once-monthly s.c. dosing of IBN plus once-daily oral eldecalcitol (ELD), an active vitamin D3 derivative, using aged ovariectomized (OVX) rats. Treatment was started the day after OVX, and analyses were performed 4, 8, and 12 weeks thereafter by determination of bone markers, bone mineral density, biomechanical properties, and histomorphometry. The combination treatment showed a synergistic effect in increasing both lumbar and femoral BMD, and resulted in a significant increase in bone ultimate load. The combination of IBN plus ELD acted synergistically to reduce bone resorption, whereas bone formation did not decrease any more than with monotherapy with either IBN or ELD. Bone formation independent of bone resorption (a process known as ‘minimodeling’) was not changed in vehicle treated OVX rats despite the increase in bone turnover. ELD upregulated minimodeling, which was however not diminished in the combination treatment. In conclusion, treatment with the combination of IBN plus ELD was beneficial in the treatment of osteoporosis in aged OVX rats. It exhibited a synergistic inhibitory effect on bone resorption and keeps bone formation at the level of sham controls. This uncoupling of bone resorption/bone formation was affected, to some extent, by minimodeling-based bone formation which is independent of bone resorption. This combination regimen which showed synergistic effect on BMD and bone ultimate load without inhibition of bone formation may be beneficial in long-term osteoporosis treatment to prevent bone fractures.

© 2015 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Keywords:
Osteoporosis treatment
Combination therapy
Ibandronate
Eldecalcitol
Ovariectomized (OVX) rats

Abbreviations: IBN, ibandronate; ELD, eldecalcitol; OVX, ovariectomized; MCT, medium-chain triglyceride; OCN, osteocalcin; DPD, deoxypiridinolircon; BV/TV, bone volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; Oc.S/Bv, osteoclast surface; Es/S, eroded surface; N.Oc.S/Bv, osteoclast number; Oc.S/Bs, osteoblast surface; N.Ob/Bs, osteoblast number; M.S/Bs, mineralizing surface; O.S/Bs, osteoid surface; B.Vr/Bs, bone formation rate; MAR, mineral apposition rate; Ac.f, activation frequency; N.ML/Bs, minimodeling number; N.Bv/Bs, minimodeling surface; MBV/TV, minimodeling volume.

* Corresponding author.
E-mail addresses: sakaisdo@chugai-pharm.co.jp (S. Sakai),
takedasts@chugai-pharm.co.jp (S. Takeda), m-sugimoto@taisho.co.jp (M. Sugimoto),
shimizums@chugai-pharm.co.jp (M. Shimizu), shimonakays@chugai-pharm.co.jp (Y. Shimonaka),
yogoky@chugai-pharm.co.jp (K. Yogo),
hashimotojnk@chugai-pharm.co.jp (J. Hashimoto),
frieder.bauss@roche.com (F. Bauss),
endokius@chugai-pharm.co.jp (K. Endo).

1. Introduction

Ibandronate (IBN) is a highly potent inhibitor of bone resorption which was selected from among 300 bisphosphonate molecules [1]. IBN inhibits farnesyl diphosphate synthase (an enzyme correlated with efficacy of bone resorption) in osteoclasts more efficiently than alendronate, another widely used bisphosphonate [2]. IBN can be administered at extended between-dose intervals both intravenously and orally, and is widely used in the treatment of osteoporosis [3–5]. IBN exhibits its anti-osteoporotic effect in a dose-dependent manner [6], and the efficacy of treatment is related to the total dose of IBN, independent of the treatment regimen [7].
Eldecalcitol (ELD) is an active vitamin D₃ derivative used for the treatment of osteoporosis in Japan [8]. Active vitamin D₃ derivatives such as ELD are frequently used in osteoporosis treatment in combination with other anti-osteoporotic drugs, not only because of their effect in correcting the uptake of calcium from the intestine but also because of their effect in reducing the incidence of bone fractures. However, even under appropriate treatment, many patients with osteoporosis nevertheless experience bone fractures, necessitating the establishment of a more effective regimen.

In this study, we investigated the effect of a combination treatment with IBN plus ELD on BMD, bone ultimate load, and bone turnover using aged ovariectomized (OVX) rats. We also attempted to explain a phenomenon of the uncoupling of bone formation/bone resorption that was observed in the combination treatment by focusing on mimimodeling, which is defined as bone formation independent of bone resorption [9,10].

2. Materials and methods

2.1. Animal experiments

Female Wistar–Imamichi rats were purchased from the Institute for Animal Reproduction (Ibaraki, Japan) and sham-operated or ovariectomized (OVX) when 8 months old to make a model for the study of estrogen deficiency. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd. Animals were allowed free access to feed (CE-2; CLEA Japan, Tokyo, Japan) in stainless steel feeders. Drinking water was provided to the animals ad libitum via an automatic watering system. Glass water bottles fitted with sipper tubes were used during urine collection.

2.1.1. A dose-finding experiment

From the day after surgery, OVX rats were treated with IBN (subcutaneously, once every 4 weeks, 3 times in total; 1, 3, 10, and 30 μg/kg) for 12 weeks. Isotonic sodium chloride solution (Otsuka Pharmaceutical Factory, Tokushima, Japan) was used as the vehicle. At 12 weeks after treatment, 24-h urine and serum from jugular vein blood were collected. Lumbar vertebrae and femurs were collected at the end of the experiment.

2.1.2. A combined therapy experiment

From the day after surgery, OVX rats were treated with either IBN (subcutaneously, once every 4 weeks, 3 times in total; 3 μg/kg), ELD (orally, once daily, 15 mg/kg), or their combination for 12 weeks. Isotonic sodium chloride solution was used as the vehicle for IBN and medium-chain triglyceride (MCT) (The Nissin OilliO Group, Tokyo, Japan) was used as the vehicle for ELD. Every 4 weeks, 24-h urine and serum from jugular vein blood were collected. Seven days prior to necropsy, 20 mg/kg tetracycline (Sigma-Aldrich, MO, USA) was subcutaneously administered; 2 days prior to necropsy, 10 mg/kg calcein (Dojindo Laboratories, Kumamoto, Japan) was subcutaneously administered. Lumbar vertebrae and femurs were collected at the end of the experiment.

2.2. Serum and urine analysis

Calcium and phosphorus in serum and creatinine (Cr) in urine were measured using an automatic analyzer (Clinical Analyzer Model 7180; Hitachi High-Technologies Corporation, Tokyo, Japan). Serum osteocalcin (OCN) and urine deoxypyridinoline (DPD) were measured by ELISA (OCN: GE Healthcare Japan, Tokyo, Japan; DPD: DS Pharma Biomedical, Osaka, Japan). The concentration of urinary DPD was normalized to the concentration of urinary creatinine (Cr).

2.3. Bone mineral density measurement

Bone mineral density (BMD) of the second to fourth lumbar vertebrae (L2–L4) and right femur was determined by dual-energy X-ray absorptiometry (DCS-600EX-IIR; Hitachi Aloka Medical, Tokyo, Japan). After dual-energy X-ray images were obtained, the long axis of the femur was divided into 10 parts numbered F1 to F10 from the proximal to distal ends, then BMD of the whole (F1–F10), proximal (F1–F3), middle (F4–F7), and distal (F8–F10) femur was calculated.

2.4. Bone mechanical property measurement

To assess the bone mechanical properties of trabecular and cortical bone, a compression test [11] for the L5 lumbar vertebra and a 3-point bending test [12] for the middle femur were performed. The vertebral arch and disk were removed from each L5 lumbar vertebra and vertebral bodies were trimmed to a length of 5 mm on the vertical axis. The trimmed vertebral bodies were placed in a bone strength tester (TK-252C; Muromachi Kikai, Tokyo, Japan), and compressive strength was measured by pressure at a speed of 2.5 mm/min. The 3-point bending test of the femur was performed by the same tester. The midpoint of the left femur was placed on a holding device, and the supports were located 12 mm apart. The bending force was calculated at a speed of 20 mm/min until fractures occurred. From the load–displacement curve, the ultimate load (N), stiffness (N/mm), and energy (mJ) were obtained.

2.5. Bone histomorphometry analysis

Bone histomorphometry analysis was performed as previously reported [13]. Briefly, the third lumbar vertebra body (L3) and right femur were fixed in 70% ethanol and stained according to the method of Villanueva [14]. After dehydration with ethanol and acetone, the samples were embedded in methyl methacrylate (Wako Pure Chemical Industries, Osaka, Japan). Midsagittal sections of L3, 5 μm thick, were obtained with a microtome (SuperCut 2050; Reichert-Jung, Heidelberg, Germany). The cancellous bone within 1.7 mm from the growth plates was excluded from the measurements. The image obtained under a fluorescence microscope was recorded with a digital camera and the primary histomorphometric parameters were measured with an imaging analyzing system (Cosmozone 1SA; Nikon, Tokyo, Japan). Nomenclature, symbols, and units used in this study are those described in the Report of the American Society for Bone and Mineral Research Nomenclature Committee [15]. New formed bones on smooth cement lines were defined as mimimodeling.

2.6. Statistical analysis

All data are presented as means ± standard deviation (SD). Statistical analysis was performed by analysis of variance (ANOVA) on the SAS statistical analysis software package (SAS institute Inc., Cary, NC, USA). Statistical differences between Sham and Vehicle groups were analyzed by unpaired t-test. In the dose-finding test, analysis of the sham group vs. other groups was performed by Dunnett’s multiple comparison test. In the combination treatment test, analyses between the Vehicle group and all other groups were analyzed by Tukey’s multiple comparison test. A value of p < 0.05 was considered significant for all statistical analyses.

3. Results

3.1. A dose-finding experiment

Prior to researching the IBN plus ELD combination treatment, we performed the dose-finding experiment of IBN in OVX rats. Urinary DPD, a bone resorption marker, and serum OCN, a bone formation
marker, were increased 12 weeks after OVX operation. Rats treated with IBN showed a significant decrease in DPD and OCN (Fig. 1A). In femur and lumbar vertebrae, BMD was decreased by OVX and it was inhibited by IBN. High dose of IBN even showed significant increase of BMD compared with sham (at 30 μg/kg in lumbar and at over 10 μg/kg in femoral BMD) a decrease in BMD was not only inhibited but also increased (Fig. 1B). So that 3 μg/kg IBN treatment resulted in a BMD in the lumbar vertebrae and femur comparable to that of the Sham group.

3.2. A combined therapy experiment

3.2.1. Body weight, serum calcium or phosphorous after combination treatment

After the dose-finding study, we investigated the effects of combination treatment with IBN plus ELD under conditions of estrogen deficiency using OVX rats. The dose of 3 μg/kg for IBN was used in this experiment because it increased lumbar and femoral BMD in OVX rats to a level comparable to that of the Sham group (Fig. 1B). Rats were allocated into 5 groups (Table 1) as follows: vehicle-treated sham-operated rats (administered saline and ELD vehicle); vehicle-treated OVX rats (administered saline and ELD vehicle); OVX rats treated with IBN (administered IBN and ELD vehicle); OVX rats treated with ELD (administered saline and ELD); and, combination treatment with IBN plus ELD. IBN or saline was subcutaneously administered once every 4 weeks, and ELD or MCT was orally administered every day.

Body weight and concentrations of calcium and phosphorus in serum are shown in Table 2. The body weight of OVX rats in the Vehicle group was significantly higher than rats in the Sham group throughout the course of the experiment. Treatment with IBN, ELD, or the combination had no effect on body weight. ELD increased serum calcium and phosphorus as previously reported [13], and serum phosphorus in the Combination group at Week 4 showed no significant change as compared to that in the Sham group. Serum calcium and phosphorus were increased in the ELD group at all sampling times. In the Combination group, no differences were seen in any of these parameters other than the concentration of phosphorus at Week 4.

3.2.2. Bone turnover markers, lumbar and femur BMD and mechanical properties

The bone resorption marker DPD in urine was increased in the Vehicle group throughout the experimental period (Fig. 2A). Monotherapies with IBN or ELD significantly inhibited the OVX-related rise in DPD within 4 weeks, and the values were decreased below those of the Sham group.
Table 2

| Body weight (g)   | Sham       | Vehicle    | IBN        | ELD        | Combination |
|------------------|------------|------------|------------|------------|-------------|
| Week 4           | 314 ± 11   | 367 ± 28*  | 359 ± 20   | 376 ± 14   | 374 ± 18    |
| Week 8           | 325 ± 13   | 380 ± 27*  | 373 ± 22   | 395 ± 16   | 395 ± 21    |
| Week 12          | 339 ± 15   | 400 ± 30*  | 393 ± 17   | 416 ± 17   | 413 ± 19    |

| Serum calcium (mg/dL) |
|----------------------|
| Week 4               | 9.77 ± 0.19 | 9.90 ± 0.26 | 10.12 ± 0.22 | 10.68 ± 0.34§ | 10.83 ± 0.25§ |
| Week 8               | 9.85 ± 0.25 | 9.77 ± 0.29 | 9.69 ± 0.19  | 10.79 ± 0.29§ | 10.77 ± 0.30§ |
| Week 12              | 9.89 ± 0.28 | 9.64 ± 0.30 | 9.60 ± 0.12  | 10.59 ± 0.29§ | 10.38 ± 0.22§ |

| Serum phosphorus (mg/dL) |
|-------------------------|
| Week 4                  | 4.05 ± 0.43 | 4.79 ± 0.68* | 5.22 ± 0.48  | 5.71 ± 0.63§  | 5.36 ± 0.37  |
| Week 8                  | 4.05 ± 0.48 | 4.40 ± 0.65  | 4.20 ± 0.31  | 5.61 ± 0.58§  | 5.72 ± 0.59§ |
| Week 12                 | 3.97 ± 1.11 | 3.98 ± 0.79  | 4.24 ± 0.94  | 5.79 ± 1.10§  | 5.51 ± 0.55§ |

Data are presented as mean ± S.D. (n = 10).

IBN, ibandronate; ELD, eldecalcitol.

* p < 0.05 Sham group vs. Vehicle group by unpaired t-test.

§ p < 0.05 vs. Vehicle.

\( ^{\dagger} \) p < 0.05 vs. IBN by Tukey’s multiple comparison test.

Approximately 4% of bone formation marker, serum OCN, was increased in the Vehicle group, it was not remarkably changed following administration of IBN or ELD alone or even in combination (Fig. 2B).

At the end of the 12 week treatment, BMD of the lumbar spine was significantly decreased in the Vehicle group as compared to that in the Sham group (Fig. 3A). A synergistic increase was observed in the Combination group. The same result was observed with femoral BMD, both in the proximal and distal femur, which consist of abundant trabecular bone, and in the middle femur, which consists of cortical bone (Fig. 3B, C).

To assess bone mechanical properties, compression tests of the L5 lumbar vertebra and a 3-point bending test of the middle femur were performed. In the Vehicle group, the ultimate load of the lumbar vertebra and femur were significantly diminished (Fig. 4A and B). Treatment of OVX rats with either IBN or ELD alone prevented the decrease in lumbar ultimate load. Combination treatment did not interfere with the effects of these drugs on the ultimate load of the lumbar vertebra (Fig. 4A). With respect to the ultimate load of cortical bone, monotherapy groups showed a slight tendency for bone ultimate load to increase, and when compared to Vehicle group, combination treatment had significantly increased femoral ultimate load as assessed by 3-point bending test (Fig. 4B).

3.2.3. Bone histomorphometry analysis

To examine the way of increasing BMD and bone ultimate load seen in combination therapy with IBN plus ELD, we focused on the uncoupling of bone formation from bone resorption shown in Fig. 2. To investigate more precisely the state of bone turnover in the Combination group, histomorphometric analysis of trabecular bone was performed in the third lumbar vertebra (L3). Typical photos of bright field and fluorescence image were demonstrated in Fig. 5. Fluorescence imaging can visualize bone mineralizing surface as bright lines. Bone volume was decreased in Vehicle group compared with Sham group (white area in bright field image or black area in fluorescence image). The reduction was prevented by IBN or ELD, and the combination of IBN plus ELD had a synergistic effect on trabecular thickness (width of bone area) (Fig. 5).

The quantitative analysis of bone histomorphometry was performed. In all experimental groups, including the Combination group, bone mineralization was maintained on the surface of trabecular bone. Bone resorption parameters (Oc.S/BS, Es/BS, N.Oc/BS) were increased in the Vehicle group, and monotherapy with IBN or ELD decreased these parameters (Table 3).

The combination treatment showed greater inhibition of bone resorption than did IBN alone. Bone formation parameters (Ob.S/BS, Ob.N/BS, Ocn.S/BS, Oc.S/BS, Mar, B.Fr/BS, Ocn/BS, Ac.f) were also increased in the Vehicle group and were reduced in the IBN group and the ELD group. While combination treatment facilitated the reduction of bone resorption, no significant changes in bone formation parameters were observed in the Combination group as compared to the IBN group or the ELD group; this result was identical to that found with serum and urine bone turnover markers (Fig. 2). Interestingly, “bud” shaped bone formation was also observed in ELD-treated rats (the ELD group and the IBN plus ELD combination group) (Fig. 6A). The formation of bud-shaped bone on the smooth cement lines of intact lamellar bone is called “minimodeling” [9] and is independent of bone resorption [16]. In the Vehicle group, different from the increased remodeling and high bone turnover, there was no increase in the number of min模特ling sites, length of min模特ling surfaces, or bone volume of min模特ling (Fig. 6B). Min模特ling parameters were not affected by the inhibition of bone resorption in the IBN group. Although the same level of reduction in bone resorption was observed in the ELD group as in the IBN group (Table 3), minimMODELING in the ELD group was remarkably upregulated as previously reported [10]. Furthermore, the combination treatment with IBN plus ELD did not interfere with the bone formation by minimmodeling as compared to the ELD-treated group (Fig. 6B).

4. Discussion

In the present study we demonstrated that combination treatment with IBN plus ELD—a nitrogen-containing bisphosphonate plus an active vitamin D₂ derivative—had an advantageous effect on BMD and bone ultimate load in both the lumbar vertebrae and femur in OVX aged rats. Since the rats were 8 months of age at start of the study, growth-dependent effects, which may have influenced the results, can be neglected. We chose the dose of 3 μg/kg for the combination experiment because that dose was the maximal dose which didn’t show the significant increase BMD compared to sham operated rats. We chose 15 ng/kg of ELD in the combination treatment on the basis of our previous results [13]. ELD is administered at 0.75 μg/kg per day in clinical treatment, so 15 ng/kg is almost the same dose as in clinical use.

Interestingly, at the doses used in this study, IBN showed almost the same efficacy as ELD with respect to femoral ultimate load. It is reported that IBN exerts its anti-osteoporotic effect, and a greater beneficial result
is reported at higher doses of IBN corresponding to clinical use [17]. In
our detailed investigation of combination treatment with IBN plus
ELD, IBN and ELD acted synergistically to reduce bone resorption
in OVX rats, in which both bone resorption and bone formation is
accelerated. These two drugs have different mechanisms of action in
the inhibition of bone resorption, and we consider that this is the reason
behind this enhanced effect of combination treatment on bone resorp-
tion. Although higher doses of each monotherapy, in particular with

Fig. 2. Combination treatment with IBN plus ELD had inhibitory effects on bone resorption, with the level of bone formation being comparable to that of the Sham group. (A) Monotherapy with IBN or ELD significantly decreased urinary DPD, and the combination of IBN plus ELD showed greater inhibition throughout the experiment. (B) Serum OCN was increased in the Vehicle group. Treatment with IBN, ELD, or the combination of IBN plus ELD did not markedly suppress the increased OCN. n = 10, * p < 0.05 vs. Sham by t-test; — p < 0.05 by Tukey’s multiple comparison test. IBN, ibandronate; ELD, eldecalcitol; DPD, deoxypiridinol; Cr, creatinine; OCN, osteocalcin.

![Diagram](image-url)
IBN, had demonstrated higher increases in BMD than the combination therapy, the combination therapy has the advantage to combine both modes of action at lower doses, with the benefit of vitamin-related increased calcium absorption from the intestine.

It has been reported that bisphosphonates bind to the bone surface and are released under low pH conditions during bone resorption whereupon they are incorporated into osteoclasts and, at high concentrations, induce apoptosis [18]; it is also reported that they stimulate the expression of bone resorption inhibitors from osteoblasts in vitro [19]. Farnesyl diphosphate synthase is the target of nitrogen-containing bisphosphonates in osteoclasts, and it is reported that the effect of IBN on the inhibition of this enzyme is greater than that of alendronate [2]. Since IBN has a more convenient dosing regimen than e.g. alendronate, with a higher compliance, adhesion and persistence to therapy, IBN is the more preferred combination partner for ELD than other bisphosphonates [20]. ELD, on the other hand, inhibits osteoclast differentiation directly through inhibition of NFATc1 amplification, which is the critical transcription factor in osteoclastogenesis. ELD upregulates the mRNA expression of IFN-β [21] and inhibits the c-Fos translation which is the transcription factor of NFATc1 in osteoclast progenitor cells [22]. In addition to direct inhibition of osteoclastogenesis, ELD controls the migration of osteoclast precursors through regulation of sphingosine-1-phosphate signaling in osteoclast precursor cells [23]. ELD also reduces the expression of RANKL (receptor activator of NF-κB ligand) in bone tissue, a stimulator of osteoclast differentiation and bone resorption [24]. In this way, IBN attaches to the bone surface and is released to exert its anti-bone resorptive capacity at the bone resorption stage, whereas ELD acts at the osteoclast differentiation stage. This temporal difference in the mode of action on osteoclastogenesis may be the reason why these two drugs exert a synergistic effect on the reduction of bone resorption.

Despite of its synergistic effect on bone resorption, the combination treatment did not show greater inhibition of bone formation than did either monotherapy. In general, in animals or humans with accelerated bone turnover, inhibition of bone resorption causes downregulation of bone formation because bone formation is induced by bone resorption in the remodeling cycle. Stronger inhibition of bone resorption parallels the effect on BMD in osteoporosis patients, so the balance between reducing bone resorption and maintaining bone turnover is the key...
issue of effective treatment of osteoporosis. It is reported that ELD administered simultaneously with alendronate showed a synergistic effect on the reduction of bone resorption and a dose-dependent increase in bone formation [13]. In the present study, we focused on minimodeling as a cause of this uncoupling of bone formation/bone resorption observed in the combination treatment with bisphosphonate plus active vitamin D₃ derivative, because it is reported that calcitriol and ELD both stimulated minimodeling based bone formation in distal femur of OVX rats even though the efficacy of ELD on minimodeling was much greater than calcitriol [25].

Fig. 4. Combination treatment with IBN plus ELD increased lumbar and femoral bone strength. (A) Monotherapy with IBN or ELD increased lumbar ultimate load. Combination treatment did not interfere with the effect of monotherapy and enhanced bone strength as compared to the Vehicle group. (B) Monotherapy with IBN or ELD showed a tendency to increase femoral bone strength, and combination treatment significantly increased femoral midshaft strength. n = 10. *, p < 0.05 vs. Sham by t-test; —, p < 0.05 by Tukey's multiple comparison test. IBN, ibandronate; ELD, eldecalcitol.
Minimodeling is a process of bone formation on quiescent bone surfaces without bone resorption that continues throughout life [16]. Minimodeling observed in human iliac bone is reported to account for less than 1% of the trabecular bone volume and about 2% of the entire bone surface [9]. In this study, minimodeling in sham operated rats was calculated to account for 0.14% of bone volume and 2.13% of bone surface in the lumbar vertebrae. Thus, there is no discrepancy between the previous report of minimodeling in human iliac bone and our present data on minimodeling in lumbar cancellous bone of rats [9]. In the Vehicle group, although the markers of bone resorption and formation in urine and serum and the parameters of bone resorption and formation in bone histomorphometry were increased, minimodeling was not shown to be significantly different from that in the Sham group. This fact indicated that minimodeling is not affected by accelerated bone turnover and so is different from remodeling in this respect. In the ELD group, the number of minimodeling sites, length of minimodeling

Fig. 5. Mineralizing surfaces were seen in all groups. Representative images of trabecular bone (L3) from all experimental groups are shown. Scale bar, 100 μm.
surfaces, or bone volume of minimodeling were increased as has been previously reported [10], and combination treatment did not interfere with this upregulation.

In conclusion, we demonstrated the benefit of combination treatment with IBN plus ELD. The combination treatment showed synergistic inhibition of bone resorption, with bone formation at the same level as in the Sham group. Minimodeling—bone formation independent of bone resorption—may play a part in the uncoupling of bone resorption/bone formation seen with the combination treatment. This combination regimen of IBN plus ELD will help in the efficient long-term

|               | Sham     | Vehicle | IBN     | ELD     | Combination |
|---------------|----------|---------|---------|---------|-------------|
| BV/TV (%)     | 29.5 ± 4.4 | 22.7 ± 3.1* | 32.0 ± 4.8* | 35.8 ± 6.8* | 38.6 ± 4.7† |
| Tb.Th (μm)    | 84.0 ± 5.6 | 71.7 ± 8.6* | 87.6 ± 15.4 | 97.3 ± 17.1* | 103.7 ± 14.4* |
| Tb.Sp (μm)    | 205.4 ± 37.8 | 245.0 ± 22.8* | 186.9 ± 23.4* | 178.3 ± 39.5* | 165.0 ± 18.6* |
| Tb.N (/mm)    | 3.51 ± 0.45 | 3.17 ± 0.20* | 3.67 ± 0.35* | 3.69 ± 0.49* | 3.74 ± 0.28* |
| Oc.S/BS (%)   | 4.86 ± 1.32 | 7.52 ± 2.14* | 2.52 ± 1.14* | 1.22 ± 0.84* | 0.43 ± 0.21† |
| Es/BS (%)     | 19.7 ± 4.0  | 28.2 ± 6.1* | 9.2 ± 3.9* | 4.0 ± 3.4† | 1.2 ± 0.7† |
| N.Oc/BS (/mm) | 1.65 ± 0.39 | 2.57 ± 0.74* | 0.84 ± 0.35* | 0.49 ± 0.26* | 0.24 ± 0.11† |
| Ob.S/BS (%)   | 3.17 ± 1.82 | 14.39 ± 3.31* | 5.15 ± 2.23* | 6.29 ± 1.84* | 6.19 ± 3.11† |
| N.Ob.S/BS (/mm) | 2.87 ± 1.43 | 11.21 ± 2.28* | 4.79 ± 1.75* | 6.58 ± 2.26* | 6.24 ± 2.98* |
| Ms/BS (%)     | 5.55 ± 3.46 | 23.48 ± 5.35* | 6.94 ± 3.27* | 7.89 ± 1.68* | 5.58 ± 2.47* |
| Os/BS (%)     | 4.89 ± 2.76 | 21.27 ± 4.75* | 8.22 ± 3.36* | 10.62 ± 3.20† | 10.48 ± 5.42† |
| BFR/BS (mm³/mm²/year) | 0.029 ± 0.020 | 0.163 ± 0.038* | 0.038 ± 0.020* | 0.044 ± 0.013† | 0.030 ± 0.015§ |
| MAR (μm/day)  | 1.38 ± 0.21 | 1.90 ± 0.10* | 1.48 ± 0.24* | 1.50 ± 0.22* | 1.41 ± 0.22§ |
| Ac.f (/year)  | 1.20 ± 0.71 | 9.08 ± 2.07* | 1.91 ± 0.94* | 2.04 ± 0.64* | 1.36 ± 0.69* |

Data are presented as mean ± S.D. (n = 10).

IBN, ibandronate; ELD, eldecalcitol.

* p < 0.05 vs. Sham group by unpaired t-test.
§ p < 0.05 vs. Vehicle.
† p < 0.05 vs. IBN by Tukey’s multiple comparison test.

Fig. 6. (A) Representative images of minimodeling in cancellous bone (IBN plus ELD combination treatment). Minimodeling formed budding on quiescent bone surfaces. Bright-field, polarized, and fluorescence images are shown. (B) Minimodeling-based bone formation was stimulated in the combination treatment with IBN plus ELD. In the Vehicle group, the level of minimodeling was not changed from that in the Sham group despite increased bone formation markers in serum and increased bone formation parameters in the bone morphometric analysis. Bone formation by minimodeling was significantly increased in the ELD group and Combination group. n = 10. †, p < 0.05 vs. Sham by t-test; —, p < 0.05 by Tukey’s multiple comparison test. IBN, ibandronate; ELD, eldecalcitol; N.ML/BS, minimodeling number; MLBS, minimodeling surface; MLBV/TV, minimodeling volume.
treatment of osteoporosis without inducing severe suppression of bone turnover in osteoporosis patients.

Conflict of interest

SS, ST, MSh, YS, KY, KE are employees of Chugai Pharmaceutical Co., Ltd.

MSh is an employee of Taisho Pharmaceutical Co., Ltd.

FB is an employee of Roche Diagnostics GmbH.

Authorship

Conception and design of study: SS, ST, MSh, JH, FB, KE.

Acquisition and analysis of data: SS, ST, MShu.

Drafting article: SS, JH, FB, KE.

Final approval of submitted version: SS, YS, KE.

Acknowledgment

The authors acknowledge Dr. Daiva Masanauskaite of F. Hoffmann-La Roche Ltd. for discussing the results.

References

[1] R.C. Muhlhauser, F. Bauss, R. Schenk, M. Janner, E. Bosies, K. Strein, H. Fleisch, BM 21.0955, a potent new bisphosphonate to inhibit bone resorption, J. Bone Miner. Res. 6 (1991) 1003–1011.

[2] J.F. Danford, K. Thompson, F.P. Coxon, S.P. Luckman, F.M. Hahn, C.D. Poulter, F.H. Ebertino, M.J. Rogers, Structure–activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates, J. Pharmacol. Exp. Ther. 286 (2001) 235–242.

[3] J.A. Eismann, R. Civitelli, S. Adami, E. Czerwinski, C. Recknor, R. Prince, J.V. Regnster, M. Zaidi, D. Felsenberg, C. Hughes, N. Mairon, D. Masanauskaite, D.M. Reid, P.D. Delmas, R.R. Recker, Efficacy and tolerability of intravenous ibandronate injections in postmenopausal osteoporosis: 2-year results from the DIVA study, J. Rheumatol. 35 (2008) 488–497.

[4] J.Y. Regnster, S. Adami, P. Lakatos, M. Greenwald, J.J. Stepan, S.L. Silverman, C. Christiansen, L. Rowell, N. Mairon, B. Bonvoisin, M.K. Drezer, R. Emkey, D. Felsenberg, C. Cooper, P.D. Delmas, P.D. Miller, Efficacy and tolerability of once-monthly oral ibandronate in postmenopausal osteoporosis: 2 year results from the MOBILE study, Ann. Rheum. Dis. 65 (2006) 654–661.

[5] T. Nakamura, T. Nakano, M. Ito, H. Hagino, J. Hashimoto, M. Tobinai, H. Mizunuma, M.S. Group, Clinical efficacy on fracture risk and safety of 0.5 mg or 1 mg/month intravenous ibandronate versus 2.5 mg/day oral risedronate in patients with primary osteoporosis, Calcif. Tissue Int. 93 (2013) 137–146.

[6] F. Bauss, S. Lalla, R. Endele, L.A. Hothers, Effects of treatment with ibandronate on bone mass, architecture, biomechanical properties, and bone concentration of ibandronate in ovariectomized aged rats, J. Rheumatol. 29 (2002) 2200–2208.

[7] F. Bauss, M. Wagner, L.H. Hothers, Total administered dose of ibandronate determines its effects on bone mass and architecture in ovariectomized aged rats, J. Rheumatol. 29 (2002) 990–998.

[8] T. Matsuzomo, M. Ito, Y. Hayashi, T. Hirota, Y. Tanigawara, T. Sone, M. Fukunaga, M. Shirakai, T. Nakamura, A new active vitamin D3 analog, eldecalcitol, prevents the risk of osteoporotic fractures—a randomized, active comparator, double-blind study, Bone 49 (2011) 605–612.

[9] S. Kobayashi, H.E. Takahashi, A. Ito, N. Saito, M. Nawata, H. Horiuchi, H. Ohta, A. Ito, R. Iorio, N. Yamamoto, K. Takaoaka, Trabecular mimimodeling in human iliac bone, Bone 32 (2003) 163–169.

[10] P.H. de Fretas, T. Hasegawa, S. Takeda, M. Sasaki, C. Tabata, K. Oda, M. Li, H. Saito, N. Amizuka, Eldecalcitol, a second-generation vitamin D analog, drives bone minimodeling and reduces osteoclastic number in trabecular bone of ovariectomized rats, Bone 49 (2011) 335–342.

[11] L. Modekilde, C.C. Danielsen, U.B. Knudsen, The effect of aging and ovariectomy on the vertebral bone mass and biomechanical properties of mature rats, Bone 14 (1993) 1–6.

[12] T. Katsumata, T. Nakamura, H. Ohnishi, T. Sakurama, Intermittent cyclc etidronate treatment maintains the mass, structure and the mechanical property of bone in ovariectomized rats, J. Bone Miner. Res. 10 (1995) 921–931.

[13] S. Sakai, K. Endo, S. Takeda, M. Mihara, A. Shiraishi, Combination therapy with eldecalcitol and alendronate has therapeutic advantages over monotherapy by improving bone strength, Bone 50 (2012) 1054–1063.

[14] A.R. Villanueva, A bone stain for osteoid seams in fresh, unembedded, mineralized bone, Stain. Technol. 49 (1974) 1–8.

[15] F. Bauss, M.P. Drezer, F.H. Glorieux, J.A. Kanis, H. Malluche, P.J. Meunier, S.M. Ott, R.R. Recker, Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee, J. Bone Miner. Res. 2 (1987) 595–610.

[16] W.S. Lee, X.Y. Tian, R.B. Setterberg, Canine ovariectomized bone minimodeling-based formation: a Frost, Takahashi legacy, J. Musculoskelet. Neuronal Interact. 7 (2007) 232–239.

[17] F. Bauss, D.W. Dempter, Effects of ibandronate on bone quality: preclinical studies, Bone 40 (2007) 265–273.

[18] D.E. Hughes, K.R. Wright, H.L. Uy, A. Sasaki, T. Yoneda, G.D. Roodman, G.R. Mundy, B.F. Boyce, Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo, J. Bone Miner. Res. 10 (1995) 1478–1487.

[19] C. Vitte, H. Fleisch, H.L. Guenter, Bisphosphonates induce osteoblasts to secrete an inhibitor of osteoclast–mediated resorption, Endocrinology 137 (1996) 2324–2333.

[20] F.E. Cotte, P. Fardellone, F. Mercier, A.F. Gaulin, C. Roux, Adherence to monthly and weekly oral bisphosphonates in women with osteoporosis, Osteoporos. Int. 21 (2010) 145–155.

[21] S. Sakai, H. Takahashi, K. Matsuzaki, H. Kaneko, M. Furukawa, Y. Miyaeuchi, A. Shiraishi, K. Saito, A. Tanaka, T. Taniguchi, T. Suda, T. Miyamoto, Y. Toyama, 1-Alpha, 25-dihydroxy vitamin D3 inhibits osteoclastogenesis through IFN-beta-dependent NFATc1 suppression, J. Bone Miner. Res. 27 (2010) 1452–1458.

[22] H. Takasu, A. Sugita, Y. Uchiyama, N. Katagiri, M. Okazaki, E. Ogata, K. Ikeda, C-Fos protein as a target of anti-osteoclastogenic action of vitamin D, and synthesis of new analogs, J. Clin. Invest. 116 (2006) 528–535.

[23] J. Kikuta, S. Kawamura, F. Okiji, M. Shirakai, S. Sakai, H. Saito, M. Ishii, Sphingosine-1-phosphate–mediated osteoclast precursor monocyte migration is a critical point of control in antinbone-resorptive action of active vitamin D, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 7009–7013.

[24] S. Harada, T. Mizoguchi, Y. Kobayashi, Y. Nakamichi, S. Takeda, S. Sakai, F. Takahashi, H. Saito, H. Yasuda, N. Udagawa, T. Suda, N. Takahashi, Daily administration of eldecalcitol (ED-71), an active vitamin D analog, increases bone mineral density by suppressing RANKL expression in mouse trabecular bone, J. Bone Miner. Res. 27 (2012) 461–473.

[25] H. Saito, S. Takeda, N. Amizuka, Eldecalcitol and calcitriol stimulates ‘bone mimimodeling’ focal bone formation without prior bone resorption, in rat trabecular bone, J Steroid Biochem. Mol. Biol. 131 (2013) 178–182.