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Antiremodeling Agents Influence Osteoblast Activity Differently in Modeling and Remodeling Sites of Canine Rib

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Abstract. Antiremodeling agents reduce bone loss in part through direct actions on osteoclasts. Their effects on osteoblasts and bone formation activity are less clear and may differ at sites undergoing modeling vs. remodeling. Skeletally mature intact beagles, 1–2 years old at the start of the study, were treated daily with clinically relevant doses of alendronate (0.10 or 0.20 mg/kg), risedronate (0.05 or 0.10 mg/kg), raloxifene (0.50 mg/kg), or vehicle (1 mL/kg). Dynamic bone formation parameters were histologically assessed on periosteal, endocortical/trabecular, and intracortical bone envelopes of the rib. Raloxifene significantly increased periosteal surface mineral apposition rate (MAR), a measure of osteoblast activity, compared to all other treatments (+108 to +175%, $P < 0.02$), while having no significant effect on MAR at either the endocortical/trabecular or intracortical envelope. Alendronate (both 0.10 and 0.20 doses) and risedronate (only the 0.10 dose) significantly ($P \leq 0.05$) suppressed MAR on the endocortical/trabecular envelope, while none of the bisphosphonate doses significantly altered MAR at either the periosteal or intracortical envelopes compared to vehicle. Based on these results, we conclude that (1) at clinically relevant doses the two classes of antiremodeling agents, bisphosphonates and selective estrogen receptor modulators, exert differential effects on osteoblast activity in the canine rib and (2) this effect depends on whether modeling or remodeling is the predominant mechanism of bone formation.

Key words: Bisphosphonate — Raloxifene — Alendronate — Risedronate — Periosteal

Antiremodeling agents significantly reduce vertebral fracture risk in part through their direct effects on osteoclasts. By inducing osteoclast apoptosis and suppressing activity/proliferation/differentiation [1–5], the number of newly initiated remodeling sites is reduced

[6]. Due to the coupling of resorption and formation activities during remodeling, antiremodeling agents effect a systemic decrease in bone-formation activity [7, 8]. It is unclear whether these agents have separate effects on osteoblasts independent of their suppression of remodeling.

Numerous studies have attempted to determine the effects of antiremodeling agents on osteoblasts. *In vitro* data show positive effects of bisphosphonates on osteoblast proliferation and differentiation [9–11] as well as suppression of apoptosis [12, 13]. These data are contrasted by *in vivo* animal studies that have shown bisphosphonate suppression of mineral apposition rate (MAR), indicative of reduced osteoblast activity, at both modeling and remodeling sites [14–17]. Selective estrogen receptor modulators (SERMs) suppress osteoblast apoptosis [18] and increase osteoblast differentiation [19] and proliferation [20] *in vitro*. *In vivo*, SERMs have produced conflicting results with respect to effects on MAR [21–23].

Antiremodeling agents could exert differential effects on osteoblasts depending on whether the cells are involved in remodeling or modeling bone-formation activity. Remodeling-associated formation activity, the predominant form on endocortical, trabecular, and intracortical envelopes [24, 25], is coupled to osteoclasts. Antiremodeling agents may indirectly reduce the individual activity of osteoblasts through reductions in basic multicellular unit-level osteoclast activity (i.e., reduced erosion depth) [26]. These agents may also directly influence osteoblasts *in vivo*. This effect would be most evident at sites undergoing modeling, where formation occurs without previous resorption. Modeling is the predominant mechanism of formation activity on periosteal surfaces, although it can occur on other surfaces [24, 25].

The goal of this study was to determine the effect of bisphosphonates (alendronate and risedronate) and a SERM (raloxifene) on MAR, an indicator of osteoblast activity. Following 1-year treatment of intact beagle

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dogs with clinically relevant doses of these agents, ribs were histologically assessed for bone formation variables. MAR was used as an indicator of osteoblast activity and assessed separately at sites that predominantly undergo remodeling (endocortical/trabecular and intracortical envelopes) or modeling (periosteal) bone formation. We hypothesized that these three antiremodeling agents would suppress osteoblast activity (MAR) at both modeling and remodeling sites.

Materials and Methods

Animals

Seventy-two skeletally mature female beagles (1.3 ± 0.02 years old) were purchased from Marshall Farms (North Rose, NY). Upon arrival, lateral X-rays of all dogs were obtained to confirm skeletal maturity (closed proximal tibia and lumbar vertebra growth plates). Animals were housed two per cage in environmentally controlled rooms at Indiana University School of Medicine's Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility and provided standard dog chow and water. All procedures were approved prior to the study by the Indiana University School of Medicine Animal Care and Use Committee.

Experimental Design

Following 2 weeks of acclimatization, animals were assigned to treatment groups ($n = 12/\text{group}$) by matching body weights. All dogs were treated daily for 1 year with oral doses of vehicle (saline, 1.0 mL/kg), risedronate sodium (0.05 or 0.10 mg/kg; Procter & Gamble Pharmaceuticals, Norwich, NY), alendronate sodium (0.10 or 0.20 mg/kg; Merck, RAHWAY, NJ), or raloxifene (0.50 mg/kg; Lilly Research Labs, Indianapolis, IN). The bisphosphonate doses (risedronate 0.10 and alendronate 0.20) are equivalent to those used for treatment of postmenopausal osteoporosis (on a mg/kg basis), and the lower doses of each drug correspond to approximately half the clinical treatment dose. The raloxifene dose was chosen to produce serum levels approximately equivalent to those documented in postmenopausal women. Risedronate and alendronate were dissolved in saline; raloxifene was diluted in 10% hydroxypropyl- β -cyclodextrin made with distilled water. All agents were administered orally with a syringe each morning after an overnight fast and at least 2 hours prior to feeding. Prior to necropsy, animals were injected with calcein (0.20 mL/kg, intravenous) using a 2-12-2-5 labeling schedule ($n = 9$ animals/group) or a 2-5-2-5 ($n = 3$ animals/group). The shorter interlabel duration was due to a scheduling error. Animals were killed after 1 year by intravenous administration of sodium pentobarbital (0.22 mg/kg). After death, the right ninth rib (~20 mm) was dissected, placed in 10% neutral buffered formalin for 72 hours, and then transferred to 70% ethanol for processing.

Histology

Using an automatic tissue processor (Shandon/Lipshaw, Pittsburgh, PA), specimens were cycled through a graded series of ethanols, cleared using xylene, and infiltrated with methyl methacrylate (MMA; Aldrich, Milwaukee, WI) using routine embedding procedures. Transverse sections (80–100 μm) were cut using a diamond wire saw (Histosaw; Delaware Diamond Knives, Wilmington, DE). Histological measurements were made using a semiautomatic analysis system (Bioquant OS-

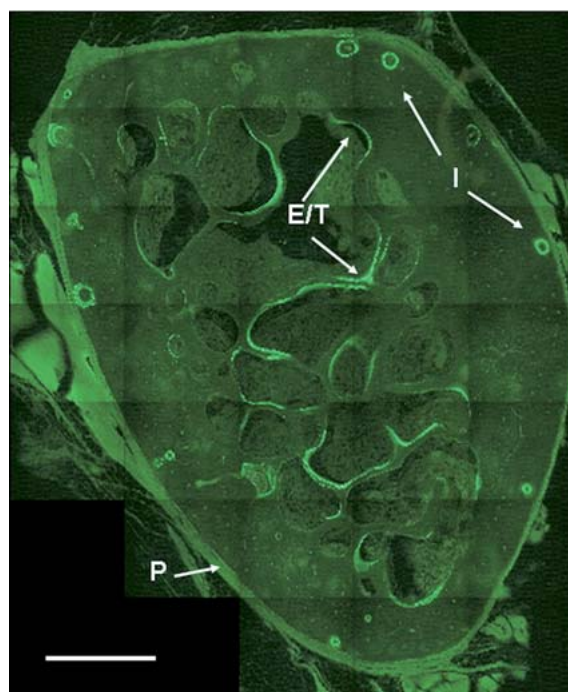


Fig. 1. Photomicrograph of rib cross section. Calcein labeling was separately quantified on periosteal (P), endocortical/trabecular (E/T), and intracortical (I) bone envelopes. Scale bar = 1 mm.

TEO 7.20.10; Bioquant Image Analysis, Nashville, TN) attached to a microscope equipped with an ultraviolet light source (Nikon Optiphot 2; Nikon, Tokyo, Japan). Measurements were made on one cross section per animal (Fig. 1). Periosteal, endocortical/trabecular (mineral surface/bone surface [MS/BS], MAR, bone formation rate [BFR]/BS), and intracortical (MAR, labeled osteon number, activation frequency) dynamic bone formation parameters were separately analyzed as previously described [16] and conformed to standard ASBMR nomenclature [27]. Endocortical and trabecular surfaces were analyzed as a single entity because it was not possible to consistently define the boundary between two surfaces across all tissue sections.

Statistics

Statistical tests were performed using SAS software (SAS Institute, Cary, NC). Differences among treatment groups were evaluated using one-way analysis of variance. When a significant overall F value ($P \leq 0.05$) was present, differences between individual group means were tested using Fisher's protected least-significant difference *post-hoc* test. For all tests, $P \leq 0.05$ was considered statistically significant. Data are presented as mean \pm standard error.

Results

The two classes of drugs, bisphosphonates (alendronate and risedronate) and SERMs (raloxifene), exerted envelope-specific effects on MAR of nonovariectomized dog ribs (Table 1, Fig. 2). Raloxifene-treated animals had significantly higher MAR (+108%, $P = 0.019$) on the periosteal surface compared to animals treated with

Table 1. Rib bone-formation parameters on periosteal, endocortical/trabecular, and intracortical envelopes

| | | | Risedronate | | Alendronate | | |
|---|--------------------------|-------------------------------|---------------------|--------------------------|--------------------------|------------------------|----------|
| | Vehicle (1 mL/kg/day) | Raloxifene (0.5 mg/kg/day) | (0.05 mg/kg/day) | (0.10 mg/kg/day) | (0.10 mg/kg/day) | (0.20 mg/kg/day) | <i>P</i> |
| Periosteal | | | | | | | |
| MS/BS (%) | 7.7 ± 1.5 | 17.6 ± 2.5 | 14.0 ± 2.3 | 13.6 ± 3.9 | 16.1 ± 2.7 | 13.8 ± 2.8 | 0.22 |
| BFR (μm ³ /μm ² /day) | 5.4 ± 1.8 | 20.8 ± 4.1 ^a | 8.0 ± 3.2 | 9.6 ± 3.5 | 11.1 ± 3.2 | 9.4 ± 3.6 | 0.05 |
| Endocortical/trabecular | | | | | | | |
| MS/BS (%) | 22.1 ± 3.3 | 26.6 ± 3.2 ^b | 16.6 ± 3.5 | 9.4 ± 2.3 ^c | 9.0 ± 2.4 ^c | 7.5 ± 1.6 ^c | < 0.0001 |
| BFR (μm ³ /μm ² /day) | 28.7 ± 4.8 | 36.7 ± 4.9 ^b | 18.6 ± 5.5 | 4.7 ± 2.7 ^{c,d} | 6.0 ± 2.6 ^{c,d} | 6.5 ± 1.6 ^c | < 0.0001 |
| Intracortical | | | | | | | |
| MAR (μm/day) | 0.93 ± 0.14 | 0.98 ± 0.14 | 1.07 ± 0.09 | 0.97 ± 0.14 | 1.02 ± 0.06 | 0.89 ± 0.13 | 0.89 |
| L.On.N (<i>n</i> /mm ²) | 1.43 ± 0.21 | 1.80 ± 0.42 | 1.88 ± 0.27 | 1.45 ± 0.31 | 2.10 ± 0.49 | 1.59 ± 0.45 | 0.78 |
| Ac.f (<i>n</i> /year) | 13.9 ± 3.1 | 14.8 ± 3.1 | 15.1 ± 2.9 | 13.0 ± 3.3 | 17.0 ± 4.2 | 12.9 ± 3.5 | 0.96 |

Data are means ± standard error of *n* = 12/group. *P* < 0.05 vs.

^a all other treatments,

^b all bisphosphonate-treated groups,

^c vehicle,

^d risedronate 0.05, and

L.On.N, labeled osteon number; Ac.f, activation frequency.

vehicle (Fig. 2). Neither alendronate nor risedronate significantly altered periosteal MAR at either treatment dose compared to vehicle-treated animals. Periosteal surface MAR was significantly higher in raloxifene-treated animals compared to all bisphosphonate-treated groups (+112% to +175%, all *P* < 0.05). Endocortical/trabecular MAR was similar between vehicle- and raloxifene-treated animals but significantly higher in raloxifene-treated animals compared to all bisphosphonate-treated groups (+53% to +464%, all *P* < 0.05) (Fig. 2). MAR was significantly lower than vehicle in animals treated with alendronate at the 0.10 dose (−59%) and the 0.20 dose (−41%), as well as risedronate at the 0.10 dose (−80%). There was no difference in MAR between vehicle and the lower dose of risedronate (0.05) on the endocortical/trabecular surface. Intracortical MAR was not altered by any of the treatments.

Periosteal mineralizing surface (MS/BS) was not significantly different (*P* = 0.22) among the treatment groups (Table 1). On the endocortical/trabecular surface, there was no significant effect of raloxifene on MS/BS compared to vehicle. However, raloxifene-treated animals had significantly higher MS/BS on this surface compared to all bisphosphonate-treated groups (+60% to +254%, all *P* < 0.05). Endocortical/trabecular MS/BS was significantly lower than vehicle in groups treated with alendronate (both 0.10 and 0.20 doses) or risedronate (only the 0.10 dose). Labeled osteon number, the surrogate to MS/BS within the intracortical envelope, was not significantly altered by any of the treatments (Table 1).

Periosteal surface BFR was significantly higher in raloxifene-treated animals compared to both vehicle (+285%, *P* < 0.003) and all bisphosphonate-treated groups (Table 1). There was no significant difference between vehicle and any of the bisphosphonate-treated

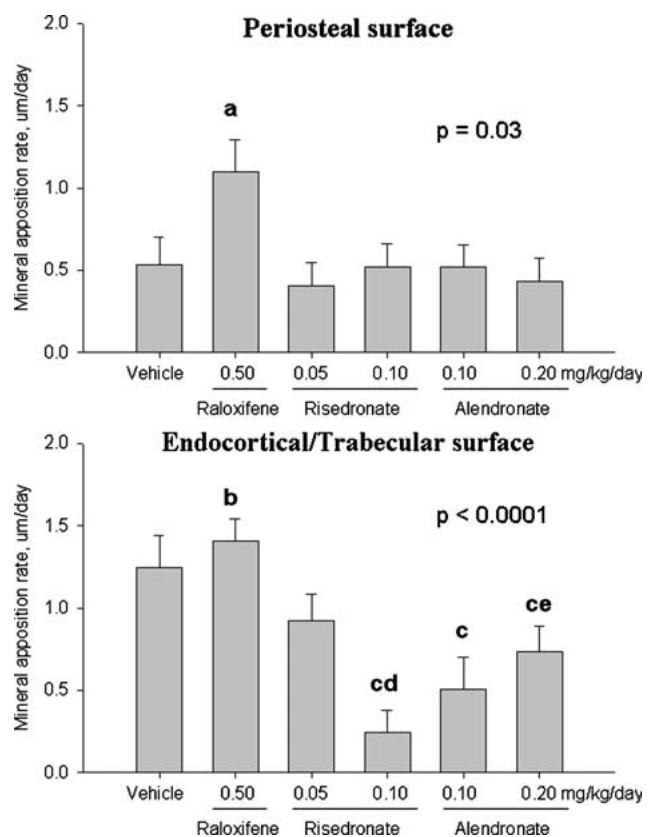


Fig. 2. Envelope-specific effects of antiremodeling agents on MAR of dog ribs following 1-year treatment with clinically relevant doses of raloxifene, alendronate, or risedronate. (A) Periosteal MAR. (B) Endocortical/trabecular envelope MAR. Data presented as mean ± standard error. *P* < 0.05 vs. ^aall other treatments, ^ball bisphosphonate-treated groups, ^cvehicle, ^drisedronate 0.05, ^erisedronate 0.10.

groups. Endocortical/trabecular surface BFR was unchanged with raloxifene compared to vehicle; bisphosphonate-treated animals (alendronate 0.10 and

0.20, risedronate 0.10) had significantly lower BFR compared to vehicle. Intracortical activation frequency was not different among the groups.

Discussion

In addition to direct effects on osteoclasts, antiremodeling agents may influence osteoblasts. It is well accepted that these agents reduce bone formation, but whether this is due to a decrease in the number of formation sites, a decrease in formation at each individual site, or both is unclear. *In vitro* studies have shown that both bisphosphonates and SERMs have direct positive effects on osteoblasts [9–13, 18–20]; however, *in vivo* data have produced conflicting results. Our data show that following 1-year treatment of intact dogs with clinically relevant doses, raloxifene significantly stimulates osteoblast activity on periosteal surfaces without an effect on endocortical/trabecular surfaces. In contrast to raloxifene, bisphosphonates do not alter periosteal osteoblast activity while significantly suppressing MAR on endocortical/trabecular surfaces.

In the mature skeleton, periosteal bone formation occurs predominantly through formation without prior resorption [24]. Our data show that raloxifene significantly increases osteoblast activity (assessed by MAR) on the rib periosteal surface compared to vehicle-treated animals. This is consistent with *in vitro* data showing that raloxifene can positively affect osteoblast activity. In culture, raloxifene stimulates type I collagen secretion and alkaline phosphatase activity [19, 20] and suppresses osteoblast apoptosis [18]. In our study, there was no significant effect of raloxifene (positive or negative) on MAR within either the endocortical/trabecular or intracortical envelopes of the rib, both sites that predominantly undergo coupled formation and resorption in the adult skeleton. These data suggest that raloxifene has a direct stimulatory effect on osteoblasts associated with formation on a surface that primarily undergoes modeling, while this effect is negated on surfaces that primarily undergo remodeling-associated formation activity.

The mechanism through which raloxifene stimulates osteoblast activity on the periosteal surface is unclear. Estrogen receptor- α (ER α) is more highly expressed in cortical bone [28] and appears to be a major regulator of bone modeling on the periosteal surface. Mice lacking ER α receptors exhibit reduced periosteal diameter [29, 30] and an attenuation of loading-induced periosteal formation [31]. Animals and cells lacking ER β are minimally affected with respect to periosteal geometry or cellular activity [32]; this receptor appears to play a greater role in endocortical and trabecular sites. Our findings may be explained by the selective modulation of ER α or ER β by raloxifene, which may differentially

regulate osteoblast-related genes [33] at the various bone envelopes depending on the ER population expressed. It is also possible that factors associated with osteoclasts and/or resorption offset the positive effect of raloxifene at remodeling sites.

Periosteal expansion significantly increases bone strength [34]. The relationship between periosteal dimensions and bone strength is exponential; increases of periosteal radii enhance section modulus (an estimator of bone strength) by the fourth power [35]. Thus, small increases in periosteal apposition are mechanically advantageous as limited amounts of new bone can substantially increase fracture resistance and can mechanically offset loss of endocortical/trabecular bone. The increases in MAR (and BFR) with raloxifene would be expected to significantly increase nonvertebral bone cross-sectional area and reduce nonvertebral fracture risk, yet neither of these effects has been confirmed in clinical trials. Raloxifene did not enhance periosteal expansion beyond that of placebo-treated postmenopausal women after 3 years [36], although the technique used to assess bone size (dual-energy X-ray absorptiometry) may not have sufficient resolution to detect small differences. Additionally, clinical trials with raloxifene have not shown a significant reduction in nonvertebral fractures [37] (in contrast to both risedronate [38] and alendronate [39]). Increased trabecular BMD with bisphosphonates is largely responsible for the reduced fracture risk. Because raloxifene is a less potent antiremodeling agent, it has less effect on trabecular BMD, which may explain its failure to significantly reduce nonvertebral fracture risk. Still, it is entirely possible that periosteal expansion, although known to increase bone strength, is not sufficient with raloxifene treatment to decrease fracture risk in the absence of a more significant trabecular bone mineral density response.

Bisphosphonate treatment (either alendronate or risedronate) did not significantly alter periosteal surface MAR but did significantly suppress MAR on endocortical/trabecular surfaces. These effects on different bone surfaces are consistent with previous studies in dogs [16, 17, 40, 41] and nonhuman primates [42]. In growing [14] and skeletally mature [15] rats, however, treatment with either alendronate or risedronate significantly suppresses osteoblast activity on both periosteal and endocortical surfaces. Our data suggest that bisphosphonates negatively influence the work rate of osteoblasts associated with remodeling-associated formation. High concentrations of bisphosphonates are known to be liberated from the bone matrix during resorption, and osteoblasts have been shown to internalize bisphosphonates [43]. Since bisphosphonates cannot be metabolized, sufficient uptake in osteoblasts would compromise their function via inhibition of protein prenylation [44]. We therefore hypothesize that

decreases in formation with bisphosphonates are due to both a decrease in the number of forming sites and the osteoblast activity at each site. However, human data quantifying MAR with bisphosphonate treatment generally show no effect at any bone envelope [6, 26, 45, 46]. Although these human data are limited by relatively small sample sizes and confined to the iliac crest, until similar drug effects on osteoblast activity can be confirmed in humans our interpretations remain hypothetical.

Periosteal bone formation can be a compensatory mechanism to maintain bone strength in situations where bone loss occurs from trabecular and endocortical surfaces [47]. At their respective clinical doses, raloxifene is known to suppress remodeling less than the bisphosphonates [48]. In these same dogs, vertebral bone turnover was suppressed ~70% with alendronate (0.20) and risedronate (0.10) and only ~20% with raloxifene compared to vehicle [49]. It is therefore possible that bisphosphonates did not increase periosteal formation in our study because the suppression of remodeling on endocortical/trabecular surfaces was sufficient to increase bone mass to some critical level. If this were the case, one might expect that a bisphosphonate dose that had less suppressive effect on the endocortical/trabecular surface would have a more significant anabolic effect on the periosteal surface. However, we did not find this to be the case as the lower dose of risedronate (0.05) suppressed endocortical/trabecular bone formation significantly less than the higher dose (0.10), yet the two had similar periosteal bone formation parameters. These data suggest that the level of turnover suppression on the endocortical/trabecular surface is not a main determinant of periosteal osteoblast activity.

Our data describing bisphosphonate suppression of MAR at remodeling sites may provide some explanation for results from clinical trials using combination treatments. Treatment with alendronate, either concurrently or sequentially with teriparatide, blunts teriparatide-induced increases in bone formation biomarkers and bone mineral density of postmenopausal women [50, 51] and men with low bone mass [52]. This blunting was hypothesized to be related to the reduction in the number of remodeling sites by alendronate, leaving few active formation sites for teriparatide to stimulate. Based on our data, an alternative hypothesis may be that bisphosphonate suppression of osteoblast activity directly offsets the osteoblast stimulatory effect of teriparatide. Additionally, a suppressive effect of bisphosphonates on remodeling-associated bone-formation activity could help explain the failure to consistently find increased trabecular bone volume in bisphosphonate clinical trials [6, 53].

One aim of this study was to compare the effects of raloxifene and bisphosphonates at doses corresponding to those used for treatment of postmenopausal women.

Poststudy analyses of serum from three raloxifene-treated dogs, for reasons unrelated to this study, revealed that the serum concentration of raloxifene was approximately half that predicted from the original dosing calculations. These serum levels were still within the range quantified in postmenopausal women receiving the 60 mg/day dose of raloxifene (Lilly, unpublished data). We therefore compared the raloxifene dose to both the clinical and half-clinical dose of each bisphosphonate. Based on the similarity among the four different bisphosphonate-treated groups with respect to periosteal MAR, the significantly greater MAR with raloxifene appears to represent a true difference in the biological activity of the drug administered at clinically relevant doses. We cannot discount the possibility, however, that bisphosphonate doses lower than those used in this study could potentially stimulate periosteal formation to levels comparable with raloxifene.

The current study has various limitations that should be noted. We analyzed only one bone site of intact, nonovariectomized beagle dogs and therefore cannot be certain whether similar changes would occur in the absence of estrogen (i.e., postmenopausal women) or at other bone sites. The endocortical/trabecular surfaces were not separately assessed as the boundaries are difficult to differentiate and in our experience the surfaces respond in similar ways. Although raloxifene dosing levels provided serum values within the range quantified in postmenopausal women receiving the 60 mg/day dose of raloxifene, we cannot discount the possibility that administering the drug at a higher dose would produce different results. Although there are no data to suggest the raloxifene carrier has any effect on bone formation (10% hydroxypropyl- β -cyclodextrin), we cannot exclude this possibility. Finally, although all growth plates are generally closed in the dog by 12 months [54], we did not assess the rib growth plates prior to treatment and therefore do not know whether continued growth occurred in the ribs of these animals during the course of the study.

In conclusion, we have shown that 1 year of treatment with clinically relevant doses of antiremodeling agents significantly influences osteoblast activity in the rib of beagle dogs. Raloxifene stimulates modeling-associated osteoblast activity on the periosteal surface while maintaining remodeling-associated activity on endocortical/trabecular surfaces at levels similar to untreated controls. In contrast, bisphosphonates suppress remodeling-associated osteoblast activity on the endocortical/trabecular surface while having no significant effect on periosteal osteoblast activity.

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