Development of a low-dose anti-resorptive drug regimen reveals synergistic suppression of bone formation when coupled with disuse

Shane A.J. Lloyd,1,2 Neil D. Travis,2 Teng Lu,2 and Ted A. Bateman2

1Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, British Columbia, Canada; and 2Department of Bioengineering, Clemson University, Clemson, South Carolina

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Lloyd SAJ, Travis ND, Lu T, Bateman TA. Development of a low-dose anti-resorptive drug regimen reveals synergistic suppression of bone formation when coupled with disuse. J Appl Physiol 104: 729–738, 2008. First published January 3, 2007; doi:10.1152/japplphysiol.00632.2007.—Safe and effective countermeasures to spaceflight-induced osteoporosis are required to mitigate the potential for mission-critical fractures and ensure long-term bone health in astronauts. Two anti-resorptive drugs, the bisphosphonate zoledronic acid (ZOL) and the anti-receptor activator of NF-κB ligand protein osteoprotegerin (OPG), were investigated to find the minimal, comparable doses that yield a maximal increase in bone quality, while minimizing deleterious effects on turnover and mineralization. Through a series of five trials in normally loaded female mice (n = 56/trial), analysis of trabecular volume fraction and connectivity using microcomputed tomography, along with biomechanical testing, quantitative histomorphometry, and compositional analysis, was used to select 45 μg/kg ZOL and 500 μg/kg OPG as doses that satisfy these criteria. These doses were then examined for their ability to mitigate bone loss following short-term unloading through hindlimb suspension (HLS). Seventy-two mice were prophylactically administered ZOL, OPG, or PBS and assigned to loaded control or 2-wk HLS groups (n = 12 for each of 6 groups). Both anti-resorptives were able to preserve trabecular microarchitecture and femoral elastic and maximum force in HLS mice (+30–40% ZOL/OPG vs. PBS). In HLS mice, anti-resorptive dosing reduced resorption perimeter at the femoral endocortical surface by 30% vs. PBS. In loaded control mice, anti-resorptives produced no change in bone formation rate; however, reductions in bone formation rate brought about by HLS were exacerbated by anti-resorptive treatment, suggesting synergistic inhibition of osteoblasts during disuse. Refined anti-resorptive dosing will tend to preserve trabecular microarchitecture and femoral elastic and maximum force in HLS mice in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Spaceflight countermeasures currently employed, such as exercise and resistance training, have been shown to effectively prevent muscle atrophy, but they have not translated successfully to the prevention of bone loss (38). Bisphosphonates were the first class of drugs approved for the treatment of osteoporosis. To exert their pharmacological effect, these drugs are thought to act as analogs to pyrophosphate and are incorporated into bone following their addition to adenine nucleotides (30). Bisphosphonates help maintain bone mass, inhibit osteoclast-mediated bone resorption, and reduce the risk of both vertebral and nonvertebral fractures (13). Although the use of bisphosphonates as a countermeasure is under consideration by NASA, the etiology of spaceflight-induced osteoporosis is fundamentally different from the osteoporosis commonly found in the postmenopausal women for whom these drugs were originally developed (14).

Bone remodeling is a continual process that includes both the resorption and formation of bone. This homeostatic process allows for maintenance of normal bone mass and quality, thus ensuring skeletal competence under dynamic loading conditions. In a phenomenon referred to as “coupling”, the activities of bone-building osteoblasts and bone-resorbing osteoclasts are intimately linked and balanced (17, 25). During spaceflight, osteoclast-mediated bone resorption has been shown to increase dramatically, and the resultant bone loss is compounded by the effects of reduced calcium consumption, reduced intestinal calcium absorption, and increased calcium excretion (33). In microgravity, there is an uncoupling of bone resorption and formation that is not seen in postmenopausal osteoporosis (34). Analysis of specific alkaline phosphates and osteocalcin has shown that, during spaceflight, bone formation actually re-
A receptor-related protein that acts as a decoy receptor for receptor activator of NF-κB ligand (3). Through coupling, bisphosphonates and other anti-resorptive therapies will tend to suppress both bone resorption and formation (16). However, suppression of bone turnover postflight, which can be exacerbated by the long half-life of bisphosphonates following incorporation into bone, could adversely affect astronaut recovery. Of course, recovery following spaceflight may not follow the coupling mechanism seen during normal remodeling and may instead resemble that observed during pathological conditions, such as fracture repair, where formation increases independent of resorption (27). Furthermore, clinical studies have demonstrated that bisphosphonates are capable of blunting the anabolic effect of parathyroid hormone (PTH) (4, 12). Analogous postflight anabolic suppression would not be favorable, given that this response is required to recover bone mass lost during spaceflight unloading.

As countermeasures to the consequences of spaceflight, NASA and the National Space Biomedical Research Institute have proposed the use of alendronate and two other anti-resorptives (23): zoledronic acid (ZOL) (Reclast, Novartis Pharmaceuticals, Basel, Switzerland) and the osteoprotegerin (OPG) analog AMG 162 (Denosumab, Amgen, Thousand Oaks, CA) (2, 26). Endogenous OPG is a novel, secreted, TNF (OPG) analog AMG 162 (Denosumab, Amgen, Thousand Oaks, CA) (2, 26). Endogenous OPG is a novel, secreted, TNF receptor-related protein that acts as a decoy receptor for receptor activator of NF-κB ligand, a cytokine required for osteoclast activation (6). ZOL is a bisphosphonate that is Food and Drug Administration (FDA) approved for use in treating hypercalcemia of malignancy (5) and postmenopausal osteoporosis (3). However, these drugs and their recommended therapeutic doses are not optimized for microgravity-associated bone loss in astronauts. Regardless of the agent selected, the formulation of specific countermeasures is critical to optimizing recovery by expeditiously returning bone turnover to preflight levels.

Administration of supramaximal doses of anti-resorptive agents can lead to unnecessary inhibition of bone formation postflight, while the use of subtle dosing can target the therapy to a desired timeframe (i.e., during the mission) and can ensure that a maximal effect is achieved with the administration of a minimal dose. For osteoporosis, this approach will limit suppression of osteoblast activity and bone turnover. This is particularly relevant given the pharmacokinetics of a protein-based treatment such as Denosumab, which is not incorporated into bone like bisphosphonates and is metabolized in a relatively short duration. Denosumab is a fully human, monoclonal antibody that mimics the action of the naturally occurring protein OPG; however, it should be noted that Denosumab could not be used in the present animal studies because it is specific to the human receptor activator of NF-κB ligand.

We propose that it is advantageous and medically beneficial to determine the minimum, comparable, efficacious doses of ZOL and OPG in mice. In this paper, the regimen-development process is presented as a series of iterative studies in a murine model that gradually refines the target dose for each drug as a “proof-of-concept”. Microcomputed tomography (microCT), compositional analysis, and quantitative histomorphometry were used to evaluate bone quality and indicators of bone resorption and formation. In later iterations of this dose-development phase, the range of bone assays was expanded, and the target doses were more precisely refined.

These doses were then tested for their ability to maintain skeletal competence during limited periods of disuse, while minimizing the aforementioned effects on bone formation. ZOL and OPG were administered prophylactically to mice before unloading through hindlimb suspension (HLS), the accepted ground-based model for simulating the adverse effects of microgravity on bone and muscle in both rats (15) and mice (1, 31, 32). By placing the animal at an incline, its hindlimbs are free of mechanical loading, and a cephalic fluid shift occurs. Although not without its limitations, HLS does sufficiently simulate two of the most important physiological effects of microgravity.

This paper represents the first in a series of investigations, with the ultimate aim being to establish an anti-resorptive treatment plan that is effective in preventing bone loss in combination with other anabolic countermeasures, such that bone turnover may be returned to normal levels as soon as possible after the mission. Given the uncoupling of bone turnover that occurs during spaceflight, it is hypothesized that the development of subtle, refined, anti-resorptive dosing will allow bone resorption to be effectively inhibited, independent of bone formation. Such an approach would tend to target the countermeasure to the period of disuse and lead to less adverse effects on the skeletal health of astronauts.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) with identical, exact date of birth (6 or 12 wk, depending on trial) were utilized. Animals were weighed and divided into groups to achieve an approximately equivalent mean mass (15.3 ± 0.1 g) and minimally equivalent standard deviation. Animals were group housed (3 mice/cage), given food and water ad libitum, and allowed a 1-wk acclimatization period before the start of a study. Each treatment group was composed of 8–12 animals, depending on the trial. The biotechnology company Amgen (Thousand Oaks, CA) provided all of the drugs utilized in these studies. It should be noted that, primarily due to concerns regarding the development of an immunogenic response to human OPG in mice, all studies were limited to 2 wk in duration. All animal protocols were approved by the Animal Care and Use Committee at Clemson University and were carried out under the supervision of certified veterinary and veterinary-assistant staff.

Maximal Dose Study

To establish the maximal anti-resorptive response, a preliminary study was conducted with the relatively large doses of 10 mg/kg ZOL and 20 mg/kg OPG. On day 0, mice were subcutaneously (SC) injected with phosphate-buffered saline (PBS) or one of the anti-resorptives.

Dose Development Study

The process for determining the minimum, comparable, efficacious dose of ZOL and OPG involved a series of five iterative trials. In the early phases, a limited number of assays were applied to each trial; principally, trabecular microarchitecture through microCT was employed. At this stage, it was critical to establish a range of efficacious doses rather than to concentrate on analysis of a large number of bone assays. In each iteration, mice were administered a SC injection of PBS, ZOL, or OPG. Three doses of each anti-resorptive were chosen: low (0.1×), medium (1×), and high (10×). For the first iteration, Trial I, doses of ZOL (0.3, 3, 30 µg/kg) and OPG (1, 10, 100 µg/kg) were administered.
Trabecular bone parameters were examined with microCT, and the minimum dose to produce maximal response vs. PBS (i.e., 30 μg/kg ZOL and 100 μg/kg OPG) was set as the median dose for the next iteration, Trial II.

The procedure for Trial II was identical, with treatment groups (n = 8/group) of PBS, ZOL (15, 30, 100 μg/kg), and OPG (50, 100, 1,000 μg/kg). MicroCT analysis of tibial trabecular bone properties revealed that ~30 μg/kg ZOL and 100 μg/kg OPG produced an optimal response. This process continued with each iteration, narrowing the range of the bracketing doses as outlined in Table 1. As the optimal dose became more refined, assays were expanded from microCT to include compositional analysis, quantitative histomorphometry, and biomechanical testing. This allowed for comparison of the various aspects of bone formation and resorption.

**HLS Study**

Following the dose-development process, optimal doses of 45 μg/kg ZOL and 500 μg/kg OPG were selected for evaluation in a model of simulated microgravity called HLS. Animals were randomly assigned to one of six treatment groups (n = 12/group): loaded control (LC; PBS, 45 μg/kg ZOL or 500 μg/kg OPG) or HLS (PBS, 45 μg/kg ZOL or 500 μg/kg OPG). LC mice were housed in groups of three within standard vivarium cages, whereas HLS mice were maintained in individual cages. On day 0, all animals were weighed and given a SC injection of PBS, ZOL, or OPG.

The HLS apparatus is composed of a 2-cm plastic dowel and attached swivel hook that was attached to the animal’s tail with hypoallergenic tape, such that when the hook was attached to the cage’s guide wire, the animal was suspended at a 30° angle with its forelimbs reaching the wire mesh floor. The condition of all animals and their weight was recorded daily.

**Study End Point**

On day 14 of the maximal, dose-development, and HLS experiments, all mice were anesthetized with 2% isoflurane and killed by cardiac puncture and cervical dislocation. Hindlimbs were removed, and both tibiae and femora were cleaned of nonosseous tissue. The left femur of each animal, required for mechanical testing and compositional analysis, was allowed to air-dry for 48 h. The right femur and both tibiae and femora were cleaned of nonosseous tissue. The left femur, required for histomorphometry and microCT assays, respectively, were fixed in a 10% neutral buffered formalin solution for 48 h, rinsed with distilled water, and stored in 70% ethanol.

**MicroCT**

Tibiae were randomized and loaded four at a time into a sample tube filled with 70% ethanol. MicroCT analysis (μCT20, Scanco Medical, Bassersdorf, Switzerland) with a voxel size of 9 μm (15-μm resolution) in all three spatial directions was conducted. Trabecular bone parameters were obtained with scans of 0.9-mm trabecular bone sections at the proximal end of the tibia, immediately distal to the epiphyseal plate. Examined parameters included trabecular bone volume (BV), total volume (TV), and connectivity density (Conn.Dens) of trabecular struts. Trabecular volume fraction was calculated from BV/TV. These parameters have been established as being critical to bone quality (18).

**Biomechanical Testing**

For biomechanical testing of the femora, bones were rehydrated in PBS for 90 min before evaluation to simulate in vivo properties (7). Three-point bending tests were performed using an Instron 5582 (Blue Hill 2 software, Instron, Norwood, MA). Femora were tested to failure with an 8-mm span length and a deflection rate of 5 mm/min. The maximal force (P, N) and deflection (δ, mm) were measured for all mechanically tested bones. These two properties were also determined at the elastic limit and the failure point. Stiffness (N/mm) was calculated from P/δ

**Quantitative Histomorphometry**

Fixed femora were allowed to air-dry and then were embedded with non-infiltrating Epo-Kwick epoxy (Buehler, Lake Bluff, IL). The formed disks were sectioned with a low-speed saw (Buehler, 12.7 cm × 0.5 mm diamond blade) at the mid-diaphysis of the femur. These sections were wheel-polished to a flat, smooth surface using 600-, 800-, and 1,200-grit carbide paper, followed by polishing with a cloth impregnated with 6-μm diamond paste. This allowed micrographs at ×50 magnification to be taken of the bone cross sections under UV light (400 nm) with an FS filter. All animals in dose-development Trial V and in the HLS study had been given a SC injection of calcine bone label (10 mg/kg) on day 7, which produced a visible green label, indicating the bone formation sites during the period of the study. Quantitative histomorphometric analysis was performed using these photographs and SigmaScan Pro software (SPSS, San Rafael, CA).

Measurements of bone morphology (29) included total bone area enclosed by periosteal perimeter and endocortical area. Cortical area (CA) was calculated as total bone area enclosed by periosteal perimeter minus endocortical area. The area between the calcine label and the cortical perimeter was measured as bone formation area, and linear content of the labeled perimeter was defined as active mineralizing perimeter (AMPm). Bone formation rate (BFR = bone formation area/7 days) and mineral apposition rate (MAR = BFR/AMPm) were calculated separately in the periosteal and endocortical areas. The proportional endocortical eroded surface (Ec.ES) was measured by quantifying the portion of the nonlabeled surface with a rough/ruffled border and dividing it by the length of the endocortical perimeter.

**Compositional Analysis**

For both maximal and dose-development studies, mineral-content analysis was performed on whole fractured femurs (37). For the HLS study, analysis of the diaphysis, epiphysis, and whole femur was performed separately. A properly calibrated analytic scale was used for all measurements. Dry mass (Dry-M) was measured after heating the bones to 105°C for 24 h. Mineral mass (Min-M) was measured after the bones had been ashed by baking at 800°C for another 24 h. Percent mineralization (%Min) was calculated by the formula %Min = Min-M/Dry-M × 100%.

**Statistics**

Statistical analysis of results was completed using SigmaStat software version 8.2 (Systat Software, San Jose, CA). For the maximal and dose-development studies, comparisons were made using a one-way ANOVA with a Student-Newman-Keuls post hoc test to reveal
significance between groups. A 95% level of significance (type I error) was utilized for each of these tests. For the HLS study, a two-way ANOVA, with a Tukey test for follow-up comparisons, was used to pool the data appropriately and to determine the overall effects of anti-resorptive treatment and HLS. Differences for the HLS study were determined with a $P < 0.05$. Trends were used to evaluate high throughput dose-development data and represent a $P < 0.1$. Data are presented as means ± SE.

RESULTS

MicroCT: Maximal Dose Study

Administration of relatively large doses of ZOL (10 mg/kg) and OPG (20 mg/kg) to 12-wk-old mice was carried out to establish their maximal effect (Fig. 1). BV/TV was greater in mice treated with ZOL (+34%) and OPG (+38%) vs. PBS ($P < 0.05$ for both). Conn.Dens was also greater than PBS in ZOL- (+32%) and OPG-treated (+27%) mice ($P < 0.05$ for both).

Dose Development Study

MicroCT: dose-development study Trial I. Analysis of trabecular microarchitecture was completed for mice tibiae from Trial I (Fig. 2). Although it represents a nonsignificant trend, the maximally responding ZOL dose was 30 μg/kg, with a mean BV/TV 27% greater than PBS ($P = 0.08$). Conn.Dens from this group was 81% greater than PBS ($P < 0.05$). The optimal OPG dose was 100 μg/kg, resulting in a trend toward greater BV/TV (+12%) and Conn.Dens (+38%) ($P = 0.09$ for both). On the basis of these results, doses of 30 μg/kg ZOL and 100 μg/kg OPG were selected as the median doses for the next iteration (i.e., Trial II).

Data trends and statistical significance were used when necessary to select the appropriate doses for subsequent iterations. The high throughput nature of this “proof-of-concept” investigation prevented the large $n$ values that are often required to elucidate significant differences. The iterative process continued with interim Trials II-IV, utilizing this same refinement process (Table 1).

MicroCT: dose-development study Trial V. For the final iteration in the dose-development process, Trial V, a transition to younger animals with greater bone turnover was made to increase the likelihood of observing significant differences. Six-week-old mice were treated with PBS, ZOL (0.3, 3, 30 μg/kg), or OPG (1, 10, 100 μg/kg) and killed 14 days later. Values are means ± SE. *Data are significantly different vs. PBS ($P < 0.05$).

![Fig. 1. Maximal dose study. Mean trabecular volume fraction (bone volume/total volume (BV/TV)) and connectivity are shown as measured through microcomputed tomography of 0.9-mm sections of mouse tibiae immediately distal to the epiphyseal plate. PBS, phosphate-buffered saline; ZOL, zoledronic acid; OPG, osteoprotegerin. Twelve-week-old mice were administered a subcutaneous injection of either PBS or anti-resorptive (10 mg/kg ZOL or 20 mg/kg OPG) and killed 14 days later. Values are means ± SE. *Data are significantly different vs. PBS ($P < 0.05$).](image1)

![Fig. 2. Dose development study Trial I. Mean trabecular volume fraction (BV/TV) (A) and connectivity (B) are shown as measured by microcomputed tomography of 0.9-mm sections of mouse tibiae immediately distal to the epiphyseal plate. Twelve-week-old mice were administered a subcutaneous injection of PBS, ZOL (0.3, 3, 30 μg/kg), or OPG (1, 10, 100 μg/kg) and killed 14 days later. Values are means ± SE. *Data are significantly different vs. PBS ($P < 0.05$).](image2)
previous low-dose treatments (Fig. 2), although these trials were conducted in older animals.

**Biomechanical testing: Trial V.** Mechanical properties of mice in Trial V were assessed through three-point bending (Table 2). Generally, ZOL and OPG did not produce significant changes in femoral maximum strength or stiffness. However, 500 μg/kg OPG did result in greater stiffness than PBS (+36%, P < 0.05).

**Quantitative histomorphometry: Trial V.** Histomorphometric analysis revealed a minimal effect on bone-formation parameters with the administration of low-dose anti-resorptives in Trial V (Table 3). For most treatment groups, no significant change in BFR, AMPm, or MAR was observed (P > 0.05). There was, however, significantly greater Ct.Th (+8%) and Ct.Ar (+7%) vs. PBS for 500 μg/kg OPG (P < 0.01 for both). These results compliment the significantly greater maximal force observed for this dose during biomechanical testing (Table 2). There was a greater Ct.Ar (+5%, P < 0.05) observed for both 10 and 30 μg/kg ZOL, although no other significant difference was found in any other histomorphometric parameter for ZOL (P > 0.05).

**Composition analysis: Trial V.** Compositional analysis allowed the determination of whole femur percent mineralization of mice treated in Trial V. Percent mineralization of femora from anti-resorptive-treated mice was consistent with PBS control (61–63%), with no significant difference between groups (P > 0.05).

**HLS Study**

**Effects of HLS.** HLS had an effect on the mechanical strength of mouse tibiae, resulting in significantly lower mechanical properties examined compared with LC mice (Fig. 4). For PBS-treated mice, this included lower elastic force (−29%), maximal force (−28%), and stiffness (−40%) (P < 0.05 for all).

MicroCT analysis (Fig. 5) revealed that HLS produced significantly lower Conn.Dens (−64%) and BV/TV (−37%) for PBS-treated mice compared with LC (P < 0.05 for all).

Histomorphometric analysis of the PBS-treated mice revealed lower parameters for HLS mice compared with LC (Table 4). This included lower total BFR (Ti.BFR, −45%), cortical thickness (Ct.Th, −15%), and total MAR (−27%) (P < 0.05 for all). The ratio of endocortical eroded surface to total endocortical perimeter in HLS mice was greater than LC (+240%) (P < 0.01).

Percent mineralization in the femoral epiphysis was 7% lower (P < 0.01) in HLS mice treated with PBS compared with LC. Additionally, there was a trend toward lower whole bone femoral percent mineralization (P = 0.08) (Table 5).

**Effects of anti-resorptives on LC mice.** For LC mice, ZOL was found to result in significantly greater stiffness (+20%) and maximal force (+17%) (P < 0.05 for both) (Fig. 4). OPG was found to produce a significant change in stiffness (+20%) (P < 0.05), while a trend of greater maximal force (+10%, 8.7 ± 0.2 vs. 9.6 ± 0.3 N) (P = 0.08) was also observed. No significant difference in elastic force was found in LC mice treated with OPG compared with PBS (P > 0.05 for both).

Administration of ZOL to LC mice resulted in significantly greater BV/TV (+199%) vs. PBS, while OPG in LC mice

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**Table 2. Dose development study Trial V: Femora mechanical properties as tested in a 3-point bending setup**

<table>
<thead>
<tr>
<th>Femoral Measurement</th>
<th>ZOL</th>
<th>OPG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>10 μg/kg 30 μg/kg 45 μg/kg</td>
</tr>
<tr>
<td>Stiffness, N/mm</td>
<td>29.0±2.3</td>
<td>35.2±1.7</td>
</tr>
<tr>
<td>Elastic force, N</td>
<td>6.3±0.3</td>
<td>6.6±0.4</td>
</tr>
<tr>
<td>Maximum force, N</td>
<td>8.7±0.5</td>
<td>10.0±0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. PBS, phosphate-buffered saline. Mechanical testing was conducted with an Instron 5582 following 90 min of rehydration in PBS. Six-week-old mice were administered a subcutaneous injection of PBS, ZOL (10, 30, 45 μg/kg), or OPG (100, 250, 500 μg/kg) and killed 14 days later. *Data are significantly different vs. PBS (P < 0.05).
resulted in a similar 180% greater BV/TV compared with PBS ($P < 0.01$ for both) (Fig. 5A). Conn.Dens was even more greatly effected, with 443 and 445% greater Conn.Dens for ZOL and OPG, respectively ($P < 0.01$), compared with PBS within LC mice (Fig. 5B).

There was an absence of significant histomorphometric differences in LC mice treated with either ZOL or OPG compared with PBS, including a BFR that was not significantly lower than PBS ($P > 0.05$) (Table 4).

Determination of percent mineralization revealed no significant difference for any part of the femur following treatment with OPG or ZOL in PBS-treated LC mice ($P > 0.05$) (Table 5).

**Effects of anti-resorptives on HLS mice.** Anti-resorptive treatment ameliorated the deleterious effects of unloading on the mechanical strength of the mouse femurs (Fig. 4). All mechanical properties were significantly greater in HLS mice than PBS, including a BFR that was not significantly lower than PBS ($P > 0.05$). The endocortical region (endocortical BFR) had lower TlBFR ($−66−74\%$, $P < 0.05$) for anti-resorptive-treated HLS mice vs. PBS, while the periosteal surface (periosteal BFR) saw no significant difference ($P > 0.05$). In HLS mice, MAR was not significantly different for anti-resorptive treatment compared with PBS ($P > 0.05$). Bone resorption on the endocortical surface was over twofold greater for HLS mice treated with PBS ($P < 0.05$) (Table 2); however, prophylactic treatment with ZOL or OPG reduced this response by one-third ($P < 0.05$).

For HLS mice, there was no significant difference in percent mineralization for anti-resorptive treatment compared with PBS ($P > 0.05$).

### DISCUSSION

**Dose Development**

Based on data from microCT, mechanical testing, quantitative histomorphometry, and compositional analysis, the results of the dose-development study indicated that 45 μg/kg ZOL and 500 μg/kg OPG are the minimum, comparable, efficacious anti-resorptive doses in mice for a 2-wk duration. Analysis of trabecular microarchitecture and mechanical testing alone would have indicated 30 μg/kg ZOL and 250 μg/kg OPG as the optimal doses, as generally no difference was found between parameters for these and the higher 45 μg/kg ZOL and 500 μg/kg OPG doses (Fig. 3, Table 2). However, the value of expanded assays was highlighted by the results of compositional analysis, which suggested a trend toward greater percent mineralization at the larger doses. Based on the combination of these data and quantitative histomorphometry (Table 3), which indicated greater Ct.Th for 500 μg/kg OPG vs. PBS, the decision was made to recommend these two relatively higher doses.

The iterative development process presented here attempts to refine the maximal dose (Fig. 1) into a regimen that will achieve the desired effect: optimizing bone quality, while limiting adverse effects on recovery. This dose development has not been without challenges and is presented here as a “proof-of-concept”. The iterative process is dependent on both result consistency and animal response. During the course of

### Table 3. Dose development trial V: Quantitative histomorphometry parameters obtained from micrographs of mid-diaphysis femur cross sections

<table>
<thead>
<tr>
<th>Measurement</th>
<th>PBS</th>
<th>10 μg/kg</th>
<th>30 μg/kg</th>
<th>45 μg/kg</th>
<th>100 μg/kg</th>
<th>250 μg/kg</th>
<th>500 μg/kg</th>
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<tbody>
<tr>
<td>Ec.Ar, mm²</td>
<td>0.90±0.02</td>
<td>0.91±0.02</td>
<td>0.90±0.02</td>
<td>0.90±0.02</td>
<td>0.89±0.01</td>
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<td>Ct.Ar, mm²</td>
<td>0.58±0.01</td>
<td>0.61±0.01*</td>
<td>0.61±0.01*</td>
<td>0.60±0.01</td>
<td>0.56±0.01</td>
<td>0.57±0.02</td>
<td>0.62±0.01†</td>
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<tr>
<td>Ct.Th, mm</td>
<td>0.134±0.004</td>
<td>0.143±0.003</td>
<td>0.144±0.004</td>
<td>0.143±0.005</td>
<td>0.135±0.004</td>
<td>0.137±0.005</td>
<td>0.145±0.004*</td>
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<tr>
<td>Ec.BFR, 10⁻³ mm³/day</td>
<td>6.85±0.62</td>
<td>7.28±0.38</td>
<td>5.60±0.58</td>
<td>5.53±0.61</td>
<td>7.16±0.58</td>
<td>5.67±0.82</td>
<td>6.62±0.40</td>
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<tr>
<td>Ps.BFR, 10⁻³ mm³/day</td>
<td>3.25±0.38</td>
<td>3.66±0.27</td>
<td>3.73±0.15</td>
<td>3.19±0.39</td>
<td>2.65±0.37</td>
<td>3.12±0.40</td>
<td>3.80±0.39</td>
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<tr>
<td>Ts.BFR, 10⁻³ mm³/day</td>
<td>10.10±0.72</td>
<td>10.93±0.47</td>
<td>9.33±0.60</td>
<td>8.72±0.72</td>
<td>9.81±0.69</td>
<td>8.79±0.91</td>
<td>10.42±0.56</td>
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<tr>
<td>Ec.AMPm, mm</td>
<td>2.23±0.11</td>
<td>2.31±0.07</td>
<td>2.10±0.14</td>
<td>2.13±0.14</td>
<td>2.26±0.09</td>
<td>1.99±0.12</td>
<td>2.39±0.18</td>
</tr>
<tr>
<td>Ps.AMPm, mm</td>
<td>1.46±0.09</td>
<td>1.64±0.08</td>
<td>1.82±0.07</td>
<td>1.55±0.13</td>
<td>1.43±0.11</td>
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<td>1.70±0.08</td>
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<tr>
<td>Ts.AMPm, mm</td>
<td>3.69±0.14</td>
<td>3.95±0.11</td>
<td>3.92±0.16</td>
<td>3.67±0.19</td>
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<tr>
<td>Ec.MAR, 10⁻³ mm/day</td>
<td>3.06±0.23</td>
<td>3.15±0.13</td>
<td>2.64±0.19</td>
<td>2.55±0.22</td>
<td>3.15±0.18</td>
<td>2.77±0.28</td>
<td>2.83±0.14</td>
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<td>Ps.MAR, 10⁻³ mm/day</td>
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<td>2.23±0.14</td>
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<td>2.01±0.14</td>
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<td>1.94±0.16</td>
<td>2.21±0.15</td>
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<td>Ts.MAR, 10⁻³ mm/day</td>
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<td>5.39±0.19</td>
<td>4.69±0.20</td>
<td>4.56±0.26</td>
<td>4.95±0.27</td>
<td>4.71±0.32</td>
<td>5.04±0.21</td>
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</table>

Values are means ± SE. Six-week-old mice were administered a subcutaneous injection of PBS, ZOL (10, 30, 45 μg/kg), or OPG (100, 250, 500 μg/kg) and killed 14 days later. Ec, endocortical; Ps, periosteal; Ts, total; Ar, area; BFR, bone formation rate; AMPm, active mineralizing perimeter; MAR, mineral apposition rate; Th, thickness. Data are significantly different vs. PBS (*$P < 0.05$; †$P < 0.01$).
In this study, the importance of animal age was highlighted through the use of 6- and 12-wk-old animals. The initial maximal and dose development trials were conducted in older animals to more accurately model the mature physiology of the astronaut population; however, resolving fine changes in trabecular architecture and bone composition over a 14-day period proved to be a challenge as the doses became more refined. At the suggestion of Amgen personnel, Trial IV was repeated with younger, 6-wk-old mice (i.e., Trial V). The metabolism of young animals is higher, and this increased growth rate translates into higher bone turnover. Thus it is easier to observe differences in the 2-wk trial period required for this high-throughput study.

The observed trabecular BV/TV and Conn.Dens for the lowest ZOL dose (10 μg/kg) in Trial V (Fig. 3) was indeed anomalous in the context of the previous results. However, in combination with other assays, the specific response in regard to trabecular microarchitecture is consistent with an anabolic effect. While quantitative histomorphometry revealed that administration of 10 μg/kg ZOL resulted in the greatest bone formation and MAR (Table 3), the percent mineralization produced by the 10 μg/kg dose was lower than PBS. This suggests an increase in turnover consistent with an anabolic therapy, such as PTH (9, 28). It is more likely, however, that this is a unique, species-specific, low-dose bisphosphonate effect in particularly young animals. Given that this effect was not observed in earlier assays of trabecular microarchitecture in mature animals, there is limited value in further consideration of the response. Therefore, these results were eliminated from the determination of the minimally efficacious anti-resorptive dosing.

MicroCT results (Fig. 3) revealed greater trabecular parameters for both 45 μg/kg ZOL and 500 μg/kg OPG vs. PBS in Trial V, indicating that these doses were able to successfully inhibit osteoclast-mediated resorption. However, there was no difference in BFRs for these subtle doses (Table 3). This is in contrast to previous studies, in which administration of high-dose anti-resorptives (i.e., 0.3 mg·kg⁻¹·day⁻¹) mitigated bone resorption at the cost of a relatively large and deleterious impact on BFR (1). Clearly, such a response would pose an unacceptable risk to astronaut health postflight.

HLS

Through the iterative process outlined above, we demonstrated that minimal doses of the anti-resorptives ZOL and OPG were sufficient to ameliorate bone loss in 6-wk-old mice. Mean trabecular volume fraction (BV/TV) (A) and connectivity (B) are shown as measured though microcomputed tomography of 0.9-mm sections of mouse tibiae immediately distal to the epiphyseal plate. Six-week-old mice were administered a subcutaneous injection of PBS or anti-resorptive (45 μg/kg ZOL or 500 μg/kg OPG) and killed after 14 days of loaded control (LC) or HLS conditions. *Significant difference between anti-resorptive (ZOL or OPG, P < 0.05) and PBS within the LC or HLS groups. †Significant effect of loading condition (LC vs. HLS, P < 0.05) within PBS, ZOL, or OPG treatment groups.

Fig. 5. HLS study. Mean trabecular volume fraction (BV/TV) (A) and connectivity (B) are shown as measured though microcomputed tomography of 0.9-mm sections of mouse tibiae immediately distal to the epiphyseal plate. Six-week-old mice were administered a subcutaneous injection of PBS or anti-resorptive (45 μg/kg ZOL or 500 μg/kg OPG) and killed after 14 days of LC or HLS conditions. Values are means ± SE. *Significant difference between anti-resorptive (ZOL or OPG, P < 0.05) and PBS within the LC or HLS groups. †Significant effect of loading condition (LC vs. HLS, P < 0.05) within PBS, ZOL, or OPG treatment groups.
Hindlimb suspension study: Quantitative histomorphometry parameters from hindlimb-suspended and loaded-control mice obtained from micrographs of mid-diaphysis femur cross sections

<table>
<thead>
<tr>
<th>Measurement</th>
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<th>OPG</th>
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<td>Ec.BFR, 10⁻³ mm³/day</td>
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<td>Ps.BFR, 10⁻³ mm³/day</td>
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<tr>
<td>Tt.BFR, 10⁻³ mm³/day</td>
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<td>Ec.AMPm, mm</td>
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<tr>
<td>Ps.AMPm, mm</td>
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<td>Tt.MAR, mm</td>
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</tr>
<tr>
<td>Ec.ES</td>
<td>0.14±0.02 †</td>
<td>0.11±0.02 †</td>
<td>0.14±0.02 †</td>
</tr>
</tbody>
</table>

Values are means ± SE. Six-week-old mice were administered a subcutaneous injection of PBS or anti-resorptive (45 µg/kg ZOL or 500 µg/kg OPG) and killed after 14 days of loaded-control or hindlimb-suspension conditions. Ec.ES, endocortical eroded surface. *Significant difference between anti-resorptive (ZOL or OPG, P < 0.05) and PBS within the loaded-control or hindlimb-suspended groups. †Significant effect of loading condition (loaded vs. hindlimb suspended, P < 0.05) within PBS, ZOL, or OPG treatment groups.

As mentioned previously, the ability of anti-resorptives to effectively inhibit bone resorption is ideal, but the concomitant inhibition of bone formation is of special concern, particularly at high doses of anti-resorptive. This concept can be seen in our analysis of bone histomorphometry parameters for loaded and unloaded mice (Table 4). Although anti-resorptive treatment was able to reduce osteoclast-mediated endocortical resorption brought about by HLS, it also further potentiated the suppression of endocortical bone formation rate. Of course, it is important to note that treatment with OPG and ZOL in LC mice did not result in suppression of bone formation, Ct.Th, or AMPm. Despite their different mechanisms of action, there was no difference between the suppressing effects of ZOL and OPG. These findings highlight the uncoupling of bone formation and resorption that occurs during unloading (34) and the fact that unloading may act cooperatively with anti-resorptive treatments to potentiate inhibition of osteoblasts via coupling. Alternatively, the observed response may be a form of mechanical adaptation, whereby anti-resorptive-induced decreases in bone resorption maintain bone strength during unloading, thereby resulting in a decrease in osteoblast-mediated bone formation. Future studies might investigate multiple time points in animal recovery to confirm these findings and elucidate the mechanism behind the response. Although not investigated in the present study, it would be reasonable to hypothesize that the magnitude and duration of the suppression of bone-formation rate following reloading would be reduced compared with higher doses. Furthermore, the 2-wk duration of the studies outlined presently is an important limitation, especially considering the varying mechanisms of action of the two anti-resorptives investigated and their rise to maximal efficacy. Considerations, including the development of an immunogenic response to human OPG in mice and previous data from 2-wk shuttle missions, were our guiding motivation for selecting this duration. Despite this, both the consistency and significance of the findings suggest that the administration of relatively large doses of anti-resorptive may not be necessary to maintain the integrity of trabecular bone during extended microgravity exposure.

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results throughout this “proof-of-concept” study highlight the applicability of the results.

**Therapeutic Translation**

The development of an effective therapeutic countermeasure to spaceflight-induced osteoporosis should not be undertaken without regard for the long-term health of the astronaut population. Excessive anti-resorptive treatment and severe inhibition of bone resorption may not be necessary to protect an astronaut’s skeletal system from the temporary stress of the space environment. Compared with PBS control, administration of high-dose anti-resorptives (i.e., 10 mg/kg ZOL, 20 mg/kg OPG) in the present study was found to result in greater trabecular volume fraction and connectivity, which both increased by one-third compared with PBS (Fig. 1). One of the most important properties of a protein-based treatment, such as Denosumab or the PTH analog Teriparatide (Forteo, Eli Lilly, Indianapolis, IN), is that they do not become incorporated into bone like bisphosphonates. These drugs are also cleared from the body more quickly and are less likely to adversely suppress bone turnover postflight. Despite these beneficial properties, the concomitant issue of bone turnover inhibition with either OPG or ZOL is important to consider.

One could administer an arbitrarily high dose of ZOL, OPG, or another anti-resorptive and effectively inhibit bone resorption and minimize bone loss in microgravity. However, the serious effects on bone turnover and detrimental long-term effects on recovery could result in a net negative balance for the astronaut. Previous studies have highlighted the blunted anabolic response to agents such as PTH when given following anti-resorptive therapies (3, 11). These postflight recovery considerations are especially important considering the relative youth of astronauts, who would be expected to have at least 40 more productive years of life following their mission. A refined dosing regimen that incorporates the desirable response in regard to bone microarchitecture, with minimal adverse effects on turnover, is clearly required.

Although ZOL has the potential to be administered infrequently as once a year (3), the relative ease and safety of a SC Denosumab injection in microgravity is a consideration. Alternatively, oral bisphosphonates, such as alendronate, may prove to be the most convenient option, as they have fewer risks of complication during administration (8). Furthermore, it would not be prudent to discount other FDA approved bisphosphonates, such as ibandronate and risedronate. In particular, risedronate has a less profound effect on bone turnover than other agents in this class (10).

The refinement of this minimally efficacious dosing regimen clearly has application only to the specific animal model for which it was developed. Ultimately, a human clinical trial, with an objective similar to the present study, would need to be conducted in normally loaded patients. The relative dosing ratios recommended here (~1:10 for ZOL/OPG) would be a reasonable starting point for administration. Such a study would utilize dual-energy X-ray absorptiometry, peripheral quantitative computed tomography, serum markers, and ideally a bone biopsy to evaluate outcomes and combined effects on trabecular microarchitecture, cortical strength, and bone formation. The next logical step would be a follow-up bed-rest study to evaluate the ability of the minimally efficacious anti-resorptive dosing to ameliorate the effects of prolonged disuse in humans.

**Conclusion**

The determination of 45 μg/kg ZOL and 500 μg/kg OPG as the minimum, comparable, efficacious, anti-resorptive regimen demonstrates that bone quality can be preserved with low-dose anti-resorptive therapy. The fact that significant increases in bone microarchitecture can be achieved without reducing BFRs alludes to the potential of this type of regimen to ameliorate the effects of long-duration spaceflight without inhibiting bone turnover postflight. Such countermeasures could help to ensure astronaut health, mission safety, and the success of future long-duration space exploration.

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**DISCLOSURES**

Amergen Inc. provided the zoledronic acid and osteoprotegerin that was utilized in this study. T. Bateman is the recipient of a monetary gift from Amergen Inc.

**REFERENCES**


