ORIGINAL ARTICLE

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# Modulation of murine bone marrow-derived CFU-F and CFU-OB by *in vivo* bisphosphonate and fluoride treatments

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**Structured Abstract**

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***Objectives –*** Bisphosphonates (BPN) have actions on a variety of cell types including: osteoclasts, osteoblasts, osteocytes, and endothelial cells. The objectives of this report are to review the current state of understanding of the effects of BPNs on orthodontic tooth movement and to provide evidence on BPN's *in vivo* effects on bone marrow-derived osteoprogenitor cells.

***Material and Methods –*** Mice from the C3H/HeJ (C3H), C57BL/6J (B6), FVB/NJ (FVB), and BALB/cByJ (BALB) strains were treated for 3 weeks with 0, 3, 30, or 150 mcg/kg/week alendronate (ALN) administered subcutaneous alone or in combination with 50 ppm fluoride (F). Bone marrow cells were harvested and subjected to *in vitro* colony-forming unit fibroblast (CFU-F) and colony-forming unit osteoblasts (CFU-OB) assays.

***Results –*** Baseline differences in CFU-F, CFU-OB/ALP+, and CFU-OB/total were observed among the four strains. Strain-specific responses to ALN and F treatments were observed for CFU-F, CFU-OB/ALP+, and CFU-OB/total. F treatment alone resulted in decreases in CFU-F ($p = 0.013$), CFU-OB/ALP+ ($p = 0.005$), and CFU-OB/total ($p = 0.003$) in the C3H strain. CFU-F ($p = 0.036$) were decreased by F in the B6 strain. No significant (NS) effects of F were observed for FVB and BALB. ALN treatment resulted in a significant decrease in CFU-F ($p = 0.0014$) and CFU-OB/total ($p = 0.028$) in C3H only. ALN treatment had NS effect on CFU-OB/ALP+ in all four strains.

***Conclusion –*** Genetic factors appear to play a role in ALN's effects on CFU-F and CFU-OB/total but not on CFU-OB/ALP+.

**Key words:** alendronate; bisphosphonates; colony-forming unit fibroblast; mesenchymal stem cells; osteoprogenitors

## Introduction

Nitrogen-containing bisphosphonates (BPN) are a major class of bone seeking compounds that are used for the treatment of bone diseases, such as Paget's disease of bone, multiple myeloma, bone metastases, osteoporosis (adults), and pediatric bone diseases (i.e. osteogenesis imperfecta). BPNs are stable analogs of naturally occurring inorganic

pyrophosphate-containing two phosphonate groups attached to a single carbon atom, forming a 'P-C-P' structure. BPNs have high affinity for hydroxyapatite (HAP) crystals and have multiple direct effects on HAP, including inhibition of calcification, crystal growth, and crystal dissolution. BPNs have highly selective localization and retention in bone preferentially compartmentalizing to bone undergoing resorption or formation and can achieve high local levels (1–3).

Bisphosphonates can inhibit of bone resorption indirectly through impairment of osteoclast function. The non-nitrogenous BPN can disrupt osteoclast cellular metabolism and induce apoptosis (4, 5). In long-term bone marrow cultures BPNs inhibit osteoclast differentiation (6, 7) and appear to act through osteoblasts to inhibit osteoclast function (7–9). Despite these actions, BPN's greatest effects on osteoclast function come from inhibition of farnesyl pyrophosphate synthase (FPPS) within the melavonate pathway (10–12). FPPS generates isoprenoid lipids during the post-translational modification of small GTP-binding proteins (i.e. Rho, Rac, cdc42, and Rab) important for osteoclast formation, function, and survival.

As pointed out above BPN can have actions on osteoblast function. BPN exhibit biphasic effects on osteoblast precursors *in vitro* – stimulatory at low doses and inhibitory at higher doses (13, 14). At a lower dose/exposure, ALN inhibits osteoblast apoptosis (15, 16) via connexin 43 (17, 18). Several investigators also noted that low dose/exposure of BPN stimulates osteoblast proliferation and differentiation *in vitro* (14, 19, 20). On the other hand, at a higher dose/exposure, BPN have been shown to exhibit no stimulatory effect on osteoblasts (21, 22) and suppress bone formation independently of bone resorption (23). Effects of BPNs, such as ALN, have been observed to be dose-dependent and animal model dependent (24). However, no study has investigated the dose-dependent modulation of formation of early- and late-osteoblastic cell precursors by *in vivo* ALN treatments with genetic background as a factor. Finally, bisphsophonates have actions on angiogenesis. Clodronate, risedronate, ibandronate, pamidronate, and zoledronic acid have anti-angiogenic actions (25–27). BPN can inhibit *in vitro* proliferation, chemotaxis, circulation, and capillary formation of bone marrow endothelial cells via VEGF and VEGF receptors (28–30). Furthermore, BPN can lead to transient reduction in circulating levels of VEGF, bFGF, and Mmp2 following zolendronate infusion (31).

#### Bisphosphonates and orthodontic tooth movement

The systemic effects of BPNs on orthodontic tooth movement. ALN administered subcutaneously (s.c.) inhibited tooth movement in rats to 40% of the control (32). A single intraperitoneal dose of 1500 $\mu$g/kg of pamidronate prior to orthodontic tooth movement resulted in impaired osteoclast structure and decreased expression of vacuolar-type $H^+$-ATPase and cathepsin K (33). Systemic pamidronate administered shortly before removal of orthodontic force resulted in decreases the extent of initial relapse of orthodontically moved rat molars (34). Similarly, s.c. administration of pamidronate inhibited molar tooth movement in rats (35). The topical application of risedronate, ALN, or clodronate in the subperiosteum of following tooth movement prevented relapse of the moved teeth as well as root resorption (32, 36–38). Finally, there is *in vitro* evidence using isolated primary human periodontal ligament (PDL) cells subjected to mechanical induced stress (compression) that clodronate is capable of inhibiting prostaglandin $E_2$, cyclo-oxygenase-2, and receptor activator of NF-kappa B ligand gene expression (39).

Awareness to potential risks of BPN in orthodontics has been raised (40–42). However, there are very few case reports in literature of orthodontic treatment of patients who were taking BPN, one of which involved only two patients (41).

#### Fluoride

Fluoride (F) is an important micronutrient which, similar to BPN, preferentially compartmentalizes to bone and accumulates with deposition of newly formed bone (43, 44). F is known for its anabolic effects on bone and its use as a therapeutic agent for post-menopausal osteoporosis has been investigated with mixed results (45, 46). F can affect osteoblasts anabolically *in vivo* (47) and *in vitro* (43) through an undetermined mechanism and results in increased bone mass (43, 48). It has been demonstrated using inbred mouse strains that genetic factors play a role in the effects of F both in dental fluorosis (49) and in variation in bone properties in response to F exposure (50, 51). Yan et al. (52) used B6

and C3H inbred strains of mice to show that genetic background influences F's effect on osteoclastogenesis. As F and BPN target the same physiological compartment, it is possible that there may be some interaction between these two agents.

#### Bone marrow-derived MSCs and osteogenic potential

In 1970s, Friedenstein et al. reported that marrow stromal cells/mesenchymal stem cells (MSC), from the bone marrow, possess the potential to differentiate along multiple mesenchymal cell lineages, including osteoblast precursors (53–55). A standard liquid culture system was developed to isolate MSC by their adherence to the plastic of tissue-culture plates, where clonal populations expand from single precursors – colony-forming unit fibroblasts (CFU-F). CFU-F is recognized as the early-osteoblastic cell precursors and the CFU-F assay is a useful method to enumerate the number of MSCs in bone marrow (53–56). With the addition of ascorbic acid and dexamethasone, the differentiation of the plastic adherent cells can be modified *in vitro* at the colony level to give rise to cells capable of forming mineralized nodules – colony-forming unit osteoblasts (CFU-OB) (56).

The use of CFU-F and CFU-OB assays provides an opportunity for the assessment of the effects of *in vivo* ALN treatment, alone and with F, on early- and late-osteoblastic cell precursors from different inbred strains of mice.

## Materials and methods

#### Animals

Male mice of the C57BL/6J (B6), BALB/cByJ (BALB), C3H/HeJ (C3H), and FVB/NJ (FVB) inbred strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) at 5 weeks of age. Food and water were provided *ad libitum*. A laboratory rodent diet LabDiet® 5001 (PMI® Nutrition International, Richmond, IN, USA) was provided and contained 0.95% calcium, 0.67% phosphorous, 4.5I U/g vitamin D3, and an average [F] of 6.56 ± 0.28 $\mu$g/g. Mice from each strain were caged in trios and housed in the Division of Lab Animal Medicine facility within the Dental Research Center, a fully AAALAC accredited unit, at an ambient temperature of 21°C and maintained on a 12:12 h light/dark cycle. All experimental procedures were approved by the IACUC at the University of North Carolina at Chapel Hill.

#### ALN and F treatments

A total of 64 mice per strain were used for this study. After 1 week of acclimation, eight mice per strain were randomly assigned to one of eight treatment groups (group 1 = ALN 3 $\mu$g/kg/week, F 0 ppm; group 2 = ALN 3 $\mu$g/kg/week, F 50 ppm; group 3 = ALN 30 $\mu$g/kg/week, F 0 ppm; group 4 = ALN 30 $\mu$g/kg/week, F 50 ppm; group 5 = ALN 150 $\mu$g/kg/week, F 0 ppm; group 6 = ALN 150 $\mu$g/kg/week, F 50 ppm; group 7 = ALN 0 $\mu$g/kg/week, F 0 ppm; and group 8 = ALN 0 $\mu$g/kg/week, F 50 ppm).

Fluoride ion (0 or 50 ppm) was provided as NaF (CAS 7681-49-4; Sigma-Aldrich, Inc., St Louis, MO, USA) in the drinking water. ALN (alendronate sodium, a gift from Merck Research Laboratories, Rahway, NJ, USA) was prepared in 0.9% w/v NaCl and administered s.c. The 3 and 30 $\mu$g/kg/week was administered as a single dose and the 150 $\mu$g/kg/week was administered as a split dose twice a week. The 3 $\mu$g/kg/week dose has been previously shown to significantly increased the number of CFU-F colonies in the bone marrow from young and old animals and better permit assessment of bone-forming effects of low-dose ALN in osteoporosis (14). The 30 $\mu$g/kg/week dose was shown to stop bone loss in ovariectomized rats (57). The 150 $\mu$g/kg/week was considered an equivalent dose for mice to have a maximal effect on osteoclasts without toxic effects. After 3-week treatment mice were euthanized. Bone marrow cells were flushed and collected from the tibia and femur from one hind leg of each animal.

#### CFU-F assays

Bone marrow cells were flushed from the femurs and tibiae of mice and plated in triplicate cultures (six-well plates) at two different densities ($0.5 \times 10^6$ or $1.0 \times 10^6$ cells/well) using complete media prepared with MesenCult™ Basal Medium and Mesenchymal stem cell Stimulatory Supplements (StemCell Technologies, Vancouver, BC, Canada). The formation of CFU-F was evaluated after 14 days of culture in a humidified 5% $CO_2$/37°C environment. Cultures were washed with calcium and magnesium-free Dulbecco's

phosphate buffered saline (PBS) twice and then fixed with cold ethanol. CFU-Fs were stained with Giemsa stain and colonies with >50 cells counted using light microscopy.

#### CFU-OB assays

The bone marrow cells obtained from the mice were plated in triplicate cultures (six-well plates) at two different densities ($0.5 \times 10^6$ or $1.0 \times 10^6$ cells/well) as described above. The complete media used will be prepared from MesenCult$^{TM}$ Basal Medium and Mesenchymal stem cell Stimulatory Supplements (StemCell Technologies) plus 50 $\mu$g/ml of ascorbic acid and $10^{-8}$ M dexamethasone. After 14 days, the cultures were terminated by washing with PBS twice and then fixed with cold ethanol. Formation of osteoblast progenitors was detected using an alkaline phosphatase assay (86-R; Sigma-Aldrich). Alkaline phosphatase positive colonies were counted. Afterwards the plates were washed with borate buffer and stained with borate buffer containing 1% w/v methylene blue for total colonies.

#### Statistics

Results of the CFU-F and CFU-OB assays were reported as mean ± SD of triplicate cultures. For each variable, effects across treatment groups were compared using one-way ANOVA. For comparison of treatment groups in pairs, Student's *t*-test was used. Adjusted *p*-values ≤ 0.05 were considered significant.

## Results

Strain-dependent differences in CFU-F, CFU-OB/ALP+, and CFU-OB/total were observed at baseline (Table 1). BALB mice demonstrated significantly higher mean CFU-F compared with B6 ($p < 0.0001$), FVB ($p < 0.0001$), and C3H ($p < 0.001$). FVB had the lowest mean CFU-F and this difference was significant when compared to C3H ($p = 0.002$) and BALB ($p < 0.0001$) but not B6. BALB also displayed a significantly higher number of ALP+ CFU-OB than B6 ($p < 0.0001$), C3H ($p < 0.001$), and FVB ($p < 0.001$). When the total numbers of CFU-OB were counted, a greater difference between the strains was noted. BALB had significantly higher number of CFU-OB/total compared with B6 ($p < 0.0001$) and FVB ($p < 0.001$); C3H also displayed significantly higher number of CFU-OB/total com-

***Table 1.*** **Colony-forming units (CFUs) fibroblast and osteoblast following alendronate and fluoride treatment**

| Treatment group* | CFU-F† | | CFU-OB/ALP+ | | CFU-OB/total | |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD |
| C3H-1 | 16.56 | 5.50 | 8.89 | 5.13 | 17.11 | 8.71 |
| C3H-2 | 15.78 | 2.77 | 5.67 | 2.24 | 14.44 | 3.54 |
| C3H-3 | 17.22 | 7.36 | 8.56 | 6.29 | 18.11 | 11.44 |
| C3H-4 | 15.33 | 5.34 | 6.22 | 5.33 | 13.89 | 10.95 |
| C3H-5 | 11.22 | 2.44 | 5.11 | 1.96 | 10.33 | 4.06 |
| C3H-6 | 12.56 | 1.51 | 5.67 | 2.55 | 11.56 | 5.34 |
| C3H-7 | 20.33 | 5.66 | 7.22 | 3.70 | 19.56 | 8.05 |
| C3H-8 | 13.11 | 5.23 | 2.89 | 1.36 | 9.00 | 4.53 |
| B6-1 | 9.89 | 4.01 | 2.78 | 2.39 | 6.89 | 3.02 |
| B6-2 | 6.67 | 5.57 | 1.44 | 1.24 | 4.67 | 2.40 |
| B6-3 | 9.56 | 9.58 | 4.22 | 4.87 | 9.33 | 8.69 |
| B6-4 | 7.33 | 6.08 | 2.89 | 2.93 | 8.00 | 5.48 |
| B6-5 | 7.44 | 6.31 | 3.56 | 4.25 | 7.67 | 6.60 |
| B6-6 | 6.00 | 3.57 | 1.00 | 1.12 | 3.33 | 3.08 |
| B6-7 | 13.00 | 6.14 | 3.56 | 4.00 | 8.33 | 4.42 |
| B6-8 | 7.89 | 2.71 | 3.22 | 2.73 | 7.56 | 5.13 |
| FVB-1 | 11.33 | 3.84 | 4.78 | 3.99 | 9.11 | 6.75 |
| FVB-2 | 11.44 | 5.20 | 5 | 4.44 | 6.78 | 5.87 |
| FVB-3 | 13.56 | 8.85 | 6.56 | 5.83 | 12.44 | 10.85 |
| FVB-4 | 16.11 | 5.11 | 5.33 | 3.67 | 9.33 | 6.63 |
| FVB-5 | 10.78 | 4.06 | 4.33 | 2.65 | 6.89 | 3.72 |
| FVB-6 | 10.11 | 6.43 | 5.56 | 4.59 | 8.33 | 6.86 |
| FVB-7 | 10.56 | 4.82 | 7.33 | 5.48 | 12.22 | 8.53 |
| FVB-8 | 9.11 | 5.73 | 5.89 | 6.11 | 10.33 | 9.35 |
| BALB-1 | 35.00 | 14.02 | 20.33 | 9.11 | 31.89 | 12.59 |
| BALB-2 | 34.44 | 12.74 | 18.78 | 6.85 | 31.22 | 10.50 |
| BALB-3 | 34.78 | 4.79 | 18.44 | 3.81 | 31.89 | 3.95 |
| BALB-4 | 32.78 | 6.04 | 18.56 | 2.70 | 31.00 | 3.00 |
| BALB-5 | 29.44 | 3.75 | 17.89 | 2.71 | 28.22 | 4.18 |
| BALB-6 | 29.44 | 3.78 | 17.67 | 3.32 | 29.56 | 2.96 |
| BALB-7 | 28.22 | 7.12 | 15.78 | 5.93 | 26.00 | 7.70 |
| BALB-8 | 29.89 | 6.17 | 16.78 | 2.95 | 26.44 | 3.47 |

*Group 1 = ALN 3 $\mu$g/kg/week, F 0 ppm; group 2 = ALN 3 $\mu$g/kg/week, F 50 ppm; group 3 = ALN 30 $\mu$g/kg/week, F 0 ppm; group 4 = ALN 30 $\mu$g/kg/week, F 50 ppm; group 5 = ALN 150 $\mu$g/kg/week, F 0 ppm; group 6 = ALN 150 $\mu$g/kg/week, F 50 ppm; group 7 = ALN 0 $\mu$g/kg/week, F 0 ppm; and group 8 = ALN 0 $\mu$g/kg/week, F 50 ppm.
†Mean CFU colonies/$1 \times 10^6$ BMCs from triplicate cultures.

pared with B6 ($p = 0.003$) and FVB ($p = 0.042$). These strain-dependent differences in bone marrow-derived CFUs were consistent with reports from others (58, 59). Intrinsic differences were again observed in the ratios of the total CFU-OB that were ALP+ at baseline for each individual strain. BALB and FVB showed higher ALP+/total CFU-OB ratios compared with B6 (BALB, $p = 0.004$; FVB, $p = 0.006$) and C3H (BALB, $p = 0.011$; FVB, $p = 0.017$).

Fluoride alone did not significantly alter the frequency of CFU-F, CFU-OB/ALP+, or CFU-OB/total for BALB and FVB mice. Significant decreases in mean CFU-F were found in B6 ($p = 0.036$) and C3H mice ($p = 0.013$) treated with 50 ppm [F]. Only the C3H strain demonstrated significant reductions in CFU-OB/ALP+ ($p = 0.005$) and CFU-OB/total ($p = 0.003$).

Systemic ALN had no significant effect on the frequencies of CFU-F, CFU-OB/ALP+, or CFU-OB/total for the BALB, B6, and FVB strains. ALN treatment significantly reduced CFU-F ($p = 0.0014$) and CFU-OB/total ($p = 0.028$) in the C3H strain without affecting the frequency of CFU-OB/ALP+.

## Discussion

In our review of the literature, we found that little is known regarding BPNs' action during orthodontic tooth movement beyond reductions in movement magnitude and reducing relapse predominantly in animal models (32, 34–38). BPN have actions on a variety of cell types including those coordinating angiogenesis. Whereas the majority of studies have focused upon BPNs' actions on osteoclasts and osteoblasts, we chose to investigate potential effects of the nitrogen-containing BPN, ALN in the context of another bone seeking agent F, on the bone marrow MSC pool. This study investigated the inter-strain responses to ALN treatment (+/− F). Four strains of mice, C57BL/6J (B6), BALB/cByJ (BALB), C3H/HeJ (C3H), and FVB/NJ (FVB) were selected based on differences in genetics, bone biology, and wound healing characteristics (49–52, 60, 61). This study tested the hypothesis that systemic ALN and F would interact and affect the bone marrow pool of MSCs capable of forming early- (CFU-F) or late- (CFU-OB) osteoprogenitor colonies in a strain-specific manner. While the four strains differed in baseline frequencies of CFU-F, CFU-OB/ALP+, and CFU-OB/total their responses to F or ALN were limited. F treatment of 50 ppm in the drinking water would raise the serum [F] to a physiologically relevant level of approximately 6–10 $\mu$M/l (52, 62) and had the most pronounced effect on the C3H strain by reducing CFU-F, CFU-OB/total, and CFU-OB/ALP+ frequency. B6 was the only other strain that responded to F with a reduction in CFU-F. ALN across a wide range of doses resulted in only a modest reduction in CFU-F and CFU-OB/total in the C3H strain. Given the relatively small number of strains investigated it would appear that systemic ALN has minimal effects on bone marrow MSC pool and no effect on the frequency of ALP+ osteoblast precursor potential.

## Clinical relevance

There is a rapidly growing population of patients receiving oral and parenteral BPN to manage/treat diseases of the bone and in cancer. Concerns have emerged towards unwanted and potentially serious side-effects of BPN therapy. Successful orthodontics relies upon orchestrated function of many of the same cells affected by BPN. There is great interest to better understand to what extent BPN therapy may have on orthodontic treatment and outcome as well as any possibility that orthodontic treatment may trigger events capable of leading to serious side-effects like osteonecrosis of the jaw.

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