EFFECTS OF ENOXACIN ON OSTEOBLASTS

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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To my wife, family, friends, and colleagues, who helped me along the way

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Introduction: Numerous bone debilitating diseases such as osteoporosis are linked to osteoclastic bone resorption. Current conventional treatments with bisphosphonates inhibit osteoclast bone resorption by inducing apoptosis. Unfortunately, this also impedes osteoclasts from signaling the reparative bone forming function of osteoblasts. Interaction between actin filaments and vacuolar H+-ATPases (V-ATPases) in osteoclasts is required for transport of V-ATPase to the plasma membrane which is required for bone resorption. Computational docking programs were used to identify enoxacin as a small molecule that binds to the actin –binding site on the B-subunit of V-ATPase and competitively inhibits the V-ATPase-actin interaction. Enoxacin is potentially the first example of a new class of small molecule therapeutic agents for the treatment of osteoclast-mediated diseases that target V-ATPase transport.

Methods: This study complements ongoing analysis of the effects of enoxacin on osteoclasts by testing the effects of enoxacin on osteoblast formation, bone mineralization, and cytokine and chemokine production. The working hypothesis is that

enoxacin will have no effect on the presence of osteoblasts, osteoblast mineralization or cytokine production at concentrations that inhibit osteoclastic bone resorption.

Results: Osteoblasts were observed in concentrations of enoxacin that inhibited osteoclasts. The results of the two experiments testing osteoblast mineralization were contradictory. There was no significant difference in osteoblast cytokine and chemokine production.

Conclusion: Enoxacin may be of therapeutic benefit in the treatment of osteoclastic diseases or as an adjunct to orthodontic tooth movement. This study demonstrated that enoxacin inhibited osteoclast bone resorption at certain concentrations (IC50=10uM) in vitro while having limited effects on bone mineralization of osteoblasts.

CHAPTER 1
INTRODUCTION

Osteoblasts and Bone Remodeling

Bone contains three specialized cell types, osteoblasts, osteoclasts, osteocytes, that tend an extracellular matrix mineralized by calcium hydroxyapatite deposits. Bone remodeling is a constant and dynamic process by which the functions of osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells) are interdependent. Osteoblasts produce factors that regulate osteoclast differentiation and function.[1] In humans, a given amount of bone will take two to three weeks to resorb but at least three months to reform.[2, 3] A primary mechanism by which osteoblasts regulate bone resorption is through the expression of receptor activator of nuclear factor kappa B ligand (RANKL). This ligand binds to RANK on the surface of pre-osteoclast cells, stimulating differentiation of these cells into mature osteoclasts. RANKL also activates bone resorption.[1] A decoy receptor, osteoprotegerin (OPG), is also produced by osteoblasts and, acting as an antagonist, binds RANKL, compeititvely-inhibiting its interaction with RANK and thus preventing osteoclast formation and activity.[4, 5, 6, 7, 8] This relationship was demonstrated when deletion of the RANKL gene in mice resulted in the absence of osteoclasts and the presence of osteopetrosis due to osteoblasts being unable to stimulate osteoclastogenesis.[9] Likewise, over-expression of OPG in mice also resulted in osteopetrosis; and OPG-deficient mice exhibited osteoporosis.[10] Mature osteoblasts express greater levels of OPG versus RANKL, thus supporting bone formation; whereas, pre-osteoblastic cells express more RANKL, stimulating osteoclast formation and bone resorption.[11]

Osteoclasts Stimulate Osteoblasts in Bone Formation

As previously discussed, osteoblasts have been demonstrated to regulate osteoclast activity by means of RANKL and OPG. More recently it has been suggested that osteoclasts, the bone-resorbing cells, produce factors that control bone formation (Figure 1-1). Mutations in mice resulting in the loss of macrophage colony stimulating factor (M-CSF) or the *c-fos* gene (a transcription factor affecting osteoblasts and osteoclasts) caused the absence of osteoclasts which led to negative effects on bone formation.[12] Deletion of the *c-src* gene in transgenic mice resulted in increased numbers of osteoclasts that were unable to resorb bone because of a defect in the formation of the resorptive sealing zone. This resulted in increased bone formation despite decreased resorption.[13, 14] Signaling between osteoblasts and osteoclasts is also demonstrated in relation to the anabolic effect of parathyroid hormone (PTH).[15, 16] *C-fos* -/- animals (absence of osteoclasts) treated intermittently with PTH showed no anabolic effects.[18] *C-src* -/- mice with increased nonresorbing osteoclasts showed normal anabolic effect with PTH treatment.[18] These results indicate the presence of osteoclasts is needed for an anabolic response to PTH. Pederson *et alia* discovered that mature osteoclast conditioned medium stimulated human mesenchymal stem (hMS) cells migration and differentiation toward the osteoblast lineage and enhanced mineralization.[19] During differentiation osteoclasts produce sphingosine-1-phosphate (S1P) which stimulates osteoblast migration and survival.[19] Osteoclasts also secrete Wnt10b and BMP6 which promote osteoblast mineralization.[19] Therefore, osteoclast's activities may be divided into two categories: bone resorption, and the production of anabolic factors to osteoblasts.[12]

This suggested the possibility that anabolic therapeutic agents might be produced by identifying molecules that allow osteoclasts to differentiate, but prevent them from activating to resorb bone. Based on this rationale, efforts are underway to produce therapeutically useful inhibitors of c-src.[20] As described in detail below, data suggests that the actin binding activity of subunit B2 of V-ATPase is also required for mature osteoclast to resorb bone, and thus represents an attractive target for drug development.

V-ATPase Actin Interaction in Osteoclasts

Osteoclasts attach to bone and form rings of tight adhesion (sealing zones), thereby creating resorption compartments.[21] Bone resorption by osteoclasts involves the formation of subcompartments of the plasma membrane called ruffled membranes, which are rich in V-ATPases.[22] Resorption of bone requires acidification of the resorption compartment by V-ATPases in the ruffled membrane.

V-ATPases are enzymes that acidify compartments of the endocytic pathway in all cells. They are composed of more than ten subunits (Figure 1-2). They feature an alternating heterohexamer formed by three copies of subunit A and three copies of subunit B.[23, 24, 25] The A subunit is the site of ATP hydrolysis, which drives rotation of a central stalk. This is coupled to proton transport against an electrochemical gradient.[23, 24, 25] V-ATPase activity is required in the ruffled membranes of osteoclasts to acidify extracellular resorption compartments.[26] This solubilizes bone mineral and provides an environment favorable for the activity of the acid cysteine proteinase, cathepsin K, which is secreted by osteoclasts into the resorption compartment and degrades the organic matrix of the bone.[27] Subunit B has two isoforms that are expressed by eukaryotic cells, and both B subunit isoforms (B1 and B2) contain high affinity actin-

binding sites.[28] B2 is ubiquitously expressed at low levels and is expressed at much higher levels in certain cells, including neurons, proximal tubules, and osteoclasts. Multiple subsets of V-ATPase occur in eukaryotic cells. For example, even in yeast, one subset is responsible for normal acidification of the endocytic pathway, while a second acidifies vacuoles.[25] These subsets are distinguished by the expression of isoforms of the a-subunit. All functional V-ATPases contain one a-subunit. In yeast there are two isoforms, one which is associated with the endosomal pathway, the other is a component of V-ATPases in vacuoles. Likewise, high level expression of V-ATPases is linked to the formation of a specialized subset of V-ATPases that are targeted to ruffled membranes which exist in addition to the subset of V-ATPases responsible for "housekeeping" functions.[29] Lee, *et alia* reported B2 containing V-ATPases are targeted to ruffled membranes of osteoclasts.[30] This suggests that the B2 subunit that mediates the actin-binding activity of V-ATPases which is responsible for directing the V-ATPases to the plasma membrane is no different than B2 subunit found in osteoclast housekeeping V-ATPases. The sequences between amino acids 23 and 67 in B1 and 29 and 73 in B2 have since been identified as responsible for the actin binding activities.[31]

In addition to the B subunit, osteoclasts also contain both the a1 and a3 isoforms of V-ATPase subunit a.[32] Subunit a3 is linked to V-ATPases that were bound to F-actin, whereas V-ATPases containing subunit a1 were not found bound to F-actin (Figure 1-3). It is known that mutations in subunit a3 in humans and mice caused severe osteopetrosis because osteoclasts were not able to resorb bone.[33] The aforementioned suggests that in addition to the binding site of the B subunit, subunit a3 is also required

by osteoclasts and is present in V-ATPases that are bound to actin filaments. However, the mechanistic basis explaining why only a3-containing V-ATPases express B2-mediated actin binding activity has yet to be uncovered.

V-ATPases are not normally present in the plasma membrane of most cells, but are plentiful in the ruffled membrane of osteoclasts.[22] Evidence suggest that transport of V-ATPases to the ruffled membrane, retrieval of V-ATPases from ruffled membranes after resorption, and maintenance of sealing zones requires interaction with the actin-based cytoskeleton.[34, 31, 35] The most direct evidence was derived from studies of exogenously expressed B-subunit with or without actin binding activity in osteoclasts. Adeno-associated virus vectors were used to transduce mouse osteoclasts with wild type (B1) or a mutant B1 (B1 mut) form that contained minor alterations that disrupted actin-binding activity. B1 does not co-assemble with B2 when they are expressed in the same cell. This allowed B1 targeting to be studied on the background of normal B2-mediated V-ATPase targeting. Wild type B1 was transported to the ruffled membrane of resorbing osteoclasts, but the B1 mut was not associated with the actin cytoskeleton and was not transported efficiently to the ruffled membranes.[35] Therefore, Zuo, *et alia* suggested that the actin binding activity of subunit B of V-ATPase is involved in proper targeting to ruffled membranes of osteoclasts and the interaction between V-ATPase and microfilaments is required for efficient bone resorption.[35] This binding site may be useful as a therapeutic target to inhibit resorption in osteoclastic diseases.

Bisphosphonates Treatment

The current treatments of osteoclastic diseases do not specifically target the aforementioned actin-VATPase binding site. Bisphosphonates are the drugs most commonly prescribed for limiting bone loss associated with bone pathologies. It was

originally proposed that by their affinity for the solid-phase calcium phosphate, bisphosphonates reduce the precipitation and dissolution of calcium phosphate from bone thus inhibiting bone resorption.[36, 37, 38] This mechanism was demonstrated in vitro, and therefore was hypothesized to be effective in vivo. Schenk, *et alia* used bisphosphonates to block bone and cartilage degradation and stop the remodeling of metaphyses resulting in club shaping and denser bone.[39] Gasser, *et alia* found using bisphosphonates resulted in an increase in calcium balance and mineral content of bone.[40] However, in vivo, nitrogen-containing bisphosphonates were found to be active, not due to reduction of precipitation and dissolution of calcium as earlier proposed, but by inhibiting the mevalonate pathway, thereby inhibiting farnesyl pyrophosphate synthase which results in a decrease in the formation of isoprenoid lipids like farnesylpyrophosphate and geranylpyrophosphate.[36, 41, 42] These lipids are needed for the prenylation of small GTPases of the Ras-superfamily (Ras, Rho, Rac, and Rab) which are important for many cellular functions.[43, 44] Consequently, bisphosphonates have been shown to inhibit osteoclast recruitment, shorten osteoclast life span by apoptosis, and inhibit osteoclast activity.[45]

Unfortunately bisphosphonates have also been associated with osteonecrosis of the jaw following certain invasive dental treatments, including extractions, osteotomies, tori removal, and bone resections. Factors that place patients at risk for osteonecrosis of the jaw may include the particular drug (the more potent nitrogen-containing bisphosphonates, such as zoledronate, carry increased risk compared with lower activity non-nitrogen containing bisphosphonates, such as alendronate), route of administration (intravenous is higher risk than oral), cumulative dosage, low bone

turnover when bisphosphonates are initiated, and the degree of reduction in bone turnover induced by bisphosphonates.[46] The incidence of the disease seems to be relatively low in patients receiving oral bisphosphonates for osteoporosis or Paget's disease and considerably higher in patients with a malignancy receiving high doses of intravenous bisphosphonates.[46]

Osteoporosis is due to a loss of ovarian sex steroids in postmenopausal women results in an acceleration of bone turnover with predominance of bone resorption over bone formation.[47] The related negative calcium balance promotes bone loss, increases bone fragility, and thereby the risk of future fractures.[48] A rational approach to counter these unwanted processes is to inhibit bone resorption, which until now also has led to inhibition of bone formation, caused by the coupling between these cellular events.[49] In existing pharmacological treatments for osteoporosis, as with bisphosphonates, a decrease in bone formation rate is observed secondary to the reduction in bone resorption. The lack of anabolic bone formation and the added risk of osteonecrosis, indicate a need for an alternative drug therapy that can target the actin-VATPase binding site.

Enoxacin / Bis-enoxacin

The emerging methodologies of computational chemistry are becoming increasingly important in drug discovery. Structure based virtual screening techniques are widely used to discover new therapeutic agents.[50, 51] One key methodology is computer docking of small molecules to known protein binding sites. The docking process involves the prediction of ligand conformation and orientation within a targeted binding site.[52] The two aims of docking studies are accurate structural modeling and correct prediction of activity.[52] An advantage of virtual screening is that it affords an

opportunity to newly identify active compounds at a site without chasing pre-conceived biases.[52]

Using docking simulations, Ostrov, *et alia* tested approximately 300,000 small molecules in the selected structural pockets for the V-ATPase B2 subunit model and scored them based on predicated polar and non-polar interaction (Figure 1-4).[53] From the initial virtual screening, 100 small molecules were identified and ranked regarding best fit. Of those, the top forty small molecules were obtained from the repository at the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Forty of these small molecules were tested for their ability to block interaction between recombinantly-expressed B2 subunit and pure rabbit muscle F-action using a pelleting assay. Several molecules were found to block the interaction between B subunit and microfilaments in the test tube. Of those, Binhib7 (enoxacin) and Binhib16 at concentrations of 100 µm blocked greater than 90 per cent of the B subunit-F-actin binding. The Holliday lab also found that enoxacin concentrations as low as 1 µM significantly reduced the formation of osteoclasts and ruffled membrane and blocked bone resorption (Figures 1-5, 1-6)[53]

Enoxacin (Penetrex) is a member of a family of molecules called fluoroquinolones and has been used clinically as an antibiotic. Quinolones inhibit bacterial DNA gyrase or the topoisomerase II enzyme, thereby inhibiting DNA replication and transcription. Enoxacin has the potential to be an effective therapeutic agent to inhibit bone resorption and might also stimulate of anabolic bone regeneration because it does not induce apoptosis in osteoclasts. When considering the long-term use of the antibiotic enoxacin to treat diseases like osteoporosis, bacterial tolerance and side-effects must be considered. Systemic long-term use of antibiotics has been demonstrated to result in

antibiotic resistant bacteria. This can render a class of antibiotic less effective against certain bacteria, jeopardizing the patient to future bacterial infections. Additionally, disruption of the normal flora existing in the patient can result in opportunistic infections or diseases normally kept in check by competing bacteria and the body's own immune system. One potential possibility to avoid this is to administer locally instead of systemically. Areas of load-bearing bone or joints that suffer degeneration, such as the pelvis, can receive locally administered doses to enhance the regeneration of bone in the vicinity. This may prevent systemic uptake and limit the aforementioned side-effects.

Etidronate (Didronel, brand-name) (Figure 1-7) is an oral bisphosphonate with a methyl group attached to its phosphate "backbone" instead of an attached nitrogen group as in the bisphosphonate, alendronate (Figure 1-8). As stated above, an advantage of bisphosphonates in treating osteoclastic diseases is that by binding to calcium the bisphophonate molecule is incorporated into the patient's bone structure and released when osteoclasts degrade that portion of bone.[36, 54, 39, 38, 41, 42] This property results in long-lasting, bone specific, therapeutic dosing. As previously stated, enoxacin is an antibiotic (Figure 1-9). Long-term, repeated, systemic doses of antibiotics have been widely associated with developed bacterial resistance. Therefore, combining the anti-osteoclastic property of enoxacin with the pharmocodynamic advantages of a bisphosphonate may result in a more effective therapeutic compound. Thus, bis-enoxacin was developed. Bis-enoxacin (Figure 1-10) is the name given to a bisphophonate with an enoxacin moiety instead of a methyl or nitrogen-containing group.[55]

Another potential benefit of a localized administered agent that regulates bone resorption and generation would be in the assistance of orthodontic tooth movement. Selective application of enoxacin to the root areas of certain teeth to resist tooth movement can increase anchorage and hasten desirable orthodontic tooth movement to correct malocclusions. Numerous potential benefits may be derived from influencing the bone resorption/generation system.

Purpose

Enoxacin has been identified as an inhibitor of osteoclast resorption that has a different mechanism of action from currently available anti-osteoclastic agents. This study examined the effects of enoxacin on osteoblasts, as a first step toward determining whether enoxacin, and agents with similar activities, may be useful as bone anabolic therapeutic agents and potential adjuncts to orthodontic tooth movement. Our hypothesis is that enoxacin treatment yields no significant difference in the presence of osteoblasts, mineral deposition, and cytokine / chemokine production compared with vehicle controls.

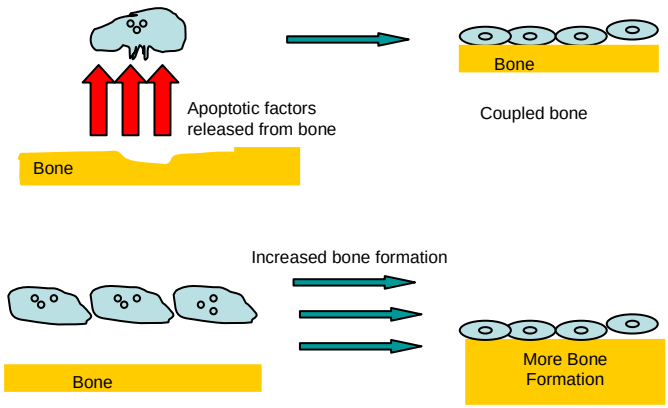


Figure 1-1. Osteoclast bone resorption releases factors that limit osteoclast numbers and stimulate bone formation.

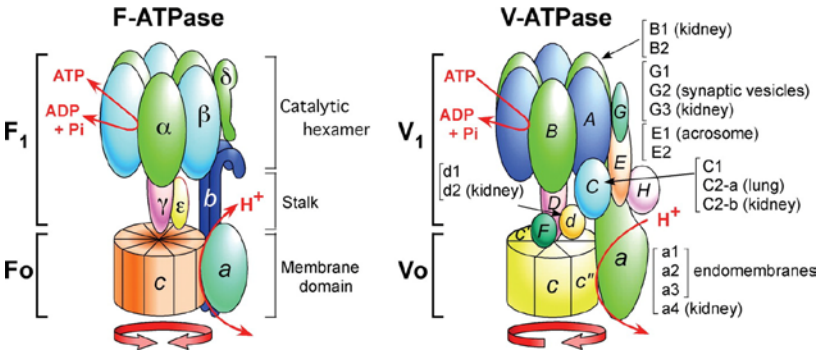


Figure 1-2. Osteoclast V-ATPase structure.

Figure 1-3. Subunit a3 detected in primary osteoclasts. Primary osteoclasts were extracted with 1% Triton X-100 and subjected to ultra-centrifugation at 100 Kxg/45 min. Supernatants (S) and pellets (P) were collected, separated by SDS-PAGE, blotted and probed with anti-a1 or anti-a3 antibodies. Subunit a3, but not a1, was associated with the detergent-insoluble cytoskeleton in osteoclasts.

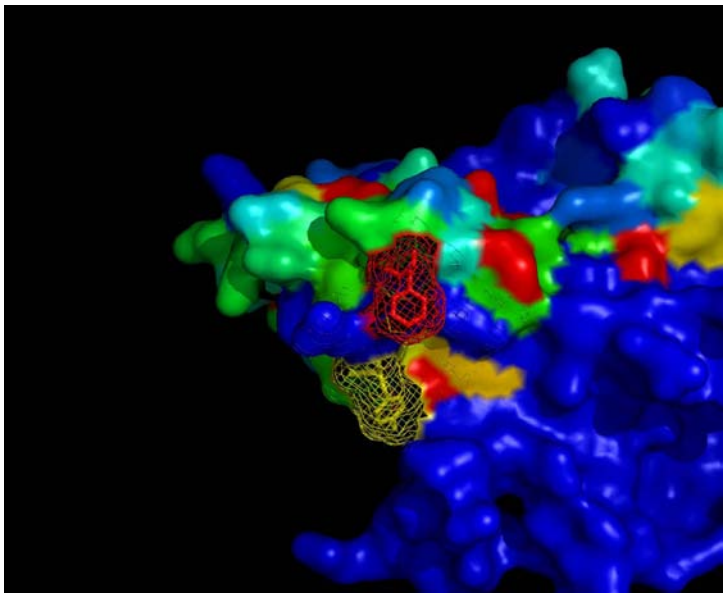


Figure 1-4. Computational docking. Small molecules (Binhib2 is yellow, a second is shown in red) docked in the most favorable location of the actin binding site of subunit B2. The structure of the actin binding site was predicted from the crystal structure of the alpha subunit of F-ATPase. Additional information and guidance was obtained from examination of the crystal structure of profilin 1 bound to actin.

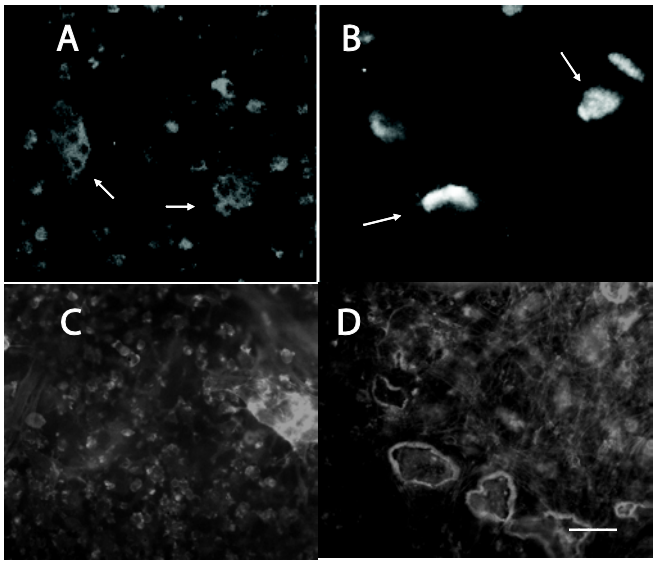


Figure 1-5. Treatment of mature osteoclasts with enoxacin reduced actin ring and ruffled membrane formation. Mouse marrow cultures on tissue culture plates were stimulated with calcitriol to for 6 days to produce osteoclasts. The cells were then scraped free and loaded atop bone slices and treated with vehicle or enoxacin as indicated. After 3 days the cells were fixed and stained with anti-E subunit antisera to detect ruffled membranes (A, B) and with phalloidin to detect actin rings (C,D). B and D are typical fields from vehicle control cultures. A and C are fields from cultures treated with 100 μM enoxacin. Tabulation of actin rings and ruffled membranes show that both were reduced in a dose dependent manner by enoxacin.

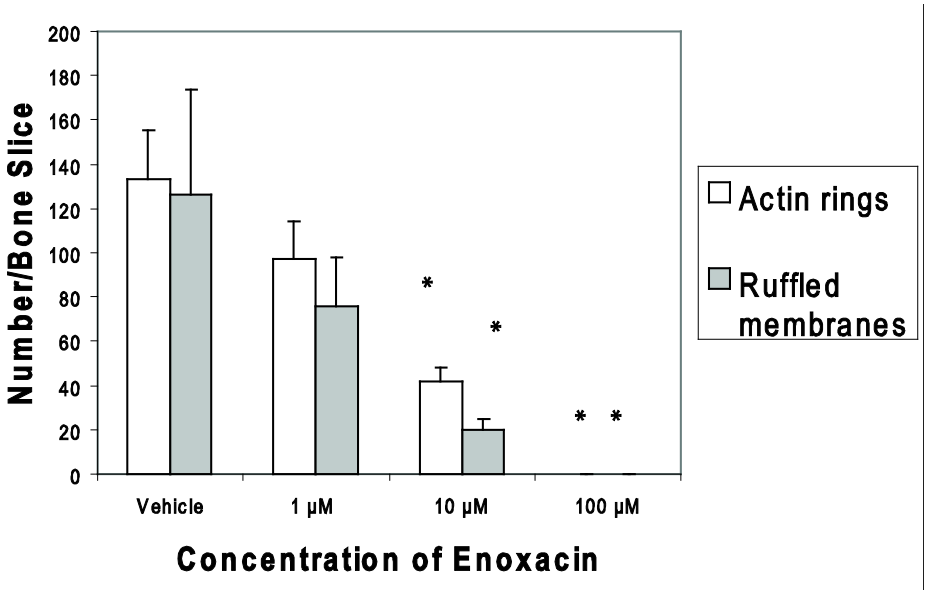


Figure 1-6. Actin rings and ruffled membranes at different concentrations of enoxacin. Asterisks indicate $p < 0.05$ compared with control value by Student's T test.

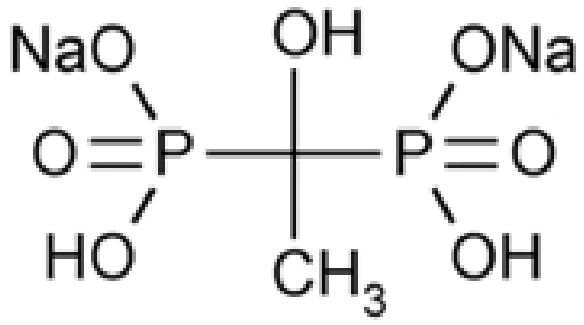


Figure 1-7. Etidronate.

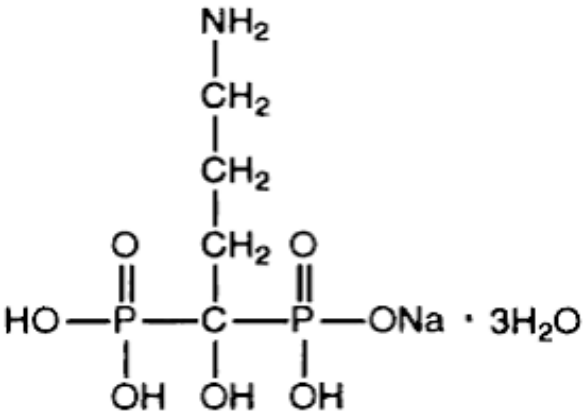


Figure 1-8. Alendronate.

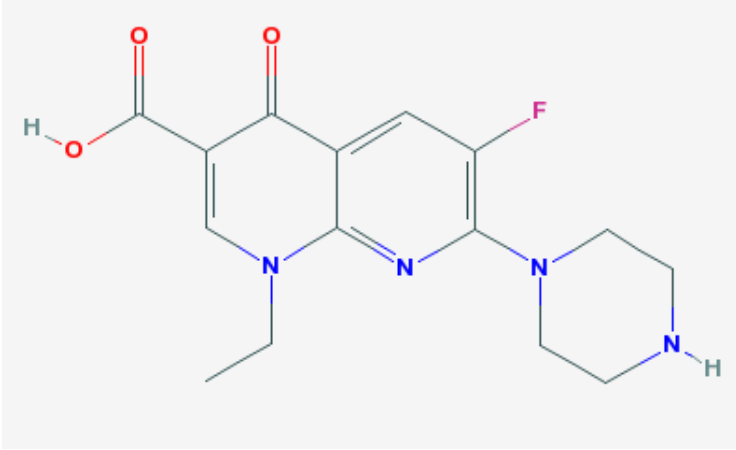


Figure 1-9. Enoxacin chemical structure.

CHAPTER 2
MATERIALS AND METHODS

Presence of Osteoblasts—Alkaline Phosphatase

Swiss Webster mice weighing between 8 to 20g were sacrificed by cervical dislocation. Femora and tibia bones were dissected from the mice. After cutting the bones at both ends, αMEM (minimum essential medium) plus 10% fetal bovine serum (αMEM D10) in a 25 gauge needle syringe was used to flush out the marrow. The marrow was washed twice with MEM D10 and then plated at a density of 1×10^6 cells/cm^2 on tissue culture plates for 5 days in αMEM D10. To stimulate osteoblast and osteoclast growth, 10^{-8} M of 1,25-dihydroxyvitamin D_3 ($1,25D_3$) was added.[31] Cultures were fed on day three by replacing half the media per plate and adding fresh $1,25D_3$. The mouse marrow cells that had been cultured for five days with 10^{-8}M $1,25D_3$ on tissue culture plates were scraped and plated onto bovine bone slices in 24-well dishes. Different concentrations of enoxacin (from 1 to 100 uM) in dimethyl sulfoxide (DMSO) solvent were added from a 100 mM stock to individual cultures, as well as a control without enoxacin but with DMSO. The cultures were fixed with 2% formaldehyde, and stained for alkaline phosphatase (using Alkaline Phosphatase Kit #86R-1KT from Sigma-Aldrich, St. Louis, MO) following the manufacturer's directions. Alkaline phosphatase activity is a well established marker for osteoblasts.[56] Therefore, this assay indicates the presence or absence of osteoblasts. A "blinded" examiner then visually inspected the cultures and indicated positively or negatively if stained osteoblasts were present.

This experiment was a positive or negative qualitative preliminary test useful to indicate whether more quantative experiments were warranted. Therefore statistics were not utilized.

Mineralization Assay—Von Kossa

Mouse MC3T3- E1, subclone 4 preosteoblastic cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in α-MEM supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). The cells were seeded at 1 x 10^4 cells per cm^2 and incubated at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. 2 mM L-glutamine and penicillin/streptomycin (25 mg/mL) were added to all cell cultures. At day 5, α-MEM with L-ascorbic acid and β-glycerol-2-phosphate were added to stimulate osteoblast mineralization.[53] Different concentrations of enoxacin (from 1 to 100 μM) in dimethyl sulfoxide (DMSO) solvent were added from a 100 mM stock to individual cultures, as well as a control without enoxacin but with DMSO. At day 10, mineralization was determined by the Von Kossa assay.[57] The cells were rinsed twice with 1 X phosphate buffer saline and fixed in 4% (v/v) paraformaldehyde at room temperature for 10 min. The cells were then rinsed three times in deionized distilled water, incubated with 5% (w/v) $AgNO_3$ under ultraviolet light (254 nm) for 1 hour. The cells were washed twice with deionized distilled water and fixed with 5% sodium thiosulfate for 5 minutes. Photographs were randomly taken in bright field, transferred to Image J software (NIMH, Bethesda, MD), and mineral flecks for three random micrographs per well were quantified for number.

The non-parametric data was transformed into parametric correlated logs. A one-way ANOVA analysis using Bonferroni's method was performed. Fleiss' intraclass classification coefficient was also determined to assess intra-examiner reliability.

Mineralization Assay—OsteoImage

As in Experiment #2, mouse MC3T3- E1, subclone 4 preosteoblastic cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in α-MEM supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). The cells were seeded at 1×10^4 cells per cm^2 and incubated at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. 2 mM L-glutamine and penicillin/streptomycin (25 mg/mL) were added to all cell cultures. At day 5, α-MEM with L-ascorbic acid and β-glycerol-2-phosphate was added to all groups except the unstimulated control group (34). Fifty μM concentrations of enoxacin, bis-enoxacin, or etidronate (a non-nitrogen bisphosphonate) in dimethyl sulfoxide (DMSO) solvent were added to individual cultures, as well as a control without any treatment but with DMSO. After twenty-one days of mineralization, the cells were rinsed twice with 1 X phosphate buffer saline and fixed in 4% (v/v) paraformaldehyde at room temperature for 10 minutes. The cells were then rinsed again with diluted (1X) Wash Buffer provided in the OsteoImage Mineralization Assay (Lonza PA-1503, Walkersville, MD). The cells were then stained with OsteoImage Staining Reagent and incubated away from light at room temperature for 30 minutes. Each well was then washed three times with diluted Wash Buffer. The wells were then analyzed with a fluorescent plate reader at 458/528 excitation and emission wavelength setting. The measure of green fluorescent staining is proportional to the amount of mineralization present.

Utilizing Prism software (Graphpad Software), a one-way ANOVA analysis using Bonferroni's method was performed. Additional, individual a two-tailed t-test was performed comparing two treatments.

Cytokines and Chemokines Test

Mouse MC3T3- E1, subclone 4 preosteoblastic cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in α-MEM supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). The cells were seeded at 1 x 10^4 cells per cm^2 and incubated at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. Two mM L-glutamine and penicillin/streptomycin (25 mg/mL) were added to all cell cultures. At day two, the cells were then transferred to a 12 well plate and α-MEM w/ L-ascorbic acid was added to stimulate osteoblast maturation.[34] Cultures were fed every three days by replacing half the media per well and adding fresh L-ascorbic acid. On day 28, the wells were washed with DMSO. Different concentrations of enoxacin (10 and 50 µM) in dimethyl sulfoxide (DMSO) solvent were added from a 100 mM stock to individual cultures, as well as a control without enoxacin but with DMSO. After six hours of incubation, 50 µL of supernatant was removed from each well and the cytokines isolated by means of dialysis. Utilizing the Milliplex Mouse Cytokine / Chemokine kit (Millipore, Billerica, MA, #MPXMCYTO70KPMX32) and the Luminex xMAP system (Luminex, Austin, TX), various cytokines and chemokines, including IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin, GM-CSF, G-CSF, INF-gamma, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1a, MIP-1b, MIP-2, RANTES, TNFa, VEGF, were then "blindly" qualified and quantified from these cultures (Figure 2-1). Briefly, tiny beads, called microspheres are color-coded into 100 distinct

sets. Each of these beads is coated with a specific reagent for a particular assay. The Luminex has two lasers that excite the internal dyes of the microspheres and a reported dye that is captured during the assay. Multiple readings are performed on each bead set, increasing the assays validity. Due to the different distinct color sets, multiplexing of multiple assays can be achieved with a single sample.

Due to the small sample number, ANOVA results were not informative. Therefore multiple t-tests between one control and one concentration were performed to indicate potential trends. Unpaired, one-tailed t-tests with Welch's correction were performed. Equal variance was assumed.

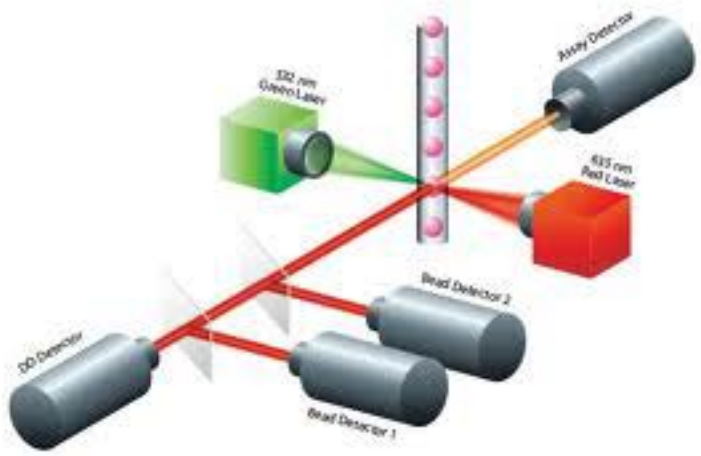


Figure 2-1. Luminex laser array.

CHAPTER 3
RESULTS

Alkaline Phosphatase—Results

When examining bone slices with stimulated mouse marrow, treated with enoxacin concentrations at 1 and 10 μM, and stained for alkaline phosphatase activity, we distinguished no detectable effect on osteoblast number or morphology (Figure 3-1). Enoxacin at 100 μM did reduce the number of osteoblasts. At these concentrations enoxacin has been demonstrated to sharply reduce osteoclast numbers.[53] Therefore, in this experiment, enoxacin did not block osteoblast formation at concentrations where osteoclast numbers were sharply reduced, specifically at 1 and 10μM.

Von Kossa Mineralization Assay—Results

Figure 3-2 depicts the number of mineralization flecks quantified at each concentration of enoxacin and Figure 3-3 illustrates a typical field. The data was converted to logs to obtain parametric data and a two-tail, one way ANOVA was performed. No statistical difference was determined among the concentrations of enoxacin and the number of flecks quantified. A slightly higher number was noted for the control group, but this again was not statistically different.

Eighteen random samples were then recalculated using the ImageJ software to determine intra-examiner reliability. In terms of intra-class classification coefficient,[43] the intra-examiner reliability coefficient was 0.902 for “count variability.”

OsteoImage Mineralization Assay—Results

As expected, the stimulated osteoblast cultures at 21 days demonstrated much greater mineralization than the unstimulated control, a mean of 7.5 to 1.0 RFU respectively (Figure 3-4). Stimulated treatment was also statistically significant

compared to unstimulated and all of the other treatments. The cells treated with enoxacin demonstrated a mean of 1.83 RFU. Bis-enoxacin treatment had a mean of 3.16 RFU. Etidronate treatment had a mean of 1.17 RFU. ANOVA determined no significant difference among the three treatments. However, a t-test between Bis-enoxacin and etidronate treatment was significant, indicating greater mineralization was achieved with bis-enoxacin versus etidronate.

Luminex Assay—Results

Figure 3-5 demonstrates the results of the Luminex Assay. When comparing control, supernatants from mineralizing osteoblasts treated without enoxacin, to supernatants from mineralizing osteoblasts treated with 10µM concentration of enoxacin, out of the thirty-two cytokines tested, IL-G, IP-10, KC, MCP-1, and Rantes were detected at higher levels. However, only KC was detected at a statistically significantly higher level than control. When comparing control to 50µM concentration, GMCSF, IL-6, IP-10, KC, MCP-1, and Rantes were detected at higher levels. However, only GMCSF, IL-6, and KC were determined to be statistically significant.

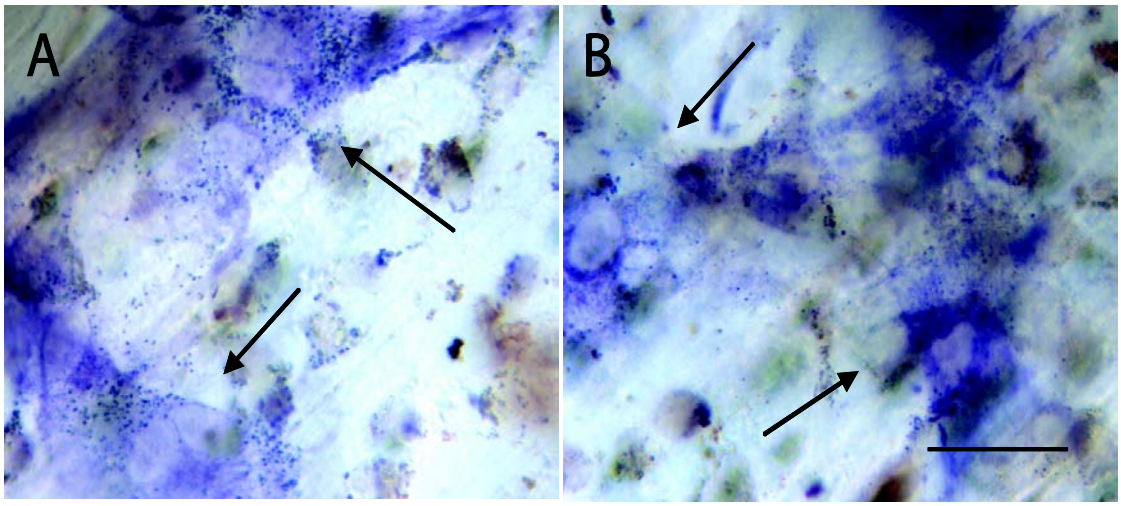


Figure 3-1. Presence or absence of osteoblasts. A) Typical field from a bone slice with mouse marrow atop a bone slice and cultured in the presence of vehicle fixed and stained after 3 days for alkaline phosphatase activity to detect osteoblasts (Blue arrows). B) Typical field from a bone slice in which the cultures were handled identically to A, but in the presence of 10 µM enocacin. Scale bar = 20 microns.

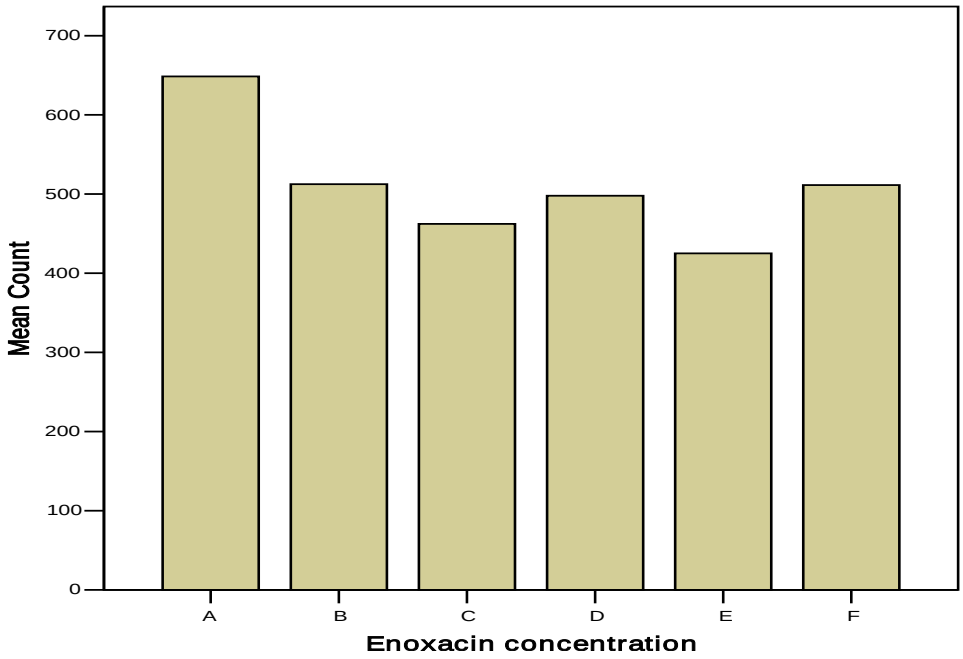


Figure 3-2. Mean count. A=control, B=100uM, C=50uM, D=25uM, E=10uM, F=5uM. P value < 0.05 ANOVA analysis using Bonferroni's method yielded no significant difference among the concentrations.

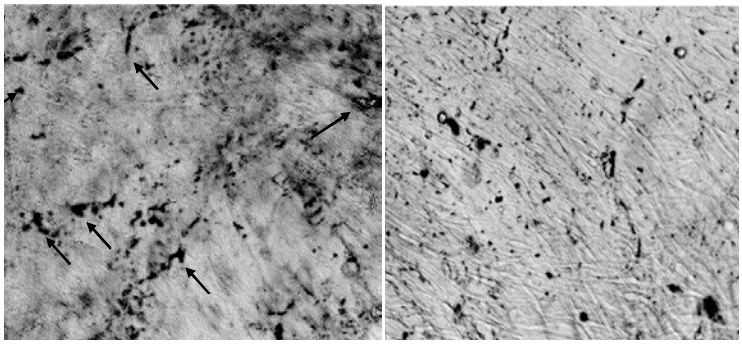
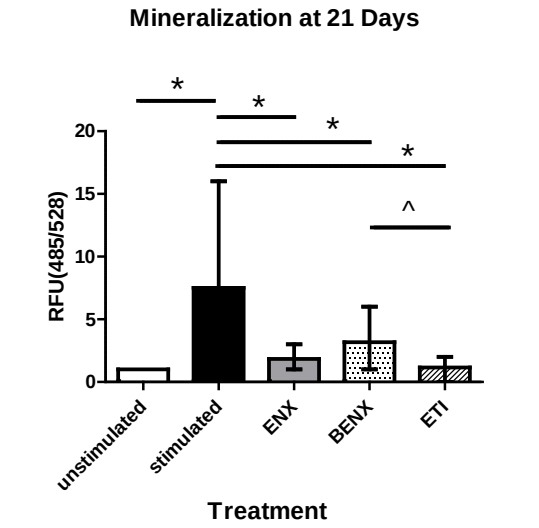
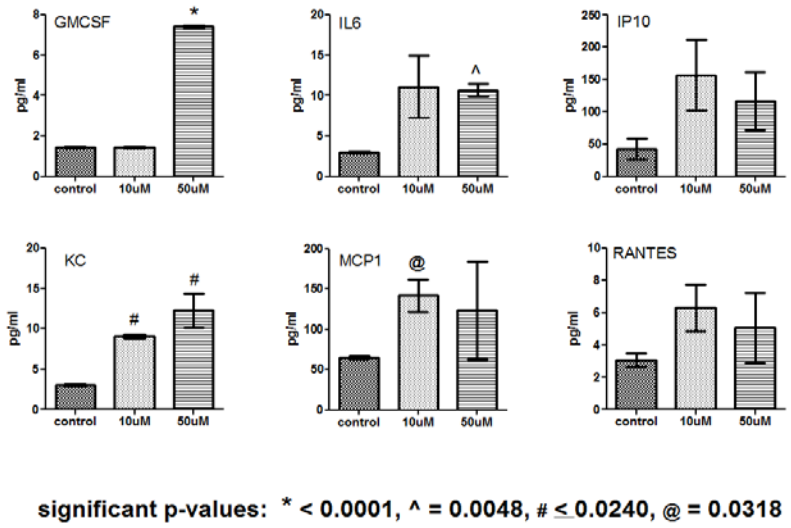


Figure 3-3. Mineral flecks. Vehicle control (on left) and 100uM enoxacin (on right).



* p value < 0.05 1 way ANOVA with Bonferonni's Multiple Comparison Correction
^ p value = 0.0117 Student's t test

Figure 3-4. OsteoImage assay results.



significant p-values: * < 0.0001, ^ = 0.0048, # ≤ 0.0240, @ = 0.0318

Figure 3-5. Luminex assay results.

CHAPTER 4
DISCUSSION

Osteoporosis continues to debilitate large numbers of post-menopausal women[58] The use of bisphosphonates has decreased the number of bone fractures but the levels of osteoporotic fractures remain unacceptably high.[58] In addition, current bisphosphonates are associated with side effects including chemo-osteonecrosis.[46] Bone repair and even orthodontic tooth movement is impaired due to the lack of remodeling of bone due to bisphosphonate treatment.[47, 59] Since bisphosphonates induce osteoclasts to undergo apoptosis, damaged bone cannot be replaced and osteoblasts are not as active.[45] This impedes bone repair and basic remodeling of bone that is part of a body's regular adaptive changes and essential to orthodontic tooth movement.

As the cellular structure and mechanisms of osteoclasts are elucidated, new strategies for altering the resorption activity of osteoclasts are being explored. Osteoclasts secrete acid by means of V-ATPase pumps.[26] These pumps are transferred to the outer resorption compartment region of the cell by means of the cell's actin filaments which organize intracellular structures.[35] By utilizing a small molecule, enoxacin, to prevent the interaction between the actin filaments and the B subunit of V-ATPase pumps, we can prevent osteoclasts from secreting acid and resorbing bone.[53] If this occurs while still maintaining the viability of the osteoclasts and their signaling to osteoblasts for bone formation, it is possible that remodeling can still take place. Potentially such agents may be bone anabolic. Essentially, if a therapeutic agent can be utilized to control bone resorption without eliminating the means for bone remodeling

and formation, it may be more useful for the treatment of osteoporosis and other bone pathologies than current treatments.

First and foremost, this novel small molecule, enoxacin, must experimentally demonstrate the ability to impede bone resorption by osteoclasts. Ostrov *et alia* tested whether enoxacin inhibited bone resorption.[53] Mouse marrow cultures were treated with 0 – 100μM of enoxacin for five days and then loaded onto bovine bone slices with continued enoxacin treatment. After another five days, no resorption was detected in cultures treated with 100 μM enoxacin (Figure 4-1). At 25 μM of enoxacin, both the resorptive area and the number of pits formed on the bone was significantly reduced, thus demonstrating enoxacin's effectiveness in impeding bone resorption.

Since enoxacin has demonstrated an ability to impede bone resorption by osteoclasts, the next step was to investigate its effect on osteoblasts. First, we had to investigate whether osteoblasts would be viable in the presence of enoxacin. Our Experiment #1, utilizing alkaline phosphotase staining, did detect the presence of osteoblasts in 1μM and 10μM concentration of enoxacin. 100 μM of enoxacin demonstrated a noticeable reduction of the presence of osteoblasts. Due to the numerous, different, and overlapping cell types with various configurations, it was difficult to quantify the number of osteoblasts present. A more quantifiable experiment should be repeated for a more definitive measure of osteoblast presence. However, for the purpose of developing experimental research, we merely wanted to investigate whether osteoblasts would even be present in concentrations of enoxacin that would inhibit osteoclasts. Therefore, we did establish osteoblasts can survive in the presence of concentrations of enoxacin which inhibited osteoclasts.

Next whether enoxacin impedes the main function of osteoblasts, mineralization, needed to be explored. Our Experiment #2 demonstrated that in the presence of varying concentrations of enoxacin, osteoblasts still produced mineralization flecks. The number of mineralization flecks was not significantly different in enoxacin concentrations from 0 – 100μM. Therefore, at concentrations that inhibit osteoclast bone resorption, osteoblast mineralization was not reduced in this assay.

One of the difficulties in Experiment #2 was developing a means to quantify the amount of mineralization. As previously mentioned, we developed a method of photographing random areas of each well and importing the images into Image J software (National Institutes of Health). The software was then utilized to quantify the number of flecks present. The operator converts and manipulates the images to contrast the particles. These contrasted particles are then quantified by the software. A disadvantage of this process is that it does not distinguish among different contrasted particles which may or may not be mineralization flecks.

An additional disadvantage of von Kossa staining is that it is not specific to hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), the mineralization product of osteoblasts. Instead, it reacts to the anionic portion of phosphates.[60] Since phosphate is a component of hydroxyapatite, the von Kossa assay is assumed to be indirectly detecting mineralization of osteoblasts. A new alternative assay is the OsteoImage Assay from Lonza Walkersville, Inc. The OsteoImage Assay binds a fluorescent staining reagent specifically to the hydroxyapatite portion of mineralization nodules. The amount of fluorescence can then be quantified quickly by a plate reader, thereby more accurately measuring the amount of mineralization by an osteoblast cell culture. Therefore, to

better measure the amount of mineralization of osteoblasts in the presence of enoxacin treatment, an OsteoImage Assay was performed. In addition to enoxacin, bis-enoxacin and etidronate treatments were also added to the experiment.

Experiments performed in the Holliday lab demonstrated that when bovine bone was treated with Bis-enoxacin or enoxacin, fewer resorption pits were detected on the bis-enoxacin treated bone.[55] The IC-50 (inhibitory concentration) was also determined to be significantly lower for bis-enoxacin compared to enoxacin.[55] This is likely due to the phosphate portion of bis-enoxacin improved binding to bone. However, tartrate resistant acid phosphatase (TRAP) staining experiments performed by the Holliday lab have demonstrated that treatment with the same concentrations of enoxacin or bis-enoxacin result in the same amount of TRAP implying the same level of anti-osteoclastic activity.[55] This result indicates that the actual inhibition of osteoclast resorption by bis-enoxacin treatment is likely due to the enoxacin molecule and not the result of the bisphosphonate structure. Furthermore, additional experiments performed in the Holliday lab compared osteoclast treatment with etidronate, a non-nitrogen bisphosphonate, to bis-enoxacin. Bis-enoxacin treatment resulted in significantly less TRAP staining than etidronate.[55] This again indicates that the increased inhibition of osteoclast activity by bis-enoxacin is likely due to the enoxacin structure and not the bisphosphonate portion.

Therefore, experiment #3 compared the effects of enoxacin, bis-enoxacin, and etidronate on osteoblast mineralization. The experiment also more accurately quantified the amount of mineralization produced by osteoblasts under these treatments. Although, there were no statistically significant differences among the

treatments regarding mineralization, the data did indicate enoxacin and bis-enoxacin treatments resulted in twice the amount of mineralization than etidronate.

As previously mentioned in relation to RANK-L, OPG, *c-fos* and M-CSF, signaling to and from osteoblasts and osteoclasts is essential for bone remodeling activity. We must also investigate whether enoxacin affects cytokine production from osteoblasts. In our Experiment #4, we demonstrated that in the presence of 0, 10, 50 µM of enoxacin, only GMCSF, IL-6, and KC out of thirty-two cytokines were determined to be at statistically significantly different levels. These increases were at very small amounts (<10 pg/mL) and involve cytokines that may likely be anabolic in effect. These cytokines are all involved in the immune response. GMCSF (Granulocyte Macrophage Colony Stimulating Factor), for example, is a cytokine released by macrophages, T-cells, and fibroblasts that stimulate stem cells to differentiate into granulocytes. However, cytokine actions cannot be considered in isolation because they can interact with each other resulting in differing responses. Consequently, the activities of all these cytokines have not been completely elucidated.

The results of Experiment #3 demand further investigation. The number of wells in the experiment was low and the specific effects of the statistically significant cytokines need to be demonstrated on osteoclasts. A likely follow-up experiment would be to compare resorption pits of osteoclasts in cultured medium containing the cytokines from the enoxacin treated MC3T3 cells.

Currently, an experiment is underway to test the anabolic mineralization effects of the cytokines released by osteoclasts in the presence of varying concentrations of enoxacin. Primary osteoclasts have been obtained from mouse femur marrow and

induced to maturation with glutamate and Vitamin D_3 in the presence of enoxacin and without. The cultured media from these osteoclasts will be then combined with MC3T3 osteoblasts induced to mineralization. The resulting formation of mineralized nodules of inorganic hydroxyapatite will be quantified utilizing fluorescent microscopy by means of the OsteoImage Mineralization Assay by Lonza. This experiment will demonstrate the crux of the benefit of enoxacin as compared to bisphosphonates, namely the ability to inhibit osteoclast resorption while still promoting osteoclast signaling to osteoblast enabling bone formation.

In conclusion, enoxacin has been identified as an inhibitor of osteoclast resorption that is believed to have a different mechanism of action from currently available anti-osteoclastic agents.[53] Based on our results, our hypothesis that enoxacin treatment yields no significant difference in the presence of osteoblasts, mineral deposition, and cytokine / chemokine production compared with vehicle controls has to be rejected. Therefore, it is possible that binding of enoxacin to V-ATPase, which is ubiquitous in all cells including osteoblasts, [25] may be affecting cellular functions of osteoblasts. Additionally, enoxacin was also recently reported to stimulate the activity of microRNAs, important regulators of gene expression.[61] This may also be another means by which enoxacin affects osteoblasts. Nevertheless, this does not mean that enoxacin may not be of therapeutic benefit. This study demonstrated that in vitro at certain concentrations enoxacin inhibits osteoclast bone resorption while not affecting bone mineralization of osteoblasts. Whereas, the currently commonly prescribed bisphoshonate, alendronate, is known to effectively inhibit osteoclast bone resorption, but also greatly inhibit bone remineralization.[62, 63] Additionally, according to our results, an alternative

bisphosphonate, etidronate, does not impede osteoblasts mineralization as much as alendronate, but also is much less effective in impeding osteoclast resorption than enoxacin.[55] In summary, enoxacin is a more effective inhibitor of bone resorption than etidronate and impedes osteoblasts less than alendronate. Further in vivo animal studies are now indicated. These studies should include bone remodeling examinations as well as orthodontic tooth movement measurements to further analyze enoxacin as a potential bone anabolic therapeutic agent and an adjunct to orthodontic tooth movement.

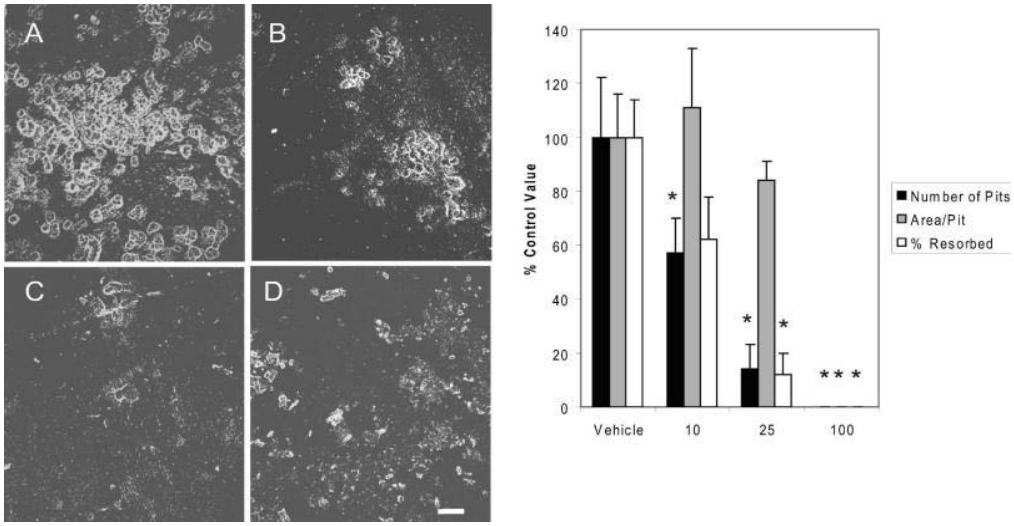


Figure 4-1. Resorption pits. Left panel: Scanning electoron micrographs of bone slices resorbed by mouse marrow cultures in the presence of A) 0 µM enoxacin. B) 10 µM, C) 25 µM, D) 100µM. The scale bar = 25 µm. Right panel: Tabulation of the per cent of the control amount of pit numbers, area per pit, and per cent of total area resorbed.

LIST OF REFERENCES

1. Takahashi N, Udagawa N, Suda T. A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. Biochem Biophys Res Commun 1999;256(3):449-55.

2. Harada S, Rodan G. Control of osteoblast function and regulation of bone mass. Nature 2003;423:349-355.

3. Rodan G. Mechanical loading, estrogen deficiency, and the coupling of bone formation to bone resorption. J Bone Miner Res 1991;6**:**527–530.

4. Gori F, Hofbauer L, Dunstan C, Spelsberg T, Khosla S, Riggs B. The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. Endocrinology 2000;141:4768-4776.

5. Khosla S. Minireview: the OPG/RANKL/RANK system. Endocrinology 2001;142:5050-5055.

6. Atkins G, Kostakis P, Pan B. RANKL expression is related to the differentiation state of human osteoblasts. J Bone Miner Res 2003;18:1088-1098.

7. Yamada Y, Ando F, Niino N, Shimokata H. Association of polymorphisms of the osteoprotegerin gene with bone mineral density in Japanese women but not men. Mol Genet Metab 2003;80: 344-349.

8. Theoleyre S, Wittrant Y, Tat S, Fortun Y, Redini F, Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev. 2004;15:457-475.

9. Kong Y, Yoshida H, Sarosi I, Tan H, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos A, Van G, Itie A, Khoo W, Wakeham A, Dunstan C, Lacey D, Mak T, Boyle W, Penninger J. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 1999;Jan 28;397(6717):315-23.

10. Kartsogiannis V, Zhou H, Horwood N. Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. Bone 25:525-534.

11. Krane SM. Identifying genes that regulate bone remodeling as potential therapeutic targets. J Exp Med 1999;201:841-843.

12. Karsdal M, Martin T, Bollerslev J, Christiansen C, Henriksen K. Are nonresorbing osteoclasts sources of bone anabolic activity? Journal of Bone and Mineral Research 2007;22(4):487-494.

13. Soriano P, Montgomery C, Geske R, Bradley A. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 1991;64**:**693–702.

14. Horne W, Neff L, Chatterjee D, Lomri A, Levy J, Baron R. Osteoclasts express high levels of pp60c-src in association with intracellular membranes. J Cell Biol 1992;119**:**1003–1013.

15. Burr D. Does early PTH treatment compromise bone strength? The balance between remodeling, porosity, bone mineral, and bone size. Curr Osteoporos Rep 2005;3**:**19–24.

16. Martin T, Sims N. Osteoclast-derived activity in the coupling of bone formation to resorption. Trends Mol Med 2005;11**:**76–81.

17. Demiralp B, Chen H, Koh A, Keller E, McCauley L. Anabolic actions of parathyroid hormone during bone growth are dependent on c-fos. Endocrinology. 2002;143**:**4038–4047.

18. Koh A, Demiralp B, Neiva K, Hooten J, Nohutcu R, Shim H, Datta N, Taichman R, McCauley L. Cells of the osteoclast lineage as mediators of the anabolic actions of parathyroid hormone in bone. Endocrinology 2005;146**:**4584–4596.

19. Pederson L, Ruan M, Westendorf J, Khosla S, Oursler M. Regulation of bone formation by osteoclasts involves Wnt/BMP signaling and the chemokine sphingosine-1-phosphate. PNAS 2008;105:20764-20769.

20. Rucci N, Susa M, Teti A. Inhibition of protein kinase c-Src as a therapeutic approach for cancer and bone metastases. Anticancer Agents Med Chem 2008;Apr;8(3):342-9.

21. Lakkakorpi P, Vaananen H. Kinetics of the osteoclast cytoskeleton during the resorption cycle in vitro. J Bone Miner Res 1991;6:817-826.

22. Blair H, Teitelbaum S, Ghiselli R, Gluck S. Osteoclastic bone resorption by a polarized vacuolar proton pump. Science 1989;Aug 25;245(4920):855-7.

23. Nishi T, Forgac M. The vacuolar (H+)-ATPases: Nature's most versatile proton pumps. Nature Rev Mol Cell Biol 2002;3:94-103.

24. Imamura H, Takeda M, Funamoto S, Shimabukuro K, Yoshida M, Yokoyama K. Rotation scheme of V1-motor is different from that of F1-motor. Proc Natl Acad Sci U S A 2005;Dec 13;102(50):17929-33.

25. Forgac M. Vacuolar ATPases: Rotary Proton Pumps in Physiology and Pathophysiology. Nature Reviews Molecular Cell Biology 2007;Nov;8(11):917-29.

26. Baron R, Neff L, Louvard D, Courtoy P. Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. J Cell Biol 1985;Dec;101(6):2210-22.

27. Gelb B, Moissoglu K, Zhang J, Martignetti J, Brömme D, Desnick R. Cathepsin K: isolation and characterization of the murine cDNA and genomic sequence, the homologue of the human pycnodysostosis gene. Biochem Mol Med 1996;Dec 59(2):200-6.

28. Holliday L, Lu M, Lee B, Nelson R, Solivan S, Zhang L, Gluck S. The amino-terminal domain of the B subunit of vacuolar H+-ATPase contains a filamentous actin binding site. J Biol Chem 2000;Oct 13;275(41):32331-7.

29. Gluck S, Lee B, Wang S, Underhill D, Nemoto J, Holliday L. Plasma membrane V-ATPases in proton-transporting cells of the mammalian kidney and osteoclast. Acta Physiol Scand Suppl 1998;643:203-212.

30. Lee B, Holliday L, Ojikutu B, Krits I, Gluck S. Osteoclasts express the B2 isoform of vacuolar H(+)-ATPase intracellularly and on their plasma membranes. Am J Physiol 1996;270:C382-C388.

31. Chen S, Bubb M, Yarmola E, Zuo J, Jiang J, Lee B, Lu M, Gluck S, Hurst IR, Holliday L. Vacuolar H+-ATPase binding to microfilaments: regulation in response to phosphatidylinositol 3-kinase activity and detailed characterization of the actin-binding site in subunit B. J Biol Chem 2004;Feb 27;279(9):7988-98.

32. Toyomura T, Oka T, Yamaguchi C, Wada Y, Futai M. Three subunit a isoforms of mouse vacuolar H(+)-ATPase. Preferential expression of the a3 isoform during osteoclast differentiation. J Biol Chem 2000;Mar 24;275(12):8760-5.

33. Kornak U, Schulz A, Friedrich W, Uhlhaas S, Kremens B, Voit T, Hasan C, Bode U, Jentsch T, Kubisch C. Mutations in the a3 subunit of the vacuolar H(+)-ATPase cause infantile malignant osteopetrosis. Hum Mol Genet 2000;Aug 12;9(13):2059-63.

34. Lee B, Gluck S, Holliday L. Interaction between vacuolar H(+)-ATPase and microfilaments during osteoclast activation. J Biol Chem. 1999;Oct 8;274(41):29164-71.

35. Zuo J, Jiang J, Chen S, Vergara S, Gong Y, Xue J, Huang H, Kaku M, Holliday L. Actin binding activity of subunit B of vacuolar H+ -ATPase is involved in its targeting to ruffled membranes of osteoclasts. Journal of Bone and Mineral Research 2006;22(5):714-721.

36. Francis M, Russell R, Fleisch H. Diphosphonates inhibit formation of calcium phosphate crystals in vitro and pathological calcification in vivo. Science 1969;165:1264-1266.

37. Jung A, Bisaz S, Fleisch H. The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. Calcif Tissue Res 1973;11:269-280.

38. Masarachia P, Weinreb M, Balena R, Rodan G. Comparison of the distribution of 3H-alendronate and 3H-etidronate in rat and mouse bones. Bone 1996;19:281-290.

39. Schenk R, Merz W, Mühlbauer R, Russell R, Fleisch H. Effect of ethane-1-hydroxy-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl2MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis. Calcif Tissue Res 1973;12;11(3):196-214.

40. Gasser A, Morgan D, Fleisch H, Richelle L. The influence of two diphosphonates on calcium metabolism in the rat. Clin Sci 1972;43:31-45.

41. Van Beek E, Pieterman E, Cohen L, Löwik C, Papapoulos S**.** Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. Biochem Biophys Res Commun 1999;264:108-111.

42. Bergstrom J, Bostedor R, Masarachia P, Rewszka A, Rodan G. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. Arch Biochem Biophys 2000;373:231-241.

43. Fleisch H. Development of Bisphosphonates. Breast Cancer Res 2002;4:30-34.

44. Jaffe A, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 2005;21:247-69.

45. Hughes D, Wright K, Uy H, Sasaki A, Yoneda T, Roodman G, Mundy G, Boyce B. Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. J Bone Miner Res 1995;10:1478-1487.

46. Khosla S (Chair), Burr D, Cauley J, Dempster D, Ebeling P, Felsenberg D, Gagel R, Gilsanz V, Guise T, Koka S, McCauley L, McGowan J, McKee M, Mohla S, Pendrys D, Raisz L, Ruggiero S, Shafer D, Shum L, Silverman S, Van Poznak C, Watts N, Woo S, Shane E. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the american society for bone and mineral research. Journal of Bone and Mineral Research 2007;22(10):1480-1491.

47. Rodan G. Mechanical loading, estrogen deficiency, and the coupling of bone formation to bone resorption. J Bone Miner Res 1991;6**:**527–530.

48. Garnero P, Sornay-Rendu E, Claustrat B, Delmas P. Biochemical markers of bone turnover, endogenous hormones and the risk of fractures in postmenopausal women: The OFELY study. J Bone Miner Res 2000;15**:**1526–1536.

49. Ravn P, Hosking D, Thompson D, Cizza G, Wasnich R, McClung M, Yates A, Bjarnason N, Christiansen C. Monitoring of alendronate treatment and prediction of effect on bone mass by biochemical markers in the early postmenopausal intervention cohort study. J Clin Endocrinol Metab 1999;84**:**2363–2368.

50. Bajorath J. Integration of virtual and high-throughput screening. Nature Rev Drug Discov 2002;1:882-894.

51. Gohlke H, Klebe G. Approaches to the description and prediction of the binding affinity of small-molecule ligands to macromolecular receptors. Angew Chem Int Ed. 2002;41:2644-2676.

52. Kitchen D, Decornez H, Furr J, Bajorath J. Docking and scoring in virtual screening for drug discovery: methods and applications. Nature Reviews Molecular Cell Biology 2004;Nov; 3:935-949.

53. Ostrov D, Magis A, Wronski T, Chan E, Toro E, Donatelli R, Sajek K, Haroun I, Nagib M, Piedrahita A, Harris A, Holliday L. Identification of Enoxacin as an Inhibitor of Osteoclast Formation and Bone Resorption by Structure-Based Virtual Screening. J. Med. Chem 2009;52,5144–5151.

54. Jung A, Bisaz S, Fleisch H. The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. Calcif Tissue Res 1973;11:269-280.

55. Toro E, Holliday L. unpublished.

56. Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science 2000;289:1501-1504.

57. Jin D, Chen L, Yan, J. Effects of IGF-I and BMP-2 combined application on promoting proliferation, differentiation and calcification of MC 3T3-E1 and NIH 3T3 cells. Zhejiang Da Xue Xue Bao Yi Xue Ban 2006;35, 55–63.

58. Bacon W. Secular trends in hip fracture occurrence and survival: age and sex differences. J Aging Health 1996;8(4):538-53.

59. Adachi H, Igarashi K, Mitani H, Shinoda H. Effects of topical administration of a bisphosphonate (risedronate) on orthodontic tooth movements in rats. J Dent Res 1994;73(8):1478-86.

60. Bonewald L, Harris S, Rosser J, Dallas M, Dallas S, Camacho N, Boyan B, Boskey A. Von Kossa staining alone is not sufficient to confirm that mineralization in vitro represents bone formation. Calcif Tissue Int 2003;72(5):537-47.

61. Shan G, Li Y, Zhang J, Li W, Szulwach E, Duan R, Faghihi M, Khalil A, Lu Lianghua, Paroo Z, Chan A, Shi Z, Liu Q, Wahlestedt C, He C, Jin P. A small molecule enhances RNA interference and promotes microRNA processing. Nature Biotechnology 2008;26 (8):933-940.

62. Siris E, Weinstein R, Altman R, Conte J, Favus M, Lombardi A, Lyles K, McIlwain H, Murphy W, Reda C, Rude r, Seton M, Tiegs R, Thompson D, Tucci J, Yates A, Simering M. Comparative study of alendronate versus etidronate for the

treatment of Paget’s disease of bone. J Clin Endocrinology and Metablolism 1996;81:961-967.

63. Idris A, Rojas J, Greig I, Van't Hof R, Ralston S. Aminobisphosphonates cause osteoblast apoptosis and inhibit bone nodule formation in vitroj. Calcif. Tissue Int 2008;82:191–201.

BIOGRAPHICAL SKETCH

Richard Egidio Donatelli was born in Queens, New York. He was born to Richard U. and Eunicia Donatelli. Three years after his birth, his family moved to south Florida, where his younger sister, Donna, and younger brother, Paul, were born. Richard grew up in Ft. Lauderdale and Jupiter, Florida. He graduated Valedictorian from Jupiter Christian School. He earned B.A. degrees in both political science and biological science from Florida Atlantic University, graduating with honors and being invited to join Pi Sigma Alpha and Phi Kappa Phi honor societies. In 2000, Richard earned a doctorate in dental medicine from the University of Florida. After graduation, Richard married his wife, Yvonne. He then practiced general dentistry in Naples and West Palm Beach, Florida until returning to the University of Florida to specialize in orthodontics in 2008. Upon completion of his residency, Richard earned a Master of Science degree and a Certificate of Orthodontics.