JOURNAL OF UNDERGRADUATE RESEARCH

Novel Small Molecule Inhibitor of Osteoclast Differentiation

Macey L. Martin, David A. Ostrov and L. Shannon Holliday

College of Agriculture and Life Sciences (Nutritional Sciences), University of Florida

L. Shannon Holliday, Department of Orthodontics

Abstract

Osteoporosis results in over a million bone fractures each year despite current therapeutics that inhibit bone resorption. The need for better anti-osteoporotic therapeutics is clear. Recently, extracellular vesicles (EVs) containing RANK that are released by osteoclasts and serve as novel regulators of bone remodeling were identified. These RANK-containing EVs both block bone resorption by osteoclasts and stimulate a RANKL reverse signaling pathway in osteoblasts that promotes bone formation. Small molecules with the same activity could prove to be a new class of therapeutic for treatment of bone disease. To seek such small molecules, a virtual screen identified small molecules that were predicted to bind RANKL in the same location as RANK, and an initial cell culture screen showed eighteen of the candidates reduced osteoclast formation *in vitro* at a concentration of 100 µM. In this project, one candidate, 3-Nitro-4-phosphobenzoic acid (NPA), was examined in greater detail. NPA does-dependently reduced recombinant RANKL-stimulated differentiation of RAW 264.7 cells into osteoclast-like cells with an IC_{50} of 38 µM. In calcitriol-stimulated mouse marrow, primary cultures that produce both osteoblasts and osteoclasts, NPA (50 µM) reduced osteoclast formation by 88%, while alkaline phosphatase positive osteoblast numbers were higher. These data show that NPA, in cell culture, is able to simultaneously inhibit osteoclast differentiation and promote osteoblast formation. Thus, NPA is a candidate to be a lead molecule for novel dual-function, small molecule, therapeutic agents to treat osteoporosis.

Keywords: RANK, RANKL, osteoclast, osteoblast, EV

Introduction

Bones must maintain their shape and structural properties throughout the life cycle, as they are the structural foundation of the human body (Clarke, 2008; Florencio-Silva et al., 2015). Osteoporosis is a common and serious disease, impacting both men and women as they age, although women are more susceptible. Osteoporosis arises from an imbalance in bone formation and bone resorption, resulting in weakened bone due to more resorption than formation. This structurally compromised bone results in fractures that can lead to morbidity and mortality (Compston et al., 2019). Treatment for osteoporosis include inhibitors of bone resorption, such as bisphosphonates (zoledronate, ibandronate, alendronate), and the monoclonal anti-receptor

activator of nuclear factor kappa B-ligand (RANKL) antibody, denosumab (Compston et al., 2019). Bone forming strategies including intermittent parathyroid hormone therapy, and the anti-sclerostin antibody, romosozumab are also used clinically (Compston et al., 2019). Despite these therapeutics, osteoporotic fractures are common and cause high levels of morbidity and mortality.

A central and essential regulatory network in bone remodeling involves receptor activator of nuclear factor kappa B (RANK) and RANK-Ligand (RANKL) (Boyce & Xing, 2008; Honma et al., 2020; Lacey et al., 1998). RANKL, a transmembrane protein found on osteocytes and osteoblasts, stimulates RANK found on osteoclasts and their precursors, initiating regulatory pathways resulting in osteoclastogenesis, osteoclast survival and osteoclast bone resorptive activity (Kong et al., 1999; Lacey et al., 1998; Martin & Sims, 2015; Yasude et al., 1998). Recent data showed that RANK is found in extracellular vesicles (EVs) shed by osteoclasts (Huynh, 2016). These RANK-containing EVs were initially shown to block osteoclast formation by competing with RANK in cells for binding RANKL. Subsequently, RANK-EVs were shown to stimulate osteoblastic bone formation through a RANKL reverse signaling pathway (Figure 1) (Ikebuchi et al., 2018). This opens the door to finding pharmacological methods that utilize agents that simultaneously inhibit bone resorption by blocking binding between RANKL with RANK on the osteoclast plasma membrane, while stimulating RANKL reverse signaling to promote bone formation. This may serve as a better way to combat bone diseases including osteoporosis.

A supercomputer-based virtual screen, which sought small molecules that bind the RANK-interaction pocket in RANKL, was performed that identified eighteen small molecule candidates (D.A. Ostrov and L. S. Holliday, unpub data). The current study tests a small molecule, 3-Nitro-4-phosphobenzoic acid (NPA), that was selected in the supercomputer-based virtual screen as a possible mimic of RANK-containing EVs. RAW 264.7 cells were treated with NPA to observe osteoclast differentiation, and it was found that NPA reduced recombinant RANKL stimulated differentiation of RAW 264.7 cells into osteoclast-like cells. NPA was further tested on calcitrol-stimulated mouse marrow to observe the effects on both osteoclast and osteoblast differentiation,

and it was found that NPA both increased osteoblast numbers while also decreasing osteoblast differentiation.

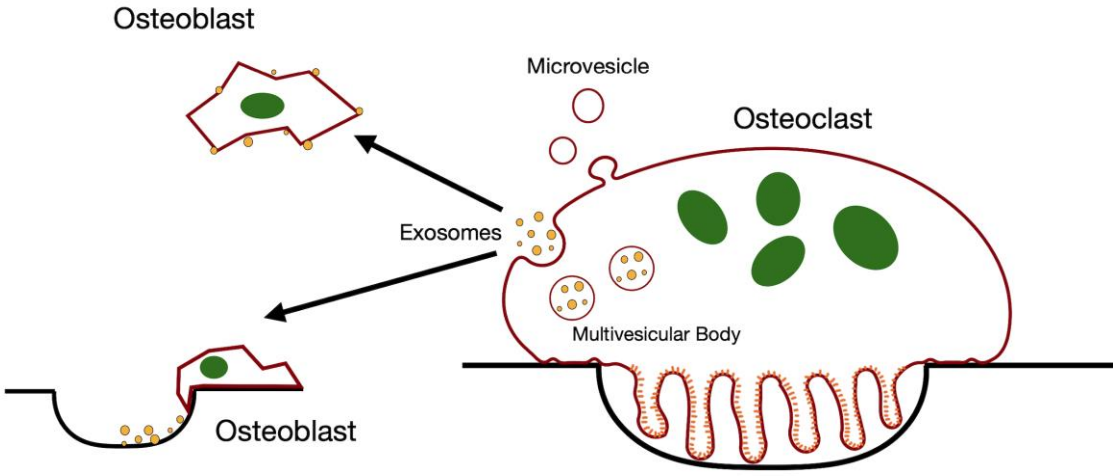


Figure 1. RANK-containing EV stimulation of RANKL reverse signaling pathway. Microvesicles and exosomes are two types of EVs that can be released by osteoclasts. RANK may be present on exosomes or microvesicles. Once released, RANK-containing EVs can either first bind to the bone, and then bind RANKL on osteoblasts, or interact directly with osteoblasts. By binding RANKL, RANK-containing EVs block RANKL's ability to bind RANK on the osteoclast surface, which inhibits osteoclast activity. Simultaneously, the RANK-containing EVs stimulate RANKL reverse signaling, which triggers the osteoblast to form bone. The ability to both block bone resorption and stimulate bone formation makes RANK-containing EVs potent regulators of bone remodeling. Small molecules that mimic the regulatory activity of RANK-containing EVs would likewise be powerful bone anabolic agents.

Materials and Methods

Identification of NPA

A supercomputer-based virtual screen was performed making use of the crystal structure of RANK in complex with RANKL (Ostrov et al., 2009; Liu et al., 2010). The top fifty scoring candidates were obtained from the National Cancer Center small molecule repository and tested at a concentration of 100 μM for their ability to inhibit osteoclast formation (David A. Ostrov and L. Shannon Holliday, unpub. Data). NPA was among the eighteen small molecules that tested positive in the initial screen. Other small molecules from this screen are also separately under investigation.

Preparation of NPA

3-Nitro-4-phosphobenzoic acid (NPA), with the molecular formula $C_7H_6NO_7P$ and a molecular weight of 247.1 grams per mole, was solubilized in Dimethylsulfoxide (DMSO) to generate a 5 millimolar stock solution. This was used in the study, and DMSO was used as the vehicle control.

Materials

Unless otherwise noted, all reagents were from Sigma-Aldrich and were the highest available grade. 1,25-$(OH)_2D_3$ (calcitriol) was from Sigma-Aldrich.

Cell Culture

RAW 264.7 cells (ATTC), a mouse hematopoietic cell line that has been used extensively as a model for macrophages and osteoclasts (Vracar et al., 2018; Yamaba et al., 2015), was used to generate osteoclast-like cells. A recombinant fusion protein composed of glutathione-S-transferase attached to amino acids 158 to 316 of mouse RANKL was used to stimulate RAW 264.7 cells to differentiate into osteoclasts-like cells (Hurst et al., 2004). Cells were plated at a density of 20,000-40,000 cells/cm^2 in 24 well plates in Dulbecco's modified eagle medium with 10% fetal bovine serum. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. To generate osteoclast-like cells, 5 ng/mL of RANKL was added and was replenished after 3 days of culture. Each group was run in quadruplicate.

Calcitriol-stimulated mouse marrow was prepared as described previously (Holliday et al., 1995). All protocols using of animals were approved by the University of Florida Institutional Animal Care and Usage Committee. All procedures were performed in accordance with guidelines of the National Institutes of Health of the United States. Cervical dislocation was performed to sacrifice Swiss-Webster mice. Femora and tibia were dissected from the mice, and marrow was flushed using α-MEM plus 10% fetal bovine serum (α-MEM D10). Marrow was washed and plated at a density of 4×10^4 or 8×10^4 cells/cm^2 on 24-well plates for 5 d in α-MEM D10 with 10^{-8} M calcitriol plus NPA as noted. Cultures were fed on day 3 by replacing half the media per plate and adding fresh calcitriol.

Cell fixation and permeabilization

Cells were fixed in 2% formaldehyde in PBS for 20 min on ice, then washed 3X with PBS and permeabilized for 10 min with PBS containing 1% Triton X-100. Cells were washed in PBS and histological staining as described below was performed.

Detection of Osteoclasts and Osteoblasts:

Tartrate- resistant Acid Phosphatase Leukocyte (TRAP) and Alkaline Phosphatase staining kits were purchased from Sigma-Aldrich (catalog No. 387A-KT, and 85L-KT). They were used to detect osteoclast and osteoblast differentiation, respectively as described previously (Holliday et al., 1997).

Counting of TRAP+ cells

TRAP is a specific marker for osteoclasts in these cultures. Osteoclasts were detected as cells staining positive for TRAP activity. Because osteoclasts can undergo fusion to form multinuclear cells, quantitation requires assessing the degree of cell fusion. Here, TRAP+ cells were counted and classified according to the number of nuclei present as mono- or multinuclear (2–10 nuclei) or as giant cells (more than 10 nuclei) as has been described previously (Toro, et al., 2012).

Statistical Analysis

All quantitative data was analyzed by one tail ANOVA followed by T tests where appropriate. Trial 1 treated with 50 μM NPA had T test P values of 0.011, 0.0227, and 0.0041 for giant cells, multinuclear cells, and mononuclear cells respectively. These P values less than 0.05 show a statistical significance in the 50 μM NPA data when compared with the control data. The trial 2 50 μM NPA mononucleated cells also resulted in a P value less than 0.05, again showing statistical significance when compared with the control.

In the calcitriol-stimulated mouse marrow, the 10 μM NPA treatment had T test p values of 0.0331, 0.001, and 0.0061 for giant, multinucleated, and mononucleated cells respectively.

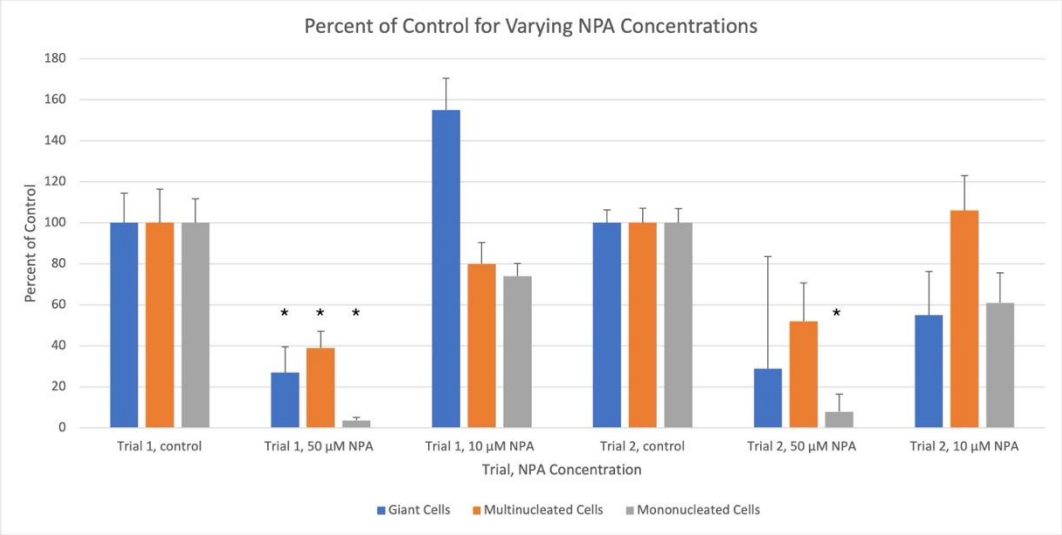
Results

To determine whether NPA blocks osteoclast differentiation directly, we tested it on RAW 264.7 cells that were stimulated with recombinant RANKL to form osteoclasts. NPA (10 and 50 µM) was added to cells on days 1 and 3 of a 5 day culture. The cells were then fixed and TRAP stained. The TRAP stained RAW cells were then quantified by counting (Figure 2A) and representative images are shown in Figure 2 B-I. NPA dramatically reduced osteoclast numbers at 50 µM, but displayed modest reduction at 10 µM. However, note that the intensity of the TRAP stain was reduced at 10 µM. This suggests that while 10 µM NPA did not inhibit the fusion of osteoclast precursors to form multinuclear and giant cells, it did reduce TRAP activity.

Next we tested the effects of NPA on calcitriol-stimulated mouse marrow. Marrow has both mesenchymal and hematopoietic stems that differentiate in response to calcitriol into osteoclasts and osteoblasts. Crucial to this process, calcitriol stimulates expression of RANKL by osteoblasts. This has proven a useful model for the bone microenvironment in many studies.

NPA (50 µM), a high dose to test the effects on both osteoclast and osteoblast formation, was added to calcitrol-stimulated mouse marrow on day 1 and day 4 of a 6-day culture period. The cells were fixed and first stained for TRAP activity, and TRAP+ cells were counted (Figure 3). The number of TRAP+ cells mononuclear, multinuclear and giant cells were each reduced by more than 80%.

The cultures were then stained for alkaline phosphatase activity, a marker for osteoblasts (Figure 4). Note that a large number of alkaline phosphatase positive cells were present in NPA treated cultures, even though osteoclasts were mostly absent. Both osteoclasts and osteoblasts were present in control cultures.



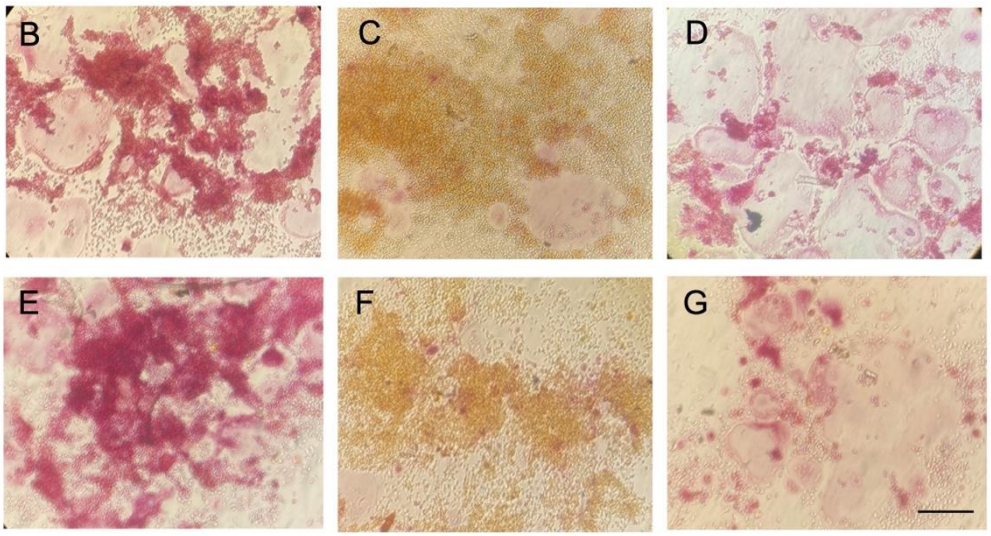


Figure 2. NPA dose-dependently inhibits osteoclast formation in RANKL-stimulated RAW 264.7 cell cultures. A.) Quantitation of osteoclast numbers when treated with 10 and 50 µM NPA. We estimated the IC50 for giant cells to be 38 µM. Asterisk indicates P< 0.05 by T test. B, E) Osteoclasts detected by TRAP staining in control cultures. C, Trial 1, F Trial 2.) Osteoclasts treated with 50 µM NPA. D, G.) Osteoclast treated with 10 µM NPA. Scale bar equals 25 microns.

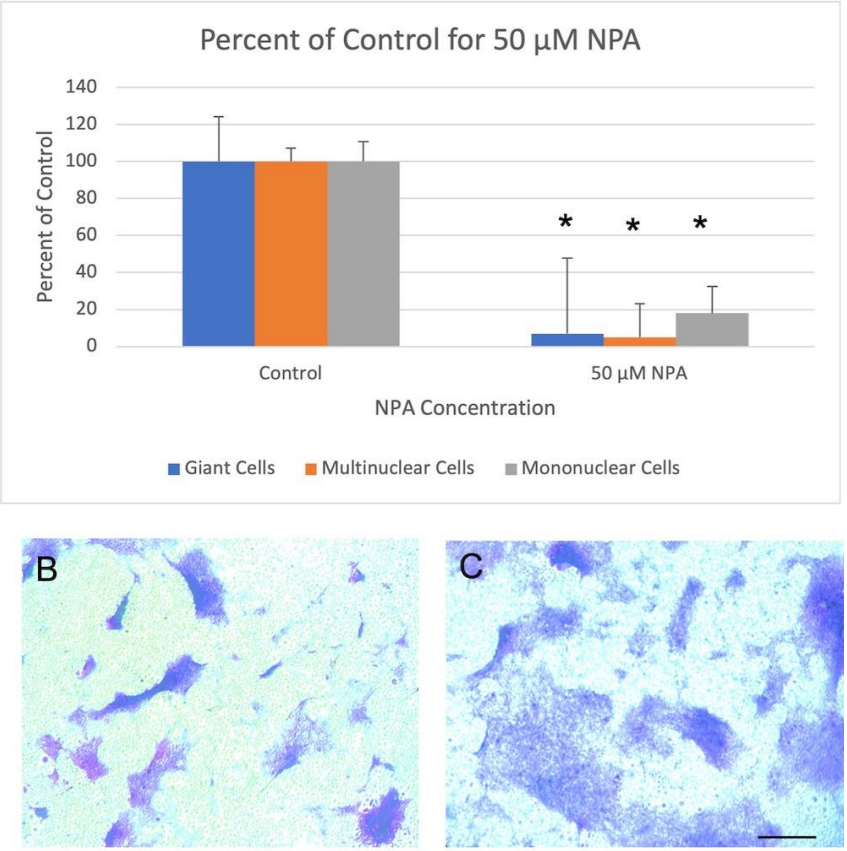


Figure 3. NPA inhibits osteoclast formation and stimulates osteoblasts differentiation. A). NPA (50 µM) inhibits osteoclast formation in calcitriol-stimulated mouse marrow. Asterisk indicates P< 0.05 by T test for all cell types. B.) Osteoblasts, detected by alkaline phosphatase staining, in control cultures. C.) Osteoblasts in culture treated with 50 µM NPA. Scale bar equals 20 microns in B and C.

Discussion

In this study, we tested a small molecule (NPA) that inhibited osteoclast formation without disrupting osteoblast formation. This is the first identification of NPA as a bone active molecule. NPA blocked osteoclast formation both pure osteoclast cultures where differentiation was stimulated with recombinant RANKL, or in calcitriol-stimulated mouse marrow.

NPA was first identified as part of a virtual screen. Initial characterization of the top 50 candidates identified in the virtual screen showed that NPA inhibited osteoclast formation at a concentration of 100 µM (D.A. Ostrov and L. S. Holliday, unpub. Data). The current project is part of the ongoing characterization of the small molecules identified in the screen.

The ability of NPA to inhibit osteoclast formation in pure cultures is consistent with it binding RANKL and preventing it interacting with RANK. Because the affinity between recombinant RANKL and RANK, the number of RANK receptors on osteoclasts and the possible affinity of NPA for RANKL, and recombinant RANKL are not known, these data are not representative of *in vivo* circumstances.

That NPA both blocked osteoclast formation and that osteoblasts appeared more abundant after NPA treatment, could possibly indicate stimulation of the RANKL-reverse signaling pathway by binding RANKL. Most agents that inhibit osteoclast formation in calcitriol-stimulated mouse marrow cultures also reduce osteoblasts likely because of loss of anabolic signals produced by the differentiating osteoclasts. Future studies will examine the effects of NPA directly on osteoblasts.

The IC_{50} for osteoclast differentiation is in the low micromolar range. Most therapeutics have activities in the nanomolar range, therefore NPA is not likely to be directly useful as a therapeutic. It may serve as a lead molecule for the identification of more potent small molecules for simultaneously stimulating bone formation and blocking bone resorption. Typically, after lead molecules with the right activity are identified, various chemical modifications of the lead molecule are tested empirically to identify the best candidate. For example, bisphosphonates have been through several generations of therapeutics that range in potency over several orders of magnitude (Pan et al., 2004).

We do not yet know whether NPA is acting through binding RANKL. Although we screened molecules based on identifying small molecules that would stimulate RANKL, and the activities of NPA *in vitro* are consistent with that mechanism, several additional tests are required to confirm this idea. This includes direct demonstration that NPA blocks interaction between RANKL and RANK. Finally, tests in *in vivo* models will be required to ensure that the activity we have detected *in vitro* is present *in vivo*. In addition, tests of toxic effects will be required for further development of the molecule as a therapeutic.

In conclusion, we have tested a small molecule for its ability to inhibit osteoclast differentiation and promote osteoblast formation using cell culture models. We found that it both directly inhibits osteoclast formation, and that it both inhibits osteoclast formation and stimulates osteoblast formation in co-culture *in vitro*. The molecule, NPA, does not have these effects with

at sufficiently low concentrations to be considered attractive directly as a therapeutic. However, if further experiments confirm that it acts by binding RANKL and that it stimulates RANKL reverse signaling, it would provide proof-in-principle evidence that a small molecule of this type is possible. It then could serve as a lead molecule. Modifications of the base molecule, using the virtual model to guide the modifications so as to preserve the interaction with the RANKL binding site, may yield molecules with higher affinity for RANKL, and which would represent a new type of therapeutics to treat osteoporosis and other types of bone disease.

References

Boyce, B. F. & Xing, L. (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys,* 473, 139-146. doi:10.1016/j.abb.2008.03.018

Clarke, B. (2008). Normal bone anatomy and physiology. *Clin J Am Soc Nephrol,* 3 Suppl 3, S131-139. doi: 10.2215/CJN.04151206

Compston, J. E., McClung, M. R. & Leslie, W. D. (2019). Osteoporosis. *Lancet,* 393, 364-376. doi: 10.1016/S0140-6736(18)32112-3

Florencio-Silva, R., Sasso, G. R., Sasso-Cerri, E., Simones, M. J. & Cerri, P. S. (2015). Biology of bone tissue: Structure, function, and factors that influence bone cells. *Biomed Res Int,* 2015, aricle ID 421746. doi: 10.1155/2015/421746

Holliday, L. S., Dean, A. D., Greenwald, J. E., & Glucks, S. L. (1995). C-type natriuretic peptide increases bone resorption in 1,25-dihydroxyvitamin D3-stimulated mouse bone marrow cultures. *J Biol Chem,* 270(32), 189983-18989. doi: 10.1074/jbc.270.32.18983

Holliday, L. S., Welgus, H. G., Fliszar, C. J., Veith, G. M., Jeffery, J. J., & Gluck, S. L. (1997). Initiation of osteoclast bone resorption by interstitial collagenase. *J Biol Chem*, 272(35), 22053-22058. doi: 10.1074/jbc.272.35.220

Honma, M., Ikebuchi, Y. & Suzuki, H. (2020). RANKL as a key figure in bridging between the bone and immune system: Its physiological functions and potential as a pharmacological target. *Pharmacol Ther,* 107682. doi:10.1016/j.pharmthera.2020.107682

Hurst, I. R., Zuo, J., Jiang, J., & Holliday, L. S. (2004). Actin-related protein 2/3 complex is required for actin ring formation. *J Bone Min Res,* 19(3), 499-506. doi: 10.1359/JBMR.0301238

Huynh, N., VonMoss, L., Smith, D., Rahman, I., Felemban, M. F., Zuo, J., Rody Jr, W. J., McHugh, K. P. & Holliday, L. S. (2016). Characterization of regulatory extracellular vesicles from osteoclasts. *Journal of Dental Research,* 95, 673-679. doi: 10.1177/0022034516633189

Ikebuchi, Y., Aoki, A., Honma, M., Hayashi, M., Sugamori, Y., Khan, M., Kariya, Y., Kato, G., Tabata, Y., Penninger, J. M., Udagawa, N., Aoki, K., & Suzuki, H. (2018). Coupling of bone resorption and formation by RANKL reverse signalling. *Nature,* 561, 195-200. doi:10.1038/s41586-018-0482-7

Kong, Y. Y., Boyle, W. J. & Penninger, J. M. (1999). Osteoprotegerin ligand: a common link between osteoclastogenesis, lymph node formation and lymphocyte development. *Immunol Cell Biol,* 77, 188-193. doi:10.1046/j.1440-1711.1999.00815.x

Lacey, D. L., Timms, E., Tan, H.-L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y.-X., Kaufman, S., Sarosi, I., … Boyle, W. J . (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell,* 93, 165-176. doi:10.1016/s0092-8674(00)81569-x

Liu, C., Walter, T. S., Huang, P., Zhang, S., Zhu, X., Wu, Y., Wedderburn, L. R., Tang, P., Owens, R. J., Stuart, D. I., Ren, J., & Gao, B. (2010). Structural and functional insights of RANKL-RANK interaction. *The Journal of Immunology*, 184(12), 6910-6919. doi: 10.4049/jimmunol.0904033

Martin, T. J. & Sims, N. A. (2015). RANKL/OPG; Critical role in bone physiology. *Rev Endocr Metab Disord,* 16, 131-139. doi:10.1007/s11154-014-9308-6

Ostrov, D. A., Magis, A. T., Wronski, T. J., Chan, E. K. L., Toro, E. J., Donatelli, R. E., Sajek, K., Haroun, I. N., Nagib, M. I., Piedrahita, A., Harris, A., & Holliday, S. H. (2009). Identification of enoxacin as an inhibitor of osteoclast formation and bone resorption by structure-based virtual screening. *Journal of Medicinal Chemistry*, 52(16), 5144-5151. doi: 10.1021/jm900277z

Pan, B., Farrugia, A. N., To, L. B., Findlay, D. M., Green, J., Lynch, K., & Zannettino, A. C. W. (2009). The nitrogen-containing bisphosphonate, zoledronic acid, influences RANKL expression in human osteoblast-like cells by activating TNF-alpha converting enzyme (TACE). *J Bone Miner Res*, 19(1), 147-154. doi: 10.1359/jbmr.2004.19.1.147

Prestwood, K. M., Pilbeam, C. C. & Raisz, L. G. (1995). Treatment of osteoporosis. *Annual Review of Medicine,* 46, 249-256. doi: 10.1146/annurev.med.46.1.249

Toro E. J., Zuo, J., Ostrov, D. A., Catalfamo, D., Bradaschia-Correa, V., Arana-Chavez, A. R., Neubert, J. K., Wronski, T. J., Wallet, S. M., & Holliday, L. S. (2012). Enoxacin directly inhibits osteoclastogenesis without inducing apoptosis. *Journal of Biological Chemistry*, 287(21), 17894-17904. doi:10.1074/jbc.M111.280511

Vracar, T. C., Zuo, J., Park, J., Azer, D., Mikhael, C., Holliday, S. A., Holsey, D., Han, G., VonMoss, L., Neubert, J. K., Rody Jr., W. J., Chan, E. K. L., & Holliday, L. S. (2018). Enoxacin and bis-enoxacin stimulate 4T1 murine breast cancer cells to release extracellular vesicles that inhibit osteoclastogenesis. *Scientific Reports*, 8, 16182. doi: 10.1038/s41598-018-34698-9

Yamaba, S., Yamada, S., Kajikawa, T., Awata, T., Sakashita, H., Tsushima, K., Fujihara, C., Yanagita, M., & Murakami, S. (2015). PLAP-1/Asporin regulates TLR2- and TLR4- induced inflammatory responses. *Journal of Dental Research*, 94(12), 1706-1714. doi: 10.1177/0022034515606859

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N., & Suda, T. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci, U S A* 95, 3597-3602. doi:10.1073/pnas.95.7.3597