Effects of a New Conjugate Drug in a Rat Model of Postmenopausal Osteoporosis

By

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Postmenopausal osteoporosis is a disease characterized by bone loss and increased risk of fracture, and represents a significant burden on the Canadian health care system. Current treatments lack the ability to simultaneously address the therapeutic needs for promoting bone formation and inhibiting resorption. Our approach employs a novel conjugate drug in which an anabolic agent (EP4 receptor agonist) is reversibly joined with an anti-resorptive agent (alendronate) through a linker. This allows the bone-targeting ability of alendronate to deliver the EP4 agonist to bone sites, thereby mitigating the side effects associated with systemic administration of the EP4 agonist. This study investigated the in vivo efficacy of this drug in a curative experiment to treat postmenopausal osteoporosis using an ovariectomized rat model. Results showed that conjugate treatment dose-dependently stimulated bone formation and restored ovariectomy-induced bone loss, and conjugation between alendronate and the EP4 agonist was crucial to the drug’s anabolic effect.
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# Table of Contents

Acknowledgements ......................................................................................................................... iii  
Table of Contents.......................................................................................................................... iv 
List of Tables.................................................................................................................................... vi  
List of Figures.................................................................................................................................... vii 
List of Abbreviations....................................................................................................................... ix 

1 Introduction .................................................................................................................................. 1  
  1.1 Bone Biology .......................................................................................................................... 1  
    1.1.1 Structure and Composition ............................................................................................... 1  
    1.1.2 Bone Cells ....................................................................................................................... 4  
    1.1.3 Bone Remodeling ............................................................................................................ 6  
    1.1.4 Menopause ...................................................................................................................... 8  
  1.2 Osteoporosis ............................................................................................................................ 9  
    1.2.1 Postmenopausal Osteoporosis ......................................................................................... 10  
    1.2.2 Animal Models of Osteoporosis ..................................................................................... 12  
  1.3 Osteoporosis Treatment ........................................................................................................... 13  
    1.3.1 Bisphosphonates ............................................................................................................. 13  
    1.3.2 Parathyroid Hormone ...................................................................................................... 19  
    1.3.3 Other ............................................................................................................................. 21  
    1.3.4 Combination Therapy ..................................................................................................... 21  
  1.4 Prostaglandin E2 ...................................................................................................................... 23  
    1.4.1 Molecular Structure and Biosynthesis ............................................................................ 23  
    1.4.2 Biological Functions ....................................................................................................... 25  
    1.4.3 Effects on Bone .............................................................................................................. 26  
    1.4.4 Mechanism of Action on Bone ..................................................................................... 27  
  1.5 Synthetic EP4 Receptor Agonists ............................................................................................. 30  
  1.6 New Approach: Conjugate ....................................................................................................... 31  
    1.6.1 Mechanism of Action ..................................................................................................... 32  
    1.6.2 In vivo Uptake and Release ............................................................................................ 35  
  1.7 Bone Quality ............................................................................................................................ 36  
    1.7.1 Bone Mass and Density .................................................................................................. 36  
    1.7.2 Geometry and Architecture ......................................................................................... 37  
    1.7.3 Remodeling ................................................................................................................... 39  
    1.7.4 Mineralization ............................................................................................................... 39  
    1.7.5 Mechanical Properties ................................................................................................. 40  

2 Study Hypothesis .......................................................................................................................... 42  

3 Materials and Methods ............................................................................................................... 43  
  3.1 In Vivo Experiments ............................................................................................................... 43  
    3.1.1 Animals ......................................................................................................................... 43  
    3.1.2 Treatment ..................................................................................................................... 43  
    3.1.3 Sacrifice and Dissection ............................................................................................... 46  
  3.2 Evaluation of Treatment Effects: Overview ........................................................................... 46  
    3.2.1 Tissue-Level Remodeling ............................................................................................... 47  
    3.2.2 Bone Mineral Density and Microarchitecture .............................................................. 51  
    3.2.3 Mechanical Properties ................................................................................................. 53
List of Tables

Table 1–1. Chemical characteristics of various BPs in clinical use................................................................. 17
Table 1–2. Commonly used bone turnover markers .......................................................................................... 19
Table 1–3. Distribution of EP receptors and their physiological impact .......................................................... 25
Table 1–4. Inhibitory constant K_i of ligand-binding for different EP receptor agonists .............................. 29
Table 1–5. Binding affinities of synthetic EP4 receptor agonists ................................................................... 31
Table 3–1. Treatment groups .......................................................................................................................... 44
Table 3–2. Chemical formula and molecular weight of compounds used in this study .................................. 44
Table 3–3. List of bone specimens used in each experiment ............................................................................... 47
Table 4–1. Animal reactions to treatment ...................................................................................................... 62
Table 5–1. Summary of experimental findings in this study ........................................................................... 94
List of Figures

Figure 1–1. Illustration of cortical (compact) bone and cancellous (trabecular) bone. ........................................2
Figure 1–2. Illustration of different regions of a long bone. .................................................................................. 2
Figure 1–3. Illustration of osteons within cortical bone ......................................................................................... 3
Figure 1–4. The structure of hydroxyapatite ........................................................................................................... 4
Figure 1–5. Multinucleated primary rat osteoclast. Reproduced from reference 8 ................................................. 4
Figure 1–6. Mesenchymal cell differentiation ......................................................................................................... 6
Figure 1–7. Illustration of the bone remodeling cycle. ............................................................................................. 8
Figure 1–8. Comparison of normal (left) and osteoporotic (right) trabecular bone structure .................................... 9
Figure 1–9. Schematic illustration of vertebral compression fracture ................................................................. 10
Figure 1–10. WHO definitions for diagnosis of postmenopausal osteoporosis .................................................... 11
Figure 1–11. Chemical structure of pyrophosphate and bisphosphonate. ................................................................. 15
Figure 1–12. Structure of BPs ............................................................................................................................... 15
Figure 1–13. 3D computational models of different N-BPs ................................................................................... 18
Figure 1–14. Structures of prostanoids. ................................................................................................................ 24
Figure 1–15. Biosynthesis pathway for prostaglandins. ........................................................................................... 24
Figure 1–16. Mechanism of action of ALN-LK-EP4a conjugate ......................................................................... 32
Figure 1–17. Chemical structure of ALN-PGE2 along with PGE2 and ALN ......................................................... 33
Figure 1–18. Chemical structures of ALN-LK-EP4a conjugate and its constituents ............................................. 34
Figure 1–19. In vivo hydrolysis of the ALN-LK-EP4a conjugate ......................................................................... 35
Figure 1–20. In vivo uptake an release of radiolabeled ALN-LK-EP4a conjugate. ................................................ 36
Figure 1–21. Cross-sectional resistance to bending in cortical bone. ................................................................. 38
Figure 1–22. Load-displacement curve from biomechanical testing of bone specimen ...................................... 40
Figure 1–23. Force-displacement curve of normal and diseased bone .............................................................. 41
Figure 1–24. Illustration of an ideal treatment strategy for improving bone fragility ....................................... 41
Figure 3–1. Study timeline ........................................................................................................................................ 43
Figure 3–2. Overview of bone quality indicators and the experimental techniques ........................................... 47
Figure 3–3. Sample image of bone section stained using Goldner’s Trichrome. .................................................. 48
Figure 3–4. Regions of interest (ROI) for analysis of proximal tibia ............................................................... 49
Figure 3–5. Sample image of unstained slide under fluorescent microscopy ............................................... 49
Figure 3–6. TRAP-positive osteoclast appears red under immunohistological staining .................................. 50
Figure 3–7. MicroCT scanning field-of-view at 11.6 µm voxel size ...................................................................... 52
Figure 3–8. Regions of interest for microCT analysis of femurs ....................................................................... 53
Figure 3–9. Regions of interest for microCT analysis of vertebrae ................................................................. 53
Figure 3–10. Typical results from biomechanical testing of bone specimens ...................................................... 54
Figure 3–11. Illustration of three-point bending test .......................................................................................... 55
Figure 3–12. Illustration of femoral neck fracture test ....................................................................................... 57
Figure 3–13. Illustration of vertebral compression test ..................................................................................... 58
Figure 3–14. Sample BSE image showing contrast difference due to mineralization .................................... 59
Figure 3–15. Regions of interest for analyzing BSE images .............................................................................. 60
Figure 3–16. Histogram from BSE imaging ........................................................................................................ 61
Figure 4–1. Treatment effects on animal weight .................................................................................................. 64
Figure 4–2. Treatment effects on bone size .......................................................................................................... 64
Figure 4–3. Representative BSE images of proximal tibia ................................................................................. 65
Figure 4–4. Osteoid formation in the proximal tibial metaphysis ................................................................. 66
Figure 4–5. Proximal tibial metaphysis under dynamic histomorphometry ...................................................... 67
Figure 4–6. Bone formation parameters from dynamic histomorphometry ..................................................... 68
Figure 4–7. Representative images of proximal tibial metaphysis .................................................................. 69
Figure 4–8. Osteoclast number measured in TRAP-stained decalcified histomorphometry ........................................... 70
Figure 4–9. Osteoclast surface measured in TRAP-stained decalcified histomorphometry ........................................... 71
Figure 4–10. Trabecular microarchitecture .................................................................................................................. 72
Figure 4–11. Structural indices derived from microCT images of vertebrae ................................................................. 73
Figure 4–12. Trabecular structural indices from undecalcified histomorphometry ......................................................... 74
Figure 4–13. DEXA results for lumbar vertebrae ......................................................................................................... 75
Figure 4–14. Volumetric bone mineral density (vBMD) via microCT ......................................................................... 76
Figure 4–15. Vertebral compression results using L6 vertebrae ................................................................................. 77
Figure 4–16. Vertebral compression results using L6 vertebrae ................................................................................. 78
Figure 4–17. Degree of mineralization results for trabecular bone ............................................................................ 79
Figure 4–18. Representative images of the tibial diaphysis ......................................................................................... 79
Figure 4–19. Intracortical bone formation parameters ................................................................................................. 80
Figure 4–20. Representative images of coronally sectioned tibial diaphysis ............................................................... 81
Figure 4–21. Axial and coronal sections of cortical bone ............................................................................................. 81
Figure 4–22. Representative 3D models of femoral mid-diaphysis ............................................................................ 82
Figure 4–23. Geometrical parameters of femoral mid-diaphysis measured via microCT ...................................... 82
Figure 4–24. Cortical thickness and porosity measured using microCT .................................................................. 83
Figure 4–25. Volume and vBMD of endocortical bone in the non-vehicle-treated groups ........................................... 84
Figure 4–26. DEXA results for femur ......................................................................................................................... 84
Figure 4–27. Volumetric BMD of cortical bone ........................................................................................................... 85
Figure 4–28. Three-point bending results using left femurs ....................................................................................... 86
Figure 4–29. Three-point bending results using left femurs ....................................................................................... 86
Figure 4–30. Femoral neck fracture results using left femurs .................................................................................... 87
Figure 4–31. Degree of mineralization results for cortical bone ................................................................................. 88
Figure 4–32. Images of endocortical marrow region in undecalcified histomorphometry ........................................... 89
Figure 4–33. Effects of high dose conjugate treatment ................................................................................................. 91
Figure 5–1. Theoretical time course of conjugate uptake and EP4a release .............................................................. 107
Figure 5–2. Illustration of crossover between cAMP and canonical Wnt pathways ................................................. 128
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-HPA</td>
<td>4-hydroxyphenylacetic acid</td>
</tr>
<tr>
<td>aBMD</td>
<td>Areal bone mineral density</td>
</tr>
<tr>
<td>ALN</td>
<td>Alendronate</td>
</tr>
<tr>
<td>ALN-LK</td>
<td>Alendronate joined with linker moiety; one of two components in the EA group</td>
</tr>
<tr>
<td>ALN-LK-EP4a</td>
<td>Conjugate drug in current study</td>
</tr>
<tr>
<td>ALN-PGE₂</td>
<td>Conjugate drug in previous study</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BFR/BS</td>
<td>Surface-referent bone formation rate</td>
</tr>
<tr>
<td>BFR/BV</td>
<td>Volume-referent bone formation rate</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone mineral content</td>
</tr>
<tr>
<td>BP</td>
<td>Bisphosphonates</td>
</tr>
<tr>
<td>BRU</td>
<td>Bone remodeling units</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoprotein</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Bone volume fraction</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CH</td>
<td>Treatment group, OVX rat given conjugate high dose</td>
</tr>
<tr>
<td>CL</td>
<td>Treatment group, OVX rat given conjugate low dose</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COOH</td>
<td>Carboxyl moiety</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>dLS</td>
<td>Double label surface</td>
</tr>
<tr>
<td>EA</td>
<td>Treatment group, OVX rat given separate EP4a and ALN-LK</td>
</tr>
<tr>
<td>EP4a</td>
<td>EP4 agonist used in conjugate</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl diphosphate, also known as farnesyl pyrophosphate</td>
</tr>
<tr>
<td>FPPS</td>
<td>Farnesyl diphosphate synthase</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half max</td>
</tr>
<tr>
<td>GGPP</td>
<td>Geranylgeranyl diphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>hPTH</td>
<td>Human parathyroid hormone</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>Ir.L.Wi</td>
<td>Interlabel width</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LK</td>
<td>Linker molecule used in conjugate</td>
</tr>
<tr>
<td>MAR</td>
<td>Mineral apposition rate</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>microCT</td>
<td>Micro-computed tomorgraphy</td>
</tr>
<tr>
<td>MS</td>
<td>Mineralizing surface</td>
</tr>
<tr>
<td>MS/BS</td>
<td>Percent mineralizing surface</td>
</tr>
<tr>
<td>MS/BV</td>
<td>Volume-referent mineralizing surface</td>
</tr>
<tr>
<td>N.Oc</td>
<td>Osteoclast number</td>
</tr>
<tr>
<td>N.Oc/BS</td>
<td>Osteoclast number normalized for total bone surface</td>
</tr>
<tr>
<td>Oc.S</td>
<td>Osteoclast surface</td>
</tr>
</tbody>
</table>
Oc.S/BS  Osteoclast surface normalized for total bone surface
OH   Hydroxyl moiety
OPG  Osteoprotegrin
OPN  Osteopontin
OV   Treatment group, OVX rat given vehicle
PG   Treatment group, OVX rat given PGE₂
PGE₂ Prostaglandin E₂
PNS  Peripheral nervous system
PKA  Protein kinase A
PLC  Phospholipase C
PTH  Parathyroid hormone
PTHrP Parathyroid hormone related hormone
RANK receptor activator of NF-κB
RANKL receptor activator of NF-κB ligand
ROI  Region of interest
SD   Standard deviation
SERM Selective estrogen receptor modulator
SFRP1 Secreted Frizzled-related protein 1
sLS  Single label surface
SV   Treatment group, sham-operated rat given vehicle
Tb.N Trabecular number
Tb.Sp Trabecular separation
Tb.Th Trabecular thickness
TNF  Tumor necrosis factor
TRAP Tartrate-resistant acid phosphatase
vBMD Volumetric bone mineral density
VEGF Vascular endothelial growth factor
VOI  Volume of interest
1 Introduction

In Canada, incidences of fractures due to osteoporosis are more common than that of heart attack, stroke, and breast cancer combined*. According to Osteoporosis Canada, at least 1 in 3 women and 1 in 5 men in this country will develop an osteoporotic fracture in their lifetime*. As of 2010, the annual cost to the Canadian healthcare system for treating osteoporosis and its associated fractures is $2.3 billion, which constitutes a significant burden on the healthcare system*. Osteoporosis is characterized by decreased bone mass and increased risk of fracture, and postmenopausal osteoporosis is the most common form of pathological bone loss, often occurring in women after the age of 50. Since bone loss is frequently not detected until an individual develops an osteoporotic fracture, many patients are not diagnosed until after significant bone loss has occurred. Effective interventions are thus needed to slow the rate of bone loss and restore bone mass in patients with postmenopausal osteoporosis.

1.1 Bone Biology

The human skeleton has several important functions, including structural support for the body, protection for the internal organs, storage of minerals for maintaining mineral homeostasis, and enabling movement by serving as levers for the muscles¹. In addition, the bone marrow also provides an environment for the generation of blood cells as well as lymphocytes for the immune system².

There are different categories of bone, such as long bones, short bones, flat bones, and irregular bones. Examples of different types of bone include the femur and tibia for long bones, the patella for short bone, the skull and ribs for flat bone, and the vertebra for irregular bone¹. In this thesis, the discussion will focus on the femur, tibia, and vertebra.

1.1.1 Structure and Composition

There are two different types of bone structure. Cortical or compact bone is dense and hard, and forms the outer shell of most bones (Figure 1–1). In contrast, cancellous or trabecular bone is highly porous, and consists of a meshwork of rods and plates known as trabeculae. It is also known as spongy bone, and forms the inner compartment of many bones such as the femur and vertebra. The

trabecular structures are interspersed with bone marrow, and have a much greater surface area than cortical bone. This provides increased opportunity for biological activity in trabecular bone.

![cancellous bone](image)

**Figure 1-1. Illustration of cortical (compact) bone and cancellous (trabecular) bone.** Image taken from https://courses.stuqmul.ac.uk/smd/kb/microanatomy/bone/answers/index.htm.

In long bones, the middle section of the bone is a hollow shaft consisting of cortical bone, and is known as the diaphysis (Figure 1–2). The region adjacent to the diaphysis is known as the metaphysis, and consists of a dense cortical wall surrounding an inner compartment containing trabecular bone. The ends of the bone are known as the epiphysis, the surface of which is covered by articular cartilage. The epiphyseal growth plate separates the metaphysis from the epiphysis, and is the site of endochondral bone formation which facilitates the elongation of long bones. The outer cortical surface is covered by a layer of fibrous connective tissue known as the periosteum, while the inner surface of cortical bone is known as the endoseum.

The adult human skeleton contains approximately 80% cortical and 20% trabecular bone. Depending on its function, different areas of the skeleton have different proportions of cortical and trabecular bone. For instance, the vertebra in healthy young adults consists of approximately 25:75 cortical and trabecular bone, while the ratio is 95:5 in the radial diaphysis. The femoral head contains approximately 50:50 cortical and trabecular bone.

![Regions of a long bone](image)

**Figure 1–2. Illustration of different regions of a long bone.** Image from http://www.studyblue.com/notes/n/bones/deck/5808495.
The fundamental functional unit of cortical bone is the osteon or Haversian system, which consists of cylindrical structures that are approximately 400 µm in length and 200 µm in width \(^3\) (Figure 1–3). Each osteon is composed of concentric layers of compact bone surrounding a central canal, the outer boundary of which is known as the cement line. The layers of an osteon are known as the lamellae, while the central canal is known as the Haversian canal and contains the bone’s nerves and blood supply. It is estimated that there are 21 million cortical osteons in healthy human adults\(^1\). In trabecular bone, the fundamental functional units are known as packets. Although they are also composed of layers of lamellae, these packets are semilunar in shape. There are an estimated 14 million trabecular packets in healthy human adults\(^1\).

![Figure 1–3. Illustration of osteons within cortical bone. Image from http://upload.wikimedia.org/wikipedia/commons/3/34/Illu_compact_spongy_bone.jpg.](http://upload.wikimedia.org/wikipedia/commons/3/34/Illu_compact_spongy_bone.jpg)

Bone is a composite material consisting of mineralized organic matrix, and approximately 60-70% of its mass is mineral. The organic phase of bone is composed primarily of type I collagen, with trace amounts of other collagensous proteins such as types III, V, and X\(^1\). The collagen fibers are arranged in an organized fashion within each lamella. In addition, a variety of noncollagensous proteins are also present in the bone matrix, and constitute approximately 10-15% of total bone protein\(^4\). Some examples of noncollagenous matrix proteins in bone are proteoglycans, osteonectin, fibronectin, osteopontin, bone sialoprotein (BSP), and bone morphogenic protein (BMP). The organic phase of bone provides elasticity to the material and enables it to absorb energy upon impact.

The inorganic phase of bone is a nano-crystalline structure consisting primarily of a calcium phosphate mineral known as hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_{6}(\text{OH})_2]\)\(^1\) (Figure 1–4). This structure is highly substituted with other minerals such as magnesium and potassium. Depending on the particular diet and environment, there may also be trace amounts of other elements\(^5\). The mineral phase of bone provides strength and mechanical resistance, and enables the bone to bear load.
1.1.2 Bone Cells

There are three different types of bone cells, each with a unique function in the maintenance of bone. Osteoclasts are responsible for breaking down old bone in a process known as resorption, while osteoblasts are involved in the formation of new bone matrix. Osteocytes are terminally differentiated osteoblasts which have become buried within the bone matrix, and are believed to be involved in sensing and signaling of mechanical stresses in the bone.

1.1.2.1 Osteoclasts

Osteoclasts are the exclusive bone-resorbing cell (Figure 1–5), and are members of the monocyte/macrophage family derived from hematopoietic stem cells. Activated multinucleated osteoclasts are formed by the fusion of multiple mononuclear precursor cells. Osteoclastogenesis is the process of osteoclast differentiation, and is regulated by numerous cytokines such as IL-1, IL-6, and parathyroid hormone (PTH). However, the most important regulators of osteoclastogenesis are macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL), which are both secreted mainly by marrow stromal cells and their derivative osteoblasts.

M-CSF has been shown to be involved in the differentiation, proliferation, and survival of osteoclasts and their precursors. In addition, RANKL is a member of the tumor necrosis factor (TNF)
superfamily, and is secreted along with osteoprotegrin (OPG) to regulate osteoclast formation. RANKL binds to the RANK receptor on osteoclast precursors to stimulate their differentiation, while OPG acts as a decoy ligand for RANK and inhibits the effects of RANKL. The ratio of RANKL to OPG is thus an important determinant of osteoclast differentiation.

During bone resorption, osteoclasts bind to bone matrix via the integrin receptors on their membranes, with the main receptor facilitating bone resorption being the \( \alpha_v\beta_3 \) integrin, which binds to osteopontin and BSP. This binding causes the osteoclasts to become polarized, and results in the formation of a microenvironment underneath the osteoclast known as the sealing zone. The basal membrane of the osteoclast then becomes scalloped in the formation of a ruffled border, as vesicles containing matrix degradation enzymes such as cathepsin K fuse with the membrane. The ruffled border secretes hydrogen and chloride ions via a proton pump and chloride channel, respectively, which acidifies the microenvironment to achieve a pH of approximately 4.5. This acidic environment dissolves bone mineral and exposes the underlying organic matrix for cleavage by enzymes such as cathepsin K.

To facilitate the resorption process, the cytoskeleton of the osteoclast reorganizes upon its binding with bone matrix. The fibrillar actin reorganizes into an actin ring, which promotes formation of the sealing zone. Disruptions in the formation of the ruffled border or the actin ring have been shown to hinder bone resorption.

1.1.2.2 Osteoblasts

Osteoblasts are derived from mesenchymal stem cells, are involved in the synthesis of new bone matrix. Mesenchymal stem cells are pluripotent cells that have the potential to differentiate into a variety of cell types, such as osteoblasts in bone, chondrocytes in cartilage, and adipocytes in fat. Osteoblast differentiation is controlled by a number of cytokines such as BMP, TGF-\( \beta \), IL-3, and IL-6. The differentiation of osteoblasts occurs in several stages, including the proliferation of preosteoblasts, maturation of immature osteoblasts, and the terminal differentiation of mature osteoblasts into osteocytes, quiescent bone lining cells, or those undergoing apoptosis (Figure 1–6). Osteoblasts express alkaline phosphatase (ALP) during the early stages of differentiation, whereas osteocalcin is considered to be a late marker of osteoblast differentiation.
Figure 1–6. Schematic of the mesenchymal cell differentiation toward the osteoblastic lineage and the effect of various transcription regulators. Reproduced from reference\textsuperscript{11}.

1.1.2.3 Osteocytes

Osteocytes are terminally differentiated osteoblasts that have become embedded in the bone matrix, and lie inside lacunae within layers of mineralized bone. They have extensive filopodial processes that extend through canaliculi, which are channels in the lamellae. These enable osteocytes to communicate with each other and establish connection with the bone surface, and it has been shown that osteocytes are involved in sensing mechanical stresses in the bone as well as initiating the biological response to damage\textsuperscript{7,14-16}. Osteocytes normally do not express ALP, but do express osteocalcin and several matrix proteins that support cell adhesion\textsuperscript{1}.

1.1.3 Bone Remodeling

Bone remodeling, or turnover, is a natural process of renewal in which discrete packets of old bone are resorbed and replaced with newly formed bone matrix which then becomes gradually mineralized over time. It has several important roles in maintaining bone health, including reparation of microdamage, response to mechanical loading, healing after fracture, and maintenance of mineral homeostasis. Since the skeleton is continually subjected to wear and tear in providing structural support for the body and facilitating its movement, microdamage is sustained as a result of normal function. Bone remodeling enables the reparation of microdamage by replacing old bone with new matrix which then becomes mineralized, and is essential to preserving the mechanical integrity of bone\textsuperscript{17}. In addition, remodeling also enables the bone to respond to functional demands of mechanical loading, such that the shape of bone may be changed due to addition of new bone in places of increased mechanical demand\textsuperscript{18}. Moreover, remodeling is required to complete the healing process following a fracture, as it allows the fracture callus to be modified to restore the structure of the
original bone\textsuperscript{18}. Since the skeleton serves as a mineral reservoir for the body, the level of resorption is partially dependent upon the calcium levels in blood plasma, as low plasma concentrations require replenishment via calcium release from its storage in bone. This is demonstrated by one of the physiological roles of parathyroid hormone (PTH) in maintaining calcium homeostasis, as it promotes osteoclastic resorption in response to reductions in plasma calcium levels, which results in the release of skeletal calcium back into the blood\textsuperscript{19}.

Bone remodeling occurs sequentially in several stages consisting of activation, resorption, reversal, and formation (\textit{Figure 1–7}). During activation, mononuclear osteoclast precursors are recruited to form multinucleated osteoclasts, which then bind to bone surface and form a sealing zone. The osteoclasts become activated with the formation of the ruffled border and begin resorbing bone, and the process of osteoclastic resorption occurs over a period of 2-4 weeks during each remodeling cycle. As mentioned earlier, osteoclastic resorption involves the acidification of the sealing zone and the enzymatic digestion of bone matrix proteins, and actively resorbing osteoclasts secrete many digestive enzymes such as cathepsin K, tartrate-resistant acid phosphatase (TRAP), matrix metalloproteinase 9, and lysosomal gelatinase\textsuperscript{1}. The resorption of bone matrix results in formation of a resorption pit, or Howship’s lacunae, on the surface of trabecular bone, while the resorption of cortical bone results in longitudinal tunneling through the cortical osteon\textsuperscript{1}. Near the end of the resorption phase, the multinucleated osteoclasts undergo apoptosis, and the resorption phase is completed by mononuclear cells\textsuperscript{20}.

During the reversal phase, there is a transition from resorption to formation. Many cells are present in the resorption cavity, including a variety of mononuclear cells and preosteoblasts. Since the maintenance of bone balance requires coupling between the resorption and formation processes, such that osteoclasts may signal the osteoblasts regarding the amount of bone resorbed, much research has been conducted in search of a coupling factor. Recent studies have shown that there may be several coupling factors from a variety of different sources\textsuperscript{21}. For instance, cardiotrophin-1 is a cytokine secreted by osteoclasts that stimulates bone formation \textit{in vivo}, and may allow the communication between osteoclasts and osteoblasts\textsuperscript{22}. In addition, Semaphorin 4D is another osteoclast-secreted factor that has been shown to inhibit bone formation \textit{in vivo}\textsuperscript{23}, while a third potential candidate is EphrinB2, which is expressed on the surface of osteoclasts and has been shown to interact with EphrinB4 receptors on the surface of osteoblasts \textit{in vitro}\textsuperscript{24}. These new findings suggest that there may be many channels of coupling between resorption and formation in bone remodeling, which enables the balance between the two processes to be tightly controlled.
The formation stage of bone remodeling occurs over a period of 3 months\(^1\). Osteoblasts synthesize new organic matrix within the resorption cavity, while also releasing small surface-bound vesicles that concentrate calcium and phosphate ions to promote mineralization\(^1\). In addition, these vesicles also help destroy mineralization inhibitors such as pyrophosphate\(^25\). Some osteoblasts become buried in the new bone matrix and transform into osteocytes, with networks of canaliculi connecting them with each other and the bone surface. Others become quiescent bone lining cells. However, the majority of osteoblasts (50-70%) undergo apoptosis upon the completion of bone formation\(^1\).

Osteoclasts and osteoblasts that are tightly coupled together within each remodeling cycle are known as bone remodeling units (BRU). The number of new BRUs created per unit volume of bone within a unit time is known as the activation frequency.

![Figure 1-7. Illustration of the bone remodeling cycle. Image obtained from http://www.ns.umich.edu/Releases/2005/Feb05/bone.html.](http://www.ns.umich.edu/Releases/2005/Feb05/bone.html)

1.1.4 Menopause

Menopause is the cessation of menstruation, and occurs at approximately 48-50 years of age in healthy women. The deficiency of ovarian hormones such as estrogen leads to significant bone loss in postmenopausal women, and is a great contributor to the development of osteoporotic fractures in older women.

In normal postmenopausal women, there is an increase in the proportion of active resorption surfaces in bone, as well as increased depth of resorption pits. These are the result of greater activation frequency in the remodeling units as well as increased resorption time within each cycle. Bone resorption indices are doubled relative to that of premenopausal women, while formation is increased by less than half\(^26\). Thus, bone loss following menopause is due to increased turnover with negative bone balance in each remodeling cycle, which leads to the perforation and loss of trabeculae in cancellous bone. In cortical bone, menopause leads to greater porosity and increased endosteal resorption, which results in cortical thinning\(^27\).

The effects of menopause on bone may be partially accounted for by examining the role of estrogen in bone physiology. Estrogen receptors are expressed in both osteoclasts and osteoblasts,
and its effects favor formation over resorption. In particular, estrogen promotes the preferential differentiation of mesenchymal precursors cells into osteoblasts over adipocytes, increases osteoblast proliferation, suppresses the apoptosis of osteoblasts and osteocytes, and stimulates the production of various osteoblast proteins such as IGF-1, type I procollagen, TGF-β, and BMP-6. In contrast, the effects of estrogen on bone resorption are inhibitory, as exemplified by its stimulation of osteoclast apoptosis, and reduction of RANKL production by osteoblasts while simultaneously increasing the production of OPG. In addition, estrogen also decreases the production of osteoclastic cytokines such as IL-1, IL-6, and TNFα in bone marrow.

Postmenopausal bone loss occurs rapidly during the initial decade following menopause, and is slower thereafter. It impacts trabecular bone more than cortical due to the greater surface to volume ratio in trabecular bone, which exposes more bone surface to osteoclastic resorption.

1.2 Osteoporosis

Osteoporosis is a skeletal disease characterized by decreased bone mass, damage to bone architecture, and increased risk of fracture. Osteoporotic fractures frequently occur in the hip, spine, and wrist, with hip fractures being particularly devastating. Of the 30,000 annual hip fractures in Canada, 70-90% occur as a result of osteoporosis. These fractures carry approximately 20% risk of mortality within the first 12 months, and 40% of the patients lose the ability to walk independently after the fracture. As many as 33% of all patients are moved to long-term care facilities one year following a hip fracture.

![Comparison of normal (left) and osteoporotic (right) trabecular bone structure](image)

Osteoporosis is classified into primary and secondary types. While secondary osteoporosis is often associated with an underlying disease or medical treatment and affects approximately 10% of patients, the majority of osteoporotic patients belong to the primary disease category. Primary

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Osteoporosis is further classified into idiopathic osteoporosis, which occurs in children and young adults, and involutional osteoporosis, which is found in adults and aging individuals\textsuperscript{36}. There are 2 main forms of involutional osteoporosis: Postmenopausal or type I osteoporosis, and senile or type II osteoporosis. Postmenopausal osteoporosis predominantly affects women and is associated with the loss of ovarian hormones following menopause, in which bone loss is more prominent in trabecular than cortical bone\textsuperscript{37}. In contrast, senile osteoporosis is associated with aging in both men and women, with greater impact on cortical bone\textsuperscript{38}.

### 1.2.1 Postmenopausal Osteoporosis

Postmenopausal osteoporosis is the most common form of pathological bone loss, and mainly affects women after menopause. It is characterized by fractures of the vertebrae (spine), proximal femur (hip), and distal forearm (wrist), although there is increased risk of all types of fractures due to the systemic effects of osteoporotic bone loss. Individuals with low bone mineral density are at increased risk of fracture, and those who develop one fracture are at increased risk of sustaining another\textsuperscript{39}.

Although hip fractures are considered the most severe form of osteoporotic fracture, vertebral fractures are also devastating (Figure 1–9), as they cause significant pain and lead to increased thoracic kyphosis, or curling of the spine. While approximately 25\% of vertebral fractures result from moderate trauma such as falls, the majority of these fractures result from routine activities such as bending or lifting light objects\textsuperscript{39}. These low-trauma fractures are known as fragility fractures, and studies have shown that the development of one vertebral fracture in a patient leads to a 10-fold increase in the risk of developing a subsequent fracture\textsuperscript{40}.

![Figure 1-9. Schematic illustration of vertebral compression fracture, which leads to deformity of the spine. Image obtained from http://umm.edu/programs/spine/health/guides/lumbar-compression-fractures.](image)

Bone loss in postmenopausal osteoporosis is generally diagnosed using categories defined by the World Health Organization (WHO), and is based on areal bone mineral density (aBMD) measurements of the hip (Figure 1–10). These are obtained using dual-energy X-ray absorptiometry
(DEXA), and an individual’s result is compared to that of a reference population consisting of young healthy Caucasian women. The resulting T-scores are divided into 3 categories: 1) Hip aBMD higher than 1 SD below the mean of the reference population is considered to be normal; 2) hip aBMD in the range of 1 SD to 2.5 SD below the reference mean is considered to be osteopenic, or having low bone mass; 3) hip aBMD more than 2.5 SD below the reference mean is deemed to be osteoporotic. There is a fourth category of severe osteoporosis, which is defined as the combination of an osteoporotic T-score with the presence of one or more fragility fractures.

Since the T-scores do not facilitate prediction of fracture risk, more recent diagnosis of osteoporosis has included the assessment of fracture risk using specific tools. These tools include the WHO Fracture Risk Assessment Tool (FRAX), Garvan Fracture Risk Calculator, and Qfractur algorithm, which aim to assist clinicians in determining patient’s 5-year or 10-year fracture risk based on a combination of known risk factors such as BMD, age, prior fracture, body mass index, weight, and femoral neck T-score.

[Figure 1-10. Schematic illustration of the WHO definitions for diagnosis of postmenopausal osteoporosis. Reproduced from reference.]

Similar to normal postmenopausal bone loss, the bone loss in postmenopausal osteoporosis also occurs rapidly in the initial decade following menopause, and becomes slower in later decades. The bone loss occurs as a result of increased turnover with resorption exceeding formation, leading to the perforation of trabeculae and loss of whole trabecular elements. There is a conversion of the trabecular structure from a plate-like to a more rod-like appearance, and decreased connectivity between adjacent trabeculae. Although trabecular thickness is mostly unaffected during the initial period of rapid bone loss, there is gradual thinning of remaining trabeculae with age.

Although bone loss occurs in normal postmenopausal women as well as in women with postmenopausal osteoporosis, it is still unclear how these conditions differ. Researchers have compared the histomorphometric indices in the iliac crest of normal postmenopausal women and those with postmenopausal osteoporosis (osteoporosis defined as having radiographic evidence of
vertebral deformities). Results showed that the trabecular bone volume was 35% lower with 36% decrease in trabecular number in the osteoporotic women, and the trabecular separation was also 34% greater. However, bone remodeling parameters were similar between the two groups, such as mineralizing surface which measures the extent of mineralization in bone, and mineral apposition rate which measures the activity of osteoblasts.

There are many factors that influence the development of postmenopausal osteoporosis, such as genetics, nutrition and exercise, estrogen, systemic hormones, and various local factors. Genetics are a significant determinant of the peak bone mass in life as well as bone architecture, while good nutrition with adequate intake of calcium and vitamin D, plus sufficient exercise, are important to general bone health. The deficiency of sex hormones such as estrogen are important determinants of net bone balance in remodeling as well as the rate of remodeling, while systemic hormones such as PTH and growth hormone also control the resorption and formation processes in remodeling. In addition, the tissue-level interactions between osteoclasts and osteoblasts, as well as various local factors such as interleukin and TGF-β may all play a role in the pathogenesis of postmenopausal osteoporosis.

1.2.2 Animal Models of Osteoporosis

Research investigating the pathophysiology and treatment of osteoporosis requires an effective animal model of the disease. There are currently two well-established small animal models of osteoporotic bone loss: the immobilized rat model and the ovariectomized (OVX) rat model.

The immobilization model involves the induction of bone loss through disuse of one or more limbs in the rat. This may be achieved by nerve or tendon resections, casting or bandaging of the limb, or suspension of hind limbs. The resulting bone loss is site-specific, with greater bone loss occurring in the weight-bearing lower extremities than the non-weight-bearing upper extremities. The cancellous compartment of the caudal vertebrae loses more bone than the weight-bearing bones, and animals with greater peak bone mass also lose more bone in this model. In addition, the loss of trabecular bone is more rapid than that of cortical due to the greater surface to volume ratio in trabecular bone.

In contrast, the OVX rat model induces bone loss by the bilateral surgical removal of ovaries, which leads to ovarian hormone deficiency. Just as in humans, this results in rapid loss of cancellous bone mass and strength, which is followed by a slower rate of bone loss until steady state is reached. The bone loss occurs by decreased trabecular bone volume with perforative resorption leading to decreased trabecular number and increased trabecular separation, which mimics postmenopausal...
bone loss in humans. There is greater impact on trabecular than cortical bone due to the increased surface to volume ratio in trabecular bone\textsuperscript{51}.

OVX-induced bone loss is site-specific, and affects some regions more rapidly than others. For instance, the proximal tibial metaphysis undergoes significant trabecular bone loss within approximately 14 days following OVX, and 50\% of the bone is lost after approximately 30-60 days\textsuperscript{52,53}. However, in the lumbar vertebrae, significant bone loss is not detected until approximately 2 months following OVX, with 50\% bone loss occurring 6-9 months post-OVX\textsuperscript{54}. In contrast, the effect of ovarian hormone deficiency is not significant in the distal tibial metaphysis of OVX rats\textsuperscript{50}.

In immobilization bone loss, resorption is increased while formation is decreased, leading to negative bone balance. In contrast, OVX leads to overall increase in turnover, with resorption exceeding formation. This also results in negative bone balance, but more closely resembles the postmenopausal bone loss in humans, as the rate of remodeling is also increased in humans as a result of ovarian hormone deficiency. As such, the OVX rat model of postmenopausal osteoporosis will be used in this study.

1.3 Osteoporosis Treatment

1.3.1 Bisphosphonates

Among pharmacological agents for the treatment of osteoporosis, the class of compounds known as bisphosphonates (BP) is the most widely used and the most extensively studied\textsuperscript{55,56}. BPs have been employed in medical interventions for metabolic bone diseases since the late 1960’s, with wide-ranging applications in many conditions such as heterotopic ossification, osteogenesis imperfecta, Paget’s disease, bone loss, hypercalcemia, and skeletal involvement with metastatic cancers\textsuperscript{57}. As treatment for osteoporosis, BPs’ efficacy for inhibiting osteoclastic resorption provides many therapeutic benefits, such as mitigating bone loss, improving bone mineral density (BMD), and reducing incidences of fracture\textsuperscript{58}. In fact, numerous randomized controlled trials to date have demonstrated that, in addition to increasing BMD and decreasing bone loss, BPs also exhibit anti-fracture efficacy in many of the skeletal sites most commonly affected by osteoporosis, including the vertebrae, hip, and non-vertebral sites such as the distal radius\textsuperscript{59-67}.

One of the most striking and unique characteristics of BPs is their ability to target bone mineral. When administered systemically, BPs rapidly and preferentially deposit on bone surfaces, and are removed quickly from blood plasma\textsuperscript{57}. In addition, since BPs are also resistant to biological degradation and cannot be metabolized in the body, the portion that is not adsorbed onto bone
surfaces is excreted unaltered by the kidneys\textsuperscript{68}. Pharmacological studies have demonstrated that the oral bioavailability of BPs is about 1-2%, and of this amount, approximately 40-60% distributes to bone sites, while the rest is excreted unchanged by the kidneys\textsuperscript{69-71}. In fact, researchers have used rats to demonstrate the significant and rapid re-distribution of BP from non-calcified tissues to bone after intravenous administration of the BP alendronate\textsuperscript{71,72}. While non-calcified tissues contained 63% of the initial alendronate dose at 5 minutes post-injection, this amount is dramatically decreased to 5% after 1 hour. In contrast, the alendronate concentration in bone continuously increased after injection, and reached its peak at 1 hour\textsuperscript{71}. Moreover, other studies have used radiolabeling to show that subcutaneous administration of alendronate also results in significant localization of the drug on bone surfaces one day after injection\textsuperscript{73}.

Due to its strong bone-binding affinity, BP forms one of the two pharmacological components in the conjugate drug being examined in this study. To facilitate a better understanding of the conjugate drug’s potential mechanism of action, the molecular structure and biological activity of BPs will now be discussed.

\subsection*{1.3.1.1 Molecular Structure}

Bisphosphonates are similar in structure to the naturally occurring inorganic pyrophosphate, which is involved in inhibiting mineralization in bone. While pyrophosphate consists of two phosphate groups joined by an oxygen atom, BPs replace the central oxygen with a carbon, resulting in a P-C-P structure (Figure 1–11). This substitution determines several important properties of BPs: 1) The P-C-P moiety is resistant to enzymatic hydrolysis, giving BPs their tremendous stability\textsuperscript{70}; 2) the P-C-P component forms a “bone hook” in attaching to the crystalline structure of hydroxyapatite (HA), and is responsible for BPs’ strong affinity in binding to bone mineral\textsuperscript{58}; 3) the P-C-P structure is essential for BPs to be pharmacologically active, as modifications to one or both phosphonate groups can lead to dramatic reductions in the compound’s affinity for bone mineral as well as its anti-resorptive potency\textsuperscript{74-76}; and 4) the P-C-P configuration allows for many variations in chemical structure depending upon the R\textsubscript{1} and R\textsubscript{2} side chains attached to the central carbon, which gives the class of BPs its wide range of biochemical and therapeutic properties\textsuperscript{68}. In fact, even small modifications of these side chains can lead to substantial changes in the BP’s molecular and pharmacological characteristics\textsuperscript{70}. 
To date, many different bisphosphonates have been synthesized (Figure 1–12), and much has been learned about their structure-activity relationship. For instance, the presence of a hydroxyl (OH) group on the \( R_1 \) chain enhances the molecule’s affinity for HA, since it allows the BP to chelate calcium ions by tridentate rather than bidentate binding\(^{68}\). In addition, BPs that contain a nitrogen atom on the \( R_2 \) chain while maintaining the OH on \( R_1 \) show greater anti-resorptive potency, and are collectively known as nitrogen-containing BPs (N-BPs)\(^ {58}\). Moreover, altering the \( R_2 \) chain of N-BPs from alkyl to a heterocyclic configuration further improves their anti-resorptive potency. In fact, the anti-resorptive potency of a compound can be maximized when the nitrogen atom in its \( R_2 \) chain is a critical distance away from its P-C-P group, and is in a particular spatial configuration\(^ {68,77}\). With the understanding of these principles, researchers have been able to design BPs with improved potency and pharmacology.

Figure 1–11. Chemical structure of pyrophosphate and bisphosphonate. The shared oxygen atom in pyrophosphate is replaced by a carbon atom in bisphosphonate, with two side chains \( R_1 \) and \( R_2 \) attached. Modified from reference\(^ {69}\).

Figure 1–12. Structure of BPs. Compounds are classified based on whether they contain a nitrogen atom in their \( R_2 \) chain (N-BP), and N-BPs are further divided based on the configuration of their \( R_2 \) chain. Modified from reference\(^ {58}\).
1.3.1.2 Mechanism of Action

Because BPs selectively bind to bone mineral, their site of adsorption brings them into close proximity of osteoclasts. During bone resorption, acidification of the subcellular space underneath the osteoclast causes dissolution of the HA mineral, while the bone matrix is broken down by proteolytic enzymes such as cathepsin K. BPs that are bound to bone mineral are released into the low pH environment inside the sealing zone\textsuperscript{74,78,79}, and are internalized by the osteoclast via fluid-phase endocytosis\textsuperscript{58}. In fact, the localization of BPs inside intracellular endocytic vesicles has been demonstrated using both fluorescently labeled and radiolabeled BP compounds\textsuperscript{73,80}. Moreover, despite the structural variations among BPs, evidence thus far suggests that their uptake into osteoclasts all occur via a similar endocytic mechanism\textsuperscript{58}.

The various BPs can be classified into two major groups depending on their mode of action once inside the osteoclast. The first group comprises non-nitrogen-containing BPs such as etidronate and clodronate, which closely resemble naturally occurring pyrophosphate in structure. Since pyrophosphates serve as additional phosphate groups in the formation of adenosine triphosphate (ATP) from adenosine monophosphate (AMP)\textsuperscript{68}, these BPs replace pyrophosphate in the formation of ATP, resulting in non-hydrolyzable ATP analogues which then accumulate in the osteoclast and induce osteoclast apoptosis\textsuperscript{81,82}.

The second group comprises nitrogen-containing BPs such as alendronate and risedronate, which interfere with the mevalonate pathway involved in the biosynthesis of essential isoprenoids such as cholesterol\textsuperscript{58,83}. Studies have shown that the main mechanism of action of N-BPs is the inhibition of farnesyl diphosphate synthase (FPPS), which prevents the synthesis of farnesyl diphosphate (FPP) and results in decreased substrates for the production of geranylgeranyl diphosphate (GGPP)\textsuperscript{84-86}. Both FPP and GGPP are isoprenoid metabolites required for the post-translational prenylation of small GTPases such as Ras, Rho, Rab, and Rac, which are modified via the addition of a lipid prenyl group\textsuperscript{58,68}. Since this prenyl group allows small GTPases to anchor to the cell membrane and may also be involved in protein-protein interactions\textsuperscript{87}, prenylation is essential to the correct functioning of these proteins. Small GTPases are important signaling proteins involved in regulating many aspects of osteoclast function, including cell morphology, cytoskeletal arrangement, membrane ruffling, vesicular trafficking, and apoptosis\textsuperscript{88-91}. By interfering with the proper function of GTPases, N-BPs cause impaired osteoclast function and increased apoptosis\textsuperscript{92-94}.

In addition to their effects on osteoclasts, early studies in the 1970’s demonstrated that BPs also impede mineralization. In particular, BPs were found to inhibit the precipitation of calcium
phosphate from solution, slow the structural conversion from amorphous to crystalline HA, and hinder both the aggregation and dissolution of HA crystals\textsuperscript{41-43}. These effects were more pronounced at high BP concentrations.

Since osteoporosis therapy at the time involved non-nitrogen-containing BPs with relatively lower potency, treatment typically required higher doses to achieve anti-resorptive efficacy, leading to clinical concerns associate with impaired mineralization\textsuperscript{58}. However, with the advent of more potent, nitrogen-containing BPs such as alendronate and zoledronate, the doses that reach anti-resorptive efficacy are now substantially lower than that required to inhibit mineralization\textsuperscript{68}. Moreover, since osteoporosis treatment today most commonly involves the more potent N-BPs, the detrimental effect of BPs on mineralization no longer constitutes a major clinical concern\textsuperscript{69}.

1.3.1.3 Binding to Hydroxyapatite

Many BPs are currently in clinical use, and vary widely in anti-resorptive potency (Table 1-1). These compounds differ not only in their ability to inhibit FPPS, but also in their binding affinity to HA. Until recently, it was widely believed that BP binding to HA was solely determined by the P-C-P moiety, with added effects from the OH group in R\textsubscript{1} when applicable\textsuperscript{58}. However, some studies found that BPs with identical configurations in their P-C-P and R\textsubscript{1} components still exhibit differences in binding affinity to HA, suggesting that additional factors such as three-dimensional (3D) conformation and molecular charge may also contribute to their binding\textsuperscript{56,95-97}.

Table 1-1. Chemical characteristics of various BPs in clinical use. Anti-resorptive potency is measured relative to that of etidronate. Data from references\textsuperscript{56,70,98}.

<table>
<thead>
<tr>
<th>N-BP</th>
<th>Compound</th>
<th>Proprietary Name</th>
<th>Anti-Resorptive Potency</th>
<th>Adsorption Constant for Binding to HA (K\textsubscript{L}/10\textsuperscript{6} L mol\textsuperscript{-1})</th>
<th>Inhibition of FPPS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Etidronate</td>
<td>Didronel</td>
<td>1</td>
<td>1.19</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Clodronate</td>
<td></td>
<td>10</td>
<td>0.72</td>
<td>17</td>
</tr>
<tr>
<td>✓</td>
<td>Pamidronate</td>
<td>Aredia</td>
<td>100</td>
<td>2.94</td>
<td>61</td>
</tr>
<tr>
<td>✓</td>
<td>Alendronate</td>
<td>Fosamax</td>
<td>100 – 1000</td>
<td>2.36</td>
<td>80</td>
</tr>
<tr>
<td>✓</td>
<td>Ibandronate</td>
<td>Boniva</td>
<td>1000 – 10,000</td>
<td>3.47</td>
<td>99</td>
</tr>
<tr>
<td>✓</td>
<td>Risedronate</td>
<td>Actonel</td>
<td>1000 – 10,000</td>
<td>4.19</td>
<td>94</td>
</tr>
<tr>
<td>✓</td>
<td>Zoledronate</td>
<td>Zometa</td>
<td>&gt;10,000</td>
<td>3.47</td>
<td>99</td>
</tr>
</tbody>
</table>

To investigate HA binding, studies have used 3D computational modeling to examine potential interactions between the nitrogen moiety of N-BPs and the crystalline structure of HA\textsuperscript{99,100}. After accounting for the known interactions associated with the P-C-P “bone hook” and the OH domain of the R\textsubscript{1} chain, these studies revealed that some N-BPs can also form N-H-O hydrogen bonds at two sites.
on the HA surface. Moreover, these hydrogen bonds require an N-H-O angle of ≥125° and an N-O distance of approximately 3 Å for optimal binding.

These results provide new insight into the variations in BPs’ binding affinity to HA. As shown in Figure 1–13, alendronate and zoledronate both have N-H-O angles of >125° and are able to form hydrogen bonds, leading to their greater adsorption constant to HA relative to other N-BPs (Table 1–1). In contrast, risedronate does not meet the 3D conformational requirements for hydrogen bonding and can only form weak electrostatic attractions, while the structure of ibandronate exhibits steric hindrance that interferes with hydrogen bonding. These structural properties thus lead to lower HA binding affinities for risedronate and ibandronate.

![Image](image.png)

**Figure 1–13.** 3D computational models of different N-BPs showing their N-H-O bond angles. A) Schematic illustration of the location of N-H-O hydrogen bond in binding of BP to hydroxyapatite (HAP). Interactions are shown for both the hydrogen bond and the P-C-P and R1 components. B) 3D models of 4 major N-BPs currently in clinical use. Alendronate and zoledronate have N-H-O bond angles of >125° and can form hydrogen bonds with HA, while risedronate has insufficient bond angle for hydrogen bond formation. Ibandronate structure results in steric hindrance, which may interfere with hydrogen bonding. Reproduced from reference30.

### 1.3.1.4 Disadvantages

Despite the well-established efficacy of BPs for mitigating bone loss and improving bone strength, these drugs do have significant drawbacks. Due to the coupling between resorption and formation in bone remodeling, the inhibition of resorption by BPs also results in the eventual suppression of formation, leading to decreased bone turnover. Since remodeling is essential to maintaining healthy bone, this reduced turnover compromises bone quality.

The suppression of bone turnover under BP treatment has been well-documented clinically using biomarkers101, some of which are shown in Table 1–2. Resorption markers decline rapidly within 3 months of initiating treatment, while formation markers decrease more gradually over 6 months. Both resorption and formation markers remain dose-dependently decreased for the duration of treatment102-104.
In addition to biomarkers, histomorphometric analysis has also been employed to examine the tissue-level effects of BP treatment on postmenopausal women. One study found that, relative to normal young women, the percent mineralizing surface in patients treated with alendronate was drastically reduced by 92% after 2 years, and by 87% after 3 years. Moreover, another study found that, after 6.5 years of BP treatment, mineralizing surface was dramatically decreased in all patients, and one third of the women showed a mineralizing surface of zero. These results indicate that, not only do BPs inhibit resorption, but they also significantly suppress formation of new bone.

Table 1–2. Commonly used bone turnover markers. Modified from reference 101,107-110.

<table>
<thead>
<tr>
<th>Formation Markers</th>
<th>Resorption Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum osteocalcin (OC)</td>
<td>Urinary hydroxyproline (HYP)</td>
</tr>
<tr>
<td>Serum bone-specific alkaline phosphatase (BSAP)</td>
<td>Urinary deoxypyridinoline (DPD)</td>
</tr>
<tr>
<td>Serum procollagen type I C-terminal propeptide (P1CP)</td>
<td>Urinary collagen type I cross-linked N-telopeptide (NTX)</td>
</tr>
<tr>
<td>Serum procollagen type I N-terminal propeptide (P1NP)</td>
<td>Serum collagen type I cross-linked C-telopeptide (CTX)</td>
</tr>
</tbody>
</table>

Since bone remodeling is required to repair microdamage and maintain mechanical properties in bone, researchers have also investigated the impact of suppressed turnover on bone quality. Studies using dogs showed that, after daily BP treatment for 12 months at high dose (orally administered 1.0 mg/kg/day alendronate and 0.5 mg/kg/day risedronate), there was an accumulation of microdamage and reduction of toughness in both the cortical and trabecular bone of the animals, which compromised the bone’s capacity for absorbing energy. Moreover, concerns have also been raised regarding the extreme suppression of bone turnover in human patients treated with BPs such as alendronate, as both formation and resorption were severely diminished and, in some cases, absent. This seems to be implicated in serious complications such as atypical fracture, which is seen in some patients.

1.3.2 Parathyroid Hormone

Parathyroid hormone (PTH) is a single-chain polypeptide hormone with 84 amino acids, and is produced almost exclusively by the parathyroid gland. The principal physiological role of PTH is the maintenance of blood calcium levels, as the calcium sensing receptors on chief cells in the parathyroid gland detect plasma calcium levels, and mediates PTH production accordingly. There is a negative-feedback loop in which PTH secretion is increased with decreasing calcium concentration, but decreased as blood calcium increases. In addition, PTH also stimulates renal reabsorption of calcium and excretion of phosphate, as well as increasing the renal production of the active vitamin D metabolite 1,25(OH)2 vitamin D, which also stimulates renal reabsorption of calcium.
Studies have shown that intermittent administration of human parathyroid hormone (hPTH) results in net bone gain\textsuperscript{115,116}. The anabolic effects of intermittent PTH have been demonstrated using both rats and monkeys\textsuperscript{115-117}. Effects of intermittent PTH include increased cancellous bone volume and trabecular thickness in OVX rats\textsuperscript{118,119}, enhanced osteogenic capacity of the bone marrow\textsuperscript{120}, as well as increased cortical porosity\textsuperscript{117}.

The ability of intermittent PTH to promote new bone formation enables restoration of bone mass and microarchitecture, and is being used as an anabolic therapy for osteoporosis. Various human trials have demonstrated promising anabolic effects of intermittent hPTH. For instance, one study showed a dose-dependent increase in aBMD of the spine after 1 year of daily subcutaneous hPTH (1-84)\textsuperscript{121}, while another study demonstrated decreased risk of vertebral fractures, but not non-vertebral fractures, after 18 months of daily subcutaneous hPTH (1-84) in 2532 postmenopausal women\textsuperscript{104}.

Moreover, another study also employed a treatment regime consisting of 1 month of daily subcutaneous injections followed by 11 months of weekly subcutaneous injections\textsuperscript{122}. After 12 months of treatment, lumbar spine aBMD was increased by 2.1% in the PTH-treated groups compared to control, while vertebral trabecular volumetric BMD increased by 3.8% as a result of PTH treatment.

Along with the parathyroid hormone related hormone (PTHrP) which binds to the same target receptors, hPTH binds to specific G-protein-coupled receptors on the cell surface known as the type I PTH/PTHrP receptor. It stimulates activation of adenylate cyclase and phospholipases, leading to increased levels of intracellular cyclic adenosine monophosphate (cAMP) and calcium\textsuperscript{123}.

The significant anabolic efficacy of hPTH led to its approval by the FDA in 2002 as treatment for osteoporosis\textsuperscript{124}. It is marketed as Teriparatide, and is the only anabolic drug currently available in the market\textsuperscript{125}. However, despite its effects in promoting bone formation, there are also potential complications with hPTH therapy. A study examining the effects of long-term treatment using daily subcutaneous injections of hPTH in rats showed that, while there were substantial increases in bone mass due to treatment, there was also a dose-dependent increase in the development of bone cancers such as osteosarcoma, osteoma, and osteoblastoma\textsuperscript{126}. In addition, a follow-up study was conducted by the same group to investigate the effects of dose and treatment duration on the development of bone cancer\textsuperscript{127}. Results showed that treatment using daily subcutaneous hPTH at 30 μg/kg for 20 or 24 months both induced significant increases in bone cancer, such as osteosarcoma, osteoma, and osteoblastoma. These findings highlight the serious potential complications that may arise from long-term treatment using high dose hPTH.
The treatment of human postmenopausal osteoporosis using hPTH typically employs a treatment regime consisting of subcutaneous injections of hPTH at doses of 100 μg per injection, as it has been shown that anabolic efficacy cannot be achieved at doses below this level\textsuperscript{121}. In fact, this dose was used in all the human studies mentioned earlier which reported anabolic effects of hPTH. Although the per kilogram dosing is different between rats and humans, and no incidences of bone cancer have been reported in humans to date, the hPTH-induced bone cancer in rats still constitutes a warning regarding the possibility of cancer as a result of hPTH therapy.

Although short-term hPTH also exhibits anabolic effects \textit{in vivo}, studies have shown that the withdrawal of treatment leads to loss of the added bone, which renders treatment redundant\textsuperscript{128}. Thus, better alternatives are needed as anabolic therapy in osteoporosis.

\subsection{1.3.3 Other}

In addition to bisphosphonate and PTH, alternative approaches for treating osteoporosis include hormone replacement therapy (HRT) with estrogen and progestin\textsuperscript{129}, selective estrogen receptor modulators (SERM)\textsuperscript{130}, and cathepsin K inhibitors\textsuperscript{131-134}. HRT aims to replace lost endogenous ovarian hormones with exogenous analogues, and has been shown to increase BMD and decrease fracture risk\textsuperscript{129}. However, its use is also hampered by serious side effects, such as increased risks for heart disease, stroke, breast cancer, and venous thromboembolism\textsuperscript{129}. Similarly, SERMs such as Raloxifene selectively activate or deactivate estrogen receptors on cell surfaces, and induce conformational changes in estrogen receptors. This leads to activation of downstream estrogenic pathways in cells\textsuperscript{135}, but its use is also hampered by serious adverse effects, such as deep vein thrombosis and pulmonary embolism\textsuperscript{135}.

Unlike the other two approaches that focus on estrogen, cathepsin K inhibitors such as Odanacatib and ONO-5334 suppress action of the cathepsin K enzyme, which is one of the main enzymes involved in the digestion of bone matrix during osteoclastic resorption. This approach has been shown to improve aBMD of the lumbar spine and total hip\textsuperscript{136}, but cannot stimulate formation of new bone. An ideal therapy for osteoporosis should promote bone formation to facilitate the recovery of lost bone, while also exhibiting few side effects.

\subsection{1.3.4 Combination Therapy}

Given the separate anabolic and anti-resorptive effects of various treatments, studies have investigated the potential of combination therapy in improving treatment outcome. These include both concurrent and sequential study designs, and have been used in both animals and humans.
The use of hPTH with anti-resorptive agents has shown mixed results in human clinical trials. Black et al. conducted a study in 238 women with previously untreated postmenopausal osteoporosis, and investigated the effects of 12-month daily treatment via subcutaneous hPTH (100 μg) and oral alendronate (10 mg) alone or in combination. The aBMD and volumetric BMD (vBMD) of the total hip and spine were measured using DEXA and quantitative computed tomography (QCT), respectively, and results for combination therapy and hPTH alone were compared to that of alendronate. Areal BMD of the hip was 1.9% increased in the combination group, but did not increase for hPTH alone. However, vBMD of the hip did not increase in either hPTH or combination groups relative to alendronate. There were also comparable increases in aBMD and vBMD of the spine between the groups, while vBMD of the vertebral trabecular bone showed significantly greater improvement in hPTH alone compared to the combination group (25.5% vs 12.6%). Combination treatment also did not change cortical vBMD in the hip, while hPTH alone reduced hip vBMD by 1.7%. These results do not provide clear evidence for additive or synergistic effects in the use of combination therapy using hPTH and alendronate, as the effects of hPTH seems to have been blunted by simultaneous use of alendronate in vertebral trabecular bone, but this is not reflected in other regions.

Finkelstein et al. also conducted a combination study using daily subcutaneous hPTH (40 μg) and daily oral alendronate (10 mg) in men. Alendronate treatment was given for 30 months, while hPTH was begun at month 6. Areal BMD of the lumbar spine, proximal femur, and radial shaft, as well as the total body was measured every 6 months using DEXA, and the outcome of hPTH alone and in combination were compared with that of alendronate alone. Results showed that aBMD of the lumbar spine was increased in the combination group compared to alendronate treatment alone, and the aBMD was the greatest under hPTH treatment alone. In addition, the aBMD of the femoral neck was also significantly increased in the hPTH treatment alone compared to both other groups. Thus, the findings of this study also do not support the combined use of hPTH and alendronate.

More recent efforts have been focused on sequential therapy using hPTH and bisphosphonates. Black et al. conducted another study to examine the effects of one year of daily subcutaneous hPTH (100 μg) followed by one year of alendronate therapy (10 mg daily, Fosamax), and measured aBMD and vBMD of the hip and spine. In particular, patients were divided into 4 different groups: 1) hPTH for 1 year followed by placebo for 1 year (hPTH-placebo); 2) hPTH for 1 year followed by alendronate for 1 year (hPTH-ALN); 3) combination treatment for 1 year followed by alendronate for 1 year (combination-ALN); and 4) alendronate for 2 years. Results after 2 years showed that hPTH-ALN led to significantly increased total spine aBMD compared to all other groups, while that of the
total hip was increased in all groups compared to hPTH-placebo. In addition, the vBMD of the vertebral trabecular bone showed 31% increase relative to baseline, while the increase was only 14% in the hPTH-placebo group. Results for vBMD also showed that there were no significant changes in any of the groups for cortical bone at the hip. For subjects receiving placebo in year 2, there was substantial loss in vBMD of vertebral trabecular bone, which is not surprising given the transient effects of hPTH-induced bone formation. This study showed improved outcome with alendronate therapy following hPTH, which exceeded the results for combination-ALN.

Although the study using sequential therapy shows promising results, this approach may have limited long-term potential. Since the effects of alendronate are based on the total accumulated dose rather than the quantity of drug in each administration\textsuperscript{58}, the accumulation of alendronate in bone over time as a result of long-term use may lead to oversuppression of bone turnover. However, if alendronate is withdrawn, it is possible that the hPTH-induced bone formation will be lost over time, which would be similar to results in the hPTH-placebo group. Thus, despite the successful maintenance of newly formed bone with the use of sequential hPTH and alendronate therapy, the long-term outcome remains uncertain.

1.4 Prostaglandin E\textsubscript{2}  
Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) is an alternative compound to PTH that has also been shown to exhibit bone-anabolic effects \textit{in vivo}. Prostaglandins (PG) are members of the prostanoid class of messenger molecules, and are involved in regulation of various physiological functions such as contraction and relaxation of smooth muscles, secretion and motility in the gastrointestinal tract, as well as transport of water and ions in the kidney\textsuperscript{140}. In contrast to hormones such as PTH that are produced in discrete glands in the body and secreted into the bloodstream to target other tissues, PGs are produced throughout the body and act locally within their site of secretion. They are thus involved in autocrine and paracrine signaling, and have short metabolic half-lives\textsuperscript{141}.

1.4.1 Molecular Structure and Biosynthesis  
Prostaglandins consist of a 20-carbon chain with one cyclopentane ring attached to two side chains known as $\alpha$ and $\omega$, and are classified into different types depending on structural variations in the cyclopentane ring (Figure 1–14). Among these, three types do not occur naturally and are only derived artificially during extraction processes (PG types A, B, C), while others are chemically unstable under physiological conditions (PG types G, H, I). The remaining prostaglandins (PG types D, E, F), though chemically stable, are metabolized quickly\textsuperscript{140}.
In addition to classification by type, prostanoids are further divided into three series according to the number of double bonds in their side chains. The series 1 PGs have one double bond, the series 2 PGs have two double bonds, and the series 3 PGs have three double bonds.

All prostanoids are derived from fatty acids in response to physiological and pathological stimuli. Most PGs are produced from arachidonic acid, which is the most common precursor fatty acid in mammals. Upon initiation of PG biosynthesis, arachidonic acid is released from membrane phospholipids by the phospholipase A₂ enzyme. It is then sequentially converted to PGG₂ and PGH₂ by the cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2) enzymes, with PGH₂ then undergoing synthase-driven modifications to form the final PG products (Figure 1–15).

Figure 1–14. Structures of prostanoids. Only the series 2 PGs are depicted. PGG₂ and PGH₂ are unstable intermediates. TXA₂ denotes thromboxane, which is another member of the prostanoid family. Reproduced from Narumiya et al.

Figure 1–15. Biosynthesis pathway for prostanoids. Phospholipase A₂ drives the release of arachidonic acid from membrane phospholipids, then cyclooxygenase (COX-1 and COX-2) catalyzes the conversion of arachidonic acid into PGG₂ followed by PGH₂. Finally, PGH₂ is modified to form different types of PG products by various synthases.
1.4.2 Biological Functions

Among the various PG products, PGE₂ is the most widely produced in the human body, and has the most significant impact on tissues. It is a key regulator of many biological functions essential to survival, such as the inflammatory response and associated sensitivity to pain, dilation and contraction of the vasculature, fetal and newborn respiration, and contraction and relaxation of the uterus.

PGE₂ binds to specific cell surface receptors known as G-protein-coupled receptors, and activates intracellular signaling and gene transcription. This involves 4 receptor subtypes EP1-4, which are present in a variety of tissues in the body. Since the signal transduction pathways differ for these receptors, their biological distribution and ligand-binding affinity are critical to the particular actions of PGE₂ at any tissue site. For instance, the EP3 receptor is widely expressed in the CNS, while EP1 is expressed in several organs such as the kidney, lung, and stomach. In fact, the gastrointestinal tract has been shown to contain multiple EP receptors, with studies suggesting that EP1 is involved in the local movement and folding of the stomach mucosa while EP3 and EP4 are involved in secretion of acid and mucus. Similarly, the uterus has also been shown to express all 4 EP receptors, which mediate the effects of PGE₂ in pregnancy.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>G Protein</th>
<th>Intracellular Effects</th>
<th>Receptor Expression</th>
<th>Physiological Impact</th>
</tr>
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<tr>
<td>EP1</td>
<td>G₉⁺</td>
<td>Ca²⁺ mobilization</td>
<td>Lung, gastrointestinal tract, kidney, cardiovascular system, CNS, PNS, uterus</td>
<td>Cardiovascular homeostasis; neurovascular transmission; hypertension; inflammation; inflammatory pain and hyperalgesia; neuropathic pain; movement of gastric mucosa</td>
</tr>
<tr>
<td>EP2</td>
<td>G₅</td>
<td>↑ cAMP</td>
<td>Uterus, lung, respiratory tract, kidney, cardiovascular system, CNS</td>
<td>Uterine distension during labor; fertilization and implantation; labor suppression; bronchodilation; relaxation of vascular smooth muscle; neuronal excitation and depression; pain transmission; pro-inflammatory neurotoxicity;</td>
</tr>
<tr>
<td>EP3</td>
<td>G₅</td>
<td>↓ cAMP</td>
<td>CNS (abundant in sensory neurons), cardiovascular system, reproductive system, kidney, bladder, gastrointestinal tract</td>
<td>Hyperalgesia; febrile response; sympathetic nerve activity leading to tachycardia and hypertension; platelet aggregation; vasoconstriction; bladder contractions;</td>
</tr>
<tr>
<td>EP4</td>
<td>G₅</td>
<td>↑ cAMP</td>
<td>Bone, cardiovascular system, gastrointestinal tract, cervix</td>
<td>Bone formation and resorption; vasodilation; cardiac hypertrophy; cervical relaxation at parturition; acid secretion;</td>
</tr>
</tbody>
</table>

Table 1–3. Distribution of EP receptors and their physiological impact. *Though some evidence suggests that EP1 receptor may couple to Gq, this has not been conclusively established. Data from reference 151.

To date, studies investigating the PGE₂ mechanism of action have identified two main intracellular secondary messenger systems. EP2 and EP4 receptors have been shown to activate adenylate cyclase and increase the intracellular cyclic adenosine monophosphate (cAMP), while
EP1 activates the phospholipase C (PLC) pathway involved in calcium mobilization\textsuperscript{148,151}. In contrast, depending on the alternatively spliced isoform, EP3 can either lower the intracellular cAMP levels or activate the PLC\textsuperscript{156-158}.

1.4.3 Effects on Bone

PGE\textsubscript{2} is produced in bone tissue mainly by osteoblasts\textsuperscript{159,160}, and early studies using organ cultures showed that it stimulates bone resorption in vitro\textsuperscript{160-164}. Akatsu et al co-cultured mouse marrow mononuclear cells with PGE\textsubscript{2} for 8 days, and found that higher concentrations of PGE\textsubscript{2} produced greater numbers of cells that exhibit osteoclast-like characteristics, such as positive staining for TRAP and formation of ruffled borders\textsuperscript{165}. Moreover, PGE\textsubscript{2} has also been shown to promote bone resorption in fetal rat long bone cultures\textsuperscript{166}, and is involved in the mechanism of osteoclastogenesis induced by both IL-1 and IL-6\textsuperscript{167,168}. However, despite these catabolic actions in vitro, more recent studies have shown that the effect of PGE\textsubscript{2} in vivo is predominantly anabolic\textsuperscript{169}.

To date, numerous studies have demonstrated the anabolic effects of PGE\textsubscript{2} in intact animals. One study utilized local intraosseous infusion via a cannula implanted into the proximal tibial metaphysis of young male rats\textsuperscript{170}. Compared to the vehicle-treated contralateral limbs that served as control, two weeks of daily PGE\textsubscript{2} administration stimulated new woven bone formation, and resulted in significant and dose-dependent increase in metaphyseal trabecular bone volume. In addition, systemic administration of PGE\textsubscript{2} via subcutaneous injection in growing rats also led to increased trabecular bone volume\textsuperscript{171} as well as increased number of osteoblasts on bone surfaces\textsuperscript{41,43}.

Given PGE\textsubscript{2} ’s effects in promoting bone formation in healthy animals, other studies have investigated the potential of PGE\textsubscript{2} as an anabolic treatment for osteoporosis. Using a curative experimental design, these studies typically subject rats to bilateral OVX followed by a bone loss period before beginning PGE\textsubscript{2} treatment. For instance, Mori et al subjected 3-month-old rats to OVX, then started PGE\textsubscript{2} treatment 4 months post surgery. After 30 days of daily subcutaneous injections, they showed that PGE\textsubscript{2} at 3 and 6 mg/kg doses increased trabecular bone volume in both the lumbar vertebrae and proximal tibial metaphysis, and stimulated formation of new woven trabeculae with elevated bone turnover\textsuperscript{172,173}. In fact, PGE\textsubscript{2} at 6 mg/kg completely restored trabecular bone volume in both regions to sham levels\textsuperscript{173}. Moreover, PGE\textsubscript{2} treatment also activated intracortical remodeling and enhanced cortical bone mass in the tibial shaft in OVX osteopenic rats\textsuperscript{46}. Not only did PGE\textsubscript{2} promote bone formation on the periosteal and endosteal surfaces of the tibial shaft, but it also stimulated formation of woven bone within the endocortical marrow region\textsuperscript{174,175}. Furthermore, these anabolic
effects of PGE$_2$ have been shown to translate into improved mechanical strength in both the distal femoral metaphysis and the lumbar vertebrae of intact and osteopenic rats$^{176}$.

In addition to its anabolic effects in curative experiments, other studies have demonstrated that PGE$_2$ treatment can also protect against bone loss due to OVX-induced ovarian hormone deficiency. When PGE$_2$ treatment was commenced immediately following OVX and continued for 90 days, daily subcutaneous injection of low dose PGE$_2$ at 1 mg/kg successfully prevented OVX-induced cancellous bone loss in the proximal tibial metaphysis, while high dose PGE$_2$ at 6 mg/kg also increased trabecular bone volume and decreased trabecular separation compared to sham-operated controls$^{177}$. In addition, immediate administration of PGE$_2$ following OVX has also been shown to increase cortical bone mass in the tibial shaft, and prevent OVX-induced changes such as increased marrow area and endosteal eroded perimeter$^{178}$.

Despite PGE$_2$ ’s bone-anabolic effects, its therapeutic potential in osteoporosis is hampered by significant drawbacks. The broad distribution of EP receptors throughout the body leads to multisystem adverse effects when PGE$_2$ is administered systemically. These side effects include headache, lethargy, uterine contraction, flushing, gastrointestinal disturbance (e.g. abdominal pain, nausea, vomiting, and diarrhea), and cardiovascular symptoms (e.g. hypotension, peripheral edema),$^{179-182}$ Such serious adverse reactions preclude the clinical application of PGE$_2$ as a therapeutic agent for osteoporosis.

**1.4.4 Mechanism of Action on Bone**

In order to exploit PGE$_2$ ’s therapeutic potential for osteoporosis while mitigating other unwanted effects, it is crucial to better understand its mechanism of action on bone. Experiments using calvarial and bone marrow cultures have shown that PGE$_2$ exerts numerous effects on osteoblasts and their precursors: 1) PGE$_2$ increases osteogenic potential of the bone marrow by promoting transition from nonadherent to adherent osteoblast precursors$^{183-185}$; 2) PGE$_2$ stimulates nodule formation and elevates alkaline phosphatase (ALP) activity, which indicates increased osteoblast differentiation$^{186,187}$; 3) PGE$_2$ treatment suppresses apoptosis in rat bone marrow stromal cells as well as osteoblast cell lines$^{183,188}$; 4) PGE$_2$ exerts its intracellular effects by increasing cAMP and elevating levels of protein kinase A (PKA)$^{154,189}$; and 5) PGE$_2$ induces early-response genes such as $c$-fos, $c$-jun, and $egr$-1 in rat bone tissue$^{190}$. In fact, *in-situ* hybridization experiments have shown that, as early as 15 minutes after subcutaneous injection of 6 mg/kg PGE$_2$, these early response genes become elevated in both the calvariae and proximal tibial metaphysis of rats$^{190}$. 

27
In addition to its effects on formation, PGE₂ also impacts resorption. It has been shown to promote osteoclastogenesis via stimulation of RANKL production by osteoblasts and decreasing production of OPG\textsuperscript{191-194}. Moreover, PGE₂ has also been shown to inhibit the bone-resorbing activity of mature, multinucleated osteoclasts in vitro\textsuperscript{195}. In fact, the expression of EP2 and EP4 receptors on bone marrow macrophage osteoclast precursors was found to be downregulated during RANKL-induced osteoclast differentiation\textsuperscript{196}.

In light of these insights, studies have been conducted to identify which EP receptor is involved in mediating the PGE₂ effects on bone. For instance, Miyaura et al\textsuperscript{197} cultured cells from calvariae of wild type and knockout mice, and examined PGE₂-induced resorbing activity by measuring the calcium content in culture media. They found that, among mice lacking each of the four EP receptors (EP1\textsuperscript{−/−}, EP2\textsuperscript{−/−}, EP3\textsuperscript{−/−}, EP4\textsuperscript{−/−}), only EP4\textsuperscript{−/−} mice exhibited impaired resorbing activity under PGE₂ treatment, while other knockout mice were comparable to wild type control. In addition, another study demonstrated that, when calvarial cells from wild type mice were cultured with agonists specific to each of the four EP receptors, the stimulatory effect on resorbing activity was highest for the EP4 agonist and was minimal for EP2, while the EP1 and EP3 agonists failed to induce bone resorption\textsuperscript{159}. These results thus suggest that PGE₂-induced resorbing activity is mediated mainly by the EP4 receptor.

Results from other in vitro studies indicate that the EP4 receptor is also involved in PGE₂-induced osteoclastogenesis. For example, Ono et al\textsuperscript{198} used agonists of different EP receptors, and showed that agonists of EP1 and EP3 receptors did not induce formation of TRAP-positive, multinucleated osteoclasts in culture. In contrast, EP4 agonist significantly stimulated osteoclastogenesis, while the EP2 agonist showed very little stimulatory response. In addition, another study examined co-cultures of osteoclast precursors with primary osteoblasts, and showed that the expression of EP4 receptors in the primary osteoblasts is crucial to PGE₂-induced osteoclastogenesis\textsuperscript{199}.

In identifying the EP receptor involved in PGE₂-stimulated bone formation, Yoshida et al conducted a seminal study involving both knockout mice and specific EP receptor agonists\textsuperscript{200}. In the first experiment, they locally infused PGE₂ in the femurs of mice lacking each of the four EP receptors (EP1\textsuperscript{−/−}, EP2\textsuperscript{−/−}, EP3\textsuperscript{−/−}, EP4\textsuperscript{−/−}), and showed that PGE₂ stimulated bone formation in all knockout mice except EP4\textsuperscript{−/−}. Next, they locally infused agonists specific to each EP receptor in the femurs of wild type mice, and found that bone formation at the site of infusion occurred only in mice treated with the EP4 receptor agonist. In addition, the authors also demonstrated that PGE₂-induced mineralized nodule formation was mediated by the EP4 receptor, as no mineralized nodules were formed when bone
marrow cells from EP4<sup>-/-</sup> mice were treated with PGE<sub>2</sub>. In this study, all agonists were highly selective for their corresponding EP receptors, as binding affinity between each agonist and the matching receptor was at least 100 times higher relative to all other EP receptors expressed in the same cell (Table 1–4).

In the same study, Yoshida et al also investigated the effect of subcutaneous injection of the EP4 agonist to OVX rats in preventing OVX-induced bone loss. After treating rats 3 times per day for 70 days, they found that the EP4 agonist dose-dependently prevented OVX-induced bone loss in the femoral metaphysis relative to vehicle-treated controls. Moreover, EP4 agonist treatment also dose-dependently increased trabecular bone density as well as mechanical strength compared to vehicle-treated OVX controls. Together, these results provide compelling evidence that PGE<sub>2</sub> exerts its anabolic effects on bone via the EP4 receptor.

### Table 1–4. Inhibitory constant \( K_i \) of ligand-binding for the different EP receptor agonists used by Yoshida et al. \( K_i \) is inversely proportional to binding affinity. Ligand binding was measured between each agonist and the respective EP receptors expressed in Chinese hamster cells. PGE<sub>2</sub> binds to all receptors with low \( K_i \), while \( K_i \) is lowest only between each agonist and its corresponding EP receptor. Values expressed in \( \mu M \), referenced from 159.

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<tr>
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<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>0.038</td>
<td>0.005</td>
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<td>&gt;10</td>
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<td>EP2 Agonist</td>
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<tr>
<td>EP3 Agonist</td>
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<tr>
<td>EP4 Agonist</td>
<td>&gt;10</td>
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</table>

In addition to work using EP4 agonists, others studies have examined the effects of EP4 receptor antagonists \( \textit{in vitro} \) and \( \textit{in vivo} \). The antagonist used in these studies is highly selective in binding with the EP4 receptor \( (K_i = 0.032 \ \mu M) \), and is 200-2000 times less selective for the other EP receptors \( (K_i \text{[EP1]} = 61 \ \mu M, K_i \text{[EP2]} = 58 \ \mu M, \text{and } K_i \text{[EP3]} = 7.25 \ \mu M) \). \( \textit{In vitro} \) experiments showed that co-treating osteoblasts with PGE<sub>2</sub> and the EP4 antagonist resulted in suppression of numerous PGE<sub>2</sub>-induced effects, including increase in intracellular cAMP<sup>201,202</sup> and bone nodule formation<sup>202</sup>, increased percent of adherent cells<sup>202</sup>, and suppression of osteoblast apoptosis<sup>201</sup>. Further experiments also showed that the same antagonist also suppressed PGE<sub>2</sub>-induced osteoclastogenesis \( \textit{in vitro} \)<sup>203</sup>.

The \( \textit{in vivo} \) studies using the EP4 antagonist examined the effect of pre-treating rats with the antagonist 45 minutes prior to each injection of PGE<sub>2</sub>. In 6-week old rats pre-treated with the EP4 antagonist in a 12-day study, results showed that the antagonist prevented PGE<sub>2</sub>-induced increases in both mineralized surface as well as trabecular bone volume in the proximal tibial metaphysis<sup>201</sup>. In addition, a similar method was used to treat 4-week old rats in a 2-week study, and results showed
that the EP4 antagonist significantly decreased the cancellous bone area in the proximal tibial metaphysis compared to PGE$_2$ treatment alone. Furthermore, when bone marrow stromal cells from the treated animals were cultured, they found that percent-mineralized area is also decreased due to EP4 antagonist, indicating the prevention of PGE$_2$-induced nodule formation. Together, results from these studies demonstrate that antagonists of the EP4 receptor suppress PGE$_2$ effects \textit{in vitro} and \textit{in vivo}.

Since both EP2 and EP4 receptors activate the downstream cAMP/PKA pathway\textsuperscript{182}, some studies have suggested that the EP2 receptor also plays a role in mediating PGE$_2$-induced effects on bone\textsuperscript{204-206}. However, much evidence exists that attribute greater importance to the EP4 receptor: 1) Primary cultures of human osteoblasts and osteoclasts express mRNA for the EP4 receptor but not EP2\textsuperscript{207,208}; 2) the EP4 receptor, but not EP2, is expressed in murine osteoblastic cell lines, rabbit primary osteoblasts, as well as isolated mature osteoclasts in rabbits\textsuperscript{146,195}; 3) the EP4 receptor is abundantly expressed in adult rat bone tissue and is positively self-regulated by its ligand PGE$_2$ \textsuperscript{146}; 4) EP4$^{-/-}$ mice shows greater suppression of response to PGE$_2$ than EP2$^{-/-}$ mice\textsuperscript{194}, and 5) aged EP4 knockout mice exhibit osteopenia and impaired fracture healing, indicating that the EP4 receptor also mediates anabolic effects under physiological conditions\textsuperscript{209}. Therefore, the current literature presents strong evidence that the effects of PGE$_2$ on bone are mediated primarily through the EP4 receptor.

\subsection*{1.5 Synthetic EP4 Receptor Agonists}

Since PGE$_2$ has been shown to exert its anabolic effects via the EP4 receptor, many highly selective synthetic EP4 receptor agonists have been developed in the past decade that mimic the effects of PGE$_2$ on bone (Table 1-5)\textsuperscript{159,206,210-212}. Further research using these agonist compounds has demonstrated their strong anabolic effects \textit{in vitro} and \textit{in vivo}. For instance, treatment with one of the synthetic EP4 agonists increases bone mineral content and BMD in the distal femoral metaphysis of OVX rats\textsuperscript{210}. In addition, the agonist CP432 has been shown to promote trabecular bone formation in the lumbar vertebrae of aged OVX rats, which not only restored the bone volume lost due to OVX, but also increased the mechanical strength\textsuperscript{213}. Moreover, several studies using the agonist ONO-4819 have demonstrated a variety of anabolic effects, such as stimulation of osteoblast differentiation\textsuperscript{214,215}, increased cortical bone formation in response to mechanical loading\textsuperscript{216}, and accelerated cortical bone repair after femoral drill-hole injury\textsuperscript{217}. Furthermore, ONO-4819 can also improve fixation of hydroxyapatite (HA)-coated implants in OVX rats by increasing the osteoconductivity of the HA coating and strengthening the bone-implant attachment\textsuperscript{218}. 

30
However, despite their anabolic effects, EP4 agonists also have drawbacks. Because the EP4 receptor is also expressed in other body tissues such as the gastrointestinal tract, systemic administration of synthetic EP4 agonists still results in side effects. These adverse effects include diarrhea, hypotension, and thickening of intestinal epithelium, and present further obstacles in the application of these agents as potential treatment for osteoporosis.

Table 1-5. Binding affinities of synthetic EP4 receptor agonists. $K_i$ = inhibitory constant (reciprocal of binding affinity). $IC_{50}$ = half max inhibitory concentration (inversely related to binding affinity). *Original publication did not provide a name for the agonist compound other than its chemical formula, which has been excluded from this table due to space constraints.

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<td>&gt;3200</td>
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1.6 New Approach: Conjugate

Synthetic EP4 agonists exhibit excellent anabolic properties for the treatment of osteoporosis, but their use is hindered by side effects when the compounds are administered systemically (Section 1.5). Since they act locally on bone, a better approach would be to non-invasively deliver these agonists to bone sites and limit systemic exposure. In addition, bisphosphonates (BP) are a class of compounds with unique bone-targeting characteristics, such that they bind rapidly to bone mineral when administered systemically, and are cleared quickly from blood plasma (Section 1.3.1). Therefore, in order to exploit the bone-binding property of BPs in delivery of EP4 agonists to bone sites, our approach involves the creation of a conjugate drug in which the EP4 agonist and BP are linked together. This enables systemic administration of the conjugate drug while achieving local delivery.

The BP component of our conjugate was chosen to be alendronate (ALN) based on its well-established pharmacological properties (Table 1–1, page 17). It is a nitrogen-containing BP, and has high anti-resorptive potency relative to etidronate (100-1000X). It inhibits farnesyl diphosphate synthase (FPPS) by 61%, which achieves good suppression of osteoclast function but does not reach prohibitive levels. In contrast, other N-BPs such as risedronate and zoledronate suppress FPPS by 94-99%, which may not be desirable in our conjugate. Moreover, ALN also exhibits excellent adsorption to hydroxyapatite (HA) relative to other N-BPs, and its binding affinity to HA is the second highest among all clinically used BPs, below only zoledronate. Since the main objective in linking the EP4 agonist with
BP is the rapid delivery of the agonist to bone sites, ALN’s high binding affinity with HA makes it an excellent candidate for conjugation.

The other component of our conjugate, the EP4 agonist (EP4a), was developed by Billot et al.\textsuperscript{211}, and exhibits good anabolic efficacy as well as high specificity in binding with the EP4 receptor (Table 1–5). In our approach, the EP4a and ALN components are reversibly joined via a linker molecule (LK) to create an ALN-LK-EP4a conjugate drug\textsuperscript{221}.

1.6.1 Mechanism of Action

The hypothesized mechanism of action of the ALN-LK-EP4a conjugate drug is illustrated in Figure 1–16. After the systemic administration of the conjugate compound, the ALN component binds to bone mineral and “delivers” EP4a to bone sites. Hydrolytic enzymes in the local bone environment then cleave the conjugation links to liberate both EP4a and LK, which allows the freed EP4a to promote bone formation while leaving ALN bound to bone to inhibit resorption. This mechanism of action then enables the conjugate drug to have dual therapeutic benefits, where the EP4a component acts as the anabolic agent and the ALN exerts anti-resorptive effects.


A similar approach was employed by Gil et al.\textsuperscript{222} in a previous study, in which they developed an ALN-PGE\textsubscript{2} conjugate drug (Figure 1–17). Due to the metabolic instability of PGE\textsubscript{2}, the preparation of ALN-PGE\textsubscript{2} required alterations to the structure of ALN, such that it retained its bone-targeting ability but was no longer biologically active as an anti-resorptive agent. The authors demonstrated that this conjugate drug was metabolically stable, underwent efficient uptake into bone, and that its PGE\textsubscript{2} component was gradually released over a 7-day period via cleavage of the conjugation link.
In addition to in-vitro experiments, the authors also investigated the conjugate’s effects in vivo in a curative study using ovariectomized (OVX) rats. The animals were OVX at 3 months of age, and were allowed to lose bone for 2 months before being treated. Rats were given weekly intravenous (IV) injections of PGE$_2$-ALN at low dose (10mg/kg) and high dose (100mg/kg) for 4 weeks. Histomorphometric analysis of the tibial metaphysis showed that PGE$_2$-ALN led to increased mineralizing surface relative to vehicle-treated OVX animals. In addition, bone formation rate was dose-dependently increased under conjugate treatment. However, neither treatment dose was able to achieve significant recovery of OVX-induced bone loss relative to sham.

Given the instability of PGE$_2$ and its short metabolic half-life (under 10 minutes)\textsuperscript{140}, it is possible that the freshly liberated PGE$_2$ was quickly inactivated by enzymes in the bone environment before exerting a substantial anabolic effect. In addition, since the ALN component of the conjugate was not an active anti-resorptive agent, it is also possible that the elevated resorption due to OVX overwhelmed the increase in bone formation, resulting in the lack of differences in trabecular bone volume despite increased formation at the tissue level.

The chemical structure of the current ALN-LK-EP4a conjugate is shown in Figure 1–18. In comparison to the earlier ALN-PGE$_2$ conjugate, the ALN-LK-EP4a compound has two critical advantages. First, the EP4a component is a synthetic agonist with greater metabolic stability than PGE$_2$, and has a much longer half-life in vivo. In fact, the half-life of EP4a is approximately 2 hours in rats and 1 hour in mice (private communication with Dr. Robert Young), allowing the EP4a to exert a greater anabolic effect compared to PGE$_2$. In addition, the ALN component of the current drug has not been significantly altered during conjugation, and retains its biological activity. It should therefore be able to inhibit resorption following conjugate cleavage in vivo, allowing the drug to have dual-acting therapeutic effects.
The linker for joining ALN and EP4a was chosen to be 4-hydroxyphenylacetic acid (4-HPA in Figure 1–19), which is a natural compound found in foods such as olive oil. In the final conjugate, the linker molecule LK is joined to the EP4a at the C-15 hydroxyl group, and to ALN via the terminal amine. Since the C-15 hydroxyl group is required for the EP4a to be biological active at the EP4 receptor, and the terminal amine group is important to the efficacy of ALN, the conjugate drug is not expected to exert any biological effects in its uncleaved state. Upon hydrolytic cleavage, both the hydroxyl group of EP4a and the amine of ALN would be freed, allowing the respective components to become active.

The simplified schematic for the in vivo hydrolysis of ALN-LK-EP4a is shown in Figure 1–19. The ester bond between EP4a and LK is first cleaved by esterases to liberate the EP4a component, leaving ALN attached to LK. Peptidases then cleave the carbamate bond to release 4-hydroxyphenylacetic acid (4-HPA) and ALN, with ALN attached to a carboxyl (COOH) group. Since this COOH is not stable, the ALN residue will then lose CO₂ to become free ALN. Both the ester and carbamate moieties on either end of LK are expected to have good chemical reactivity in allowing hydrolytic cleavage.

There is one additional note of clarification regarding the difference in C-1 terminal group between the original EP4a and the ALN-LK-EP4a conjugate. While the original EP4a has a carboxyl group (COOH) at its C1 terminal, the conjugate carries an ester group (CO₂Et) to ease the chemistry during drug preparation. By incubating the conjugate in rat plasma, Arns et al demonstrated that the ester group is rapidly cleaved by hydrolytic enzymes in the metabolic environment, yielding a COOH terminal on the conjugate. This cleavage occurs rapidly in vivo, and is expected to take place either before the conjugate binds to bone or soon after.
1.6.2 \textit{In vivo} Uptake and Release

Arns et al.\textsuperscript{221} conducted several experiments to determine the conjugate’s metabolic characteristics. First, they incubated the conjugate in fresh rat plasma at 37°C to assess total hydrolysis, and showed that there was a 6.2% hydrolysis of the conjugate after 24 hours. They then boiled rat plasma to denature the enzymes, incubated the conjugate for 24 hours, and showed that the drug underwent a 3.1% non-enzymatic hydrolysis. In addition, they also showed that the EP4a alone does not bind to bone powder, confirming the necessity of conjugation with ALN for delivering EP4a to bone sites.

The authors also investigated the conjugate’s properties \textit{in vivo}. In particular, they radiolabeled the EP4a component of the conjugate (Figure 1–20, A), and intravenously injected female Sprague-Dawley rats with a single dose of 10 mg/kg. Serial blood sampling showed that the conjugate was eliminated from blood plasma with a half-life of under 30 minutes, indicating rapid removal from systemic circulation. The authors then sacrificed the animals at different times points leading up to 2 weeks, and measured the radioactivity in the tibia and femurs to determine the conjugate’s bone-binding and gradual release profile (Figure 1–20, B). Since only the EP4a component was radiolabeled, the measure of radioactivity on bone provides information about the amount of EP4a that remains bound to bone relative to the initial dose. Results showed that the conjugate’s uptake into bone was 5.9%
after 6 hours, which then gradually decreased over the next two weeks. The \textit{in vivo} half life of EP4a release from bone was found to be 5 days, corresponding to a rate of 15 μg/kg/day.

Following development of the ALN-LK-EP4a conjugate and demonstration of its metabolic characteristics, the objective of the current study is to investigate the \textit{in vivo} efficacy of this conjugate drug using the OVX rat model of postmenopausal osteoporosis. In particular, this study will treat OVX rats with established osteopenia, and examine conjugate effects on bone quality following treatment.

\begin{figure}[h]
\begin{center}
\includegraphics{figure1.png}
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\caption{\textit{In vivo} uptake an release of radiolabeled ALN-LK-EP4a conjugate. A) Rats were treated with IV injection of the conjugate, in which the EP4a component was radioactively labeled. The rats were then sacrificed at different time points after injection (6 hours, 2 days, 7 days, and 14 days), and the radioactivity measured in their femurs and tibia. (B) Percent of initial dose of conjugate, as determined by radioactivity measurements on bone. Cleavage of the conjugate over time resulted in decreased radioactivity in the bone. Data obtained from reference \cite{221}.
}
\end{figure}

### 1.7 Bone Quality

Since one of the primary functions of bone is to support the body, its ability to bear loads is an essential determinant of bone health. In skeletal diseases, bone fracture or failure is the most important clinical indicator of pathology, and treatment strategies generally aim to reduce the risk of fractures\cite{39,224,225}. Though bone fragility, or the susceptibility to fracture, is often considered a key indicator of the health or quality of bone, the term “bone quality” actually refers to many characteristics that contribute to its load-bearing ability. These include bone mass, density, geometry, architecture, rate of turnover, tissue composition, mineralization, and mechanical properties\cite{224,226,227}. In assessing bone quality following osteoporosis treatment, these different areas must be addressed.

#### 1.7.1 Bone Mass and Density

Bone mass refers to the total tissue quantity in bone, and is represented differently for cortical and trabecular bone. In cortical bone, bone mass is measured as bone mineral content, and is reflected in geometric properties such as cross-sectional area, moment of inertia, and section modulus. In trabecular bone, the structure is more complex, and bulk cancellous bone mass is quantified as the bone volume fraction within a total tissue volume (BV/TV)\cite{226}. Researchers have found that low bone
mass measurements are important contributors to future fracture risk in elderly white women, as the 6-year fracture risk of the hip and forearm were both negatively correlated with radial bone mass \(^{228}\).

While bone mass attempts to capture total tissue quantity, bone mineral density (BMD) is a measure of the density of hydroxyapatite in bone, and has also been found to be an important contributor to bone fragility \(^{229,230}\). For instance, BMD measurements of the lumbar spine and distal femur have been found to be correlated with incidental fracture risk in both young and elderly women, with incidental fractures arising from moderate trauma such as falling from a standing height \(^{231}\). These results were obtained after adjusting for the effects of age, and help illustrate the importance of BMD as an indicator of bone quality.

Clinical assessment of BMD typically employs dual-energy X-ray absorptiometry (DEXA), which is a method of noninvasive imaging \(^{232}\). By bombarding the area of interest with two X-rays of different energies and quantifying the amount of energy absorbed, this technique provides a measure of the amount of mineral per unit area, which is known as areal BMD (aBMD) \(^{233}\). This can be used to evaluate many different regions in the body, such as the hip, lumbar spine, forearm, and heel \(^{41,232}\). DEXA results are frequently interpreted using the World Health Organization’s definitions of osteoporosis and osteopenia (Figure 1–10), and is an important tool in diagnosing osteoporosis as well as in monitoring its treatment \(^{232}\).

However, despite its widespread clinical use, DEXA also has significant disadvantages \(^{234}\). Since it is a two-dimensional measurement, this technique can only account for the areal length and width of a specimen, and cannot detect its depth. As bone increases in size during growth, there is also a proportional increase in the total amount of mineralized bone within the periosteal envelope. However, the volumetric BMD of the whole bone remains constant or increases only slightly during this process. A bone with greater depth than another as a result of growth will absorb more energy under DEXA, which results in a higher aBMD measurement. However, since DEXA cannot account for differences in depth, this erroneously suggests an increase in bone mineral density.

In contrast to DEXA, micro-computed tomography (microCT) can detect differences in specimen depth, and provides a three-dimensional volumetric measurement of bone mineral density (vBMD). This technique is particularly useful in evaluating trabecular bone, as the network of trabeculae produces a complicated structure that cannot be properly assessed in two dimensions \(^{41}\).

### 1.7.2 Geometry and Architecture

In addition to bone mass and BMD, the load-bearing ability of bone is also determined by its geometry and architecture. In cortical bone, both the bone size and distribution of tissue influence its
response to applied forces. Tissue distribution is dependent upon geometric parameters such as cross-sectional area, moment of inertia, and section modulus, with the relevance of different geometric parameters dependent upon the particular mode of applied load. In bending and torsion, resistance to loading can be increased if the tissue is located farther away from the plane of bending or the axis of rotation, such that changes in bone mass at critical locations can have a disproportionate effect on the structural performance of bone\textsuperscript{226}. For instance, the outer surface of a solid cylinder better resists bending forces than its inner core, such that the loss of bone mass in the center only slightly reduces the bone’s strength in bending (Figure 1–21). In contrast, expanding the bone’s outer diameter by 25% while maintaining the cross-sectional bone mass will increase the bone strength by 70%\textsuperscript{226}. Thus, bone resorption along the endosteal surface enlarges the marrow cavity and leads to some loss of bone strength, but periosteal bone formation can compensate for this by increasing bone strength.

![Cross-sectional Resistance to Bending](image)

**Figure 1–21. Cross-sectional resistance to bending in cortical bone.** Variations in the size and distribution of bone mass within a cortical cross-section affect the section modulus, which is proportional to the failure strength of whole bone in bending. Compared to the reference bone (center), a hollow bone with the same periosteal diameter but 25% less material is only 6% weaker (left), but another bone with the same amount of material distributed farther away from the center is 70% stronger in resistance to bending (right). Reproduced from reference\textsuperscript{226}.

In cancellous bone, the architectural contributions to bone strength are determined by the size and spatial distribution of trabeculae, with the indices collectively known as microarchitecture. These indices represent measurements of the thickness and number of trabeculae in the trabecular compartment, the separation between adjacent trabeculae, and their structural connectivity.

Various studies have examined the contribution of microarchitecture on bone strength. For instance, a study using sheep femoral bone showed that differences in architectural indices accounted for 10% to 70% of the variation in bone strength under compression\textsuperscript{235}. In addition, there was a difference of up to 50% in the strength and stiffness of bone with similar BMD but different microarchitectural characteristics\textsuperscript{236,237}. Moreover, when human vertebrae are loaded along the axis of the spine (superior-inferior direction), the bone strength is significantly increased if the alignment of trabeculae is also in the superior-inferior direction\textsuperscript{238}. In fact, compared to trabecular alignment along any other direction (i.e. medial-lateral, anterior-posterior), alignment along the axis of loading nearly doubles the bone strength.
Microarchitecture and structural characteristics in small animal models is typically evaluated using microCT, which noninvasively provides 3-dimensional high-resolution images of the interior bone architecture\textsuperscript{239}.

### 1.7.3 Remodeling

In providing structural support for the body and enabling its movement, the skeleton is continually subjected to wear and tear throughout its life. This results in microdamage to the bone, which must be repaired by the natural renewal process of remodeling in order for bone to remain healthy\textsuperscript{17}. If remodeling does not occur, microdamage accumulates in bone over time, and its load-bearing ability is compromised.

One example of the importance of remodeling in bone quality is the oversuppression of bone turnover in long-term bisphosphonate treatment, which is a major clinical concern. As discussed in Section 1.3, the accumulation of microdamage due to severely depressed turnover compromises the bone’s ability to absorb energy, which increases its risk of fracture. Treatments that aim to improve bone quality must therefore ensure that adequate bone turnover can take place, so that the skeleton’s natural reparation and renewal mechanism is not impaired.

Bone remodeling is assessed via undecalcified and decalcified histomorphometry, through the chemical staining of particular tissue and cellular components. Undecalcified histomorphometry allows the measurement of osteoid and mineralized bone in evaluating formation, while decalcified histomorphometry enables the assessment of osteoclasts in examining resorption\textsuperscript{240}.

### 1.7.4 Mineralization

The degree of mineralization of bone provides important information about its age and health. Peak mineralization is shifted toward lower densities during periods of rapid turnover, while decreased remodeling leads to higher mineral densities\textsuperscript{241}. These effects have been shown to have important clinical implications. For instance, sodium fluoride treatment results in increased peak mineralization due to the stabilization of the hydroxyapatite crystal lattice, which reduces the mineral solubility. In contrast, strontium treatment decreases the stability of the crystal lattice and increases the solubility of the mineral. In addition, bisphosphonate treatment also increases the peak mineralization due to its inhibition of resorption, while diseases such as osteomalacia results in altered mineralization profiles\textsuperscript{241}. Bone mineralization can be effectively assessed using back-scattered electron microscopy (BSE)\textsuperscript{242}. 
1.7.5 Mechanical Properties

Bone fracture occurs when the loading force exceeds the load-bearing capacity of the bone. In assessing bone quality, the most direct evaluation of its fragility is through the measure of its mechanical properties. These biomechanical indices provide information about the bone’s ability to withstand loading forces and absorb energy without failure, which are dependent upon both the structural characteristics of the bone (extrinsic) as well as the material properties of the tissue itself (intrinsic)\textsuperscript{227}.

Biomechanical testing of whole bone commonly employs monotonically increasing forces. Specimens are subjected to incrementally larger loading forces until bone failure occurs, typically via fracture or structural collapse\textsuperscript{226}. Mechanical properties are then derived by plotting the applied load against the deformation or displacement in bone, which results in a load-displacement curve (Figure 1–22). These parameters provide information about the bone’s behavior under loading, such as its strength, stiffness, and ability to absorb energy (work to failure). Since bone specimens vary in size and geometry, these measurements represent geometry-dependent properties.

![Load-displacement curve from biomechanical testing of bone specimen](Image)

Figure 1–22. Load-displacement curve from biomechanical testing of bone specimen. The height of the curve represents the specimen strength (ultimate force), the area under the curve work to failure (U), and the maximum slope of the initial linear region the stiffness (S). The width of the curve is the ultimate displacement (d.), Reproduced from reference\textsuperscript{227}.

After normalizing for variations in specimen geometry, another curve can also be derived. This is known as the stress-strain curve, and yields mechanical properties that parallel those of the load-displacement curve, but instead characterize the material properties of bone tissue. For instance, the “stiffness” of the material is known as its elastic modulus, the “strength” of the material its ultimate stress, and the “work to failure” of the material its toughness. These mechanical properties will be discussed further in Section 3.2.3.

Skeletal diseases can impact bone mechanical properties in different ways. For example, osteopetrosis and osteomalacia are diseases that both result in increased fracture risk, but their differing pathophysiologies lead to grossly different biomechanical characteristics in bone (Figure 1–23).
Osteopetrosis involves excessive mineralization that results in bones that are strong but brittle, which cannot absorb energy by deforming under load. These bones are therefore susceptible to fractures following trauma. In contrast, osteomalacia arises from impaired mineralization, and leads to weak, ductile bones that can deform considerably before fracture. However, its strength is significantly compromised due to insufficient mineralization. Though the work to failure is below normal in both osteopetrosis and osteomalacia, the former is the result of reduced ductility or deformation under load, while the latter is due to decreased bone strength.

![Diagram](image1.png)

**Figure 1–23. Force-displacement curve of normal and diseased bone.** While osteopetrotic bones are strong and have high ultimate load, osteomalacic bones are weak and ductile, and have reduced ultimate load. In addition, osteopetrotic bones are brittle and have low ultimate displacement, while osteomalacia results in increased ultimate displacement. Reproduced from reference 227.

Treatment strategies that aim to reduce bone fragility may do so in several ways: 1) Bone mass may be increased, which improves the load-bearing ability since bigger bones can carry more load; 2) strategically distribute bone tissue such that areas with the greatest mechanical demands may be fortified, which exploits geometrical properties in improving bone strength; and 3) improve the material properties of bone such that it is stronger at a tissue level. In an ideal treatment, the bone strength would be improved without compromising its ductility (Figure 1–24).

Mechanical properties of bone are typically measured via three-point bending, vertebral compression, and femoral neck fracture.

![Diagram](image2.png)

**Figure 1–24. Illustration of an ideal treatment strategy for improving bone fragility.** The bone strength is improved with greater ultimate force, while the ductility is also improved by increasing displacement at failure. Reproduced from reference 227.
2 Study Hypothesis

Systemically administered ALN-LK-EP4a conjugate drug will promote bone formation and improve bone quality in the ovariectomized (OVX) rat model of postmenopausal osteoporosis.

In particular, the bone quality indicators used in this study are as follows:

1. Tissue-level remodeling
2. Architecture
3. Bone mineral density
4. Mechanical properties
5. Mineralization
3 Materials and Methods

3.1 In Vivo Experiments

In this study, a curative experimental design was used to investigate the efficacy of osteoporosis treatment (Figure 3–1). The animals were subjected to bilateral ovariectomy (OVX) at 3 months of age, and were given 7 weeks to lose bone to establish OVX-induced osteopenia before being treated for 6 weeks. They were sacrificed at the end of the study period at 6 months of age, and their bones were excised for evaluation of treatment effects. All animal work was approved by the University Animal Care Committee at the University of Toronto.

Figure 3–1. Study timeline. Study period begins at week 0.

3.1.1 Animals

Seventy-one, 3-month-old female virgin Sprague-Dawley rats were purchased from Charles River Laboratories (St-Constant, Quebec). Sixty animals were subjected to bilateral ovariectomy (OVX) at Charles River, and 11 had sham surgery. Animals were housed 2/cage at the Division of Comparative Medicine at the University of Toronto for the duration of the study.

The animal facility provided a light and dark cycle of 12 hours each, and the rats were given free access to reverse-osmosis water and a standard rodent diet containing 1.0% calcium (Teklad Global 2018, Harlan Laboratories, Mississauga, Ontario).

3.1.2 Treatment

Following a bone-loss period of 7 weeks, the animals were divided into 6 groups and treated as shown in Table 3–1. Sham (SV) and PGE₂ (PG) groups were injected subcutaneously in a daily dosing regime to maintain consistency with other PGE₂ studies in literature¹⁶⁹,¹⁷⁰,¹⁷⁴,¹⁷⁷,²⁴³–²⁴⁶. All other groups were injected intravenously to deliver the treatment directly to circulation. Since other administration methods such as subcutaneous and intramuscular injections result in partial absorption of the drug to circulation that likely also varies among animals, intravenous injection enables the quantity of absorbed drug to be precisely controlled, allowing consistent per-kilogram dosing among all animals within each treatment group. The molecular weights used to calculate the molar doses are shown in Table 3–2 along with chemical formulas.
ALN-LK-EP4a conjugate was administered at two different doses. The low dose (CL) was chosen to be 5 mg/kg, which is equivalent to a molar dose of 5.69 μmol/kg (Table 3–1). This was selected based on the previous ALN-PGE₂ conjugate study, in which tissue-level anabolic effects were achieved with an in vivo release rate of 12 μg/kg/day for PGE₂, or 0.034 μmol/kg/day²²². The current ALN-LK-EP4a conjugate, when dosed at 5 mg/kg, provides an in vivo release rate of 15 μg/kg/day for EP4a, which results in a comparable molar rate of 0.036 μmol/kg/day²²¹. In addition, since EP4a is released from the conjugate with an in vivo half-life of 5 days, a weekly dosing interval was used²²¹.

Table 3–1. Treatment groups. SQ=subcutaneous injection; IV=intravenous injection. *CH group was given 25 mg/kg injection in week 7, but dosage was reduced to 15 mg/kg at biweekly intervals in weeks 8, 10, 12 due to side effects.

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</tr>
<tr>
<td>Conjugate, High Dose (CH)</td>
<td>9</td>
<td>OVX</td>
<td>15</td>
<td>17.07</td>
<td>IV</td>
<td>Biweekly</td>
<td>17.07</td>
</tr>
<tr>
<td>Separate EP4a + ALN-LK (EA)</td>
<td>12</td>
<td>OVX</td>
<td>2.5 (EP4a) 2.5 (ALN-LK)</td>
<td>5.97 (EP4a) 5.28 (ALN-LK)</td>
<td>IV</td>
<td>Weekly</td>
<td>5.97 (EP4a) 5.28 (ALN-LK)</td>
</tr>
<tr>
<td>Prostaglandin E2 (PG)</td>
<td>12</td>
<td>OVX</td>
<td>4</td>
<td>11.35</td>
<td>SQ</td>
<td>Daily</td>
<td>56.75</td>
</tr>
</tbody>
</table>

ALN-LK-EP4a conjugate was administered at two different doses. The low dose (CL) was chosen to be 5 mg/kg, which is equivalent to a molar dose of 5.69 μmol/kg (Table 3–1). This was selected based on the previous ALN-PGE₂ conjugate study, in which tissue-level anabolic effects were achieved with an in vivo release rate of 12μg/kg/day for PGE₂, or 0.034 μmol/kg/day²²². The current ALN-LK-EP4a conjugate, when dosed at 5mg/kg, provides an in vivo release rate of 15μg/kg/day for EP4a, which results in a comparable molar rate of 0.036 μmol/kg/day²²¹. In addition, since EP4a is released from the conjugate with an in vivo half-life of 5 days, a weekly dosing interval was used²²¹.

Table 3–2. Chemical formula and molecular weight of compounds used in this study. All compounds other than PGE₂ were provided in salt form, and the molecular weights include the molar mass of Na.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>Molecular Weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP4a</td>
<td>C₂₁H₂₂F₂NNaO₄</td>
<td>418.43</td>
</tr>
<tr>
<td>ALN-LK</td>
<td>C₁₃H₁₉NO₁₁Na₂P₂</td>
<td>473.22</td>
</tr>
<tr>
<td>ALN-LK-EP4a</td>
<td>C₃₂H₴₈F₂N₂O₄Na₂P₂</td>
<td>878.69</td>
</tr>
<tr>
<td>PGE₂</td>
<td>C₂₀H₁₂O₆</td>
<td>352.5</td>
</tr>
</tbody>
</table>
The high dose (CH) group was initially treated weekly at 25mg/kg in order to determine the maximal anabolic effect at extremely high dose, which is equivalent to 28.45 μmol/kg. However, the animals became very ill after the first dose, and one rat died two days after injection. The dose was then reduced to 15mg/kg (17.07 μmol/kg) at weekly dosing, but this still did not sufficiently alleviate the problems in tolerability. The CH dosing was finally modified to 15mg/kg biweekly, with vehicle treatment during the non-dosing weeks. The final treatment regime for CH was thus 25mg/kg in week 7, 15 mg/kg in weeks 8, 10, 12, and PBS in weeks 9, 11 (Figure 3-1). The adverse reactions due to treatment will be discussed further in Section 4.1.

There were two negative controls for this study, and both were given vehicle treatment. One group was OVX to account for the osteopenic condition (OV), while the other was sham-operated to serve as a healthy control (SV). In addition, PGE₂ treatment at 4mg/kg served as positive control for bone formation (PG), which is equivalent to 11.35 μmol/kg. Previous studies have shown that PGE₂ has significant anabolic effects in vivo when dosed at 3mg/kg or higher, but the maximal dose can be no more than 6 mg/kg due to issues with tolerability\(^\text{172,173,175,176}\). The 4-mg/kg dose for PGE₂ is thus sufficient for bone formation but still within the window of tolerance.

In comparing dosing between groups, it should be noted that the total weekly molar dose of PG is significantly greater than both CL and CH. This is due to differences in molecular weight and frequency of injection between PGE and the conjugate. Though the dosing weight per injection is only 20% different between PG and CL, PGE₂’s much smaller molecular weight results in a much greater molar dose per injection (2X compared to CL, and 66% compared to CH). In addition, PGE₂ is dosed daily to be consistent with previous studies demonstrating its anabolic effects in vivo, while the conjugate can be dosed weekly given its longer half-life for EP4a release. The total weekly dosing in PG is therefore 10X greater compared to CL, and 3X greater relative to CH (Table 3–1).

The final group for this study consisted of a mixture of EP4a and ALN-LK, in which the two components were not conjugated (EA). This is designed to control for the effect of conjugation between EP4a and ALN-LK in the CL group, and the dosing weight of each of the two components is thus half that of CL. However, an ideal control for the effect of conjugation should have equal molar quantities between EA and CL, but this is not the case due to slight differences in molecular weight. In fact, the molar dose of EA is 4.9% higher than CL for its EP4a component, and 7.2% lower for its ALN-LK component. Though this is not ideal, the difference in molar dose is less than 8%, and should not constitute a major concern in experimental design.
In examining the dosing for EA, it should also be noted that there is 12% difference in molar dose between EP4a and ALN-LK, which could lead to a slightly uneven effect between the two components. However, this is not a substantial difference.

In preparing the drugs for treatment, PGE$_2$ (Cayman Chemical, Ann Arbor, Michigan, USA) was first dissolved in 100% ethanol, then diluted in sterile PBS to produce 4 mg/L concentration stock solution. This was injected into the animals at 1 mL/kg volume. The solutions for the conjugate and EA mixture were both prepared by dissolving the drugs in sterile PBS at the appropriate concentrations, and injected at 1 mL/kg volume. Vehicle consisted of normal sterile PBS in this study.

3.1.3 Sacrifice and Dissection

At the end of the study, animals were placed under isofluorane anesthesia and sacrificed by exsanguination from cardiac puncture. Left and right femurs and 5$^{th}$ and 6$^{th}$ lumbar vertebrae (L5 and L6, respectively) were dissected, stripped of adherent soft tissue, and immediately wrapped in saline-soaked gauze and frozen at-20°C. The vertebral processes were also removed. Prior to any experimental testing, samples were thawed to room temperature and re-hydrated.

The left and right tibia were excised, cleaned of adherent soft tissue, and cut at mid-shaft. The distal halves were discarded, and the proximal halves were immediately immersed in tissue-fixing solutions in preparation for embedding. The left tibiae were fixed in 70% ethanol for embedding in Spurr resin, while the right tibiae were fixed in 10% neutral buffered formalin for later embedding in paraffin wax. All samples were fixed at room temperature for 5 days.

3.2 Evaluation of Treatment Effects: Overview

Following animal sacrifice, a variety of *ex vivo* experiments were performed on excised bone samples to evaluate treatment effects (Figure 3–2). The bone specimens used in each experiment are listed in Table 3–3.
Figure 3–2. Overview of bone quality indicators and the experimental techniques used to measure them.

Table 3–3. List of bone specimens used in each experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Bone Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undecalcified Histomorphometry</td>
<td>Tibia (Left)</td>
</tr>
<tr>
<td>Decalcified Histomorphometry</td>
<td>Tibia (Right)</td>
</tr>
<tr>
<td>DEXA</td>
<td>Femur (Left &amp; Right)</td>
</tr>
<tr>
<td></td>
<td>Vertebra (L5 &amp; L6)</td>
</tr>
<tr>
<td>MicroCT</td>
<td>Femur (Left)</td>
</tr>
<tr>
<td></td>
<td>Vertebra (L6)</td>
</tr>
<tr>
<td>3-Point Bending</td>
<td>Femur (Left)</td>
</tr>
<tr>
<td>Femoral Neck Fracture</td>
<td>Femur (Left)</td>
</tr>
<tr>
<td>Vertebral Compression</td>
<td>Vertebra (L6)</td>
</tr>
<tr>
<td>BSE</td>
<td>Tibia (Left)</td>
</tr>
</tbody>
</table>

3.2.1 Tissue-Level Remodeling

Tissue-level remodeling is assessed via histomorphometry, which involves the quantitative histological examination of preserved bone specimens that have been embedded in a suitable medium and processed into thin sections. These sections are then chemically stained to allow visualization of specific tissue and cellular components, and viewed under a microscope at high magnification. Results are quantified using specialized computer software. Histomorphometry may be performed on both mineralized bone (undecalcified histomorphometry) and unmineralized bone (decalcified histomorphometry).
3.2.1.1 Undecalcified Histomorphometry

Undecalcified histomorphometry allows the differentiation of mineralized from unmineralized tissue, which is crucial when measuring osteoid formation as well as actively mineralizing bone for the assessment of bone turnover\textsuperscript{240,249,250}.

Following a 5-day fixation in 70% ethanol, left tibias were defatted in ascending concentrations of acetone, then infiltrated with ascending concentrations of acetone-diluted Spurr resin in preparation for embedding. The processing time at each step was 5 days. The samples were then embedded in Spurr resin, which was polymerized at 60°C over 48 hours.

3.2.1.1.1 Goldner’s Trichrome Staining

Spurr-embedded samples were cut coronally into 5-μm sections using a Leica RM2265 microtome (Leica Microsystems, Richmond Hill, Ontario), and mounted onto gelatinized slides with 48-hour incubation at 60°C. They were then stained using Goldner’s Trichrome by sequential incubations in Weigert’s Iron Hematoxylin, Ponceau Acid Fuchsin, Phosphomolybdic Acid/Orange G, and Light Green for 15, 15, 8 and 15 minutes each, respectively. This allows osteoid to be stained red while mineralized bone is stained green\textsuperscript{249} (Figure 3–3).

![Figure 3–3. Sample image of bone section stained using Goldner’s Trichrome. Osteoid is stained red, while mineralized bone is stained green. 100X magnification.](image)

The slides were viewed under light microscopy at 100X magnification, and the results were quantified using the Bioquant Osteo 11.2.6 MIR software (Bioquant Image Analysis Corporation, Nashville, Tennessee, USA). Two regions of interest (ROI) were defined for analysis of trabecular and cortical bone (Figure 3–4). For trabecular bone, only the secondary spongiosa in the proximal tibial metaphysis was analyzed, with the ROI starting 1 mm from the distal end of the growth plate, extending for 2 mm into the metaphysis, and 0.5 mm from the endosteal surface of the cortex. Primary spongiosa near the growth plate was excluded since it is not representative of metaphyseal trabecular bone\textsuperscript{52,251,252}. For cortical bone, the cortical shaft of the diaphysis was analyzed, starting 5 mm from the distal end of the growth plate, and extending for 7 mm into the diaphysis. The
parameters measured include osteoid surface (OS/BS, %), percent osteoid volume (%), osteoid width (μm), and eroded surface (%).

![Trabecular ROI and Cortical ROI](image)

**Figure 3–4.** Regions of interest (ROI) for analysis of proximal tibia stained with Goldner’s Trichrome.

### 3.2.1.1.2 Fluorescent Labeling

Fluorescent labeling is used to perform dynamic histomorphometry for the measurement of kinetic bone formation parameters. This involves the use of fluorochromes, which strongly chelate calcium ions and are thus incorporated into bone during mineralization. These molecules fluoresce under specific wavelengths and can be viewed under fluorescent light to detect bone surfaces that are undergoing active mineralization. Among various fluorochrome markers, calcein green is frequently used due to its non-toxicity, efficacy at low dose, and robust labeling which is resistant to fading.

Animals were injected with 10 mg/kg Calcein Green (Product Number C0875-25G, Sigma-Aldrich Canada, Oakville, Ontario) at 12 and 2 days before sacrifice, which allows for the formation of two different fluorescent labels as mineralization occurs over the 10-day period. If only one of the two injected doses is incorporated into bone, a *single label* is formed (Figure 3–5). If both injections are incorporated into bone, a *double label* is formed. By measuring the distance between the two labels (*interlabel width*) and accounting for the time difference between the two injections, the rate of bone formation may be determined.

![Single Label, Double Label, Interlabel Width](image)

**Figure 3–5.** Sample image of unstained slide under fluorescent microscopy. Mineralizing surfaces labeled with calcein green fluoresce green under ultraviolet light. 100X magnification.

Spurr-embedded tibia samples were cut coronally into 7-μm sections, and were cover-slipped unstained using Paramount mounting medium. They were then viewed under UV light (470/509 nm wavelength) at 100X magnification, and quantified using Bioquant Osteo 11.2.6 MIR software. The
ROIs for analysis were identical to those in Goldner’s Trichrome-stained slides. Several parameters were measured: 1) single label surface (sLS, mm); 2) double label surface (dLS, mm); 3) mineralizing surface (MS, formula = dLS + sLS/2, mm); 4) percent mineralizing surface (MS/BS, %), 5) mineral apposition rate (MAR, formula = interlabel width/labeling period, μm/day), and 3) bone formation rate (BFR/BS, formula = MS/BS*MAR, μm/day/mm)\textsuperscript{48}.

### 3.2.1.2 Decalcified Histomorphometry

Decalcified histomorphometry is performed on unmineralized bone, and is used to measure osteoclasts for the assessment of bone resorption\textsuperscript{240}. Since the tartrate-resistant acid phosphatase (TRAP) enzyme is strongly expressed in osteoclasts\textsuperscript{255-257}, it is a commonly used osteoclastic marker in bone histomorphometry\textsuperscript{253}. In addition, researchers have found that the expression of TRAP by osteoclasts is also positively correlated with their resorptive activity\textsuperscript{258}, further supporting the use of TRAP staining as a marker of resorption. In histomorphometric experiments, TRAP appears red under immunohistological staining (Figure 3–6).

![Figure 3–6. TRAP-positive osteoclast appears red under immunohistological staining.](image)

After a 5-day fixation at room temperature, the proximal halves of right tibiae were immersed in ethylenediaminetetraacetic acid (EDTA, 0.5M, pH 7.4) for decalcification at 4°C, with continual agitation and solution changes every other day. After 7 weeks, complete decalcification was confirmed by faxitron imaging, and the samples were sequentially processed using increasing concentrations of ethanol followed by xylene and paraffin, and embedded in bone-specific paraffin. Coronal 5-μm thick sections were prepared using a Leica Reichart Jung 2030 microtome (Leica Microsystems Canada Inc., Richmond Hill, Ontario), mounted on Superfrost Plus glass slides, and incubated at 60°C for 24 hours to ensure adhesion to slide.

Slides were stained for the TRAP enzyme using the Acid Phosphatase Leukocyte kit (Sigma-Aldrich Canada, Oakville, Ontario). This involves the sequential incubations in Tris buffer (0.2M Trizma base, pH 9, Sigma-Aldrich Canada, Oakville, Ontario) and TRAP stain for 1 hour each at 37 °C, followed by counter-staining with Acid Hematoxylin for 2 minutes.
Slides were viewed under light microscopy at 250X magnification, and histomorphometric analysis was performed using the Bioquant Osteo 11.2.6 software. Trabecular bone of the proximal tibial metaphysis was analyzed using the same ROI as Goldner’s Trichrome analysis, and osteoclast number (N.Oc/BS, 1/mm) and surface (Oc.S/BS, %) were quantified.

3.2.2 Bone Mineral Density and Microarchitecture

3.2.2.1 Dual-Energy X-Ray Absorptiometry

Dual-energy X-ray absorptiometry (DEXA) enables the examination of tissue density and mineral content in vivo. It bombards the tissue sample with two simultaneous beams of γ radiation of different energies (40 KeV and 70 KeV), and measures the energy transmitted through the sample. Since energy attenuation is different between bone and soft tissue, the scanner determines the amount of energy absorbed by bone and thereby calculates the amount of bone mineral in the sample. The results are expressed as bone mineral content (g) or areal bone mineral density (aBMD, g/cm²)\textsuperscript{233}. However, since DEXA cannot detect specimen height, this is not a true measure of volumetric BMD\textsuperscript{234}. After thawing the samples to room temperature, DEXA was performed on the left and right femurs as well as the L5 and L6 vertebrae using the Lunar PIXImus Bone Densitometer (GE Healthcare Canada, Mississauga, Ontario), which is designed for small animal analysis. Prior to each session, the PIXImus was calibrated by scanning a test phantom. Bone samples were positioned on a polystyrene plate for each scan, and care was taken to ensure that the sample orientation was consistent across sessions. After scanning, the bones were re-wrapped in saline-soaked gauze and stored at -20°C.

The results were analyzed using the PIXImus software. For each animal, data were averaged for the left and right femurs as well as the L5 and L6 vertebrae to obtain single measurements for the femur and vertebra.

3.2.2.2 Micro-Computed Tomography

First introduced in the 1980’s, micro-computed tomography (microCT) has become the gold standard for the ex vivo evaluation of volumetric BMD (vBMD) and bone architecture in rats and other small animals\textsuperscript{239}. It bombards the sample with X-ray beams that are collimated and filtered to achieve a narrow energy spectrum, and records the transmitted X-rays on a 2D charge-coupled device. By rotating the sample on a central platform and recording the X-ray transmissions, the scanner obtains a series of projections which are then processed offline to reconstruct an image of the original 3D object\textsuperscript{259}. Modern desktop microCT scanners are capable of achieving extremely high resolutions, with
isotropic voxels that are as small as ~6 μm in size. Since rat trabeculae are typically ~100 μm in thickness, this facilitates the detailed study of trabecular microarchitecture in rats.

Left femurs and L6 vertebrae were scanned using the Skyscan 1174 compact desktop Micro-CT (Bruker-microCT, Kontich, Belgium). Each sample was thawed to room temperature, re-wrapped tightly in saline-soaked gauze, and placed inside a 10 mL microtube in preparation for scanning. The microtubes were securely fastened onto the rotating platform to prevent motion during the scan, and samples were carefully positioned such that the orientation of the femoral shaft and the vertebral body were as vertical as possible (Figure 3–7). Imaging was performed at 50-kVp source voltage, 3000-ms exposure time, and an isotropic voxel size of 11.6 μm for both femurs and vertebrae. Two hydroxyapatite standards of known densities were scanned daily to allow calibration of BMD measurements.

Given the large size of the rat femurs relative to the scanning field-of-view, only the femoral diaphysis could be captured in the images. In order to locate the mid-diaphysis accurately, a custom marker was made using a staple and wrapped around the bone, which would be visible in the images (Figure 3–7, B). This marker was positioned 16 mm from the femoral condyles, to be used a landmark in locating the mid-diaphysis during image analysis. This distance was chosen so that the marker would be visible within the scanning field-of-view, but was far enough from the mid-diaphysis to avoid contamination of the analytical volume of interest (VOI).

The images were reconstructed using the NRecon software (Version 1.6.3.2) analyzed using CTAn (Version 1.10.0.1). Femoral scans were reconstructed using consistent settings for all samples (smoothing = 3, beam hardening correction = 30%, ring reduction = 6, defect pixel masking = 50%). To determine the mid-diaphyseal ROI for analysis, the bone lengths were first measured using a digital caliper as the distance between the greater trochanter and the femoral condyles. The mid-point was then calculated, and the offset distance determined relative to the staple at 16mm. All samples were measured in a consistent manner, and the VOI for analysis was selected to be a 1-mm thick section at

Figure 3–7. MicroCT scanning field-of-view at 11.6 μm voxel size. A) L6 vertebra. B) Left femur. A staple was curved into horseshoe shape and wrapped around the femoral shaft at 16 mm from the femoral condyles. The staple was carefully positioned to be perpendicular to the femoral shaft, and offset distances for determining mid-diaphysis were measured from the proximal end of the staple. Red box denotes volume of interest (VOI) for analysis of diaphyseal cortical bone.

52
the mid-diaphysis (Figure 3–7). Within this volume, separate ROIs were defined for measuring structural parameters, consisting of femoral cortex, endocortical cavity, and total femoral cross-section (Figure 3–8). The structural indices obtained include vBMD (mg/cm$^3$), cross-sectional area (mm$^2$), cortical thickness (mm), and cortical porosity (%). In addition, geometrical parameters were also obtained, such as anterior-posterior (AP) diameter, medial-lateral (ML) diameter, and minimum principal moment of inertia (MMI$_{min}$). These parameters would later be used in deriving tissue material properties from specimen mechanical measurements, as they allow the mechanical measurements to be normalized for sample geometry.

![Figure 3–8. Regions of interest for microCT analysis of femurs. ROIs are shown in red, and consist of whole femoral cross-section (A), endocortical cavity (B), and cortex (C). A was used to measure cross-sectional bone area, B endocortical bone formation, and C vBMD and cortical porosity.](image)

Vertebral images were reconstructed using the same settings as femurs, and were analyzed using the CTAn software. In defining the VOI for analysis, primary spongiosa near the growth plate were excluded, and only portions of the vertebral body containing secondary spongiosa were analyzed. Within this volume, regions of interest were then defined corresponding to the vertebral cortical wall, metaphyseal trabecular bone, and whole vertebra (Figure 3–9). These were used to measure structural indices for trabecular microarchitecture, including vBMD (mg/cm$^3$), trabecular number (1/mm), trabecular thickness (mm), trabecular separation (mm), structure model index (SMI), and connectivity density (1/mm). In addition, geometrical parameters such as vertebral cross-sectional area (mm$^2$) were also obtained, which were used to normalize mechanical measurements.

![Figure 3–9. Regions of interest for microCT analysis of vertebrae. ROIs are shown in red, and consist of whole vertebral cross-section (A), metaphyseal trabecular bone (B), and vertebral cortical wall (C). A was used to measure vertebral cross-sectional area, B trabecular structural properties for microarchitecture, and C cortical wall vBMD.](image)

3.2.3 Mechanical Properties

Bone is a composite material containing both organic matrix and inorganic mineral, and each component contributes differently to its mechanical properties. While the strength and stiffness of
bone are attributed primarily to its mineral component\textsuperscript{261-263}, the collagen content of the organic matrix is critical in determining its toughness and deformation under load\textsuperscript{262,264,265}. Healthy bone exhibits an optimal balance between strength and ductility, such that the mechanical performance of the overall composite tissue exceeds either of its individual constituents\textsuperscript{226}.

Different mechanical tests are performed on bone specimens to characterize their behavior under loading\textsuperscript{266}, and the force-deformation relationships recorded during these tests can be used to derive a variety of mechanical properties (Figure 3–10). Extrins\textit{ic} properties of bone are dependent upon the specimen geometry, and include architectural contributions to bone strength. In contrast, intrin\textit{sic} properties of bone characterize the behavior of the tissue material irrespective of geometrical differences. While the \textit{load} measurements represent forces applied to the bone, the \textit{stress} measurements correspond to the force per unit area experienced by the tissue. Similarly, \textit{displacement} corresponds to the absolute deformations in the tissue under applied load, while \textit{strain} is a measure of the relative deformation after normalizing for specimen dimensions.

Numerous measurements are derived from the load-displacement and stress-strain curves (Figure 3–10). The \textit{stiffness} or \textit{modulus} of the bone denotes its resistance to applied loads, while the \textit{ultimate load} or \textit{ultimate stress} denotes its strength. In addition, the area under the curve represents the total amount of energy absorbed by the bone during the test, and corresponds to the \textit{work to failure} for the sample or \textit{toughness} of the tissue material. The end of the test is marked by the \textit{failure point}, which corresponds to specimen fracture and is characterized by the bone’s load/displacement and stress/strain measurements.

\textbf{Figure 3–10. Typical results from biomechanical testing of bone specimens.} A) Load-displacement curve used to derive extrinsic properties of bone, which are geometry-dependent. This is produced from force-deformation data recorded during the tests. B) Stress-strain curve used to derive intrinsic properties of bone tissue, which are independent of sample geometry. This is produced from normalizing the load-displacement curve. The green line denotes the linear region in which the slope is calculated to obtain stiffness and modulus measurements, respectively. The red dotted line separates the elastic and plastic regions of the curves.
In addition to mechanical parameters, these curves also provide information about specimen behavior. In particular, the curves are divided into elastic and plastic regions, which are separated by the yield point. The yield point is determined by drawing a line parallel to the linear region of the curve, but with a 0.2% offset relative to the curve. The point at which the load-displacement curve intersects with the line is defined as the yield point, as it delineates a change in the slope of the curve. The behavior of the bone prior to the yield point is known as elastic behavior, and resembles that of a spring in that it is capable of returning to its original position once the applied load is removed. However, after the yield point, the bone enters the plastic region and undergoes permanent deformation. The ability of the bone to deform under load represents its ductility, and is crucial to the bone’s capacity to safely absorb energy upon impact. In fact, pathologically brittle bone exhibits little ductility and is susceptible to fracture under trauma, which is exemplified by diseases such as osteopetrosis.

Due to the anisotropic nature of bone, its performance in mechanical tests varies depending upon the method and direction of loading. As such, different tests are employed in this study to evaluate the bone’s mechanical properties.

### 3.2.3.1 Three-Point Bending

Three-point bending is used to evaluate the mechanical properties of cortical bone, and is often applied to the diaphysis of long bones. The bone is positioned flat on supporting struts, and a compressive force is applied vertically from above at the midpoint between the supports (Figure 3–11). This creates compressive stresses in the bone at the point of applied force, while tensile stresses are produced on the opposite side.

![Figure 3–11. Illustration of three-point bending test.](image)

Left femurs were subjected to three-point bending using the Instron 4465 mechanical testing machine (Instron, Norwood, Massachusetts, USA), with a 1 kN load cell. Prior to testing, geometrical properties of the femurs were obtained via microCT imaging, and femoral midpoints were measured using a digital caliper and marked. Samples were thawed to room temperature, re-hydrated in saline, and were placed on two supports that were 15 mm apart at the tip. Bones were positioned with the
posterior side facing downwards, with consistent orientation for all samples. A pre-load of approximately 1 N was applied to the midpoint of the femur, and the force applicator was lowered at a rate of 0.5 mm/min. This exerts an incrementally greater compressive force upon the sample, and the test was continued until specimen fracture. Load versus time data were recorded using the LabVIEW 5.0 data acquisition software (National Instruments corp., Austin, Texas, USA) at a sampling rate of 10 Hz. After the test, the distal portions were discarded and the proximal portions were frozen at -20 °C for future use.

Load-displacement curves were produced by plotting the load vs. time data and accounting for the force applicator’s rate of movement. From this curve, the ultimate load (N), work to failure (mJ), ultimate displacement (mm), and stiffness (N/mm) were calculated. The failure point was determined to be the location of a sudden and significant drop in applied load, as the fractured bone was no longer able to resist applied forces.

The load-displacement curves were converted to a stress-strain curve using the mathematical relationships below. This allows the data to be normalized for specimen geometry. Material properties were then derived from the stress-strain curve, including Young’s modulus or elastic modulus (MPa), ultimate stress (MPa), toughness (MPa), and failure strain (%).

**Stress:**

\[ \sigma = \frac{F \cdot L \cdot d_{AP}}{8 \cdot I_{xx}} \]

where \( \sigma = \text{stress (MPa)} \),

\( F = \text{applied load (N)} \)

\( L = \text{gauge length (15 mm)} \)

\( d_{AP} = \text{AP diameter (mm)} \)

\( I_{xx} = \text{Moment of inertia (mm}^4) \).

**Strain:**

\[ \varepsilon = \frac{6 \cdot d_{AP} \cdot D}{L^2} \cdot 100 \]

where \( \varepsilon = \text{strain (\%)} \),

\( D = \text{displacement (mm)} \)

\( L = \text{gauge length (15 mm)} \)

\( d_{AP} = \text{AP diameter (mm)} \).
3.2.3.2 Femoral Neck Fracture

Femoral neck fracture is a mechanical test designed to mimic osteoporotic hip fractures, and provides a clinically relevant assessment of femoral neck strength. In this technique, the proximal end of the femur is secured in an inverted position, and a compressive force is applied such that the femoral head is pushed against a lower contact platen (Figure 3–12). The applied force is incrementally increased until the femoral neck fails by fracture. This test produces compressive, tensile, as well as shear stresses in the bone.

![Figure 3–12. Illustration of femoral neck fracture test. In this experiment, the diaphyseal end of the specimen was mounted inside a custom jig using PMMA.](image)

The Instron 4465 mechanical testing machine (Instron, Norwood, Massachusetts, USA) was used for femoral neck fracture with a 1 kN load cell. The proximal halves of left femurs that had been fractured using three-point bending were used for this test. Samples were thawed to room temperature and re-hydrated prior to testing, and were mounted inside a custom-built jig using polymethylmethacrylate (PMMA). Care was taken to ensure that the femoral shaft was as vertical as possible, with approximately 3.5 mm protruding above the PMMA. All samples were mounted in a consistent fashion.

After positioning the specimen inside the custom jig, the PMMA was allowed to cure for 10 minutes at room temperature while the sample was covered with saline-soaked gauze to maintain proper hydration. The jig was then mounted onto the Instron system with a pre-load of approximately 1N, then tested by lowering the crosshead at a rate of 2.5 mm/min until failure was achieved. Load and time data were recorded using the LabVIEW 5.0 data acquisition software (National Instruments Corp., Austin, Texas, USA), with a sampling rate of 10 Hz.

Load-displacement curves were generated to allow the derivation of mechanical properties, which consisted of ultimate load (N), stiffness (N/mm), and work to failure (mJ). Similar to three-point bending, specimen failure for femoral neck fracture was also determined by a sudden, significant drop in the measured load.

In order to obtain normalized material properties for a given biomechanical test, the geometry at the site of fracture must be considered. However, given the irregular geometry of the femoral neck
as well as the tremendous variability in the direction and angle of fracture, it is extremely difficult to obtain accurate information about the geometrical properties involved. Thus, normalized material properties were not derived for the femoral neck fracture experiment, and only the structural properties are used. This is consistent with other studies in literature\textsuperscript{268,269}.

### 3.2.3.3 Vertebral Compression

Vertebral compression is a mechanical test that exerts compressive loads upon the vertebral body, and mimics the spine compression fractures that occur in osteoporosis. In this technique, the vertebral body is affixed to a holding plate on the bottom, while a compression plate on top is lowered (Figure 3–13). The loading force is incrementally increased until specimen failure.

![Figure 3–13. Illustration of vertebral compression test. In this experiment, superglue was used to affix the vertebra to the holding plate, while PMMA was employed to create a flat surface at the compression plate interface.](image)

Vertebral compression was performed on the L6 vertebrae using the Instron 4465 mechanical testing machine (Instron, Norwood, Massachusetts, USA) and a 1000N load cell. Vertebral geometrical properties were determined using microCT images, including both vertebral height and cross-sectional area. Vertebral height was measured as the distance between growth plates at the proximal and distal end of the vertebrae. Prior to testing, samples were thawed to room temperature and re-hydrated. Each sample was trimmed to remove soft tissue at the intervertebral discs, and the distal end of the vertebra was affixed to a metal holding plate with superglue. Care was taken to ensure that the bottom of the vertebra was as flat as possible, such that its length was perpendicular to the plate. Since the proximal end of the vertebra was slanted at an angle, PMMA was used to create a flat surface for contact with the compression plate, which facilitates even distribution of load across the vertebral cross-section.

A pre-load of approximately 1N was applied to the vertebra, and the sample was left in place for 10 minutes while the PMMA cured. The compression plate was then lowered at a rate of 1 mm/min until specimen failure. Data were recorded using the LabVIEW 5.0 data acquisition software (National Instruments Corp.; Austin, Texas, USA).
Load-displacement curves were generated using the recorded data, and mechanical properties were determined similar to those in three-point bending. Stress-strain curves were then derived from load-displacement curves using the relationships below. Since the compression of vertebrae does not result in full fracture, failure point was determined as the location of a 8-10 % reduction in load.

Stress: \[ \sigma = \frac{F}{A} \]

where \( \sigma \) = stress (MPa),
\( F \) = applied load (N)
\( A \) = cross-sectional area (mm²)

Strain: \[ \varepsilon = \frac{D}{h} \cdot 100 \]

where \( \varepsilon \) = strain (%),
\( D \) = displacement (mm)
\( h \) = vertebral height (mm).

### 3.2.4 Degree of Mineralization

Scanning electron microscopy (SEM) is an imaging technique that bombards specimens with high-energy electrons, and provides information about the sample based on the electron-sample interactions. Back-scattered electron microscopy (BSE) is a type of SEM in which the back-scattering, or reflection, of these electrons by the sample allows the characterization of its chemical composition. Because heavier elements with higher atomic numbers scatter electrons more strongly than those with lower atomic numbers, the contrast of BSE images reflects differences in elemental content. In addition, the distribution of heavy elements within a sample also influences the electron-sample interactions, such that tissue areas more densely populated with heavy elements also appear brighter in BSE images. Since bone tissue consists of an organic matrix populated by hydroxyapatite mineral, BSE is well-suited to examining mineral distribution in bone (Figure 3–14).²⁷⁰,²⁷¹

![Figure 3–14. Sample BSE image of bone showing differences in contrast due to variation in mineralization.](image-url)
After sectioning to obtain samples for histomorphometry, the Spurr-embedded left tibias were used for BSE imaging. Samples were polished to a 1 μm diamond finish using a Phoenix BETA Grinder/Polisher (Buehler, Lake Bluff, Illinois, USA), then mounted securely on 8.7 cm x 8.7 cm plexiglass plates using Fimo polymer clay (Fimo Classic, Eberhard Farber). Blocks were positioned with the exposed bone facing up, and care was taken to ensure that the top surface was level across all blocks on each plate. In order to facilitate electron conduction across the blocks, they were joined by carbon tape and coated with an even layer of carbon. Specimens were then imaged using a Philips XL300ESEM system, which is a solid state BSE detector (FEI company, Hillsboro, Oregon, USA). Beam parameters were set to 20 kV with a working distance of 15 mm, and all images were obtained at 100X magnification. A calibration standard consisting of joint silicon dioxide (SiO₂) and magnesium fluoride (MgF₂) was scanned prior to obtaining data for each sample, and scanner settings were adjusted accordingly to minimize variation and drift in contrast levels as well as maintain consistency across samples.

Two different regions in the tibia were imaged to obtain measurements for trabecular and cortical bone. The trabecular region focused on the proximal tibia, and covered 2 mm extending from the proximal end of the growth plate. In contrast, the cortical region focused on the tibial shaft, and consisted of a 1 mm section beginning 6 mm from the distal end of the growth plate. Results were analyzed using in-house software, and the regions of interest for analysis for trabecular and cortical bone were the proximal tibial metaphysis and the tibial shaft, respectively (Figure 3–15). Histograms of grey level pixel intensities were generated for each ROI, which provide sample mineralization profiles. The parameters measured include maximum grey value, which represents peak mineralization in the sample, and full width at half max (FWHM), which represents the spread of mineralization (Figure 3–16).

Figure 3–15. Regions of interest for analyzing BSE images of trabecular and cortical bone. ROIs are shown in red. Trabecular ROI started 1 mm from the growth plate and extended for 2 mm into the metaphysis while maintaining 0.5 mm separation from the endosteal surface. Cortical ROI covered the entire cortex within the imaged region. Original magnification 100X.
Figure 3–16. Histogram from BSE imaging. Baseline intensity (red) is subtracted from the histogram data before calculating parameters. Max grey value represents peak mineralization in the sample, while FWHM is a measure of the spread of mineralization.

3.3 Statistical Analysis

Statistical analysis was performed using SPSS software (Version 21). Results were compared using one-way analysis of variance (ANOVA), with Bonferroni correction for post-hoc multiple comparisons. Data with inhomogeneous variance were compared using non-parametric Kruskal-Wallis test. Significance was defined as $p < 0.05$ for two-tailed probability at 95% confidence. Data are presented as mean ± standard deviation.
4 Results

4.1 Treatment Side Effects

Since one of the main objectives in designing the ALN-LK-EP4a conjugate drug was to mitigate side effects due to treatment, the animal reactions during the study were carefully recorded. No adverse reactions were observed in the vehicle-treated groups (SV and OV), and the reactions of other groups are shown in Table 4–1.

Table 4–1. Animal reactions to treatment. Numerical results are displayed as number of affected animals / total number of animals in group. Since animals were doubly housed, the animal numbers for diarrhea were determined based on cages rather than individual animals, as it was impossible to trace which animal had the reaction.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>PG</th>
<th>CL</th>
<th>CH</th>
<th>EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhea</td>
<td>Mild (8/12)</td>
<td>None</td>
<td>None</td>
<td>Mild (10/12)</td>
</tr>
<tr>
<td>Lethargy (Lack of Movement)</td>
<td>Severe (12/12)</td>
<td>Mild (8/12)</td>
<td>Moderate (11/11)</td>
<td>Mild (12/12)</td>
</tr>
<tr>
<td>Short Gasps of Breath</td>
<td>12/12</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Closed Eyes</td>
<td>12/12</td>
<td>12/12</td>
<td>11/11</td>
<td>12/12</td>
</tr>
<tr>
<td>Puffy Fur</td>
<td>12/12</td>
<td>None</td>
<td>11/11</td>
<td>12/12</td>
</tr>
<tr>
<td>Hunched Posture</td>
<td>12/12</td>
<td>None</td>
<td>11/11</td>
<td>None</td>
</tr>
<tr>
<td>Porphyrin Around Eyes/Nose</td>
<td>1/12</td>
<td>None</td>
<td>11/11</td>
<td>None</td>
</tr>
<tr>
<td>Urine Staining</td>
<td>None</td>
<td>None</td>
<td>9/11</td>
<td>None</td>
</tr>
<tr>
<td>Swollen/Purple Legs/Feet</td>
<td>None</td>
<td>None</td>
<td>11/11</td>
<td>None</td>
</tr>
<tr>
<td>Swollen Tail</td>
<td>None</td>
<td>None</td>
<td>11/11</td>
<td>None</td>
</tr>
<tr>
<td>Duration of Effects</td>
<td>1 Day or more</td>
<td>1 Hour</td>
<td>2 Weeks</td>
<td>1 Day</td>
</tr>
</tbody>
</table>

A number of side effects were observed during the treatment. Symptoms included mild diarrhea, lethargy or lack of movement, immediate symptoms of physical distress such as puffy fur and shortness of breath, as well as more long-term effects such as swelling and lack of grooming. The most common side effect was lethargy, which was observed in all non-vehicle-treated groups.

Among treatment groups, PG exhibited the most severe symptoms of immediate distress, which included shortness of breath, closing of the eyes, puffy fur, hunched poster, and severe lethargy. They also experienced mild diarrhea within 1 hour after injection, which is consistent with other PGE₂ studies. Despite these immediate symptoms, animals in PG did not exhibit serious, long-term effects such as swelling in the legs and tail. With a few exceptions, most were able to recover fully within 1 day.

In contrast, CL presented the least number of adverse reactions (2/10) relative to the other non-vehicle treated groups. They showed some mild lethargy and kept their eyes closed after injection, but were able to return to normal behavior within 1 hour. In comparison, EA showed slightly
more symptoms than CL (4/10), and exhibited mild diarrhea and slightly puffy fur in addition to lethargy and eye closure. However, the severity of their reactions was much less than PG, and they were able to recover within 24 hours.

On the other hand, CH exhibited the greatest number of side effects among all groups, and the symptoms persisted for up to 2 weeks. They were moderately lethargic following injection, and other symptoms such as puffy fur and hunched posture appeared more gradually over ~24 hours. Compared to PG, animals in CH were more alert following injection, and their immediate symptoms were not as severe. They also did not experience diarrhea, unlike the animals in PG.

Other symptoms in CH included more severe and long-term effects, such as swelling in the hind legs, feet, and tail. Their feet appeared tender to the touch, and they were extremely reluctant to put weight on them. In fact, they dragged their hindquarters while moving about the cage, as if paralyzed. However, they were able to move their feet when gently probed, so their lack of movement was due to reluctance rather than incapacity. In addition, these animals also showed signs of lack of grooming, including porphyrin staining around the eyes and nose as well as urine staining on their fur. Porphyrin in rats is a natural red pigment secreted by the Harderian gland near the eyes, and is normally removed during grooming. The appearance of porphyrin stains around the eyes and nose is therefore an indicator of improper grooming and illness\textsuperscript{272,273}.

The adverse reactions in CH generally appeared 1 day following injection, gradually worsened over the next several days, and became the most severe 3-4 days after injection. There were some alleviation of these symptoms beginning at approximately 5 days, but the animals never completely recovered within the 2-week interval between doses. Of the 12 animals originally in this group, one died 24 hours after the first injection, and another two animals died one day before sacrifice. The final sample size in CH at the end of the study period was therefore 9 animals.

Following animal sacrifice, gross necropsy was performed by a veterinary pathologist (Dr. Richard Renlund, Department of Comparative Medicine) to evaluate animal health in the non-vehicle treated groups. Three animals from each group were examined. The internal organs of animals in PG did not exhibit gross abnormalities, and their livers had “sharp” edges indicating proper health and function. Similarly, there were also no gross liver abnormalities in EA, although the edges of their livers were slightly rounded compared to PG. On the other hand, the livers of animals in CL were somewhat enlarged with pale coloring, which indicates potential compromise in function.

The animals in CH had some abnormality in both liver and kidney. The livers were enlarged, and one showed yellow coloration indicating possible fat infiltration. In addition, the kidneys also showed brown discoloration, with parts of the internal tissue appearing white. Samples were taken
from both the liver and kidney for histological examination by Dr. Renlund, who did not find any gross abnormalities at the tissue level.

4.2 Gross Effects

Animal weights were measured at treatment start and sacrifice, and the results are shown in Figure 4-1. Weights were comparable among all O VX groups prior to treatment, and weight gains over the treatment period were similar for all groups except CH and PG, which did not increase as much as the others. At both time points, animals in SV showed significantly lower weight compared to O VX groups, which was expected due to O VX-induced weight gain.

![Animal Weights Graph](image)

*Figure 4-1. Treatment effects on animal weight.* Blue graph shows animal weights in week 7 prior to treatment start, while red graph shows animal weights at sacrifice in week 13. Comparison was made among groups at each time point. *p<0.002 compared to all other groups.

Treatment effects on bone size are shown in Figure 4-2. Bone sizes are comparable across except CH, which are significantly larger the others. In fact, the right femurs in CH had significantly greater length as well as mid-shaft cross-sectional area compared to all other groups.

![Bone Size Graph](image)

*Figure 4-2. Treatment effects on bone size.* Top image shows photograph of representative right femurs for all the groups, while bottom images show measurements for femoral length and cross-sectional bone area at the mid-diaphysis. Cross-sectional bone area was measured using microCT. *p<0.05, **p<0.001 compared to all other groups.
4.3 Effects on Trabecular Bone

Treatment effects in trabecular bone will be discussed in this section. To provide an overview, representative images of the proximal tibia are shown in Figure 4–3. OVX (OV) exhibits decreased trabecular bone volume compared to sham (SV), which confirms OVX-induced bone loss in our disease model. In addition, conjugate treatment led to dose-dependent formation of trabecular bone in the proximal tibial metaphysis, with the low dose (CL) comparable to PGE$_2$ control (PG). This appears to restore OVX-induced loss of trabecular bone up to sham levels. However, bone formation under high dose treatment (CH) is excessive. There appears to be a slight increase in trabecular bone volume in EA relative to OV, but this is not to the same extent as CL or PG. The following sections will present results of the various *ex vivo* experiments that characterize treatment efficacy in trabecular bone.

![Representative BSE images of proximal tibia. Original magnification 100X.](image)

4.3.1 Tissue-Level Remodeling

4.3.1.1 Formation

To investigate tissue-level remodeling, bone formation in the proximal tibial metaphysis was examined using undecalcified histomorphometry. Two different techniques were employed: 1) Goldner’s Trichrome, which allows differentiation between mineralized and unmineralized bone; and 2) calcein green fluorescent labeling, which was used to measure dynamic bone formation parameters.

Results for Goldner’s Trichrome staining are shown in Figure 4–4. As osteoblasts form new bone, they produce an organic matrix which is then gradually mineralized over time. The unmineralized bone matrix is known as osteoid, and examination of the extent of osteoid formation provides information about the bone remodeling taking place. In particular, osteoid surface (OS, mm) and percent osteoid surface (OS/BS, %) represent the extent of bone undergoing new matrix formation. Similarly, osteoid volume (mm$^3$) and percent osteoid volume (%) are measures of the total volume of new matrix
formed. Osteoid width (O.Wi, μm) is a measure of its thickness, while percent eroded surface (ES/BS, %) is a measure of the extent of bone surface undergoing active osteoclastic resorption.

Results show that OS is comparable across all the groups except in OV, which is significantly increased. In fact, the osteoid surface in CH is 3.6-fold greater than that of any other group. However, after normalizing for total bone surface, OS/BS is comparable across most of the groups with the exception of OV, which is increased compared to EA and CL. Measurements for osteoid volume show a dose-dependent increase in conjugate-treated groups relative to OV, although this is not significant for CL. However, the dose-dependent effect disappears after normalizing for total bone volume, and both CL and CH show comparable percent osteoid volume. In addition, osteoid width is comparable across most groups except in CL, which is increased compared to OV and EA. Although both conjugate-treated groups seem to be increased relative to other groups, this difference is not statistically significant at the high dose.

There is high variability in the eroded surface measurements that resulted in little statistical significance among groups. Although CL, CH, and PG groups seem increased relative to others, the only comparison that approaches statistical significance is CL, which is increased compared to EA.

Results also show that the normalized osteoid surface and volume measurements for OV are both increased compared to SV, which is consistent with the elevated turnover seen in O VX-induced bone loss\textsuperscript{252}. However, neither osteoid width nor eroded surface is different between OV and SV.

Dynamic histomorphometry was performed on the proximal tibial metaphysis using fluorescently labeled unstained sections. Representative images are shown in Figure 4–5. Fluorescent labels incorporated into bone during mineralization appear as thin, bright lines, and this was seen in all

Figure 4–4. Osteoid formation in the proximal tibial metaphysis. Measurements were performed at 100X magnification, on 5 μm thick, undecalcified slides stained with Goldner’s Trichrome. Blue graph shows absolute measures, while red graph shows normalized measures. *p<0.05 as indicated; **p<0.01 compared to all other groups; ***p<0.05 compared to EA, SV.
groups except CH, which showed significant diffuse labeling. This prevented quantification of samples from the CH group, and it was excluded from measurement of bone formation parameters.

Figure 4–5. Proximal tibial metaphysis under dynamic histomorphometry. Calcein green was injected at 12 and 2 days before animal sacrifice, and undecalcified samples were embedded in Spurr and sectioned to 7-μm thickness. They were viewed unstained at 100X magnification under fluorescent microscopy. White arrows show areas of diffuse labeling.

Measurements of dynamic bone formation indices are shown in Figure 4–6. The parameters include: 1) Single label surface (sLS); 2) double label surface (dLS); 3) mineralizing surface (MS, formula = dLS+sLS/2), which represents total labeled surface; 4) interlabel width (Ir.L.Wi), which is the distance between labels; 5) percent mineralizing surface (MS/BS); 6) mineral apposition rate (MAR, formula = Ir.L.Wi/labeling period); and 7) surface referent bone formation rate (BFR/BS, formula = MAR*MS/BS). Parameters 1-4 are absolute measurements, while 5-7 have been normalized.

Results show that CL is increased compared to all other groups in the absolute measurements of bone formation parameters, and exceeds PG in all parameters except dLS. After normalizing for total bone surface, CL remains increased relative to all other groups in MAR and BFR/BS, but is comparable to PG for MS/BS. When compared to OV, CL led to 69% increase in MAR, 35% increase in MS/BS, and 132% increase in BFR/BS. These results indicate that conjugate treatment at low dose significantly increased normalized bone formation parameters compared to OV, and its efficacy is comparable to or exceeding that of PG.

Interestingly, the results of EA treatment were opposite to those of CL. Rather than increasing bone formation compared to OV, EA significantly decreased MS/BS, MAR, and BFR/BS relative to OV. In fact, EA was significantly reduced compared to all other groups for MAR, and all groups except SV for MS/BS and BFR/BS. These results show that bone formation under EA treatment was significantly decreased compared to CL, which indicates that conjugation between EP4a and ALN-LK is critical to the conjugate drug’s anabolic efficacy.
In comparing between O VX and sham-operated groups, OV is comparable to SV in all absolute measurements, but is increased relative to SV in most normalized measurements except MAR. These results indicate that the relative proportion of bone surface undergoing formation is increased in OV compared to SV, and the rate of bone formation is also increased in OV compared to SV. This is consistent with OVX-induced increase in remodeling.

Figure 4–6. Bone formation parameters from dynamic histomorphometry. Blue graphs show absolute measurements; red graph shows normalized measurements. *p<0.05 as indicated; **p<0.05 compared to all other groups; ***p<0.01 compared to all groups except PG; @p<0.001 compared to all groups except SV; *p<0.01 as indicated if exclude CL due to high variability.

4.3.1.2 Resorption

Bone resorption was assessed by staining for osteoclasts using the TRAP enzyme as a marker, and histomorphometric analysis was performed on TRAP-stained, decalcified sections of the proximal tibial metaphysis. Representative images are shown in Figure 4–7. Positive staining for TRAP appears red or pink in color, and this was seen at the growth plates of all treated groups. In addition, positive TRAP staining was also seen in the metaphyseal secondary spongiosa of SV, OV, and PG groups. However, the majority of “osteoclast-like” cells in the metaphysis of conjugate-treated groups, and some of the “osteoclast-like” cells in the metaphysis of EA, did not stain positive for TRAP. Instead, these cells were purple or blue in color. Nevertheless, they did exhibit several other characteristics of osteoclasts: 1) They were large in size relative to other surrounding cells; 2) they were attached to the bone surface; 3) they were rounded in shape and appeared to “spread out” along the bone; and 4) they displaced other surrounding marrow cells. Although these cells do not express the TRAP enzyme conventionally used to identify osteoclasts, they are considered to be “osteoclast-like” in the current analysis based on their secondary characteristics. Nonetheless, to more definitely establish whether these cells are osteoclasts, additional histochemical staining should be performed, such as staining for calcitonin receptors or RANK.
Figure 4–7. Representative images of proximal tibial metaphysis. Samples were decalcified, embedded in wax, and cut into 7-μm thin sections. They were stained for TRAP and viewed under light microscopy at 200X magnification. A) Growth plate region. B) Metaphyseal secondary spongiosa on the same sample slide as A. Black arrows indicate TRAP-positive osteoclasts (red/pink); red arrows indicate osteoclast-like cells (purple). All images are of the same scale.

In order to distinguish between cells that are confirmed to be osteoclasts based on their positive TRAP stain and those that are osteoclast-like based on their secondary characteristics, they were quantified separately in the analysis. Osteoclasts were classified as TRAP-positive (red/pink color) and TRAP-negative (purple/blue color). A further parameter was created to account for the total number of “osteoclast-like” cells, which include both TRAP-positive and TRAP-negative cells. Both the number of cells and their coverage of bone surface were measured.

Results for osteoclast number are presented in Figure 4–8. Measurements for both absolute number (N.Oc) and normalized number (N.Oc/BS) are shown, with N.Oc referring both to confirmed osteoclasts expressing TRAP and those that are considered osteoclast-like based on their secondary characteristics. Only TRAP-positive osteoclasts were found in the control groups (SV, OV, PG), while TRAP-negative, osteoclast-like cells were seen in the conjugate-treated groups as well as EA.

In comparing SV and OV, the results showed that SV was increased relative to OV in TRAP-positive N.Oc. However, this was due to the greater total bone surface in SV rather than a higher rate of turnover, as OV was increased relative to SV after normalizing for bone surface. This is consistent with the increased remodeling seen in OVX-induced bone loss.
Conjugate treatment decreased the TRAP-positive N.Oc relative to other groups including OV, but this is not significant for CH. However, after normalizing for total bone surface, both CL and CH significantly decreased TRAP-positive N.Oc/BS relative to all other groups. In contrast, conjugate treatment dose-dependently increased TRAP-negative N.Oc relative to other groups, such that the total N.Oc is comparable across all groups except CH, which is significantly increased. Interestingly, the dose-dependent effect in conjugate-induced increase in TRAP-negative N.Oc disappears after normalizing for total bone surface, such that both CL and CH led to comparable N.Oc/BS. This suggests that, despite the 3X dose difference between CH and CL, their effects on tissue-level remodeling are comparable.

Unlike those in conjugate-treated groups, the majority of osteoclasts in EA did express the TRAP enzyme, and the number of TRAP-positive osteoclasts in this group was comparable to PG for both absolute and normalized measurements. However, some of the cells in EA were also TRAP-negative, and were considered osteoclast-like based on their secondary characteristics. The TRAP-negative N.Oc and N.Oc/BS were both much lower in EA than in CL, indicating that the suppression of TRAP expression in these osteoclast-like cells is dependent upon the conjugation between the EP4a and ALN-LK components.
Osteoclast surface is a measure of the amount of bone surface covered by osteoclasts, and provides information about the extent of bone surface undergoing resorption. Results for osteoclast surface are shown in Figure 4–9, and measurements for both absolute surface (Oc.S) and normalized surface (Oc.S/BS) are presented. Oc.S refers to bone coverage by TRAP-expressing osteoclasts as well as by cells that do not express TRAP, but that are osteoclast-like based on their secondary characteristics.

In examining results for SV and OV, SV exhibited greater absolute osteoclast surface due to its higher total bone surface relative to OV. However, after normalizing for bone surface, OV shows greater Oc.S/BS compared to SV, which is consistent with elevated bone turnover seen in OVX-induced bone loss.

Conjugate treatment did not significantly alter absolute measures of TRAP-positive Oc.S relative to other treated groups with OVX-induced bone loss, but did significantly decrease normalized measures of Oc.S/BS relative to all other groups. In contrast, conjugate treatment resulted in dose-dependent increase in TRAP-negative Oc.S compared to other groups, which led to dose-dependent increase in total Oc.S. However, similar to the results for osteoclast number, this dose-dependent effect also disappears after normalizing for total bone surface. Consequently, both CL and CH resulted in comparable Oc.S/BS, suggesting that the effects of both doses on bone remodeling at the tissue level were comparable despite CH being 3X higher than CL.

In examining osteoclast surface for EA, the results follow a similar pattern as those of osteoclast number. While mostly TRAP-positive, some of the cells covering bone surface in EA were also TRAP-negative and exhibiting osteoclast-like secondary characteristics. Both Oc.S and Oc.S/BS

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**Figure 4–9. Osteoclast surface measured in TRAP-stained decalcified histomorphometry.** Measurements include TRAP-positive (TRAP+), TRAP-negative (TRAP-), and total number. Blue graphs show absolute measurements [Oc.S], while red graphs show measurements normalized for total bone surface (Oc.S/BS). *p<0.05 as indicated; **p<0.05 compared to all other groups. ***p<0.01 compared to EA, PG, SV if exclude OV due to high variability.
were significantly lower in EA compared to CL, suggesting that the induction of TRAP-negative osteoclast surface in CL was dependent upon conjugation between EP4a and ALN-LK.

4.3.2 Microarchitecture

4.3.2.1 Three-Dimensional Measures

Trabecular microarchitecture of the lumbar vertebrae was assessed using microCT, and the results are shown in Figure 4–10. The percent bone volume (BV/TV) was significantly decreased in OV relative to SV, which confirms OVX-induced bone loss in the disease model. In addition, the trabecular number (Tb.N) was also decreased in OV with simultaneous increase in trabecular separation (Tb.Sp), while trabecular thickness (Tb.Th) was unchanged. These results are consistent with the microarchitectural changes following OVX, in which bone loss occurs primarily through reduction in trabecular number rather than thickness\textsuperscript{172,275}.

Relative to OV, conjugate treatment dose-dependently increased the trabecular bone volume as well as trabecular number, which decreased the trabecular separation. However, the trabecular thickness was not changed, suggesting that the conjugate-induced increase in bone volume was due to formation of new trabeculae rather than thickening of existing ones. In fact, CL and CH restored the OVX-induced bone loss by 63% and 120%, respectively, with CH exceeding sham levels. In addition, despite the lack of statistical significance in the bone volume and trabecular number results for CL, these results are comparable to both EA and PG. Interestingly, the vertebral architectural parameters are comparable between CL and EA despite differences in their tissue-level bone formation parameters.

![Figure 4-10. Trabecular microarchitecture. L6 vertebrae were imaged using microCT at 11.6-μm voxel size, and the secondary spongiosa was analyzed. *p<0.05 as indicated; **p<0.01 compared to all other groups; ***p<0.01 compared to OV, SV if exclude CH due to high variability; @p<0.05 compared to OV.](image)
Both osteoporotic bone loss in humans and OVX-induced bone loss in rats are characterized by the conversion of trabeculae from a plate-like to rod-like structure due to elevated bone turnover, which eventually leads to loss of trabecular connectivity. Thus, assessment of the structure and connectivity of cancellous bone provides important information about the effects of treatment on trabecular bone architecture. In particular, structure model index (SMI) is an estimate of the plate-like or rod-like nature of trabecular bone, and is 0 for ideal plates and 3 for ideal rods. In addition, connectivity density is a measure of the number of trabecular connections per unit volume. These structural indices were obtained from microCT data, and the results are shown in Figure 4–11.

![Figure 4–11. Structural indices derived from microCT images of vertebrae. Trabecular secondary spongiosa of the L6 vertebrae were used to obtain these results. *p<0.05 as indicated; **p<0.001 compared to all other groups; ***p<0.01 compared to all groups except EA.](image)

In examining the results for SMI, it is interesting to observe that CH is not only much lower than all other groups, but that it is actually negative. This is due to the technical constraints of the algorithm used to derive the SMI, which is influenced by the number of enclosed cavities in the trabecular architecture. This is more prevalent in instances where the bone volume fraction (BV/TV) is high, resulting in a negative measure for SMI. All other groups show positive SMI between 0 and 3, as expected.

The SMI of OV is significantly increased compared to all groups except EA, which suggests that the structural characteristics of the trabeculae are more rod-like in OV than in SV, CL, CH, or PG. In addition, the connectivity density of OV is also significantly decreased compared to SV, indicating that the density of trabecular connections was also reduced due to OVX. These results are consistent with the osteoporotic degradation of bone, which results in a transition from plate-like to rod-like state and decreased trabecular connectivity.

In contrast, the treated groups including CL, EA, and PG showed comparable SMI relative to SV, indicating that the OVX-induced structural transition has been halted as a result of treatment. In addition, while CL did not produce a significant increase in connectivity density relative to OV, CH exceeded all other groups. Together, these results suggest that the microarchitectural effects of
conjugate treatment also led to structural changes in trabecular bone, with increased connectivity and resulted in a shift towards a more plate-like state.

4.3.2.2 Two-Dimensional Measures

Microarchitecture was also assessed via undecalcified histomorphometry, which measures 3-dimensional structural indices by extrapolating from a 2-dimensional plane using the principles of stereology. Results are shown in Figure 4–12. Trabecular bone volume (BV/TV) and number (Tb.N) are both decreased in OV compared to SV, along with increased trabecular separation (Tb.Sp). However, trabecular thickness is unchanged as a result of OVX. This is consistent with the 3D measurements obtained from microCT, and confirms OVX-induced bone loss in the secondary spongiosa.

There is a dose-dependent increase in BV/TV and Tb.Th under conjugate treatment, which restored the OVX-induced bone loss to sham levels. Tb.Sp is dose-dependently decreased in conjugate-treated groups, and is decreased compared to all groups except SV. Although both CL and CH are comparable to PG in Tb.N, they are both increased in BV/TV and decreased in Tb.Sp compared to PG. These findings differ from the microCT results presented earlier, which may be due to differences in the sampling site as well as the measurement techniques employed.

![Figure 4–12. Trabecular structural indices for proximal tibial metaphysis. **p<0.05 compared to all other groups. The alphabetical symbols for statistical comparisons are described in the following table:](attachment:image.png)

<table>
<thead>
<tr>
<th></th>
<th>BV/TV</th>
<th>Tb.Th</th>
<th>Tb.Sp</th>
<th>Tb.N</th>
</tr>
</thead>
<tbody>
<tr>
<td>a: p&lt;0.05 compared to all other groups except CL</td>
<td>f: p&lt;0.05 compared to CL, CH</td>
<td>k: p&lt;0.05 compared to EA, PG, OV</td>
<td>o: p&lt;0.05 compared to all groups except PG</td>
<td></td>
</tr>
<tr>
<td>b: p&lt;0.05 compared to all other groups except EA</td>
<td>g: p&lt;0.01 compared to PG, CL, CH</td>
<td>l: p&lt;0.05 compared to all groups except SV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c: p&lt;0.05 compared to all other groups except SV</td>
<td>h: p&lt;0.05 compared to all groups except PG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d: p&lt;0.05 compared to SV, CL, CH</td>
<td>i: p&lt;0.05 compared to PG, CL, CH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e: p&lt;0.05 compared to all other groups except EA</td>
<td>j: p&lt;0.05 compared to OV, EA, SV</td>
<td>m: p&lt;0.05 compared to all other groups except EA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.3 Bone Mineral Density

4.3.3.1 Dual-Energy X-Ray Absorptiometry

Dual-energy X-ray absorptiometry (DEXA) imaging was performed on the lumbar vertebrae to assess bone densitometry, and the results are shown in Figure 4–13. Bone mineral content (BMC) measures the amount of bone mineral in the vertebral body, while areal bone mineral density (aBMD) measures the density of bone mineral per unit area. Bone area measures the area of mineralized bone in the coronal cross-section of the vertebral body.

![Figure 4–13. DEXA results for lumbar vertebrae. L5 and L6 vertebrae were imaged under DEXA, and the two measurements were averaged for each animal. *p<0.001 as indicated; **p<0.01 compared to all other groups; ***p<0.01 compared to SV,OV,CH.](image)

All vertebrae were of statistically comparable size in coronal cross-sectional area except those in CH, which were significantly larger than the others. Conjugate treatment led to dose-dependent increase in BMC and aBMD relative to OV, with statistical significance at both doses. In addition, while the low dose conjugate resulted in comparable BMC and aBMD relative to both EA and PG, the high dose group was significantly increased compared to all other groups. While BMC is a reliable measure of the total quantity of bone mineral within the specimen, the increased aBMD may not be an accurate reflection of the true volumetric BMD given DEXA’s inability to detect specimen depth.

4.3.3.2 Micro-Computed Tomography

Bone mineral density of the L6 vertebrae was also assessed via micro-computed tomography (microCT), which provides information about the sample’s volumetric bone mineral density (vBMD) and is thus more accurate than DEXA. The vBMD of both trabecular secondary spongiosa and the vertebral cortical wall was measured, and the results are shown in Figure 4–14.
Figure 4–14. Volumetric bone mineral density (vBMD) via microCT. L6 vertebrae were imaged via microCT at 11.6 μm voxel size. A) vBMD of the secondary spongiosa. Blue graph shows vBMD of whole trabecular tissue volume including marrow space, while green graph shows vBMD of trabeculae only. B) vBMD of the vertebral cortical wall. *p<0.001, ***p<0.01 as indicated; **p<0.05 compared to all other groups; @p<0.05 relative to OV.

In examining whole-tissue trabecular vBMD (blue graph), OV was significantly decreased relative to SV, indicating a reduction of volumetric BMD as a result of OVX-induced bone loss. However, this loss was partially or completely restored under conjugate treatment, which dose-dependently increased vBMD compared to OV. In particular, CL recovered 51% of the vBMD lost after OVX, while CH exceeded sham levels. In comparison, PG also resulted in 55% recovery of the OVX-induced loss in vBMD, while EA did not significantly alter vBMD compared to OV. This indicates that the volumetric BMD was increased under conjugate treatment, with the high dose exceeding all other groups, and the low dose achieving partial recovery of the bone loss due to OVX, which is comparable to PG control.

Given the changes seen in total tissue vBMD of the trabecular volume, further analysis was performed to determine whether these changes could be attributed to differences in the densities of individual trabeculae. Results show that there were no group-level significant differences in the density of trabecular bone excluding marrow space, indicating that the vBMD changes seen in total tissue were due to structural differences among groups. Moreover, analysis of the cortical wall surrounding the vertebral body indicates that there were also no significant differences across groups.

4.3.4 Mechanical Properties

Vertebral compression was used to evaluate the behavior of L6 vertebrae under compression, and mechanical indices were determined corresponding to the bone’s strength, resistance to load, deformation under load, and ability to absorb energy (work to failure/toughness). Both extrinsic and intrinsic properties were measured. Extrinsic properties incorporate contributions from the architectural makeup of the bone, and are specimen-specific. In contrast, intrinsic properties characterize the bone tissue irrespective of sample geometry.
Results for bone strength and resistance to load are shown in Figure 4–15. Bone and tissue strength are represented by the ultimate load and ultimate stress, respectively. Similarly, the bone and tissue resistance to load are represented by the stiffness and modulus measurements.

There were no statistically significant differences in the mechanical properties of SV and OV despite the changes in remodeling and architecture seen earlier. However, conjugate treatment did result in dose-dependent increase compared to OV for ultimate load, which is a measure of the maximum force that the sample could safely withstand. Nevertheless, after normalizing for sample geometry, ultimate stress was comparable across groups. This suggests that the changes in ultimate load were due to differences in sample architecture and geometry rather than the bone tissue itself.

**Figure 4–15. Vertebral compression results using L6 vertebrae.** Blue graph shows extrinsic properties that are architecture-dependent, and red graph shows intrinsic properties that have been normalized for sample geometry. *p<0.05 as indicated; **p<0.05 compared to all other groups; ***p<0.05 compared to all groups except PG.

Stiffness and modulus measure the ability to resist mechanical loading. Stiffness incorporates geometrical contributions of the bone specimen, while modulus refers to the bone tissue only. Stiffness was not significantly different across groups except in CH, which was decreased. Similarly, Young’s modulus was also decreased in CH, but was comparable among the others. These results indicate that, while low dose conjugate treatment did not significantly alter the stiffness and modulus of the vertebrae, the high dose treatment compromised the vertebral resistance to applied load, which affected both the specimen and the bone tissue.

Results for work to failure and toughness are shown in Figure 4–16, and represent the ability of the vertebrae to safely absorb energy. Work to failure is a property of the specimen and includes contributions from sample geometry, while toughness is tissue property. Work to failure was increased in CH compared to the others, but the material toughness was unchanged across groups. This suggests that the increase in work to failure was due to the larger vertebral size (also shown in Figure 4–16) and other architectural differences in CH rather than changes in bone tissue.
In addition, failure displacement and strain were also measured, and represent the bone’s ability to undergo deformation under load. Displacement is a specimen-specific measure, while strain corresponds to the tissue property. CH is significantly increased compared to all other groups in deformation of the specimen, while it is only increased compared to SV and OV for the material deformation. As the bone strength is also increased in CH due to its larger size, this suggests that there is a corresponding increase in the ability of these vertebrae to deform under load. This is also true for the tissue material, suggesting that the ductility is increased in the tissue material of the vertebral trabecular and cortical bone.

![Graphs showing work to failure, failure displacement, cross-sectional bone area, toughness, and failure strain](image)

Figure 4–16. Vertebral compression results using L6 vertebrae. Blue graph shows extrinsic properties that are geometry-dependent, and red graph shows intrinsic properties that have been normalized for sample geometry. The cross-sectional bone area was measured using microCT. *p<0.05 as indicated; **p<0.05 compared to all other groups; ***p<0.05 compared to SV, OV, EA, PG.

### 4.3.5 Degree of Mineralization

The degree of mineralization of trabecular bone was measured using BSE, and the results are shown in Figure 4–17. OV was decreased compared to SV in maximum grey value, which is a measure of the peak mineralization in bone. In addition, OV was also decreased relative to SV in full width at half max (FWHM), which is a measure of the spread of mineralization. Given the OVX-induced bone loss seen in other experiments earlier, these results suggest that the trabecular bone loss in OV also resulted in decreased peak mineralization in the bone. Consequently, the overall reduction in bone mineral may have also narrowed the spread in mineralization for OV, as there is less mineral to spread around.

Among groups with OVX-induced bone loss, conjugate treatment did not significantly affect the peak mineralization in trabecular bone, but did result in dose-dependent increase in FWHM compared to OV (although not significant at the low dose). In addition, CH was also significantly increased compared to PG control. This increase in the spread of mineralization under conjugate treatment suggests that the elevation in trabecular bone remodeling not only affected mineralization
on the surface of trabeculae, but also impacted the mineralization of the cancellous structure as a whole. In addition, conjugate treatment also seems to recover the spread of mineralization in OV towards sham levels, which is not seen in other groups.

![Graph showing trabecular maximum grey value and trabecular full width half max height (FWHM)](image)

**Figure 4–17. Degree of mineralization results for trabecular bone.** Spurr-embedded left tibia were scanned under BSE at 100X magnification, and the secondary spongiosa of the proximal tibial metaphysis was analyzed. The maximum grey value is a measure of peak mineralization, while FWHM is a measure of the spread of mineralization. *p<0.05 as indicated; ***p<0.05 compared to OV, EA, PG.

### 4.4 Effects on Cortical Bone

This section will discuss the effects of conjugate treatment on cortical bone. To provide an overview of the results, representative images of the tibial diaphysis are shown in [Figure 4–18]. No obvious differences are visible between the OV and SV groups, suggesting that OVX-induced bone loss within the 12-week study period has not significantly impacted cortical bone. However, conjugate treatment did result in dose-dependent increase in cortical porosity compared to OV, with the high dose group also exhibiting significant endocortical and periosteal bone formation. In fact, the marrow cavity appears to be entirely occluded in CH. In contrast, neither EA nor PG appeared to produce any prominent changes in cortical bone compared to OV, although there may be a slight increase in cortical porosity for PG. These effects were further characterized in various ex vivo experiments, the results of which will be presented in the following sections.

![Representative images of the tibial diaphysis](image)

**Figure 4–18. Representative images of the tibial diaphysis.** Spurr-embedded tibia samples were scanned under BSE at 100X magnification, and 1-mm section of the diaphysis is shown. White arrows indicate periosteal bone formation.
4.4.1 Tissue-Level Remodeling

Since coronal sections of the tibia had been created for evaluation of trabecular bone remodeling in the proximal tibial metaphysis, the same sections were also used to examine cortical bone turnover in the tibial diaphysis. Remodeling was assessed in the cortex as well as the periosteal and endosteal surfaces. However, given the extensive endocortical bone formation in CH, it was impossible to clearly define endosteal boundaries for measuring the different regions, and this group was excluded from these analyses.

Results for intracortical bone formation parameters are shown in Figure 4–19. There were no significant differences among the groups for percent osteoid volume (OV/BV). In addition, there were also no significant differences between OV and SV for any of the dynamic bone formation parameters, which included volume-referent mineralizing surface (MS/BV), mineral apposition rate (MAR), and volume-referent bone formation rate (BFR/BV). However, CL did lead to significant increases in all dynamic bone formation parameters relative to both OV and SV, and is comparable to PG. This suggests that, while OVX-induced bone loss did not lead to significant changes in cortical bone remodeling within the 12-week study period, both low dose conjugate treatment and PGE$_2$ resulted in elevated cortical bone remodeling compared to OV and SV.

**Figure 4–19. Intracortical bone formation parameters from histomorphometric analysis of tibial diaphysis.** Spurr-embedded, undecalcified tibia were sectioned coronally and stained with Goldner’s Trichrome for the measurement of OV/BV, and viewed unstained under fluorescent microscopy for the measurement of dynamic bone formation parameters (MS/BV, MAR, BFR/BV). **p<0.01 compared to all other groups; ***p=0.002 compared to SV, EA.

Prior to quantitative analysis, bone formation on the periosteal and endosteal surfaces was examined visually for osteoid and dynamic labeling, and representative images are shown in Figure 4–20. Differences were not observed among groups based on this visual assessment. However, this may not be an accurate reflection of the physiological effects due to challenges in the technique, which will be explained below.
## 4.4.2 Microarchitecture

Cortical bone microarchitecture in the femoral mid-diaphysis was evaluated using microCT, and 3D models are shown in Figure 4–22. There appears to be no obvious changes between SV and OV, suggesting that OVX-induced bone loss has not resulted in significant alterations to femoral mid-diaphyseal cortical bone. However, conjugate treatment did lead to a dose-dependent increase in endocortical woven bone formation compared to OV, with CH resulting in complete occlusion of the
marrow region. Moreover, CH also exhibited significant periosteal bone formation. Further experiments were performed to characterized these effects.

![Representative 3D models of femoral mid-diaphysis with color-coding for density.](image)

**Figure 4–22.** Representative 3D models of femoral mid-diaphysis with color-coding for density. Left femurs were scanned under microCT at 11.6 μm, and a 1-mm volume was analyzed in the mid-diaphysis.

Geometrical parameters were measured using microCT, and results are shown in **Figure 4–23.** The cortical diameters were measured along the medial-lateral (dML) and anterior-posterior (dAP) directions, along with the cross-sectional area. The principle moment of inertia along the ML axis (MMI$_{\text{min}}$) was also determined, which is a measure of the resistance of the structure to bending, and larger MMI$_{\text{min}}$ allows the bone to better resist bending. Results show that both the AP and ML diameters are increased in CH relative to the others, along with increases in cross-sectional area and MMI$_{\text{min}}$. In contrast, no significant differences were found in the cross-sectional geometry of the other groups.

![Geometrical parameters of femoral mid-diaphysis measured via microCT.](image)

**Figure 4–23.** Geometrical parameters of femoral mid-diaphysis measured via microCT. *p<0.05 compared to EA, PG, SV; **p<0.05, ***p<0.001 compared to all other groups.
Cortical thickness and porosity in the femoral mid-diaphysis were also measured. To minimize contamination of the thickness and porosity results due to endocortical woven bone, the marrow cavity was excluded from the region of interest (ROI), and only the femoral cortex was measured. Since the occlusion of the marrow cavity in CH prevented the clear definition of the endocortical boundaries, the ROI had to be approximated in measuring cortical porosity (Figure 4–24, C), and CH was excluded from measurements for cortical thickness. Results are shown in Figure 4–24. Both CL and CH led to significantly increased cortical porosity compared to other groups, with the porosity in CH exceeding others by over 10X. CL also resulted in significant increase in cortical thickness compared to all other groups (p<0.05).

![Cortical thickness and porosity measured using microCT.](image)

**Figure 4–24.** Cortical thickness and porosity measured using microCT. Region of interest (ROI) was defined to exclude endocortical cavity to minimize contamination of the results by endocortical woven bone. A. Representative cross-sectional images of femoral mid-diaphysis. B. The ROI for CH was approximated given its extensive endocortical bone formation (red). *p<0.001 compared to all other groups; **p<0.05 compared to SV, OV, EA, PG.

The volume and density of endocortical bone were quantified using microCT. Since endocortical bone formation was found only in the non-vehicle-treated groups, SV and OV were excluded from these measurements. Results are shown in Figure 4–25. The endocortical bone in CL was comparable to both EA and PG in volume and vBMD. In contrast, CH was significantly increased compared to all other groups in endocortical bone volume, and was also increased in vBMD compared to EA.
4.4.3 Bone Mineral Density

4.4.3.1 Dual-Energy X-Ray Absorptiometry

Cortical bone densitometry was measured using dual-energy X-ray absorptiometry (DEXA), and the results are shown in Figure 4–26. Although the bone mineral content (BMC) was comparable between SV and OV, the areal bone mineral density (aBMD) was decreased in OV compared to SV. This seems to suggest that there was a reduction in cortical BMD as a result of OVX, but it should be noted that, since DEXA cannot detect specimen depth, this decrease may not be a true reflection of the volumetric BMD in the specimen.

Examination of conjugate treatment shows that there is a dose-dependent increase in both BMC and aBMD relative to OV. In addition, both EA and PG groups also exhibited elevated BMC and aBMD relative to OV. While the BMC result suggests that the total mineral quantity in bone is dose-dependently increased under conjugate treatment, it should be noted that the bone sizes are also increased, particularly in CH. Thus the apparent differences in aBMD may be due to changes in specimen size rather than true mineral density, and should be interpreted with caution.

Figure 4–26. DEXA results for femur. Left and right femurs were imaged under DEXA, and the two measurements were averaged for each animal. *p<0.05 as indicated; **p<0.001, ***p<0.05 compared to all other groups; @p<0.001 compared to OV.
4.4.3.2 Micro-Computed Tomography

Volumetric BMD (vBMD) of cortical bone was measured in the femoral mid-diaphysis using micro-computed tomography (microCT), and the results are shown in Figure 4–27. All groups were comparable in vBMD except CH, which was significantly decreased relative to the others.

![Volumetric Bone Mineral Density (vBMD) graph](image)

**Figure 4–27. Volumetric BMD of cortical bone.** Left femurs were imaged under microCT at 11.6 μm, and a 1-mm volume in the mid-diaphysis was analyzed. To exclude the influence of endocortical woven bone on measurements, the ROI included only the femoral cortex. The ROI for CH was approximated due to its extensive endocortical bone formation (B, red). **p<0.005 compared to all other groups.

4.4.4 Mechanical Properties

4.4.4.1 Three-Point Bending

The mechanical properties of cortical bone were assessed in three-point bending at the femoral mid-diaphysis. Similar to vertebral compression earlier, both architecture-dependent specimen properties (*extrinsic*) and geometry-independent material properties (*intrinsic*) were measured. These mechanical properties correspond to the bone’s strength, resistance to load, and ability to absorb energy.

Results bone strength (ultimate load and ultimate stress) and resistance to load (stiffness and modulus) are shown in Figure 4–28. There were no significant differences between SV and OV in any of the measures, indicating that OVX-induced bone loss has not impacted the mechanical properties of cortical bone during the 12-week study period. In addition, the ultimate strength and stiffness are comparable across all groups, but the ultimate stress and modulus are decreased in CH. These results suggest that conjugate treatment did not modify the specimen strength or resistance to load, but decreased the strength and resistance to load of the tissue material.
The bone's ability to absorb energy (work to failure and toughness) and deformation under load (ultimate displacement and strain) was not found in work to failure or toughness. However, while failure displacement was also comparable among groups, failure strain was increased in CH. These measures reflect the bone's ductility, and are important indicators of the bone's susceptibility to fractures following trauma. Given the increased size of CH, there should also be a corresponding increase in work to failure as well as failure displacement. However, this is not the case, suggesting that the specimens in CH are compromised in energy absorption as well as deformation under load. Nonetheless, CH was increased in the material deformation relative to other groups, suggesting that its bone tissue is more ductile. These results suggest that the architectural changes in the bone specimens of CH led to increased material ductility without increasing the specimen ductility, while the energy absorption is unchanged for both the sample and the material.
4.4.4.2 Femoral Neck Fracture

The mechanical properties of the femoral neck were measured in a fracture test, and the results are shown in Figure 4–30. Due to the irregularity of its geometry and the unpredictability of the direction of fracture, material properties could not be derived for this experiment, and only specimen properties were measured. There were no significant differences between SV and OV in any of the measured parameters, suggesting that OVX-induced bone loss within the 12-week study period has not yet significantly impacted the femoral neck. However, both the ultimate load and work to failure of CL was increased relative to SV, while its stiffness was unchanged compared to all other groups except CH. This suggests that the femoral neck bone of CL is both stronger and able to absorb more energy compared to sham, but its resistance to load was not altered. The failure displacement is not changed across groups, suggesting that the ability of the femoral neck to deform under load is not altered as a result of conjugate treatment.

Interestingly, CH led to decreased ultimate load and work to failure relative to CL, and its stiffness was reduced compared to PG. This suggests that the elevated remodeling in CH detrimentally affected the mechanical properties of the femoral neck relative to CL, such that the bone is both weaker and less capable of energy absorption, and the resistance to load is compromised relative to PG control.

Figure 4–30. Femoral neck fracture results using left femurs. *p<0.05 as indicated; **p<0.01 compared to PG, EA, CL.

4.4.5 Degree of Mineralization

As in trabecular bone, the degree of mineralization was also assessed in the cortical bone of the tibial shaft via BSE. Results are shown in Figure 4–31. As mentioned earlier, the maximum grey value is a measure of the peak mineralization, while the full width at half max (FWHM) is a measure of the
spread of mineralization. There were no significant differences between SV and OV in either of these measures. In addition, there were also no significant differences across all other groups for maximum grey value, while only CH was increased relative to others for FWHM. This indicates that the elevated remodeling in CH led to an overall change in the spread of mineralization of cortical bone, and suggests that conjugate treatment at high dose impacted the overall mineralization state of the entire cortical structure. However, these effects were not observed for low dose conjugate treatment.

![Cortical Maximum Grey Value and Cortical Full Width Half Max Height (FWHM)](image)

**Figure 4–31. Degree of mineralization results for cortical bone.** Spurr-embedded left tibia were scanned under BSE at 100X magnification, and the tibial diaphysis was analyzed. The maximum grey value is a measure of peak mineralization, while FWHM is a measure of the spread of mineralization. **p<0.001 compared to all other groups.**

### 4.4.6 Marrow Effects

The marrow region was qualitatively examined using the tibial sections for undecalcified histomorphometry. Representative images are shown in Figure 4–32. Large, circular white cells represent adipocytes, and OV exhibits increased number of adipocytes relative to SV, which is consistent with OVX-induced increase in marrow adiposity. Interestingly, low dose conjugate treatment appears to have decreased the OVX-induced marrow adiposity, as the number of adipocytes in CL seems decreased compared to OV. In fact, this effect appears the most prominent in CL compared to both EA and PG, as neither of the other two groups shows any obvious decrease in marrow adipocytes relative to OV. However, the marrow region of the high dose group appears permeated with mineralized bone, and the marrow cells show little trace of adipocytes. The presence of mineralized bone in the marrow region of CH indicates abnormal response due to excessive bone formation.
4.4.7 Effects in High Dose Group

Animals in CH exhibited tremendous elevation in cortical bone remodeling in response to conjugate treatment. The cortical porosity is increased over 10X compared to CL, while endocortical bone formation resulted in complete occlusion of the marrow cavity in most animals. However, undecalcified histomorphometry showed that there are also some differences in tissue-level response among animals in CH, particularly with respect to periosteal bone formation. To illustrate, images from 8 out of 9 animals are shown in Figure 4–33. The 9th animal was excluded due to space constraints, and the response is similar to CH5 in Figure 4–33.

Images show the endocortical marrow cavity and periosteal bone surface from undecalcified histomorphometry as well as cross-sections of the femoral mid-diaphysis from microCT. Both Goldner’s Trichrome-stained slides and unstained, fluorescently labeled slides are presented. All but 2 of the animals had entirely occluded marrow cavities due to extensive endocortical bone formation (all except CH5 and CH9). Approximately 5 out of the 9 animals showed both osteoid formation and dynamic labeling on the periosteal surface (CH4, 5, 6, 7, 8, 9). In contrast, 2 animals exhibited little osteoid formation (CH1 and CH3), with CH1 also showing little dynamic labeling while CH3 has significant and diffuse fluorescent labeling despite exhibiting little osteoid formation. Interestingly, another animal showed significant osteoid formation on the bone surface, but did not have much dynamic labeling (CH2). These different physiological effects in CH suggest that there are variations in tissue-level periosteal bone formation in response to conjugate treatment despite most of the animals exhibiting significant endocortical bone formation, as seen in the microCT images.

Comparison of the microCT images shows that 3 animals have relatively “intact” cortical walls with woven endocortical bone filling the marrow cavity (CH1, 4, 8, white arrows). Though some porosity is visible as dark spots within the brighter color of the cortex in these animals, the cortical...
structure is easily distinguishable from the woven bone in the marrow region. In contrast, the periosteal cortical boundary of 4 other animals appears to be “smeared” with significant periosteal bone formation (CH2, 3, 6, 7), such that the cortical structure is no longer clearly distinguishable from the endocortical and periosteal bone formation. Instead, their cortex appears to be more blended with other regions, and this is the most prominent in CH6. The only animal with noticeable marrow space is CH5 (and CH9 which is not shown), and its cortical wall appears to be mostly intact with some slight blending towards the periosteal surface.

On their periosteal surfaces, none of the animals showed the thin lines of fluorescent labeling typically expected of normal mineralizing surfaces. Instead, the animals in CH exhibited either tremendous diffuse labeling or no labeling at all, indicating that the mineralization is either occurring with extraordinary rapidity or not at all. These observations suggest there may be variations in the physiological response of these animals to treatment, which should be investigated further.
Figure 4–33. Effects of high dose conjugate treatment. 8 out of 9 animals in the CH group are shown. A. Endocortical marrow cavity in Goldner’s Trichrome stained slides, viewed under 200X magnification. B. Periosteal surface in Goldner’s Trichrome stained undecalcified slides of tibial shaft, 200X magnification. Osteoid is stained red while mineralized bone is stained green. C. Periosteal surface in dynamically labeled, unstained tibial shaft, 100X magnification. Fluorescent labels indicate mineralizing surfaces. D. MicroCT cross-sectional images of femoral mid-diaphysis, 11 μm voxel size. White arrows denote “intact” cortical wall, while red arrows denote “smearing” of cortical wall.
5 Discussion

This discussion will be organized into several sections. The study objectives will be reviewed first, followed by a discussion of the validity of OVX as an in vivo model of postmenopausal osteoporosis. The subsequent sections will focus on results of conjugate treatment, starting with the tissue-level effects and a discussion of the conjugate drug’s potential mechanism of action. These findings will then be used to interpret the conjugate’s effects on architecture and mechanical properties, in order to determine whether the tissue-level effects translate into macroscopic changes in bone. Finally, the side effects of treatment will be discussed to evaluate whether the design of the conjugate drug successfully alleviated the side effects associated with systemic administration of PGE$_2$ and EP4a. Given the large quantity of data presented, this discussion will end with a section that summarizes the findings and presents issues to be considered for further development of the conjugate drug.

To facilitate the discussion, the main experimental results from this study are summarized and presented in Table 5–1, after reviewing the study objectives.

5.1 Review of Study Objectives

This study aimed to determine the effects of a new conjugate drug in treating postmenopausal osteoporosis using a rat model of the disease. Since osteoporosis is characterized by excessive bone loss and increased fracture risk, an important therapeutic objective is to promote new bone formation. Prostaglandin E$_2$ (PGE$_2$) is an endogenous ligand known to have bone-anabolic effects in vivo$^{169}$, but its use in clinical therapy is hindered by significant side effects when administered systemically$^{179}$. Previous studies have shown that PGE$_2$ acts on bone primarily through the EP4 receptor in osteoblasts$^{200}$, and stimulates osteoblast differentiation as well as osteoblastic induction of osteoclastogenesis$^{186,192}$. Moreover, synthetic agonists of the EP4 receptor have also been developed that promote bone formation and improve bone strength in rat models of osteoporosis, and are highly selective in binding with the EP4 receptor$^{213-215}$. However, these agonists still produce side effects when administered systemically, which hampers their clinical utility$^{200}$.

Bisphosphonates (BP) are a class of synthetic compounds that have long been employed as anti-resorptive agents in therapeutic interventions for osteoporosis, as they bind strongly to bone mineral and inhibit critical cellular pathways that interfere with the function and survival of osteoclasts$^{57,58}$. However, their suppression of resorption also eventually decreases formation due to the natural coupling between resorption and formation in bone remodeling. Oversuppression of bone
turnover impairs the natural renewal process required to repair microdamage and maintain healthy bone, which may detrimentally affect the mechanical properties of bone and increase the risk of fracture\textsuperscript{111,112}. This constitutes an important clinical concern in the treatment of osteoporosis, particularly in cases of high dose BP therapy.

Given the bone-targeting ability of BP, our novel approach employs a conjugate drug in which the EP4 agonist (EP4a) is reversibly joined with the BP alendronate (ALN) via a linker molecule (LK) to create an ALN-LK-EP4a conjugate drug. The hypothesized mechanism of action is that the ALN may “deliver” the attached EP4a component to bone sites when the conjugate drug is administered systemically. Once bound to bone, local hydrolytic enzymes can cleave the chemical links and liberate both the EP4a and LK components, leaving only ALN bound to bone. This approach enables the EP4a to be locally delivered to bone sites, which should help to mitigate the side effects associated with its systemic administration. Moreover, the liberated EP4a can promote bone formation while ALN remains bound to bone to inhibit resorption, enabling the drug to have dual-acting effects. A previous study had established the metabolic stability of ALN-LK-EP4a as well as the gradual release of EP4a from the bone-bound conjugate \textit{in vivo}\textsuperscript{221}, setting the stage for the current work to investigate its \textit{in vivo} efficacy.

In order to examine the \textit{in vivo} therapeutic effects of the new drug in treating postmenopausal osteoporosis, an animal model of the disease is required. The ovariectomized (OVX) rat is a well-established animal model that mimics many bone changes occurring in human postmenopausal osteoporosis, such as the pronounced loss of cancellous bone and increased rate of turnover\textsuperscript{51}. It was thus employed as the disease model in this study.

The current study used a curative experimental design to determine treatment effects in animals with established bone loss. Animals were OVX at 3 months of age, and were given 7 weeks to lose bone before being treated for 6 weeks. Rats were sacrificed at 6 months of age, and effects on bone remodeling, density, architecture, mechanical properties, and mineralization were evaluated.

The bone loss duration in this study was selected based on previous research examining the time course of OVX-induced bone loss, which showed that significant bone loss occurs in many sites such as the proximal tibial metaphysis, lumbar vertebrae, and femoral neck after approximately 30 days\textsuperscript{52,54,277,278}. The 7-week initial period thus provides sufficient bone loss within a relatively short time frame, as the current study was not intended to be a long-term examination of treatment effects.
Table 5-1. Summary of experimental findings in this study.

<table>
<thead>
<tr>
<th>Bone Type</th>
<th>Bone Quality Indicator</th>
<th>OV (Relative to SV)</th>
<th>CL and CH (Relative to OV)</th>
<th>EA (Relative to OV)</th>
<th>PG (Relative to OV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular</td>
<td>Remodeling</td>
<td>↑ Turnover</td>
<td>↑ Formation; ↓ TRAP expression, but ↑ TRAP-negative osteoclast-like cells; dose-dependence disappears after normalizing</td>
<td>↓ Formation; did not affect absolute TRAP expression but ↓ normalized TRAP expression; slight ↑ osteoclast-like cells</td>
<td>↑ Formation, but less than CL; did not suppress TRAP expression</td>
</tr>
<tr>
<td></td>
<td>Bone Architecture</td>
<td>↓ Bone volume due to ↓ Tb.N; no effect on Tb.Th; ↓ vBMD</td>
<td>↑ Bone volume, dose-dependent; due to ↑ Tb.N in vertebra but ↑ Tb.Th in proximal tibial metaphysis; ↑ vBMD</td>
<td>No effect on bone volume in vertebra or tibial metaphysis; no effect on vBMD</td>
<td>↑ Bone volume by ↑ Tb.N in vertebra, ↑ Tb.Th in proximal tibial metaphysis; ↑ vBMD</td>
</tr>
<tr>
<td></td>
<td>Mechanical Properties</td>
<td>No difference</td>
<td>Dose-dependently ↑ bone strength without affecting material strength</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Mineralization</td>
<td>↑ Peak mineralization and spread of mineralization</td>
<td>No effect on peak mineralization; dose-dependently ↑ spread of mineralization</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Cortical</td>
<td>Remodeling</td>
<td>No difference</td>
<td>↑ Intracortical remodeling</td>
<td>No effect on intracortical remodeling</td>
<td>↑ Intracortical remodeling</td>
</tr>
<tr>
<td></td>
<td>Bone Architecture</td>
<td>No difference</td>
<td>Dose-dependent effects: ↑ periosteal apposition and bone size; ↑ endocortical bone formation; ↑ cortical porosity; ↓ vBMD only for CH</td>
<td>No effect on endocortical bone formation, cortical porosity, cortical geometry, or periosteal apposition; no effect on vBMD</td>
<td>Slight ↑ in endocortical bone formation; no effect on cortical geometry, periosteal apposition, porosity, or vBMD</td>
</tr>
<tr>
<td></td>
<td>Mechanical Properties</td>
<td>No difference</td>
<td>Dose-dependently ↑ bone strength but ↓ material strength for CH</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>Mineralization</td>
<td>No difference</td>
<td>No effect on peak mineralization; ↑ spread of mineralization for CH</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>
5.2 OVX Model

The effects of OVX in this study were assessed by comparisons between OVX and sham-operated groups that were both given vehicle treatment (OV and SV, respectively), and different aspects of bone quality were evaluated in trabecular and cortical bone.

5.2.1 Trabecular Bone Effects

Since the effects of OVX on the rat skeleton vary depending on skeletal site, the measurement site for trabecular bone remodeling was selected based on sensitivity to estrogen deficiency. The proximal tibial metaphysis exhibits statistically significant bone loss as early as 14 days post-OVX, and achieves 50% bone loss after approximately 30-60 days\textsuperscript{277,279}. This site is more sensitive to OVX-induced bone loss than either the lumbar vertebra or the femoral neck\textsuperscript{52,53}, and was used for the evaluation of trabecular bone remodeling.

Results show that OVX resulted in increased turnover relative to sham, as evidenced by greater osteoid surface (OS/BS, p=0.001), osteoid volume (p=0.002), percent mineralizing surface (MS/BS, p<0.001), bone formation rate (BFR/BS, p<0.001 if CL is excluded due to high variability), osteoclast number (N.Oc/BS, p<0.001), and osteoclast surface (Oc.S/BS, p<0.05). These measures have been normalized for total bone quantity in the analysis region, and reflect the proportion of bone undergoing remodeling. The increased remodeling in OV relative to SV is consistent with OVX-induced bone loss seen in other studies\textsuperscript{52,173,251,252}. Although the absolute measurements for these remodeling indices are not increased in OV compared to SV, they are influenced by the total quantity of bone in the sample, and do not accurately reflect the extent of turnover activities at the tissue level.

The OVX-induced remodeling effects also produced macroscopic changes in bone architecture. In both the lumbar vertebra and the proximal tibial metaphysis, OVX resulted in decreased trabecular number (Tb.N, p<0.05 and p<0.001, respectively) without affecting trabecular thickness (Tb.Th), and led to increased trabecular separation (Tb.Sp, p<0.05 and p<0.001). These effects resulted in decreased trabecular bone volume (BV/TV) in both sites (p<0.05 for vertebra and p<0.001 for tibial metaphysis). In the lumbar vertebra, trabecular bone loss also resulted in reduced density of connections between adjacent trabeculae (p<0.05). These results indicate that bone loss was due to disappearance of whole trabecular elements rather than thinning of individual trabeculae, and are consistent with other studies\textsuperscript{45,172,275}.

The effects of OVX on lumbar vertebral trabecular microarchitecture led to a conversion of trabecular structures from a plate-like to a more rod-like nature, which is evidenced by increased structure model index (SMI, p<0.05). This is also consistent with the effects of osteoporosis and OVX,
as bone loss in postmenopausal osteoporosis occurs via increased depth of osteoclastic resorption cavities as a result of elevated bone turnover, which leads to focal perforation of trabecular plates. These perforations are then gradually enlarged with further resorption, resulting in the conversion of trabecular plates to rods.\textsuperscript{45} The cumulative changes due to perforative resorption eventually lead to the loss of trabeculae and increased discontinuity in the cancellous bone network.\textsuperscript{46}

The trabecular volumetric BMD in the lumbar vertebrae was also decreased as a result of OVX-induced bone loss, which reduced the tissue vBMD including marrow space (p<0.001) but did not affect the vBMD of individual trabeculae. This suggests that the mineral density of the remaining trabecular elements was not significantly affected by OVX, and the impact of estrogen deficiency was due primarily to the reduction in trabecular number. The areal BMD and bone mineral content were not changed, but these may not be accurate as DEXA is unable to detect specimen depth.\textsuperscript{234}

To assess the mechanical properties of trabecular bone, the entire vertebral body was compressively loaded until specimen failure. Since the specimens were not trimmed to remove the primary spongiosa or the cortical wall, this was not a true reflection of the mechanical properties of trabecular secondary spongiosa. However, trimming the primary spongiosa or the cortical shell would have compromised the structural integrity of the specimen, and would not have provided an accurate measure of the sample’s mechanical properties. In addition, compression of the whole vertebral body is also a more accurate representation of the vertebral compression fractures seen in clinical presentations of postmenopausal osteoporosis, since human spine fractures occur \textit{in vivo} while the entire vertebral body is loaded.

There were no statistically significant differences between OV and SV in the mechanical strength of the vertebral body. However, given the known correlations between trabecular microarchitecture and bone strength\textsuperscript{46,280-282}, this lack of difference is likely due to the current study’s relatively short time frame, as this study is only 3 months in duration while OVX-induced bone loss in the lumbar vertebrae does not reach 50% until 6-9 months post-OVX\textsuperscript{54}. In fact, another study has shown that the vertebral strength is significantly decreased in 12-month-old rats after they had been allowed to lose bone for 8.5 months following OVX\textsuperscript{213}.

The OVX-induced bone loss also impacted the degree of mineralization in trabecular bone, as both the peak mineralization and the spread of mineralization were decreased in OV compared to SV (p=0.052 and p<0.001, respectively). The reduction in peak mineralization likely occurred due to increased turnover following OVX, while the spread of mineralization may have been reduced as a result of the overall loss of bone mineral due to estrogen deficiency, as there is less mineral for distribution within the trabecular compartment. This suggests that the elevated remodeling following
OVX affected not only mineralization on the surface of individual trabeculae, but also altered the degree of mineralization in the entire trabecular structure.

5.2.2 Cortical Bone Effects

In the tibial shaft, intracortical remodeling was not changed due to OVX, as there were no significant differences in MS/BS, MAR, BFR/BS, OS/BS, or osteoid volume between OV and SV. This is also reflected in cortical bone architecture, as no differences were found in geometrical parameters such as cross-sectional diameters or architectural indices such as cortical porosity. No differences were found in cortical vBMD, mechanical properties, or the degree of mineralization between OV and SV. These results are consistent with other studies demonstrating that OVX leads to more pronounced changes in trabecular than cortical bone.

OVX is known to stimulate periosteal bone growth in the cortical diaphysis of long bones. In addition, resorption is also known to be increased on the endosteal surface of long bones following OVX, which leads to enlargement of the marrow cavity. However, these aspects of remodeling could not be effectively evaluated using histomorphometric techniques in the current study due to the lack of axial bone sections, as other studies of cortical bone remodeling have all employed transverse bone sections for appropriate sampling of the cortex. Though an effort was made to histomorphometrically examine both periosteal and endosteal bone remodeling using the available coronal sections of proximal tibia, no obvious differences could be found.

No differences were found in the mechanical properties of the femoral neck, but this may be due to the relatively short time frame in the current study, as OVX-induced bone loss in the femoral neck does not reach 50% until 6-9 months post-OVX.

5.2.3 Secondary Effects

In addition to bone loss, OVX-induced estrogen deficiency also has well-documented secondary effects, including weight gain and increased adiposity. OVX-induced weight gain was also observed in the current study, as the animal weights were increased in OV relative to SV both before and after treatment (p<0.001 and p=0.001, respectively). In addition, the proportion of adipocytes in the marrow space also appeared to be increased in OV relative to SV, which is consistent with the increase in marrow adiposity following OVX.

These secondary effects of estrogen deficiency are due to its important role in regulating food intake and adiposity. For instance, one role of estrogen is to strengthen satiation signals during meals by enhancing the effects of cholecystokinin (CCK), which is a peptide molecule released from the small intestines.
intestine during meals and binds to receptors on the stomach and duodenum\footnote{293}. An abnormal increase in appetite thus results from estrogen deficiency, and is known as hyperphagia\footnote{274}. In addition, because osteoblasts and adipocytes both originate from mesenchymal stem cells, and estrogen promotes the preferential differentiation of marrow mesenchymal stem cells into osteoblasts over adipocytes\footnote{28}, estrogen deficiency results in increased adipogenesis relative to osteoblastogenesis. This leads to a greater number of marrow adipocytes in OVX relative to sham-operated rats.

5.3 Conjugate Treatment

Conjugate treatment consisted of low dose (CL) and high dose (CH) groups. These results were compared to sham and OVX negative controls, along with PGE\textsubscript{2} treatment (PG) as a positive control for bone formation. In addition, there was also another control group treated with a mixture of unconjugated EP4a and ALN-LK (EA), which were given in nearly identical molar quantities as the dose in CL, so that the effect of conjugation may be examined.

5.3.1 Tissue-Level Remodeling

5.3.1.1 Formation

Conjugate treatment dose-dependently stimulated trabecular bone formation in the proximal tibial metaphysis compared to OV, as evidenced by increases in the absolute measures for osteoid volume (p<0.001 for CH) and osteoid surface (OS, p<0.05 for CH). However, after normalizing for total bone quantity, the conjugate-treated groups were no longer increased compared to OV. In addition, the dose-dependent effect also disappeared, and both doses led to comparable results despite CH being 3X higher than CL.

In measuring bone formation kinetics using fluorescent labels, normal mineral deposition creates labels along the bone surface that are thin, well-defined lines. While these lines were observed in most groups, samples from the CH group showed numerous clouds of diffuse fluorescence. This prevented the accurate measurement of labels in CH, and this group was excluded from quantitative analysis. However, the presence of diffuse labels does suggest that there is widespread mineralization in the high dose group.

Quantitative results show that, relative to OV, kinetic bone formation indices were also increased under low dose conjugate treatment. This is demonstrated by the 69% increase in mineral apposition rate (MAR, p<0.001), 35% increase in percent mineralizing surface (MS/BS), and 132% increase in surface-referent bone formation rate (BFR/BS, p<0.05). In fact, CL is significantly increased relative to all other groups including PG for MAR (p<0.001) and BFR/BS (p<0.05), and is comparable to
PG for MS/BS. Since MAR is a measure of osteoblast activity and BFR/BS is a measure of the bone formation rate\(^4\), these findings indicate that conjugate treatment at low dose stimulates osteoblast activity and increases bone formation rate relative to OV, and the efficacy exceeds that of PG. In addition, since MS/BS is an indicator of osteoblast recruitment\(^{173,174,209,294}\), these results also suggest that the stimulatory effect of CL on osteoblast recruitment, although not statistically significant relative to OV, is comparable to that of PG. Moreover, the anabolic effects of conjugate treatment can also be seen in the absolute measurements of fluorescent labels, as CL is also increased compared to OV for single label surface (sLS), double label surface (dLS), interlabel width (Ir.L.Wi), and mineralizing surface (MS).

Interestingly, the effects of EA were opposite to those of CL. Rather than increasing the dynamic bone formation parameters, EA treatment significantly deceased MAR, MS/BS, and BFR/BS relative to OV (p<0.05 for all). In addition, EA was also significantly decreased compared to CL and PG for all 3 parameters (p<0.05 for all). Since the molar doses between EA and CL are nearly identical (4.9% difference for EP4a and 7.2% difference for ALN-LK components), this suggests that conjugation is critical to the anabolic efficacy of ALN-LK-EP4a. Given the well-documented bone-targeting actions of ALN and its rapid clearance from circulation upon systemic administration\(^{57,58}\), both CL and EA are expected to have similar binding to bone for their ALN components following administration. However, while CL also “delivers” the EP4a component to bone sites with the conjugation between ALN-LK and EP4a, EA must rely upon systemic circulation to distribute its EP4a component throughout the body. As such, the proportion of EP4a that reaches bone sites in EA must be less than that of CL. As EP4a is the anabolic agent due to its stimulation of bone formation in vivo\(^{200,206,210-212}\), this discrepancy in the delivery of EP4a to bone sites may account for the difference between the anabolic efficacies of the two groups.

A significant finding in this study is that, while the effect of CL is comparable to PG in MS/BS, it is 55% increased relative to PG for MAR, and 58% increased for BFR/BS. This highlights the crucial importance of conjugation between the EP4a and ALN components for local delivery of EP4a to bone sites, as the anabolic potency is tremendously improved by doing so. Even though the molar dose in PG is double that of CL for each injection, and the overall molar dose per week is 10X higher in PG than CL, the impact of PG on dynamic bone formation parameters is much lower than CL. This may be partly due to the improved potency of EP4a as a synthetic agonist of the EP4 receptor relative to the natural compound PGE\(_2\), as \(K_i\) is 1.2 nM for EP4a (private communication from Dr. Robert Young) while that of PGE\(_2\) is 2 nM for binding with the EP4 receptor\(^{295}\). Nonetheless, this is still an important indicator of the significant anabolic efficacy achieved through the conjugate drug approach.
The difference in total administered dose between PG and EA may also account for the reduced anabolic efficacy seen in EA relative to PG despite the higher binding affinity of EP4a\textsuperscript{211,212}. Because the molar dose of the EP4a component in EA is 10X lower than that of PG, and EP4a is the anabolic portion of the drug mixture in EA, this most likely resulted in the reduction of anabolic efficacy in EA relative to PG.

5.3.1.2 Resorption

In examining resorption via staining for the TRAP enzyme, results showed that cells in SV, OV, and PG groups exhibited red coloring indicating positive enzyme activity\textsuperscript{253}. However, most of the cells in CL and CH, along with some of the cells in EA, do not show positive TRAP staining. Instead, they are purple or blue in color. However, these cells do exhibit several other characteristics of osteoclasts: 1) They are large in size relative to other surrounding cells; 2) they are attached to the bone surface; 3) they are rounded in shape and appear to spread out along the bone surface; and 4) they displace other surrounding marrow cells. In the histomorphometric analysis, these cells were quantified separately from the TRAP-positive cells, as they were considered to be “osteoclast-like” cells based on their secondary characteristics rather than confirmed osteoclasts based on their TRAP activity.

Results show that, relative to other groups, conjugate treatment led to suppression of TRAP activity in osteoclast-like cells. This is evidenced by the reduction in both normalized and un-normalized measures for TRAP-positive osteoclast number (TRAP+ N.Oc and N.Oc/BS) and surface (TRAP+ Oc.S and Oc.S/BS), although the reduction in un-normalized osteoclast surface is not significant. Interestingly, conjugate treatment also stimulated formation of osteoclast-like cells that do not express TRAP enzyme activity, as TRAP-negative osteoclast number (TRAP- N.Oc) and surface (TRAP- Oc.S) are dose-dependently increased compared to other groups. However, this dose-dependent effect disappears after normalizing for total bone surface, and both CL and CH are comparable. Consequently, the total osteoclast-like cell number and surface are also comparable between the two doses.

Osteoclast number measures the size of the osteoclast population in the sample, while osteoclast surface represents the proportion of the bone surface undergoing resorption. It is interesting that the TRAP-positive osteoclast surface is not significantly decreased in conjugate-treated groups compared to OV, despite conjugate-induced suppression of TRAP-positive osteoclast number. This suggests that the TRAP-positive osteoclasts in CL and CH exhibit greater coverage of bone surface than those in OV. Since larger cells tend to have more nuclei, this may be an indication of greater nuclear number in osteoclasts in the conjugate-treated groups. However, this cannot be ascertained in
the current experiment, as only 2-dimensional slices of the cells can be visualized in histomorphometry, and the size of the cell on the section also depends upon the orientation of the osteoclast and the particular angle and placement of the section through the cell. Thus, while osteoclasts in OV and SV appear smaller than those of conjugate-treated groups, this may be influenced by the technique in histomorphometry. As a result, even though the number of nuclei in an osteoclast is correlated with its resorptive activity, nuclear counting was not performed in this experiment since it may not be representative of the true number.

TRAP plays an important role in osteoclastic resorption, and the lack of TRAP staining in the conjugate-treated groups is an important result in this study. This will be discussed below.

TRAP is an iron-containing enzyme expressed by cells of the monohistiocytic lineage, which includes macrophages and dendritic cells. It is also known as type 5 acid phosphatase, and was originally identified based on its mobility at pH 4 and resistance to inhibition by L(+)-tartrate. TRAP is a single-chain proenzyme with low activity when first synthesized, and requires cleavage by cysteine proteinases to convert to its highly active, two-subunit form. TRAP is widely expressed in its latent form in many tissues such as the skin, thymus, spleen, liver, lung, and gastrointestinal linings, but its level of activity in these tissues is low under normal circumstances and thus histochemically undetectable. In contrast, osteoclasts exhibit high levels of TRAP activity, leading to the use of this enzyme as a histochemical marker of osteoclasts.

In addition to being expressed during osteoclast differentiation, TRAP is also secreted by osteoclasts during active bone resorption, and its secretion is positively correlated with osteoclastic resorptive behavior. Experiments using knock-out mice have shown that deletion of the TRAP gene results in increased bone volume, suggesting that the enzyme is involved in the resorption process. Some studies have shown that osteoclasts secrete TRAP from their lysosomes into the resorption pit beneath their ruffled border, while others demonstrated that TRAP is also present in vesicles that transport bone matrix degradation products back into the osteoclast. Nevertheless, the importance of the TRAP enzyme to bone resorption is well-documented, and the serum TRAP activity has been shown to be positively correlated with the level of resorption in metabolic bone diseases.

TRAP is involved in the hydrolysis of nucleotides and aryl phosphates, and contains a catalytically active di-iron-oxo cluster that is critical to its enzymatic activity. It partially dephosphorylates osteopontin (OPN) and bone sialoprotein (BSP), which are bone matrix proteins that promote osteoclast adhesion. Following partial dephosphorylation by TRAP, these proteins no longer support osteoclast binding. In addition, TRAP also produces oxygen radicals in the presence
of hydrogen peroxide and it has been suggested that these reactive oxygen radicals help to destroy the collagen and other matrix degradation products inside the transport vesicles in which TRAP has been found.

Given that TRAP activity is positively correlated with osteoclastic resorptive behavior and the histochemical staining targets the activity of TRAP rather than its expression, the results from the current experiment suggest that conjugate treatment suppresses TRAP activity and thereby exerts inhibitory effects on osteoclastic resorption. However, since TRAP expression is often used as a marker of osteoclasts, the lack of TRAP staining by cells in CL, CH, and EA groups leaves room for doubt in the identification of these cells as osteoclasts. Despite the fact that they exhibit many other characteristics of osteoclasts such as large size and attachment to bone surface, these are not definitive osteoclastic markers. To confirm the osteoclastic identify of these TRAP-negative cells, further histochemical staining must be performed that target other known markers of osteoclasts, such as the calcitonin receptor and RANK. Nevertheless, as additional experiments are beyond the scope of the current study, the TRAP-negative cells that express secondary osteoclast characteristics are considered to be “osteoclast-like” in the current discussion, and the results are interpreted accordingly. Thus, the lack of TRAP activity in cells that otherwise show osteoclast-like characteristics is considered to be due to the inhibitory effect of treatment, although this must be confirmed with further staining.

One additional note is that staining experiments were repeated multiple times with appropriate controls to ensure that differences were not due to technical problems. Slides were also divided such that each batch of stain contained slides from all groups. Results were repeatable, and slides from CL and CH consistently stained purple for their osteoclast-like cells while others from the same staining batch appeared red for osteoclasts. Moreover, some cells from the growth plate region of the same slides in CL and CH would stain more pink or red while those from the metaphysis region consistently stained purple or blue. Together, these observations indicate that the color differences in staining were due to physiological changes rather than technical problems.

Most of the osteoclast-like cells in CL stained negative for TRAP activity, with few TRAP-positive cells. In contrast, most of the osteoclasts in EA stained positive for TRAP activity, with few that are TRAP-negative. This suggests that conjugation is crucial to the suppression of TRAP activity in osteoclast-like cells in CL. In addition, PG did not exhibit suppression of TRAP activity in osteoclasts, indicating that this is not induced by PGE₂.
5.3.1.3 Mechanism of Action

The stimulation of bone formation under conjugate treatment is likely due to the tissue-level anabolic effects of EP4a (and PGE₂), as the increases in MAR, BFR/BS, and MS/BS are all consistent with the *in vivo* effects of PGE₂ and EP4a in literature¹⁷²,¹⁷³,²¹³. However, the conjugate effects on resorption are more complex, as both the EP4a and ALN components can impact resorption.

In examining the potential effect of EP4a on osteoclastic resorption, the lack of TRAP activity in osteoclast-like cells of the conjugate-treated groups suggests possible reduction in resorptive activity, which is consistent with the inhibitory effects of PGE₂ on osteoclastic resorptive behavior *in vitro*²⁴⁵. In addition, the overall increase in the number of osteoclast-like cells under conjugate treatment may be explained by the stimulatory effect of EP4a (and PGE₂) on osteoclastogenesis via increased RANKL production by osteoblasts and decreased OPG¹⁹²,¹⁹₃. Nevertheless, this interpretation is predicated on the assumption that the “osteoclast-like” cells that do not stain positive for TRAP are, in fact, true osteoclasts with low TRAP activity rather than being cells of another type entirely. This interpretation is reasonable given their highly osteoclast-like secondary characteristics, but must be confirmed with additional immunohistochemistry in order to be certain.

Since the chemical reactivity of ALN requires its terminal amine group which is used to join ALN and LK²²¹, the ALN-LK component of EA is pharmacologically inactive until LK is cleaved by hydrolytic enzymes in the bone environment. Although the carbamate bond between ALN and LK is expected to have good chemical reactivity²²¹, radiolabeling assays have not yet been performed to establish the rate of LK release *in vivo*. Thus, the extent of potential anti-resorptive effect by ALN is unknown, and must be evaluated by comparing the tissue-level resorption results with those of other ALN studies. Seedor et al³¹⁷ treated OVX rats for 3 months in a preventative study, and showed that subcutaneous injection of ALN resulted in a dose-dependent anti-resorptive effect, which was significant at doses as low as 0.056 mg/kg per month. The 0.056 mg/kg/month dose produced significant reductions in normalized osteoclast number, osteoclast surface, as well as eroded surface. In comparison, the current study uses *weekly injections* of 2.5 mg/kg ALN, with a total monthly dose of 10 mg/kg which is much greater than that of the Seedor et al study. Since the pharmacological effect of ALN is based on the cumulative dose within a treatment period rather than the dose of individual administrations⁶⁸, there should be a tremendous suppression of osteoclastic resorption in EA based on the dose used. However, this is not the case. Only the normalized total osteoclast number is decreased compared to OV, while the normalized total osteoclast surface is not affected by EA treatment relative...
to OV. Thus, it is likely that the ALN component in EA was not exerting much effect, which indicates that LK must not have been cleaved *in vivo*.

The chemical reactivity of EP4a occurs through its hydroxyl group, and this group is also used to join EP4a to LK\(^{221}\). EP4a is therefore similar to ALN in its pharmacological inactivity prior to cleavage from the bone-bound conjugate, making the conjugate a prodrug that must be cleaved *in vivo* before becoming active. The binding of ALN to bone mineral is known to be robust\(^{58}\), and deposits the absorbed portion of the inactive conjugate on bone surfaces following administration. In addition, the cleavage of EP4a via enzymatic action *in vivo* has been shown to occur gradually over 2 weeks with a 5-day half-life\(^{221}\), which releases small quantities of EP4a at a time. Given these conditions, the local release of EP4a in bone sites thus more closely resembles continuous administration rather than the intermittent dosing applied during the study. However, based on the resorption results for EA, the ALN component of the conjugate was likely not exerting an anti-resorptive effect due to the failure of LK cleavage *in vivo*.

While the EP4a component of CL is released locally in a sustained fashion, the EP4a component of EA is distributed systemically through circulation with each dose of treatment. Since the metabolic half-life of EP4a is approximately 2 hours in rats (private communication with Dr. Robert Young), the biological activity of EP4a does not persist for more than a few hours in the body after each IV administration, and no more EP4a activity occurs until the next weekly dose. This more closely conforms to the weekly dosing regime intended by the experimental design.

In comparing the effects of CL and PG, there are several advantages to the conjugate drug approach versus PGE\(_2\) alone. First, since the synthetic agonist EP4a is a highly selective activator of the EP4 receptor, its use improves the conjugate drug’s local efficacy at the EP4 receptor relative to the natural compound PGE\(_2\)\(^{210}\). In addition, and perhaps more importantly, the conjugate approach allows a large portion of the absorbed EP4a to be delivered directly to bone, while PGE\(_2\) must rely upon systemic circulation for distribution throughout the body. Moreover, the metabolic half-life of PGE\(_2\) is no more than 10 minutes, while that of EP4a is 2 hours, which allows the EP4a to exert a more sustained effect in bone sites following cleavage. Thus, the efficient delivery of EP4a to bone sites, its sustained release via enzymatic action, plus its significantly longer metabolic half-life, all come together to produce a significant anabolic effect which far exceeds that of PGE\(_2\). Thus, despite the weekly molar dose of PGE\(_2\) being 10X that of CL, the anabolic efficacy of PGE\(_2\) is much lower.

Upon qualitative examination of the cortical marrow space (Figure 4–32, pp89), there is an apparent reduction in the number of adipocytes in CL and CH compared to OV. This suggests that conjugate treatment decreased the OVX-induced marrow adiposity, and CL also seems to have slightly
reduced marrow adiposity relative to EA and PG. Since osteoblasts and adipocytes both originate from mesenchymal stem cells, studies have found that adipogenesis is inversely correlated with osteoblastogenesis, and that the proportion of marrow adipocytes is inversely related to bone formation in osteoporosis. The appearance of fewer adipocytes in the marrow of the low dose animals thus supports the finding that treatment likely improved the osteogenic capacity of the bone marrow. Nonetheless, this effect was excessive in CH, which showed overwhelming suppression of marrow adiposity and increased bone formation, such that the marrow space is mostly occluded due to presence of mineralized tissue.

5.3.1.3.1 Dose-Dependent Effect

It is interesting to find that the normalized, tissue-level effects of CL and CH on bone remodeling are comparable despite CH being much higher than CL. This is true for normalized osteoid volume and surface, which represent conjugate effects on bone formation, and normalized TRAP-negative osteoclast-like cell number and surface, which represent effects on bone resorption. This suggests that, at the tissue level, there is a “saturation” in the response of bone tissue to the effects of conjugate treatment, such that, beyond a certain point, the proportion of bone undergoing remodeling activities no longer increased with increasing dosage.

Since measurements were performed at study endpoint, it is possible that a dose-dependent effect could have been observed in the proportion of bone undergoing remodeling at earlier time points during the course of treatment, which produced greater bone volume in CH relative to CL. However, saturation was reached at some point, and this effect could no longer be observed at study endpoint. Thus, although the proportion of bone undergoing remodeling was not different between CL and CH at the end of treatment, a dose-dependent effect in absolute remodeling activities could be observed at study endpoint due to the accumulation of formation and resorption over time.

This saturation effect is surprising given the tremendous difference between the molar dose for CH relative to CL, as CH ranges from being 5X to 3X that of CL at each injection. PGE₂ has been shown to exhibit auto-amplification effects by upregulating the osteoclastic expression of EP4 receptors in adult rat bone tissue. As PGE₂ is believed to act on bone primarily through the EP4 receptor, it is reasonable to assume that the effects of the EP4 receptor agonist would parallel those of PGE₂. As such, it may be possible that the sustained release of EP4a in vivo due to conjugate treatment resulted in the saturation of the osteoblastic expression of EP4 receptors, such that further increases in drug dosage can no longer enhance the pharmacological effects. If the osteoblasts have reached their maximum capacity for expression of EP4 receptors, and all the receptors are already
interacting with available EP4a, then the use of additional agonists can no longer have an effect because no new receptors are available for agonist binding.

The location of the saturation point cannot be precisely determined based on the available data in this study, as data were only collected at study endpoint. Moreover, both CL and CH showed comparable effects in normalized remodeling, suggesting that even low dose conjugate produced saturation by the end of the 6-week treatment. It is possible that the proportion of bone undergoing remodeling was much higher in CH than CL at the beginning of treatment, and CH reached saturation much sooner than CL. However, more data must be collected to investigate this further and determine the location of the saturation point. New studies should explore a wide range of doses, particularly those below that of CL. In addition, data should be sampled at regular points throughout treatment to examine the time course of response, which may allow the time of saturation to be determined.

5.3.1.3.2 Dosing Regime

Since the conjugate is a prodrug that requires enzymatic cleavage in vivo before becoming pharmacologically active, the physiologically active doses of EP4a in CL and CH are different from the nominal doses intended by the study design. With gradual cleavage by local hydrolytic enzymes, the amount of freed EP4a varies with time after administration of the conjugate. As demonstrated by Arns et al.\textsuperscript{221} (Figure 1–20), approximately 6% of the initial dose binds to bone, which then undergoes rapid cleavage for release of EP4a. Approximately 66% of the bone-bound conjugate is cleaved within the first week after administration. However, the cleavage then becomes much slower in the second week, in which only 8% of the bone-bound conjugate is cleaved. It should be noted that the pharmacokinetic data collected by Arns et al were from healthy rats with normal bone remodeling.

With each successive administration of the conjugate drug, part of the administered dose becomes bone-bound and is then gradually cleaved via enzymatic action. However, as not all bone-bound conjugate can be cleaved prior to the next dose, the remaining portion becomes gradually embedded in the bone as layers of new matrix are formed by osteoblasts. These buried molecules of conjugate must then be exposed via osteoclastic bone resorption prior to enzymatic cleavage and pharmacological activation. Thus, depending on the administered dose and the quantity of uncleaved conjugate which then becomes buried in the bone matrix, the release of EP4a is also influenced by the rate of remodeling in bone. In fact, studies have shown that BPs preferentially adsorb to areas of active resorption in the bone due to the greater exposure of bone mineral at these sites\textsuperscript{73,80}, which further strengthens the connection between conjugate binding and bone turnover.
When examining the dosing regime for CL, approximately 0.34 μmol/kg of the initial 5.69-μmol/kg dose binds to bone (Figure 5–1). Of this, 0.23 μmol/kg is released within the first week, while the remainder becomes gradually embedded within the bone. With the administration of the second dose of conjugate the following week, another 0.34 μmol/kg binds to bone and becomes gradually cleaved by enzymes. However, depending upon the rate of bone formation, a portion of the previous dose may remain exposed on the bone surface and continue to be cleaved along with drug molecules from the second dose. Thus, the rate of bone formation influences the amount of conjugate that accumulates on bone surface with each successive dose, and higher bone formation will embed more of the conjugate molecules in bone matrix and lead to less accumulation on bone surface.

Histomorphometric analysis indicates that formation is increased in CL, suggesting that more of the bone-bound conjugate would likely be buried in bone. However, the precise rates of conjugate embedding in bone versus release of EP4a cannot be conclusively determined from the current data, and more studies need to be conducted in order to establish the detailed pharmacokinetics of the conjugate.

Another means by which remodeling influences the release of EP4a in CL is that, while the increase in bone formation probably results in more of the conjugate being embedded in bone matrix, the rate of resorption will help control the exposure of these buried conjugate molecules. This then determines their susceptibility to cleavage and EP4a activation. Since histomorphometry shows that the majority of osteoclast-like cells in CL do not stain positive for TRAP activity despite increased osteoclast population in the metaphysis, it is difficult to determine how the overall resorption is changed in CL compared to other groups. Based on the suppression of TRAP activity in these osteoclast-like cells, it is likely that the effect of conjugate treatment on individual osteoclast is inhibitory. Since the data suggest ALN is not active in the conjugate, this suppression is likely due to
the EP4a component, and is also consistent with previous research demonstrating the inhibitory effect of PGE$_2$ on the activity of mature, multinucleated osteoclasts$^{195}$. In fact, despite the compensatory increase in number of TRAP-negative, osteoclast-like cells in CL, the normalized, total number of osteoclast-like cells in CL remains decreased compared to OV. This suggests that the cumulative resorptive activity in CL may also be decreased compared to OV, although it would depend upon the relative resorptive capacity of individual osteoclasts in OV and CL, which the current data cannot elucidate.

The release of EP4a from the conjugate is more complex in CH due to its more complicated treatment regime. Though this group had been designed to undergo weekly administration with 5X the dose in CL, the severe reaction of the animals necessitated multiple changes to treatment, ultimately resulting in the biweekly, 15-mg/kg dosage which is 3X higher than that of CL. With the first injection, approximately 1.70 μmol/kg of the conjugate drug binds to bone out of the total 28.45 μmol/kg administered (Figure 5–1). Of this, 1.14 μmol/kg is cleaved within the first week, leaving the remaining 0.56 μmol/kg as bone-bound conjugate by the next injection. The second injection of conjugate in the following week provided a dose of 11.35 μmol/kg, of which 0.68 μmol/kg binds to bone. Depending on the rate of remodeling at this time, a portion of the uncleaved drug remaining from the previous treatment may still be exposed on the surface while the rest is embedded within the bone matrix. The surface-bound conjugate would then be added to the conjugate that binds to bone from the newly administered dose, thus slightly increasing the total surface-bound conjugate. This takes place with each new dose of the drug, while the cleavage of EP4a occurs according to the rate established by Arns et al, such that 66% of the initial bone-bound conjugate would be cleaved in the first week, while another 8% is cleaved in the second week after injection. During the non-dosing weeks, there is no new binding of conjugate to bone surface, and the quantity of surface-bound conjugate is determined by the amount of uncleaved drug from the previous doses that remains on bone surface. Thus, the theoretical time course of conjugate binding to bone and EP4a release is much more varied compared to that of CL. The slight recovery of the CH animals in the non-dosing weeks during treatment is therefore likely due to the low levels of surface-bound conjugate as well as EP4a release during those weeks, as the theoretical amount of bone-bound conjugate is only approximately 0.11 μmol/kg with 0.009 μmol/kg of EP4a release. This is significantly lower than the amount of surface-bound drugs during the dosing weeks of treatment.

Examination of the theoretical pharmacokinetic data in Figure 5–1 shows that the time course of conjugate binding to bone surface is relatively stable for CL. This leads to a relatively flat time course of EP4a release throughout the treatment period. In contrast, the complex dosing of CH results in a much
more varied time course, with high amounts of conjugate binding and EP4a release during the initial weeks, which almost reach zero in weeks 10 and 12 (the actual values are 0.08 and 0.05 μmol/kg). This significant reduction in the non-dosing weeks is likely due to the low proportion of conjugate binding to bone with the rapid release during the first week. These significant fluctuations likely contributed to the differences seen in the physiological responses of animals in CH.

One additional note is that the amount of surface-bound conjugate at any given time depends partly upon the proportion of uncleaved conjugate that becomes embedded in the bone matrix. In Figure 5–1, it is assumed that half of the uncleaved conjugate is buried in bone while the other half remains surface-bound and available for cleavage. However, additional calculations were also made for different possibilities in the proportion of embedded drug. These include: 1) None embedded, meaning that all uncleaved conjugate remains surface-bound; 2) 25% embedded, such that 75% of uncleaved conjugate remains surface-bound; 3) 75% embedded, such that 25% of uncleaved conjugate remains surface-bound; and 4) 100% embedded, such that none of the uncleaved conjugate remains surface-bound. Time courses were created for all these scenarios, but the differences due to relative embedding of conjugate are negligible, and all plots look nearly identical. Thus, only one time course plot is shown for CL and CH.

The final issue to be considered is the biological effect of the linker molecule LK after liberation of both EP4a and ALN. The link is 4-hydroxyphenylacetic acid (4-HPA), which is a natural compound found in foods such as olive oil. As such, it is not expected to exert any pharmacological effects in vivo, and would likely be metabolized by the natural processes in the body.

5.3.2 Architecture and Mechanical Properties

Given their tissue-level effects, experiments were performed to investigate whether these changes translate into effects on macroscopic properties of bone, such as its structural characteristics and performance in mechanical testing. These will be discussed below, and due to fundamental differences in their architecture, trabecular and cortical bone will be examined separately.

5.3.2.1 Trabecular Bone

5.3.2.1.1 Microarchitecture and Bone Mineral Density

Conjugate treatment led to significant changes in the trabecular microarchitecture of the lumbar vertebrae and proximal tibial metaphysis. Relative to OV, both sites showed dose-dependent increases in bone volume (BV/TV, p<0.05 for CH in vertebra, p<0.05 for both doses in tibia) and reductions in trabecular separation (p<0.05 for both doses in the vertebra and tibia), but this seems to
be due to differing mechanisms. In the vertebra, there was a concomitant, dose-dependent increase in trabecular number (Tb.N, p<0.01 if CH is excluded due to high variability), without impacting trabecular thickness. In contrast, the proximal tibial metaphysis showed a dose-dependent increase in Tb.Th (p<0.05 for both doses), while Tb.N was not dose-dependently increased. Nonetheless, CH exceeds all other groups in bone volume for both sites (p<0.05 for all), while also exceeding other groups in connectivity density (p<0.001 for all). Given the 10X dose difference between CL and PG, this provides further evidence for the strong anabolic potency of the conjugate drug compared to PGE₂, as all architectural indices of CL are either comparable to or exceeding those of PG in both sites.

Additional results in the lumbar vertebra show that conjugate treatment also induced a conversion of the trabecular structure from a rod-like to a more plate-like state, as the structure model index (SMI) is dose-dependently decreased compared to OV (p<0.01 for CL and p<0.001 for CH). While a similar structural conversion also occurred in PG, it was not seen in EA.

In the vertebrae, the results suggest that de novo bone formation occurred within the marrow space, which is consistent with other studies using PGE₂ in OVX rats. This is also consistent with the increase in osteoblastogenesis due to conjugate treatment, as more marrow progenitor cells are recruited to become osteoblasts, which then form new trabeculae. This led to increased density of connections between adjacent structural elements, and decreased separation between them. Given the pathological structural conversion of trabecular plates to rods in osteoporosis, it is promising that conjugate treatment seems to reverse this effect. Moreover, since treatments that only thicken existing trabeculae may be less effective if OVX-induced bone loss results in the disappearance of a majority of trabecular elements, the de novo formation of new trabeculae under conjugate treatment helps to increase the number of trabecular elements and restore the trabecular bone structure irrespective of the number of trabeculae that remain.

It is interesting that results in the proximal tibial metaphysis do not resemble those of the lumbar vertebrae, which suggests that the effects of conjugate treatment may vary depending on skeletal site. Since different measurement techniques were used in the two (microCT in the vertebra, histomorphometry in the tibia), differences in their sampling of the bone should be examined. While histomorphometric analysis is performed using 2-dimensional bone sections, microCT creates a 3-dimensional model of the entire trabecular compartment, and is thus less prone to sampling bias. However, the discrepancy between results obtained from these techniques is unlikely to be due to differences in sampling, as the results for OVX-induced changes in trabecular bone microarchitecture are the same for both techniques. If the differences are due to sampling bias, then the same bias should apply to the OV and SV groups, but the measurement outcome was not affected in these
groups. Thus, the differences in the lumbar vertebrae and tibial metaphysis are more likely due to the effects of conjugate treatment on the two sites. These discrepancies pose an interesting possibility regarding the potentially site-specific effects of the conjugate, which should be investigated further.

It is promising that the conjugate-induced anabolic effects on turnover in the proximal tibial metaphysis also translated into architectural changes in the lumbar vertebrae, the former has a much higher rate of turnover and also responds more quickly to anabolic treatment compared to the latter. In fact, studies have shown that the proximal tibial metaphysis undergoes significant bone loss within only 14 days following OVX, while the lumbar vertebra requires approximately 60 days post-OVX before noticeable bone loss can be detected. In addition, OVX-induced bone loss in the proximal tibial metaphysis reaches 50% in 1-2 months, while the lumbar vertebrae does not show 50% bone loss until 6-9 months post-OVX. Given the short study duration of 3 months, it is promising that conjugate low dose treatment successfully restored 63% of the bone lost due to OVX, while high dose treatment led to 120% recovery of lost bone.

Despite differences in tissue-level remodeling between EA and CL, there are no significant differences between the two groups in vertebral architecture. This is likely due to the short study duration given the slower rate of remodeling in this site, and it is likely that a longer duration study will be able to detect some differences. The same can also be said for CL and PG.

The results of EA and OV may be compared to examine the potential efficacy of ALN in EA, as sufficient cleavage of LK from ALN-LK in EA should lead to greater bone volume in EA relative to OV. However, this is not the case, as both groups are comparable for trabecular BV/TV in both the lumbar vertebra and the proximal tibial metaphysis. This indicates that the ALN-LK conjugation link was likely not sufficiently cleaved in vivo, and that the effects due to conjugate treatment are primarily due to the EP4a component.

5.3.2.1.2 Bone Mineral Density

Conjugate-induced changes in trabecular bone also improved the volumetric BMD (vBMD) of the lumbar vertebrae, as evidenced by dose-dependent increases in whole tissue vBMD of the trabecular compartment (p<0.05 for CH), which was due to formation of new trabeculae rather than increases in the density of individual trabeculae. This provides further evidence for the improved bone quality under conjugate treatment, as remodeling effects have been shown to produce architectural changes that increase the volumetric density of trabecular bone, which help to facilitate potential improvements in mechanical properties. However, given the potential differences in the conjugate’s mechanism of action between the lumbar vertebrae and the proximal tibial metaphysis, it would be
interesting to examine the vBMD of the proximal tibia metaphysis, as thickening of the trabeculae may have altered vBMD of the trabecular elements.

Additional results suggest that the conjugate-induced effects also led to overall changes in the quantity of bone mineral in the lumbar vertebrae, as the bone mineral content (BMC) was also dose-dependently increased relative to OV (p<0.05 for CL and p<0.001 for CH). However, because these measurements were performed on whole vertebral bodies, they include both the cortical wall and the trabecular compartment. Thus, it is difficult to determine based on these results whether the changes in total mineral content are due to increased trabecular bone volume or effects on cortical bone, which will be explored separately.

5.3.2.1.3 Mineralization

The degree of mineralization was assessed using two measures, which provide information about the peak mineralization in bone (maximum grey value) and the spread of mineralization (full-width at half max, or FWHM). High turnover leads to decreased peak mineralization but increased spread of mineralization, as the remodeling activities result in breakdown of older, highly mineralized bone and production of new bone matrix. Results show that the conjugate-induced effects led to changes in mineralization throughout the trabecular structure, as the spread of mineralization is dose-dependently increased relative to OV (p<0.05 for CH). However, peak mineralization was not significantly affected.

Since OVX-induced bone loss also resulted in decreased FWHM in OV compared to SV, the conjugate-induced increase indicates that treatment helps to recover some of the loss in mineralization spread toward sham levels, suggesting that conjugate treatment leads to overall restoration of mineralization in the bone. This recovery of mineralization spread is not seen in PG.

5.3.2.2 Cortical Bone

5.3.2.2.1 Tissue-Level Remodeling

Conjugate low dose treatment resulted in elevated intracortical remodeling compared to all groups except PG, as evidenced by increases in the normalized dynamic bone formation indices (p<0.01 for all). CH was also excluded from quantitative analysis due to excessive endocortical bone formation, which precluded the clear definition of endosteal boundaries. Since bone formation within the cortex cannot occur without prior resorption, these results suggest that conjugate treatment activated intracortical remodeling.
The increase in intracortical remodeling seen in PG is consistent with other studies demonstrating the effects of PGE on cortical bone\textsuperscript{174}. Since the skeleton of young adult rats (<8 months old) lack the Haversian system necessary for cortical bone remodeling\textsuperscript{51}, the induction of intracortical remodeling by exogenous PGE\textsubscript{2} is an important consequence of treatment. As the main pharmacological agent in CL is EP4a, which is known to mimic the effects of PGE\textsubscript{2} on bone, it is not surprising that conjugate treatment also induces intracortical remodeling. Interestingly, despite the increased anabolic potency of CL relative to PG in trabecular bone, their effects on intracortical remodeling appear to be comparable. However, given that the total weekly molar dose of CL is less than 10% that of PG, their similar effects are actually an indication of the enhanced efficacy of CL relative to PG, as a much lower dose is needed to produce the same result. This is in agreement with the increased efficacy of CL relative to PG seen in trabecular bone.

The endosteal and periosteal bone formation could not be measured histomorphometrically in this study due to the lack of appropriate axial bone sections. However, given the extensive periosteal bone formation seen in CH, it is likely that conjugate treatment also results in periosteal bone formation. This is not surprising since PGE\textsubscript{2} treatment is known to stimulate periosteal bone formation in rats\textsuperscript{178,246,287}. Nonetheless, no quantitative histomorphometric measurements could be performed to evaluate periosteal bone formation.

5.3.2.2.2 Microarchitecture and Bone Mineral Density

Conjugate treatment resulted in dramatic alterations in cortical bone architecture compared to OV, which are particularly prominent at the high dose. These include: 1) endocortical bone formation, as evidenced by dose-dependent increase in endocortical bone volume (p<0.001 for CH); 2) periosteal apposition, as evidenced by increased anterior-posterior (not significant) and medial-lateral diameters (p<0.05 for CH), along with increased cross-sectional bone area (p<0.001 for CH); and 3) dose-dependent increase in cortical porosity, for which CL exceeds all other groups (p<0.05 for all), and CH is 14X greater than any other group (p<0.001 for all). The significant elevation in cortical porosity in CH also reduced the cortical vBMD compared to all other groups (p<0.005). Together, these conjugate effects in CH resulted in such dramatic transformation of the femoral architecture that very little marrow cavity can be found, and the cortical structure is no longer recognizable in some animals. The porous nature of the cortex is clearly visible in cross-sectional microCT images, and the density of the cortex, as indicated by its brightness in the images, is significantly lower in CH than any other group.
The formation of endocortical woven bone in PG is consistent with other studies\textsuperscript{171,178,246}, and is also consistent with the effect of PGE\textsubscript{2} in stimulating osteoblastogenesis and increasing the osteogenic capacity of the bone marrow\textsuperscript{184,185,189,321}. As EP4a mimics the effects of PGE\textsubscript{2} on bone, the increased endocortical bone formation due to conjugate treatment is also consistent with the anabolic effects of PGE\textsubscript{2} and EP4 receptor agonists. However, the significant differences in the volume of endocortical bone formed between conjugate treatment and other groups are not due to the anabolic effects of EP4a alone. Instead, these effects may also be attributed to the particular design of the conjugate drug, which creates a sustained anabolic effect due to the sustained release of EP4a.

The conjugate-induced periosteal expansion is not noticeable at the low dose, but is excessive at the high dose. Although bone also undergoes periosteal apposition with endosteal resorption as it ages\textsuperscript{38,322,323}, the effect of CH is periosteal expansion with endocortical formation, which significantly modified the cortical structure.

The gain in cortical bone mass can also be seen in the dose-dependent increases in bone mineral content (BMC, \( p<0.05 \) for CL and \( p<0.001 \) for CH), aBMD (\( p<0.001 \) for CL and CH), and bone area (\( p<0.001 \) for CH) relative to OV. However, these results should be interpreted with caution due to DEXA’s inability to detect sample height, and the increases could also be due to changes in bone size.

5.3.2.2.3 Mineralization

Low dose conjugate treatment did not affect the degree of mineralization relative to OV, while high dose conjugate treatment increased the spread of mineralization without affecting peak mineralization. These results indicate that the tremendous remodeling in the high dose group altered the overall spread of mineralization in the cortical structure, while the lack of differences in peak mineralization is likely due to the relatively short study duration.

5.3.3 Mechanical Properties

Despite the significant bone loss that occurs in rats following OVX, the rat skeleton is not susceptible to the fragility fractures that occur in human postmenopausal osteoporosis\textsuperscript{51}. Since the mechanism of skeletal loading is different between rats and humans, the lack of fragility fractures in rats may be due to their different natural postures compared to humans. Thus, in order to assess the skeletal fragility of rats, biomechanical testing must be performed\textsuperscript{227}. These tests allow characterization of the performance of rat bone under various forms of mechanical loading, which may be used to determine the effects of treatments on reducing bone fragility. Standard mechanical properties include: 1) Ultimate load and ultimate stress, which correspond to the strength of the bone
specimen and tissue material, respectively; 2) Stiffness and modulus, which correspond to the resistance to loading of the respective bone specimen and tissue material; 3) work to failure and toughness, which measure the ability to absorb energy for the bone specimen and tissue, and 4) failure displacement and strain, which measure the ductility of the specimen and tissue material, respectively. Specimen properties are extrinsic, and are influenced by the architecture and geometry of each bone sample, while material properties are intrinsic and geometry-independent.

Because the purpose of most treatment therapies for osteoporosis is to reduce skeletal fragility and minimize the risk of fracture, the mechanical properties of bone represent the culmination of all architectural and tissue-level changes resulting from treatment. Any treatment-induced stimulation of bone formation and improvements in bone architecture are contributors to the bone's mechanical properties. However, these structural and tissue-level changes are ultimately secondary to the true impact of the treatment on patients, as effective therapies for osteoporosis should always improve the bone's load-bearing ability and reduce its risk of fracture. Thus, biomechanical testing is a critical component of any study that aims to evaluate the efficacy of a new treatment for osteoporosis.

5.3.3.1 Three-Point Bending

Results of the three-point bending test show that the material strength (ultimate stress) and modulus are both decreased in CH (p<0.05 for all), likely due to its tremendous porosity. However, the specimen strength (ultimate load) is actually increased in CH (p<0.001 for all), likely due to changes in bone geometry and architecture. In fact, the bones in CH showed nearly 2-fold increase in their resistance to bending forces compared to other groups, as evidenced by their increased principal moment of inertia (MMI\textsubscript{min}, p<0.001).

Although none of the mechanical testing results for CL showed significant differences despite architectural changes such as increased cortical porosity, this was likely due to the short study duration, as elevated intracortical remodeling has been demonstrated in this group. Nevertheless, it is of note that, while increased porosity can disproportionately reduce bone strength\textsuperscript{227}, the location of porosity within the cortex can also influence its effects on bone strength, as periosteal and endosteal regions of the cortex differ significantly in their load-bearing when subjected to bending. While the endosteal region of the bone carries the lowest stress under bending, the periosteal region faces the greatest stresses. This difference has been demonstrated in studies that treated monkeys with PTH\textsuperscript{117}, which found that an 18-month treatment using high dose PTH resulted in 12% increase in the cortical porosity of the humerus. However, the mechanical strength of the bone was not significantly reduced, as the distribution of the porosity was mostly near the endosteal boundary, with very few near the
periosteal surface. Though the location of porosity within the cortex was not determined in this study, it may be worthwhile to examine this in a future study to assess the effects of treatment on mechanical properties of cortical bone.

It is interesting that the architectural changes in CH led to increased strength but did not impact the stiffness. This is likely due to the material being much weaker in CH, as the highly porous cortex is not able to provide much resistance to loading. In fact, the tissue resistance to loading in CH is less than half that of any other group.

The results also show that the tissue material in CH is more ductile compared to other groups, as the material deformation under load (failure strain) is increased in CH (p<0.05 for all). However, the architectural changes in CH must have decreased its ability to deform under load, as the specimen ductility (failure displacement) was not increased in CH. The total energy absorption of the tissue is not changed due to decreased strength of the bones in CH despite increased ductility.

5.3.3.2 Femoral Neck Fracture

Since the femoral neck contains cortical and trabecular bone which both contribute to the overall mechanical properties, the results reflect conjugate effects on trabecular as well as cortical bone. However, the irregular geometry of the femoral neck and the unpredictability of the direction of fracture prevent the accurate calculation of the material properties of bone tissue in this test. Thus, only specimen-specific properties can be determined, and the results are dependent upon the geometry and architecture of each sample.

Results that CH is somewhat decreased compared to other groups in strength, resistance to load, and energy absorption capacity, but the differences seen are not as prominent as those of 3-point bending. Though these results are somewhat consistent with the results of 3-point bending and seem to suggest that the mechanical properties are all decreased due to high dose conjugate treatment, it should be noted that there are many factors that contribute to these differences. Depending on the direction of fracture, the proportion of cortical and trabecular bone may be different in the plane of fracture, which is then reflected in the resulting mechanical properties measured for each sample. In addition, the architecture of the femoral neck also has an impact on the outcome of the test, and this is particularly important when interpreting the results of CH given its unusual geometry. As shown in Figure 4–2 (pp64), while the main femoral shaft of CH is significantly larger than other groups, the femoral neck region does not appear to be increased in size as much as the other parts. This results in an architecture that is unusual compared to that of other groups, such that the femoral neck appears to be disproportionate to the rest of the femur. This change in
geometry and architecture is likely also reflected in the test results, as the location of the fracture relative to the femoral head may be different in CH than in other groups due to its unusual geometry.

The increase in ultimate load and work to failure in CL relative to SV suggests that conjugate low dose treatment improves the strength and energy absorption above sham levels. However, as the bones in CL generally tend to be larger than those of SV (though not statistically significant at the group level), this may also be due in part to the greater bone size in CL. In addition, it should be noted that the geometry of the bones in CL does not appear to be as distorted as that of CH, allowing the fractures to occur in a manner more consistent with that seen in other groups.

Despite the significant increase in cortical and trabecular bone remodeling relative to OV, conjugate treatment did not produce any significant changes in the mechanical properties of the femoral neck compared to OV. However, this may also be due to the relatively short study duration of 3 months, as bone loss in the femoral neck does not reach 50% until 6-9 months post-OVX\textsuperscript{50}. It is possible that a longer-duration study will be able to detect statistically significant differences.

### 5.3.3.3 Vertebral Compression

In compression testing, conjugate treatment led to mixed results on vertebral mechanical properties, as the bone strength was dose-dependently increased compared to OV (p<0.05 for CL and CH), but the material strength was not changed. In addition, both the stiffness and modulus were decreased in CH (p<0.05 for all), indicating significantly compromised mechanical resistance to load despite larger bone sizes in this group.

These results suggest that conjugate treatment improved the load-bearing strength of the whole vertebral body without changing its material strength, while stiffness and modulus were decreased. However, because both cortical and trabecular bone were tested, it is difficult to determine whether the improvements are due primarily to trabecular or cortical bone. Because the vertebra consists of approximately 75:25 ratio of trabecular to cortical bone\textsuperscript{1}, effects on either component will contribute to its overall performance. Thus, the increased trabecular bone volume in CH may improve its stiffness and strength. However, the conjugate effects on the cortical wall have not been measured, and given the induction of intracortical remodeling and increased cortical porosity seen in the femoral cortex of CH animals, it is possible that the effects of CH on the vertebral cortical wall are similar. This may in turn result in compromised stiffness in the whole vertebrae.

Interestingly, while the deformation under load (failure displacement) is comparable between CH and other groups in the femoral diaphysis, it is increased relative to other groups in the lumbar vertebrae (p<0.05 compared to all except CL). This suggests that the trabecular portion of the bone
may have absorbed some of the energy during compression, such that the vertebral body was able to extend further than the femoral cortical bone. In fact, the combination of trabecular and cortical bone in the vertebrae likely also led to the increase in failure strain for CH compared to the vehicle-treated groups, as trabecular bone can deform more under load and is thus more ductile than cortical bone. The increased work to failure is likely also due to the greater size of the vertebrae in CH, as it allows the bone to absorb more energy.

The lack of differences in the overall material strength, toughness, and ductility suggests that, despite individual effects on trabecular and cortical bone due to conjugate treatment, there are no overall changes when examining the combination of the two in the vertebral body.

5.3.4 Adverse Reactions

Since one of the main objectives in designing the ALN-LK-EP4a conjugate drug is to mitigate the side effects associated with systemic administration of PGE$_2$ and EP4a, the adverse reactions experienced by the animals during treatment represent an important component of the results in this study. These include: 1) gastrointestinal effects such as diarrhea, 2) general system effects such as lethargy and lack of movement, 3) immediate distress symptoms such as shortness of breath, eye closure, puffy fur, and hunched posture, 4) long-term system effects such as lack of grooming indicated by porphyrin and urine staining, and 5) serious long-term effects such as severe swelling in the legs and feet.

PG and CH groups exhibited the most severe adverse reactions to treatment, with PG showing serious immediate reactions that lasted for 1 day (e.g. diarrhea, extremely hunched posture, puffy fur, shortness of breath), while CH showed serious long-term reactions to treatment that persisted for 2 weeks (e.g. tissue edema in legs and tail). These findings may be explained by the different biological effects of PGE$_2$ versus the conjugate. Because PGE$_2$ acts on all 4 EP receptors, which have widespread distribution in many body tissues, the immediate symptoms of distress are likely due to the effects of PGE$_2$ on the various body tissues. Given the mode of injection was subcutaneous, the immediate site of action for the compound is likely skin tissue. Since prostaglandins such as PGE$_2$ are synthesized abundantly in the epidermis, and the keratinocytes of the epidermis express all 4 EP receptors$^{324}$, some of the immediate symptoms such as puffy fur may be due to the effects of PGE$_2$ on the skin. In addition, the gastrointestinal tract has also been shown to express multiple EP receptors including EP4$^{140,151}$, leading to diarrhea in the PGE$_2$-treated animals. Moreover, the EP2 receptor is expressed in the respiratory tract, while all 4 EP receptors are expressed in the cardiovascular system, which may have led to the shortness of breath seen in the PGE$_2$-treated animals$^{151}$. Finally, given the known
effects of PGE\textsubscript{2} on inducing uterine contraction\textsuperscript{179}, the hunched posture may be a sign of PGE\textsubscript{2} effects on internal organs which are difficult to observe from the outside. Similarly, the eye closure and lethargy may be the result of general discomfort of the animals following treatment, and are a cumulative outcome of the variety of effects PGE\textsubscript{2} has on body tissues.

In contrast, the relatively short duration of the symptoms in PG animals may be due to the short metabolic half-life of PGE\textsubscript{2}, which is under 10 minutes (private communication from Dr. Robert Young). Thus, although the animals experience significant side effects immediately following injection, these effects do not persist for more than a few hours, giving them a chance to recover significantly prior to the next injection.

It should be noted that the different routes of administration between the conjugate and PGE\textsubscript{2} may have exacerbated some of the symptoms seen in PG relative to the conjugate, as subcutaneous administration exposes large portions of the skin to immediate effect by PGE\textsubscript{2}, whereas intravenous injection allows the conjugate to be more quickly distributed through the body, thereby mitigating some of the effects due to concentrated exposure in particular areas of the body. The treatment route of PGE\textsubscript{2} was selected based on previous studies using systemic administration of the compound, all of which employed subcutaneous rather than intravenous injection\textsuperscript{172,173,175,177,286,287,325-330}. However, the intravenous injection route was chosen for the conjugate in order to achieve precise dosing to circulation, as subcutaneous injection results in variable absorption of the drug into circulation, which cannot be precisely controlled for consistency between animals. Nevertheless, a future study to examine the difference in side effects between systemic administration of PGE\textsubscript{2} and the conjugate should employ consistent routes of administration for more accurate comparison.

In comparing PG with CH, it is interesting to observe that, while there were several long-term symptoms in CH that were not seen in PG, CH did not show any diarrhea whereas mild diarrhea was observed in PG. This likely results from the effect of PGE\textsubscript{2} on the EP receptors expressed in the gastrointestinal system, whereas gastrointestinal effects were not observed in either of the conjugate-treated groups due to the local delivery of EP4a to bone sites.

In contrast to PG and CH, the CL group exhibited the least number of adverse reactions, and returned to normal behavior after only 1 hour. Interestingly, the EA group showed slightly more symptoms than CL, and exhibited mild diarrhea and slightly puffy in addition to the symptoms observed in CL. Since the routes of administration are the same for EA and CL, and the molar doses of EP4a and ALN-LK components in EA are nearly identical to that of CL, the additional symptoms of EA are likely due to the lack of conjugation between the two components. Since both the gastrointestinal tract and the epidermis are known to express the EP4 receptor, the diarrhea and puffy fur seen in EA
are consistent with the effects of EP4a on these regions. In contrast, the EP4a in CL is conjugated to ALN-LK, and is deposited on bone surfaces as a result of ALN binding to bone mineral. Thus, the EP4a in CL does not have as much opportunity to exert effects on other regions compared to EA.

The adverse reactions in CH are much more severe than those of CL. Since intravenous administration bypasses first pass metabolism in the liver\textsuperscript{331}, the drug is not metabolized in the liver before reaching various tissues in the body for action. This approach can lead to depression of cardiovascular function, also known as cardiogenic shock, which may be particularly relevant in CH given the extremely high dose of treatment\textsuperscript{331}. In addition, as the drugs do eventually reach the liver via systemic circulation, and esterase and peptidase enzymes involved in conjugate cleavage are also present in the liver, a small portion of the drug does become metabolized in the liver as well (private communication with Dr. Robert Young). However, given the administered dose in CL is much lower than that of CH, very few systemic effects are induced in CL, while there are dramatic effects in CH. Moreover, these side effects likely also impacted the other internal of the animals in CH, which could not be properly assessed in external observation, although liver discoloration and rounding of the livers edges were observed on necropsy.

Given the mechanism of sustained EP4a release from the bone-bound conjugate, the extremely high local concentrations of EP4a in CH led to dramatic and abnormal alterations to bone physiology, which likely contributed to the animals’ extreme tissue edema and unwillingness to move their legs. In addition, their illness may also be attributed to the near depletion of the bone marrow due to occlusion of the marrow cavity by mineralized tissue, as the bone marrow is responsible for the production of a variety of cells, and is critical to survival. These cells include erythrocytes, granulocytes, monocytes, lymphocytes, and platelets, making the bone marrow an important component of both hematopoiesis and the lymphatic immune response\textsuperscript{332}. The lack of adequate supply of erythrocytes likely led to anemia in these animals, which would have contributed to their sluggishness.

5.3.5 Overall Discussion

This study aimed to investigate the \textit{in vivo} efficacy of the ALN-LK-EP4a conjugate drug in treating postmenopausal osteoporosis using the OVX rat model. The drug is specifically designed to mitigate the side effects due to systemic administration of synthetic agonists of the EP4 receptor, which mimic the bone effects of the natural compound PGE\textsubscript{2}. The EP4 receptor agonist (EP4a) is linked to the bisphosphonate alendronate (ALN) via the linker molecule 4-hydroxyphenylacetic acid (LK), which allows the bone-binding property of ALN to deliver the EP4a component directly to bone sites following systemic administration. In the hypothesized mechanism of action, enzymatic cleavage then
releases both EP4a and LK, allowing EP4a to promote bone formation while leaving ALN bound to bone for inhibiting resorption.

In a curative experiment, 3-month-old rats were OVX and allowed to lose bone for 7 weeks before undergoing treatment for 6 weeks. The effects of OVX-induced bone loss were clearly demonstrated in elevated turnover as well as reduced trabecular bone volume via decreased number of trabeculae. In addition, there was also a conversion of trabecular elements from a plate-like to a more rod-like structure. These effects are consistent with OVX-induced bone loss in other studies as well as bone loss in human postmenopausal osteoporosis45,51,251,252, and confirm the validity of the disease model in our curative experiment.

The side effects due to treatment were carefully observed, and the reactions of the PGE2-treated (PG) animals were consistent with those seen in other studies179. Low-dose conjugate (CL) treatment resulted in decreased number of side effect reactions compared to all other non-vehicle-treated groups. This includes the mixture group EA which was treated with unconjugated EP4a and ALN, and was designed to control for the effect of conjugation between the components. This suggests that the design of the conjugate drug effectively alleviated the symptoms associated with systemic administration of EP4a, and was much better tolerated compared to PGE2.

The tissue-level effects in trabecular bone showed significantly increased bone formation under CL, which exceeded that of PG despite the dose in CL being less than 10% that of PG. This indicates that the anabolic potency of the conjugate is significantly greater than that of PGE2. In addition, conjugate treatment led to the formation of osteoclast-like cells that did not express positive TRAP activity, but exhibited secondary characteristics of osteoclasts (ie. large size, attachment to bone surface, rounded shape, spreading out on bone surface, displacement of other marrow cells). These cells were dose-dependently formed in the conjugate-treated groups, with a few also present in the EA group, but none were found in the other groups. The identity of these cells as osteoclasts needs to be confirmed via additional histochemical staining of other osteoclast markers such as RANK and calcitonin receptor, but they were considered to be “osteoclast-like” cells in the current analysis based on their secondary characteristics. Under this assumption, these cells are deemed to be osteoclasts with reduced TRAP activity, and given that TRAP expression is positively correlated with osteoclastic resorptive behavior258, this suggests that conjugate treatment led to the suppression of resorptive activity in the TRAP-negative, osteoclast-like cells.

These tissue-level effects also translated into microarchitectural changes in the bone, as the lumbar vertebral trabecular bone volume is dose-dependently increased under conjugate treatment, with concomitant increase in trabecular number. In addition, there was a conversion of the trabecular
structure from a rod-like to a more plate-like architecture, which reversed the architectural changes resulting from OVX. These effects led to the dose-dependent increase in vBMD of the trabecular tissue, which slightly improved the mechanical properties of the vertebrae at the low dose but compromised the mechanical performance at the high dose. Given the tissue-level remodeling effects seen in CL, the lack of significant differences in mechanical properties may also be due to the relatively short study duration.

In cortical bone, there was dose-dependent intracortical remodeling due to conjugate treatment, which significantly increased the cortical porosity. In addition, the tremendous remodeling due to CH dramatically altered the cortical bone architecture, as the marrow cavity was entirely occluded in 7 out of 9 animals. Though the increased cortical porosity in CL did not detrimentally affect its mechanical properties, the excessive remodeling in CH led to brittle bone that showed increased strength due to its large size, but was significantly compromised in material properties.

The anabolic effects of the conjugate are consistent with other studies using PGE₂. In trabecular bone, PGE₂ has been shown to increase osteoblast number as well as fluorescently labeled bone surfaces, leading to increased bone formation. There is greater bone turnover under intermittent PGE₂ treatment with formation exceeding resorption, resulting in increased trabecular bone volume. In cortical bone, PGE₂ has been shown to elevate intracortical remodeling and promote woven bone formation within the endocortical cavity as well as on the periosteal surfaces.

On examining its mechanism of action on bone, studies have shown that PGE increases the osteogenic potential of the bone marrow by promoting the differentiation and proliferation of osteoblasts, and acts mainly via the cAMP/PKA intracellular pathway. In addition, PGE₂ has also been shown to promote osteoclastogenesis by increasing the osteoblastic production of RANKL and decreasing OPG, while its effects on mature, multinucleated osteoclasts in vitro are inhibitory. Because numerous studies have demonstrated that PGE₂ exerts its effects on bone primarily through the EP4 receptor, these results may also be extended to EP4 receptor agonists. In fact, various synthetic agonists of the EP4 receptor have also been shown to exert anabolic effects in vivo.

The nitrogen-containing bisphosphonate ALN is known to strongly inhibit resorption by interfering with the mevalonate pathway in osteoclasts. ALN inhibits farnesyl diphosphate synthase (FPPS), which prevents the synthesis of farnesyl diphosphate (FPP) and impairs the post-translational
prenylation of small GTPases critical to osteoclast function and survival. ALN thus strongly impairs osteoclast function and leads to increased osteoclast apoptosis.

Together, these findings provide insight into the effects of conjugate treatment in this study. The increased trabecular bone volume is due to the anabolic effects of EP4a in promoting osteoblastogenesis and increasing bone turnover, while the increased number of TRAP-negative, osteoclast-like cells is due to the effect of EP4a in stimulating osteoclastogenesis. Although both ALN and EP4a may have anti-resorptive effects in the conjugate, the anti-resorptive action of ALN depends on the in vivo cleavage of LK by local enzymes, which has not been established using radiolabeling experiments. The effects of EA on resorption are thus crucial to determining the potential anti-resorptive efficacy of ALN in vivo, but results do not show significant suppression of osteoclast surface compared to OV. As other studies have shown that ALN has significant anti-resorptive effects in suppressing both osteoclast number and surface at doses over 100X lower than that of EA, the lack of significant reductions in osteoclast surface in EA relative to OV indicates that the ALN component of the conjugate is mostly not active. Thus, the lack of TRAP activity in osteoclast-like cells in the conjugate-treated groups is more likely due to the inhibitory effect of EP4a on mature osteoclasts.

The growth plate region appears to be thin in both OVX and sham-operated rats (Figure 4-3), and is slightly thicker in the PGE₂-treated animals. However, there is significant thickening of the growth plate in the EA group, and this is even more pronounced in the conjugate-treated groups. Significant thickening of the growth plate has been observed in other studies using risedronate, which is another bisphosphonate, while PGE₂ has been shown to thicken the growth plate only slightly. Lin et al. systemically administered PGE₂ and risedronate to rats, and demonstrated that there is a dose-dependent increase in the thickness of the primary spongiosa in rats treated with risedronate alone. The relative thickness of the growth plate in the EA group seems greater than that of the risedronate-treated rats in the Lin et al study, which may be explained by the combined effects of EP4a and ALN, as Lin et al also found that the growth plate thickening is increased with combined risedronate and PGE₂ relative to risedronate alone. Moreover, since the dose of PGE₂ in the other study was 6 mg/kg/day or 85 μmol/kg/week while the dose of EP4a in EA was much lower at 5.97 μmol/kg/week, it is unlikely the growth plate thickening effect seen in EA was due to EP4a alone. Thus, although the ALN component of the conjugate is mostly not active due to insufficient LK cleavage, a very small portion of it likely was cleaved, and contributed to the effect of EP4a at the growth plate.

Another observation regarding the growth plate is that its thickness in the conjugate-treated groups is dose-dependently increased relative to EA. This may be explained by the conjugate drug design, as the conjugate drug locally deposits the EP4a in bone, and releases EP4a locally in a sustained
fashion with enzymatic cleavage. The thickening effect on the growth plate is thus amplified, and the significant effect in CH relative to CL is the result of its much higher dose.

The effects of ALN on thickening of the growth plate is primarily due to its interference with endochondral bone formation, which involves chondrocyte proliferation and hypertrophy, followed by mineralization of the cartilage\textsuperscript{336}. This calcified cartilage is then resorbed by osteoclasts as part of the remodeling process required to transform the primary spongiosa into secondary spongiosa\textsuperscript{337,338}. Since ALN acts on osteoclasts to interfere with their function and promote apoptosis, it decreases the osteoclastic resorption of calcified cartilage, leading to growth plate thickening. In addition, ALN has also been shown to interfere with vascular invasion into the cartilage by inhibiting the expression of vascular endothelial growth factor (VEGF) in chondrocytes, which also interferes with endochondral ossification\textsuperscript{336}.

Another interesting observation in this study is the apparent saturation of the conjugate effects on tissue-level remodeling. While the absolute measurements of various remodeling indices showed a dose-dependent effect between CL and CH, this was not seen in the normalized measures after accounting for the total quantity of bone in each group. This effect was consistent in both the formation and resorption measures, and indicates that there may have been a “saturation” in the tissue-level response to conjugate treatment. Since PGE\textsubscript{2} has been shown to self-amplify its effect by upregulating the expression of EP4 receptors on osteoblasts\textsuperscript{146}, it is possible that the sustained release of EP4a at bone sites resulted in saturation of the osteoblastic capacity to express the EP4 receptor. If the expression of EP4 receptor reaches its maximum, and all the receptors are already interacting with available agonists, then the addition of new agonists would not increase the effect of EP4a. This may be a possible mechanism of the saturation effect seen in the tissue-level response.

The comparable rates of remodeling between CL and CH at the end of the study suggests saturation of the tissue-level response at some point during the study. Although the precise location of this saturation point could not be determined based on the available data, it is possible that CH did show greater rate of remodeling than CL near the beginning of treatment, which resulted in dose-dependent increase in bone volume. Although the proportion of bone undergoing remodeling was comparable between the two groups after saturation, the absolute volume of bone formed is greater in CH due to its overall greater bone volume.

The animals in CH exhibit somewhat different periosteal bone formation in response to treatment. Some animals show significant periosteal bone formation with concomitant increase in diffuse fluorescent labeling, while others exhibit very little periosteal bone formation with virtually no fluorescent labeling.
In the animals with significant periosteal bone formation, the femoral cortex appears to be “smeared” due to the presence of low density periosteal bone, and in some instances, the femoral cortical structure becomes nearly unrecognizable. However, all but 2 of the animals showed occlusion of the marrow cavity due to endocortical bone formation, and the endocortical cavities appear identical in all animals irrespective of periosteal differences. This suggests that the main effects of the conjugate in inducing endocortical bone formation are present in all animals, but some of the animals seem to exhibit different tissue-level responses on the periosteal surface. It should be noted that the sample images were taken from the same relative location in all slides, so that the variability due to sampling location would be minimized. However, upon visual inspection of the slides, it was observed that the responses are not uniform throughout the tibial shaft. Depending on the location, all animals exhibit significant periosteal osteoid formation with concomitant diffuse fluorescent labeling in parts of their tibial shaft, but this response was not seen in the entire tibial shaft for all animals. Thus, the differences observed may also be due in part to the sampling location of the images.

Nonetheless, it is interesting that there are slight differences in the physiological response of the CH animals to the conjugate. The animals quickly became very ill after treatment was started, although the extent of illness varied somewhat among them. Given the significant fluctuation in the dosing of CH and the variability in animal reactions, this may have contributed to the differences seen. However, since no serum samples were taken in the current study to monitor animal responses during the course of treatment, it is difficult to determine the precise mechanism of these differences. It would be interesting to investigate this further in a future study, perhaps by examining the serum and urine markers of bone remodeling.

Since the mechanism of EP4a release at bone sites resembles continuous administration of the compound, it is worthwhile to examine other studies using continuous administration of PGE2. Tian et al. treated 6 month old rats with continuous IV administration of PGE2 at doses of 1 or 3 mg/kg/day, and showed that 21 days of treatment led to bone loss in both the proximal tibial metaphysis, tibial shaft, and the lumbar vertebrae. While both intermittent and continuous administrations of PGE2 stimulated bone remodeling, intermittent administration led to positive bone balance with formation exceeding resorption, while continuous administration resulted in negative bone balance with resorption exceeding formation. Under continuous administration, there was cancellous bone loss due to shortened formation period. In addition, although there was some gain of cortical bone mass via periosteal bone formation, this gain was less than the loss of bone mass due to negative bone balance on the cortical endosteal surface and increased intracortical porosity. In contrast, intermittent PGE2 administration at the same doses of 1 mg/kg and 3 mg/kg resulted in
cancellous bone gain via positive bone balance and shortened resorption period. Moreover, intermittent administration also led to net gain of cortical bone mass by endocortical formation exceeding any intracortical loss. Although intermittent PGE₂ also induces intracortical remodeling, the extent is not nearly as high compared to continuous administration.

In comparison with the studies using continuous PGE₂ administration, the significantly increased intracortical porosity induced by conjugate treatment may be partially the result of its mode of EP4a release, which resembles continuous administration. While the intracortical porosity due to conjugate treatment is significantly higher than that of PGE₂, the induction intracortical remodeling likely occurs via a similar mechanism for both the conjugate and continuous PGE₂ administration. However, given previous studies have demonstrated that continuous PGE₂ leads to net bone loss in both cancellous and cortical bone, it is interesting that the current study shows net bone gain due to conjugate treatment. Although there are two components with potential for pharmacological action in the conjugate, earlier results have shown that the ALN component is mostly inactive due to lack of LK cleavage. However, the local delivery of EP4a to bone sites may have somehow facilitated an anti-resorptive effect which is seen in the lack of TRAP activity in these osteoclasts. This may have in turn contributed to a reversal of the net bone loss resulting from continuous PGE₂.

The effects of conjugate treatment were also compared to those of continuous and intermittent PTH. Although studies have led to mixed results regarding the precise mechanism of the effects on bone in the two modes of administration, there have been consistent findings of the net anabolic effect in intermittent PTH exceeding that of continuous administration. In trabecular bone, Hock et al.\textsuperscript{115} showed that intermittent administration of PTH increased trabecular bone volume by increasing trabecular thickness and number, while continuous infusion of PTH did not alter trabecular bone volume, thickness, or number relative to vehicle-treated control. In contrast, Uzawa et al.\textsuperscript{116} showed that both continuous and intermittent administration led to increased trabecular bone volume, while continuous administration also increased trabecular thickness. The trabecular number was not reported in the Uzawa et al study.

In cortical bone, both Uzawa et al and Hock et al reported increased cortical bone mass with intermittent PTH. However, Uzawa et al.\textsuperscript{116} found marked increase in cortical porosity in continuous PTH administration, which led to decreased cortical bone mass relative to vehicle control. However, Hock et al found comparable cortical bone mass relative to vehicle control, and did not report cortical porosity. Nonetheless, although remodeling is increased in both modes of administration, the net anabolic effect in intermittent PTH exceeds that of continuous administration.
The mechanism of action on bone is similar between PTH and PGE$_2$. Both increase intracellular cAMP and PKA, with PTH also increasing intracellular calcium while PGE$_2$ can increase intracellular calcium depending on the EP receptor (of which the EP4 receptor mainly activates cAMP/PKA)$^{123190}$. However, studies have shown that PTH also activates the canonical Wnt pathway, which involves the binding of Wnt ligands to a complex of Frizzled and LRPS/LRP6 receptors leading to stabilization of β-catenin$^{341}$. The β-catenin then moves into the nucleus to activate gene transcription$^{341}$. It has been shown that PTH-induced ALP was significantly decreased by the knockdown of β-catenin in osteoblast cell lines in vitro$^{342}$. Moreover, PGE$_2$ has also been shown to activate the canonical Wnt pathway$^{341}$.

The Wnt pathway is involved in the osteoblastic induction of osteoclastogenesis via production of OPG$^{343}$, and antagonists of the Wnt receptor such as secreted frizzled-related protein-1 (SFRP1) has been shown to control osteoblast and osteocyte apoptosis$^{344}$. In addition, the Wnt antagonist sclerostin is also involved in maintaining normal bone mass, as sclerostin knockout mice have been shown to exhibit excess bone growth$^{345,346}$. Researchers have recently demonstrated a crossover between the Wnt and cAMP pathway, as the Frizzled receptor can also activate downstream cAMP/PKA activity (Figure 5–2)$^{347}$. In examining the effect of PGE$_2$ on Wnt, Genetos et al$^{341}$ found that PGE$_2$ suppressed sclerostin but not Dkk1, which is another Wnt antagonist. In contrast, PTH suppressed both sclerostin and Dkk1. These findings may help to explain potential differences between the mechanism of action for PGE$_2$ and PTH. In addition, although the same study also suggested that these effects of PGE$_2$ occur through the EP2 receptor, this may have been influenced by the higher expression of EP2 relative to EP4 receptors in the particular osteoblast cell line used in the study.

Although the tremendous anabolic efficacy of the conjugate drug in trabecular bone is promising, this must be balanced with its effects in cortical bone. In an ideal therapy for osteoporosis, trabecular bone volume should be restored without inducing overwhelming effects in cortical bone that compromise its mechanical and structural integrity. In the conjugate drug, the trabecular bone volume is successfully restored, but there is also a simultaneous increase in cortical porosity due to elevated intracortical remodeling as well as the increased endocortical bone formation inside the marrow cavity. This may compromise the structural and mechanical integrity of cortical bone. In fact, this is seen in the high dose group, in which the tremendous endocortical bone formation led to the occlusion of the marrow cavity. Perhaps future work using the conjugate should investigate lower doses of the drug to mitigate the cortical bone effects due to treatment, as the highly potent effects of this conjugate already appear to saturate the tissue-level response in the bone at the dose used in CL.
Given the sustained release of EP4a in bone which is facilitated by the conjugate drug’s mechanism of action, it is likely that a much lower dose of the conjugate drug will still achieve anabolic efficacy. This may also enable the cortical bone effects to be managed, such that balance may be found between trabecular bone formation and the induction of endocortical bone formation and intracortical remodeling.

![Illustration of crossover between cAMP and canonical Wnt pathways](image)

**Figure 5-2. Illustration of crossover between cAMP and canonical Wnt pathways.** This is activated when a Wnt protein activates the Frizzled receptor bound to a trimeric G protein. The G-protein hydrolyzes GTP to GDP, which releases the Gs subunit to activate adenylate cyclase, resulting in the production of cAMP. The cAMP then binds to PKA, which changes the conformation of PKA to release the catalytic subunit. The catalytic subunit then moves into the nucleus to phosphorylate the transcription factor CREB, which binds to genes that contain a cAMP response element. Reproduced from reference 348.

Even though the ALN component of the conjugate drug did not exert much anti-resorptive effect, this could be an unintended advantage. Since prolonged treatment of high dose ALN leads to oversuppression of turnover and compromised structural and mechanical properties of bone, the excessive inhibitory effect of fully active high dose ALN in the treatment might have suppressed the potential for anabolic response. Since EP4a has effects on both resorption and formation, perhaps the current outcome with minimal contributions from ALN is better than a fully dual-acting conjugate. However, this remains to be further investigated.

The idea of combining anabolic and anti-resorptive therapies in the treatment of osteoporosis is not new. As discussed in the Introduction, clinical trials have investigated the efficacy of combination PTH and ALN therapy, with mixed results. In addition, a previous study has also been conducted to investigate the combined effects of ALN and PGE2 in treating OVX rats with established osteopenia. Using 6-month-old rats that were OVX and then allowed to lose bone for 2 months, the researchers treated the rats for 25 days and showed that the bone formation due to combined therapy was less...
than that of PGE₂ alone. This was true for both dynamic bone formation parameters in trabecular bone and bone volume in the proximal tibial metaphysis\textsuperscript{349}. In contrast, the conjugate drug in the current study demonstrates strong anabolic effects in both the proximal tibial metaphysis and the lumbar vertebrae, and appears to show both anti-resorptive and anabolic effects.
6 Conclusion

• Conjugate treatment led to significant increase in trabecular bone formation, with tissue-level anabolic effects that exceed PGE$_2$.

• Conjugate treatment led to dose-dependent increase in trabecular bone volume in both the proximal tibial metaphysis and lumbar vertebrae. This is due to trabecular thickening in the proximal tibial metaphysis without increasing trabecular thickness, while the lumbar vertebrae shows increased trabecular number without changing trabecular thickness. This led to partial and complete recovery of OVX-induced bone loss.

• The ALN component of the conjugate did not exert much anti-resorptive effect, likely due to insufficient cleavage of LK.

• Conjugate treatment led to dose-dependent intracortical remodeling and endocortical bone formation, with the high dose showing excessive endocortical bone formation that led to occlusion of the marrow cavity.

• Conjugate treatment dose-dependently increased the vertebral strength in compression, but the tissue material was unchanged. The strength of cortical bone in the mid-diaphysis is also dose-dependently increased under conjugate treatment, while the material strength is decreased due to greater porosity.

• There appears to be suppression of TRAP activity in the conjugate-treated groups, with cells expressing secondary osteoclast markers. This must be confirmed with histochemical staining for additional osteoclast markers such as RANK or calcitonin receptor.
7 Future Work

Based on the current study, several experiments should be conducted in the future:

1. Radio-labeling study to determine the time course of LK release, as this provides information about the extent of ALN activity in the conjugate.

2. Osteoclast staining using additional osteoclast markers such as calcitonin receptor and/or RANK to ascertain the identity of the TRAP-negative osteoclast-like cells.

3. Further in vivo study determine the efficacy of the conjugate drug at lower doses. This should be designed to cover a range of doses for examination of the dose-response relationship as well as determination of the location of “saturation” in tissue-level response.

4. Investigate the time course of metabolic responses of the animals, such as collecting serum samples during the course of treatment. This helps to characterize the metabolic response of the animals to the conjugate.

5. Investigate the long-term in vivo effects of conjugate treatment in OVX rats, particularly at lower doses of the conjugate to examine the balance between trabecular and cortical bone effects. A long-term still will also facilitate the examination of differences in mechanical properties.

6. Investigate the effects of conjugate treatment on osteocytes.
8 References


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