Investigating *In Vivo* Efficacy of Novel ALN-EP4a Conjugate Drugs for the Treatment of Post-Menopausal Osteoporosis

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Current treatments for post-menopausal osteoporosis are mainly anti-resorptive, which decrease osteoclastic bone resorption but show little bone-building effects to reverse bone loss. The current study employs a conjugate approach in which an anabolic agent (EP4 receptor agonist) is covalently linked to an anti-resorptive agent (alendronate) to create two novel conjugate drugs: C1 and C2. The rationale is that when administered systemically, C1 and C2 will be target delivered to bone through alendronate’s ability to bind to bone mineral, thereby decreasing side effects associated with systemic administration of the EP4 agonist. This study investigated the *in vivo* efficacy of the C1 and C2 conjugates for the treatment of post-menopausal osteoporosis in a curative experiment using the ovariectomized rat model. Results showed that the C1 conjugate dose-dependently stimulated new bone formation in both cortical and trabecular bone, while the anabolic effects of the C2 conjugates were not observed in any skeletal sites.
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LIST OF ABBREVIATIONS

aBMD Areal Bone Mineral Density
ALN Alendronate
ALN-LK Alendronate covalently linked to linker molecule
ALN-PGE2 Alendronate covalently linked to PGE2, conjugate drug in previous study
ALP Alkaline phosphatase
ANOVA Analysis of variance
ATP Adenosine triphosphate
BFR/BS Bone formation rate normalized over bone surface
BFR/BV Bone formation rate normalized over bone volume
BMD Bone mineral density
BMC Bone mineral content
BMP Bone morphogenic protein
BMU Basis multicellular unit
BP Bisphosphonate
BSE Back-scattered electron microscopy
BV/TV Percent Bone Volume
C1 Conjugate drug with the structure: ALN-LK-EP4a
C1H Treatment group: OVX rats given C1 high dose treatment
C1L Treatment group: OVX rats given C1 low dose treatment
C2 Conjugate drug with the structure: ALN-EP4a
C2H Treatment group: OVX rats given C2 high dose treatment
C2L Treatment group: OVX rats given C2 low dose treatment
C2M Treatment group, unconjugated EP4a and ALN in a drug mixture
cAMP Cyclic adenosine monophosphate
CCK Cholecystokinin
COOH Carboxyl moiety
DEXA Dual-energy X-ray absorptiometry
dLS Double label surface
EDTA Ethylenediaminetetraacetic acid
EP4a Synthetic EP4 receptor agonist used in the conjugate drugs
EP4a-LK EP4 receptor agonist connected to the linker molecule
FPP  Farnesyl diphosphate, also known as farnesyl pyrophosphate
FPPS  Farnesyl diphosphate synthase
FWHMH  Full width at half maximum height
GGPP  Geranylgeranyl diphosphate
HA  Hydroxyapatite
Ir.L.Wi  Interlabel width
IV  Intravenous
LK  Linker molecule used in the C1 conjugate
LK-ALN  Linker molecule connected to alendronate
MAR  Mineral apposition rate
MicroCT  Micro-computed tomography
MS  Mineralizing surface
MS/BS  Percent mineralizing surface
N  Nitrogen atom
N-BPs  Nitrogen containing bisphosphonates
N.Ob  Osteoblast number
N.Ob/BS  Osteoblast number normalized over bone surface
N.Oc  Osteoclast number
N.Oc/BS  Osteoclast number normalized over bone surface
N.Ot  Osteocyte number
N.Ot/BS  Osteocyte number normalized over bone surface
Oc.S  Osteoclast surface
Oc.S/BS  Percent osteoclast surface
OH  Hydroxyl moiety
OVX  Ovariectomy, also represent treatment group: OVX rats given vehicle treatment
PBS  Phosphate buffered saline
PGE\textsubscript{2}  Prostaglandin E\textsubscript{2}
PMMA  Polymethylmethacrylate
PTH  Parathyroid hormone
ROI  Region of interest
SEM  Standard error of the mean
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SH</td>
<td>Sham ovariectomy procedure, also represent treatment group: sham rats given vehicle</td>
</tr>
<tr>
<td>sLS</td>
<td>Single label surface</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Tb.N</td>
<td>Trabecular number</td>
</tr>
<tr>
<td>Tb.Sp</td>
<td>Trabecular separation</td>
</tr>
<tr>
<td>Tb.Th</td>
<td>Trabecular thickness</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>vBMD</td>
<td>Volumetric bone mineral density</td>
</tr>
<tr>
<td>VOI</td>
<td>Volume of interest</td>
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1 INTRODUCTION

Osteoporosis is a metabolic bone disease characterized by low bone mineral density (BMD), deterioration of the cancellous bone microarchitecture leading to enhanced bone fragility and increased risk of fracture\(^1\). By the year 2020, it is estimated that 14 million people in the United States will be suffering from the disease in addition to 47 million people with established osteopenia, or low bone mass*. In Canada, treatment of osteoporosis and associated fractures is costing the health care system over $2.3 billion annually as of 2010**. Furthermore, this number is expected to greatly increase over the next twenty years due to our aging population. There is therefore an urgent need for effective treatments of osteoporosis. Unfortunately, osteoporosis is often not diagnosed until after a patient experiences a fracture, at which point significant bone loss has already occurred. Thus effective treatment of osteoporosis requires not only slowing the progression of the disease but also recovering lost bone in osteoporotic patients.

1.1 BIOLOGY OF BONE

1.1.1 BONE STRUCTURE

Bone serves important functions in the body including providing protection of vital organs and structural support for the body, act as attachment sites for muscles, serve as mineral reservoir for ions\(^2\) and as sites for blood cell formation\(^3\). The bones of the adult skeleton consist of two major types: cortical bone (or compact) and cancellous (or trabecular) bone (Figure 1-1). Cortical bone is dense, rigid and is mainly found in shafts of long bone. Furthermore, cortical bone accounts for about 80% of the skeletal bone mass\(^4\). A detailed diagram depicting the

*Information obtained from National Osteoporosis Foundation Website: http://www.nof.org/.
**Information obtained from Osteoporosis Canada Website: http://www.osteoporosis.ca/osteoporosis-and-you/osteoporosis-facts-and-statistics/.
hierarchical structure of the human cortical bone is shown in Figure 1-2. The fundamental functional unit of cortical bone is the osteon, which are vascular channels surrounded by concentric layers of lamellae\textsuperscript{5}. The lamellae are consisted of collagen fibers, which are made up of bundles of collagen fibrils. Furthermore, collagen fibrils are composed of organized collagen molecules with mineral occupying gaps in between the collagen molecules. While this is true in humans, cortical bone in rats has a rotated plywood structure composed of primary lamellar bone\textsuperscript{6}.

In contrast to cortical bone, cancellous bone accounts for 20\% of the skeletal mass and is composed of a three-dimensional network of struts (trabeculae) oriented along areas of stress\textsuperscript{4}. It is predominately found in the vertebrae as well as proximal ends of long bones and ribs. The functional unit of cancellous bone are known as packets, which are also composed of layers of lamellae but are semilunar in shape\textsuperscript{2}. Furthermore, cancellous bone has much greater porosity than cortical bone, where marrow, blood vessels as well as other macromolecules are found within the pores of cancellous bone. Also due to this greater porosity, cancellous bone has a much greater surface area than cortical bone, accounting for more than 80\% of bone’s total surface area\textsuperscript{4}. As a result of the large surface area, cancellous bone is more dynamic and metabolically active than cortical bone.
Figure 1-1: The two types of structures of bone: compact (cortical) bone and cancellous (trabecular bone). Image take from https://courses.stu.qmul.ac.uk/smd/kb/microanatomy/bone/answers/index.htm.

Figure 1-2: The hierarchical structure of bone. The fundamental functional unit of cortical bone is the osteon made up of concentric layers of lamellae. Lamellae are consisted of collagen fibers, which are made up of bundles of collagen fibrils, and are in turn composed of organized collagen molecules with mineral occupying gaps in between the molecules. Image adapted from Lakes, 1993."
1.1.2 BONE COMPOSITION

Bone tissue is a composite material comprised of an organic matrix component and an inorganic mineral phase. In fact, bone is composed of 60-70% mineral, 5-8% water and solutes, while the organic phase accounts for the rest. The organic matrix is primarily Type I collagen in a triple helix form, as well as small amounts of other collagen proteins including Type III, V and X collagens. The collagen fibers are aligned in parallel to each other and stabilized by inter and intramolecular crosslinks. Furthermore, a number of noncollagenous proteins such as proteoglycans, fibronectin, osteopontin, bone morphogenic protein (BMP) and others are also present in the bone matrix, which account for approximately 10-15% of the total bone protein.

The mineral phase of the bone is composed of a poorly crystalline calcium phosphate salt in the form of hydroxyapatite (HA) with the formula \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \). Substitutions by other minerals such as magnesium and potassium are common in hydroxyapatite, which can alter the bone’s biological properties. In the bone, there is a strong interface between the collagen and the mineral phase, with the long axis of the mineral crystals oriented parallel to the collagen fibers. Both the organic phase and the mineral component are important in establishing the unique biomechanical properties of bone. The mineral phase of the bone provides strength and mechanical resistance while the organic phase gives bone its ductility and increases its toughness.

1.1.3 BONE CELLS

There are three types of bone cells involved in the maintenance of bone: osteoblasts, osteocytes and osteoclasts. Osteoclasts are bone-resorbing cells with a hematopoietic origin. These are large multinucleated cells which are formed by fusion of multiple mononuclear precursor cells during osteoclast differentiation. During bone resorption, osteoclasts adhere to
bone surface by binding to integrins and create a sealing zone over the bone. The basal membrane of the osteoclasts then form a ruffled border, which contain proton pumps that pump hydrogen ions into the sealing zone to dissolve the bone mineral underneath. Furthermore, lysosomal enzymes such as Cathepsin K are released from the osteoclasts to degrade the underlying organic matrix.

Osteoblasts are bone forming cells that are derived from mesenchymal stromal cells. Mesenchymal stromal cells are originated from mesenchymal stem cells (MSCs), which gives rise to a number of other cell types including adipocytes, chondrocytes and myoblasts. Osteoblasts line bone surfaces and produce matrix elements such as type I collagen, osteocalcin, proteoglycans, alkaline phosphatases, (ALP), and etc. Compared to osteoclasts, osteoblasts are smaller in size and contain a single nucleus. In addition to secreting bone matrix protein, osteoblasts are also responsible for mineralizing the bone matrix to form new bone.

Osteocytes are terminally differentiated osteoblasts that have been trapped within the mineralized bone matrix. They have long processes which connect to other osteocytes and osteoblasts through small channels in the bone called canaliculi. These connections allow them to communicate with each other in order to mediate the skeleton’s response to mechanical stress. Furthermore, osteocytes are thought to take part in cell signalling events that lead to the initiation of the bone remodeling cycle.

1.1.4 BONE REMODELLING

The adult skeleton is in a dynamic state of bone remodelling or turnover, where old bones are continually broken down by osteoclasts and replaced by osteoblasts. This is a very important process which allows bone to adapt to stress, repair microcracks, replace old or damaged bone, as well as function in the maintenance of mineral homeostasis. Bone remodelling occurs at
discrete sites throughout the skeleton and is carried out by Basic Multicellular Units (BMU). BMU are bone remodelling units composed of tightly coupled groups of osteoclasts and osteoblasts that carry out the sequential action of old bone resorption and new bone formation.

Bone remodelling follow a series of successive phases: activation, resorption, reversal and formation\textsuperscript{17} (Figure 1-3). During activation, mononuclear osteoclast precursors are recruited to the remodelling site, where they fuse to form mature multinucleated osteoclasts and bind to bone surface to form a sealing zone. The osteoclasts become activated with the formation of the ruffled border and begin resorbing bone by pumping protons and lysosome enzymes on the bone surface underneath\textsuperscript{17}. During each remodelling cycle, the resorption phase takes about 2 to 4 weeks, after which a resorption pit or Howship’s lacunae is left behind on the surface of trabecular bone. Resorption of cortical bone results in longitudinal tunnelling of the cortical osteon. Osteoclasts undergo apoptosis upon completion of the bone resorption phase. During the reversal phase, transition from resorption to formation occurs by the secretion of coupling factors from osteoclasts to osteoblasts, resulting in the tight coupling of bone resorption and formation\textsuperscript{18}. Scientists have investigated a number of osteoclast secreted factors including cardiotrophin-1\textsuperscript{19}, semaphorin 4D\textsuperscript{20} and Ephrin B2\textsuperscript{21}, all of which may have important roles in the coupling process. Lastly, the remodelling process is completed by formation, where osteoblasts secrete collagen matrix and promote mineralization of the matrix\textsuperscript{17}. The formation phase is the longest and occurs over a period of 3 months\textsuperscript{2}. As mineralization proceeds, some osteoblasts become trapped in the bone matrix and become osteocytes while the majority of osteoblasts undergo apoptosis upon completion of the bone remodelling cycle.
Since bone remodelling begins on the surface of bone, the rate of bone remodelling differs for cortical and trabecular bone. In cortical bone, remodelling occurs on the periosteal, endosteal and Haversian canal surfaces whereas in trabecular bone, remodelling occurs on the trabeculae. Since trabecular bone has a much greater surface area than cortical bone, the remodelling rate in trabecular bone can be 5 to 10 times higher than the cortical remodelling rates\textsuperscript{22}. As a result, disease and drug treatments which affect bone remodelling often lead to more pronounced effects in trabecular bone than in cortical bone.

The process of bone remodelling is regulated by a number of factors such as cytokines, mechanical stimuli as well as hormones including growth hormone or sex hormone\textsuperscript{23}. In order to maintain normal bone health, bone remodelling needs to be a balanced process between resorption of old bone and formation of new bone. This balance shifts as one ages\textsuperscript{4}. During childhood, bone formation exceeds bone resorption resulting in increased bone mass. During adulthood of healthy individuals, the process of bone remodelling is balanced and there is no net
change in bone mass. After 30-40 years of age, the balance shifts such that resorption exceeds formation, resulting in gradual loss of bone mass as an individual ages\textsuperscript{24}. Bone diseases which lead to imbalance in the bone remodelling process such as osteoporosis can also result in net bone loss.

1.2 **OSTEOPOROSIS**

Osteoporosis is a metabolic bone disease characterized by decreased bone mass leading to changes in bone microarchitecture and increased risk of fracture\textsuperscript{1}. Osteoporosis is classified as either primary or secondary. Secondary osteoporosis occur as a result of another recognizable disease such as hyperthyroidism and account for about 10% of total osteoporotic conditions. Primary osteoporosis accounts for the majority of osteoporosis cases, and can be further divided into two main categories: postmenopausal osteoporosis (Type I) or age-related osteoporosis (Type II). Postmenopausal osteoporosis mainly affects postmenopausal women due to the large reduction in estrogen production after menopause and is the most common form of primary osteoporosis. It is characterized by accelerated cancellous bone loss and increased risk of vertebral fracture\textsuperscript{25}. Age-related osteoporosis is associated with aging in both men and women due to the normal loss of bone over time. It is characterized by bone loss of both cancellous and compact bone, and most frequently leads to hip fractures\textsuperscript{24}.

1.2.1 **POSTMENOPAUSAL OSTEOEPROSIS**

Postmenopausal osteoporosis is the most common form of osteoporosis and affects mostly women after menopause. It is characterized by low bone mineral density (BMD), deterioration of the cancellous bone microarchitecture leading to enhanced bone fragility and increased risk of fracture of the spine and wrist\textsuperscript{25}.
At menopause, women experience a significant reduction in estrogen production, which leads to an overall increase in bone remodelling activity\textsuperscript{26}. However, the remodelling process becomes unbalanced where bone resorption exceeds formation, resulting in overall bone loss. Due to the much larger surface area of trabecular bone vs cortical bone and greater rate of bone remodelling, trabecular bone is more negatively affected by this type of osteoporotic bone loss. Increased osteoclastic activity leads to larger eroded cavities in the trabeculae, which can lead to loss of trabecular elements and decrease in trabecular bone volume. In turn, this results in the deterioration of the trabecular microarchitecture and increased risk of fracture\textsuperscript{27}.

The diagnosis of postmenopausal osteoporosis is based on the determination of areal bone mineral density (aBMD) measurements of the spine and hip obtained using dual-energy X-ray absorptiometry (DEXA)\textsuperscript{28,29}. The aBMD of an individual is compared to that of a reference healthy population in order to generate his or her T score. The T score is used to categorize the individual’s disease state as defined by the World Health Organization (WHO): 1) a T-score within 1 standard deviation (SD) less than the reference population’s mean is considered normal; 2) T-score between 1 to 2.5 SD below the reference mean is considered osteopenic; and 3) T-score more than 2.5 SD below the reference mean is defined as osteoporotic\textsuperscript{30}.

1.2.2 THE OVX RAT MODEL OF POSTMENOPAUSAL OSTEOPOROSIS

Animal models are often required to effectively study the pathogenesis and treatment of osteoporosis. The rat is an established model for osteoporosis as they are economical, easy to handle and exhibit similar skeletal biology compared to humans. However, it must be pointed out that rats lack the Haversian remodelling system and so may not be a good model for the investigation of cortical remodelling in humans\textsuperscript{31}.
The ovariectomized (OVX) rat model is a commonly adopted animal model for postmenopausal osteoporosis. In the OVX rat model, animals are subject to bilateral ovariectomy where both ovaries are removed. This leads to an estrogen deficiency similar to that in postmenopausal women, which induce significant bone loss\(^\text{32}\). Past studies have shown that OVX induced bone loss in rats share many similar characteristics with postmenopausal bone loss in humans, thus making it a suitable animal model for the current study. OVX rats exhibit increase in levels of bone turnover where bone resorption exceeds bone formation, leading to rapid initial bone loss which eventually plateaus. Just as in humans, trabecular bone are more negatively affected by OVX induced bone loss than cortical bone due to its greater surface area. Past studies have shown that OVX in rats lead to deterioration of trabecular microarchitecture, such as decreased trabecular bone volume, decreased trabecular number and increased trabecular separation\(^\text{33,34}\). However, it should be noted that OVX rats do not develop fractures that occur in human and therefore the disease state in OVX rats is termed osteopenia rather than osteoporosis\(^\text{32}\). Furthermore, it should also be noted that menopause in women is actually a gradual process that is proceeded by a perimenopausal period lasting 2 to 8 years\(^\text{35}\), during which estrogen production gradually declines resulting in accelerated bone loss\(^\text{36}\). This gradual period of decline in estrogen level is absent in OVX rats. As a result, the OVX rat model more closely resembles a condition where women undergo bilateral oophorectomy and experience a sudden drop in estrogen rather than the actual gradual process of menopausal development in women.

1.3 TREATMENT OF OSTEOPOROSIS

Most current treatment of osteoporosis can be grouped into two categories: anti-resportive or anabolic. Anti-resportive drugs act to decrease bone resorption in order to prevent
further osteoporotic bone loss while anabolic agents work to promote bone formation for the purpose of restoring lost bone.

1.3.1 BISPHOSPHONATES

Bisphosphonates (BP) are a class of anti-resortive agents widely used for the treatment of osteoporosis. They are synthetic compounds that have the ability to bind to HA in the bone, which allows them to concentrate selectively in the skeleton and decrease bone resorption by inhibiting osteoclastic bone resorption. Randomized controlled trials of various BPs have demonstrated that BP significantly reduces osteoporotic bone loss, improves BMD and reduce incidences of fracture at the spine, hip and other skeletal sites in treated individuals. Currently, a number of BPs including alendronate, ibandronate, risedronate, and zoledronate are approved for the treatment of osteoporosis worldwide.

1.3.1.1 PHARMACOKINETICS

BPs are typically given orally or systemically by intravenous (IV) injection. The intestinal absorption of BPs is poor (1%) and the absorption rate is decreased by the presence of food and mineral supplements. Once inside the circulatory system, BPs are rapidly cleared from the circulation system with an elimination half-life of a few hours or less. Of the total dose reaching the circulatory system, about 50% concentrate in bone primarily at sites of active bone remodelling while the rest is excreted unmetabolized by the kidney. Once bound to bone, BPs inhibit bone resorption and become deposited under bone minerals. The rate of elimination of BPs from bone is very long with a half-life of around 10 years, and is dependent on the rate of bone remodelling. BPs’ slow rate of release from the skeleton causes them to have long lasting effects on bone resorption following cessation of treatments.
1.3.1.2 MOLECULAR STRUCTURE

BPs are synthetic analogs of inorganic pyrophosphate, which are natural circulating inhibitors of bone mineralization in the blood and urine\textsuperscript{49}. In BPs, the oxygen atom in the P-O-P structure of pyrophosphates is replaced by a carbon atom, giving rise to a P-C-P core structure\textsuperscript{50}. The P-C-P structure is resistant to biological degradation, which makes BPs suitable for clinical use. In addition to their stability, the P-C-P moiety is also required for BP’s anti-resorptive activity as well as the ability to bind to HA. Past studies have shown that modifications to one or both of the phosphonate groups can significantly decrease BP’s affinity for bone mineral and reduce its biochemical potency\textsuperscript{37}.

In addition to the P-C-P core structure, BPs have two additional side chains (R1 and R2) attached to the carbon atom, which allow for the synthesis of a variety of BPs with differential pharmacological properties\textsuperscript{37}. A hydroxyl substitution (OH) at R1 greatly enhances BPs’ affinity to bind to bone mineral while the addition of a nitrogen (N) atom on the R2 side chain enhances BPs’ biochemical potency\textsuperscript{50}. In fact, a number of such BPs collectively termed N-containing BPs (N-BPs) have been synthesized, many of which have demonstrated significant clinical efficacy in reducing osteoporotic bone loss\textsuperscript{40-42}. For example, previous randomized trials of alendronate (ALN), a nitrogen containing BP showed that women treated with ALN over a period of 3 to 5 years had significantly higher BMD and lower vertebral fracture risks\textsuperscript{40,51-53}. Furthermore, increasing the degree of substitution of the N moiety also increases the antiresorptive potency of the BP while the incorporation of the N atom in a heterocyclic ring produces BPs with the highest potency\textsuperscript{37}. To date, a number of different BPs have been synthesized (Figure 1-4)\textsuperscript{37}. These BPs exit differential potency for inhibiting bone resorption due to their varied R2 side chains (Table 1-1)\textsuperscript{54}. For example, zoledronate and risedronate are two of the most potent
antiresorptive BPs due to their nitrogen containing heterocyclic rings. Ibandronate is more potent than alendronate and pamidronate due to its more highly substituted nitrogen atom\textsuperscript{37}.

**Figure 1-4: BP Structures** Compounds are classified based on their side chains (R1 and R2). Some BPs contain a nitrogen atom on their R2 side chains while others do not, giving rise to BPs with different antiresorptive potencies. The N-BPs can be further differentiated by the degree of substitution of the N atom and whether it is in a heterocyclic ring. Image adapted from reference\textsuperscript{37}. 
1.3.1.3 MECHANISM OF ACTION

As mentioned previously, BPs have the ability to bind to HA, which brings them in close proximity to bone cells such as osteoclasts and osteoblasts. During bone resorption, the subcellular space beneath the osteoclast is acidified by proton pumps located in the ruffled border of the osteoclasts, which causes dissolution of the HA. This leads to the release of the BPs from the bone mineral underneath the osteoclasts, which are then internalized by osteoclasts most likely by fluid phase endocytosis\textsuperscript{37}. This is supported by previous studies using fluorescent labeled and radiolabeled BPs, which demonstrated BP localization to intracellular endocytic vesicles in osteoclasts\textsuperscript{55,56}.

Based on previous literature, there appear to be two distinct molecular mechanisms by which BPs decrease osteoclastic resorption\textsuperscript{57}. The first is adopted by non-N containing BPs such

<table>
<thead>
<tr>
<th>Drug</th>
<th>Relative potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etidronate</td>
<td>1</td>
</tr>
<tr>
<td>Clodronate</td>
<td>10</td>
</tr>
<tr>
<td>Tiludronate</td>
<td>10</td>
</tr>
<tr>
<td>Pamidronate</td>
<td>100</td>
</tr>
<tr>
<td>Neridronate</td>
<td>100</td>
</tr>
<tr>
<td>Alendronate</td>
<td>100 -- &lt; 1000</td>
</tr>
<tr>
<td>Clamadronate</td>
<td>100 -- &lt; 1000</td>
</tr>
<tr>
<td>EB-1053</td>
<td>100 -- &lt; 1000</td>
</tr>
<tr>
<td>Olpadronate</td>
<td>100 -- &lt; 1000</td>
</tr>
<tr>
<td>Ibandronate</td>
<td>1000 -- &lt; 10,000</td>
</tr>
<tr>
<td>Risedronate</td>
<td>1000 -- &lt; 10,000</td>
</tr>
<tr>
<td>YH 529</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Zoledronate</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

Table 1-1: Anti-resorptive potency of various BPs Table taken from reference\textsuperscript{54}.
as etidronate, clodronate and tiludronate. Due to their close resemblance to the structure of naturally occurring pyrophosphates, they can become incorporated into adenosine triphosphate (ATP) to make β, γ-methylene analogs of the regular ATP\textsuperscript{58,59}. These ATP analogues are non-hydrolyzable and accumulate intracellularly to induce osteoclast apoptosis\textsuperscript{58}.

The N containing BPs such as alendronate and ibandronate do not become incorporated into ATP analogous but instead interfere with the mevalonate pathway of osteoclasts\textsuperscript{37,60}. The mevalonate pathway is essential for the production of cholesterol and other sterols\textsuperscript{61,62}. Several enzymes in this pathway uses isoprenoid diphosphates as a substrate, for which N-BPs can act as a substrate analogs for one or more of the enzymes and in turn inhibit their enzymatic activity. Past studies have shown that N-BPs’ major target is farnesyl diphosphate synthase (FPPS)\textsuperscript{63,64}, which is the enzymes that synthesizes farnesyl pyrophosphate (FPP). FPP is the substrate of geranylgeranyl-pyrophosphate (GGPP) synthase, and so the reduction in the intracellular availability of FPP in turn results in decreased production of GGPP. Both FPP and GGPP are isoprenoid metabolites required for prenylation of small GTPases, which allow GTPases to anchor to the cell membrane and attain correct subcellular membrane localization\textsuperscript{57,65,66}. Failure of prenylation of GTPases causes the GTPases to accumulate intracellularly and unable to exert their regular function. Since GTPases are important proteins involved in a number of signalling pathways that control the regular functioning and survival of osteoclasts\textsuperscript{67-70}, N-BPs’ interference with the prenylation of GTPases therefore lead to impaired osteoclast function and increased apoptosis\textsuperscript{60,71}.

1.3.1.4 DRAW BACKS

Despite the significant clinical benefits of BPs in reducing bone loss associated with osteoporosis, long term treatment with these drugs have significant drawbacks. Due to the
natural coupling of bone resorption and bone formation\textsuperscript{72}, emerging evidence suggest that long-term use of BP not only suppress bone formation but is followed by a slow decrease in the rate of bone formation and overall decrease in bone turnover\textsuperscript{73,74}. Since bone remodelling is an essential mechanism for the maintenance of bone health by which old or damaged bone is replaced by new healthy bone, reduction in bone turnover can lead to accumulation of microdamage in the bone and may be implicated in the development of atypical fracture seen in some BP treated individuals\textsuperscript{75,76}.

1.3.2 PARATHYROID HORMONE (PTH)

Since osteoporosis is often diagnosed after significant bone loss has occurred, effective treatment of osteoporosis requires not only inhibiting bone resorption but also increasing bone formation. Thus effective anabolic agents are needed in order to restore bone loss in osteoporotic patients. The only clinically approved anabolic therapy for the treatment of osteoporosis is parathyroid hormone (PTH). PTH is a single chain polypeptide hormone secreted by the parathyroid glands in humans. Its regular function in the body is to increase blood calcium ion concentration by increasing calcium absorption by the intestine, reabsorption by the kidney as well as enhancing the release of calcium from bones\textsuperscript{77}. When administered exogenously, it has differing functions on the bones depending on its treatment regime\textsuperscript{78,79}. When administered continuously, it exerts an overall resorptive effect on bone. However when administered in low doses intermittently, it stimulates bone formation without major effects on resorption, resulting in an overall anabolic effect on bone\textsuperscript{80,81}. For example, one study showed that intermittent PTH injection in rats led to increased cancellous bone volume and improved mechanical strength\textsuperscript{82}, while another study demonstrated that daily injection of PTH increases bone mass and reduces fracture incidence in osteoporotic patients\textsuperscript{83}. The significant anabolic efficacy of PTH led to its
approval by the FDA in 2002 for the treatment of osteoporosis and is the only anabolic drug clinically available at this time. Unfortunately, long term treatments with PTH have been associated with osteogenic sarcoma in rats\textsuperscript{84-86}, thus its use may be limited due to safety concerns.

1.3.3 PROSTAGLANDIN E2 (PGE\textsubscript{2})

An alternative anabolic bone agent that is currently under investigation is prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). PGE\textsubscript{2} is an arachidonic acid derivative that is widely produced within the body. It is involved in a number of physiological and pathophysiological responses including bone metabolism, embryo implantation, induction of labour, as well as acts as an important mediator in inflammation, pain and fever\textsuperscript{87,88}. Past studies have shown that PGE\textsubscript{2} is a potent multifunctional regulator of bone turnover \textit{in vitro}, which can stimulate osteoclastogenesis and bone resorption in organ and marrow cultures\textsuperscript{89-92}, as well as increase bone formation by stimulation of osteoblast differentiation in stromal cell and primary calvarial cell cultures\textsuperscript{93-96}. Furthermore, PGE\textsubscript{2} has been shown to have an overall anabolic effect \textit{in vivo}, with effects such as increasing rat cortical and trabecular bone mass\textsuperscript{97-99}, preventing OVX-induced cancellous bone loss\textsuperscript{100,101}, as well as induction of periosteal and endosteal bone formation\textsuperscript{98,102}.

1.3.3.1 STRUCTURE AND SYNTHESIS

Prostaglandins belong to a family of signalling molecules called prostanoids, which are derived from the enzymatic modifications of C-20 unsaturated fatty acids called arachidonic acid by the activity of cyclooxygenase (COX)\textsuperscript{103}. The structure of prostaglandin is that of a 20-carbon chain containing a cyclopentane ring attached to two side chains called $\alpha$ and $\omega$. Based on the modifications of the cyclopentane ring, prostaglandins are classified into different types by the
letters A to I (Figure 1-5). Types A, B, and C do not occur naturally but are only produced artificially during extraction procedures. Types G, H and I are chemically unstable in physiological conditions while types D, E, and F are relatively more stable\textsuperscript{103}. In addition, prostanoids are further divided into series 1 to 3 based on the number of double bonds in their $\alpha$ and $\omega$ side chains. For example, PGE\textsubscript{2} contains two double bonds in its side chain while prostaglandin E\textsubscript{1} (PGE\textsubscript{1}) contains one double bond in its side chain.

\textbf{Figure 1-5: Structure of different types of prostanoids} Prostanoids are derived from modification of arachidonic acid by the enzyme cyclooxygenase (COX) and are classified based on modifications of the cyclopentane ring. Only series 2 prostanoids (with 2 double bonds in the side chains) are depicted here. TXA\textsubscript{2} denotes thromboxane, which is another member of the prostanoids family. Image taken from reference\textsuperscript{103}.
1.3.3.2 DOWNSTREAM RECEPTORS AND SIGNALLING PATHWAY

PGE₂ functions are mediated through binding to four cell surface G-protein coupled receptors, EP1-EP4\textsuperscript{104}, which in turn activate secondary messenger systems to mediate downstream effects (Table 1-2)\textsuperscript{103}. Specifically, EP2 and EP4 receptors mediate increase in intracellular cyclic adenosine monophosphate (cAMP) concentration, while EP1 activation leads to an elevation of cytosolic free calcium concentration\textsuperscript{87,104}. EP3 has multiple splicing variants but it is mainly regarded as an “inhibitory” receptor whose function downstream is to decrease cAMP production\textsuperscript{105,106}. Furthermore, these receptors have wide distributions in the body: EP3 and EP4 receptors are distributed throughout the body and their mRNAs have been found to be expressed in almost all mouse tissues examined\textsuperscript{107-109}; EP1 expression is mainly restricted to the kidney, lung and stomach\textsuperscript{110}; and EP2 is the least abundant but can be induced in response to stimuli, such as in macrophages and in epithelial cells of the uterus\textsuperscript{111-113}. Unfortunately as a result of the receptors’ wide distribution, systemic administration of exogenous PGE₂ can affect multiple systems in the body to cause a number of adverse effects including headaches\textsuperscript{114}, gastrointestinal problems (abdominal pain, nausea, vomiting, and diarrhea), lethargy, flushing\textsuperscript{107,115,116} and uterine contraction\textsuperscript{116}. Owing to these side effects, PGE₂ may not be an ideal therapeutic option for the treatment of osteoporosis.
**Table 1-2: Summary of signal transduction by prostanoids receptors.** EP shown in the red box represent receptors subtypes of PGE$_2$. EP3 has multiple isoforms which can elicit different responses of secondary messenger systems. Image taken from reference$^{103}$.

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtype</th>
<th>Isoform</th>
<th>G Protein</th>
<th>Second Messenger</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP</td>
<td>EP$_1$</td>
<td>Unidentified</td>
<td>$G_s$</td>
<td>cAMP ↑</td>
</tr>
<tr>
<td>EP</td>
<td>EP$_2$</td>
<td>$G_s$</td>
<td>cAMP ↑</td>
<td></td>
</tr>
<tr>
<td>EP</td>
<td>EP$_4$</td>
<td>$G_s$</td>
<td>cAMP ↑</td>
<td></td>
</tr>
<tr>
<td>EP</td>
<td>EP$_3$</td>
<td>$G_i$, $G_s$, $G_q$</td>
<td>cAMP ↓, cAMP ↑, PI response</td>
<td></td>
</tr>
</tbody>
</table>

1.3.3.3 EP4 RECEPTOR MEDIATED EFFECTS ON BONE

Among the four receptor subtypes of PGE$_2$, past studies have shown that the EP4 receptor subtype is the most abundantly expressed PGE$_2$ receptor in bone, including in primary cultures of human osteoblasts$^{117,118}$, purified rabbit mature osteoclasts$^{119}$, as well as in various osteoblastic cell lines and bone marrow stromal cell cultures of rats and mice$^{120-122}$. In fact, studies using EP1-EP4 specific knockout (KO) mice have shown that it is indeed the EP4 receptor that is responsible for mediating PGE$_2$ stimulatory effects on bone resorption and bone formation$^{123,124}$. Specifically, cultured calvarial cells from EP4 KO mice showed no increase in bone resorption when exposed to PGE$_2$,$^{123}$ and when PGE$_2$ was locally infused onto the periosteal surface of femurs, local bone formation was observed in wild type mice but not in EP4 KO mice$^{124}$. Furthermore, a number of synthetic EP4 agonists (EP4a) with high affinity for the EP4 receptor have been shown to strongly mimic PGE$_2$ effects on bone, including 1) stimulation
of bone resorption in mouse calvarial cultures\textsuperscript{123}; 2) induction of woven bone formation by local infusion in mice\textsuperscript{124}; 2) restoring trabecular and cortical bone mass and bone mechanical strength lost due to ovariectomy (OVX) in rats\textsuperscript{125} and 4) accelerating bone repair of rat femoral cortex after drill-hole injury\textsuperscript{126}. Taken together, these findings confirm that PGE\textsubscript{2} effects on bone are mediated through the EP4 receptor. However, despite their anabolic effects, systemic administration of EP4a also produces unwanted side effects including thickening of intestinal epithelium, hypotension and diarrhea in rodents\textsuperscript{124}, possibly limiting the clinical application of these agents as anabolic therapies for osteoporosis.

1.3.4 THE NEW CONJUGATE APPROACH

To decrease adverse effects associated with systematic administration of EP4a, the current study adopts a conjugate approach where a synthetic, stable EP4 agonist is covalently linked to the bisphosphonate ALN\textsuperscript{127}. The rationale is that when administered systemically, the conjugate drugs will be target delivered to bone through ALN’s ability to bind to hydroxyapatite(HA)\textsuperscript{37}, where local hydrolytic enzymes liberate the EP4a components to exert anabolic effects on bone (Figure 1-6). Additionally, ALN will remain attached to bone and exert anti-resorptive effects. There is thus a potential for the conjugates to exert dual effects on bone.

A similar conjugate approach was explored by Gil et al\textsuperscript{128} in a previous study, where PGE\textsubscript{2} and biologically inactive bisphosphonate carriers were covalently linked in a conjugate drug. However due to the chemical instability of PGE\textsubscript{2}, bioactive bisphosphonates such as ALN could not be used to prepare ALN-PGE\textsubscript{2} conjugates\textsuperscript{128}. Instead, an ALN molecule with slight alterations in structure was used in that conjugate, which retained its ability to bind to HA but did not exhibit anti-resorptive activities. The authors showed that this ALN-PGE\textsubscript{2} conjugate drug was metabolically stable and that the PGE\textsubscript{2} component was gradually released over a 7-day
period after binding of the conjugate to bone and cleavage of the conjugate link\textsuperscript{128}. In OVX rats treated with the conjugate for 4 weeks, histomorphometric analysis of the tibia metaphysis showed that ALN-PGE\(_2\) treatment led to increased mineralizing surface as well as bone formation rate compared to OVX controls. Unfortunately, the ALN-PGE\(_2\) conjugate treatment was unable to achieve significant recovery of OVX-induced bone loss back to sham levels. As previous studies have shown, PGE\(_2\) has a very short metabolic half-life of around 30 seconds\textsuperscript{103,129}, such that PGE\(_2\) is rapidly converted to inactive metabolites (13, 14-dihydro-15-keto PGE\(_2\)) \textit{in vivo} by the prostaglandin 15-dehydrogenase pathway\textsuperscript{130}. Thus it is possible that the liberated PGE\(_2\) was inactivated too quickly by enzymes in the bone environment to exert its full anabolic effects. Furthermore, since the modified ALN component used in the conjugate could not exert anti-resptive effects, PGE\(_2\) mediated increase in bone formation may also have been overwhelmed and masked by elevated levels of bone resorption in the OVX rats, resulting in no substantial anabolic effects in treated rats. In the current approach, stable synthetic EP4a with a longer \textit{in vivo} half-life is used in conjunction with the original, bioactive ALN in a conjugate for the purpose of achieving both robust anabolic and anti-resptive effects on bone.
Mechanism of Action

1. Delivery to bone due to ALN binding to hydroxyapatite
2. Local hydrolytic enzymes cleave conjugate to free EP4A
3. EP4A free to exert anabolic effect while ALN remains bound to bone to inhibit resorption

Figure 1-6: Simplified schematic of the conjugate mechanism of action bone. 1) Systemic administration of the conjugate will lead to target delivery of the conjugate to bone through ALN’s ability to bind to HA; 2) local hydrolytic enzyme cleave the conjugate linkage to free EP4a and 3) EP4a becomes activated and exert anabolic effects on bone while ALN remains attached to bone to exert anti-resorptive effects. Image created based on information from reference 127.

1.3.4.1 CONJUGATE COMPONENTS: ALN AND EP4A

In the current conjugate approach, the nitrogen contain BP - alendronate (ALN) is used as the bone targeting agent. ALN was chosen based on these reasons: 1) it is well characterized clinically as an anti-resorptive agent for osteoporosis therapy; 2) it demonstrates high anti-resorptive potency compared to etidronate (100-1000X)\textsuperscript{131}, 3) it leads to the inhibition of farnesyl diphosphate synthase (FPPS) by 61% rather than 94-99% as seen by zoledronate or risedronate treatment\textsuperscript{132}, which means it can effectively decrease osteoclastic resorption but not completely prohibit it, therefore may exhibit less long term side effects associated with the over
suppression of bone turnover; and 4) second to zoledronate, ALN exhibits one of the highest affinities for binding to HA, making it an excellent bone targeting agent\textsuperscript{133}.

The synthetic EP4 receptor agonist used in our conjugate is a synthetic compound developed by Billot et al\textsuperscript{134} (Figure 1-7). Compared to PGE\textsubscript{2} which has an \textit{in vivo} half-life of around 30 seconds\textsuperscript{103}, this EP4a is more metabolically stable and has \textit{in vivo} half-life around 2 hours\textsuperscript{134}. Furthermore, the EP4a’s potency for binding to the EP4 receptor as determined by radioligand binding assay is 1.2nM vs 1.1 nM (Ki) for PGE\textsubscript{2}\textsuperscript{135}, while cell efficacy assays showed that EP4a has an EC\textsubscript{50} of 2.5±1.0 nM, versus the EC\textsubscript{50} of PGE\textsubscript{2} at 3.0±0.4 nM\textsuperscript{134}. These data suggest that the EP4a has comparable or even slightly higher potency than PGE\textsubscript{2} itself \textit{in vitro}. Nonetheless, it is possible that the EP4a may have greater potency than PGE\textsubscript{2} \textit{in vivo} due to its greater stability, although this has not been explicitly investigated in experiments and so conclusions cannot be made regarding their relative potencies \textit{in vivo}.

Last but not least, the greater chemical stability of EP4a allows bioactive bisphosphonates to be used in the current conjugates instead of inactive BPs with diminished antiresorptive activities, thereby allowing these conjugates to have the potential to exert dual activities on bone\textsuperscript{127}. Taken together, these evidence strongly support that EP4a is the more preferable anabolic candidate in the current conjugates than PGE\textsubscript{2}.

It should be noted that while the original EP4a has a carboxyl group (COOH) at its carbon-1 terminal, the EP4a used in the conjugates carries an ester group (COOEt)\textsuperscript{127}. This was done to ease the chemistry during conjugate preparation. However as Arns et al\textsuperscript{127} have shown in their study, the ester group is quickly converted to the original EP4a compound within 30 minutes incubation of the compound in rat plasma. Thus it is expected that this conversion would take place quickly \textit{in vivo} before the conjugate drugs bind to bone or soon after.
1.3.4.2 CONJUGATE STRUCTURES

The present study aims to investigate the in vivo efficacy of the conjugate drugs as anabolic therapy for post-menopausal osteoporosis. Specifically, two conjugate drugs - C1 and C2 were tested in the study (Figure 1-8A)\textsuperscript{127}. Note that although C1 and C2 both have the same ALN and EP4a components, C1 has a short linker molecule (LK) joining the two components while C2 does not. The LK molecule is a 4-hydroxyphenylacetic acid, which is a natural compound found in foods and is not expected to have any pharmacological effects in vivo\textsuperscript{136}. In the C1 conjugate, the ALN’s terminal amine group is covalently linked to the LK molecule’s one end through a carbamate bond while the other end of the LK is attached to EP4a’s C-15 hydroxyl group through an ester bond. In the C2 conjugate, the ALN’s terminal amine group is directly linked to EP4a’s C-15 hydroxyl group through a carbamate bond. Since the terminal amine group and the C-15 hydroxyl group are required for the biological functions of ALN\textsuperscript{37} and EP4a\textsuperscript{134} respectively, the conjugate drugs are not expected to exert any drug effects until they are
cleaved to yield the original ALN and EP4a components. Nonetheless, since esterases are highly abundant in vivo, it is safe to expect that the EP4a in the C1 conjugate would be quickly released from the LK. However, the same could not be said about the less common carbamate linkage present in the C1 and C2. In past studies, phenol carbamates are thought to be cleaved by cholinesterases, carboxypeptidases and P450 enzymes in the liver. However, it is unclear if these activities are available in the bone environment to cleave the carbamate bonds present in the conjugates. Nonetheless, if enzymatic cleavage does occur at the carbamate linkage, we expect that this leaves ALN attached to an unstable carbamic acid, which would rapidly and spontaneously loses a CO₂ molecule to yield the original ALN molecule (unpublished data).

As a result of the differential structures of C1 and C2, these two conjugates exhibit different stability in vitro. By incubation in fresh rat plasma at 37°C, it was determined that approximately 6.2% hydrolysis of the C1 conjugate occurs over 24 hours to release EP4a from the conjugate, while the C2 conjugate is very stable and essentially no liberation of EP4a was observed over a 96 hour period. Furthermore, in vivo radiolabelling study using EP4a–H³ labeled conjugates showed that upon systemic administration of the conjugates, 5.9% of total administrated C1 and 9.4% of C2 were taken up by bone, while the rest are excreted in the feces and urine. Furthermore, EP4a was released from the C1 conjugate with a half-life of 5 days and much more slowly from the C2 conjugate, with a half-life of about 28 days (Figure 1-8B). A more recent radiolabelling study further showed that in contrast to fast EP4a release in the C1 conjugate, the LK is cleaved much more slowly with a half-life of approximately 22 days (unpublished data), most likely due to shortage of carboxypeptidases or other suitable enzymes in the local bone environment to cleave the carbamate bond between ALN and the LK molecule.
Figure 1-8: Differing structures of C1 and C2 lead to their distinct in vivo stability. A) Molecular structures of C1 and C2. In the C1 conjugate, ALN is covalently linked to the LK molecule through a carbamate bond at ALN’s terminal amine group, while the other end of the LK is covalently linked to EP4a through an ester bond at EP4a’s C-15 atom. In the C2 conjugate, the ALN and EP4a components are directly linked through a carbamate bond at ALN’s terminal amine as well as EP4a’s C-15. Carbamate bonds are shown in dotted black circle and ester bond is shown in red solid circle. B) In vivo uptake and release of radiolabelled conjugates into rat long bones (EP4A-H³ labelled) performed in a previous study. The initial uptake rate of C1 and C2 into long bones were 5.9% and 9.4% respectively. The in vivo release half-life of EP4a from the conjugates was 5 days from C1 and 28 days from C2.
Based on these information, a simplified schematic for the \textit{in vivo} hydrolysis of the C1 and C2 conjugates is shown in \textbf{Figure 1-9}. In the C1 conjugate, abundant esterases first cleave the bond between LK and EP4a to liberate the EP4a component, leaving ALN attached to the LK. Enzymes such as carboxypeptidases then cleave the carbamate bond to release the linker and the ALN-COOH, where ALN spontaneously loses a CO$_2$ molecule to become the original ALN molecule. Nonetheless, it is possible that both cleavage steps can occur simultaneously or in the reverse order. However, the \textit{in vivo} radiolabelling data mentioned previously suggest that the EP4a-LK ester bond is cleaved significantly faster than the LK-ALN carbamate bond. In the C2 conjugate, enzymes such as carboxypeptidases cleave the carbamate bond to yield the free EP4a and ALN-COOH components spontaneously, where ALN quickly loses a CO$_2$ molecule to become the original ALN molecule.
Figure 1-9: Simplified schematic illustrating \emph{in vivo} hydrolysis of the C1 and C2 conjugates. In the C1, esterases first cleave the bond between LK and EP4a to liberate the EP4a component, leaving ALN attached to the LK. Enzymes such as carboxypeptidases then cleave the carbamate bond to release ALN from the LK molecule. In the C2 conjugate, enzymes such as carboxypeptidases cleave the carbamate bond to release EP4 and ALN molecules from the conjugate spontaneously.

The current study adopts the ovariectomized (OVX) rat model as the animal model for post-menopausal osteoporosis to study the \emph{in vivo} efficacy of the C1 and C2 conjugates. Mentioned previously, past studies have shown that OVX induced bone loss in rats share many similar characteristics with postmenopausal bone loss in humans, making it a suitable animal model for the current study\textsuperscript{32}. Previously, our laboratory conducted a 6 week short term study examining solely the effects of the C1 conjugate in OVX rats, which was shown to have robust anabolic effects on bone in the study time period (unpublished data). The current study aims to
confirm and further characterize the effects of C1 conjugate on bone in a longer term study of 3 months, as well as investigating the efficacy of the more stable conjugate C2 in comparison to C1 in recovering bone loss due to osteoporosis.

1.4 **BONE QUALITY**

Traditionally, an individual’s susceptibility to bone fracture has been assessed by measurements of bone mass or bone mineral density (BMD). However, this does not take into account of bone’s material properties and microarchitecture, and so measurements of this parameter alone is not an adequate assessment of bone quality and determinant of fracture susceptibility. In 1993, Heaney introduced a more complete definition of what constitutes bone quality\(^{138}\), in which material properties such as bone mineralization, structural properties such as bone structure as well as bone remodelling are all included in the assessment of bone quality.

1.4.1 **BONE MINERAL DENSITY (BMD)**

Bone mineral density is a measurement of the density and amount of minerals in bone. BMD is strongly correlated with fracture risk\(^{139}\) and bone strength\(^{140,141}\), although bone strength is also associated with a number of other predictors. Traditional measurements of BMD is performed by dual-energy-x-ray absorptiometry (DEXA), which is a non-invasive method that employs two x-rays of different energies to assess amount of energy that is absorbed by a specific bone sample\(^{142}\). This gives the measured parameter areal bone mineral density or aBMD, which is the detected total bone mineral content (BMC) normalized by the two dimensional projected area of the bone. This method of determining aBMD has been widely used clinically for the diagnosis of osteoporosis using the World Health Organization’s T-score guidelines as well as for monitoring its treatment\(^{143}\). Unfortunately, BMD as determined by DEXA is an areal
measurement rather than a volumetric measurement, which does not take into account the depth of a bone specimen.

In recent years, the use of high-resolution micro–computed tomography (MicroCT) imaging has grown immensely. A MicroCT machine uses x-ray to take 2D scans of an object at different rotations and uses computer to reconstruct the accurate 3-D structure of the object\textsuperscript{144}. Unlike DEXA, MicroCT provides a three-dimensional volumetric measurement of a bone specimen to give its volumetric bone mineral density (vBMD). Furthermore, this technique is particularly useful in assessing trabecular and cortical bone microarchitecture, which could not be properly assessed by two dimensional measurements\textsuperscript{30}.

1.4.2 GEOMETRY AND MICROARCHITECTURE

Both bone geometry and microarchitecture are important aspects of bone quality and bone mechanical properties. In cortical bone, bone size as well as the distribution of bone tissue influence bone strength\textsuperscript{145}. Tissue distribution refers to the distribution of bone around the central axis, which is dependent on geometric parameters such as cross-sectional area, moment of inertia, anterior-posterior diameter and medial-lateral diameter.

In trabecular bone, the size, orientation and spacing of trabeculae as well as their connectivity all contribute to mechanical properties of the bone\textsuperscript{146}. Thus the microarchitecture of trabecular bone are defined by structural indices such as trabecular number, trabecular thickness trabecular separation, and connectivity density of the trabeculae. Past studies have shown that bones with similar BMD but different microarchitectural characteristics can have very different mechanical properties\textsuperscript{147}, and that differences in architectural indices such as in sheep femoral trabecular bone accounted for a large percentage in the variation of bone strength under
Bone geometry and microarchitecture in small animals can be determined by the use of MicroCT to reconstruct an accurate 3-D structural representation of the bone specimen\textsuperscript{148}.

1.4.3 BONE MECHANICAL PROPERTIES

The mechanical properties of bone is the most direct predictor of its susceptibility to fracture and is therefore the best assessment of bone quality. Mechanical testing of a bone provides information about the bone’s load bearing capacity and ability to absorb energy without failure, which are dependent on the extrinsic geometry of the bone as well as its intrinsic material properties\textsuperscript{149}. Unfortunately since mechanical testing is invasive and destructive, it is limited to testing of animal models only but cannot be employed clinically.

In the biomechanical testing of bones, specimen are usually subject to an increasing load until failure occurs. Failure is often defined by a fracture as seen in bending tests of femurs or a sudden large drop in load as seen in compression tests of the vertebrae\textsuperscript{145}. The deformation or displacement in the bone can be plotted as a function of the load applied to generate load-displacement curves, which are used to calculate the unnormalized or extrinsic mechanical properties of the bones, including the ultimate load, failure displacement, stiffness and work to failure\textsuperscript{150}. Furthermore, stress-strain curves can be generated by normalizing the load-displacement curves over the external geometry of the tested specimen. The stress-strain curves can be used to determine the intrinsic material properties of the bone including ultimate stress, failure strain, Young’s modulus as well as toughness\textsuperscript{150}. Since the current study focuses on evaluating the \textit{in vivo} effects of novel conjugate drugs on bone including effects on their mechanical properties, it should be noted that treatment strategies can therefore improve bone’s mechanical properties by either increasing the bone mass and/or improving the intrinsic material properties of the bone.
1.4.4 BONE MINERALIZATION

Due to the process of bone remodelling where old bone is continually broken down and replaced by new bone formation, at any given time bone has a heterogeneous distribution of packets of new bone and old bone. Since bone becomes more mineralized as they age, this means that the mineralization profile of bone is heterogeneous as well. In general, the mineralization profile of the bone provides important information about bone health, and can be affected by a number of factors including age of the bone, certain bone disease and treatments. For example, in periods of rapid bone remodelling, the peak mineralization of the bone is shifted downwards while the heterogeneity of the mineralization profile may increase. Furthermore, anti-resorptive treatments such as BPs increases peak mineralization by inhibiting osteoclastic bone resorption and bone turnover, while the heterogeneity of bone mineralization decreases. The bone mineralization distribution of a particular specimen such as its peak mineralization and heterogeneity can be determined by back-scattered electron microscopy (BSE).

1.4.5 BONE REMODELLING

Bone remodelling is an important predictor of bone quality as it is a crucial process for the maintenance of healthy bone. If bone remodelling is impaired, microdamage accumulates in bone over time and can lead to decrease in bone strength and increased risk of fracture. Bone remodelling is assessed by undecalcified and decalcified histomorphometry analysis of bone sections stained with a variety of histology stains. Static histomorphometry can be performed on plastic sections stained with Goldener’s Trichrome to assess tissue level bone formation by quantification of osteoid formation and osteoblast numbers. Calcein green labels on unstained sections can be quantified as a measurement of the rate of new bone formation.
Furthermore, tissue level bone resorption can be assessed by quantification of osteoclasts numbers on tartrate resistant acid phosphatases (TRAP) stained sections\textsuperscript{156}.
2 STUDY OBJECTIVES AND HYPOTHESIS

The current study aims to investigate and compare the \textit{in vivo} efficacy of the C1 and C2 conjugates on bone in an OVX rat model of postmenopausal osteoporosis. We hypothesize that:

1) Systemic administration of C1 and C2 will promote bone formation in treated rats over the course of the treatment;

2) The conjugates C1 and C2 may exert differential levels of anabolic effects as the two conjugates differ slightly in structure and have differential stabilities in \textit{vivo}^{127}.

Specifically, treatment effects on bone quality are evaluated by assessing the following parameters:

1) Areal and volumetric bone mineral density

2) Bone geometry and microarchitecture

3) Bone mechanical properties

4) Tissue-level bone remodelling

5) Mineralization profile
3 MATERIALS AND METHODS

3.1 ANIMAL HUSBANDRY

The present study used the OVX rat model as the animal model for post-menopausal osteoporosis. 70 three month old outbred female virgin Sprague-Dawley rats were obtained from Charles Rivers Laboratories (Quebec, Canada). Of these, 60 were subjected to bilateral ovariectomy (OVX) while 10 were sham operated. They were housed three per cage at the Division of Comparative Medicine (DCM) at the University of Toronto, where they were fed a rodent maintenance diet containing 0.6% calcium (Harlan Laboratories, Diet # 2014). Prior to the start of drug treatments, animals were allowed to lose bone for 3 months to achieve established osteopenia.

During the entire animal experiment, all animals were housed in the same room where temperature and humidity in the room were monitored daily by animal technicians at the animal facility. All animals’ cages were lined with cornmeal bedding and contained a red hide-away tube, and each cage had a water pipe supplying the rats with unlimited supply of reverse-osmosis water. Animals were fed by refilling feeder bins inside each case with food pellets and care was taken to ensure that food pellets were always available in feeder bins. All animal procedures were reviewed and approved by the University of Toronto Animal Care Committee.

3.2 TREATMENTS

At the end of the bone loss period, OVX animals were randomized into 6 groups of 10, and the sham operated animals were assigned to the sham vehicle treatment group (SH) as healthy controls. The rats then received tail vein injections of various drug treatments (Table 3-1) for a period of 3 months. Treatment groups consisted of high and low dosages for both the
C1 and C2 conjugates (C1H, C1L, C2H, and C2L, respectively), as well as vehicle-treated OVX negative controls (OVX), vehicle treated SH healthy controls (SH), and an unconjugated mixture of ALN and EP4a in identical concentration to control for the effect of conjugation between the components (C2M). Note that this group was named the C2 Mixture (C2M) since it contained the exact same drug components as the C2 conjugates but in their separate forms. Sterile phosphate buffered saline (PBS) at PH 7.2 was used for vehicle treatments. All drug treatments were synthesized in-house and dissolved in sterile PBS to achieve desired concentration. Molecular weights of all drug compounds used in the study are shown in Table 3-2.

### Treatment Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Animals</th>
<th>Treatment</th>
<th>Dosage (mg/kg)</th>
<th>Molar Dosage (µmole/kg)</th>
<th>Frequency</th>
<th>Total Dosage (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (SH) Sham Control</td>
<td>10</td>
<td>Sham</td>
<td>Vehicle (PBS)</td>
<td>-</td>
<td>-</td>
<td>biweekly</td>
<td>-</td>
</tr>
<tr>
<td>2 (OVX) OVX control</td>
<td>10</td>
<td>OVX</td>
<td>Vehicle (PBS)</td>
<td>-</td>
<td>-</td>
<td>biweekly</td>
<td>-</td>
</tr>
<tr>
<td>3 (C1H) C1 High Dose</td>
<td>10</td>
<td>OVX</td>
<td>C1</td>
<td>5</td>
<td>5.69</td>
<td>weekly</td>
<td>60</td>
</tr>
<tr>
<td>4 (C1L) C1 Low Dose</td>
<td>10</td>
<td>OVX</td>
<td>C1</td>
<td>5</td>
<td>5.69</td>
<td>biweekly</td>
<td>30</td>
</tr>
<tr>
<td>5 (C2H) C2 High Dose</td>
<td>10</td>
<td>OVX</td>
<td>C2</td>
<td>15</td>
<td>20.20</td>
<td>biweekly</td>
<td>90</td>
</tr>
<tr>
<td>6 (C2L) C2 Low Dose</td>
<td>10</td>
<td>OVX</td>
<td>C2</td>
<td>15</td>
<td>20.20</td>
<td>monthly</td>
<td>45</td>
</tr>
<tr>
<td>7 (C2M) Unconjugated Mixture</td>
<td>10</td>
<td>OVX</td>
<td>ALN + EP4a</td>
<td>EP4a: 0.75 ALN: 0.75</td>
<td>EP4a: 1.79 ALN: 2.54</td>
<td>biweekly</td>
<td>EP4a: 4.5 ALN: 4.5</td>
</tr>
</tbody>
</table>

Table 3-1: Study Treatment Groups (n =10/group, 7 groups). SH and OVX are healthy and negative controls, respectively. C1 and C2 both have high and low dose treatment groups. C2M is a mixture of ALN and EP4a to examine the effects of conjugation. All solutions were administered at 1mL/kg and via tail vein injections.
Table 3-2: Chemical formula and molecular weight of treatment compounds used in the study. All compounds were provided in a solid salt form. EP4a and ALN were provided in a single drug mixture but unconjugated.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Formula</th>
<th>Molecular Weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1: ALN-LK-EP4a</td>
<td>C_{36}H_{48}F_{2}N_{2}Na_{2}O_{12}P_{2}</td>
<td>878.70</td>
</tr>
<tr>
<td>C2: ALN-EP4a</td>
<td>C_{28}H_{40}F_{2}N_{2}Na_{2}O_{12}P_{2}</td>
<td>742.55</td>
</tr>
<tr>
<td>EP4a</td>
<td>C_{21}H_{27}F_{2}NaNaO_{4}</td>
<td>418.43</td>
</tr>
<tr>
<td>ALN</td>
<td>C_{2}H_{13}NNa_{2}O_{3}P_{2}</td>
<td>295.08</td>
</tr>
</tbody>
</table>

As mentioned previously, a high and low dosage for each conjugate drug was used to investigate the dose dependent effects of the drugs. The C1H was dosed at 5mg/kg weekly while C1L was dosed at 5mg/kg biweekly. This dosage was calculated based on previous radiolabelling data which showed a 5.9% uptake rate into bones 6 hours after initial dosing, as well as an EP4a release half-life of approximately 5 days\textsuperscript{127}. This allows calculation that a 5mg/kg weekly dose should provide a sustained release of EP4a at 12-15 µg/kg/day, which is comparable to the rate of PGE\textsubscript{2} previously shown to be effective in reversing bone loss in OVX rats\textsuperscript{128}. In comparison, the C2 conjugate has an initial update rate of 9.4% and a slow rate of EP4a release from the conjugate, with a release half-life of approximately 28 days\textsuperscript{127}. This means approximately 7.5mg/kg of the C2 conjugate is needed in order to maintain a sustained EP4a release of 12-15µg/kg/day. Therefore a dosage of 15mg/kg biweekly for C2H and 15mg/kg monthly for C2L were chosen in the current study. As for the dose of the C2M group, this was chosen with the purpose of matching the highest dosage of ALN and EP4a in the C1 or C2 conjugates. Since C2H is dosed at 15mg/kg biweekly with approximately 10% uptake rate into bones, only about 1.5mg/kg of the conjugate would be realistically taken up by bone. This gives an ideal dosage of approximately 0.75 mg/kg of each of the two components, which is the dosage
we chose for ALN and EP4a in the unconjugated mixture. It should be noted that this calculation is based on the assumption that all of the C2 components are being cleaved once it is taken up by bone. However since the release half-life of EP4a and ALN from C2 were about 28 days, in fact only half of the 0.75mg/kg that was absorbed could have been realistically released in a month. Therefore the dosage for the C2M group is actually about four times the possible maximum dose released by C2. This was done to avoid any possibility of underdosing the C2M control group. Furthermore, it should also be noted that although the C2H was dosed at 15mg/kg biweekly, this actually calculates to a dosage of 8.45 mg/Kg of EP4a and 5.96 mg/kg of ALN biweekly rather than exactly 7.5 mg/kg for each of the two components due to differences in the molecular weight of ALN and EP4a. At around 10% uptake rate, this means that the ideal dosage for C2M should have been 0.845 mg/kg of EP4a and 0.596 mg/kg of ALN in order to match the dosage of C2H. However since C2M was actually overdosed compared to C2 due to C2’s slow cleavage in vivo as mentioned previously, a dose at 0.75mg/kg each for ALN and EP4a for C2M should not constitute a major concern in the experimental design.

3.3 SACRIFICE AND DISSECTION

At the end of treatment period, animals were sacrificed via cardiac puncture under isofluorane anesthesia and followed by cervical dislocation. Immediately following sacrifice, femurs as well as the 5th and 6th lumbar vertebrae were dissected and stripped clean of soft tissue. They were then wrapped in saline-soaked gauze and frozen at -20ºC for storage. Furthermore, tibia were excised, cleaned and cut at mid-shaft. Proximal regions of left tibia were fixed in 70% ethanol for 7 days prior to Spurr processing, while the right tibia were fixed in 10% neutral buffered formalin for 7 days prior to decalcification with ethylenediaminetetraacetic acid (EDTA).
3.4 EVALUATION OF TREATMENT EFFECTS ON BONE

A number of techniques were employed in the current study to investigate drug treatment effects on the bone. Specifically, DEXA analysis was performed on L6 vertebrae and left femurs to determine their aBMD; MicroCT analysis was conducted on L6 vertebrae and left femurs to assess their vBMD, geometry and microarchitecture; mechanical testing including three-point bending and femur neck fracture tests were performed using left femurs while vertebral compression test were conducted using the L6 vertebrae to determine their mechanical properties; undecalcified histomorphometry analysis was conducted on sections cut from left tibia embedded in Spurr blocks while decalcified histomorphometry analysis were conducted on sections cut from right tibia embedded in paraffin blocks; BSE analysis of mineralization profiles were conducted on left tibia embedded in Spurr blocks. L5 and right femurs were stored at -20ºC as backup bones. Refer to Table 3-3 for analysis overview.

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Bone Specimen Used</th>
<th>Bone Quality Indicator Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEXA</td>
<td>Left Femurs, L6 vertebrae</td>
<td>aBMD</td>
</tr>
<tr>
<td>MicroCT</td>
<td>Left Femurs, L6 vertebrae</td>
<td>vBMD, geometry and microarchitecture</td>
</tr>
<tr>
<td>Three-Point-Bending</td>
<td>Left Femurs</td>
<td>Mechanical Properties</td>
</tr>
<tr>
<td>Femoral Neck Fracture</td>
<td>Left Femurs</td>
<td>Mechanical Properties</td>
</tr>
<tr>
<td>Vertebral Compression</td>
<td>L6 vertebrae</td>
<td>Mechanical Properties</td>
</tr>
<tr>
<td>Undecalcified Histomorphometry</td>
<td>Left Tibia in Spurr blocks</td>
<td>Bone Remodelling: Tissue Level Formation</td>
</tr>
<tr>
<td>Decalcified Histomorphometry</td>
<td>Right Tibia in Paraffin blocks</td>
<td>Bone Remodelling: Tissue Level Resorption</td>
</tr>
<tr>
<td>BSE</td>
<td>Left Tibia in Spurr blocks</td>
<td>Mineralization</td>
</tr>
</tbody>
</table>

Table 3-3: List of techniques employed, bones used for each technique and associated bone quality indicators assessed in the study are listed in the table.
3.4.1 DUAL-ENERGY X-RAY ABSORPTIOMETRY (DEXA)

DEXA analysis of left femurs and the L6 vertebrae were conducted. After thawing bone samples at room temperature overnight, DEXA was performed using a small animal PIXIImus densitomer (Lunar; GE Corp, Mississauga, Ontario). Phantom was scanned prior to each batch to calibrate the PIXIImus machine. During scanning, bone samples were carefully positioned on a polystyrene plate to ensure consistent sample orientation across scans. For left femurs, the region of interest was a rectangular box measured 100 by 270 pixels. This was large enough to include even the largest of femurs individually, and was kept consistent for all femur scans. For the L6 vertebrae, the region of interest was a box measured at 100 by 110 pixels and was kept consistent for all scans of the vertebrae. Following scanning, results were analyzed using the PIXIImus software to determine BMC, bone area, as well as areal BMD (aBMD = BMC / bone area).

3.4.2 X-RAY MICRO-COMPUTED TOMOGRAPHY (MICROCT)

Following DEXA analysis, L6 lumber vertebrae and left femurs were scanned using the SkyScan 1174 Compact Desktop MicroCT machine (Bruker-MicroCT, Belgium) to determine volumetric bone mineral density (vBMD) and bone microarchitecture. Prior to scanning, samples were thawed overnight at room temperature, re-wrapped tightly in two layers of saline soaked gauze, and placed inside a 10mL microtube. The microtubes were position vertically onto the scanning platform and secured tightly on the bottom with double sided tape to prevent unwanted tube movement during scanning. Care was taken to ensure that femurs and vertebrae were positioned on the platform as vertically as possible (Figure 3-1A). All femurs were scanned in the vertical orientation with the condyles away from the scanning plate, and heads of the femur adjacent to the plate. The scans were performed using the following settings: x-ray voltage = 50-kV, x-ray current = 800μA, frame averaging = 2. A 0.25mm aluminum filter was applied to
remove image noise and an isotropic voxel size of 11.6μm was used for both femurs and vertebrae. Hydroxyapatite phantoms of 750 and 1300 mgHA/cm³ were scanned at the end of each day for the calibration of BMD measurement.

Due to the large size of femurs, only parts of the femoral diaphysis could be included in the scanning field-of-view at 11.6μm. To locate the mid-diaphysis accurately on the scans, a marker was made using oil-free Fimo polymer clay (Fimo Classic, Eberhard Farber) and was wrapped around the femur bone at a specific distance (around 5mm) distal to the femur mid-diaphysis. Actual distance between the most proximal edge of the Fimo marker to the femur mid-diaphysis was measured using a standard caliper and recorded. During scanning, the Fimo marker is visible on the scans and is used as a landmark to locate the femur mid-diaphysis (Figure 3-1B).

![Figure 3-1: MicroCT scanning field-of-view at 11.6μm voxel size. A) L6 vertebrae. B) Left femur. A Fimo polymer clay marker was carefully positioned on the femur and is visible on the MicroCT scan. Distance between the proximal end of the Fimo marker to the femur mid-diaphysis was measured by a standard caliper before scanning and is used to determine the location of the femur mid-diaphysis. Blue box denotes the VOI used for femurs, which is a 1mm thick region in the femur mid-diaphysis.](image-url)
After scanning, images were reconstructed using Skyscan NRecon software (Version 1.6.3.2) and analysed using the Skyscan CTAN software (version 1.10.0.1). All scans were reconstructed with the following settings: smoothing = 1, ring reduction = 6, beam hardening correction = 30%, defective pixel masking = 50%.

For femoral scans, the femur mid-diaphysis was located by method mentioned above, and the volume of interest (VOI) for analysis was chosen to be 0.5mm above and below the mid-diaphysis, which gives a total VOI of 1-mm thick section. Analysis of this VOI was performed to determine a number of parameters including anterior-posterior diameter (dAP), medial-lateral diameter (dML), minimum principal moment of inertia (MMI\text{min}), cross-sectional bone area, cortical thickness, cortical porosity, as well as cortical vBMD. For AP, ML, MMI\text{min} and cross-sectional bone area, the region of interest (ROI) used was the whole femoral cross section. For analysis of cortical thickness, the ROI used encompassed the cortex only. Note that due to encortical formation in the C1H and C1L treated groups, boundaries between cortex and endocortical bone could not be clearly delineated and so these groups were not included in the data analysis. For cortical vBMD and cortical porosity, a ring approximately 0.5mm thick drawn from the outer edge of the cortex was used as the ROI. The 0.5mm thickness was chosen by evaluating the cortical thickness of all femur samples with clear boundaries of cortex and marrow to determine the smallest femoral cortical thickness and then subtract 15%. This was done to avoid any possibility of including endocortical bone in the calculation of cortical vBMD and cortical porosity, since boundary between endocortical bone and the femoral cortex were ambiguous in some C1 treated samples. Refer to Figure 3-2 for sample images of ROIs used for femur analysis.
Figure 3-2: ROIs used for analysis of femur MicroCT scans. Bone included in ROI are highlighted in blue while marrow included in ROI is highlighted in red. Images are taken directly from CTAN program. A) Whole femur cross-sectional ROI is used for the measurement of cross-sectional bone area, dAP, dMP and MMI$_{min}$. B) ROI of the femoral cortex is used for the measurements of cortical thickness. C) For cortical vBMD and cortical porosity, a ring approximately 0.5mm thick drawn from the outer edge of the cortex was used as the ROI.

For vertebral scans, only portions of the vertebral body containing secondary spongiosa were analyzed while primary spongiosa near the growth plate were excluded. Parameters including cross-sectional bone area, percent bone volume (BV/TV), trabecular vBMD, trabecular number, trabecular thickness, trabecular separation, connectivity density, as well as structure model index(SMI) of the trabeculae were determined from MicroCT analysis. Since cross-sectional bone area is used for the normalization of vertebral compression data, whole vertebral cross section was used as the ROI. For the other parameters, a circle with diameter of 1.8mm placed next to the flat end of the vertebrae was used as the ROI. This was done for consistency purposes and to avoid including any cortex in the calculation of trabecular parameters, since it is difficult to delineate the boundaries between cortex and trabecular bone in some C1 treated samples. Diameter of 1.8mm was chosen by examining all samples which have clear boundaries of cortex and trabeculae and determining the diameter of the circle which encompasses the
trabecular region of the smallest vertebrae and then subtract 15%. Refer to Figure 3-3 for sample images of ROI used for vertebral analysis.

![Figure 3-3: ROI used for analysis of vertebral MicroCT scans.](image)

Bone included in ROI are highlighted in blue while marrow included in ROI is highlighted in red. Images are taken directly from CTAN program. A) Whole vertebral cross-sectional ROI is used for the measurement of cross-sectional bone area B) For consistency, a circle with diameter of 1.8mm placed adjacent to the flat end of the vertebrae was used as the ROI for the calculations of trabecular vBMD, percent bone volume (BV/TV), trabecular number, trabecular thickness, trabecular separation, trabecular connectivity density, structure model index and trabecular vBMD.

### 3.4.3 MECHANICAL TESTING

Three-point bending tests and femoral neck fracture test of the femurs as well as vertebral compression tests of the L6 lumbar vertebrae were performed to investigate the biomechanical properties of the specimen. All tests were performed using an Instron 4465 testing machine (Instron, Canton, MA) with a 1000N load cell. All time points and load data were recorded with LabVIEW Acquisition software (LabVIEW 5.0; National Instruments, Austin, TX).

From the unnormalized mechanical testing data, load-displacement curves can be generated and used to determine the extrinsic mechanical properties of the bone. Furthermore, by normalizing the mechanical data over the external geometry of the specimen determined from MicroCT analysis, stress-strain curves can be constructed to determine the intrinsic bone
material properties (Figure 3-4). On these curves, the ultimate load or ultimate stress denotes the strength of the bone specimen and is the highest load or stress value on the curves. The stiffness or modulus represents the bone’s resistance to applied loads and is equal to the slope of the linear region of the curves. The yield point is the point at which the behavior of the bone changes from elastic to inelastic, and begin to experience permanent deformation. The area under the curves denotes either the work to failure or toughness of the bone, which represents the energy required to break the bone\textsuperscript{150}. Furthermore, the end of the test is denoted by fracture of the specimen as seen in three-point-bending tests or femoral neck fracture tests or a large drop in load as seen in vertebral compression tests.
Figure 3-4: Sample curves from biomechanical testing of bones. A) Load-displacement curve constructed from unnormalized mechanical testing data. Extrinsic properties of the bone can be determined from the curve. B) Stress-strain curve constructed from normalized mechanical testing data. Intrinsic properties of the bone can be determined from the curve. Stiffness or modulus is equal to the slope of the linear region of the curves as shown in red whereas the area under the curves is equal to the work to failure or toughness of the bone.

3.4.3.1 THREE POINT BENDING

Biomechanical properties of femoral cortical bone was investigated by the three-point bending test. Prior to testing, geometrical properties of the femurs including the anterior-posterior diameter (dAP) as well as the minimum principal moment of inertia (MMI_{min}) were determined by MicroCT analysis. Mid-points of the femurs were determined by a standard caliper and marked with a permeant marker. Femurs were thawed overnight at room temperature and re-hydrated in saline. During the test, left femurs were supported on two struts at either end
with a gauge length of 15mm between the struts. Care was taken to ensure that all femurs were placed onto the struts in the same orientation, with the posterior side of the femur facing down. The femur was then loaded with a force by a force applicator from above at the previously marked midpoint (Figure 3-5). The force applicator continuously moves downward at a bulk head speed of 0.5mm/min to gradually exert a greater force until fracture of the femur. Load and time data of the tests were recorded by LabVIEW 5.0 data acquisition software at a sampling rate of 10 Hz.

![Diagram of femur and force applicator](image)

**Figure 3-5: Typical set-up of the three-point-bending test.** Femur is placed on two supporting struts set at a specific gauge length. The gauge length used in the current study is 15mm. An increasing force is applied from above at the midpoint of the femur until fracture of the femur.

From the data obtained from in the three-point-bending test, load-displacement curves were generated and was used to determine a number of parameters including ultimate load (N), stiffness (N/mm), failure displacement (mm) and work to failure (mJ). On the curve, failure of the bone was represented by a sudden and significant drop in the measured load. Furthermore, by normalizing the mechanical testing data over the dAP, MMI\textsubscript{min} and gauge length using the equations stated below, stress and strain data were determined and used to generate stress-strain curves. Parameters including ultimate stress (MPa), Young’s modulus (MPa), failure strain (%),
and toughness (MPa) were determined from the curve. Following completion of the tests, the proximal portions of the femurs were stored at -20°C for use in the femoral neck fracture tests.

Normalization equations:

Stress:

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

Where:

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

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

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

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

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

Strain:

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

Where:

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

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

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

\( d_{ap} = \text{AP diameter (mm)} \)
3.4.3.2 FEMORAL NECK FRACTURE

Since hip fractures occur in some osteoporotic patients, the femoral neck fracture test is a way to evaluate femoral neck strength and associated risk of hip fracture\textsuperscript{150}. Unfortunately while this test is clinically relevant, no normalized data can be obtained from the test due to the complex geometry of the femoral neck and complex loading condition.

Prior to the test, samples were thawed at room temperature and rehydrated with saline. The proximal femora was mounted vertically in aluminum cylinders and fixed in place with polymethylmethacrylate (PMMA, Patterson Dental, Canada). Care was taken when placing the samples in the cylinder such that the femoral shaft was as vertical as possible and approximately 3mm protruding above the PMMA. The PMMA was allowed to cure for 10 minutes to ensure proper fixation of the sample in the cylinder. The cylinders were then mounted into the instron testing system upside down with the femoral neck inverted vertically. The cylinders were then lowered towards a metal plate below where the femoral head is eventually pushed against the metal plate (Figure 3-6). Test was conducted by lowering the crosshead at a speed of 1.0mm/min until fracture of the neck was achieved. Load and time data were recorded by the LabVIEW software with a sampling rate of 10 Hz.

![Figure 3-6: Typical set-up of the femoral neck fracture test.](image)

In the test, the diaphyseal end of the specimen was mounted inside a custom aluminum cylinder using PMMA. The femoral head is loaded against a bottom metal plate by an applied force above until fracture of the neck.
From the un-normalized data, force-displacement curves were generated and was used to determine ultimate load (N), stiffness (N/mm), failure displacement (mm) and work to failure (mJ) of the samples. Failure of the bone was denoted by a sudden and significant drop in the measured load on the curve. Data normalization and intrinsic properties of the bone could not be obtained from the femoral neck fracture test as mentioned previously.

3.4.3.3 VERTEBRAL COMPRESSION TEST

Biomechanical properties of the L6 vertebrae was investigated by the vertebral compression test. Prior to testing, vertebral height as well their mean cross-sectional bone area were determined by MicroCT analysis for the normalization of mechanical testing data. L6 vertebrae were thawed overnight at room temperature and re-hydrated. In the vertebral compression test, the flat end of each vertebra was super-glued vertically to a metal plate while a small amount of PMMA was used to create a flat surface on the opposite end. Care was taken to ensure that the vertebrae were glued to the plate as vertically as possible. During the test, a force was applied from above by a compression plate onto the PMMA surface to compress the vertebra in the longitudinal axis until failure (Figure 3-7). A 1000N load cell and a bulkhead speed of 1.0mm/min was used.

![Figure 3-7: Typical set-up of the vertebral compression test](image)

In the test, the flat end of the vertebra is superglued to the bottom metal plate while a PPMA hat is used to create a flat surface on the other end. A load is applied from above by a compression plate until failure.
Load-displacement curves were generated from the recorded data and were used to calculate the specimen’s ultimate load (N), stiffness (N/mm), failure displacement (mm) and work to failure (mJ). On the load-displacement curve, failure of the bone was represented by a 10% drop in load since compression of the vertebrae does not result in full fracture. Furthermore, stress and strain data were generated using the equations listed below and were used to generate stress-strain curves. Ultimate stress (MPa), young’s modulus (MPa), failure strain (%) and material toughness (MPa) were determined from the stress-strain curves.

**Normalization equations:**

**Stress:**

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

Where:

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

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

\( A = \text{cross-sectional bone area (mm}^2) \)

**Strain:**

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

Where:

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

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

\( h = \text{vertebral height (mm)} \)
3.4.4 TISSUE-LEVEL BONE REMODELLING

Effects of drug treatments on tissue-level remodelling are assessed by undecalcified and decalcified histomorphometry analysis. All histomorphometric parameters were measured following the guidelines of the American Society of Bone and Mineral Research for bone histomorphometry\textsuperscript{156}.

3.4.4.1 UNDECALCIFIED HISTOMORPHOMETRY

Undecalcified bone histomorphometry was conducted to evaluate tissue level bone formation. Prior to sacrifice, rats were injected with calcein green (10mg/kg, Sigma-Aldrich, Oakville, ON) subcutaneously at 12 days and 2 days before sacrifice. Directly following sacrifice, left tibia were immediately cleaned of adherent soft tissue and fixed in 70% ethanol for 7 days, dehydrated in ascending concentration of acetone, and embedded in Spurr resin. Five \(\mu\)m and seven \(\mu\)m thick coronal sections were cut using a Leica RM2265 rotary microtome (Leica, Wetzlar, Germany) and mounted onto gelatinized slides. Slides were then incubated in a 60\(^\circ\)C oven for 48 hours to ensure proper adherence of the sections to the slides.

3.4.4.1.1 GOLDENER’S TRICHOmE STAIN

Five \(\mu\)m thick Spurr slides prepared as mentioned previously were stained with Goldener’s Trichrome stain. Specifically, slides were stained by sequential incubations in Weigert’s Iron Hematoxylin (15 minutes), Ponceau Acid Fuchsin (15 minutes), Phosphomolybdic Acid/Orange G (8 minutes) and Light Green (15 minutes). On this stain, mineralized bone is stained green, osteoid is stained red while cell nuclei are stained dark brown to black\textsuperscript{157} (Figure 3-8). After staining, all slides were cover-slipped with Paramount mounting medium.
Figure 3-8: Sample image of bone section stained with Goldner’s Trichrome. Osteoid is stained red and appear on the surface of bone while mineralized bone is stained green. Image scanned at 200X magnification.

The slides were viewed under a light microscope and imaged at 200X magnification. Static histomorphometric analysis of these slides were performed using the Bioquant Osteo 11.2.6 MIR software (Bioquant Image Analysis Corporation). The region of interest was defined as the tibial proximal metaphysis beginning 1mm from the distal end of the growth plate, extending 2mm into the metaphysis, and 0.5mm from the endosteal surface of the cortex (Figure 3-9). This ensures that primary spongiosa near the growth plate was excluded in the analysis of the trabecular bone since it is not representative of metaphyseal trabecular bone\textsuperscript{158,159}. Bone formation parameters including percent osteoid surface (OS/BS, %), percent osteoid volume (OV/BV, %), and osteoid width (µm) were measured.
Figure 3-9: Region of interest (ROI) for the analysis of trabecular bone on Goldener’s Trichrome stained sections. The region of interest was defined as starting at 1mm from the distal end of the growth plate, extending 2mm into the metaphysis, and 0.5mm from the endosteal surface of the cortex to exclude primary trabecular spongiosa in the analysis. Image scanned at 200X magnification.

3.4.4.1.2 CALCEIN GREEN LABELS

Calcein green is a fluorochrome, which strongly chelates calcium ions and thus gets incorporated into mineralization fronts in bone. Fluorochromes absorb light energy of a specific wavelength and re-emit light at a longer wavelength. When viewed under ultraviolet (UV) light, calcein green labels can be detected at bone surfaces undergoing active mineralization\textsuperscript{160,161}.

As mentioned previously, calcein green was injected at a 10 day interval prior to sacrifice (12 days and 2 days prior to sacrifice). This allows two different fluorescent labels to form as mineralization occurs over the 10 day period. When viewed under UV light, double labels mean that both doses of calcein green were incorporated into bone during mineralization while single labels means that only a single dose of calcein green was incorporated into bone at those
locations (Figure 3-10). Furthermore, the rate of bone formation can be determined by measuring the distance between double labels to determine the interlabel width and then dividing by the time interval (10 days).

**Figure 3-10: Sample image of unstained Spurr section viewed under UV light.** Mineralizing surfaces labeled with calcein green appear as double labels or single labels. Imaged at 100X magnification.

For quantification of calcein labels, seven μm unstained Spurr slides prepared previously were left unstained, cleared in xylene and then cover-slipped with Paramount mounting medium. They were then viewed under UV light and imaged at 100X magnification. Dynamic histomorphometric analysis of these slides were performed using the Bioquant Osteo 11.2.6 MIR software (Bioquant Image Analysis Corporation). The region of interest was identical to the ROI used in Goldener’s Trichrome analysis. Parameters measured were: single label surface (sLS, mm); double label surface (dLS, mm); mineralizing surface (MS, formula = dLS + sLS/1, mm); interlabel width (Ir.L.Width, μm), percent mineralizing surface (MS/BS, %); mineral apposition
rate (MAR, formula = interlabel width/labeling period, μm/day), bone formation rate normalized over bone surface (BFR/BS, formula = MS*MAR/BS, μm/day/mm); and bone formation rate normalized over bone volume (BFR/BV, formula = MS*MAR/BV, μm/day/mm²).

3.4.4.2 DECALCIFIED HISTOMORPHOMETRY

Decalcified bone sections stained with tartrate resistant acid phosphate (TRAP) is used to assess tissue level bone resorption. Past studies have shown that the TRAP enzyme is strongly expressed in osteoclasts¹⁶²,¹⁶³, and that TRAP expression is positively correlated with osteoclasts’ resorptive activity¹⁶⁴. As a result, TRAP is commonly used as an osteoclastic marker as well as a marker for tissue level bone resorption.

Directly after sacrifice, right tibia were fixed in 10% neutral buffered formalin for 7 days and then decalcified in 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 7.4) for 2 months at room temperature. Specimens were then dehydrated through ascending grades of ethanol and then infiltrated with successive melted wax steps before being embedded in paraffin blocks. Five μm thick coronal sections were cut with a Leica Reichert June 2030 microtome and stained for TRAP using the Acid Phosphatase Leukocyte Kit (Sigma-Aldrich) and then counterstained with Acid Hematoxylin for nuclei. All sections were then cover-slipped with an aqueous mounting medium. On these sections, osteoclasts appear red while nuclei are stained dark purple (Figure 3-11). Furthermore, sections were viewed under a light microscope and imaged at 200X magnification. Analysis was performed using the Bioquant Osteo 11.2.6 MIR software (Bioquant Image Analysis Corporation). The region of interest was kept identical to Goldener’s Trichrome analysis. Parameters measured include normalized osteoclast number (NOc/BS, 1/mm) and percent osteoclast surface (OcS/BS, %). In addition, marrow adiposity of the proximal tibia were assessed on these slides.
Furthermore, osteoblast and osteocytes were quantified on TRAP stained sections to further assess tissue-level bone formation. On these sections, osteoblasts appear to be cuboidal in shape and dark blue in colour from the Hematoxylin counterstain. To be consistent, only cells that follow these three criteria were counted: 1) they were as long as they were wide; 2) one side of the cell was immediately touching bone surface; and 3) they were in close proximity to resorption surfaces. On the other hand, osteocytes are terminally differentiated osteoblasts that become embedded in mineralized bone. On the stained sections, cells that were counted as osteocytes were: 1) oval in shape; 2) stained dark blue; and 3) are embedded in mineralized bone. For consistency, cells that were on the surface of the bone were not counted, and empty lacuna in the bone where osteocytes may have resided were not counted. Sample images of osteoblasts and osteocytes as measured on TRAP stained sections are shown in Figure 3-12.
Figure 3-12: Sample image of osteoblasts and osteocytes on bone sections stained with TRAP. A) Sample osteoblasts. Quantified osteoblasts were cuboidal in shape, immediately adjacent to bone surface and near resorption pits. B) Sample osteocytes. Quantified osteocytes were oval, dark blue cells that were embedded in mineralized bone. Imaged scanned at 200X magnification.
Finally, growth plate thickness were measured on TRAP stained sections. On these sections, the growth plate has a glassy appearance due to the hyaline cartilage in the growth plate, and is found directly above the primary spongiosa which is heavily stained with TRAP positive osteoclasts. Since the thickness of the growth plate is not uniform along its length, 20 measurements at locations spread out along the entire length of the growth plate were made for each sample to derive the average growth plate thickness of the sample. A zoomed in image of a sample growth plate is shown in Figure 3-13.

**Figure 3-13: Sample image of growth plate measurement performed on bone sections stained with TRAP.** On these sections, growth plate has a glassy appearance due to the hyaline cartilage in the growth plate, and is found directly above the primary spongiosa which is heavily stained with TRAP+ osteoclasts. The border between the growth plate and the bone above is clearly delineated by a boundary that is stained light purple. 20 measurements at locations spread out along the entire length of the growth plate were made for each sample to derive the average growth plate thickness of the sample. Imaged scanned at 200X magnification.
3.4.5 BACK-SCATTERED ELECTRON MICROSCOPY (BSE)

Back-scattered electron microscopy (BSE) is a type of scanning electron microscopy, where the specimens are hit with high-energy electrons and a BSE detector is used to collect the scattered electrons for characterization of the samples’ chemical composition\textsuperscript{166,167}. Since the probability of scattering an electron by collision increases with greater number of protons (atomic number), the intensity of detected electrons is therefore related to the atomic number of the constituent. Furthermore, since calcium is the heaviest atom in bone mineral, the intensity of detected electrons is related to the calcium concentration and therefore the degree of mineralization of the bone. The current study uses BSE to evaluate the effects of drug treatments on the mineralization profile of the bone. Note that on the BSE scans of bone, regions with higher levels of mineralization will scatter more electrons, leading to a higher intensity of electrons being detected by BSE detectors and will appear brighter on BSE images. Regions of lower mineralization will lead to less scattering of electrons and will appear darker on BSE images (Figure 3-14A).

After sectioning to obtain samples for undecalcified histomorphometry, Spurr blocks were polished to a 1-\(\mu\)m finish with diamond polishing fluid (Phoenix BETA Grinder/Polisher; Buehler, Canada). Samples were mounted securely on plexiglass BSE plates using Fimo polymer clay (Fimo Classic, Eberhard Farber) with the surface of the exposed bone facing up, and care was taken to ensure a leveled surface across all blocks on the plate. The edges of the blocks on each plate were then taped with carbon tape and the surfaces were coated with a thin layer of carbon to ensure proper conduction of electron current across all samples. Each plate of blocks were then placed on the stage of a scanning electron microscope (FEI XL30 SEM; FEI, Best, Netherlands) under an incident beam set at an accelerating voltage of 20-kV and a beam spot size
of 6.3. A BSE detector (Solid state BSE detector, FEI Company, Hillsboro, OR, USA) was set at a working distance of 15mm and all images were captured at 100X magnification. Two calibration standards consisting of silicon dioxide (SiO$_2$) and magnesium fluoride (MgF$_2$) were scanned prior to obtaining data for each sample, and scanner settings were calibrated against the grey level peaks generated by the standards. The region of interest was kept consistent with bone histomorphometry analysis (Figure 3-14B). Histograms of grey level intensities from 0 to 255 were generated for each ROI and used for analysis of mineralization profiles of the samples by in-house analysis program (Figure 3-15). Prior to the calculation of BSE parameters from the histograms, the baseline was determined by averaging the intensity values of the first 100 data points, and was subtracted from the histogram data during calculations of BSE parameters. The parameters measured were maximum grey value, which represents peak mineralization of the sample; as well as full width at maximum height (FWHMH), which represents the spread of bone mineralization of the sample.

**Figure 3-14: Sample BSE images.** A) Regions of bone with higher mineralization are brighter than regions that are less mineralized on BSE images. B) ROI of BSE image used for analysis. The ROI starts from 1mm below the growth plate and extends 2mm into the metaphysis and 0.5mm away from the endosteal surface. This is consistent with ROI used for histomorphometry analysis.
Figure 3.15: Sample BSE histogram. The baseline intensity is subtracted from the histogram prior to calculating BSE parameters. The maximum grey value represents peak mineralization of the sample while full width at maximum height (FWHMH) represents the spread of bone mineralization.

3.5 STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS statistical software (Version 21, IM). Results were compared using one way analysis of variance (ANOVA), and Bonferroni post hoc tests were performed for pair-wise comparisons only if statistical significance was obtained from results of the ANOVA test. Kruskal Wallis non-parametric test was performed on samples with a non-normal distribution. Significance was defined as p<0.05 for two tailed probability at 95% confidence. Data are presented as mean ± standard error of the mean (SEM).
4 RESULTS

4.1 TREATMENT SIDE EFFECTS

One of the important goals of the study is to observe the in vivo tolerance of the C1 and C2 conjugates as well as compare their drug-related side effects in treated rats. Animal reactions during and after drug treatments were therefore carefully followed and documented. As expected, no adverse reactions were observed in groups treated with PBS. C1 treatment via tail-vein injections resulted in noticeable discomfort in treated rats during injection. Directly after treatment, animals showed mild lethargy and eye closure but recovered within a few hours after injection. Furthermore, 3 out of 10 animals in the C1 high dose group developed swollen feet approximately 4 weeks into the treatment period. Affected rats were lethargic and exhibited weight loss but did recover on their own within 2 weeks of initial development of symptoms. Based on suggestions from the attending veterinarian at the animal facility where rats were housed, drug volume was increased based on a 1 : 1.5 PBS dilution at 6 weeks of treatment. After drug dilution, pain during injection significantly decreased and no further case of swollen feet were observed in C1 treated rats.

Tail-vein injection of rats with the C2 conjugate also caused visible pain and discomfort upon injection. Directly after injection, a number of immediate side effects including porphyrin staining, mild lethargy, redness and swelling of the tail were observed in treated animals. Furthermore, 4 out of 10 rats in the high dose group developed visible necrosis of the tails within 24 hours after the second C2 injection was administrated. The rats recovered on their own within two weeks without external treatments, although visible scar tissue developed on the tail in all four rats at the necrotic locations. At 6 weeks the C2 conjugate was also administered based on a
1:1.5 PBS dilution. After dilution, pain during injection significantly decreased and new cases of tail necrosis did not arise.

In the C2M group, animals did not show visible discomfort during injection but exhibited prolonged lethargy for up to 1 day after injections. Furthermore, slight discoloration and swelling of the tails were observed in treated rats after treatment. The swelling subsided within 48 hours while tail discoloration took 1 or 2 weeks to fade. Other EP4a related symptoms reported previously such as diarrhea and puffy fur were not observed in the current study, most likely due to low concentrations of EP4a used in the C2M treatment group. No loss of animals was recorded from any treatment groups in the current study.

4.2 TREATMENT GROSS EFFECTS

Animal weights were carefully measured weekly, and weights directly before start of drug treatment and prior to sacrifice were compared (Figure 4-1). Before treatments, rats in the SH group weighed significantly less than rats that received the OVX operation. After treatment, weight changes were comparable across all groups with the exception of C1H and C1L, which showed less weight increase in the 3 month treatment period.
Figure 4-1: Animal Weight Before vs. After Treatment. Blue bar represents animal weight prior to the start of drug treatments and red bar represents animal weight after the three month treatment period. Significant relationship: ^a^ significantly different compared to weight of SH prior to treatment; ^A^ significantly different compared to weight of SH after treatment period. Mean± SEM, p<0.05 as indicated.

Some femurs in the C1H group appeared to be larger and yellower than femurs in the other treatment groups, although statistical analysis did not show an overall significant difference between the average femur lengths across treatment groups (Figure 4-2). Furthermore, height of the L6 vertebrae were also comparable across all groups.

Figure 4-2: Treatment gross effects on femurs. A) Representative images of femurs from treatment groups. Some femur from the C1H treatment group appear to be slightly larger and yellower compared to femurs from other groups. B) Femur Length. No significant difference between the average femur lengths of the seven treatment groups was found by one way ANOVA test. Mean ± SEM.
4.3 DEXA ANALYSIS

DEXA provides a two-dimensional analysis of the bone mineral status of a specimen and is a clinically relevant technique as it is commonly used to determine the bone mineral density in humans. In the current study, DEXA was used to evaluate the bone mineral density at two skeletal sites: the rat L6 vertebrae and left femurs. The areal bone mineral density (aBMD) and bone mineral content (BMC) were measured and compared between treatment groups. BMC measures the amount of bone mineral in the sample, which is normalized over the projected bone area to give the aBMD, which is the density of the bone mineral per unit area.

In the L6 vertebrae (Figure 4-3), aBMD and BMC of the OVX control group was significantly lower than that of the SH. Compared to the OVX control group, C1H and C2M had significantly greater aBMD and BMC while C1L had a significantly greater aBMD but not BMC. C2 treated groups including both C2H and C2L had comparable aBMD and BMC to OVX control. Furthermore, results showed that C1H had significantly greater values for both aBMD and BMC compared to C2H, and higher aBMC versus C2L.

![Figure 4-3: Results from DEXA analysis of L6 vertebrae. Parameters measured were L6 bone mineral content (BMC) and L6 areal BMD (aBMD). \(^a\)significantly different vs SH; \(^b\)significantly different vs OVX; \(^c\)significantly different vs C1H; Values represent mean ± SEM, \(p<0.05\) as indicated.](image)
In the femurs (Figure 4-4), the aBMD of OVX but not its BMC was significantly lower than the SH. C1H led to significant increase in aBMD and BMC compared to all other groups, including C1L. The BMC for C1L was significantly higher than all other groups except C1H and C2M, while its aBMC was significantly higher than that of the OVX, C2H, C2L and C2M groups. Furthermore, neither C2H or C2L led to significant increase in aBMD and BMC levels compared to OVX. In contrast, aBMD of C2M was significantly increased compared to OVX and C2L.

**Figure 4-4:** Results from DEXA analysis of left femurs. Parameters measured were femur bone mineral content (BMC) and femur areal BMD (aBMD). \( ^a \)significantly different vs SH; \( ^b \)significantly different vs OVX; \( ^c \)significantly different vs C1H; \( ^d \)significantly different vs C1L; \( ^e \)significantly different vs C2L. Values represent mean ± SEM, p<0.05 as indicated.

### 4.4 MICROCT ANALYSIS

Femurs and L6 vertebrae were assessed by MicroCT analysis to determine their volumetric BMD (vBMD) and three-dimensional microarchitecture.
4.4.1 L6 VERTEBRAE

Sample MicroCT cross-sectional images of the L6 vertebrae are shown in **Figure 4-5**. Qualitative analysis of the images showed that OVX led to loss of trabecular elements in the vertebral column, whereas C1H led to apparent increase in trabecular bone formation in the vertebral column. Anabolic effects associated with C1L is less apparent when examined qualitatively. In contrast, C2 and C2M treatment did not lead to trabecular bone formation in the vertebrae.

![Sample MicroCT cross-sectional images of the L6 vertebrae.](image)

**Figure 4-5: Sample MicroCT cross-sectional images of the L6 vertebrae.** OVX led to decrease in trabecular bone elements while C1H led to apparent trabecular bone formation in the vertebral column.

Quantitative MicroCT analysis of the L6 vertebrae confirmed that OVX did in fact compromise trabecular microarchitecture compared to SH (**Figure 4-6A-D**). This is demonstrated by decreased percent bone volume (BV/TV), decreased trabecular number and increased trabecular separation compared to SH. C1H led to significant recovery of the trabecular structural indices, including significantly greater BV/TV, trabecular thickness,
trabecular number as well as decreased trabecular separation compared to OVX. C1L however only led to significant increase in BV/TV and trabecular number compared to OVX. In addition, C2M had significantly greater BV/TV and trabecular number compared to OVX control.

In contrast to C1 treatment, C2 conjugate treatment did not lead to any improvement in the trabecular structural indices compared to OVX control. Between the conjugate treatment groups, C1H showed significantly greater BV/TV and trabecular thickness compared to both C2H and C2L, as well as greater trabecular number compared to C2L. C1L also led to greater trabecular thickness compared to C2H.

The volumetric BMD (vBMD) of the L6 trabecular bone was also determined by MicroCT analysis (Figure 4-6E). Compared to SH, OVX led to significant decrease in trabecular vBMD. C1H, C1L and C2M showed significantly greater trabecular vBMD values compared to OVX, but not back to SH levels. In contrast, C2 treated groups had comparable trabecular vBMD to OVX. Furthermore, C1H group’s trabecular vBMD was also significantly greater than that of the C2L group.
Figure 4-6: Quantitative MicroCT analysis of the L6 vertebrae. A)-D) Vertebral structural indices as determined by quantitative MicroCT analysis. A) Percent bone volume (BV/TV); B) Trabecular thickness (Tb. Th); C) Trabecular number (Tb. N); D) Trabecular separation (Tb.Sp). E) vBMD of the L6 trabecular bone. *significantly different vs SH; †significantly different vs OVX; ‡significantly different vs C1H; §significantly different vs C1L. Values represent mean ± SEM, p<0.05 as indicated.
In addition, osteoporotic bone loss in both humans and OVX rats are further characterized by changes in the structure of the trabeculae from a plate-like structure to a rod-like structure and eventual loss of trabecular connectivity\(^{168,169}\). Therefore structure model index (SMI) and connectivity density of the trabecular bone were also assessed by MicroCT analysis of the L6 vertebrae. Connectivity density measures the number of trabecular connections per unit volume\(^{169}\), while SMI is an assessment of the rod-like or plate like structure of the trabeculae, with 0 being ideal plates and 3 being ideal rods\(^{170}\). The SMI and connectivity density results are shown in **Figure 4-7**.

**Figure 4-7**: Structure model index and connectivity density of the L6 trabecular bone as determined by quantitative MicroCT analysis. A) Structure Model Index (SMI). B) Connectivity Density.  
\(^{a}\)significantly different vs SH; \(^{b}\)significantly different vs OVX; \(^{c}\)significantly different vs C1H. Values represent mean ± SEM, p<0.05 as indicated.

It should be pointed out that in the results of the current study, SMI values of SH and C1H were not only significantly decreased compared to the other groups, but were actually negative. This is due to the inherit limitations in the algorithm used to calculate the SMI, which can lead to negative SMI values often in cases where the percent bone volume (BV/TV) is
However this should not interfere with the interpretation of the data as negative SMI values should be interpreted in relationship to other groups.

Compared to SH, OVX led to a significant increase in SMI, indicating that OVX caused the trabeculae of the L6 vertebrae to change from a plate-like structure to a more rod-like structure. Of the drug treatment groups, only C1H significantly decreased SMI compared to OVX, while all of the other drug treated groups showed comparable levels of SMI to OVX. Furthermore, SMI of C1H was also comparable to SH, and is significantly smaller than all of the other drug treated groups. This indicates that out of all of the drug treatment groups, only C1H treatment had effects which caused the trabeculae to shift towards a more plate-like structure.

In addition, OVX led to a significant decrease in connectivity density compared to SH, which was rescued by C1H and C2M back to SH level. Together, these results suggest that C1H drug treatment not only have effects which caused the trabeculae to shift towards a more plate-like structure but also increased the connectivity of the trabeculae. In contrast, C2M only increased the connectivity of the trabeculae but did not improve their actual structure. Other drug treatments including C1L, C2H and C2L did not have these effects.

4.4.2 FEMURS

Three-dimensional geometry and vBMD of the left femurs were assessed by MicroCT analysis. Sample MicroCT cross-sectional images of the femur mid-diaphysis are shown in Figure 4-8. It can be seen from the cross-sectional images that C1 treatment stimulated endocortical bone formation in the femoral midshaft in a dose dependent manner. The endocortical bone appear to be highly porous compared to the surrounding femur cortex, and the amount of endocortical bone formation vary largely between rats even within the same treatment group. Specifically, C1L induced moderate amount of endocortical bone formation in the
femoral mid-diaphysis: 2/10 samples had zero endocortical bone formation; 4/10 samples had low amount of endocortical bone formation (<10% occlusion of marrow space by bone formation); 4/10 samples had moderate amount of endocortical bone formation (10% - 50% marrow space occlusion), and no sample had its marrow space completely occluded by endocortical bone formation. In contrast, C1H induced greater amount of endocortical bone formation than C1L: 1 sample had low amount of endocortical bone formation (<10% occlusion of marrow space); 3/10 samples had moderate amount of endocortical bone formation (10% - 50% occlusion of marrow space) and 6/10 samples had more than 50% of its marrow space occluded by endocortical bone formation, which included 4 samples which had near complete occlusion of their marrow space by endocortical bone formation. No visible periosteal bone formation was observed on MicroCT analysis of either C1 treated groups. In contrast, MicroCT cross-sectional images of C2 and C2M femoral mid-diaphysis were comparable to that of SH and OVX controls, with no visible periosteal or endocortical bone formation observed in these groups.
Figure 4-8: Sample MicroCT cross-sectional images of left femoral mid-diaphysis. C1 treatment led to visible endocortical bone formation in the femur mid-diaphysis. The endocortical bone appear to be highly porous compared to the femoral cortex and its amount vary largely between rats even within the same group. Samples images of femurs with high, moderate and low amount of encortical bone formation from the C1H treatment group are shown on the bottom row. C2 and C2M treated femurs show no visible endocortical bone formation.
Quantitative MicroCT analysis showed that the femur’s cortical vBMD were comparable across all treatment groups (Figure 4-9A). However, significant increase in the cross-sectional femur bone area was observed in C1 treated groups due to the endocortical bone formation as mentioned above (Figure 4-9B). Specifically, C1H had a greater femur bone area compared to all other treatment groups, while C1L had a greater bone area compared to OVX control and C2L. In contrast, femur bone area of all other treatment groups were comparable.

Furthermore, we found no difference in the anterior-posterior diameter (dAP), medial-lateral diameter (dML), minimum principal moment of inertial (MMI$_{min}$), or the cortical thickness of femurs across treatment groups (Figure 4-9B-E), indicating that the external geometry of the femurs were not affected by the drug treatments. Note that the cortical thickness of the C1H and C1L treated groups were not included in the data analysis due to the formation of endocortical bone in these groups, which make delineating the boundary between femoral cortex and endocortical bone difficult and ambiguous. In addition, C1H and C1L led to significant increase in the cortical porosity of treated femurs versus SH, but not OVX (Figure 4-9G).
Figure 4-9: Quantitative MicroCT analysis of the left femurs. A) cortical vBMD; B) Cross-sectional bone area; C) Anterior-posterior diameter (dAP); D) Medial-lateral diameter (dML); E) Minimum principal moment of inertia (MMI_{min}); F) Cortical thickness; G) Cortical porosity. a) significantly different vs SH; b) significantly different vs OVX; c) significantly different vs C1H; d) significantly different vs C1L. Values represent mean ± SEM, p<0.05 as indicated.

4.5 MECHANICAL PROPERTIES

Three-point bending tests and femoral neck fracture tests of the femurs as well as vertebral compression tests of the L6 lumbar vertebrae were performed to investigate the mechanical properties of the specimen. Both extrinsic and intrinsic properties of the femurs and L6 vertebrae were assessed.

4.5.1 VERTEBRAL COMPRESSION

Mechanical properties of the L6 vertebrae were evaluated by vertebral compression tests. Results for the vertebrae’s extrinsic and intrinsic bone strength and resistance to load are shown in Figure 4-10. Extrinsic bone strength of the vertebrae is represented by ultimate load while the intrinsic bone strength of the bone tissue is represented by ultimate stress. Furthermore, the
vertebrae’s extrinsic resistance to load is represented by stiffness while the bone tissue’s intrinsic resistance to load is represented by Young’s modulus.

OVX led to a significant decrease in the ultimate load and stiffness of the L6 vertebrae compared to SH. C1H and C2M led to significant increase in the ultimate load compared to the OVX control, but not the stiffness. This indicates that C1H and C2M increased the bone strength of the L6 vertebrae but not their resistance to load. Furthermore, C1L as well as both C2 treated groups had comparable levels of ultimate load and stiffness to OVX control. After normalizing for the external geometry of the L6 vertebrae, the ultimate stress and Young’s modulus were comparable across all treatment groups. This suggests that the observed increase in bone strength in the C1H and C2M groups were due to differences in vertebral geometry or architecture rather than improvement in the bone material properties.
Figure 4-10: L6 vertebrae’s bone strength and resistance to load as determined by the vertebral compression test. Blue bars represent extrinsic properties that are geometry-dependent, and red bars represent intrinsic properties of the bone material that have been normalized. Extrinsic properties are ultimate load and stiffness, while intrinsic properties are ultimate stress and Young’s modulus. a significantly different vs SH; b significantly different vs OVX. Values represent mean ± SEM, p<0.05 as indicated.

The vertebrae’s ability to absorb energy before failure is represented by its work to failure and toughness (Figure 4-11). Work to failure is an extrinsic property of the vertebrae while toughness is an intrinsic property representing the bone material’s ability to absorb energy. There was no significant difference in the work to failure and toughness between SH and OVX, indicating that OVX did not significantly compromise the vertebrae’s ability to absorb energy.
Compared to OVX, C1H and C2M groups had significantly greater work to failure values. Neither C2 treated groups showed increase in work to failure compared to OVX. On the other hand, the normalized work to failure or toughness is comparable across all treatment groups except C2M, which was significantly increased compared to OVX. In fact, toughness of the C2M group was significantly greater compared to multiple treatment groups including SH, OVX, C1L and C2H. Taken together, these results suggest that the increase in work to failure as observed for C1 was most likely due to changes in the vertebral geometry or microarchitecture but not due to improved material property of the vertebral trabecular bone. However, C2M most likely improved both the overall ability of the vertebrae to absorb energy as well as the material toughness of the bone.

The vertebrae’s ability to undergo deformation before failure is represented by its failure displacement and failure strain (Figure 4-11). Failure displacement is an extrinsic property of the vertebrae while failure strain is an intrinsic property of the bone tissue to absorb energy, or its ductility. Again, there was no significant difference in the failure displacement and failure strain between OVX and SH, indicating that OVX did not significantly compromise the vertebrae’s ability to undergo deformation. Only C2M led to an increase in failure displacement compared to SH and OVX controls, while all other treatment groups had comparable failure displacement values to SH and OVX. Furthermore, the failure strain of C2M was also significantly increased compared to OVX, indicating that C2M treatment not only increased the vertebrae’s ability to undergo deformation under compression, but also improved the ductility of the bone tissue.
L6 vertebrae’s ability to absorb energy and undergo deformation before failure as determined by the vertebral compression test. Green bars represent extrinsic properties that are geometry-dependent, and yellow bars represent intrinsic properties of the bone material that have been normalized. Extrinsic properties are work to failure and failure displacement, while intrinsic properties are toughness and failure strain. \(^{a}\)significantly different vs SH; \(^{b}\)significantly different vs OVX; \(^{c}\)significantly different vs C1L; \(^{d}\)significantly different vs C2H; \(^{e}\)significantly different vs C2L. Values represent mean ± SEM, p<0.05 as indicated.

4.5.2 THREE-POINT BENDING

Mechanical properties of left femurs were evaluated by three-point bending tests. Both extrinsic and intrinsic properties were measured. Results for bone strength and resistance to load are shown in Figure 4-12. Similarly to vertebral compression, the extrinsic bone strength of the
femurs is represented by ultimate load while the intrinsic bone strength of the bone tissue is represented by ultimate stress. Femur’s extrinsic resistance to load is represented by stiffness while the bone tissue’s intrinsic resistance to load is represented by Young’s modulus.

Based on the results, OVX and SH had comparable ultimate load and stiffness, indicating that OVX did not significantly compromise femur’s bone strength and its resistance to load. However, C1H significantly increased the femur ultimate load compared to SH, OVX, C2H and C2L treated groups, while C1L only significantly increased the femur ultimate load compared to OVX and C2L. This indicates that C1 conjugate dose dependently increased femur strength. Furthermore, stiffness of C1H was also significantly increased compared to C2L, indicating a greater resistance to load than C2L. After normalizing for the specimen geometry of the femurs, the ultimate stress and Young’s modulus were comparable across all treatment groups. This suggests that the observed increase in bone strength and resistance to load in C1H is most likely due to change in the femur geometry but not due to improvement in the bone material properties of the femur.
Figure 4-12: Femurs’ bone strength and resistance to load as determined by the three-point bending test. Blue bars represent extrinsic properties that are geometry-dependent, and red bars represent intrinsic properties of the bone material that have been normalized. Extrinsic properties are ultimate load and stiffness, while intrinsic properties are ultimate stress and Young’s modulus. a significantly different vs SH; b significantly different vs OVX; c significantly different vs C1H; d significantly different vs C1L. Values represent mean ± SEM, p<0.05 as indicated.

Similarly to the vertebral compression test, the femurs’ ability to absorb energy before failure is represented by its work to failure and toughness (Figure 4-13). There was no significant difference in the work to failure and toughness values between SH and OVX, indicating that OVX did not significantly compromise the femurs’ ability to absorb energy. C1H and C2M groups did show significantly greater work to failure values compared to SH.
However, the normalized work to failure or the toughness values were comparable across all treatments group. This indicates that the observed increased in the femurs’ ability to absorb energy is due to changes in their external geometries but not due to improvement in the bone material properties.

In addition, the femurs’ ability to undergo deformation before failure is represented by its failure displacement and failure strain (Figure 4-13). Again, there was no significant difference in the failure displacement and failure strain between OVX and SH, indicating that OVX did not significantly compromise the vertebrae’s ability to undergo deformation. Only C2M led to a significant increase in failure displacement compared to SH, as well as significantly greater failure strain compared to SH and C1H after normalizing for femur external geometry. This suggests that the observed increase in the femurs’ ability to absorb energy without failure in the C2M group may be due to either improvement in the bones’ external geometry or improvement in the intrinsic material properties of the femurs. Other treatments including C1H, C1L, C2H and C2L did not show this effect.
Figure 4-13: Femurs’ ability to absorb energy and undergo deformation before failure as determined by the three-point bending test. Green bars represent extrinsic properties that are geometry-dependent, and yellow bars represent intrinsic properties of the bone material that have been normalized. Extrinsic properties are work to failure and failure displacement, while intrinsic properties are toughness and failure strain. \(^a\)significantly different vs SH; \(^c\)significantly different vs C1H. Values represent mean ± SEM.

### 4.5.3 FEMORAL NECK FRACTURE

Mechanical properties of the femoral neck were evaluated by femoral neck fracture tests.

As mentioned previously, no normalization of the mechanical data was performed due to the irregular geometry of femoral necks and the unpredictability of the direction of fracture.
Unnormalized mechanical testing data representing extrinsic properties of the femoral necks are shown in Figure 4-14.

![Bar charts showing Ultimate Load, Stiffness, Failure Displacement, and Work to Failure for SH, OVX, C1H, C1L, C2H, C2L, C2M.](image)

**Figure 4-14: Results of the femoral neck fracture test.** Only extrinsic properties of the femoral neck were evaluated while no normalization of the mechanical data was performed. No significant difference was observed for ultimate load and stiffness. \(^b\)significantly different vs OVX. Values represent mean ± SEM, p<0.05 as indicated.

There was no significant difference in the ultimate load and stiffness between any treatment group, indicating the bone strength and resistance to load of the femoral necks were comparable across all groups. However, failure displacement of C1H and C1L was significantly
greater than that of OVX, indicating that C1 treatment increased the bone’s ability to undergo deformation. Furthermore, C1H, C1L and C2M groups all had significantly greater work to failure compared to OVX. In contrast, C2H and C2L treatments did not significantly improve the mechanical properties of the femoral neck.

4.6 HISTOMORPHOMETRY ANALYSIS OF TISSUE-LEVEL REMODELLING

4.6.1 TISSUE-LEVEL FORMATION

To investigate tissue-level formation, the proximal tibial metaphysis trabecular bone was examined by undecalcified histomorphometry analysis. Specifically, dynamic histomorphometry analysis was conducted on unstained Spurr slides with calcein green fluorescent labeling and static histomorphometry analysis was conducted on Goldener’s Trichrome stained sections.

Sample images of dynamic histomorphometry analysis are shown in Figure 4-15. Calcein green labels become incorporated into bone during mineralization and is used as an surrogate measurement for bone formation\textsuperscript{171}. These labels appear as thin, bright green lines at locations of new bone formation, and were quantified to derive the following parameters: 1) single label surface (sLS, mm); 2) double label surface (dLS, mm); 3) mineralizing surface (MS, mm); 4) interlabel width (Ir.L.Width, μm); 5) percent mineralizing surface (MS/BS, %); 6) mineral apposition rate (MAR, μm/day); 7) bone formation rate normalized over bone surface (BFR/BS, μm/day/mm); and 8) bone formation rate normalized over bone volume (BFR/BV, μm/day/mm\textsuperscript{2}). Parameters 1 to 4 are unnormalized measurements of calcein labels while 5 to 8 are normalized parameters. Results of the analysis is shown in Table 4-1.

While the unnormalized data represent the primary measurements of the calcein green labels and is thus shown in the data table, the normalized data is much more meaningful as they
account for the difference in bone volume or bone surface between the samples. Based on the normalized data, OVX led to a significant increase in the dynamic parameters compared to SH, including increased MS/BS, MAR, BFR/BS and BFR/BV, indicating increased tissue-level bone formation. Dynamic parameters of C1H were comparable to that of OVX with the exception of BFR/BV, which was significantly decreased compared to OVX. Furthermore, dynamic parameters of C1L with the exception of MAR were significantly decreased compared to OVX, while all the dynamic parameters of the C2H, C2L and C2M treated groups were significantly decreased compared to OVX control. This indicates that tissue-level formation of the all drug treated groups with the exception of C1H were significantly decreased compared to OVX.
Figure 4-15: Sample images of unstained undecalcified bone sections of the proximal tibia viewed under UV light. A) Representative images of the proximal tibial metaphysis of different treatment groups. B) Zoomed in images showing double and single calcein green labels. All samples were imaged at 200X magnification.
Table 4-1: Results of dynamic histomorphometry analysis. Parameters measured were: 1) single label surface (sLS, mm); 2) double label surface (dLS, mm); 3) mineralizing surface (MS, mm); 4) interlabel width (Ir.L.Width, μm); 5) percent mineralizing surface (MS/BS, %); 6) mineral apposition rate (MAR, μm/day); 7) bone formation rate normalized over bone surface (BFR/BS, μm/day/mm); and 8) bone formation rate normalized over bone volume (BFR/BV, μm/day/mm²). Parameters 1 to 4 are unnormalized measurements of calcein labels while 5 to 8 are normalized parameters. a significantly different vs SH; b significantly different vs OVX; c significantly different vs C1H; d significantly different vs C1L; e significantly different vs C2H. Values represent mean ± SEM, p<0.05 as indicated.
Static histomorphometry analysis was performed on Spurr sections stained with Goldner’s Trichrome stain. Sample images of Goldner’s Trichrome stained sections are shown in Figure 4-16. On these slides, mineralized bone is stained green while osteoid is stained red. Osteoid is the organic matrix of bone secreted by osteoblasts, which gradually becomes mineralized over time. Therefore analysis of the osteoid seam provides information about tissue-level bone formation. Specifically, the parameters measured were: 1) osteoid surface (OS, mm); 2) osteoid volume (OV, mm$^2$); 3) percent osteoid surface (OS/BS, %); 4) percent osteoid volume (OV/BV, %); and 5) osteoid width (O.Wi, µm). Osteoid surface and osteoid volume are primary measurements of the osteoid seam, which are normalized over total bone surface or total bone volume to derive normalized parameters. Osteoid width measures the thickness of the osteoid seam.

Results of static histomorphometry analysis are shown in Table 4-2. OVX significantly increased osteoid formation as shown by significantly greater OS/BS and OV/BV compared to SH. Among the drug treated groups, C1H had significantly greater OS/BS compared to C2H, C2L and C2M. However, all drug treated groups including C1H had significantly lower OS/BS and OV/BV compared to OVX control, indicating that osteoid formation in these drug treated groups are significantly decreased.

In addition, there was no significant difference in the osteoid width between SH and OVX control. All drug treated groups with the exception of C2M had comparable levels of O.Wi to OVX, indicating that the decrease in osteoid formation as observed in these groups are not due to reductions in the osteoid width. Furthermore, it should be noted that the O.Wi of C1H was significantly greater than that of C2H, C2L and C2M, but not OVX.
Figure 4-16: Sample images of undecalcified sections stained with Goldener’s Trichrome
A) Representative images of the proximal tibia metaphysis of different treatment groups. Mineralized bone is stained green while osteoid is stained red. B) Zoomed in images showing osteoid on the surface of bone, which is red and has a glassy appearance. All samples were imaged at 200X magnification.
Table 4-2: Results of static histomorphometry analysis. Parameters measured were: 1) osteoid surface (OS, mm); 2) osteoid volume (OV, mm$^2$); 3) percent osteoid surface (OS/BS, %); 4) percent osteoid volume (OV/BV, %); and 5) osteoid width (O.Wi, µm). Osteoid surface and osteoid volume are primary measurements of the osteoid seam, while percent osteoid volume and percent osteoid surface are normalized parameters of osteoid formation. Osteoid width measures the thickness of the osteoid seam. $^a$significantly different vs SH; $^b$significantly different vs OVX; $^c$significantly different vs C1H; $^d$significantly different vs C1L; $^e$significantly different vs C2H; $^f$significantly different vs C2L. Values represent mean ± SEM, p<0.05 as indicated.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>SH (Vehicle)</th>
<th>OVX (Vehicle)</th>
<th>C1H</th>
<th>C1L</th>
<th>C2H</th>
<th>C2L</th>
<th>C2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS (mm)</td>
<td>0.335 ± 0.030</td>
<td>0.919 ± 0.064$^a$</td>
<td>0.835 ± 0.115$^a$</td>
<td>0.327 ± 0.028$^{b,c}$</td>
<td>0.202 ± 0.012$^{b,c}$</td>
<td>0.210 ± 0.006$^{b,c}$</td>
<td>0.200 ± 0.023$^{b,c}$</td>
</tr>
<tr>
<td>OV (mm$^2$)</td>
<td>0.00076 ± 0.00014</td>
<td>0.00232 ± 0.00016$^a$</td>
<td>0.00289 ± 0.00039$^a$</td>
<td>0.00090 ± 0.00012$^{b,c}$</td>
<td>0.00046 ± 0.00003$^{b,c}$</td>
<td>0.00047 ± 0.00003$^{b,c}$</td>
<td>0.00047 ± 0.00007$^{b,c}$</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>0.457 ± 0.036</td>
<td>3.997 ± 0.255$^a$</td>
<td>1.189 ± 0.094$^{a,b}$</td>
<td>0.931 ± 0.046$^b$</td>
<td>0.623 ± 0.030$^{b,c}$</td>
<td>0.645 ± 0.040$^{b,c}$</td>
<td>0.576 ± 0.038$^{b,c}$</td>
</tr>
<tr>
<td>OV/BV (%)</td>
<td>0.025 ± 0.006</td>
<td>0.210 ± 0.021$^a$</td>
<td>0.061 ± 0.001$^b$</td>
<td>0.054 ± 0.004$^b$</td>
<td>0.034 ± 0.002$^b$</td>
<td>0.034 ± 0.004$^b$</td>
<td>0.028 ± 0.003$^b$</td>
</tr>
<tr>
<td>O.Wi (µm)</td>
<td>1.848 ± 0.142</td>
<td>2.059 ± 0.121</td>
<td>2.371 ± 0.067$^a$</td>
<td>2.082 ± 0.043</td>
<td>1.916 ± 0.047$^c$</td>
<td>1.893 ± 0.023$^c$</td>
<td>1.487 ± 0.061$^{b,c,d,e,f}$</td>
</tr>
</tbody>
</table>
In addition, osteoblast and osteocytes were quantified on decalcified bone sections of the proximal tibial metaphysis stained for tartrate-resistant acid phosphates (TRAP) to further assess tissue-level bone formation. On these sections, osteoblasts are dark blue, cuboidal cells that are located on the surface of the bone while osteocytes are oval shaped, dark blue stained cells that are embedded in the mineralized bone. The measured parameters were: 1) osteoblast number (N.Ob, #); 2) osteocyte number (N.Ot, #); 3) normalized osteoblast number (N.Ob/BS, /mm); and 4) normalized osteocyte number (N.Ot/BS, 1/mm). Parameters 1 and 2 are absolute measures of osteoblast and osteocytes numbers while parameters 3 and 4 are data normalized over bone surface.

Results of the analysis are summarized in Table 4-3, and bar graphs of the normalized parameters are shown in Figure 4-17. Based on results of the normalized data, N.Ob/BS of OVX was significantly increased compared to SH. However, all of the drug treated groups including C1H showed significantly decreased N.Ob/BS compared to OVX, although N.Ob/BS of C1H was significantly greater than that of the other drug treated groups.

There was no significant difference in the N.Ot/BS between SH and OVX. Furthermore, there was no difference in the N.Ot/BS of C2H, C2L and C2M treated groups compared to SH or OVX. However, N.Ot/BS of both C1H and C1L were significantly increased compared to OVX, C2L and C2M.
### Table 4-3: Results of quantification of osteoblast and osteocyte numbers.

Parameters measured were: 1) osteoblast number (N.Ob, #); 2) osteocyte number (N.Ot, #); 3) normalized osteoblast number (N.Ob/BS, /mm); and 4) normalized osteocyte number (N.Ot/BS, /mm). Parameters 1 and 2 are absolute measures of osteoblast and osteocytes numbers while parameters 3 and 4 are data normalized over bone surface. \(^{a}\)significantly different vs SH; \(^{b}\)significantly different vs OVX; \(^{c}\)significantly different vs C1H; \(^{d}\)significantly different vs C1L. Values represent mean ± SEM, p<0.05 as indicated.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>SH (Vehicle)</th>
<th>OVX (Vehicle)</th>
<th>C1H</th>
<th>C1L</th>
<th>C2H</th>
<th>C2L</th>
<th>C2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.Ob (#)</td>
<td>34.286 ± 2.884</td>
<td>32.500 ± 6.910</td>
<td>40.333 ± 6.884</td>
<td>13.400 ± 1.500(^{a,b,c})</td>
<td>8.200 ± 1.632(^{a,b,c})</td>
<td>7.556 ± 1.564(^{a,b,c})</td>
<td>4.286 ± 0.369(^{a,b,c})</td>
</tr>
<tr>
<td>N.Ot (#)</td>
<td>1186.286 ± 72.470</td>
<td>418.125 ± 48.486(^{a})</td>
<td>1568.333 ± 93.329(^{a,b})</td>
<td>731.625 ± 37.169(^{a,b,c})</td>
<td>523.800 ± 52.056(^{a,b,c})</td>
<td>385.444 ± 40.388(^{a,b,c,d})</td>
<td>443.875 ± 60.785(^{a,b,c,d})</td>
</tr>
<tr>
<td>N.Ob/BS (1/mm)</td>
<td>0.674 ± 0.048</td>
<td>1.209 ± 0.134(^{a})</td>
<td>0.792 ± 0.098(^{b})</td>
<td>0.406 ± 0.035(^{b,c})</td>
<td>0.307 ± 0.057(^{a,b,c})</td>
<td>0.328 ± 0.053(^{a,b,c})</td>
<td>0.219 ± 0.044(^{a,b,c})</td>
</tr>
<tr>
<td>N.Ot/BS (1/mm)</td>
<td>21.287 ± 1.354</td>
<td>17.110 ± 0.790</td>
<td>34.802 ± 2.344(^{a,b})</td>
<td>24.722 ± 1.225(^{b,c})</td>
<td>19.039 ± 1.170(^{c})</td>
<td>15.560 ± 0.743(^{c,d})</td>
<td>16.703 ± 0.849(^{c,d})</td>
</tr>
</tbody>
</table>
Figure 4-17: Graphs representing measurements of osteoblast and osteocyte numbers. 

\( a \) significantly different vs SH; \( b \) significantly different vs OVX; \( c \) significantly different vs C1H; 
\( d \) significantly different vs C1L. Values represent mean ± SEM, \( p<0.05 \) as indicated.

#### 4.6.2 TISSUE-LEVEL RESORPTION

Tissue level bone resorption was assessed by decalcified histomorphometry analysis of the proximal tibial metaphysis trabecular bone. Specifically, wax sections of decalcified right tibia were stained for the enzyme tartrate resistant acid phosphatase (TRAP), which are highly expressed in osteoclasts. Sample images of TRAP stained sections are shown in Figure 4-18. On these slides, osteoclasts appear to be large, round, multinucleated cells that are red and are attached to the surface of the bone. In the analysis of these sections, only cells with more than one distinguishable nuclei was counted as an osteoclast. The measured parameters for bone resorption were: 1) osteoclast number (N.Oc, #); 2) osteoclast surface (Oc.S, mm); 3) osteoclast number normalized over bone surface (N.Oc/BS, 1/mm); and 4) percent osteoclast surface (Oc.S/BS, %). Parameters 1 and 2 are primary measures of osteoclast number and osteoclast surface, which are then normalized to derive parameters 3 and 4.
Results of histomorphometry analysis of TRAP stained sections are shown in Table 4-4. Compared to SH, normalized resorption parameters including N.Oc/BS and Oc.S/BS are significantly increased in OVX control, indicating an increase in tissue-level resorption. Compared to OVX control, all drug treated groups had significantly lower N.Oc/BS and Oc.S/BS, indicating reduction of tissue-level bone resorption by all of the drug treatments.

Between the drug treatment groups, N.Oc/BS and Oc.S/BS of C1H were significantly greater than that of C1L. Furthermore, both C1H and C1L had significantly greater N.Oc/BS and Oc.S/BS compared to C2H, C2L and C2M, indicating severe suppression of tissue-level bone resorption in C2H, C2L and C2M.
Figure 4-18: Sample images of decalcified sections stained with TRAP. A) Representative images of the proximal tibial metaphysis of different treatment groups. B) Zoomed in images showing osteoclasts on the surface of bone, which are large, multinucleated cells that are stained bright red. All sample were imaged at 200X magnification.
Table 4-4: Results of histomorphometry analysis of TRAP stained sections. Parameters measured were: 1) osteoclast number (N.Oc, #); 2) osteoclast surface (Oc.S, mm); 3) osteoclast number normalized over bone surface (N.Oc/BS, 1/mm); and 4) percent osteoclast surface (Oc.S/BS, %). Parameters 1 and 2 are primary measures of osteoclast number and osteoclast surface, while parameters 3 and 4 are normalized parameters of bone resorption. Significant differences vs SH: a) significantly different; vs OVX: b) significantly different; vs C1H: c) significantly different; vs C1L: d) significantly different. Values represent mean ± SEM, p<0.05 as indicated.
4.6.3 EFFECTS ON MARROW ADIPOSITY AND GROWTH PLATE THICKNESS

The marrow adiposity of the proximal tibia was examined qualitatively on TRAP stained sections. Representative images are shown in Figure 4-19. On these sections, fat pockets which were previously occupied by adipocytes appear as large and white round regions. Compared to SH, marrow of OVX control appeared to be higher in adiposity. Furthermore, it seems that all drug treatment appeared to have decreased marrow adiposity compared to OVX, with C1H showing the most prominent decrease in marrow adiposity amongst the drug treatment groups.

![Figure 4-19](image)

Figure 4-19: Sample images of the proximal tibial marrow region on TRAP stained sections. OVX led to increase in marrow adiposity compared to SH, which appear to be decreased by all drug treatments, most prominently by C1H.
In addition, the growth plate thickness of the proximal tibia were also measured on TRAP stained sections. Samples images are shown in Figure 4-20A. Since the thickness of the growth plate is not uniform along its length, 20 measurements at locations spread out along the entire length of the growth plate were made for each sample to derive the average growth plate thickness of the sample. The results are shown in Figure 4-20B. There was no significant difference between the growth plate thickness of SH and OVX. However, the growth plate thickness of C1H and C2M were significantly increased compared to both SH and OVX.
Figure 4-20: Analysis of growth plate thickness on TRAP stained sections. A) Sample images of the proximal tibial growth plate above the primary spongiosa. B) Results from quantitative analysis of growth plate thickness between treatments groups. *significantly different vs SH; †significantly different vs OVX. Values represent mean ± SEM, p<0.05 as indicated.
4.7 BSE ANALYSIS OF THE DEGREE OF MINERALIZATION

The degree of mineralization of tibial trabecular bone were evaluated by BSE analysis of Spurr embedded tibial blocks. Two parameters including the maximum grey value and the full width at half max height (FWHMH) were accessed. Max grey value measures the bone’s peak mineralization while FWHMH measures the spread of the mineralization, or the heterogeneity of bone mineralization. Representative BSE images and results are shown in Figure 4-21. The maximum grey values of all groups were comparable, indicating that there was no significant difference in the peak mineralization levels between any of the treatment group. However, OVX’s FWHMH value was significantly decreased compared to SH, indicating a decreased heterogeneity in bone mineralization in the OVX control group. Of the drug treated groups, only C1H significantly increased FWHMH compared to OVX control, while all the other drug treated groups had comparable FWHMH values to OVX. Furthermore, FWHM of C1H group was significantly increased compared to all treatment groups except SH. Together, these results indicate that only C1H treatment increased the heterogeneity of bone mineralization while all the other drug treatments including C2H and C2L conjugate treatments had no effect on the spread of mineralization.
Figure 4-21: Analysis of bone mineralization by BSE. A) Representative BSE images of tibial proximal metaphysis embedded in Spurr blocks. B) Results of BSE analysis. Maximum grey value represent sample’s peak mineralization level, and full width at half max height (FWHMH) represent the spread of bone mineralization of the sample. *significantly different vs SH; **significantly different vs OVX; ***significantly different vs C1H. Values represent mean ± SEM, p<0.05 as indicated.
5 DISCUSSION

The goal of the present study was to investigate and compare the in vivo efficacy of two novel EP4a-ALN conjugate drugs: C1 and C2. The conjugates were designed to facilitate target delivery of EP4a to bone through ALN binding to hydroxyapatite for the purpose of decreasing adverse side effects associated with EP4a systemic administration. The hopes were that by local enzymatic hydrolysis of the conjugates in the local bone environment, the ALN and EP4a components will exert anti-resorptive and anabolic effects simultaneously on bone to not only stop further osteoporotic bone loss but also rebuild lost bone. The C1 and C2 conjugates differ structurally with the presence or absence of a 4-hydroxyphenylacetic acid linker (LK) molecule, leading to their distinct in vivo and in vitro stabilities\textsuperscript{127}. In the C1 conjugate, the LK is covalently attached to ALN on one end through a carbamate bond while the other end of the LK is attached to EP4a through an ester bond. Radiolabelling studies have shown that EP4a is released from the rest of the conjugate with a half-life of approximately 5 days while the LK molecule is released from the ALN with a half-life of 22 days. In the C2 conjugate, the ALN is directly linked to EP4a through a carbamate bond. Radiolabelling studies showed that ALN and EP4a are released from each other with a half-life of about 28 days.

The current study employed the ovariectomized (OVX) rat model as the animal model for post-menopausal osteoporosis. Past studies have shown that OVX induced bone loss in rats share many similar characteristics with postmenopausal bone loss in humans, making it a suitable animal model for the current study\textsuperscript{32}.

A curative experimental design was adopted in the current study. Specifically, rats were subjected to bilateral ovariectomy at 3 months of age and were then allowed to lose bones for a period of 3 months prior to the start of drug treatments to achieve established osteopenia\textsuperscript{172}. At
the end of the bone loss period, the rats received tail vein injections of various drug treatments for a period of 3 months. Seven treatment groups including SH healthy control, OVX negative control, C2M control for conjugation effects, as well as high and low doses for C1 and C2 conjugates were included in the study. Rats were sacrificed at 9 months of age, and their bones were dissected and used for analysis of drug effects on a number of bone quality parameters including bone mineral density (BMD), microarchitecture, mechanical properties, degree of mineralization and tissue-level remodelling.

5.1 OVX MODEL

The OVX model is a well-established model for post-menopausal osteoporosis which closely mimics many aspects of post-menopausal osteoporotic bone loss in humans including increased rate of bone turnover with resorption exceeding formation and a greater loss of cancellous bone than cortical bone\textsuperscript{32,172-175}. Based on a study by Wronski et al\textsuperscript{173}, Sprague-Dawley rats which have received the OVX procedure first experience a period of rapid cancellous bone loss for up to 100 days after surgery, which is then followed by a relatively more stable period of bone loss until 270 days after surgery, at which point the cancellous bone volume of the proximal tibia reaches about only 5-7\% of the original bone volume. Increased levels of bone turnover is associated with both of these phases of bone loss. In the current study, Sprague Dawley rats also received the OVX procedure at 3 months of age and were sacrificed 6 months (180 days) later. Based on the previous data, this would predict the rats in the current study were in the steady phase of cancellous bone loss and were exhibiting elevated levels of turnover at the time of sacrifice. In order to validate the OVX model of the current study, skeletal changes of the OVX control rats were carefully documented and compared to the SH control.
In the current study, OVX led to significant increase in animal weight compared to SH both before and after treatments. This is consistent with previous reports of weight gain in OVX rats and is most likely directly related to estrogen deficiency in these rats\textsuperscript{158,176,177}. Past studies have shown that estrogen has important functions in regulating food intake, including enhancing effects of the peptide hormone cholecystokinin (CKK) that is responsible for providing the sensation of satiation after food intake\textsuperscript{178}. Thus estrogen deficiency is associated with reduced satiating potency of CKK and increased appetite, leading to weight gain in OVX rats\textsuperscript{179}.

In addition to weight gain, OVX rats also exhibited a number of skeletal changes both at the macroscopic level and at the tissue-level. In the L6 vertebrae, OVX led to significant decrease in aBMD and BMC compared to SH. Furthermore, the microarchitecture of the L6 trabecular bone was significantly compromised due to significantly decreased trabecular number leading to increased trabecular separation, although trabecular thickness was not significantly affected. These changes were further translated into significantly decreased trabecular connectivity density, percent bone volume (BV/TV) and vBMD. In addition, OVX led to a conversion of the lumbar trabeculae from a plate-like to a more rod-like structure, as shown by an increased structure model index (SMI) value compared to SH. This is consistent with previously reported effects of OVX induced bone loss which increases depth of resorption cavities on the trabeculae due to heightened levels of bone turnover, resulting in focal perforation of trabecular plates and the eventual conversion of the plates to rods\textsuperscript{168}. The perforative resorption accumulates and eventually leads to loss of trabecular elements and decrease in trabecular connectivity, thereby compromising the trabecular microarchitecture\textsuperscript{180}. Together, these results indicate that the observed bone loss in the OVX rats was due to loss of whole
trabecular elements rather than thinning of individual trabeculae and are consistent with previous reports of OVX induced cancellous bone loss in rats\textsuperscript{32,168,175,181}.

In addition, OVX led to significant reduction in the bone strength and resistance to load of the L6 vertebrae as demonstrated by decreased ultimate load and stiffness compared to SH, respectively. This is consistent with a previous study conducted by Ke et al which showed that OVX in Sprague Dawley rats significantly decreased the ultimate load and stiffness of the lumbar vertebrae\textsuperscript{125}. However, the same paper also reported significant reduction in the toughness and Young’s modulus of the OVX vehicle groups compared to SH. In the current study, no significant difference was observed in the normalized parameters representing intrinsic mechanical properties of the bone, including Young’s modulus or toughness. This data indicates that in the current study, the observed decrease in the ultimate load and stiffness of the vertebrae in the OVX group was actually due to loss of trabecular bone volume and connectivity as described previously, and not due to compromised bone material properties. It should be noted although this is different than results reported by Ke et al, the OVX rats in that study were allowed to lose bones for a period of 8.5 months before sacrifice\textsuperscript{125}, which is longer than the 6 months in the current study. Therefore this time difference may account for the changes in the material properties of the bone in OVX rats.

In the femurs, some studies have reported that OVX stimulated periosteal bone growth in the cortical diaphysis\textsuperscript{182,183}. Since periosteal bone growth is difficult to visualize on MicroCT, this is often assessed by histomorphometry analysis of transverse axial bone sections of the cortex of long bones\textsuperscript{184,185}. However in the present study, this could not be done due to technical difficulties in preparing axial bone sections. Thus explicit observations on periosteal formation could not be made. However, MicroCT analysis showed no significant difference in the femur
geometry between OVX and SH control, as shown by comparable medial-lateral diameter, anterior-posterior diameter, principal moment of inertia, cortical thickness and cortical bone area between the two groups. Furthermore, no difference in the vBMD of the cortical bone was observed between OVX and SH. In addition, mechanical properties of the femurs as assessed by the three-point-bending tests were also comparable between OVX and SH. Together, these data indicate that OVX did not induce significant changes in the geometry or the intrinsic material properties of the femoral cortical bone.

Based on these observations, it is reasonable to conclude that OVX associated bone loss was more prominent in cancellous bone than cortical bone in the present study. This is consistent with previous studies of the OVX model\textsuperscript{32,172,174}, which reported elevated levels of bone turnover in trabecular bone, leading to pronounced cancellous bone loss.

Changes in tissue-level bone remodelling were evaluated by histomorphometry analysis of the proximal tibial metaphysis trabecular bone. Results showed that OVX led to significant increase in dynamic parameters of bone formation including mineralizing surface (MS/BS), mineral apposition rate (MAR), bone formation rate (BFR/BS), as well as static bone formation parameters including percent osteoid surface (OS/BS), and normalized osteoblast number (N.Ob/BS) compared to SH. Furthermore, parameters of bone resorption including normalized osteoclast number (N.Oc/BS) and percent osteoclast surface (Oc.S/BS) were also significantly increased by OVX. Together, these data suggest that OVX significantly increased tissue-level bone turnover by increasing both bone formation and resorption. These observations are consistent with previous results of histomorphometry analysis on tissue-level turnover in OVX rats\textsuperscript{125,159,172}.
In addition, the mineralization profile of the proximal tibial trabecular bone was assessed by BSE. Results showed that there was no significant difference between the maximum grey value between SH and OVX, indicating that the level of mineralization of the trabecular bone between the two groups were comparable. In contrast, MicroCT results of the current study showed that the L6 vertebral trabecular bone of the OVX control groups had a significantly lower vBMD compared to SH. Although these results may seem conflicting at first, it should be noted that the maximum grey value and trabecular vBMD actually measure two different things: the MicroCT analysis of vBMD measures the density of bone mineral over the total tissue volume and thus reflect the total amount of bone mineral in a specimen whereas the maximum grey value represents the mineralization level of the trabecular bone only. Therefore, this data suggests that while the trabecular vBMD of the L6 vertebrae is decreased in OVX most likely due to loss of trabecular elements and bone volume, the actual level of mineralization of the trabecular bone is not significantly different than that of SH. On the other hand, BSE results showed that OVX’s FWHMH values was significantly decreased compared to SH, indicating a decreased heterogeneity in the spread of bone mineralization of the OVX control group. This is most likely due to OVX’s significantly smaller amount of trabecular bone volume compared to SH.

Also from histomorphometry analysis, the marrow space of the proximal tibia of the OVX group appeared to be higher in adiposity compared to SH. As past studies have shown, estrogen promotes the differentiation of mesenchymal stem cells towards the osteoblastic lineage over adipocytes\textsuperscript{186}. Therefore the observed increase in marrow adiposity is most likely due to effects of estrogen deficiency as a result of OVX in the rats.
5.2 C2M

Prior to the discussion of conjugate drug effects, the skeletal effects of the C2M will be briefly discussed. The C2M contains the EP4a and ALN drug component in their original unconjugated form and will therefore be used as a control for evaluating conjugation effects between the two components in the conjugate drugs.

The C2M was dosed at 0.75mg/kg biweekly for each of the two components, ALN and EP4a. As mentioned previously, this dosage was chosen to match the highest amount of ALN and EP4a that is possibly released by C2 in the bones. Previous pre-clinical studies using OVX rats showed that a dose as small as 0.015mg/kg of ALN twice per week by subcutaneous injection or 0.06mg/kg equivalent dose biweekly can significantly increase bone mass and strength in OVX rats\textsuperscript{187-189}. Based on these findings, we would expect a dose of 0.75mg/kg of ALN in the C2M group to exert robust anti-resportive effects for preventing OVX induced bone loss. On the other hand, previous studies have shown that a dose of 3-6mg/kg PGE\textsubscript{2} by daily subcutaneous injections are required to significantly increase bone formation and bone mass in OVX rats\textsuperscript{98,101,190,191}. Therefore in the current study, it is possible that a dose of 0.75mg/kg EP4a biweekly was too low to produce any robust anabolic effects but is necessary for fair comparison with the conjugates to control for drug conjugation effects.

In terms of treatment side effects, animals treated with the C2M did not show visible pain during injection but exhibited prolonged lethargy for up to 1 day after injections. Slight discoloration and swelling of the tails were also observed in treated rats after treatment. This is most likely due to the local acidity of the ALN in the tails. In contrast, EP4a related symptoms reported previously such as diarrhea and puffy fur were not observed in the current study. This is
likely due to the low concentration of EP4a used in the C2M group compared to previous studies, leading to less associated side effects.

In the lumbar vertebrae, DEXA analysis showed that C2M treated bones had significantly greater aBMD and BMC compared to OVX. MicroCT analysis further showed that C2M led to significant increase in the BV/TV and trabecular number of the vertebral trabecular bone compared to OVX. Together, these results indicate that either one or both components of the C2M drug mixture is effective in increasing trabecular bone volume compared to OVX.

In the femurs, previous studies have shown that PGE\textsubscript{2} and EP4a have effects which stimulate activation of intracortical remodeling leading to increased cortical porosity and stimulation of endocortical bone formation in rats\textsuperscript{98,107,121,192}. In the current study, MicroCT analysis showed that C2M did not lead to significant changes in the vBMD and geometry of the femurs compared to OVX. Furthermore, endocortical bone formation in the femoral midshaft as well as increase in cortical porosity was not observed in C2M. This indicates that EP4a effects in the C2M treatment may be non-significant and that the observed skeletal phenotypes in the vertebrae are due to effects of ALN alone to prevent OVX induced bone loss.

In the mechanical testing of the vertebrae by vertebral compression, the ultimate load but not the ultimate stress was increased in C2M compared to OVX. This indicates that the observed increase in ultimate load is due to the increase in vertebral trabecular bone volume by C2M but not improvement in the intrinsic load bearing ability of the bone material. Furthermore, C2M’s failure displacement, failure strain, work to failure and toughness were all significantly increased compared to OVX in the vertebral compression test. In the femurs, no changes in the ultimate load was observed but the work to failure, failure displacement and failure strain were significantly increased compared to OVX. Together these results suggest that C2M not only
increased the extrinsic ability of the femurs and vertebrae to deform and absorb energy without failure, but also improved the material toughness and ductility of the treated bones. Currently, there is no literature which has reported that PGE$_2$ or EP4a increases the toughness or ductility of bone. However, it is possible that the observed changes in bone mechanical properties in the C2M group is due to short term effects of ALN. Past studies have shown that short term bisphosphonate treatment enhanced collagen gene expression and secretion by osteoblasts$^{193,194}$. Since the collagen component is generally considered to be the major contributor to toughness and ductility in bone$^{195-198}$, it is possible that such a change in the collagen matrix also occurred in C2M treated bones in response to short term ALN treatment, leading to an increase in material toughness and ductility.

In histomorphometry analysis, significant reduction was observed in tissue-level bone formation in the tibial proximal metaphyseal trabecular bone, which was shown by decreased mineralization parameters, decreased osteoid formation parameters, and decreased normalized number of osteoblasts. Furthermore, decrease in osteoclast number and surface was also observed. Together, this indicates that C2M led to a significant decrease in tissue-level bone turnover compared to OVX. These results are consistent with previous reports that bisphosphonate treatment inhibit osteoclast resorption as well as lead to suppression of overall bone turnover$^{74,75,199}$. Taken together, it is reasonable to conclude that the observed effects of the C2M treatment are mostly due to the robust anti-resorptive effects of ALN, and that the anabolic effects of EP4a are not seen in this treatment group due to its low concentration.

It should also be noted that a decrease in marrow adiposity of the proximal tibial metaphysis was observed in C2M compared to OVX. Recent studies have reported that ALN can promote osteoblast differentiation over adipogenesis from mesenchymal stem cells by inhibition
of adipogenic differentiation, thereby decreasing marrow adiposity\textsuperscript{200,201}. Furthermore, other studies have shown that PGE\textsubscript{2} also have effects which stimulate osteoblast differentiation from mesenchymal stromal cells as well as inhibit adipocyte differentiation from pre-adipocytes \textit{in vitro}\textsuperscript{96,202}. These findings suggest that both the ALN and EP4a from the C2M drug mixture can lead to decreased marrow adiposity. However based on the other skeletal effects observed for C2M, ALN is most likely the major contributor for this change in marrow adiposity observed in the proximal tibial metaphysis.

In the C2M, the growth plate thickness was significantly increased compared to SH and OVX. In past studies, significant thickening of the growth plate has been observed in bones treated with risedronate\textsuperscript{203}, another bisphosphonate, or PGE\textsubscript{2}\textsuperscript{100}. Given the low concentration of EP4a used in the C2M group, the observed increase in growth plate thickness may most likely be due to effects of ALN in the C2M mixture.

5.3 CONJUGATE TREATMENT

The conjugate treatments consisted of a high dose and a low dose for each of the two conjugate drugs: C1 and C2. The C1H and C1L were dosed at 5mg/kg weekly and biweekly, respectively, while the C2H and C2L were dosed at 15mg/kg biweekly and monthly. The high doses of the two conjugates were chosen based on the uptake rates of the conjugates into bone as well as the \textit{in vivo} release rates of EP4a from the conjugates, such that both C1H and C2H will provide a sustain release of EP4a at 12-15\textmu g/kg/day. This is the release rate of PGE\textsubscript{2} shown previously required to achieve robust bone anabolic effect \textit{in vivo}\textsuperscript{127}. The low doses were exactly half of the high doses, and were included for the investigation of dose-dependent effects of the conjugates. For this part of the discussion, the effects of the conjugate drugs on the rat skeleton will be compared to OVX, SH and C2M controls.
5.3.1 CONJUGATE TREATMENT RELATED SIDE EFFECTS

In the current study, conjugate treatment related side effects were carefully documented. During tail vein injection of either of the conjugate treatments, rats experienced visible pain and discomfort. Extreme care had to be taken to restraint the rats in order to prevent tail movement during injection. Directly after injection, tail redness and swelling was observed in C2H treated rats but not in C1H treated rats. Furthermore, several cases of tail necrosis was also observed in the C2H treated rats after the administration of the third dose. Based on past experiments of the conjugates, it has been shown that upon tail vein injections, the conjugates precipitated when it sees calcium in the blood vessel, which is then quickly resolubilized by albumin in the blood (private communication with Dr. Robert Young). However at a higher concentration, a small amount of precipitation of the conjugate would occur, leading to local toxicity effects in the tail. Since C2H was dosed at three times the concentration of C1H during each injection, the possibility of the C2 conjugate precipitating in the blood is therefore higher. After increasing the volume of the drug based on a 1:15 PBS dilution, pain during injection of the conjugates was significantly decreased while no additional cases of tail necrosis were observed. This suggests that the observed local toxicity in the tails of the rat associated with C2 injection may possibly be due to partial precipitation of the drug in the blood vessel, and a slower infusion of the drug over a greater volume may be able to decrease these effects.

Several cases of swollen feet were observed in rats treated with the C1H dose. Affected rats were lethargic and exhibited weight loss but did recover on their own within 2 weeks. A previous study have reported that PGE$_2$ treatment is associated with paw edema in mice$^{204}$. In that study, intraplantar injection of PGE$_2$ into the hindpaw of mice elicited a dose-dependent edema formation in the mice. In the current study, conjugate drugs were directly injected into the
circulatory system. However, only about 5.9% of the C1 conjugate and 9.4% of the C2 conjugate has been shown to localize to bone after 6 hours of injection\textsuperscript{127}. While most of the conjugates are excreted in feces and urine, it is possible that a small percentage of the conjugates will reach local tissue including the rat feet through the circulatory system. Due to the abundance of esterases \textit{in vivo}, it is possible that the EP4\textsubscript{a} component is cleaved from the C1 conjugate in the feet, leading to tissue edema in some C1H treated rats. On the other hand, enzymes such as carboxypeptidases or P450 that are required to release EP4\textsubscript{a} from the C2 conjugate may be less available in local tissues. This may explain why no cases of swollen feet was observed in C2 treated rats though the concentration of C2 injection was greater than C1. Nonetheless, the C1 conjugate was administered based on 1: 1.5 PBS dilution at 6 weeks of treatment, after which point no more cases of swollen feet was observed. It is unclear whether this was due to effects of the dilution or increase in local tolerance of the rats to the conjugate treatment.

In the current study, no diarrhea was observed in C1 or C2 treated rats. Since diarrhea is a frequent side effect due to EP4\textsubscript{a} systemic administration\textsuperscript{115,116}, this indicates that the conjugation between ALN and EP4\textsubscript{a} is beneficial for facilitating the target delivery of the EP4\textsubscript{a} to bone and mitigating its associated side effects due to systemic administration.

\subsection*{5.3.2 \textbf{GEOMETRY AND MICROARCHITECTURE}}

Conjugate drug effects on bone geometry and microarchitecture was evaluated by MicroCT analysis of the L6 vertebrae and left femurs. In the L6 vertebrae, qualitative analysis of vertebral cross-sectional images showed that C1H conjugate treatment led to apparent trabecular bone formation in the vertebral column. This was confirmed by quantitative MicroCT analysis of the vertebral trabecular bone, which showed that C1 led to a dose-dependent recovery of the trabecular structural indices compared to OVX. Specifically, C1H led to increase in percent bone
volume, trabecular thickness, trabecular number, decreased trabecular separation and increased connectivity density compared to OVX, while C1L only led to significantly increased percent bone volume and trabecular number compared to OVX. Furthermore, the BV/TV of C1H was also significantly greater than that of C2M control. This suggests a dose-dependent effect of the C1 conjugate. Additionally, C1H significantly decreased the structure model index, indicating that the conversion of the trabecular structure from plates to rods is effectively reversed by C1 high dose treatment. Taken together, these results strongly suggest that C1H treatment restored trabecular bone mass lost due to ovariectomy, which is consistent with previously reported anabolic effects of PGE$_2$ in OVX rats. 

In contrast, C2’s anabolic effect in the L6 vertebrae was less apparent. The structural indices of C2 treated vertebrae were comparable to OVX control, while the percent bone volume and trabecular thickness of the C1H was significantly greater than both C2H and C2L. This indicates that although C1H and C2H were both dosed to provide a sustained release of EP4a at 12-15$$\mu$$g/kg/day, C1H was able to exert robust anabolic effects in the L6 vertebrae while C2H was not.

In the femurs, conjugate C1 led to endocortical bone formation in a dose dependent manner. In C1L treated femurs, relatively low amount of endocortical bone formation was observed, which ranged from 0 to 50% marrow space occlusion by this new bone formation. This was translated into a significant increase in femoral bone area compared to OVX control. In the C1 high dose, the majority of rats had greater than 50% marrow space occlusion by this endocortical bone formation, which led to significant increase in the femoral bone area compared to OVX, SH, C2M and C1L. However, even within the same treatment group, a large variation in the amount of encortical bone formation was observed. A number of possible reasons may
have contributed toward the observed variation. Firstly, the observed variation in the amount of endocortical bone formation may be due to differential animal response to the drug treatment. Previous drug studies in rats have shown that there can be responders and non-responders to a specific drug treatment, leading to high levels of variance within the same treatment group\textsuperscript{205}. It is therefore possible that some rats were less responsive than others to the C1 conjugate, leading to less endocortical bone formation in these rats. Second, the observed variation may possibly be due to potential formation of precipitates from the C2 conjugate solution during injection. As mentioned previously, the C2 conjugate drug can precipitate when it meets calcium in the blood but is then quickly solubilized by albumin (private communication with Dr. Young). However depending on the speed of administration of the conjugate during IV injection, it may be possible that small amount of precipitates formed in the tail vein of the rats and do not become fully resolubilized the blood, resulting in loss of drug being delivered to the rest of the body. Although care was taken to administer the drug solution at similar speeds for each rat, the administration of the drug was performed manually and was therefore subject to human errors. It is possible that in some cases the drug was administered slightly quicker leading to loss of drugs in some animals due to precipitation and thus potentially contribute toward the observed variation in drug effects in the animals. Furthermore, another possible cause for the observed variation may be due to loss of drug solution during injection. Since animals experienced noticeable pain and discomfort during injection, there are cases where significant movement in the tails caused shifting of the needle. In these cases, the needles were quickly removed and then reinserted to complete the injection. It is possible that loss of drug solution may have occurred in the process. However, careful documentation of these incidences were made and no correlation was found between
individuals animals which have experienced such incidences and their level of endocortical formation.

In contrast to C1 treated femurs, no endocortical bone formation was observed in C2 treated groups, including both high and low doses. The formation of endocortical bone has been shown to be an anabolic effect of PGE$_2$ treatments in rats$^{98,107,121,192}$, and is consistent with the effect of PGE$_2$ in stimulating osteoblastogenesis and increasing the osteogenic capacity of the bone marrow$^{96,206,207}$. Since EP4a mimics the effects of PGE$_2$, the observed endocortical bone formation in C1 is thus most likely due to anabolic effects of EP4a in the conjugate structure. Furthermore, these results also suggest that the EP4a component in the C2 conjugate did not exert the same robust anabolic effects in treated femurs compared to C1, since no endocortical bone formation was observed in C2 treated femurs.

Past studies have shown that periosteal bone formation was also associated with PGE$_2$ treatment$^{98}$. In the current study, no periosteal formation was observed by MicroCT analysis in any of the treatment groups. This may be due to the lack of resolution of the MicroCT machine, which may have been unable to detect small amounts of periosteal bone formation on the surface of the femurs. Unfortunately due to difficulties with producing transverse sections of axial bone, this could not be confirmed by histomorphometry analysis. Nonetheless, MicroCT analysis showed no significant difference in the femur geometry between conjugate treated groups versus OVX, as evidenced by comparable medial-lateral diameter, anterior-posterior diameter and minimum principal moment of inertia between the groups.

In addition, increase in cortical porosity was observed in C1H and C1L treated femoral cortices compared to SH. This is consistent with previously reported effects of PGE$_2$ in the stimulation of intracortical remodelling leading to a porous cortex$^{98,192,208}$. Again, this effect was
not observed in C2H or C2L treated femurs, further confirming that EP4a downstream effects are only seen in the C1 conjugate and not in the C2.

5.3.3 BONE MINERAL DENSITY AND MINERALIZATION

In the vertebrae, both C1H and C1L treatment led to significant increase in the vertebrae’s aBMD compared to OVX, while only C1H significantly increased the BMC compared to OVX. This again suggests a dose-dependency of the C1 conjugate treatment. Furthermore, MicroCT analysis showed that the vBMD of the vertebral trabecular bone was improved in both C1H and C1L treated groups compared to OVX. Given that the C1 conjugate treatment also led to increase in trabecular bone volume and trabecular number, this increase in the vBMD of the trabecular bone may therefore be due to formation of new trabeculae and the overall increase in trabecular bone volume in the L6 vertebrae.

In the BSE analysis of the proximal tibial metaphyseal trabecular bone, results showed no change in the peak mineralization of the trabeculae but did show an increase in the spread of mineralization of C1H treated group compared to OVX. This indicates that C1H did not increase the mineralization of individual trabeculae, but did lead to increase in the heterogeneity of the bone mineral in the trabecular department. This is most likely due to EP4a associated anabolic effects which led to new bone formation in the C1H treated tibial metaphysis. Since these new bones are less mineralized than the old bone present in the metaphysis, an increase in overall heterogeneity of bone mineralization spread was detected. Compared to OVX, which had significantly decreased FWHMH vs. SH, the current results demonstrate that C1H conjugate treatment helped to recover this loss of mineralization spread from OVX. This increase in FWHMH was not observed in the C1L treated tibia, suggesting that the amount of new bone formed in response to C1L was not enough to significantly change the mineralization.
heterogeneity of the trabecular bone in the tibial metaphysis. In C2 treated vertebrae, the aBMD, BMC and vBMD were all comparable to that of OVX. These results indicate that unlike C1, the C2 conjugate treatments were not able to induce new bone formation in the vertebrae.

In the femurs, the aBMD and BMC of the C1 treated femurs were significantly increased compared to OVX as measured by DEXA analysis. This is most likely due to the increased bone volume in the C1 treated femurs via endocortical formation, leading to an overall increase in the amount of bone mineral present in the femurs. In addition, the cortical porosity of the femoral cortex in the C1H (1.57% ± 0.69%) and C1L (1.57 ± 0.88%) treated groups were significantly increased compared to SH (0.36% ± 0.29%) but not OVX (0.51% ±0.33%). This increase in cortical porosity did lead to slight decrease in the cortical vBMD of the C1H (1.38±0.02) and C1L (1.36±0.01) treated femurs compared to the cortical vBMD of SH (1.45±0.05) and OVX (1.39 ± 0.16), although the levels of reduction were not statistically significant. This indicates that the increase in cortical porosity associated with C1 conjugate treatments did not compromise the cortical vBMD of the treated femurs.

5.3.4 MECHANICAL PROPERTIES

Mechanical properties of bone represent the culmination of all architectural and tissue-level changes resulting from a treatment, and is thus the best assessment of drug effects on bone quality.

5.3.4.1 VERTEBRAL COMPRESSION

In the vertebral compression test, the mechanical properties of the vertebrae depends on both its cortical bone and trabecular bone. Since the ratio of trabecular bone to cortical bone in the vertebrae is approximately 75:25, effects of either component will contribute to its
performance in the vertebral compression test, although the trabecular component will likely have an overall greater effect on the mechanical properties of the vertebra\(^2\).

Results of the vertebral compression test showed that C1H led to a significant increase in the ultimate load compared to OVX, but not ultimate stress. This suggests that the increase in bone strength was most likely due to changes in the vertebral geometry or architecture rather than improvement in the bone material properties. Since the vertebral cortical bone was not explicitly assessed, much cannot be said about its geometry. Assuming that conjugate C1’s effects on vertebral cortical bone was similar to its effects in the femoral cortex, then the increase in trabecular volume and connectivity is most likely the major contributor to this increase in bone strength.

In addition, work to failure of the C1H treated vertebrae was also significantly increased compared to OVX while toughness was not. This suggests that the intrinsic properties of the bone material to absorb energy was not improved by C1H treatment, and the observed increase in work to failure was likely due to improvement in the vertebral microarchitecture. Interestingly, the material toughness and ductility of the bone was improved by C2M, which as discussed before was most likely due to the short term effects of ALN in enhancing collagen gene expression and secretion by osteoblasts\(^{193,194}\). Since this effect was not seen in C1H treated group, this suggests that ALN associated downstream effects may be greater in C2M than in the C1 conjugate.

Parameters of mechanical properties of the C2 treated vertebrae including ultimate load, stiffness, failure displacement, work to failure and their corresponding normalized parameters were all unchanged compared to OVX. Since mechanical properties are a direct predictor of
bone quality, these results suggest that the C2 conjugate exhibited no overall beneficial effect in improving the bone quality of the L6 vertebrae compared to OVX.

5.3.4.2 THREE POINT BENDING

In the femurs, results of the three-point bending test showed that mechanical properties of OVX and SH were comparable. However, the ultimate load of the C1H and C1L but not their corresponding ultimate stress were significantly increased compared to OVX. This suggests that the increase in bone strength observed in these groups are due to changes in the femoral geometry likely owing to the encortical bone formation in these groups, but not due to improvement in the intrinsic material properties of the femurs.

It is interesting that in addition to ultimate load, the stiffness and failure strain of C1H and C1L were comparable to SH and OVX despite significant increase in the cortical porosity of the femurs. Past studies have shown that while increased porosity can reduce bone strength, the location of the porosity can also influence its effects on bone strength. In fact, the periosteal region of the cortex is the region responsible for withstanding the greatest amount of stress while the endosteal region of the cortex carries the lowest stress under bending\(^\text{149,209}\). In the C1 treated groups, the increase in porosity was mostly near the endosteal region of the cortex. This may explain why the observed increase in cortical porosity did not compromise the overall mechanical properties of the bone.

The extrinsic and intrinsic parameters representing the mechanical properties of C2 femurs were all comparable to their corresponding OVX parameters. This is not surprising given the lack of effects of the C2 conjugate on femoral geometry and bone mineral density, and further indicate that the C2 conjugate did not improve the bone quality of the treated vertebrae.
5.3.4.3 FEMORAL NECK FRACTURE

In the femoral neck fracture test, both cortical and trabecular bone contribute to the overall mechanical properties of the femoral neck. Due to the irregular geometry of the femoral neck and the unpredictability of the direction of fracture, normalization of the femoral neck mechanical properties cannot be achieved. Thus only extrinsic properties of the femoral neck can be assessed, which are dependent on the geometry of each sample.

Results showed that there was no significant difference in the ultimate load or stiffness of the conjugate treated groups, while the work to failure and failure displacement were increased by C1H. Since normalized parameters could not be determined, conclusions cannot be made about whether this increase in the femoral necks’ ability to deform and absorb energy without failure is due to changes in the geometry of the femoral necks or due to improvement in the material properties of the bone. Assuming C1’s effects on trabecular and cortical bone material properties are consistent with what has been shown by vertebral compression and three-point bending tests, the observed change in the work to failure and failure displacement are most likely due to changes in the geometry of the femoral neck by C1’s anabolic effects in the skeletal site, and less likely due to improvement in the material properties of the bone.

5.3.5 REMODELLING

5.3.5.1 RESORPTION

Tissue-level bone resorption was assessed by histomorphometry analysis of the proximal tibial trabecular bone stained with TRAP. Analysis of these sections showed that normalized osteoclast surface and normalized osteoclast number were both significantly decreased in all conjugate treated groups compared to OVX control, indicating that tissue-level resorption were
significantly depressed by all conjugate treatments. Furthermore, the level of reduction of these two parameters were significantly greater in the C2 treated groups compared to C1. The observed reduction in tissue-level bone resorption is most likely due to effects of the ALN component in the conjugates, which has been shown to have robust effects in decreasing osteoclastic bone resorption\textsuperscript{37,38}. Since C2 treated groups showed a greater reduction in bone resorption parameters, this indicates that ALN effects in the C2 conjugates are more prominent than their effects in C1 at the tissue-level.

In the assessment of bone resorption parameters, osteoclast number measures how many osteoclasts are present in the sample while osteoclast surface represents the proportion of the bone surface undergoing resorption. Since osteoclasts that occupy less bone space tend to have less nuclei and that the number of nuclei is correlated with an individual osteoclast’s resorptive activity\textsuperscript{210}, therefore a decreased overall osteoclast surface without change in osteoclast number can suggest a decrease in individual osteoclast activity. However since both of these parameters were significantly decreased in the current study, no conclusion can be made regarding the individual activity of the osteoclasts. Nonetheless, past studies have shown that ALN reduces bone resorption by decreasing both osteoclast number through induction of osteoclast apoptosis\textsuperscript{37,50}, and by reducing individual osteoclast activity\textsuperscript{211}. This suggests that the reduction in tissue-level resorption observed in the conjugate treated groups may be the result of both of these effects by ALN.

5.3.5.2 FORMATION

Tissue-level bone formation was also assessed by histomorphometry analysis of the proximal tibial trabecular bone. Quantification of calcein green labels showed that while OVX led to an overall increase in tissue-level bone formation, the dynamic parameters of C1H
including MS/BS, MAR, BFR/BS were comparable to the OVX control. However, dynamic parameters of C1L, as well as both C2 treated groups were significantly decreased compared to OVX. Interestingly, analysis of osteoid seam on Trichrome stained sections showed that both C1H and C1L, as well as C2H and C2L led to significant suppression of osteoid formation compared to OVX. Results of osteoid formation was confirmed by quantification of osteoblast number, which was significantly decreased in all conjugate treated groups including C1H compared to OVX.

Results in C1L, C2H and C2L strongly indicate that tissue-level bone formation was suppressed in these groups. This is consistent with previously reported effects of bisphosphonate, which have been shown to not only decrease tissue-level bone resorption, but can also lead to overall reduction in tissue-level turnover including bone formation\textsuperscript{74,75,199}. Specifically, studies of ALN effects in rats have shown that ALN treatment for as short as 12 weeks can significantly decrease tissue-level bone formation parameters as measured by histomorphometry analysis including MAR, MS/BS, BFR/BS, OWi, and percent osteoblast surface, which is consistent with the observations in the current study\textsuperscript{212-216}. Therefore the ALN component in the conjugate drugs are most likely responsible for the observed decrease in tissue-level bone formation. In C1H, the results are less straightforward: there was a decrease in osteoid formation and osteoblast number but the mineralizing surface of the bone was comparable to OVX. Although this data may seem conflicting, it should be noted that osteoid formation and mineralizing surface actually measure two different things. Osteoid formation is the earliest step of bone formation where new collagen matrix is laid down, which is then mineralized to form new bone\textsuperscript{2}. These two steps thus occur at different time points, with osteoid formation occurring before mineralization. In the analysis of bone formation, mineralizing surface represent new bone
formation that is occurring at the moment of sacrifice, whereas osteoid formation is a measurement of how much new bone formation will occur at a later time point. Thus, the decrease in osteoid formation in the C1H may be an early indication of decreased bone formation in the treated rats if the experimental duration was longer. Together, this suggests that the effect of ALN mediated suppression of bone formation is delayed in C1H, which is surprising given that the dose of ALN used in C1H was two times higher than in C1L, yet a greater level of suppression of bone formation was observed in C1L. However, it should be noted that the concentration of EP4a in the C1H was also two times higher than C1L. Given the anabolic effects of PGE$_2$ on bone in vivo in promoting osteoblastogenesis and de novo new bone formation$^{93-96,98}$, it is possible that the EP4a was able to in part decrease ALN associated reduction in bone formation by stimulating osteoblastogenesis in treated bones. However, since the effective dose of ALN is determined by its cumulative dose as ALN remains bound to bone for a prolonged period of time$^{50}$, it is possible that total dose of ALN administered will eventually reach a sufficient dose as the treatment period progresses to overcome effects of EP4a and lead to significant suppression of bone formation in C1H.

In the analysis of bone formation, the activity of individual osteoblasts can be determined by considering the total amount of new bone formation and the number of osteoblasts present in the sample. Unfortunately, since an overall reduction in bone formation as well as decrease in osteoblast number was observed, no explicit conclusion can be made regarding the activity of individual osteoblasts in these groups. However, past studies have suggested that osteoid width (OWi.) may be correlated with osteoblast activity$^{217}$. In the present study, the osteoid width of all conjugate treated groups with the exception of C1H were comparable to OVX. This suggests that the osteoblasts in these samples may have similar levels of activities compared to OVX.
Interestingly in C1H, the bone formation rate as measured by dynamic histomorphometry analysis was comparable to OVX while the osteoblast number was significant decreased. Furthermore, the O.Wi of the C1H was also significantly greater than all other treatment groups. Taken together, these observations may suggest that the activities of individual osteoblasts in C1H may be increased compared to other groups. Although they are no literature which explicitly report that PGE$_2$ or EP4a enhance individual osteoblast activity, there has been one study which found that PGE$_2$ stimulate collagen and non-collagen protein synthesis of osteoblasts independently of its effects on osteoblast proliferation \textit{in vitro}.$^{218}$ This study suggests that the observed increase in O.Wi of the C1H group may in fact be due to EP4a activation of individual osteoblasts to secrete more collagen. Interestingly, although C2H was dosed to release equivalent concentration of EP4a with C1H in the bone (12-15\(\mu\)g/kg/day), such increase in O.Wi was not observed in C2H. Given that C1 and C2 both have the same ALN and EP4a drug components, the difference in their conjugate structure may therefore be responsible for the observed differences in tissue-level remodelling.

\textbf{5.3.6 MARROW ADIPOSITY AND GROWTH PLATE}

Qualitative analysis of the proximal tibial metaphysis showed that all conjugates treatments led to decrease in marrow adiposity compared to OVX. As mentioned previously, both ALN$^{200,201}$ and PGE$_2$$^{96,202}$ have effects which can stimulate osteoblast differentiation over adipocyte differentiation from mesenchymal stem cells. Therefore either drug in the conjugate treatments may have contributed to this observed effect.

In addition, an increase in the growth plate thickness was observed in C1H treated group compared to SH and OVX. In a study conducted by Lin et al investigating the effects of risedronate and PGE$_2$ in bone, it has been reported that both of these drugs can increase growth
plate thickness. Furthermore, the same study also reported that combined risedronate and PGE\(_2\) treatment can lead to a greater effect on increasing growth plate thickness than risedronate or PGE\(_2\) alone. In the current study, the observed increase in growth plate thickness may therefore be due to either the effect of ALN or EP4a alone, or their combined effects.

5.4 SUMMARY OF CONJUGATE EFFECTS AND PHARMACOLOGY

Results so far indicate that C1 conjugate demonstrated robust anabolic effects in the vertebrae and femurs, while drug anabolic effects associated with the C2 conjugate are not seen at the macroscopic level. At the tissue-level, results of histomorphometry analysis reveal a far more complex picture regarding the pharmacology of the two conjugates. In this section, findings for each conjugate drug will be considered in conjunction with their conjugate structures in order to understand their pharmacology.

5.4.1 C1 CONJUGATE

On the macroscopic level, C1 conjugate treatment led to dose-dependent anabolic effects in the vertebrae and the femurs. This is evidenced by increased percent bone volume in the vertebrae and visible encortical bone formation in the femurs. The observed effects in the bones are consistent with previously reported effects of PGE\(_2\), which include stimulation of cancellous bone formation, increased intracortical remodelling leading to a porous cortex in the femurs, and \textit{de novo} woven bone formation on cortical endosteal surface\(^9\)\(^8\),\(^9\)\(^9\),\(^1\)\(^2\)\(^4\),\(^1\)\(^9\)\(^2\),\(^2\)\(^0\)\(^8\). Since EP4a mimics the effects of PGE\(_2\) \textit{in vivo}, these results suggest that the EP4a component in the C1 conjugate treated groups is responsible for the observed bone anabolic effects. Furthermore, since the dosage of EP4a in the C1H was about two times greater than that in the C1L, the conjugate associated anabolic effects was greater in the C1H treated group than the C1L treated group.
At the tissue level, C1L led to significant reduction in both tissue-level bone resorption and formation vs. OVX. This is mainly due to effects of ALN, which has been shown to decrease osteoclastic resorption as well as formation in long term treatments\textsuperscript{74,75,199}. In C1H, a significant reduction in tissue-level resorption was observed, while tissue-level formation as evidenced by dynamic parameters were comparable to OVX. However, analysis of osteoid seam showed that C1H led to decrease in osteoid formation. Considering that osteoid formation is the earliest step of bone formation where new collagen matrix is laid down prior to bone mineralization, this may be an early indication of subsequent decrease in bone formation due to longer C1 treatment. It is possible that the study window was not long enough in the present study to observe these effects.

As mentioned previously, past studies have reported that long term ALN treatment not only decreases osteoclastic bone resorption, but can also lead to suppression of bone formation\textsuperscript{74,75,199}. Given the anabolic effects of PGE\textsubscript{2} on bone \textit{in vivo} in promoting osteoblastogenesis and \textit{de novo} new bone formation\textsuperscript{93-96,98}, it is possible that long term ALN treatment may have conflicting effects with EP4a which can lead to reduction of EP4a’s anabolic effects \textit{in vivo}. Currently, combination therapy using bisphosphonate and PGE\textsubscript{2} has been performed mostly using risedronate with PGE\textsubscript{2}\textsuperscript{203,219,220}, while one study uses alendronate and PGE\textsubscript{2}\textsuperscript{221}. All of these studies reported that co-treatment with PGE\textsubscript{2} and bisphosphonate led to similar levels of anabolic effects in OVX rats compared to PGE\textsubscript{2} treatment alone. While these studies seem to suggest that bisphosphonate do not negatively affect the anabolic effects of PGE\textsubscript{2} \textit{in vivo}, attention must be paid to the type of bisphosphonates as well as the dosage of PGE\textsubscript{2} and bisphosphonates used in these studies. Since risedronate has different \textit{in vivo} drug potency and ability to bind to hydroxyapatite compared to alendronate, its exact interaction with PGE\textsubscript{2} will not be equivalent to alendronate. In the one study which used ALN and PGE\textsubscript{2} combination
therapy, the dosage used was 3mg/kg/day for PGE$_2$ and 0.8 micrograms/kg/day for ALN, which means that the daily dosage for PGE$_2$ was almost 3750 times greater than ALN$^{221}$. Therefore this particular study only shows that at this dosage, ALN does not interfere with the anabolic effects of PGE$_2$ in vivo but does not show how ALN may affect the effects of EP4a when the dosage ratio between the two drugs is lower. Unfortunately there is currently no literature on combination therapies conducted with ALN and EP4a dosed at a lower ratio.

Since the C1 conjugate contains both of these drug components, its pharmacology may not be as straight forward as previously thought. A carefully examination of its conjugate structure and in vivo stability may provide some insight into the pharmacology of the C1 conjugate. In the C1 conjugate, the ALN and EP4a components are covalently linked through a short LK molecule$^{127}$. The EP4a component is linked to the LK through an ester bond, which has been shown to have a release half-life of approximately 5 days$^{127}$. In contrast, the ALN component is jointed to the LK molecule through a stable carbamate bond, which has a release half-life of approximately 22 days (unpublished data). In vivo experiments have shown that this LK linkage to ALN severely interferes with ALN’s anti-resorative activities, and that cleavage of the LK is required for ALN to exert its full effects on bone (unpublished data). However due to the different release half-lives of the carbamate bond vs the ester bond at two ends of the LK, the ALN component becomes freed of the LK much slower than the EP4a component, where the rate of release for the two components is approximately 4 to 1 for EP4a vs. ALN. This means that at any given time, the amount of EP4 released in the bone environment is four times greater than that of ALN being freed from the LK molecule. Based on observations of C1 treated bones in the current study, EP4a’s anabolic effects are most likely not significantly affected by ALN at this dosage ratio. Therefore during this early part of the treatment period, EP4a was able to exert
robust anabolic effects on treated bones, which is translated into increased bone volume in the vertebrae and femurs as observed. However, since pharmacological effect of ALN is determined by its cumulative dose within a treatment period as ALN remains bound to bone for a prolonged period of time, it is possible that the cumulative dose of ALN eventually reaches a sufficient level at later time points of the study to hamper effects of EP4a. This led to the suppression of bone resorption as well as bone formation as observed in histomorphometry analysis. Based on these results, it is possible that if a longer treatment window was used, a greater level of suppression of bone formation would be observed due to greater accumulation of ALN on treated bones.

5.4.2 C2 CONJUGATE

Unlike the C1 conjugate which stimulated bone formation in the rat lumbar vertebrae and femurs, C2 did not show apparent anabolic effects in these skeletal sites. MicroCT analysis and mechanical testing showed that C2 treated vertebrae and femurs had comparable vBMD, bone structural indices and mechanical strength to OVX controls. In histomorphometry analysis, C2 conjugate led to significant suppression of tissue level bone resorption and tissue level bone formation. Taken together, these results suggest that the EP4a component in the C2 conjugate was unable to exert its anabolic effects on bone while the ALN component was successful in suppressing tissue level bone remodelling.

Compared to the conjugate structure of C1, the C2 conjugate lacks the LK molecule, where ALN and EP4a are directly linked to each other through a stable carbamate bond. Therefore in the C2 conjugate, cleavage of the carbamate bond leads to the release of both EP4a and ALN simultaneously in a 1 to 1 ratio. This means that the concentration of newly freed EP4a and ALN are the same at any given time. At this dosage ratio of EP4a vs ALN, results of the
current study suggest that ALN’s inhibitory effects on bone remodelling may suppress EP4a’s stimulatory effects on bone formation, leading to no anabolic effects associated with EP4a as observed in the vertebrae and femurs. Furthermore, it is also interesting to note that since C2 treated vertebrae show compromised microarchitecture and mechanical strength similar to that of OVX controls including decreased trabecular bone volume and load bearing ability versus SH or C2M, this suggests that the ALN component in C2 was not able to successfully prevent OVX-induced bone loss. As mentioned previously, other pre-clinical studies using OVX rats showed that a dose as small as 0.015mg/kg of ALN twice per week by subcutaneous injection or 0.06mg/kg equivalent dose biweekly can significantly increase bone mass and strength in OVX rats\textsuperscript{187-189}. In the current study, the C2H was dosed at 15mg/kg biweekly, which calculates to an administered dose of approximately 0.013 mg/kg of ALN daily or 0.182 mg/kg biweekly based on C2’s \textit{in vivo} uptake rate and cleavage half-life\textsuperscript{127}. These numbers suggest that the dose of ALN used in the C2 conjugate should have been sufficient to prevent OVX-induced bone loss. However since significant cancellous bone loss was observed in C2 treated bones despite a sufficient ALN dose, this suggests that there was a reduction in the inherent ability of ALN to prevent bone resorption. Given that EP4a is the only other drug component present in the C2 conjugate, it is possible that EP4a may be responsible for the reduction in ALN effects. Past studies have shown that although the effects of PGE2 \textit{in vivo} is predominantly anabolic\textsuperscript{98}, PGE\textsubscript{2} and EP4 receptor agonists are also strong stimulators of osteoclastic differentiation and bone resorption \textit{in vitro}\textsuperscript{119,123,222-224}. For example, one study showed that an EP4 receptor agonist stimulated bone resorption in organ culture of rodent calvaria\textsuperscript{225} while another study showed that EP4 antagonist AH23848B inhibited osteoclastogenesis in mouse marrow culture\textsuperscript{226}. Thus in the C2 conjugate, EP4a’s stimulatory effects on osteoclastogenesis and bone resorption may have
decreased ALN’s inhibitory effects on osteoclastic resorption, leading to overall bone loss due to OVX. Together, these results seem to suggest that at a 1 to 1 dosage ratio, ALN and EP4a may have antagonistic effects on their respective antiresorptive and anabolic actions respectively, although future in vitro experiments are needed to explicitly confirm this possibility. Nonetheless, it seems that at later time points of the study, ALN eventually reached a sufficient cumulative dose to overcome effects of EP4a and robustly suppress bone remodelling, which was translated to decreased levels of bone resorption and bone formation as observed in histomorphometry analysis. However at this time point, significant bone loss in the vertebrae and femurs have already occurred.

5.5 CONCLUSION

The current study aimed to investigate the in vivo anabolic effects of the novel ALN-EP4a conjugate drugs, C1 and C2, in a rat model of post-menopausal osteoporosis. The results of the study showed that the C1 conjugate dose-dependently stimulated new bone formation in both cortical and trabecular bone. The lack of apparent anabolic effects seen in the C2M treatment suggest that conjugation between the EP4a and ALN-LK components are crucial to the anabolic efficacy of the drug. Instead of relying on the systemic circulation to distribute EP4a in the body, the conjugate takes advantage of ALN’s bone targeting properties\textsuperscript{37} to deliver EP4a to bone sites. In addition to decreasing systemic side effects associated with EP4a administration, this likely also led to higher concentration of EP4a being released locally in the bone, leading to more robust anabolic effects. Furthermore, since EP4a is eliminated rapidly from the bloodstream with a half-life of 1-2 hours\textsuperscript{127}, the conjugation method allows longer exposure of EP4a to the bone by slow cleavage of the conjugate link and gradual release of EP4a in the local bone environment.
By comparing the *in vivo* efficacy of C1 and C2, results of the current study clearly demonstrate that C1 exerted stronger anabolic effects than C2. This is most likely due to the difference in the structure of the two conjugates. In the C2 conjugate, cleavage of the conjugate bond leads to the release of the ALN and EP4a components simultaneously from the conjugate in a 1:1 ratio. At similar concentrations, our results suggest that ALN may strongly inhibit EP4a mediated effects on bone, resulting in no observable anabolic effects of treated bones *in vivo*. In the C1 conjugate, cleavage of the EP4a-LK ester bond is more than 4 times quicker than cleavage of the ALN-LK bond. This results in a 4:1 release ratio of functional EP4a vs. ALN. As a result, the EP4a is able to overcome the inhibition of ALN during this “window of opportunity” to exert robust anabolic effects on bone before ALN eventually reaches a sufficient cumulative dose to inhibit bone remodelling. Thus even though the two conjugates were dosed to provide an equivalent release rate of EP4a, a much greater anabolic effect was observed for C1 vs. C2. Furthermore, since the initial goal for the synthesis of the two conjugates was to mitigate adverse effects associated with systemic EP4a treatment, the C1 conjugate structure is therefore much more favourable as it minimizes the dose level of EP4a needed to achieve desired anabolic efficacy. Taken together, results of the current study suggests that the C1 conjugate may be a more promising future therapy for reversing bone loss caused by post-menopausal osteoporosis.

### 5.6 FUTURE CONSIDERATIONS

In future investigations and development of the C1 conjugate, a number of issues and questions should be addressed. First, further *in vivo* studies using different concentrations of the C1 conjugate should be conducted to cover a range of doses between or above the doses of C1H and C1L used in the current study. In the current study, although C1 conjugate treatment led to dose-dependent bone formation in the L6 vertebrae and femurs, C1L did not lead to actual
improvement in the mechanical properties of these bones. It is possible in C1L, the rate of sustained release of EP4a was below a threshold needed to overcome ALN’s suppression of tissue-level bone turnover and to exert a robust anabolic effect in bone. Therefore futures studies would be needed to determine an optimal dose of C1 that is able to achieve the desired anabolic effects while limiting associated side effects in vivo. In addition, since C1 treatment led to significant amount of encortical bone formation in the femurs and near occlusion of the marrow space in some high dose treated animals, this may have led to a number of internal side effects in the rats. Unfortunately since this was an unexpected effect associated with C1 treatment, whole blood samples were not taken from the animals prior to sacrifice to rule out possible anemia in the rats. However it should be noted that in the current study, yellowing of femurs in the C1H treated group was observed, which may possibly be due to the endocortical bone formation in these femurs leading to marrow depletion and subsequent reduction in blood cell formation. Nonetheless, further in vivo studies should be conducted to carefully examine potential side effects that may arise due to C1 mediated endocortical bone formation. Finally, since histomorphometry analysis results indicate that C1 treatment significantly suppressed bone resorption and may in long term decrease bone formation, C1’s long term effects on overall bone turnover remains to be investigated. Furthermore, the effects of such depression of bone turnover on the microarchitecture or mechanical properties of the bone is also unknown. Based on a similar study focused on the combined effects of PGE2 and risedronate, such inhibition on bone remodelling may actually be beneficial, as the study showed that risedronate helped to maintain newly added bone in cortical bone site after withdrawal of PGE2 treatment\textsuperscript{221}. Nonetheless, longer term studies in rats are needed to accurately examine these effects.
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