V-ATPase \( a_3-d_2 \) and \( a_3-B_2 \) Subunit Interaction in Osteoclasts are Viable Targets for Anti-Resorptive Therapeutics

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
Graduate Department of Dentistry
University of Toronto

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2012

ABSTRACT

For bone resorption, vacuolar-type H$^+$-ATPases (V-ATPases) on the plasma membranes of osteoclasts acidifies the extracellular milieu adjacent to the bone surface. The V-ATPase $a_3$ and $d_2$ subunits are enriched in osteoclasts. B2 subunit is also expressed on the osteoclast plasma membrane. Disruption of genes encoding subunits $a_3$ and $d_2$ impairs bone resorption. In this study, we have shown an interaction between the $a_3$-$B_2$ and $a_3$-$d_2$ subunits. Luteolin and KM91104 were found to be effective inhibitors of the $a_3$-$d_2$ and $a_3$-$B_2$ interactions respectively. Secondary assays revealed luteolin and KM91104 were not toxic to cells, did not affect osteoclastogenesis yet inhibited bone resorption. Furthermore luteolin did not affect V-ATPase subunit formation or assembly. Inhibitors of osteoclast resorption that do not affect osteoclast viability, preserve osteoclast–osteoblast signalling are desirable than existing anti-resorptives. Therefore, V-ATPase $a_3$–$d_2$ and $a_3$-$B_2$ interactions are viable targets for anti-resorptive therapeutics for osteoporosis.
To Cristian Frusina,

Who offered me unconditional love and support
ACKNOWLEDGEMENTS

I am grateful to Dr. Norbert Kartner without who this study would never be possible. He “showed me the ropes” of research. His guidance and constant encouragement helped me through the toughest time. He has been extremely supportive throughout the study and I forever will be in debt. His sense of humour kept the days going and his love for spicy food helped me discover the best eateries in town.

I would also like to thank my supervisor, Dr. Morris Manolson for cheerfully encouraging me along, his constant support and reminders to maintain a balanced home-school life will always be remembered. I specially want to THANK him for giving me room to grow, presenting me with plenty of opportunities to expand my horizons by participating in conferences, committee’s, panels and teaching me soft skills that I will never forget. However he hasn’t succeeded in convincing me to take up Kayaking… Yet!
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List of Abbreviations

ARO, autosomal–recessive osteopetrosis

BMM, bone marrow mononuclear (cells)

BMD, bone mineral density

CLC-7, chloride channels- 7

CTR, calcitonin receptor

CTSK, cathepsin K

EC₅₀, effective concentration (half-maximal)

ELISA, enzyme-linked immunosorbent assay

GST, glutathione-S-transferase

IC₅₀, inhibitory concentration (half-maximal)

HRP, horseradish peroxidase

IPTG, isopropyl β-D-1-thiogalactopyranoside

KM91104, 3,4-dihydroxy-N’-(2-hydroxy- benzylidene)benzohydrazide

M-CSF, macrophage colony stimulating factor

MMP9, matrix metalloproteinases 9

MTS,3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

OPG, osteoprotegrin

OVX, ovariectomized

PMSF, phenylmethylsulfonylfluoride

PYK2, proline-rich tyrosine kinase 2
RANKL, receptor activator of NF-κB ligand

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

SYK, spleen tyrosine kinase

TMB, 3,3’,5,5’-tetramethybenzidine

TRAP, tartrate-resistant acid phosphatase

TRX, thioredoxin

V-ATPase, vacuolar H^+-ATPase.
List of Thesis Publications


List of Abstracts


2. **Crasto, G.J.**, Kartner N., Li, K., Yao, Y., Manolson, M.F. The benzyhydrazide derivative KM91104 inhibits osteoclast mineral resorption at µM concentrations that do not affect osteoclast differentiation or fusion. Conference ASBMR, 2010 Toronto October 15-19


Invited Public Lecture talks:

1. Canadian Arthritis Network Training Workshop, Gatineau, Quebec. October 28th, 2010

2. Canadian Arthritis Network Training Workshop, Quebec City, Quebec. October 27th, 2011
Unique Contributions to Science

The current accepted model of V-ATPase structure and activity, assumes $a3$ and $d2$ only interact when $V_1$ is dissociation from $V_0$. Our original hypothesis was based on the assumption that inhibitor of $a3-d2$ would inhibit V-ATPase activity by affecting $V_1V_0$ dissociation and reassociation. Our results did not confirm our original hypothesis. Rather our results suggest that $a3$ and $d2$ subunits interact during a proposed “transient dwell time”. Based on current literature and our findings we can speculate that the $a$ and $d$ subunits interact to align all the V-ATPase subunits in a complex in anticipation of the subsequent 120° turn or the interaction acts as a regulatory mechanism during association or disassociation, alternatively the interaction may prevent reverse rotation of the barrel of $c$ subunits during proton transport.

Nevertheless our results indicated that inhibiting specific V-ATPase protein-protein interaction $a3-d2$ did not result in disassociation of $V_1$ and $V_0$ domains rather it inhibited V-ATPase activity, which lead to decrease in overall osteoclast resorptive activity without affecting either osteoclast formation, maturation or activation.

The work presented in this thesis seeks to further clarify the structure of V-ATPase, specifically elucidating the role of protein-protein interaction within the V-ATPases subunits


   Dr. Norbert Kartner and Dr. Morris Manolson provided intellectual guidance, support and subsequent editing of the manuscript. Dr. Yeqi Yao and Dr. Liv Bullock contributed to Fig 1A and Fig 1B, respectively. Dr. Keying Li completed the experimental work in Fig 6A. The screening of chemical compounds was carried out at the Samuel Lunefeld Research Institute- Simple Modular Assay & Robotic Technology (SMART) facility under the direction of Dr. Alessandro Datti. I wrote the first draft of manuscript, completed all the experiments and statistics in the paper unless listed otherwise.


   Dr. Norbert Kartner and Dr. Morris Manolson provided intellectual guidance and editing of the manuscript. I wrote the first draft of the bulletin, completed all the experiments and statistics reported in the technical bulletin.


   As a co-author in this publication. I completed all experiments and statistics required for Figure 6, 7 and 8, which involved secondary screening assays for KM9110
CHAPTER1: INTRODUCTION

1.1 Literature Review

1.1.1 Bone and Bone-Related Diseases

Bone is a dynamic tissue that provides mechanical and structural strength that protects vital organs and maintains mineral homeostasis in the body (1). Chemically bone is made up of both inorganic and organic elements. The organic phase is composed predominantly of type I collagen (approximately 98% by weight) and the inorganic phase is composed of calcium hydroxyapatite. This unique combination provides the structural strength and flexibility required by the body. Bone remodelling serves to remove damaged regions of bone. In adulthood, bone undergoes constant remodelling at approximately 1-2 million sites per adult skeleton to maintain bone structural strength and repair damaged bone (2). In adults, bone remodelling is balanced by maintaining a steady state between bone resorption and formation. At a cellular level, bone remodelling is highly regulated by both local and systemic factors to maintain bone homeostasis. An imbalance in bone remodelling can lead to onset and progression of diseases such as osteoporosis, osteopetrosis, osteopenia and rheumatoid arthritis (3,4). Therefore it is important to understand the underlying molecular mechanisms in bone remodelling for the development of therapeutics.

1.1.1.1 Osteoporosis

Osteoporosis is a skeletal disease that is defined by a decrease in bone mass and deterioration of the micro-architecture of bone tissue that results in a natural increase in bone fragility and susceptibility to bone fractures. For patients suffering from osteoporosis the reported fracture risk is 40% in their lifetime. Although these fractures commonly occur at the spine, hip and or wrist, bone at other locations have also been shown to be affected leading to chronic pain, hospitalization and surgery thereby decreasing the quality of life for patients
suffering from osteoporosis. Worldwide, the number of elderly people is growing yearly with the “baby boomers” pushing towards retirement there is a substantial rise in the number of fractures and osteoporosis cases. Bone loss in osteoporosis takes place either due to estrogen deficiency in postmenopausal women or due to age related conditions (5,6). In 2005, 11.4 million cases of osteoporosis have been reported in North America with an additional 38 million at risk (osteoporosis.ca and nof.org).

Significant side effects and problems reported on efficacy for bisphosphonates and PTH therapy have led to development of new therapies that target specific steps in bone remodeling process (4,7).

1.1.1.2 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an inflammatory disease that manifests itself in synovial joints, leading to degradation of collagen rich structures such as tendons, cartilage and bone erosions (8). Bone loss has been one of the main diagnostic tools for patients with RA (9). The majority of patients suffering with RA, have rapid bone and cartilage damage within the first year of the onset of disease (10), which lead to limited mobility and physical pain (11). TNF blockers have been used as a therapeutic to treat RA, which stops further structural damage. However, the use of TNF blockers does not address several aspects such as joint repair or bone erosion healing in these patients (9).

There is strong evidence implicating osteoclasts in the development and progression of inflammatory and degenerative bone and joint disease in RA and osteoarthritis. Patients with RA form a higher number of osteoclasts in vitro from peripheral blood mononuclear cells (12). This suggests that disturbances in bone remodeling, specifically in osteoclasts can lead to diseases which necessitates better understanding of osteoclast biology to develop improved therapeutics (13).
1.1.2 Current therapeutics and effects of Bisphosphonates

Several therapeutics are administered as treatment against bone loss diseases such as the use of bisphosphonates, estrogen replacement therapy, denosumab, teriparatide.

Bisphosphonates, the current gold standard therapy for bone loss treatments are analogues of pyrophosphates P-C-P where the central carbon atom is replaced with oxygen and forms a P-O-P structure. Bisphosphonates bind to hydroxyapatite bone mineral surface that is subsequently internalized selectively by resorbing osteoclasts. Nitrogen-bisphosphonates such as zelandronate target the mevalonate pathway leading to the loss of prenylated proteins and defective intracellular vesicle transport. This process causes apoptosis of osteoclasts leading to a significant decrease in bone resorption (14). However, it is now recognized that osteoclasts and osteoblasts are interdependent and apoptosis of osteoclasts uncouples the bone remodelling cycle leading to decreased bone formation. Cases of bisphosphonates-induced osteonecrosis of the jaw have been reported (15-17) and subtrochanteric fractures have also been observed (18).

Estrogen replacement therapy has been utilized against preventing osteoporosis in women. Several estrogens are orally administered to women in combination with progestin to prevent uterine cancer in women. Treatment with estrogen can increases the bone mineral density, while in early postmenopausal women estrogen has been shown to increase spine and hip BMD by approximately 4% (19). However, estrogen treatment has been associated with an established risk of breast tumours, uterine cancer and also rare side effects such as thromboembolism.

Denosumab, monoclonal antibody, which is a recently approved FDA treatment against osteoporosis binds to RANKL and prevents it from activating the RANK receptor on osteoclasts precursor cells leading to a decrease in osteoclast formation.

Anabolic drugs have also been shown to increase bone mass. For example, Teriparatide (synthetic/recombinant parathyroid hormone) is FDA approved for treatment against osteoporosis where it increases bone mass. Side effects have been reported with the use of Teriparatide. 45% rats treated with high doses of teriparatide developed aggressive form of bone cancer. Human patients have also been reported to develop osteosarcoma (20).
1.1.3 Bone remodelling

Bone is composed of an inorganic phase comprising of hydroxyapatite (Ca$_{10}$[PO$_4$]$_6$[OH]$_2$) and an organic phase, primarily of type I collagen, as well as other proteins and cells. Bone includes highly porous trabecular bone and cortical bone also known as compact bone. Bone homeostasis is maintained by mesenchymal derived cells: osteoblasts, osteocytes, and hematopoietic derived cells: osteoclasts. Bone remodelling is coordinated, where osteoclasts resorb old bone and osteoblasts deposit new bone in a process known as coupling (21,22). Osteocytes are present in numerous locations in the bone, approximately 10,000 cells per cubic millimeter (23). Micro-cracks in the bone severe osteocyte processes resulting in osteocyte apoptosis (24), which has been shown to precede osteoclastogenesis where they signal the location of damage (25). RANKL triggers M-CSF committed proliferative osteoclast precursor cells to differentiate to osteoclasts (26).

The differentiated osteoclasts resorb damaged bone. Products secreted from osteoclast resorption and independent products regulate osteoblastogenesis and bone formation (27-29). Consequently following resorption, osteoblasts deposit osteoid followed by mineralization. Osteoclasts undergo apoptosis after resorption. Some osteoblasts undergo apoptosis, while others become bone lining cells and few others become trapped within the bone matrix and form osteocytes (30). The remodeling process is complex and intricate and not completely defined. Generally, bone resorption facilitates the removal of damaged bone and is coupled with an equal amount of bone formation and therefore is not harmful, unless it becomes unbalanced.
1.1.4 Osteoclasts and osteoblasts cross talk

The current working hypothesis suggests that osteoclast and osteoblasts are interdependent for normal bone remodelling. The signalling pathways from osteoblasts to osteoclasts is well defined and involves OPG and RANKL (see section 1.1.5). However the reverse signalling, osteoclast to osteoblasts is not clear and is currently under investigation.

Osteoclast to osteoblast signalling

Recently, ephrin ligands and Eph receptors have been identified as mediators between osteoclasts and osteoblasts (31,32). Eph receptors are tyrosine kinase receptors that are divided into class A and B. The EphB4 receptor prefers the ligand ephrinB2. Differentiating osteoclasts express ephrinB2 and osteoblasts express EphB4. EphrinB2 in a negative feedback loop suppresses osteoclast differentiation and in osteoblasts, binding of ephrinB2 to EphB4 triggers osteoblast differentiation (32). In vivo, experiments conducted using transgenic mice expressing EphB4 showed increased bone formation and decreased bone resorption relative to the wild type mice (32). The ephrinB2-EphB4 interaction facilitates the bone remodelling cycle.

Effect of bisphosphonates and PTH on cross talk

Bisphosphonates have been shown to reduce bone turnover and increase overall mineralization by suppressing excessive bone remodelling. However decrease in remodelling results in an increase in accumulation of microcracks or microdamage. Bisphosphonates accentuate the damage because they impair normal bone remodelling as compared to reduction in stochastic remodelling. A task force report published by The American Society for Bone and Mineral Research (ASBMR) on the association of atypical subtrochanteric femoral fractures and bisphosphonates stated that, “preliminary estimates of atypical femoral fracture incidence increased progressively from 2 per 100,000 cases per year for 2 years of BP use to 78 per 100,000 cases per year for 8 years of BP use. These data suggest that atypical femoral fractures are rare in both the general population and BP-treated patients, but their incidence may increase
with increasing duration of BP exposure” (33). In addition, short-term clinical trials show that bisphosphonates reduce the efficacy of teriparatide (34,35).

Parathyroid hormone is marketed as “Forteo” and has the U.S. Food and Drug Administration (FDA) approval for prescription. However the FDA warns patients that the use of 750 µg/3ml injection could cause an increase in incidence of osteosarcoma. The FDA reports, that patients be prescribed teriparatide only “for whom the potential benefits are considered to outweigh the potential risk”. Teriparatide is also not recommended for patients with Paget’s disease, unexplained elevations of alkaline phosphatase, prior implant radiation therapy involving the skeleton or who are at increased baseline risk for osteosarcoma (www.fda.gov).

Micro-crack accumulation, atypical subtrochanteric femoral fractures and reduced efficacy in combination therapy are few concerns that have risen with the long-term use of bisphosphonates. PTH therapy has several limitations as well and the FDA does not recommend the use of teriparatide beyond two years, since there is lack in information regarding Forteo benefits and safety for long-term use.

Therefore there is dire need to investigate bone biology further to develop new therapies that target specific steps in the remodelling cycle.
1.1.5 Osteoclast

Osteoclast differentiation

Osteoclasts are multinucleated bone resorbing cells. Increased osteoclast activity causes decreased bone mass leading to diseases such as osteoporosis. Decreased osteoclast activity causes increased bone mass seen in patients diagnosed with osteopetrosis (36). Macrophage colony stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B ligand (RANKL) expressed on osteoblasts and stromal cells are required for proliferation and differentiation of pre-osteoclasts, whereas, RANKL is critical for osteoclastogenesis (Fig. 1). Osteoprotegerin (OPG) acts as a decoy receptor that competitively binds to RANK. Mutations in genes encoding RANKL and RANK genes result in diseases such as autosomal–recessive osteopetrosis (ARO) (37,38).

Osteoclast activation

Osteoclasts polarize on the bone, where the plasma membrane forms three distinct areas: basolateral membrane, the sealing zone which is attached to the bone, and a ruffled border that is adjacent to the bone matrix. Polarization of osteoclasts involves the formation of an F-actin ring, which upon completion results in the formation of ruffled borders only on mineralized matrix and not on plastic or glass. Active osteoclasts attach to bone and form a ruffled border that is a highly convoluted membrane, where hydroxyapatite is dissolved by the translocation of protons (Fig. 2).

Differentiated osteoclasts use $\alpha_v\beta_3$ integrins to attach to extra cellular matrix (ECM) proteins such as osteopontin, bone sialoprotein and vitronectin via formation of a signalling complex composed of c-Src, tyrosine kinases, proline-rich tyrosine kinases 2 (PYK2), spleen tyrosine kinase (Syk) and scaffold proteins (39). Osteoclasts from $\beta_3^{-/-}$ mice do not form actin rings, have abnormal ruffled membranes, and fail to resorb bone in vivo. Further these mice have low levels of calcium and increased bone mass, thereby confirming the role of $\alpha_v\beta_3$ integrin’s in osteoclast activity (40).
Osteoclasts are multinucleated cells that are formed by differentiation of haematopoietic precursor cells. Maturation of osteoclasts occurs on the bone surface from peripheral blood mononuclear cells in presence of M-CSF and RANKL. OPG acts as a decoy receptor that binds to RANKL neutralizing it, thus acting as a negative regulator of osteoclastogenesis and maturation. Tartrate- resistant acid phosphatase (TRAP) is a highly expressed in osteoclasts and is recognized as a marker for preosteoclasts and osteoclasts. Calcitonin receptor (CTR) is also a marker that distinguishes osteoclasts from macrophages. Figure adapted from Boyle et al (41).
Osteoclast activity

Vacuolar H\(^+\)-ATPases (V-ATPase) present on the ruffled border acidify the resorption lacunae to a pH of approximately 4.5 (42). Mutations in the \(a3\) subunit of the multimeric V-ATPase cause autosomal recessive osteopetroses (43-45). Carbonic anhydrase II supplies protons and electroneutrality is maintained by chloride channel -7 (CLC-7) and Cl/\(H^+\) antiporters (46) (Figure 2). Defects in CLC-7 gene has been shown to block proton secretion, wherein osteoclasts are formed but are unable to resorb leading to osteopetrosis (47-49). The exposed organic phase of demineralized bone matrix is further degraded by cathepsin K and matrix metalloproteinases 9 (MMP) (50,51). Rise of calcium concentration (40 mM) leads to inhibition in osteoclast activity and subsequent podosomal disassembly and actin reorganization, resulting in osteoclast apoptosis (52). Factors that control osteoclast activity are potential targets for the development of anti-resorptive therapeutics.
Osteoclasts are multinucleated cells that polarize when attached to bone and form a highly convoluted ruffled membrane that is in contact with the bone. Degradation of bone matrix is facilitated by matrix degrading proteinases (MMP 9 and Cathepsin K) and protons pumped by V-ATPases localized on the ruffled border membrane. Carbonic anhydrase II (CAII) supplies protons and electroneutrality is maintained by chloride channels (CLC-7) and Cl⁻/H⁺ antiporters. Osteoclasts create proton impermeable resorption lacunae by forming sealing zones with the bone matrix (53-55)
1.1.6 V-ATPases

V-ATPases are ATP-dependent, multi-subunit proton pumps that are ubiquitous and essential for the function of cells (56-61). These proteins are localized on a variety of eukaryotic membranes and organelles such as endosomes, lysosomes, golgi membranes, clarithin coated vesicles, secretory granules and vacuoles of plants and yeast. In each case, an organelle requires a specific internal pH which is regulated by V-ATPase activity (59,62). V-ATPases are essential for several cellular functions such as post-translational modifications, protein degradation, receptor mediated endocytosis and secondary transport (63,64). In specialized cells, they are targeted to the plasma membrane, where they translocate protons into the extracellular space. This translocation of protons can have a wide variety of functions including demineralization of bone by osteoclasts as part of resorption (65) or to maintain sperm in a quiescent state during maturation in the epididymis (66). V-ATPases are structurally related to F-ATPases with common evolutionary ancestry (67-69). F-ATPases are found on the inner membrane of mitochondria and predominantly operate as ATP-synthases, while V-ATPases pump protons across membranes (58). V-ATPase activity is regulated, in part, by the dissociation/reassociation of its V₁ and V₀ domains (70-73) (see section 1.1.7).

Mammalian V-ATPases are comprised of 14 subunits with multiple isoforms that are organized within two functional domains: an integral membrane spanning domain V₀ and a peripheral bound cytoplasmic domain V₁. Considering multiple subunit isoforms, specialized V-ATPases isocomplexes assembled can be tissue specific or cell specific. Fig. 3 shows the current model of V-ATPase based on studies conducted using single particle electron microscope EM, protein cross-linking studies and studies carried out by other members in the Manolson lab (70,74-78).

Based on the F-ATPases, the V-ATPases structure is divided into counter-rotating stator and rotor (79,80) (The rotor and stator subunits are listed in the table 1). ATP hydrolysis in the V₁ domain provides the torque that results in the rotation of the V₀ domain. The V-ATPase has three ATP hydrolyzing sites in the V₁ domain and six proton binding sites in the V₀ domain. Under normal conditions, every 360° rotation results in the hydrolysis of three ATP’s and the transport of six protons across the membrane. The rotation of the “rotor” is relative to the “stator”. Thus, V-ATPases can be perceived as “nano-machines”, where ATP hydrolysis and proton pumping are coupled together in a rotary mechanism.
The V-ATPase structure is a summary of studies carried by single particle EM, protein crosslinking studies, physical description of subunits, correlation with F-ATPase data and work conducted in our own lab. V-ATPases are composed of two domains: catalytic $V_1$ domain that has the empirical subunit composition $A_3B_3CDE_3FG_3H$ and integral membrane spanning domain $V_0$ that has the composition $ac_5c'c''de(Ac45)$. The cytoplasmic $V_1$ domain is composed of the catalytic head group comprising of three alternating A and B subunits (blue and orange, respectively), the central rotor shaft comprised of subunits D and F (purple and brown, respectively) and components of the stator: three peripheral stalks consisting of EG heterodimers (dark green and dark blue, respectively), the H subunit (transparent purple) and the C subunit (yellow). The bifurcated NTa / CTa domain of the a subunit (transparent green) is part of the $V_0$ domain. The mammalian hexameric $c_5c''$ proteolipid barrel (yellow), and subunit d (pink). Accessory proteins, e (orange) and Ac45 (red) are also shown. Image produced by Dr. Norbert Kartner.
Table 1. V-ATPase subunits and domain organization:
(Osteoclast enriched V-ATPase subunits are coded in red)

<table>
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<th>Subunit Isoforms</th>
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</tr>
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</tr>
<tr>
<td>B₁, B₂</td>
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<td>approximately 100</td>
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</tr>
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<td></td>
</tr>
<tr>
<td>d₁, d₂</td>
<td>1</td>
<td>38-42</td>
<td></td>
</tr>
<tr>
<td>Accessory</td>
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</tr>
<tr>
<td>e₁, e₂</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Ac₄₅</td>
<td>1</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>
1.1.6.1 General Structure of $V_1$ domain

The peripheral $V_1$ domain of the V-ATPase is composed of eight different subunits and has the empirical subunit composition $A_3B_3CDE_3FG_3H$ (70). Both subunits $A$ and $B$ are involved in ATP hydrolysis, where subunit $A$ has the catalytic function and $B$, the regulatory function (81-83). ATP hydrolysis in the catalytic headpiece drives the central stalk connected to the $V_0$ domain barrel of $c$ subunits for proton translocation. Functionally, the hexamer $A_3B_3$ is connected to the $V_0$ domain via a central stalk formed by subunits D and F. The E and G subunits form the peripheral stalks and attached to H and C together with $A_3B_3$ form the “stator”(58).

1.1.6.2 General Structure of $V_0$ domain

The integral membrane $V_0$ domain of the V-ATPase consists of $a$, $c$, $c''$, $d$, $e$ and $Ac45$ subunits and is involved in proton translocation (70). The $a$ subunit is the largest (100 kDa), with a hydrophobic C-terminal domain (CT$a$; 50 kDa) within the $V_0$ sector and a hydrophilic N-terminal domain (NT$a$; 50 kDa) that interacts with the $V_1$ sector (78,84-87). The central stalk from the stator is attached to the hydrophobic barrel of $c$ subunits and forms the rotor.

1.1.7 Regulation of V-ATPases: dissociation and reassociation

V-ATPases regulate the pH of both intracellular compartments and extracellular compartments for specialized cells. The proton flux activity is regulated by reversible dissociation of the complex, where the $V_1$ domain detaches of the $V_0$ domain rendering the complex inactive. The process of dissociation and reassociation is well defined in yeast and occurs in mammalian cells as well, however its not clearly defined in mammalian cells (61,88,89).

In yeast cells, V-ATPases dissociate in response to glucose depletion from the media (90). The mechanism of reversible dissociation is regulated by the $a$ isoform and is dependent on the availability of glucose in the cellular environment and regulated by luminal pH (91). In yeast, V-ATPase subunits are all encoded by single genes except the $a$ subunit, is encoded by
two genes VPH1 and STVI. Vph1p is targeted to the vacuoles and Stv1p is targeted to the yeast golgi (86,92). Dissociation occurs rapidly and involves cytosolic microtubular network (93) and reassembly/reassociation requires a novel protein cytosolic protein complex – RAVE (Rav1p, Rav2p, and Skp1). RAVE complex stabilizes the dissociated V₁ domain. In yeast the V-ATPases that contain Vph1p disassociate in response to glucose depletion but V-ATPases that contain Stv1p (ortholog of a subunit) do not dissociate. When Stv1p V-ATPase are re-directed to the vacuole, dissociation is observed (86).

Reversible dissociation of V-ATPases has been shown in renal cells and dendritic cells (94,95). In mammalian renal cells, phosphatidylinositol 3- kinase (PI3K)- dependent signalling has been shown to mediate glucose-dependent assembly (96).
Figure 4. Predicted mechanism of dissociation and reassociation in V-ATPases

V-ATPases are proton pumping “nano-motors”. ATP hydrolysis drives clockwise rotation of the central stalk, the rotation further pushes protons from the cytosol to the lumen. In response to depletion in glucose, ATP hydrolysis ceases and the $V_1$ domain dissociates from the $V_0$ domain. The $a$ subunit collapses on to the $V_0$ domain preventing further proton leak. Image produced by Dr. Norbert Kartner.
1.1.8 Function of intracellular membrane V-ATPases

V-ATPases play an important role in several intracellular functions. For instance, they provide acidic endosomal pH in cells for receptor mediated endocytosis (61), they facilitate the formation of endosomal carrier vesicles (97) and are also involved in membrane trafficking (98). In lysosomes and other digestive organelles, V-ATPases acidify the environment for the degradation of proteins and other molecules. Similar to lysosomes in secretory vesicles, V-ATPases can activate acidic proteases that process hormones to their mature forms. For example in pancreatic islet cells, V-ATPase activity is necessary for the processing of pro-insulin to insulin (91).

V-ATPases play a critical role in viral and bacterial infections by acidifying endosomal compartments. In certain viral infections such as that of the influenza virus, the virus is internalized via clatherin-coated vesicles after it binds to cell surface receptors. These vesicles are delivered to acidic early endosomes, where hemagglutinin (viral coat protein) undergoes a conformational change and exposes a hydrophobic domain, which is used to insert itself into an endosomal membrane. The fusion of the viral and endosomal membrane triggers the release of viral RNA into the cellular cytoplasm of the cell causing a viral infection. Similarly, infection caused by diphtheria is initiated with the help of V-ATPases. This bacterium has two protein chains A and B, where the B chain forms a pore in the endosomal membrane for the A chain to enter and inhibit protein synthesis. The formation of B chain is triggered by V-ATPase activity, where the B chain undergoes a conformational change upon exposure to low pH (99).

1.1.9 Functions of plasma membrane V-ATPases

V-ATPases are present on the plasma membranes of several different cell types such as the intercalated cells of the distal renal tubule and collecting ducts of the kidney, osteoclasts, the vas deferens and epididymis. In the kidney, alpha-intercalated cells contain V-ATPases at the apical membrane and Cl/HCO₃⁻ at the basolateral membrane, where V-ATPases secrete protons from the cytoplasm into the lumen of the tubules whereas Cl/HCO₃⁻ release HCO₃⁻ across the basolatreal membrane (Figure 2). V-ATPases maintain low pH in vas deferens and epididymis for sperm maturations and maintenance of sperm in a quiescent state. As noted previously, V-
ATPases localize on the plasma membrane of active osteoclasts and pump protons across the ruffled membrane onto to the bone in order to facilitate bone resorption.

Mutations in plasma membrane V-ATPases cause several diseases. Distal renal tubule acidosis is caused by a mutation in renal specific V-ATPase V₁ domain B1 subunit. The mutation in the B1 subunit impedes V-ATPase function in the intercalated cells of the distal renal tubule, thereby preventing acid secretion that is required to maintain normal plasma pH (100). Mutations in V₀ domain subunit a4 has also been implicated in renal tubule acidosis (101), mutations in the a2 gene results in cutis laxa (102) and mutations in osteoclasts specific V-ATPase a3 isoforms result in osteopetrosis.
1.1.10  Structure and Function of V-ATPases in Osteoclasts

V-ATPases targeted to the ruffled border of osteoclasts have a unique combination of subunit isoforms specific to osteoclasts. Subunits $a3$, $d2$ and $B2$ are highly enriched in V-ATPases at the ruffled border of osteoclasts (65,103,104). Specific targeting of these subunits can be an effective approach against bone loss diseases without serious side effects (See Table 1 for list of all V-ATPase subunits and isoforms).

1.1.10.1  V-ATPase $V_0$ domain $a3$ subunit

Mammalian V-ATPases have four isoforms for the 100 kDa $V_0$ domain $a$ subunit: $a1$, $a2$, $a3$ and $a4$. Mutations in $a3$ and $a4$ lead to human diseases such as osteopetrosis and renal tubule acidosis (105,106). In breast cancer MB231 cells, treatment of cells with siRNA to both $a3$ and $a4$ subunits significantly inhibited invasion (107,108). In osteoclast precursor cells, $a3$ is localized in the lysosomes, which upon differentiation to mature osteoclasts, $a3$ was shown to move to the plasma membrane (109). The expression of $a3$ in osteoclasts is 100-fold greater than that seen in other tissues such as liver, kidney, brain, lung, spleen and muscle (65,104,110). Considering the impact of subunit $a$ and its specificity on tissue or cell types, the $a$ subunit has gained attention as a molecular target for treatment of pathological bone loss and cancer (59,111). However, in order to design compounds that specifically target the V-ATPase $a$ subunit, the structure and mechanism of action of the $a$ subunit is critical. Currently very little is known about the structure and the protein-protein interactions. The work described in this thesis is focused on elucidating the protein-protein interaction of $a3$ in V-ATPases and applying this knowledge to discover novel targets to inhibit V-ATPase osteoclast activity specifically.
Figure 5. Predicted structure of V-ATPase a3 subunit

V-ATPases are composed of two domains: catalytic $V_1$ domain that has the empirical subunit composition $A_3B_3CDE_3FG_3H$ and integral membrane spanning domain $V_0$ that has the composition $ac_5c^{\prime}de(Ac45)$. The bifurcated NTa / CTa domain of the $a$ subunit (transparent green) is part of the $V_0$ domain and was shown to interact with the B subunit of the $V_0$ domain. Image obtained from Dr. Norbert Kartner.
1.1.10.2 V-ATPase $V_1$ domain $B2$ subunit

The $V_1$ cytosolic domain constitutes the catalytic domain where three copies of each $A$ subunit and $B$ subunits function in ATP hydrolysis. The 56 kDa $B$ subunit has two homologous isoforms encoded by two different genes ATP6V1B1 (B1 subunit) and ATP6V1B2 (B2 subunit). The $B1$ subunit is expressed at high levels in intercalated cells of the kidney (112,113), the ear (100) and epididymis (66). Although the $B2$ isoform is ubiquitously expressed (114), it is expressed at higher levels in osteoclasts relative to $B1$. Lee et al showed that an osteoclast-rich mouse bone marrow culture model expressed the V-ATPase $B2$ subunit only. Human marrow cultures and monocyte-derived macrophages used as models for osteoclasts were also shown to express only the $B2$ subunit supporting the view that the V-ATPase containing $B2$ subunit mediates osteoclast V-ATPase activity (115). Holliday et al showed that the N-terminal domain of the $B2$ subunit interacts with F-actin with Kd value of 190 nM (116). We have recently shown that the C-terminal domain of the $B2$ subunit interacts with the $a3$ subunit of $V_0$ domain (78). Therefore, given the interaction between $a3$ and $B2$ subunits and relatively higher expression of both subunits in osteoclasts, inhibition of this interaction is an attractive target to reduce V-ATPase activity targeting osteoclast resorption.
1.1.10.3 V-ATPase $V_0$ domain $d2$ subunit

In V-ATPase, the $V_0$ domain $d$ subunit has two known isoforms $d1$ and $d2$. The $d2$ gene is predominantly expressed in the kidney and osteoclasts as opposed to the $d1$ gene that is ubiquitously expressed (117,118). Previously, Bauerle et al suggested in their study that the $d2$ protein is associated with the $V_0$ domain by protein-protein interaction and is not directly anchored to the membrane domain. The $d2$ and $a3$ subunits have been shown to be co-localized in osteoclasts (118). Wu et al used lentivirus-mediated RNA interference knockdown of $d2$ expression in vitro at different stages of osteoclast differentiation. This study showed that $d2$ in osteoclasts has dual functions that include differentiation of osteoclasts from osteoclast precursors and in extracellular acidification in mature osteoclasts. Depletion of $d2$ in osteoclast precursors impaired fusion while depletion of $d2$ expression at a later stage of differentiation did not affect osteoclast maturation but inhibited bone resorption by inhibiting V-ATPase specific extracellular acidification (119). Kim et al further confirmed that ATP6v0d2 is limited to the control of bone homeostasis. These studies further confirmed that $d2$ can be a potential target against excessive bone loss (120).
Figure 6. Predicted structure of V-ATPase a3 and d2 subunits

V-ATPases are composed of two domains: catalytic $V_1$ domain that has the empirical subunit composition $A_3B_3CDE_3FG_3H$ and integral membrane spanning domain $V_0$ that has the composition $acsc''de(Ac45)$. The $d$ (pink) subunit is part of the $V_0$ domain. Image obtained from Dr. Norbert Kartner.
1.1.11 V-ATPases as Novel Targets

V-ATPases are necessary for osteoclast bone resorption; targeting activity of specific osteoclast V-ATPases may be a viable approach against excessive bone loss diseases. V-ATPase inhibitors such as bafilomycin A1, SB242784 and FR167356 have been previously shown to decrease osteoclast resorption. SB242784, a derivative of bafilomycin A1 has been shown to prevent bone loss in ovariectomized (OVX) rats (121). Similarly, FR167356 has been shown to inhibit bone resorption in vitro and to prevent bone loss in OVX rats (122). Diphyllin was also identified as an inhibitor of V-ATPase activity, where diphyllin inhibited V-ATPase activity with an IC$_{50}$ value of 17 nm compared with bafilomycin at 4 nm. Diphyllin was shown to inhibit bone resorption by human osteoclast (generated from CD14+ monocytes) in vitro (123). Enoxacin, a fluoroquinolone antibiotic inhibited binding of B2 -actin and was also shown to inhibit osteoclast formation (124). Studies show that enoxacin treated MC3T3-E1 osteoblastic cells did not have problems mineralizing at concentrations where osteoclast activity was inhibited (125). These studies further confirm the potential of V-ATPase as therapeutic targets and encourage discovery of new inhibitors that specifically target osteoclast V-ATPase activity.
CHAPTER 2: STATEMENT OF PROBLEM, HYPOTHESIS AND OBJECTIVES

2.1 Statement of Problem

Maintaining cross-talk between osteoclasts and osteoblasts is crucial to maintain normal bone remodelling that is essential for skeletal integrity and calcium homeostasis. Bisphosphonates are clinically the gold standard for treatment against bone loss diseases such as osteoporosis. They target molecular pathways in osteoclasts leading to osteoclast apoptosis. This prevents further signalling of the osteoclasts to the osteoblasts to deposit bone, hence breaking the bone remodeling cycle. Bisphosphonates have been proven to be ineffective in combinational therapies where they are administered with Teriparatide (parathyroid hormone) demonstrating the importance of osteoclast-osteoblast cross talk. It is therefore essential to investigate alternative anti-resorptive therapies that not only prevent excessive bone loss caused by osteoclasts, but also maintain osteoclast integrity, required to encourage osteoblast activity.
2.2 General Hypothesis

Inhibition of \(a_3-d_2\) and \(a_3-B_2\) protein-protein interactions in osteoclast specific V-ATPase subunits leads to a decrease in bone resorption without affecting osteoclastogenesis.

The current accepted model of V-ATPase structure and activity suggests that the V-ATPase a3 and d2 subunits only interact when the \(V_1\) domain is dissociated from \(V_0\) domain. Therefore we hypothesize that an inhibitor of a3-d2 interaction would target V-ATPase activity by affecting dissociation/reassociation of \(V_1\) from \(V_0\)

2.3 Objectives

1. To elucidate the V-ATPase subunit \(a_3-d_2\) interaction and investigate the role of V-ATPase \(a_3-d_2\) inhibitor luteolin as a potential anti-resorptive therapeutic

2. To examine the effect of \(a_3-B_2\) V-ATPase inhibitor KM91104 on cell viability and growth, osteoclast formation, maturation and resorptive activity
2.4 Significance of Research

Reducing bone resorption, while increasing bone formation is an attractive strategy to not only prevent pathological bone loss, but also, at the same time, restore bone that has been lost. Bisphosphonate therapy, the gold standard for bone loss disease suppresses the efficacy of parathyroid hormone-related protein (teriparatide) therapy for increasing bone formation (126-129). Bisphosphonates induce apoptosis in osteoclasts eliminating osteoclast-osteoblast signalling (130) that is essential for bone formation and continued bone remodelling. A therapeutic that inhibits osteoclast bone resorption without affecting osteoclastogenesis would be advantageous over the current therapies available. Studies conducted in our lab and by others suggest that V-ATPases are viable targets against excessive bone loss diseases. Luteolin and KM91104 are model compounds that inhibit bone resorption without affecting osteoclastogenesis. Although these compounds may not show sufficient specificity, they serve as proof of principle.

The work published in this thesis provides significant insight into the structure and function of the V-ATPase subunit isoforms and has the potential to impact the quality of life for patients suffering from bone loss diseases.
CHAPTER 3: MATERIALS AND METHODS

3.1 Materials and reagents

Table 2: Materials and reagents used in research

<table>
<thead>
<tr>
<th>Materials</th>
<th>Catalogue Number</th>
</tr>
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<tr>
<td>RAW 264.7 murine macrophage cells</td>
<td>ATCC accession no. TIB-71</td>
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<td>DMEM with high D-glucose (4.5 g/l), L-glutamine and sodium pyruvate (110 mg/L)</td>
<td>Invitrogen, cat. no. 11995</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
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<td>α-MEM</td>
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<td>Penicillin and Streptomycin</td>
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<td>Macrophage colony stimulating factor (M-CSF)</td>
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<td>Triton X-100</td>
<td>Sigma-Aldrich, cat. no. T8787</td>
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<td>Gelatin for blocking in pulldown assays and ELISA was EIA grade</td>
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<td>Ni-NTA agarose</td>
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<td>CellTiter 96 Aqueous One Solution Cell</td>
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<td><strong>proliferation assay</strong></td>
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<tr>
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<td>Formalin</td>
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<tr>
<td>Fast Red Violet LB salt</td>
<td>Sigma-Aldrich, cat. no. F-3381</td>
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<td>Cell dissociation buffer</td>
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<td>Sigma-Aldrich, cat. no. 43665</td>
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<td>Entellan new rapid mounting medium</td>
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<tr>
<td>Western Lightning ECL detection solution</td>
<td>PerkinElmer, cat. no. NEL102001</td>
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**Table 3: Antibodies used in research**

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<th><strong>Antibodies used in research</strong></th>
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<tr>
<td>Anti-GST antibody was rabbit polyclonal GST(Z-5)</td>
<td>Santa Cruz Biotechnology, cat. no. sc-459</td>
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<tr>
<td>Anti-His-tag antibody was His-Probe (H-3)</td>
<td>Santa Cruz Biotechnology, cat. no. sc-8036</td>
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<tr>
<td>Horse radish peroxidase (HRP)-conjugated anti-rabbit second antibody (HRP-GAR) was polyclonal goat anti-rabbit IgG</td>
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<tr>
<td>HRP conjugated anti-mouse second antibody (HRP-GAM) was polyclonal goat anti-mouse</td>
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<tr>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>anti-a3 and anti-E antibodies</strong></td>
<td></td>
</tr>
<tr>
<td><strong>anti-d2 antibody</strong></td>
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<tr>
<td><strong>Luteolin: dissolved in water and 1N NaOH.</strong></td>
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</table>

<table>
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<tr>
<th>Donation from Dr. Beth Lee</th>
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</thead>
<tbody>
<tr>
<td>Donation from Alethia Biotherapeutics.</td>
</tr>
<tr>
<td>Sigma-Aldrich, cat. no. L9283</td>
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</table>
3.2 Cell culture

RAW 264.7 cells were cultured in DMEM with high D-glucose (4.5 g/l), L-glutamine and sodium pyruvate (110 mg/L), supplemented with 10% (v/v) FBS. RAW 264.7 cells were differentiated into osteoclasts in α-MEM supplemented with 10% (v/v) FBS and 100 ng/ml soluble recombinant RANKL for 5 days with change of media on day 3.

Bone marrow mononuclear (BMM) cells were isolated from tibias and femurs of six-week-old CD-1 mice. The cells were seeded in 96 well microtiter plates at $1 \times 10^5$ cells/well in α-MEM supplemented with 15% (v/v) FBS, 100 u/ml penicillin, 100 µg/ml streptomycin and 50 ng/ml M-CSF and grown for two days. Cells were differentiated into osteoclasts in the presence of 100 ng/ml soluble recombinant RANKL and 50 ng/ml M-CSF for an additional 5 days. Cell culture was at 37 °C in a humidified 5% CO₂ incubator.

3.3 Constructs

(Constructs were prepared by Dr. Norbert Kartner, Dr. Keying Li and Dr. Yeqi Yao )

The construct expressing the N-terminal thioredoxin (with His-tag) fusion of the N-terminal domain of the mouse V-ATPase a3 subunit, TRX-NTa3, was constructed as described in Kartner et al (78). Constructs expressing N-terminal glutathione-S-transferase (GST) fusions with full-length mouse V-ATPase d1 and d2 subunits were prepared using RT-PCR strategies described previously (78), from mouse brain and mouse kidney mRNA with the oligonucleotide primers listed in Table 4.
Table 4: Primers, plasmid constructs and protein expression products

<table>
<thead>
<tr>
<th>Subunit (domain)(^a)</th>
<th>Expression plasmid(^b)</th>
<th>Product (a.a.)(^c)</th>
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</thead>
<tbody>
<tr>
<td>(a3) (NT(a3) domain)</td>
<td>pET32a-NT(a3)</td>
<td>TRX(H(_6))-G(_2)-NT(a3)(^{(1–393)})</td>
</tr>
<tr>
<td>[ pcDNA 3.1-a3, ] &lt;br&gt;5(^\prime)-gaattcggtggtatgggctctatgttccggagtgaagag-3(^\prime) &lt;br&gt;5(^\prime)-gtgcacattaggtagggagcagggttaacttccc-3(^\prime) ]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(d1) (full-length)</td>
<td>pGEX-4T1-(d1)</td>
<td>GST-G(_2)-d(_1)(^{(1–351)})</td>
</tr>
<tr>
<td>[ mouse brain cDNA, ] &lt;br&gt;5(^\prime)-gaattcggaggtatgtcgttctt-3(^\prime) &lt;br&gt;5(^\prime)-gtcgactaaaagatgggatgtgtagttgctc-3(^\prime) ]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(d2) (full-length)</td>
<td>pGEX-4T1-(d2)</td>
<td>GST-G(_2)-d(_2)(^{(1–350)})</td>
</tr>
<tr>
<td>[ mouse kidney cDNA, ] &lt;br&gt;5(^\prime)-gaattcggagttagagctgagcaagctgtacttcaatgtgg-3(^\prime) &lt;br&gt;5(^\prime)-gtgcactattataaaatggagaatgtagttgatgttcgcc-3(^\prime) ]</td>
<td></td>
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</tbody>
</table>

\(^a\)The \(a3\) subunit was cloned partially, as its hydrophilic amino-terminal (NT) cytoplasmic domain; \(d\) subunits were full length; all V-ATPase subunits were of mouse origin; all PCR primers had a small, random 5\(^\prime\) extension (not shown) to improve restriction enzyme cleavage.

\(^b\)pET constructs were in pET32a(+), pGEX constructs in pGEX-4T-1; PCR products were EcoRI/SalI digested and ligated into EcoRI/SalI digested vectors; all constructs were verified by full-length sequencing of inserts and junctions.

\(^c\)Expressed protein domain organization is indicated—TRX(H\(_6\)) and GST are N-terminal \(E.\ coli\) thioredoxin (with His-tag and S-tag domains) and \(S.\ japonicum\) glutathione-S-transferase fusions, respectively; G\(_2\) is a Gly-Gly coupler; in superscript parentheses are shown the amino acid ranges (a.a.) of the expressed subunits (homologous target V-ATPase subunit sequence only, numbered with respect to the natural subunit N-terminal methionine).
3.4 Purification of fusion proteins

(Purification were carried out by Dr. Norbert Kartner, Dr. Keying Li and Dr. Yeqi Yao)

Fusion proteins were purified essentially as described in Kartner et al (78). Briefly, transformed, exponentially growing bacteria were induced with 0.2 mM IPTG and incubated for 16 h at 16°C. Cells were harvested and resuspended in PBS containing 0.2 mg/ml lysozyme per 200 ml of original culture volume, incubated on ice for 30 min and mixed with 2.5 volumes of ice cold 0.2 % (w/v) Triton X-100 and sonicated. DNase (5 µg/ml) and RNase (10 µg/ml) were added and the mixture was incubated on ice for an additional 10 min. After centrifugation at 20,000g for 15 min., the supernatant was extracted and mixed, at 10 ml per liter of starting culture, with a 50% slurry of Glutathione Sepharose 4B in PBS and allowed to incubate for 1 h at 4°C with rocking. Beads were collected by centrifuging at 500g for 5 min. and were transferred to a Poly-Prep chromatography column (Bio-Rad) and eluted with 10 mM glutathione in 50 mM Tris-HCl. Fractions were collected and analyzed by SDS-PAGE.
3.5 Pulldown assays

Glutathione affinity pulldown assays were done with 40 µg of GST fusion protein (d1, d2, or GST control) in 600 µl PBS, 0.05% Triton X-100 and 2 mM DTT. To this was added 150 µl of 50% Gultathione Sepharose 4B equilibrated with PBS, 0.05% Triton X-100 on a rocking platform at 8°C for 1 h. After several washes with PBS and 0.05% Triton X-100 at 4 °C, 40 µg of TRX-NTa3 protein was added to 600 µl PBS, 0.05% Triton X-100 and 2 mM DTT and rocked gently at 8°C for 1 h. After several washes with PBST, proteins were eluted with elution buffer containing 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. Samples were run on SDS-PAGE and immunoblotted with a 1:2500 dilution of mouse anti-His-tag antibody, or anti-GST antibody, followed by secondary HRP-GAM, or HRP-GAR, respectively. Blots were developed using Western Lightning-ECL enhanced chemiluminescence substrate, imaged with a chemiluminescence ECL detector (Bio Rad), and quantified with Quantity one 4.6.9 software. A similar procedure was used for Ni-NTa pulldown assays.
3.6 In vitro ELISA-based solid-phase binding assays

Protein coating buffer
For \( a3 \), 10mM Sodium phosphate, pH 7.0
For \( d2 \), 10mM Sodium phosphate, pH 7.4

Tris buffered saline with Triton X-100 (TBST), pH 7.4
20 mM Tris-HCl
0.9% (w/v) NaCl
0.05% (w/v) Triton X-100

Washing buffer
Tris buffered saline with Triton X-100 (TBST), pH 7.4
20 mM Tris-HCl
0.9% (w/v) NaCl
0.05% (w/v) Triton X-100

Blocking Buffer
3% w/v Gelatin in PBS containing 0.1% phenol

TMB stock
1 mg/ml TMB in DMSO/ethanol 1:9

Developer solution
80 µg/ml 3,3’’,5,5’’-tetramethylbenzidine (TMB)
0.01% hydrogen peroxide
0.1M sodium acetate, pH 6.0

Stop solution
1N H\(_2\)SO\(_4\)

Methods for obtaining protein-binding saturation curves were essentially as previously reported by Kartner et al. (78) Briefly high protein-binding polystyrene 384-well plates (Greiner Microlon 781097) were coated with ligand protein 10 µg/ml TRX-NT\( a3 \) in protein coating buffer overnight at 4 °C. Coated plates were washed twice with TBST, pH 7.4 and blocked for 1 h with blocking buffer and washed twice again with washing buffer. Wells were then incubated with analyte protein, GST-\( d1 \), or GST-\( d2 \), in blocking buffer for an hour, followed by three consecutive washes. Anti-GST antibody in blocking buffer was added to the wells and allowed to further incubate for 30 min. Plates were washed again and incubated with secondary HRP-GAR antibody in blocking buffer for 30 min. Plates were washed again, developer solution was freshly prepared and added for 15 min., and the reaction was stopped with stop solution and absorbance was quantified using a Perkin Elmer Envision Multilabel Reader at 450 nm, with subtraction of an optical reference absorbance at 600 nm.
3.7 High-throughput chemical library screening

This assay was developed to identify compounds that inhibit the \( a3-d2 \) V-ATPase interaction. Briefly, the same protocol was carried out as outlined in the ELISA-based solid-phase binding assay, above, except wells were pre-incubated with a compound of interest before the GST-\( d2 \) analyte protein was added. Typically 320 compounds were screened per plate with two positive controls (no library compound, vehicle only) and two negative controls (no ligand). The assays were carried out at the Samuel Lunefeld Research Institute, SMART facility. Hits obtained were assigned a score after data analysis was carried out using B score statistics (explained in section 3.18) (131). The hits were ranked based on negative deviation from the mean B score. Positive selected hits were confirmed by repeating the primary assay screen. Hits that were reproducible were tested in secondary cellular screening assays for toxicity.

3.8 Cell toxicity assay

To evaluate the effect of luteolin on metabolic activity, the modified mitochondrial reductase activity assay based on MTT was used. Cells were seeded at 5 x 10\(^3\) cells in a 150 µl volume per well of a 96-well plate and allowed to grow for two days in the presence and absence of luteolin. 20 µl of a modified tetrazolium dye was added to individual wells and absorbances were read at 490 nm after overnight incubation with the dye.

3.9 Protein assay for determining cell growth

Lysis buffer
90mM trisodium citrate
10mM NaCl (adjusted to pH 4.8 with HCl)
0.1% Triton X-100 (from 10% stock solution)

5 x 10\(^3\) cells in 200 µl volumes were seeded in 96-well plates and allowed to grow for three days in the presence and absence of luteolin. Cells were washed with PBS and lysed with protein lysis buffer. Protein concentration was determined using the Pierce 660 nm BCA protein assay.
3.10 Tartrate-resistant acid phosphatase (TRAP) staining

TRAP buffer
50 ml acetate buffer
10 ml 0.3 M sodium tartrate
1 ml 10 mg/ml naphtol AS-MX phosphate disodium salt
0.10 ml Triton X-100
38.9 ml distilled water
0.3 mg Fast Red Violet LB salt

Cells were stained according to the BD BioSciences TRAP staining protocol no. 445. Briefly, cells were washed with PBS and fixed with 200 µl/well formalin (Sigma-Aldrich; cat. no. HT501128) for 15 min. at 37 °C and washed again with PBS three times. Cells were incubated in 200 µl/well TRAP buffer for 5 minutes at 37 °C. TRAP stain was aspirated and stained cells were washed and stored in PBS at 4°C. Photomicrography was done using brightfield illumination after cells were warmed to room temperature.

3.11 Total solubilized (TRAP) assay

Lysis buffer
90mM trisodium citrate
10mM NaCl (adjusted to pH 4.8 with HCl)
0.1% Triton X-100 (from 10% stock solution)

Substrate solution
0.1g disodium p-nitrophenyl phosphate
25 ml distilled water
Tartaric Acid Buffer
40mM L (+)- tartaric acid added to Citrate Buffer (final pH 4.0)

Cells were differentiated into osteoclasts for 5 days in 96-well cell culture plates, medium was aspirated on day 5, and cells were washed twice with PBS and wells were aspirated, then 200 µl/ well of lysis buffer was added to the plate. After lysis, 20 µl samples were withdrawn and added to microplate wells on ice, containing 50 µl each of ice cold substrate solution and Tartaric acid buffer. The assay plate was mixed and then incubated for 30 minutes at 37 °C. The reaction was stopped with 80 µl of 2 N NaOH. Absorbances were measured at 405nm and compared with standards consisting of 1-100 µM p-nitrophenol (78).
3.12 Hydroxyapatite resorption assay

Resorption assays on the Corning Osteo Assay Surface were performed as described (42). Briefly, RAW 264.7, or BMM cells, were differentiated into osteoclasts on Corning Osteo Assay Surface 96-well plates. Cells were allowed to attach to plates for 2 h at 37 °C, then 100 µl of medium containing 100 ng RANKL and varying concentration of luteolin was added to each well. A complete change of medium was carried out on day 3. On day 5, cells were stripped from plates with 150 µl of 1.2% sodium hypochlorite for 5 min. The plates were then aspirated, washed thoroughly with water and air dried, prior to staining. The modified von Kossa staining protocol was used to improve the contrast for resorbed pit image analysis and quantification. For brightfield visualization, von Kossa staining greatly increases the image contrast, facilitating imaging and quantification of resorption pits. For microscopy, the multiple well plate was oriented with the bottom of the wells closest to the objective lens of an inverted microscope. Digital images were captured using resident image capture software. NIH ImageJ software was used (132).

Modified Von Kossa Staining

For von Kossa staining, 100 µL of 5% (w/v) aqueous silver nitrate solution was added to each of the bleached wells of a 96 well plate. Plates were incubated for 30 minutes at room temperature in the dark (covered with foil). The silver nitrate solution was discarded into a hazardous waste container and the plates were then soaked in distilled water for 5 minutes. The water was discarded into a hazardous waste container. The mineral surface appears yellow after this step. The ionic silver (I) was reduced to metallic silver, developing a dark color, by adding 100 µL of 5% sodium carbonate (w/v in commercial buffered formalin) and incubated for 4 minutes at room temperature. The carbonate/ formalin solution was discarded into a hazardous waste container and the plates were dried at 50°C for 1 hour.
3.12 **Ivory Resorption assay**

An Isomet slow speed saw with a 4 in. diameter diamond wafering blade was used to cut 100–150 μM thick ivory slices that were then 0.25 in. hole-punched to fit into wells of a 96-well plate. Ivory discs were stored in 20% (v/v) ethanol. For resorption assays, discs were washed with 70% (v/v) ethanol, followed by sterile PBS, incubated overnight in medium at 37 °C in a tissue culture incubator. BMM cells were isolated and grown in the presence of α-MEM and 50 ng M-CSF for 2 days. Cells were differentiated into osteoclasts on day 3 in α-MEM containing 50 ng M-CSF and 100 ng/ml soluble recombinant RANKL. On day 4, osteoclasts were dissociated using cell dissociation buffer and were replated onto ivory in the presence of varied concentrations of luteolin until day 9 or 10. Ivory discs were washed with 70% (v/v) ethanol and cold PBS twice each, sonicated in PBS, and washed several times in PBS to remove cells and cell debris.

3.13 **Picrosirius Red staining of ivory slices to visualize resorption pits**

Resorbed ivory slices were washed in distilled water three times and immersed in 0.1 M phosphate buffer, pH 7.4, then incubated in 12 units of papain for 90 min. at 37 °C, followed by picro-sirius red stain for 60 min. at room temperature. After staining, slices were washed twice with 0.1 M HCl for 2 min. and dehydrated consecutively in 70%, 90% and 100% alcohol baths, followed by consecutive immersion in 70%, 90% and 100% xylene baths. Entellan new rapid mounting medium was used to mount slices on glass slides and they were stored at 4 °C. A Zeiss 100M LSM 510 confocal microscope was used to evaluate pit depth and pit area on ivory slices. Three independent experiments were performed in which 15 zones were measured for pit depth and pit area for each group. Confocal microscopy of resorption pits resulted in several images that were slices 2 microns thick. The images were processed in ImageJ (NIH) and Volocity (Perkin Elmer) software were used to quantify total pit depth and total pit area.
3.14 Phalloidin staining

Briefly, BMM-differentiated osteoclasts were cultured on ivory slices, the osteoclast cell cultures were rinsed with PBS, then fixed and permeabilized, as described by McMicheal et al., (133). Rhodamine phalloidin (5 µl of stock solution) was used to stain F-actin and cell staining was visualized using a Zeiss 100M LSM 510 confocal microscope.

3.15 Osteoclast membrane preparations

Homogenization buffer, pH 7.0
230 mM sucrose
40 mM KCl
2.0 mM HEPES-KOH
2 mM DTT

Resuspension buffer, pH 7.4
150 mM KCl
20 mM HEPES-KOH
2 mM DTT

Microsomal membranes from RAW 264.7 cell-derived osteoclasts were prepared according to the modified method Baron et al. (134). Briefly, RAW264.7 cells were differentiated into osteoclasts in the presence and absence of luteolin for five days. Cells were washed with homogenization buffer and homogenized using 50 strokes in a glass homogenizer. Homogenate was centrifuged for 10 min at 4000 x g 4 °C to pellet unbroken cells and mitochondria. Supernatants were collected on ice and centrifuged at 100,000 x g for 40 min. and the pellet was resuspended in buffer and placed on ice for 40 min. to form vesicles. Membranes were stored at -20°C and used subsequently for immunoblotting.
3.17 Western blot analysis

Microsomal membrane preparation from osteoclast membranes were used to conduct western blot analysis to identify if luteolin targets V-ATPase subunits formation and assembly. Anti-E, anti-\(a_3\), anti-\(d_2\), anti-\(\beta\) actin were used at 1:1000 dilution, secondary antibody was HRP-GAR. Images were developed with Western Lightning ECL detection solution (PerkinElmer, cat. no. NEL102001) and bands were photographed and quantified in a Bio-Rad Molecular Imager ChemiDoc™ XRS system (cat. no. 170-8070, 8071) and using Quantity One 4.6.9 software.

3.18 Statistical Analysis

Repetition of experiments is given in figure legends. Determination of IC\(_{50}\) values for dose response curves, or half-maximal binding in protein interaction assays, was done using GraphPad Prism (v4.02) curve-fitting software. Hits resulted from primary screening of compounds was analyzed using B-score statistics (131) and ranking the inhibitory compounds according to negative deviation from the mean. Selected hits were confirmed by repeating the primary assay.

Standard deviations were calculated and are shown as ±SD wherever values are quoted, or in error bars in histograms and graphs. Non-parametric tests (GraphPad v4.02) were used to test significance of differences, as appropriate.
CHAPTER 4: Luteolin Inhibition of V-ATPase a3-d2 Interaction Decreases Osteoclast Resorptive Activity


4.1 Introduction

General V-ATPase inhibitors reduce bone resorption (135-137), but they are cytotoxic, as V-ATPases are essential for a variety of intracellular housekeeping functions. Targeting specific V-ATPases may be possible, as it is hypothesized that specialized V-ATPases may have unique combinations of subunit isoforms. For example, the combination of a3, d2, and B2 subunit isoforms is thought to be a signature feature of V-ATPases in the osteoclast plasma membrane. In previous work, we identified a small molecule benzohydrazide derivative that inhibited the a3–B2 subunit interaction, and this compound was found to reduce bone resorption in vitro (78). The a3 and d2 subunits are also known to interact (119,120). In the present work we further characterize a3–d1 and a3–d2 interactions, using purified fusion proteins, and implement a high-throughput ELISA screening assay to identify compounds that inhibit the interaction. A hit discovered in this screen was the flavonoid Luteolin.

Flavonoids are bioactive phenols that have been shown to have antioxidant (138), anti-inflammatory (139), anti-allergic (140), anti-carcinogenic (141) antimicrobial (142) and anticancer (143,144), anti-tumour (145) activity. Flavonoids have been shown to inhibit enzymes like hydrolases, alkaline phosphatase, arylsulphatase, lipases and kinases. Flavonoids have C6-C8-C6 carbon Skelton, with substituents in C2-C3 position. Flavonoids are widely distributed, found in vegetables, fruits, plants, seeds, flowers, tea etc. The dietary intake of flavonoids is estimated to be 1-2 g/day.

Luteolin belongs to the flavone class of six flavonoids commonly found in fruits, vegetables and medicinal herbs. It occurs in glycosylated form at high concentrations in celery, green pepper, perilla leaf and chamomile (146) and aglycone in perilla seeds (147).
Lee et al. (148) Kim et al. (149) and have previously shown that luteolin inhibits bone resorption. In the former study, Lee et al. suggest that luteolin specifically inhibits the RANKL-induced signalling pathway, but the proximal molecular target of luteolin within osteoclasts was not identified (148). Here we show that luteolin prevents the V-ATPase $a3-d2$ protein–protein interaction without affecting V-ATPase subunit formation or assembly and decreases bone resorption significantly.
4.2 Results

V-ATPase a3 and d2 subunits are highly enriched in osteoclasts and are required for their bone resorption activity (62,65,111,118,119,150). Considering this, it is possible that a specific inhibitor of the a3-d2 interaction could therapeutically decrease bone resorption in bone loss pathologies. In previous work, we characterized an interaction between V-ATPase a3 and B2 subunits, which are also enriched in osteoclasts. We then used an ELISA-based binding assay to screen small molecule libraries for a3–B2 protein interaction inhibitors and discovered a benzohydrazide derivative that not only inhibited the protein interaction, but also decreased osteoclast resorption without significantly affecting cell viability or differentiation (see Chapter 5) (78). To assess whether this approach can be generally applied to osteoclast-specific subunit interactions we asked whether inhibitors of the known a3–d2 subunit interaction would yield similar results. Here we express the a3, d1 and d2 subunits as fusion proteins to investigate their binding characteristics. Using this information we exploit the developed solid-phase binding assays to screen chemical libraries for inhibitors. Finally, we ask what the consequences are of inhibiting the a3–d2 interaction in osteoclasts, with respect to cell viability, osteoclast differentiation and osteoclast resorptive activity.

4.2.1 Interaction of V-ATPase a3 and d2 subunit isoforms

Affinity pulldown assays using recombinant fusion proteins demonstrated that the N-terminal domain of the V-ATPase a3 subunit (NTa3) could pull down the d2 subunit from solution (Fig. 7A–C). This qualitatively demonstrated an interaction between a3 and d2, but to quantitatively assess relative binding affinities of a3–d interactions we used an ELISA-based binding assay, as previously described for NTa3–B interactions (78). V-ATPase d1 and d2 subunits (analyte proteins, as GST fusions) were tested against bound NTa3 (ligand fused with TRX), as shown in Fig. 7B. The half-maximal binding values calculated from saturation curves for NTa3-d1 and NTa3–d2 were not significantly different, at 3.1±0.04 nM and 3.9±0.06 nM, respectively. Binding to GST was negligible, as were the signals obtained when either analyte or ligand protein were omitted (Fig. 7B).
Figure 7. NTa3 interacts with both d1 and d2 with similar affinities.

A. For the left and center panels, glutathione affinity beads were coated with GST-d2 or GST alone (as indicated above the blots), then incubated with TRX-NTa3. After extensive washing of beads, SDS-eluted protein was run on SDS-PAGE and immunoblotted with anti-His-tag or anti-GST antibodies, as indicated below the blots (note that TRX contains a His-tag). For the right panel Ni(II) affinity beads were coated with TRX-NTa3, TRX alone, or were control treated with buffer only, then incubated with GST-d2, further processed like the glutathione beads and probed with anti-GST antibody. Ticks on left margin of blots represent positions of molecular weight markers indicated in left-most blot. Note that blots are not in exact horizontal alignment with respect to molecular weight. B. ELISA saturation binding curves for NTa3–d1 (●) and NTa3–d2 (■) interactions (note log-scale abscissa). ELISA plates were coated with TRX-NTa3 (ligand) and were probed with GST-d1 and GST-d2 (analytes) from 1.5 µM down in a 2-fold serial dilution. Absorbance at 450 nm (A450) indicates amount of analyte bound (see “Experimental Procedures”). Negative controls were: NTa3 ligand with GST analyte (△); NTa3 ligand with no analyte (▽); no ligand with d1 (○), d2 (◇) or GST (□) analyte. Results are represented as means, error bars are ±S.D. (n=4, in quadruplicate).
4.2.2 Luteolin inhibits the NTa3–d2 interaction

Having modeled the $a_3$–$d_2$ interaction in an ELISA-based binding assay, we adapted this assay for high-throughput screening for small molecule inhibitors. This screen yielded several primary hits for inhibition of the $a_3$–$d_2$ interaction (hits being defined as having B-score greater than 3 standard deviation below the mean). Rescreening of primary hits was done to determine dose response in the protein interaction assay and toxicity in cellular assays. Only one compound (Fig. 8A) luteolin, a naturally occurring flavonoid was found to be significantly inhibitory without issues of irreproducibility or overt cytotoxicity. The dose response for luteolin in $a_3$–$d_2$ inhibition is shown in Fig. 8B. The half-maximal concentration ($IC_{50}$) for luteolin inhibition of the $a_3$–$d_2$ interaction in the solid-phase binding assay was $1.9 \pm 0.7 \mu M$. Secondary screens were subsequently performed to determine the effect of luteolin on cell viability, osteoclast differentiation and osteoclast resorptive activity.
Figure 8

A

[Chemical structure of luteolin]

luteolin

$M=286.2$ g/mol

B

[Graph showing the concentration of luteolin vs. $A_{450}$]

$A_{450}$

$[\text{luteolin}]$
Figure 8. Luteolin inhibits V-ATPase a3–d2 interaction.

A, Structure of luteolin, 3',4',5,7-tetrahydroxyflavone. B, In an ELISA-based binding assay TRX-NTa3 ligand was probed with GST-d2 analyte in the presence of luteolin (2-fold serial dilution from 80 µM to 0.3 nM). Absorbance at 450 nm (A_{450}) indicates amount of analyte bound (see “Experimental Procedures”). Results are represented as the mean; error bars are ±S.D. (n=4, in quadruplicate).
4.2.3 Luteolin is not toxic to NIH/3T3 and RAW264.7 cells

To assess the effect of luteolin on cell viability, the RAW 264.7 mouse macrophage cell line and the NIH/3T3 mouse fibroblast cell lines were grown in the presence of a concentration range of luteolin for three days (0.3 - 40 µM, 2 fold serial dilution). Cells were subsequently tested in a mitochondrial reductase-based MTS assay to assess metabolic activity, and were assayed for total protein to assess growth. According to the MTS assay RAW 264.7 and NIH/3T3 cells were not significantly affected at any luteolin concentration used, suggesting that luteolin, at concentrations up to 40 µM, is not toxic to either cell line (Fig. 9A). Furthermore, under the same growth conditions, total protein content was unaffected at up to 40 µM luteolin (Fig. 9B). These data strongly suggests an IC$_{50}$$>>$ 40 µM for luteolin cytotoxicity in the proliferation assays employing either the RAW 264.7 or NIH/3T3 cellular models.
Figure 9
Figure 9. Luteolin is not toxic to NIH3T3 and RAW 264.7 cells.

Cells were cultured in the presence of luteolin (2-fold dilution series from 40 µM to 0.3 µM). A, After 3 days of continuous exposure, mitochondrial reductase activity was assayed using MTS tetrazolium dye reduction to assess metabolic activity. No significant differences were observed. B, In a separate assay, total cellular protein was measured as an indicator of cell growth. Control untreated cells (C) were not exposed to luteolin. As with the MTS assay, no significant differences were observed. Results are represented as means; error bars are ±S.D. (n=3, six replicates each).
4.2.4 Luteolin does not affect osteoclastogenesis in RANKL-induced RAW 264.7 and BMM cells

We further investigated whether there were any adverse effects of luteolin on osteoclast differentiation and maturation by assaying TRAP. This early enzyme marker of osteoclastogenesis is readily detectable in mononuclear preosteoclasts through to mature osteoclasts (151). Fig. 10A and B show the effect of luteolin on the number of TRAP-positive multi-nucleated osteoclasts differentiated from RAW 264.7 cells in the presence of recombinant RANKL and BMM cells in the presence of recombinant RANKL and M-CSF, respectively. Histograms are shown normalized to untreated samples. The osteoclast counts were subdivided into three subpopulations: 2–5 nuclei, 6–12 nuclei, and ≥13 nuclei to determine the effect of luteolin on osteoclast size, which is contributing factor for osteoclast resorptive activity. Osteoclast counts for RAW 264.7 and BMM cells appeared to decrease marginally up to 40 µM, but this was not statistically significant and all subpopulations were similarly affected.

Fig. 10C shows example images of BMM-derived osteoclasts, grown in the absence or presence of 5 µM luteolin on ivory and Osteo Assay surface, respectively. Osteoclast morphology varied on these surfaces, having rounded appearance on ivory and a flattened, fibroblastoid appearance on the synthetic hydroxylapatite of the Osteo Assay surface; however, the sizes of the osteoclasts did not significantly differ, whether untreated or treated with luteolin, on either surface. In RAW 264.7 cells the level of intracellular TRAP measured was not significantly different between treated and untreated samples, as shown in Fig. 10D. The histogram is normalized to untreated samples and again suggests that in the TRAP assay for differentiation luteolin has an IC$_{50}$$>>$ 40 µM.
Figure 10

A

TRAP+ cell count

μM luteolin

C 0.6 1.2 2.5 5 10 20 40

2–5 nuclei
5–12 nuclei
≥13 nuclei

RAW 264.7 osteoclasts

B

TRAP+ cell count

μM luteolin

C 0.6 1.2 2.5 5 10 20 40

2–5 nuclei
5–12 nuclei
≥13 nuclei

BMM osteoclasts
Figure 10. Luteolin does not affect osteoclastogenesis in RANKL-induced RAW 264.7 and BMM cells.

RAW 264.7 and BMM cells were differentiated in the presence of 0.3 to 40 µM luteolin and RANKL, or RANKL and M-CSF, respectively, for 5–7 days (see “Experimental Procedures”). TRAP-stained, fixed cultures were counted for number of nuclei per cell and counts were subdivided into three groups: 2–5 nuclei per cell, 5–12 nuclei per cell and ≥13 nuclei per cell. Results are represented as means; error bars are ±S.D. (n=3, in quadruplicate). A, RAW 264.7 cells and B, primary mouse BMM cells. C, (top row) TRAP-stained BMM osteoclasts differentiated in the absence (C) and presence of 5 µM luteolin on ivory; (bottom row) TRAP-stained BMM osteoclasts differentiated in the absence (C) and presence of 5 µM luteolin on Corning Osteo Assay Surface. Total TRAP activity was assayed (bottom panel) as p-nitrophenol (PNP) released by TRAP activity in solubilized RAW 264.7 cells, prepared as described in “Experimental Procedures” and normalized to untreated cells (C). Error bars are ±S.D. (n=3, in triplicate).
4.2.5 Luteolin suppresses osteoclast resorptive activity in RAW 264.7 and BMM derived osteoclasts

To assess the efficacy of luteolin as a potential anti-resorptive agent, RAW 264.7 and BMM derived osteoclasts were plated on a synthetic hydroxyapatite mineral surface (Corning Osteo-Assay Surface) and on dentin (elephant ivory) in the presence and absence of luteolin. For resorption on synthetic hydroxyapatite, both RAW 264.7 and BMM cells were plated directly on Corning Osteo-Assay plates and differentiated into osteoclasts for 5 days with exposure to 1.25 µM to 40 µM luteolin. Figs. 11A and B shows photomicrographs of Osteo-Assay surface partially resorbed by RAW 264.7 – derived osteoclasts at various luteolin concentrations. Fig. 10B shows quantification of the luteolin dose-dependent inhibition of resorption of Osteo Assay surface by osteoclasts derived from either RAW 264.7 and BMM cells, as indicated. Approximate EC\textsubscript{50} values for luteolin inhibition of osteoclast resorption were 1.2 µM for RAW 264.7- derived cells and 2.5 µM for BMM- derived cells.

By direct observation, isolated resorption pits formed by BMM- derived osteoclasts on the Osteo Assay surface in the presence of luteolin appeared shorter than those of untreated controls. Thus, to assess more precisely how resorption pit morphology is affected by luteolin, we differentiated BMM cells on dentin and observed pits using Z-stacking confocal microscopy. An example of a resorption pit and its 3D –rendered image is shown in Fig. 11C. Pit lengths, areas and depths were quantified and pit volumes were calculated. These data are represented graphically in Fig. 11D. We observed a highly significant decrease even at 0.6 µM, relative to controls, but no dose-dependence across samples treated with 0.6 to 20 µM luteolin (Fig. 11D, upper left). Although luteolin decreased resorption pit depth overall compared to untreated controls, pit depth paradoxically increased with higher luteolin concentrations (Fig. 11D, lower left). Because of the opposite slopes of the pit depth and pit areas in response to increasing luteolin concentration, the calculated pit volumes fall off sharply at 0.6 µM luteolin, relative to control, but show no further significant decrease up to 20 µM luteolin.
Figure 11

A

B

RAW 264.7 osteoclasts

% area resorbed

% area resorbed

µM luteolin

µM luteolin
**Figure 11. Luteolin suppresses osteoclastic resorptive activity.**

A, RAW 264.7 osteoclasts were grown on Corning Osteo Assay plates with continuous exposure to luteolin for 5 days, as indicated (C, control without luteolin). Remaining mineral was von Kossa stained (*black*) and imaged, as described in “Experimental Procedures”. Note that area of resorption (*white*) decreases with increased luteolin concentration. B, Quantification of experiments similar to those in panel A. *Upper panel*, RAW 264.7 cells were differentiated into osteoclasts on Osteo Assay plates in the absence of (C) or continuous exposure to 0.6 µM to 40 µM luteolin for 5 days. *Lower panel*, BMM cells were differentiated into osteoclasts in the absence of (C) or continuous exposure to 1.2 µM to 40 µM luteolin for 5 days. In both panels, percent area resorbed (white area divided by total area) was quantified using NIH ImageJ after stripping cells and von Kossa staining plates for photomicrography. C, *left panel*, shows a typical isolated resorption pit in ivory, made by a BMM osteoclast, imaged by confocal fluorescence microscopy after staining with Picro-Sirius Red. *Right panel*, shows the 3D rendering (NIH ImageJ) of the confocal Z-stack of the pit shown in the left panel. D, BMM cells were plated and differentiated into osteoclasts on ivory in the absence of (C) or continuous presence of 0.6 to 20 µM luteolin for 8 days, after which cells were removed and ivory slices stained with Picro-Sirius red to visualize pits, as in panel C. Pit lengths, areas and depths were measured in confocal images using ImageJ and Volocity software. Pit volumes were estimated as the products of pit areas and pit depths. All data from images were normalized to untreated controls (C) and are represented as means ±S.D.
4.2.6 Luteolin has no effect on V-ATPase subunit expression, or V-ATPase assembly

Luteolin was identified in a screening assay as an inhibitor of the V-ATPase $a3-d2$ subunit interaction and was subsequently shown to inhibit osteoclast resorption. This connection however does not identify the precise mechanism of action of luteolin on osteoclast acid secretion. To address mechanism, we first investigated the effect of luteolin on V-ATPase expression and or assembly in osteoclasts. RAW 264.7 osteoclasts, differentiated for 5 days in the presence of 1.2, 10 and 20 μM luteolin were lysed and microsomal membranes were obtained by differential centrifugation. Immunoblots of microsomal membrane proteins were probed with antibodies specific to the V-ATPase $V_0$ subunits $a3$ and $d2$, and the $V_1$ subunit $E$ and quantified with signals normalized to β-actin (Fig. 12A). These data (band intensities) were quantified as the ratios of $a3/d2$ ($V_0/V_0$), $a3/E$ ($V_0/V_1$) and $d2/E$ ($V_0/V_1$), all normalized to β-actin (Fig. 12B). None of these ratios, across the range of luteolin concentrations changed significantly relative to controls. These results indicate that the luteolin-dependent reduction of resorptive activity is not due to an effect on osteoclast V-ATPase subunit expression or assembly.
Figure 12

A

<table>
<thead>
<tr>
<th></th>
<th>cell lysate</th>
<th>microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-ATPase a3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-ATPase d2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-ATPase E</td>
<td></td>
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</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
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</tbody>
</table>

\[ \mu M \text{ luteolin} \]

B

\[ \text{ratio} \]

\[ \mu M \text{ luteolin} \]

- a3/d2
- a3/E
- d2/E

- microsomal membrane
- whole cell lysate
Figure 12. Luteolin does not affect V-ATPase subunit protein expression or complex assembly.

RAW 264.7 cells were differentiated into osteoclasts in the presence of luteolin (1.2, 10 and 20 µM) for 5 days. Whole cell lysates and microsomal membranes were then immunoblotted to evaluate the effect of luteolin on V-ATPase assembly and subunit expression. A, Immunoblots of microsomal membranes and whole cell lysates were probed with antibodies specific to the V-ATPase V₀ subunits a3 and d2, and V₁ subunit E, as indicated; anti-β-actin staining served as a control. B, Quantitative analysis of bands from blots in panel A, the ratios of a3/d2 (both V₀ sector), a3/E (V₀ sector vs. V₁ sector) and d2/E (V₀ sector vs. V₁ sector) were calculated. No significant differences (at P≤0.05) were observed between untreated (C) and treated osteoclasts. Results were normalized to β-actin; error bars are ±S.D. (n=3).
4.2.7 Luteolin does not inhibit actin ring formation

As V-ATPase assembly was unaffected by luteolin, we asked whether it acted on the ability of osteoclast to form an osteoclast-bone interface. Actin rings are regarded as markers of osteoclast activation, remodelling their membranes to create sealing zones between the cell and bone during resorption (152). BMM-differentiated osteoclasts were fixed and probed with the F-actin stain, phalloidin, to observe the effect of luteolin on actin ring formation (examples shown in Fig. 13A). Luteolin, up to 20 µM, did not have any observable effect on actin ring formation or sealing zone width (Fig. 13B). These data indicate that luteolin does not prevent osteoclast actin ring formation and bone attachment and correlate with similar observations by other workers after downregulating α3 expression (152), or for mutations.
Figure 13

A

width

control

20 μM luteolin

B

sealing zone width

μm

μM luteolin

C  0.6  1.2  2.5  5  10  20
Figure 13. Luteolin does not inhibit osteoclast actin ring formation.

BMM cells were differentiated into osteoclasts in the absence (control, C) or continuous presence of 0.6 to 20 µM luteolin for 7 days on ivory (see “Experimental Procedures”). A, Actin rings of typical phalloidin-stained BMM osteoclasts are shown. Qualitatively, F-actin ring formation in both control and luteolin-treated osteoclasts was not different. Typical measurement of ring is indicated by arrowheads (“width”, top panel) B, Sealing zone width was measured to quantitatively evaluate the effect of luteolin at different concentration on osteoclast F-actin ring formation. No significant differences were observed between untreated (C) and treated osteoclasts. Results are represented as means, error bars are ±S.D. (n=3, in duplicate).
4.3 Discussion

Luteolin, a naturally occurring compound was identified as an inhibitor of the V-ATPase $a3-d2$ interaction. Secondary assays were conducted to identify the effect of luteolin on cell toxicity, proliferation, osteoclastogenesis and bone resorption. These assays revealed luteolin did not inhibit cell growth and proliferation at concentrations below 40µM yet inhibited bone resorption without affecting osteoclastogenesis at concentrations below 20 µM. The mechanism of luteolin action against V-ATPase activity was further investigated. Luteolin was shown to not effect V-ATPase subunit assembly and expression.

The results obtained in this study will be further clarified in the general discussion section presented later (Chapter 6).
CHAPTER 5: Inhibition of Osteoclast Bone Resorption by Disrupting Vacuolar H+-ATPase a3-B2 Subunit Interaction


5.1 Introduction

V-ATPases are highly expressed in the ruffled border of bone resorbing osteoclasts. Proper function of V-ATPase is required for proton secretion into the extracellular resorption lacunae of osteoclasts (42). Disruption of the V-ATPase leads to bone disorders such as osteoporosis and osteopetrosis (153-155). Therefore in order to develop new therapeutics for the treatment of bone loss diseases, the structural and function of the V-ATPase is studied in great detail. The a3 and B2 subunits are highly enriched in osteoclasts (65) and are molecular targets against V-ATPase activity that leads to excessive bone loss. Kartner et al., have discovered protein-protein interactions with the N-terminal of a3 (NTa3) subunit and the C-terminal domains of B1 and B2 subunits with equal affinity. They further characterized the interaction using affinity pulldown assays and solid-phase binding assays. Affinity pull down assays showed that a1, a2, a3 and a4 (V-ATPase a subunit isoforms) were efficiently pulled down with subunits B1 and B2. To quantitatively determine the relative protein binding, an ELISA based solid phase binding assay was used to obtain saturation curves on NTa3 against B1 and B2. The curves were used to calculated half-maximal binding values and indicated that NTa3-B1 and NTa3-B2 were not significantly different. Further experiments confirmed a higher affinity for a3-B2 interaction as opposed to other a-B subunit interactions. We speculate that the a3-B2 interaction stabilizes the V-ATPase structure since the a3 subunit is part of the V₀ domain and the B subunit is part of the V₁ domain. Therefore small molecule inhibitors of the a3-B2 interaction might target the structure of the V-ATPase, destabilizing the V₁V₀ complex. This might possibly regulate V-ATPase activity, specifically targeting the plasma membrane osteoclast V-ATPases that contain a3 and B2 subunit isoforms (78).
5.2 Results

ELISA-based solid phase binding assay was used in a high throughput robotics platform to screen synthetic chemical libraries for possible V-ATPase a3-B2 subunit inhibitors. Synthetic chemical screening yielded primary hits at a rate of 0.2% and rescreening resulted in model compound KM91104 with a B-score of minus 14.8. Another analog of KM91104 was also tested in the 10,000 compound library screen and it failed to reproducibly give a low B-score on rescreening (Experimental work carried out by Dr. Keying Li and Dr. Norbert Kartner).

Five compounds were obtained and tested, except KM91104 the other four compounds were found to be overtly toxic to mammalian cells in growth and cytotoxicity assays. KM91104 was further screened, where its effect on osteoclastogenesis and resorption were investigated.

5.2.1 KM91104 is not toxic to RAW264.7 macrophagic cells

To assess the effect of KM91104 on cell viability and growth, cells were grown in presence of 0.3 - 40 µM (2 fold serial dilution) KM91104. Cells were subsequently tested in a mitochondrial reductase-based MTS assay to assess metabolic activity, and were assayed for total protein to assess growth. Fig. 14A, shows that 5 day growth of undifferentiated RAW 264.7 cells was negligibly affected by KM91104 up to 20 µM with an IC\textsubscript{50} ≥ 40 µM (P < 0.05) and mitochondrial reductase based MTS assay (Fig. 14B) showed that KM91104 was not toxic to 2.5 µM with an IC\textsubscript{50} of approximately 20 µM (n = 3, in triplicate; error bars are ±SD, P < 0.05) (78).
Figure 14

A

Protein (µg)

KM91104 concentration (µM)

B

MTS assay absorbance

KM91104 concentration (µM)
Secondary cellular assays were used to screen primary hits to determine their effect on growth (total cellular protein) and metabolic status (MTS tetrazolium dye reduction) after 5 days of continuous exposure. The test cell line was undifferentiated RAW 264.7 cells and results are shown for the subject compound, KM91104. Panel A, total cell protein was determined in cultures exposed to a concentration range of KM91104, from 0.3 to 40 µM, as indicated (n=3, in duplicate; error bars are ±SD). C indicates control, vehicle (DMSO) only added. Concentration of KM91104 up to 20 µM had negligible effect on cell growth, with an IC$_{50}$ >40 µM. Panel B, as in panel A, but MTS reduction was determined to assess metabolic status. KM91104 had negligible effect up to 2.5 µM, with an IC$_{50}$ of approximately 20 µM (n=3, in triplicate; error bars are ±SD).
5.2.2 KM91104 does not affect osteoclastogenesis in RANKL- induced RAW 264.7 and BMM cells

It was of interest to determine the effect of KM91104 on osteoclastogenesis. We assayed TRAP enzyme a marker for osteoclastogenesis. Fig. 15A, TRAP was not significantly affected at up to 20 µM for cells treated with KM91104 in comparison to control. The estimated IC$_{50}$ > 40 µM, reflecting the growth inhibition results in Fig. 6A (n=3, in duplicate; error bars are ±SD, P < 0.05). Fig. 15B shows a small, but significant drop in osteoclast formation at 0.3 µM of KM91104 and a steady decline at higher concentration up to 20 µM (IC$_{50}$ approximately 20 µM). Osteoclasts sub-population counts are shown (2-5 nuclei, 6-12 nuclei, and ≥13 nuclei) in comparison with total osteoclasts (≥ 2 nuclei). Histograms are shown normalized to control (n=3, in triplicate; error bars are ±SD, P < 0.05). Fig. 14B illustrates that up to 20 µM KM91104 does not significantly alter the distribution of small vs large osteoclasts, relative to total osteoclasts. The inset panel in Fig. 14B shows TRAP- positive osteoclasts with >20 nuclei derived from mouse bone marrow mononuclear (BMM) cells in primary tissue culture exposed to 1.2 µM KM91104. There did not appear to be any inhibition of differentiation or maturation of authentic osteoclasts (BMM derived osteoclasts) compared with untreated cultures (data not shown) (78).
Figure 15

A

![Graph A showing p-Nitrophenol released (nM) vs. KM91104 concentration (μM).]

B

![Graph B showing % normalized cell count vs. KM91104 concentration (μM).]
A secondary cellular assay was used to determine the effect of KM91104 on osteoclast differentiation and fusion after 5 days of continuous exposure. TRAP is a marker enzyme for osteoclast differentiation that converts $p$-nitrophenylphosphate to $p$-nitropheno under acidic conditions. The test cell line was RAW 264.7 cells differentiated in the presence of 100 ng/ml soluble recombinant RANKL. Panel A, total solubilized TRAP assay was used to determine the effect of compound KM91104 on RANKL-mediated differentiation of RAW 264.7 cells (see Experimental Procedures). The concentration range of compound tested was 0.3 to 40 µM, as indicated. C indicates control, vehicle only added. Amount of $p$-nitrophenol released indicates the degree of differentiation after 5 days of growth with RANKL. Differentiation was not significantly affected up to 20 µM KM91104 concentration (n=3, in duplicate; error bars are ±SD). Panel B, shows quantitative analysis of TRAP-stained fixed cultures (see Experimental Procedures). Cell counts for TRAP-positive (red) multinucleated cells with ≥2 nuclei after treatment with compound KM91104 are plotted. Controls are normalized to 100%. (n=3, in duplicate; error bars are ±SD). Inset panel shows primary culture mouse BMM cells (TRAP stained, red) differentiated with M-CSF and RANKL and exposed continuously to 1.2 µM KM91104 for 5 days. Ability of primary BMM cells to form large osteoclasts (>20 nuclei) has not been impaired at this concentration.
5.2.3 KM91104 inhibits osteoclast resorptive activity in RAW 264.7 and primary BMM derived osteoclasts

Finally it was of great interest to determine whether KM91104 inhibited osteoclast resorptive activity. RAW 264.7 cells were differentiated to osteoclasts in presence and absence of varied concentrations of KM91104 on Corning Osteo-Assay surface and dentin (elephant ivory). Fig. 16A shows photomicrographs of synthetic hydroxyapatite mineral Corning Osteo-Assay surface, post stained black with metallic silver (Von kossa staining). Resorption areas (white areas) are visible due to active acid secretion by RAW 264.7 differentiated osteoclasts in presence of RANKL. Fig. 16B shows quantified resorption areas, indicated that KM91104 does inhibit osteoclast mineral resorptive activity with and EC$_{50}$ of approximately, 1.2 µM (n=3, in duplicate, 5 fields per well imaged; error bars are ±SD, P <0.05). This suggests that there is an acceptable ratio (approximately 20 fold) between the concentration of KM91104 that is efficacious in reducing mineral resorption (approximately 1 µM) and the concentration that toxic, as measured either by cytotoxicity assays or assays of cell differentiation. In order to truly quantify resorption, dentin (approximately 150 µM thick elephant ivory slices) was used to assess the effect of KM91104 as an anti-resorptive agent. Quantified resorption area is shown in Fig. 16D (average of results from 3 independent slices, error bars are ±SD). These data suggest that the efficacy of KM91104 in inhibiting authentic bone resorption is greater than in inhibiting resorption of the artificial mineral surface.
Figure 16

A

i

ii

iii

iv

v

vi

vii

viii

B

Relative resorption areas

KM91104 concentration (μM)

C  0.6  1.2  2.5  5.0  10  20  40
Figure 16. Secondary screening for inhibition of hydroxyapatite resorption by RANKL-differentiated RAW 264.7 cells.

Panel A, Cells were differentiated in the presence of 100 ng/ml RANKL on synthetic mineralized surfaces of Corning Osteo-Assay Surface 96-well plates. Cells were cultured for 5 days with continuous exposure to RANKL and KM91104. The complete medium was changed on the third day. On day 5, cells were stripped with 1.2% sodium hypochlorite solution and the mineral surface was stained using a modified von Kossa method (see Experimental Procedures). Plates were air-dried and imaged using digital brightfield photomicrography. Images shown are NIH ImageJ processed for quantification. Concentrations of KM91104 (µM) in medium were as follows: i, control, vehicle only; ii, 0.6; iii, 1.2; iv, 2.5; v, 5.0; vi, 10; vii, 20; viii, 40. Panel B, shows quantitative image analysis of resorption areas (white areas shown in panel A), using NIH ImageJ software. The concentration range of KM91104 was from 0.6 to 40 µM, as indicated (n=3, in duplicate, 5 fields per well imaged; error bars are ±SD). C indicates control, vehicle only added. Highly significant reduction in resorption (P<0.0001) was observed even at the lowest concentration. Approximate IC₅₀ of KM91104 for resorption was 1.2 µM, a concentration which was not cytotoxic, and which did not significantly affect osteoclast differentiation or fusion to form large osteoclasts (see Figs. 6 & 7). Panel C, Osteoclasts derived from RAW 264.7 cells were seeded on dentin (elephant ivory) slices. After 3 days resorption, slices were stained with Picro-Sirius Red and viewed by epifluorescence (GFP filters). Left column is representative fluorescence image, right column is NIH ImageJ processed to quantify fluorescence (white) on a black background. i and ii, control (vehicle only); iii and iv, 1.25 µM KM91104; v and vi, 40 µM KM91104. Panel D, Quantified fluorescence from panel C (average of results from 3 independent slices; error bars are ±SD).
5.3 Discussion

Our lab previously identified compound KM91104 as a potential V-ATPase a3-B2 inhibitor. I further characterized compound KM91104 using secondary screening assays to elucidate its potential as an anti-resorptive agent. Cellular growth and proliferations assays revealed KM91104 was not toxic to macrophagic RAW 264.7 cells. KM91104 did not affect osteoclastogenesis at concentrations 20µM and below yet inhibited bone resorption at those concentrations.

The results of the experiments reported here will be further discussed in the general discussion (Chapter 6).
CHAPTER 6: SUMMARY AND GENERAL DISCUSSION

6.1 Characterization of the a3–d interaction

The V-ATPase complexes in the ruffled borders of osteoclasts are thought to contain specifically the a3 and d2 subunit isoforms that are highly enriched in osteoclasts. Furthermore, the a3 and d2 subunits are known to interact, as we and others have shown, and both are required for bone resorption (65,104,118,119,156). We hypothesized that small molecule inhibitors of the a3–d2 interaction might have an impact on ruffled border V-ATPase function resulting in reduced osteoclast bone resorption. To test this hypothesis we first set out to characterize the specificity and affinity of the a3–d2 interaction.

Wu et al. (119) previously compared interactions of a3, d1 and d2 subunits and reported a “more extensive interaction” for a3–d2, compared with a3–d1. This observation was based on the ability of the recombinantly-expressed, His-tagged a3 subunit to co-immunoprecipitate more GST-d2 than GST-d1. In contrast, using an ELISA-based solid-phase binding assay, we have shown here that the a3–d1 and a3–d2 interactions are indistinguishable (Fig. 7B). This discrepancy likely reflects the limitation of comparing gel band intensities from co-immunoprecipitations to assess differences in binding affinities. Indeed, we found similar discrepancies in our own previous work when characterizing a–B subunit interactions (78). While solid-phase binding assays also have limitations with respect to determining true binding affinities, they have the advantage of being quantitative, and half-maximal binding values can be used to directly compare apparent affinities between subunit isoforms. Thus, based on saturation binding curves, we conclude that a3–d1 and a3–d2 interactions in V-ATPases are essentially identical.

6.2 V-ATPase subunit a3-d2 interaction and inhibitor luteolin

6.2.1 High throughput screening for inhibitors of the a3-d2 interaction

By adapting the above described solid-phase binding assay to high throughput screening, we were able to perform a pilot-scale screen of natural and synthetic compounds for inhibitors of the a3-d2 interaction. A promising hit that was identified in this screen was the natural product flavonoid, luteolin. Performing a dose-response in the binding assay revealed that
luteolin had an IC$_{50}$ in the low micromolar range, suggesting that it might have therapeutic potential, and this notion was further reinforced by studies demonstrating its lack of toxicity in the therapeutic range, and its ability to inhibit osteoclast bone resorption with an EC$_{50}$ of approximately 2.5 µM.

6.2.2 Biological significance of $a$-d interaction and possible mechanism of inhibition

The apparent affinities observed for $a3$-d interactions (half maximal binding at 3-4nm) were similar to those previously observed for $a3$-B interactions (2-3nM) (78). These are relatively high apparent affinities, suggesting biological significance. In comparison, affinities obtained for $B2$-F-actin interaction (K$_d$=55 nM) and $a2$-ARNO (K$_d$=310 nM) are approximately 20-fold and 100-fold lower respectively, yet those interactions are known to be of biological importance (157,158).

What the biological significance of $a$-d subunit interaction might be remains largely speculative. Since the $a$ subunit is part of the V-ATPase stator and the $d$ subunit is part of the rotor it seems likely that any $a$-d interaction must be transient in the functional complex. Alternatively, it could be argued that the interaction is important for accurate alignment of the $V_1$ and $V_0$ sectors during regulatory dissociation and reassociation of the V-ATPase complex. It has also been speculated that the NT$a$ domain of the $a$ subunit collapses onto the $d$ subunit after $V_1$-$V_0$ dissociation to prevent reverse rotation of the rotor c-barrel from dissipating the established proton gradient. These alternative scenarios seem unlikely, as we did not observe any effect of luteolin on V-ATPase assembly or dissociation.

Single beam particle electron microscopy has revealed that V-ATPases are closely related to F$_1$F$_0$ –ATP synthases (F-ATPases) (159-161). F-ATPases carry out sequential catalysis at three sites, radially separated by 120° around the rotor axis (162). Shimabukuro et al showed that each step of F$_1$ rotor rotation is divided into two substeps, 80° for ATP binding, and 40° for ADP/phosphate release (163). In contrast, Yokoyama and co-workers showed that, irrespective of ATP concentration, $V_1$ dwells at 120° intervals, or once per catalytic cycle, without substeps (164). It seems possible that $a$-d subunit interaction occurs during the “transient dwell time” of the proton translocation cycle. Moreover, it has been noted that for
optimum function of V-ATPases, all the different subunits in the V₁ and V₀ sectors must be in correct alignment. We speculate that the transient a-d interaction may serve to align the complex during the dwell time. Interference with this putative alignment mechanism could significantly impair the efficiency of rotation of the rotor complex, impacting the tightly coupled catalytic function of the V₁ sector.

6.2.3 Discrepancies with existing literature on luteolin inhibition of osteoclast function

Luteolin has been previously characterized for its inhibitor effect upon osteoclasts (148,165) and its ability to decrease ovariectomy-induced bone loss (149). The results that we have presented here and those of the existing literature are in agreement with respect to the anti-resorptive properties of luteolin, yet there are significant differences in proposed mechanism. We show that at low micromolar concentrations luteolin inhibits osteoclast resorptive activity without affecting osteoclast differentiation. In contrast, Lee et al., (148) and Kim et al., (149) both conclude that anti-resorptive properties of luteolin are due to its inhibitory effect on osteoclast differentiation.

There are numerous potential explanations for the differences within the existing literature and with the present work. One variable is the source of luteolin itself. Zhang et al., (165) purified luteolin from the plant Halenia comiculata, while Lee et al., (148) and Kim et al., (149) do not mention its source. Zhang et al., (165) and Kim et al., (149) used DMSO as the carrier and none of the three papers comment on the purity. Here we use ≥98% pure luteolin (Sigma catlog. No. L9283) in an aqueous stock solution. The difficulty in comparing the existing literature with our work is compounded by the differences in methodology and source of osteoclasts (e.g. vitamin D-induced co-cultures; or M-CSF and RANKL-induced bone marrow macrophages; or RANKL and PD98059-induced RAW 264.7 cells).

When direct comparisons are possible, substantial differences are seen within the published literature. For example, Lee et al., (148) and Kim et al., (149) report that when assaying differentiation of BMM-derived mouse osteoclasts, 1 μM luteolin results in 5% or 75% inhibition, respectively. In contrast, at 1 μM luteolin, we found no significant inhibition of differentiation. The difference with respect to BMM-derived osteoclasts most likely results from
differences in culture conditions, and the timing of luteolin addition to the cultures. In the present work, BMM cells were first cultured for 2 days with M-CSF prior to RANKL differentiation in presence of luteolin. It has been previously noted that this 2 day pre-incubation period in M-CSF is essential for optimal osteoclast differentiation (166). In contrast, Kim et al., (149) include both RANKL and luteolin at day 1 culture. Furthermore, in their discussion, the authors note that addition of luteolin one day after the cultures were initiated did not significantly affect TRAP activity (data not shown), in complete agreement with our present results.

With reports to the use of RAW 264.7 cells, Lee et al. differentiated RAW 264.7 cells in the presence of both 100 ng/ml RANKL and PD98059, an inhibitor of the mitogen-activated protein kinases, MEK1 and MEK2 (167); the addition of PD98059 was to enhance osteoclastogenesis. We do not use PD98059 and obtain comparable amounts of RAW 264.7 derived osteoclasts with 100 ng/ml RANKL alone. Although one might expect the presence of PD98059 to attenuate any inhibitor effect on osteoclastogenesis, mitogen-activated protein kinases are involved in numerous other cellular processes such as cell proliferation, motility and apoptosis (168), and could possibly augment the effect of luteolin. As Kim et al., do not present any histological data with their ovariectomy-induced bone loss model, the ultimate question of whether luteolin protects against bone loss by inhibiting just osteoclast activity, or osteoclastogenesis, in vivo remains unanswered.

6.2.4 Antiresorptive therapeutic potential of luteolin

Although a3 has the same apparent affinity for either d1 or d2, lack of specificity may not be an issue affecting therapeutic targeting. The differential expression patterns and functional differences between the two d isoforms. The amino acid identity between the two murine d isoforms is only 68%; this difference is reflected in the fact that in yeast, the murine d1 can functionally complement yeast d ortholog (Vma6p) deletion, at 30 °C, while d2 could only partially complement at 25 °C (169). While d1 is ubiquitously expressed, d2 expression is limited to bone and at lower levels in kidney, heart, spleen and testis (119,169). Within bone, d2 is restricted to osteoclasts where expression levels increase over 50-fold during differentiation (119). Finally, while ablation of d1 is embryonically lethal (170) knocking out d2 in mice results
in only a mild osteopetrotic phenotype (156). The absence of d2 in mice not only resulted in decreased osteoclast maturation and activity, but also increased osteoblast formation, an ideal outcome for an anti-resorptive therapeutic (171). Using a solid phase ELISA-based binding assay adapted for high throughput screening, we identified luteolin as a compound that could inhibit the a3-d2 interaction and subsequently confirmed that it decreases osteoclast resorptive activity.

### 6.2.5 V-ATPase as the proximal target for multiple effects of luteolin

V-ATPase inhibition by luteolin may explain some of the other potentially therapeutic effects that have been reported for the compound, including its anti-cancer, anti-viral and anti-inflammatory properties. Luteolin has been shown to decrease the viability and inhibit the invasive potential of human breast cancer cell line MDA-MB231 (144). Hinton et al., (107) in their studies reveal that MDA-MB231 cells express higher levels of V-ATPase a3 and a4 isoforms. They also show that siRNA knockdown of either a3 or a4 significantly inhibits the invasiveness of MDA-MB231 breast cancer cells is by targeting V-ATPase activity.

Similarly, luteolin has been previously shown to have the properties of an anti-viral agent preventing influenza virus infection (172). V-ATPase-mediated lysosomal acidification is required for influenza virus infection, enabling the release of the virus from its receptor and translocation into the cytoplasm. V-ATPase specific inhibitors have been shown to inhibit replication/transcription of viral RNAs (173). Thus, the proximal target of luteolin as an anti-viral agent may be the lysosomal V-ATPase.

### 6.1.6 Other cellular effects of luteolin

Osteolytic diseases create an imbalance between bone resorption and formation, and an ideal therapeutic would target osteoclast activity without affecting osteoclastogenesis (174,175). Luteolin did not affect RANKL-induced osteoclast differentiation and maturation. We examined the effect of luteolin on osteoclast genes nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), matrix metallopeptidase 9 (MMP9), cathepsin K (CTSK) and found no significant
difference between untreated and luteolin at concentrations 1.2, 10 and 20 µM (data not shown). Typically, during bone resorption, osteoclasts pump protons onto the bone surface to dissolve microcrystalline hydroxylapatite and activate acid proteases to enzymatically degrade bone. Osteoclasts resorb bone to a certain depth and then move on or undergo apoptosis. With time, there is an increase in number of resorption pits created by multiple osteoclasts thereby leading to an increase in total resorbed surface. In the case of luteolin, our studies suggest that in its presence osteoclasts continue to resorb bone; however, the size of the resorption pits are significantly diminished. In addition, this occurs with a decrease in the number of total resorptive events. We report that luteolin not only decreases the total resorption carried out by osteoclasts, but also limits the depth of the resorptive events that occur by inhibiting the V-ATPase $a3-d2$ interaction. Pit depth and pit area were both significantly reduced in comparison to untreated cells in the presence of luteolin; however, estimated pit area was shown to significantly decrease, while an increase was observed for pit depth with increasing concentration. The inverse correlation was an interesting observation, since the trend for compounds that inhibit osteoclast activity is that pit depth and pit area are both significantly reduced. We postulate that reduction of pit area is due to reduction in osteoclast resorption activity, and a concomitant increase in pit depth might be observed due to the effect that luteolin may have on osteoclast migration.

Our initial hypothesis was based on the current accepted model of V-ATPase structure and activity. We hypothesized that the $a3$ and $d2$ subunits do not interact under normal conditions and interact when $V_1$ domain and $V_0$ domain dissociate. However our studies suggest otherwise. We expected to see, in presence of luteolin the $V_1$ domain and $V_0$ domain would dissociate. However, immunoblots of osteoclast membrane revealed that V-ATPase subunits $a3$, $d2$ and $E$ are synthesized normally and are not retained in the ER, or rapidly degraded. Based on our results, we therefore concluded that our initial hypothesis was wrong. In order to further characterize luteolins action on V-ATPase additional experiments will be required to be performed. Experiments such as ATP hydrolysis assay and proton transport assay to further elucidate the role of luteolin.
6.2 V-ATPase subunit a3-B2 interaction inhibitor KM91104

Previously our lab showed that the a3-B2 interaction has a moderately higher affinity than other subunit pairs. This may drive a3-B2 formation in preference to other combinations when the two subunits are available. The functional significance of the NTa3-B2 interaction remains to be determined, but speculatively it may be important to the stator function of the a subunit, its role in docking of the V1 to the V0 sector and possibly in intra-complex regulatory signal transduction mediated by the a subunit in response to pH or cellular energy status (58,84,87,176). We have exploited the knowledge of the a3-B2 interaction to screen for inhibitors, using a simple in vitro a3-B2 ELISA binding model. It was largely speculative that such an inhibitor might also disrupt V-ATPase function in cells; however, one compound, KM91104, identified as an inhibitor in the primary screen has proved to inhibit bone resorption by osteoclasts, which is entirely dependent on inhibiting V-ATPase-mediated acid secretion without affecting osteoclastogenesis. The data of Fig. 14-16 lend support to the initial assumption that small molecule inhibitors of a3-B2 interaction might destabilize the V1/V0 association of the V-ATPase complex that is required for active proton translocation in vivo. It is also noteworthy that the IC50 for inhibition of osteoclast resorption (approx. 1.2 µM) is very similar to the IC50 seen for in vitro inhibition of a3-B2 protein interaction (2.3 µM).

The broader cell biological mechanism of action of KM91104 inhibition of a3-B2 remains to be determined; it could be via disruption of catalytic function, disruption of proton translocation, interference with V-ATPase trafficking to the plasma membrane, interfering with actin ring formation, or other possible scenarios. These scenarios can be further investigated carrying out additional experiments: F-actin staining and acridine orange staining can elucidate the effect KM91104 has on actin ring formation or acidification in osteoclasts. Western blot analysis on osteoclast membrane preparation can further clarify the effect of KM91104 on osteoclast V-ATPase subunit formation and assembly. ATP hydrolysis and proton transport assay can further be utilized to observe the effect of KM91104 on proton translocation. Additionally experiments can be carried out to test the effect of KM91104 on osteoblasts.

Nevertheless, the data presented here suggest that development of a targeted inhibitor of a3-B2 interaction that exploits differences among the a subunit polypeptide sequences may be possible and might be useful in regulating osteoclast bone resorption activity at doses that preclude cytotoxicity or have appreciable effect upon osteoclast differentiation or maturation.
6.3 Summary

In summary, we show here for that the V-ATPase subunits $a3$ and $d2$ interact. A compound selected to inhibit the $a3$-$d2$ interaction (luteolin) and $a3$-$B2$ interaction (KM91104) reduced osteoclast resorption at concentrations that had no appreciable effect on osteoclastogenesis. A therapeutic that blocks resorption but not osteoclastogenesis would be advantageous over bisphosphonates, by preserving osteoclast-osteoblast signalling, and thus could potentially be used concurrently and synergistically with osteoblast bone formation therapies. Our data also validates our approach for screening chemical libraries for potential osteoclast V-ATPase targeted bone-loss therapeutics, and supports the notion that the $a3$-$d2$ interaction is biologically important.

Bisphosphonates accumulate in bone and cause apoptosis in resorbing osteoclasts, breaking the bone remodelling cycle. In addition, bisphosphonates have also been shown to cause problems with gastrointestinal tract of patients leading to side effects such as oesophageal ulcers and gastrointestinal infections, they have also been shown to reduce the efficacy of Teriparatide, a parathyroid hormone related protein that encourages bone formation. Luteolin also has been shown to decrease ATP production in cancer cells in vitro, thereby decreasing biosynthesis and proliferation. Accumulating evidence suggests that luteolin has a wide range of biological activities and mechanisms of actions. Some of these effects indirectly are mediated by V-ATPases activity. Luteolin might be targeting V-ATPases first upstream before the downstream processes occur.
CHAPTER 7: FUTURE PLANS

Luteolin and KM91104 in this study have been validated as good anti-resorptives. Although luteolin and KM91104 were identified as inhibitors of the V-ATPase a3-d2 and a3-B2 ELISA inhibitor screening, there are limitations to their potential applications. Luteolin cannot be developed as a specific therapeutic since its current applications are widespread. Researchers have identified luteolin to have several different properties such as anti-inflammatory, anti-tumour, anti-carcinogenic, anti-microbial to name a few. Although others and we have identified luteolin has as a excellent anti-resorptive, its application as a drug will most likely not be explored since it lacks specificity. KM91104, is a chemical compound obtained through a library screen and Dr. Rima Al-Awar (OICR) researched its potential stability. Her findings concluded that KM91104 would rapidly be degraded in the gut; its approximate half-life was estimated roughly to be 2 hours. The stability of KM91104 therefore limits its potential application as an anti-resorptive agent.

However regardless of the limitations of the two compounds discovered here, the technique used to discover these compounds can be utilized to discover additional compounds by screening several other large libraries that have not been investigated yet. The natural extension of this project can be to discover several other compounds with low B scores against either the a3-B2 interaction or the a3-d2 interaction and carry out secondary screening assays to screen against toxicity, osteoclastogenesis and resorption. Ideally these compounds should display characteristics that enhance osteoblast differentiation and reduce excessive osteoclast resorption at low micromolar or nanomolar concentrations.

After initial characterization of the high impact compounds, in-vivo models such as OVX-induced mice or IL-1 induced mice that have extensive calvarial resorption can be utilised to further gauge the potential of these compounds as anti-resorptives. The compounds can be further validated in human osteoclasts differentiated from CD14+ monocytes sorted from peripheral blood mononuclear cells.

This thesis seeks to establish an alternative approach towards discovering novel anti-resorptives. Proof of principle has been established here and it serves as a basis for discovery of new compounds that are specific, stable and exhibit high efficacy as anti-resorptive agent.
CHAPTER 8: REFERENCES


171. Choi, E. M. (2011) *Toxicol In Vitro*