Targeting Histone Deacetylases to Suppress Bone Loss in Similar Chronic Inflammatory Diseases, Periodontitis and Rheumatoid Arthritis

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Table of Abbreviations

HDAC	Histone deacetylase
HAT	Histone acetyltransferase
HDACi	Histone deacetylase inhibitors
PD	Periodontitis
RA	Rheumatoid arthritis
TNF-α	Tumour necrosis factor alpha
IL	Interleukin
M-CSF	Macrophage colony stimulating factor
RANKL	Receptor activator of nuclear factor kappa B ligand
RANK	Receptor activator of nuclear factor kappa B
NFATc1	Nuclear Factor of Activated T cells
NF-κB	Nuclear factor kappa B
OPG	Osteoprotegerin
TRAF-6	TNF receptor factor-6
AP-1	Activator protein-1
IKK	IκB kinase
CTR	Calcitonin receptor
TRAP	Tartrate Resistant Acid Phosphatase
OSCAR	Osteoclast-associated receptor
CAIA	Collagen antibody induced arthritis
LPS	Lipopolysaccharide
Micro CT	Micro Computed Tomography
DMARDs	Disease modifying anti-arthritic drugs
MTX	Methotrexate

Cath-K	Cathepsin K
mAb	Monoclonal antibody
SAHA	Suberoxylanilide hydroxamic acid
TSA	Trichostatin A
PBMCs	Peripheral blood mononuclear cells
MCP-1	Monocyte Chemotactic Protein 1
MIP-1α	Macrophage Inflammatory Protein 1α
CIA	Collagen induced arthritis
PTH	Parathyroid hormone
IFN-β	Interferon Beta
IFN-γ	Interferon Gamma
LPS	Lipopolysaccharide
MMP	Matrix metallo-proteinase
BMD	Bone mineral density

Abstract

Rheumatoid arthritis (RA) and periodontitis are two common chronic inflammatory diseases characterized by soft tissue inflammation and associated bone loss. Despite the high prevalence of these conditions and our growing knowledge of the mechanisms involved in the disease processes, the control of bone destruction is still a challenging problem. For this reason it is important to identify anti-resorptive agents that may also inhibit inflammation which can be delivered orally upon diagnosis. Histone deacetylase inhibitors (HDACi) are one such potential therapeutic agent. The aim of this research was to use *in vitro* human peripheral blood mononuclear cells and human osteoclast assays in conjunction with animal models of periodontitis and inflammatory arthritis to determine the effects of novel HDACi (1179.4b which targets class I and II HDACs and NW-21 targets HDAC 1) on both inflammation and bone loss. The results of this thesis have identified that both RA and periodontitis are interrelated diseases, however, the specific HDACs involved in regulating the inflammatory and resorptive processes may be distinct. It is evident that, in arthritis, HDAC 1 is important in tissue inflammation, in periodontitis HDAC 1, 5, 8 and 9 are important and in osteoclasts HDAC 5 and 8 are up regulated. HDACi such as 1179.4b, NW-21 and MS-275 (class I specific HDACi) have been shown to have the potential to treat inflammatory bone loss. Further studies are necessary to elucidate the roles of each HDAC in RA and periodontitis and better target HDACi therapy.

Student Declaration

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***Inhibitors of histone deacetylases in Class I and Class II suppress human osteoclasts in vitro.** MD Cantley, DP Fairlie, PM Bartold, KD Rainsford, GT Le, AJ Lucke, CA Holding, DR. Haynes. *Journal of Cellular Physiology* 2011;226(12):3233-41

Histone deacetylase inhibitors and periodontal bone loss. MD Cantley, PM Bartold, V Marino, DP Fairlie, GT Le, AJ Lucke, DR Haynes. *Journal of Periodontal Research* 2011;46(6):697-703

Pre-existing periodontitis exacerbates experimental arthritis in a mouse model.
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Histone deacetylase inhibitors as suppressors of bone destruction in inflammatory diseases. MD Cantley, PM Bartold, DP Fairlie, KD Rainsford, DR Haynes. *J Pharmacy and Pharmacology* 2012;64(6):763-74

Epigenetic regulation of inflammation: progressing from broad acting histone deacetylase (HDAC) inhibitors to targeting specific HDACs. MD Cantley, DR Haynes. *Inflammopharmacology* 2013;21(4):301-7.

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Date: / /2013

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List of Jointly Authored Papers Included in Thesis

Chapter 2. Pre-existing periodontitis exacerbates experimental arthritis in a mouse model.
MD Cantley, DR Haynes, V Marino, PM Bartold.
Journal of Clinical Periodontology 2011;38:532–541

Chapter 3. Inhibitors of histone deacetylases in Class I and Class II suppress human osteoclasts *in vitro.*
MD Cantley, DP Fairlie, PM Bartold, KD Rainsford, GT Le, AJ Lucke, CA Holding, DR Haynes.
Journal of Cellular Physiology 2011;226(12):3233-41

Chapter 4. Histone deacetylase inhibitors and periodontal bone loss.
MD Cantley, PM Bartold, V Marino, DP Fairlie, GT Le, AJ Lucke, DR Haynes.
Journal of Periodontal Research 2011;46(6):697-703

Chapter 5. Class I and I histone deacetylase (HDAC) expression in human periodontitis.
MD Cantley, TN Crotti, PM Bartold, DR Haynes.
(Manuscript in preparation)

Chapter 6. Targeting HDAC 1 to suppress both inflammation and bone loss in arthritis.
MD Cantley, DP Fairlie, PM Bartold, V Marino, DR Haynes.
(Manuscript in preparation)

Papers Included in Appendices

Appendix 1. Histone deacetylase inhibitors as suppressors of bone destruction in inflammatory diseases.
MD Cantley, PM Bartold, DP Fairlie, KD Rainsford, DR Haynes.
J Pharmacy and Pharmacology 2012;64(6):763-74

Appendix 2. Epigenetic regulation of inflammation: progressing from broad acting histone deacetylase (HDAC) inhibitors to targeting specific HDACs.
MD Cantley, DR Haynes.
Inflammopharmacology 2013;21(4):301-7.

Chapter 1. Histone Deacetylase Inhibitors as Suppressors of Bone Destruction in Inflammatory Diseases

Chapter 1 incorporates review paper: Histone deacetylase inhibitors as suppressors of bone destruction in inflammatory diseases. **MD Cantley**, PM Bartold, DP Fairlie, KD Rainsford, DR Haynes. *J Pharmacy and Pharmacology* 2012;64(6):763-74 (Appendix 1)

1.1 Introduction

Chronic inflammatory diseases such as periodontitis (PD) and rheumatoid arthritis (RA) are commonly associated with destruction of the bone due to stimulation of tissue damaging cells and enzymes by inflammatory mediators. These two diseases share many similarities, with both demonstrating an exuberant chronic inflammatory reaction associated with high numbers of pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α) and interleukins (IL), along with eventual destruction of both the soft and hard tissues. Progressive joint destruction is a hallmark of RA and ultimately results in a painful debilitating condition (73, 84). Alveolar bone loss which is characteristic of PD can eventually result in tooth loss, if left untreated. Despite the high prevalence of these conditions, and a growing knowledge of the mechanisms involved in the disease processes, the control of bone destruction is still a challenging problem (72, 74, 77, 130, 178). The aim of most pharmacological therapies in RA is to resolve the inflammation, however whilst this may have a positive effect on disease activity it does not always control the bone loss (49, 83). It may also take a considerable time for clinicians to find an appropriate treatment tailored for individuals. During this time there is likely to be continued destruction of the bone (78, 193). In PD, current treatment focuses mainly on targeting the infection and inflammation aspects of the disease. It is therefore clear that administration of an anti-resorptive therapy early in the disease process would be most beneficial suppressing the destruction of bone and treating these debilitating diseases.

1.2 Normal Bone Remodeling

Bone is a specialized type of connective tissue and is one of the few tissue types able to undergo mineralization (110). Skeletal tissue is composed of both organic and mineral (inorganic) components (117). The inorganic component consists of calcium hydroxyapatite providing strength and hardness, whilst the organic component includes the cells residing in bone along with a variety of extra cellular matrix proteins (110). Bone is a dynamic tissue with its constituents being continually remodeled to ensure a maintenance of a constant bone mass (170). The continuous remodeling also enables maximal effectiveness for mechanical adaptation and also helps to maintain plasma calcium levels for normal homeostatic functioning (181). It is estimated that a volume equalling 10% of the total human skeleton is completely remodeled every year (128, 157).

During bone remodelling, both formation and resorption occur concurrently ensuring a balance exists along with a constant bone mass. Remodeling is a complex process involving a series of highly regulated steps that are regulated by both paracrine and autocrine factors and also by external factors such as mechanical stresses (Figure 1.1) (45, 157). The many hormones, such as calcitonin, parathyroid hormone (PTH), and cytokines including TNF-α and interleukins, that play a role in regulating this process act upon the bone cells, the osteoclasts and osteoblasts (79).

Osteoblasts are derived from mesenchymal precursors located on the surface of bone and are responsible for bone deposition. Metabolically active osteoblasts live for approximately 3 months and then undergo apoptosis or become surrounded by matrix forming osteocytes, which are the most numerous cells in bone. Osteocytes communicate with one another via a network of narrow tunnels known as cannaliculi and play an important role in regulating the bone remodeling process (149). Osteoclasts are large multinucleated cells (50 to 100

μM diameter) derived from hematopoietic progenitor cells, and are responsible for bone resorption using a combination of acid secretions to dissolve the mineral components and cysteine proteinases to degrade the organic component (128).

In cortical bone, remodeling is carried out in a unique complex structure known as a basic multicellular unit (BMU), consisting of osteoclasts and osteoblasts functioning in a highly coordinated sequence along with connective tissue, blood vessels and nerves (128, 157). These BMUs in cortical bone have an average lifespan of 6 to 9 months (128, 157). During the activation phase, osteoblasts and osteoclast precursor cells interact with one another, resulting in the formation of mature osteoclasts that degrade bone during the resorption phase. During the reversal stage, bone resorption is completed and osteoclasts release signals that initiate the formation of functional osteoblasts that subsequently lay down the bone matrix (128, 157). Osteoclasts also undergo apoptosis during this stage. The formation stage involves the deposition of new, healthy osteoid that is unmineralised collagen matrix by osteoblasts, followed by deposition of a mineralized component, hydroxyapatite. The mineralisation stage is also regulated by osteoblasts through control over local calcium and phosphate concentrations (128, 157). The resorption phase is short (2 to 4 weeks), whilst the formation phase continues for approximately 3 to 6 months, as shown in Figure 1.1 (128). If the balance between bone formation and resorption is not maintained and there is enhanced bone resorption or decreased bone formation that is not compensated for, then pathological bone loss occurs.

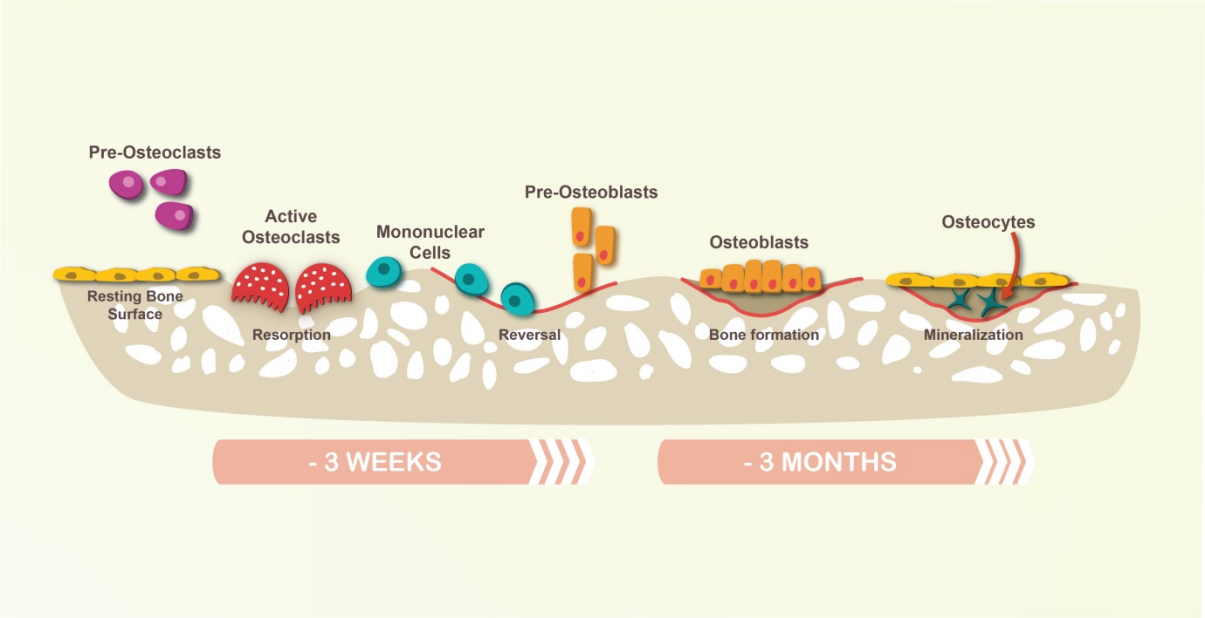

Figure 1.1. The process of bone remodeling in healthy bone (134). The resorption stage continues for 2 to 4 weeks whereas the formation phase lasts for 3 to 6 months. (Adapted from (134)).

1.3 Pathological Bone Loss

Pathological bone loss associated with inflammation is a common feature of many chronic inflammatory diseases, including periodontitis (PD) and rheumatoid arthritis (RA). In both these diseases an intimate relationship between the inflammation and bone resorption exists.

1.3.1 Periodontitis

Periodontitis (PD) is a common destructive inflammatory disorder of the tissues that surround and support the teeth known as the periodontium. Periodontal diseases, can range from the relatively benign form of gingivitis with 90% of individuals affected, 60% having chronic PD and 5–15% having aggressive PD (154). If PD remains untreated the tissue damage results in a loss of tooth support and can ultimately lead to loss of teeth. The main features of PD include destruction of the periodontal attachment apparatus, loss of crestal

alveolar bone, apical migration of the epithelial attachment and formation of periodontal pockets (151, 155). Inflammation and associated alveolar bone loss are the hallmark features (35). Disease progression is associated with environmental conditions, such as poor oral hygiene, cigarette smoking, systemic disease (rheumatoid arthritis, cardiovascular disease and diabetes), medications (i.e. steroids, anti-epilepsy drugs), ill-fitting bridges, crooked teeth and loose fillings (153).

PD is initiated by a microbial challenge, resulting in a host response to this foreign material. The microbial components of the bacteria, in particular lipopolysacchardide (LPS) which is a cell wall constituent of gram negative organisms, is known to induce leukocyte infiltration, oedema and vascular dilation in the inflamed area (51). This stimulates immune cell secretion including monocyte extravasation into the tissues and activates macrophages resulting in the synthesis of pro-inflammatory molecules, including IL-1β and TNF-α, within the inflamed gingival tissue and it is this process that initiates the soft and hard tissue destruction. The anaerobic gram negative bacterium *Porphyromonas gingivalis (P. gingivalis)* is the main species associated with PD along with others including *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* (4). Bacteria are necessary for disease initiation, however they are though not sufficient on their own to initiate the disease if there is no inflammatory reaction (154). The immune and inflammatory responses are critical in the pathogenesis of PD and are impacted on by a number of host related factors, including genetics (35). There are a large number of cytokines, cell signalling molecules and matrix metalloproteinases (MMPs) involved in the pathogenesis of PD (6). The cytokines produced during the host response to bacteria, such as IL-1β, IL-6, IL-11, IL-17, and TNF-α, up regulate expression of receptor activator of nuclear factor kappa B ligand (RANKL), both membrane bound on osteoblasts and the soluble form, by fibroblasts and lymphocytes as demonstrated in Figure 1.2 (6, 74, 124).

This leads to an enhanced formation of osteoclasts that degrade alveolar bone and it is this signalling process that is becoming an important therapeutic target. The role of RANKL will be discussed in more detail later.

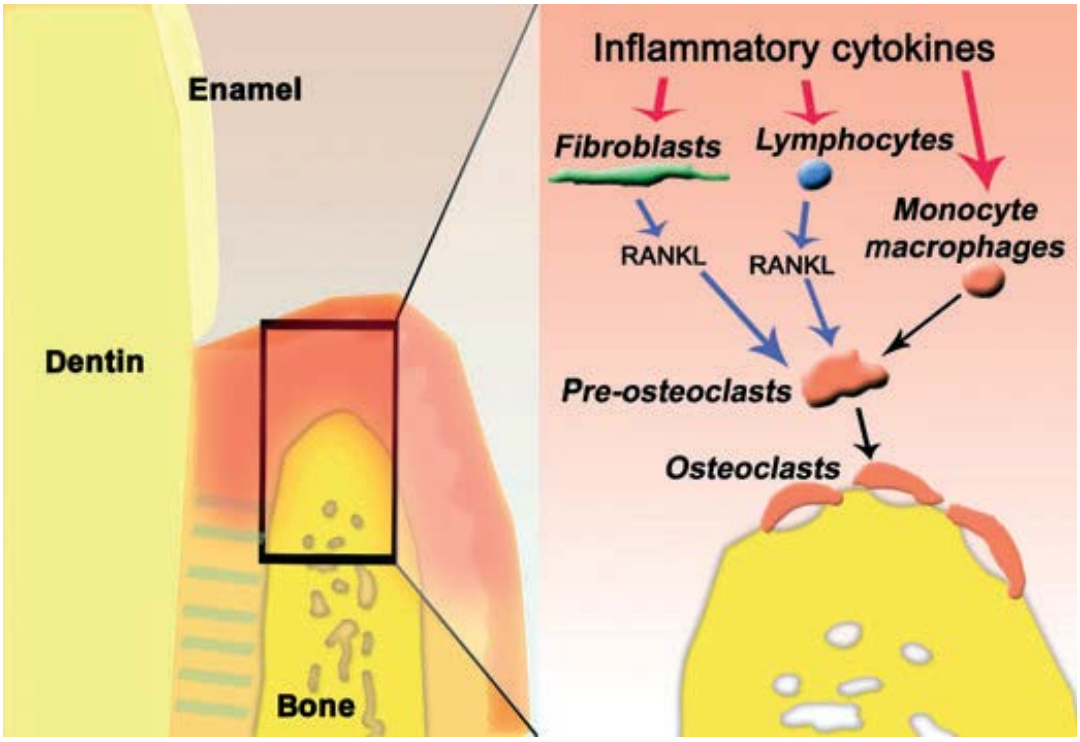

Figure 1.2. The mechanisms of bone destruction in periodontitis (6). The production of large numbers of inflammatory cytokines up regulate production of RANKL by lymphocytes and fibroblasts which binds to its receptor RANK on osteoclast precursor cells resulting in the formation of multinucleated osteoclast cells that resorb the alveolar bone. The cytokines can also directly activate monocytes or macrophages to differentiate into osteoclasts that resorb bone.

1.3.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common systemic inflammatory disease that is characterized by destruction of synovial joints. It commonly affects joints in the hand, such as, the metacarpo-phalangeal, proximal interphalangeal, mid-carpal, radio-carpal and distal radioulnar joints. Joints in the feet, such as, metatarso-phalangeal, proximal interphalangeal and the intertarsal joint are also commonly affected. This condition has a major impact on the normal functioning of the affected joints. RA is known to be the most

common autoimmune disease in Australia (203) affecting 400,000 Australians and currently affects 1% of the world's population (204). According to the Australian Institute of Health and Welfare "*In 2004–05, rheumatoid arthritis accounted for 4% ($175 million) of total expenditure on arthritis and other musculoskeletal conditions. Of this, 53% was spent on prescribed pharmaceuticals and the remainder mostly on out-of-hospital services (25%) and hospital services for admitted patients (20%)"* (203). This demonstrates the significant burden that RA places on not only the economy but also in the well-being and quality of life of the individuals affected.

RA is characterized by joint inflammation, synovial hyperplasia and associated destruction of bone and cartilage with the hallmark feature being progressive joint destruction (59). Figure 1.3 demonstrates a radiograph revealing areas of bone loss.

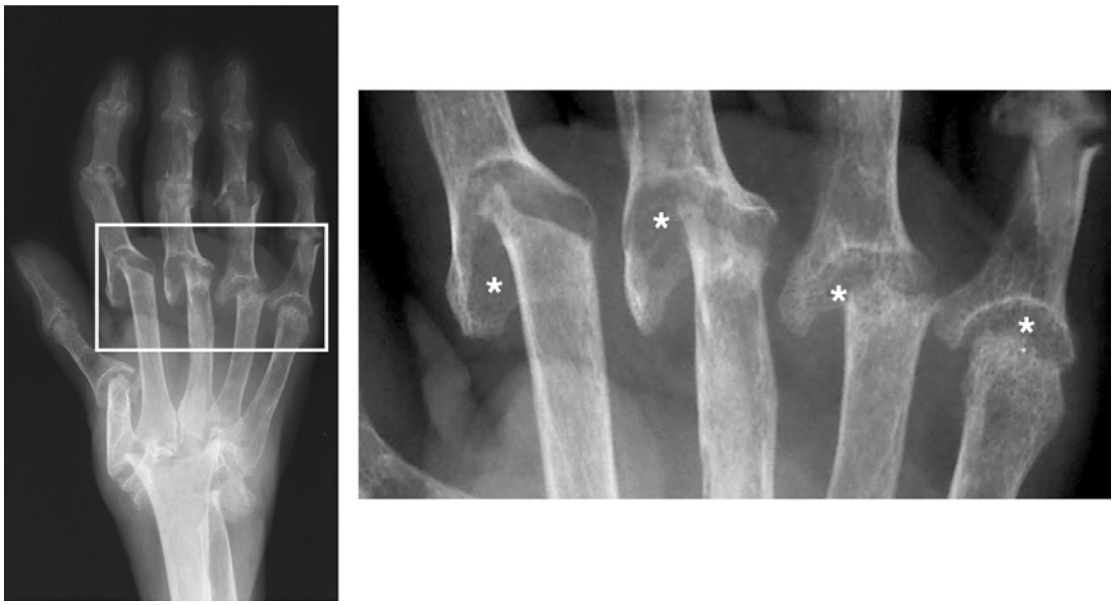

Figure 1.3. Radiograph of a hand with RA (177). Asterisks demonstrate areas of bone erosion.

The disease process and inflammation cause pain, stiffness and swelling of the joints. The recurring inflammation results in destruction of both the cartilage and bone impacting on the individual's physical functioning causing both short and long term morbidity (113).

The synovial membrane is the main site of immune cell infiltration and proliferation resulting in inflammation of the membrane. The expansion of the synovial tissue results in the formation of the pannus that invades the bone and cartilage (201). Similar to PD an array of cytokines are produced within the synovial tissues which stimulate expression of RANKL by fibroblasts and lymphocytes resulting osteoclastogenesis (21, 172). This increase in bone resorbing osteoclasts leads to focal articular bone erosion at the site of pannus formation (42).

Systemic bone loss very similar to osteoporosis is often observed in RA (171, 201). Patients with RA have generalised bone loss, known as osteopenia that results in a loss of bone mineral density (BMD) and an elevated risk of osteoporotic fractures (47). Clinical studies have demonstrated a reduction in BMD and disease severity in the hands of patients with early RA (67, 68). This increased systemic bone loss is attributed to the effects of pro-inflammatory cytokines, such as TNF-α, released from the site of synovial inflammation that act systemically (67, 68). Long term use of glucocorticoids as a treatment for RA has also been known to be associated with the development of systemic bone loss. Increased risk of fracture in the hip and vertebra have been dose dependently associated with glucocorticoid treatment (44, 200). Glucocorticoids have also been shown to suppress osteoblast formation, hence this could explain the observed side effect of decreased BMD (178). Figure 1.4 demonstrates the features of both local and systemic bone loss in RA.

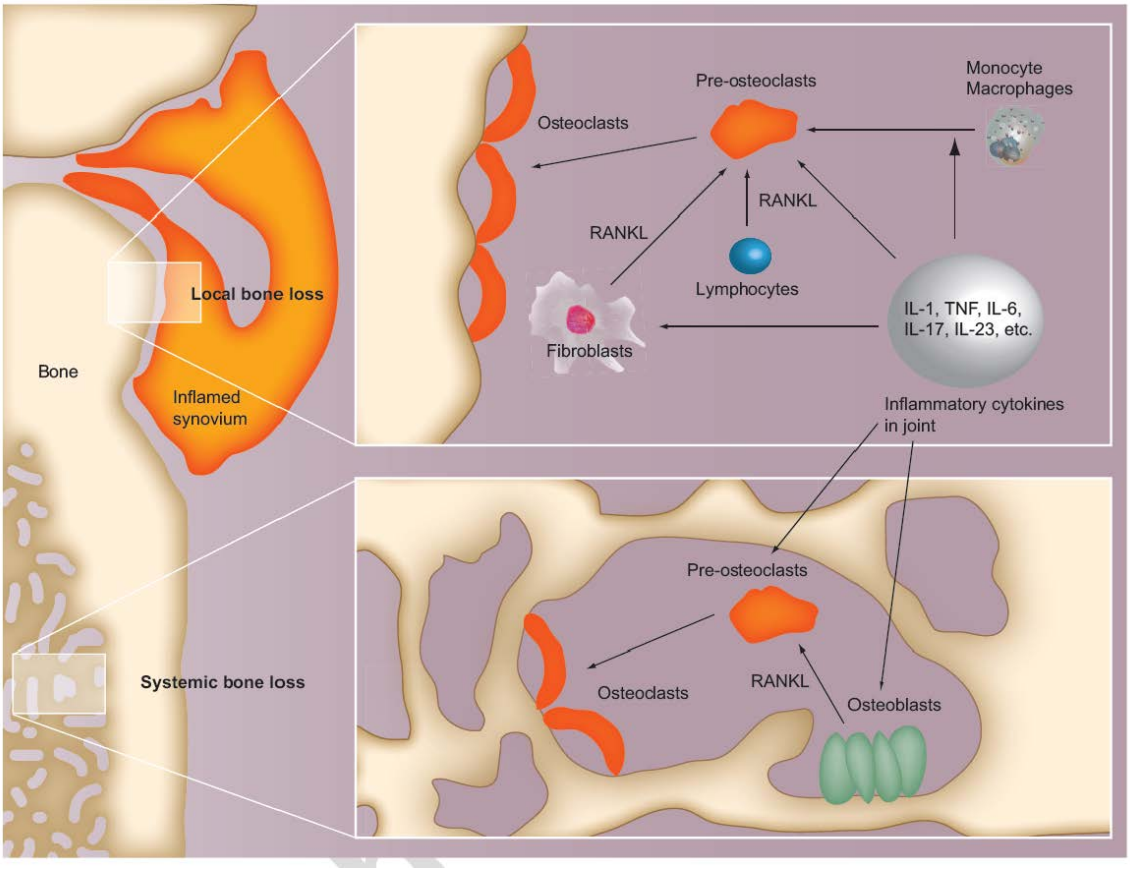

Figure 1.4. Mechanisms involved in the pathogenesis of bone destruction both locally and systematically in RA (26). In localized bone loss, the pro-inflammatory cytokines e.g. IL-1 and TNF-α result in up regulation of RANKL expression by fibroblasts and lymphocytes. RANKL binds its receptor RANK on pre-osteoclast cells, resulting in formation of mature osteoclasts that are able to resorb bone. There can also be direct activation of macrophages and monocytes promoting differentiation into osteoclasts (191). With systemic bone loss, the inflammatory cytokines activate osteoblasts leading to membrane bound RANKL expression and subsequent increased osteoclast bone resorption (80). Cytokines can also act directly on the pre-osteoclast cells in both types of bone loss as reviewed in (97, 201, 202).

1.3.3 Relationship between PD and RA

Traditionally RA and PD have been considered to be independent diseases but it is becoming more apparent that a relationship may exist between these two diseases. Both RA and PD are characterized by an exuberant inflammatory response with production of a large numbers of pro-inflammatory cytokines, such as, TNF-α and IL-1, 4, 6, 11 and 17 (6, 26). These cytokines stimulate destruction of both the soft and hard tissues of the joints in RA and the periodontium in PD (6, 26, 59). PD is initiated by a microbial challenge,

resulting in a host response whereas RA is an autoimmune disease with the causes being unknown and hence there are differences in the initiation phases of both these diseases.

An increasing number of studies have reported a relationship between PD and RA (139). A significantly higher incidence of tooth loss and alveolar bone loss have been found in patients with RA (2, 138) whilst other studies have also shown that PD may be a risk factor for developing RA or enhancing the severity of RA (71, 168). More recently, studies have also shown that treatment of periodontitis can reduce the severity of arthritis (1, 152, 168). Enhanced osteoclast formation and activity is a characteristic feature in both PD and RA as shown in figure 1.5. For this reason, in order to understand the bone loss processes and how it might be treated it is important to understand the relationship between the two diseases.

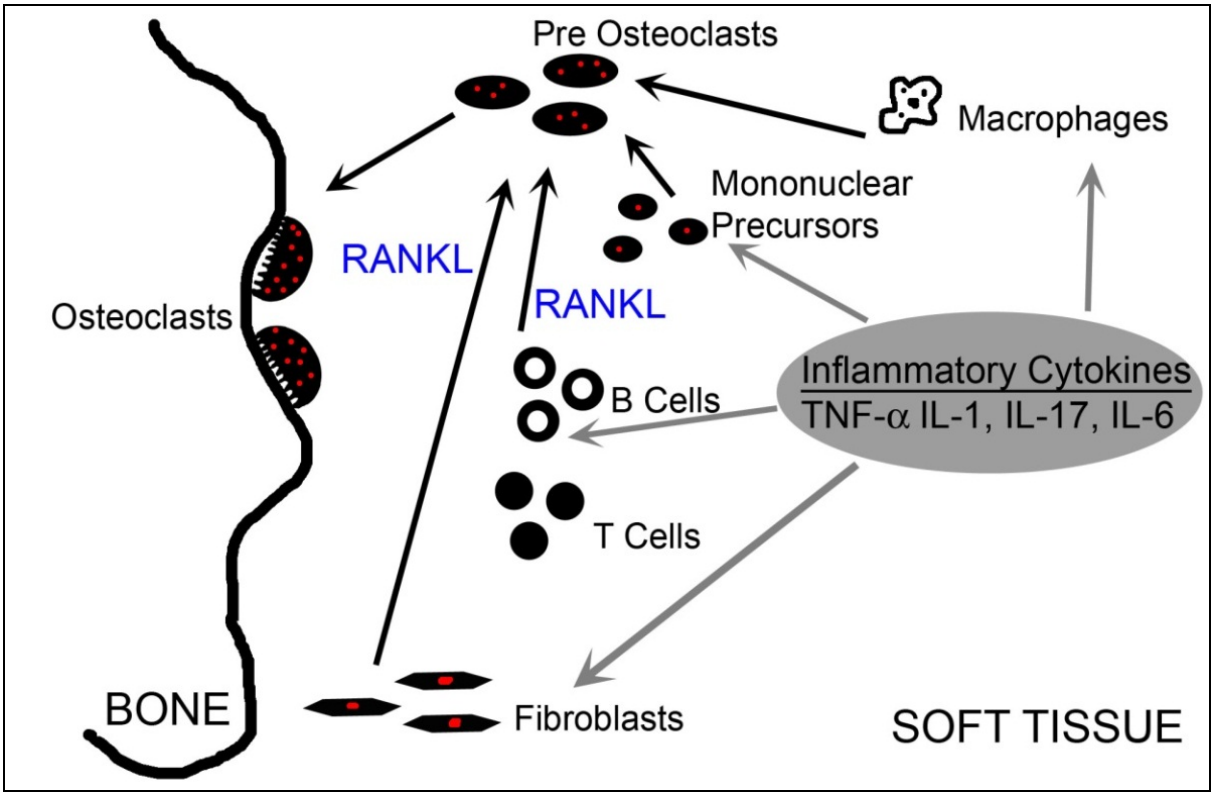

Figure 1.5. High levels of inflammatory cytokines including TNF-α and the IL 1, 6 and 17 drive expression of soluble RANKL by fibroblasts and lymphocytes. This results in increased osteoclast formation and hence bone loss.

1.4 Inflammation, Bone Loss and Osteoimmunology

Inflammation and bone loss are the hallmark features of both PD and RA. The involvement of the immune system appears to be fundamental to the excessive bone destruction in these diseases. In recent decades, this point has become increasingly recognized, with the relationship between the immune system and bone metabolism now being termed "osteoimmunology" as reviewed in multiples publications (123, 167, 188, 190). An understanding of this relationship is needed in order to develop ways of preventing as well as treating this pathological bone loss.

1.4.1 Osteoclasts

Osteoclasts are giant multinucleated cells derived from the haematopoietic lineage that are responsible for resorbing bone during both normal and pathological bone resorption (20, 117, 174). RANKL has recently been recognised as the key mediator of osteoclast differentiation, activation and survival (112, 207). RANKL is usually a membrane bound protein of the TNF family that is expressed by osteoblasts but may also exist in a soluble form produced by fibroblasts and activated T cells. A crucial step in osteoclast formation in both health and disease is the ligation of RANKL with its receptor RANK on osteoclast precursor cells resulting in activation of NF-κB along with other intercellular signalling molecules, leading to development of mature osteoclasts (see Figure 1.6) (174). RANKL has been shown to have an important link between immunology and bone physiology with inflammatory cytokines, such as TNF-α and IL-1, known to stimulate its production (82). The elevated expression of RANKL by inflammatory cells is known to be associated with common chronic inflammatory diseases including both RA and PD (39, 40, 72). Consequently, the interaction between RANKL and RANK along with the signal transduction mechanisms are becoming important targets for therapeutic intervention.

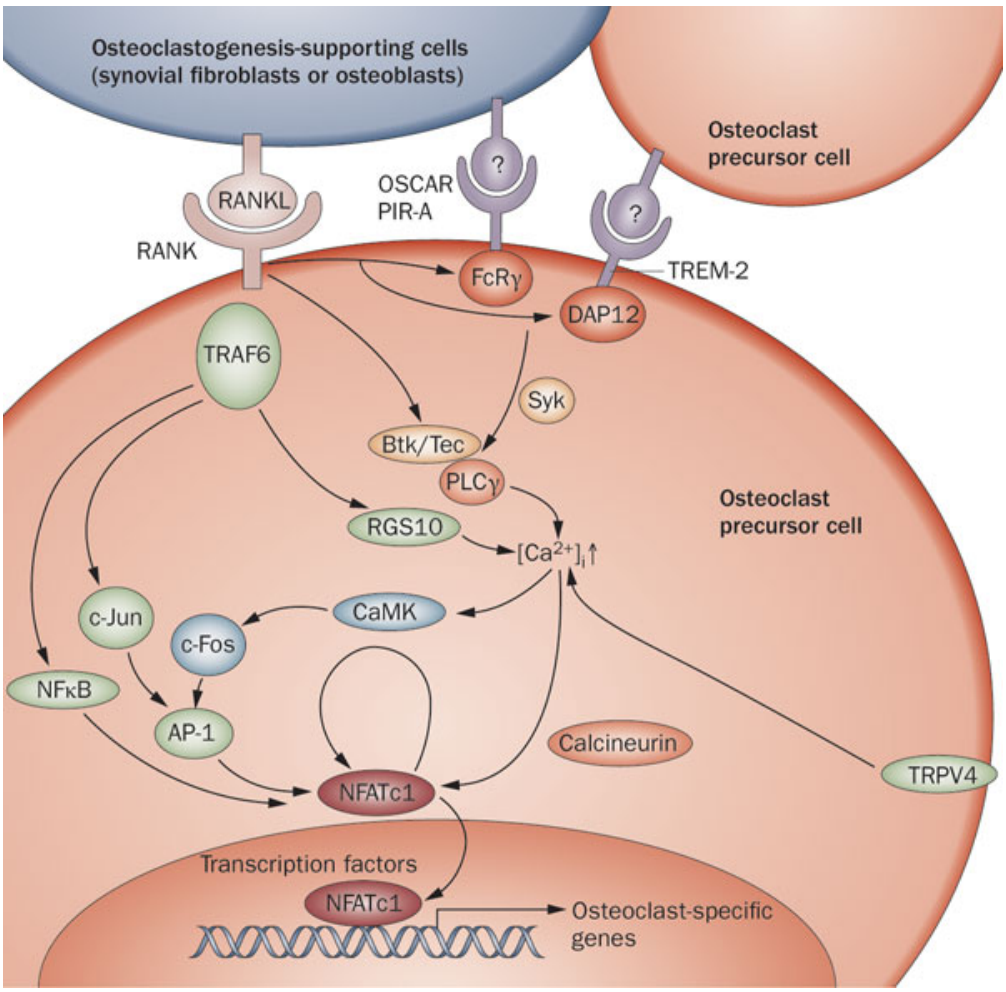

Figure 1.6. The process of osteoclast formation (189). RANKL binds to its receptor RANK on the osteoclast precursor cells, an essential step for osteoclast formation. This binding activates the intercellular signalling pathways which results in the expression of osteoclast specific genes including TRAP, cathepsin K and Osteoclast Associated Factor (OSCAR).

1.4.1.1 Osteoclast Bone Resorption

The process of osteoclast bone resorption is achieved over a series of stages with the osteoclasts initially attaching firmly to the bone surface via integrins such as αvβ3. This allows the formation of an F-actin ring enabling the development of a sealed zone. This sealed zone ensures enzymes and ions do not readily escape during the resorption process. Acid secretion as a result of electrogenic hydrogen ion transporting is responsible for dissolving the mineral component of bone. The generation of bicarbonate ions by carbonic anyhydrase and the release of H+ ions regulates in the intracellular pH. The organic component of bone is degraded by secretion of acid cysteine proteinases. Cathepsin K is

the major enzyme responsible for matrix degradation of the triple helix of native collagen at an acidic pH (16, 129). The degraded mineral is then transported in vesicles out of the cell and into the extra cellular environment. During the process of bone resorption the cell surface area is increased, forming the characteristic “ruffled border” of osteoclast as shown in Figure 1.7.

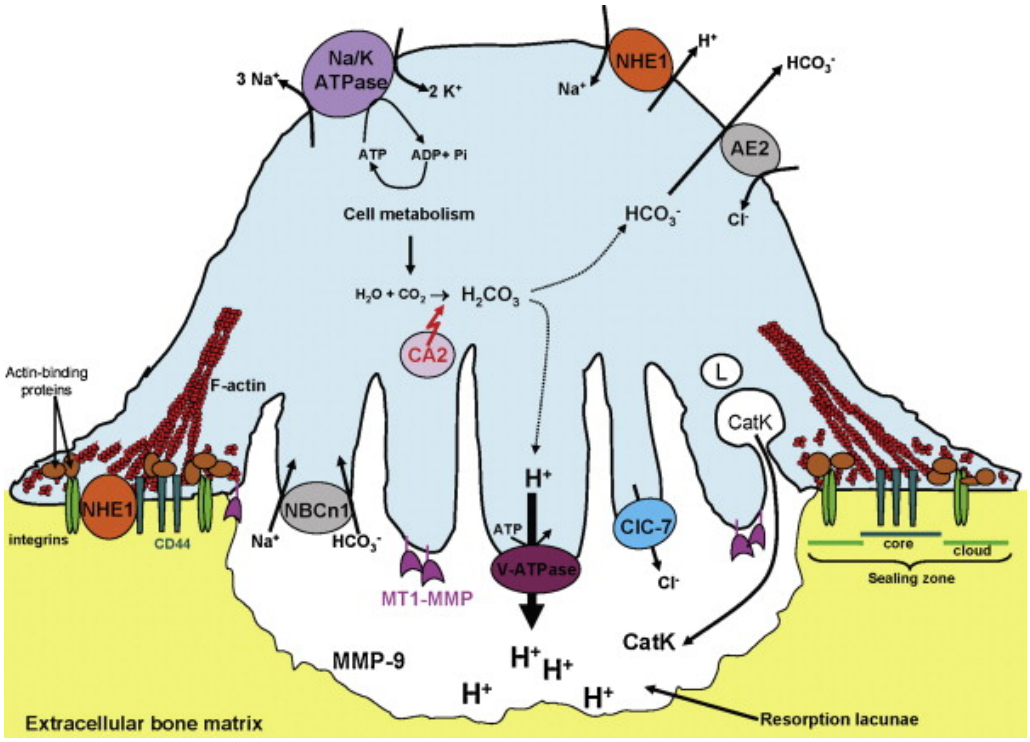

Figure 1.7. The process by which osteoclasts attach to and degrade bone (24). The organic component is degraded by secretion of acid cysteine proteinases whilst the mineral component is dissolved by acid secretion.

1.4.1.2 Role of Osteoclasts in Pathological Bone Loss

Enhanced osteoclast formation and resorption is the predominant feature in both RA and PD. The role of these cells has been demonstrated in a number of studies involving animal tissues, with mice lacking osteoclasts shown to be resistant to arthritis induced bone loss (159, 166). Enhanced osteoclast formation has also been demonstrated in human RA tissues (63, 75, 186, 198). In both animal models of inflammatory arthritis and human RA tissues, large multinucleated osteoclastic cells resorbing the subchondral bone have been

detected at sites of bone loss in the joints (111, 185). In a serum transfer model of arthritis, RANKL knockout and control mice developed inflammation, however, it was found that mice lacking osteoclasts through RANKL knockout were resistant to arthritis induced bone destruction (159). In PD tissue, multinucleated TRAP positive osteoclasts have also been detected (39). These studies emphasise the important role osteoclasts play in bone loss pathologies and highlight that they are appropriate targets for therapeutics.

1.4.1.3 Osteoclastogenesis

Osteoclastogenesis is the process by which mononucleated cells of the hematopoetic lineage differentiate into mature osteoclasts that are able to resorb bone. As they develop, osteoclasts develop specific phenotypical features including expression of calcitonin receptor (CTR), enzymes such as tartrate-resistant acid phosphatase (TRAP), carbonic anhydrase II, Cathepsin K (Cath-K) and more specifically the ability to resorb bone (28). These features can be useful in identifying osteoclasts in both *in vitro* and *in vivo* studies. Other markers include vitronectin receptor (VNR) and a vacuolar-type proton pump (28).

Macrophage colony stimulating factor (M-CSF), produced by osteoblastic bone marrow stromal cells, is a factor necessary for osteoclast formation. M-CSF binds to its receptor, Colony stimulating factor 1 (CSF-1) on early osteoclast precursors (28, 194) and enhances both the proliferation and survival of the precursor cells along with survival of mature osteoclasts (28). The essential role of M-CSF (known as CSF-1 in mice) in osteoclastogenesis has been demonstrated by studies in M-CSF knockout mice (op/op) who are osteopetrotic as a result of an inhibition of bone resorption. Daily injections of M-CSF reversed the osteopetrosis, further highlighting the importance of this factor in osteoclast formation (106). Previous studies have also demonstrated that M-CSF was able

to induce substantial osteoclast formation and bone resorption in cultures of human bone marrow stromal cells (176).

1.4.1.4 RANKL/OPG

RANKL forms an important link between immune and bone functions and is a crucial factor for osteoclast formation, function and survival (56). RANKL is expressed by osteoblasts but can also be released in a soluble form by activated T cells, fibroblasts and lymphocytes (72, 173, 195). During inflammation, RANKL is produced in response to inflammatory cytokines such as TNF-α and IL-1, as evidenced through the high expression of RANKL by inflammatory cells, such as synovial fibroblasts and activated T cells (40, 42, 72, 86).

The critical step in the process of osteoclast formation is RANKL binding to its receptor RANK, which was first identified in dendritic cells, responsible for processing and presenting antigens to T cells (174) (see Figure 1.7). RANK is a transmembrane protein of the TNF super family and is expressed by osteoclast progenitor cells as well as mature osteoclasts. Studies have demonstrated complete inhibition of osteoclast formation in RANK knockout mice resulting in the development of osteopetrosis (120). Reintroduction of RANK into these animals resulted in osteoclast formation (120).

RANKL-RANK binding activates the c-jun terminal kinase sending signals to NF-κB that stimulates the formation of active osteoclasts able to degrade bone (174). The importance of RANKL in the formation of osteoclasts was demonstrated in a RANKL knockout mice model in which mice were found to exhibit an osteopetrotic phenotype due to a lack of osteoclasts (159). Conversely, excessive administration of recombinant RANKL in mice

has been found to result in increased osteoclast formation resulting in bone resorption and hence the mice become osteoporotic (112).

Osteoprotegerin (OPG) is the natural decoy receptor that binds to RANKL preventing it from binding to its receptor RANK and hence inhibits osteoclast formation (see Figure 1.8). OPG which is also a member of the TNF superfamily was first reported on in 1997, with over expression found to inhibit osteoclastogenesis and it subsequently shown OPG knockout resulted in accelerated osteoclastogenesis and hence the development of osteoporosis (182). Over expression of OPG in transgenic mice was also found to inhibit osteoclast formation with the mice developing severe osteopetrosis (140). Administration of OPG to these mice was able to reduce the number of osteoclasts and hence prevent TNF-α mediated bone destruction (165).

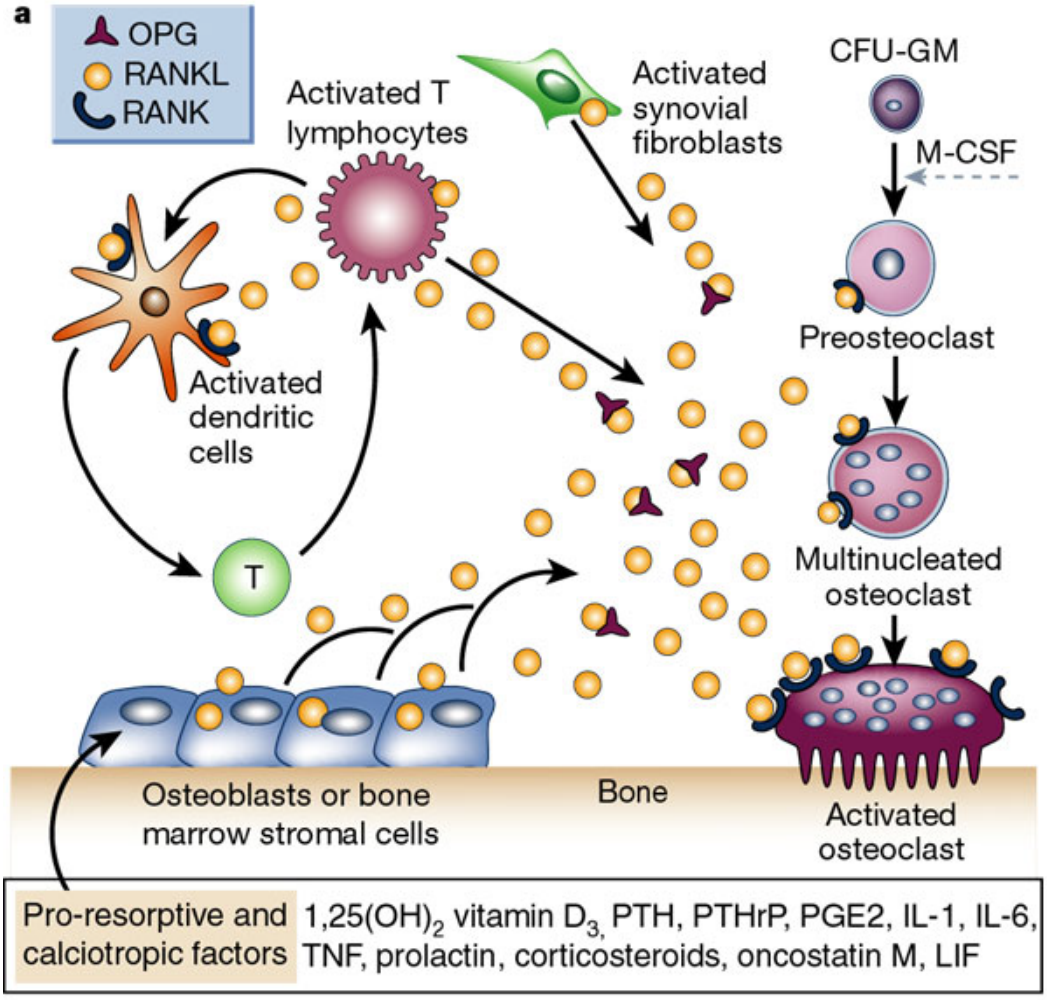

Figure 1.8. The role of RANKL and RANK in osteoclastogenesis (19).

Studies have demonstrated high levels of RANKL expression and low levels of OPG in human periodontitis tissues (39, 124, 141). In a murine model of PD, induced by *Actinobacilllius actinomyecetemcomitans*, there was increased RANKL production by CD4+T cells and in turn enhanced osteoclastic alveolar bone resorption (196). Similarly, in another study using PD induced by *P. gingivalis*, there was enhanced expression of RANKL in osteoblasts that led to the induction of osteoclastogenesis (30). The role of RANKL in RA has also been demonstrated using human tissues with elevated expression found in the synovium (40, 42). Higher levels of RANKL in the mononuclear aggregates and fibroblast like cells has been observed in the subintimal regions of the synovial membrane (42). The high expression of RANKL was shown to be largely confined to sites of bone resorption at the pannus-bone interface and subchondral bone erosion (160).

1.4.1.5 Intracellular Mediators of Osteoclast Formation

The binding of RANKL to its receptor RANK results in the activation of a number of intracellular mediators that play an important role in the formation of osteoclasts (see Figure 1.9). TRAF-6 is a member of the TNF receptor factor (TRAF) family of proteins that forms the important initial link between the ligation of RANK and the adaptor proteins downstream (197). The downstream targets of TRAF-6 include transcription factors such as NF-κB, activator protein-1 (AP-1) and NFATc1, cascades of mitogen-activated protein kinases (MAPKs) such as p38 stress kinase, c-Jun N-terminal kinase (JNK), ERK and Pi3K/AKT pathways (197). NF-κB is a pleiotropic transcription factor critical in RANK signalling, with knockout mice shown to develop osteopetrosis as a results of defective osteoclast formation (94). NF-κB proteins are located in the cytoplasm of non-stimulated cells and once RANK is activated it rapidly enters the nucleus. C-fos, another transcription factor that plays a role in the process of osteoclastogenesis, is a member of the fos family, whose expression is induced by M-CSF and also by RANKL/RANK interaction. Mice deficient in c-fos also develop a severe osteopetrotic phenotype confirming the role of c-fos in osteoclast formation (66, 96).

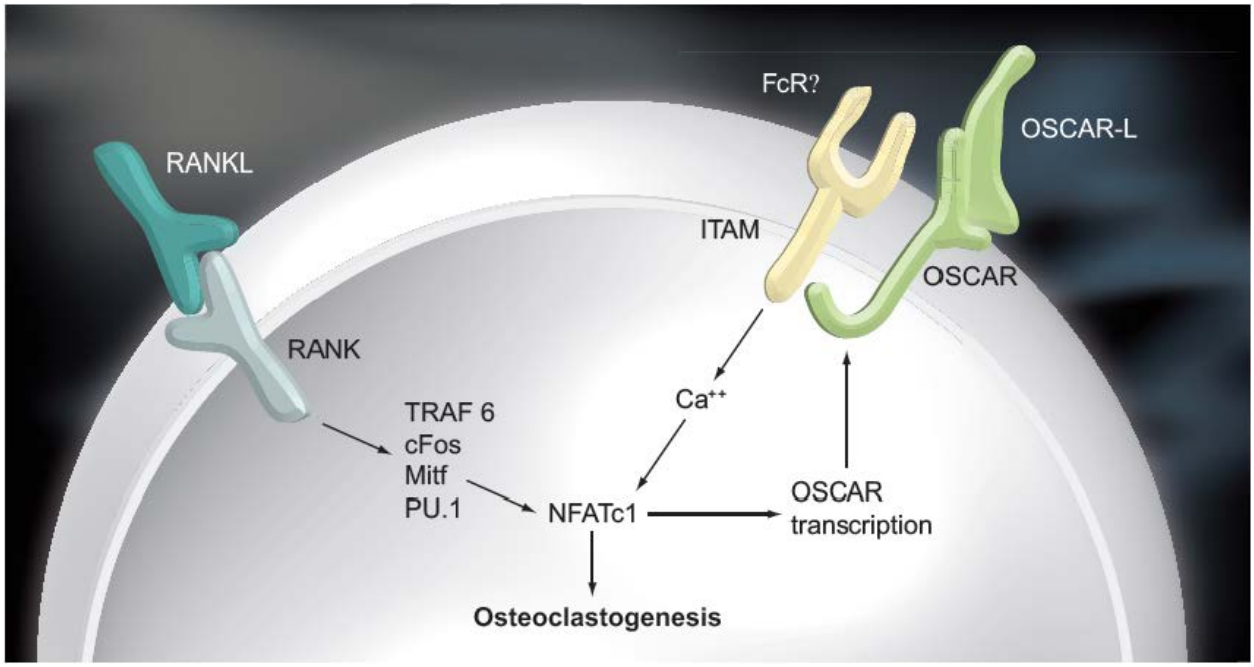

Figure 1.9. Intracellular mediators involved in osteoclast formation (26). RANKL binds to its receptor RANK present on the surface of osteoclast precursor cells. The binding activates a number of intracellular adaptor proteins such as the TRAF and c-fos family. This results in activation of the key transcriptional factor in osteoclast formation NFATc1 which is known to induce expression of osteoclast genes such as TRAP, Cathepsin K and OSCAR.

1.4.1.6 Nuclear Factor of Activated T Cells (NFATc1)

Nuclear factor of activated T cells (NFATc1) is the key transcriptional factor in osteoclast formation. It is a member of the NFAT family of transcription factor genes that is strongly induced following RANKL stimulation (164) via the TRAF-6 and c-fos signalling pathways. RANKL is also known to activate NFATc1 through calcium signalling. NFATc1 is able to regulate its own activity by autoamplifying its own gene, thought to be achieved by it binding to its own promoter (188). During the final stages of osteoclast differentiation, NFATc1 cooperates with AP-1, a dimeric transcription factor complex consisting of c-fos and jun family members playing a vital role in the process of osteoclastogenesis as shown in Figure 1.10. NFATc1 directly induces osteoclast genes such as the calcitonin receptor (CTR), Cath-K, tartrate resistant acid phosphatase (TRAP) and the β3 integrin, in addition to osteoclast-associated receptor (OSCAR) (131, 132, 180)

(Figure 1.10). Increased expression of NFATc1 has been shown in synovial tissues from patients with RA (41).

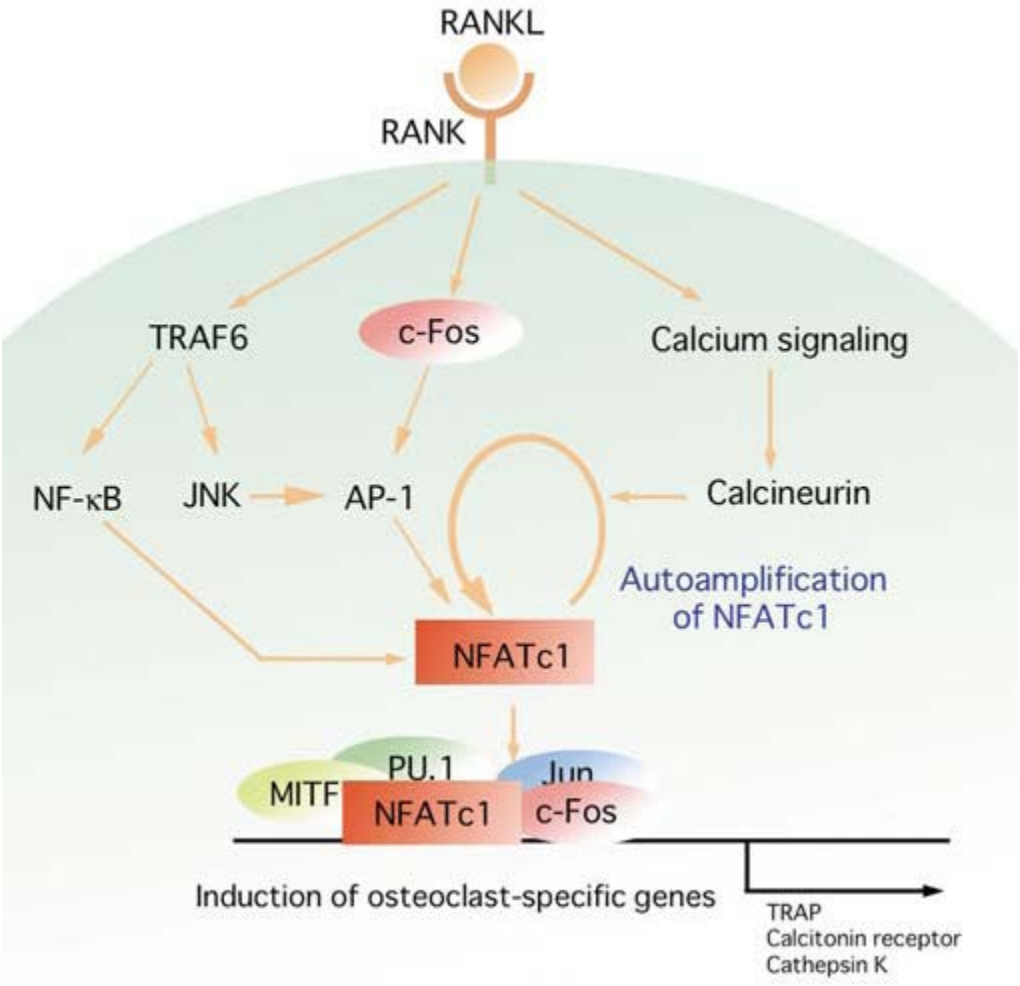

Figure 1.10. NFATc1 is the master regulator of osteoclastogenesis resulting in expression of key osteoclast genes including TRAP, calcitonin receptor and cathepsin K (188).

1.5 Treatment of Pathological Bone Loss Diseases

Most of the current treatments available for RA target the inflammatory process and have shown limited effects on halting the bone destruction. In PD, treatment focuses on reducing the infection and hence reducing the soft tissue inflammation (6). Over the past decade, there have been major advances in the understanding of bone metabolism and the mechanisms of pathological bone loss. This knowledge, in combination with the recent development of therapies that target bone loss, now gives the ability to directly treat the destructive bone loss. Although modern anti-inflammatory therapies have resulted in a

remarkable improvement in disease activity, there is still a need to prevent joint destruction in RA and alveolar bone loss in PD. In the case of RA, it takes considerable time for clinicians to find appropriate treatments for patients that target the inflammation and hence during this time there is continuing destruction of the joint.

1.5.1 Anti-Inflammatory Treatments

1.5.1.1 Conventional Treatments

Conventional therapies that target the soft tissue inflammation are the most common treatments for RA. Non-steroidal anti-inflammatory drugs (NSAIDs) have remained the first line of treatment for a considerable time. NSAID reduce inflammation by preventing prostaglandin release via suppression of the cyclooxygense (COX) enzymes. In general, NSAIDs principally have anti-inflammatory actions whilst having little if any beneficial effects on bone destruction (49). Aspirin, which was one of the most commonly used NSAIDs to treat RA, has been reported to provide no prevention of joint destruction (49). NSAIDs targeting COX-1 were found to be associated with adverse gastrointestinal side effects which led to the development of selective COX-2 inhibitors. Although both conventional NSAIDs and COX-2 inhibitors have positive effects on inflammation, their effects on bone are ambiguous (11, 55, 99). There is even some evidence to suggest that select NSAIDs may actually accelerate bone destruction (11, 55, 99). Under pathological conditions it is known that prostaglandins such as PGE_2 can stimulate osteoclast mediated bone resorption, (64, 93, 192) but conversely prostaglandins have also been reported to increase bone formation by stimulating osteoblast formation (121, 156). Although NSAIDs do continue to be commonly utilised and demonstrate positive effects on disease activity, their benefits to bone are dubious. Furthermore, the application of NSAIDs, although useful in mild PD, has not been particularly successful due to the associated side effects (87, 199).

Glucocorticoids have been another common choice of treatment, particularly in RA, and when given intra-articularly have shown mixed results. Although demonstrating a positive effect in controlling inflammation, long term use of glucocorticoids has been shown to result in the appearance of unwanted side effects such as osteoporosis, hypertension, peptic ulcer diseases, accelerated atherosclerosis and vascular disease (49). Despite improvements in practice (69) the direct effect of these drugs on bone is essentially catabolic, with deleterious effects on bone through suppression of osteoblast mediated bone formation (178) and their administration is usually limited to a few weeks or months depending on the arthritic state (69). Glucocorticoids could play a role in suppressing early inflammation; however, as a result of their associated adverse side effects with long term use and suppression of bone formation, it is clear that they are unlikely to be useful in preventing structural damage in common chronic inflammatory diseases.

The traditional "disease-modifying anti-rheumatic agents" (DMARDs), such as methotrexate, leflunomide, sulphasalazine and cyclosporine, have been beneficial with radiological evidence of improvement in joint space narrowing in RA and related conditions (32, 50, 60, 184). Methotrexate (MTX) is one of the most widely used DMARDs and is the standard of care for patients with moderate to severe arthritis as it is relatively safe and well tolerated at therapeutic doses (5, 38). Complications with Long term use of MTX have been noted related to gastrointestinal liver toxicities and malignancies although overall with low doses MTX does still appear to be relatively safe as demonstrated in a recent systematic review (175).

MTX is often administered to RA patients in combination with other biologic agents such as etanercept (53, 105), adalimumab (101), anakinra (37), leflunomide (108) and rituximab

(54). MTX is known to provide additional anti-inflammatory mechanisms and/or can act synergistically with the other therapies (178). The use of MTX has resulted in substantial improvements in disease activity in RA. Although DMARDs have been shown to improve disease activity, not all patients will respond favourably to DMARD treatment with a significant number having adverse side effects (5). As a result, it can often take time clinicians to find a more effective treatment during which there is continuing destruction of the bone.

1.5.1.2 Biologic DMARDS

During the inflammatory process there is elevated production of an array of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-17. These are known to be responsible for the up regulation of osteoclasts that resorb bone (77). A number of therapeutics have been developed to target these inflammatory cytokines including Anti-TNF-α therapy which has become an increasingly popular option due to its rapid onset of action. There are currently five TNF-α antagonists approved for treatment of RA including Adalimumab - human monoclonal antibody, Entanercept - TNF receptor (p75): FclIgG construct, Infliximab - chimeric monoclonal antibody, Golimumab (CNTO148) - fully human monoclonal anti-TNF-α antibody and Certolizumabpegol (Cimzia) - humanized anti-TNF-α antibody. The difference in each of the anti-TNFs is related to the method of delivery and the frequency of administration. Anti-TNF-α therapy has been found to reduce synovitis and indices of joint deterioration in RA and psoriatic arthritis, however, its ability to immediately halt bone erosion is not noted in a large proportion of patients (76, 146, 178, 206). Moreover, in recent years there have been concerns about the development, albeit rarely, of serious adverse effects from anti-TNF-α therapies. Among these is a demyelenating reaction (142) and other effects (109, 126) leading to activation or development of infections such as, tuberculosis (100, 114, 205), coccidiomycosis (14) and

Listeria monocytogenes (183). These treatments also come at a very high cost to patients and it is known that not all individuals treated will respond (5). These drugs need to be administered parentally which can mean access can be restricted for some individuals. It can also take a considerable amount of time for clinicians to identify appropriate treatments tailored to individuals and so during this time there is continuing bone erosion that can strongly impact on joint function. Even if treatment is commenced at the onset of disease it still may not suppress joint damage as there is recent evidence to suggest that bone damage can still occur during anti TNF-α therapy (83). Over expression of TNF-α has been shown to be associated with periodontitis with TNF antagonists shown to suppress inflammation and bone loss in a *Macacafascicularis* primate model of experimental periodontitis (4). However, there does appear to be conflicting evidence as to whether TNF inhibitors can effectively suppress periodontal parameters in periodontitis (48, 133, 152).

Other inflammatory cytokines such as IL-1, IL-6, IL-12, IL-15, IL-17 are also known to play a role in pathological bone loss diseases. For this reason, a number of therapeutics developed to target these cytokines. For instance, IL-1 has been shown to increase expression of both RANKL and RANK and to facilitate osteoclast differentiation (209). Anakinra, is a recombinant IL-1 receptor antagonist (IL-1Ra), that is approved for treatment of RA (178). Anakinra given as a monotherapy and in combination with MTX has been found to reduce focal bone erosion in arthritis (22, 37, 95, 150). A role for IL-1 in periodontitis bone destruction has also been demonstrated (4) and inhibition of IL-1 has also been reported to reduce inflammation and bone resorption in a primate model of periodontitis (46).

IL-6 (reviewed by (61)) is a pro-inflammatory cytokine that has been found in elevated levels in synovial fluid from RA patients (107). Tocilizumab is a humanized monoclonal antibody against IL-6 receptor that has been suggested to be effective in treating RA (61). IL-15 is a pro-inflammatory innate response cytokine expressed by macrophages in RA tissue and has been demonstrated to promote cytokine and chemokine release through its effects on synovial T cells (10, 29, 137). HuMaxIL-15 is a high affinity fully human monoclonal antibody against IL-15 that has been tested in phase I-II clinical trials. It has been well tolerated in these studies and has shown a considerable improvement in disease activity (10).

Whilst the newly developed biologic DMARDs do have positive effects on reducing disease activity, they do not directly inhibit osteoclast mediated bone destruction and so the bone and cartilage loss may continue, at least to some extent, despite treatment. There can also be a number of disadvantages with biologic DMARDS including high cost, the need for parental administration and also the fact that not all patients will respond; only 50% of patients treated will reach remission.

A variety of inflammatory cytokines have been shown to stimulate RANKL expression (112, 195, 207) and enhance its ability to induce the formation of resorptive osteoclasts (84). Despite this, it is clear that therapies targeting the osteoclast mediated bone loss, in combination with anti-inflammatory treatments are likely to be most beneficial in improving quality of life for disease sufferers, preventing the joint and bone destruction and so halting disease progression.

1.5.2 Anti-Resorptive Therapies

1.5.2.1 Osteoclast Inhibition

Osteoclasts are becoming an important target for development of therapeutics to treat pathological bone loss diseases including PD and RA. These treatment can involve reducing the rate of osteoclast formation, decreasing the number of resorptive cells, inhibition of the resorptive activity or reducing the life span of osteoclasts and as a result reducing the capacity for resorption (145).

Bisphosphonates are a class of anti-resorptive drugs that inhibit osteoclast activity and are used to treat a wide variety of bone disorders, including osteoporosis, tumour-associated osteolysis and arthritis (77). Despite having benefits of reducing generalized bone loss in osteoporosis, their effectiveness in suppressing focal bone erosions specifically observed in RA is questionable (23), 12). Reports of osteonecrosis of the jaw in patients treated with high levels of bisphosphonates have also led to their use being questioned, particularly in relation to dental disease and treatments (18, 58).

1.5.2.2 Inhibiting RANK/RANKL Signalling - Denosumab

The RANKL/RANK signalling pathway is becoming an important target for treatment of pathological bone loss. A single subcutaneous injection of OPG fused to the human immunoglobulin G in women with postmenopausal osteoporosis was found to profoundly reduce bone turnover (12). Chronic use however was associated with the formation of anti-OPG antibodies that could neutralize endogenous OPG activity so it was determined inappropriate to use exogenous OPG administration to prevent bone loss (3).

Amgen has successfully developed a “humanised” monoclonal antibody to RANKL; Denosumab (formally known as AMG 162). Denosumab mimics the action of natural OPG

by binding to RANKL thereby preventing it from interacting with RANK as shown in Figure 1.11. Denosumab was effective in clinical trials of postmenopausal women with low bone mineral densities (119, 135). In June 2010 the FDA approved Denosumab for use in postmenopausal women at risk of osteoporosis (158). A single dose of Denosumab has also been shown to reduce bone resorption for up to 6 months (13). Injections of Denosumab twice yearly with ongoing MTX treatment inhibited structural damage in patients with RA with no increased rate of side effects (36). Denosumab did not however have an effect on RA disease activity, on the occurrences of RA flares, or on joint space narrowing and had no significant effect on reducing cartilage erosion (36).

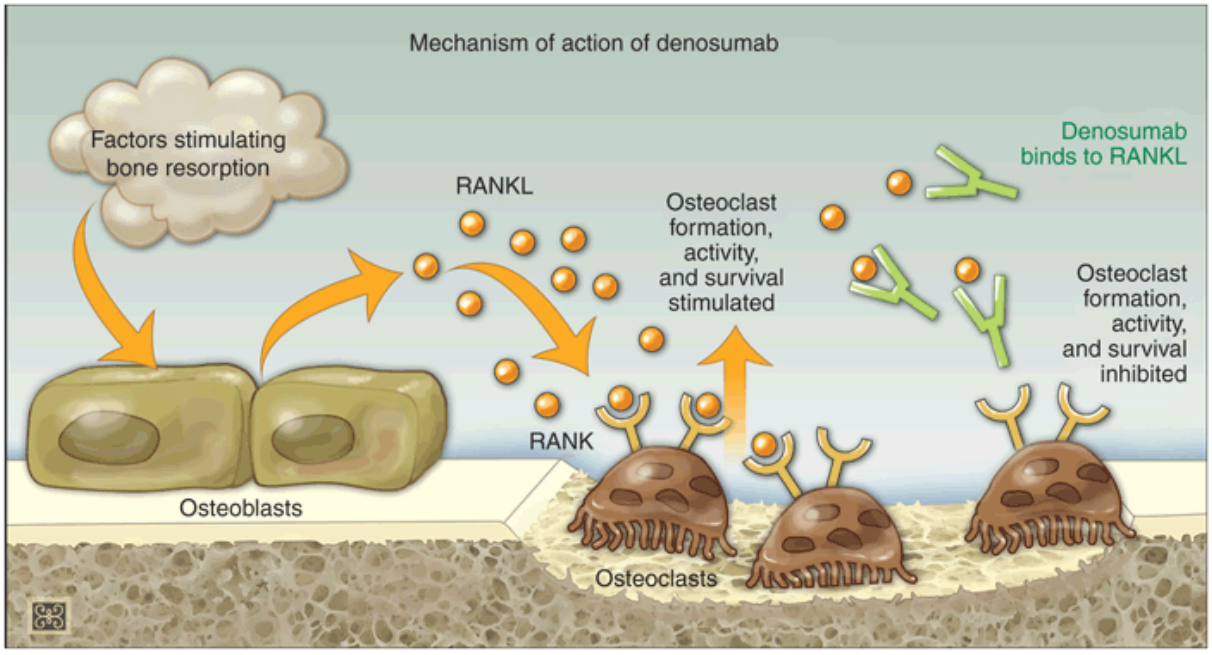

Figure 1.11. The action of Denosumab (118). Denosumab mimics the action of OPG and binds to RANKL preventing it from binding to RANK and so blocks the subsequent formation of osteoclast.

1.6 Novel Targets for Treating Bone Loss

Despite many advances being made in understanding the cellular and molecular processes of bone destruction in arthritic and periodontal diseases, there are few safe and effective therapies available, aside from those used for osteoporosis (72, 178, 206). Research into potential treatments to suppress none loss is now focused on small molecules (130). New

small molecule compounds that target specific cytokine-mediated processes (e.g. RANK and its RANKL) (116) seem likely to prove more effective in specifically controlling bone destruction in a variety of diseases (130). Small molecules have the benefit of being cheaper to manufacture, can be administered orally, thereby enhancing patient compliance, concentrations can also be more easily monitored and correlated with disease in patients receiving treatment. One such new class of small molecules are histone deacetylase inhibitors (HDACi) that have potential for treating both bone loss and inflammation.

1.6.1 Histone Deacetylase Inhibitors (HDACi)

1.6.1.1 Background of HAT and HDACs

A novel approach for the treatment of localized bone loss in inflammatory diseases is via the epigenetic control of bone resorption. Epigenetic alterations are described as DNA modifications without any changes to the base sequences with two major types including DNA methylation and histone acetylation that modulate the access of transcription factors (90). There has been considerable interest in the gene-regulated events involving post-translational modification of histone N-terminal acetylation and subsequent regulation of the intracellular signalling pathways that control production of inflammatory mediators and cell cycle activity (17, 31, 65). The process of histone acetylation, which is key in regulating these gene-mediated events, is controlled by two key enzymes. Histone acetyl-transferase (HAT) enzymes are responsible for adding acetyl groups to lysine side chains in histones, leading to exposure of DNA to transcription factors, resulting in gene expression as shown in Figure 1.12. Histone deacetylases (HDAC) on the other hand are zinc metalloenzymes that counteract this by removing acetyl groups from histones, resulting in gene repression. There can also be deacetylation of non-histone signal transduction proteins that are important in various inflammatory events (65).

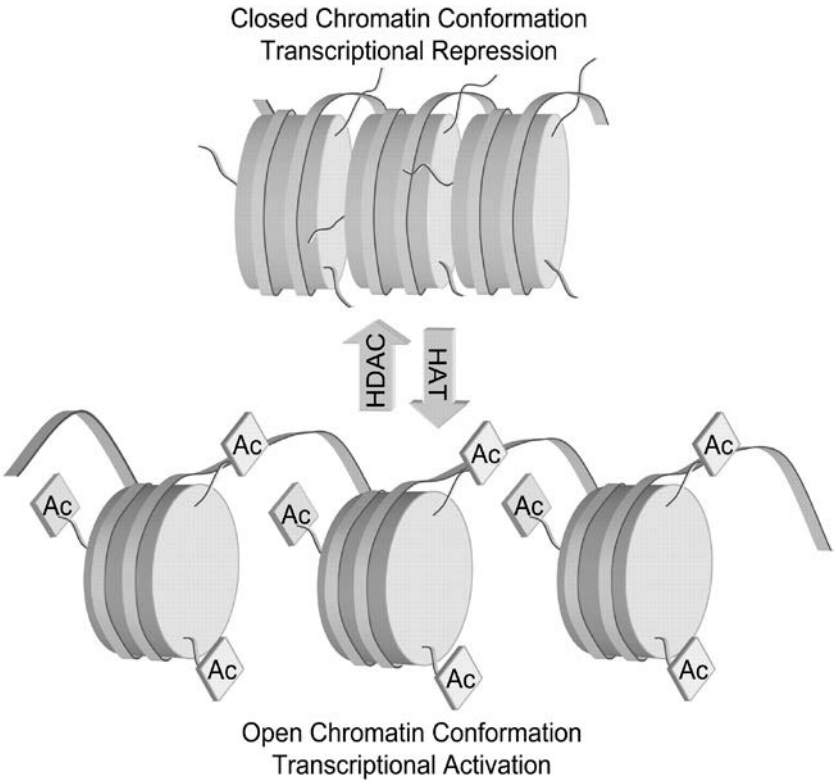

Figure 1.12. Histone acetyltransferases (HAT) and Histone deacetylases (HDAC) and their role in the acetylation status of histones (81). HATs add acetyl groups to the lysine molecules which reduces the affinity of the histone for the DNA backbone which opens up the chromatin allowing transcription factors to access. HDACs are responsible for removing these acetyl groups increasing the lysine affinity for the DNA backbone condensing the chromatin leading to gene regression.

1.6.1.2 Biological Properties of HDACi

The inhibition of HDAC isoforms is considered to be a viable therapeutic strategy and is being investigated to treat a wide variety of chronic inflammatory and malignant diseases (34, 62, 70, 91, 92, 208). Particular interest has centred on the roles of HDAC enzymes in controlling expression of metalloproteinases (MMPs) and pro-inflammatory cytokines that mediate cartilage and bone destruction in RA (34, 62, 70, 91, 92, 208). The mechanism of HDAC control over MMPs and cytokines, while not completely understood yet, is thought to be via suppression of the NF-κB pathway with subsequent reduction in the production of pro-inflammatory cytokines, nitric oxide and other inflammogens (62, 70, 92), as well as the production of TNF-α receptors (92).

Histone deacetylase inhibitors (HDACi) have been found to have anti-inflammatory effects and to reduce cancer cell growth by inhibiting differentiation of cells both *in vitro* and *in vivo* (17, 25, 57, 88, 92). They are known to inhibit the expression of several important genes, particularly inflammatory cytokines, such as TNF-α and interferon-γ (IFN-γ) (92, 147), the expression of cyclo-oxygenase-2 (57, 88), and to co-operate with Fas-signalling to induce apoptosis (144) and down-regulate the expression of hypoxia-induced endothelial growth factor in rheumatoid synoviocytes (127).

1.6.1.3 Expression and Inhibition of Specific HDACs

Among the factors that could influence histone acetylation are variations in the activities of HAT and HDAC enzymes in diseased tissues (89). Moreover, the existence of 11 human isoforms of zinc-containing HDACs (HDAC 1 to 11) and a further 7 NAD^+-dependent enzymes (SIRT 1 to 7) (98) raises issues about the relative activities of the different isoforms in different cellular systems. Class I HDACs include HDACs 1, 2, 3 and 8 which are primarily found in the nucleus. Class II include HDACs 4, 5, 7 and 9 belonging to Class IIa HDACs, and HDACs 6 and 10 belonging to Class IIb HDACs. Class II HDACs are able to shuttle between the nucleus and cytoplasm as shown in Figure 1.13. Class III HDAC are found in the cytoplasm require a co-factor NAD^+ for activation. These are also called sirtuins (SIRT 1-7) and act via a different mechanism to Class I and II. HDAC 11 is the only member of Class IV and this is similar to Classes I and II HDACs (15, 43, 143).

Recent studies have demonstrated that HDACs are expressed differently in RA tissue, with higher nuclear activity of HDACs being demonstrated in RA synovial tissue compared to osteoarthritis (OA) tissues (98). HDAC 1 has been shown to be highly expressed in synovial fluid from RA patients compared to OA (85). High levels of HDAC 1 have also recently been observed in RA tissues and these levels also correlate with higher TNF-α

expression (85, 98). Interestingly, Huber and colleagues demonstrated hyperacetylation in RA patients with HDAC activity being lower, particularly HDAC 1 and 2 (89). This is suggested to promote the transcription of genes of several pro-inflammatory cytokines (89). These conflicting results might be explained by differences in experimental methods or it could be related to differences in the patient population selected for the studies. Interestingly, HDAC 5 has also been reported to be elevated in other bone pathologies, such as in patients with primary osteoporosis (169). Suppression of HDAC 3 expression via short interfering RNA (siRNAs) in osteoclasts isolated from mouse bone marrow and RAW 264.7 c4 cells has been recently shown to inhibit osteoclast differention (161). Wheras supression of HDAC 7 in these cells actually accelerated osteoclast differentiation (161). Overexpression of HDAC 5 has also recently been shown to reduce RANKL mediated acetylation of NFATc1 suggesting that the balance between HATs and HDACs may play an important role in regulation NFATc1 in osteoclasts (104). These studies suggest that different HDACs may play a role in the overall differentiation and activity of osteoclasts. In light of conflicting reports and the lack of studies looking at HDAC expression in diseased states further investigations into HDAC expression in both RA and other bone resorption diseases are required to determine the potential of targeting individual HDAC as treatments.

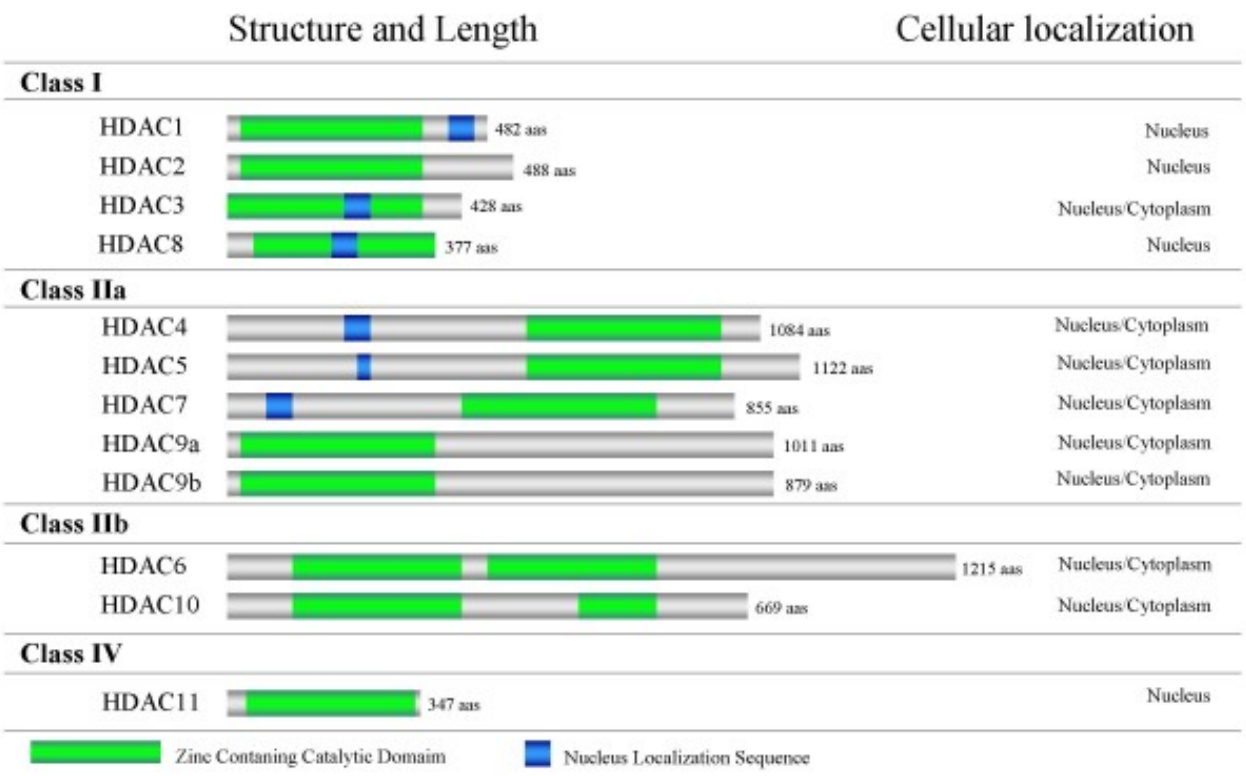

Figure 1.13. Three major classes of HDACs – Class I, Class II (IIa and IIb) and Class IV (125).

1.6.1.4 HDACi and Osteoclasts

Studies investigating the effects of HDACi on osteoclasts and in *in vivo* disease models suggest that HDACi hold promise for treatment of bone destruction in chronic inflammatory diseases. Various HDACi have shown potential to regulate the effects of RANKL on inflammatory cells and osteoclasts via a number of key factors involved in osteoclastogenesis (102, 147, 163, 187). In mouse macrophages, HDACi mediate their effects through inhibiting RANKL induced osteoclastogenesis by suppressing the NF-κB pathway (187). Several HDACi have been reported to suppress osteoclastogenesis in murine cell lines (102, 147, 163). Two very non-specific HDACi, trichostatin (TSA) and sodium butyrate (NaB), have been shown to suppress RANKL mediated osteoclastogenesis in a rat and mouse bone marrow cultures (163). TSA was recently demonstrated to suppress RANKL induced osteoclast formation from primary bone marrow-derived macrophages *in vitro* with an associated significant decrease in the expression of c-Fos and

NFATc1 (102). Nakamura *et al* (147) showed that a cyclic dipsipeptide, HDACi, FR901228 (also known as FK228), inhibited osteoclast differentiation from mononuclear cells derived from rat bone marrow cells. This effect was due to blockade of the nuclear translocation of NF-κB and was accompanied by induction of β-interferon (INF-β).

1.6.1.5 HDACi for the Treatment of Bone Resorption

Various HDACi compounds have been shown to inhibit inflammation and joint destruction in arthritis animal models (122, 147, 148). The cyclic dipsipeptide HDACi, FR901228 (also known as FK228), suppressed osteoclasts *in vitro* and was also found to have both prophylactic and therapeutic effects in adjuvant-induced arthritis in rats (147). However, this compound is a peptide and, as a therapeutic entity, is unlikely to be developed as a drug as it needs to be injected intravenously in oil to be effective. The development of a more stable non-peptide HDACi would be a more appropriate strategy for targeting bone destruction in RA and PD. Suberoxylanilidehydroxamic acid (SAHA) has been shown to reduce paw swelling as a result of suppressed inflammation and decreased bone erosion but could not prevent the onset of arthritis in rat and mouse collagen induced arthritis (CIA) models. MS-275 was also found to strongly suppress paw swelling and bone erosions in these models (122). Using a rat model of adjuvant arthritis, phenylbutyrate and TSA were also found to suppress joint swelling, reduce subintimal mononuclear cell infiltration, inhibit synovial hyperplasia, suppress pannus formation and cartilage and bone destruction (33). These studies highlight the positive effects of HDACi on both bone and inflammation in models of arthritis and are further discussed later in this thesis. To date, no studies have assessed the effects of HDACi on bone loss and inflammation in PD. Recently, the use of HDACi in restorative dentistry has also been suggested (52). Given the close relationship between RA and PD with similarities in regards to the chronic inflammation and bone destruction by osteoclasts (7-9, 27) such treatments maybe useful.

Interestingly, some inhibitors of HDACs have been found to affect bone formation (103) by stimulating osteoblast maturation (115, 179). In contrast to this, SAHA stimulated individual osteoblast activity but reduced osteoblast numbers overall. The negative effect could be due to the high concentration (100 mg/kg) used in this study (136). Specifically targeting individual HDACs may be important in various diseases and different effects may be seen under different conditions.

1.6.1.6 Potential Side Effects of HDACi Treatment

HDACi have been reported to be well tolerated as chemotherapeutic agents but they may have some adverse effects due to their wide spread effects in a variety of cells and tissues (162). The possibility of side effects can be reduced by topical administration and to this end topical Trichostatin A (TSA) and phenylbutyrate has been found to reduce paw swelling and joint destruction in an adjuvant arthritis model (33). This method of application could potentially reduce the risk of adverse events due to the systemic nature of the inhibitors and widespread expression of select HDAC enzymes. In addition, developing compounds that specifically target only those HDACs involved in the disease process may markedly reduce side effects.

Although showing potential to treat arthritis more research is needed to elucidate the benefits of HDACi in treating bone resorption in these chronic inflammatory diseases. To date no studies have assessed the effects of HDACi on bone resorption in periodontitis. With the knowledge of HDACs differentially expressed in disease states such as PD and RA, and the development of selective HDAC targeting inhibitors, it is likely that the inhibition of selective HDAC(s) could eventually be used as a clinical therapy.

1.7 Conclusion

Bone destruction is a ubiquitous feature of chronic inflammatory diseases such as RA and PD. Many of the current treatments for RA target the inflammation and have limited effects on bone. The major treatment target in PD is on the infection and reducing inflammation via mechanical debridement of affected areas. Therapies directly targeting bone destruction could be administered immediately to patients upon diagnosis for bone protection, and later in combination with current ant-inflammatory treatments. In this way, both inflammation and bone destruction can be targeted. HDACs are one new target to control bone destruction in chronic inflammatory diseases. Although HDACi hold promise for treatment of pathological bone loss, more research is required to understand the roles of specific HDACs in diseased states and which HDAC(s) need to be targeted to be effective. Overall identification of safe and effective drugs that target bone resorption will not only improve our treatment of PD and RA but also other bone loss pathologies becoming more prevalent in our ageing populations.

1.8 Hypotheses

This thesis can be defined by two hypotheses.

1. Pre-existing periodontitis will result in more severe arthritis in a mouse model.

2. Treatment with histone deacetylase inhibitors (HDACi) will suppress both inflammation and bone loss in periodontitis and inflammatory arthritis.

1.9 Aims

Aim 1: To develop a novel mouse model to determine if there is a relationship between periodontitis and rheumatoid arthritis.

Aim 2: To determine the effects of a novel HDACi (1179.4b) that targets both Class I and I HDACs on human osteoclast formation and activity *in vitro*.

Aim 3: To determine the effects of 1179.4b on both inflammation and bone loss in a mouse model of periodontitis.

Aim 4: To assess Class I and II HDAC expression in human periodontitis gingival tissues compared to non-periodontitis gingiva.

Aim 5: To determine the effects of a novel HDACi (NW-21) that targets HDAC 1 on bone loss and inflammation *in vitro* and in a mouse model of collagen antibody induced arthritis (CAIA).

The results of this study will investigate the use of HDACi, on inflammation and bone loss. It will further our knowledge in the relationship between the inflammatory processes and bone along with the relationship between PD and RA.

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Chapter 2. Pre-existing Periodontitis Exacerbates Experimental Arthritis in a Mouse Model

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Chapter Summary

Previous clinical studies have demonstrated a potential relationship between the periodontitis and rheumatoid arthritis. The aim of this study was therefore to develop a novel mouse model that would allow assessment of the effects pre-existing periodontitis has on the severity of inflammatory arthritis and determine if there is a relationship. It was hypothesized that mice with pre-existing PD would develop more severe arthritis.

STATEMENT OF AUTHORSHIP

Pre-existing periodontitis exacerbates experimental arthritis in a mouse model

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Certification that the statement of contribution is accurate

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STATEMENT OF AUTHORSHIP

Pre-existing periodontitis exacerbates experimental arthritis in a mouse model

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Marino V.

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Helped with animal work, data analysis and manuscript evaluation.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed .....*Date*...14/1/2013.....

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Journal of Clinical Periodontology

Pre-existing periodontitis exacerbates experimental arthritis in a mouse model

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Abstract

Aims: Previous studies have shown a higher incidence of alveolar bone loss in patients with rheumatoid arthritis (RA) and that patients with periodontitis are at a greater risk of developing RA. The aim of this study was to develop an animal model to assess the relationship between pre-existing periodontitis and experimental arthritis (EA).

Methods: Periodontitis was first induced in mice by oral gavage with *Porphyromonas gingivalis* followed by EA using the collagen antibody-induced arthritis model. These animals were compared with animals with periodontitis alone, EA alone and no disease (controls). Visual changes in paw swelling were assessed to determine clinical development of EA. Alveolar bone and joint changes were assessed using micro-CT, histological analyses and immunohistochemistry. Serum levels of C-reactive protein were used to monitor systemic inflammation.

Results: Mice with pre-existing periodontitis developed more severe arthritis, which developed at a faster rate. Mice with periodontitis only also showed evidence of loss of bone within the radiocarpal joint. There was also evidence of alveolar bone loss in mice with EA alone.

Conclusions: The results of this study indicate that pre-existing periodontitis exacerbated experimental arthritis in a mouse model.

Key words: bone; chronic inflammation; *P. gingivalis*; periodontal disease; RANKL; rheumatoid arthritis

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Periodontitis and rheumatoid arthritis (RA) are two of the most common chronic inflammatory diseases affecting humans (Albandar & Rams 2002, Silman & Pearson 2002). In the past, these two diseases have been studied independently with attempts focused on understanding the individual disease mechanisms and potential therapeutic strategies. However, with close examination, it is clear that both RA and periodontitis have many similarities that warrant further investigation (Bartold et al. 2005, de Pablo et al. 2009).

Both diseases demonstrate an exuberant chronic inflammatory reaction and the presence of large numbers of immune cells including T and B lymphocytes, neutrophils and monocytes. They also have similar cytokine profiles including high numbers of pro-inflammatory cytokines such as TNF-α and interleukins (Bozkurt et al. 2000, Ogrendik et al. 2005). It is this inflammatory reaction that eventually leads to destruction of both the soft and hard tissues of the joint and alveolar bone in RA and periodontitis, respectively. One important common pathway involves the upregulated expression of receptor activator of nuclear factor κB ligand (RANKL) by fibroblasts and lymphocytes, which is an essential factor for osteoclast formation (Crotti et al. 2003, Haynes et al. 2003, Cantley et al. 2009b, Bartold et al. 2010a).

Numerous studies have reported that the relationship between periodontitis and RA may be bidirectional. For example, while many studies have demonstrated significantly higher incidence of tooth loss and alveolar bone loss in patients with RA (Mercado et al. 2000, 2001, Al-Shammari et al., 2005), others have reported that periodontitis is a risk factor for developing RA or even enhancing the severity of RA (Ribeiro et al. 2005, Havemose-Poulsen et al. 2006). More recently, studies have demonstrated that treatment of periodontitis may reduce the severity of arthritis (Ribeiro et al. 2005, Al-Katma et al. 2007, Ortiz et al. 2009). While many possibilities exist to explain these inter-relationships, one process gaining interest is the role that autoim-

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munity to citrullinated proteins might play in the development of RA. Of particular interest is the role that *Porphyromonas gingivalis* might play because this periodontal pathogen can citrullinate proteins through the release of peptidylarginie deiminase (Rosenstein et al. 2004, Wegner et al. 2010).

Our group has recently demonstrated, using a rat model, that the presence of a pre-existing chronic inflammatory reaction induced by a heat killed (non-infective) form of the periodontal pathogen *P. gingivalis*, exacerbated the development of adjuvant arthritis in female DA rats (Bartold et al. 2010b). This established that an extra synovial inflammation, similar to but not identical to periodontitis, exacerbated experimental arthritis (EA). In the present investigation we have extended these studies to include an assessment of an infective component (*P gingivalis*-induced periodontitis) to the development of EA.

Recently a model in which periodontitis and arthritis were both co-induced in an inflammation-prone mouse strain [acute inflammatory reactivity maximum (AIRmax)] and (AIRmin) using the pristane-induced arthritis (PIA) model has been described (Trombone et al. 2010). They found that co-induction in AIRmin mice did not alter the course of both pathologies. Clinical investigations by our group have suggested that patients with severe RA are more likely to have advanced periodontitis and vice versa. Patients with RA taking NSAIDs, which are likely to reduce periodontal inflammation still have signs of periodontal destruction indicating that periodontitis develops and may not be detected before the RA development (Bartold et al. 2005). Hence, to further understand this relationship, an animal model was developed to assess the relationship between pre-existing periodontitis followed by induction of arthritis.

Methods

Animals

Eight-week old female Balb/c mice were obtained from the Institute of Medical and Veterinary Science (IMVS) Animal Services Division and approval was obtained from the Animal Ethics Committees of the University of Adelaide and the IMVS, Adelaide, South Australia. All experiments were carried out according to the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997). For this study a total of 14 mice were used. Group 1 ($n = 4$) consisted of mice induced with periodontitis alone, group 2 ($n = 4$) mice were induced with arthritis only, group 3 ($n = 4$) mice were first induced with periodontitis and subsequently with arthritis, and the control group received no disease ($n = 2$). All animals received kanamycin (1 mg/ml in drinking water) *ad libitum* for a period of 7 days to reduce the native flora and support colonization of the *P. gingivalis* bacteria as has been reported previously (Baker et al. 2000, Bendyk et al. 2009, Kuula et al. 2009).

Periodontitis induction

Three days after antibiotic treatment ceased periodontitis was induced by oral inoculations of *P. gingivalis* bacteria over a period of 44 days (Cantley et al. 2009a). Cultures of *P. gingivalis* bacteria (W50) were grown on anaerobic blood agar plates and stored at 37°C in an atmosphere of $N_2/CO_2/H_2$ (90:5:5) for 3 days, before harvesting. Bacterial cells were suspended in 2 ml of carboxymethyl-cellulose [2% carboxymethyl-cellulose in phosphate-buffered saline (PBS)] as described previously (Bendyk et al. 2009, Cantley et al. 2009a). The viable count of the bacteria was determined to be 24.3×10^{10} colony-forming units/ml and the dry weight was 12.5 mg/ml. The bacteria (0.1 ml in carboxymethyl-cellulose) were swabbed directly on the gingivae of the mice using a small brush. Mice not receiving periodontitis were swabbed with carboxymethyl-cellulose alone. Following each inoculation mice were kept without any food or water for a period of 1 h to ensure effectiveness of the inoculation. Mice were also housed in an environment void of antibacterial products and were fed monitored powdered food throughout the entire experiment. Live *P. gingivalis* bacteria was recovered from gingival tissue as described previously (Bendyk et al. 2009).

Collagen antibody-induced arthritis induction

To induce arthritis, mice were injected intravenously via the tail vein with 150 μl (1.5 mg) of a monoclonal antibody against type II collagen (Chondrex Inc., Arthrogen-CIA® Arthritogenic Monoclonal Antibodies, Redwood WA, USA). For the periodontitis and arthritis group this injection was carried out on day 44 after the induction of periodontitis. Two days later mice were given an intraperitoneal (i.p.) injection of 20 μl (5 μg) of lipopolysaccharide (LPS) (Chondrex Inc.). The dose of LPS used in this study was lower than the dose recommended by the manufacturers. In pilot studies 10 μg of LPS was used to induce disease. The reason for using a lower dose arose from these initial studies, which indicated that the full dose produced a florid arthritic reaction, which prevented the assessment of any co-morbidity factors. By reducing the dose we could still induce a noticeable arthritic response, which then allowed us to fully assess the impact of pre-existing periodontitis on the arthritic response. Control animals (non-arthritis groups) were injected with PBS alone. Following antibody injection mice were monitored daily by two experienced observers for a period of 10 days. This time point was determined from preliminary studies to be the best to assess bone loss and inflammation. Recording of body weight, monitoring of other factors (including dull/ruffled coat, a change in temperament, reduced food/water intake or a reluctance to move) and clinical scoring of the paws for swelling were all conducted daily. To assess the clinical paw swelling each paw was given a score from 0 to 4, to make a total score of 16 for each animal. 0 = normal paw, 1 = mild but definite redness and swelling of the wrist/ankle, 2 = moderate swelling and redness of the wrist/ankle with digit involvement, 3 = severe swelling of the wrist/ankle with multiple digit involvement and 4 = maximum inflammation within the entire paw, wrist/ankle with many digits involved.

Live animal micro-CT scanning

Throughout the experiment, mice were scanned using a live animal micro-CT scanner situated in Adelaide Microscopy (SkyScan 1076, Kontich, Belgium). The specifications used for scanning and machine details have been published previously (Cantley et al. 2009a, b). Mice were scanned at 74 kV/136 mA with a pixel size of 18 μM, 1 mm aluminium filter and frame averaging of 1. These parameters were chosen to minimize the radiation exposure to the animals and significantly reduce scanning time to around 12 min. per animal. Scanning was conducted initially before beginning periodontitis induction to form baseline measurements, again at day 44 after inducing periodontitis but before collagen antibody-induced arthritis (CAIA) induction and finally at the completion of the

study (10 days after inducing CAIA). Before scanning, the mice were anaesthetized via i.p. injection [rat/mouse anaesthetic – 1 ml xylazine, 2 ml ketamine (100 mg/ml), 17 ml of water in the injection, 0.3 ml for a 30 g mouse]. For each scan the mouse was positioned to ensure that both the head and the two front paws were within the scanning area of interest allowing analysis of both alveolar bone and the paws.

CT scans were then re-constructed using SkyScan N Recon program and bone volume (BV) analysis was carried using SkyScan's CTAn program (SkyScan, Kontich, Belgium). For the heads, the three molars and surrounding alveolar bone of the maxilla was chosen as the region of interest. For the paws, 200 slices either side of the radiocarpal joint of the front paws was used as the region of interest for BV analysis. For each area of interest, BV (mm^3), was calculated using CTAn software. The length of the cemento-enamel junction (CEJ) to alveolar bone crest (ABC) in micrometres was also measured at two locations – between the first and second molars and between the second and third molars on three slices for each mouse using CTAn as described previously (Park et al. 2007).

Histological analysis

At the completion of the study (day 54) mice were humanely killed via CO_2 inhalation. All paws and heads were collected, skinned and placed in fixative solution (10% PBS-buffered formalin) for 48 h. Following this, decalcification was carried out for a period of 2 weeks using 5% formic acid and then the specimens were processed for paraffin embedding in preparation for histological analysis. Sections (7 μm) were cut and stained with haematoxylin and eosin (H&E). Histological sections were imaged using the NanoZoomer Digital Pathology (NDP) (Hamamatsu Photonics K.K., Hamamatsu City, Shizuoka Pref., Japan) at $\times$ 40 magnification. Assessments of the radiocarpal joints in the paws and three molars of the maxilla were made. Two histological sections per mouse were scored based on a method of Tak et al. (1997). Two independent observers, blinded to the tissue type, used a 4-point scale. Scoring was based on the numbers of inflammatory cells (lymphocytes, plasma cells neutrophils or macrophages). Normal tissue ($<5\%$ inflammatory cells) was scored a 0, mild inflammation (5–20% inflammatory cells) was scored a 1, moderate inflammation (20–50% inflammatory cells) was scored a 2 and severe inflammation with a massive immune cell infiltration ($>50\%$ of cells) was scored a 3. Bone and cartilage destruction was assessed by: 0 = normal, 1 = mild cartilage destruction, 2 = evidence of both cartilage and bone destruction, 3 = severe cartilage and bone destruction. Pannus formation: 0 = no pannus, 1 = pannus formation.

Gingival tissues of the three molars of the maxilla were also assessed for inflammation and alveolar bone destruction as assessed by measuring the length (μm) of the CEJ to ABC between the first and second molars of the maxilla. This was conducted using the Nanozoomer program (NDP View) length was measured using the linear measurement tool with images at $\times$ 5 magnification.

C-Reactive Protein (CRP) ELISA

Blood samples were collected from mice on the final day of the experiment via cardiac puncture. Samples were left at room temperature to clot for 2 h, followed by centrifugation at 1000 *g* for 20 min. Serum was collected and stored at $-80^{\circ}C$. A commercially available CRP kit (Mouse CRP Elisa test kit, Life Diagnostics, West Chester, PA, USA) was used to assess the CRP levels in the serum samples. Samples were diluted 1 in 100 and manufacturer's instructions were followed.

Immunohistochemistry

Immunohistochemistry was conducted on paraffin embedded sections to determine the expression of RANKL in the radiocarpal joints and periodontal tissues. Sections were stained with a goat polyclonal antibody (2 μg/ml) against RANKL (Santa Cruz sc-7628, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Negative controls consisted of normal goat serum. A ready to use kit (Vectastain, Vector laboratories, Burlingame, CA, USA) was used for staining sections followed by a brief counterstaining with haematoxylin and lithium carbonate and mounting using aquatex. RANKL staining was scored by a semi-quantitative analysis, by two blinded individuals. A 0–4 scoring system was used as previously described (Kraan et al. 1999). Three areas were scored for each stained section of both periodontal tissues and the radiocarpal joints. The score 0 = 0–5% positive stained cells, 1 = 6–10%, 2 = 11–25% 3 = 26–50% and 4 > 50% positive staining cells.

Statistics

Unpaired *t*-tests were used to analyse statistical differences between the groups. Statistical significance was accepted when $p<0.05$.

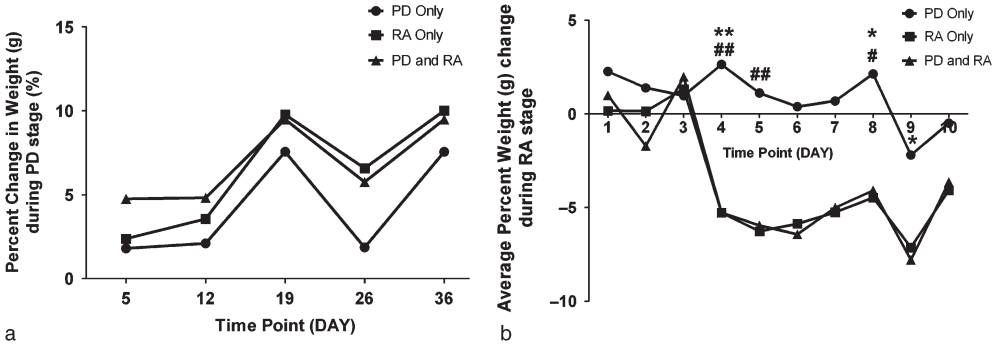

Fig. 1. Average percentage change in weight (grams). (a) The periodontitis induction stage from day 0 to day 36. Weight change relative to day 0. No significant differences between groups at any time points and (b) the collagen antibody-induced arthritis [experimental arthritis EA] stage, (days 0–10) weight change relative to day 0. Data represented as mean ± SEM. ($n = 4$ per group) $^{**}p<0.01$, $^{*}p<0.05$ periodontitis and EA compared with periodontitis alone, $\#\#p<0.01$ $\#p<0.05$, EA alone *versus* periodontitis alone.

Results

Weight changes

Body weight was monitored during induction of both the periodontitis and arthritis. There were no significant differences in the per cent weight change between the groups during the periodontitis stage (Fig. 1a). Following induction of arthritis there was a greater percentage of weight loss in the two groups that had the CAIA (Fig. 1b). Although, there was a greater weight loss in the group with periodontitis and arthritis compared with arthritis alone from day 4, this was not significant ($p > 0.05$).

Joint inflammation

The macroscopic clinical images of the paws shown in Fig. 2 are representatives of mice within each group at day 8 (post-arthritis induction) when disease was found to be at its maximum. More severe redness and swelling was seen in the front and rear paws of mice with both diseases compared with mice that received only arthritis. It was also more common for mice in the combined periodontitis and arthritis group to demonstrate signs of disease in their rear paws compared with mice with arthritis only.

Paws were scored for signs of redness and swelling as described in the 'Methods' (Fig. 3). In pilot studies (data not shown) paw scores with the 5 μg dose of LPS were not significantly different to those when 10 μg of LPS ($p > 0.05$) was used. With the 5 μg dose at day 3 (LPS injections), all mice with pre-existing periodontitis were showing signs of disease with average scores 3.5 times higher than that of the arthritis alone group. There were significantly higher paw scores for the periodontitis and arthritis group at days 6, 8, 9 and 10 ($p < 0.05$) using a 16-point method of scoring. Similar observations were also made in mice receiving only the CAIA antibody with no LPS. Mice in the group with periodontitis and arthritis (no LPS) similarly to the LPS mice developed arthritis at a greater rate compared with arthritis alone (no LPS). When combining the results of mice pre-existing periodontitis that received arthritis both with and without LPS at day 3 the incidence of disease was 88% compared with 28% in mice with no pre-existing periodontitis.

This observed clinical paw inflammation was also confirmed following assessment of H&E-stained histological sections of mouse front paws (radiocarpal joint) at the completion of the study (Fig. 4). In the arthritis alone group there was evidence of infiltrating polymorphonuclear and mononuclear cells. The inflammation in the periodontitis and arthritis group was more severe with extensive pathological changes seen in the bone and soft tissues. There was evidence of synovial hyperplasia, with significantly high numbers of inflammatory cells. Blinded analysis of the radiocarpal joint in the front paws demonstrated that inflammation in mice with both periodontitis and arthritis was significantly higher than in the periodontitis alone and arthritis alone groups (Fig. 5a).

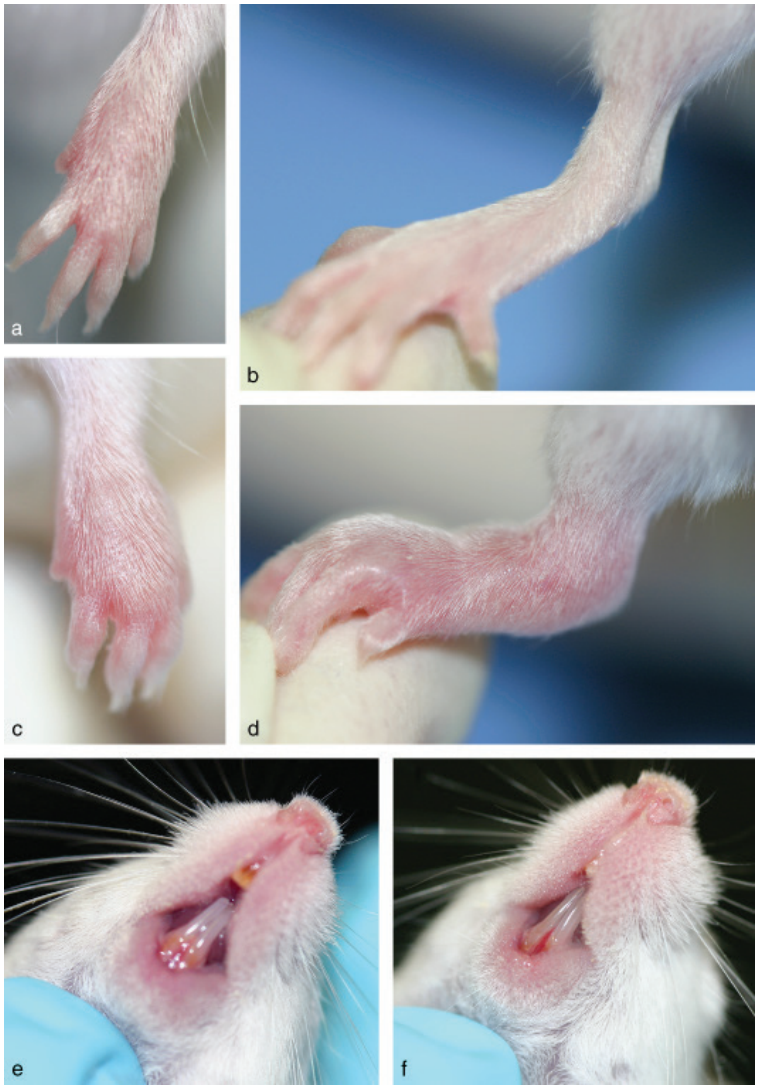

Fig. 2. Macroscopic appearance of the paws and gingival tissues. (a) Experimental arthritis (EA) only front paw; (b) EA only rear paw; (c) periodontitis and EA front paw; (d) periodontitis and EA rear paw; (e) control gingiva; (f) periodontitis gingiva. Paws were imaged at day 8 post arthritis induction.

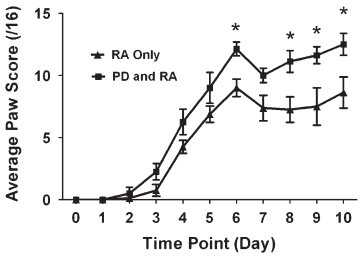

Fig. 3. (a) Average paw scoring using the /16 method as described in the 'Methods' section ($n = 4$ per group). Periodontitis and experimental arthritis (EA) score was significantly higher than RA alone $^{*}p < 0.05$ (b).

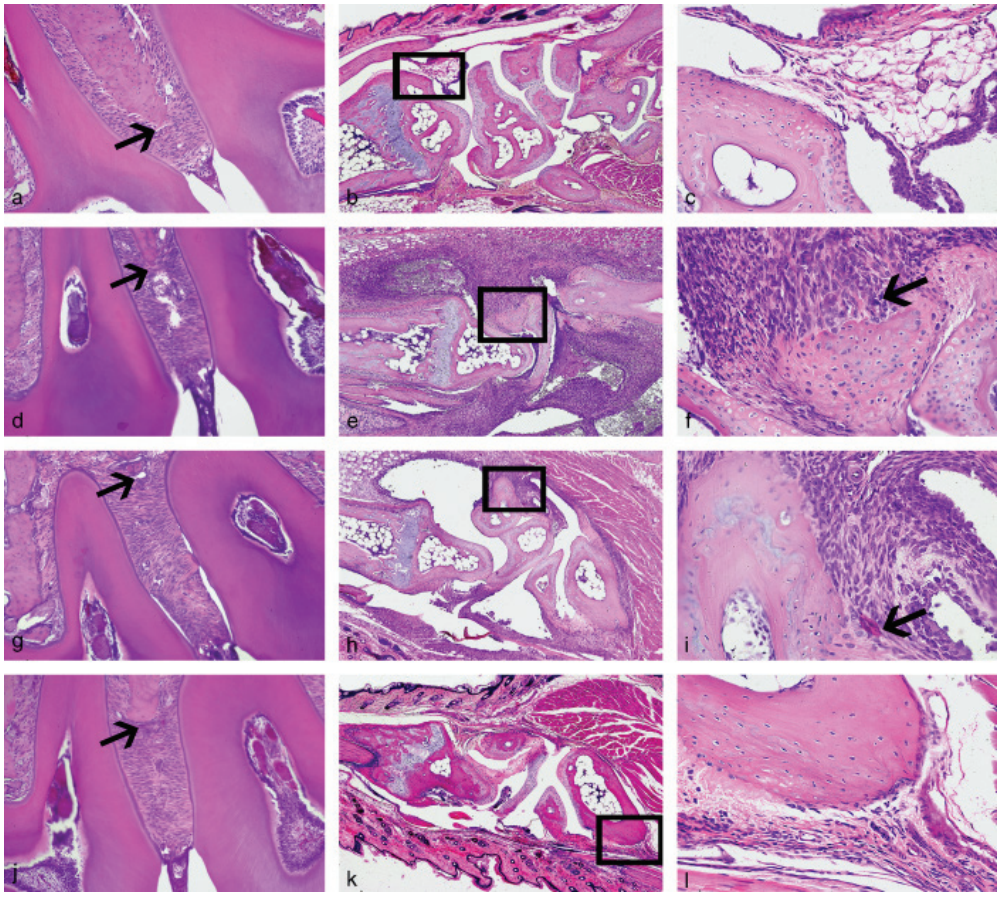

Fig. 4. Histological appearance of periodontal tissues (a,d,g,j) at × 20 magnification and front paw joints (b,e,h,k) at × 4 magnification and (c,f,i,l) at × 20 magnification. (a,b,c) Controls (d,e,f) periodontitis and experimental arthritis (EA) (g,h,i) EA only (j,k,l) periodontitis only. The boxes in the second panel highlight the areas imaged at × 20 magnification in the third panel. Arrows in the first panel indicate level of alveolar bone crest. In the third panel arrows indicate the presence of multinucleated osteoclast cells.

Inflammation in the periodontal tissues

Macroscopic assessment of the incisors indicated that the gingival tissues were red and inflamed and confirmed that a gingival response had been induced (Fig. 2) although, this can be difficult to appreciate due to the nature of this model. The molars, which are the main affected sites, cannot be imaged macroscopically in a longitudinal study of this kind. Micro-CT analysis at the day 44 confirmed alveolar bone loss consistent with our previous studies (Bendyk et al. 2009, Cantley et al. 2009a). The increased CEJ to ABC length at scan 2 also confirms disease induction (Fig. 6). Histological assessment demonstrated a robust inflammatory infiltrate in the periodontitis and combined periodontitis and arthritis groups (Fig. 5). Interestingly, the arthritis alone group manifested some histological evidence of gingival inflammation (Figs 4 and 5). Further analysis and scoring of the periodontal tissues confirmed that inflammation was present in all three of the test groups with the arthritis alone group and combined periodontitis and arthritis groups having greater inflammation than the periodontitis alone (Fig. 5).

Micro-CT analysis of joint and periodontal bone loss

Live animal micro-CT analysis was used to assess changes in BV in the radiocarpal joints of the paws and alveolar bone in the mouth (Fig. 6). From the initial baseline scan to scan 2 (after inducing periodontitis for 36 days) there was an average 16% increase in BV for both front and left-hand side paws as would be expected with the normal growth of these mice. The BV analysis of the front paws indicated that arthritis had been induced with a significant decrease in BV compared with controls (Fig. 6). From scan 2 to scan 3 there was an average 14% decrease in BV for both the arthritis only and combined periodontitis and arthritis groups compared with the normal 18% increase in BV observed in control mice. From scan 2 to 3 there was a decrease in BV in the paws of mice with periodontitis ($p < 0.01$). In the histological sections there was also some evidence of resorption in the periodontitis alone group but little evidence of inflammation (Fig. 4). There was no significant difference in the percentage change in BV from scan 2 to 3 in the arthritis group and the

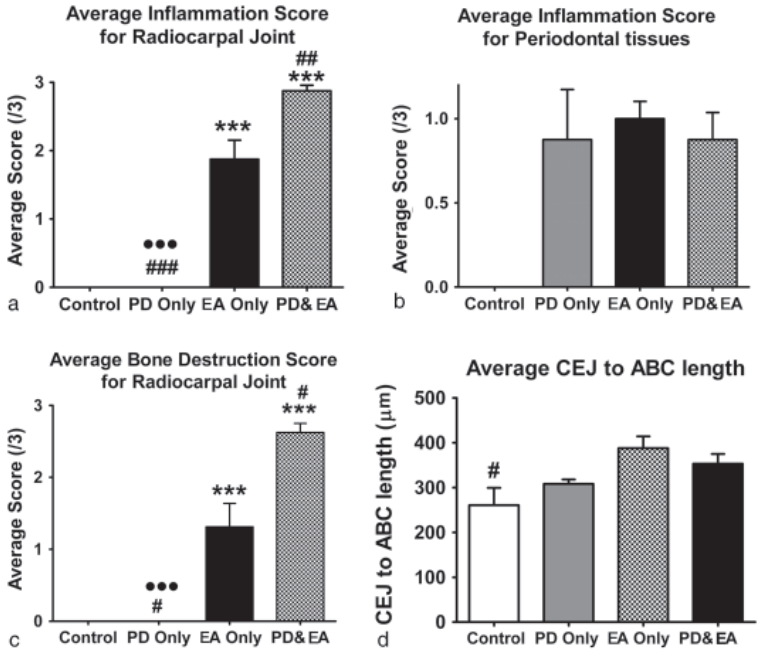

Fig. 5. Average total histological scores for inflammation in (a) the radiocarpal joints (b) periodontal tissues. Bone destruction in (c) radiocarpal joints (d) average cemento-enamel junction to alveolar bone crest length in μm. $^{***}p<0.001$ compared with controls. ###$p<0.001$. ##$p<0.01$. #$p<0.05$ compared with experimental arthritis (EA) only. • • • $p<0.001$ compared with periodontitis and EA. Details of scoring can be found in the methods.

combined periodontitis and arthritis group, indicating that although there are significant differences in inflammation during this time, the extent of bone destruction is similar. Scoring of histological sections for bone and cartilage destruction demonstrated a significant loss in the group with periodontitis and arthritis compared with arthritis alone (Fig. 5).

Analysis of the alveolar bone surrounding the three maxillary left molars using micro-CT demonstrated that periodontitis had effectively been induced with a 18% decrease in BV from scan 2 (day 0 of RA induction) to scan 3 (10 days after arthritis induction). Overall, in the periodontitis alone group, from the baseline scan to the final scan there was 5% decrease in bone loss, whereas in the group that also received arthritis there was a 23% decrease in BV, indicating enhanced bone destruction in the jaw in mice with both diseases. Interestingly, there was also a 16% decrease in BV of alveolar bone in mice that only received arthritis, which is also evident in the histological image (Fig. 5c). The distance from the CEJ to ABC was increased in mice with periodontitis (Fig. 6). For scan 2 the CEJ–ABC length was significantly higher for both periodontitis groups compared with control confirming disease induction ($p<0.01$). There were no significant differences in CEJ to ABC length for all diseased groups during scan 2. For scan 3 the CEJ to ABC length was significantly higher in the arthritis alone group compared with control confirming the presence of alveolar bone destruction.

RANKL immunohistochemistry

Immunostaining for RANKL demonstrated high levels in the inflamed soft tissue of joints from mice with both periodontitis and arthritis (Figs 7 and 8a). High levels were particularly noted in the pannus region where active bone loss was apparent as would be expected in these groups. Healthy animals and animals with periodontitis alone showed no evidence of pannus in growth into the bone and lacked strong staining in the region where the synovial tissues attached to the bone cartilage interface. RANKL was also detected in the bone-lining cells as well as some chondrocytes and osteocytes of the radiocarpal joint. Similar, but slightly weaker, staining in both the soft tissue and bone was evident in the group with arthritis alone. We did note some weak staining in the synovial tissues of animals with periodontal disease alone and RANKL was only very weakly expressed in the tissues of healthy animals. RANKL was also detected in the periodontal tissues of mice with periodontitis alone and the combined periodontitis and arthritis group. There was mild RANKL staining in the periodontal tissues of the arthritis alone group and weaker staining in healthy tissues (Fig. 8a).

Serum CRP

CRP levels were assessed in serum collected from mice 10 days after arthritis induction. Significantly higher levels of CRP were detected in all groups compared with the control mice. Mice with both periodontitis and arthritis also demonstrated significantly higher CRP levels compared with mice with periodontitis alone and arthritis alone ($p<0.05$) (Fig. 8b).

Discussion

In recent years, a relationship between periodontitis and RA has become increasingly apparent. In a previous study, we established that an extra synovial inflammation, similar to but not identical to periodontitis, exacerbated RA (Bartold et al. 2010a, b). In the present investigation, we have extended these studies to include an assessment of an infective component (*P. gingivalis*-induced periodontitis) to the development of EA. The individual roles of infective viable *P. gingivalis* and its antigens (non-infective) in the interaction between periodontitis and arthritis will now require further study.

The results of this study demonstrate that, in mice, a pre-existing periodontitis significantly influences the induction and severity of CAIA. This observation is consistent with clinical studies in which individuals with periodontitis have more significant RA (Ribeiro et al. 2005, Havemose-Poulsen et al. 2006). The observed exacerbation of arthritis by pre-existing periodontitis could be related to the systemic effects of inflammatory cytokines as a result of the pre-existing chronic inflammation associated with the periodontitis (Bartold et al. 2010a, b). Recently, it has been demonstrated that in AIRmax mice co-induction of periodontal disease

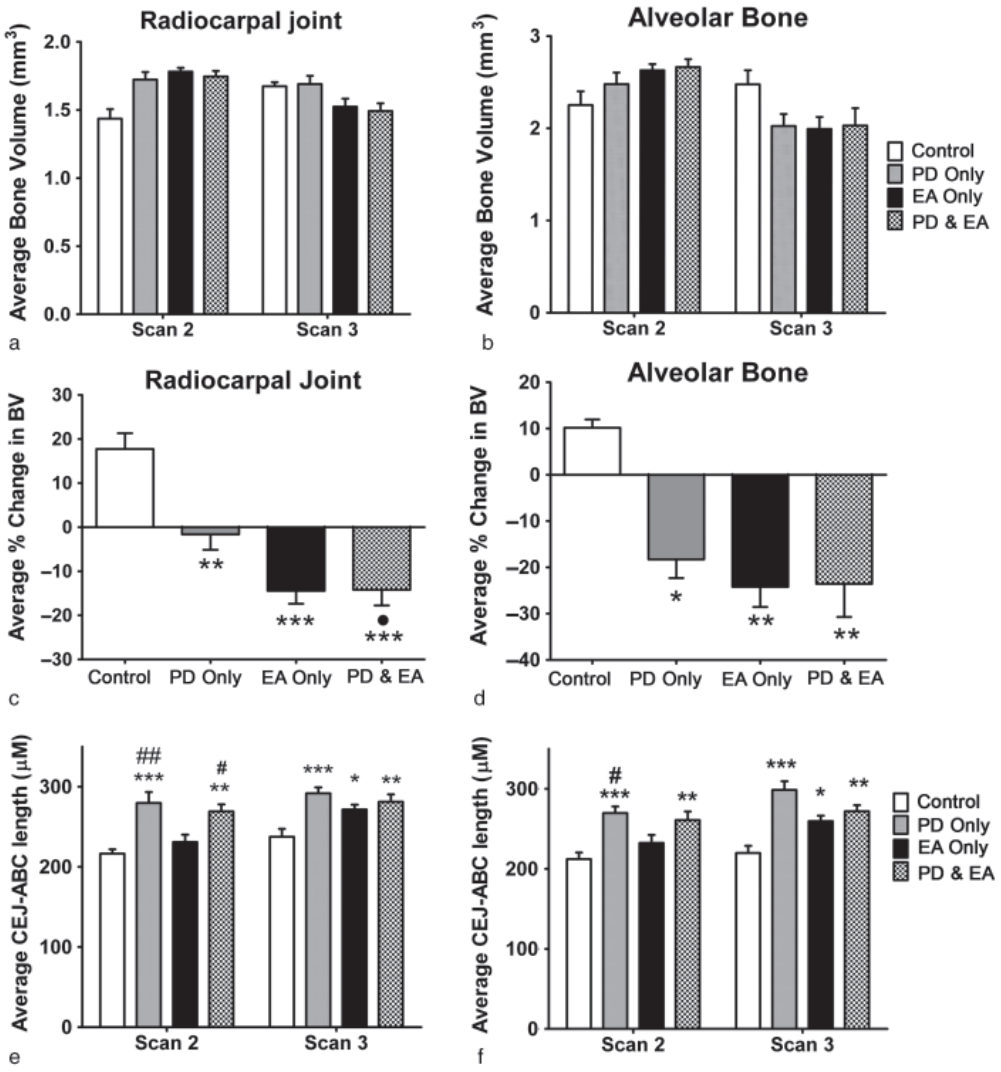

Fig. 6. (a) Absolute bone volume (BV) in mm^3 in the radiocarpal joint as assessed by micro-CT analysis for scan 2 and scan 3 (scan 2 – after inducing periodontitis, before experimental arthritis (EA) induction, scan 3 – final scan). (b) Absolute BV in mm^3 of alveolar bone as assessed by micro-CT analysis for scan 2 and scan 3. (c) Average percentage change in bone volume of the radiocarpal joint during the EA stage as determined using live animal micro-CT scanning. Bars represent mean ± SEM ($n = 4$ per group), (d) average percentage change in bone volume of the alveolar bone supporting the three molars of the maxilla on the left-hand side. Bars represent mean ± SEM ($n = 4$ per group), (e) average length of cemento-enamel junction (CEJ) to alveolar bone crest (ABC) (μm) between the first and second molars. (f) Average length of CEJ to ABC (μm) between the second and third molars. $^{***}p < 0.001$, $^{**}p < 0.01$ $^{*}p < 0.05$ compared with control. • $p < 0.05$ compared with periodontitis only. $\#\#p < 0.001$, $\#p < 0.05$ compared with RA only.

and arthritis, using the model of PIA, resulted in higher serum levels of IL-1β, IFN-γ, IL-17, RANKL and MMP-13 levels compared with the PIA alone (Trombone et al. 2010). It has been proposed that these inter-relationships may be related to dysregulation of the inflammatory system and a systemic increase in pro-inflammatory cytokines (Mercado et al. 2003, Bartold et al. 2005, 2010a, b) and a general systemic inflammation. The elevated RANKL expression in joint and periodontal tissues and increased serum CRP levels in the test groups of the present study lend support to the effect of systemic inflammation on both periodontitis and RA.

The above findings are in contrast to another study, which investigated the co-induction of arthritis and periodontitis in AIRmin mice (Trombone et al. 2010). This could be due to the differences in experimental arthritis models in which different severities of arthritis induction could be related to the development of a pre-existing periodontitis. Furthermore the sequence of disease induction differed between these two studies. In our study, periodontitis was induced before the induction of arthritis. The reasoning behind developing a pre-existing periodontitis is related to the clinical situation. Clinical investigations by our group have suggested that patients with severe RA are more likely to have advanced periodontitis and vice versa

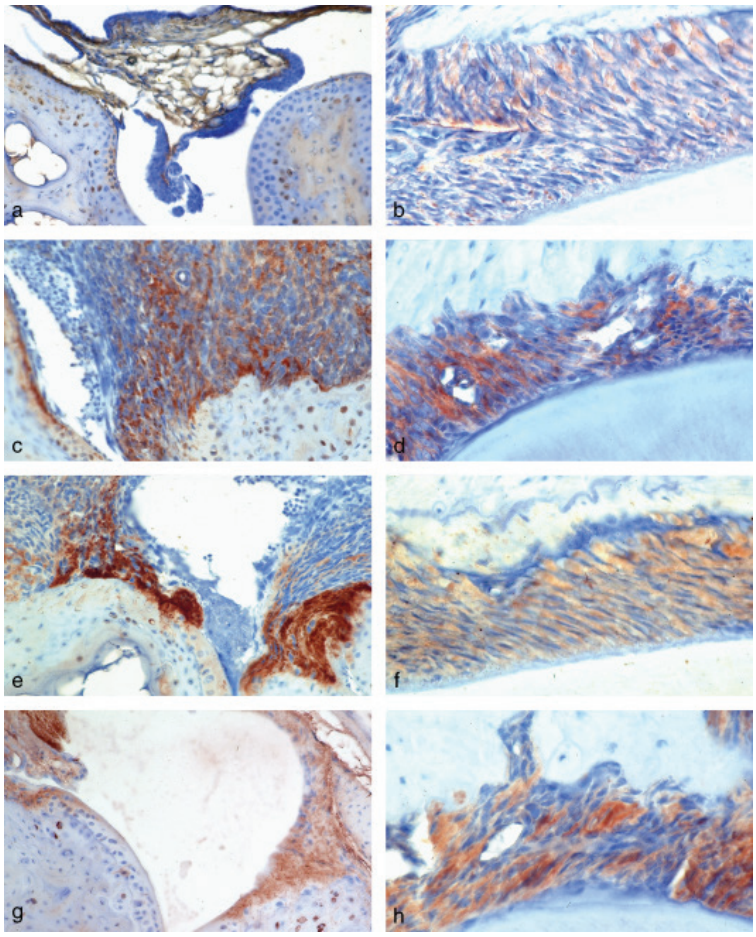

Fig. 7. Immunostaining to detect RANKL in the radiocarpal joints (a,c,e,g) and periodontal tissues (b,d,f,h). Controls (a and b), periodontitis and experimental arthritis (EA) (c and d); EA only (e and f); periodontitis only (g and h). Original magnification × 20. In the periodontal tissue images alveolar bone is at the top of the section and the tooth at the bottom.

(Mercado et al. 2000, 2001). It has also been noted that patients with RA taking anti-inflammatory medications, which are likely to reduce periodontal inflammation, still have signs of periodontal destruction indicating that periodontitis may develop early and is not detected before development of RA (Bartold et al. 2005).

In this model of CAIA, we used a lower dose of LPS, 5 μg compared with 10 μg used in previous studies. This lower dose was used to induce a noticeable arthritic response that allows us to assess the effect of pre-existing periodontitis on the response. There were no significant differences in paw scoring observed between mice given the lower 5 μg of LPS compared with 10 μg. The major difference in disease severity with the different doses included the rear paws more commonly being affected in the higher LPS group. This did not impact the results as the front paws were used for CT and histological analysis. It was interesting to note that rear paws of mice with periodontitis and arthritis were more commonly affected.

Despite significantly higher levels of inflammation in the radiocarpal joints in mice with both periodontitis and arthritis compared with arthritis alone, there was no significant difference in bone loss as detected by CT analysis. Although scoring of histological bone and cartilage destruction did demonstrate significant levels of higher bone loss in the periodontitis and arthritis group compared with arthritis alone. This could be due to methodology differences and a later time point CT scan may demonstrate differences but this was not conducted due to ethics.

In addition to noting the effect of pre-existing periodontitis on the development of arthritis, we found evidence of bone destruction and increased RANKL expression in the radiocarpal joints of mice that had periodontitis only. This finding further highlights the possibility that extra-synovial infection and/or inflammation has the potential to influence the synovial tissues (Bartold et al. 2010a, b). The mechanisms underlying this finding remain to be elucidated. Because there was no overt inflammation noted in the synovial tissues of mice with periodontitis alone, the mechanisms for these joint changes in these animals remains to be established. Nonetheless, the observation that RANKL expression was elevated in the joints of these animals indicates that some systemic influences have the potential to affect local sites. Such findings have been noted in RA whereby generalized osteopenia is noted and is thought to arise through the systemic action of inflammatory cytokines released from the site of synovial inflammation (Deodhar & Woolf 1996, Guler-Yuksel et al. 2008, 2009, Goldring 2009).

The converse finding from this study is that mice with arthritis alone showed signs of alveolar bone destruction implying that experimental arthritis may increase the severity of periodontitis. CT analysis revealed evidence of a BV loss in mice with arthritis only and a significantly longer CEJ to ABC length compared with controls as assessed by CT and histological sections. While this could not be directly assessed in this study due to the timing of disease induction, this is also a possibility. Similar findings of periodontal changes being observed following the induction of adjuvant arthritis in rats have been reported (Ramamurthy et al. 2005, Park et al. 2010). In our model, the periodontal changes observed in the arthritis only animals may be related to the LPS dosing given for the induction of CAIA, which was introduced as an i.p. injection, and thus entered the systemic circulation with the potential to have effects at sites throughout the body. However, the mechanisms through which these changes may arise are unclear and warrant further investigation. Nonetheless, the result of periodontal bone destruction observed in mice with arthritis is consistent with

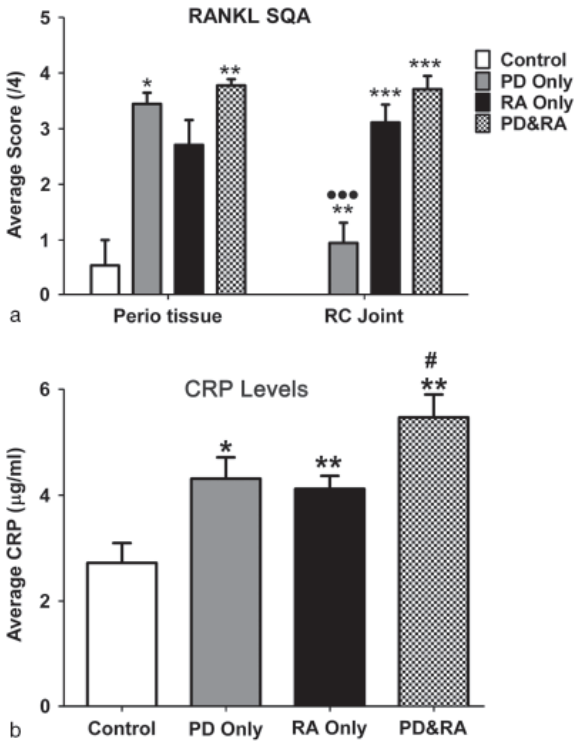

Fig. 8. (a) Semiquantitaive analysis (SQA) of RANKL positive cells for radiocarpal joints and periodontal tissues. (b) Average serum C-reactive protein (CRP) levels (μg/ml) measured using an ELISA. $^{***}p<0.001$ $^{**}p<0.01$ and $^{*}p<0.05$ compared with control. $\#p<0.05$ compared with EA only. • • • $p<0.001$ compared with periodontitis and EA group.

clinical studies that have demonstrated significantly higher incidences of tooth loss and alveolar bone loss in patients with RA (Mercado et al. 2000, 2001, Al-Shammari et al. 2005).

A number of mechanisms which may account for a relationship between periodontitis and RA have been proposed (Bartold et al. 2005). These may include vascular alterations and osseous changes related to altered RANKL/osteoprotegerin ratios, bacterial infection and enhancement of autoimmunity through citrullination of proteins. Of these, autoimmunity characterized by production of antibodies against citrullinated proteins is of particular interest because *P. gingivalis* produces deimination enzymes such as peptidyl arginine deaminase and this may lead to citrullination of proteins within the periodontal tissues. Accordingly, the potential for citrullination of proteins by *P. gingivalis* and subsequent generation of autoantigens, which could drive autoimmunity in RA has been proposed as one mechanism linking periodontitis and RA (Rosenstein et al. 2004, Lundberg et al. 2010).

In conclusion the key finding of this study indicates that pre-existing periodontitis exacerbated arthritis in a mouse model. While statistically significant, this relationship was modest. Furthermore, there is some potential that a bidirectional relationship between periodontitis and arthritis may exist. That is, not only can periodontitis influence the joint tissues but arthritis can also influence the periodontal tissues. Future studies will need to address the mechanisms through which these associations act and the model developed in this study would provide a valuable tool for such studies. In addition, this model could be used to investigate whether treatment of one condition can have any benefit on the other as well as test new therapeutic targets to determine effects on both disease states.

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Clinical Relevance

Scientific rationale for study: Previous studies have indicated a significant relationship between RA and periodontitis. In this study, we have extended our previous animal model of periodontal disease to develop an animal model in which pre-existing periodontitis is established in mice followed by induction of RA to investigate the relationship between these two prevalent conditions.
Principal findings: Pre-existing periodontitis induced by *P. gingivalis* exacerbated the onset and severity of experimental RA.
Practical implications: Control of periodontal infection and inflammation may be important in the management of RA.

Chapter 3. Inhibitors of Histone Deacetylases in Class I and Class II Suppress Human Osteoclasts *In Vitro*

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Journal of Cellular Physiology 2011; 226(12):3233-41

Chapter Summary

Bone loss is a characteristic feature in both PD and arthritis. The aim of most pharmacological therapies in RA is to resolve the inflammation, whilst having positive effects on disease activity it does not always control the bone loss. In PD, current treatment focuses mainly on targeting the infection and hence the inflammatory process. HDACi are one such potential treatment to suppress the enhanced osteoclast bone loss. Previous studies have shown that HDACi can suppress osteoclast bone resorption using cell lines. No studies have assessed effects on human cells or assessed the importance of individual HDACs (1-10) during osteoclastogenesis. For this reason, the aims of the study were to determine the effect of inhibitors targeting different HDACs on human osteoclast activity

in vitro. It was hypothesized that HDACi targeting both Class I and II HDACs would be more effective at suppressing osteoclast bone resorption *in vitro* than inhibitors targeting either Class I or Class II alone.

STATEMENT OF AUTHORSHIP

Inhibitors of Histone Deacetylases in Class I and Class II Suppress Human Osteoclasts In Vitro

Journal of Cellular Physiology, 2011; 226(12):3233-41

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Certification that the statement of contribution is accurate

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Lucke A.J

My contribution to the paper was in the design, chemical synthesis and characterization of the HDAC inhibitor 1179.4b. Critical review, comments and proofing of the paper were also contributed.

Drug design and development

I certify that the statement of contribution is accurate and permission is given for the inclusion of this paper in the thesis.

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Advice on data analysis and PCR methodology. Involved with western blot methodology.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Supervised development of work, helped in data interpretation and manuscript evaluation

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Journal of Cellular Physiology

Inhibitors of Histone Deacetylases in Class I and Class II Suppress Human Osteoclasts In Vitro

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Histone deacetylase inhibitors (HDACi) suppress cancer cell growth, inflammation, and bone resorption. The aim of this study was to determine the effect of inhibitors of different HDAC classes on human osteoclast activity in vitro. Human osteoclasts generated from blood mononuclear cells stimulated with receptor activator of nuclear factor kappa B (RANK) ligand were treated with a novel compound targeting classes I and II HDACs (1179.4b), MS-275 (targets class I HDACs), 2664.12 (targets class II HDACs), or suberoylanilide hydroxamic acid (SAHA; targets classes I and II HDACs). Osteoclast differentiation was assessed by expression of tartrate resistant acid phosphatase and resorption of dentine. Expression of mRNA encoding for osteoclast genes including RANK, calcitonin receptor (CTR), c-Fos, tumur necrosis factor (TNF) receptor associated factor (TRAF)6, nuclear factor of activated T cells (NFATc1), interferon-β, TNF-like weak inducer of apoptosis (TWEAK), and osteoclast-associated receptor (OSCAR) were assessed. Expression of HDACs 1–10 during osteoclast development was also assessed. 1179.4b significantly reduced osteoclast activity ($IC_{50} < 0.16$ nM). MS-275 (IC_{50} 54.4 nM) and 2664.12 ($IC_{50} > 100$ nM) were markedly less effective. A combination of MS-275 and 2664.12 inhibited osteoclast activity similar to 1179.4b (IC_{50} 0.35 nM). SAHA was shown to suppress osteoclast activity (IC_{50} 12 nM). 1179.4b significantly ($P < 0.05$) reduced NFATc1, CTR, and OSCAR expression during the later stages of osteoclast development. Class I HDAC 8 and Class II HDAC5 were both elevated ($P < 0.05$) during osteoclast development. Results suggest that inhibition of both classes I and II HDACs may be required to suppress human osteoclastic bone resorption in vitro.

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Pathological bone loss is associated with many common diseases including rheumatoid arthritis (RA), periodontitis, and osteoporosis. Although treatments for osteoporosis and RA now exist, there are very few effective treatments to suppress the destructive alveolar bone loss that occurs in periodontitis. A disruption to the normal bone remodeling process with enhanced bone resorption by osteoclasts is the characteristic feature of these diseases and hence factors that influence osteoclast function have become important targets for therapeutic intervention.

Osteoclasts are multinucleated cells derived from the hematopoietic lineage that are responsible for resorbing bone during both normal and pathological bone turnover (Lerner, 2000; Boyle et al., 2003; Rubin and Greenfield, 2005). Receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) is a key mediator for osteoclast differentiation, activation, and survival (Lacey et al., 1998; Yasuda et al., 1998). This is a membrane bound protein of the tumor necrosis factor (TNF) family that is expressed by osteoblasts, fibroblasts, and activated T cells. A crucial step in osteoclast formation is ligation of RANKL with its receptor RANK on osteoclast precursor cells activating NF-κB, leading to the development of osteoclast cells that resorb bone (Rubin and Greenfield, 2005). RANKL has been shown to form this important link between immunology and bone physiology, with inflammatory cytokines such as TNF-α and IL-1b known to stimulate the production of RANKL (Hofbauer and Heufelder, 2001). This elevated expression of RANKL by inflammatory cells is known to be associated with common chronic inflammatory diseases such as RA and periodontitis (Crotti et al., 2003a,b; Haynes, 2006) and hence the interaction between RANKL and RANK has been a common target for therapeutic intervention. Denosumab, a monoclonal antibody to RANKL, has undergone clinical trials of postmenopausal women with low bone mineral densities (McClung et al., 2006; Lewiecki et al., 2007). Results to date indicate that inhibition of RANKL leads to increased bone mineral density and decreased bone resorption (Bekker et al., 2001; Bekker et al., 2004). Denosumab has now been FDA approved for use in postmenopausal women at risk of osteoporosis (Perrone, 2010).

While treatments targeting RANK–RANKL interactions may prove to be effective, they may not be suitable for all situations and therefore other approaches will be needed. Emerging new targets for therapeutic intervention to treat a wide variety of disease states are enzymes known as histone deacetylases (HDACs) that are reported to regulate transcription of genes such as NF-κB (Trepel and Birrer, 2003; Chen and Greene, 2004; Imre et al., 2006). An important

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function of HDAC enzymes is to remove acetyl groups from histones that condense the chromatin leading to gene repression (Richon and O'Brien, 2002). However, when HDACs are inhibited, other non-histone proteins may be acetylated (Richon and O'Brien, 2002) and this may have a variety of effects including regulating the transcription of genes. Inhibition of HDACs has been shown to result in up-regulation of cell cycle inhibitors, down-regulation of immune stimulators, repression of inflammatory cytokines (Chung et al., 2003), and suppression of osteoclast bone resorption (Nakamura et al., 2005).

Class I HDACs include HDAC 1, 2, 3, and 8 enzymes that are found mainly in the nucleus. Class II include HDACs 4, 5, 7, and 9 which belong to class IIa HDACs and HDACs 6 and 10 belonging to class IIb HDACs. Class II HDACs are able to shuttle between the nucleus and cytoplasm (De ruijter et al., 2002; Dokmanovic and Marks, 2005; Monneret, 2005; Bhavsar et al., 2008). Most histone deacetylase inhibitor (HDACi) research is currently focusing on developing compounds that selectively inhibit different HDAC isoforms since they may have quite different anti- or pro-inflammatory properties. However, suberoylanilide hydroxamic acid (SAHA) that inhibits both classes I and II HDACs may have important anti-inflammatory and anticancer activities (Butler et al., 2002; Vernia et al., 2003; Huang, 2006) as well as causing growth arrest, differentiation, or apoptosis of transformed cells (De ruijter et al., 2002; Nakamura et al., 2005).

Select HDACi have been shown to inhibit osteoclast-mediated bone resorption in murine cells through interference with the RANKL–RANK signaling pathway (Nakamura et al., 2005). Depsipeptide, FR901228 (also known as FK228) was shown to inhibit osteoclastogenesis in rat bone marrow cultures by suppressing RANKL and also increasing expression of osteoclast inhibitor interferon-β (IFN-β; Nakamura et al., 2005). Trichostatin A (TSA) which targets classes I and II HDACs, was also shown to suppress RANKL-mediated osteoclastogenesis in a dose-dependent manner (Rahman et al., 2003). Recently, it was found that SAHA mediated its effects by inhibiting RANKL-induced osteoclastogenesis through suppression of the NF-κB pathway (Takada et al., 2006). Although these studies provide some evidence that HDACi prevent osteoclastic bone resorption it is not known whether inhibiting both classes of HDACs (i.e., classes I and II HDACs) or targeting-specific classes is most effective to inhibit osteoclast bone resorption. In addition, studies using human osteoclasts have not yet been reported and the exact mechanisms of action of these HDACi are still not clear.

This study aimed to test the hypothesis that compounds targeting both classes I and II HDACs are more effective at suppressing osteoclast bone resorption in vitro than inhibitors targeting either class I or class II alone. To test this hypothesis we compared the ability of four HDACi compounds to suppress RANKL-stimulated formation of human osteoclasts in vitro. The effects of a novel inhibitor that targets both classes I and II HDACs on the mRNA expression of key factors involved in osteoclast formation and activity were also investigated, such as RANK, calcitonin receptor (CTR), c-Fos, TNF receptor-associated factor (TRAF) 6, nuclear factor of activated T cells (NFATc1), IFN-β, TNF-like weak inducer of apoptosis (TWEAK), and osteoclast-associated receptor (OSCAR). The expression of HDACs during osteoclast development was also assessed at the mRNA and protein levels.

Materials and Methods

HDACi

Structures for the four HDACi compounds evaluated in this study are shown in Figure 1. Compound 1179.4b is a newly developed synthetic compound targeting HDACs classes I and II developed in our laboratories (compound 52; Kahnberg et al., 2006). Compound

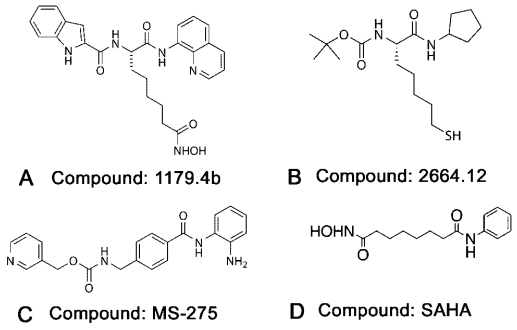

Fig. 1. Chemical structures of: (A) 1179.4b, which targets classes I and II HDACs (*S*)-*N*8-hydroxy-2-(1H-indole-2-carboxamido)-*N*1-(quinolin-8-yl)octanediamide (Compound #52; Kahnberg et al., 2006). B: 2664.12 which targets class II HDACs (*S*)-tert butyl 1-(cyclopentylamino)-7-mercapto-1-oxoheptan-2-ylcarbamate (compound 17a; Suzuki et al., 2006). C: MS-275 which targets class I HDACs *N*-(2-aminophenol)-4-(*N*-(pyridine-3-yl-methoxycarbonyl) aminomethyl) benzamide) (Suzuki et al., 1999). D: Suberoylanilide hydroxamic acid (SAHA) which targets classes I and II HDACs (Vorinostat, Zolinza®; Merck and Co.).

2664.12 targets class II HDACs with some selective affinity for HDAC-6 (compound 17a; Suzuki et al., 2006), while MS-275 is a selective inhibitor of HDAC class I that has been shown to induce hyperacetylation of nuclear histones in various tumor cell lines and is in clinical investigations for treatment of solid and hematological tumors (Ryan et al., 2005a). SAHA (Vorinostat, Zolinza®, Merck and Co., New Jersey) is a hydroxamic-based inhibitor approved for treatment of cutaneous T-cell lymphoma and targets both classes I and II HDACs (Duvic and Vu, 2007).

In vitro osteoclast assay

Human peripheral blood mononuclear cells (PBMCs) were obtained by differential centrifugation of whole blood buffy coats as previously reported (Holding et al., 2006). PBMCs were suspended in α-minimal essential medium (α-MEM; Invitrogen, Melbourne, Victoria, Australia) supplemented with 10% fetal calf serum (FCS; Invitrogen, Life Technologies, Carlsbad, CA), 1% penicillin–streptomycin (Invitrogen), and 1% L-glutamine (Invitrogen). The PBMCs were then seeded (5×10^5 cells/well) into wells of 48-well trays either onto sterilized whale dentine pieces used for pit resorption analysis or directly onto the glass slide for TRAP stain analysis as previously described (Holding et al., 2006).

Cells were maintained at 37°C with 5% CO_2 in complete medium (with 10% FCS, 1% penicillin–streptomycin, 1% L-glutamine) with 100 nM 1α, 25$(OH_2)D_3$ (vitamin D_3) (Novachem, Melbourne, Victoria, Australia), 100 nM dexamethasone (Fauldings, Adelaide, South Australia, Australia), and 25 ng/mL of recombinant human M-CSF (Chemicon International Inc, Millipore, MA) for 17 days. Cells were treated using fivefold dilutions of the inhibitors, 1179.4b, MS-275, 2664.12, and SAHA starting at 100 nM in 0.01% DMSO from day 7. Control wells were treated with 0.01% DMSO. Human recombinant RANKL (50 ng/mL; Chemicon International Inc) was added to the medium on the treatment days. Treatment with 1179.4b was also commenced on days 10 and 13 after RANKL administration to assess the effect of the compounds on the different stages of osteoclast development. On day 17, the formation of multinucleated TRAP-positive staining cells was used as an indication that osteoclasts had formed. The number of cells with three or more nuclei and expressed TRAP were considered to

be osteoclasts. The number of TRAP-positive cells for each concentration of HDACi was compared with the controls for individual donors. Osteoclast activity was assessed by determining the area of pit resorption on the dentine pieces using scanning electron microscopy. Three representative images were taken for each dentine piece at 150× magnification and, using Image J analysis software, the area of pit resorption for each concentration of the different HDACi was determined and expressed as a percentage of the total area of the dentine. This percent area of resorption in the treated cells was then represented as a percentage of the average area of resorption in the untreated cultures.

WST-1 cell viability assay

Cells grown in the presence of 1179.4b, MS-275 + 2664.12, and SAHA at all concentrations (0.16–100 nM) were assessed for cell viability using a standard WST-1 assay (Roche Applied Science, Castle Hill, NSW, Australia). On day 14, 10 μL of cell proliferation reagent WST-1 was added to each well (100 μL media) followed by incubation for 2 h at 37°C. A control blank was used which consisted of culture medium with WST-1 reagent without any cells. After 2 h incubation absorbance was measured at 450 nm. Absorbance in drug-treated wells was compared with that of controls.

RNA extraction and reverse transcription

Human PBMCs were cultured in the presence or absence of classes I and II HDACi 1179.4b for a period of 17 days. Total RNA was extracted from the cells on days 0, 7, 10, 14, and 17 using 600 μl TRIzol (Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription was carried out using a Corbett real-time PCR machine (Corbett Research Rotor Gene RG-3000; Corbett Life Science, Mortlake, NSW, Australia) with 250 ng of random hexamer (Geneworks, Adelaide, SA, Australia) and 200 U of Superscript III Reverse Transcriptase according to the manufacturer's instructions to produce cDNA.

Real-time PCR analysis

Quantitative real-time PCR was then performed to compare expression levels of mRNA for specific osteoclast genes in drug-treated cells with the untreated cells. This was achieved using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The endogenous reference gene human acidic ribosomal protein (hARP; Franssen et al., 2005) was used to allow comparison of the data for each of the genes. Each reaction mixture consisted of 1 μg cDNA, 2× supermix (has SYBR Green I dye), 300 nM of forward and reverse primer and this was made up to a total volume of 15 μL with diethyl pyrocarbonate (DEPC) water. PCR was performed in triplicate for each sample. The relative quantification of the mRNA expression for each of the genes was then calculated using the comparative C_t method – $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Effect of HDACi on osteoclast genes

Oligonucleotide primers described previously used were RANK (Untergasser et al., 2007), CTR (Granfar et al., 2005), IFN-β (Nagy et al., 2008), and NFATc1 (Granfar et al., 2005). Other primers were designed using Primer3Plus (Untergasser et al., 2007). These were c-fos (sense, 5′-CAAGCGGAGACAGACCAACT-3′ and antisense, 5′-GGGCAAGGTGGAACAGTTAT-3′), TRAF-6 (sense, 5′-TCATCAGAGAACAGATGCCTAATC-3′ and antisense, 5′-GGCATTTGAAGATAAAGAGATCC-3′), OSCAR (sense, 5′-CATGAGCTTCGTGCTGTACC-3′ and antisense, 5′-CCAGAGTCTTCCCAGCTGAT-3′), and TWEAK (sense, 5′-ATCGCTGTCCGCCCAGGAGC-3′ and antisense, 5′-CTGTCTGGGGATTCAGTTCCG-3′).

HDAC expression throughout osteoclast development

HDAC primers that were those previously used, HDAC 1 (Glozak et al., 2005), HDAC 2,4,5,7,8,9,10 (Duong et al., 2008). Others were designed using Primer3Plus, HDAC 3 (sense, 5′-GAGAGTCAGCCCCACCAATA and antisense, 5′-TGTGTAACGCGAGCAGAACT), HDAC 6 (sense, 5′-ACCTAATCGTGGGACTGCAAG and antisense, 5′-GAAAGGACACGCAGCGATCT).

Western blot analysis

Cells for Western blot analysis were seeded into 75 cm^2 flasks at a concentration of 1×10^7 cells/mL. The cells were maintained for 17 days and treatment carried out as described above (from day 7). On day 17 of culture, 1179.4b-treated and the controls cells were lysed with Radio Immuno Precipitation Assay Buffer (RIPA) buffer to extract nuclear proteins. Protein concentration was determined using a BSA assay (Thermo Scientific BCA Protein Assay kit, Thermo Fisher Scientific, MA). Samples were dissolved in non-reducing Laemmli 2× buffer, boiled, and subjected to electrophoresis on a 10% SDS–PAGE gel. Protein was transferred from the gel to a PVDF membrane (Hybond-P Amersham Life Sciences, GE Healthcare Australia Pty. Ltd. Rydalmere, NSW, Australia) and then blocked with 5% skim milk powder in TBST (5% skim milk powder in TBS + 1% Tween-20) for 1 h to reduce non-specific binding. The membrane was washed with TBST and exposed to the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, NFATc1 (7A6):sc-7294) (1 μg/ml) overnight at 4°C. It was then washed three times with TBST and incubated with the secondary sheep anti-mouse antibody (AP326A; Chemicon, Millipore, Australia) for 1 h. After washing with TBST and TBS the membrane was subjected to an ECF substrate and incubated for 5 min at room temperature. Following washing with TBS the membrane was imaged using the Typhoon scanner (Typhoon TRIO+ Variable Mode Imager; Amersham Biosciences, GE Healthcare Australia Pty. Ltd. Rydalmere, NSW, Australia). Mouse monoclonal to beta actin (mAbcam 8224, 1 μg/mL) was used as a loading control.

Immunocytochemistry

Control cells and cells treated with 1179.4b at 20 nM were fixed at days 0 and 17 with 1:1 acetone and methanol for 5 min. Immunocytochemistry was then conducted to assess the protein expression of NFATc1, HDAC 5, and HDAC 8. Antibodies used were NFATc1 (7A6:sc-7294, 4 μg/mL; Santa Cruz Biotechnology), rabbit polyclonal to HDAC 5 (ab55403, 10 μg/mL) and rabbit polyclonal to HDAC 8 (ab39664, 10 μg/mL; Sapphire Bioscience). Staining was carried out using a Vectastain ABC kit (Vector Laboratories, CA) and visualized using a Vector Perioxidase substrate kit AEC (Vector Laboratories).

Statistics

For analysis of in vitro results a one-way ANNOVA was used to compare all drug concentrations for TRAP stain and bone resorption analysis. For mRNA analysis a Mann–Whitney *U*-test was used to determine significant at different time points. Statistical significance was accepted when $P < 0.05$.

Results

In this study, the effects of a novel compound that targets both classes I and II HDACs (1179.4b), a compound targeting class I HDACs (MS-275), a selective inhibitor of class II HDACs (2664.12) and an inhibitor of classes I and II HDACs (SAHA) on osteoclast formation and activity in vitro were investigated. RANKL stimulation of non-HDACi-treated cultures consistently induced the adherent PBMCs to differentiate into mature TRAP-positive multinucleated osteoclasts (Fig. 2A) that formed numerous resorption pits (Fig. 2B).

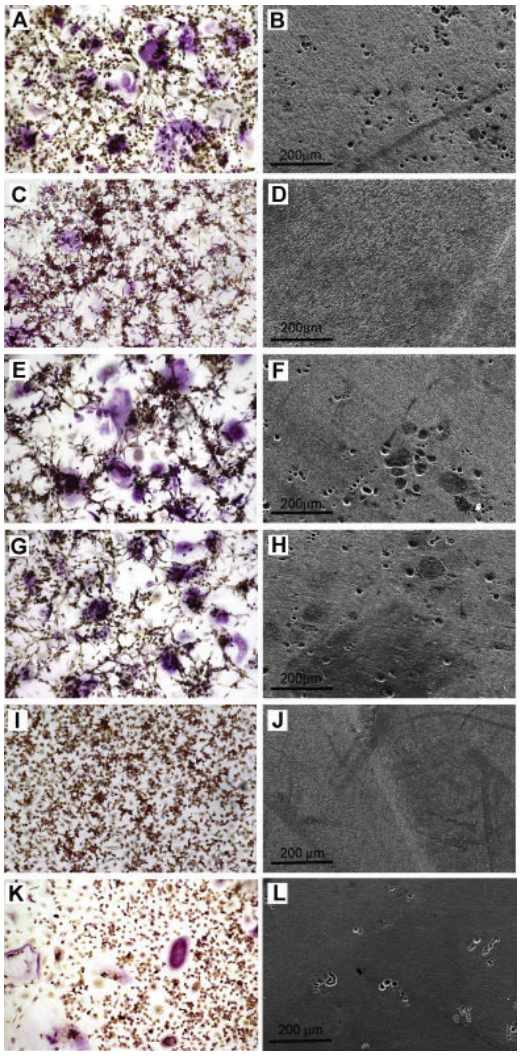

Fig. 2. The formation of TRAP expressing multinucleated cells (A,C,E,G,I,K) and resorption pit formation (B,D,F,H,J,L) were assessed as described in the methods. (A,B) No treatment; (C,D) treatment with 1179.4b at 20 nM; (E,F) treatment with MS-275 at 20 nM; (G,H) treatment with 2664.12 at 20 nM; (I,J) treatment with MS-275 and 2664.12 together at 20 nM; (K,L) treatment with SAHA at 20 nM.

Effect of HDACi on osteoclasts in vitro

In the human in vitro osteoclast assay, the WST-1 assay indicated no effect on cell viability for all concentrations of MS-275 and 2664.12 and for SAHA (0.16–100 nM). For 1179.4b at 100 nM there was a decreased cell viability compared to control ($P < 0.001$). There was no other significant effect on cell viability at any other concentrations tested (0.16–20 nM). Multinucleated osteoclast cells formed with treatment appeared smaller in size compared to controls.

At a concentration of 20 nM, 1179.4b almost completely inhibited TRAP expression (Figs. 2C and 4A) and osteoclast resorption (Figs. 2D and 4D). A decrease in the number of TRAP-positive cells was statistically significant ($P < 0.01$) at concentrations of 4 nM and above. There was also a statistically significant ($P < 0.001$) reduction in the number of pits formed with 1179.4b treatment at all concentrations tested, with a concentration-dependent response. The IC_{50} for inhibition of resorption for 1179.4b was less than 0.16 nM. This observed inhibition of bone resorption was confirmed by real-time PCR analysis of CTR expression, an osteoclast gene closely associated with the ability of osteoclasts to resorb bone. As expected there was more than a 100-fold increase, compared to day 0, in CTR mRNA in cultures at day 17 when osteoclasts were formed in untreated cultures. When cells were treated with 20 nM 1179.4b there was a markedly reduced (13-fold) CTR expression that corresponded with a marked inhibition of resorption (Fig. 5A; $P < 0.01$).

Treatment of cells with 1179.4b from day 10 following RANKL addition at day 7 did not have any effect on the formation of multinucleated TRAP-positive cells as assessed at day 17 (Fig. 4B). There was however a concentration-dependent reduction in the number of pits formed with significant inhibition at concentrations higher than 0.8 nM ($P < 0.05$, $IC_{50} = 1.1$ nM; Fig. 4E). Interestingly treatment commencement from day 13 did not have any effect on reducing the number of TRAP-positive cells and there was no significant reduction in the number of pits on dentine (Fig. 4C,F).

MS-275 had little effect on TRAP expression (Figs. 2E and 3B) and osteoclast activity (Figs. 2F and 3F) at 20 nM. MS-275 only significantly ($P < 0.001$) inhibited osteoclast formation and activity at the highest concentration tested (100 nM). The IC_{50} for inhibition of resorption for MS-275 was 54.4 nM.

2664.12 had no significant ($P > 0.05$) effect on TRAP expression or resorption at all concentrations tested (Fig. 3C,G). The IC_{50} for inhibition of resorption for 2664.12 was greater than 100 nM.

A combination of MS-275 and 2664.12 was tested to assess the effect on osteoclasts when HDAC of both classes I and II are targeted. Simultaneous MS-275 and 2664.12 treatment reduced TRAP expression and resorption at all concentrations similar to that observed for 1179.4b alone. There was a significant ($P < 0.01$) decrease in resorption at all concentrations with very few small pits seen at the 20 nM concentration (Figs. 2J and 3H). At 20 nM there was a significant difference in the number of TRAP-positive cells compared to (MS-275 $P < 0.05$, 2664.12 $P < 0.001$) and the area of bone resorption (MS-275 $P < 0.01$) when both drugs were combined compared to their individual administration. The compound combination demonstrated synergistic inhibition with more than 100-fold increase in activity with an IC_{50} of 0.35 nM.

SAHA was found to significantly reduce the formation of TRAP-positive osteoclasts at 20 nM ($P < 0.05$; Figs. 2K and 3A), and it was found to suppress pit resorption at all concentrations above 0.16 nM (Figs. 2L and 3E). It was however not as potent as 1179.4b with its IC_{50} being 12 nM.

In an attempt to establish the mechanism of action of novel HDACi compound 1179.4b, real-time PCR was carried out on RNA extracted from cells treated with and without 1179.4b at 20 nM at various time points during the assay. Throughout days 0–17 there was an increase in the expression of RANK with the formation of mature osteoclast cells (Fig. 5C). Administration of 1179.4b at a concentration of 20 nM from day 7 onwards however was shown to have no significant effect on the expression of RANK ($P > 0.05$; Fig. 5C). Cells treated with the novel classes I and II HDACi, 1179.4b, that inhibited osteoclast formation in vitro strongly suppressed NFATc1 ($P < 0.01$; Fig. 5C) and OSCAR ($P < 0.05$; Fig. 5G) mRNA expression during the late stages of osteoclast formation. This was despite

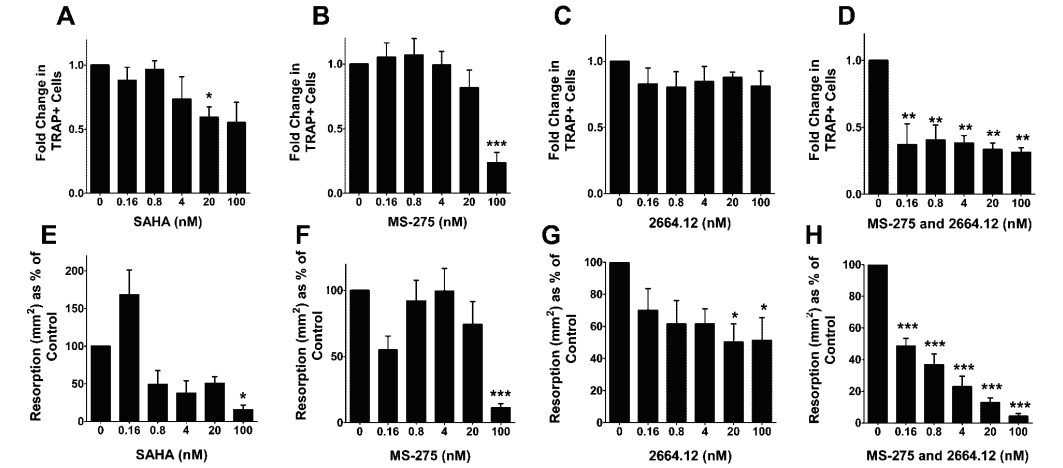

Fig. 3. A–D: Fold change in the number of multinucleated cells expressing TRAP with drug treatment. Cells with more than three nuclei expressing TRAP were classified as osteoclasts. (A) SAHA; (B) MS-275; (C) 2664.12; (D) MS-275 and 2664.12; (E–H) area of bone resorption with drug treatment expressed as a percentage of the control resorption. A: SAHA (n = 3); B: MS-275 (n = 9 100 nM, n = 8 4 nM, n = 7 0.8 nM, n = 6 0.16 nM); C: 2664.12 (n = 7 0.16 nM, n = 4); D: MS-275 and 2664.12 (n = 5 0.8 nM, n = 4, 0.16 nM, n = 3). Bars represent mean ± SEM. Significance determined using a one-way ANNOVA followed by a Tukey's post hoc test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Drug treatment compared to untreated.

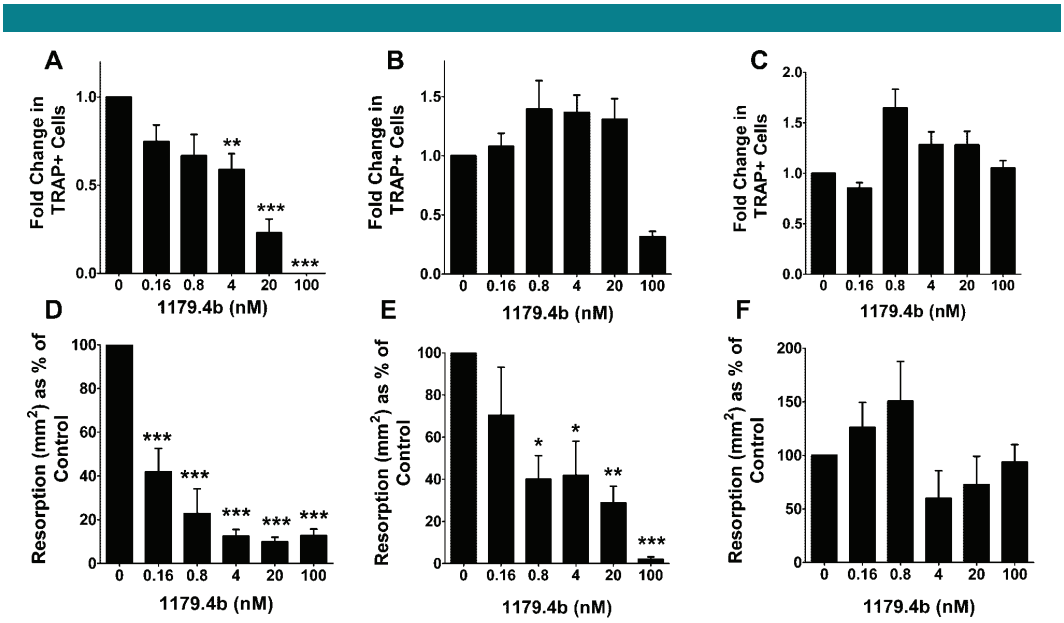

Fig. 4. A–C: Fold change in the number of multinucleated cells expressing TRAP with drug treatment. Cells with more than three nuclei expressing TRAP were classified as osteoclasts. (A) 1179.4b from day 7; (B) 1179.4b from day 10; (C) 1179.4b from day 13; (E,F) area of bone resorption with drug treatment expressed as a percentage of the control resorption. A: 1179.4b from day 7 (n = 4); B: 1179.4b from day 10 (n = 3); C: 1179.4b from day 13 (n = 3). Bars represent mean ± SEM. Significance determined using a one-way ANNOVA followed by a Tukey's post hoc test. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Drug treatment compared to untreated.

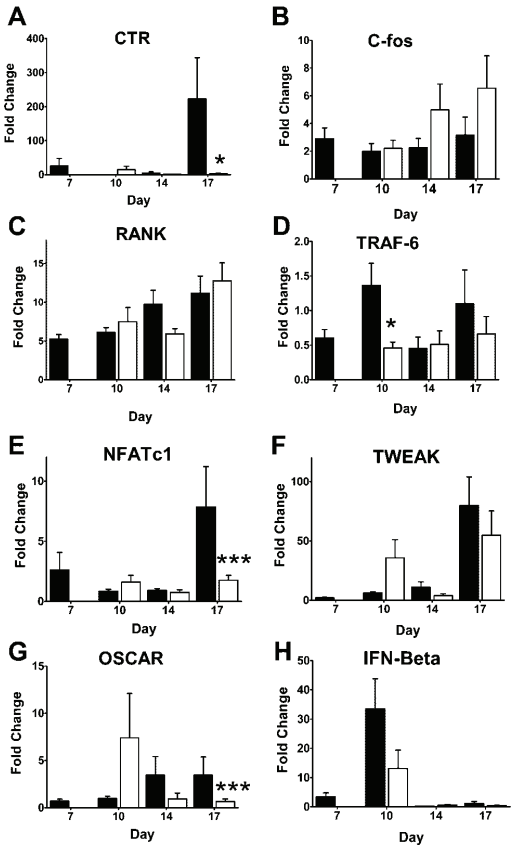

Fig. 5. Fold change in expression of osteoclast-related genes relative to day 0. Effect of treatment on expression with HDACi 1179.4b at 20 nM from day 7 onwards. Control cells—RANKL applied from day 7 but no drug treatment. (A) Calcitonin receptor (CTR) mRNA expression, (B) c-fos expression, (C) RANK expression, (D) TRAF-6 expression, (E) nuclear factor of activated T cells (NFATc1) expression (n = 6), (F) TWEAK expression, (G) OSCAR expression (n = 6), (H) IFN-β expression (n = 6). Significance determined using Mann–Whitney *U*-test. $^{***}P < 0.001$, $^{*}P < 0.05$. Expression with drug treatment compared to untreated at day 17.

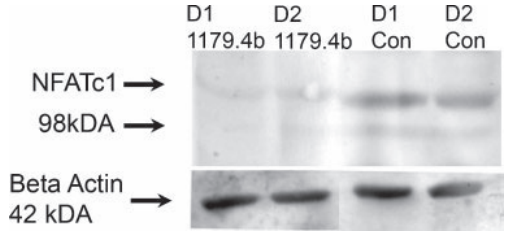

Fig. 6. Protein was extracted from 1179.4b-treated and controls cells and used for Western blot analysis as described in the Materials and Methods Section to detect NFATc1 protein at day 17. β-Actin is shown as the loading control, band at 42 kDA. D1 and D2 represent two different human donors.

having no significant effect on transcription factor c-Fos (Fig. 5B) mRNA normally expressed earlier during osteoclast development. However, there was a significant reduction in TRAF-6 (Fig. 5D) mRNA expression at day 10 with 1179.4b treatment ($P < 0.05$). 1179.4b also resulted in a decrease in expression of TWEAK (Fig. 5F) in the later stages of formation, however this was not significant ($P > 0.05$). 1179.4b did not elevate the expression of IFN-β (Fig. 5H) at any stage during osteoclast formation as previously reported (Nakamura et al., 2005).

At the level of protein expression there was a significant reduction in the expression of NFATc1 at day 17 (Fig. 6). This Western blot analysis supported the findings of mRNA expression of NFATc1 and immunocytochemistry at day 17. Large multinucleated cells strongly express NFATc1 in the controls whereas there was much smaller cells with decreased positive staining for NFATc1 as demonstrated in Figure 7.

To help evaluate the importance of different HDACs during osteoclast development, we assessed the expression of HDACs 1–10 throughout osteoclast development using real-time PCR (Fig. 8). Throughout osteoclast development there was an increase in all HDAC expression but most markedly increased compared to day 0 was expression of class I HDAC 8 and class II HDACs 5, 7, and 10 during the later stages of development (days 14 and 17). This observation was supported by immunocytochemistry of cells fixed at days 0 and 17. HDAC 8 and 5 were investigated at the protein level due to high-mRNA expression (classes I and II, respectively). At day 17, there were large multinucleated cells with positive staining for HDAC 5 (class II) in the cytoplasm whereas the positive staining for HDAC8 (class I) at day 17 seems to be concentrated around the nucleus of the cells as demonstrated in Figure 8.

Discussion

The hypothesis that compounds targeting both classes I and II HDACs are more effective at suppressing osteoclast bone resorption in vitro than inhibitors targeting either class I or class II alone was strongly supported by the findings of this study. Of most relevance were the observations of synergistic inhibition observed by combining HDACi compounds targeting classes I and II alone. This was similar to the strong suppression observed with novel compound 1179.4b and with SAHA. The less potent effect of SAHA observed in our in vitro system is consistent with reports of moderate effects of this compound in vivo (Lin et al., 2007). In contrast to SAHA, the novel potent HDACi used in this study, 1179.4b, was extremely effective at suppressing both osteoclast formation and activity. This greater activity may be due to the fact that 1179.4b has been designed to have a bulky 8-aminoquinoline substituent that is thought to bind better to the surface-binding pocket of HDACs (Kahnberg et al., 2006), resulting in more effective HDAC inhibition.

All three compounds (1179.4b, MS-275, and 2664.12) are chemically stable in aqueous buffers. The half-life of 1179.4b is unknown but it has a similar structure to SAHA that is very stable in vivo and has FDA approval (Grant et al., 2007). In clinical trials for various cancers MS-275 has been shown to have a long half-life of approximately 50 h in vivo (Ryan et al., 2005b). The half-life of 2664.12 is yet to be determined but, although it is a thiol, it is stable in aqueous solution at pH 7. These compounds have varying effects on the hyperacetylation of HDAC 1 (a class I HDAC) and HDAC 6 (a class II HDAC) in vitro. At concentrations below 0.1 μM, 1179.4b significantly

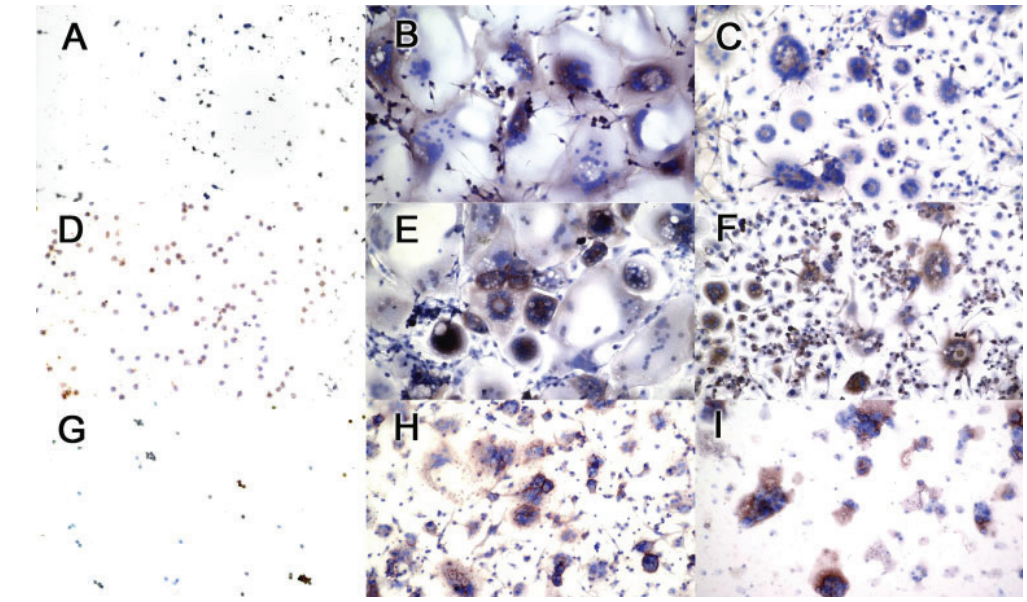

Fig. 7. Immunostaining to detect NFATc1 in (A) day 0 cells; (B) day 17 control cells; and (C) day 17 1179.4b-treated cells. To detect HDAC 5 (D) day 0 cells, (E) day 17 control cells, and (F) day 17 1179.4b-treated cells. To detect HDAC 8 (G) day 0 cells, (H) day 17 control cells, and (I) day 17 1179.4b-treated cells. Images taken at 10× magnification.

inhibits both HDAC 1 and 6, whereas MS-275 inhibits HDAC 1 at concentrations below 0.1 μM but not HDAC 6. 2664.12 only inhibits HDAC 6 at concentrations less than 0.1 μm but not HDAC 1 (Gupta et al., 2010). MS-275 has also been reported to have no inhibitory activity over HDAC8 but inhibits HDAC1 at concentrations below 100 nM (Hu et al., 2003).

The specificity of various HDACi is only partially known, most inhibitors target both class I or class II HDAC enzymes (de Ruijter et al., 2003), while there are very few inhibitors of specific HDACs currently available. Interestingly, our findings imply that compounds targeting both classes of HDACs may be more effective at suppressing osteoclasts in vitro suggesting there may be some redundancy among the HDACs expressed during osteoclast development. Real-time PCR analysis of the various HDACs throughout osteoclast development demonstrated that HDACs 5 and 8 were the most up-regulated at day 17 relative to day 0. Interesting HDAC5, a class II HDAC, and HDAC 8 a class I HDAC, were strongly expressed late in osteoclast differentiation, supporting the observation that HDACi targeting both classes are effective at suppressing osteoclast development. The specificity for which the various inhibitors investigated in this study have on HDACs 5 and 8 is not at this stage known. The lack of effect observed with MS-275 could be related to the fact that it has no inhibitory activity over HDAC 8 (Hu et al., 2003). As more selective HDACi are developed it will become possible to more comprehensively investigate the roles of individual HDACs in osteoclast formation.

Our understanding of the HDACi mechanisms is further complicated by the fact that HDAC are also now known to acetylate lysines in non-histone proteins. The fact that HDACi are known to stimulate as well as inhibit gene expression has led to the observation that HDAC modify the action of proteins, including NF-κB, that regulate transcription through reversible acetylation (Glozak et al., 2005). Another example is that p21 is induced by 1179.4b in vitro (Kahnberg et al., 2006). However, the inhibition of osteoclast formation and activity by HDACi may be due to its regulation of the key NF-κB pathway involved in the process of osteoclast differentiation and maturation (Rahman et al., 2003). It has been previously reported that the weak HDACi, sodium butyrate was able to suppress the activation of NF-κB in colon epithelial cells (Yin et al., 2001). Of particular relevance to our study is the observation that treatment of an osteoclastic RAW cell line with HDACi caused a decrease in NF-κB within the nucleus (Rahman et al., 2003). Other factors that stimulate osteoclast formation may also be involved as down-regulation of pro-inflammatory cytokines such as TNF-α by HDACi has been reported (Imre et al., 2006). The HDACi SAHA has been shown to inhibit the release of pro-inflammatory cytokines such as TNF-α and interleukins by monocytes stimulated with lipopolysaccharide (Leoni et al., 2002).

A novel and important finding of this study was the observation that, during the latter stages of osteoclast formation, 1179.4b significantly reduced the expression of osteoclast transcription factors NFATc1 and OSCAR. RANKL stimulation of osteoclastogenesis requires induction of NFATc1 (Takayanagi et al., 2002; Hirotani et al., 2004) and it is also a major regulator of immunity (Asagiri and Takayanagi, 2007). NFATc1 directly induces osteoclast genes such as CTR, cathepsin K, TRAP, and the β3 integrin in addition to OSCAR (Matsumoto et al., 2004; Sharma et al., 2007; Matsuo and Irie, 2008). RANKL also induces the activation of TRAF-6 and c-fos pathways that result in the activation of NFATc1 (Takayanagi,

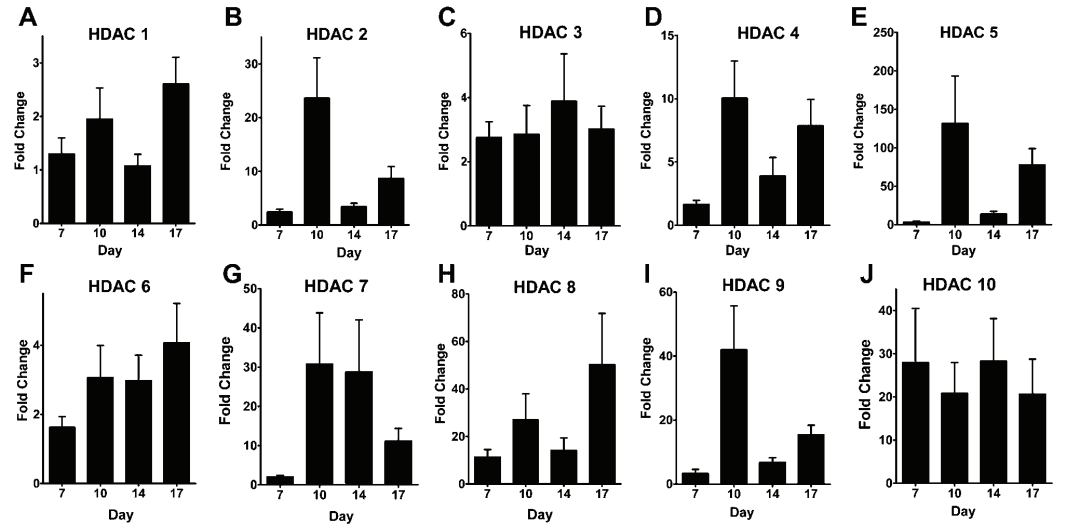

Fig. 8. Fold change in mRNA expression at days 7, 10, 14, and 17 during osteoclast development relative to day 0 for HDACs 1–10. (A) Class I HDAC 1, (B) class I HDAC 2, (C) class I HDAC 3, (D) class II HDAC 4, (E) class II HDAC 5, (F) class II HDAC 6, (G) class II DAC 7, (H) class I HDAC 8, (I) class II HDAC 9, (J) class II HDAC 10. In reference to endogenous house keeping gene hARP.

2007). In this study, we also observed that administration of 1179.4b from day 10 had little effect on the number of TRAP-positive cells formed but suppressed pit formation. The TRAP-positive cells that were evident in wells treated with 1179.4b from day 10 were unlikely to be fully active osteoclasts that resorb bone as they were unable to form pits in the dentine. This suggests 1179.4b may affect the important late stages of osteoclast development. Interestingly, 1179.4b reduced expression of TRAF-6 at day 10, as well as affecting late stage expression of NFATc1 and OSCAR. The lack of effect with treatment from day 13 could indicate that the inhibition of TRAF-6 before day 13 is needed to reduce NFATc1 and OSCAR expression, and thus, osteoclast differentiation. These results are consistent with TRAF-6 being an important intracellular regulators of osteoclast formation (Asagiri and Takayanagi, 2007).

The reduction in mRNA expression of OSCAR may be a consequence of the reduced NFATc1 expression at day 17 as both are intimately involved in the later stages of osteoclast formation (Cantley et al., 2009). Interestingly, IFN-β expression was not elevated with HDACi treatment as has been described previously with studies using animal cell lines (Nakamura et al., 2005). These differences may be due to the use of human osteoclast precursor cells and different types of in vitro assays.

Conclusion

The results of this study support our original hypothesis and demonstrate that HDACi targeting both classes I and II HDACs are more effective at suppressing osteoclast bone resorption in vitro than inhibitors targeting either class I or class II alone. This is consistent with the finding that class I HDAC 8 and class II HDAC 5, are up-regulated in the later stages of osteoclast development at the level of both protein and mRNA. Similarly, the novel compound targeting both classes I and II HDAC, 1179.4b, was a potent inhibitor of osteoclast bone resorption in vitro. The mechanism of action is likely to be due to reduced expression of the key osteoclast transcription factors, TRAF-6, NFATc1, and OSCAR during the later stages of osteoclast differentiation. This study demonstrates that targeting both classes I and II HDACs may be effective at suppressing human osteoclasts in vitro and could potentially used for treating pathological bone loss.

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Chapter 4. Histone Deacetylase Inhibitors and Periodontal Bone Loss

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Journal of Periodontal Research, 2011; 46(6):697-703

Chapter Summary

The results of Chapter 3 demonstrated that the novel HDACi 1179.4b effectively suppressed human osteoclast formation and activity *in vitro*. Given this, the aim of Chapter 4 study was to assess the effects of this HDACi on bone loss in a chronic inflammatory disease mouse model of periodontitis. It was hypothesized that 1179.4b, which targets both classes of HDACs would inhibit bone resorption in a mouse model of periodontitis. The study compared 1179.4b to HDACi MS-275, which targets Class I HDACs and has been previously shown to suppress bone loss in inflammatory arthritis mouse models. The effects of both HDACi on bone loss and inflammation was assessed in this study.

STATEMENT OF AUTHORSHIP

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Journal of Periodontal Research, 2011; 46(6):697-703

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Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Performed all experiments, analysis on all samples, interpreted data and wrote manuscript.

Certification that the statement of contribution is accurate

Signed ....*Date*13/1/13.....

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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Journal of Cellular Physiology, 2011; 226(12):3233-41

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STATEMENT OF AUTHORSHIP

Histone Deacetylase Inhibitors and Periodontal Bone Loss

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Lucke A.J

My contribution to the paper was in the design, chemical synthesis and characterization of the HDAC inhibitor 1179.4b. Critical review, comments and proofing of the paper were also contributed.

Drug design and development

I certify that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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STATEMENT OF AUTHORSHIP

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Supervised development of work, helped in data interpretation and manuscript evaluation

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Histone deacetylase inhibitors and periodontal bone loss

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Cantley MD, Bartold PM, Marino V, Fairlie DP, Le GT, Lucke AJ, Haynes DR. Histone deacetylase inhibitors and periodontal bone loss. J Periodont Res 2011; 46: 697–703. © 2011 John Wiley & Sons A/S

Background and Objective: Bone loss caused by enhanced osteoclast activity is a significant feature of periodontitis. Histone deacetylase inhibitors (HDACi) can suppress osteoclast-mediated bone loss *in vitro* and *in vivo*. This study investigated whether HDACi can suppress bone loss in experimental periodontitis.

Material and methods: Experimental periodontitis was induced in mice by oral inoculation with *Porphyromonas gingivalis* bacteria. Mice were treated orally with olive oil alone, with olive oil and a novel compound – 1179.4b – which targets both Class I and Class II histone deacetylases (HDACs) or with olive oil and MS-275, which targets Class I HDACs. Micro-computed tomography scans of live mice, stereo imaging and histological analyses were used to detect changes in bone.

Results: In the absence of treatment there was a 13.2% increase in bone volume in controls compared with a 7.4% decrease in *P. gingivalis*-inoculated mice. 1179.4b significantly reduced bone loss, with a 3.4% increase in bone volume ($p < 0.01$). MS-275 did not have a significant effect on *P. gingivalis*-induced bone loss. Histological analysis revealed that 1179.4b reduced bone loss despite having no effect on inflammation.

Conclusion: HDACi were found to effectively suppress bone loss in the mouse model of periodontitis. 1179.4b – the inhibitor of Class I and Class II HDACs – was more effective at suppressing bone loss than MS-275, which targets Class I HDACs only. These compounds may therefore have the potential to be used for the management of periodontitis.

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Key words: histone deacetylase inhibitor; periodontitis; osteoclasts; bone resorption

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A hallmark of periodontitis is pathological bone loss that, if left untreated, can ultimately result in tooth loss. This bone loss is attributed to enhanced osteoclast formation and activity (1). During the inflammatory process of periodontitis, a large array of cytokines, including interleukins 1, 6, 11 and 17, and tumor necrosis factor-α (TNF-α), are produced, which are reported to up-regulate the expression of RANKL by fibroblasts and lymphocytes (2,3). Elevated RANKL leads to enhanced production of osteoclasts, which degrade the alveolar bone. Enhanced osteoclast numbers have been reported in human and animal models of periodontitis and are associated with increased RANKL expression and corresponding low levels of its natural inhibitor, osteoprotegerin (OPG) (4–6). Enhanced degradation of bone by osteoclasts is becoming an important target to treat pathological bone loss. Denosumab, a monoclonal antibody to RANKL, has recently been approved by the US Food and Drugs Administration (FDA) for use in postmenopausal women at risk of osteoporosis (7).

Many current treatments for periodontitis target only the infection and inflammation. Despite recent advances in understanding the cellular and molecular processes of bone destruction, there are few effective treatments that directly target the alveolar bone destruction. Hence, therapeutics directly targeting osteoclastic bone

destruction may be useful adjuncts to current treatments. One potential treatment to directly target bone destruction, and possibly inflammation, in periodontitis is histone deacetylase inhibitors (HDACi) (1).

Histone deacetylases (HDACs) play a role in the regulation of histone and nonhistone proteins. There are two main classes of HDACs, Class I and Class II. Class I includes HDACs 1, 2, 3 and 8, which are primarily found in the nucleus. Class II includes HDACs 4, 5, 7 and 9 that belong to class IIa HDACs and HDACs 6 and 10 belonging to class II b HDACs, which are able to shuttle between the nucleus and the cytoplasm. These enzymes remove acetyl groups, originally added to lysine amino acids by histone acetyltransferases, causing the chromatin to become condensed, resulting in the repression of genes, including inflammatory genes and cell-cycle genes, along with numerous others that can affect a variety of cellular functions. Although this is the major function of HDACs, lysine deacetylation is now known to be a significant post-translational modification of many nonhistone proteins, such as nuclear factor-κB (NF-κB) (8); (9–11). NF-κB can be reversibly acetylated, which has positive and negative regulatory effects on its action (9). This highlights the diverse role that post-translational modifications can play in the regulation of many important proteins.

Inhibition of HDACs has been shown to up-regulate cell-cycle inhibitors, down-regulate immune stimulators and repress inflammatory cytokines, such as TNF-α (12), a major cytokine associated with periodontitis (13); and a range of HDACi [suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA) and depsipeptide (FR901228)] have also demonstrated osteoclast suppression *in vitro* (14–16). HDACi have also recently been reported to have potential applications for use in restorative dentistry (17).

Interestingly, inhibitors of HDACs have also been shown to stimulate osteoblast maturation (18,19). Using cultures of primary osteoblasts (MC3T3-E1), HDACi TSA was shown to accelerate mineralization and increase expression of osteoblast genes, including type I collagen, osteopontin, bone sialoprotein and osteocalcin (18). This property of HDACi could potentially aid in the repair of mineralized tissue in the periodontium.

Direct suppression of osteoclasts by HDACi [SAHA, TSA and depsipeptide (FR901228)] *in vitro* has been demonstrated via suppression of osteoclast factors, including c-Fos, nuclear factor of activated T cells c1 (NFATc1) and by the induction of inhibitory factors, including interferon-β (14,20,21). We have recently investigated the effects of a novel HDACi (1179.4b), which targets Class I and Class II HDACs, and the effects of MS-275, which targets Class I HDACs, on the formation and activity of human osteoclasts *in vitro*. 1179.4b was found to reduce human osteoclast formation and activity *in vitro* with a half-maximal (50%) inhibitory concentration (IC_{50}) of < 0.16 nM (20). This appeared to occur via the suppression of TNF receptor-associated factor 6 (TRAF-6), which resulted in reduced expression of nuclear factor of activated T cells 1 (NFATc1) and osteoclast associated receptor (OSCAR) during the terminal stages of osteoclast formation. However, MS-275, which targets Class I HDACs, was 100–1000 times less effective at inhibiting osteoclast formation than 1179.4b. These results show that the inhibitor of Class I and Class II HDACs suppresses resorption *in vitro* but its effects in an animal model of pathogenic bone loss are not known.

As other HDACi compounds, including TSA, have been shown to suppress osteoclast bone resorption in animal models of rheumatoid arthritis (RA) (14,22,23), it is possible that HDACi will have similar effects on the bone loss seen in periodontitis. Therefore, based on this thought and on the results of our *in vitro* studies we hypothesized that the novel HDACi, 1179.4b, which targets both classes of HDACs would inhibit bone resorption in a mouse model of periodontitis. The aim of this study was to compare the effects of 1179.4b and MS-275, which target Class I HDACs, on bone loss and inflammation in this mouse model (24) by assessing changes in the alveolar bone and soft tissues using micro-computed tomography (micro-CT) scans, histological analysis and stereo imaging.

Material and methods

Figure 1 shows the chemical structures of the HDACi compounds evaluated. 1179.4b is a synthetic compound, developed in our laboratories, which targets Class I and Class II HDACs [Compound 52 in (25)]. MS-275 is a selective inhibitor of Class I HDACs that has been shown to induce hyperacetylation of nuclear histones in various tumor cell lines (26).

Mouse model of periodontitis

Experimental periodontitis was induced in 6- to 8-wk-old female BALB/c mice

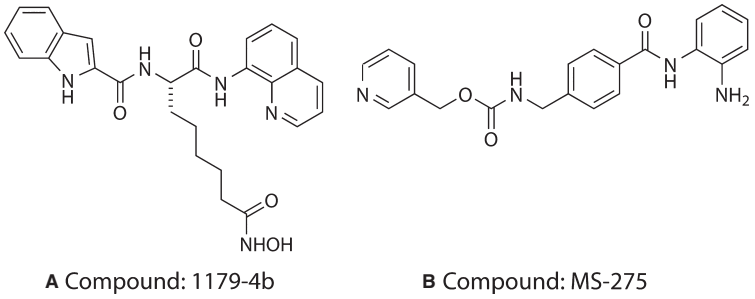

Fig. 1. Chemical structures of: (A) Histone deacetylase inhibitor (HDACi) 1179.4b which targets Class I and Class II HDACs [(S)-N8-hydroxy-2-(1H-indole-2-carboxamido)-N1-(quinolin-8-yl)octanediamide; Compound 52 in (25)] and (B) HDACi MS-275 which targets Class I HDACs [N-(2-aminophenol)-4-(N-(pyridine-3-yl-methoxycarbonyl) aminomethyl) benzamide) (39)].

($n = 18$) over a period of 81 d, as described previously (24,27). Mice were housed in the Institute of Medical and Veterinary Science Animal House, Adelaide, Australia. Ethical approval was received from the IMVS Animal Ethics Committee (Project no. 148/06).

Mice were initially treated with 1 mg/mL of kanamycin (Sigma-Aldrich, St Louis, MO, USA) in water for 7 d to reduce the native flora and to support colonization of *Porphyromonas gingivalis* (*P. gingivalis*), as previously described (24,27,28). Three days after antibiotic treatment ceased, periodontitis was induced by a monoinfection with *P. gingivalis* bacteria, as also previously described (24,28). Briefly, cultures of *P. gingivalis* (strain W50) were grown anaerobically on blood agar plates and stored for 3 d at 37°C, in an atmosphere of $N_2/CO_2/H_2$ (90 : 5 : 5), before harvesting. The bacterial viable count was found to be 24.3×10^{10} colony-forming units/mL with a dry weight of 12.5 mg/mL. *P. gingivalis* was suspended in 2 mL of carboxymethyl cellulose (2% CMC in phosphate-buffered saline) (Sigma-Aldrich), as previously described (24,27,28). A 0.1-mL sample of the bacterial suspension (i.e. 0.1 mL in CMC) was directly swabbed around the molars using a small brush. Control mice were swabbed with CMC alone. Following each inoculation, mice were kept without food or water for 1 h. The first sequence consisted of four inoculations over 8 d, followed by two inoculations a week for 2 wk, then a second sequence (four inoculations over 8 d). For the remainder of the experimental period, mice were inoculated twice a week. Live *P. gingivalis* were recovered from the gingival tissue, as described previously (28).

From day 44, mice were administered daily doses of the compounds suspended in olive oil via oral gavage. The compounds were administered in olive oil due their lipophilic nature. This dose of olive oil was well below the levels reported to have an effect on bone (29). Day 44 was determined to be an appropriate time to commence treatment from preliminary experiments as disease had been adequately induced and this was also a clinically relevant time (24). Periodontitis was induced in the following three groups of mice by inoculation with *P.gingivalis* bacteria. Group 1: *P. gingivalis* inoculations and received 0.1 mL of olive oil only ($n = 5$) (known as oil treated); group 2: *P. gingivalis* inoculations and received 10 mg/kg/d dose of MS-275 (0.1 mL) ($n = 4$); and Group 3: *P. gingivalis* inoculations and received a 1 mg/kg/d dose of 1179.4b (0.1 mL) ($n = 4$). These doses were based on the *in vitro* results (20) and on the recommendations by the developers of the compounds. Group 4 were control mice (not inoculated with *P. gingivalis* or treated with HDACi) ($n = 5$). Control mice were used to give an indication of alveolar bone changes during normal mouse growth.

Three methods of analysis were used to determine the effects of HDACi: micro-CT scans of live mice to assess changes in bone volume; stereo imaging to assess the change in the area from the cemento–enamel junction (CEJ) to the alveolar bone crest (ABC); and histological analysis to determine effects on inflammation.

Bone volume analysis

Mice were scanned using the live animal micro-CT scanner [Sky Scan 1076 high-resolution *in vivo* scanner (SkyScan, Kontick, Belgium), Adelaide Microscopy Australia], as previously described (24). Mice were scanned on day 44 (before drug treatment) and at the completion of the study (day 81). The scanning specifications and machine details have been previously published (24). Mice were scanned at 74 kV/136 mA with a pixel size of 18 μM, 1 mm aluminum filter and frame averaging of 1. These parameters were used to minimize the radiation exposure to the animals and to significantly reduce the scanning time to around 12 min per animal. Before scanning, mice were anaesthetized via intraperitoneal injection [rat/mouse anaesthetic: 1 mL of xylazine, 2 mL of ketamine (100 mg/mL), 17 mL of water in injection, 0.3 mL for a 30-g mouse]. For each scan the mouse was positioned to ensure that the head was within the scanning area of interest. Following this, CT scans were then reconstructed using the SkyScan N Recon program for analysis (SkyScan). Bone volume analysis was then carried out using SkyScan's CTAn program. The three molars of the maxilla on both the left-hand and right-hand sides were used for the region of interest. For each area of interest, bone volume (mm^3) was calculated using CTAn software. The percentage change in bone volume over time was determined for each mouse.

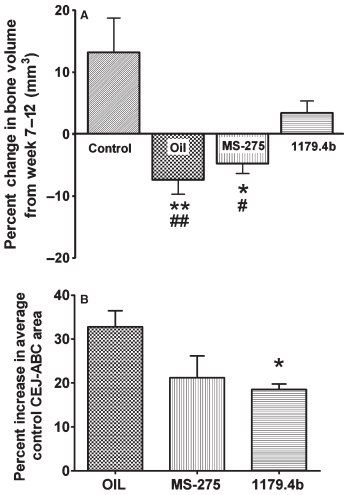

Fig. 2. (A) Percentage change in bone volume (mm^3) from week 7 (disease is evident) to week 12 (study completion). Control: mice with no disease or treatment ($n = 5$). Oil: mice ($n = 5$) treated with oil only. MS-275: mice ($n = 4$) treated orally with oil + 10 mg/kg/d of MS-275. 1179.4b: mice ($n = 4$) treated orally with oil + 1 mg/kg/d of 1179.4b. Bars represent mean ± standard error of the mean. Significance was determined using a one-way analysis of variance followed by Tukey's *post hoc* test. Significance was accepted when $p < 0.05$. $^{**}p < 0.01$, $^{*}p < 0.05$ compared to treatment with 1179.4b. $\#p < 0.01$, $\#p < 0.05$ compared with the control. (B) Average percentage increase in the control cemento–enamel junction (CEJ) to the alveolar bone crest (ABC) area. Oil: mice ($n = 5$) treated with oil only. MS-275: mice ($n = 4$) treated orally with oil + 10 mg/kg/d MS-275. 1179.4b: mice ($n = 4$) treated orally with oil + 1 mg/kg/d of 1179.4b. Significance was determined using a one-way analysis of variance followed by Tukey's *post hoc* test. Significance was accepted when $p < 0.05$. $^{*}p < 0.05$ compared with oil alone.

Bone area analysis

At the completion of the study, mice were killed, their heads were collected and defleshed, stained with Methylene Blue (0.003%) and the maxillae imaged using a stereo microscope (MZ16FA Leica Stereo microscope; Leica, Solms, Germany) (24). Right-hand and left-hand sides, and buccal and lingual surfaces were imaged using a 1.0 objective lens. The area from the CEJ to the alveolar bone crest (ABC) was measured for each molar (molars 1–3) using ImageJ analysis software, as previously described (24). This was conducted by two blinded observers and the area from the CEJ to the ABC was calculated (in mm^2) for the three molars combined. This was then presented as the percentage increase in area relative to the average control area from the CEJ to the ABC.

Histological analysis

Standard hematoxylin and eosin staining was conducted on jaw tissue fixed in 4% formalin for 24 h, decalcified in 4% formic acid for 2 wk, then paraffin embedded. Three histological sections per mouse were scored based on previous methods (24). Scoring was carried out by two independent observers, blinded to the tissue type, using a four-point scale, and was based on the total numbers of inflammatory cells present within the tissues (lymphocytes, plasma cells, neutrophils or macrophages). Normal tissue (< 5% inflammatory cells) was scored as 0; mild inflammation (5–20% inflammatory cells) was scored as 1; moderate inflammation (20–50% inflammatory cells) was scored as 2; and severe inflammation, with massive infiltration of immune cells (> 50% of cells), was scored as 3. The number of multinucleated osteoclast cells (more than three nuclei) per square millimeter was also determined as described previously (30,31). An area encompassing 4×2 mm, and which included the alveolar bone between the first and third molars, was analysed.

Statistics

A one-way analysis of variance, followed by Tukey's *post hoc* test, was conducted to assess changes in bone volume between the groups (group 1, oil only; group 2, oil + MS-275; and group 3, oil + 1179.4b). Statistical significance was accepted when $p < 0.05$.

Results

Micro-CT scans of live mice confirmed that periodontitis had been induced with significant ($p < 0.001$) bone loss in mice inoculated with *P. gingivalis*. Control mice averaged a 13.2% increase in bone volume compared with a 7.4% decrease in mice inoculated with *P. gingivalis* (oil treated) (Fig. 2A and Table 1). Bone loss was confirmed by stereo imaging of the mouse heads (Fig. 3A,B). In mice inoculated with *P. gingivalis* (oil treated), an average 32% increase was observed in the area from the CEJ to the ABC compared with controls ($p < 0.001$) (see Fig. 2B). The presence of an inflammatory infiltrate in the gingiva of both drug-treated and oil-treated animals was noted (Fig. 4). The numbers of osteoclastic cells resorbing bone were also significantly elevated in mice inoculated with *P. gingivalis* (oil treated) compared with control animals (Fig. 5A).

No side effects or weight loss were noted in the treated mice. 1179.4b (1 mg/kg/d, orally) significantly reduced bone loss induced by *P. gingivalis* inoculation (Fig. 2A). There was an average 3.4% increase in bone volume in the 1179.4b-treated mice compared with a 7.4% decrease in bone volume in mice treated with oil only ($p < 0.01$) (Fig. 2A). Stereo imaging similarly showed a 20% increase in the CEJ to the ABC in 1179.4b-treated mice compared with an increase of 32% in mice treated with oil only ($p < 0.05$) (Fig. 2B). This observation was also confirmed by a significant reduction in osteoclast numbers in treated mice. Despite this suppression of bone loss, compound 1179.4b did not suppress inflammation, as demonstrated in Fig. 4E,F ($p > 0.05$). There was still a significant inflammatory cell infiltrate present in the periodontal tissues, as assessed by semiquantitative analysis (described in the Material and methods) (Fig. 5B).

In contrast to treatment with 1179.4b, treatment with MS-275 had little effect on bone loss. The change in bone volume in MS-275-treated mice was not significantly different from that of oil-treated animals ($p > 0.05$). There was a significant ($p < 0.05$) difference in bone volume in the mice treated with 1179.4b compared to the mice treated with MS-275 (Fig. 2A). Micro-CT scans of live mice revealed an average decrease in bone volume of 4.8% in the mice treated with MS-275, demonstrating that MS-275 did not

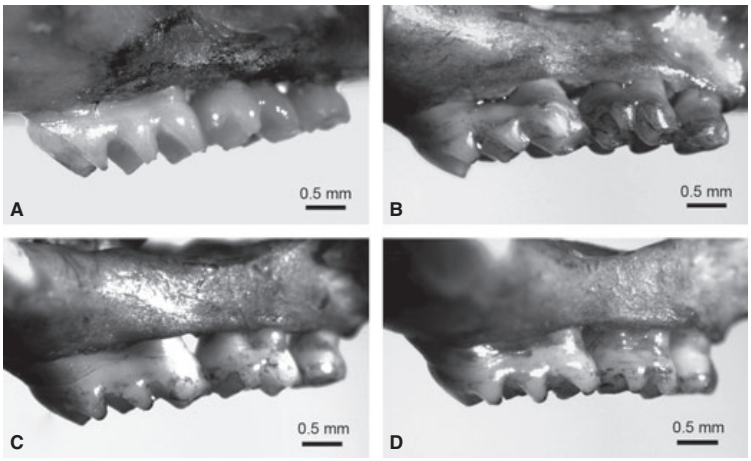

Fig. 3. Stereo images of the mouse heads at completion of the study (week 13) showing the 3 molars of the maxilla. Images were taken using a stereomicroscope, as described in the Material and methods, using a 1.0× objective lens. (A) Control mouse; (B) oil-treated mice (inoculated with *Porphyromonas gingivalis*, administered oil only, no drug treatment); (C) mouse treated with oil + 1 mg/kg/d of 1179.4b; (D) mouse treated with oil + 10 mg/kg/d of MS-275.

have a significant effect on *P. gingivalis*-induced bone loss ($p > 0.05$). However, inflammation was reduced in mice treated with MS-275, as demonstrated by semiquantitative analysis of the histological sections, but this was not significant ($p > 0.05$) (Fig. 5B).

Discussion

In this study, the bone-protective effects of two HDACi – 1179.4b and MS-275 – were investigated in a mouse model of periodontitis. In this model, periodontitis was induced by oral inoculations with *P. gingivalis* over a period of 44 d. A chronic inflammatory reaction was established with associated alveolar bone destruction (24), shown by micro-CT scans of live mice, histology and stereo imaging. Treatment with HDACi suspended in olive oil or treatment with olive oil alone was commenced once disease had been established as this is clinically relevant to the human situation.

The results of this study demonstrated that a novel HDACi, 1179.4b, administered at 1 mg/kg/d, significantly reduced alveolar bone loss compared with oil-treated mice. The other HDACi, MS-275, administered at 10 mg/kg/d, did not suppress bone loss in this model. Interestingly, inhibition of alveolar bone resorption by 1179.4b occurred even though there was no reduction in inflammation of the gingival tissues, as assessed histologically. This could suggest that the mechanism of action involves a direct effect on the bone rather than an indirect effect through suppression of inflammation.

The findings of the current study are consistent with our recent *in vitro* findings in which 1179.4b, an inhibitor of both Class I and Class II HDACs, suppressed osteoclast bone resorption.

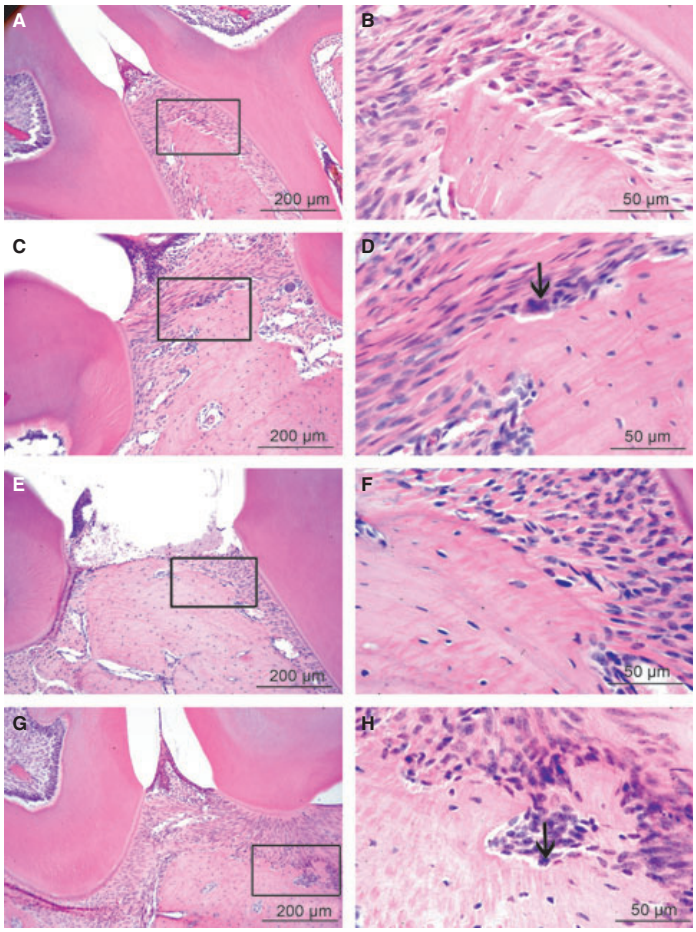

Fig. 4. Standard hematoxylin and eosin staining of the gingival tissue near the first and second molars in (A) control (10× magnification); (B) control (40× magnification of the boxed area in A); (C) oil-treated mice (inoculated with *Porphyromonas gingivalis*, administered oil only, no treatment) (10× magnification); (D) oil-treated mice (40× magnification of the boxed area in C); (E) 1179.4b (1 mg/kg/d) (10× magnification); (F) 1179.4b (40× magnification of the boxed area in E); (G) MS-275 (10 mg/kg/d) (10× magnification); (H) MS-275 (40× magnification of the boxed area in G). Arrows in (D) and (H) show multinucleated osteoclasts at resorption.

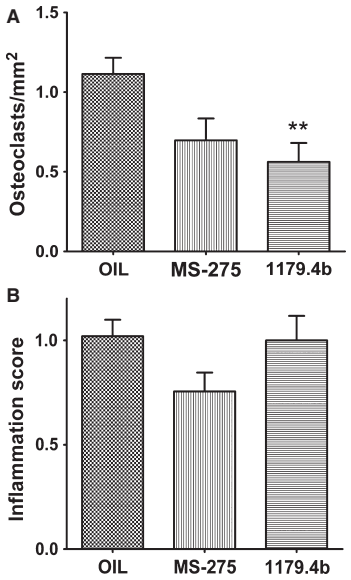

Fig. 5. (A) Average number of osteoclasts on the surface of alveolar bone supporting the 3 molars per section. Scoring details are described in the Material and methods. Three sections for each animal were quantified. Bars represent mean ± standard error of the mean. Significance was determined using a one-way analysis of variance followed by Tukey's *post hoc* test. $^{**}p < 0.01$ compared with oil. (B) Average histological scoring of the periodontal tissues for inflammation, as described in the Material and methods. Bars represent mean ± standard error of the mean. Significance was assessed using a one-way analysis of variance followed by Tukey's *post hoc* test. No significant differences were observed in inflammation.

Table 1. Average bone volume (BV) values for scan 1 (after inducing disease but before treatment) and scan 2 (after treatment), determined using micro-computed tomography of live mice

	Scan 1 BV (mm^3)	Scan 2 BV (mm^3)	Average change in BV during treatment (mm^3)
Control ($n = 5$)	1.320 ± 0.024	1.484 ± 0.030	+0.164 ± 0.037
Oil ($n = 5$)	1.208 ± 0.069	1.124 ± 0.049	−0.080 ± 0.038*
MS-275 ($n = 4$)	1.176 ± 0.026	1.119 ± 0.021	−0.057 ± 0.015*
1179.4b ($n = 4$)	1.076 ± 0.026	1.109 ± 0.020	+0.033 ± 0.027**·***

Results are given as mean ± standard error of the mean.
The final column shows the average change in bone volume for each group during the treatment period. Control: mice with no disease or treatment ($n = 5$). Oil: diseased mice (administered oil only, no drug treatment) ($n = 5$). MS-275: mice treated orally with oil + 10 mg/kg/d of MS-275 ($n = 4$). 1179.4b: mice treated orally with oil + 1 mg/kg/d of 1179.4b ($n = 4$). Significance was determined using a one-way analysis of variance followed by Tukey's *post hoc* test.
*$p < 0.05$ **$p < 0.01$ compared with control. ***$p < 0.001$ compared with oil.

Class II HDAC 5 and Class I HDAC 8 have also been shown to be up-regulated during the late stages of osteoclast development *in vitro* (20), suggesting that these HDACs may play a key role in osteoclast differentiation and activity. MS-275 has also been shown to have anti-inflammatory effects when administered at 10 mg/kg/d in a rat model of arthritis (22), with marked suppression of serum interleukin-6 in this model. In the present study, histological assessment indicated that MS-275 did appear to reduce inflammation in the gingival tissues of treated mice, although this was not statistically significant. MS-275 did not have any effect on bone destruction, confirmed by both CT analysis and histology. Although the exact activity of MS-275 is not known, it is reported to inhibit HDAC 1 at concentrations below 0.1 μM but does not affect HDAC 6 (32), indicating its selectivity towards Class I HDACs. It is also reported not to inhibit the Class I HDAC 8, suggesting its selectivity towards HDAC 1 and HDAC 3 (33). 1179.4b, on the other hand, effectively suppressed bone loss in this mouse model. This compound has been shown to inhibit HDAC 1 and HDAC 6 at concentrations of < 0.1 μM, indicating that it interacts with both Class I and Class II HDACs. This observation could explain differences in HDACs targeted by these compounds. As well as the selectivity of the compounds, there may also be differences in their potency. However, we noted significant bone-protective effects, even though 1179.4b was administered at a 10-fold lower concentration (1 mg/kg/d) than MS-275 (10 mg/kg/d). This is consistent with our previous *in vitro* findings, where 1179.4b was found to be more than 30-fold more active than MS-275 (IC_{50} of < 0.16 nM for 1179.4b vs. IC_{50} of 54.4 nM for MS-275) (20).

It has recently been demonstrated that HDAC 1 is highly expressed in synovial fluid from RA patients compared to patients with osteoarthritis (34). High levels of HDAC 1 have also been observed in RA tissues and these levels also correlate with higher TNF-α expression (34,35). Other HDACi compounds (TSA and SAHA) have demonstrated suppression of osteoclast-mediated bone resorption in various animal models of RA (14,22,23). Periodontitis shares many common features with RA (36) and there is a relationship between the two diseases (29,37) so it may not be surprising that similar results were seen in this current model of periodontitis. More extensive, detailed studies are needed to determine which specific HDACs of both classes are most important in various aspects of the periodontitis disease process (i.e. inflammation and bone resorption).

Although this class of compound is reported to be generally well tolerated as chemotherapeutic agents, they can have adverse side effects (38). While we did not observe any adverse effects at the doses used, the possibility of using these compounds in the clinic will have to be well monitored. For the management of periodontitis, side effects might be minimized by treating accessible sites in the mouth locally with these compounds. Another possibility is to specifically target only those HDACs required for osteoclast formation, with the advent of more HDAC-specific inhibitors on the immediate horizon. In addition, topical HDACi treatment may be a less toxic way of HDACi administration. Topical administration of HDACi – TSA and phenylbutyrate – was found to reduce paw swelling in an adjuvant arthritis model, and rats treated with HDACi exhibited no pannus formation or joint destruction (12). This method of application could potentially reduce any risk of systemic effects caused by the widespread expression of HDAC enzymes.

In conclusion, this study supports recent findings that HDACi prevent bone loss and demonstrates that the novel HDACi, 1179.4b, reduces alveolar bone loss in an *in vivo* model of periodontitis. The inhibition of bone loss was independent of the anti-inflammatory effects of the compounds and is likely to be a result of their ability to target osteoclast formation directly. While further studies are needed, the findings indicate that HDACi treatment could be used for the treatment of periodontitis in humans in the future.

Acknowledgements

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Chapter 5. Class I and II Histone Deacetylase (HDAC) Expression in Human Periodontitis

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Chapter Summary

The results of Chapter 4 suggested the importance of targeting both classes of HDACs in PD for suppression of bone loss. This study therefore aimed to investigate the mRNA expression of Class I HDACs (1, 2, 3 and 8) and Class II HDACs (4, 5, 6, 7, 9 and 10) in human gingival samples obtained from patients with chronic PD compared to those without PD. It was hypothesised that HDACs from both classes would be highly expressed in chronic PD gingival tissues.

STATEMENT OF AUTHORSHIP

Class I and II Histone Deacetylase (HDAC) Expression in Human Periodontitis

Cantley M.D (candidate)

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Performed PCR runs, immunohistochemistry, analysis on all samples, interpreted data and wrote manuscript.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Chapter 5. Class I and II Histone Deacetylase (HDAC) Expression in Human Periodontitis

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5.1 Abstract

Background and Objective**:** Histone deacetylase inhibitors (HDACi), originally developed as anti-cancer agents, are now being considered for use at 10-100x lower doses to treat chronic inflammatory diseases. HDACi have been demonstrated to reduce alveolar bone loss in a mouse model of periodontitis (PD). To develop more specific HDACi it is important to understand the role that the different enzymes might play in the pathogenesis of disease. It was hypothesised that both HDAC Classes I and II would be highly expressed in human periodontitis tissue. Therefore, this study aimed to determine the expression of HDACs in human gingival samples obtained from patients with chronic PD.

Material and Methods**:** Gingival biopsies were obtained from healthy/mild inflammation (no bone loss) sites (n=8) and inflamed sites affected by chronic PD with associated bone loss (n=9). RNA was isolated from frozen samples for real time QPCR to determine the mRNA expression of HDACs 1-10. Protein distribution of HDAC 5 and 8 was detected by immunohistochemistry and assessed by semi-quantitative analysis (SQA).

Results**:** Real time QPCR detected a 2 fold increase in the Class I HDACs 1 and 8 mRNA expression, a 2 fold increase in the Class II HDAC 5 and a 3 fold increase in HDAC 9 mRNA expression in chronic PD compared to non-PD samples ($p<0.05$). HDAC 5 and 8 immunostaining of gingival biopsies was not significantly different between tissues from chronic PD compared to non-PD samples. Of note, both HDAC 5 and 8 were expressed strongly by the microvasculature in chronic PD gingival tissues. 63% of the PD samples stained intensely for HDAC 5 and 8 protein compared to only 17% of the non-PD samples.

Conclusion**:** This study shows that at the mRNA level HDACs within both Class I and II are highly expressed in the inflamed gingiva of chronic PD. Further studies are necessary

to determine whether targeting select HDAC up regulated in PD could be used as a potential treatment.

5.2 Introduction

Periodontitis (PD) is a chronic inflammatory disease affecting up to 60% of the world's population. It is characterised by gingival tissue inflammation and associated loss of the supporting structures, including the periodontal ligament and alveolar bone (36, 38). Current treatments for this disease involve the use of a combination of anti-microbial treatments and mechanical debridement of affected sites. The effects of these treatments on the alveolar bone can be varied, and hence anti-resorptive medications (reviewed (2)) that can also reduce inflammation would be valuable adjuncts for treatment. Histone deacetylase inhibitors (HDACi) are one such option. HDAC enzymes were originally identified as functioning to counteract the actions of acetyl transferase enzymes (HATs) and removing the acetyl groups that have been added to lysine groups of histone proteins. This results in a condensed chromatin structure leading to gene repression. More recently HDACs have also been shown to regulate expression of a number of key cytoplasmic proteins (27, 28).

There are two main classes of HDAC enzymes. Class I includes HDACs 1, 2, 3 and 8 located within the nucleus of cells and Class II that includes HDACs 4, 5, 7 and 9, which belong to Class IIa HDACs, and HDACs 6 and 10 belonging to Class II b HDACs. The Class II enzymes are able to shuttle between the nucleus and cytoplasm (18, 48).

HDACi have been highly utilised to treat malignancies (31), but more recently have been demonstrated to have the potential to treat a range of chronic inflammatory diseases, including rheumatoid arthritis (RA) and PD (9, 11, 12). This is due to their ability to alter

the acetylation status of cytoplasmic proteins including inflammatory cytokines, which might modulate and suppress inflammation and associated bone loss (10, 27, 28). The majority of HDACi (trichostatin A (TSA), phenylbutyrate, vorinostat (SAHA) and givinostat) are broad acting as they target a number of HDAC enzymes across the two main classes. A range of HDACi have also shown promise in suppressing osteoclast activity *in vitro* (5, 34, 39), as well as bone loss and inflammation *in vivo* (21, 29, 35). Recently HDACi that target both Class I and II HDACs have been identified as being potential treatments for periodontitis using a mouse model (4).

As new more specifically acting HDACi are being developed, knowledge of the HDACs involved in human diseases will help identify which targeted treatments will be best for different diseases. At present, there is only limited knowledge of the types of HDACs expressed in various tissues and diseases. Studies in cancer have demonstrated elevated Class I HDACs compared to Class II in breast cancer tissues (45). In a range of cancers, such as in the stomach, oesophagus, colon, prostate, breast, ovary, lung, pancreas and thyroid, all Class I HDACs were shown to be ubiquitously expressed (33). Expression of a selection of HDACs has also been shown in healthy tissues including brain, thymus, testis, pancreas, liver, skeletal muscle, uterine cervix, fallopian tube and bladder wall, lung, and breast (46).

To date, the only investigations assessing HDAC expression in inflammatory diseases have been in RA tissues using osteoarthritic tissues as controls. Higher overall nuclear activity of most HDACs has been demonstrated (23) with HDAC 1 being highly expressed in synovial fibroblasts from RA patients compared to osteoarthritis (OA) (17). High HDAC 1 levels have also been shown to correlate with elevated TNF-α expression in RA tissues. In contrast, another study using tissues from human RA demonstrated that HDAC activity

was suppressed (23). Recently the expression of HDAC enzymes has been demonstrated by human osteoclasts cultured *in vitro*, with Class I HDAC 8 and Class II HDAC 5 found to be significantly up regulated during the later stages of osteoclast development (5). Since increased pre-osteoclasts and osteoclasts are associated with bone erosion in PD (16), it was hypothesised that HDAC 5 and 8 would be elevated in PD tissues.

The development of isoform specific inhibitors enables the ability to target individual HDACs that may offer therapeutic benefits with improved efficacy and reduced side effects compared to broad-acting inhibitors. In order to identify which treatment would be most beneficial, further elucidation of the important HDACs in the inflammatory process and bone loss is needed.

It was hypothesised that both HDAC Classes I and II would be highly expressed in human PD tissues. Therefore the aim of this study was to determine the tissue expression and distribution of Class I and II HDACs 1-10 in human gingival tissues obtained from patients with chronic PD using real time QPCR and immunohistochemistry.

5.3 Materials and Methods

5.3.1 Patient Samples

Human gingival tissues from chronic PD patients visiting the periodontal clinic of the Adelaide Dental Hospital (n=9) were compared with non-PD gingival tissues obtained during wisdom teeth removal and crown lengthening procedures (n=8). Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. This study was approved by the University of Adelaide Human Ethics Committee. Details of the patient tissue samples collected are shown in Table 1. The PD samples were all collected from patients diagnosed with chronic PD with moderate to severe levels of inflammation

assessed histologically and evidence of bone loss as shown radiographically. The non-PD samples were from patients undergoing crown lengthening procedures, who demonstrated no or only mild levels of inflammation and had no evidence of bone loss.

5.3.2 Sample Preparation

Gingival tissue samples were collected and half of each portion was placed into 10% normal buffered formalin overnight before processing and embedding. 5 µM sections were dewaxed for immunohistochemistry. The remaining half was snap frozen in Tissue Tek OCT (Pro Sci Tech, Thuringowa, Qld, Australia) using liquid nitrogen and stored at -80˚C before RNA isolation using Trizol (Invitrogen Life Technologies, Carlsbad, CA). H&E staining was conducted on two of the paraffin embedded sections to enable screening of tissue to ensure good tissue morphology. Following staining, sections were scored for signs of inflammatory cell infiltrate in the soft tissues as previously described (22). The chronic PD samples all demonstrated signs of moderate to severe levels of inflammation, representative histology is shown in Figure 5.1. Patient and sample details, including the level of bone loss at these sites (assessed using radiographs) are shown in Table 1.

5.3.3 RNA Expression of HDAC 1-10

Frozen sections (5 µM) were cut using a cryostat and placed in Trizol (Invitrogen Life Technologies, Carlsbad, CA) for RNA extraction. Reverse transcription (RT) was conducted using the Corbett real time PCR machine (Corbett Research Rotor Gene RG-3000; Corbett Life Science, Mortlake, NSW, Australia). The RT reaction to produce cDNA consisted of 1 µg RNA, 250 ng of random hexamer (Geneworks, Adelaide, SA, Australia) and 200 U of Superscript III Reverse Transcriptase, according to the manufacturer's instructions, as previously described (5). Real time quantitative PCR was conducted using

Platinum SYBR Green qPCR Supermix-UDG (Invitrogen Life Technologies, Carlsbad, CA) (according to the manufacturer's instructions). Each PCR reaction consisted of 1 µg cDNA, 2x supermix (containing SYBR Green 1 dye), 300 nM of forward and reverse primer and this was made up to a total volume of 15 µL with diethyl pyrocarbonate (DEPC) water. PCR was performed in triplicate for each sample. mRNA expression was determined relative to endogenous reference gene human acidic ribosomal protein (hARP(8)). HDAC primers were those previously used as shown in Table 2 (5). The relative quantification of the mRNA expression for each of the genes was then calculated using the comparative Ct method $2^{-\Delta\Delta Ct}$(30)).

Statistical analysis was conducted using a Mann Whitney U test and significance accepted when $p<0.05$.

5.3.4 Immunohistochemical Detection of HDAC 5 and 8

HDAC 5 and 8 protein distribution was assessed in gingival tissues using immunohistochemistry with commercially available antibodies. Rabbit polyclonal antibodies (Abcam, Cambridge, MA, USA) raised against HDAC 5 (ab55403, at 1.25 µg/ml), HDAC 8 (ab39664, at 1 µg/ml) were used. Positive controls included human breast cancer tissue for HDAC 5 and human lung cancer tissue for HDAC 8. Negative controls were carried out for all sections using normal rabbit serum at equivalent concentrations. A RTU kit (Vector Laboratories, Burlingame, CA, USA) was used in a manner similar to that previously described (14).

Endogenous peroxide was blocked with 1% H_2O_2 in methanol for 10 minutes following washing with PBS. A blocking serum of normal horse serum, supplied in the RTU kit (Vector Laboratories, Burlingame, CA, USA), was then applied to sections for 30 minutes.

Without washing, sections were incubated with the primary antibody (HDAC 5 or HDAC 8) in PBS overnight at RT. The following day, sections were washed in PBS then incubated with biotinylated secondary antibody (supplied in the RTU kit), for 45 minutes. After washing in PBS, sections were incubated with streptavidin/peroxidase complex reagent for 45 minutes. Following washing in PBS, the colour reaction was developed using AEC (AEC Peroxidase Substrate Kit, Vector Laboratories) for 30 minutes in the dark. The positive cells were stained red. Sections were washed in MilliQ H_2O following counterstaining with haematoxylin and lithium carbonate before washing and mounting with Aquatex (Merck, Whitehouse Station, NJ, USA).

5.3.5 Semiquantitative Analysis (SQA) of IHC

Semiquantitative analysis was conducted on sections scanned using the Nanozoomer Digital Pathology imaging system (Hamamatsu, Shizouka, Japan). Up to 6 random areas (2 mm x 2 mm) were selected and assessed by two independent observers. Scoring was based on previous methods and involved scoring the proportion of positive cells in the sub epithelial region of the gingival tissues (7, 25). A score of 1; 0-5% of positive cells, 2; 6-10%, 3;11-25%, 4; 26-50% and a score of 5 indicated >50% positive cells. Statistical significance of the difference in HDAC 5 and 8 staining was analysed using the Mann-Whitney test. A value of $p < 0.05$ was considered statistically significant.

5.4 Results

5.4.1 mRNA Expression of HDAC 1-10

Expression of HDAC 1-10 mRNA was examined in chronic PD gingival tissues and compared to non-PD controls using real time QPCR. Expression of HDACs 1, 5, 8 and 9 ($p<0.05$) was significantly higher in chronic PD samples compared to non-periodontitis samples (Figure 5.2). Although expression of HDACs 2, 3, 4, 7 and 10 was higher, this

was not statistically significant. HDAC 6 expression was not increased in PD tissues. There was a 2 fold increase in Class I HDACs 1 and 8 expression, a 2 fold increase in Class II HDAC 5 and a 3 fold increase in HDAC 9 expression in chronic PD samples compared to non-PD gingiva samples.

5.4.2 Expression of HDAC 5 and 8 Protein

Based on the significant increase in HDAC 5 and 8 mRNA expression, and previous studies demonstrating high expression of these enzymes during the later stage of human osteoclast differentiation (5), immunohistochemistry was conducted to determine the protein distribution of HDAC 5 and 8. The proportion of HDAC 5 and 8 positive cells was not significantly higher in the sub epithelial cells between tissues from chronic PD and non-PD samples as assessed by SQA (Table 3). Of particular note, both HDAC 5 and 8 were expressed strongly by the microvasculature in chronic PD tissue. This intense staining was observed in 5 out of the 8 chronic PD tissues samples. In comparison, only 1 of the total 6 samples stained intensely for HDAC 5 and 8 in the non-PD samples (Table 4). Representative images in Figure 5.3 demonstrate this intense staining by blood vessel lining cells in the chronic PD tissues.

5.5 Discussion

The hypothesis that both HDAC Classes I and II would be highly expressed in chronic PD tissues was confirmed in this study. Class I HDACs 1 and 8 along with Class II HDACs 5 and 9 were significantly elevated in human chronic PD gingival tissues at the mRNA level. To date, studies assessing expression of HDACs in human diseases has only been carried out in RA tissues. In these studies HDAC 1 was shown to be highly expressed in synovial fibroblasts from patients with RA compared to OA controls (17, 23) and to correlate with

TNF-α expression (23). There are many similarities between PD and arthritis (1, 15, 32, 40) with high levels of inflammatory cytokines involved in the diseases process of both conditions (3, 37). The current results suggest that despite these similarities between PD and RA there are differences in HDAC expression. HDAC 1 has been demonstrated to be highly up regulated in both diseases, however in PD other Class I (HDAC 8) and Class II HDACs (5 and 9) are also highly expressed at the mRNA level.

The results in this study demonstrating high expression of both HDAC classes in PD were consistent with our previous study using a mouse model of PD that demonstrated suppressed alveolar bone loss with a broad acting HDACi (4). In this study, the novel HDACi 1179.4b effectively suppressed bone loss, whereas MS-275, which is known to be a Class I specific inhibitor and have high selectivity over HDAC 1, had no effect (4). The alveolar bone loss was suppressed by 1179.4b despite no reduction in inflammation levels, whereas MS-275 did reduce inflammation but had little effect on bone erosion. This suggests that there may be some redundancy in the role of the specific HDACs in PD, as more than one HDAC needs to be inhibited to observe positive effects.

In arthritis studies, MS-275 has been shown to prevent the onset of arthritis and reduce bone resorption as well as suppressing Il-1β and Il-6 levels in serum (29). In this study, the more specific Class I inhibitor, MS-275, demonstrated marked anti-arthritic effects compared to broad acting HDACi SAHA (29). This could be related to MS-275 strongly targeting HDAC 1, which has been shown to be highly up regulated in RA (13). It is also possible that different HDACs may also be important in regulating the inflammatory and bone destruction processes in these diseases.

Alveolar bone destruction is a characteristic feature of PD, with high levels of inflammatory cytokines driving expression of the key osteoclast factor RANKL expression by inflammatory cells (6, 7, 43, 44). The high RANKL expression results in increased formation and activity of osteoclasts leading to degradation of alveolar bone. It has been shown previously that HDAC 5 and 8 are highly expressed throughout human osteoclastogenesis *in vitro* and most significantly during the later stages (5). The gingival tissues used in this study were obtained from sites affected by PD demonstrating evidence of associated bone loss. It is likely that there would be pre-osteoclasts cells present in the gingival tissues, as previous studies have demonstrated high levels of RANKL in PD gingival tissues (7). The positive effects of 1179.4b in suppressing bone loss in a mouse periodontitis model (4) could be a result of targeting HDACs 5 and 8 as these specific HDACs appear to be important in osteoclast mediated bone loss. Previous studies have demonstrated the role of HDAC 5 in bone, with its over expression shown to reduce RANKL-mediated acetylation of NFATc1 (24). In addition, the HDAC 5 gene has been shown to be at a locus linked to bone mineral density in a genome wide association study (41).

HDAC 8 expression has been shown by others in a range of normal tissues including brain, thymus, testis, pancreas, liver, skeletal muscle, uterine cervix, fallopian tube and bladder wall, lung (46). It was concluded that HDAC 8 was expressed in tissues where smooth muscle differentiation occurs. Interestingly, immunohistochemical staining in this study revealed HDAC 8 positive microvasculature in the gingival tissues which lack smooth muscle. Although this was seen in both PD and non-PD samples, the staining was more intense in the PD samples. Class II HDAC 5 was also strongly expressed by blood vessel lining cells in both groups, however was more commonly seen in PD samples. The implications for the higher levels of HDAC 5 and 8 shown via immunohistochemistry are

not clear. Previous studies have however shown that HDAC 5 plays a role in regulating immune cell adhesion (47). A broad acting HDACi, TSA, was shown to inhibit monocyte adhesion to the endothelium and this occurred via reducing expression of vascular cell adhesion molecule-1 (VCAM-1) (20). Although the numbers of positive staining cells was not statistically significantly elevated in the periodontitis tissues, the RNA expression of HDAC 5 and 8 was found to be significantly higher. This is likely to be related to the more intense staining of the blood vessel lining cells that was noted in the periodontitis tissues. The observation that HDAC 5 and 8 were associated with vascular endothelium demonstrates that they are not just involved in osteoclast formation, but may also be involved with inflammation in PD. Further studies are clearly necessary to elucidate whether HDAC 5 and 8 in the gingival vasculature play a role in the disease process.

The future use of isoform selective inhibitors will be vital to further elucidate the roles of individual HDAC in the pathogenesis of periodontitis. Research is now progressing with the development of isoform selective inhibitors, including those targeting HDAC 1 (26) and 8 (19, 42) that could be considered as potential treatments for PD.

Table 1. Patient Data

	Gender	Age	Diagnosis/surgery	Inflammation
CHRONIC PERIODONTITIS				
1	M	40	CP	Moderate to severe
2	M	45	CP	Severe
3	M	61	CP	Moderate to severe
4	M	43	CP	Moderate to severe
5	F	39	CP	Moderate to severe
6	F	45	CP	Severe
7	F	29	CP	Severe
8	M	65	CP	Moderate to severe
9	F	45	CP	Severe
NON-PERIODONTITIS				
1	M	58	Crown lengthening	Minimal
2	M	52	Crown lengthening	Minimal
3	F	64	Crown lengthening	Mild
4	M	56	Crown lengthening	None
5	M	56	Crown lengthening	None
6	F	45	Healthy gingiva	None
7	F	83		Mild gingivitis
8	F	64	Healthy gingiva	Mild inflammation
9	F	64	Healthy gingiva	Mild inflammation
10	F	46	Healthy gingiva/crown lengthening	None
11	M	35		
12	M	71	Crown lengthening	Mild gingivitis
13	F	48	Crown lengthening	None
14	M	65	Healthy gingiva	None

Table 2. HDAC Primers used for RT QPCR.

HDAC Gene	Primers
HDAC 1	Sense 5'- AGCCAAGAGAGTCAAAACAGA-3' Antisense 5'-GGTCCATTCAGGCCAACT-3'
HDAC 2	Sense 5'- GCTCTCAACTGGCGGTTCAG-3´ Antisense 5'-AGCCCAATTAACAGCCATATCAG-3´
HDAC 3	Sense 5'- GAGAGTCAGCCCCACCAATA-3´ Antisense 5'-TGTGTAACGCGAGCAGAACT-3´
HDAC 4	Sense 5'- GACCTGACCGCCATTTGC-3' Antisense 5' -GGGAGAGGATCAAGCTCGTTT-3'
HDAC 5	Sense 5'- CAACGAGTCGGATGGGATGT-3' Antisense 5'-GGGATGCTGTGCAGAGAAGTC-3'
HDAC 6	Sense 5'- ACCTAATCGTGGGACTGCAAG-3' Antisense 5'-GAAAGGACACGCAGCGATCT-3'
HDAC 7	Sense 5'- AGCAGCTTTTTGCCTCCTGTT-3' Antisense 5'-TCTTGCGCAGAGGGAAGTG-3'
HDAC 8	Sense 5'- CGGCCAGACCGCAATG-3' Antisense 5'-CACATGCTTCAGATTCCCTTT-3'
HDAC 9	Sense 5'- AGGCTCTCCTGCAGCATTTATT-3' Antisense 5'-AAGGGAACTCCACCAGCTACAA-3'
HDAC 10	Sense 5'- ATGACCCCAGCGTCCTTTACT-3' Antisense 5'-CGCAGGAAAGGCCAGAAG-3'

Table 3. Semiquantitative analysis of HDAC 5 and 8 expression in the sub-epithelial tissue.

Protein	Non-periodontitis	Chronic Periodontitis
HDAC 5	2.25 (1.07)	1.73 (0.77)
HDAC 8	2.85 (0.47)	2.65 (0.68)

SQA Grades for proportion of positive cells 1; 0-5% of positive cells, 2; 6-10%, 3; 11-25%, 4; 26-50% and a score of 5 indicated >50% positive cells. Mean +/- SD.

Table 4. Proportion of samples with HDAC 5 and 8 positive vessels.

Group	Non-periodontitis		Chronic periodontitis	
Protein	HDAC 5	HDAC 8	HDAC 5	HDAC 8
Negative	0/6 (0%)	2/6 (33%)	0/6 (0%)	0/6 (0%)
Mild	5/6 (83%)	3/6 (50%)	3/8 (38%)	3/8 (38%)
Intense	1/6 (17%)	1/6 (17%)	5/8 (63%)	5/8 (63%)

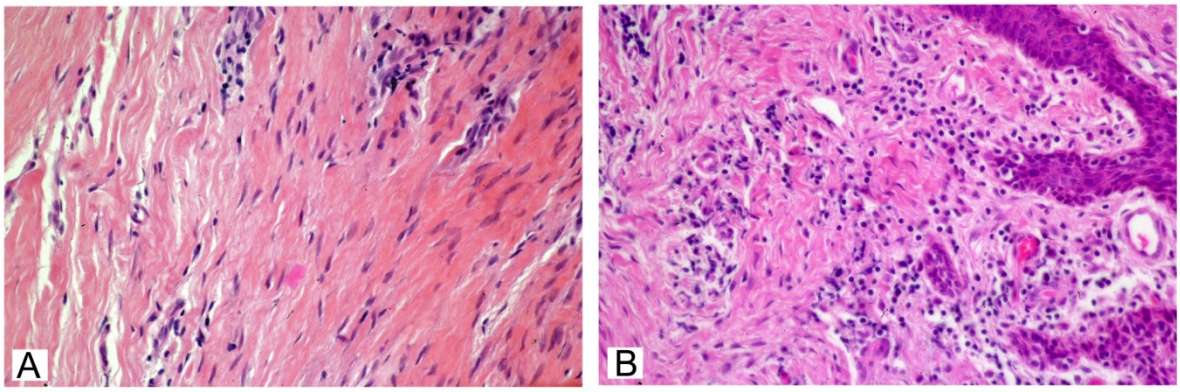

Figure 5.1. Representative H&E stained images (100x magnification) of gingival tissues from A) non-periodontitis samples (no bone loss, healthy or mildly inflamed gingiva). B) Chronic periodontitis gingival tissues (taken from sites with both inflammation and bone loss).

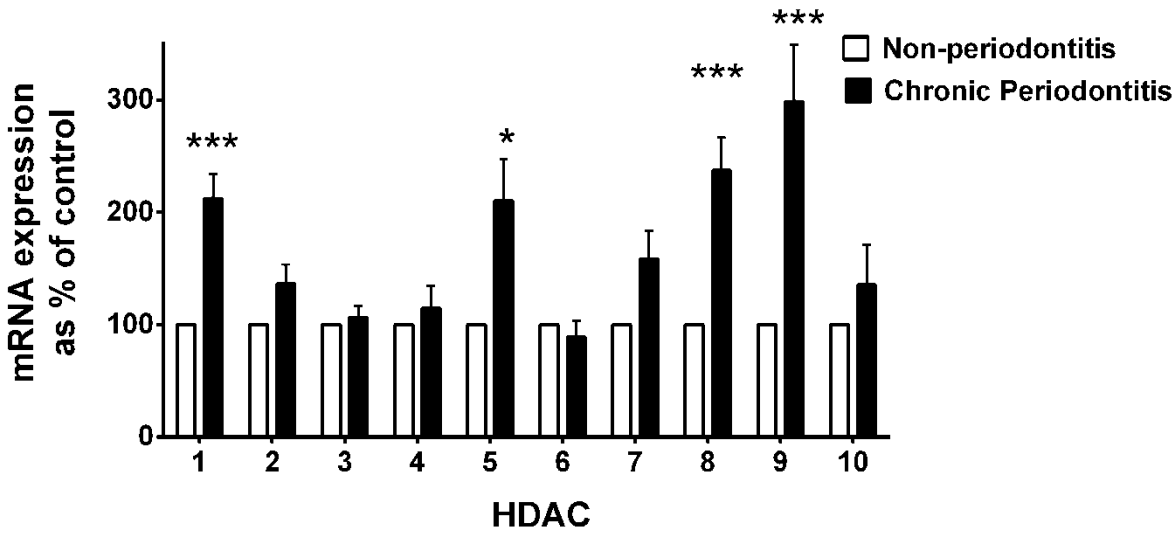

Figure 5.2. Fold change in the mRNA expression of HDAC 1-10 relative to the non-PD tissues. mRNA expression in both groups is relative to endogenous gene hARP. Bars represent mean±SEM. *** $p<0.001$, ** $p<0.01$ expression in chronic periodontitis samples relative to non-periodontitis gingival tissues**.**

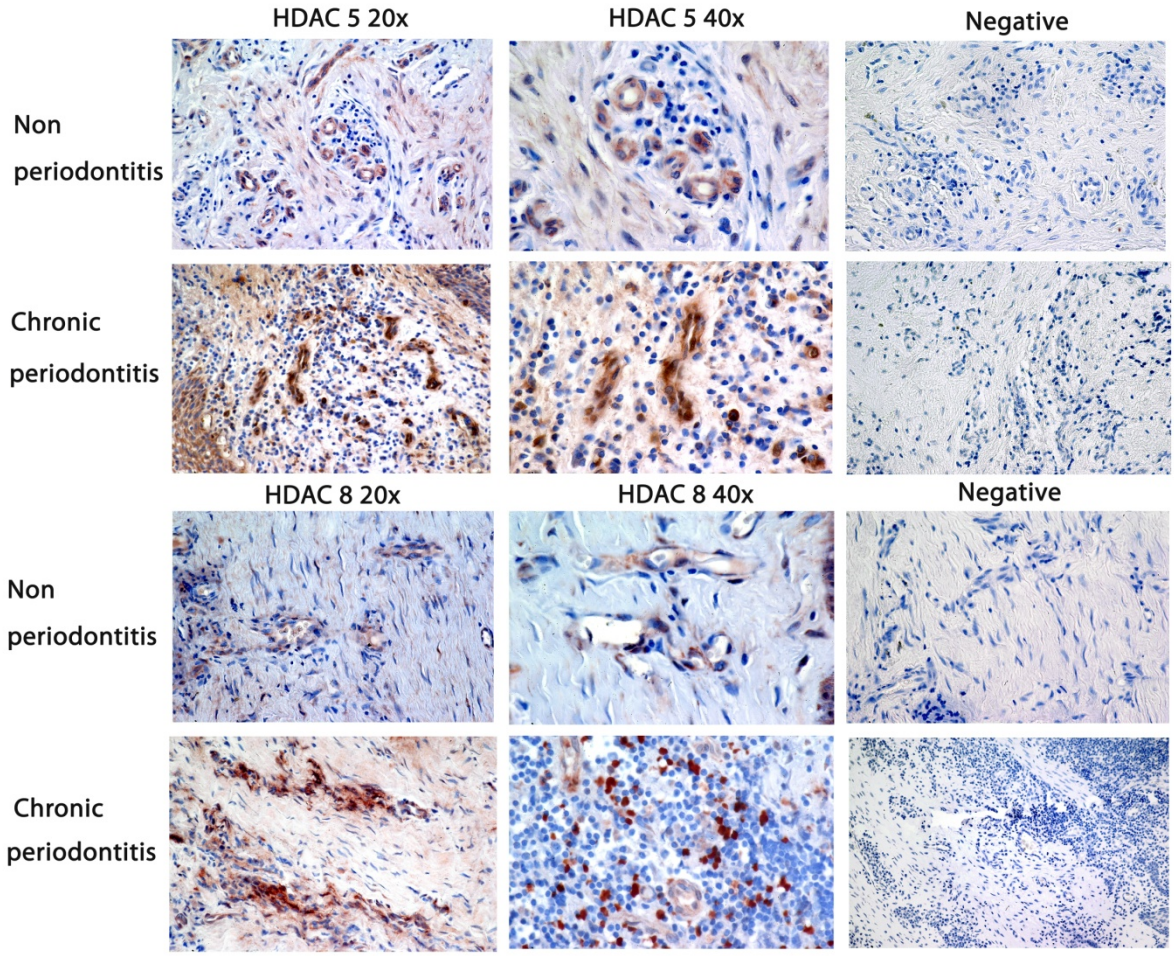

Figure 5.3. Representative images of immunohistochemistry detecting HDAC 5 (ab55403, at 1.25 µg/ml), HDAC 8 (ab39664, at 1 µg/ml) in non-periodontitis and chronic periodontitis gingival tissues. Positive cells are red. Negative controls were carried out for all sections using normal rabbit serum at equivalent concentrations as shown in the final column. HDAC staining was imaged at 20x (first column) and 40x magnification (middle column).

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Chapter 6. Inhibiting Histone Deacetylase 1 (HDAC 1) Suppresses Both Inflammation and Bone Loss in Arthritis

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Chapter Summary

In Chapter 4, the novel broad acting HDACi, 1179.4b suppressed bone loss in a mouse model of periodontitis despite no effect on inflammation. This suggests mechanisms controlling inflammation and bone loss may be different. HDAC 1 has also been shown by others to be highly expressed in human tissues from RA patients. Hence the aim of this study was to determine the effects of a novel inhibitor (NW-21) designed to target HDAC 1 on inflammation and bone *in vitro* and in a collagen antibody induced arthritis (CAIA) model. NW-21 was compared to 1179.4b and MS-275 as a positive control.

STATEMENT OF AUTHORSHIP

Inhibiting Histone Deacetylase 1 (HDAC 1) Suppresses Both Inflammation and Bone Loss in Arthritis

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Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Chapter 6. Inhibiting Histone Deacetylase 1 (HDAC 1) Suppresses Both Inflammation and Bone Loss in Arthritis

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Key words

Histone Deacetylase 1 (HDAC 1), osteoclasts, bone loss, collagen antibody induced arthritis

6.1 Abstract

Objectives: Histone Deacetylase 1 (HDAC 1) is highly expressed in synovial tissues from rheumatoid arthritis (RA) patients. Hence, this study aimed to determine the effects of a novel HDACi (NW-21) in three model systems: osteoclast formation and activity *in vitro*, cytokine and chemokine expression (TNF-α, IL-1β, Macrophage inflammatory protein-1α (MIP-1α), Monocyte chemotactic protein 1 (MCP-1) and RANTES) by human monocytes stimulated with TNF-α or *E. coli* LPS *in vitro*, inflammation and bone loss in collagen antibody induced arthritis (CAIA) in mice.

Methods: Human peripheral blood mononuclear cells (PBMCs) stimulated with receptor activator of nuclear factor kappa B ligand (RANKL) were used to test the effect of NW-21 on osteoclast formation and activity. Anti-inflammatory activity was assessed using human monocytes stimulated with either tumor necrosis factor α (TNF-α) or lipopolysaccharide (LPS). Real time PCR was used to determine mRNA expression of inflammatory factors important in osteoclast formation. Activity *in vivo* was monitored using the CAIA in mice with daily oral administration of NW-21 at 5mg/kg/day. NW-21 was compared to Class I HDACi, MS-275, and a broad acting HDACi, 1179.4b. Effects on inflammation and bone were assessed using paw inflammation scoring, histology and live animal micro CT of the radiocarpal joints.

Results: HDACi NW-21 suppressed human osteoclast formation and activity. NW-21 also significantly reduced the mRNA expression of chemokines monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) in PBMCs stimulated by LPS and TNF-α. Only HDACi that targeted HDAC 1, NW-21 and MS-275, reduced inflammation and bone loss *in vivo*.

Conclusion: The results indicate that inhibitors targeting HDAC 1, such as NW-21 and MS-275, may be useful for treating RA as they reduce both inflammation and bone loss.

6.2 Introduction

Soft tissue inflammation, focal bone erosions and systemic bone loss are all characteristic features of rheumatoid arthritis (RA), which currently affects approximately 1% of the world's population (1). RA commonly affects the synovial joints, particularly the knees and fingers, resulting in joint inflammation and synovial hyperplasia associated with destruction of bone and cartilage. A variety of treatments exist, ranging from the basic non-steroidal anti-inflammatory drugs (NSAIDs) to the more recently developed biologic anti-rheumatic drugs (DMARDs), such as the anti-TNF agents infliximab and adalimumab. Although these treatments have positive effects in suppressing inflammation, their actions on the focal bone erosions are variable (2-3). Many of these current treatments can also have adverse side effects (4-9) when taken for a long time period and it can also take a considerable time for clinicians to identify the appropriate treatment(s) for individual patients (10-11). During this delay in finding an effective treatment regime, there can be continuing erosion of the bone that strongly impacts on normal joint function both in the short and longer term. The importance of timely treatments is highlighted by a recent report showing that patients, who delayed for 12 weeks to see a rheumatologist and commence therapy, had a 1.3 times higher rate of joint damage than patients who were seen before 12 weeks (12). Therefore, treatments that inhibit both bone erosion and joint inflammation could be beneficial. Histone deacetylase (HDAC) inhibitors might be one such drug class with the potential to both suppress the inflammation and halt damage to the joints.

HDAC enzymes play an important role in regulating gene transcription, in part by altering the acetylation status of histone proteins resulting in gene repression. These enzymes can also deacetylate many other non-histone signal transduction proteins that are important in multiple inflammatory events (13-14). HDAC enzymes with a catalytic zinc ion are

classified into two main classes. Class I includes HDACs 1, 2, 3 and 8 which are primarily found in the nucleus (15). Class II includes HDACs 4, 5, 7 and 9 which belong to Class IIa and HDACs 6 and 10 which belong to Class IIb. Class II enzymes are able to shuttle between the nucleus and cytoplasm. The inhibition of HDAC enzymes is considered to be a favourable therapeutic strategy for a wide variety of diseases, including both malignancies and inflammatory diseases. The positive effects accompanying HDAC inhibitor (HDACi) treatment in inflammatory diseases appears to be related to a reduced production of inflammatory cytokines, such as TNF-α and IL-1β (16). Anti-inflammatory activity has been observed at much lower doses (10-100x lower) than those used to treat malignancies, making them attractive therapeutic options (17).

A number of studies have demonstrated positive benefits for various HDACi in animal models of inflammatory arthritis (18-21). Using an adjuvant arthritis model, cyclic dipsipeptide HDACi, FR901228 (FK228) demonstrated both prophylactic and therapeutic effects (21). Phenylbutyrate and trichostatin A (TSA), both broad acting HDACi, have been shown to suppress joint swelling, reduce subintimal mononuclear cell infiltration, inhibit synovial hyperplasia, and suppress pannus formation, cartilage damage and bone destruction in an adjuvant arthritis model (22). More specific acting inhibitors, such as MS-275, have also been reported to have significant anti-arthritic effects (19). At relatively low doses (3 mg/kg/day), MS-275 inhibited inflammation and bone loss while reducing IL-1β and IL-6 serum levels (19). MS-275 has been reported to suppress HDAC 1 at concentrations below 0.1 μM (23) and is more preferential for HDAC 1 (IC_{50}0.3 μM) compared to HDAC 3 (IC_{50}8 μM) (24). By comparison, a broad acting HDACi, such as suberoxylanilide hydroxamic acid (SAHA), requires higher doses (10mg/kg/day) to reduce inflammation and bone damage. SAHA was shown to suppress both inflammation and bone erosion, but could not prevent the onset of arthritis (19). These studies demonstrate

the potential of selective HDACi for treating arthritis. Broad acting inhibitors target a range of HDAC enzymes in both classes and in many cases it is unclear exactly which HDACs are targeted and to what extent. The broad inhibitory action increases the possibility of side effects during long-term administration due to the widespread expression of these enzymes in a variety of tissues and organs. Research is now progressing towards using more selective compounds that target specific HDACs involved in disease processes.

To date, only one HDACi, givinostat, has progressed to humans for the treatment of juvenile arthritis. In that study, 17 children with juvenile idiopathic arthritis (JIA) were treated with givinostat for up to 12 weeks (25). No systemic toxicities or organ dysfunctions were observed and only mild to moderate side effects were reported. Patients treated with givinostat reported improvements in mobility and wellbeing and there were also reductions in number of joints with active arthritis (25).

Recent studies have demonstrated elevated nuclear activity of HDACs in RA synovial tissue compared to osteoarthritis (OA) tissues (26) and HDAC 1 is highly expressed in synovial fibroblasts from RA patients (27). High levels of HDAC 1 observed in RA tissues were found to correlate with TNF-α expression (26-27), suggesting a link between HDAC activity and synovial inflammation. The high expression of HDAC 1 in RA along with the positive effects observed for MS-275 in animal models of arthritis suggests that targeting these enzymes could reduce disease activity. In this study, it was hypothesised that inhibitors targeting HDAC 1 would suppress inflammation and bone loss *in vitro* and in an animal model of inflammatory arthritis. Therefore, this study aimed to determine the effects of a novel HDACi (NW-21) in three model systems: osteoclast formation and activity *in vitro*, cytokine and chemokine expression (TNF-α, IL-1β, Macrophage

Inflammatory protein-1α (MIP-1α), Monocyte chemotactic protein 1 (MCP-1) and RANTES) by human monocytes stimulated with TNF-α or *E. coli* LPS *in vitro*, inflammation and bone loss in collagen antibody induced arthritis (CAIA) in mice. HDACi NW-21 designed to target HDAC 1 was compared with another Class I HDACi, MS-275, and a novel broad-acting HDACi, 1179.4b, previously shown to suppress bone loss *in vitro* and in a mouse model of periodontitis (28-29). In addition, expression of important factors involved in osteoclast formation and inflammation *in vitro* assays was assessed to gain further insights to mechanisms by which NW-21 inhibits osteoclast resorption and inflammation. The results of this study indicate the importance of HDAC 1 along with other Class I HDACs for both anti-inflammatory and anti-resorptive activity.

6.3 Methods

6.3.1 Histone Deacetylase Inhibitors (HDACi)

HDACi NW-21 is a novel compound initially developed by researchers at the University of Queensland to inhibit cancer (compound 51 in (30)) and malaria (compound ASU-13 in (31)). It was designed to specifically target Class I over Class II HDACs (e.g. inhibition of HDAC activity: IC_{50} = 0.021±0.02 µM for HDAC 1 (Class I) , 0.042±0.02 µM for HDAC 2 (Class I), 0.3 µM for HDAC 3 (Class I), >10 µM for HDAC 4 (Class IIa) 0.88±0.06 for HDAC 6 (Class IIb), >1 µM for HDAC 7 (Class IIa), >3 µM for HDAC 8 (Class 1) ((32) and unpublished data). MS-275 and 1179.4b were also obtained from the University of Queensland and concentrations used were based on previous studies (28). MS-275 is also a Class I selective inhibitor (IC_{50} = 0.181 µM for HDAC 1, 1.16 µM for HDAC 2, 2.31 µM for HDAC 3, >10 µM for HDAC 4, >10 µM for HDAC 6, >10 µM for HDAC 7 and >10 µM for HDAC 8 (32).

6.3.2 *In vitro O*steoclast Assay

Peripheral Blood Mononuclear cells (PBMCs) were obtained by differential centrifugation from whole blood buffy coats obtained from the Australian Red Cross Blood Service (n=9) as previously described (29). Ethical approval was obtained from the University of Adelaide Human Ethics Committee. Briefly, cells were seeded at $2x10^6$ cells per mL on day 0 in α-minimal essential medium (a-MEM; Invitrogen, Melbourne, Victoria, Australia) supplemented with 10% fetal calf serum (FCS; Invitrogen, Life Technologies, Carlsbad, CA), 1% penicillin–streptomycin (Invitrogen), and 1% L-glutamine (Invitrogen) with 100 nM 1a, 25(OH2)D3 (vitamin D3) (Novachem, Melbourne, Victoria, Australia), 100 nM dexamethasone (Fauldings, Adelaide, South Australia, Australia), and 25 ng/mL of recombinant human M-CSF (Chemicon International Inc, Millipore, MA). Cells were maintained at 37˚C with 5% CO_2 for a total of 17 days. From day 7 onwards, human recombinant receptor activator of nuclear factor kappa B (RANKL) (50 ng/mL; Chemicon International Inc.) was added to the medium. Cells were treated with 5 fold dilutions of NW-21 starting at 20 nM commencing on either day 7 (coinciding with RANKL addition), day 10 or day 13. Serial dilutions of NW-21 were achieved in DMSO and hence controls were treated with the final DMSO concentration 0.01%.

6.3.3 Cell Viability

The effect of NW-21 on cell viability was assessed over a concentration range of 0.16 nM-20 nM using a WST-1 assay (Roche Applied Science, Castle Hill, NSW, Australia). On day 14, 10 µL of cell proliferation reagent WST-1 was added to each well (100 mL media) followed by incubation for 2 h at 37˚C. A control blank was used consisting of culture medium with WST-1 reagent without any cells. Absorbance was measured at λ = 450 nm

following a 2 h incubation period. Absorbance in NW-21 treated wells was compared with that of controls.

6.3.4 Osteoclast Formation

Cells were grown in the presence of NW-21 (0.16 nM– 20 nM) commencing from either day 7, 10 or 13 in 16 well chamber slides (LabTek, Nunc International, Rochester, NY, USA). On day 14, cells were fixed with 4% glutaraldehyde in Hank's balanced salt solution (HBSS) and stained with a TRAP kit (Sigma, St. Louis, MO) according to the manufacturer's instructions and as previously described (29). Cells were counterstained using 0.5% w/v Methyl Green. TRAP staining was imaged using a light microscope (Nikon Microphot FXA Photomicroscope, Nikon Instruments Inc., Melville, NY, USA). Three representative areas were imaged for each concentration. The number of TRAP-positive multi-nucleated (more than 3 nuclei) cells for each concentration of NW-21 was compared with the controls (0.01% DMSO) for individual donors.

6.3.5 Osteoclast Activity

Cells for activity assessment were grown on 5 mm sterile whale dentine slices. On day 17, cells were trypsonised and washed with milliQ water to remove cells before mounting onto SEM stubs for analysis. Dentine slices were coated using carbon gold and then imaged with the Philips XL-20 Scanning Electron Microscope (Adelaide Microscopy). Three representative images were taken for each dentine piece at 150x magnification and quantitated using Image J analysis software (National Institutes of Health, USA). The area of pit resorption for each concentration of NW-21 was expressed as a percentage of the total area of the dentine. Resorption in treated wells was represented as a percentage of the average area of resorption in the control well (0.01% DMSO).

6.3.6 Inflammatory Gene Expression *In Vitro*

PBMCs were obtained and seeded at $2x10^6$ cells per mL in 48 well trays on day 0 in α-MEM with the supplements as described above. Cells were incubated at 37˚C with 5% CO_2 for 2h (33). Following this, cells were treated with either human recombinant *E. coli* lipopolysaccharide (LPS) (100 ng/ml) (ChondrexInc., Arthrogen-CIAs Arthritogenic Monoclonal Antibodies, Redwood WA, USA) or tumor necrosis factor α (TNF-α) (10 ng/ml) (Recombinant Human TNF-alpha, RnD Systems, Minneapolis, MN USA) with and without NW-21 (20 nM). Controls were treated with 0.01% DMSO. Cells were maintained at 37˚C with 5% CO_2 for a further 24h before RNA collection as described below.

6.3.7 Real Time PCR to Determine mRNA Expression

RNA was extracted from cells grown in the presence of HDACi NW-21 and control wells at different time points throughout the osteoclast culture (days 0,7,10, 14 and 17) using Trizol (Invitrogen Life Technologies, Carlsbad, CA). For the inflammation assay, RNA was collected after 24 hours of incubation with either TNF-α or LPS with or without HDACi NW-21. Reverse transcription was conducted using a Corbett real time PCR machine (Corbett Research Rotor Gene RG-3000; Corbett Life Science, Mortlake, NSW, Australia). The RT reaction included 250 ng of random hexamer (Geneworks, Adelaide, SA, Australia) and 200 U of Superscript III Reverse Transcriptase according to the manufacturer's instructions to produce cDNA. Real Time PCR was carried out using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The mRNA expression of osteoclast related genes nuclear factor of activated T cells (NFATc1), TNF receptor factor-6 (TRAF-6) and osteoclast associated receptor (OSCAR) were compared between treated and control

cells. Primers were those previously used: NFATc1 (29), TRAF-6 (29) and OSCAR (34). For the *in vitro* inflammatory assay cytokines included TNF-α, IL-1β and chemokines MCP-1, MIP-1α (designed using Primer3Plus) and RANTES (35). Reaction mixtures consisted of 1mg cDNA, 2x supermix (containing SYBR Green 1 dye), 300 nM of forward and reverse primer and this was made up to a total volume of 15µL with diethyl pyrocarbonate (DEPC) water. PCR was performed in triplicate for each sample. mRNA expression was determined relative to endogenous reference gene human acidic ribosomal protein (hARP; (36)). The relative quantification of the mRNA expression for each of the genes was then calculated using the comparative Ct method $2^{-\Delta\Delta Ct}$ (37).

6.3.8 Collagen Antibody Induced Arthritis (CAIA) Model

Female Balb/c mice (29 animals) at 6-8 weeks of age were used. Mice had access to food and water *ad litem*. Animal ethics approval was obtained through the University of Adelaide Animal Ethics Committee. The experiment consisted of 5 groups; group 1 controls no disease (n=6), group 2 CAIA with vehicle treatment (n = 6), group 3 CAIA with HDACi 1179.4b treatment at 5 mg/kg/day (n = 5), group 4: CAIA with HDACi treatment NW-21 at 5 mg/kg/day (n = 6) and group 5 CAIA with HDACi MS-275 treatment at 10 mg/kg/day (n = 6).

6.3.9 CAIA Induction

Four days after the initial CT scan was conducted, CAIA was induced by injecting mice intravenously via the tail vein with 150 µL (1.5 mg) of a monoclonal antibody against type II collagen (ChondrexInc, Arthrogen-CIAs Arthritogenic Monoclonal Antibodies, Redwood WA, USA) as previously described (39). Control mice were injected with PBS only. Two days later mice were given an intraperitoneal (i.p.) injection of 20 µL (10 µg) of

LPS (ChondrexInc). Control animals were injected with only PBS. Mice were monitored daily by two experienced observers for a total of 14 days. Clinical record sheets were maintained throughout the study detailing changes in body weight, dull/ruffled coat, temperament, reduced food/water intake or a reluctance to move). Paws were assessed for signs of redness and swelling daily by two experienced observers. To assess inflammation each paw was given a score from 0 to 4 giving a total maximum score of 16 for each animal (39). 0=normal paw, 1=mild but definite redness and swelling of the wrist/ankle, 2=moderates welling and redness of the wrist/ankle with digit involvement, 3=severe swelling of the wrist/ankle with multiple digit involvement and 4=maximum inflammation within the entire paw, wrist/ankle with many digits involved.

6.3.10 Live Animal Micro CT Scanning

A live animal micro CT scan was initially conducted to obtain baseline measurements (day 0), followed by a final CT scan at the completion of the study (day 18). Scans were conducted using an *in vivo* animal micro-CT scanner (Bruker formally known as SkyScan, SkyScan1076, Kontich, Belgium) situated in Adelaide Microscopy. The specifications used for scanning and machine details have been published previously (28,38-39). Scanning was conducted at 74 kV/136 mA with a pixel size of 18 mM, 1 mm aluminium filter and frame averaging of 1. Mice were initially anaesthetized with an i.p. injection (rat/mouse anaesthetic – 1 m Lxylazine, 2 mL ketamine (100 mg/mL), 17 mL of water in the injection, 0.3 mL for a 30 g mouse). Mice were aligned using polystyrene holders with the front paws being included in the scanning region of interest. Scans were reconstructed using SkyScanN' Recon program and then realigned to allow for the same area to be analysed using SkyScan program Data Viewer. Bone Volume analysis of the radiocarpal joints were conducted using SkyScan's CTAn program (SkyScan, Kontich, Belgium). The same reference point with the radiocarpal joints was utilised for all analysis with 100 slices

either side of this reference point being included in the region of interest to be analysed as previously described (29).

6.3.11 Histological Analysis

At the conclusion of the experiment, front paws were collected and fixed in 10% NBF for 48 h before decalcification in 10% EDTA for 8 weeks. Specimens were then processed and paraffin embedded. Sagittal sections (7 µM) of the radiocarpal joints were prepared. Routine H&E staining was conducted and semiquantitative analysis was carried out by two blinded observers using a 4-point scale as previously described (39). Scoring was based on the numbers of inflammatory cells within the radiocarpal joint at 10x magnification (lymphocytes, plasma cells neutrophils or macrophages). Normal tissue (<5% inflammatory cells) was scored a 0, mild inflammation (5–20% inflammatory cells) was scored a 1, moderate inflammation (20–50% inflammatory cells) was scored a 2 and severe inflammation with a massive immune cell infiltration (>50% inflammatory cells) was scored a 3. Bone and cartilage destruction was assessed by: 0=normal, 1 =mild cartilage destruction, 2=evidence of both cartilage and bone destruction, 3= severe cartilage and bone destruction. Pannus formation: 0=no pannus, 1=pannus formation. Tartrate resistant acid phosphatase (TRAP) staining of the radiocarpal joints was conducted to detect osteoclasts on the bone surface and in the soft tissues. TRAP staining methods were adapted from (40). Slides were stained in TRAP for 20 minutes at 37◦C before rinsing and counterstaining with haematoxylin. TRAP staining was imaged with a light microscope.

6.4 Results

6.4.1 Osteoclast Formation and Activity

Cells treated for 24 h with HDACi NW-21 showed no significant changes in cell viability at all concentrations tested (0.16– 20.0 nM) (data not shown). RANKL stimulation resulted

in the formation of TRAP positive multinucleated cells detected at day 14 and these cells resorbed pits in the mineralised substrate by day 17 (Figure 6.1). Treatment of the cells with HDACi NW-21, commencing from day 7 coinciding with RANKL addition, resulted in a significant reduction in the number of multinucleated TRAP positive cells for the highest concentrations (20 nM ($p<0.001$) and 4 nM ($p<0.01$)) (Figure 6.2). This reduction was associated with a similar reduction in resorption activity (Figure 6.1). At all concentrations above 0.16 nM there was a significant reduction in the area of pit resorption ($p<0.01$) (Figure 6.2). There was a marked reduction with increasing concentrations, 1.1% of the control area resorbed with 20 nM treatment and 21% with 4 nM NW-21 treatment. Experiments were carried out with treatments commencing on day 10 and day 13 to assess effects on the later stages of osteoclast differentiation. Interestingly, treatment from either day 10 or day 13 did not significantly reduce either the formation of TRAP multinucleated cells or resorption (Figure 6.2).

6.4.2 Gene Expression in Osteoclasts

Effects of NW-21 treatment commencing at day 7 on osteoclast related genes were assessed using real time PCR (Figure 6.3). NW-21 treatment resulted in a 1.4 fold reduction in the mRNA expression for TRAF-6 ($p<0.001$) at day 10. NFATc1 mRNA expression was also significantly decreased at day 10 ($p<0.05$) and at day 17 which was not significant ($p>0.05$). OSCAR mRNA expression was significantly reduced by 1.3 fold at day 10. At day 10 there was a 2.3 fold increase in MCP-1 mRNA expression relative to day 7, and this expression was reduced throughout osteoclast differentiation. At day 10, HDACi NW-21 resulted in a 1.7 fold reduction in the MCP-1 mRNA expression ($p<0.05$).

6.4.3 Inflammatory Cytokines and Chemokine Gene Expression

Monocytes stimulated with LPS or TNF-α were treated with NW-21 (20 nM) which induced a significant ($p<0.05$) reduction in mRNA expression of chemokines MIP-1α and MCP-1. For TNF-α treated cells, there was a 1.7 fold reduction in the relative mRNA expression and in LPS stimulated cells there was a 4.6 fold reduction. In both cases there was no significant change in the mRNA expression of cytokines TNF-α, IL-1β or RANTES as shown in Figure 5.4 ($p>0.05$). There was a 1.7 fold reduction in the relative mRNA expression of RANTES in both TNF-α and LPS stimulated monocytes although this was not significant. NW-21 treatment caused a 4.1 fold decrease in IL-1β mRNA expression in LPS stimulated cells and a 2.7 fold decrease in IL-1β mRNA in TNF-α stimulated cells ($p>0.05$).

6.4.4 Collagen Induced Arthritis

The effects of NW-21 (5 mg/kg/day) daily oral treatment commencing after LPS administration on day 4 was assessed in a CAIA model. These effects were compared to broad acting inhibitor 1179.4b and Class I selective inhibitor MS-275.

6.4.5 Weight Changes

Mice were monitored daily for changes in weight and all the disease groups lost weight (9%) at day 3 associated with the LPS injection as shown in Figure 5.5A. Although there was no statistically significant difference, there was less weight loss by all HDACi treatment groups.

6.4.6 Inflammation

Induction of inflammatory arthritis resulted in significant swelling and redness in the paws as shown in Figure 5.5C. Paw inflammation was first noted from day 4 following the LPS injections, which peaked at day 10 and began to reduce over the following 4 days. There was no significant effect of 1179.4b treatment on the paw scoring at any of the time points ($p>0.05$). Both NW-21 and MS-275 treated groups had significantly reduced paw scores compared to untreated mice, as depicted in the macroscopic images in Figure 6.6 (G-J). The histology of the tissues was consistent with paw inflammation seen macroscopically (Figure 6.6) with evidence of extensive inflammatory cell infiltration in both the vehicle treated (oil) and 1179.4b treated mice. There was very little inflammatory cell infiltration in the paws of mice treated with NW-21 or MS-275. There was also evidence of pannus formation in the mice given the vehicle or 1179.4b that was mostly absent in the NW-21 and MS-275 treated groups. In the NW-21 treated group, 1 of the 6 mice failed to respond to NW-21 treatment in any way and this caused a lack of statistical significance other than on days 10 and 11.

6.4.7 Bone Loss

Analysis using live animal CT also revealed evidence of bone loss throughout the 14 days in the diseased mice (Figure 6.5E). There was an expected normal growth of the mice with a 8.9% increase in bone volume in the controls over the experimental period. In diseased (oil) mice there was on average a 1% decrease in bone volume. In 1179.4b treated mice there was greater bone loss than the oil treated mice with an average 4.4% reduction in bone volume overtime. There was an average 8.7% increase in bone volume in NW-21 treated mice and a 12% increase in MS-275 treated mice as shown in Figure 6.5E. This did not reach statistical significance when compared to untreated mice (Figure 6.5E). There

was a marked reduction in bone and cartilage destruction following NW-21 and MS-275 treatments as assessed on H&E stained radiocarpal joints. In both the diseased group and the 1179.4b treated mice there was evidence of large numbers of TRAP positive cells on the bone surfaces and in the inflamed soft tissues as depicted in Figure 5.6. Very few TRAP positive cells were found in the joints from mice treated with either NW-21 or MS-275 (Figure 6.6).

6.5 Discussion

HDAC 1 is highly expressed in synovial tissues from RA patients. Hence, this study aimed to determine the effects of a novel HDACi (NW-21) in three model systems: osteoclast formation and activity *in vitro,* cytokine and chemokine expression (TNF-α, IL-1β, MIP-1α, MCP-1 and RANTES) by human monocytes stimulated with TNF-α or *E. coli* LPS *in vitro,* inflammation and bone loss in CAIA in mice. These studies helped to elucidate the roles of HDAC 1 and other class I HDACs in inflammation and bone loss.

The *in vitro* results of this study support the original hypothesis that inhibitors targeting HDAC 1 would suppress bone loss. NW-21 reduced the formation of TRAP positive osteoclasts and their resorptive activity in a concentration-dependent manner. Many previous studies have demonstrated that HDACi can suppress osteoclast differentiation and activity i*n vitro,* but they used broad acting inhibitors of HDACs including TSA (41-42), SAHA (29), ITF2357 (21) and 1179.4b (29). We have previously shown that MS-275, which inhibits HDAC 1 and other Class I HDACs (32) was able to suppress human osteoclasts (29). However, MS-275 was only effective at relatively high concentrations (100 nM). The suppression of osteoclasts with NW-21 was surprising given that HDAC 1 expression was not shown to be significantly increased during osteoclast development while HDACs 5 and 8 were highly up-regulated (29). These results could be related to the

potency of NW-21 or could suggest that although HDAC 1 expression is not markedly increased in osteoclastogenesis, it still plays a role in osteoclast differentiation. Other Class I HDACs such as HDAC 2 and HDAC 3 may also be important in this process. Studies by others have focussed on the roles of HDAC 3 and HDAC 7, with suppression of HDAC 7 accelerating osteoclast differentiation and suppressing HDAC 3 shown to inhibit differentiation (43). NW-21 can inhibit HDAC 3 (IC_{50} = 0.3 μM) and this may be a mechanism by which NW-21 inhibits osteoclastogenesis observed in this study. Over-expression of HDAC 5 has been shown to reduce RANKL-mediated acetylation of NFATc1 (NFATc1), indicating HDACs can regulate NFATc1 activity (44). While these studies show the importance of HDACs in osteoclast differentiation, it is still unclear which HDACs are the most important. It may well be that it is a combination of HDACs that influence these processes, some being more important than others as supported by the results of this study.

An interesting observation in this study of NW-21 was that commencing HDACi treatment later than day 10 did not result in a decrease in osteoclast activity. This is similar to the effect of HDACi 1179.4b, which can suppress both osteoclast formation and activity when treatment is commenced from day 7, but not afterwards (29). In order to better understand the mechanisms involved, mRNA analysis was conducted. NW-21 treatment from day 7 resulted in a small reduction in the mRNA expression of TRAF-6 and NFATc1, similar to that for 1179.4b. The reduction in TRAF6 at day 10 may be responsible for the reduced NFATc1 expression seen later as TRAF-6 signalling, initiated soon after RANK/RANKL interaction, is up stream of NFATc1 elevation essential during the terminal stages of osteoclast formation (45). This is consistent with the lack of effect of NW-21 after day 10 (3 days after RANKL administration).

NW-21 caused a significant reduction in expression of chemokines MCP-1 (also referred to as Chemokine (C-C motif) ligand 2 (CCL2)) and MIP-1α (also referred to as Chemokine (C-C motif) ligand 3 (CCL3). MCP-1 is known to recruit monocytes, memory T cells, and dendritic cells to sites of inflammation. It is also reportedly produced by synovial cells and infiltrating monocytes in RA (46). RA synovial tissue macrophages were found to constitutively express MCP-1 (46). TNF-α and IL-1 stimulation of synovial cells resulted in increased MCP-1 mRNA expression (47). Anti-TNF drug etanercept was shown in a study of RA patients to significantly reduce serum levels of MCP-1 and these levels correlated with disease activity, confirming a role for MCP-1 in the pathogenesis of RA inflammation (48). MCP-1 was also reported to be expressed by mature osteoclasts (49) and shown to promote human osteoclast formation in absence of RANKL; however, these cells were unable to resorb bone (49). Macrophage inflammatory protein 1α (MIP-1 α) is known to be produced by macrophages following stimulation, by bacterial endotoxins (50). It is known to be chemotactic for PMNs, macrophages and T cells (50). Significantly higher levels of MIP-1α have been shown in synovial fluids from patients with RA compared to OA. Mononuclear cells from synovial fluid produce MIP-1α along with synovial fibroblasts and this can be stimulated by TNF-α or LPS (51). MIP-1α has also been shown to induce osteoclasts (52). Both MCP-1 and MIP-1α were significantly reduced by NW-21 treatment in this study. A lack of MCP-1 in osteoclasts has been shown to result in a down-regulation of NFATc1 and osteoclast suppression (53). This is consistent with the notion that inhibition of NFATc1 and osteoclast formation occurs downstream of MCP-1 suppression initiated by inhibition of HDAC 1.

The second part of the study hypothesis, that HDACi which target HDAC 1 would suppress bone loss, was also supported by our findings in the CAIA model. While these effects were not found to be statistically significant, this is attributed to the lack of

response in one mouse within the treated group. In this model, 1179.4b did not reduce inflammation in paws. Surprisingly, the effects of both 1179.4b and MS-275 were opposing in a model of periodontal disease, with 1179.4b suppressing bone loss despite having no effect on inflammation and MS-275 reducing inflammation but having no effect on bone loss (28). Given the similarities between periodontitis and RA and the relationship between the two pathologies (54-58), it was expected that 1179.4b would also be able to suppress bone loss in RA. However, this lack of effect is most likely due to differences in HDACs expressed and active in the two diseases. 1179.4b also resulted in greater bone loss compared to the oil treated mice, however this was not found to be significant. Several studies point to the importance of HDAC 1 in RA with increased expression in disease (26-27) and hence its suppression will inhibit inflammation and bone resorption. Although the Class I selective HDACi MS-275 targets HDAC 1 (IC_{50}=181 nM), it also targets to a much lesser extent HDAC 2 (IC_{50}=1155 nM) and HDAC 3 (IC_{50}=231 1nM) (32). Overall, the importance of targeting HDAC 1 in RA inferred from this current study is also consistent with a report showing that MS-275 has greater anti-arthritic activity than the broader acting HDACi SAHA (19).

6.6 Conclusion

These results support the importance of HDAC 1 in the pathogenesis of RA. The HDACi, NW-21, designed to target HDAC 1 (Class I) over HDAC 6 (Class II) was able to suppress osteoclast resorption and inflammation *in vitro* and *in vivo*. Some preliminary mechanistic analysis has associated these properties with suppression of the expression of MCP-1 and possibly MIP-1α. These chemokines are important in both inflammation and osteoclast mediated bone resorption. In addition, the inhibitory effects of HDACi NW-21 on osteoclasts occur upstream of NFATc1 expression and are likely to be connected to suppression of TRAF6 and/or MCP-1 that is elevated soon after RANKL/RANK

interaction. Overall, the results support the development of inhibitors of HDAC 1, such as NW-21, for treating RA as they have the capacity to simultaneously target both inflammation and bone resorption.

6.7 References

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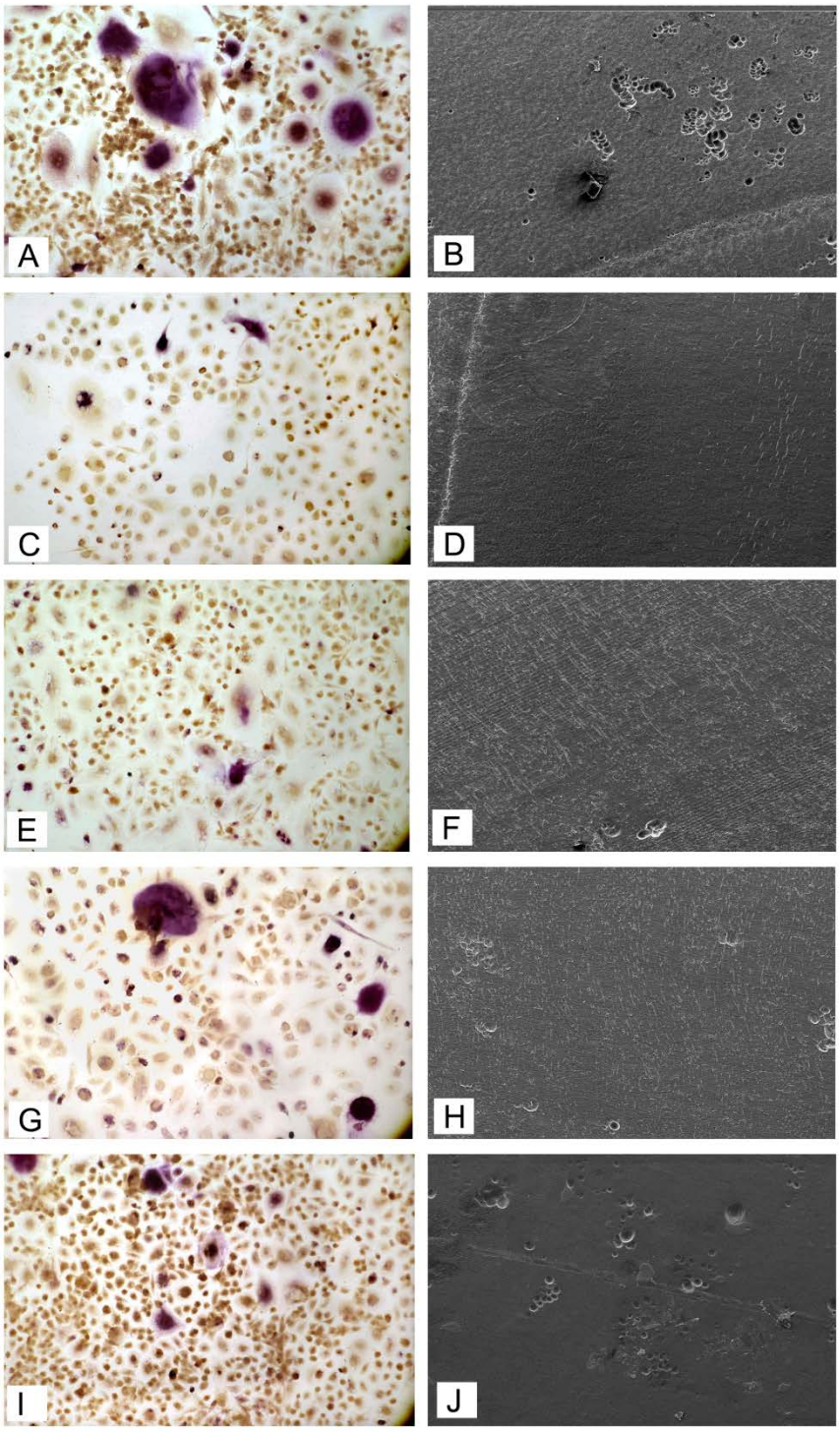


Figure 6.1. Representative TRAP stained sections (10x mag) and SEM images of dentine (150x mag). (A, B): Control (no treatment 0.01% DMSO).NW21 at (C, D): 20 nM; (E, F): 4nM; (G, H): 0.8nM; and (I, J): 0.16nM.

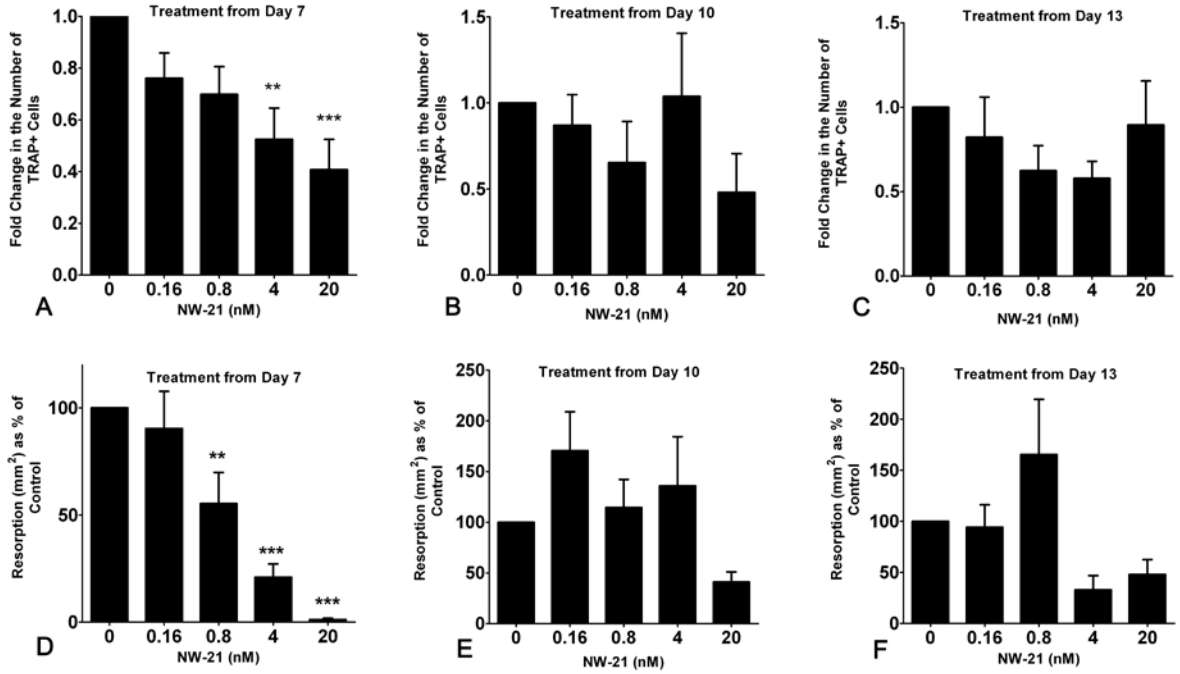


Figure 6.2. Fold change in the number of TRAP positive multinucleated (>3 nuclei) cells relative to control (0.01% DMSO). (A) NW-21 treatment from day 7 (n=10) (B) NW-21 treatment from day 10 (n=4) (C) NW-21 treatment from day 13 (n=4).
Percentage area of resorption expressed as a percentage of the control area (0.01% DMSO). (D) NW-21 treatment from day 7 (n=10) (E) NW-21 treatment from day 10 (n=4) (F) NW-21 treatment from day 13 (n=4).
Bars represent mean±SEM. ** $p<0.01$ and *** $p<0.001$ compared to control.

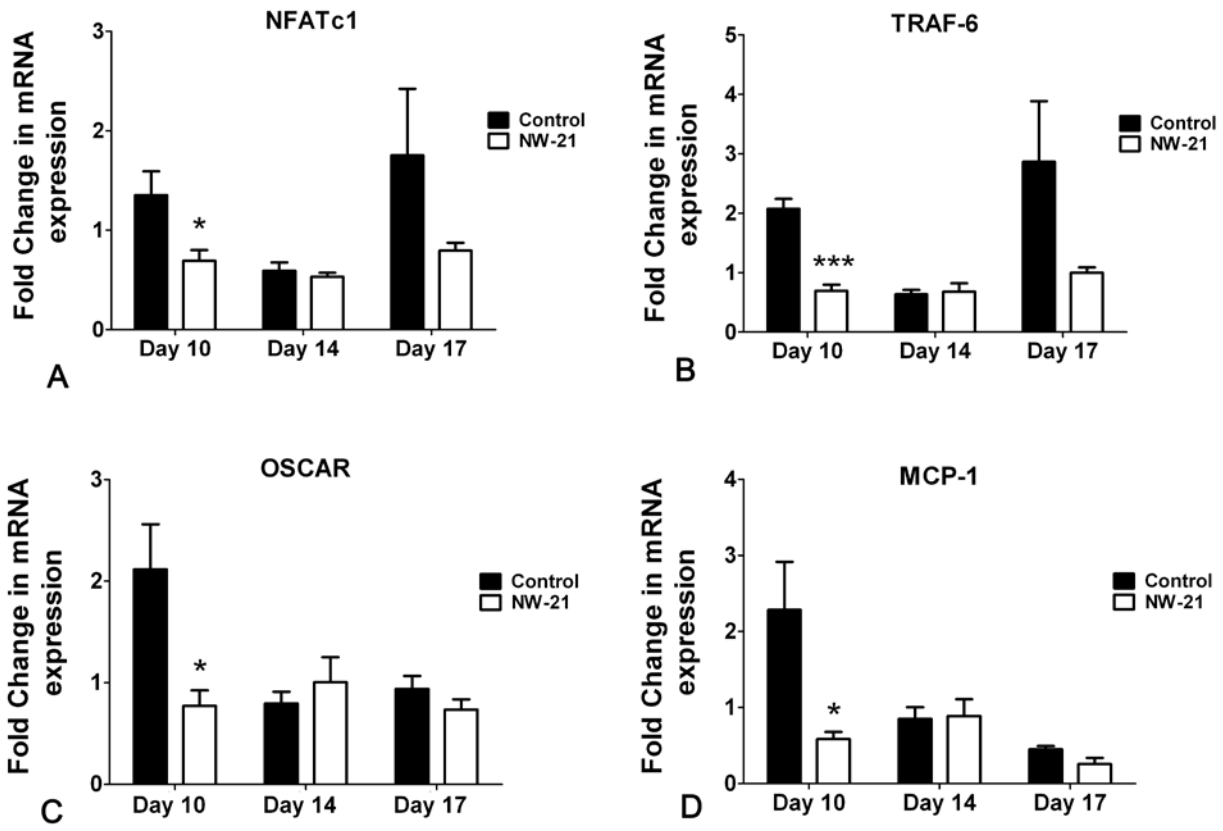


Figure 6.3. Fold change in the relative mRNA expression assessed using real time PCR analysis. Relative to endogenous gene hARP. Fold change at days 10, 14 and 17 relative to day 7. n=3 healthy donors.
(A) NFATc1 (B) TRAF-6 (C) OSCAR (D) MCP-1. Bars represent mean±SEM. * $p<0.05$ *** $p<0.001$ compared to control (0.01% DMSO).

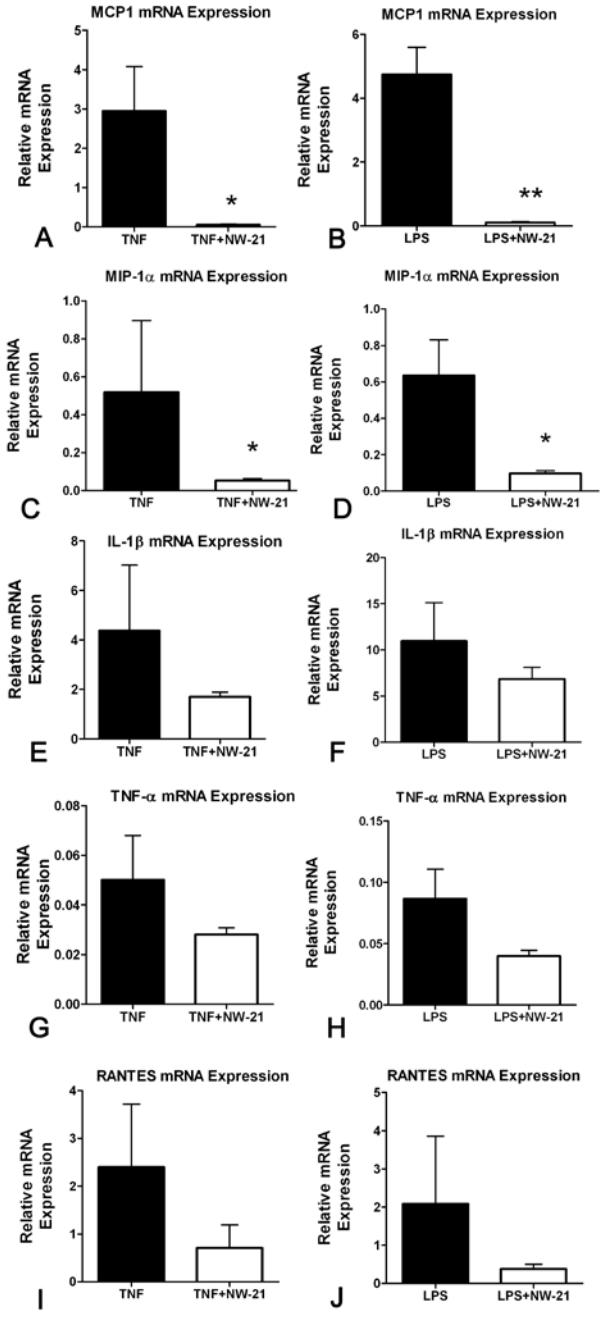


Figure 6.4. Relative mRNA expression of chemokines and cytokines by TNF-α or *E.coli* LPS stimulated monocytes for 24 hours. Cells treated with and without NW-21 at 20nM for 24 h. n=4 healthy donors.
TNF-α stimulated (A) MCP-1 (C) MIP-1α (E) IL-1β (G) TNF-α (I) RANTES
LPS stimulated (B) MCP-1 (D) MIP-1α (F) IL-1β (H) TNF-α (J) RANTES. Bars represent mean±SEM. * $p<0.05$ compared to control (0.01% DMSO).

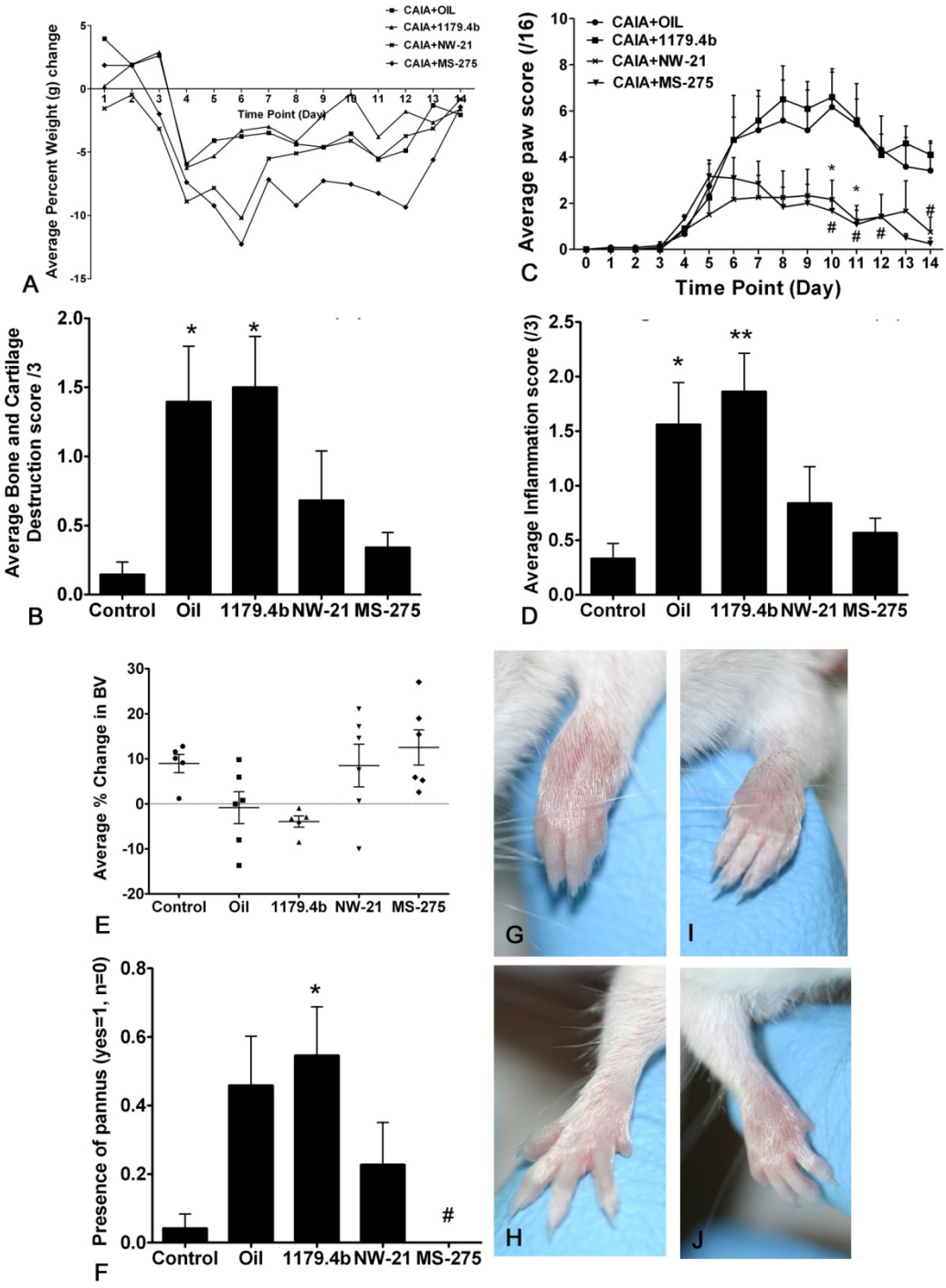


Figure 6.5. Effects of HDACi on inflammation and bone loss. A) Average change in weight (g) relative to day 0. B) Average Bone and Cartilage destruction score of H&E stained sections total score /3 C) Average paw scores out of a total of 16. * $p<0.05$ NW-21 compared to oil. # $p<0.05$ MS-275 compared to oil. D) Average inflammation score of H&E stained sections out of a total of 3. E) Average percentage change in bone volume from scan 1 to scan 3 (day 14) using CT scanning. F) Presence of pannus on H&E stained sections.

Controls n=6, CAIA+Oil n=6, CAIA+1179.4b n=5, CAIA+NW-21 n=6, CAIA+MS-275 n=6.

Representative macroscopic images of the front paws (G-J) G) CAIA +Oil H) CAIA+NW-21 I) CAIA+1179.4b J) CAIA+MS-275.

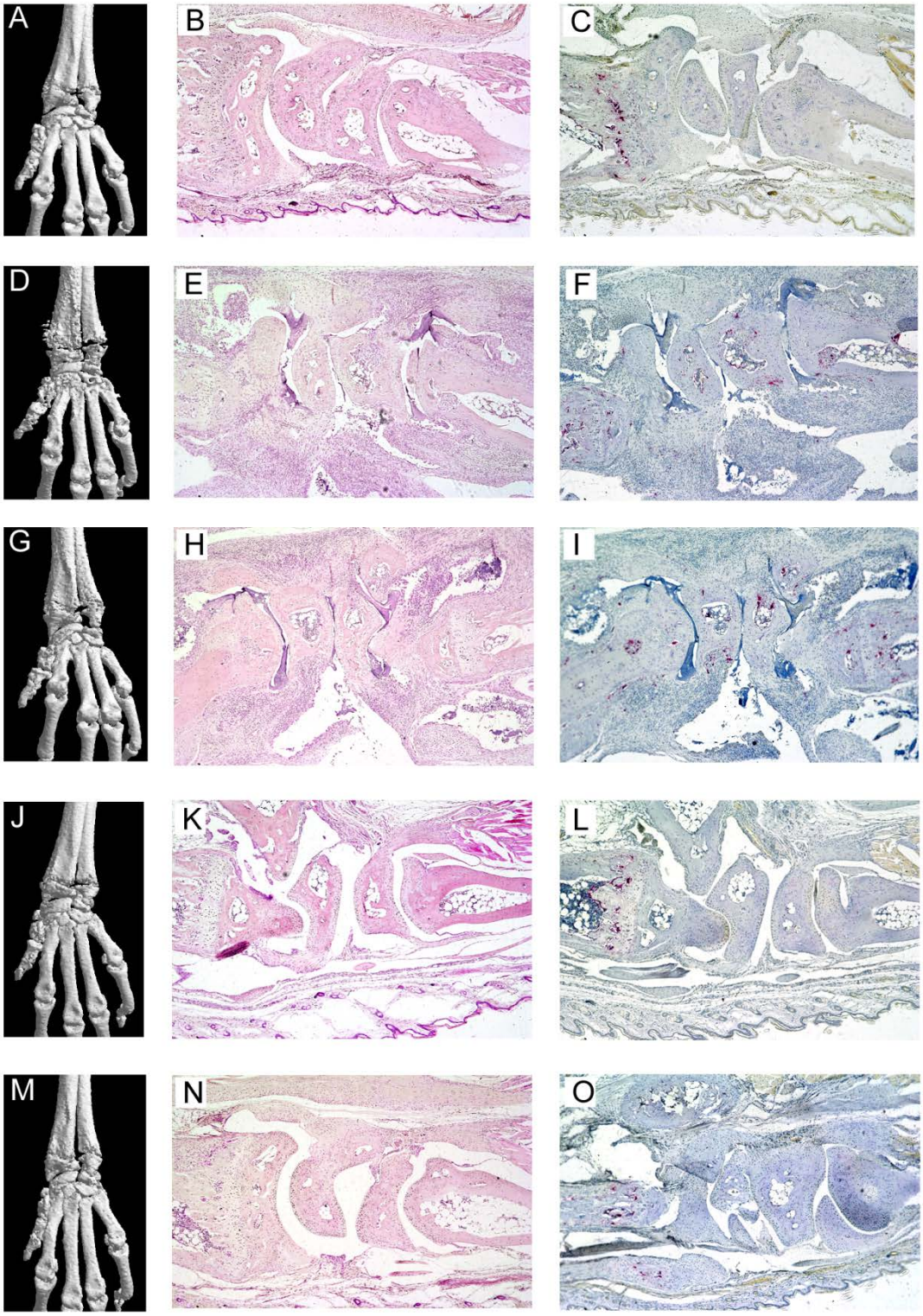
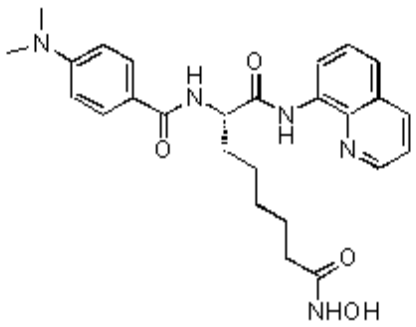


Figure 6.6. Representative images of the front left-hand side paws. 3D models created from CT scans conducted at day 14 (final day) using SkyScan program ANT (A, D, G, J, M).
H&E stained sections of the corresponding radiocarpal joint imaged at 4x magnification. (B, E, H, K, N). TRAP stained images of the corresponding radiocarpal joints at 4x magnification (C, F, I, L, O).
(A) (B) (C) Control mouse, (D) (E) (F) CAIA+oil, (G) (H) (I) CAIA+1179.4b at 5 mg/kg/day, (J) (K) (L) CAIA+NW-21 at 5mg/kg/day, (M) (N) (O) CAIA+MS-275 at 10mg/kg/day.



Supplementary Figure 6.S1. Chemical structure of HDACi NW-21 designed to target HDAC 1. Developed at the Institute of Molecular Bioscience at the University of Queensland. Chemical formula: $C_{26}H_{31}N_5O_4$.Molecular Weight: 477.56

Chapter 7. Discussion

7.1 Discussion

Traditionally developed as anticancer agents, HDACi are now being studied for their ability to treat a range of chronic inflammatory diseases. The majority of research into these drugs has involved broad acting HDACi that target several isoforms or classes of HDACs. Isoform specific inhibitors are beginning to be developed and can be used to help elucidate the roles HDACs play in disease in order to improve treatment efficacy and reduce side effects (12) (as reviewed in (7)). The particular focus of this thesis involved identifying the role(s) of individual HDACs in both the bone loss and inflammatory processes of two similar chronic inflammatory diseases, periodontitis (PD) and rheumatoid arthritis (RA). The two hypotheses that defined this body of work are: *Pre-existing periodontitis resulting in more severe arthritis in a mouse model. Histone Deacetylase Inhibitors (HDACi) will suppress both inflammation and bone loss in periodontitis and inflammatory arthritis.* These hypotheses were confirmed by the results of the study.

Bone loss as a consequence of increased osteoclast numbers and activity occurs in both PD and RA (2, 5). Previous studies have demonstrated that broad acting HDACi can suppress bone resorption in murine cells or cell lines *in vitro* (49, 54, 63). In order to elucidate important HDACs involved in human osteoclast differentiation the effects of a range of inhibitors known to target different HDACs were compared using an *in vitro* osteoclast assay. The results (Chapter 3) include findings demonstrating a novel HDACi, 1179.4b that targets both Class I and II HDACs, suppressed human osteoclast differentiation and activity. 1179.4b was compared with another previously studied broad acting inhibitor, SAHA, along with a more specific HDAC Class I inhibitor, MS-275, and a novel Class II inhibitor, 2664.12 (4). SAHA has been previously shown to inhibit osteoclastogenesis via

suppression of NF-κB (63) and in this current study, although suppressing osteoclast activity, was less potent than 1179.4b (1179.4b IC_{50}<0.16 nM, SAHA IC_{50}=12 nM). These moderate effects are consistent with other studies using SAHA (41). MS-275 is a Class I selective inhibitor reported to target HDAC 1, and to a lesser extent targets HDACs 2, 3 and 8 (19). In a human *in vitro* assay, MS-275 only suppressed human osteoclast formation and activity at the highest concentration (100 nM) tested. This is consistent with it being less effective than 1179.4b (MS-275 IC_{50}=54.4 nM). Interestingly, MS-275 in combination with 2664.12 (a novel inhibitor designed to target Class II HDACs) resulted in suppression at similar concentrations to 1179.4b treatment. The synergetic effect of MS-275 and 2664.12 (IC_{50}=0.35 nM) suggests the importance of both HDAC classes in osteoclast differentiation and activity. In contrast to this observation, results with a novel HDACi NW-21, shown to be most active against HDAC 1 and to a lesser extent HDAC 2 and 3, demonstrated a concentration dependent reduction in both TRAP positive multinucleated cell formation and pit resorption activity (NW-21 IC_{50}=1.2 nM) (Chapter 6)**.** These results suggest there could be redundancy of individual HDACs required for osteoclast differentiation and activity. The potency of HDACi may be dependent upon what combination of HDACs they target in human osteoclasts.

In order to understand the important HDACs involved in osteoclastogenesis, the expression of HDACs 1-10 throughout human osteoclast formation was assessed *in vitro* by real time PCR (4). mRNA expression of HDACs in both classes (Class I HDAC 8 and Class II HDAC 5) were found to be markedly up regulated throughout differentiation and significantly increased during the later stages of development. Evidence for the importance of these HDACs in bone is supported by studies showing that over expression of HDAC 5 reduced RANKL-mediated acetylation of key osteoclast transcription factor NFATc1 (33). Furthermore, HDAC 5 has been identified to affect bone mineral density in a genome wide

association study (56). In regard to other aspects of bone metabolism, HDAC 5 has been shown to be important in osteoblast bone formation with a novel micro RNA targeting HDAC 5 inducing degradation of the transcription factor Runx-2, a promoter of osteoblast differentiation (24). This would suggest that suppression of HDAC 5 with a selective HDACi would improve the balance between bone resorption and formation in pathological states such as RA and PD. To date, there are no reports on the role of HDAC 8 in bone metabolism, although germ line deletion of HDAC 8 has revealed an essential role for this HDAC in skull formation (20). Real time PCR analysis did not identify a significant up regulation of HDAC 1 during osteoclastogenesis (Chapter 3). This raises the question of how NW-21, which was designed to target HDAC 1, was able to suppress osteoclast activity. It could be due to the relative potency of the inhibitor with effects observed at relatively low concentrations (0.8 nM) and suggests that HDAC 1 activity, although not elevated at the mRNA level, may still be required for osteoclastogenesis. The observation that MS-275 could suppress osteoclastogenesis at relatively high concentrations supports this contention (Chapter 3). Other Class I HDACs including HDAC 2 and 3 could also be important in the differentiation process. A recent study has also revealed that suppression of HDAC 7 accelerated osteoclast differentiation and inhibition of HDAC 3 resulted in suppression of osteoclast differentiation (52). These findings and our results suggest that several HDACs regulate osteoclastogenesis and further studies are needed to elucidate the exact roles the various HDACs, in particular 5 and 8, play in this process. Studies using isoform selective inhibitors rather than only assessing mRNA expression will be important to determine which HDACs are important. In assessing the appropriate HDACs to target with therapy it is important to consider the species of the cells or tissues studied as there may be differences between humans and animal models.

A key finding of the *in vitro* study described in Chapter 3 (4) was that 1179.4b reduced the mRNA expression of key osteoclast genes necessary for differentiation. Importantly, TRAF-6, normally activated early following RANKL/RANK interaction (11), was found to be down regulated at day 10. NFATc1, a crucial transcription factor for osteoclast formation and activity (66), was also down regulated at day 17 (final day of culture) at both the gene and protein level. NFATc1 is known to induce expression of key osteoclast genes, such as, TRAP, integrin β3, OSCAR, Cathepsin K and CTR, during the terminal stages of osteoclastogenesis (45, 46, 62). 1179.4b also resulted in a significant decrease in OSCAR expression during the later stages which was not surprising given NFATc1 directly induces OSCAR (35).

Treatment with HDACi NW-21, which targets HDAC 1 along with HDAC 2 and 3 (Chapter 6), also resulted in a significant reduction in the mRNA expression of both TRAF-6 and NFATc1 at day 10. Given that HDAC 1 has a nuclear localization sequence but lacks a nuclear export signal (70), it is likely that the reduction in cytoplasmic TRAF-6 was due to its direct effect on transcription. NFATc1 was also reduced at day 17, similar to that induced by 1179.4b (Chapter 3) although not statistically significant.

Consistent with a reduction in TRAF-6 being a key effect, 1179.4b treatment commenced on day 13 did not affect formation of pre-osteoclasts/osteoclasts or their activity. Similarly, NW-21 treatment commencing on either day 10 or day 13 did not reduce osteoclast formation or activity. However, in contrast to 1179.4b, NW-21 significantly suppressed NFATc1 at day 10 (Chapter 6). NFATc1 has the ability to auto amplify its own expression by binding to the NFATc1 promoter (66) resulting in a marked elevation in NFATc1 at the terminal stages of osteoclast formation. The lack of effect with NW-21 at day 13 and later could be related, at least in part, to the activation of NFATc1 occurring before day 13.

Results with NW-21 and 1179.4b confirm that a variety of HDACs are likely to be involved in regulating osteoclast formation and activity. Treatment with both HDACi confirmed the importance of TRAF-6 and indicate that the other effects, such as NFATc1 inhibition, occur downstream of TRAF-6 suppression. NW-21 also reduced the initial NFATc1 activation that was not observed with the broad acting HDACi 1179.4b. This indicates that there are a number of key pathways that specific HDACs can regulate the differentiation process. HDACi have been reported to demonstrate a range of actions in osteoclasts, including targeting TRAF-6 (4), c-fos (32), NF-κB (which leads to proteosomal degradation of the NFκB – IκB complex via the ubiquination pathways), transcription factor NF-κB (63) and transcription factor NFATc1 (4, 49) (summarised in Figure 7.1). In a previous study, broad acting HDACi TSA was shown to suppress osteoclasts by inducing expression of IFN-β (49), which is known to inhibit osteoclastogenesis by interfering with the RANKL mediated induction of c-fos signalling (67). Interestingly this was not replicated, with 1179.4b treatment in the human osteoclast assay (Chapter 3) but could be related to the fact that human cells were used in the experiments presented in this thesis.

Overall the results obtained using the human osteoclast assay demonstrates the importance of HDACi mediated suppression of TRAF-6 and possibly NFATc1. The HDACs involved in the activation of these two important intercellular mediators are still not known.

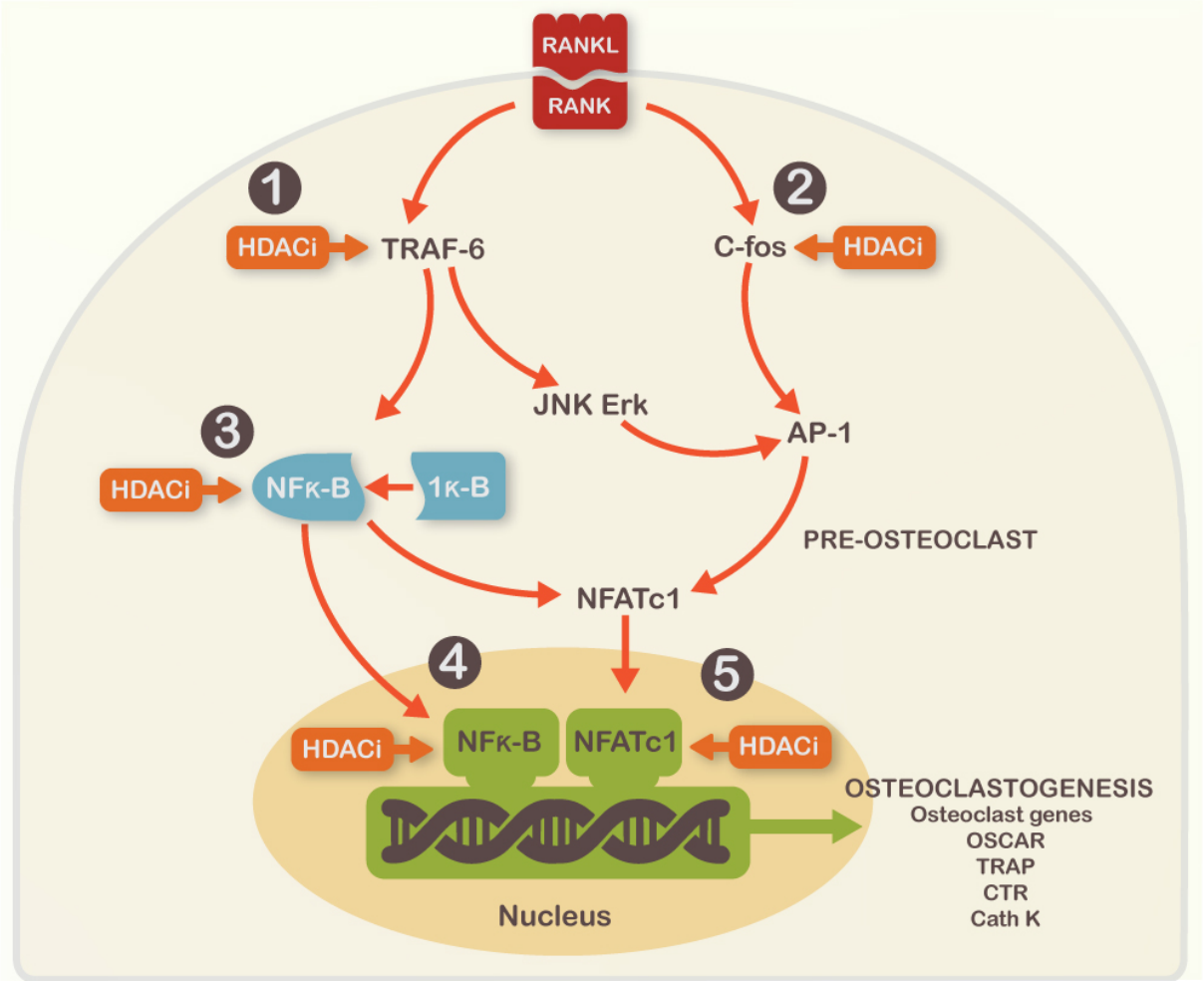


Figure 7.1. Actions of histone deacetylase inhibitors (HDACi) on RANKL-activated bone resorption by osteoclasts (7).
1) TRAF-6 (4), 2) c-fos (32), 3) NF-κB, which leads to proteosomal degradation of the NFκB – IκB complex via the ubiquination pathways, 4) transcription factor NF-κB (63) and 5) transcription factor NFATc1 (4, 49).

Based on the promising *in vitro* effects of HDACi 1179.4b, studies were carried out to assess its effects on bone loss in an animal model of human disease (Chapter 4) (6). It was hypothesised that 1179.4b would reduce alveolar bone loss as a result of suppressed osteoclast activity in a mouse model of periodontitis. It is important to note that treatment was commenced once inflammation had been established to mimic the clinical situation. 1179.4b treatment was compared to Class I selective HDACi MS-275. Consistent with the *in vitro* findings, 1179.4b was markedly more effective with significant suppression of alveolar bone loss observed. An interesting observation was that 1179.4b reduced bone loss despite having no effect on inflammation, indicating a direct effect on the bone

metabolism. The exact mechanism behind this, however, was not clear although it is consistent with the earlier *in vitro* studies showing 1179.4b inhibited expression of osteoclast genes TRAF-6 and NFATc1 during differentiation (Chapter 3).

The study described in Chapter 4 was the first to demonstrate the potential of HDACi as a treatment for periodontal bone loss. The majority of current treatments including mechanical debridement, antibiotics and anti-inflammatory drugs may effectively reduce the infection and inflammation but have limited effects on the bone (2). Accordingly, the results from this mouse model suggest that HDACi, such as 1179.4b, have the potential to be used as an adjunct treatment for periodontitis to directly target the bone loss.

The expression and activity of HDACs and inhibitors however still remains to be a complex issue, with further research necessary to understand their overall effects on all cells involved in bone metabolism including osteoblasts and osteocytes. Studies to date suggest, regulation of HDACs can increase osteoblast proliferation, expression of genes associated with bone formation mineralization, alkaline phosphatase and the transcriptional activity of RUNX-2 (27, 60, 61). For example, HDAC 1 activity declines during osteoblast maturation and hence suppression of HDAC 1 stimulates osteoblast maturation (38).

In both PD and RA, there is a clear link between inflammation and bone loss (44, 55, 64, 65) with elevated expression of RANKL by inflammatory cells including fibroblasts and lymphocytes observed in both diseases (9, 10, 21). Despite this, the majority of current treatments, such as NSAIDs used to suppress the inflammation, have a varied effect on the bone (13, 22), suggesting that a link to inflammation may not be so clear.

In order to determine which HDAC might be important in human PD, HDAC expression in inflamed gingiva tissues from PD patients was assessed in Chapter 5. The results showed Class I HDACs 1 and 8, and Class II HDACs 5 and 9 to be significantly up regulated in chronic PD tissues. This was consistent with the observation that HDAC 5 and 8 were elevated during osteoclast development *in vitro* (Chapter 3)**.** Immunohistochemistry was used to assess protein expression of these enzymes in inflamed periodontitis gingival tissues. In both the PD and non-PD groups, HDAC 5 and HDAC 8 were expressed by cells lining blood vessels. While there was no overall difference in the numbers of positively stained cells there was a marked increase in the intensity of staining in the blood vessel lining cells in the chronic PD samples. These results suggest that HDAC 5 and 8 may be important in regulating the immune cell adhesion and hence migration into the gingival tissues. Previous studies have shown that the HDACi TSA reduced monocyte adhesion to the endothelium via reducing expression of vascular cell adhesion molecule-1 (VCAM-1) (26). HDAC 5 has also been shown to play a role in regulating immune cell adhesion (69). The high expression of HDAC 5 and 8 proteins by the blood vessel lining cells was consistent with the high mRNA expression seen in these tissues.

A limitation of the study was that only soft tissue samples could be ethically obtained from human subjects and it would have been more informative to assess the expression of HDAC 5 and 8 where osteoclasts are more active, at the surface and in alveolar bone. The findings noted in the inflamed periodontal soft tissues indicate that these HDACs may be important in regulating the inflammation and as evidenced by the *in vitro* studies they also regulate bone loss. Other HDACs highly expressed in human PD were HDAC 1 and 9; hence these may also play a significant role in the inflammation process. The observed reduction in inflammation with MS-275 which targets HDAC 1 in the PD mouse model is consistent with this but further studies are required to elucidate this point.

Recent studies have shown HDAC 1 is highly expressed in synovial fibroblasts from patients with RA compared to those from OA (48). These high levels of HDAC 1 were also found to correlate with TNF-α suggesting a link between HDAC 1 and inflammation (15, 48). A number of studies have shown that HDACi can reduce disease activity in animal models of inflammatory arthritis (29, 41, 50). The majority of HDACi that have been tested were broad acting inhibitors, such as TSA and SAHA (41, 50). Although showing potential, to date only one HDACi has progressed to clinical trials (14, 68), with the broad acting HDACi, Givinostat, used to treat juvenile arthritis. Interestingly, Givinostat treatment resulted in improvements in mobility and wellbeing along with reduced numbers of active arthritic joints (68).

Given the high HDAC 1 expression in RA (25, 31), studies in Chapter 5 were conducted to determine the effects of the novel HDACi designed to target HDAC 1 (NW-21) on inflammation *in vitro* and in a collagen antibody induced arthritis model (CAIA). To investigate the anti-inflammatory effects, PBMCs were stimulated with either TNF-α or *E. coli* LPS and treated with NW-21 for 24 hours (Chapter 6)**.** TNF-α was chosen as it is a key inflammatory cytokine involved in the pathogenesis of both PD (18) and RA (57). The importance of HDAC 1 and possibly other Class I HDACs (2 and 3) in inflammation was evident as treatment of both TNF-α and *E. coli* LPS stimulated PBMCs with HDACi NW-21 markedly reduced expression of inflammatory chemokines MCP-1 and MIP-1α.

MCP-1 is reported to recruit monocytes, memory T cells, and dendritic cells to sites of inflammation and has been shown to play a role in the pathogenesis of both PD and RA (16, 36, 53). In PD patients, MCP-1 has been found to be significantly higher at both the mRNA and protein level (16, 53). MCP-1 has also been reported to be expressed by endothelial cells in inflamed gingival tissues (71). In the context of RA, MCP-1 has been

shown to be produced by synovial cells, with synovial macrophages from RA patients constitutively expressing MCP-1 (36). MCP-1 levels correlate with disease activity and treatment with anti-TNF drug Etanercept significantly reduced the serum levels (30).

Immune related molecules, such as MCP-1, are also expressed by mature osteoclasts (34) and osteoblasts in PD (28) and RA (42, 43) suggesting a linkage between inflammation and the bone. MCP-1 in combination with RANTES was shown to promote human osteoclastic cell formation in the absence of RANKL, however these cells were unable to resorb bone (34). In this human osteoclast *in vitro* assay, expression of MCP-1 peaked at day 10, consistent with its reported role in osteoclast formation (59) (Chapter 6). Overall, MCP-1 appears to be a key osteoclastogenic factor suppressed by HDACi that target HDAC 1. HDAC regulation of inflammation may be mediated by other inflammatory factors (17, 39, 40). For example, the broad acting HDACi, Givinostat, reduced TNF-α, IL-1α and IL-1β and IFN-γ expression in LPS stimulated PBMCs (39, 40). Macrophage inflammatory protein 1α (MIP-1α), is expressed by macrophages and has also been shown to induce osteoclast formation (59). Given MIP-1α also has a role in osteoclastogenesis this provides further evidence for a connection between bone metabolism and inflammation. MIP-1α was suppressed by NW-21 treatment (Chapter 6) and is reported to be highly expressed in synovial fluid from patients with RA (37).

NW-21 reduced inflammation *in vitro* as well as *in vivo* in a murine model of inflammatory arthritis. In the CAIA model, NW-21 was compared to broad acting HDACi 1179.4b, previously shown to reduce bone loss in the PD model (Chapter 4)**,** and MS-275. NW-21 treatment resulted in significant anti-arthritic effects with reductions in both inflammation and bone loss. The broad acting HDACi, 1179.4b, administered at doses that inhibited bone loss in the PD model unexpectedly had no significant effect on either

inflammation or bone loss in the arthritis model (CAIA). This may be due to differences in the HDAC expression in the different disease models. This is further supported by the results in Chapter 4 showing high expression of HDAC of both classes in the human PD tissues. The lack of effect with 1179.4b in the arthritis model could be explained by its inability to strongly inhibit HDAC 1. On the other hand, MS-275, which also strongly targets HDAC 1, had little effect on the PD model (Chapter 4) but was effective in the arthritis model. This is consistent with the previously reported anti-arthritic effects of MS-275 (41). Interestingly despite having little effect on bone loss, MS-275 was able to reduce inflammation in the PD model. The ability of MS-275 to inhibit inflammation in this model is consistent with its ability to treat experimental autoimmune prostatitis (72) and experimental autoimmune neuritis (72). The positive effects with NW-21 and MS-275, both targeting HDAC 1, also highlight the importance of HDAC 1 in the pathogenesis of inflammatory disease and arthritis in particular. This is in line with the observed high HDAC 1 expression in human patients with RA (23, 31). Both MS-275 and NW-21 may target other Class I HDACs (including HDAC 2 and 3) making it possible that these may also be important.

The relationship between periodontitis and inflammatory arthritis was explored in Chapter 2 (8). Obvious similarities between the two include an exuberant chronic inflammatory reaction with high levels of pro-inflammatory cytokines such as TNF-α and interleukins (3, 51). There is also associated destruction of the soft and hard tissues in both diseases. Previous studies have demonstrated an association between RA and PD with a higher incidence of alveolar bone loss and tooth loss found in patients with RA (1, 47, 48).

To investigate the relationship between RA and PD a mouse model which combined both the periodontitis and inflammatory arthritis was established. PD was induced for a period

of 44 days before arthritis induction using the collagen antibody induced arthritis method to mimic the clinical situation in which the PD is believed to precede the RA. Clinical investigations have suggested patients with severe RA are more likely to have advanced PD and vice versa (47, 48).

The results of this study (Chapter 2) revealed that mice with pre-existing PD developed more severe arthritis. Despite this increased inflammation, there was no significant difference in bone loss between mice with and without pre-existing PD. An interesting observation in this study was that mice with only PD showed signs of bone loss in the radiocarpal joints. There was also evidence of bone loss in the jaws of mice with arthritis alone. Taking into account the results in the previous chapters it seems that although there is a bi-directional relationship between the two diseases the mechanisms, at least in regard to HDAC regulation and activity, are different. The mechanisms behind the bone loss in both sites could be related to the increased systemic cytokine levels or could be as a result of protein citrullination induced by the inflammation (58). Further studies are required to elucidate the mechanisms and to determine if the bone loss seen at other sites is due to systemic osteoporosis or bone loss at the specific site. The overall results suggest that while these diseases are closely related in terms of their pathology, there are subtle differences in the pathogenic processes reflected in the differences in the key HDAC activities.

In summary, this thesis confirms there is a bidirectional relationship between PD and inflammatory arthritis (Chapter 2). However, it also demonstrates that regulation of these diseases is different, particularly in regard to the role and expression of individual HDAC. While the broad acting HDACi (1179.4b) suppressed bone loss in the PD mouse model (Chapter 4), it had little effect in the arthritis model (Chapter 6). This could be explained

by the high expression of both HDAC classes in human chronic PD gingival tissues (Chapter 5). In arthritis, compounds targeting Class I HDACs and HDAC 1 in particular were most effective (Chapter 6). It would have been interesting to investigate the effect of NW-21 in the PD model, however as this novel drug was only developed recently this study could not be included in this thesis. The results of the studies described in this thesis shed light on the mechanism of HDACi action in bone loss and inflammation as summarized in Figure 7.2. TRAF-6 was identified as being the key osteoclast activated early on which is regulated by HDACi *in vitro*. Suppression of inflammation occurred via reduction in chemokine levels and MCP-1 provides an important linkage between the inflammatory process and bone loss.

As more studies are carried out with specific HDACi and more are developed, the differences in the roles of various HDAC will be better understood.

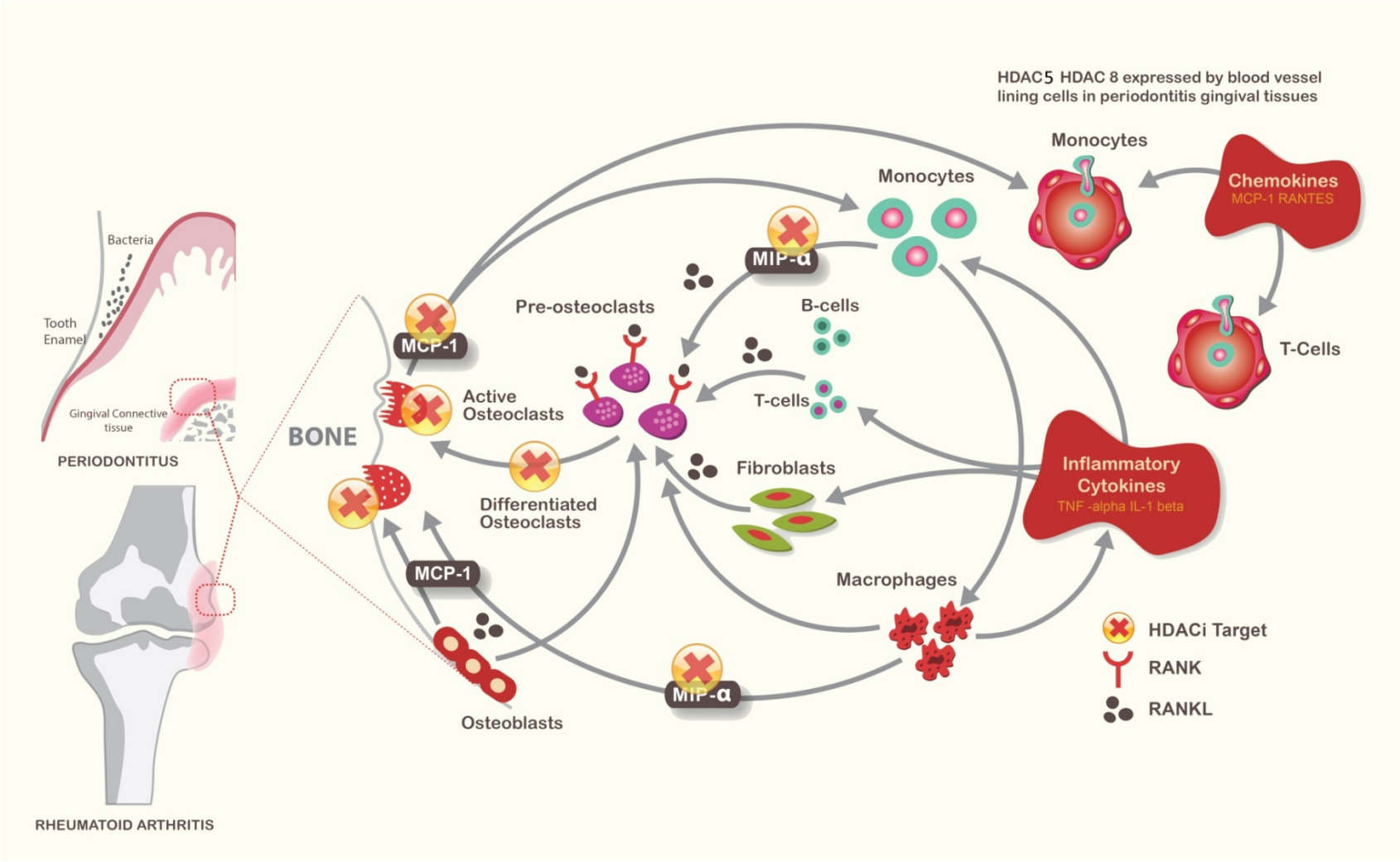


Figure 7.2. Mechanisms of inflammation and bone loss and HDACi targets in related diseases RA and PD. HDACi suppress chemokines MCP-1 and MIP-1α. They can also directly suppress osteoclasts by targeting the intercellular signalling pathway.

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Chapter 8. Conclusions and Future Directions

Overall the results of the studies described in this thesis show that HDACs play a key role in soft tissue inflammation and bone destruction in both PD and RA. An interesting finding was that targeting both HDAC classes is necessary to suppress alveolar bone destruction in PD, whereas, targeting Class I HDACs alone suppressed both inflammation and bone destruction in inflammatory arthritis. While this may be due to differences in the way the diseases develop it also highlights the difficulty in studying the way HDAC may regulate disease. Studies of HDAC regulation are complicated by the large number of enzymes and the lack of specificity of inhibitors available. This is further complicated by the pharmacology of HDACi with the specific enzymes targeted likely to be dependent on the drug concentration. In addition, inhibitors may target different HDACs in different cell types, tissues or disease states. Despite this complexity, isoform selective HDACi are proving useful in elucidating the role of individual HDACs in various diseases. For example, some important observations can be made from the studies described in this thesis. In arthritis, HDAC 1 and other Class I HDACs (HDAC 2 and 3) are likely to be important in tissue inflammation and bone loss. In PD, both HDAC classes appear important in the disease process with HDAC 1, 5, 8 and 9 up regulated at the mRNA level. In addition, HDACi such as 1179.4b, NW-21 and MS-275 have been shown to have the potential to treat inflammatory bone loss depending on the pathology.

A number of key future directions can be identified by the work presented in this thesis:

- More studies are necessary to assess HDAC activity during osteoclastogenesis to determine if this correlates with HDAC expression noted at the mRNA and protein levels.
- Appropriate acetylation assays need to be developed to determine which HDACs are suppressed by HDACi in a particular tissue or cell types.

- It would be interesting to determine which HDACs are expressed in patients with both PD and RA compared those with periodontitis or RA alone.
- The ability of HDACi, particularly 1179.4b and NW-21, to suppress PD and arthritis in combination using the novel model developed in this thesis should be determined.
- There is a need to determine if novel isoform selective inhibitors for HDAC 5 and 8 will suppress bone loss *in vitro* and *in vivo* models of disease.
- It is important to determine the effects of HDACi on bone metabolism more broadly by investigating their effects on other important bone cells such as osteoblasts and osteocytes.

The results of this thesis have demonstrated the significant potential of epigenetic therapies, in particular targeting HDACs. Even though our knowledge of HDACi is limited, the data presented here, and the work of others, demonstrates that the regulation of osteoclasts and/or inflammation by appropriate HDACi can be effective. The development of HDACi is proving to be a fast growing area of drug development with the exciting prospect that novel specific HDACi, administered alone or in combination, can be used to treat a range of human diseases, not just PD and arthritis as studied here. However, before these novel HDACi can be used for treatment further studies of disease pathology are needed to identify the best HDAC or combination of HDACs to target in human disease.

Appendices Included As Part of the Thesis

Appendix 1: Histone deacetylase inhibitors as suppressors of bone destruction in inflammatory diseases.
MD Cantley, PM Bartold, DP Fairlie, KD Rainsford, DR Haynes
J Pharmacy and Pharmacology 2012 Jun;64(6):763-74

Appendix 2: Epigenetic regulation of inflammation: progressing from broad acting histone deacetylase (HDAC) inhibitors to targeting specific HDACs.
MD Cantley, DR Haynes
Inflammopharmacology 2013;21(4):301-7.

Appendix 3: List of other Publications, Awards and Presentations during Candidature

Appendix 1. Histone Deacetylase Inhibitors as Suppressors of Bone Destruction in Inflammatory Diseases

MD Cantley[1], PM Bartold[2], DP Fairlie[3], KD Rainsford[4], DR Haynes[1]

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Journal of Pharmacy and Pharmacology 2012 Jun; 64(6):763-74

STATEMENT OF AUTHORSHIP

Histone Deacetylase Inhibitors as Suppressors of Bone Destruction in Inflammatory Diseases.

Journal of Pharmacy and Pharmacology, 2012 Jun; 64(6):763-74

Cantley M.D (candidate)

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Extensive research of literature and wrote review paper

Certification that the statement of contribution is accurate

Signed ....*Date*.....7/1/13.....

STATEMENT OF AUTHORSHIP

Histone Deacetylase Inhibitors as Suppressors of Bone Destruction in Inflammatory Diseases.

Journal of Pharmacy and Pharmacology, 2012 Jun; 64(6):763-74

Bartold P.M

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Review and corrections of manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed ....*Date* 22.1.13

STATEMENT OF AUTHORSHIP

Histone Deacetylase Inhibitors as Suppressors of Bone Destruction in Inflammatory Diseases.

Journal of Pharmacy and Pharmacology, 2012 Jun;64(6):763-74

Fairlie D.P

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Review and corrections of manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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STATEMENT OF AUTHORSHIP

Histone Deacetylase Inhibitors as Suppressors of Bone Destruction in Inflammatory Diseases.

Journal of Pharmacy and Pharmacology, 2012 Jun; 64(6):763-74

Rainsford K.D

Statement of contribution (in terms of the conceptualization of the work, its realization and its documentation)

Helped with initial drafting and guidance/advice re: journal submission

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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed ..... *Date* 14/1/13

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Review

Histone deacetylase inhibitors as suppressors of bone destruction in inflammatory diseases

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Keywords
arthritic diseases; bone resorption; histone deacetylase (HDAC) inhibitors; osteoclasts; periodontitis

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Abstract

Objectives Despite progress in developing many new anti-inflammatory treatments in the last decade, there has been little progress in finding treatments for bone loss associated with inflammatory diseases, such as rheumatoid arthritis and periodontitis. For instance, treatment of rheumatic diseases with anti-tumour necrosis factor-alpha agents has been largely successful in reducing inflammation, but there have been varying reports regarding its effectiveness at inhibiting bone loss. In addition, there is often a delay in finding the appropriate anti-inflammatory therapy for individual patients, and some therapies, such as disease modifying drugs, take time to have an effect. In order to protect the bone, adjunct therapies targeting bone resorption are being developed. This review focuses on new treatments based on using histone deacetylase inhibitors (HDACi) to suppress bone loss in these chronic inflammatory diseases.

Key findings A number of selected HDACi have been shown to suppress bone resorption by osteoclasts in vitro and in animal models of chronic inflammatory diseases. Recent reports indicate that these small molecules, which can be administered orally, could protect the bone and might be used in combination with current anti-inflammatory treatments.

Summary HDACi do have potential to suppress bone destruction in chronic inflammatory diseases including periodontitis and rheumatoid arthritis.

Introduction

Chronic inflammatory diseases, such as periodontitis (PD) and rheumatoid arthritis (RA), are commonly associated with destruction of the bone and connective tissues due to stimulation of tissue-damaging cells and enzymes by inflammatory mediators.[1–4] Progressive joint destruction is a hallmark of RA and ultimately results in a painful debilitating condition,[2,5] while alveolar bone loss associated with PD can eventually result in sufferers losing their teeth. Despite the progress in recent years, with many effective pharmacological therapies targeting the inflammatory processes, the control of bone destruction in these diseases is still a challenging problem.[1,6–9] This review will look at the currently available treatments for chronic inflammatory diseases, including PD and RA, with a focus on the potential of new treatments using histone deacetylase inhibitors (HDACi) to suppress bone loss in these chronic inflammatory diseases.

Osteoclasts in Chronic Inflammatory Diseases

Osteoclasts are large multinucleated cells derived from the haematopoietic lineage and are responsible for resorbing bone during both normal and pathological states.[10–12] Osteoclasts resorb the mineral component of bone via acid secretion and the organic component is degraded by secretion of cysteine proteinases. Under physiological states osteoclast play a critical role in maintaining bone mass by resorbing bone during the process of bone remodelling, following which new bone is laid down by the osteoblast cells. This normal balance becomes altered in diseased states resulting in excessive bone loss that cannot be compensated for as in the case of RA and PD.

Both RA and PD are characterized by an exuberant inflammatory response with production of large numbers of

pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukins (IL) 1, 4, 6, 11 and 17.[13,14] These cytokines stimulate the destruction of both soft and hard tissues of the joint in RA and the periodontium in PD.[6,13–15] Increased numbers of the bone-resorbing osteoclasts have been reported in both RA and PD in human studies and animal models of disease.[16–23] Osteoclasts erode bone, which reduces joint function in RA and can result in tooth loss in individuals with PD. The osteoclast has become an important target for controlling bone destruction in a variety of bone diseases with many new treatments targeting signalling processes during osteoclast formation.

The role of nuclear factor-κB (NF-κB) signalling in joint inflammation and cartilage destruction in RA and in bone loss in PD has been recently highlighted in a number of publications.[1,3,6,24,25] The up-regulation of receptor activator of NF-κB ligand (RANKL) in arthritic joints and periodontal diseases is a major feature in their pathogenesis.[6,10,26–28] RANKL is a membrane-bound member of the TNF-α family expressed by osteoblasts, fibroblasts and activated T cells.[10] A crucial step in osteoclast formation in both health and disease is the ligation of RANKL with its receptor RANK on osteoclast precursor cells resulting in activation of NF-κB, leading to development of osteoclasts that resorb bone.[10] A number of intracellular mediators are activated following RANKL and RANK interactions, which result in the formation of active multinucleated osteoclasts. RANKL has been found to form an important link between immunology and bone physiology with pro-inflammatory cytokines seen in RA and PD, such as TNF-α and IL 1, 4, 6, 11 and 17, known to stimulate the its production.[6,27,28] In view of the central role of RANKL in osteoclast formation and activation, therapies designed to regulate RANKL are becoming increasingly popular. These potential anti-resorptive therapies could be administered early in the disease process to prevent continued destruction and maintain joint and tooth integrity. Anti-resorptive medications/agents could also be given in combination with current ant-inflammatory treatments to improve clinical outcomes.

Current Treatments for Inflammatory Diseases

Most of the current treatments available for RA and PD target the inflammatory process with some of the common targets shown in Figure 1. Despite being effective at inhibiting inflammation, most treatments have shown limited effects in halting the bone destruction. Many of the disease-modifying anti-rheumatic drugs (DMARDs) and newer biological agents (e.g. anti-TNF-α, anti-CD20) effectively control cytokines associated with T and B cell primed inflammatory reactions.[7,9] However, while often effectively reducing inflammation their ability to stop bone destruction appears to be limited, particularly during the early stages of treatment.[32–34] Anti-TNF-α and other anti-cytokine therapies have been found to reduce synovitis and indices of joint deterioration in RA and psoriatic arthritis, although their ability to immediately halt bone erosion is not noted in a large proportion of patients.[9,35–37] Moreover, in recent years there have been concerns about the development, albeit rarely, of serious adverse effects from anti-TNF-α therapies. Among these is a demyelinating reaction[38] and other effects[39,40] leading to activation or development of infections such as tuberculosis,[41–43] coccidiomycosis[44] and listeriosis.[45] Other disadvantages of the newer biologics include the high cost, a need for parental administration and also the fact that not all patients will respond.

The established DMARDs, such as methotrexate, leflunomide, sulfasalazine and cyclosporin, have been beneficial with radiological evidence of improvement in joint-space narrowing in RA and related conditions.[46–49] This improvement in symptoms is not immediate and not all patients will respond favourably to DMARD treatment, with a significant number having adverse side effects.[50] For this reason it can take a considerable amount of time for clinicians to develop an appropriate treatment regimen using DMARDs and during this time there is continuing destruction of the bone. DMARD treatment can also result in problems after long-term use causing many individuals to discontinue therapy due to adverse events.[51]

Corticosteroids (glucocorticoids) given intra-articularly have, over the years, shown mixed responses. Despite improvements in practice[52] the direct effect of these drugs on bone is essentially catabolic[9] and their administration is usually limited to a few weeks or months depending on the arthritic state.[52] The incidence of glucocorticoid-induced osteoporosis is dramatically increased with long-term use and is associated with increased risk of hip and vertebrae fractures.[53,54]

In general, non-steroidal anti-inflammatory drugs (NSAIDs) principally have anti-inflammatory actions in synovial inflammation while having little, if any, beneficial effects on bone destruction. There is even some evidence to suggest that selected NSAIDs may actually accelerate bone destruction.[55–57] Furthermore, the application of NSAIDs though useful in mild PD has not been particularly successful due to side effects.[58,59]

Bisphosphonates have been used clinically in the past to control bone destruction in diseases such as osteoporosis, tumour-induced osteolysis and arthritis. Despite having benefits of reducing generalized bone loss in osteoporosis their effectiveness in suppressing focal bone erosions specifically observed in RA is questionable.[60] Reports of osteonecrosis of the jaw in patients treated with high levels of bisphosphonates has also led to their use being questioned, particularly in relation to dental disease and treatments.[61,62]

Denosumab, formerly known as AMG 162, is a monoclonal antibody to RANKL and has recently been shown to

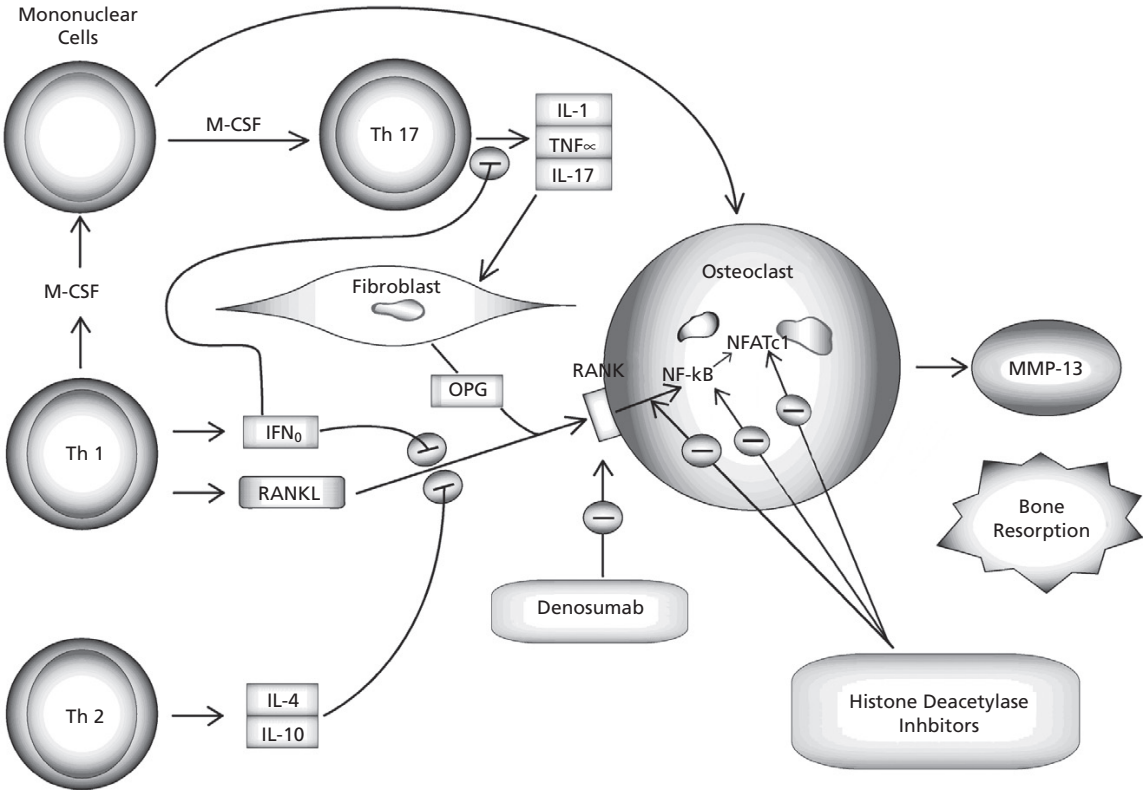

Figure 1 Cellular sites of action of T-cell specific cytokines and inhibitory effects of clinically available and potential agents affecting bone resorption. IFN, interferon; IL, Interleukin; M-CSF, macrophage colony stimulating factor; MMP, matrix metalloproteinases; NF-kB, nuclear factor-kB; OPG, osteoprotegerin; RANKL, receptor activated nuclear factor-kB (NFkB) ligand; Th, T helper cell; TNF-α, tumour necrosis factor-alpha.

reduce local and generalized bone loss in clinical trials of patients with RA.[63,64] In June 2010 the FDA approved denosumab for use in postmenopausal women at risk of osteoporosis.[65] Clinical studies demonstrated that two injections of denosumab per year, along with ongoing methotrexate treatment, resulted in suppression of structural damage in patients with RA without an increased rate of side effects.[66] However, there was no effect on RA disease activity, occurrences of RA flares and joint-space narrowing or significant effect on reducing cartilage erosion.[66] Denosumab also needs to be administered intravenously, making patient compliance difficult.

Novel Targets for Treating Bone Loss

Despite many advances being made in understanding the cellular and molecular processes of bone destruction in arthritic and periodontal diseases there are few safe and effective therapies available aside from those used for osteoporosis.[1,9,35] The research focus of potential treatments in many areas, and bone loss in particular, is now focused on small molecules with specific actions.[8] New small molecule compounds that target specific cytokine-mediated processes (e.g. RANK and its RANKL)[24] seem likely to prove more effective in specifically controlling bone destruction in a variety of diseases.[8] Small molecules have the benefit of being cheaper to manufacture, can be administered orally thereby enhancing patient compliance, and concentrations can be more easily monitored and correlated with disease in patients receiving treatment. One such new class of small molecules is histone deacetylase inhibitors (HDACi) that have potential for treating bone loss and possibly inflammation in PD and RA.

Histone Deacetylase Inhibitors

A novel approach for the treatment of localized bone loss in inflammatory diseases is via the epigenetic control of bone resorption. Epigenetic alterations are described as modifications of DNA without any changes to the base sequences with there being two major groups – DNA methylation and

histone acetylation – that modulate the access of transcription factors.[67] There has been much interest focusing on the gene-regulated events involving post-translational modification of the histone N-terminal acetylation and subsequent control of the intracellular signalling pathways that control production of inflammatory mediators and cell cycle activity.[68–70] The process of histone acetylation, which is key to regulating these gene-mediated events, is controlled by two key enzymes. Histone acetyl-transferase (HAT) enzymes are responsible for adding acetyl groups to lysine side chains in histones leading to exposure of DNA to transcription factors, resulting in gene expression. Histone deacetylases (HDAC) on the other hand are zinc enzymes that counteract this by removing acetyl groups from histones resulting in gene repression. There can also be deacetylation of non-histone signal transduction proteins that are important in various inflammatory events.[70]

Biological properties of histone deacetylase inhibitors

The inhibition of the activity of HDAC isoforms is considered to be a favourable therapeutic strategy and is being investigated to treat a wide variety of chronic inflammatory and malignant diseases.[71–76] Particular interest has centered on the roles of HDAC enzymes in controlling expression of matrix metalloproteinases (MMPs) and pro-inflammatory cytokines that mediate cartilage and bone destruction in RA.[71–76] The mechanism of HDACs control over MMPs and cytokines, while not completely understood yet, is thought to be through suppression of the NF-κB pathway with subsequent reduction in the production of pro-inflammatory cytokines, nitric oxide and other inflammogens,[72,73,75] as well as the production of TNF-α receptors.[75]

Histone deacetylase inhibitors (HDACi) have been found to have anti-inflammatory effects and suppress cancer cell growth by inhibiting differentiation of cells both *in vitro* and *in vivo*.[68,75,77–79] They are known to suppress the expression of several important genes, particularly inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interferon γ (IFN-γ),[75,31] the expression of cyclo-oxygenase-2,[78,79] cooperate with Fas-signalling to induce apoptosis[80] and down-regulate the expression of hypoxia-induced endothelial growth factor in rheumatoid synoviocytes.[81,82]

Expression and inhibition of specific histone deacetylases

Among the factors that could influence histone acetylation are variations in the activity of HAT and HDAC in diseased tissues.[83] Moreover, the existence of 11 human isoforms of the zinc-containing HDACs (HDAC 1–11) and a further 7 NAD^+-dependent enzymes (SIRT 1–7)[84] raises issues about the relative activity of HDACi on these structurally and functionally different isoforms and their relative effects on different cellular systems. Class I HDACs include HDAC 1, 2, 3 and 8, which are primarily found in the nucleus. Class II includes HDAC 4, 5, 7 and 9, which belong to class IIa HDACs and HDAC 6 and 10 belonging to class IIb HDACs, which are able to shuttle between the nucleus and cytoplasm. Class III HDACs, found in the cytoplasm, require a co-factor NAD^+ for activation. These are also called sirtuins (SIRT 1–7) and act via different mechanisms to class I and II. HDAC 11 is the only member of Class IV and this is similar to classes I and II HDACs.[85–87] Recent studies have demonstrated that HDACs are expressed differently in arthritic tissue with higher nuclear activity of HDACs being demonstrated in RA synovial tissue than in osteoarthritis tissues.[84] HDAC 1 has been shown to be highly expressed in synovial fluid from RA patients compared with synovial fluid from osteoarthritis patients.[88] High levels of HDAC 1 have also recently been observed in RA tissues and these levels also correlate with higher TNF-α expression.[84,88] Interestingly Huber and colleagues demonstrated hyperacetylation in RA patients with HDAC activity being lower, particularly HDAC 1 and 2.[83] This is suggested to promote the transcription of genes encoding for several pro-inflammatory cytokines.[83] These conflicting results might be explained by differences in experimental methods or it could be related to differences in the patient population selected for the different studies. Interestingly, HDAC 5 has also been reported to be elevated in other bone pathologies, such as in patients with primary osteoporosis.[89] We have recently demonstrated that in cultured human osteoclasts there is upregulation of class I HDAC 8 and class II HDAC 5 during the late stages of osteoclast development.[29] Other groups have also recently demonstrated that suppression of HDAC 3 expression via siRNAs in osteoclasts isolated from mouse bone marrow and RAW 264.7 c4 cells inhibited osteoclast differention, whereas suppression of HDAC 7 in these cells actually accelerated osteoclast differentiation. Overexpression of HDAC 5 has also recently been shown to reduce RANKL-mediated acetylation of nuclear factor of activated T cells c1 (NFATc1), suggesting that the balance between HATs and HDACs may play an important role in regulation NFATc1 in osteoclasts.[90] These studies suggest that different HDACs may play a role in the overall differentiation and activity of osteoclasts. In light of conflicting reports and the lack of studies looking at HDAC expression in diseased states, further investigations into HDAC expression in both RA and other bone-loss diseases is required to determine the potential of targeting individual HDAC as treatments.

Histone deacetylase inhibitors and osteoclasts

Studies investigating the effects of HDACi on osteoclasts and in *in vivo* disease models suggest that this class of compound, holds promise for treatment of bone destruction in chronic

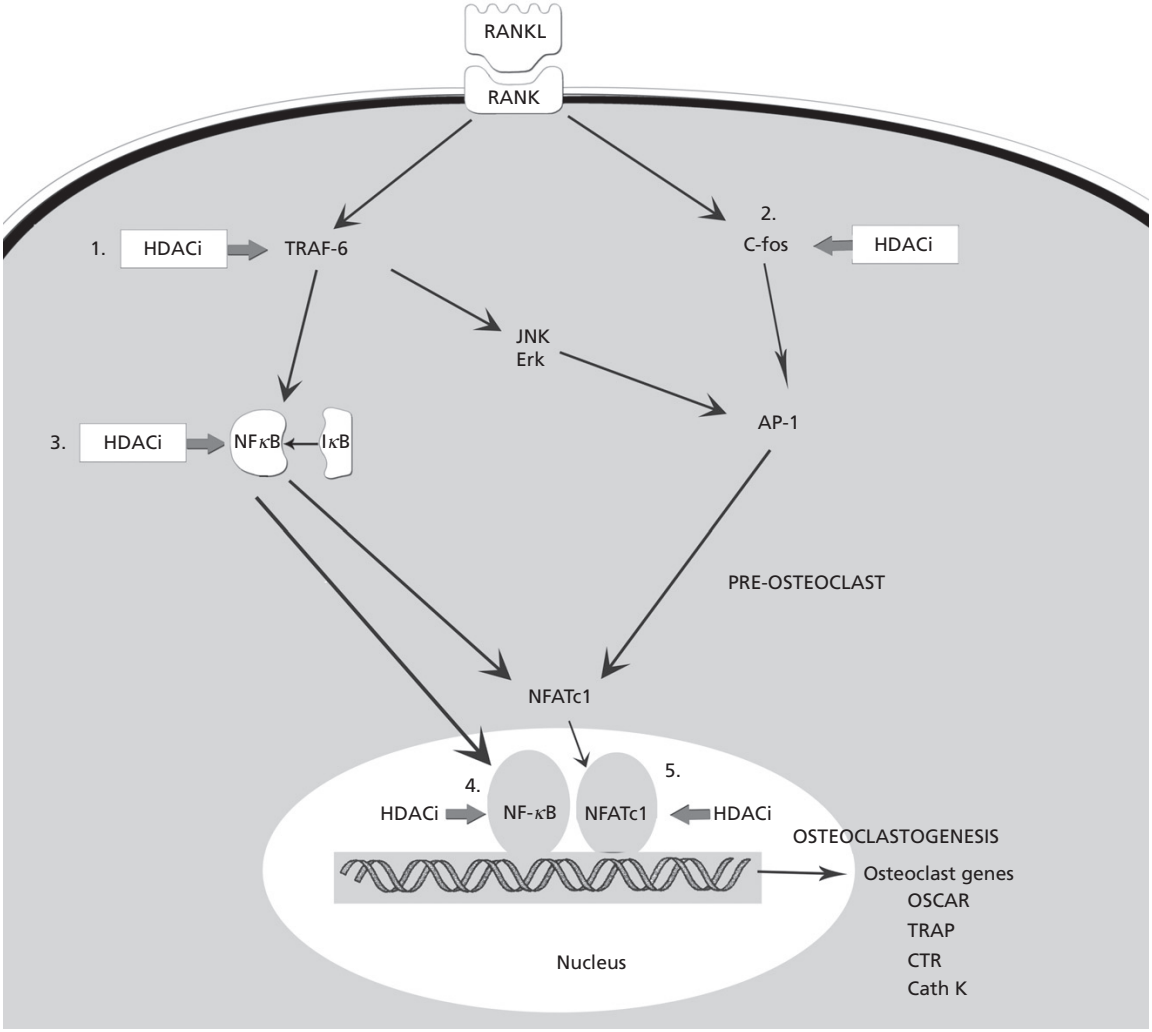

Figure 2 Concepts of the mode of *epigenetic* actions of histone deacetylase inhibitors (HDACi) on receptor activated nuclear factor-κB (NFκB) ligand (RANKL)-activated bone resorption by osteoclasts. Following binding of RANKL to its receptor RANK found on the surface of pre-osteoclast cells there is activation of a number of intracellular pathways. This includes activation of TRAF-6, which is a member of the tumour necrosis factor (TNF) receptor factor (TRAF) family and c-fos. Downstream factors of TRAF-6 are transcription factors nuclear factor κB (NFκB) and activator protein 1 (AP-1). NFκB is then activated with its dissociation from the inactive complex with IκB; the active NFκB being then translocated to the nucleus where it then binds to its promoter sites. Nuclear factor of activated T cells c1 (NFATc1) is the key intracellular regulator of terminal osteoclast differentiation. This transcription factor results in expression of osteoclast genes including tartrate resistant acid phosphatase (TRAP), cathepsin K (Cath K), calcitonin receptor (CTR) and osteoclast associated receptor (OSCAR). HDACi have been shown to have effects on a number of intracellular factors involved in osteoclast formation. **1.** TRAF-6,[29] **2.** c-fos,[30] **3.** NFκB, which leads to proteosomal degradation of the NFκB – IκB complex via the ubiquination pathways, **4.** transcription factor NFκB, **5.** transcription factor NFATc1.[29,31]

inflammatory diseases such as PD and RA. Various HDACi have shown the potential to regulate the effects of RANKL on inflammatory cells and osteoclasts via a number of key factors involved in osteoclastogenesis (Figure 2). In mouse macrophages, HDACi mediate their effects through inhibiting RANKL-induced osteoclastogenesis by suppressing the NF-κB pathway.[82] Several HDACi have been reported to suppress osteoclastogenesis in murine cell lines.[30,31,91] Two very non-specific HDACi, trichostatin A and sodium butyrate, have been shown to suppress RANKL-mediated osteoclastogenesis in rat and mouse bone marrow cultures.[91] Trichostatin A was more recently demonstrated to suppress

RANKL-induced osteoclast formation from primary bone marrow-derived macrophages *in vitro* with an associated significant decrease in the expression of c-Fos and NFATc1.[30] Nakamura *et al.*[31] showed that a cyclic dipsipeptide, HDACi, FR901228 (also known as FK228), inhibited osteoclast differentiation from mononuclear cells derived from rat bone marrow cells. This effect was due to blockade of the nuclear translocation of NF-κB and was accompanied by induction of β-interferon (INF-β).

Our recent publication[29] demonstrated the suppression of human osteoclasts *in vitro* by a novel HDACi, 1179.4b. This is a newly developed synthetic inhibitor that targets HDAC class I and II (compound 52 in Kahnberg *et al.*[92]). 1179.4b treatment coinciding with RANKL addition (from day 7) resulted in a concentration-dependent suppression of osteoclast formation and activity with an IC_{50} (half maximal inhibitory concentration) of < 0.16 nM indicating its high potency. The likely mechanism of action was via osteoimmunogenic factors through a down-regulation of both NFATc1 and osteoclast-associated receptor (OSCAR) mRNA expression during the late stages of osteoclast formation in treated cells. There was a reduction in mRNA expression of upstream factor TNF receptor-associated factor 6 (TRAF-6) expression at day 10 (see Figure 2). Treatment with 1179.4b from day 13 did not suppress osteoclast formation and activity, indicating that the inhibition of TRAF 6 before day 13 is required to reduce NFATc1 and OSCAR expression. Notably there was no elevation of INF-β expression, as described previously with cell line studies.[31] Suberoylanilide hydroxamic acid (SAHA), which is currently in clinical trials for cancer treatment, was also found to suppress osteoclast formation and activity. However, SAHA was much less potent when compared with compound 1179.4b. MS-275, which targets class I HDACs, was also only effective at relatively higher concentrations. A new compound, 2664.12, that targets class II HDACs with some selective affinity for HDAC-6 (compound 17a in Suzuki *et al.*[93]), was not effective unless combined with MS-275.[29] These results indicate that there is redundancy is the role various HDAC play in the development of osteoclasts and that HDACs of class I and class II need to be inhibited to suppress osteoclast formation.

Histone deacetylase inhibitors for the treatment of periodontitis and rheumatoid arthritis

Various HDACi have shown promise for treating RA by inhibiting inflammation and joint destruction in arthritis animal models with a number of different targets, as shown in Figure 3.[31,94,95] The cyclic dipsipeptide histone deacetylase inhibitor, FR901228 (also known as FK228), suppressed osteoclast *in vitro* and was also found to have both prophylactic and therapeutic effects in adjuvant-induced arthritis in rats.[31] However, this compound is a peptide and, as a therapeutic, it is unlikely to be developed as a drug as it needs to be injected intravenously in oil to be effective. The development of non-peptide HDACi would be a more appropriate strategy for targeting bone destruction in RA and PD. SAHA has been shown to reduce paw swelling as a result of suppressing inflammation and decreasing bone erosion, but could not prevent the onset of arthritis in rat and mouse collagen induced arthritis (CIA) models. MS-275 was also found to strongly suppress paw swelling and bone erosions in these models.[94] Using a rat model of adjuvant arthritis, phenylbutyrate and trichostatin A (TSA) were also found to suppress joint swelling, reduce subintimal mononuclear cell infiltration, inhibit synovial hyperplasia, suppress pannus formation and cartilage and bone destruction.[95] These studies highlight the positive effects of HDACi on both bone and inflammation in models of arthritis. We have recently shown, using live animal micro computed tomography (CT) analysis,[104] that the novel HDACi 1179.4b also suppresses bone loss in a mouse model of PD,[102] consistent with its *in vitro* effects (see Figure 3). Interestingly, this occurred despite the inhibitor having no effect on reducing inflammation. This study highlights the potential of HDACi to suppress bone loss. Recently, the use of HDACi in restorative dentistry has also been suggested.[105] As there is a close relationship between RA and PD, with many similarities in the chronic inflammation and bone destruction carried out by osteoclasts,[106–109] such treatments may used effectively in both RA and PD.

Interestingly, inhibitors of HDACs have been found to affect bone formation[110] by stimulating osteoblast maturation.[111,112] In contrast, SAHA stimulated individual osteoblast activity but reduced osteoblast numbers overall. The negative effect could be due to the high concentration (100 mg/kg) used in this study.[113] Specifically targeting individual HDACs may be important in various diseases and different effects may be seen under different conditions.

Potential side effects of histone deacetylase inhibitor treatment

HDACi have been reported to be well tolerated as chemotherapeutic agents but they may have some adverse effects due to their widespread effects in a variety of cells and tissues.[114] The possibility of side effects can be reduced by topical administration and to this end topical TSA and phenylbutyrate have been found to reduce paw swelling and joint destruction in an adjuvant arthritis model.[95] This method of application could potentially reduce the risk of adverse events due to the systemic nature of the inhibition and widespread expression of select HDAC enzymes. In addition, developing compounds that specifically target only those HDACs involved in the disease process may markedly reduce side effects.

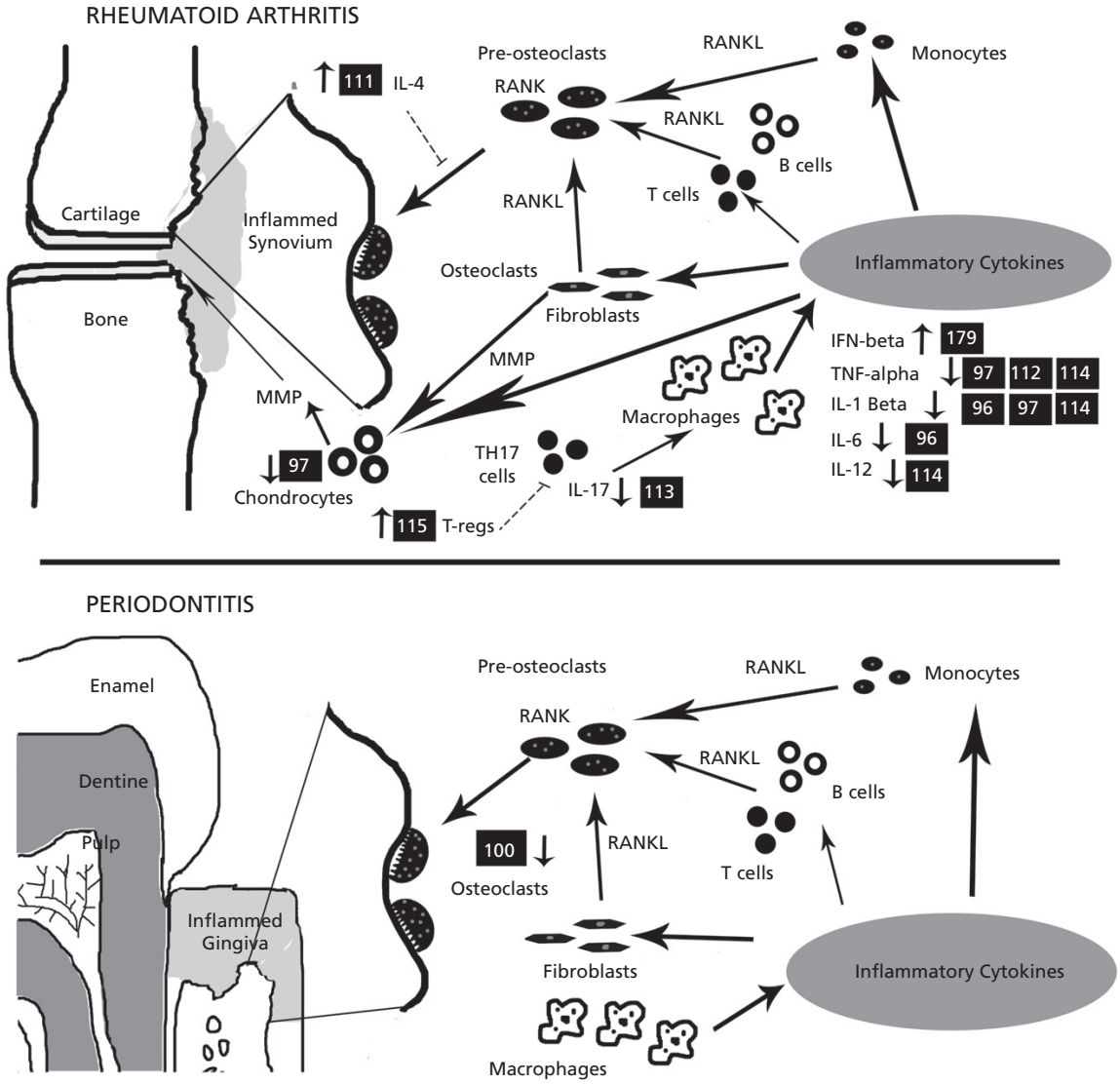

Figure 3 The sites of action of different histone deacetylase inhibitors (HDACi) in (a) rheumatoid arthritis and (b) periodontitis. The references in black boxes correspond to those described in Table 1 and selected studies are described in detail within the text. IFN, interferon; IL, Interleukin; MMP, matrix metalloproteinases; RANK, receptor activated nuclear factor-kB (NFkB); RANKL, receptor activated nuclear factor-kB (NFkB) ligand; Th,T helper cell; TNF-α, tumour necrosis factor-alpha.

Although more research is needed into HDACi and their benefits in treating bone loss in chronic inflammatory diseases, it is clear that they do hold great potential. With the knowledge of HDACs differentially expressed in diseased states such as PD and RA and the development of selective HDAC-targeting inhibitors it is likely that these could eventually be used as a clinical therapy.

Conclusions

Bone destruction is a ubiquitous feature of chronic inflammatory diseases such as RA and PD. Many of the current treatments for these conditions target inflammation and have limited effects on bone. Therapies directly targeting bone destruction could be administered immediately to patients

Table 1 The mechanisms of action of different histone deacetylase inhibitors as identified in the literature – in animal models of rheumatoid arthritis, periodontitis and osteoclast in-vitro models

HDACi	HDACs targeted	Concentration/Dose	Model	Mechanism of action	Mediators targeted	Reference
Rheumatoid arthritis						
Trichostatin A	Class I and II HDACs	2 mg/kg	Collagen-induced arthritis	Suppresses a Th1 response by inducing apoptosis and up regulates IL-4 expression.	Up regulation of IL-4	Zhou *et al*. 2011[96]
Trichostatin A	Class I and II HDACs	0.5, 1.0, and 2.0 mg/kg	Collagen antibody-induced arthritis	Suppressed synovial inflammation and cartilage destruction.	Reduced number of MMP-3 and MMP-13-positive chondrocytes. Suppression of IL1-β and TNF-α stimulated up-regulation of MMP-3.	Nasu *et al*. 2008[97]
Suberoylanilide hydroxamic acid	Class I and II	50 and 15 mg/kg	Mouse and rat collagen-induced arthritis	Reduced inflammation and bone destruction but could not inhibit disease onset.		Lin *et al*., 2007[94]
MS-275	Class I HDACs	10 and 3 mg/kg	Mouse and rat collagen-induced arthritia	Prevented paw swelling by reducing inflammation and disease onset. Reduced bone erosion.	Decreased serum IL-6 and IL-1β	Lin *et al*. 2007[94]
Trichostatin A	Class I and II HDACs	1% ointment at a dose of 100 mg/paw	Adjuvant arthritis	Reduced inflammation, no pannus formation or joint destruction.	Reduced expression of $p16^{INK4}$, $p21^{Cip1}$, and TNF-α.	Chung *et al*., 2003[98]
Phenylbutyrate		10% cream at a dose of 200 mg/paw	Adjuvant arthritis	Reduced inflammation, no pannus formation or joint destruction.	Reduced expression of $p16^{INK4}$, $p21^{Cip1}$, and TNF-α.	Chung *et al*., 2003[98]
FR901228 (depsipeptide	Class I and II HDACs	0.1, 0.2, and 0.5 mg/kg	Adjuvant arthritis	Prophylactic and therapeutic effects, inflammation and bone destruction were markedly suppressed.	Induces production of IFN-β	Nakamura *et al*., 2005[31]
Trichostatin A	Class I and II HDACs	8 mg/kg	SKG model with Zymosan	Prevented arthritis induction.	Reduced Th17 cells and induced regulatory T cells in lymph nodes	Misaki *et al*., 2011[99]
ITF2357 (givinostat)	Class I and II HDACs	5,10,50 mg/kg	*Streptococcus pyogenes* cell wall arthritis, rat adjuvant arthritis and mouse collagen-induced arthritis	Reduced inflammation Pro-inflammatory cytokines production suppressed. Suppressed both acute and chronic arthritis.	Down regulation of TNFα Reduction in IL-1β, IL-6 and IL-12	Joosten *et al*., 2011[100]
Valproic acid	Class I and II HDACs	400 mg/kg	Collagen-induced arthritis	Reduced disease incidence and severity.	Increased the suppressive function of CD4 + CD25 + Tregs and the numbers of CD25 + FOXP3 + Tregs	Saouaf *et al*., 2009[101]
Periodontal disease						
1179.4b	Class I and II HDACs	1 mg/kg	Oral inoculation mouse model	Suppressed alveolar bone loss despite no effect on inflammation.		Cantley *et al*., 2011[102]
MS-275	Class I HDACs	10 mg/kg	Oral inoculation mouse model	No suppression of bone, slight reduction in inflammation.		Cantley *et al*., 2011[102]
Osteoclasts						
1179.4b	Class I and II HDACs	0.16–100 nM	Human *in vitro* osteoclast assay	Concentration-dependent reduction in osteoclast formation and resorption.	Down-regulation of TRAF-6, NFATc1 and OSCAR.	Cantley *et al*., 2011[29]
Trichostatin A	Class I and II HDACs	10 nM	Co culture system	Inhibited osteoclast formation in the co culture systems.	Suppression of c-fos induction	Kim *et al*., 2009[30]
			Mouse bone marrow macrophages	Suppressed RANKL induced osteoclast formation from bone marrow macrophages.		
Trichostatin A	Class I and II HDACs	Bone marrow cultures 5 nM RAW cells 1 nM	Rat and mouse bone marrow cultures and a murine macrophage cell line RAW264	Suppress osteoclast differentiation, but not macrophage differentiation.	Suppression of the NF-κB signaling pathway	Rahman *et al*., 2003[91]
Sodium butyrate	Class I and II HDACs	Bone marrow cultures 0.5 mM Raw cells 0.1 mM	Rat and mouse bone marrow cultures and a murine macrophage cell line RAW264	Suppress osteoclast differentiation, but no macrophage differentiation.	Suppression of the NF-κB signaling pathway	Rahman *et al*., 2003[91]
FR901228 (depsipeptide)	Class I and II HDACs	0.8 ng/ml	Rat bone marrow cells	Suppress osteoclast differentiation.	Suppressed RANKL-induced nuclear translocation of NFATc1 but increased mRNA expression of IFN-beta	Nakamura *et al*., 2005[31]
Suberoylanilide hydroxamic acid	Class I and II HDACs	1 or 3 uM	Mouse macrophage Raw 264.7 cells	Potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis.	Suppression of NF-κB induction.	Takada *et al*., 2006[82]
Trichostatin A	Class I and II HDACs	1–100 nM	Mouse bone marrow macrophages	Induction of osteoclast apoptosis.	Up regulation of p21 (WAF1)	Yi *et al*., 2007[103]

HDACi, histone deacetylase inhibitors; HDACs, histone deacetylase; IL, interleukin; MMP, matrix metalloproteinases; NF-kB, nuclear factor-kB; NFATc1, nuclear factor of activated T cells c1; OSCAR, osteoclast associated receptor; Th, T helper; TNF, tumour necrosis factor; TRAF-6, TNF receptor-associated factor 6; p21, Waf.

upon diagnosis, for bone protection, and later in combination with current ant-inflammatory treatments. In this way both inflammation and bone destruction can be targeted. HDACs are new targets to control bone destruction in chronic inflammatory diseases. Although HDACi hold promise for treatment of pathological bone loss, more research is required to understand the roles of specific HDACs in diseased states and to elucidate which ones need to be targeted for best effect. Overall, identification of safe and effective drugs that target bone resorption will not only improve our treatment of PD and RA, but also other bone loss pathologies that are becoming more prevalent in our ageing populations.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Appendix 2. Epigenetic Regulation of Inflammation: Progressing From Broad Acting Histone Deacetylase (HDAC) Inhibitors to Targeting Specific HDACs

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REVIEW

Epigenetic regulation of inflammation: progressing from broad acting histone deacetylase (HDAC) inhibitors to targeting specific HDACs

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Abstract Inhibition of histone deacetylases (HDAC) is emerging as a novel approach to treat a variety of diseases. Recently, broad acting inhibitors of HDAC have been shown to have anti-inflammatory effects both in vitro and in vivo. It is significant that these anti-inflammatory effects are observed at 10–100 fold lower concentrations than their anti-cancer effects. The broad action of these compounds makes it difficult to determine which HDAC enzymes are important in inflammation. Although showing promise it is unlikely that these drugs will progress to the clinic for treating inflammatory diseases due to number of HDACs they affect and the widespread activity of the enzymes throughout the body. Accordingly, research is now progressing to targeting specific HDAC enzymes to improve efficacy of treatment as well as reduce the risk of any unwanted side effects. Understanding the role specific HDACs play in inflammatory disease will help us to identify novel anti-inflammatory treatments. This manuscript is designed to review our limited knowledge in this field.

Keywords Inflammation · Histone deacetylase inhibitors · Anti-inflammatory treatments

Special Issue: Dedicated to the "Life and work of Professor Barrie Vernon-Roberts".

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Introduction

Epigenetic regulation of gene expression is now recognized as a novel approach to treat disease. In particular, inhibition of histone deacetylase enzymes (HDAC) is emerging as an exciting approach. Although initially used in the treatment of cancer, research now suggests inhibitors of HDAC (HDACi) could be utilized in treating diseases, ranging from neurodegenerative conditions to inflammatory diseases such as asthma, rheumatoid arthritis (RA), and also viral infections. HDACi are orally active compounds that have anti-inflammatory effects at 10–100 fold lower doses than those used in treating malignancies (Dinarello et al. 2011). HDACi are also reported to be very well tolerated in a number of animal models and, more recently, used to treat children with juvenile arthritis (Vojinovic et al. 2011). Multiple HDACi including trichostatin A (TSA), phenylbutyrate, vorinostat (SAHA) and givinostat, have demonstrated anti-inflammatory effects both in vitro and in vivo. The majority of these inhibitors are described as broad acting or commonly known 'pan' inhibitors (Grabiec et al. 2010, 2012; Halili et al. 2010; Joosten et al. 2011; Leoni et al. 2005; Lin et al. 2007; Nasu et al. 2008). This broad action indicates an ability to target HDAC enzymes within the two main classes (class I: HDAC 1, 2, 3, and 8; class II: HDAC 4, 5, 6, 7, 9, and 10). In most cases, the exact HDACs inhibited and the degree they are inhibited are not known making it difficult to understand their mechanism(s) of action. Although showing promise it is unlikely that many of these HDACi will be used as treatments in humans due to the broad activity of the drugs and the ubiquitous distribution of class I HDACs throughout the body. Hence, new drugs targeting specific HDAC enzymes in disease are being developed to reduce the risk of any unwanted side effects. This review will discuss the

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limited progress made from broad acting HDACi to isoform selective inhibitors in the context of inflammatory disease. In addition, this review will highlight our current understanding of the role of individual HDACs in inflammatory disease processes.

HDAC classes

HDACs traditionally regulate gene transcription by altering the acetylation status of histone proteins. Histone acetyltransferase (HAT) enzymes add acetyl groups to the lysine molecules resulting in an open chromatin confirmation leading to gene expression. HDACs remove acetyl groups resulting a condensed chromatin structure reducing access by transcription factors leading to gene repression (Huber et al. 2007). However, it is now recognized that HDAC can also regulate acetylation of non-histone proteins and it is the function that may also be associated with their activities. HDAC enzymes can be classified into four main groups. Class I HDAC which includes HDAC 1, 2, 3, and 8 are predominately found within the nucleus, due to the presence of a nuclear localization sequence and the absence of nuclear export signal sequence within HDAC 1, 2, and 8 (Yang and Seto 2008). However, HDAC 3 has both a nuclear import and export signal so it is able to localize the cytoplasm and nucleus (de Ruijter et al. 2003). Class I HDACs have a ubiquitous tissue distribution (Yang and Seto 2008).

Class II HDAC is subdivided into two sub groups, IIA (HDAC 4, 5, 7, 9) and IIB (HDAC 6 and 10), and are predominately found in the cytoplasm. These HDACs are able to shuttle between the nucleus and cytoplasm but have a more tissue-specific distribution than class I HDAC (Hu et al. 2003; Yang and Seto 2008).

Class III HDACs are also called sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7) and are found in the cytoplasm. These act via different mechanisms to class I and II and require a co-factor NAD+ for activation. HDAC 11 is the only member of Class IV and shares similarities with both Class I and II HDACs (Yang and Seto 2008).

This review will focus on the more widely investigated Class I and II HDAC enzymes.

Inflammatory effects with broad acting HDACi

Anti-cancer agents SAHA and ITF2357 reduce production of tumor necrosis factor-α (TNF-α), interleukin 1α (IL-1α) and IL-1β, and IFN-γ in LPS stimulated peripheral blood mononuclear cells (PBMCs) (Leoni et al. 2002, 2005). TSA treatment suppressed IL-6 production by RA fibroblast-like synoviocytes (FLS) stimulated with IL-1β (Grabiec et al. 2012). Similarly, treatment of LPS stimulated macrophages with TSA reduced IL-6, TNF-α and IL-1β expression. TSA was found to be more potent than SAHA and other HDACi, including sodium butyrate and valproic acid (Han and Lee 2009).

In an experimental colitis model, SAHA induced apoptosis of lamina propria lymphocytes and suppressed pro-inflammatory cytokines levels (Glauben et al. 2006). SAHA also reduced LPS-induced septic shock in rodents by reducing expression of TNF-α and IL-1β (Soltanoff et al. 2009). Consistent with this, SAHA was found to reduce inflammation and decrease bone erosion in rodent models of arthritis (Lin et al. 2007). TSA also suppressed the ability of IL-1β and TNF-α to up-regulate MMP-3 consistent with a cartilage protective effect (Nasu et al. 2008). Another broad acting inhibitor, ITF2357, reduced joint inflammation in rat and mouse models and in contrast to SAHA was able to prevent joint destruction as well as inflammation (Joosten et al. 2011).

Neither SAHA or TSA has progressed to clinical studies; however, the HDACi, givinostat (formally ITF2357), has been tested in a human trial involving 17 children with JIA with treatment administered for up to 12 weeks (Vojinovic et al. 2011). No systemic toxicities or organ dysfunctions were noted, and any side effects reported were only mild to moderate. Patients reported improvements in mobility and wellbeing along with reductions in number of joints with active arthritis (Vojinovic et al. 2011). We have also recently shown that a novel HDACi 1179.4b, which targets class I and II HDACs, was able to effectively suppress bone loss in a mouse model of periodontitis although not markedly reducing inflammation (Cantley et al. 2011a). Consistent with a lack of anti-inflammatory effect, TSA was able to reduce airway constriction but surprisingly had no effect on cytokine levels including IL-4 in models of asthma (Banerjee et al. 2012). The divergent effects of broad acting HDACs in inflammation and related processes highlight the fact that HDACs may play differing roles in disease processes. In order to better target specific HDAC, the roles of the individual enzymes need to be understood.

Progression to specific HDAC inhibition

To date a number of class specific inhibitors (Haus et al. 2011; Hu et al. 2003; Khan et al. 2008) and isoform selective inhibitors have been developed to target HDAC 1 (Lee et al. 2008), HDAC 3 (Gillespie et al. 2012), HDAC 6 (Gupta et al. 2010; Inks et al. 2012; Smil et al. 2009), and HDAC 8 (Huang et al. 2012; Tang et al. 2011). The majority of these studies have focused on the molecular interactions of these compounds (reviewed in (Dallavalle et al. 2012; Pan et al. 2012) for use in the treatment of malignancies (Balasubramanian

et al. 2009; Gryder et al. 2012). Progress in developing selective inhibitors that target the other HDACs has been more limited until recently.

HDACs in inflammation

Figure 1 summarizes the present knowledge in regard to the roles of individual HDACs in inflammation.

HDAC 1

HDAC 1 has been shown to be highly expressed in synovial fluid from patients with RA compared to osteoarthritis (OA) (48). High levels of HDAC 1 have also been observed in RA synovial tissues, and these levels correlated with higher TNF-α expression (15, 48). This suggests a link between HDAC activity and inflammatory cytokines. Interestingly, another study has shown that pro-inflammatory signals, such as TNF-α or IL-1, result in proteasomal degradation of HDAC 1 (Gopal and Van Dyke 2006). Conversely, conditional deletion of HDAC 1 in airway disease was shown to enhance inflammation and Th2 cytokine production (Grausenburger et al. 2010).

No specific inhibitors targeting HDAC 1 have been reported for the treatment of RA. However, MS-275, a benzamide HDACi known to strongly inhibit HDAC 1 (Hu et al. 2003) is reported to have anti-inflammatory effects in several inflammation models including a rat experimental autoimmune prostatitis (Zhang et al. Zhang and Schluesener 2012), mouse model of periodontitis (Cantley et al. 2011a), rat experimental autoimmune neuritis (Zhang et al. 2010), and mouse models of arthritis (Lin et al. 2007). MS-275 demonstrated significant anti-arthritic effects in comparison to a broader acting HDACi, SAHA (Lin et al. 2007). Our recent study in a mouse model of periodontitis revealed that MS-275 was unable to reduce inflammation induced alveolar bone destruction in comparison to novel broad acting HDACi 1179.4b. This could indicate that different HDACs may be involved in different inflammatory diseases (Cantley et al. 2011a).

HDAC 2

HDAC 2 is also a member of class I HDACs and considered structurally similar to HDAC 1 (Yang and Seto 2008). It has been demonstrated to play a role in chronic obstructive pulmonary disease (COPD) (Barnes 2009). HDAC 2 levels were significantly reduced in the lungs of patients with COPD with expression being reported as less than 5 % of that in normal lungs (Barnes 2009). Protein expression of HDAC 2 was also shown to be reduced in

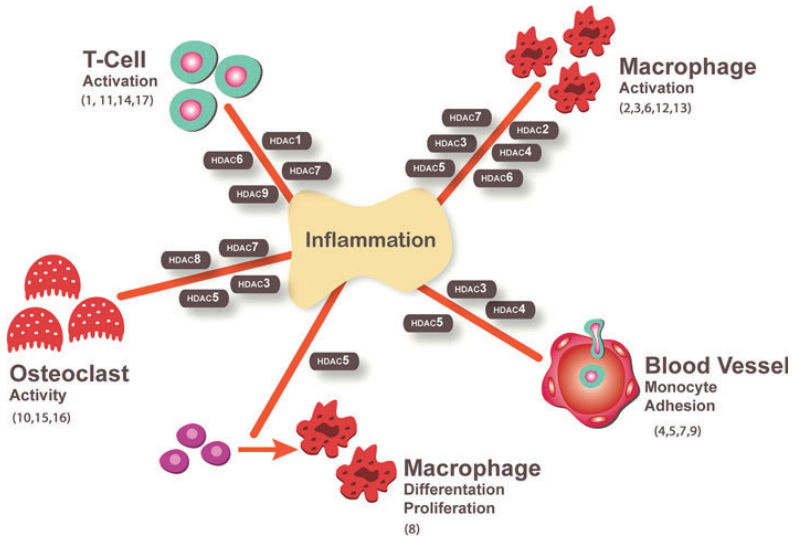

Fig. 1 The roles of HDACs 1–10 in inflammation based on current knowledge. *1* Grausenburger et al. (2010) *2* Barnes (2006) *3* Chen et al. (2012) *4* Kim et al. (2012) *5* Inoue et al. (2006) *6* Mullican et al. (2011) *7* Usui et al. (2012) *8* Baek et al. (2009) *9* Wang et al. (2010) *10* Kim et al. (2011) *11* de Zoeten et al. (2011) *12* Halili et al. (2010) *13* Aung et al. (2006) *14* Kasler et al. (2011) *15* Pham et al. (2011) *16* Cantley et al. (2011b) *17* Yan et al. (2011). Multiple HDACs including HDAC 4, 5, and 7 were shown to be increased at 24 h following LPS stimulation of bone marrow macrophages (Aung et al. 2006). HDAC 1 was rapidly induced in BMMs stimulated with LPS (8 h after stimulation) (Aung et al. 2006)

asthmatic versus normal subjects (Ito et al. 2002). Similarly, HDAC 2 levels were also reported to be reduced in smoking asthmatics compared to nonsmoking asthmatics (Ahmad et al. 2008). Transfection of HDAC2 in alveolar macrophages has been shown to restore corticosteroid resistance (Barnes 2006). To date no specific acting inhibitors targeting HDAC 2 have been reported.

HDAC 3

HDAC 3 is reported to be important in monocyte recruitment to sites of inflammation and in macrophage cytokine production. HDAC 3 deficient macrophages stimulated with LPS demonstrated reduced inflammatory cytokine expression (Chen et al. 2012). Using a mouse model of triphasic cutaneous anaphylaxis and passive cutaneous anaphylaxis, Kim et al. 2012 also showed that HDAC 3 reduced skin inflammation by reducing monocyte chemotactic protein 1 (MCP-1) expression (Kim et al. 2012). MCP-1 is known to play an important role in attracting monocytes to sites of inflammation that then differentiate and produce an array of inflammatory cytokines. Similarly, siRNA knockdown of HDAC 3 in human endothelial cell line (Huvec) was shown to reduce VCAM-1 expression and hence suppress monocyte adhesion (Inoue et al. 2006). Recently, knockdown of HDAC 3 was also shown to restore insulin secretion by beta cells in the presence of TNF-α and IL-1β. In this study, class I specific inhibitor MS-275, which targets HDAC 3 along with other HDACs, was found to effectively reduce the cytokine induced apoptosis of beta cells (Chou et al. 2012). MS-275 preferentially targets HDAC 1 over HDAC 3 suggesting that HDAC 1 could also be important in the apoptosis of beta cells (Hu et al. 2003). HDAC 3 siRNA has also been shown to inhibit osteoclast differentiation in both mouse bone marrow and RAW 264.7 c4 cells (Pham et al. 2011).

Smoke exposure has been shown to reduce HDAC 3 activity in human macrophages and hence increase the inflammatory response (Winkler et al. 2012). siRNA knockdown of HDAC 3 was shown to increase production of IL-8 and IL-1β in response to LPS (Winkler et al. 2012). Similar to these results, Mullican et al. (2011) also showed that macrophages lacking HDAC 3 were hyperresponsive to IL-4 stimulation. Contradictory evidence therefore exists for the role of HDAC 3 in inflammation. Inconsistencies in these studies maybe explained by differing models and the factors used to induce inflammation (Winkler et al. 2012). Recently, HDAC 3 selective inhibitor, known as MI192, was found to reduce TNF production and dose dependently suppressed IL-6 production in PMBCs from patients with RA (Gillespie et al. 2012). To date, the majority of the studies looking at selective HDAC 3 inhibition have been in vitro and studies testing effects of selective HDAC 3 inhibitors, such as MI192 in vivo, are likely to be informative.

HDAC 4

HDAC 4 has been demonstrated to play a role in repressing transcription of IL-5, a key mediator of inflammatory processes in allergic conditions (Han et al. 2006). HDAC 4 was also shown to promote reactive species (ROS)-dependent vascular inflammation likely via VCAM-1 expression. siRNA for HDAC 4 was able to inhibit TNF mediated monocyte adhesion (Usui et al. 2012). Similarly, siRNA to HDAC 4 was found to reduce the transcription activity and protein expression of Hypoxia-inducible factor 1 α (HIF-1 α) (Qian et al. 2006) which have been shown to be important in rheumatoid arthritis (Taylor and Sivakumar 2005). To date no specific HDACi targeting HDAC 4 have been reported.

HDAC 5

HDAC 5 is a member of class IIa HDACs that has been implicated in inflammation particularly in activated monocytes and macrophages (Baek et al. 2009). HDAC 5 has also been shown to regulate immune cell adhesion similar to that of HDAC 4 (Wang et al. 2010). Over expression of HDAC 5 reduces RANKL-mediated acetylation of NFATc1 (NFATc1), consistent with a role in osteoclastogenesis and NFATc1 regulation generally (Kim et al. 2011). This is in line with the HDAC5 gene being identified as a locus affecting bone mineral density in a genome wide association study (Rivadeneira et al. 2009). Consistent with these studies, we have recently shown that HDAC 5 is significantly up-regulated during the later stages of human osteoclast development in vitro. HDAC 5 expression was induced by RANKL induction of osteoclasts in vitro. High levels of RANKL have been shown to be associated with bone loss pathologies such as periodontitis and RA (Crotti et al. 2002, 2003).

HDAC 6

HDAC 6 is a member of the class IIb HDAC found predominately within the cytoplasm of cells uniquely expressing two catalytic domains known to bind to α-tubulin. To date a number of HDAC 6 selective inhibitors have been developed (Gupta et al. 2010; Inks et al. 2012; Smil et al. 2009) with effects on inflammation reported. For instance, HDAC 6 has been recently shown to

play a role in LPS developed tolerance of astrocytes cells with HDACi tubacin found to suppress this tolerance (Beurel 2011). Other studies have also identified HDAC 6 as playing a role in regulating T regulatory cells (de Zoeten et al. 2011). In models of inflammation and autoimmunity such as experimental colitis and cardiac allograft rejection, HDAC6 inhibition was found to promote Treg suppressive activity (de Zoeten et al. 2011). However, other studies have also shown that in HDAC−/− 6 macrophages exhibit normal inflammatory responses suggesting that other HDACs could also be important in the macrophage inflammatory response (Halili et al. 2010). This observation was confirmed in a study showing that HDAC 4, 5, and 7 expression increased in bone marrow macrophages following LPS stimulation (Aung et al. 2006).

HDAC 7

HDAC 7, a member of class IIa HDACs known to play a key role in development of thrombocytes within the immune system. CD8/CD4 double positive thrombocytes are known to express HDAC 7 that appears to regulate cell survival and T cell receptor signaling (Kasler et al. 2011). HDAC 7 has also been identified as controlling the transcription activator HIF-1 α (Kato et al. 2004). The important role that T cells play in RA pathogenesis suggests that HDAC 7 could be important. In the context of bone, however, suppression of HDAC 7 has recently been shown to result in accelerated differentiation of osteoclasts which would suggest that HDAC 7 is able to reduce osteoclast formation (Pham et al. 2011). Interestingly, activity of HDAC 7 within the nucleus has been shown to be linked to HDAC 3 (Fischle et al. 2001). These studies highlight the complexity of these enzymes and the difficulty in understanding the roles of specific HDACs in inflammation.

HDAC 8

HDAC 8 is a member of class I HDACs thought to be found predominately within the nucleus (Yang and Seto 2008). A number of HDAC 8 specific inhibitors have been developed (Huang et al. 2012; Tang et al. 2011). Lower expression of HDAC 8 has also been reported to be expressed in patients who have increasing severity of COPD (Ito et al. 2005). We have demonstrated that class I HDAC 8 is up-regulated during the later stages of human osteoclast development in vitro (Cantley et al. 2011b), this is following RANKL addition. Consistent with a role in bone metabolism, germ line deletion of HDAC 8 has been demonstrated as essential in normal skull formation (Haberland et al. 2009).

HDAC 9

Like HDAC 7, HDAC 9 is known to play a role in the development and differentiation of a number of cell types particularly regulatory T cells (Yan et al. 2011). To date no specific inhibitors for HDAC 9 have been reported on inflammation.

HDAC 10

As yet nothing in regard to inflammation and HDAC 10 has been reported.

Conclusion

Research is now progressing to develop specific HDACi that can be utilized for treating a variety of diseases including inflammatory diseases such as RA. These specific inhibitors may be more effective and also have less side effects than the currently used broad acting inhibitors. A number of isoform selective inhibitors have been developed targeting HDACs 1, 3, 6, and 8. There is also some contradictory evidence in regards to the effects of HDACi that needs to be investigated further with specific inhibitors. Overall, progression from broad acting HDACi to specific HDACis will greatly help to fill the major gaps in knowledge about the role of HDACs in inflammatory diseases.

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Appendix 3. List of Other Publications, Awards and Presentations During Candidature

Publications

Effect of *Porphyromonas gingivalis*-induced inflammation on the development of rheumatoid arthritis.
PM Bartold, V Marino, MD Cantley, DR Haynes.
J Clin Periodontol 2010 37(5):405-11.

Mechanisms and control of pathologic bone loss in periodontitis.
PM Bartold, MD Cantley, DR Haynes.
Periodontol 2000 2010;(53):55-69.

Azithromycin suppresses human osteoclast formation and activity *in vitro*.
SG Gannon, MD Cantley, DR Haynes, R Hirsch, PM Bartold
J Cell Physiol 2012 Oct 12. [Epub ahead of print]

Regulation of ITAM adaptor molecules and their receptors by inhibition of calcineurin-NFAT signalling during late stage osteoclast differentiation.
MS Zawawi, AA Dharmapatni, MD Cantley, DR Haynes, KP McHugh, TN Crotti.
Biochem Biophys Res Commun 2012;427(2):404-9

Comparison of the ability of chondroitin sulfate derived from bovine, fish and pigs to suppress human osteoclast activity *in vitro*.
MD Cantley, KD Rainsford, DR Haynes.
Inflammopharmacology 2013 May 4. [Epub ahead of print] DOI: 10.1007/s10787-013-0171-y

Effects of Osteochondrin® S and select connective tissue ribonucleinate components on human osteoclasts *in vitro*.
MD Cantley, KD Rainsford, DR Haynes.
Journal of Pharmacy and Pharmacology 2013;65(8):1214-22

Conference Presentations During Candidature

2013

Australian Rheumatology Association National Scientific Meeting, Perth, Oral Presentation entitled: Targeting Histone Deacetylase 1 (HDAC 1) to suppress both inflammation and bone loss in arthritis.

2012

Adelaide Bone Group Meeting, Adelaide, invited speaker, Invited to present my work entitled: Histone Deacetylase Inhibitors and Pathological Bone Loss to the Adelaide Bone Group.

Australian and New Zealand Bone and Mineral Society as part of the Australian Health and Medical Research Congress, Adelaide, Oral presentation entitled: Epigenetic Control of Inflammation and Bone Loss using Histone Deacetylase Inhibitors.

Australian and New Zealand Bone and Mineral Society as part of the Australian Health and Medical Research Congress, Perth, Poster presentation: Epigenetic Control of Inflammation and Bone Loss using Histone Deacetylase Inhibitors.

Australian and New Zealand Bone and Mineral Society (ANZBMS) Annual Scientific Meeting, Perth, Poster presentation entitled: Histone deacetylase (HDAC) 1 as a target for suppressing both inflammation and bone loss in chronic inflammatory diseases.

Australian and New Zealand Orthopedic Research Society (ANZORS) Annual Scientific Meeting, Perth, Oral presentation in PhD Session: Targeting HDAC1 to suppress bone loss in peri-prosthetic osteolysis.

Colgate Dental Research Day, Adelaide, Oral presentation entitled: Targeting HDAC 1 to Suppress Inflammation and Bone Loss.

SA Branch Australian Rheumatology Association Annual Meeting, Adelaide, Oral presentation entitled: Targeting Histone Deacetylase (HDAC) 1 to suppress inflammation and bone loss in arthritis.

2011
Centre for Orofacial Research and Learning (CORAL) Annual Workshop, Adelaide, Invited Speaker, Oral presentation entitled: A Relationship Between Periodontitis and Inflammatory Arthritis.

Australian Society for Medical Research South Australian Branch Annual Meeting, Adelaide, Oral presentation entitled: Pre-Existing Periodontitis Exacerbates Experimental Arthritis. I presented this as part of the Ross Wishart Memorial Award for most outstanding young investigator.

SA Branch of the Australian Rheumatology Association (ARA) Annual meeting, Adelaide, Oral presentation entitled: A relationship between periodontitis and arthritis. Received the SA Branch ARA Arthritis SA Philip Alpers Award for Best Basic Science presentation.

Colgate Dental Research Day, Adelaide, Oral presentation entitled: Histone Deacetylase Expression and Inhibition in Periodontal Disease.

American Society for Bone and Mineral Research Annual Meeting, San Diego, USA, Poster presentation entitled: Histone deacetylase (HDAC) expression and HDAC inhibition in different pathogenic bone loss diseases.

American Society for Bone and Mineral Research Annual Meeting, San Diego USA, Poster presentation entitled: Relationship between Periodontitis and Arthritis. Awarded American Society for Bone and Mineral Research President's Poster Competition Award.

2nd Asia-Pacific Osteoporosis and Bone Meeting being held in conjunction with ANZBMS Annual Scientific Meeting, Gold Coast, Poster presentation entitled: Inhibiting histone deacetylases expressed in human periodontal tissues prevents bone loss in an animal model of periodontal disease.

2010

Australian Rheumatology Association (ARA) Meeting, Melbourne, Poster presentation entitled: Inflammation induced by periodontal pathogens exacerbates rheumatoid arthritis in an experimental model.

Faculty of Health Sciences Postgraduate Conference, Adelaide, Poster presentation entitled: Novel Animal Models to Investigate a Relationship Between Periodontitis and Rheumatoid Arthritis. Received certificate for best poster presentation.

Australian and New Zealand Bone and Mineral Society (ANZBMS) Annual Meeting, Adelaide, Poster presentation entitled: Both Class I and II HDACs are important during osteoclast development.

Australian and New Zealand Orthopedic Research Society (ANZORS) annual meeting, Melbourne, Poster presentation entitled: Histone deacetylase (HDAC) expression in Human Peri-prosthetic tissue and the effect of a HDAC inhibitor on bone loss.

Australian Society for Medical Research South Australian Branch Annual Meeting, Adelaide, Oral presentation entitled: Pre-existing chronic inflammation induced by periodontal pathogen exacerbates rheumatoid arthritis in an experimental rat model.

Colgate Dental Research Day, Adelaide, Oral presentation entitled: A novel animal model demonstrating that pre-existing periodontitis exacerbates rheumatoid arthritis. Awarded first prize in the PhD Category.

Osteoarthritis Research Society International (OARSI) Annual meeting, Belgium, Poster presented by Professor KD Rainsford entitled: Effects of Ribonucleinate Components of Osteochondrin® S Osteoarthritis Therapy on Formation and Activity of Human Osteoclasts in Vitro.

MipTec Scientific Meeting, Switzerland, Poster presentation by Professor KD Rainsford entitled: Functional osteoclast bioassay *in vitro* using human mononuclear precursors for determining control of bone resorption by mass spectrometrically–defined Ribonucleinate.

Awards Received During Candidature

2013

- Australian Rheumatology Association (ARA) National Education Travel Grant

2012

- Australian and New Zealand Bone and Mineral Society (ANZBMS) best of the best poster as part of Australian Health and Medical Research Congress (AHMRC)
- SA Branch Australian Rheumatology Association (ARA) Arthritis SA Philip Alpers Award for Best Basic Science Presentation
- Fresh Science National Finalist
- University of Adelaide School of Medical Sciences Domestic Travel Award (2012)
- Best PhD Presentation at the Australian and New Zealand Orthopaedic Research Society (ANZORs) Annual Scientific Meeting
- First prize in the PhD category for oral presentation at Colgate Dental Research Day
- Fresh Science SA State finalist
- Australian and New Zealand Bone and Mineral Society (ANZBMS) Travel Award to attend Annual Scientific Meeting
- Australian and New Zealand Orthopaedic Research Society (ANZORs) Travel Award to attend Annual Scientific Meeting

2011

- SA Branch Australian Rheumatology Association (ARA) Arthritis SA Philip Alpers Award for Best Basic Science Presentation
- University of Adelaide Faculty of Health Sciences Postgraduate Traveling Fellowship
- American Society for Bone and Mineral Research (ASBMR) President's Poster Competition Award
- FHS Postgraduate Research Conference – School Certificate of best poster
- BUPA University of Adelaide – University wide Postgraduate Travel Award
- University of Adelaide School of Medical Sciences Postgraduate Travel Award
- Australian and New Zealand Bone and Mineral Society (ANZBMS) Travel award to attend Annual Scientific Meeting
- Australian Society for Medical Research (ASMR) Ross Wishart Memorial Award finalist for most outstanding young investigator

2010

- Australian and New Zealand Orthopaedic Research Society (ANZORs) Travel Award to attend Annual Scientific Meeting
- University of Adelaide Faculty of Health Sciences Postgraduate Research Expo certificate of best poster presentation
- First prize in the PhD category for oral presentation at Colgate Dental Research Day

2010-2012

- Australian Postgraduate Award