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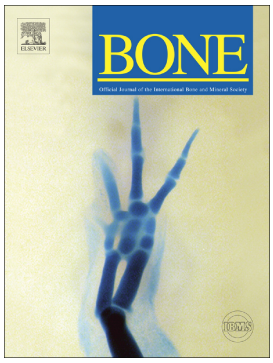

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PII: S8756-3282(16)30361-1
DOI: doi: 10.1016/j.bone.2016.11.028
Reference: BON 11200

To appear in: *Bone*

Received date: 7 September 2016
Revised date: 31 October 2016
Accepted date: 28 November 2016

Please cite this article as: M.D. Cantley, A.C.W. Zannettino, P.M. Bartold, D.P. Fairlie, D.R. Haynes , Histone deacetylases (HDAC) in physiological and pathological bone remodelling. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Bon(2016), doi: 10.1016/j.bone.2016.11.028

Histone Deacetylases (HDAC) in Physiological and Pathological Bone Remodelling

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Abstract

Histone deacetylases (HDACs)[1] play important roles in the epigenetic regulation of gene expression in cells and are emerging therapeutic targets for treating a wide range of diseases. HDAC inhibitors (HDACi)[2] that act on multiple HDAC enzymes have been used clinically to treat a number of solid and hematological malignancies. HDACi are currently being studied also for their efficacy in non-malignant diseases, including pathologic bone loss, but this has necessitated a better understanding of the roles of individual HDAC enzymes, particularly the eleven zinc-containing isozymes. Selective isozyme-specific inhibitors currently being developed against class I HDAC (1, 2, 3 and 8) and class II HDAC (4, 5, 6, 7, 9 and 10) will be valuable tools for elucidating the roles played by individual HDACs in different physiological and pathological settings. Isozyme-specific HDACi promise to have greater efficacy and reduced side effects, as required for treating chronic disease over extended periods of time. This article reviews the current understanding of roles for individual HDAC isozymes and effects of HDACi on bone cells, (osteoblasts, osteoclasts and osteocytes), in relation to bone remodelling in conditions characterised by pathological bone loss, including periodontitis, rheumatoid arthritis and myeloma bone disease.

Key words: Histone deacetylases (HDAC), osteoclasts, osteoblasts, periodontitis, rheumatoid arthritis, myeloma bone disease.

[1] Histone deacetylases (HDACs)
[2] HDAC inhibitors (HDACi)

1. Introduction

Epigenetic regulation of gene expression in cells is an important process enabled, in part, by a mechanism involving histone deacetylases (HDAC). Modulation of these enzymes using HDAC inhibitors (HDACi) is emerging as a promising treatment not only for cancer [1-3] but also for neurodegenerative diseases, asthma, rheumatoid arthritis, viral infections and malaria [4-11]. A number of HDACi have progressed to the clinic, or are in clinical trials, for treating solid and haematological tumours [1-3]. These include vorinostat (SAHA) and romidepsin (Istodax), both FDA approved for cutaneous T-Cell lymphoma (CTCL); panobinostat (LBH589, phase III, CTCL and approved for the treatment of multiple myeloma in 2015); quisinostat (JNJ-26481585, phase II, CTCL), belinostat (PXD101, phase II, ovarian, Tcell lymphoma), entinostat (MS-275, phase II, melanoma, Hodgkin's lymphoma, lung, breast), resminostat (4SC-201, Hodgkin's lymphoma, hepatocellular carcinoma), mocetinostat (MGCD0103, phase II, B cell cancers, lymphomas), abexinostat (phase II, FL), givinostat (ITF2357, phase II, leukemias, myelomas), chidamide (solid tumours) and practinostat (prostate cancer).

The vast majority of HDACi are 'pan' inhibitors, targeting multiple HDACs in both class I and II. In most cases, the exact HDAC inhibition profile of these broad-spectrum drugs is unknown or varies with cell type and context, which can lead to possible side effects. Furthermore, there is likely to be some redundancy between HDAC enzymes that could complicate the development of inhibitors with target-specific effects or the interpretation of their *in vivo* properties. Research in this area is increasingly focussing on development and application of class- or isozyme-specific HDACi in order to improve efficacy and reduce potential side effects. More selective HDACi can be valuable tools for understanding the cellular distribution and actions of individual HDAC isozymes in both physiological and pathological settings.

Broad acting HDACi, such as trichostatin A (TSA), vorinostat (suberanilohydroxamic acid) and romidepsin (FK228, Istodax), have been used clinically in the areas of psychiatry and cancer. More recently, the effects of several broad acting HDACi on bone metabolism have been examined

in a number of *in vitro* and *in vivo* studies with effects on osteoclasts and osteoblasts identified [12-20]. HDACi have also been shown to suppress inflammation and disease progression in a number of animal models of rheumatoid arthritis (RA) [7, 21-23]. Despite this, only one HDACi, Givinostat, has so far progressed to clinical trials for the treatment of juvenile arthritis [24]. Importantly, HDACi can be used at lower, non-cytotoxic, doses to treat non-malignant disease than is required to treat malignancy [25]. However, they need to be used long-term for the treatment of chronic inflammatory diseases and residual or cumulative toxicity may have contributed to their lack of progression to the clinic. The 18 HDAC enzymes are widely expressed in tissues throughout the body and deacetylate both histone and non-histone proteins, making it challenging to determine the precise mechanism of action of different HDACi. Despite the ubiquitous nature of class I HDACs, differences in expression of these enzymes have been observed in a number of malignancies and also in rheumatoid arthritis (RA) [26-29]. This review focuses on HDACs and HDACi in both physiological and pathological bone turnover. Specifically, it will focus on the current understanding of the roles played by HDACs in osteoclast, osteoblast and osteocyte development and function. In addition, we evaluate the limited studies to date that show effects of some HDACi (broad acting, class-specific and isozyme-specific) *in vitro* and *in vivo* in models of pathological bone loss, including rheumatoid arthritis, periodontitis and myeloma bone disease.

1.1 Histone deacetylase enzymes

There are 18 human HDACs grouped into 4 classes. Eleven contain a catalytic zinc (HDACs 1 to 11) while seven are NAD^+-dependent enzymes (SIRT 1 to 7). Class I is made up of HDACs 1, 2, 3 and 8 that are primarily restricted to the nucleus. HDACs 4, 5, 7 and 9 belong to class IIa and HDACs 6 and 10 belong to class IIb (see Table 1). Class II HDACs are able to shuttle between the nucleus and cytoplasm [30]. Class III HDACs, also called sirtuins (SIRT 1-7), are found in the cytoplasm and require a co-factor NAD^+ for activation. These enzymes do not have a catalytic zinc and act by a different mechanism to class I and II HDACs. HDAC 11 is the only member of Class IV HDACs and is similar to classes I and II HDACs in both sequence and presence of a catalytic

zinc ion [4, 30, 31]. Given Class III HDACs function by a very different mechanism to class I and II they will not be considered in this review.

1.2 Histone Deacetylase Inhibitors

HDACi have a metal binding moiety that interacts with the zinc dependent catalytic domain of class I and II HDACs. Inhibitors also have a capping group that interacts with residues found at the entrance to the active site and a linking structure that helps to align the binding moiety and capping group within the active site [32]. There is a high structural similarity between the HDAC enzymes and hence a large number of the HDACi researched to date are pan inhibitors. Class- or isozyme-specific HDACi are designed in the same manner as pan inhibitor but this requires accurate knowledge of the protein structures of the individual isozymes to enable determination of subtle differences that may exist within the active sites [33]. It is also important to consider how these enzymes function with other proteins within cells. For instance, HDAC 1 and 2 function within multi-protein complexes including Sin3A, NuRD and CoREST [34] whilst HDAC 3 functions within SMRT and N-CoR complexes [35]. Class IIa HDACs have a number of levels of regulation (transcriptional control, translational control including micro RNAs, proteolytic control and phosphorylation) and they also function in multi-protein complexes (as reviewed in [36]). These different levels of control and the protein complexes can complicate the trafficking and availability of specific HDAC targets but doesn't usually result in any modification to the catalytic sites of the enzymes. The high structural similarity and sequence conservation between HDAC isozymes in the same class has also limited the design and development of isozyme-specific inhibitors. Possible mechanisms that have been used or proposed to contribute in the development of isozyme-specific inhibitors include modifications to the binding moiety, capping region and linkers [32]. Combinations of these modifications are useful in developing class- or isozyme-specific inhibitors [32].

2. Osteoclasts, HDACs and HDACi

Osteoclasts are large multinucleated cells derived from the haematopoietic lineage that function to resorb bone tissue during bone maintenance, repair and remodelling. In bone loss diseases, including osteoporosis, RA, periodontitis and multiple myeloma, increased numbers of osteoclasts and high levels of Receptor Activator of Nuclear Factor Kappa B ligand (RANKL) have been reported [37-42]. Over the past decade, numerous studies have shown that RANKL, produced by cytokine-activated lymphocytes, fibroblasts and plasma cells, drives excessive osteoclast-mediated bone resorption in RA, periodontitis and multiple myeloma, respectively [37, 38, 40, 43]. Initial studies in 2003 demonstrated that broad spectrum HDACi (TSA and sodium butyrate) could inhibit osteoclast formation and activity using rat bone marrow cells with concentrations of 5 nM TSA or 0.5 mM NaB shown to inhibit TRAP cell formation [20]. Since then, a number of studies have reported on suppression of osteoclastogenesis in murine cell lines and primary human osteoclasts with broad acting HDACi including TSA, FR901228 and 1179.4b [15, 19, 20, 44, 45].

Osteoclastogenesis suppression is thought to occur through targeting of the osteoclast intercellular signalling factors following RANK/RANKL interaction, such as early activation of tumour necrosis factor (TNF) receptor associated factor-6 TRAF-6 (1179.4b) [19] and the transcription factor c-fos (TSA) [13]. HDACi (FR901228 and 1179.4b) have also been shown to induce proteosomal degradation of the NF-κB-IκB complex via ubiquination pathways and to directly act on key osteoclast transcription factors NF-κB and nuclear factor of activated T cells (NFATc1) [15, 19]. Other studies have demonstrated that HDACi (FR901228) can induce expression of osteoclast inhibitory factors such as interferon β (IFN-β) [15]. Although these broad acting HDACi have promising effects in suppressing osteoclast differentiation and activity, the significance of individual HDACs in this process remains to be fully elucidated and is the subject of considerable investigation. To this end, both class specific HDACi and/or knockdown of the specific isozymes

with small interfering RNA (siRNA) or short hairpin RNA (shRNA) have enabled roles of individual HDACs in osteoclast differentiation to be investigated.

2.1 Role of Class I HDACs in osteoclast differentiation and bone resorption.

HDAC 1 and 2, both members of class I HDACs, are structurally similar enzymes that function together in a protein complex [46]. For this reason, it is thought that there is redundancy between the two enzymes [34]. We and others have shown that the HDAC1-selective inhibitor MS-275 (IC_{50} 181 nM HDAC 1, 1160 nM HDAC 2; Table 2) suppresses osteoclast formation in a concentration dependent manner [19]. In *vitro*, 100 nM MS-275 significantly suppressed both the formation of tartrate resistant acid phosphatase (TRAP) positive human osteoclasts and their subsequent resorptive ability [19]. Using mouse bone marrow-derived macrophages (BMMs), MS-275 (20-100 nM) was shown by others to suppress RANKL-induced osteoclast formation and activity by inhibiting expression of c-fos and NFATc1 in a concentration-dependent manner. Furthermore, an equipotent inhibitor *in vitro* of both enzymes HDAC 1 and 2, NW-21 (IC_{50} 21 nM HDAC1, 42 nM HDAC2; Table 2) developed at the University of Queensland, has been found to suppress human osteoclast formation and activity in a concentration-dependent manner (0.16-100 nM) as well as reduce osteoclast formation via suppression of TRAF-6 and NFATc1 [19]. The higher potency of NW-21 (IC_{50} 4 nM) [19] over MS-275 (IC_{50} 54 nM) [19] in cells supports a role for both HDAC 1 and 2 in osteoclast differentiation and activity.

In addition to HDAC 1 and 2, HDAC 3 and 8 are also members of class I HDACs. To date, only one study has examined the role of HDAC 3 in osteoclasts using shRNA in murine BMMs [47]. HDAC 3 shRNA resulted in down regulation of NFATc1, cathepsin K and DC-STAMP [47]. Similarly, HDAC 8 mRNA expression was shown to be significantly higher during the later stages of human osteoclast differentiation *in vitro* [19]. To date, no studies have reported the effects of selective HDAC 8 inhibitors on osteoclast differentiation and function.

A recent study showed that MPT0G009, a non-specific HDACi that targets HDACs of both class I (HDAC 1, 2, 3, 8) and class IIb (HDAC 6) (Table 1), suppressed the formation of TRAP+ multinucleated cells from RAW264.7 mouse macrophages at concentrations as low as 5 nM [21]. This compound was compared to vorinostat, a similarly broad acting but less potent HDACi, which had no effect on osteoclast formation at 50 nM. In this study, MPT0G009 suppressed osteoclast differentiation by targeting key osteoclast transcription factors NF-kB and NFATc1. These effects suggest that it may be important to target multiple HDACs to effectively suppress osteoclasts.

Collectively, these studies have begun to elucidate the roles of class I HDACs in osteoclast formation and activity. Notably, suppression of key osteoclast transcription factors NF-κB and NFATc1 appear to be common mechanisms of action for HDACi studied to date. NFATc1 is a key transcription factor required for osteoclast formation and induces expression of osteoclast genes during late stages of osteoclast formation, including TRAP, calcitonin receptor (CTR), cathepsin K, tartrate resistant acid phosphatase (TRAP) and β3 integrin [48-50]. Similarly, NF-κB is an important transcription factor involved in osteoclast formation, activation and survival [51].

2.2 Role of Class II HDACs in osteoclast differentiation and bone resorption.

Class II HDACs have the ability to shuttle between the cell nucleus and the cytoplasm. HDAC 5 is a class IIa HDAC that has been shown to induce NFATc1 deacetylation, leading to down-regulation of its transcriptional activity. In BMM, overexpression of HDAC 5 impairs RANKL-induced osteoclast differentiation [52]. Recent studies have shown that in human osteoclasts, HDAC 5 mRNA is significantly increased during the later stages of differentiation *in vitro [19]*. To date, no studies have reported on inhibitors targeting HDAC 5 and their effects on osteoclasts.

HDAC 6 is a mainly cytoplasmic protein of class IIb HDACs that act on both histones and the cytoplasmic protein tubulin [53]. It also plays an important role in the process of autophagy by controlling fusion of autophagosomes to lysosomes [54]. A novel HDACi, 2664.12, that targets

class II HDACs has been shown to have selective affinity for HDAC 6 (IC_{50} 29 nM HDAC 6) (compound 17a in [55]) was able to suppress the activity of human osteoclasts *in vitro* at 20-100 nM [19]. The broad spectrum HDACi, MPT0G009, that inhibits multiple HDACs including HDAC 6 (IC_{50} 8 nM, Table 2), was shown to suppress osteoclast differentiation and activity in human and murine RAW264.7 cells, respectively [21]. Recent studies support the idea that inhibition of both class I (HDAC 1) and II (HDAC 6) HDACs may be necessary to suppress osteoclast differentiation and activity. For example, using human osteoclasts, differentiated from PBMCs, the combination of MS-275 (targets HDAC 1) and 2664.12 (targets HDAC 6) suppressed multinucleated TRAP cell formation and activity far more effectively (IC_{50} 0.4 nM) than when used on their own [19]. Furthermore, the effects observed with the combination of both compounds were similar to those observed for a broader spectrum HDACi (1179.4b) that targets both class I and II HDACs [19].

HDAC 7, a class IIa HDAC, may also have a role in regulating osteoclast differentiation and activity. Overexpression of HDAC 7 in mouse BMMs was shown to inhibit the fusion of osteoclast precursors through suppression of Mitf transcriptional activity [47]. In a subsequent study, overexpression of HDAC 7 in mouse bone marrow cells inhibited both the number and size of TRAP positive multinucleated cells formed. Notably, knockdown of HDAC 7 (HDAC 7 flox/flox; Ly-cre) has been found to enhance osteoclast differentiation. In this study, HDAC 7 was found to attenuate NFATc1 suppression of β-catenin in the presence of RANKL [56]. These studies suggest that inhibitors targeting HDAC 7 alone may not be sufficient to suppress the enhanced osteoclast differentiation and activation observed in a number of bone loss diseases.

HDAC 9 expression has been recently shown to be down-regulated following RANKL stimulation of mouse bone marrow cells. Moreover, osteoclast differentiation and bone resorption was significantly elevated in HDAC 9 KO mice, suggesting that inhibitors targeting HDAC 9 could potentially have negative effects on bone [57].

To date there have been no reports on elucidating the roles of class II HDACs 4 or 10 in osteoclast differentiation. The majority of studies on osteoclasts have focussed on inhibitors that have some affinity for class I HDACs, most likely due to the lack of class II specific inhibitors. Select studies demonstrating effects of targeting select class II HDACs and the use overexpression studies does suggest they may be appropriate targets to suppress osteoclasts that require further investigation as more selective acting HDACi are developed.

3. Osteoblasts, HDACs and HDACi

Osteoblasts are cells derived from the mesenchyme cell lineage that form bone. These cells are a major source of RANKL, which binds to its receptor RANK on pre-osteoclasts, resulting in the formation of active multinucleated osteoclasts [58]. The coupling of osteoblasts and osteoclasts is vital for homeostatic bone remodelling and maintenance of bone mass [59]. In pathological bone loss, there is an uncoupling of bone formation and resorptive processes leading to excessive bone loss. While diseases such as osteoporosis, periodontitis, RA and multiple myeloma are characterised by enhanced osteoclast resorption there are also reports of reduced osteoblast bone formation [60-62]. For this reason, osteoblasts have been targeted with anabolic agents including parathyroid hormone (PTH), bone morphogenetic proteins (BMPs), as well as sclerostin neutralising antibody, in order to stimulate bone formation [63].

As highlighted in other review articles [64, 65], a number of studies have suggested a role for specific HDACs in regulating osteoblast differentiation and maturation. Several studies have shown that broad acting HDACi (Valproate, TSA, butyrate) can increase osteoblast proliferation and the transcriptional activity of Runx2, leading to increased alkaline phosphatase (ALP) expression and mineralization *in vitro* [17, 66, 67] (Table 2).

3.1 Role of Class I HDACs in osteoblast differentiation and bone formation.

Butyrate, a broad acting but very weak HDACi, has been shown to accelerate osteogenesis [14]. HDAC 1 is thought to be a key target of butyrate and has been reported to play a role in osteoblast differentiation, with its activity declining during osteoblast maturation [14]. This notion is supported by studies showing that siRNA-mediated suppression of HDAC 1 can stimulate osteoblast maturation [14]. Treatment of MC3T3-E1 cells, primary osteoblasts and mouse calvarial cultures with HDAC1 inhibitor MS-275 (500 nM for 3 days) stimulated cell proliferation and promoted ALP production [17], suggesting that class I HDAC 1 functions to suppress osteoblast differentiation.

HDAC 3 (Class I) has been shown to interact with Runx2, repressing the activation of the osteocalcin promoter. HDACi (TSA) and shRNA to HDAC3 were able to reverse this repression [16]. In these studies, suppression of HDAC 3 in MC3T3 pre-osteoblasts accelerated expression of Runx2 target genes, osteocalcin, osteopontin, and bone sialoprotein. Mineralisation also occurred earlier in these cells [16]. In subsequent studies, conditional knockout of HDAC 3 in mature osteoblasts resulted in a decrease in postnatal cortical and trabecular bone mass [68]. Osteoblasts from these mice demonstrated reduced functional capabilities both *in vitro* and *in vivo*. These contrasting findings demonstrate the complex nature of HDACs and their role in bone cell development and function. Effects of suppressing HDAC 3 may be dependent upon the stage of osteoblast development and hence further studies are necessary to investigate the potential of selective acting HDAC 3 inhibitor. Roles for other class I HDACs, including HDAC 2 and 8, in osteoblast differentiation have not yet been reported.

Effects of a selective class I HDACi, largazole, on osteogenic capacity were assessed using murine pluripotent mesenchymal precursor C2C12 cells. Treatment with largazole was shown to decrease total HDAC activity and, at a concentration of 50 nM, stimulated increased expression of ALP, osteopontin and Runx2, along with inducing expression of BMP-2, 4, 6, 7, and 9 [69].

Collectively, these studies highlight the importance of inhibiting class I HDACs, particularly HDAC 1, in enhancing osteoblast differentiation and hence mineralization. Further studies to assess effects of HDAC 1 selective inhibitors versus other HDACi on osteoblasts would be of considerable interest.

3.2 Role of Class II HDACs in osteoblast differentiation and bone formation.

Several studies have investigated the role of class II HDACs in osteoblast differentiation. For example, HDAC 4 is suppressed by miR-29b, resulting in induction of osteogenesis [70]. In contrast, studies utilizing HDAC 4 KO mice demonstrated that HDAC 4 regulates chondrocyte hypertrophy and endochondral bone formation. This was shown to be via inhibition of Runx2 activity. The effect of inhibiting HDAC 4 could be dependent upon the stage of development [71].

Interestingly, a recent meta-analysis of five genome-wide association studies of femoral neck and lumbar spine BMD demonstrated that HDAC 5 is a bone mineral density locus [72]. This is consistent with the observation that a novel miRNA targeting HDAC 5 promotes osteoblast differentiation in primary mouse osteoblasts [73] and is further highlighted by the observation that adolescents suffering from primary osteoporosis exhibit mutations resulting in a loss of miR-2861 [73]. Notably, bone samples from these patients had increased HDAC 5 levels and correspondingly low Runx2 levels. These studies are consistent with HDAC 5 suppressing bone formation and selective HDAC 5 inhibitors elevating bone formation, although this has yet to be investigated. In contrast, a recent study revealed that HDAC 5 global knockout mice have reduced numbers of osteoblasts and hence low bone density [74]. This study focused on the fact that HDAC 5 knockout increases SOST expression by osteocytes. Studies using HDAC 5 selective inhibitors on the different stages of osteoblast development and subsequent bone formation are very much needed. It is also important that studies utilize *in vivo* models to determine effects of these selective acting HDACi on the osteoclasts and osteocytes as well.

HDAC 6 is a class IIb HDAC that is reported to bind to Runx2 in differentiating osteoblasts [75]. HDAC 7 has been shown to suppress Runx2 activity during osteoblast maturation with RNAi suppression of HDAC 7 increasing osteoblast differentiation [12].

To date, no studies have reported HDAC 4, 5, 6, or 7 selective inhibitors to investigate the roles of these enzymes in osteoblast differentiation and mineralization. It is likely that these inhibitors will have potential to stimulate osteoblast bone formation given the interactions of HDAC 5, 6 and 7 with transcription factor Runx2. Studies to date, however, have suggested conflicting results in regard to the roles of select HDACs in bone formation, for instance HDAC 4 and 5. Hence further studies are necessary to unravel the roles of these enzymes in the different stages of osteoblast development and function.

3.3 General effects of HDACi on bone formation

NFATc1 expression is suppressed in osteoclasts treated with HDACi, 1179.4b and FR901228 [15, 19]. Activation of NFATc1 has been previously shown to significantly inhibit osteoblast differentiation and activity via repression of osteocalcin. [76]. Thus HDACi that suppress NFATc1 may be able to both inhibit bone resorption and enhance osteoblast bone formation.

In contrast, a recent study suggests that the broad acting HDACi vorinostat can lead to bone loss, however doses were very high (100 mg/kg), equivalent to those used in pre-clinical models to treat malignancies. Using an *in vivo* model, it was demonstrated that vorinostat resulted in bone loss and reduced osteoblast numbers in mice [77]. Despite a negative effect on the immature osteoblasts, an increase in local bone formation was observed, with mature osteoblasts possibly being resistant to HDACi-induced apoptosis [77]. Selective targeting and lower doses (10-100x lower) of HDACi have been shown to be better tolerated in animal models and have different effects on bone cells without the induction of apoptosis. This is supported by a recent *in vitro* study demonstrating that

higher doses of vorinostat induced apoptosis of mesenchymal stem cells, but was not observed when lower doses were used [78].

While our current understanding of the roles of HDAC 1 and 3, along with a number of class II HDACs, in osteoblast differentiation remains limited, it is clear that it is essential to determine whether targeting one HDAC alone is more effective or whether a combination of HDACs need to be targeted to affect osteoblast proliferation and differentiation. Of particular interest are the effects that isozyme-specific HDACi have on various stages of osteoblast differentiation and function.

4. HDACs and inhibitors: Osteocytes

Osteocytes are the predominant cell type residing in bone, comprising up to 90-95% of all bone cells. The importance of these cells and the roles they play in all aspects of bone remodelling is becoming increasingly evident [59, 79]. The important anabolic effects of these cells is highlighted by their role in osteoid mineralisation, with the rate of mineralisation thought to be controlled by these cells [80]. Osteocytes have also been recently identified as a major source of the osteoclast differentiation factor, RANKL [81, 82]. In addition, osteocytes which have undergone apoptosis have been shown to be the major stimulator of osteoclast resorption, with apoptotic bodies released from the cells expressing RANKL [83]. Sclerostin (SOST), which is expressed by osteocytes, also plays an important role in stimulating osteoclasts with studies demonstrating that osteocyte cell line MLO-Y4 cells treated with sclerostin produce increased levels of RANKL which can stimulate osteoclast resorption in co-cultures. [81]. To date, only two studies have attempted to elucidate the roles of class I and II HDACs in SOST regulation [84] [74]. HDAC 1, 2 and 5 mRNA were found to be strongly expressed in an osteoblast cell line, UMR106, used to assess SOST gene regulation by PTH [84]. Combinations of siRNA to HDAC 1, 2 and 3 resulted in 79% inhibition of SOST expression [84]. In a recent study, shRNA to class IIa HDAC 5 was shown to increase SOST expression in cell line Ocy454 osteocytes. Osteocyte marker DMP-1 was also increased with

HDAC 5 knockdown but there was no effect on expression of PHEX. shRNA to HDAC 4 or 7 in these cells did not increase SOST expression [74]. In subsequent studies, HDAC 5 global knock out mice were shown to have higher numbers of sclerostin secreting osteocytes particularly the peri-endosteal osteocytes. In these mice, histomorphometric analysis also revealed a defect in bone formation and reduced osteoblast numbers but no changes in osteoclast parameters [74]. These results would suggest that HDACi targeting HDAC 5 may in fact have negative effects in regards to bone formation, by increasing osteocyte SOST expression. It is important to note though that HDAC 5 was absent in all cells not just osteocytes in the knockout mouse studies. Targeting class I HDAC (1,2,3) may however have opposite effects and hence further studies are necessary using selective acting HDACi to elucidate their potential.

5. Cross-talk between bone cells

The majority of *in vitro* studies conducted using HDACi have focussed on individual bone cells e.g. osteoclasts, osteoblasts or osteocytes demonstrating direct effects on the cell differentiation or activity. In osteoclasts, HDACi have been shown to suppress key transcription factors such as NFATc1 and NF-κB [15, 20, 85]. HDACi (FR901228) was shown to prevent the nuclear translocation of NFATc1 via induction of osteoclast inhibitory factor IFN-β [15]. Other studies have only assessed the expression of gene and protein NFATc1 in response to HDACi treatment and hence it is not clear exactly how HDACi alter acetylation levels leading to NFATc1 suppression. In regards to NF-κB, studies have revealed that select HDACi have the ability to suppress NF-κB p65 nuclear accumulation by enhancing its acetylation [20]. In osteoblasts, a number of HDACs and inhibitors have been shown regulate Runx2 expression along with other key osteoblast genes such as osteocalcin. The mechanisms by which HDACi alter acetylation levels in osteoblasts affecting runx2 expression is not known.

Effects of HDACi *in vitro* on the crosstalk between bone cells has not yet been investigated. Given that select HDACi suppress NFATc1 expression and the ability of this transcription factor to suppress osteoblasts suggests that these compounds could also stimulate osteoblasts. It is likely however that it will be necessary to target the different HDACs that are important for individual cell differentiation and activity. Studies focussing on osteoclast-osteoblast-osteocyte communication in the presence of HDACi will be very valuable. *In vivo* studies using HDAC knock out models have revealed potential roles for HDACs in the interactions between the bone cells. For instance, in HDAC 5 global knock out mice there was an increase in sclerostin producing osteocytes with reductions in osteoblast number and defects in bone formation [74]. Other studies using global HDAC 5 knock out mice demonstrated increased bone resorption with higher numbers of osteoclasts covering the bone surface due to significantly high levels of RANKL produced by osteoblasts [86]. Similar observations of high bone resorption and RANKL levels were also evident in mice with HDAC4 knocked out in osteoblasts suggesting a role for HDAC4 in the communications between osteoblasts and osteoclasts [86]. It is important to note in studies using HDAC global knockout mice that all bone cells would be affected making it difficult to determine the exact mechanisms of the changes in bone parameters. To date, no studies have assessed effects of HDACi on the cross talk between bone cells.

6. HDACs and HDACi in bone diseases

6.1 Periodontitis

Periodontitis is a chronic inflammatory disorder affecting the tissues of the periodontium, including the gingiva, periodontal ligament, and alveolar bone. Characteristic features of this disease include soft tissue inflammation in response to gram-negative anaerobic bacteria that collect in the gingival pockets and associated alveolar bone loss [87]. In periodontitis gingival tissues, increased numbers of multinucleated TRAP positive osteoclasts have been detected along with high levels of RANKL and corresponding low levels of its inhibitor osteoprotegerin (OPG) [37]. Osteoblast numbers are also reported to be suppressed in periodontitis lesions harbouring *Porphyromonas gingivalis* (*P.gingivalis)*, a common bacterium associated with periodontitis known to inhibit the differentiation and maturation of osteoblasts *in vitro* [88]. The alveolar bone loss characteristic of periodontitis leads to a loss of tooth support, and if untreated, can lead to tooth loss. The current treatment for periodontitis involves removal of the bacterial load through plaque removal, mechanical debridement and deep tissue scaling. Other treatments include the use of anti-microbials and anti-inflammatories. Although a number of anti-resorptive agents have been investigated in disease models, to date none have progressed to the clinic (as reviewed in [89]).

The potential of HDACi to treat periodontitis has been recently demonstrated using a novel HDACi, 1179.4b, shown to suppress alveolar bone loss in periodontitis [90]. Using a mouse model of *P. gingivalis*-induced periodontitis, oral administration of this broad acting HDACi 1179.4b (1 mg/kg/day for 36 days) suppressed alveolar bone loss with an accompanying reduction in the number of TRAP positive cells in the gingiva and alveolar bone [90]. Interestingly, whilst inhibiting bone resorption, 1179.4b did not suppress gingival inflammation. It is important to note that the dosage used in this model was 100x lower compared to other broad acting HDACi such as vorinostat used in the treatment of malignancies. It is interesting that in the same periodontitis animal model, the HDAC-1 selective inhibitor MS-275 (Table 3) administered orally at 10 mg/kg/day reduced levels of inflammation but had no effect on bone loss [90]. We have also

recently investigated the expression of HDACs 1-10 in gingival tissues collected from either patients with periodontitis and evidence of radiological bone loss, or patients with mild inflammation but no bone loss (non-periodontitis). At the mRNA level, HDACs 1 and 8 (class I) and HDAC 5 and 9 (class II) were all significantly up-regulated in periodontitis gingival tissues compared to the non-periodontitis group. Similarly, these HDACs were strongly expressed at the protein level in both tissue groups based on immunohistochemistry. HDAC 1 was strongly expressed by the CD-3 and TNF-α positive inflammatory cells, whilst HDAC 5, 8 and 9 were strongly expressed by endothelial cells in the gingival tissues from patients with periodontitis [91]. However, further studies are needed to determine the best combination of inhibitors to use and whether other mechanisms of drug delivery may be possible including direct application to the gingiva.

6.2 Rheumatoid Arthritis (RA)

The chronic inflammatory disease, RA, is a very common destructive autoimmune disorder affecting about 1% of the world's population [92]. RA is characterized by joint inflammation, synovial hyperplasia and associated destruction of bone and cartilage and progressive joint destruction. Excessive osteoclast-associated bone resorption is a characteristic feature in RA with studies demonstrating that osteoclast knock out mice are resistant to arthritis-induced bone loss [93, 94]. Enhanced osteoclast formation has also been demonstrated in human RA synovial tissues [40, 95-97]. In animal models of inflammatory arthritis and in human RA tissues, large multinucleated osteoclastic cells that resorb the subchondral bone, have been detected at sites of bone loss in synovial joints [40, 95-97]. Normal osteoblast function is impaired in arthritis, at sites of focal bone erosions, with reduced formation of mineralised bone at sites adjacent to synovial inflammation [60]. In these tissues, there was an abundance of cells expressing the early osteoblast marker Runx2, but these same cells did not express ALP and osteocalcin, suggesting that these cells were immature osteoblasts [60]. Current treatments for RA include non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDS) and biologic DMARDS such as the

anti-TNF agents, infliximab and adalimumab. Although having beneficial effects on inflammation and disease activity, their abilities to suppress focal bone erosions were variable. Notably, it can take time for an effective treatment to be identified, during which structural joint damage can occur [98].

The fact that HDACi are a new class of drugs that might help combat the ongoing bone damage associated with RA, has prompted a number of *in vitro* and *in vivo* RA related studies [7, 21-23, 99-101]. The first study to assess effects of HDACi using an animal model of inflammatory arthritis was reported in 2003 [85]. It was found that topical treatment with the broad acting HDACi's, phenyl butyrate (PB) (1% and 10% ointments) and TSA (1% ointment) suppressed joint swelling with significant reduction in synovial joint levels of TNF-α and no evidence of joint destruction in a rat adjuvant arthritis model (AIA) [99]. Subsequently, a number of other studies have demonstrated positive effects of broad acting HDACi to reduce RA disease activity. Vorinostat has been evaluated in rodent models of arthritis and anti-inflammatory activity was observed at doses of 50 mg/kg/day via subcutaneous injection [7] and 200 mg/kg/day via oral gavage [21]. However, these represent extremely high doses and raise concerns that long-term treatment of patients with chronic disease may reveal considerable unwanted off-target effects. A similarly broad acting HDACi, valproic acid (VPA), was also shown at 400mg/kg via i.p for 39 days to reduce joint destruction in a collagen-induced arthritis model in mice, with both bone and cartilage damage significantly reduced [100]. A more recent study demonstrated that a novel HDACi, S-(ε)- 3-(1-(1-(benzo[d]oxazol-2-yl)-2-methylpropyl)-1H-1,2,3-triazol-4-yl)-N-hydroxyacrylamide (referred to as NK-HDAC-1), which is more potent than vorinostat (10mg/kg and 30 mg/kg p.o every other day) suppressed bone erosion in collagen-induced arthritic mice [102]. The HDAC inhibitory profile of this inhibitor has not yet been reported. Another HDACi, MPT0G009 (3-[1-(4-methoxybenzenesulfonyl)-2,3-dihydro-1*H*-indol-5-yl]-*N*-hydroxyacrylamide), was recently shown at 25 mg/kg p.o. to decrease both cartilage and bone destruction in a murine model of adjuvant-induced arthritis and prevented reduction in bone mineral density and bone

mineral content. MPT0G009 is a non-selective HDACi that inhibits both class I (HDAC 1, 2, 3, 8) and class IIb (HDAC 6) HDACs (Table 1) [21]. Treatment with MPT0G009 at 25 mg/kg resulted in similar effects to that seen with vorinostat at 200 mg/kg. A more recent study showed that pan-HDACi, including vorinostat, are quite toxic above 10 μM concentrations in cells and even at a 10 mg/kg dose in rodents with collagen-induced arthritis, but were tolerated and optimally anti-inflammatory at a lower *in vivo* dose of 1 mg/kg [25]. This suggests that the higher doses mentioned above should not be considered safe and efficacious, especially in already immune-compromised individuals suffering arthritis.

In contrast to studies where broad acting HDACi have been used at high doses (50-100 mg/kg), class- and isozyme-selective HDACi, such as NW-21 (targets HDAC 1 and 2) and MS-275 (targets HDAC 1), BML-275 (targets HDAC 6) have been found to have anti-arthritic activity in rodent models at much lower doses (1-25 mg/kg/day) [7, 25]. For instance, in a collagen-induced arthritis (CIA) model, prophylactic treatment with MS-275 (3 and 10mg/kg/day s.c.) demonstrated significant anti-resorptive effects on the bone [7]. Therapeutic treatment with MS-275 (5 mg/kg/day) also reduced joint narrowing and bone erosion in a rat adjuvant model of arthritis. In this study, treatment with vorinostat reduced bone loss, although its effects were not as marked as that observed with MS-275 [7]. Recent studies using a collagen antibody-induced arthritis (CAIA) model revealed that HDACi targeting HDAC 1 (NW-21 at 5 mg/kg/.day p.o and MS-275 at 10 mg/kg/day p.o.) suppressed the formation of histologically and micro-CT-detectable bone erosions [103]. In contrast, the broad acting HDACi (1179.4b at 5 mg/kg/day p.o.) did not reduce bone erosions or disease activity in this model. The ability of selective-HDACi to inhibit HDAC activity at lower doses will accordingly result in fewer side effects when used for extended periods, as would be necessary in a chronic disease such as RA. Furthermore, lower doses of the HDACi, NW-21 and MS-275, show significant benefits on bone remodeling.

Consistent with the positive effects of HDACi that inhibit HDAC 1, studies have shown that HDAC 1 is highly expressed in synovial tissues from patients with RA [28]. Importantly, this high

expression of HDAC 1 was correlated with expression of inflammatory cytokine TNF-α [29]. More recent studies have confirmed a role for HDAC 1 in the inflammatory tissue destruction in RA. Use of siRNA against HDAC 1 in a CIA model was shown to suppress joint inflammation and destruction [57]. In this study, HDAC 1 mRNA expression levels were also confirmed to be high in RA synovial fibroblasts (RASFs). Interestingly, in these studies, the addition of TNF-α to the RASFs was shown to reduce HDAC 1 expression. These findings contrast those of others, which showed a correlation of HDAC 1 and TNF-α. This difference could be related the use of an isolated cell population compared to total synovial tissues where HDAC 1 and TNF-α levels were shown to be correlated.

The studies described above, highlight the potential of HDACi to reduce disease activity and focal bone erosions in RA.

6.3 Myeloma Bone Disease

Multiple myeloma (MM) is a haematological malignancy of immunoglobulin (Ig) producing plasma cells. Each year approximately 100,000 people are diagnosed with multiple myeloma worldwide. Osteolytic bone disease is a characteristic feature of more than 90% of patients with MM [104]. The imbalance between osteoblast bone formation and osteoclast bone resorption is responsible for the lytic disease observed [104]. The RANKL/RANK pathway has been shown to be important with high levels of RANKL and corresponding low levels of OPG correlating with disease activity [43, 105]. Current treatment for MM bone disease involves the use of bisphosphonates that are effective at inhibiting osteoclast activity. Bisphosphonates have however been shown to be associated with osteonecrosis of the jaw, but this is dependent upon a number of factors including route of administration, use with other agents and time of exposure to BPs [106].

A number of HDACi, including CR2408 (broad acting), JNJ-26481585 (broad acting) ACY-1215 (targets HDAC 6) and panobinostat (broad acting), have shown promise for the treatment of MM with some inhibitors now in clinical trials, or used in the clinic [107-110]. However, to date, there

have been few studies investigating the effects of HDACi on MM bone disease. A novel broad acting hydroxamate HDACi, JNJ-26481585 (20 mg/kg/day via s.c. every other day), significantly reduced the formation of osteolytic lesions by more than 70% in a 5T2MM myeloma mouse model [111]. Reductions in both trabecular bone volume and number, along with reductions in the percentage of osteoclasts, were noted in mice treated with JNJ-26481585. Interestingly, the mineralizing surface and bone formation rate were increased in treated mice [111]. In subsequent studies, JNJ-26481585 (1.25 mg/kg via s.c. every other day) was tested in combination with bortezomib, a drug currently approved in the first line treatment for MM [109]. Effects on MM bone disease in the 5T2MM mouse model were assessed and the combination treatment was shown to cause a significant reduction in osteoclast numbers along with increased osteoblast numbers and increased trabecular bone volume [109].

In the context of MM and MM bone disease, HDACi would appear to have both chemotherapeutic and anti-resorptive potential, as they may be able to suppress both MM plasma cell proliferation and osteoytic bone lesions. However, it is important to determine appropriate dosages required not only to target the cancer cells, but also to suppress osteoclasts without deleterious effects on healthy bone metabolism in the absence of cancer cells. It may also be important to target the individual HDACs important in the different aspects of disease.

7. Conclusions

Numerous studies using both *in vitro* assays and animal models have shown that HDACi can have beneficial effects in the regulation of bone remodelling by suppressing osteoclast resorption and promoting osteoblast bone formation. *In vitro*, both broad acting and isozyme-selective inhibitors have been shown to regulate osteoclasts by targeting a number of key intercellular signalling mechanisms particularly in the RANKL/RANK pathway. In osteoblasts, HDACi have been demonstrated to promote osteoblast proliferation and mineralisation. Various

HDACi have also been revealed to reduce bone loss by targeting osteoclast cells in animal models of periodontitis, RA and myeloma bone disease. Translation of these pre-clinical findings to the clinic has however been limited most likely due to the complexity of physiological activities exhibited by different HDAC enzymes. It is likely that more isozyme-specific inhibitors, exemplified here to an extent by NW-21 and MS-275, will be needed to advance the development of HDACi as clinical therapies. Although their effects have only been observed in rodent models of bone loss to date, selective HDACi such as MS-275 and NW-21 have been found to suppress bone loss. The further development of these novel isozyme-selective HDACi is critical to gain an understanding of the roles that all HDACs play in physiological and pathological bone remodelling. Furthermore, this will be important in identifying which HDAC or HDACs should be targeted in a given disease. Another important finding is that many HDACi, both isozyme-selective and broad spectrum inhibitors, can be more beneficial *in vivo* at much lower doses than have often been investigated to date. It is important that compounds be reinvestigated in animal models such as those above at much lower, sub-toxic HDACi doses where they are likely to be better tolerated in chronic disease settings. More pharmacokinetic studies are also needed for many compounds to ensure that blood levels of HDACi do not exceed cytotoxic concentrations. As new selective inhibitors are developed and our understanding of their pharmacokinetics is improved, this area of epigenetic regulation is poised to reveal exciting new opportunities for therapeutic modulation of bone pathologies and other related inflammatory diseases.

Abbreviations

HDAC – histone deacetylase

HDACi – histone deacetylase inhibitors

TSA - trichostatin A

SAHA - suberanilohydroxamic acid

CTCL - T-Cell lymphoma

RA – rheumatoid arthritis

RANKL - receptor activator of nuclear factor kappa B ligand

TRAF-6 - tumour necrosis factor (TNF) receptor associated factor-6

NFATc1 - nuclear factor of activated T cells

siRNA - small interfering RNA

shRNA - short hairpin RNA

TRAP - tartrate resistant acid phosphatase

BMM bone marrow macrophages

PTH - parathyroid hormone

BMPs - bone morphogenetic proteins

ALP - alkaline phosphatase

SOST - sclerostin

MM – multiple myeloma

RASFs - rheumatoid arthritis synovial fibroblasts

TNF-α – tumour necrosis factor alpha

Acknowledgements

MC acknowledges the National Health and Medical Research Council (NHMRC) for a Peter Doherty Early Career Fellowship **(**1070880). DF acknowledges the National Health and Medical Research Council (NHMRC) for a Senior Principal Research Fellowship (1027369) and the ARC Centre of Excellence for Advanced Molecular Imaging (CE140100011).

Table 1: Classes and locations of zinc-containing HDAC enzymes

HDAC CLASS[a]	HDAC	CELLULAR LOCATION
Class I	HDAC 1	nucleus
Class I	HDAC 2	nucleus
Class I	HDAC 3	nucleus/cytoplasm
Class I	HDAC 8	nucleus/cytoplasm
Class IIa	HDAC 4	nucleus/cytoplasm
Class IIa	HDAC 5	nucleus/cytoplasm
Class IIa	HDAC 7	nucleus/cytoplasm
Class IIa	HDAC 9	nucleus/cytoplasm
Class IIb	HDAC 6	mainly cytoplasm
Class IIb	HDAC 10	mainly cytoplasm
Class IV	HDAC 11	nucleus

[a] Class III involves 7 HDACs that do not use a catalytic zinc but rather NAD^+ to effect lysine deacetylation.

Table 2: Effects of HDACi on osteoclasts and osteoblasts

HDACi	HDACs targeted	HDAC IC_{50}	Cell type	Effect shown	Dosage	Reference
1179.4b	Class I and II HDAC 1 HDAC 6	 48 nM 107nM	Osteoclasts – human peripheral blood mononuclear cells (PBMCs)	Suppressed osteoclast formation and activity via suppression of TRAF-6 and NFATc1	0.16-100 nM IC_{50}<0.16 nM	Cantley et al, 2011 [19]
vorinostat (SAHA)	Class I and II HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 4 HDAC 6 HDAC 7 HDAC 9 [112]	 68 nM 164 nM 48 nM 1524 nM 101 nM 90 nM 104 nM 107 nM	Primary bone marrow derived macrophages. Co-culture system with calvarial osteoblasts	Suppressed osteoclast formation in co-culture system. Suppressed osteoclastogenesis in BMMs.	300 nM	Kim et al, 2009 [13]
trichostatin (TSA)	Class I and II HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 4 HDAC 6 HDAC 7 HDAC 9 [112]	 2 nM 3 nM 4 nM 456 nM 6 nM 3 nM 5 nM 6 nM	Primary bone marrow derived macrophages. Co-culture system with calvarial osteoblasts	Suppressed osteoclast formation in co-culture system. Suppressed osteoclastogenesis in BMMs via suppression of c-fos.	10 nM	Kim et al, 2009 [13]
			Mouse bone marrow cultures and RAW-D macrophage cell line	Suppressed osteoclast differentiation (reduced number of TRAP positive (TRAP+) multinucleated cells) RAW-D cells – reduction in formation of TRAP+	5 nM, 10 nM, 20 nM (BMMs) 1 nM (RAW-D cells)	Rahman et al, 2003 [20]

				multinucleated cells. Reduced nuclear translocation of NF-KB p65.		
			C3H10T1/2 mouse mesenchymal cell line	Induced osteopontin expression	50 ng/ml TSA	Sakata et al, 2004 [67]
FR901228 (Romidepsin)	Class I HDAC 1 and 2 inhibitor HDAC 1 HDAC 2 HDAC 4 HDAC 6 [113]	 36 nM 47 nM 510 nM 14,000 nM	Rat bone marrow cells RAW-D cells	Dose dependent suppression of the formation of TRAP+ multinucleated cells. RAW-D – inhibited TRAP cell formation – stimulated IFN-β gene expression	0.2, 0.4, 0.6, 0.8, 1.6 ng/ml	Nakamura et al, 2005 [15]
sodium phenyl butyrate (NaB)	Class I HDACs HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 4	 8.3 μM 7 μM 4.8 μM 10.5 μM 5725 μM	Mouse bone marrow cultures and RAW-D macrophage cell line	Suppressed osteoclast differentiation (reduced number of TRAP+ multinucleated cells). RAW-D cells – reduction in formation of TRAP+ multinucleated cells.	0.5 mM 0.1 mM (RAW-D cells)	Rahman et al, 2003 [20]

	HDAC 5 HDAC 6 HDAC 7 HDAC 9 [114]	6403 μM 5881 μM 4380 μM 5614 μM	MC3T3-E1 cells Mouse bone marrow macrophages	Increased ALP production. Suppressed formation of TRAP+ multinucleated cells.	0.5 mM and 1 mM 0.5 mM	Iwami et al, 1993 [66]
MPT0G009	Class I and II HDACs HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 6 [21]	 4.6 nM 5.2 nM 1.9 nM 22 nM 8 nM	RAW264.7 macrophages	Suppressed TRAP cell formation via suppression of NF-kB and NFATc1.	5 nM	Hsieh et al, 2014 [21]
largazole	Class I selective HDAC 1 HDAC 6 [115]	 25 nM 5700 nM	C2C12 cells into osteoblasts	Increased expression of ALP and Osteopontin. Increased Runx2 expression at mRNA level.	Up to 50 nM	Lee et al, 2011 [69]
MS-275 (entinostat)	HDAC 1 Other HDACs [116]	181 nM >1000 nM needed	Osteoclasts – human PBMCs	Suppressed osteoclast formation and activity.	100 nM IC_{50}=54 nM	Cantley et al, 2011 [19]
			Mouse bone marrow-derived macrophages (BMMs)	Inhibited osteoclast formation in co-culture. Direct effect on osteoclast precursors – suppressed c-fos and NFATc1. Inhibited bone resorbing activity of mature osteoclasts.	20 nM, 50 nM, 100 nM	Kim et al, 2012 [44]

			MC3T3-E1 cells and primary osteoblasts	Stimulate cell proliferation and increased alkaline phosphatase (ALP) production.	500 nM	Schroeder, 2005 [17]
NW-21	HDAC 1 HDAC 2 Other HDACs [116]	21 nM 42 nM >300 nM	Osteoclasts – human PBMCs	Suppressed osteoclast formation and activity via suppression of NFATc1 and TRAF-6	0.16 nM-100 nM IC_{50} 3.6 nM	Cantley et al, 2015 [103]
2664.12	HDAC 6 Other HDACs [55] [116]	29 nM > 100 nM	Osteoclasts – human PBMCs	Suppress osteoclast activity.	20 nM and 100 nM IC_{50}>100 nM	Cantley et al, 2011 [19]

Table 3: Effects of HDACi in animal models with pathogenic bone loss.

Drug/Compound name	**HDACs targeted**	**HDAC IC_{50}**	**Disease type**	**Bone Effects**	**Dosages Tested**	**Reference**
1179.4b	Class I and II HDAC 1 HDAC 6	 48 nM 107 nM	Periodontitis – oral inoculation model in balb/c mice	Suppressed alveolar bone loss (assessed via micro computed tomography (CT) and histology). Reduced numbers of TRAP+ cells.	1mg/kg/day p.o. for 36 days (oral gavage)	Cantley et al, 2011 [90]
JNJ-26481585	Class I and II HDAC 1 HDAC 2 HDAC 10 HDAC 11 [117]	 0.11 nM 0.33 nM 0.46 nM 0.37 nM	5T2MM myeloma mouse model – therapeutic effects.	Reduced formation of osteolytic lesions by more than 70%. Reductions in both trabecular bone volume and number along with the percentage of osteoclasts.	5T2MM – treatment following serum paraprotein detection. 20 mg/kg, every other day, s.c. for 3.5	Deleu et a, 2009 [111]

				Mineralizing surface and bone formation rate both increased.	weeks.	
MPT0G009	Class I and II HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 6 [21]	 4.6 nM 5.2 nM 1.9 nM 22 nM 8 nM	Adjuvant induced arthritis (AIA)	Ameliorated bone destruction and prevented decrease in bone mineral density and bone mineral content.	25 mg/kg p.o.	Hseih et al, 2014 [21]
sodium phenyl butyrate (PB)	Class I HDACs HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 4 HDAC 5 HDAC 6 HDAC 7 HDAC 9 [114]	 8.3 μM 7 μM 4.8 μM 10.5 μM 5725 μM 6403 μM 5881 μM 4380 μM 5614 μM	AIA	Treated groups – no pannus formation or joint destruction	Topical application 1% and 10% PB ointment	Chung et al, 2003 [99]
trichostatin A (TSA)	Class I and II HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 4 HDAC 6 HDAC 7	 2 nM 3 nM 4 nM 456 nM 6 nM 3 nM 5 nM 6 nM	AIA	Treated groups – no pannus formation or joint destruction	Topical application 1% TSA ointment	Chung et al, 2003 [99]

	HDAC 9 [112]					
vorinostat (SAHA)	Class I and II HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 4 HDAC 6 HDAC 7 HDAC 9 [112]	 68 nM 164 nM 48 nM 1524 nM 101 nM 90 nM 104 nM 107 nM	Mouse collagen induced arthritis (CIA) – prophylactic treatment. Rat CIA - prophylactic treatment and therapeutic treatment.	Mouse - Assessed via radiological score on x rays, micro CT of tibae (systemic bone loss) and histology. At both doses (high and low) demonstrated some bone protective effects. Rat CIA – high dose Vorinostat attenuated bone erosion	High – 50mg/kg/day s.c. Low – 5mg/kg/day s.c. After 40 days Rat CIA High dose 50mg/kg/day p.o.	Lin et al, 2007 [7]
			C57BL/KaLwRij mice	No bone loss observed in naïve mice increase in number of osteogenic colonies, increased serum osteocalcin, increased osteoblast numbers in endocortical and trabecular bone surfaces	100mg/kg i.p. 3 times per week for 3 weeks	Xu et al, 2013 [78]
			Adjuvant induced arthritis (AIA)	Ameliorated bone destruction and prevented decrease in bone mineral density and bone mineral content.	200 mg/kg p.o. daily from day 2 to 21.	Hseih et al, 2014 [21]
			Rat CIA	Reduced osteoclast	1mg/kg s.c	Lohman et

				like cells in the joint (ED1+ cell) at 1mg/kg/day, but increased numbers with 5mg/kg/day.	daily 5mg/kg s.c daily	al, 2016 [25]
valproic acid (VPA)	Class I and II HDAC 1 HDAC 2 HDAC 3 HDAC 8 HDAC 6, 7, 9 [112]	 1.58 mM 3.068 mM 3.071 mM 7.442 mM > 10,000 mM	Mouse CIA	Histology revealed well-preserved proximal interphalangeal joints with negligible cartilage and bone destruction.	400mg/kg via i.p. starting on day 21 until day 60.	Saouaf et al, 2009 [100]
ITF-2357/ givinostat	Class I and II HDAC 1 HDAC 2 HDAC 3 HDAC 4 HDAC 6 HDAC 7 [112]	 28 nM 56 nM 21 nM 52 nM 27 nM 163 nM	Rat AIA Mouse CIA	Prophylactic and Therapeutic – suppressed joint destruction in both AA and CIA	10mg/kg p.o.	Joosten et al, 2011 [22]
FR901228 (romidepsin)	Class I HDAC 1 and 2 inhibitor HDAC 1 HDAC 2 HDAC 4 HDAC 6 [113]	 36 nM 47 nM 510 nM 14000 nM	AIA	Prophylactic treatment – suppressed disease development, no bone destruction. Therapeutic treatment – bone destruction less severe and number of TRAP+ cells was significantly	Prophylactic - 0.5 mg/kg Therapeutic – 1 mg/kg.	Nakamura et al, 2005 [15]

				decreased	intradermally injected into tail	
largazole	Class I selective HDAC 1 HDAC 6 [115]	 25 nM 5700 nM	A mouse calvarial bone formation assay and the rabbit calvarial bone fracture healing model	Mouse calvarial assay - induced woven bone formation, more significant at lower concentration than higher concentration. Rabbit calvarial fracture assay - macroporous biphasic calcium phosphate scaffold with largozole – newly formed bone with combination.	Mouse - Lower 10 μM vs. 50 μM. Collagen sponges with largazole 10 μM or 50 μM) Rabbit – 100nM or 250nM. Added to the bone graft substitute (10 mg).	Lee et al, 2011 [69]
MS-275 (entinostat)	HDAC 1 selective HDAC 1 (181 nM) Other HDACs [116]	 181 nM >1000 nM	Periodontitis – oral inoculation model in mice.	No effect on alveolar bone loss as assessed via micro CT and histology.	10 mg/kg/day p.o. for 36 days (oral gavage)	Cantley et al, 2011 [90]
			IL-1-induced mouse calvarial bone loss	Attenuated IL-1 induced bone destruction of calvaria.	2 mg/kg/day i.p for 8 days	Kim et al, 2012 [44]
			Mouse collagen induced	Mouse CIA - Assessed via radiological score on x	High dose – 10 mg/kg/day s.c.	Lin et al, 2007 [7]

			arthritis (CIA) - prophylactic treatment. Rat CIA - prophylactic treatment and therapeutic treatment.	rays, micro CT of tibae (systemic bone loss) and histology. Low doses – demonstrated strong anti-erosion effects. No bone erosion in high dose. Protection against systemic bone loss. Rats – 3 mg/kg – MS-275 prevented bone erosion. At 1 mg/kg – strongly suppressed bone erosion. Rat CIA therapeutic – at 5 mg/kg only slight reduction in bone erosion and joint narrowing evident.	Low dose – 3 mg/kg/day s.c. After 40 days Rat CIA 0.3, 1, 3 mg/kg/day s.c.	
NW-21	Class I Selective for HDAC 1 and 2 HDAC 1 HDAC 2 Other HDACs [116]	 21 nM 42 nM >300 nM	Collagen antibody induced arthritis (CAIA) mouse model	Suppressed bone destruction in radiocarpal joints (assessed via micro CT and histology). Significant reduction in numbers of TRAP+ cells on bone surface	5mg/kg/day p.o. for 10 days (oral gavage)	Cantley et al, 2015 [103]
BML-281	Selective HDAC 6 inhibitor HDAC 1 HDAC 2 HDAC 3	 271 nM 252 nM 0.42 nM	Rat CIA	Reduced osteoclast like cells in the joint (ED1+ cell) at 1mg/kg/day, but increased numbers with 5mg/kg/day.	1mg/kg s.c daily 5mg/kg s.c daily	Lohman et al, 2016 [25]

	HDAC 8 HDAC 6 HDAC 10	6851 nM 0.002 nM 90.7 nM				
NK-HDAC-1 S-(ε)- 3-(1-(1-(benzo[d]oxazol-2-yl)-2-methylpropyl)-1H- 1,2,3-triazol-4-yl)-N-hydroxyacrylamide	Unknown		Mouse CIA – therapeutic effects	Inhibited pannus formation. Suppression of joint erosion assessed via histology.	Treatment from day 26. 10 mg/kg and 30 mg/kg p.o. every other day for 24 days.	Li et al, 2013 [102]

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Highlights

- HDACi can regulate bone remodelling by suppressing osteoclasts and promoting osteoblasts via targeting key intercellular signalling molecules and transcription factors.
- HDAC isozymes can have differing roles in the differentiation and function of bone cells - osteoclasts, osteoblasts and osteocytes.
- Novel isozyme-specific HDACi are critical to elucidate HDAC roles in bone remodelling and the communication between bone cells.
- For non-malignancies, such as periodontitis and inflammatory arthritis, HDACi can be more beneficial *in vivo* at much lower doses.