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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

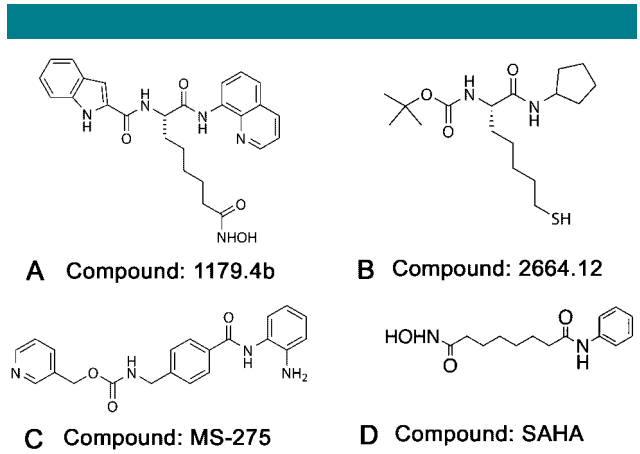

Fig. 1. Chemical structures of: (A) 1179.4b, which targets classes I and II HDACs (*S*)-*N*8-hydroxy-2-(1H-indole-2-carboxamido)-*N*1-(quinolin-8-yl)octanediamide (Compound #52; Kahnberg et al., 2006). B: 2664.12 which targets class II HDACs (*S*)-tert butyl 1-(cyclopentylamino)-7-mercapto-1-oxoheptan-2-ylcarbamate (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).

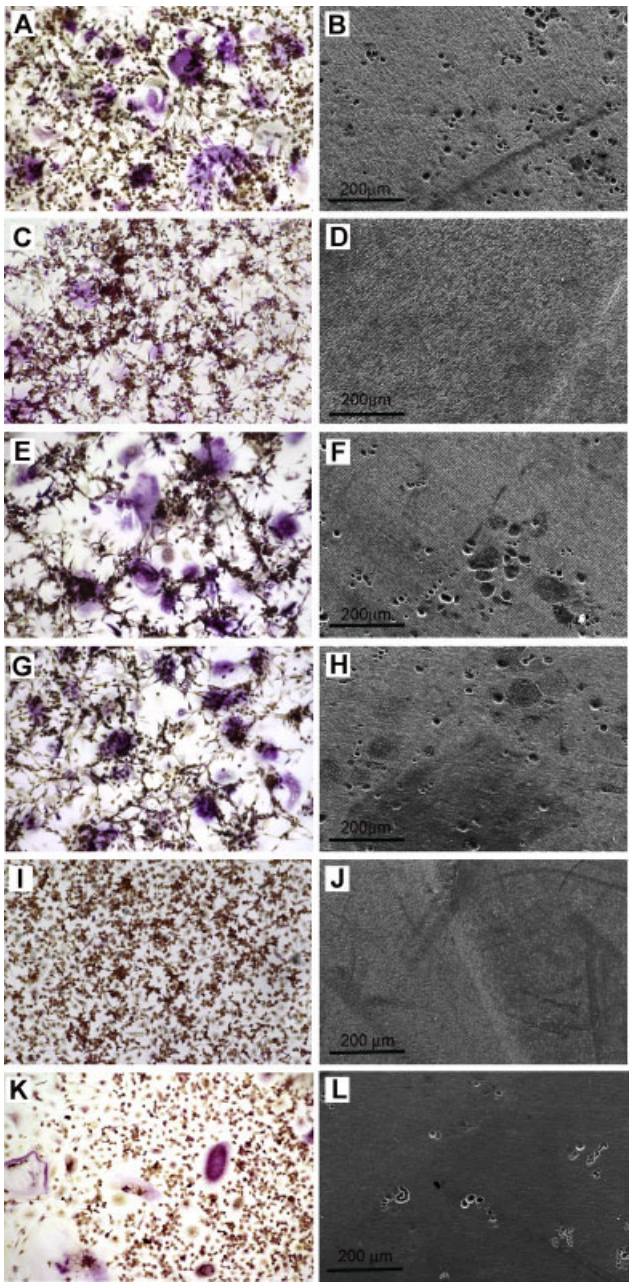

Fig. 2. The formation of TRAP expressing multinucleated cells (A,C,E,G,I,K) and resorption pit formation (B,D,F,H,J,L) were assessed as described in the methods. (A,B) No treatment; (C,D) treatment with 1179.4b at 20 nM; (E,F) treatment with MS-275 at 20 nM; (G,H) treatment with 2664.12 at 20 nM; (I,J)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

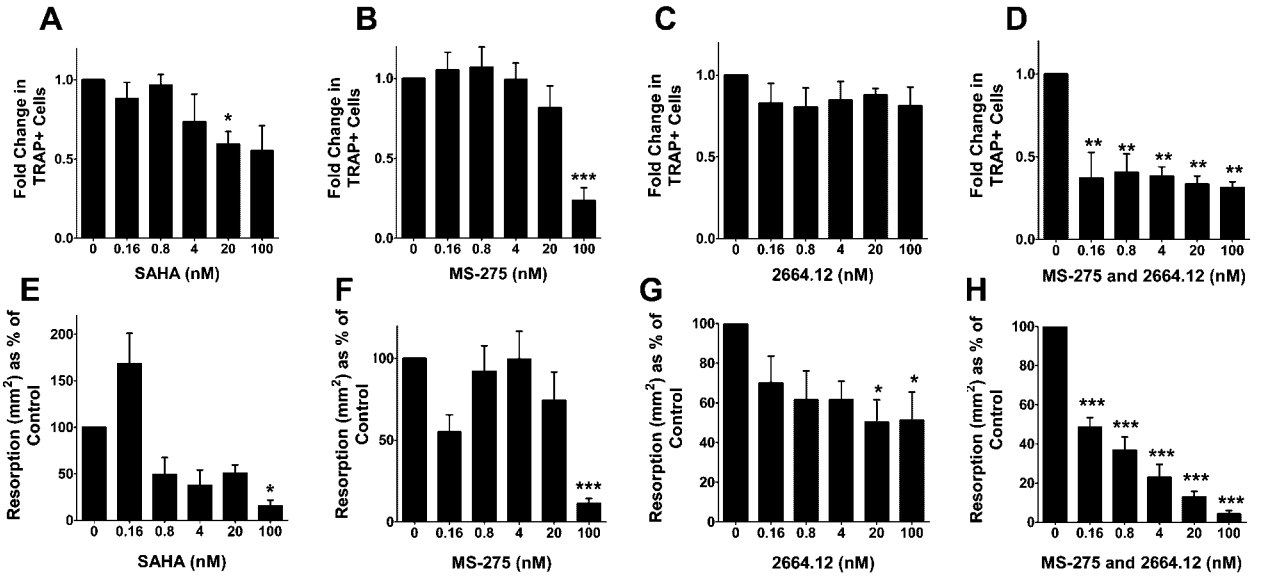

Fig. 3. A–D: Fold change in the number of multinucleated cells expressing TRAP with drug treatment. Cells with more than three nuclei expressing TRAP were classified as osteoclasts. (A) SAHA; (B) MS-275; (C) 2664.12; (D) MS-275 and 2664.12; (E–H) area of bone resorption with drug treatment expressed 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.

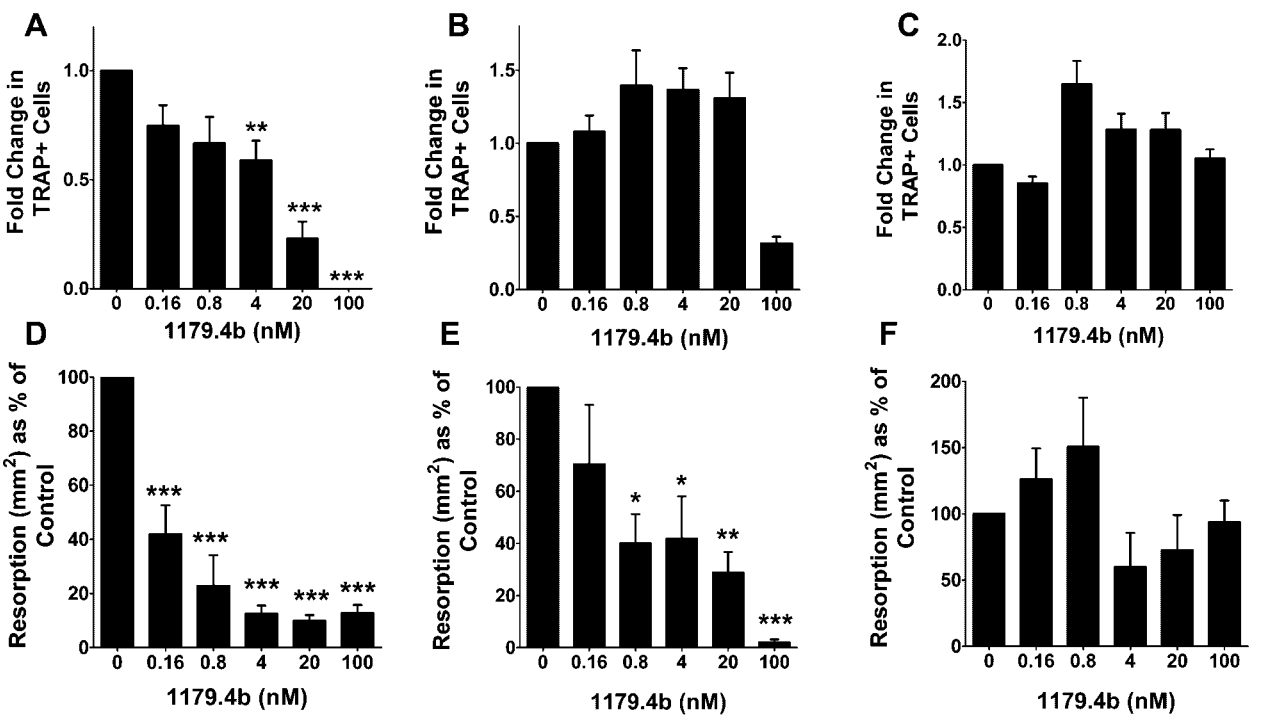

Fig. 4. A–C: Fold change in the number of multinucleated cells expressing TRAP with drug treatment. Cells with more than three nuclei expressing TRAP were classified as osteoclasts. (A) 1179.4b from day 7; (B) 1179.4b from day 10; (C) 1179.4b from day 13; (E,F) area of bone resorption with drug treatment 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.

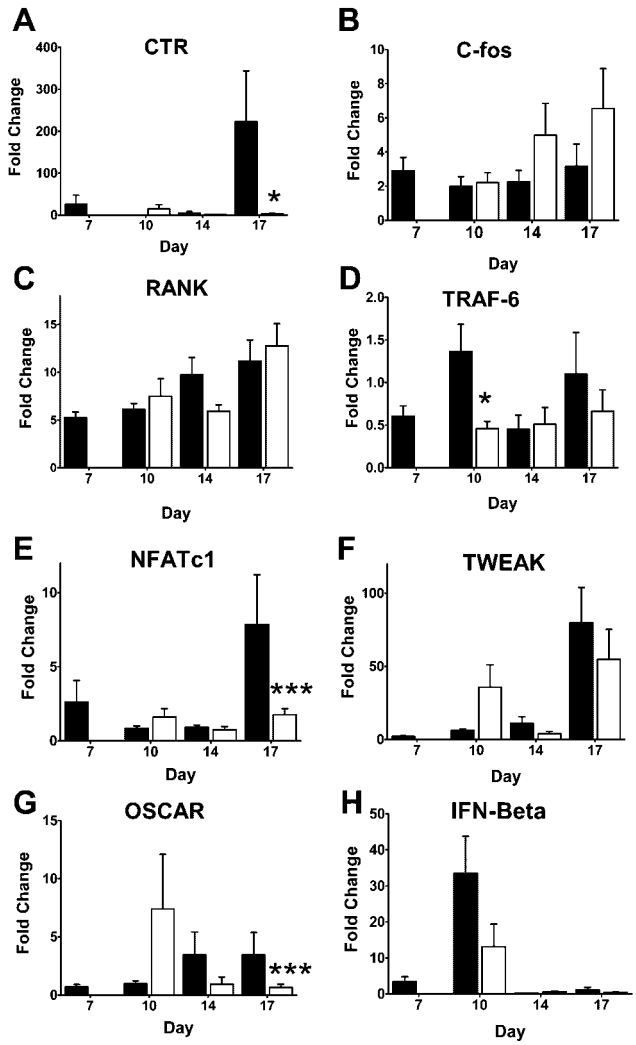

Fig. 5. Fold change in expression of osteoclast-related genes relative to day 0. Effect of treatment on expression with HDACi 1179.4b at 20 nM from day 7 onwards. Control cells—RANKL applied from day 7 but no drug treatment. (A) Calcitonin receptor (CTR) mRNA expression, (B) c-fos expression, (C) RANK 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.

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

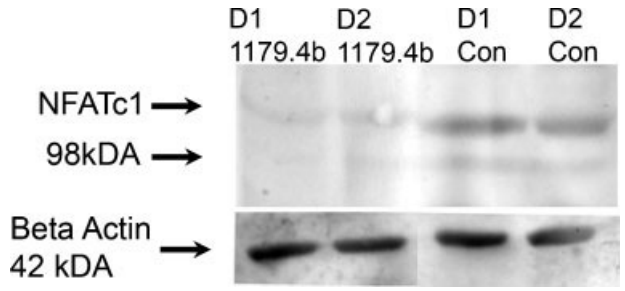

Fig. 6. Protein was extracted from 1179.4b-treated and controls cells and used for Western blot analysis as described in the Materials and Methods Section to detect NFATc1 protein at day 17. β-Actin is shown as the loading control, band at 42 kDA. D1 and D2 represent two different human donors.

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

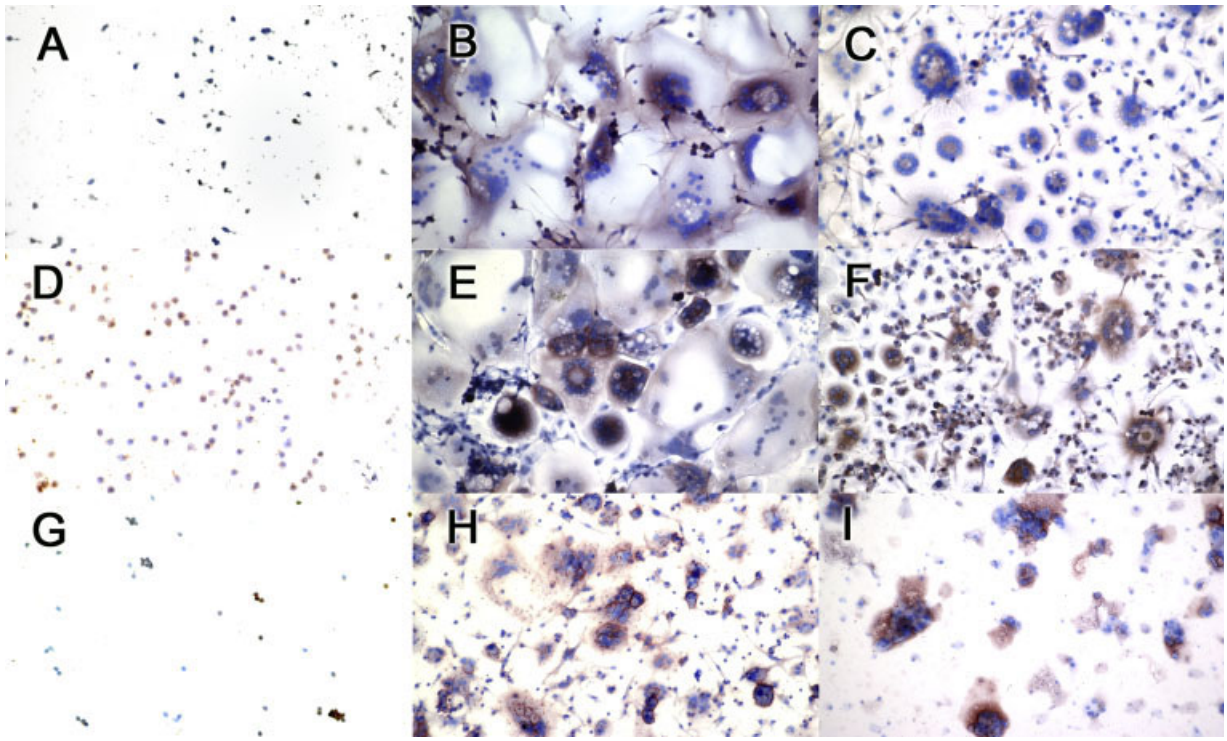

Fig. 7. Immunostaining to detect NFATc1 in (A) day 0 cells; (B) day 17 control cells; and (C) day 17 1179.4b-treated cells. To detect HDAC 5 (D) day 0 cells, (E) day 17 control cells, and (F) day 17 1179.4b-treated cells. To detect HDAC 8 (G) day 0 cells, (H) day 17 control cells, and (I) day 17 1179.4b-treated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

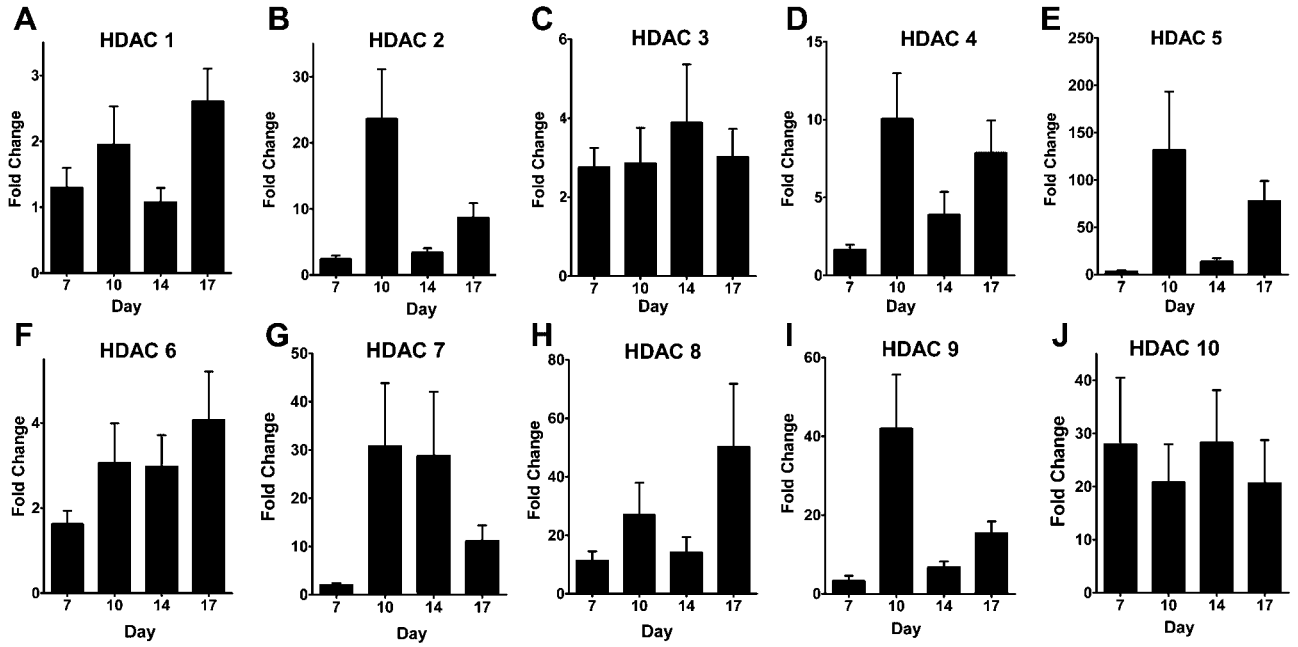

Fig. 8. Fold change in mRNA expression at days 7, 10, 14, and 17 during osteoclast development relative to day 0 for HDACs 1–10. (A) Class I HDAC 1, (B) class I HDAC 2, (C) class I HDAC 3, (D) class II HDAC 4, (E) class II HDAC 5, (F) class II HDAC 6, (G) class II DAC 7, (H) class I HDAC 8, (I) class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