Published in final edited form as:

Bone. 2011 May 1; 48(5): 1117–1126. doi:10.1016/j.bone.2011.01.007.

Suberoylanilide hydroxamic acid (SAHA; vorinostat) causes bone loss by inhibiting immature osteoblasts

Meghan E. McGee-Lawrence¹, Angela L. McCleary-Wheeler¹, Frank J. Secreto¹, David F. Ravidlo¹, Minzhi Zhang¹, Bridget A. Stensgard¹, Xiaodong Li¹, Gary S. Stein², Jane B. Lian², and Jennifer J. Westendorf¹

Meghan E. McGee-Lawrence: mcgeelawrence.meghan@mayo.edu; Angela L. McCleary-Wheeler: mcclearywheeler.angela@mayo.edu; Frank J. Secreto: secreto.frank@mayo.edu; David F. Ravidlo: razidlo.david@mayo.edu; Minzhi Zhang: zhang.minzhi@mayo.edu; Bridget A. Stensgard: stensgard.bridget@mayo.edu; Xiaodong Li: li.xiaodong@mayo.edu; Gary S. Stein: gary.stein@umassmed.edu; Jane B. Lian: jane.lian@umassmed.edu; Jennifer J. Westendorf: westendorf.jennifer@mayo.edu

¹ Mayo Clinic, Rochester, MN USA

² University of Massachusetts Medical School, Worcester, MA USA

Abstract

Histone deacetylase (Hdac) inhibitors are used clinically to treat cancer and epilepsy. Although Hdac inhibition accelerates osteoblast maturation and suppresses osteoclast maturation in vitro, the effects of Hdac inhibitors on the skeleton are not understood. The purpose of this study was to determine how the pan-Hdac inhibitor, suberoylanilide hydroxamic acid (SAHA; a.k.a. vorinostat or ZolinzaTM) affects bone mass and remodeling in vivo. Male C57BL/6 mice received daily SAHA (100 mg/kg) or vehicle injections for three to four weeks. SAHA decreased trabecular bone volume fraction and trabecular number in the distal femur. Cortical bone at the femoral midshaft was not affected. SAHA reduced serum levels of P1NP, a bone formation marker, and also suppressed tibial mRNA levels of type I collagen, osteocalcin and osteopontin, but did not alter Runx2 or osterix transcripts. SAHA decreased histological measures of osteoblast number but interestingly increased indices of osteoblast activity including mineral apposition rate and bone formation rate. Neither serum (TRAcP 5b) nor histological markers of bone resorption were affected by SAHA. P1NP levels returned to baseline in animals which were allowed to recover for four weeks after four weeks of daily SAHA injections, but bone density remained low. In vitro, SAHA suppressed osteogenic colony formation, decreased osteoblastic gene expression, induced cell cycle arrest, and caused DNA damage in bone marrow-derived adherent cells. Collectively, these data demonstrate that bone loss following treatment with SAHA is primarily due to a reduction in osteoblast number. Moreover, these decreases in osteoblast number can be attributed to the deleterious effects of SAHA on immature osteoblasts, even while mature osteoblasts are resistant to the harmful effects and demonstrate increased activity in vivo, indicating that the response of osteoblasts to SAHA is dependent upon their differentiation state. These studies suggest that clinical use of SAHA and other Hdac inhibitors to treat cancer, epilepsy or other conditions may potentially compromise skeletal structure and function.

Corresponding author: Jennifer J. Westendorf, Ph.D., Mayo Clinic, 200 First Street SW, Rochester, MN 55905, Phone: 507-538-5651, Fax: 507-284-5075, westendorf.jennifer@mayo.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Histone deacetylase inhibitor; Hdac; osteoblasts; Zolinza; γ H2AX

1. Introduction

Histone deacetylases (Hdacs) are crucial modulators of gene expression and chromatin structure. Hdacs condense chromatin and limit the accessibility of transcription factors and co-factors that regulate gene expression by removing acetyl groups from lysine residues in histones, thus manipulating transcription in an epigenetic manner [1]. Hdacs can also deacetylate non-histone proteins [2–4] such as the transcription factors Runx2 [5], p53 [6,7], and Stat3 [8], making them less stable and/or reducing their nuclear localization. Compounds aimed at altering epigenetic-regulating enzymes like Hdacs are being rapidly developed as new clinical therapies. Pharmaceutical Hdac inhibitors including valproate and suberoylanilide hydroxamic acid (SAHA; vorinostat, ZolinzaTM) are already used to treat epilepsy, bipolar disorder, and cancer [9,10]. Research aimed at expanding the usage of these and other Hdac inhibitors for treating a wide variety of diseases or clinical conditions (e.g., rheumatoid arthritis, traumatic brain injury, cystic fibrosis) is ongoing [11–14]. In October 2010, there were more than 140 ongoing clinical trials testing the therapeutic effects of vorinostat and other Hdac inhibitors [15].

There is strong evidence that Hdacs contribute to the development and maintenance of the bone cells and tissues [15,16]. Hdac inhibitors caused osteoclast apoptosis [17] but promoted osteoblast differentiation and maturation in vitro [18,19]. Treatment of murine cell lines or primary calvarial osteoblasts with the pan-Hdac inhibitor trichostatin A (TSA) increased matrix mineralization, alkaline phosphatase activity, Runx2-mediated transcriptional activity, and expression of osteoblastic genes including Runx2, alkaline phosphatase, osteopontin, and osteocalcin [18,19]. Similarly, treatment of human mesenchymal stem cells with valproate, sodium butyrate, or TSA during osteogenic induction dose-dependently increased calcium deposition and upregulated expression levels of osteoblastic genes [20,21]. Hdacs 3, 4, 6, and 7 bound and inhibited transcriptional activation by Runx2 [22–24]. Suppressing individual Hdacs with RNAi, as was done for Hdac1 [25], Hdac3 [22], Hdacs 4/5 [5,26], and Hdac7 [24], enhanced osteoblast maturation in vitro. Moreover, genetic deletion of the class II Hdacs *Hdac4* or *Hdac6*, increased bone density by promoting endochondral ossification and trabecular bone formation [27,28]. Most recently, *Hdac5* was identified as a new locus affecting BMD in a genome-wide association study (GWAS) [29], and levels of Hdac5 were found to be elevated in two juveniles with primary osteoporosis. Increasing Hdac5 levels (via antagonizing a natural Hdac5 repressor, miR-2861) decreased bone formation and caused bone loss in animal models [30]. Paradoxically, genetic ablation of class I Hdacs has deleterious effects on bone formation. In mouse models, deletion of *Hdac8* was detrimental to skull bone formation [31]. Likewise, our laboratory recently generated a mouse line in which *Hdac3* was conditionally deleted in cells of the osteoblastic lineage. These mice are severely osteopenic due to a reduction in bone formation rate, decreased osteoblast number, and increased marrow adipocyte number compared to wildtype mice [32]. Together, these data indicate that several Hdacs are important regulators of bone metabolism.

Biochemical studies indicate that class I Hdacs (Hdacs 1, 2, 3, and 8) are the primary targets of existing pan Hdac inhibitors because of structural features of their enzymatic pockets and because the deacetylase domain of class II Hdacs is not necessary for their activity [33,34]. Small molecule Hdac inhibitors include hydroxamic acids (e.g., SAHA, trichostatin A), cyclic peptides (e.g., depsipeptide, apicidin), benzamides (MS-275), and short-chain fatty

acids (e.g., sodium butyrate and valproate). Only valproate and SAHA are currently FDA-approved for use in the United States. Long-term administration of valproate for the treatment of epilepsy or mood disorders is associated with a reduction in bone mineral density and increased fracture risk [35–37], and children born to mothers treated with valproate are susceptible to developing craniofacial bone defects [38]. In animal models, the adverse effects of valproate on bone appear dependent on an unknown genetic component [39]. The mechanisms behind valproate's induction of bone loss *in vivo* are unclear due to conflicting reports of its effects on bone formation and bone resorption [35,36,40–42]. Furthermore, valproate can inhibit other enzymes (e.g., succinate semialdehyde-dehydrogenase and -reductase) [43,44], thus it is uncertain that its capacity to stimulate bone loss *in vivo* is specific to deacetylase inhibition.

In this study we sought to determine the consequences of another clinically-relevant histone deacetylase inhibitor, SAHA (vorinostat or ZolinzaTM), on bone mass and bone turnover *in vivo*. SAHA is a synthetic compound that belongs to different chemical class than valproate and is FDA-approved to treat cutaneous T cell lymphomas [45]. SAHA caused trabecular bone loss in C57BL/6 mice by decreasing osteoblast number, even while increasing the activity levels of existing osteoblasts, but did not affect cortical bone structure. SAHA induced DNA damage and cell cycle arrest of bone marrow stromal cells (BMSCs), and consequently suppressed osteogenic colony formation *in vitro*. These results provide further evidence that inhibition of class I Hdacs is largely detrimental to the trabecular skeleton, possibly due to negative effects on highly proliferating and metabolically active progenitor cells. Our results also provide insights into understanding the disparity between previously reported *in vitro* and *in vivo* effects of Hdac inhibitor function.

2. Materials & Methods

2.1 *In vivo* studies

2.1.1 Animals—The Mayo Clinic Institutional Animal Care and Use Committee approved all handling and experimental procedures. Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were maintained on a 12-h light / 12-h dark cycle, and were permitted *ad libitum* access to food and water.

2.1.2 Treatment procedures and tissue collection—SAHA was obtained from the Cancer Therapy Evaluation Program (CTEP) at the National Cancer Institute (NCI). In a pilot experiment, animals (n = 8/group) received intraperitoneal injections of 100 mg/kg/d SAHA or vehicle (10% DMSO / 45% PEG400 in water) for three weeks beginning at six weeks of age (Figure 1A). In the second experiment, mice were treated with 100 mg/kg/d SAHA or vehicle for four weeks beginning at seven weeks of age (n = 20 SAHA, 20 vehicle). Dosage, delivery schedule, and administration route were chosen based on previous experiments by our group [46] and are consistent with previous studies demonstrating SAHA's anti-cancer effects in mice [47, 48]. Animals were weighed daily. All mice in the pilot experiment and one-half of the animals in the second experiment were sacrificed 24 hours following the last SAHA injection ("*Treatment*" groups). The remaining animals (n = 10 per group) in the second experiment were allowed to recover for four weeks with no additional treatment injections prior to sacrifice ("*Recovery*" group) (Figure 1B). Mice received subcutaneous injections of calcein (10 mg/kg) five days and 24 hours before euthanasia to label mineralizing bone surfaces. Mice were sacrificed by carbon dioxide asphyxiation, and terminal serum samples were collected at sacrifice. Right femurs were fixed in 10% neutral buffered formalin and stored in 70% ethanol. Left tibias and spleens were flash frozen in liquid nitrogen and stored at –80 °C.

2.1.3 Histone 3 acetylation—Spleen explants were minced, placed in modified RIPA buffer on ice, and sonicated to generate protein extracts that were resolved by SDS-PAGE. Western blotting was performed with antibodies recognizing acetylated histone 3 (1:2000, Millipore, #06-599) and actin (1:1000, Santa Cruz, I-19 SC-1616).

2.1.4. Serum bone remodeling markers—Circulating levels of procollagen type 1 amino-terminal propeptide (P1NP; bone formation marker) and tartrate-resistant acid phosphatase 5b (TRAcP5b; bone resorption marker) were measured with colorimetric assays (Rat/Mouse P1NP EIA #AC-33F1, Immunodiagnostic Systems, Fountain Hills AZ; Mouse TRAP ELISA #SB-TR103, Immunodiagnostic Systems, Fountain Hills AZ). All samples were tested in duplicate within each assay.

2.1.5 Bone structure and mineralization in the femur—Bone architecture and mineralization were evaluated in the right femur using micro-computed tomography. The central portion of the femoral diaphysis (0.7 mm long) and distal femoral metaphysis (1.6 mm to 0.9 mm proximal to the growth plate) of each bone were scanned in 70% ethanol on a μ CT35 scanner (Scanco Medical AG, Basserdorf, Switzerland) at 7 μ m resolution. Tissue volume (TV, mm³; entire volume within periosteal border), cortical bone volume (Ct. BV, mm³; volume enclosed between periosteal and endocortical borders), cortical bone thickness (Ct.Th, mm), cortical bone material mineral density (Ct.Mat.Mn.Dn, mg hydroxyapatite (HA)/ccm), trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N, mm⁻¹), trabecular thickness (Tb.Th, mm), and trabecular separation (Tb.Sp, mm), were computed using the manufacturer's software [49].

2.1.6 Trabecular bone remodeling indices in the distal femur—Thin (5 μ m) trabecular bone sections were prepared from the right distal femoral metaphysis. Sections were mounted unstained for dynamic histomorphometry, stained with VonKossa/MacNeal's tetrachrome to highlight osteoblast surfaces, or stained with TRAP/Fast green for identification of osteoclast surfaces. Slides were digitized using a microscope and digital camera, and analyzed using image analysis software (OsteoMeasures, Osteometrics Inc., Decatur, GA). Histomorphometric remodeling indices were measured in an area of tissue (1.5 mm²) beginning 450 μ m proximal to the growth plate. Mineralizing surface (MS/BS, %), mineral apposition rate (MAR, μ m/day), bone formation rate (BFR/BV, %/day), osteoblast surface/bone surface (Ob.S/BS, %), osteoblast number/bone perimeter (N.Ob/B.Pm, #/mm), osteoclast surface/bone surface (Oc.S/BS, %), and osteoclast number/bone perimeter (N.Oc/B.Pm, #/mm) were quantified at 200X or 400X magnification in the secondary spongiosa [50].

2.1.7 Gene expression in the tibia—Whole tibias were homogenized in TRIzol using a Spex liquid nitrogen freezer mill (Model #6750, Edison, NJ). RNA was extracted and purified from the ground tissue with TRIzol reagent (Invitrogen) according to the manufacturer's protocol and was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Expression levels of mRNAs associated with osteoblast maturation (Runx2, osterix, type I collagen, alkaline phosphatase, osteopontin, and osteocalcin) and various candidate pathways were quantified using real-time PCR. Primer sequences are reported in Table 1. Real time reactions were performed using 37.5 ng of cDNA per 15 μ l with Bio-Rad iQ SYBR Green Supermix and the Bio-Rad MyiQ Single Color Real-Time PCR Detection System. Transcript levels were normalized to the reference gene Ywhaz. Gene expression levels were quantified using the 2^{-($\Delta\Delta$ Ct)} method [51].

2.2 In vitro studies

2.2.1 BMSC isolation and plating—Bone marrow was flushed from femurs and tibias of untreated C57BL/6 mice. Cells were immediately seeded into 6-well plates at 1×10^7 cells per well or on glass coverslips at 4×10^6 cells per coverslip in either regular culture medium (alpha-MEM, 20% FBS, 1% antibiotic/antimycotic (Invitrogen #15240-062), 1% non-essential amino acids (Mediatech Inc., #25-025-Cl)) or culture medium supplemented with 50 µg/ml ascorbic acid, 10 mM beta glycerol phosphate, and 10^{-8} M dexamethasone (“osteogenic medium”). Media were changed every three days after seeding. BMSCs were identified by adherence.

2.2.2 BMSC mineralization assay—BMSCs seeded in osteogenic medium were cultured for 28 days to promote calcified matrix production. SAHA (1 µM) or DMSO vehicle was present in the medium at different time points to compare SAHA’s effects on immature and mature osteoblast-lineage committed cells, respectively. Cells were fixed in 10% neutral buffered formalin and stained with 2% alizarin red. Calcified matrix production was quantified as the percentage of alizarin red positive area within each well using image analysis software (Bioquant Osteo, Nashville, TN)

2.2.3 BMSC gene expression—BMSCs seeded in regular culture medium were expanded for 10 days and then switched to osteogenic medium supplemented with DMSO vehicle or 1 µM SAHA. RNA was harvested with TRIzol after 3 or 7 days of osteogenic differentiation. RNA was reverse transcribed and gene expression was quantified as described above.

2.2.4 Cell cycle analysis—BMSCs seeded in regular culture medium were expanded for 10 days, and then incubated in reduced-serum culture medium (2% FBS) for 18 hours to promote cell cycle synchronization. Cells were then placed in culture medium (20% FBS) containing DMSO vehicle or 1 µM SAHA for 48 hours, after which they were harvested by trypsinization, washed with ice-cold PBS, and fixed with 85% ethanol. DNA was labeled with 50 µg/ml propidium iodide in the presence of 100 µg/ml RNase A for 30 minutes. Cell cycle distribution was analyzed by flow cytometry (FACSCalibur).

2.2.5 DNA damage—BMSCs seeded in regular culture medium on glass coverslips were expanded for 10 days and then incubated in culture medium containing DMSO vehicle or SAHA (1 µM) for 48 hours. Cells were rinsed with PBS, fixed with a 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT), washed three times with PBS, and permeabilized with 0.2% Triton-X in PBS for 10 minutes. Cells were then incubated with 5% milk in PBS for 1 hour at RT to reduce non-specific binding, followed by immunostaining with an anti-γH2AX-Alexa Fluor® 488 conjugated antibody (Biolegend #613405, San Diego, CA; 1 µg/ml) for 1 hour at RT in the dark. After three washes in PBS, cells were refixed with 4% PFA for five minutes, washed twice with deionized water, and mounted with Vectashield containing DAPI (Vector Laboratories Inc., Burlingame, CA). Cells were viewed using a confocal microscope (Zeiss LSM 510). DNA damage was quantified by assessing the percentage of cells demonstrating nuclear γH2AX staining and the percentage of γH2AX-positive foci within each cell using image analysis software (Bioquant Osteo, Nashville TN). More than 100 cells were measured in each condition.

2.3 Statistics

Bone properties, serum remodeling markers, and histomorphometric remodeling indices were compared within each in vivo experiment phase between vehicle- and SAHA-treated mice with a student’s t-test. A significance of $p < 0.05$ was used for all comparisons.

3. Results

3.1 SAHA increased histone 3 acetylation and induced a temporary decrease in body mass

To determine the effects of SAHA on the skeleton, mice ($n = 7$ to 10 per group) were injected with SAHA or vehicle daily for three to four weeks (Figure 1). During the course of the 3-week study, one mouse (SAHA-treated) died after nine days of treatment from unknown causes. No other unanticipated deaths occurred in either study. In both experiments, SAHA reduced normal weight gain (Figure 2A–B). These results are consistent with weight loss observed in human patients and other animal models receiving vorinostat therapy [52–54]. Following four weeks of recovery, there were no differences in body mass between groups (vehicle body mass: 28.0 ± 1.1 g, SAHA body mass: 27.1 ± 1.8 g, $p = 0.200$) (Figure 2B). SAHA-treated mice had increased levels of acetylated histone 3 (Ac-H3) as compared to vehicle-treated mice (Figure 2C), indicating that histone deacetylase activity was suppressed.

3.2 SAHA caused trabecular but not cortical bone loss

The effects of SAHA on trabecular and cortical bone were assessed by micro-computed tomography. Trabecular bone volume fraction was lower in mice receiving SAHA for three weeks as compared to vehicle-treated mice (Table 2). Four weeks of SAHA administration significantly decreased (-22% , $p = 0.0002$) the bone volume fraction (BV/TV) as compared to vehicle-treated mice. This reduction in bone volume fraction was due to decreased trabecular number (Tb.N); trabecular thickness (Tb.Th) was unaffected (Table 2). No differences in cortical bone properties were detected between SAHA- and vehicle-treated mice after either three or four weeks of treatment (Table 3).

3.3 SAHA decreased osteoblast number but increased localized osteoblast activity in vivo

The effects of SAHA on osteoblast numbers and activity were measured by static and dynamic histomorphometry. Four weeks of SAHA treatment decreased the overall osteoblast population in the distal femoral metaphysis. Both osteoblast surface (Ob.S/BS) and osteoblast number (N.Ob/B.Pm) were significantly decreased (-35% and -38% , respectively, $p < 0.036$) in SAHA treated mice as compared to controls (Table 4). Osteoclast surface (Oc.S/BS) and osteoclast number (N.Oc/B.Pm) were also lower in SAHA-treated mice, although these differences did not reach statistical significance ($p < 0.15$; Table 4). Interestingly, both mineral apposition rate (MAR) and bone formation rate (BFR/BV) were increased in mice receiving SAHA for three or four weeks as compared to vehicle-treated mice (Table 4). Mineralizing surface (MS/BS) was not affected by SAHA treatment (Table 4).

3.4 SAHA decreased levels of P1NP, a circulating marker of bone formation

Serum levels of P1NP, a marker of bone formation, were lower (-23% to -32%) in mice receiving SAHA for three or four weeks as compared to vehicle-treated mice (Figure 3A–B). There were no differences in serum TRAcP5b levels (bone resorption/osteoclast number marker) between vehicle- and SAHA-treated mice (Figure 3C). These data are consistent with the histomorphometry results and suggest that prolonged exposure to SAHA progressively decreases trabecular bone volume by decreasing type I collagen synthesis or secretion.

3.5 SAHA-induced bone loss may be reversible

To determine if SAHA-induced bone loss is reversible, a group of mice in experiment 2 were allowed four weeks of recovery after one month of SAHA injections. Most bone structural properties including bone volume fraction, trabecular number, and trabecular

apparent mineral density remained lower ($p < 0.10$) in the SAHA-treated mice as compared to vehicle-treated mice (Table 2). As with the “*Treatment*” phase of the study, there were no differences in trabecular thickness or any cortical bone properties when comparing the recovery groups (Tables 1 and 2). However, four weeks after ceasing SAHA treatment, P1NP levels were comparable between groups (Figure 3B), suggesting a recovery of bone formation activity.

3.6 SAHA reduced expression of mature osteoblast genes

The effects of SAHA on gene expression in vivo were assessed using mRNA isolated from whole tibias. As shown in Figure 4, SAHA did not alter the expression Runx2, osterix or alkaline phosphatase. However, SAHA reduced type 1 collagen, osteopontin and osteocalcin expression by 50 to 90% in vivo. Other osteoblast genes, namely RANKL, osteoprotegerin, Axin2, Sost, Sphk1, and p21 were not substantially altered by SAHA (data not shown).

3.7 SAHA decreased osteogenic colony formation of immature mouse BMSCs, but not that of osteogenically differentiated BMSCs

To gain a better understanding of how SAHA reduces osteoblast numbers while also enhancing osteoblast activity, we added SAHA to in vitro CFU-Ob assays for three or seven days at various times during the course of a 28-day experiment (Figure 5A). Osteogenic colony formation and calcified matrix production were substantially decreased when SAHA was added to the cultures during the first two weeks (Figure 5B–5E). Examination of these cultures under a microscope revealed a reduction in both cell numbers and matrix accumulation following SAHA treatment, suggesting cell death was likely a contributing factor in the overall decrease in matrix production. In contrast, BMSCs exposed to SAHA later in the experiments were resistant to or modestly stimulated by the drug (Figure 5B–5E).

Consistent with the in vivo data (Figure 4), early and continuous exposure to SAHA did not alter the expression of Runx2 in the BMSC cultures, but reduced type I collagen, alkaline phosphatase, and osteocalcin (Figure 6). In contrast to what was observed in tibial extracts, osteopontin mRNA expression was not affected by SAHA in BMSC cultures. SAHA increased osterix expression in BMSCs.

3.8 SAHA caused DNA damage in mouse BMSC

To determine how SAHA affected BMSCs, we analyzed the cell cycle distribution and DNA integrity in BMSCs exposed to SAHA for 48 hours. SAHA increased the number of cells in the S and G2/M phases of the cell cycle (Figure 7A and 7B). SAHA also induced DNA damage in mouse BMSCs, as evidenced by an increase in both percentage γ H2AX positive nuclei (Figure 7D and 7E) and γ H2AX positive nuclear area (Figure 7D and 7F). Together these data demonstrate that SAHA induces DNA damage and causes cell cycle arrest of BMSCs.

4. Discussion

Hdacs are promising new targets for medical therapies, especially for reducing tumor burden. Their ability to alter gene expression through epigenetic and cell signaling pathways provides opportunities to reactivate silenced genes or direct cell fate. However, because Hdacs are widely expressed throughout the body, usage of broad-spectrum Hdac inhibitors might have non-specific consequences. Indeed, widely reported side effects of SAHA in cancer clinical trials include fatigue, thrombocytopenia, nausea, diarrhea, and weight loss [54–56]. SAHA treatment increases lysine acetylation of not only histone proteins, as we observed in this study, but also other molecular targets including transcription factors and

proteins involved in cell structure, cell cycle, and differentiation [4]. The long-term effects of SAHA on continuously regenerating organs like bone remain unknown. A key finding of the present studies is that SAHA caused trabecular bone loss by reducing the number of osteoblasts on trabecular bone surfaces in adolescent male C57BL/6 mice.

Several lines of evidence from this study support the conclusion that SAHA-induced trabecular bone loss was due to direct effects on osteoblasts. First, serum P1NP levels were lower in SAHA-treated mice. Osteoblasts secrete P1NP, a procollagen cleavage fragment, when they produce osteoid during bone formation. The reduced P1NP levels following SAHA treatment were complemented by gene expression data showing that type I collagen expression was downregulated in the tibia from SAHA-treated mice as well as in BMSC cultures. Second, decreases in osteoblast number and osteoblast surface were seen histologically and were consistent with the reduced expression of osteocalcin and osteopontin in bone tissue and BMSC cultures. Neither osterix nor Runx2 expression was decreased by SAHA treatment in vitro or in vivo, suggesting that osteoblasts may be most susceptible to the deleterious effects of SAHA after the onset of expression of these genes. SAHA induced DNA damage and cell cycle arrest in undifferentiated mouse BMSC, which is consistent with previous literature showing that human MSCs treated with Hdac inhibitors exhibited decreased self-renewal capacity [20], decreased cell number [57], and increased DNA damage, leading to cell cycle arrest and apoptosis [58,59].

Although our results indicate that SAHA decreases net bone mass by targeting the immature subpopulation of Runx2- and osterix- expressing osteoblasts, some gene expression data are inconsistent with this conclusion and require further investigation. For example, SAHA reduced alkaline phosphatase expression in vitro but not in vivo. The discrepancy may be due to the fact that other cell types in the marrow (e.g., B-cells [60]) produce alkaline phosphatase. Another example is *Sost*. Unlike late differentiation osteoblast markers (e.g., osteocalcin), *Sost* (a gene expressed in osteocytes, which are terminally differentiated osteoblasts) was unchanged in SAHA treated subjects. One explanation for this result may be that cortical bone, which is where the majority of *Sost*-expressing osteocytes reside, was unaffected by SAHA treatment (Table 3). Alternatively, SAHA may increase the expression of the *Sost* promoter and/or enhancer by increasing histone or transcription factor acetylation. Increased *Sost* expression on a per cell basis may compensate for reduced osteocyte numbers and result in no net change in *Sost* mRNA levels. Although our data show that SAHA does not affect BMSCs that were cultured with osteogenic stimuli for more than 2 weeks (Fig 5), understanding the direct effects of SAHA on osteocytes and *Sost* regulatory elements requires further investigation.

Despite reducing osteoblast number in vivo, SAHA surprisingly increased local mineral apposition and bone formation rates (Table 4). These data suggest that SAHA stimulated the activity of mature osteoblasts already present in the animal. These changes bear a notable similarity to effects reproducibly seen following exposure of osteoblast lineage-restricted cell populations to Hdac inhibitors in vitro. Many groups reported that in vitro treatment of committed osteoblast-like cell lines with Hdac inhibitors increases osteoblastic differentiation and measures of osteoblast activity like matrix calcification [18,20,21,61]. Unlike the strong stimulatory effect of Hdac inhibitors on calcified matrix production reported in previous in vitro studies [20,21], we observed modest increases in matrix production by BMSC treated with SAHA after prolonged culture with osteogenic stimuli (Fig 5). Differences between results in various laboratories are likely explained by technical factors such as specific time courses, treatment regimens, and Hdac inhibitor chemical classes. Regardless, both the current study and previous literature [20,58,59] support the idea that SAHA suppresses an early subpopulation of osteoblast lineage cells. In vivo, this effect over time would overshadow any increases in localized activity of more differentiated

populations of osteoblasts (that are not negatively affected by SAHA), causing bone loss due to a decrease in osteoblast number (Tables 2 and 4).

SAHA is FDA-approved to treat cutaneous T cell lymphomas and is currently in dozens of clinical cancer trials throughout the world. Our results suggest that bone density and fractures should be monitored in cancer patients receiving vorinostat therapy. In independent investigations, we found that SAHA is effective at slowing the growth of breast and prostate cancers in the skeleton [46]. In these studies, SAHA decreased bone density and trabecular bone mass in both tumor-bearing and tumor-free immunocompromised mice. Our *in vivo* results are also consistent with reports of decreased bone mineral density and increased fracture risk following long-term administration of another widely-used, clinically-approved Hdac inhibitor, valproate [37,42,62–65]. Valproate reduced bone mineral content of the total femur in young Wistar rats [66], and lowered trabecular bone volume fraction and trabecular number in the proximal tibia of C3H/HeJ mice [39]. In humans, prolonged exposure to valproate decreases bone mineral density in both axial and appendicular sites in children and adults [65,67,68], leading to an increased risk of fracture [37]. Thus it appears that both the hydroxamic acid SAHA and the short-chain fatty acid valproate cause bone loss *in vivo*. Bone loss observed following SAHA exposure might be reversible, as we observed a recovery of circulating PINP levels after ceasing SAHA treatment (Figure 3B). Although structural measures of bone density were not fully recovered in four weeks, there were indications of bone regeneration during this time period. This is consistent with epidemiological studies in epileptic patients showing that fracture risk decreases after stopping valproate therapy [69].

In conclusion, our data demonstrate that SAHA causes bone loss *in vivo* by reducing osteoblast number, even while increasing activity of pre-existing mature osteoblasts. The results support the notion that Hdac inhibitors negatively affect the proliferation and survival of immature osteoblasts, which explains the bone loss observed both in our study and in patients receiving Hdac inhibitor therapy. These data underscore the need to closely monitor bone mass in these patients.

Acknowledgments

The NIAMS supported this work (R01 AR48147, T32 AR056950, F32 AR60140). The authors thank the Mayo Clinic Biomaterials and Quantitative Histomorphometry Core Laboratory for their help with preparation of the histomorphometry specimens.

References

1. Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet.* 2009; 10:32–42. [PubMed: 19065135]
2. Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. *Gene.* 2005; 363:15–23. [PubMed: 16289629]
3. Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science.* 2009; 325:834–40. [PubMed: 19608861]
4. Zhou Q, Chaerkady R, Shaw PG, Kensler TW, Pandey A, Davidson NE. Screening for therapeutic targets of vorinostat by SILAC-based proteomic analysis in human breast cancer cells. *Proteomics.* 2010; 10:1029–39. [PubMed: 20049865]
5. Jeon EJ, Lee KY, Choi NS, Lee MH, Kim HN, Jin YH, Ryoo HM, Choi JY, Yoshida M, Nishino N, Oh BC, Lee KS, Lee YH, Bae SC. Bone morphogenetic protein-2 stimulates Runx2 acetylation. *J Biol Chem.* 2006; 281:16502–11. [PubMed: 16613856]

6. Juan LJ, Shia WJ, Chen MH, Yang WM, Seto E, Lin YS, Wu CW. Histone deacetylases specifically down-regulate p53-dependent gene activation. *J Biol Chem.* 2000; 275:20436–43. [PubMed: 10777477]
7. Luo J, Su F, Chen D, Shiloh A, Gu W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature.* 2000; 408:377–81. [PubMed: 11099047]
8. Yuan ZL, Guan YJ, Chatterjee D, Chin YE. Stat3 dimerization regulated by reversible acetylation of a single lysine residue. *Science.* 2005; 307:269–73. [PubMed: 15653507]
9. Kumagai T, Wakimoto N, Yin D, Gery S, Kawamata N, Takai N, Komatsu N, Chumakov A, Imai Y, Koeffler HP. Histone deacetylase inhibitor, suberoylanilide hydroxamic acid (Vorinostat, SAHA) profoundly inhibits the growth of human pancreatic cancer cells. *Int J Cancer.* 2007; 121:656–65. [PubMed: 17417771]
10. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem.* 2001; 276:36734–41. [PubMed: 11473107]
11. Lin HS, Hu CY, Chan HY, Liew YY, Huang HP, Lepescheux L, Bastianelli E, Baron R, Rawadi G, Clement-Lacroix P. Anti-rheumatic activities of histone deacetylase (HDAC) inhibitors in vivo in collagen-induced arthritis in rodents. *Br J Pharmacol.* 2007; 150:862–72. [PubMed: 17325656]
12. Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, Wells AD, Hancock WW. Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med.* 2007; 13:1299–307. [PubMed: 17922010]
13. Shein NA, Grigoriadis N, Alexandrovich AG, Simeonidou C, Loubopoulos A, Polyzoidou E, Trembovler V, Mascagni P, Dinarello CA, Shohami E. Histone deacetylase inhibitor ITF2357 is neuroprotective, improves functional recovery, and induces glial apoptosis following experimental traumatic brain injury. *Faseb J.* 2009; 23:4266–75. [PubMed: 19723705]
14. Hutt DM, Herman D, Rodrigues AP, Noel S, Pilewski JM, Matteson J, Hoch B, Kellner W, Kelly JW, Schmidt A, Thomas PJ, Matsumura Y, Skach WR, Gentzsch M, Riordan JR, Sorscher EJ, Okiyoneda T, Yates JR 3rd, Lukacs GL, Frizzell RA, Manning G, Gottesfeld JM, Balch WE. Reduced histone deacetylase 7 activity restores function to misfolded CFTR in cystic fibrosis. *Nat Chem Biol.* 2010; 6:25–33. [PubMed: 19966789]
15. McGee-Lawrence ME, Westendorf JJ. Histone deacetylases in skeletal development and bone mass maintenance. *Gene.* 2010
16. Jensen ED, Nair AK, Westendorf JJ. Histone deacetylase co-repressor complex control of Runx2 and bone formation. *Crit Rev Eukaryot Gene Expr.* 2007; 17:187–96. [PubMed: 17725488]
17. Yi T, Baek JH, Kim HJ, Choi MH, Seo SB, Ryoo HM, Kim GS, Woo KM. Trichostatin A-mediated upregulation of p21(WAF1) contributes to osteoclast apoptosis. *Exp Mol Med.* 2007; 39:213–21. [PubMed: 17464183]
18. Schroeder TM, Westendorf JJ. Histone deacetylase inhibitors promote osteoblast maturation. *J Bone Miner Res.* 2005; 20:2254–63. [PubMed: 16294278]
19. Haberland M, Carrer M, Mokalled MH, Montgomery RL, Olson EN. Redundant control of adipogenesis by histone deacetylases 1 and 2. *J Biol Chem.* 2010; 285:14663–70. [PubMed: 20190228]
20. Lee S, Park JR, Seo MS, Roh KH, Park SB, Hwang JW, Sun B, Seo K, Lee YS, Kang SK, Jung JW, Kang KS. Histone deacetylase inhibitors decrease proliferation potential and multilineage differentiation capability of human mesenchymal stem cells. *Cell Prolif.* 2009; 42:711–20. [PubMed: 19689470]
21. Cho HH, Park HT, Kim YJ, Bae YC, Suh KT, Jung JS. Induction of osteogenic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. *J Cell Biochem.* 2005; 96:533–42. [PubMed: 16088945]
22. Schroeder TM, Kahler RA, Li X, Westendorf JJ. Histone deacetylase 3 interacts with runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. *J Biol Chem.* 2004; 279:41998–2007. [PubMed: 15292260]
23. Westendorf JJ, Zaidi SK, Cascino JE, Kahler R, van Wijnen AJ, Lian JB, Yoshida M, Stein GS, Li X. Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. *Mol Cell Biol.* 2002; 22:7982–92. [PubMed: 12391164]

24. Jensen ED, Schroeder TM, Bailey J, Gopalakrishnan R, Westendorf JJ. Histone deacetylase 7 associates with Runx2 and represses its activity during osteoblast maturation in a deacetylation-independent manner. *J Bone Miner Res*. 2008; 23:361–72. [PubMed: 17997710]
25. Lee HW, Suh JH, Kim AY, Lee YS, Park SY, Kim JB. Histone deacetylase 1-mediated histone modification regulates osteoblast differentiation. *Mol Endocrinol*. 2006; 20:2432–43. [PubMed: 16728531]
26. Kang JS, Alliston T, Delston R, Derynck R. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. *EMBO J*. 2005; 24:2543–55. [PubMed: 15990875]
27. Vega RB, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, McAnally J, Pomajzl C, Shelton JM, Richardson JA, Karsenty G, Olson EN. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell*. 2004; 119:555–66. [PubMed: 15537544]
28. Zhang Y, Kwon S, Yamaguchi T, Cubizolles F, Rousseaux S, Kneissel M, Cao C, Li N, Cheng HL, Chua K, Lombard D, Mizeracki A, Matthias G, Alt FW, Khochbin S, Matthias P. Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. *Mol Cell Biol*. 2008; 28:1688–701. [PubMed: 18180281]
29. Rivadeneira F, Stykarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, Zillikens MC, Kavvoura FK, Amin N, Aulchenko YS, Cupples LA, Deloukas P, Demissie S, Grundberg E, Hofman A, Kong A, Karasik D, van Meurs JB, Oostra B, Pastinen T, Pols HA, Sigurdsson G, Soranzo N, Thorleifsson G, Thorsteinsdottir U, Williams FM, Wilson SG, Zhou Y, Ralston SH, van Duijn CM, Spector T, Kiel DP, Stefansson K, Ioannidis JP, Uitterlinden AG. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. *Nat Genet*. 2009; 41:1199–206. [PubMed: 19801982]
30. Li H, Xie H, Liu W, Hu R, Huang B, Tan YF, Xu K, Sheng ZF, Zhou HD, Wu XP, Luo XH. A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. *J Clin Invest*. 2009; 119:3666–77. [PubMed: 19920351]
31. Haberland M, Mokalled MH, Montgomery RL, Olson EN. Epigenetic control of skull morphogenesis by histone deacetylase 8. *Genes Dev*. 2009; 23:1625–30. [PubMed: 19605684]
32. Razidlo DF, Whitney TJ, Casper ME, McGee-Lawrence ME, Stensgard BA, Li X, Secreto FJ, Knutson SK, Hiebert SW, Westendorf JJ. Histone Deacetylase 3 Depletion in Osteo/Chondroprogenitor Cells Decreases Bone Density and Increases Marrow Fat. *PLoS ONE*. 2010; 5:e11492. [PubMed: 20628553]
33. Subha K, Kumar GR. Assessment for the identification of better HDAC inhibitor class through binding energy calculations and descriptor analysis. *Bioinformation*. 2008; 3:218–22. [PubMed: 19255637]
34. Fischle W, Dequiedt F, Hendzel MJ, Guenther MG, Lazar MA, Voelter W, Verdin E. Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. *Mol Cell*. 2002; 9:45–57. [PubMed: 11804585]
35. Guo CY, Ronen GM, Atkinson SA. Long-term valproate and lamotrigine treatment may be a marker for reduced growth and bone mass in children with epilepsy. *Epilepsia*. 2001; 42:1141–7. [PubMed: 11580761]
36. Sato Y, Kondo I, Ishida S, Motooka H, Takayama K, Tomita Y, Maeda H, Satoh K. Decreased bone mass and increased bone turnover with valproate therapy in adults with epilepsy. *Neurology*. 2001; 57:445–9. [PubMed: 11502911]
37. Vestergaard P, Rejnmark L, Mosekilde L. Fracture risk associated with use of antiepileptic drugs. *Epilepsia*. 2004; 45:1330–7. [PubMed: 15509233]
38. Vajda FJ, O'Brien TJ, Hitchcock A, Graham J, Cook M, Lander C, Eadie MJ. Critical relationship between sodium valproate dose and human teratogenicity: results of the Australian register of anti-epileptic drugs in pregnancy. *J Clin Neurosci*. 2004; 11:854–8. [PubMed: 15519862]
39. Senn SM, Kantor S, Poulton IJ, Morris MJ, Sims NA, O'Brien TJ, Wark JD. Adverse effects of valproate on bone: Defining a model to investigate the pathophysiology. *Epilepsia*. 2010
40. Kim SH, Lee JW, Choi KG, Chung HW, Lee HW. A 6-month longitudinal study of bone mineral density with antiepileptic drug monotherapy. *Epilepsy Behav*. 2007; 10:291–5. [PubMed: 17224308]

41. Tsukahara H, Kimura K, Todoroki Y, Ohshima Y, Hiraoka M, Shigematsu Y, Tsukahara Y, Miura M, Mayumi M. Bone mineral status in ambulatory pediatric patients on long-term anti-epileptic drug therapy. *Pediatr Int*. 2002; 44:247–53. [PubMed: 11982890]
42. Rieger-Wettengl G, Tuttlewski B, Stabrey A, Rauch F, Herkenrath P, Schauseil-Zipf U, Schoenau E. Analysis of the musculoskeletal system in children and adolescents receiving anticonvulsant monotherapy with valproic acid or carbamazepine. *Pediatrics*. 2001; 108:E107. [PubMed: 11731634]
43. Loscher W. Valproate induced changes in GABA metabolism at the subcellular level. *Biochem Pharmacol*. 1981; 30:1364–6. [PubMed: 6791653]
44. van der Laan JW, de Boer T, Bruinvels J. Di-n-propylacetate and GABA degradation. Preferential inhibition of succinic semialdehyde dehydrogenase and indirect inhibition of GABA-transaminase. *J Neurochem*. 1979; 32:1769–80. [PubMed: 376783]
45. Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. *Oncologist*. 2007; 12:1247–52. [PubMed: 17962618]
46. Prata J, Akech J, Wixted JJ, Szabo G, Hussain S, McGee-Lawrence ME, Li X, Bedard K, Dhillon RJ, van Wijnen AJ, Stein JL, Stein GS, Westendorf JJ, Lian JB. The histone deacetylase inhibitor, vorinostat, reduces tumor growth at the metastatic bone site and associated osteolysis, but promotes normal bone loss. *Mol Cancer Ther*. 2010; 9:3210–20. [PubMed: 21159607]
47. Butler LM, Agus DB, Scher HI, Higgins B, Rose A, Cordon-Cardo C, Thaler HT, Rifkind RA, Marks PA, Richon VM. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. *Cancer Res*. 2000; 60:5165–70. [PubMed: 11016644]
48. Campbell RA, Sanchez E, Steinberg J, Shalitin D, Li ZW, Chen H, Berenson JR. Vorinostat enhances the antimyeloma effects of melphalan and bortezomib. *Eur J Haematol*. 2010; 84:201–11. [PubMed: 19929977]
49. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res*. 2010; 25:1468–86. [PubMed: 20533309]
50. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res*. 1987; 2:595–610. [PubMed: 3455637]
51. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29:e45. [PubMed: 11328886]
52. Bradley D, Rathkopf D, Dunn R, Stadler WM, Liu G, Smith DC, Pili R, Zwiebel J, Scher H, Hussain M. Vorinostat in advanced prostate cancer patients progressing on prior chemotherapy (National Cancer Institute Trial 6862): trial results and interleukin-6 analysis: a study by the Department of Defense Prostate Cancer Clinical Trial Consortium and University of Chicago Phase 2 Consortium. *Cancer*. 2009; 115:5541–9. [PubMed: 19711464]
53. Kerr JS, Galloway S, Lagrutta A, Armstrong M, Miller T, Richon VM, Andrews PA. Nonclinical safety assessment of the histone deacetylase inhibitor vorinostat. *Int J Toxicol*. 2010; 29:3–19. [PubMed: 19903873]
54. Kelly WK, O'Connor OA, Krug LM, Chiao JH, Heaney M, Curley T, MacGregore-Cortelli B, Tong W, Secrist JP, Schwartz L, Richardson S, Chu E, Olgac S, Marks PA, Scher H, Richon VM. Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. *J Clin Oncol*. 2005; 23:3923–31. [PubMed: 15897550]
55. Duvic M, Talpur R, Ni X, Zhang C, Hazarika P, Kelly C, Chiao JH, Reilly JF, Ricker JL, Richon VM, Frankel SR. Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood*. 2007; 109:31–9. [PubMed: 16960145]
56. Olsen EA, Kim YH, Kuzel TM, Pacheco TR, Foss FM, Parker S, Frankel SR, Chen C, Ricker JL, Arduino JM, Duvic M. Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. *J Clin Oncol*. 2007; 25:3109–15. [PubMed: 17577020]

57. de Boer J, Licht R, Bongers M, van der Klundert T, Arends R, van Blitterswijk C. Inhibition of histone acetylation as a tool in bone tissue engineering. *Tissue Eng.* 2006; 12:2927–37. [PubMed: 17518660]
58. Di Bernardo G, Squillaro T, Dell'Aversana C, Miceli M, Cipollaro M, Cascino A, Altucci L, Galderisi U. Histone deacetylase inhibitors promote apoptosis and senescence in human mesenchymal stem cells. *Stem Cells Dev.* 2009; 18:573–81. [PubMed: 18694296]
59. Di Bernardo G, Alessio N, Dell'Aversana C, Casale F, Teti D, Cipollaro M, Altucci L, Galderisi U. Impact of histone deacetylase inhibitors SAHA and MS-275 on DNA repair pathways in human mesenchymal stem cells. *J Cell Physiol.* 2010; 225:537–44. [PubMed: 20458754]
60. Hossain A, Jung LK. Expression of bone specific alkaline phosphatase on human B cells. *Cell Immunol.* 2008; 253:66–70. [PubMed: 18579124]
61. Iwami K, Moriyama T. Effects of short chain fatty acid, sodium butyrate, on osteoblastic cells and osteoclastic cells. *Int J Biochem.* 1993; 25:1631–5. [PubMed: 8288032]
62. Kumandas S, Koklu E, Gumus H, Koklu S, Kurtoglu S, Karakukcu M, Keskin M. Effect of carbamazepine and valproic acid on bone mineral density, IGF-I and IGFBP-3. *J Pediatr Endocrinol Metab.* 2006; 19:529–34. [PubMed: 16759039]
63. Babayigit A, Dirik E, Bober E, Cakmakci H. Adverse effects of antiepileptic drugs on bone mineral density. *Pediatr Neurol.* 2006; 35:177–81. [PubMed: 16939856]
64. Oner N, Kaya M, Karasalioglu S, Karaca H, Celtik C, Tutunculer F. Bone mineral metabolism changes in epileptic children receiving valproic acid. *J Paediatr Child Health.* 2004; 40:470–3. [PubMed: 15265190]
65. Boluk A, Guzelipek M, Savli H, Temel I, Ozisik HI, Kaygusuz A. The effect of valproate on bone mineral density in adult epileptic patients. *Pharmacol Res.* 2004; 50:93–7. [PubMed: 15082034]
66. Nissen-Meyer LS, Svalheim S, Tauboll E, Reppe S, Lekva T, Solberg LB, Melhus G, Reinholt FP, Gjerstad L, Jemtland R. Levetiracetam, phenytoin, and valproate act differently on rat bone mass, structure, and metabolism. *Epilepsia.* 2007; 48:1850–60. [PubMed: 17634065]
67. Sheth RD, Wesolowski CA, Jacob JC, Penney S, Hobbs GR, Riggs JE, Bodensteiner JB. Effect of carbamazepine and valproate on bone mineral density. *J Pediatr.* 1995; 127:256–62. [PubMed: 7636651]
68. Elliott JO, Jacobson MP, Haneef Z. Homocysteine and bone loss in epilepsy. *Seizure.* 2007; 16:22–34. [PubMed: 17110134]
69. Mezuk B, Morden NE, Ganoczy D, Post EP, Kilbourne AM. Anticonvulsant use, bipolar disorder, and risk of fracture among older adults in the veterans health administration. *Am J Geriatr Psychiatry.* 2010; 18:24–55.

Figure 1. Timeline of in vivo experiments

A) In experiment 1, 6 week-old male C57BL/6 mice received daily intraperitoneal injections of vehicle or 100 mg/kg SAHA for 3 weeks ($n = 8/\text{group}$). All mice were sacrificed 24 hours after the last treatment injection. B) In experiment 2, 7 week-old male C57BL/6 mice received daily intraperitoneal injections of vehicle or 100 mg/kg SAHA for 4 weeks ($n = 20/\text{group}$). One-half of the mice in each group were sacrificed at 4 weeks, and remaining animals were allowed to recover for an additional 4 weeks prior to sacrifice.

Figure 2. Effect of SAHA on animal weight and histone acetylation

A and B) Weights were recorded daily for all mice to ensure proper treatment dosage. SAHA administration inhibited weight gain in Experiment 2 (panel B) but not in Experiment 1 (panel A), likely because starting body masses were not identical between groups in Experiment 1. Lost weight was regained in animals allowed to recover for 4 weeks following 4 weeks of SAHA exposure (panel B). C) Hdac inhibition *via* SAHA treatment increased acetylation of histone 3 (Ac-H3). A western blot for Ac-H3 in splenic lysates from five SAHA- and five vehicle-treated mice (*"Treatment"* phase of Experiment 2, n = 5/group) is presented. Control blots were also performed for actin. Each lane contains protein from a different mouse.

Figure 3. Serum markers of bone remodeling

A–B) Serum P1NP (bone formation) levels were measured after 3 (A) or 4 weeks (B) of SAHA or vehicle injections. C) Serum TRAcP 5b (bone resorption) levels were measured for all mice treated for 4 weeks with SAHA (Treatment group only). (n = 7 to 10 animals/group)

Figure 4. Osteoblast gene expression in the tibia of vehicle-and SAHA -treated mice

Transcript levels for the specified genes were normalized to the reference gene Ywhaz according to the $2^{-(\Delta\Delta C_T)}$ method. Genes are ordered from left to right according to the time course at which they are first expressed during osteoblastic differentiation. Data are expressed as fold change in gene expression relative to the vehicle-treated mice. A representative plot of means $\pm$ SEM is presented (n = 3 animals/group).

Figure 5. Effects of SAHA on osteogenic colony formation

A) Diagram of the 3- and 7-day window culture experiments. Cells were grown in osteogenic medium for 28 days with SAHA or DMSO vehicle present in the medium for 3 or 7 days as indicated. B) Representative alizarin red stained wells from DMSO and SAHA-treated wells for each 7-day window. C) Means $\pm$ SEM of the alizarin red-positive areal percentage for the 7-day window cultures are presented ($n = 3$ wells/group). D) Representative alizarin red stained wells from DMSO and SAHA-treated wells for each 3-day window time point. E) Means $\pm$ SEM of the alizarin red-positive areal percentage for the 3-day window cultures are presented ($n = 2$ wells/group).

Figure 6. Gene expression in BMSC treated with SAHA in vitro

Cells were grown in osteogenic medium containing DMSO vehicle or SAHA (1 μM) for 3 or 7 days. Transcript levels for the specified genes were normalized to the reference gene Ywhaz according to the $2^{-(\Delta\Delta C_T)}$ method. Data are expressed as fold change in gene expression relative to Day 0 controls. Means ± SEM are presented (n = 2 samples/group).

Figure 7. Cell cycle profile and DNA damage in undifferentiated mouse BMSC

A–B) Cells were incubated medium containing 2% FBS for 18 hours, after which cells were exposed to DMSO vehicle or SAHA (1 μ M) for 48 hours. Cell cycle profiles were determined with propidium iodide staining and flow cytometry. C–F) Cells were exposed to DMSO vehicle or SAHA (1 μ M) for 48 hours, after which DNA damage was assessed via γ H2AX nuclear staining. SAHA induced accumulation of γ H2AX foci.

Table 1

Primer sequences used in PCR reactions

Gene name	ID	Forward primer sequence	Reverse primer sequence
Runx2	NM_009820	GGCACAGACAGAAGCTTGATG	GAATGCGCCCTAAATCACTGA
Osterix/Sp7	NM_130458	GGAGGTTTCACTCCATTCCA	TAGAAGGAGCAGGGGACAGA
Col1a1	NM_007742	GCTTCACCTACAGCACCTTGT	TGACTGTCTTGCCCAAGTTC
Alkaline phosphatase	NM_007431	CACAGATTCCCAAAGCACCT	GGGATGGAGGAGAGAAGGTC
Osteopontin/Spp1	NM_009263	CCCGGTGAAAGTACTGATTCT	GATCTGGGTGCAGGCTGTAAA
Osteocalcin/Bglap1	NM_007541	CCTGAGTCTGACAAAGCCTTCA	GCCGGAGTCTGTTCACCTT
Axin2	NM_015732	CGCCACCAAGACCTACATACG	ACATGACCGAGCCGATCTGT
Sphk1	NM_011451	CTGATGCATGAGGTGGTGAAT	TGCAGTTGATGAGCAGGTCTT
Sost	NM_024449	ACTTGTGCACGCTGCCTTCT	TGACCTCTGTGGCATCATTCC
Rankl	NM_011613	GCTGGGACCTGCAAATAAGT	TTGCACAGAAAACATTACACCTG
Opg	NM_008764	CCAAGAGCCCAGTGTTCCTT	CCAAGCCAGCCATTGTTAAT
p21/Cdkn1a	NM_007669	CAAGAGGCCAGTACTTCCT	ACACCAGAGTGCAAGACAGC
Ywhaz	NM_011740	GAGCTGAGCTGTCTGAATGAG	GATGACCTACGGGCTCCTAC

Table 2

Trabecular bone architectural properties in the distal femoral metaphysis

Property	3 wks treatment			4 wks treatment			4 wks treatment + 4 wks recovery		
	Vehicle	SAHA	<i>p-value</i>	Vehicle	SAHA	<i>p-value</i>	Vehicle	SAHA	<i>p-value</i>
BV/TV, %	12.2 (3.7)	9.3 (2.2)	0.096	11.8 (1.3)	9.2 (1.3)	0.0002	11.6 (2.4)	9.7 (1.4)	0.059
Tb.N, mm ⁻¹	5.70 (0.36)	5.42 (0.36)	0.151	5.44 (0.25)	4.95 (0.30)	0.0008	5.01 (0.29)	4.79 (0.24)	0.092
Tb.Th, mm	0.035 (0.004)	0.031 (0.002)	0.056	0.036 (0.002)	0.036 (0.002)	0.713	0.038 (0.003)	0.037 (0.001)	0.531
Tb.Sp, mm	0.176 (0.011)	0.186 (0.013)	0.139	0.180 (0.009)	0.201 (0.013)	0.0006	0.195 (0.013)	0.205 (0.012)	0.084

Means (standard deviations) are presented.

Table 3

SAHA did not significantly alter cortical bone properties of the femoral midshaft

Property	3 wks treatment			4 wks treatment			4 wks treatment + 4 wks recovery		
	Vehicle	SAHA	<i>p-value</i>	Vehicle	SAHA	<i>p-value</i>	Vehicle	SAHA	<i>p-value</i>
TV, mm ³	0.568 (0.069)	0.582 (0.068)	0.710	0.559 (0.031)	0.554 (0.044)	0.770	0.581 (0.044)	0.554 (0.028)	0.119
BV, mm ³	0.535 (0.066)	0.546 (0.066)	0.735	0.532 (0.030)	0.534 (0.040)	0.890	0.555 (0.043)	0.528 (0.027)	0.112
Ct.Th, mm	0.172 (0.012)	0.173 (0.011)	0.863	0.172 (0.006)	0.171 (0.007)	0.795	0.177 (0.010)	0.172 (0.003)	0.159
Ct. Mat.M.Dn, mgHA/cm ³	1199 (15)	1196 (17)	0.739	1226 (7)	1222 (24)	0.576	1242.5 (18.7)	1236.9 (21.9)	0.545

Means (standard deviations) are presented.

Table 4

Static and dynamic histomorphometry in the distal femoral metaphysis

	Oc.S/BS, %	N.Oc/B.Pm, #/mm	Ob.S/BS, %	N.Ob/B.Pm, #/mm	MS/BS, %	MAR, $\mu\text{m/day}$	BFR/BV, %/day
Vehicle	6.01 (4.41)	4.64 (3.10)	10.71 (3.02)	8.81 (2.68)	35.7 (4.5)	1.46 (0.14)	3.52 (0.63)
SAHA	3.66 (2.21)	2.75 (1.54)	6.96 (4.28)	5.47 (2.93)	36.0 (4.3)	1.73 (0.15)	4.18 (0.60)
<i>p-value</i>	<i>0.149</i>	<i>0.101</i>	<i>0.036</i>	<i>0.016</i>	<i>0.867</i>	<i>0.0008</i>	<i>0.034</i>

Means of 10 samples (standard deviations) are shown.