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

Meghan E. McGee-Lawrence [a], Angela L. McCleary-Wheeler [a], Frank J. Secreto [a], David F. Razidlo [a], Minzhi Zhang [a], Bridget A. Stensgard [a], Xiaodong Li [a], Gary S. Stein [b], Jane B. Lian [b], Jennifer J. Westendorf [a,*]

[a] Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA
[b] University of Massachusetts Medical School, Worcester, MA, USA

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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 Zolinza™) affects bone mass and remodeling in vivo. Male C57BL/6J mice received daily SAHA (100 mg/kg) or vehicle injections for 3 to 4 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 4 weeks after 4 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.

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, Zolinza™) 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 skeletal 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

* Corresponding author at: Mayo Clinic, 200 First Street SW, MSB 3-69, Rochester, MN 55905, USA. Fax: +1 507 284 5075.
E-mail addresses: mcgeelawrence.meghan@mayo.edu (M.E. McGee-Lawrence), mcclearywheeler.angela@mayo.edu (A.L. McCleary-Wheeler), secreto.frank@mayo.edu (F.J. Secreto), razidlo.david@mayo.edu (D.F. Razidlo), zhang.minzhi@mayo.edu (M. Zhang), stensgard.bridget@mayo.edu (B.A. Stensgard), li.xiaodong@mayo.edu (X. Li), gary.stein@umassmed.edu (G.S. Stein), jane.lian@umassmed.edu (J.B. Lian), westendorf.jennifer@mayo.edu (J.J. Westendorf).

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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 wild-type 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 Zolinza™), 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/6J 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.

Materials and methods

In vivo studies

Animals

The Mayo Clinic Institutional Animal Care and Use Committee approved all handling and experimental procedures. Male C57BL/6J 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.

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/day SAHA or vehicle (10% DMSO/45% PEG400 in water) for 3 weeks beginning at 6 weeks of age (Fig. 1A). In the second experiment, mice were treated with 100 mg/kg/day SAHA or vehicle for 4 weeks beginning at 7 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 h following the last SAHA injection ("*Treatment*" groups). The remaining animals ($n=10$ per group) in the second experiment were allowed to recover for 4 weeks with no additional treatment injections prior to sacrifice ("*Recovery*" group) (Fig. 1B). Mice received subcutaneous injections of calcein (10 mg/kg) 5 days and 24 h 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.

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).

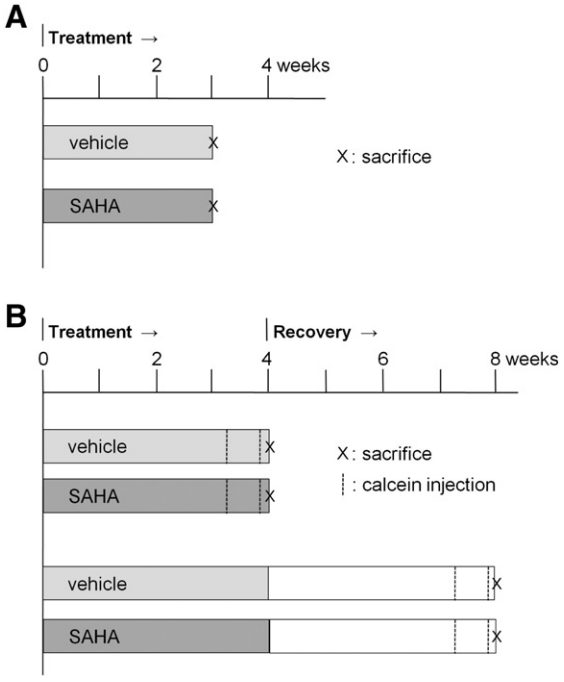

Fig. 1. Timeline of in vivo experiments. (A) In experiment 1, 6-week-old male C57BL/6 mice received daily intraperitoneal injections of vehicle or 100 mg/kg SAHA for 3 weeks ($n=8$/group). All mice were sacrificed 24 h after the last treatment injection. (B) In experiment 2, 7-week-old male C57BL/6 mice received daily intraperitoneal injections of vehicle or 100 mg/kg SAHA for 4 weeks ($n=20$/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.