The Histone Deacetylase Inhibitor, Vorinostat, Reduces Tumor Growth at the Metastatic Bone Site and Associated Osteolysis, but Promotes Normal Bone Loss

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Abstract
Vorinostat, an oral histone deacetylase inhibitor with antitumor activity, is in clinical trials for hematologic and solid tumors that metastasize and compromise bone structure. Consequently, there is a requirement to establish the effects of vorinostat on tumor growth within bone. Breast (MDA-231) and prostate (PC3) cancer cells were injected into tibias of SCID/NCr mice and the effects of vorinostat on tumor growth and osteolytic disease were assessed by radiography, micro-computed tomography, and histologic and molecular analyses. Vorinostat-treated and control mice without tumors were also examined. Tumor growth in bone was reduced ~33% by vorinostat with inhibited osteolysis in the first few weeks of the experiment. However, osteolysis became more severe in both the vehicle and vorinostat-treated groups. Vorinostat increased the expression of tumor-derived factors promoting bone resorption, including PTHrP, IL-8, and osteopontin. After 4 weeks of vorinostat therapy, the non–tumor-bearing contralateral femurs and limbs from vorinostat-treated tumor-free SCID mice showed significant bone loss (50% volume density of controls). Thus, our studies indicate that vorinostat effectively inhibits tumor growth in bone, but has a negative systemic effect reducing normal trabecular bone mass. Vorinostat treatment reduces tumor growth in bone and accompanying osteolytic disease as a result of decreased tumor burden in bone. However, vorinostat can promote osteopenia throughout the skeleton independent of tumor cell activity. Mol Cancer Ther; 9(12); 3210–20. ©2010 AACR.

Introduction
Metastasis to bone is a prevalent complication of prostate and breast cancer. Rapid autopsy programs reveal that 80% of the patients who die from advanced hormone refractory prostate cancer have bone metastases (1). Skeletal metastases are incurable and significantly decrease quality of life because they alter the normal homeostatic mechanisms within the bone microenvironment and initiate a vicious cycle that promotes tumor growth (2). Complications include fractures in the affected bones, vertebral collapse, nerve compression, hypercalcemia, and continued tumor growth and metastasis. Breast cancer and multiple myeloma preferentially induce osteolysis (2, 3). In contrast, prostate cancer–related bone diseases are predominantly osteoblastic, but originate as mixed osteolytic lesions, followed by a bone formation phase (4). Osteoblastic tumors produce an increased amount of collagenous extracellular matrix that can undergo mineralization to form pathologic bone that has a woven appearance and is structurally weak. In contrast, osteolytic tumors frequently trigger bone-resorbing cells, osteoclasts, to dissolve extracellular matrix–associated mineral and degrade bone tissue. In normal bone, there is a balanced physiologic relationship between the osteolytic and osteoblastic cycles that maintains skeletal strength and supports calcium homeostasis. Thus, the consequence of tumor colonization of bone is weakened skeletal integrity and, in severe cases, the affected bones are perforated and easily fractured.

Histone deacetylase inhibitors (HDI) are promising therapeutic agents that are in advanced clinical cancer trials (5, 6). These antagonists of epigenetic control induce cell cycle arrest, differentiation, and/or apoptosis of transformed cells in vitro and in vivo (7, 8). Normal cells are less...
susceptible to apoptosis because their cell cycle checkpoints are intact (8). Vorinostat [a.k.a. SAHA (suberoylanilide hydroxamic acid); Zolinza; Fig. 1A] is a potent HDI (9) that is being clinically evaluated in multiple clinical trials on solid tumors and leukemias. This HDI has been approved to treat cutaneous T-cell lymphomas that have failed conventional treatments because of the favorable response rate (10, 11). In contrast, trials in patients with solid tumors have produced mixed results (12, 13). To alleviate vorinostat-related side effects, including thrombocytopenia, dehydration, and fatigue, a variety of the dosing regimens are being tested (14). However, on the basis of the evidence that vorinostat stabilizes disease and/or produces partial responses in patients, the HDI remains in clinical trials, and is a component of multidrug therapies (15).

From both biological and clinical perspectives, there is a requirement to determine the effects of vorinostat or other inhibitors of class I and II histone deacetylases (HDAC) on bone metastases and tumor-associated bone disease. Class I and II HDACs (HDACs 1–11) are enzymes that remove acetyl groups from histones and nonhistone substrates (16). In some tumors, class I HDAC gene expression levels are elevated reinforcing the rationale for further evaluating HDAC inhibitors as antitumor agents (17). HDACs additionally have a crucial role in skeletogenesis. HDAC4 overexpression inhibits chondrocyte hypertrophy and skeletal development by repres-

Figure 1. Vorinostat significantly reduces tumor growth in the bone microenvironment. A, molecular structure of vorinostat. B, Western blot showing total and acetylated histone H3 levels in whole cell lysates from livers (n = 3) and tumor tissues of vorinostat-treated and control animals injected with PC3 prostate cancer cells. Total histone H3 and lamin B loading controls validated the increased acetylation. C, cross-sectional and axial MRI images from tumor-bearing legs were collected with a 7 Tesla 3D imager (gradient echo, Bruker FLASH3D, TR = 100 ms, TE = 6.5 ms, FA = 90 degrees, TT = 14 minutes, 10 mm × 10 mm × 19 mm, data size = 64 × 64,128, image resolution per pixel = 0.156 mm × 0.156 mm × 0.148 mm). D, tumor size measurements are shown for tumor volume, diameter, and number of slices that comprise the entire depth of the tumor. Parameters were obtained with ANALYZE image analysis software (n = 5 each group; *P < 0.1, **P < 0.05).
sing the Runx2 and MEF2c transcription factors (18, 19). Consistent with these findings, HDAC4 deletion induces premature ossification. Numerous HDACs are also expressed in osteoblasts (20). In vitro, HDAC inhibitors increase osteoblast differentiation and induce osteoclast apoptosis (21, 22). In this study, we tested the effects of vorinostat on tumor growth in long bones and the associated bone disease with preclinical models of breast and prostate cancer. Vorinostat blocked the growth of pre-established bone tumors and decreased tumor burden; however, it surprisingly compromised the density of contralateral, non–tumor-bearing bones. These data show that vorinostat has the potential to reduce the growth of skeletal metastases, but subsequent osteopenia or osteoporosis should be anticipated.

Materials and Methods

Cell culture and viability assay

The PC3 prostate cancer cell line was a generous gift from Leland Chung (Emory University School of Medicine, Atlanta, GA; ref. 23) and was cultured in T-medium (Invitrogen) with 5% fetal bovine serum (Atlanta Biologicals). MDA-MB-231 breast cancer cells (a highly metastatic line) obtained from American Type Culture Collection were cultured in α-MEM containing 5% fetal bovine serum. All media were supplemented with penicillin/streptomycin. Cell lines are validated for authenticity using levels of 3 gene expression markers for each cell type. The effect of vorinostat (24) on the viability of PC3 cells was determined by cell counting using a hemocytometer. Briefly, PC3 cells (1 × 10⁶) were seeded in 100-mm plates. After 48 hours, media were replaced and cells were incubated with various concentrations of SAHA (0, 0.5, 1, 1.5, 2, 2.5, 5, and 10 μmol/L). The cells were harvested and viability was determined by trypan blue exclusion. At least 3 independent analyses were done for all determinations of viability. Controls [dimethyl sulfoxide (DMSO) only] and 2.5 μmol/L of SAHA dissolved in DMSO was used for all subsequent experiments. SAHA was obtained from the Cancer Therapy Evaluation Program at the National Cancer Institute.

In vivo experiments

Groups of SCID/NCr mice (Balb/c background; n = 5 or 6 as indicated) were injected with MDA-MB-231 or PC3 cells (1 × 10⁷ cells in 100 μL PBS) in medullar cavity of tibia. Animals were injected with vorinostat (100 mg/kg body weight, intraperitoneally) daily starting 1 day after the tumor cell injections. Control mice were injected with vehicle, 50% polyethylene glycol. X-ray analysis was done on Faxitron soft X-ray machine to monitor osteolysis every week after the tumor cell injections. Serum markers for bone turnover (terminal serum samples) were obtained at sacrifice. Circulating levels of procollagen type 1 amino-terminal propeptide (P1NP; bone formation marker) and tartrate-resistant acid phosphatase 5b (TRACP5b; bone resorption marker) were quantified using colorimetric assays [rat/mouse P1NP EIA #AC-33F1, MouseTRAP ELISA #SB-TR103 (both from Immunodiagnostic Systems)]. Serum levels of osteocalcin (bone formation marker) were quantified using a single plex bead array assay (Milliplex Mouse Osteocalcin Single Plex #MBN-41K-1OC; Millipore). All samples were measured in duplicate within each assay. In all experiments, the ethics guidelines for investigations in conscious animals were followed, and experiments using animals were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Western blotting

Cells were harvested after 24 hours following exposure to SAHA. The cells were resuspended in a radioimmuno-precipitation assay buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA (pH 8.0), 1 mmol/L EGTA, 0.5% Nonidet P-40, 25 μmol/L MG132, and 1X proteinase inhibitor cocktail (Roche Diagnostics). Proteins were resolved by SDS-PAGE (10%) and transferred onto polyvinylidene difluoride (Immobilon-P) membranes (Millipore). The membranes were blocked with 5% milk in PBS containing 0.1% Tween and blotted with the following primary antibodies (1:1,000): anti-acetylated-Histone 3 (1:5,000), p21 (1:2,000), cdk2 (1:2,000), and anti-Lamin B (1:1,000) antibodies (purchased from Upstate and Santa Cruz Biotechnology). Secondary antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology.

Quantitative reverse transcription-PCR

Total RNA from PC3 and MDA-MB-231 cells exposed to SAHA or DMSO alone was isolated using Trizol reagent (Invitrogen). RNA was primed with oligo and random hexamers (histone H4) to synthesize cDNAs by using iScript cDNA synthesis kit (Bio-Rad). Primer pairs were used to detect human mRNA levels for MMP2, vascular endothelial growth factor (VEGF), osteocalcin, osteopontin, parathyroid hormone-related peptide (PTHrP), IL-8, p21, and mouse mRNA for tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (Supplementary Table) by using SYBR green chemistry (Applied Biosystems Inc.). Amplicom quantifies relative to human and mouse GAPDH were determined by using ABI Prism software (Applied Biosystems).

Histologic analysis and immunohistochemistry

Hematoxylin and eosin staining was done on paraffin sections from tibial tissues harvested 3 to 4 weeks after cancer cells were injected. These sections were fixed in 4% paraformaldehyde and decalcified in 14% EDTA solution as previously described prior to staining (25). Ki-67 (1:1,000 rabbit polyclonal; Santa Cruz) immunohistochemistry was done by using antigen retrieval with the antigen unmasking solution (DakoCytomation) diluted 1:100. The sections were incubated overnight with rabbit polyclonal Ki-67 antibody (Santa Cruz) followed by
incubation with horseradish peroxidase-conjugated secondary antibodies and developed with 3,3′-diaminobenzidine. TRAP enzyme detection for showing osteoclast activities was done using reagent kits from Sigma-Aldrich Biotechnology. Terminal uridine deoxyribo-nucleotidyl transferase dUTP nick end labeling (TUNEL) staining was done by using the In Situ Cell Death detection kit (Roche). For each mouse, 4 slides with 2 to 3 sections were examined.

**Micro-computed tomography analysis**

Three-dimensional micro-computed tomography (μCT) studies were done by the University of Massachusetts Medical School, Musculoskeletal Center for Imaging Core facility, and the Mayo Clinic Biomaterials and Quantitative Histomorphometry Core Laboratory. Bones were fixed in periodate-lysine-paraformaldehyde fixative (26) from non–tumor-bearing limbs of vorinostat-treated animals (n = 6 mice per group). After dehydration to 70% alcohol, femurs were scanned at 10-μm voxel resolution (μCT 40; Scanco Medical AG, Bruttisellen, Wangen-Bruttisellen, Switzerland). Image reconstruction was done by Scanco software Version 5.0. For trabecular bone, 100 contiguous slices below the growth plate were selected for contouring inside the endosteal edge for analyses of various bone parameters.

**Magnetic resonance imaging of tumors**

Tumor-bearing bones were analyzed in a 7 Tesla 3D MRI scanner. The bone specimens in 70% ethanol solvent were individually put in 10 mm outer diameter glass tubes for MRL Multislice spin echo MR images (1-mm thick 12 transverse to bone contiguous slices) at 25°C were obtained using a Bruker BioSpin 7T vertical bore magnet equipped with a MICRO2.5 probe and ParaVision 4.0 image acquisition and processing software. The proton-density images (TR = 2 seconds, TE = 11 ms, in-plane image resolution 78 μm/pixel) were used in tracing the tumor boundaries. ANALYZE software was used to find the tumor area in each slice and then from the contiguous slices, the entire tumor volume was estimated in each specimen. Statistical evaluation between control and vorinostat groups was done by Student’s t test.

**Results**

**Vorinostat significantly reduces tumor growth in the bone microenvironment**

Mice were treated daily with vorinostat beginning 24 hours after intratibial injection of either MDA-MB-231 breast cancer or PC3 prostate cancer cells. The efficacy of systemically delivered vorinostat throughout the time course of tumor development in bone was examined at sacrifice by determining the extent of histone acetylation in soft tissues of vital organs and in the tumor. Figure 1B shows a 4- to 5-fold increase in total acetylated histone proteins (normalized to lamin B) in the livers of vorinostat-treated animals and 2-fold increase in histone acetylation in tumor tissues. Mice that received intratibial injections of prostate cancer cells developed tumors in 3 weeks, whereas mice that were injected intratrahally with breast cancer cells did not show evidence of tumors until after 7 weeks. At that time, we observed that the vorinostat-treated group had smaller palpable tumors. MRI imaging of limbs from mice with MDA-MB-231 (data not shown) and PC3 tumors treated with vorinostat for 4 weeks confirmed that the tumors were smaller in vorinostat-treated animals (Fig. 1C). Thus, 3-dimensional quantitative values showed tumor volume and diameter was 30% to 33% lower in vorinostat-treated animals than in vehicle-treated mice (Fig. 1D). These results indicate that systemic vorinostat is effective in reducing tumor growth in bone.

Histologic evaluation of the bone tumors further confirmed the reduced tumor size in the vorinostat compared with control groups. Tumors representative of 6 animals per group are shown in Fig. 2A. Complete loss of trabecular bone and erosion of the cortex was observed. This erosion was caused by tumor stimulated osteoclastic resorption at the tumor–bone interface in which the TRAP-positive osteoclast cells are visualized on the remaining bone surface. Thus, bone erosion caused by the tumor allows tumor invasion into the surrounding muscle evident in both the vorinostat and vehicle-treated groups.

To explore a mechanism for the reduced size of bone tumors, immunohistochemical staining of tumor tissue sections was done. Vorinostat reduced the proliferative activity of the tumor cells by 44% to 50% (Fig. 2B, Ki-67 staining) and increased the number of apoptotic cells (TUNEL stain) throughout the tumor as compared with controls (Fig. 2C). In vitro 5 and 10 μmol/L doses, but not 2.5 μmol/L, significantly reduced the viable PC3 and MDA-MB-231 cell number dose dependently from 24 to 96 hours of vorinostat treatment (Supplementary Fig. S1A). Growth of PC3 cells was completely inhibited by 10 μmol/L vorinostat. Thus, vorinostat markedly inhibited cell proliferation and promoted apoptosis of the tumor cells injected into the bone, demonstrating effectiveness in reducing tumor burden of metastatic bone tumors.

**Osteolytic disease is initially reduced by vorinostat**

We addressed in vivo effectiveness of vorinostat to inhibit osteolytic diseases during rapid onset of PC3 cells tumor growth or during the slower tumor growth of the MDA-MB-231 cells. Osteolytic disease was monitored weekly by radiography (Fig. 3). Although prostate tumors grew very rapidly, the effectiveness of vorinostat was highest at 1 to 3 weeks after intratibial inoculation of the prostate cancer cells (Fig. 3A). By 4 weeks, the inhibition of osteolytic disease is less evident due to further aggressiveness of tumor activity that occurs when the tumor cells invade adjacent muscles (Fig. 3A). The radiographs comparing control and vorinostat-treated mice
with intratibial MDA-MB-231 breast cancer tumors also showed less bone loss at 5 weeks, but by 7 weeks, osteolysis increased in the vorinostat group with continued treatment (Fig. 3B). This finding is similar to the observations in prostate tumor growth in bone. Thus, whereas vorinostat can reduce tumor volume in the bone microenvironment, this HDAC inhibitor appears to be effective in preventing the associated bone resorption that occurs in the presence of prostate and breast tumors only at early stages of tumor growth (Fig. 3A and B, right panels, quantitation of lytic areas). These results suggest that a population of cells resists vorinostat and becomes more aggressive. Our findings also raise the possibility that vorinostat promotes bone resorption independent of tumor cells leading to increased osteolysis after several weeks of vorinostat treatment even though tumors remained smaller in size.

The mechanism by which vorinostat reduces bone mass and stimulates bone resorption was addressed. We focused on the extent to which vorinostat influences the expression of components of the vicious cycle of bone resorption known to stimulate osteoclast activity that contribute to tissue destruction. Following 24 hours of vorinostat exposure, tumor cells exhibited elevated levels of PTHrP, IL-8, osteopontin, and MMP2 that is expressed in both PC3 and MDA-MB-231 cells. Increased expression ranged from 1.5- to 4-fold in both prostate and breast cancer cells (Fig. 3C). The tumor cells have the expected increase in p21 mRNA (Fig. 3C) and protein (Supplementary Fig. S1B) levels as reported for vorinostat, reflecting inhibited cell proliferation in the tumors.

At the 2.5-μmol/L dose, cell growth was similar between control and vorinostat (Supplementary Fig. S1A). Decreased VEGF consistent with small tumor volume (Fig. 3C) from decreased vascularization and decreased osteocalcin, a maker of the bone osteomimetic property of these highly metastatic cell lines were significantly reduced (***P < 0.01, P < 0.001 to ****P < 0.01). However, vorinostat appears to stabilize osteolytic factors (PTHrP, IL-8, and OP) in tumor cells. These in vitro studies suggest a mechanism by which vorinostat increases bone resorption in the local environment. It also raises the possibility of systemic bone loss if tumor-
or host-derived osteolytic factors enter the circulation and promote systemic bone loss.

**Vorinostat reduces trabecular bone mass of the contralateral non–tumor-bearing limb, and in limbs of normal mice**

It is well established that metastatic breast and prostate tumors cells in the bone microenvironment secrete factors that activate a vicious cycle of osteolytic disease. To determine whether the systemic effects of the drug were altering osteoclast activity in other parts of the skeleton, we examined the contralateral femurs of the vorinostat-treated groups receiving intratibial implants of either breast (data not shown) or prostate cancer cells (Fig. 4). These contralateral limbs were not injected with tumor cells, and thus were compared between the control group (not receiving vorinostat) and vorinostat-treated groups. We did μCT analysis to quantitatively assess whether bone loss occurs in these limbs at a 4-week endpoint in the prostate tumor implant study. Three-dimensional images of the contralateral femurs show a significant decrease in trabecular bone mass in vorinostat-treated animals (Fig. 4A). Vorinostat reduced bone volume by 50%, decreased the number of trabeculae/mm², and increased trabecular spacing (Fig. 4B). We observed a similar reduction in contralateral femurs from mice at the 7-week endpoint for the breast cancer experiments treated with vorinostat compared with vehicle-treated mice (data not shown). A striking reduction of metaphyseal bone, but not cortical bone, was also evident in histologic sections (toluidine blue and TRAP staining) consistent with quantitative μCT parameters (Fig. 4C). Thus, these findings indicate preferential loss of trabecular bone in the skeleton by either systemic factors secreted from tumor cells that induce bone resorption or by systemic effects of HDAC inhibition on other cell types in bone.

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Figure 3. Osteolytic disease is modestly reduced by vorinostat. Radiographs compare osteolytic lesions after inoculation of 100,000 PC3 cells (3 and 4 weeks; A) and MDA-MB-231 cells (5 and 7 weeks; B) in the intratibial model of metastatic bone disease. Control mice received PEG and treated mice received 100 mg/kg vorinostat every day for beginning 1 day after tumor injections. Graphs to the right of the images show quantitation of osteolytic lesions in tumor-bearing limbs, which was done with ImageJ analysis software (right panels; n = 3; *P < 0.05, **P < 0.01). C, relative mRNA levels of osteolytic genes (MMP2, PTHrP, OP, IL8, VEGF, OC, and p21) in PC3 and MDA-MB-231 (MMP2, PTHrP, OP, IL8, VEGF, OC, and p21) cells exposed to vorinostat for 24 hours as examined by real-time PCR analysis. Error bars (mean ± SD) are representative of n = 3 samples analyzed in triplicates; **P < 0.01, ***P < 0.001.

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Vorinostat Reduces Tumor Growth in Bone
To address whether the effects of vorinostat in reducing trabecular bone volume were independent of the tumor-secreted factors, a study was carried out in SCID/NCr mice exposed to vorinostat for 4 weeks in the absence of tumor cell inoculation (Fig. 5). Again, approximately 50% to 60% of trabecular bone was lost in the distal femurs of tumor-free, vorinostat-treated animals as compared with control animals (Fig. 5A and B). Trabecular number and spacing were reduced and increased, respectively. No cortical bone loss by vorinostat was found (data not shown). Histochemical staining showed increased number of active osteoclasts on fewer trabeculae (Supplementary Fig. S1C), and the expression analysis of bone for TRAP confirmed increased osteoclast activity (Fig. 5C and D). Alkaline phosphatase, a marker of bone formation, was slightly increased. We also examined 2 serum markers at sacrifice and found no significant change in the procollagen Type 1, which reflects bone

Figure 4. Vorinostat reduces trabecular bone mass at the contralateral non-tumor-bearing limb. A, the contralateral femurs of the vorinostat-treated (4 weeks of daily vorinostat injections) groups receiving intratibial implants of prostate cancer cells. Trabecular region analyzed is 1 to 2 mm from distal growth plate, and cortical analysis is at 5.5 mm. B, quantitation of trabecular bone parameters on non–tumor-bearing femurs from control or vorinostat-treated animal (n = 6, *P < 0.05) injected with prostate cancer PC3 cells. C, histologic analysis of non–tumor-bearing femurs from control or vorinostat-treated animals injected with metastatic breast MDA-MB-231 cells indicates alterations in trabecular bone. Upper panels, toluidine blue staining for tissues; lower panels, tartrate-resistant acid phosphatase histochemistry for the detection of osteoclasts.
formation, but TRACP 5b levels were higher in the vorinostat group (Supplementary Fig. S1D). Thus, the histology and μCT establish that vorinostat has a direct effect selectively on trabecular bone in causing osteopenia conditions and through stimulated osteoclastic resorption.

Discussion

Skeletal metastases of breast and prostate tumors are associated with poor prognoses as morbidity and mortality are high. Breast cancers typically cause severe osteolytic lesions, whereas prostate tumors generate mixed (osteolytic and osteoblastic) lesions in bone. Several chemotherapeutic agents are currently being used to treat solid tumors to regulate growth and promote apoptosis. HDIs, including vorinostat, reactivate tumor suppressor genes that promote cell cycle arrest (e.g., p21) and/or establish competency for apoptosis. Vorinostat, an oral HDI, is in phase II and III clinical trials for hematologic and solid tumors. They were originally implicated in leukemogenesis by studies that identified them as crucial regulators of chromosomal translocation products, PML-RAR and PLZF-RAR (27). Subsequently, HDACs were linked to acute leukemia in which the AML1 (Runx1) gene is mutated by chromosomal translocations and fused to HDAC cofactors (28). In these studies, HDAC inhibition functionally inactivated expression of the translocation products and induced the re-expression of genes that inhibited tumor cell proliferation and promoted differentiation. Despite years of research, the effects of vorinostat treatment on bone metastases and normal bone tissue are unknown. Here, we show in vivo that vorinostat significantly decreases tumor cell proliferation and promotes apoptosis to suppress tumor growth within the bone in intratibial models of breast and prostate cancer bone disease. Consistent with these findings, our analyses of bone tissue showed that osteolysis is reduced and there is a lower tumor burden in vorinostat-treated mice. However, the contralateral limbs of tumor-bearing animals or femurs of
non–tumor-bearing animals treated with vorinostat exhibit significant loss of bone volume density. These findings indicate that vorinostat is effective in inhibiting cancer growth in bone, but has off-target effects that can cause osteopenic disease in the skeleton of mice beyond the metastatic site.

Inhibition of HDACs causes accumulation of acetylated histone proteins, altering the manner in which histones package the DNA to chromatin and the extent to which promoter elements are accessible to regulatory factors. We show increased acetylation of histone proteins isolated from tumor tissue of vorinostat-treated animals. HDIs induce different phenotypes in various transformed cells, including growth arrest, activation of the extrinsic and/or intrinsic apoptotic pathways, autophagic cell death, differentiation, and senescence (29). Our findings show that systemic vorinostat administration markedly inhibits prostate (PC3) and breast cancer (MDA-MB-231) cell proliferation and induces apoptosis of the tumor cells in the bone microenvironment. Vorinostat was previously shown to induce MCF7 breast cancer cells to undergo cell cycle arrest and differentiation. These cells showed a dose-dependent accumulation in the G1 and G2-M phases of the cell cycle (30). Vorinostat also inhibits the growth by induction of apoptosis and G2 cell cycle arrest of pancreatic cancer cell lines (31) and human leukemia cells (32). We find that the reduced size of bone tumors following treatment with vorinostat is because of increased apoptosis as reflected by TUNEL-positive cells. Vorinostat also induces expression of p21 in pancreatic cancer cell lines (31), consistent with our findings. Activation of genes in cancer cells by vorinostat that promote apoptosis through caspases is indicated by treatment with caspase inhibitors (33). Another contributing mechanism to decreased tumor volume by vorinostat in bone is the reduction in vascular endothelial growth factor we find in prostate and breast cells within bone. This finding is consistent with an earlier report observing VEGF inhibition in lymphoma tissue (34). Also, vorinostat blocks the production of VEGF but upregulates p21, in rheumatoid arthritis synovial fibroblastic E11 cells (35) and in mantle cell lymphoma cell lines (36). General mechanisms by which HDAC inhibitors affect gene transcription include inducing acetylation of histones, transcription factors, and other proteins (36). HDAC inhibitor–induced expression of p21 correlates with an increase in the acetylation of histones associated with the p21 promoter region (37, 38). However, HDAC inhibitors also block expression of STAT3-dependent genes and results in downregulation of several downstream genes such as androgen receptor (39). A recent report suggests that expression profile of a unique set of miRNAs is significantly influenced by SAHA in non–small cell lung cancer cells (40). These reports suggest that differential gene expression in cancer cells exposed to HDAC inhibitors is a combinatorial effect of multiple factors that can be through direct histone modifications or indirect through acetylation changes in cytokines, transcription factors, and miRNA cellular levels. Thus, our results together with previous reports suggest that vorinostat markedly inhibits cell proliferation and promotes apoptosis of the tumor cells injected into bone.

The present studies show that whereas vorinostat therapy decreases osteolysis in the bone microenvironment of the tumor-bearing limb consistent with decreased tumor size, there is significant loss of bone volume density in the contralateral femurs of tumor-bearing and non–tumor-bearing animals. Furthermore, in tumor-free mice, vorinostat treatment resulted in a 50% decrease in trabecular bone volume of the limbs. These results are somewhat surprising because several studies have examined the effects of HDIs on osteoblast differentiation using immortalized osteoblast cell lines or multipotent mesenchymal precursor cells. In vitro, HDIs and shRNAs to various HDACs promote osteoblast differentiation, matrix mineralization, and the upregulation of Runx2 target genes (41–43). Importantly, HDAC4-null mice display premature ossification of developing bones (18). The difference between systemic administration and in vitro studies of HDIs may reflect the effects on other lineages or osteoblast-support cells in the circulation or bone marrow or even other nonosseous tissues that secrete factors that impact on bone mass. We show that vorinostat increases expression of PTHrP, IL-8, and osteopontin in both PC3 and MDA-MB-231 cells in vitro, supporting an in vivo mechanism contributing to osteolysis in the tumor-bearing limb, and throughout the skeleton. Our findings show that vorinostat treatment in tumor-bearing or non–tumor-bearing animals causes bone loss possibly by upregulating the factors involved in enhanced osteoclast function. SAHA is administered systemically and many tissues are known to express bone-resorbing factors such as osteopontin (e.g., kidney and bone) and PTHrP (e.g., parathyroid gland and mammary glands). Increased levels of secreted factors (normal and cancer cell lines) that promote bone resorption as indicated by our studies might be due to effects of increased global acetylation levels.

While SAHA appears effective against solid tumors at doses well tolerated by patients (44), the administration of antiresorptive therapy in combination with tumor growth inhibitors might be effective in preventing vorinostat-induced bone loss throughout the skeleton. The bisphosphonate, zoledronic acid, administered with vorinostat induce cell death in the prostate cancer cell lines LNCaP and PC3 (45). Currently, bisphosphonates are widely used to treat malignant bone disease (46–48). They inhibit osteoclast-mediated bone resorption and can directly affect cancer cells. Our results from a preclinical animal model of breast and prostate cancer osteolytic disease suggest that treatment with bisphosphonates should be considered in combination with vorinostat as a strategy to maximize the therapeutic and
minimize treatment-related compromises in skeletal structure and function.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Victoria Richon (Merck Research Laboratories) for helpful discussions related to mouse dosages of SAHA, Dr. Douglas Adams (Department of Orthopaedic Surgery, University of Connecticut Health Center) for PET imaging in the early stages of these studies, Judy Rask for editorial assistance, and members of the participating laboratories for valuable suggestions throughout the study.

Grant Support

Studies reported were in part supported by National Institutes of Health grants P01CA082834, CA91956, AR48147, 58180RO23540, T32 AR056950, and T32 DK007302.

Received 06/17/2010; revised 09/16/2010; accepted 09/30/2010; published 12/14/2010.


**Molecular Cancer Therapeutics**

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