Targeting Tumor Angiogenesis with Histone Deacetylase Inhibitors: the Hydroxamic Acid Derivative LBH589

David Z. Qian,1 Yukihiko Kato,1 Shabana Shabbeer,1 Yongfeng Wei,1 Hendrik MW. Verheul,1 Brenda Salumbides,1 Tolib Sanni,1 Peter Atadja,2 and Roberto Pili1

Abstract

Purpose: Angiogenesis is required for tumor progression and represents a rational target for therapeutic intervention. Histone deacetylase (HDAC) inhibitors have been shown to have activity against various tumor cell types by inhibiting proliferation and inducing apoptosis both in vitro and in vivo. HDAC inhibitors have also been reported to inhibit angiogenesis. The goal of this study was to characterize the antiangiogenic and antitumor activity of a recently developed HDAC inhibitor, the hydroxamic derivative LBH589.

Materials and Methods: To evaluate the antiangiogenic activity of LBH589, we did cell cycle analysis, cell proliferation, tube formation, invasion assays in vitro, and Matrigel plug assay in vivo. To determine the antitumor activity of LBH589, we established human prostate carcinoma cell PC-3 xenografts in vivo. To evaluate the effect of LBH589 on endothelial signaling pathways, gene expression, and protein acetylation, we did Western blots and reverse transcription-PCR in human umbilical vein endothelial cells (HUVEC). Immunohistochemical analysis was done to evaluate new blood vessel formation in vivo.

Results: LBH589 induced acetylation of histone H3 and α-tubulin protein in HUVECs. Histone and nonhistone protein acetylation correlated with induction of G2-M cell cycle arrest, inhibition of HUVEC proliferation, and viability. Noncytotoxic concentrations of LBH589 inhibited endothelial tube formation, Matrigel invasion, AKT, extracellular signal-regulated kinase 1/2 phosphorylation, and chemokine receptor CXCR4 expression. In vivo dosing of mice with LBH589 (10 mg/kg) reduced angiogenesis and PC-3 tumor growth.

Conclusion: This study provides evidence that LBH589 induces a wide range of effects on endothelial cells that lead to inhibition of tumor angiogenesis. These results support the role of HDAC inhibitors as a therapeutic strategy to target both the tumor and endothelial compartment and warrant the clinical development of these agents in combination with angiogenesis inhibitors.

Histone deacetylase (HDAC) inhibitors represent an emergent class of anticancer drugs. The targets of these agents are enzymes (HDACs), which induce histone and nonhistone protein deacetylation at the lysine residues, chromatin condensation, and modulation of gene expression. Altered HDAC activity is associated with cancer, and inhibitors targeting HDACs have been shown to induce tumor cell cytostasis, differentiation, and apoptosis in various hematologic and epithelial tumors (1, 2). Five classes of HDAC inhibitors have been characterized, including short-chain fatty acids (i.e., sodium butyrate), hydroxamic acids (i.e., trichostatin A, SAHA, and LAQ824), cyclic peptides containing a 2-amino-8-oxo-9,10-epoxy-decanoyl moiety (i.e., trapoxin A), cyclic peptides without the 2-amino-8-oxo-9,10-epoxy-decanoyl moiety (i.e., FK228), and benzamides (i.e., MS-275). These agents induce a dose-dependent inhibition of either class I (1, 2, 3, and 8) or class II (4-10) HDACs, or both. Depending on the specific drug, the cancer cell line tested, the dose, and time of exposure, HDAC inhibitors can induce G1 or G2 cell cycle arrest and/or cell death (3, 4).

The reported p53-independent induction of the p21 gene and protein expression is probably due to the nature of epigenetic regulation of the p21 promoter and, along with histone lysine acetylation, represents a common molecular observation associated with drug exposure (5, 6). There have also been reports of up-regulation of proapoptotic pathways and down-regulation of prosurvival pathways associated with the HDAC inhibitors, which depend on the agent and the tumor cell line used (7, 8).

We have previously shown that the HDAC inhibitors phenylbutyrate and MS-275 induce the expression of the putative tumor suppressor gene RARβ2 and increase the retinoid sensitivity in retinoid-resistant prostate and renal cell carcinoma cell lines (9, 10).
In addition to the inhibitory effect on cancer cell proliferation, HDAC inhibitors have been reported to inhibit the process of new capillary blood vessel formation or angiogenesis (6, 11–13). Tumor-initiated angiogenesis takes place following an “angiogenic switch.” During the initial avascular tumor growth, there is an accumulation of hypoxia-inducible factor-1α (HIF-1α) protein, which activates an array of gene transcriptions, including vascular endothelial growth factor (VEGF), one of the most proangiogenic factors (14). VEGF-A (the principal isoform of VEGF) binds to VEGF receptor 2 (VEGFR2) on the surface of quiescent endothelial cells in the surrounding vessel walls and triggers the signaling pathways for endothelial activation and downstream angiogenesis (15).

During the multistep angiogenesis process, several ligand/receptor interactions are required. The interaction of angiopoietins and endothelial receptor Tie-2 affects endothelial cell survival and vessel stability (16). Stromal-derived factor-1 (SDF-1) and endothelial chemokine receptor CXCR4 influence the homing of mobilized/activated endothelial cells or endothelial progenitor cells to active sites of neovascularization (17, 18). Overall, the combined net result of ligand/receptor interaction activates intracellular pathways, leading to endothelial cell survival, proliferation, mobilization, and invasion of the extracellular matrix. This culminates in recognition and homing to the sites of new blood vessel formation.

In our previous work, we identified two different HDAC inhibitors, phenyl butyrate (short-chain fatty acid) and LAQ824 (hydroxamic acid), which have antiangiogenesis activity both in vitro and in vivo (3, 6). Angiogenesis inhibition induced by LAQ824 was associated with modulation of angiogenesis-related genes both in cancer cells (inhibition of HIF-1α and VEGF) and in endothelial cells (inhibition of Tie-2 and survivin, an inhibitor of apoptosis). Other groups have also reported that other HDAC inhibitors, including SAHA, TSA, FK228, valproic acid, and apicidin, have antiangiogenic activity (11–13).

In the current study, we investigated the antiangiogenic effect of a recently developed HDAC inhibitor, LBH589, that is currently in phase I clinical trial (19, 20). We identified altered gene expression and protein phosphorylation induced by LBH589 in endothelial cells. This data lead us to explore the potential of LBH589 as therapeutic agent with dual activity against both tumor proliferation and angiogenesis.

### Materials and Methods

**Cell lines, reagents, and animals.** Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Walkersville, MD) and cultured in complete EGM-2 medium containing EGM-2 bullet kit (Cambrex) with growth factors and 2.5% fetal bovine serum supplied by the vendor. All experiments were done using endothelial cells between passages 3 and 8. A human prostate (PC-3) carcinoma cell line was originally obtained from the American Type Culture Collection (Manassas, VA), maintained in RPMI 1640 with 1-glutamine supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). LBH589 was kindly provided by Novartis Pharma AG (Cambridge, MA). Stock solutions for in vitro assays were prepared in DMSO with final concentrations of <0.01%. For the in vivo animal experiments, LBH589 was dissolved in saline solution. Six-week-old male athymic nude mice and C57/BL6J mice (National Cancer Institute, Bethesda, MD) were housed under pathogen-free conditions. All animal studies were done according to the protocol approved by the Animal Care and Use Committee at the Johns Hopkins University.

**Cell viability assay.** The effect of LBH589 on HUVEC growth and survival was assessed by a 3,3′-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay (Roche, Indianapolis, IN) as previously described (6). In brief, 1 × 10^5 cells were seeded into 96-well plates. After an overnight incubation, the medium was replaced by fresh EGM-2 with either complete growth factors (EGM-2 bullet kit), 50 ng/mL VEGF-A (R&D Systems, Minneapolis, MN), or 50 ng/mL basic fibroblast growth factor (bFGF; R&D Systems). LBH589 was added at 0 to 800 nmol/L final concentrations. After 48 hours of incubation, the viable cells (a net result of both cell growth and cell death) were measured by 3,3′-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt reagent at UV 490 nm as described by the manufacturer’s protocol. The UV readings of solvent-treated controls were normalized to 100%, and the readings from LBH589-treated cells were expressed as % controls.

**Cell proliferation assay.** The proliferation assay was run as previously described (3, 6). Briefly, cancer and endothelial cells were seeded (1 × 10^4 per well) in six-well plates and incubated at 37°C, 5% CO_2 for 24 to 48 hours. The medium was then replaced with appropriate basal medium without growth factors. After overnight starvation, cells were counted in triplicate with a Coulter counter and designated as day 0 values. Then, cells were treated with different concentrations of LBH589 (triplicate) in complete media with growth factors and serum. Forty-eight hours later, the viable cells from each condition were harvested and counted by Coulter counter. Cell number at day 0 was normalized to 100%, and cell proliferation following 48 hours of treatment was adjusted to the % day 0 controls. The experiments were repeated three times with similar results.

**Cell cycle and cell death analysis.** Proliferating HUVECs were starved overnight and then treated with increasing concentrations of LBH589 with complete media for 24 hours. After drug treatment, both starved and treated cells were harvested, fixed, and stored in 70% ice-cold ethanol. The percentage of the cell cycle that was in G_0-G_1, S, and G_2-M phase was quantitated by using a propidium iodide–based cellular DNA flow cytometry analysis kit (Roche) following manufacturer’s instructions. For cell death analysis, Annexin V and propidium iodide costaining followed by flow cytometry was done according to manufacturer’s instructions (BD Biosciences, San Jose, CA).

**Matrigel angiogenesis assay in vitro (tube formation).** HUVECs were cultured in complete EGM-2 media before being plated in 24-well plates (5 × 10^4 per well) previously coated with 300 μL of growth factor–reduced Matrigel (BD Biosciences), in the presence of LBH589 or solvent control. The morphology of capillary-like structures formed by HUVECs 15 hours after culturing was visualized using an inverted microscope (Zeiss Axioskop) and photographed with a digital camera. Tube formation was analyzed with an imaging system (Image-Pro) as previously described (6).

**Matrigel invasion assay.** The Matrigel invasion assay was done with precoated Matrigel inserts following manufacturer’s instructions (BD Biosciences). Proliferating HUVECs were pretreated with indicated doses of LBH589 overnight, and then the cells were harvested and resuspended in basal EGM-2 media with 0.5% fetal bovine serum and the indicated dose of LBH589. An equal number of cells (4 × 10^4) were added to the invasion chambers in triplicate. The chemoattractants were either VEGF-A or SDF-1α (50 ng/mL, R&D Systems). Twenty-four hours later, the membranes containing migrated cells were fixed and stained, and cell numbers were counted under a microscope. Five random fields were chosen for each membrane, and the results were expressed as migrated cells per field for each condition.

**Reverse transcription-PCR and Western blot analysis.** The details of reverse transcription-PCR and Western blots have been described previously (6). The antibodies for AKT and extracellular signal-regulated kinase 1/2 (ERK1/2) and their phosphorylation were purchased from Cell Signaling (Beverly, MA); antibodies for HIF-1α and CXCR4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies for histone H3 and acetylated H3 were purchased from Cell Signaling (Beverly, MA); and antibodies for Bax and Bcl-2 were purchased from Cell Signaling (Beverly, MA).
Upstate (Lake Placid, NY); and antibodies for tubulin and acetylated tubulin were purchased from Sigma (St. Louis, MO).

**Matrigel angiogenesis assay in vivo.** Four to 6-week-old C57/BL6J mice were pretreated with LBH589 (10 mg/kg/d, i.p.) for 3 days and injected s.c. in the abdomen with 500 μL of Matrigel (BD Biosciences, San Jose, CA) supplemented with VEGF-A (150 ng/mL) and bFGF (50 ng/mL; R&D Systems). Treatment was continued for 10 days after the Matrigel injection. Then, the mice were sacrificed, and the plugs were retrieved for immunohistochemical analysis. The plugs were fixed in PBS-buffered 10% formalin containing 0.25% glutaraldehyde and were processed for Masson’s Trichrome staining.

**Tumor growth in vivo.** PC-3 tumor cells were resuspended in HANKS solution and mixed with Matrigel (1:1) in a final volume of 0.1 mL. Approximately two million cells were injected bilaterally and s.c. into male athymic mice. Once the tumors became palpable (50-100 mm³), mice were randomly assigned to control group (n = 10) and experimental group (n = 10). The experimental group was treated with LBH589 (10 mg/kg/d, i.p.). Control animals were given vehicle consisting of saline solution with 5% DMSO and 1% Tween 80. The mice were treated 7 days/wk. Tumor volume was measured with a caliper and calculated according to the formula:

\[
\text{Tumor volume} = A \times B \times C \times 0.5236.
\]

The efficacy of treatment was evaluated by the change in tumor volume during treatment period. Following 3 to 4 weeks of treatment, the mice were sacrificed, and the tumors were harvested for histologic studies and Western blots.

**In vivo cell death analysis.** DeadEnd Fluorometric Terminal Deoxynucleotidyl Transferase–Mediated Nick-End Labeling System (Promega, Madison, WI) was used to evaluate the cell death in sectioned tumor xenografts from control and LBH589-treated animals according to the manufacturer’s instructions. The green signal was considered being positive for cell death. The positive controls were obtained by treating cells with DNase, which causes DNA fragmentation.

**Immunohistochemistry study.** Frozen tissue was generated from each tumor to quantify differences in microvessel density between control and experimental groups. Sections were incubated (18 hours at 4°C) with anti-CD31 antibody (PharMingen, San Diego, CA), a specific marker for endothelial cells. Sections were incubated with a secondary biotin-conjugated rabbit anti-goat IgG antibody (1:100) for 30 minutes at room temperature. Sections were incubated with avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA) as per manufacturer’s instructions. Sections were then incubated with 3,3′-diaminobenzidine solution, washed, and counterstained with methyl green.

**Imaging analysis for angiogenesis.** Measurement of the level of angiogenesis in the in vivo Matrigel plug experiment was based on the Masson’s Trichrome staining, and CD31 staining was used in the PC-3 tumor experiment. The regions containing the most intense area of neovascularization (“hotspots”) were chosen for analysis. Eight hotspots were identified for each Matrigel or tumor section. The ImagePro Plus analysis system was used to quantify the percentage of area occupied by the vessel-like structures in each field. The mean and the SE of mean from each group were compared. The negative control was obtained by tissue staining with secondary antibody only.

**Statistical analysis.** Differences between the means of unpaired samples were evaluated by the Student’s t test using the SigmaPlot and SigmaStats program. Ps < 0.05 were considered statistically significant. All statistical tests were two sided.

**Results**

**LBH589 increased histone and nonhistone protein acetylation and inhibited HUVEC proliferation in vitro.** The hydroxamic acid HDAC inhibitor LBH589 was very effective in increasing the acetylation level of histone H3 protein in endothelial cells (Fig. 1A). LBH589 treatment of HUVECs also increased the acetylation level of nonhistone proteins. α-Tubulin is deacetylated by cytoplasmic HDAC6 in several cell types of mouse and human origin (21, 22). In the current study, the acetylation level of α-tubulin was increased after LBH589 treatment (Fig. 1A), and this is an indication of HDAC6 inhibition. The increase in protein acetylation was also associated with perturbation of the endothelial cell cycle. Using our experimental tissue culture conditions, growth factor starvation induced HUVECs cell cycle to arrest in the G0-G1 phase. Twenty-four hours following treatment with complete EGM-2 growth media, the previously starved HUVECs showed a decrease in the G0-G1 phase and an increase in the S-phase population (Fig. 1B). However, simultaneous incubation with increasing doses of LBH589 significantly induced G2-M arrest and decreased S-phase cells (Fig. 1B).

To investigate the effects of different growth factors on LBH589 inhibition of HUVECs, proliferating cells were maintained in either complete EGM-2 growth media, or EGM-2 basal media with 50 ng/mL bFGF/0.5% fetal bovine serum, or 50 ng/mL VEGF-A/0.5% fetal bovine serum. Under these culture conditions, cells were treated with increasing doses of LBH589 for 48 hours, and inhibition of cell viability and % G0/G1, G2/M and S Cells in triplicate experiments; bars, SE. * P < 0.05 versus solvent-treated controls.

![Fig. 1. Effect of LBH589 on HUVECs protein acetylation and cell cycle.](http://www.aacrjournals.org/clinica/2006;12(2)January15,2006)
growth was evaluated by 2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanilide inner salt assay (Fig. 2A). These results showed that LBH589 had similar dose-dependent inhibitory effects on HUVECs regardless of the growth factor used to induce cell proliferation. Using the complete EGM-2 bullet kit growth media, the inhibition of endothelial cell proliferation was also determined by counting viable cell numbers as previously described (6). Following 48 hours of treatment, the solvent-treated control HUVECs had a 1.5-fold increase in cell number, whereas ≤200 nmol/L LBH589 inhibited cell growth. Cytotoxicity was observed with ≥400 nmol/L LBH589 (Fig. 2B) as evidenced by a sharp decrease in cell number. This dose-dependent response was rather unique for endothelial cells. LBH589 inhibited cell proliferation in LNCaP and PC-3 human prostate carcinoma cells at concentrations up to 1 μmol/L (Fig. 2C) without significant cell death and induced primarily G2-M cell cycle arrest (data not shown).

This difference between HUVECs and PC-3 was confirmed by caspase assay. A significant induction of caspases was observed only in HUVECs with concentration of >400 nmol/L (Fig. 2D). Costaining of cells with Annexin V and propidium iodide followed by flow cytometry analysis confirmed a significant cell death in HUVECs but not in PC-3 following treatment with LBH589 (Fig. 2E).

**LBH589 inhibited in vitro angiogenesis.** In the presence of high concentrations of VEGF-A, endothelial cells form tube and capillary-like structures on the surface of basement membrane matrices (Matrigel), through a process involving attachment, alignment, and migration. Treatment with a noncytotoxic dose of LBH589 (50-200 nmol/L) significantly reduced this process (Fig. 3A). We also tested the effect of LBH589 on endothelial chemotaxis. Proliferating HUVECs were starved for 24 hours and pretreated with different concentrations of LBH589 for the last 12 hours of starvation. The cells were then tested in the...
Matrigel invasion assay in the presence of LBH589. The chemokines used in this assay were VEGF-A and SDF-1α, a growth factor that attracts CXCR4 receptor--positive endothelial cells and endothelial precursor cells. Both VEGF-A and SDF-1α induced solvent-treated control cells to invade and migrate through Matrigel (Fig. 3B). Noncytotoxic doses of LBH589 (50-200 nmol/L) induced a dose-dependent inhibition of HUVECs migration and invasion.

**LBH589 modulated VEGF-A signaling.** VEGF-A has been shown to be an important stimulator for endothelial cell functions, such as survival, proliferation, migration, and differentiation. Following VEGF/VEGFR binding, endothelial cell activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT signaling pathways mediates the downstream effect of VEGF (23–26). To study the effect of LBH589 on this signaling pathway, proliferating HUVECs were growth factor-starved and treated with the indicated doses of LBH589 for 24 hours. A high concentration of VEGF-A (100 ng/mL) was added to restimulate the cells for 10 minutes and 24 hours. The phosphorylation of AKT and ERK1/2 and the transcription of three VEGF-responsive genes were evaluated (Fig. 4A). VEGF rapidly induced the phosphorylation of AKT and ERK1/2 (indicative of AKT and mitogen-activated protein kinase activation) in control cells. LBH589 treatment prevented protein phosphorylation in a dose-dependent fashion but had no effect on total AKT and ERK1/2 protein stability.

To further assess the effect of LBH589 on VEGF signaling, we measured the gene expression level of angiopoietin-2 (Ang-2), survivin, and CXCR4, which have been reported to be up-regulated by VEGF stimulation in HUVECs (27–29). Following 24 hours of restimulation with VEGF-A, the mRNA level of all three genes was increased by reverse transcription-PCR. LBH589 treatment (150 nmol/L) inhibited Ang-2, survivin, and CXCR4 gene expression (Fig. 4B).

We have previously reported that LAQ824, another hydroxamic HDAC inhibitor, down-regulates Ang-2, survivin, HIF-1α, and VEGF (6). Besides VEGF, another factor capable of up-regulating the expression of CXCR4 is hypoxia, which results in HIF-1α--initiated transcription (30). CXCR4 promoter has been reported to contain hypoxia response elements. Using the hypoxia mimic cobalt chloride (CoCl₂), we first tested whether LBH589 was able to inhibit HIF-1α protein accumulation in HUVECs, similar to LAQ824 in tumor cells (Fig. 4C). Then, following the treatment of HUVECs with LBH589 (150 nmol/L) in the presence of CoCl₂, we observed the inhibition of CXCR4 at both mRNA and protein levels (Fig. 4D).

**LBH589 inhibited in vivo angiogenesis and prostate tumor growth.** The in vitro antiangiogenic property of LBH589 was tested in vivo. First, the effect on neovascularization in responses to VEGF-A and bFGF was evaluated in the Matrigel plug assay. Matrigel supplemented by VEGF-A and bFGF was injected s.c. into the abdomen of C57/BL6 mice. Ten days after the injection, the neovascularization within the Matrigel plug was evaluated by histology. The Matrigel plugs in the control groups were visually bloodier than the LBH589 (10 mg/kg/d)–treated ones, suggesting a higher level of angiogenesis (Fig. 5A). Masson’s Trichrome staining of the vasculature within the plugs identified more vessel-like structures within the control than the LBH589 treated (Fig. 5B). By imaging analysis, there was a ∼50% reduction in the number of blood vessels in mice treated with LBH589 compared with controls (Fig. 5C). These data suggested that LBH589 attenuated the angiogenic response initiated by both VEGF and bFGF.

In a prostate tumor (PC-3) mouse xenograft model, daily treatment with LBH589 (10 mg/kg/d) induced a statistically significant inhibition of the tumor growth (Fig. 6A), without overt toxicity (no statistically significant weight loss). Western blot analysis of histone protein extract recovered from the tumors revealed induction of histone acetylation in vivo following LBH589 treatment. (Fig. 6B). In vivo tumor angiogenesis was evaluated by immunohistochemical staining of the tumor blood vessel. Using a rat anti-mouse CD31/platelet/endothelial cell adhesion molecule 1 antibody, we observed that control tumors had more CD31+ vessel structures in terms of size and number per field than the LBH589–treated group (Fig. 6C). Quantitative analysis of the percentage of the CD31-positive vessel area in each high power field (∗×200) confirmed reduction of tumor angiogenesis in the LBH589–treated group (Fig. 6C). Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining of tumor sections for detection of in vivo apoptosis did not reveal significant difference between the control and LBH589–treated animals (Fig. 6D). The apoptotic index calculated based on the ratio of terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) reporter-positive endothelial cells and total endothelial cells (24). TUNEL analysis showed that LBH589 treatment reduced the degree of apoptosis in both the control and treated groups (Fig. 6D).
Fig. 4. Effect of LBH589 on HUVEC protein phosphorylation, HIF-1α stability, and CXCR4 expression. A, HUVECs were starved and treated with the indicated dose of LBH589 for 24 hours. Then, VEGF-A (100 ng/mL) was added to restimulate HUVECs for 10 minutes. Phosphorylation of AKT (Ser473) and ERK1/2 (Thr185/Tyr187) were assessed using total cellular protein lysates. AKT and ERK1/2 phosphorylation was determined in growth factor–depleted HUVECs, and following the addition of VEGF-A in untreated and 24-hour LBH589-treated cells. B, HUVECs were treated under the same conditions as in (A), VEGF restimulation was conducted for 24 hours. Total mRNA from cells under different conditions (starvation, VEGF restimulation, and VEGF + 150 nmol/L LBH589) were isolated, and gene expression for Ang-2, survivin, and CXCR4 was evaluated using reverse transcription-PCR. C, HUVECs were treated with 150 nmol/L LBH589 for 24 hours. The hypoxia mimetic CoCl2 was added to induce HIF-1α stabilization for the last 6 hours of treatment. Total cellular protein extracts were used to determine HIF-1α levels. D, proliferating HUVECs were treated with LBH589 and CoCl2 for 24 hours, and CXCR4 expression was evaluated by reverse transcription-PCR and Western blot.

Discussion

Preclinical tumor models have shown the potential therapeutic role of HDAC inhibitors. This approach has been targeting tumor cell proliferation and viability by inducing cell cycle arrest and/or cell death. In previous studies, we reported that the HDAC inhibitors phenylbutyrate and LAQ824 have inhibitory activity on endothelial cell proliferation in vitro and inhibited angiogenesis in vivo (3, 6). These initial observations and similar results from other groups suggested that targeting endothelial cells with HDAC inhibitors may directly contribute to inhibition of tumor angiogenesis and eventually cancer progression. In the current study, our results suggest that LBH589, a recently developed hydroxamic HDAC inhibitor, is also an effective inhibitor of angiogenesis. LBH589 induced G2-M cell cycle arrest in both cancer (PC-3) and endothelial cells (HUVEC) at nanomolar concentrations. The IC50 for LBH589 in HUVEC was 1-fold lower than the IC50 for the HDAC inhibitor LAQ24 (6). Under these conditions, LBH589 was also very effective in inhibiting differentiation processes of endothelial cells, such as tube formation, migration, invasion. The LBH589 concentrations used in our in vitro studies are achievable in mice. Pharmacokinetic data show that LBH589 dose at 25 mg/kg in mice bearing PC-3 tumors resulted in an AUC of 463 ng h/mL (0.96 μmol/L). In mice bearing HCT116 tumors, the AUC was 533 ng h/mL (1.11 μmol/L).3

In our study, we report for the first time that the HDAC inhibitor LBH589 attenuates VEGF-induced signaling in endothelial cells and reduces endothelial cell chemotaxis and invasion. A recent report has shown that class I HDACs regulate the expression of extracellular matrix proteins, and treatment of cancer cells with HDAC inhibitor impaired cancer cell invasion (31). Our results also show for the first time that an HDAC inhibitor inhibits HIF-1α and CXCR4 expression in human endothelial cells. HDAC inhibitor induced repression of HIF-1α not only in tumor cells but also in endothelial cells, and consequent reduction in CXCR4 expression may contribute to the antiangiogenesis properties of these agents.

The down-regulation of CXCR4 by LBH589 is of interest because of the role of this chemokine receptor in the homing of bone marrow progenitor and circulating endothelial cells to active sites of angiogenesis (32). Interestingly, HDAC activity has been reported to be involved in endothelial progenitor cell biology, and HDAC inhibition resulted in impaired endothelial progenitor cell proliferation (33). Taken together, our results provide both potential mechanisms for antiangiogenic activities of LBH589 and the rationale for clinical testing of HDAC inhibitors as angiogenesis inhibitors.

3 Unpublished data.
Although the angiogenesis-related gene modulation, such as HIF-1α, eNOS, VEGF, VEGFRs, and Tie-2 by HDAC inhibitors both in vitro and in vivo, is consistent, the underlying molecular mechanisms have yet to be elucidated (6, 34). One possibility is that through chromatin histone acetylation and gene transcription, HDAC inhibitors up-regulate tumor suppressor genes, such as p53 and VHL, which negatively regulate HIF-1α and VEGF (11).

HDAC inhibitors may also up-regulate some yet to be identified transcriptional repressors, which subsequently inhibit specific gene expression. Another possibility is that HDAC inhibitors induce acetylation of nonhistone proteins and subsequently affect the gene expression (both up-regulation and down-regulation) and protein stability. For example, the acetylation of transcription factor YY1 modifies its transcriptional activity (36), and the acetylation of androgen receptor has been associated with its increased nuclear localization and transcriptional activity (37). Therefore, it is conceivable that unidentified transcription repressors are activated upon acetylation and may contribute to angiogenesis-related gene regulation.

Protein acetylation may have a direct effect on endothelial cell biology. The acetylation of α-tubulin through HDAC6 inhibition has been associated with the change of tubulin dynamics and cell motility (37, 38). This observation may provide insights for the LBH589-induced inhibition of endothelial cell tube formation and Matrigel invasion, because both processes require cell motility.

HIF-1α acetylation by acetyl transferase ARD1 promotes HIF-1α degradation (39) and HSP90 acetylation compromises its chaperone function (40). Class I and II HDACs (HDAC1, HDAC6, and HDAC10) have been shown to physically associate with protein phosphatase 1, and the inhibition of HDAC by TSA disrupts their disassociation (41). All these events may be potentially associated with HDAC inhibitors and responsible for their biological activity.

The future direction of pharmacogenetic research on HDAC inhibitors will need to expand from chromatin acetylation and epigenetic regulation to nonhistone protein acetylation and its functional consequences. It is very likely that changes in protein acetylation and function will provide molecular links for inhibition of gene expression and attenuation of signaling pathways.

Major cellular signaling pathways, such as the phosphatidylinositol 3-kinase–dependent mechanism (43). Downstream gene activation by phosphatidylinositol 3-kinase and survivin has been shown to play a pivotal role in VEGF-mediated endothelial cell protection by preserving the microtubule network (44). Survivin up-regulation may represent a novel mechanism of endothelial cell “resistance” (43). Thus, exploiting HDAC inhibitors as antiangiogenesis agents by impairing endothelial cell survival should enhance the antiangiogenesis activity of chemotherapy agents, such as microtubule inhibitors.

Several HDAC inhibitors are currently in clinical trials both in solid and hematologic malignancies (45). These clinical studies will provide the opportunity to test the dual function (both antitumor and antiangiogenesis) of some HDAC inhibitors, such as LBH589, SAHA, depsipeptide, MS-275, and valproic acid. A therapeutic approach where a drug simultaneously targets both the tumor cell and the endothelial compartment seems to be a rational strategy.

The HDAC inhibitors currently undergoing clinical trials have a safer toxicity profile than traditional chemotherapeutic agents. These characteristics raise the possibility of combining the HDAC inhibitors with other anticancer agents for targeted therapy. For example, we recently reported that the combination of LAQ824 and the VEGFR tyrosine kinase inhibitor PTK787 has an additive inhibitory activity on VEGF-induced angiogenesis in vitro and is more effective than single agents in controlling the progression of both prostate and breast cancer without overt toxicity. The mechanisms underlying the observed additive and/or synergistic effect may due to the inhibition of multiple independent and/or converging signaling pathways (6).
The combination of HDAC inhibitors with anti-VEGF therapies (i.e., VEGF blocking agents or VEGFR tyrosine kinase inhibitors) is of particular interest. Treatment with the anti-VEGF monoclonal antibody bevacizumab in combination with chemotherapy has been shown to increase overall survival in patients with metastatic colon carcinoma (46). Moreover, molecular targeted therapies with VEGFR tyrosine kinase inhibitors have been reported to have clinical activity in metastatic renal cell carcinoma patients (47). However, there are preclinical and clinical evidences that tumor “escape” to anti-VEGF therapy occurs (48). It is conceivable that angiogenesis-related gene modulation by HDAC inhibitors may affect tumor cell and endothelial cell adaptation to anti-VEGF therapies and prevent or delay the escape.

In conclusion, the HDAC inhibitor LBH589 has shown the dual function of targeting both tumor cells and proliferating endothelial cells and to inhibit tumor angiogenesis by gene modulation. Rational clinical testing of these anticancer agents as single agents or in combination with angiogenesis inhibitors and other biological therapies is warranted and should include angiogenesis-related correlative studies as potential markers of drug efficacy.

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References


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