Histone Deacytlation Inhibition in Pulmonary Hypertension
Therapeutic Potential of Valproic Acid and Suberoylanilide Hydroxamic Acid

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Background—Epigenetic programming, dynamically regulated by histone acetylation, is a key mechanism regulating cell proliferation and survival. Little is known about the contribution of histone deacetylase (HDAC) activity to the development of pulmonary arterial hypertension, a condition characterized by profound structural remodeling of pulmonary arteries and arterioles.

Methods and Results—HDAC1 and HDAC5 protein levels were elevated in lungs from human idiopathic pulmonary arterial hypertension and in lungs and right ventricles from rats exposed to hypoxia. Immunohistochemistry localized increased expression to remodeled vessels in the lung. Both valproic acid, a class I HDAC inhibitor, and suberoylanilide hydroxamic acid (vorinostat), an inhibitor of class I, II, and IV HDACs, mitigated the development of and reduced established hypoxia-induced pulmonary hypertension in the rat. Both valproic acid and suberoylanilide hydroxamic acid inhibited the imprinted highly proliferative phenotype of fibroblasts and R-cells from pulmonary hypertensive bovine vessels and platelet-derived growth factor–stimulated growth of human vascular smooth muscle cells in culture. Exposure to valproic acid and suberoylanilide hydroxamic acid was associated with increased levels of p21 and FOXO3 and reduced expression of survivin. The significantly higher levels of expression of cKIT, monocyte chemoattractant protein-1, interleukin-6, stromal-derived factor-1, platelet-derived growth factor-b, and S100A4 in R-cells were downregulated by valproic acid and suberoylanilide hydroxamic acid treatment.

Conclusions—Increased HDAC activity contributes to the vascular pathology of pulmonary hypertension. The effectiveness of HDAC inhibitors, valproic acid, and suberoylanilide hydroxamic acid, in models of pulmonary arterial hypertension supports a therapeutic strategy based on HDAC inhibition in pulmonary arterial hypertension.

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Key Words: epigenesis, genetic ■ histone deacetylation ■ hypertension, pulmonary ■ valproic acid ■ vorinostat

The lungs of patients with advanced pulmonary arterial hypertension (PAH) exhibit a vascular remodeling involving all cellular elements of the vessel wall caused by dysregulated cell proliferation and survival, inflammation, and in situ thrombosis.1,2 Currently approved treatments target primarily an imbalance of vasoactive factors in PAH3 and at best retard the course of the disease. There is an urgent need for therapies that directly target the structural vascular pathology.

Clinical Perspective on p 467

Aberrant epigenetic changes such as the histone acetylation state influence gene expression and play a role in regulating cell proliferation, migration, and survival and inflammation in several diseases, including cancer.4,5 Histone acetylation/deacetylation balance, maintained dynamically by 2 important families of enzymes, histone acetyltransferases and histone deacetylases (HDAC),6 controls the higher-order
structure of chromatin and the resultant accessibility of transcriptional factors to their target genes.\textsuperscript{7} Histone acetyltransferases catalyze the acetylation of lysine residues, neutralizing positive charges, relaxing chromatin structure, and increasing accessibility to transcription machinery. HDACs remove acetyl groups from histones (and other nuclear proteins), inducing chromatin condensation and transcriptional repression.\textsuperscript{8,9} HDACs have emerged as key targets to reverse aberrant epigenetic changes associated with cancer and autoimmune disease, and HDAC inhibitors show promise as anticancer and antiinflammatory agents.\textsuperscript{9,10}

The present understanding of epigenetic modifications through histone acetylation in PAH is very limited. Li et\textsuperscript{11} have described pulmonary adventitial fibroblasts from chronically hypoxic calves that expressed an epigenetically altered proinflammatory phenotype; the phenotype was reversed by HDAC inhibition. Recently, selective class I HDAC inhibition has been reported to attenuate the development of hypoxia-induced pulmonary hypertension in the rat through an antiproliferative mechanism.\textsuperscript{12} Moreover, right ventricular (RV) function was preserved in contrast to experience with the pan-HDAC inhibitor trichostatin A in rat pulmonary artery banded models.\textsuperscript{13} However, neither the involvement of HDACs in human PH nor the ability of HDAC inhibitors to reverse existing PAH has been explored. Here, we show increased expression of HDAC1, HDAC4, and HDAC5 in human idiopathic PAH (IPAH) lung and the association of HDAC1 and HDAC5 with remodelled vessels. Valproic acid (VPA), a class I HDAC inhibitor, and suberoylanilide hydroxamic acid (SAHA), Vorinostat, an inhibitor of classes I, II, and IV, are effective in reversing pulmonary hypertension in the hypoxic rat and exert antiproliferative and antiinflammatory effects in human and animal vascular cells in culture. The data provide further compelling evidence that HDACs should be explored as therapeutic targets in pulmonary vascular disease.

Methods

Human Tissues

Human lung samples (lobectomy and IPAH) were obtained from the Imperial College Pulmonary Hypertension biorepository (ethics reference numbers 01–210 and 2001/6003). The patients’ characteristics have been described previously.\textsuperscript{14}

Animals and Experimental Design

Adult male Sprague-Dawley rats (body weight, 200–250 g; Charles River, UK) were used. All experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 (London, UK). Consecutive in vivo experiments were designed as follows. For chronic hypoxia–time course experiments, rats were divided into 4 groups (n=3) and exposed to either normoxia or hypoxia (normobaric; FiO\textsubscript{2} 10%) for 3:1 days, 1 week, and 2 weeks.\textsuperscript{15} Hemodynamic parameters were measured and tissues were collected for biochemical and histological examination. For the chronic hypoxia–protection study with VPA, rats were divided into 4 groups (n=6) and exposed to normoxia, hypoxia for 2 weeks, hypoxia plus VPA (sodium salt; Sigma-Aldrich) 100 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} in drinking water (low VPA), and hypoxia plus treatment with VPA 300 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} (high VPA).\textsuperscript{16} Drug treatment was started 3 days before hypoxia and continued for the 2-week duration of exposure. For the chronic hypoxia–treatment study with VPA and SAHA, rats were divided into 5 groups (n=9–12) and exposed to normoxia, hypoxia for 2 weeks, hypoxia for 4 weeks, hypoxia and VPA (300 mg · kg\textsuperscript{-1} · d\textsuperscript{-1}), and hypoxia and SAHA (50 mg · kg\textsuperscript{-1} · d\textsuperscript{-1}; Chemos GmbH).\textsuperscript{17} Drug treatment was given in drinking water, initiated after 2 weeks of hypoxia exposure and continued for the remaining 2 weeks of exposure. VPA was dissolved in distilled water and SAHA in 5 molar equivalents of 2-hydroxypyropyl-β-cyclodextrin (HOP-β-CD; Sigma-Aldrich) as previously described.\textsuperscript{18} The 4-week-hypoxia group was given HOP-β-CD solution (1.38 g · kg\textsuperscript{-1} · d\textsuperscript{-1}) as vehicle control. Animals were weighed every other day, and treatment doses were calculated accordingly.

Hemodynamic Measurements, Tissue Collection, and Histology Examination

Rats were anesthetized (Hypnorm 1 mL/kg i.m, Hypnovel 0.8 mL/kg i.p). RV systolic pressure and pulmonary arterial pressure were measured with a precured catheter inserted through the right jugular vein, and systemic blood pressure was recorded via the carotid artery cannulation using a PowerLab Data Acquisition system (ADInstruments Ltd).

The animals were euthanized and then tissues were collected, snap-frozen, and stored at ~80°C for biochemical measurements. Hearts were dissected and weighed, and the ratio of RV to left ventricle plus the septum mass was used as an index of RVH. The left lung was fixed with 10% formalin in PBS and processed for elastic van Gieson and hematoxylin and eosin staining. Vessels <100 μm in peripheral lung were counted blindly under microscope (×40), and pulmonary vascular remodeling was expressed as the proportion of vessels with double elastic lamina to total vessels counted.

Cell Culture

Adventitial fibroblasts (PH-Fibs) and morphologically distinct cells potentially of hematopoietic origin with high growth potential (rhomboidal or “R” cells) were isolated from the adventitial and medial layers of distal pulmonary arteries of chronically hypoxic calves using explant techniques as described previously.\textsuperscript{11,19} Experiments were performed on cells at passage 4 to 10 and compared with fibroblasts (CO-Fibs) and smooth muscle cells isolated from control animals (CO-SMCs) and studied at a similar passage number. For proliferation assays, cells were serum deprived and cultured for 3 days. Cell numbers were counted every day. The effects of varying concentrations of VPA (1, 2.5, and 5 mmol/L) and SAHA (10 μmol/L) on cell proliferation were also examined under serum-deprived conditions, and cell numbers were counted at day 3. For biochemical studies, serum-starved cells were treated with VPA (5 mmol/L) or SAHA (10 μmol/L) for 24 hours, and RNA was extracted for reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. Primers are listed in Table I in the online-only Data Supplement.

Human pulmonary smooth muscle cells (passages 5–9) were assessed for proliferation by hemocytometer or BrdU incorporation assay (Millipore). Cells were serum deprived and stimulated with 50 ng/mL platelet-derived growth factor (PDGF; Ebioscience) in 10% FBS with/without HDAC inhibitors treatment (VPA 1–2 mmol/L and SAHA 2.5–10 μmol/L)\textsuperscript{20} for 72 hours. Individual experiments were repeated at least 3 times. In addition, harvested cells were stained with 50 μg/mL propidium iodide (Sigma) to access the cell cycle distribution and viability by use of the Becton-Dickinson fluorescence-activated cell sorting Calibur flow cytometer system with Cell Quest software (CytoLogic version 1.2.1; Becton-Dickinson).

Western Blotting

Rat (lungs, RV, and kidneys) and human (lobectomy and IPAH lung) samples were homogenized in phosphate buffer (100 mmol/L D04 [K2HPO4·KH2PO4=3:1], 1 mmol/L EDTA, 1 mmol/L DTT supplemented with protease inhibitor; Roche). Proteins from cell pellets were extracted with TGN buffer (1 mol/L Tris, 2.5 mol/L NaCl, glycerol, 0.5 mol/L β-glycerophosphate, Tween 20, Nonidet P40, and protease inhibitor). Western blotting was performed according to the manufacturer’s suggestions (rabbit polyclonal antibodies against...
human HDACs, 1:1000, Cell Signaling; mouse polyclonal antibodies against human Bcl-2, 1:1000, BD Biosciences). Proteins were detected by the Novex ECL chemiluminescent kit (Invitrogen). Optical densities of individual bands were measured and protein expressions were standardized with α-actin for the RV and β-actin for the other tissues.

**Immunohistochemistry**

Tissue sections were processed as previously described.21 Sections were incubated with normal goat serum (1:5 dilution in PBS; DAKO, UK), followed by purified mouse antibody against HDAC1 (1:50; Cell Signaling), rabbit antibody against Ki67 (1:50; Thermo Scientific), or HDAC5 (1:50; Cell Signaling Technology) and then secondary antibody against mouse or rabbit (Cell Signaling). Peroxidase activity was visualized with diaminobenzidine tetrahydrochloride (0.5 mg/mL; Sigma, UK), and sections were counterstained with Mayer hematoxylin.

**Histone Extraction and Acetylation Assessment**

Cell pellets or tissues were washed with ice-cold PBS containing 5 mmol/L sodium butyrate. Lysis buffer (10 mmol/L Tris, 50 mmol/L sodium bisulfate, 10 mmol/L MgCl₂, sacarose 8.6%, 1% Triton X-100) was added on ice for 15 minutes. After homogenization, the pellets were washed with lysis buffer and then Tris-EDTA (10 mmol/L Tris, 13 mmol/L EDTA). Precipitated nuclei were suspended in ice-cold dH₂O and 0.4 mol/L sulfuric acid (1:1) and kept on ice for 1 hour. After centrifugation at 15 000 rpm, histone precipitation was performed with 1 mL acetone at −20°C overnight. Acetylation was detected on Western blotting with anti-acetylated H3 and H4 antibodies (Millipore). Parallel gels were executed with anti-whole H3 and H4 antibodies (Millipore) as loading control for quantification.

**RT-PCR Analysis**

Total RNA isolation from cultured cells, first-strand cDNA synthesis, and real-time RT-PCR were performed as described previously.22 mRNAs were quantified by TaqMan real-time RT-PCR using the commercially available specific primer probes (Table I in the online-only Data Supplement). Real-time PCR reactions were set following the manufacturer’s conditions. Threshold cycle (Ct) values obtained for each gene were converted to the linear form with the term $2^{-\Delta\Delta Ct}$ as a value directly proportional to the copy number of mRNA,22 where ΔΔCt was the Ct value normalized by β-actin and referenced to the values obtained for each gene under normoxic conditions.

**Statistics**

Data are presented as the mean±SEM, and variance of homogeneity was tested by the Levene test. Differences between groups were assessed by the Student t test or appropriate ANOVA, specifically the Welch ANOVA for data with unequal variances or standard ANOVA otherwise, followed by the Bonferroni post hoc test for multiple comparisons. A value of $P<0.05$ was considered statistically significant. All statistical analyses were performed with SPSS Statistics 17.0 (IBM Corp.).

**Results**

**HDAC1 and HDAC5 Expression Is Increased in Lungs From IPAH Patients and Lungs and RV From the Chronically Hypoxic Rat**

HDAC (class I: HDAC1, HDAC2, and HDAC3; class II: HDAC4, HDAC5, and HDAC7) proteins levels were measured in human and rat lung samples. Human IPAH lung exhibited increased expression of HDAC1, HDAC4, and HDAC5 and decreased expression of HDAC2 and HDAC3 compared with control lung tissue, along with a 2-fold increase in the antiapoptotic regulator Bcl-2 (Figure 1). Hypoxic rats with an elevated mean pulmonary arterial pressure and RVH (Figure 2A and 2D) exhibited a striking increase in HDAC1 (Figure 2B and 2E) and HDAC5 (Figure 2C and 2F) expression in both lung and RV, again accompanied by elevation of Bcl-2 in the lung (Figure 2G and Figure 1 in the online-only Data Supplement).

Consistent with a role in the pathology of pulmonary hypertension, HDAC1 and HDAC5 immunostaining was observed in remodeled pulmonary vessels of IPAH and hypoxic rat lungs, in contrast to control tissues (Figure 3 and Figures II and III in the online-only Data Supplement). In keeping with their known function, HDAC1 showed a predominantly nuclear distribution whereas HDAC5 was cytoplasmic. These vessels also demonstrated Ki67 expression, a marker of proliferation, in the rat lung (Figure 3C).

**VPA and SAHA Ameliorate Established Pulmonary Arterial Hypertension in Chronic Hypoxia**

To address the role of increased HDAC1 and HDAC5 levels, we investigated the effects of VPA (class I inhibitor) and SAHA (class I, II, and IV inhibitor) in the hypoxic rat. Supportive of a previous study, treatment with VPA at the start of hypoxia attenuated the development of pulmonary hypertension (Figure IV in the online-only Data Supplement). More important, treatment with VPA or SAHA after pulmonary hypertension was established reduced mean pulmonary arterial pressure (Figure 4A) and RV hypertrophy (Figure 4B) with no significant changes in heart rate or systemic blood pressure (Figure 4C). Consistent with an effect on vascular remodeling, pulmonary arteriolar muscularization was significantly reduced (Figure 4D and 5E), along with Bcl-2 and HDAC1 (Figure 5A and Figure V in the online-only Data Supplement), whereas lung p21 levels were increased by VPA and SAHA treatment (Figure 5B).

**Histone Acetylation Levels Were Augmented by SAHA and VPA Treatments**

As evidence of HDAC inhibition, VPA increased acetylated histone H3 levels, whereas both H3 and H4 acetylation levels were increased >60% in SAHA-treated lungs (Figure 5C–5E). Interestingly, total histone levels were elevated in the rat lung after hypoxia exposure, a measure of protein synthesis, and they were reduced by VPA and SAHA treatment (Figure 5F).

**HDAC Inhibitors Prevent Constitutive Growth of PH-Fibs and R-Cells**

Previous studies have shown that cells with high proliferative potential and stably increased HDAC activity can be isolated and perpetuated in culture from both the adventitia (PH-Fibs) and media (R-cells) of the hypertensive bovine pulmonary artery.11,24 It has been speculated that these cells with high proliferative capacity contribute selectively to the remodeling process in pulmonary hypertension.3 Significantly increased class I HDAC catalytic activity has been shown in PH-Fibs compared with CO-Fibs.11 In this study, class I HDAC mRNA levels were also significantly increased in R-cells compared with CO-SMCs; no differences in HDAC2 and
HDAC3 levels were detected (Figure VI in the online-only Data Supplement).

Under serum-deprived conditions, R-cells and PH-Fibs exhibited greater proliferation than CO-SMCs and CO-Fibs (Figure 6A and 6C). The HDAC inhibitor VPA, at concentrations of 2.5 and 5 mmol/L, significantly inhibited the growth of R-cells but had no effect on CO-SMC growth or viability (Figure 6B). A similar response was observed in PH-Fibs with again no effect seen on CO-Fibs (Figure 6D). SAHA (10 μmol/L) also inhibited cell growth, with the greatest sensitivity exhibited by R-cells and PH-Fibs (Figure 6B and 6D). At the concentrations tested, no significant effects on cell viability were found (Figure VII in the online-only Data Supplement).

We sought to determine mechanisms for VPA and SAHA induced growth inhibition. Constitutively activated R-cells and PH-Fibs expressed significantly higher levels of survivin and lower levels of p21 and FOXO3 under basal, serum-free conditions than their respective control cells, CO-SMCs and CO-Fibs (Figure 6E and 6F). Treatment with VPA and SAHA led to significant increases in p21 and FOXO3 mRNA levels in both R-cells and PH-Fibs and a significant decrease in survivin in both cell types (Figure 6E and 6F).
A previous study showed that R-cells expressed significantly higher levels of progenitor cell markers (cKIT), pro-inflammatory factors (monocyte chemoattractant protein-1, interleukin-6, stromal-derived factor-1), and growth factors (PDGF-b and S100A4). In this study, treatment with VPA and SAHA significantly decreased expression of these genes in R-cells (Figure 7).

HDAC Inhibitors Prevent PDGF-Induced Human Pulmonary Smooth Muscle Cell Proliferation

A direct inhibitory effect of VPA and SAHA on PDGF-stimulated human pulmonary smooth muscle cell proliferation was observed (Figure 8A and 8B). VPA and SAHA arrested cell growth at the G1-S phase, reversing the shift caused by PDGF stimulation (Figure 8C). The percentage of cells in G1 (PDGF, 55.6%) was higher in VPA-treated cells (VPA 1 mmol/L, 63.4%; VPA 2 mmol/L, 69.0%; *P<0.05) and SAHA-treated cells (SAHA 2.5 μmol/L, 71.5%; SAHA 10 μmol/L, 71.4%; **P<0.001) compared with the NC group.

Figure 2. Time course of development of pulmonary hypertension phenotype and histone deacetylase (HDAC) expression in a chronically hypoxic rat. A, Mean pulmonary arterial pressure (PAP), (B) HDAC1 in lung, (C) HDAC5 in lung, (D) right ventricular (RV) hypertrophy (RVH), (E) HDAC1 in RV, (F) HDAC5 in RV, (G) Bcl-2 in lung, and (H) representative bands. Rats were exposed to normal air (NC) or hypoxia for 2 days (2D), 1 week (1W), and 2 weeks (2W). The data are generated from optical density measurements of individual bands from Western blots and normalized to β-actin. The ratios are presented as mean±SEM of fold change relative to control (normal air), n=3 for each group. Comparisons were made with NC with the Welch ANOVA for (C) HDAC5 in lung and (F) HDAC5 in RV and standard ANOVA in the remaining analyses. *P<0.05, **P<0.001 compared with the NC group.
Discussion

This study demonstrates for the first time changes in the expression of HDAC proteins, specifically increased HDAC1 (class I) and HDAC5 (class II), in human IPAH lung. These data were replicated in lungs and RV from rats with hypoxia-induced pulmonary hypertension. The lack of change in HDAC expression in the kidneys from these animals links the observed changes in HDAC expression to the pathological vascular remodeling of pulmonary hypertension rather than the hypoxic stimulus per se. Immunohistochemical assessment of human IPAH and chronic hypoxic rat lungs confirmed increased nuclear expression of HDAC1 and cytoplasmic expression of HDAC5 in remodeled vessels. These vessels also express the proliferative marker Ki67, supporting a link between aberrant epigenetic changes and dysregulated cell proliferation. Consistent with a functional role for HDACs in pulmonary hypertension, long-term administration of VPA, a HDAC class I inhibitor, not only prevented hypoxia-induced pulmonary hypertension but also attenuated the phenotype in the rat when administered after pulmonary hypertension had become established. A similar effect was produced by the broad-spectrum HDAC inhibitor SAHA. VPA and SAHA were also effective in cell culture models. Both inhibited PDGF-stimulated human smooth muscle cell proliferation and the hyperproliferation of epigenetically altered bovine R-cells and fibroblasts in culture. The precise molecular mechanisms by which HDAC inhibition exerts its effects in these models remain to be elucidated, but the changes in Bcl-2 and p21 expression in vivo and p21, FOXO3, cKIT, PDGF-b, S100A4, and survivin in vitro strongly support a direct effect on cell division and survival. Downregulation of proinflammatory factors such as monocytic chemoattractant protein-1, interleukin-6, and stromal-derived factor-1 may also be involved.

HDACs are expressed in all eukaryotic cells and regulate many genes engaged in controlling cell proliferation, differentiation, and survival. Eighteen HDACs have been identified in humans. Eleven contain highly conserved deacetylase domains and are zinc dependent: class I (HDAC1, HDAC2, HDAC3, and HDAC8, nuclear localization); class IIa (HDAC4, HDAC5, HDAC7, and HDAC9)/class IIb (HDAC6...
and HDAC10; cytoplasm and nuclear localization), and class IV (HDAC11). Another 7 HDACs, known as class III or sirtuins, require nicotinamide adenine dinucleotide for their enzymatic activity. The precise balance between the acetylated and deacetylated states of histones is an important feature of gene regulation. HDAC expression is increased in a number of human tumors and cancer cell lines.25 In general, increased HDAC activity results in histone hypoacetylation, which has been implicated in the initiation and progression of various tumors.26–28 For example, HDAC1 is overexpressed in gastric, pancreatic, colorectal, prostate, and hepatocellular cancers and correlates with poor prognosis.26,29–31 The increase in total histone levels and HDAC1 and HDAC5 in human IPAH and lungs from pulmonary hypertensive rats, together with the increase in Bcl-2 expression, is consistent with the proliferative, apoptosis-resistant vascular pathology that characterizes pulmonary hypertension.2 The reduction in HDAC2 in lungs from IPAH patients and hypoxic rats is consistent with a compensatory reaction to HDAC1 overexpression.27

We selected 2 HDAC inhibitors on the basis of the pattern of HDAC expression in human lung and the potential to translate findings into clinical studies. VPA is used clinically as an antiepileptic drug, and for some painful neuropathies, it has a low toxicity profile.32 It has a complex pharmacology, which includes sodium channel blockade and class I HDAC inhibition (IC50, 0.4 mmol/L).33 The doses chosen for our experiments were based on published studies in the range reported to inhibit HDAC1.16 Recent studies have reported that VPA has antitumor effects in various cancers through HDAC inhibition, and clinical studies are ongoing.34,35 SAHA is a relatively broad-spectrum HDAC inhibitor (classes I, II, and IV) that was approved by the Food and Drug Administration as a therapy for cutaneous T-cell lymphoma in 2006 under the generic name vorinostat.36 In keeping with inhibition of HDAC activity, both VPA and SAHA increased lung histone H3 acetylation levels, and SAHA increased H4 histone acetylation.

Both agents reversed the increase in total histone levels in the chronic hypoxic rat lung, an indirect measure of hyper-
proliferation.37 HDAC inhibitors exert their antineoplastic effects through multiple interacting processes.38 Both VPA and SAHA have been reported to downregulate the antiapoptotic factor Bcl-2 and to activate proapoptotic factors such as Bid and Bim,39,40 thereby increasing the ratio of proapoptotic to antiapoptotic proteins. The cyclin-dependent kinase inhibitor p21 is directly transcriptionally upregulated within hours of HDAC inhibitor treatment, leading to cell cycle arrest at the G1-S phase via the tumor suppressor p53.41,42 Upregulation of p21 and downregulation of Bcl-2 expression in hypoxic rat lungs by VPA and SAHA are consistent with the notion that HDAC inhibition may reverse vascular remodeling by inhibiting proliferation and promoting apoptosis.

Further support for this comes from the measurements made on cells in vitro. Human pulmonary vascular smooth muscle cells exposed to VPA and SAHA showed increased p21 levels, and fluorescence-activated cell sorting analysis confirmed cell cycle arrest at the G1-S phase. We observed a significant attenuation of PDGF-stimulated Bcl-2 expression with SAHA but not VPA. PARP cleavage, another signature of apoptosis, was observed in cells exposed to SAHA (10 μmol/L), indicating a proapoptotic action.

The fibroblasts and R-cells (potentially of hematopoietic origin) derived from the distal pulmonary arteries of chronically hypoxic calves display a stable hyperproliferative/apoptosis-resistant phenotype, even under serum-deprived ex vivo conditions,11,19 and thus offer a cell model to study the effects of HDAC inhibition in cells displaying an epigenetically altered and stable ex vivo phenotype. The phenotypic changes displayed by the PH-Fibs are strikingly similar to those described in rheumatoid arthritis fibroblasts in which changes in HDAC activity have been shown to contribute to their inflammatory/destructive phenotype.43 HDAC1 catalytic activity is significantly elevated in PH-Fibs and R-cells compared with control cells.11 The greater growth inhibitory effect of VPA and SAHA on PH-Fibs and R-cells compared with control cells might reflect specific modulation of epigenetically altered signaling pathways in these cells. Similar results have been observed in studies of cancer in which transformed cells are highly sensitive to the apoptosis-inducing effects of HDAC inhibitors compared with normal controls.44 Here, we show that PH-Fibs and R-cells express significantly lower levels of p21 and FOXO3 and higher levels of survivin compared with cells from control animals.
Figure 6. Effects of histone deacetylase inhibition on bovine R-cells and adventitial fibroblasts (PH-Fibs) in culture. Effects on proliferation are assessed by counting (A) daily cell counts of serum-deprived R-cells and control smooth muscle cells (CO-SMCs) in 3 days; (B) cell counts of R-cells and CO-SMCs, untreated (NT) and treated with increasing concentrations of valproic acid (VPA; 1, 2.5, and 5 μM).
The data in this study come from end-stage human PAH, a rodent model, and cells in culture. Each has their limitations as a readout for the human condition but collectively support the argument that changes in HDAC activity may participate in the vascular pathology of pulmonary hypertension, both IPAH and secondary to hypoxia. RV function was not examined in the rat model, but no adverse effects on animal survival were observed. The data to date in the literature on RV function after pulmonary artery banding are conflicting and indicate caution. However, recent data on RV function and gene expression with selective class I HDAC inhibition are more encouraging. There is extensive human experience with VPA, and it is generally well tolerated. SAHA is active against HDACs in the low-nanomolar-

(CO-fibs and CO-SMC) under serum-starved conditions. The increase in p21 and FOXO3 when exposed to VPA and SAHA is compatible with the inhibition of constitutive cell growth. Survivin, a member of the “inhibitor of apoptosis” family, is associated with the development of PH etiologically. Interestingly, both VPA and SAHA reduced survivin levels in PH-Fibs and R-cells, arguing that VPA and SAHA target the apoptosis-resistant remodeling cells in PAH. The significantly higher levels of proinflammatory factors, including monocyte chemoattractant protein-1 and interleukin-6, in the R cell were downregulated by VPA and SAHA treatment, confirming the antiinflammatory mechanism of HDAC inhibition described previously.
concentration range and does not accumulate in cardiac tissue. Because the safety profile of both drugs in humans is well established, HDAC inhibition is an accessible therapeutic strategy to examine in patients with PAH.

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Disclosures
None.

References


Histone deacetylases (HDACs) have emerged as key targets to reverse aberrant epigenetic changes associated with cancer and autoimmune disease, and HDAC inhibitors show promise as anticancer and antiinflammatory agents. We examined the pattern of HDAC expression in lungs from patients with pulmonary arterial hypertension and investigated the effect of HDAC inhibition on the reversal of pulmonary hypertension in a rat model. Coupled to this, we explored the effects on mechanisms (proliferation, apoptosis, and inflammation) relevant to the pathology of pulmonary arterial hypertension in human and animal cell model systems. Our results demonstrate that increased HDAC activity contributes to the vascular pathology of pulmonary hypertension. The effectiveness of the HDAC inhibitors valproic acid and suberoylanilide hydroxamic acid in models of pulmonary arterial hypertension supports a therapeutic strategy based on HDAC inhibition in pulmonary arterial hypertension.
Histone Deacetylation Inhibition in Pulmonary Hypertension: Therapeutic Potential of Valproic Acid and Suberoylanilide Hydroxamic Acid

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**Supplemental Table 1.** Bovine primers for real time RT-PCR

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Supplemental Figures

**Figure I.** HDAC protein expression in lung extracts from rats. (A) HDAC1, (B) HDAC2, (C) HDAC3, (D) HDAC4, (E) HDAC5, (F) HDAC7 (G) Bcl-2, (H) representative bands and (I) kidney. Rats were exposed to normal air (NC) or hypoxia for 2 days (2D), 1 week (1W) and 2 weeks (2W). The data are generated from optical density measurements of individual bands from Western blots and normalised to β-actin. The ratios are presented as mean ± SEM of fold change relative to NC. n=3 in each group. Welch’s ANOVA was used for (C) HDAC3 and (E) HDAC5 and standard ANOVA for the others, comparing each group with NC. * p<0.05, ** p<0.001 compared with NC group.
Chromatically hypoxic rat

A. HDAC1 expression

B. HDAC2 expression

C. HDAC3 expression

D. HDAC4 expression

E. HDAC5 expression

F. HDAC7 expression

G. Bcl-2 expression

H. Western Blot Analysis

I. HDAC1 expression in kidney

Experiments were conducted to evaluate the expression levels of HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC7, and Bcl-2 under hypoxic conditions. The expression levels were quantified using Western Blot analysis.
**Figure II.** Distribution of HDAC1 and HDAC5 expression in IPAH lung sections, low power view. Bar = 25 µm.

**Rat, normoxia control**

**Figure III.** Distribution of HDAC1 and HDAC5 in normoxic rat lung sections, high power view. Bar = 25 µm.
**Figure IV.** Prevention study: Valproic acid (VPA) was administered during 2 weeks hypoxia. (A) PAP, mean pulmonary artery pressure (B) RV/LV+sep, right ventricular hypertrophy, (C) SBP, systolic blood pressure, (D) percentage of muscularised vessels, (E) histology figures with van Gieson’s elastic stain. NC: normoxia; HC: hypoxia for 2 weeks; low VPA: hypoxia with VPA 100 mg/kg/day; and high VPA: hypoxia with VPA 300 mg/kg/day. The data are presented as mean± SEM. n=6 in each group. * p<0.05, ** p<0.001 compared with HC, ## p<0.001 compared with NC. One way standard ANOVA was used, comparing all groups pairwise. Bar = 25 µm.
Figure V. HDAC1 protein levels in chronically hypoxic rat lungs. The data are generated from optical density measurements of individual bands from Western blots and normalised to β-actin. Data are presented as mean ± SEM of fold change relative to 4WH. n=6. 4WH: hypoxia for 4 weeks; VPA: hypoxia with VPA 300 mg/kg/day; SAHA: hypoxia with SAHA 50 mg/kg/day. * p<0.05 compared with 4WH. Welch’s ANOVA was used, comparing each group with 4WH.
**Figure VI.** HDAC-1 mRNA levels are significantly increased in R-cells compared to CO-SMC. Data is analyzed as relative expression to HPRT and presented as mean ± SEM. *, p<0.05; compared with CO-SMC using Student’s t test.
**Figure VII.** HDAC inhibitors, VPA and SAHA, do not induce significant cell death at the concentrations tested. Fibroblast cells isolated from chronically hypoxic pulmonary hypertensive calves (PH-Fibs) were treated with VPA (5mM), SAHA (10µM), or left untreated. Cell viability was measured by cell count using automatic cell counter. Data are presented as percentage of live cells to total cells.

**Figure VIII.** FACS analysis of (A) viability staining with PI and (B) cell cycle distribution in PSMC stimulated with PDGF 50µg for 72 hours, with or without VPA or SAHA treatment. The calculations of defined areas are performed by software Cyflogic v1.2.1. Each experiment was repeated at least 3 times with separate cell preparations.