Histone Deacetylation Inhibition in Pulmonary Hypertension:
Therapeutic Potential of Valproic Acid (VPA) and Suberoylanilide Hydroxamic Acid (SAHA)

Running title: Zhao et al.; Therapeutic potential of HDAC inhibitors in PH

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Abstract

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 (PAH), a condition characterised by profound structural remodelling of pulmonary arteries and arterioles.

Methods and Results - HDAC1 and HDAC5 protein levels were elevated in lungs from human idiopathic PAH and in lungs and right ventricles from rats exposed to hypoxia. Immunohistochemistry localised increased expression to remodelled vessels in the lung. Both valproic acid (VPA), a class I HDAC inhibitor, and suberoylanilide hydroxamic acid (SAHA), an inhibitor of class I, II and IV HDACs, mitigated the development and reduced established hypoxia-induced pulmonary hypertension in the rat. Both VPA and SAHA inhibited the “imprinted” highly proliferative phenotype of fibroblasts and R-cells from pulmonary hypertensive bovine vessels and PDGF-stimulated growth of human vascular smooth muscle cells in culture. Exposure to VPA and SAHA was associated with increased levels of p21 and FOXO3 and reduced expression of survivin. The significantly higher level of expression of cKIT, MCP-1, IL-6, SDF-1, PDGFb and S100A4 in the R-cells were down regulated by VPA and SAHA treatment.

Conclusions - Increased HDAC activity contributes to the vascular pathology of pulmonary hypertension. The effectiveness of HDAC inhibitors VPA and SAHA, in models of PAH, support a therapeutic strategy based on HDAC inhibition in PAH.

Key words: epigenetic modification, histone deacetylation, pulmonary hypertension, suberoylanilide hydroxamic acid, valproic acid
Introduction

The lungs of patients with advanced pulmonary arterial hypertension (PAH) exhibit a vascular remodelling involving all cellular elements of the vessel wall, caused by dysregulated cell proliferation and survival, inflammation and in-situ thrombosis \(^1,^2\). Current approved treatments target primarily an imbalance of vasoactive factors in PAH \(^3\) and at best retard the course of the disease. There is an urgent need for therapies that target directly the structural vascular pathology.

Aberrant epigenetic changes, such as 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, dynamically maintained by two important families of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDAC) \(^6\), controls the higher-order structure of chromatin and the resultant accessibility of transcriptional factors to their target genes \(^7\). HATs 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 \(^8,^9\). HDACs have emerged as key targets to reverse aberrant epigenetic changes associated with cancer and autoimmune disease and HDAC inhibitors show promise as anti-cancer and anti-inflammatory agents \(^9,^10\).

The present understanding of epigenetic modifications through histone acetylation in PAH is very limited. Li et al (2011) have described pulmonary adventitial fibroblasts from chronically hypoxic calves that expressed an epigenetically altered proinflammatory phenotype; the phenotype was reversed by HDAC inhibition \(^11\). Recently, selective class I HDAC inhibition
has been reported to attenuate the development hypoxia-induced pulmonary hypertension in the rat through an anti-proliferative mechanism. Moreover, right ventricular (RV) function was preserved, in contrast to experience with the pan-HDAC inhibitor trichostatin A (TSA) in rat PAB models. However, the involvement of HDACs in human PH has not been explored nor has the ability of HDAC inhibitors to reverse existing PAH. Here we show increased expression of HDACs 1, 4 and 5 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), an inhibitor of classes I, II and IV, are effective in reversing pulmonary hypertension in the hypoxic rat and exert anti-proliferative and anti-inflammatory 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 & 2001/6003). The patients’ characteristics have been described previously.

Animals and experimental design

Adult male Sprague-Dawley (SD) rats (body weight 200-250g) (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:

a) Chronic hypoxia - time course; rats were divided into 4 groups (n=3) and exposed either to
normoxia or hypoxia (normobaric, FIO2 = 10%) for 2 days, one week and two weeks.

Haemodynamic parameters were measured and tissues were collected for biochemical and histological examination. b) *Chronic hypoxia - prevention study with valproic acid (VPA)*; rats were divided into 4 groups (n=6) and exposed to (i) normoxia (NC), (ii) hypoxia for two weeks (HC), (iii) hypoxia plus VPA (sodium salt, Sigma-Aldrich) 100mg/kg/day in drinking water (low VPA) and (iv) hypoxia plus treatment with VPA 300mg/kg/day (high VPA). Drug treatment was started 3 days prior to hypoxia and continued for the remaining 2 weeks of exposure. c) *Chronic hypoxia - treatment study with VPA and SAHA*; rats were divided into five groups (n=9-12) and exposed to (i) normoxia (NC), (ii) hypoxia for 2 weeks (2WH), (iii) hypoxia for 4 weeks (4WH), (iv) hypoxia and VPA (300mg/kg/day) and (v) hypoxia and SAHA (Chemos GmbH) (50mg/kg/day). Drug treatment was given in drinking water and initiated after two weeks hypoxia exposure and continued for the remaining 2 weeks of exposure. VPA was dissolved in distilled water and SAHA in 5 molar equivalents 2-hydroxypropyl-β-cyclodextrin (HOP-β-CD, Sigma-Aldrich) as previously described. The 4WH group was given HOP-β-CD solution (1.38g/kg/day) as vehicle control. Animals were weighed every other day and treatment doses were calculated accordingly.

**Haemodynamic measurements, tissue collection and histology examination**

Rats were anaesthetised (Hypnorm 1ml/kg *i.m*; Hypnovel 0.8ml/kg *i.p*). Right ventricular systolic pressure (RVSP) and pulmonary arterial pressure (PAP) were measured via a pre-curved catheter inserted through the right jugular vein and systemic blood pressure (SBP) recorded in the carotid artery cannulation using a PowerLab Data Acquisition system (ADI Instruments Ltd).

The animals were then killed, 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 (RV/LV+sep). The left lung was fixed with 10% formalin in phosphate-buffered saline and processed for elastic Van Gieson (EVG) and H&E staining. Vessels less than 100 μm in peripheral lung were counted blindly under microscope (40X) and pulmonary vascular remodelling 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 hematopoetic origin with high growth potential (rhomboidal or “R”-cells) were isolated from the adventitial and the medial layer of distal pulmonary arteries of chronically hypoxic calves, using explant techniques as described previously. Experiments were performed on cells at passage 4-10 and compared to fibroblasts and smooth muscle cells isolated from control animals (CO-Fibs, CO-SMC) and studied at 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 (1mM, 2.5mM, and 5mM) and SAHA (10μM) 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 (5mM) or SAHA (10μM) for 24hrs and RNA were extracted for RT-PCR analysis. Primers are listed in **Supplemental Table 1**.

Human pulmonary smooth muscle cells (passages 5 to 9) were assessed for proliferation by hemocytometer or BrdU incorporation assay (Millipore). Cells were serum deprived and stimulated with 50ng/ml platelet-derived growth factor (PDGF, Ebioscience) in 10% FBS with/without HDAC inhibitors treatment (VPA 1-2mM and SAHA 2.5-10 μM) for 72 hours. Individual experiments were repeated at least 3 times. In addition, harvested cells were stained
with 50μg/ml propidium iodide (PI, Sigma) to access the cell cycle distribution and viability by using Beckton-Dickinson Fluorescent-activated cell sorting (FACS) Calibur flow cytometer system with Cell Quest software (Beckton-Dickison, Cyflogic version 1.2.1).

**Western Blotting**

Rat (lungs, RV and kidneys) and human (lobectomy and IPAH lung) samples were homogenized in phosphate buffer (100mM DO4 [K2HPO4:KH2PO4 = 3:1], 1mM EDTA, 1mM DTT supplemented with protease inhibitor, Roche). Proteins from cell pellets were extracted using TGN buffer (1M Tris, 2.5M NaCl, glycerol, 0.5M β-glycerophosphate, Tween 20, Nonidet P40 and protease inhibitor). Western blotting was performed as per manufactures’ suggestions (rabbit polyclonal antibodies against human HDACs, 1:1000, Cell signalling; mouse polyclonal antibodies against human Bcl-2, 1:1000, BD Biosciences). Proteins were detected by Novex® ECL chemiluminescent kit (Invitrogen). Optical densities of individual bands were measured and protein expressions were standardised 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 (DAKO, UK; 1:5 dilution in PBS), followed by purified mouse antibody against HDAC1 (1:50, Cell Signalling), or rabbit antibody against Ki67 (1:50, Thermo Scientific), or HDAC5 (1:50, Cell Signalling Technology), and then secondary antibody against mouse or rabbit (SignalStain® Boost IHC Detection Reagent HRP; Cell Signalling). Peroxidase activity was visualized with diaminobenzidine tetrahydrochloride (DAB 0.5 mg/ml; Sigma, UK), and sections were counterstained with Mayer’s haematoxylin.

**Histone extraction and acetylation assessment**
Cell pellets or tissues were washed with ice-cold PBS containing 5mM sodium butyrate. Lysis buffer (10 mM Tris, 50mM sodium bisulfate, 10 mM 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 (10mM Tris, 13mM EDTA). Precipitated nuclei were suspended in ice-cold dH2O and 0.4M sulfuric acid (1:1) and kept on ice for one hour. Following centrifugation at 15000 rpm, histone was precipitated with 1ml acetone at -20°C overnight. Acetylation was detected on Western blotting using 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

Total RNA isolation from cultured cells, first-strand cDNA synthesis, and real-time RT-PCR were performed as described previously. mRNAs were quantified by TaqMan® real-time RT-PCR using the commercially available specific primer-probes (Supplemental Table 1). Real-time PCR reactions were set following the manufacture’s conditions. Threshold cycle (Ct) values obtained for each gene were converted to the linear form using the term $2^{\Delta\Delta Ct}$ as a value directly proportional to the copy number of mRNA, where $\Delta\Delta Ct$ 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 Levene’s test. Differences between groups were assessed by Student’s t test or appropriate ANOVA; specifically, Welch’s ANOVA for data with unequal variances, otherwise standard ANOVA, followed by Bonferroni post hoc test for multiple comparisons. A value of p<0.05 was
considered statistically significant. All statistical analyses were performed using SPSS Statistics 17.0 (IBM Corporation).

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, 2, 3; Class II: HDAC 4, 5, 7) 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 HDAC 3 compared with control lung tissue, along with a two-fold increase in the anti-apoptotic regulator, Bcl-2 (Figure 1). Hypoxic rats with an elevated mean PAP and RVH (Figure 2A, 2D) exhibited a striking increase in HDAC1 (Figure 2B, 2E) and HDAC5 (Figure 2C, 2F) expression in both lung and RV, again accompanied by elevation of Bcl-2 in the lung (Figure 2G and Supplemental Figure 1).

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 Supplemental Figure 2, 3). In keeping with their known function, HDAC1 showed a predominantly nuclear distribution while 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 (Supplemental Figure 5). More important, treatment with VPA or SAHA after pulmonary hypertension was established reduced mean PAP (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 remodelling, pulmonary arteriolar muscularisation was significantly reduced (Figure 4D, 5E), along with Bcl-2 (Figure 5A), while 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 while both H3 and H4 acetylation levels were increased > 60% in SAHA treated lungs (Figure 5C-E). Interestingly, total histone levels were elevated in the rat lung after hypoxia exposure, a measure of protein synthesis, and this was 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. It has been speculated that these cells with high proliferative capacity contribute selectively to the remodeling process in pulmonary hypertension. Significantly increased class I HDAC catalytic activity has been shown in PH-Fibs compared to CO-Fibs. In this study, class I HDAC mRNA levels were also significantly increased in R-cells compared to CO-SMC; no differences of HDAC-2 and HDAC-3 levels were detected (Supplemental Figure 6).

Under serum deprived conditions, R-cells and PH-Fibs exhibited greater proliferation than CO-SMC and CO-Fibs (Figure 6A, 6C). The HDAC inhibitor VPA, at concentrations of
2.5mM and 5mM, 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 (10μM) also inhibited cell growth, with the greatest sensitivity exhibited by “R”-cells and PH-Fibs (Figure 6B, 6D). At the concentrations tested, no significant effects on cell viability were found (Supplemental Figure 7).

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-SMC and CO-Fibs (Figure 6E and F). 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, 6F).

A previous study showed that R-cells expressed significantly higher levels of progenitor cell markers (cKIT), pro-inflammatory factors (MCP-1, IL-6, SDF-1) and growth factors (PDGFb and S100A4) 19. 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, 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 (VPA 1mM: 63.4%; VPA 2mM: 69.0% p<0.05) and SAHA treated cells (SAHA 2.5μM: 71.5%; SAHA 10μM: 71.4%, p<0.05) (Supplemental Figure 8). Bcl-2 expression was reduced by SAHA treatment (Figure 8D). Both
VPA and SAHA led to increased p21 expression in cells (Figure 8E). Furthermore, SAHA treatment at 10 µM resulted in PARP cleavage to an 85kDa fragment (Figure 8F).

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 pathology/vascular remodelling 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 remodelled 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, chronic administration of the HDAC class I inhibitor, VPA, not only prevented hypoxia-induced pulmonary hypertension but 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 hyper-proliferation 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, PDGFb, S100A4 and survivin in vitro support strongly a direct effect on cell division and survival.
Downregulation of pro-inflammatory factors such as MCP-1, IL-6 and SDF-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, 2, 3 and 8, nuclear localization); class-IIa (HDACs 4, 5, 7 and 9); class-IIb (HDACs 6 and 10) (cytoplasm and nuclear localization) and class-IV (HDAC11). Another seven HDACs, known as class III or sirtuins, require NAD 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 tumours and cancer cell lines. In general, increased HDAC activity results in histone hypoacetylation, and this has been implicated in the initiation and progression of various tumours. For example, HDAC1 is overexpressed in gastric, pancreatic, colorectal, prostate and hepatocellular cancers, and correlates with poor prognosis. 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, are consistent with the proliferative, apoptosis-resistant vascular pathology that characterises pulmonary hypertension. The reduction in HDAC2 in lungs from IPAH patients and hypoxic rats is consistent with a compensatory reaction to HDAC1 overexpression.

We selected two HDAC inhibitors on the basis of (i) the pattern of HDAC expression in human lung and (ii) the potential to translate findings into clinical studies. VPA is used clinically as an antiepileptic drug and for some painful neuropathies, and has a low toxicity profile. It has a complex pharmacology, which includes sodium channel blockade and class I HDAC
inhibition (IC₅₀ 0.4mM)⁹. The doses chosen for our experiments were based on published studies in the range reported to inhibit HDAC1⁶. Recent studies have reported that VPA has antitumor effects in various cancers through HDAC inhibition and clinical studies are ongoing.³⁴ SAHA is a relatively broad spectrum HDAC inhibitor (class I, II and IV) that was approved by the FDA 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 activate proapoptotic factors, such as Bid and Bim,³⁹,⁴⁰ thereby increasing the ratio of pro- to anti-apoptotic proteins. The cyclin-dependent kinase (CDK) inhibitor, p21, is directly transcriptionally upregulated within hours of HDAC inhibitor treatment, leading to cell cycle arrest at G1/S phase via the tumour suppressor p53.⁴¹,⁴² Upregulation of p21 and downregulation of Bcl-2 expression in hypoxic rat lungs by VPA and SAHA is consistent with the notion that HDAC inhibition may reverse vascular remodelling 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 FACS 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 (10μM), indicating a pro-apoptosis action.
The fibroblasts and R-cells (potentially of haematopoetic 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\textsuperscript{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 where changes in HDAC activity have been shown to contribute to their inflammatory/destructive phenotype\textsuperscript{43}. HDAC1 catalytic activity is significantly elevated in PH-Fibs and R-cells compared to control cells\textsuperscript{11}. The greater growth inhibitory effect of VPA and SAHA on PH-Fibs and R-cells compared to control cells might reflect specific modulation of epigenetically altered signalling pathways in these cells. Similar results have been observed in studies of cancer, where transformed cells are highly sensitive to the apoptosis-inducing effects of HDAC inhibitors compared to normal controls\textsuperscript{44}. Here, we show that PH-Fibs and “R” cells express significantly lower levels of p21 and FOXO3 and higher levels of survivin compared to cells from control animals (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\textsuperscript{45}. Interestingly, both VPA and SAHA reduced survivin levels in PH-Fibs and R-cells, arguing that VPA and SAHA target the apoptosis-resistant remodelled cells in PAH. The significantly higher levels of pro-inflammatory factors, including MCP-1, SDF-1 and IL-6 in the R-cell was downregulated by VPA and SAHA treatment, confirming the anti-inflammatory mechanism of HDAC inhibition as described previously\textsuperscript{19}.

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 post pulmonary artery banding\textsuperscript{13,46} are conflicting and indicate caution. However, recent data on RV function and gene expression with selective class I HDAC inhibition are more encouraging\textsuperscript{12}. There is extensive human experience with VPA and it is generally well tolerated. SAHA is active against HDACs in the low nanomolar concentration range and does not accumulate in cardiac tissue\textsuperscript{47,48}. As 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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**Conflict of Interest Disclosures:** None

**References:**


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**Figure Legends:**

**Figure 1.** HDAC protein expression levels in human lung extracts from IPAH patients (n=12) and lobectomy (control, n=21). (A) HDAC1, (B) HDAC2, (C) HDAC3, (D) HDAC4, (E) HDAC5, (F) HDAC7 (G) Bcl-2, and (H) representative bands. 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 control. * p<0.05, ** p<0.001 compared with control group using Student’s t test.

**Figure 2.** Time course of development of pulmonary hypertension phenotype and HDAC expression in chronically hypoxic rat. (A) PAP, mean pulmonary arterial pressure, (B) HDAC1 in lung, (C) HDAC5 in lung, (D) RVH, RV hypertrophy, (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 normalised 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 using Welch’s 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 NC group.

**Figure 3.** Immunohistochemistry of HDAC1 and HDAC5 in human and rat lungs. (A) Both HDAC1 and HDAC5 exhibit relatively weak expression in control lobectomy lung sections. (B) HDAC1 exhibits predominantly nuclear distribution in perivascular and vascular cells of remodelled vessels while HDAC5 is seen mainly in cytoplasm in IPAH lung sections. (C) Prominent HDAC1 and Ki67 expression in nuclei of perivascular and vascular cells while HDAC5 has a cytoplasmic distribution in vascular cells of remodelled vessels in lung sections from rats exposed to hypoxia for 4 weeks. HPF: high power field; LPF: low power field. Bar = 25 μm.
**Figure 4.** Treatment of established hypoxia-induced pulmonary hypertension. Valproic acid or SAHA administered during the last 2 weeks of 4 week hypoxia exposure. (n≥6 for each group). (A) Mean pulmonary artery pressure, PAP, (B) right ventricular hypertrophy, RV/LV+sep, (C) systolic blood pressure, SBP, (D) percentage of muscularised vessels, and (E) Elastic van Gieson. NC: normoxia; 2WH: hypoxia for 2 weeks; 4WH: hypoxia for 4 weeks; VPA: hypoxia with VPA 300 mg/kg/day, and SAHA: hypoxia with SAHA 50 mg/kg/day. The data are presented as mean±SEM. n=12 for each group except 9 for SAHA. Data analysed by one way standard ANOVA, comparing all groups pairwise. * p<0.05, ** p<0.001 compared with 4WH. x p<0.05, xx p<0.001 compared with 2WH, ## p<0.001 compared with NC. HPF: high power field; LPF: low power field. Bar = 25 μm.

**Figure 5.** Expression of Bcl-2, p21 and acetylated histone levels in rat lung extracts from chronic hypoxia treatment study. (A) Bcl-2, (B) p21, (C) acetylated H3, (D) acetylated H4, (E) representative blots, (F) total histone levels. The data are generated from optical density measurements of individual bands from Western blots. Bcl-2 and p21 protein expression is normalised to β-actin, acetylated histone to total histone, and total histone levels to total protein level. Data are presented as mean ± SEM of fold change relative to NC. n=6. NC: normoxia; 4WH: hypoxia for 4 weeks; VPA: hypoxia with VPA 300 mg/kg/day; SAHA: hypoxia with SAHA 50 mg/kg/day. Welch’s ANOVA was used for (B) p21 and standard ANOVA for the others. Comparisons were made of all groups pairwise. * p<0.05 compared with 4WH, # p<0.05 compared with NC.

**Figure 6.** Effects of histone deacetylase inhibition on bovine R-cells and 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-SMC) in 3 days, (B) Cell counts of R-cells and CO-SMC, untreated and treated with increasing concentrations of VPA (1mM, 2.5mM, and 5mM), SAHA (10μM) for 3 days, (C) Daily cell counts of serum deprived PH-Fibs and control fibroblasts (CO-Fibs) in 3 days, (D) Cell counts of PH-Fibs and CO-Fibs, untreated and treated with increasing concentrations of VPA (1mM, 2.5mM, and 5mM), SAHA (10μM) for 3 days. The data are presented as mean ± SEM of cell numbers. NT: untreated. * p<0.05, ** p<0.001 compared with day 0 within the same cell type, ## p<0.001 compared with CO-SMC or CO-Fibs on the same day, xx p<0.001 compared with untreated on day 3. Effects of VPA (5mM) and SAHA (10μM) for 24 hours on gene expression are assessed by RT-PCR: (E) mRNA expression of genes involved in the cell cycle and apoptosis, including survivin, p21 and FOXO3. The data are presented as mean ± SEM of fold change to either CO-SMC or CO-Fibs. Two way ANOVA was used for (A) and (C) and one way standard ANOVA for the others, all comparisons of groups pairwise. * p<0.05 compared with CO-SMC or CO-Fibs, #, p<0.05 compared with untreated R-cells or PH-Fibs.

**Figure 7.** Effects of VPA (5mM) and SAHA (10μM) on gene expression of growth factor (cKIT, PDGFb, S100A4) and pro-inflammatory factor (MCP-1, SDF-1, IL-6) in serum deprived R-cells. Cells were untreated or treated with VPA (5mM) for 24 hours and mRNA expression is analysed by RT-PCT. The data are presented as mean ± SEM of fold change to either CO-SMC. * p<0.05, ** p<0.001 compared with CO-SMC, #, p<0.05 compared with untreated R-cells by one way standard ANOVA, comparing all groups pairwise.
**Figure 8.** Effect of histone deacetylase inhibition on PDGF-stimulated human pulmonary smooth muscle cells in culture. (A) cell counts; (B) BrdU incorporation assay; (C) FACS analysis for cell cycle distribution showing both VPA and SAHA arrested cell growth at G1-S phase; (D) p21 and (E) Bcl-2 protein expression. (F) PARP cleavage. Data are presented as mean ± SEM of fold change relative to control (Ctrl). Each experiment was repeated at least 3 times with separate cell preparations. Ctrl: control, PC: PDGF, PV1: PDGF+VPA 1mM, PV2: PDGF+VPA 2mM, PS2.5: PDGF+SAHA 2.5μM, PS10: PDGF+SAHA 10μM. Welch’s ANOVA was used for (D) p21 and standard ANOVA for the others, comparing all groups pairwise. * p<0.05, ** p<0.001 compared with PC, x p<0.05 compared with PS2.5, # p<0.05, ## p<0.001 compared with Ctrl.
Chronically hypoxic rat

**A**
PAP

**B**
HDAC1 in lung

**C**
HDAC5 in lung

**D**
RVH

**E**
HDAC1 in RV

**F**
HDAC5 in RV

**G**
Bcl-2 in lung

**H**

![Western Blot Images]

- HDAC1
- HDAC5
- Bcl-2
- β-actin
- α-actin

**NC**, **2D**, **1W**, **2W**
Histone Deacetylation Inhibition in Pulmonary Hypertension: Therapeutic Potential of Valproic Acid (VPA) and Suberoylanilide Hydroxamic Acid (SAHA)
Lan Zhao, Chien-Nien Chen, Nabil Hajji, Eduardo Oliver, Emanuele Cotroneo, John Wharton, Daren Wang, Min Li, Timothy A. McKinsey, Kurt R. Stenmark and Martin R. Wilkins

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## SUPPLEMENTAL MATERIAL

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

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<td>FOXO3</td>
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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.
Chronically hypoxic rat

A. HDAC1 expression levels over time:
   - NC: Low expression
   - 2D: Moderate expression
   - 1W: High expression
   - 2W: Very high expression

B. HDAC2 expression levels over time:
   - NC: Moderate expression
   - 2D: High expression
   - 1W: Very high expression
   - 2W: Moderate expression

C. HDAC3 expression levels over time:
   - NC: Low expression
   - 2D: Moderate expression
   - 1W: High expression
   - 2W: Very high expression

D. HDAC4 expression levels over time:
   - NC: Low expression
   - 2D: Moderate expression
   - 1W: High expression
   - 2W: Very high expression

E. HDAC5 expression levels over time:
   - NC: Low expression
   - 2D: Moderate expression
   - 1W: High expression
   - 2W: Very high expression

F. HDAC7 expression levels over time:
   - NC: Low expression
   - 2D: Moderate expression
   - 1W: High expression
   - 2W: Very high expression

G. Bcl-2 expression levels over time:
   - NC: Low expression
   - 2D: Moderate expression
   - 1W: High expression
   - 2W: Very high expression

H. Western blot analysis of HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC7, Bcl-2, and β-actin in kidney samples over time:
   - NC: Low expression
   - 2D: Moderate expression
   - 1W: High expression
   - 2W: Very high expression

I. HDAC1 expression levels in kidney samples over time:
   - NC: Low expression
   - 2D: Moderate expression
   - 1W: High expression
   - 2W: Very high expression
**Figure II.** Distribution of HDAC1 and HDAC5 expression in IPAH lung sections, low power view. Bar = 25 µm.

**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.
A

Control

2.85%

PDGF

3.08%

PDGF + VPA 1mM

4.32%

PDGF + VPA 2mM

2.69%

PDGF + SAHA 2.5μM

6.93%

PDGF + SAHA 10μM

8.84%

B

Control

G1: 73.40%
S: 14.89%
G2/M: 11.71%

PDGF

G1: 55.61%
S: 21.23%
G2/M: 23.16%

PDGF + VPA 1mM

G1: 63.39%
S: 17.28%
G2/M: 19.32%

PDGF + VPA 2mM

G1: 68.95%
S: 16.48%
G2/M: 14.59%

PDGF + SAHA 2.5μM

G1: 71.53%
S: 15.90%
G2/M: 12.57%

PDGF + SAHA 10μM

G1: 71.43%
S: 11.43%
G2/M: 17.14%