Selective Class I Histone Deacetylase Inhibition Suppresses Hypoxia-Induced Cardiopulmonary Remodeling Through an Antiproliferative Mechanism

Maria A. Cavasin, Kim Demos-Davies, Todd R. Horn, Lori A. Walker, Douglas D. Lemon, Nicholas Birdsey, Mary C.M. Weiser-Evans, Jules Harral, David C. Irwin, Adil Anwar, Michael E. Yeager, Min Li, Peter A. Watson, Raphael A. Nemenoff, Peter M. Buttrick, Kurt R. Stenmark, Timothy A. McKinsey

Rationale: Histone deacetylase (HDAC) inhibitors are efficacious in models of hypertension-induced left ventricular heart failure. The consequences of HDAC inhibition in the context of pulmonary hypertension with associated right ventricular cardiac remodeling are poorly understood.

Objective: This study was performed to assess the utility of selective small-molecule inhibitors of class I HDACs in a preclinical model of pulmonary hypertension.

Methods and Results: Rats were exposed to hypobaric hypoxia for 3 weeks in the absence or presence of a benzamide HDAC inhibitor, MGCD0103, which selectively inhibits class I HDACs 1, 2, and 3. The compound reduced pulmonary arterial pressure more dramatically than tadalafil, a standard-of-care therapy for human pulmonary hypertension that functions as a vasodilator. MGCD0103 improved pulmonary artery acceleration time and reduced systolic notching of the pulmonary artery flow envelope, which suggests a positive impact of the HDAC inhibitor on pulmonary vascular remodeling and stiffening. Similar results were obtained with an independent class I HDAC-selective inhibitor, MS-275. Reduced pulmonary arterial pressure in MGCD0103-treated animals was associated with blunted pulmonary arterial wall thickening because of suppression of smooth muscle cell proliferation. Right ventricular function was maintained in MGCD0103-treated animals. Although the class I HDAC inhibitor only modestly reduced right ventricular hypertrophy, it had multiple beneficial effects on the right ventricle, which included suppression of pathological gene expression, inhibition of proapoptotic caspase activity, and repression of proinflammatory protein expression.

Conclusions: By targeting distinct pathogenic mechanisms, isoform-selective HDAC inhibitors have potential as novel therapeutics for pulmonary hypertension that will complement vasodilator standards of care. (Circ Res. 2012;110:00-00.)

Key Words: histone deacetylase | pulmonary hypertension | proliferation | gene expression | signaling pathways
(LV) dysfunction (eg, β-blockers and angiotensin-converting enzyme inhibitors) are effective for RV failure, increased emphasis needs to be placed on elucidating pathogenic mechanisms in this chamber of the heart. Given the multitude of cell types and signaling cascades that govern pulmonary vascular and associated RV remodeling, it is predicted that effective therapeutic strategies for PH will involve targeting distal signaling mediators. Histone deacetylases (HDACs) may represent such targets.

HDACs control cell proliferation, inflammation, and fibrosis by catalyzing removal of acetyl groups from lysine residues in a variety of proteins. The 18 mammalian HDACs are encoded by distinct genes and are grouped into 4 classes. Two broad-spectrum HDAC inhibitors are approved for the treatment of cancer. One of these compounds, SAHA (suberoylanilide hydroxamic acid), and other “pan”-HDAC inhibitors have been shown to be efficacious in animal models of LV dysfunction, which suggests unforeseen potential for HDAC inhibitors for the treatment of heart failure. However, a recent report of findings in a model of pulmonary artery (PA) banding suggested that pan-HDAC inhibitors have

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**Figure 1. Class I HDAC inhibition in a hypoxia model of pulmonary hypertension.**

A. Rats were housed in hypobaric chambers and received intraperitoneal injection of the class I HDAC inhibitor MGCD0103 (10 mg/kg) every other day for 3 weeks. On days when compound was not delivered, animals were treated with vehicle control. Normoxic and hypoxic control rats were dosed with compound vehicle on a daily basis. B. Rats were weighed daily. C and D. Class I and class IIa HDAC enzymatic activity was quantified in lung and RV homogenates. E. Lung homogenates were immunoblotted for acetyl-α-tubulin and total α-tubulin. Lung lysates from an independent study with the pan-HDAC inhibitor SAHA were used as controls. P indicates barometric pressure; ***.
deleterious effects on the RV, which casts doubt on the utility of this class of compounds for patients with right-sided heart failure caused by diseases such as PH.

Here, we tested the hypothesis that selective inhibition of a subset of HDACs will provide a safe and effective means of treating PH and RV remodeling. MGCD0103, a small-molecule HDAC inhibitor that is in clinical development for cancer and is highly selective for class I HDACs 1, 2, and 3, was tested for efficacy in a rat model of PH induced by chronic hypoxia. Rationale for using this compound was based on prior studies that implicated class I HDACs in the control of LV remodeling, and on the well-described role for this HDAC class in the control of aberrant cell proliferation, such as is found in lung vasculature of patients with PH. The data demonstrate that class I HDAC inhibition significantly reduces pulmonary arterial pressure (PAP) through a mechanism that involves suppression of pulmonary vascular smooth muscle cell proliferation, while maintaining RV function and blocking cellular and molecular processes that contribute to RV failure. Another class I HDAC inhibitor that is in clinical development, MS-275, also effectively reduced hypoxia-induced PH. These results highlight the potential of isoform-selective HDAC inhibitors for the treatment of cardiovascular diseases.

**Methods**

**Experimental Animals**

All animal experiments were conducted in accordance with the National Institutes of Health’s “Guide for the Care and Use of Laboratory Animals” and were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. Ten-week-old male Sprague-Dawley (SD) rats weighing 250 to 280 g (Charles River Laboratories) were used for all studies. MGCD0103 and MS-275 (Selleck Chemicals, Boston, MA) were delivered every other day via daily intraperitoneal injection at concentrations of 10 and 3 mg/kg, respectively, in a 50:50 DMSO:PEG-300 vehicle. Tadalafil (Sequoia Research Products, Reading, United Kingdom; 10 mg/kg) was dosed daily by oral gavage in a 20% Cremophor/H2O vehicle (dose volume 2 mL/kg). MGCD0103, MS-275, and tadalafil administration began the day animals were placed in chambers. Normoxic and hypoxic control animals were dosed on the same schedule with compound vehicles. Animals were killed 20 hours after compound dosing, unless otherwise indicated.

**Hemodynamic Analysis**

Echocardiographic analyses were performed with a Vevo770 (VisualSonics, Toronto, Ontario, Canada). Pulse-wave Doppler of pulmonary outflow was recorded in the parasternal short-axis view at the level of the aortic valve. Baseline measurements were obtained 1 day before animals were placed in chambers. PAP was measured with a Millar catheter placed in the main PA via the RV; correct placement of the catheter was confirmed by observing a significant rise in diastolic pressure as the catheter moved out of the ventricle. Systemic blood pressure was monitored with another pressure catheter inserted in the femoral artery. Cardiac performance was assessed with a pressure-volume system (Scisense Inc, London, Ontario, Canada). For analyses, animals were anesthetized with 2% isoflurane, and their body temperature was maintained at 37°C. Hemodynamic analyses were performed 20 to 24 hours after the final dose of compound (MGCD0103 and MS-275) or 2 hours after dosing (tadalafil). Total pulmonary vascular resistance index was calculated as mean PAP/cardiac index, where cardiac index equals cardiac output divided by body weight, as described previously.

For data from all in vivo studies, GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA) was used to generate graphs and analyze data. ANOVA with Bonferroni post test was used to determine statistical differences between groups. Rats presented no health concerns associated with compound treatment. Animals were monitored daily and showed no evidence of paleness in eyes, nose, or skin, which are the most common signs of hematologic toxicities. Rats were alert and conducted normal activities such as eating, drinking, and grooming.

**Results**

To assess the role of class I HDACs in PH and RV remodeling, adult Sprague-Dawley rats were housed in a hypobaric chamber to simulate an altitude of 18 000 feet above sea level and create a hypoxic environment (10% O2).
Normoxic control rats were maintained in chambers that simulated sea level (21% O₂). Animals were treated with compound vehicle or MGCD0103, a selective inhibitor of class I HDACs that is currently in clinical trials for the treatment of cancer (Figure 1A). Normoxic and hypoxic control rats were dosed with compound vehicle alone. Rats receiving MGCD0103 gained weight at a rate similar to vehicle controls, which indicates that the compound was well tolerated (Figure 1B). Enzymatic assays with lung and RV homogenates confirmed that MGCD0103 selectively inhibited class I but not class IIa HDACs (Figures 1C and 1D). MGCD0103 did not increase tubulin acetylation in lung (Figure 1E) or heart (not shown), which indicates that the compound did not inhibit the tubulin deacetylase, HDAC6.

Three weeks of hypoxia resulted in a ~2-fold increase in PA systolic pressure, as determined by placement of a Millar catheter into the PA (Figure 2A). MGCD0103 significantly reduced PA systolic pressure and PA pulse pressure, which suggests that the compound increased arterial compliance in the lungs of hypoxic rats (Figures 2A and 2B). Mean PAP values correlated with PA systolic pressure and PA pulse pressure (Figure 2C). Systemic blood pressure was unaffected by MGCD0103 (Online Table II), and MGCD0103 had no impact on pulmonary pressures in normoxic rats (Online Figures 1A–1C). The class I HDAC inhibitor reduced PAP more effectively than tadalafil, a phosphodiesterase type 5 inhibitor used to treat patients with PH (Figure 2D).

A follow-up study assessed effects of MGCD0103 on RV function and pulmonary blood flow in hypoxic rats. Importantly, cardiac output was maintained in animals that received the class I HDAC inhibitor (Figure 3A), which rules out the possibility that the observed reduction in PAP was a consequence of compound-mediated impairment of cardiac function. Consistent with the findings shown in Figure 2, MGCD0103 also significantly reduced pulmonary vascular resistance in hypoxic rats (Figure 3B). Pressure-volume analyses confirmed that MGCD0103 did not negatively impact RV function (Table). In line with the PAP measurements, Doppler echocardiography revealed reduced PA acceleration time and velocity time integral in hypoxic rats, which was rescued by MGCD0103 (Figures 3C and 3D). Reduced pulmonary vascular compliance often causes transient cessation of forward PA blood flow during systole, which is detected by Doppler as a “notch” in the signal.17
Table. Invasive Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Normoxia+ Vehicle</th>
<th>Hypoxia+ Vehicle</th>
<th>Hypoxia+ MGCD0103</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>357±7</td>
<td>343±5</td>
<td>361±5</td>
</tr>
<tr>
<td>RV end-systolic</td>
<td>30±7</td>
<td>60±10*</td>
<td>47±8*†</td>
</tr>
<tr>
<td>pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV end-diastolic</td>
<td>4±1</td>
<td>6±3</td>
<td>4±2</td>
</tr>
<tr>
<td>pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke work, mm Hg/μL</td>
<td>4569±1034</td>
<td>8634±2048*</td>
<td>8034±2306*</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>82.6±6.4</td>
<td>82.2±11.7</td>
<td>83.7±9.3</td>
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<tr>
<td>Ejection fraction, %</td>
<td>80.6±4.6</td>
<td>71.8±4.2*</td>
<td>70.7±6.3*</td>
</tr>
<tr>
<td>dP/dt max, mm Hg/s</td>
<td>1634±295</td>
<td>2638±385*</td>
<td>2804±377*</td>
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<tr>
<td>dP/dt min, mm Hg/s</td>
<td>−1586±478</td>
<td>−2822±684*</td>
<td>−2786±394*</td>
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<tr>
<td>Preload recruitable</td>
<td>22.4±7.3</td>
<td>30.0±10.0*</td>
<td>35.4±15.4*</td>
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<td>stroke work, mm Hg</td>
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<tr>
<td>Ea, mm Hg/μL</td>
<td>0.12±0.04</td>
<td>0.26±0.07*</td>
<td>0.21±0.03*</td>
</tr>
</tbody>
</table>

Systolic notching was readily detected in hypoxic control rats but not in animals treated with MGCD0103 (Figure 3E).

Biochemical, morphological, and histological analyses were performed to further address the action of the class I HDAC inhibitor directly suppressed PA smooth muscle cell proliferation through a mechanism that involved induction of genes that promote cell cycle arrest, including FoxO3a and p27 (Figures 7C through 7E). MGCD0103 also reduced FoxO3a phosphorylation at threonine 32 in PA smooth muscle cells (supplemental Figure 5B). Reduced pulmonary vascular remodeling was associated with reduced numbers of proliferating cells (presumably smooth muscle cells) in this vessel compartment (Figure 7A, lower panel). Quantification of PA medial thickening revealed complete normalization in MGCD0103-treated rats (Figure 7C). MGCD0103 had no effect on medial thickness of pulmonary vessels from normoxic rats (supplemental Figure 5A). In contrast to MGCD0103, tadalafil failed to significantly reduce hypoxia-dependent medial thickening of PAs (supplemental Figure 5B).

Studies with MGCD0103 and cultured cells confirmed that the class I HDAC inhibitor directly suppressed PA smooth muscle cell proliferation through a mechanism that involved induction of genes that promote cell cycle arrest, including FoxO3a and p27 (Figures 7C through 7E). MGCD0103 also reduced FoxO3a phosphorylation at threonine 32 in PA smooth muscle cells cultured under hypoxic conditions (Figure 7D, lane 4); phosphorylation of this site promotes nuclear export of FoxO3a, which results in activation of genes that promote cell proliferation. Taken together, the data suggest that class I HDAC inhibition improves pulmonary hemodynamics through an antiproliferative mechanism.

**Discussion**

Here, we demonstrate in vivo efficacy of selective class I HDAC inhibitors in a preclinical model of PH. Two class I HDAC inhibitors, MGCD0103 and MS-275, reduced hypoxia-mediated PH in rats in a manner that correlated with suppression of medial thickening of PAs and inhibition of smooth muscle cell proliferation in these vessels. Reduced
Figure 4. Class I HDAC inhibition suppresses multiple pathological pathways in the RV. A, Immunoblotting of class I HDAC proteins in RV lysates. C-terminus Hsp70 interacting protein (CHIP) was immunoblotted as a control. B, HDAC1 levels were quantified with a digital imager. C, RV hypertrophy was assessed by weighing ventricular chambers at the time of necropsy. D, Quantitative polymerase chain reaction analysis of RV brain natriuretic peptide (BNP) and α-skeletal actin (α-Sk-actin) mRNA levels. E, Caspase activity was measured in RV homogenates with a fluorescent substrate that is cleaved by caspase-3 and -7. For B through E, values presented are mean plus SEM. *P<0.05 vs normoxia; #P<0.05 vs hypoxia+vehicle. F, RV sections from hypoxic rats stained for cleaved (active) caspase-3. Arrows indicate caspase-positive cells. Caspase-positive cells were not detected in RVs from normoxic controls. Scale bar=10 μm. G, Cytokine protein levels in RV homogenates. For each group, RV protein from 4 independent animals was pooled before analysis. IL indicates interleukin; CINC-2, cytokine-induced neutrophil chemoattractant-2; and CNTF, ciliary neutrophic factor.
PA smooth muscle cell proliferation on class I HDAC inhibition was caused in part by upregulation of the antiproliferative transcription factor FoxO3a. Importantly, we also demonstrated that RV function was maintained in the face of class I HDAC inhibition and that indices of adverse ventricular remodeling (eg, myocyte apoptosis and inflammation) were blunted by selective inhibition of class I HDACs. This is in contrast to what was observed previously with the pan-HDAC inhibitor trichostatin A and supports the hypothesis that isoform-selective HDAC inhibition will be safer than general HDAC inhibition in the setting of RV pressure overload. Both MGCD0103 (Mocetinostat) and MS-275 (Entinostat) are in clinical development for cancer and are well tolerated by humans, thus highlighting the translational potential of the present findings.

PH is associated with dramatic structural remodeling of the pulmonary vasculature. Remodeling of PAs is due in part to abnormal proliferation of PA smooth muscle cells, the resulting muscularization and stenosis of the vessels, and thereby increasing pulmonary vascular resistance.20 The remodeling process is exacerbated by aberrant proliferation of other cell types, including endothelial cells and fibroblasts, as well as vascular inflammation and adventitial fibrosis.23 As such, it has been proposed that antiproliferative agents should be used in combination with vasodilators for the treatment of PH, and recent clinical trials with anticancer agents such as the tyrosine kinase inhibitor imatinib are addressing this hypothesis.24 HDAC inhibitors are in use for the treatment of cancer on the basis of their ability to block proliferation and stimulate apoptosis of transformed cells.25 Class I HDACs 1, 2, and 3, which reside in the nucleus and regulate epigenetic processes through deacetylation of nucleosomal histones, appear to be the HDAC isoforms that are primarily responsible for cell cycle control.13 In addition to their anticancer actions, clinical and preclinical studies have revealed that HDAC inhibitors potently suppress inflammation, fibrosis, and restenosis and have beneficial effects on the LV in the setting of increased afterload.7 Furthermore, we found class I HDAC inhibition blocks the persistent, proinflammatory phenotype of pulmonary adventitial fibroblasts derived of hypoxic calves.26 Given these findings, HDAC inhibitors appear to be ideally suited for the treatment of PH, and the results with MGCD0103 and MS-275 described here support this notion.

Figure 5. A second class I HDAC inhibitor suppresses hypoxia-dependent pulmonary hypertension and RV hypertrophy. Rats were housed in hypobaric chambers and injected with the class I HDAC inhibitor MS-275 (3 mg/kg) every other day for 3 weeks. On days when compound was not delivered, animals were treated with vehicle control. Normoxic and hypoxic control rats were dosed with compound vehicle on a daily basis. MS-275 reduced PA systolic pressure (PASP; A), PA pulse pressure (PAPP; B), and RV hypertrophy (C). For all graphs, values represent mean plus SEM. *P<0.05 vs normoxia (ANOVA); #P<0.05 vs hypoxia+vehicle (ANOVA).

Figure 6. MGCD0103 is not an acute vasodilator. A, Rat PA strips (1.5 mm×200 μm) were hung on a “bubble plate” between 2 tungsten wires. One wire was fixed and the other attached to a force transducer. Intact strips were stimulated with high potassium (KES). MGCD0103 had no effect on the potassium-mediated contraction at any concentration (10-300 nmol/L); however, when strips were washed with normal extracellular solution (NES) and contracted again with potassium, sodium nitroprusside (SNP, 1 μmol/L) effectively relaxed the vessel. B, Rats were maintained in hypobaric chambers for 3 weeks. On the final day of the study, animals received a single injection of MGDC0103 (10 mg/kg) 2 or 20 hours before PAP was measured. Acute administration of MGDC0103 failed to lower hypoxia-induced increases in PA systolic pressure (PASP) or PA pulse pressure (PAPP).
Two reports have addressed effects of HDAC inhibitors in models of RV remodeling. Valproic acid was shown to block RV cardiac hypertrophy in response to PA banding, as well as in the setting of PH caused by monocrotaline-induced lung injury. In contrast, trichostatin A failed to block hypertrophy in response to PA banding and actually appeared to worsen RV function. Valproic acid is a weak HDAC inhibitor that has many additional pharmacological activities, including regulation of ion channels, glycogen synthase kinase-3β, and mitogen-activated protein kinases. Thus, it is unclear whether the beneficial effects of this compound on the RV were a direct consequence of HDAC inhibition. Trichostatin A is a potent pan-HDAC inhibitor. The deleterious effects of this compound on the RV (eg, decreased cardiac output, increased RV dilatation, and apoptosis) could be a reflection of a protective role for HDAC(s) in this chamber of the heart. It is interesting to note that valproic acid, which exhibits selectivity for class I HDACs, did not cause adverse effects in the PA banding model. The present findings suggest that with regard to the RV, isoform-selective HDAC inhibition is safer than nonselective suppression of HDAC activity. This is evidenced by the ability of MGCD0103 to block RV apoptosis and inflammation and maintain RV contractile function in chronically hypoxic rats (Figure 4; Table). Nonetheless, the model used for our studies (3 weeks of hypoxia in Sprague-Dawley rats) is mild with regard to RV remodeling (Table) and represents a model of PH caused by interstitial lung disease and/or hypoxemia.
(World Health Organization group III PH). It will be important to extend the current findings to more severe models of PH and RV dysfunction, such as the Sugen plus hypoxia model, to determine whether the beneficial effects of class I HDAC inhibitors are generalizable to other forms of PH, including World Health Organization group I idiopathic pulmonary arterial hypertension.

The modest effect of MGCD0103 on RV hypertrophy is surprising given the prior demonstrations of antihypertrophic activity of HDAC inhibitors in models of LV dysfunction but is consistent with the inability of trichostatin A to block hypertrophy in response to PA banding. These results may reflect differential requirements for HDACs in the control of hypertrophy in the LV and RV and, more broadly, differences in signaling and transcriptional mechanisms that control growth of the 2 chambers. Interestingly, tadalafil reduced RV hypertrophy as efficiently as MGCD0103 despite having a more modest effect on PAP than the class I HDAC inhibitor. This finding may point to a direct role for phosphodiesterase type 5 in the regulation of RV growth in response to chronic hypoxia. The data presented here suggest that class I HDACs function in multiple cell types in lungs and RV as key components of pathogenic pathways that trigger increases in pulmonary vascular resistance and culminate in right-sided heart failure. Robust efforts in industry are focused on clinical development of isoform-selective HDAC inhibitors for oncology and nononcology indications. The present results reflect differential requirements for HDACs in the control of hypertrophy in the LV and RV and, more broadly, differences in signaling and transcriptional mechanisms that control growth of the 2 chambers. Interestingly, tadalafil reduced RV hypertrophy as efficiently as MGCD0103 despite having a more modest effect on PAP than the class I HDAC inhibitor. This finding may point to a direct role for phosphodiesterase type 5 in the regulation of RV growth in response to chronic hypoxia.

The data presented here suggest that class I HDACs function in multiple cell types in lungs and RV as key components of pathogenic pathways that trigger increases in pulmonary vascular resistance and culminate in right-sided heart failure. Robust efforts in industry are focused on clinical development of isoform-selective HDAC inhibitors for oncology and nononcology indications. The present results validate a role for class I HDACs in the pathogenesis of PH and justify expanded preclinical and clinical evaluation of class I HDAC inhibitors to determine the utility of this compound class for patients with PH and RV failure.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- Small-molecule inhibitors of histone deacetylases (HDACs) are efficacious in animal models of left ventricular dysfunction.
- The roles of HDACs in right ventricular (RV) remodeling are poorly understood.
- Pulmonary hypertension (PH) is associated with remodeling of the lung vasculature due to excessive proliferation of multiple cell types.

**What New Information Does This Article Contribute?**

- Isoform-selective HDAC inhibitors (class I HDAC specific) suppress hypoxia-mediated pulmonary hypertension.
- Efficacy of these compounds is due in part to suppression of cell proliferation in the lung vasculature.
- RV function is maintained in the face of class I HDAC inhibition, and cellular events associated with adverse RV remodeling are blocked by class I HDAC inhibition.

HDAC inhibitors are efficacious in models of left ventricular failure, blocking cardiac hypertrophy and fibrosis and improving systolic and diastolic performance of the ventricle. These results have suggested unforeseen potential for this class of compounds for the treatment of heart failure. However, in a recent study, a pan-HDAC inhibitor was shown to negatively impact the RV in the setting of pressure overload. The present study was performed to address the hypothesis that selective inhibition of a subset of HDACs, rather than all HDACs, will provide a safe and efficacious means of treating PH and associated RV remodeling. In addition, because the pathogenesis of PH involves increased proliferation of cells in the pulmonary vasculature, we reasoned that the antiproliferative action of HDAC inhibitors would suppress PH. We show for the first time that small molecules that selectively block class I HDACs (HDACs 1, 2, and 3) reduce hypoxia-induced PH in a manner that correlates with blunted medial thickening of pulmonary arterials and reduce proliferation of smooth muscle cells in these vessels. Importantly, class I HDAC inhibition was well tolerated by the RV and was associated with blockade of cellular events (eg, myocyte apoptosis and inflammation) that contribute to adverse RV remodeling. These findings justify expanded evaluation of class I HDAC inhibitors to determine the utility of this compound class for patients with PH and RV failure.
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Methods

*Ex vivo* analysis of pulmonary artery contraction

Pulmonary artery strips (1.5 mm X 200 μm) from normoxic SD rats were hung on a “bubble plate” between two tungsten wires. One wire was fixed and the other attached to a force transducer. Intact strips were stimulated to contract with potassium extracellular solution (KES; 109 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 11.6 mM HEPES [pH 7.4], 1.16 mM dextrose). Strips were relaxed with normal extracellular solution (NES; 137 mM, 5.9 mM, 1.2 mM CaCl₂, 1.2 mM, 11.6 mM HEPES [pH 7.4], 1.15 mM dextrose) or sodium nitroprusside (SNP, 1 μM; Calbiochem).

Tissue procurement and processing

After hemodynamic recordings, rats were sacrificed by exsanguination and hearts and lungs were excised and placed in ice-cold saline. RV was dissected from LV by cutting along the septum and the outer wall of the LV. Lungs were cleaned from fat tissue and vessels. Fifty milligram biopsies from RV free wall and left lung were flash-frozen in liquid nitrogen for subsequent biochemical and gene expression analyses. Total RNA was isolated using Trizol (Sigma) and protein lysates were prepared in PBS (pH 7.4) containing 0.5% Triton X-100, 300 mM NaCl and protease/phosphatase inhibitor cocktail (Thermo Fisher) using a Bullet Blender homogenizer (Next Advance). A longitudinal mid-section of the left lung was fixed in 10% formalin for histology.

Protein and RNA analysis

Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes (BioRad) and probed with antibodies for α-tubulin (Santa Cruz Biotechnology, sc-23948), acetyl-tubulin (Santa Cruz Biotechnology, sc-23950), HDAC1 (Cell Signaling Technology, 5356), HDAC2 (Cell Signaling Technology, 5113), HDAC3 (Cell Signaling Technology; 3949), CHIP (Cell Signaling Technology, 2080), p27 (Cell Signaling Technology, 3686), FoxO3a (Abcam, 23683) or phospho-FoxO3a (Cell Signaling Technology, 9464) . Proteins were detected using SuperSignal West Pico chemiluminescence system (Thermo Scientific) and a FluorChem HD2 imager (Alpha Innotech). HDAC enzymatic assays were performed as previously described 1. Quantitative PCR was performed as described previously, with results presented as relative expression to HPRT using the DCT method 2. PCR primers are shown in Online Table I. For quantification of cytokine/chemokine levels, RV homogenates (n = 4/group) were pooled and 50 μg total protein was analyzed using a rat cytokine array panel (R&D Systems, Minneapolis, MN). Signals were visualized by chemiluminescence and quantified using Image J software.

Histological analysis

Effects of compounds on pulmonary arterial remodeling were quantified using longitudinal mid-sections of the left lung stained with hematoxylin and eosin. All histological analyses were carried out in a blinded manner using an Axiovert 200 inverted microscope with a digital camera equipped with AxioVision imaging software (Zeiss, Germany). Approximately 15-20 random pulmonary vessels (50 to 250 μm outside diameter) were analyzed in each sample and averaged. Percent medial thickness was calculated as follows: 1) four independent measurements of the smooth muscle layer along two axes were averaged (MT), and 2) outside diameter (D) was calculated as outside vessel perimeter divided by π. Percent medial thickness = MT/D x 100. Immunohistochemical analysis was performed using an anti-proliferating cell nuclear antigen (PCNA) primary antibody (Santa Cruz Biotechnology, sc-25280), secondary
antibody (Vector Labs, biotinylated anti-mouse IgG, an Elite ABC Kit (Vector Labs) and a VECTOR NovaRED Substrate Kit.

**Cell culture**

Rat pulmonary artery smooth muscle cells (PASMCs) were isolated as described previously. Briefly, freshly excised lobar pulmonary arteries obtained from adult male rats were stripped of adventitia under 10x magnification in a sterile cabinet. Vascular segments were cut open, and endothelium was removed by gentle scraping of the luminal surface of the vessel. Cleaned vessel segments were then cultured as explants and grown in DMEM supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (1.25 µg/ml). After 48 hours, segments were removed and adherent cells passaged every 1 – 2 wk at a 1:3 ratio. Passage 3 – 8 cells at 70% confluence were used for experiments. Cells were stained with antibodies for α-smooth muscle actin (should be positive), calponin (should be positive), smooth muscle myosin heavy chain (should be positive), collagen I (should be negative), and HSP47 (should be negative) to confirm their identity as SMCs as opposed to myofibroblasts. Cell counting and viability was assayed using a Vi-Cell Cell Viability Analyzer (Beckman Coulter). Cells were grown to subconfluence and seeded on 24-well plates (20,000 cells/well). After overnight attachment, cells were starved for 24 hrs in DMEM containing 0.1% FBS. Cells were pre-treated for 30 minutes with MGCD0103 in serum-free medium, and subsequently stimulated with 10% FBS. Cells were counted 24, 48, and 72 hours post-FBS stimulation. For immunoblotting studies, cells were cultured in DMEM containing 0.1% FBS in the absence or presence of MGCD0103 (500 nM) under normoxic or hypoxia (3% O₂) conditions for 72 hours.
Online Figure I. Class I HDAC inhibition does not reduce pulmonary arterial pressure in normoxic rats. Rats were housed in sea level chambers and were injected with MGCD0103 (10 mg/kg) every other day for three weeks. On days when compound was not delivered, animals were treated with vehicle control. Control rats were dosed with compound vehicle on a daily basis. MGCD0103 had no effect on PASP (A), PAPP (B) or mPAP (C) in normoxic rats.

Online Figure II. PDE5 inhibition suppresses hypoxia-induced RV hypertrophy. A, Rats were housed in hypobaric chambers and were treated with tadalafil (10 mg/kg) daily for three weeks via oral gavage. Normoxic and hypoxic control rats were gavaged with vehicle control every day. Tadalafil reduced RV hypertrophy, as determined by RV/LV + septum ratio. B and C, Quantitative PCR revealed that tadalafil also reduced hypoxia-induced increases in BNP and α-Sk-actin mRNA levels in the RV.

Online Figure III. Class I HDAC inhibition does not impact RV hypertrophy in normoxic rats. Rats were housed in sea levels chambers and were injected with MGCD0103 (10 mg/kg) every other day for three weeks. On days when compound was not delivered, animals were treated with vehicle control. Control rats were dosed with compound vehicle on a daily basis. A, RV expression of HDAC1 protein was not altered by MGCD0103 in normoxic rats. B, RV mass was not altered by MGCD0103 in normoxic rats. C and D, Quantitative PCR revealed that MGCD0103 did not alter RV expression of BNP or α-Sk-actin mRNA levels in normoxic rats.

Online Figure IV. Class I HDAC inhibition suppresses RV cytokine expression. Relative cytokine/chemokine protein levels in RV homogenates were quantified using a rat cytokine array. For each group, RV protein from four independent animals was pooled prior to analysis.

Online Figure V. Consequences of MGCD0103 and tadalafil on pulmonary vascular medial thickening. A, Representative images of hematoxylin and eosin-stained lung sections from normoxic rats treated with compound vehicle or MGCD0103 for three weeks. B, Medial thickness of pulmonary arteries between 50 and 250 μm in diameter was quantified using lung sections from rats treated with compound vehicle or tadalafil (10 mg/kg) daily for three weeks. Values are presented as mean +SEM. *P<0.05 vs. normoxia.

Online Table I. PCR primers. Sequences of primers used for quantitative PCR of rat mRNA transcripts are shown. α–Sk-actin (alpha-skeletal actin), BNP (brain natriuretic peptide).

Online Table II. Invasive assessment systemic blood pressure. Blood pressure was obtained at study endpoint in anesthetized rats using a catheter placed in the femoral artery. Values represent averages +/- SEM. Upper study: normoxia + vehicle (n = 5), hypoxia + vehicle (n = 7), hypoxia + MGCD0103 (n = 7); lower study: normoxia + vehicle (n = 9), hypoxia + vehicle (n = 12), hypoxia + tadalafil (n = 12); *P<0.05 vs. normoxia + vehicle.

Online Table III. RV mass measurements. RV was dissected away from LV and septum and weighed. Upper study: normoxia + vehicle (n = 8), hypoxia + vehicle (n = 10), hypoxia + MGCD0103 (n = 10); middle study: normoxia + vehicle (n = 9), hypoxia + vehicle (n = 12), hypoxia + tadalafil (n = 12); lower study: normoxia + vehicle (n = 6), hypoxia + vehicle (n = 8), hypoxia + MS-275 (n = 10). Values represent averages +/- SEM. *P<0.05 vs. normoxia; #P<0.05 vs. hypoxia + vehicle.
References


Online Fig. I. Class I HDAC inhibition does not reduce pulmonary arterial pressure in normoxic rats.

Rats were housed in sea level chambers and were injected i.p. with MGCD0103 (10 mg/kg) every other day for three weeks. On days when compound was not delivered, animals were treated with vehicle control. Control rats were dosed with compound vehicle on a daily basis. MGCD0103 had no effect on PASP (A), PAPP (B) or mPAP (C) in normoxic rats.
Online Fig. II. PDE5 inhibition suppresses hypoxia-induced RV hypertrophy. A, Rats were housed in hypobaric chambers and were treated with tadalafil (10 mg/kg) daily for three weeks via oral gavage. Normoxic and hypoxic control rats were gavaged with vehicle control every day. Tadalafil reduced RV hypertrophy, as determined by RV/LV + septum ratio. B and C, Quantitative PCR revealed that tadalafil also reduced hypoxia-induced increases in BNP and α-Sk-actin mRNA levels in the RV. *P<0.05 vs. normoxia; #P<0.05 vs. hypoxia plus vehicle.
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Online Fig. IV. **Class I HDAC inhibition suppresses RV cytokine expression.** Relative cytokine/chemokine protein levels in RV homogenates were quantified using a rat cytokine array. For each group, RV protein from four independent animals was pooled prior to analysis.
Online Figure V

**A**

Normoxia + Vehicle

Normoxia + MGCD0103

**B**

Online Fig. V. Consequences of MGCD0103 and tadalafil on pulmonary vascular medial thickening. 

**A**, Representative images of hematoxylin and eosin-stained lung sections from normoxic rats treated with compound vehicle or MGCD0103 for three weeks. 

**B**, Medial thickness of pulmonary arteries between 50 and 250 μm in diameter was quantified using lung sections from rats treated with compound vehicle or tadalafil (10 mg/kg) daily for three weeks. Values are presented as mean +SEM. *P<0.05 vs. normoxia.
Online Table I. Primers for Q-PCR of rat α-SK-actin and BNP.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Q-PCR Primer</th>
<th>Reverse Q-PCR Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Sk-actin</td>
<td>5’-TCACTTCCTACCCTCGGCAC-3’</td>
<td>5’-AGGCCAGAGCCGTTGTCACA-3’</td>
</tr>
<tr>
<td>BNP</td>
<td>5’-GGTCTCAAGACAGCGCTTC-3’</td>
<td>5’-ACAACCTCAGCCGTCACAG-3’</td>
</tr>
</tbody>
</table>
Online Table II. Invasive assessment systemic blood pressure. Blood pressure was obtained at study endpoint in anesthetized rats using a catheter placed in the femoral artery. Values represent averages +/- SEM. Upper study: normoxia + vehicle (n = 5), hypoxia + vehicle (n = 7), hypoxia + MGCD0103 (n = 7); lower study: normoxia + vehicle (n = 9), hypoxia + vehicle (n = 12), hypoxia + tadalafil (n = 12); *P<0.05 vs. normoxia + vehicle.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia + Vehicle</th>
<th>Hypoxia + Vehicle</th>
<th>Hypoxia + MGCD0103</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systolic systemic blood pressure (mmHg)</strong></td>
<td>104.22 ± 8.97</td>
<td>119.06 ± 5.41</td>
<td>103.39 ± 5.04</td>
</tr>
<tr>
<td><strong>Diastolic systemic blood pressure (mmHg)</strong></td>
<td>68.50 ± 12.41</td>
<td>89.91 ± 4.19</td>
<td>75.17 ± 3.90</td>
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<tr>
<td><strong>Mean systemic blood pressure (mmHg)</strong></td>
<td>80.40 ± 11.03</td>
<td>99.66 ± 4.51</td>
<td>84.59 ± 4.23</td>
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</table>

<table>
<thead>
<tr>
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<th>Normoxia + Vehicle</th>
<th>Hypoxia + Vehicle</th>
<th>Hypoxia + Tadalafil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systolic systemic blood pressure (mmHg)</strong></td>
<td>103.92 ± 5.37</td>
<td>122.56 ± 3.85*</td>
<td>112.28 ± 5.53</td>
</tr>
<tr>
<td><strong>Diastolic systemic blood pressure (mmHg)</strong></td>
<td>68.67 ± 3.95</td>
<td>92.80 ± 3.04*</td>
<td>86.93 ± 4.38*</td>
</tr>
<tr>
<td><strong>Mean systemic blood pressure (mmHg)</strong></td>
<td>80.41 ± 4.33</td>
<td>102.70 ± 3.26*</td>
<td>95.37 ± 4.74</td>
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</tbody>
</table>
Online Table III. RV measurements.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia + Vehicle</th>
<th>Hypoxia + Vehicle</th>
<th>Hypoxia + MGCD0103</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV weight (mg)</td>
<td>254.74 ± 12.29</td>
<td>397.27 ± 16.95*</td>
<td>360.01 ± 20.69*</td>
</tr>
<tr>
<td>RV/body weight (mg/mg)</td>
<td>0.62 ± 0.02</td>
<td>1.16 ± 0.05*</td>
<td>1.11 ± 0.06*</td>
</tr>
<tr>
<td>RV/tibia length (mg/mm)</td>
<td>6.44 ± 0.29</td>
<td>10.27 ± 0.42*</td>
<td>9.36 ± 0.52*</td>
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</tbody>
</table>

<table>
<thead>
<tr>
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<th>Normoxia + Vehicle</th>
<th>Hypoxia + Vehicle</th>
<th>Hypoxia + Tadalafil</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV weight (mg)</td>
<td>291.11 ± 8.50</td>
<td>446.33 ± 19.03*</td>
<td>385.85 ± 12.52**#</td>
</tr>
<tr>
<td>RV/body weight (mg/mg)</td>
<td>0.67 ± 0.02</td>
<td>1.25 ± 0.05*</td>
<td>1.06 ± 0.03**#</td>
</tr>
<tr>
<td>RV/tibia length (mg/mm)</td>
<td>7.19 ± 0.21</td>
<td>11.32 ± 0.48*</td>
<td>9.77 ± 0.30**#</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Normoxia + Vehicle</th>
<th>Hypoxia + Vehicle</th>
<th>Hypoxia + MS-275</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV weight (mg)</td>
<td>280.02 ± 5.89</td>
<td>545.16 ± 32.12*</td>
<td>383.83 ± 23.50**#</td>
</tr>
<tr>
<td>RV/body weight (mg/mg)</td>
<td>0.67 ± 0.02</td>
<td>1.35 ± 0.10*</td>
<td>1.07 ± 0.06**#</td>
</tr>
<tr>
<td>RV/tibia length (mg/mm)</td>
<td>6.92 ± 0.17</td>
<td>13.41 ± 0.80*</td>
<td>9.78 ± 0.59**#</td>
</tr>
</tbody>
</table>

**Online Table 3.** RV mass measurements. RV was dissected away from LV and septum and weighed. Upper study: normoxia + vehicle (n = 8), hypoxia + vehicle (n = 10), hypoxia + MGCD0103 (n = 10); middle study: normoxia + vehicle (n = 9), hypoxia + vehicle (n = 12), hypoxia + tadalafil (n = 12); lower study: normoxia + vehicle (n = 6), hypoxia + vehicle (n = 8), hypoxia + MS-275 (n = 10). Values represent averages +/- SEM. *P<0.05 vs. normoxia; #P<0.05 vs. hypoxia + vehicle.