Published in final edited form as:

Sci Transl Med. 2020 January 08; 12(525): . doi:10.1126/scitranslmed.aay7205.

HDAC inhibition improves cardiopulmonary function in a feline model of diastolic dysfunction

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Supplementary Materials

Materials and Methods

Fig. S1. Effects of SAHA in a feline model of HFpEF

Table S1. Assessment of blood count

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References (57–91)

Competing Interests:

No authors have any competing interests to declare.

Data and materials availability: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (56) with the dataset identifier PXD013663.

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Abstract

Heart failure with preserved ejection fraction (HFpEF) is a major health problem without effective therapies. This study assessed the effects of histone deacetylase (HDAC) inhibition on cardiopulmonary structure, function, and metabolism in a large mammalian (feline) model of pressure overload that recapitulates features of diastolic dysfunction common to human HFpEF. Male domestic short hair felines ($n=31$, aged 2 mo), underwent a sham procedure ($n=10$) or loose aortic banding ($n=21$), resulting in slow-progressive pressure overload during growth. Two months post-banding, animals were treated daily with suberoylanilide hydroxamic acid (b+SAHA, 10 mg/kg, $n=8$), a Food and Drug Administration (FDA)-approved pan-HDAC inhibitor, or vehicle (b+veh, $n=8$) for 2 months. Echocardiography at 4-months post-banding revealed that b+SAHA animals had significantly reduced left ventricular hypertrophy (LVH) ($P < 0.0001$) and left atrium size ($P < 0.0001$) vs b+veh animals. Left ventricular end-diastolic pressure and mean pulmonary arterial pressure were significantly reduced in b+SAHA ($P < 0.01$) vs b+veh. SAHA increased myofibril relaxation *ex vivo*, which correlated with *in vivo* improvements of LV relaxation. Furthermore, SAHA treatment preserved lung structure, compliance, blood oxygenation, and reduced perivascular fluid cuffs around extra-alveolar vessels, suggesting attenuated alveolar-capillary stress failure. Acetylation proteomics revealed that SAHA altered lysine acetylation of mitochondrial metabolic enzymes. HDAC inhibition reduces LVH, LV diastolic dysfunction, and atrial and pulmonary remodeling in a feline model of diastolic dysfunction. These results suggest that acetylation defects in hypertrophic stress can be reversed by HDAC inhibitors, with implications for improving cardiac structure and function in patients.

One Sentence Summary:

Histone deacetylase inhibition counteracts adverse left ventricular remodelling, improves pulmonary function, and reverses diastolic dysfunction.

Introduction

Heart failure (HF) with preserved ejection fraction (HFpEF) accounts for about 50% of all cases of HF and its prevalence relative to HF with reduced ejection fraction (HFrEF) is growing by 10% per decade. The HFpEF prognosis is poor, with a 5-year survival rate as low as 50%. Furthermore, the hospitalization rate is high and quality of life for patients is substantially compromised (1, 2). There are currently no FDA-approved therapies for this condition.

Inhibitors of excess neurohormonal activity have been shown to decrease mortality in patients with HFrEF in large clinical outcome trials (3). However, the effects of these same neurohormonal inhibitors in patients with HFpEF have consistently failed to reach positive primary outcomes (4). The dichotomy in the responses of the patients likely results from differences in HFpEF versus HFrEF pathophysiology and the absence of experimental HFpEF models that capture essential characteristics of this syndrome that could be used to test new HFpEF therapies (5). While all HFpEF animal models have limitations, rodent models have inherent limitations due to their small size and differences in physiological properties compared to large mammals (6). New large animal models that recapitulate critical features of human HFpEF might be more useful in identifying beneficial HFpEF therapies. Most studies using large animal HFpEF models have mainly focused on cardiac function and structure, without assessing pulmonary changes (7–9), although dyspnea, particularly during exercise, is one of the cardinal symptoms observed in patients with HFpEF. Furthermore, pulmonary hypertension (PH) is common in patients with HFpEF (36–83%) and strongly associated with morbidity and mortality (10–12). Our group previously established a feline model that has critical features of HFpEF, including left ventricular hypertrophy (LVH), elevated left ventricular filling pressures (LVEDP), left atrial (LA) enlargement, increased concentration of plasma NT-proBNP, and impaired pulmonary mechanics that contribute to functional compromises and poor oxygenation. We recently reported an in-depth structural and functional cardiopulmonary characterization of the model, which supports its translational efficacy (13).

The aim of the current study was to determine if inhibition of histone deacetylase (HDAC) catalytic activity could prevent and/or reverse the adverse structural and functional cardiopulmonary remodeling in this feline model of slow-progressive pressure overload. We used SAHA (suberoylanilide hydroxamic acid, vorinostat), a pan-HDAC inhibitor, that has FDA approval for the treatment of cutaneous T-cell lymphoma (CTCL) (14). Histone deacetylation leads to compaction of chromatin, therefore repressing mRNA synthesis and altering gene expression. HDACs also catalyze removal of acetyl groups from ϵ -amino groups of lysine residues in a variety of non-histone proteins, thus having a role in the regulation of post-translational protein modification affecting transcription and other protein functions (15, 16). In experimental models of HF (pressure overload, ischemia/reperfusion), small-molecule inhibitors of HDAC catalytic activity have demonstrated potent cardioprotective and anti-remodeling effects, as seen by a decrease in scar size, attenuated cardiac hypertrophy, and improved systolic function (17–19). A recent report showed that HDAC inhibition in rat and mouse models of diastolic dysfunction improved diastolic function by potentiating cardiac myofibril relaxation (20). These studies suggest that HDAC inhibition promotes beneficial remodeling in cardiac disease phenotypes through a non-genomic mechanism. The present study tests the hypothesis that HDAC inhibition can reverse and/or prevent adverse cardiopulmonary remodeling in a large animal model of diastolic dysfunction.

Results

The experimental protocol is displayed in Fig. 1A. Subcutaneous injections of SAHA and vehicle were administered daily and were well tolerated. SAHA did not alter total counts of

white blood cells, red blood cells (RBC), and platelets, or result in changes to RBC or platelet size (table S1). There was no difference in plasma concentrations of blood urea nitrogen (BUN) between groups, suggesting that kidney function was not negatively affected by slow-progressive pressure overload or SAHA treatment (fig. S1A). Furthermore, SAHA treatment did not cause QT interval prolongation (fig. S1B), which had been previously described in a phase I trial (21). The treatment strategy used in this study did not induce any known toxic side effects of SAHA. As previously reported (13), banded animals displayed symptoms of HF such as tachypnea and diaphragmatic breathing, particularly in response to physical exertion.

SAHA rescues HFpEF phenotype

At 2-months post-banding, before starting SAHA treatment, all banded animals exhibited a similar cardiac phenotype with significant LVH ($P < 0.0001$), LA enlargement ($P < 0.001$), and reduced radial strain values ($P < 0.05$), but with preserved LV ejection fraction (EF) when compared to sham-operated animals (Fig. 1). SAHA treatment prevented progression of LV hypertrophy, as reflected by a decrease in LV wall thickness (4mo: SAHA: 4.5 ± 0.1 mm vs. veh: 6.1 ± 0.2 mm, $P < 0.0001$) (Fig. 1B) and significantly reduced LA size, reflected by a decrease in LA volume (Fig. 1C, $P < 0.0001$) and left atrial to aortic root size ratio (LA/Ao) (Fig. 1D, $P < 0.0001$). After 2 months of SAHA treatment, LA size and LV wall thickness did not differ remarkably between SAHA-treated and sham animals, whereas veh-treated animals had progressive deterioration of these parameters. LA EF, a parameter of LA function, also improved with SAHA treatment and was significantly greater than in veh-treated animals (Fig. 1E, $P = 0.0011$). Furthermore, SAHA treatment attenuated an increase in E/e' compared to veh-treated animals at 4-months, indicative of lower left ventricular filling pressures (22) (Fig. 1F). LV EF (Fig. 1G) and left ventricular end-diastolic diameter (LVEDD) (Fig. 1H) were not affected by aortic banding and did not differ between groups at any time point, suggesting normal LV capacitance. However, speckle tracking based strain analysis, a sensitive method used to assess myocardial function (23), revealed a significant impairment in all banded animals at 2-months compared to sham-operated animals. ($P < 0.05$). By 4-months, the global radial strain further decreased in the veh-treated animals but did not decline in the SAHA group (Fig. 1I). Representative parasternal long axis b-mode echocardiogram (ECHO) images (Fig. 1J) display LV wall thickness and LA size at 4-months. Representative short axis b-mode images with superimposed regional radial strain values (Fig. 1K) show differences in wall thickness and myocardial contractility. These data document that SAHA treatment in banded animals had strong anti-hypertrophic effects (LV wall thickness and LA size), and improved LV systolic and diastolic function.

SAHA improves diastolic function and decreases LV filling pressures

Invasive hemodynamic measurements were performed at 4-months post-banding. Mixed-venous and arterial blood gas analysis (BGA), cardiac output (CO), pulmonary and cardiac pressures were recorded before (dob-) and after (dob+) intravenous infusion of dobutamine ($5 \mu\text{g/kg/min}$). Pressure gradients across the aortic band were measured using a fractional flow reserve (FFR) technique, which is conventionally used to assess coronary physiology in humans. The mean systolic aortic pressure gradient across the band did not differ between SAHA (33 ± 6 mmHg) and veh-treated (38 ± 9 mmHg, $P = 0.6$) animals (Fig. 2A), indicating

comparable pressure overload between banded groups. CO (Fig. 2B) and heart rate (HR) (Fig. 2C) did not differ significantly between groups, although SAHA-treated cats tended to have lower HRs. $dP/dt_{\max}$ was comparable between groups before dobutamine infusion, however the increase in $dP/dt_{\max}$ with dobutamine was significantly less in veh-treated (4947 ± 598 mmHg/s) compared to SAHA-treated animals (7690 ± 723 mmHg/s, $P < 0.05$) (Fig. 2D), suggesting depressed contractile reserve in veh-treated banded animals versus shams, and improved inotropic reserve after SAHA treatment. $dP/dt_{\min}$ (Fig. 2E) was reduced in both banded groups compared to sham. However, the ratio between diastolic time interval and LV isovolumic relaxation constant ($t\text{-dia}/\tau$), an index of relaxation, was significantly smaller in veh-treated (3.9 ± 0.3 , $P < 0.01$), but not in SAHA-treated banded animals (5.2 ± 0.4 , $P = 0.16$) when compared to shams (6.4 ± 0.7) (Fig. 2F). SAHA treatment also significantly reduced LVEDP ($P < 0.01$) and mean pulmonary arterial pressure (mPAP) ($P < 0.01$) compared to veh-treated banded animals (Fig. 2G and 2H). These results show that SAHA treatment improves both systolic and diastolic function in banded animals.

SAHA improves myofibril relaxation and decreases cardiomyocyte size

To determine if in vivo diastolic dysfunction in veh-treated animals was related to HDAC-dependent alterations in intrinsic cardiomyocyte relaxation, myofibril mechanical studies were performed (20). Samples of myofibrils isolated from homogenized LV tissue were mounted on a force transducer, and rapidly shifted from high (to measure Ca^{2+} -activated force) to low Ca^{2+} -containing solution (to measure relaxation kinetics). The maximal calcium-activated tension generation (myofibril maximal tension) was not different between groups (Fig. 3A). However, myofibrils from veh-treated banded animals exhibited a significant prolongation in the duration of the linear phase of relaxation when compared to sham ($P < 0.01$), which was significantly reversed by SAHA (Fig. 3B, $P < 0.05$). The linear phase of relaxation represents inactivation of thin filament regulatory proteins upon unbinding of Ca^{2+} . Linear relaxation duration significantly correlated with invasively measured $t\text{-dia}/\tau$ (Fig. 3C, $P = 0.02$) and LVEDP (Fig. 3D, $P = 0.0061$), but not with LV wall thickness (Fig. 3E, $P = 0.28$). These findings show that myofibril-autonomous impairment in banded animals correlates with in vivo diastolic dysfunction. These results also show that HDAC inhibition improved myofibril relaxation independent of the anti-hypertrophic effects of the treatment.

SAHA-treated animals (3.4 ± 0.1 kg) gained less weight than untreated sham-operated animals (4.2 ± 0.1 kg) and this difference was significant at 4-months (Fig. S1C) when compared to veh-treated animals (3.8 ± 0.1 kg, $P < 0.05$). Body weight was lower in the SAHA group and the heart weight to body weight (HW/BW) ratio was also significantly decreased in SAHA-treated compared to veh-treated animals (Fig. 3F, $P < 0.05$). Histological analysis showed that cardiomyocyte (CM) cross-sectional area in SAHA-treated animals was significantly smaller than veh-treated animals ($P < 0.05$) but still increased compared to shams (Fig. 3G, $P < 0.05$). Representative confocal micrographs show LV cardiomyocyte cross-sections (Fig. 3H) and demonstrate that decreased myocyte size is responsible for the reduced LV wall thickness in SAHA-treated banded animals shown by ECHO analysis.

LV cross sections were stained with Masson's Trichrome (MT) and the aniline blue stained area (collagen) was measured. Sub-endocardial and sub-epicardial layers were analyzed separately. Only the sub-endocardial layer from veh-treated banded animals showed an increase in LV fibrosis (Fig. 3I). SAHA-treated animals had greater fibrotic area than shams and less than veh-treated animals, but these differences were not significant in any specific region of the left ventricle wall.

Plasma concentration of NT-proBNP, a HF biomarker, was significantly higher in banded animals at 4-months when compared to shams ($P < 0.001$). SAHA-treated animals had lower plasma concentrations of NT-proBNP compared to veh-treated animals (Fig. 3J).

SAHA improves pulmonary function and preserves lung structure

Pulmonary mechanics were assessed at baseline (BL), 1, 2, 3, and 4-months post-banding using pneumotachography in spontaneously breathing animals. Banded animals had significantly lower lung compliance at 2-months post-banding compared to sham-operated animals ($P < 0.05$). Over the next 2 months, lung compliance deteriorated in veh-treated animals, but SAHA treatment prevented further stiffening of the lungs and at the end of the treatment period there was no difference compared to sham animals (Fig. 4A). Lung compliance measured during ventilation was consistent with the data collected from spontaneously breathing cats (fig. S1D). Gas exchange parameters at 4-months demonstrated that veh-treated banded animals had a significant decrease in $\text{PaO}_2/\text{F}_1\text{O}_2$ (Fig. 4B, $P < 0.01$), a greater alveolar-arterial oxygen difference (A-aDO_2) (Fig. 4C, $P < 0.01$), and greater intrapulmonary shunt fraction (Fig. 4D, $P < 0.01$) compared to sham and SAHA-treated animals. Except for PaO_2 , parameters derived from the arterial blood gas analysis were comparable between groups (table S2). These findings suggest that SAHA treatment in animals with pressure overload improves pulmonary function and oxygenation. Lung compliance negatively correlated with LV wall thickness and LA size and positively correlated with $\text{PaO}_2/\text{F}_1\text{O}_2$ (Fig. 4E–G), suggesting a link between the development of LV filling defects and impaired lung function.

We have previously shown that slow-progressive pressure overload in this model induces lung remodeling that explains the altered lung function (13). Quantitative histomorphological analyses were performed to determine whether the improved pulmonary function in SAHA-treated animals was associated with prevention or reversal of morphological changes in the lung. SAHA significantly reduced the alveolar-capillary wall thickness (Fig. 4H, $P < 0.05$) and increased both the alveolar area (Fig. 4I, $P < 0.05$) and expansion index (ratio of volume of gas exchange to parenchymal space) (Fig. 4J, $P < 0.01$) compared to veh-treated cats. Assessment of perivascular fluid cuff formation (Fig. 4K) around extra-alveolar blood vessels revealed a significant increase in veh-treated compared to SAHA-treated ($P < 0.05$) and sham animals ($P < 0.05$). No statistically significant differences were observed between SAHA-treated and sham animals in any histomorphological indices. Representative bright field micrographs of H&E stained lung sections are displayed in Fig. 4L. These results show that SAHA treatment in this animal model can improve the lung remodeling that underlies abnormal lung function, likely by normalizing LV filling pressures.

SAHA induces a switch in skeletal muscle fiber composition and improves mitochondrial function

O₂ consumption was assessed during hemodynamic studies by performing arterial and mixed venous blood gas analysis and cardiac output measurements. Veh-treated banded animals had lower O₂ consumption compared to SAHA-treated banded and sham-operated animals (Fig. 5A). Furthermore, SAHA-treated animals had a significantly lower venous oxygen return (SvO₂% (P < 0.01), PvO₂ (P < 0.01), oxygen content (P < 0.05) compared to veh-treated animals, consistent with increased O₂ extraction (table S3). The root cause of these changes was further investigated by examining whether changes in metabolic genes could explain the differences in O₂ consumption between different groups of animals. In skeletal muscle (gastrocnemius muscle) of SAHA-treated animals, mRNA expression of a transcription factor (Tfam) and coactivators (Pgc-1 α , Pgc-1 β , P < 0.05) that regulate mitochondrial function as well as genes involved in glucose metabolism (Glut4, P < 0.05) and the TCA cycle (Idh3 α , P < 0.01) were significantly increased compared to the veh-treated animals. mRNA expression of all genes tested were slightly decreased in veh-treated animals compared to sham animals, but these changes did not reach statistical significance (Fig. 5B). Furthermore, SAHA led to a switch in skeletal muscle fiber composition with a significant increase in the percentage of type 1 muscle fibers compared to veh-treated animals (P < 0.05) (Fig. 5C). Type 1 fibers have a higher oxidative capacity compared to type 2 fibers and are known to be reduced in patients with HFpEF (24). Type 2 skeletal muscle fibers can be further categorized as type 2a or type 2b. There was no difference in the percentage of type 2a fibers between groups. These findings suggest increased mitochondrial biogenesis and oxidative capacity in skeletal muscle after SAHA treatment.

The effects of SAHA on mitochondria function and structure were assessed in vitro using HeLa cells. There was no difference in basal or histamine-triggered cytosolic Ca²⁺ content observed (fig. S1E–F), indicating that SAHA does not affect the integrity of the endoplasmic reticulum. SAHA induced slight depolarization of the mitochondrial membrane (Fig. 5D) and elongation of the mitochondria structure (Fig. 5E). Basal mitochondrial Ca²⁺ was reduced with SAHA, but when treated with histamine the mitochondrial uptake of Ca²⁺ was significantly increased (Fig. 5F, P < 0.05). Furthermore, SAHA treatment led to an elevation in both basal and maximal mitochondria respiration, uncoupling, and proton leak (Fig. 5G). These studies support the idea that SAHA directly affects mitochondrial function.

To determine whether the SAHA-induced changes in mitochondrial function occur in cardiac myocytes, experiments were performed using isolated adult feline cardiomyocytes (AFVM). There was no difference in oxygen consumption rate during basal respiration between SAHA and vehicle (DMSO) treated AFVMs, but SAHA treatment led to a significant increase in the oxygen consumption rate during maximal respiration (fig. S1G, P < 0.05).

Mitochondrial metabolism changes induced by SAHA are associated with post-translational modifications

To determine if SAHA treatment alters the concentration of Acetyl-CoA and protein acetylation patterns, mass spectrometry was performed on LV tissue from SAHA-treated and

control sham-operated animals. Sham (not banded) animals were used to determine the impact of SAHA on acetylation independent of any influence from the aortic banding procedure. The concentration of Acetyl-CoA was not altered (Fig. 6A) by SAHA treatment, whereas protein acetylation was altered substantially (table S4). We found 51 acetylation sites out of 577 to be altered by SAHA when allowing two miscleavages by trypsin and another 17 out of 589 acetylation sites altered when allowing three miscleavages by trypsin. Most of the differentially acetylated proteins could also be quantified at the level of total protein by performing label free quantitative proteomics on the non-acetylated peptides in the flow-through of the immunoprecipitations of acetylated peptides, revealing no significant changes in their abundance (table S5). These findings suggest that SAHA can exert changes in protein function via post-translational modifications.

The most upregulated acetylation sites were lysine 24 (>3000 fold), 19 (>3000 fold), 15 (>100 fold), and 10 (>100 fold) on histone 3 (Fig. 6B). These sites are conserved in various species and known to activate genes (25). The different histone H3 variants could not be discriminated since they share an identical N-terminal sequence. Interestingly, proteins of complexes I, II, and V of the electron transport chain (ETC) were differentially acetylated (Fig. 6C). NDUB3 of complex I could not be quantified in all samples (marked by * in Fig. 6C), but all other proteins were unchanged in terms of total protein abundance. However, sham+SAHA samples had both hyper- and hypo-acetylation of individual ETC components, suggesting that SAHA also indirectly affects protein acetylation in addition to inhibiting lysine deacetylases. Furthermore, components of the malate-aspartate shuttle were also highly regulated by SAHA, with changes in acetylation patterns (Fig. 6D) but not total protein abundance (table S5). We discovered three proteins that were differentially acetylated on six lysines.

We also investigated the effect of SAHA treatment on enzymes involved in the TCA cycle. There was an increase in the acetylation of fumarate hydratase on lysine 292 (about 3000-fold), whereas acetylation of others was slightly downregulated (Fig. 6E). Moreover, seven sites on five proteins involved in mitochondrial fatty acid oxidation were less acetylated in SAHA-treated hearts (Fig. 6F). Taken together, many key mitochondrial enzymes had acetylation pattern alterations, which suggests SAHA affects mitochondrial metabolism via post-translational modifications.

Discussion

Using a large animal model with critical cardiac characteristics (long action potential duration and beta MHC as the primary myosin isoform) that are identical to humans and not found in rodents (26, 27), we showed that slow-progressive pressure overload causes the development of dysfunctional cardiopulmonary features also seen in humans with HFpEF. This model was used to test the hypothesis that HDAC inhibition improves/reverses these features. Our study found that the pan-HDAC inhibitor SAHA reversed LVH, reduced LA enlargement, improved LV systolic and diastolic function, and normalized LVEDP. SAHA treatment also restored lung compliance and structure, and improved oxygenation. The enhanced LV filling characteristics resulted from improved relaxation and were responsible for lower LV filling pressures. We also demonstrated that HDAC inhibition augmented the

relaxation properties of myofibrils isolated from the LVs of pressure overloaded hearts and that this mechanism likely contributes to improving diastolic function in vivo. The findings from the current study are compared to and contrasted with previously published reports that used HDAC inhibitors in animal models of pressure overload and/or heart failure in table S6.

The dose of SAHA used was based on previous studies conducted in experimental models of HF (18, 28, 29). McDonnell *et al.* (28, 30) showed that both intravenous (2 mg/kg) and oral (17 mg/kg) administration of SAHA were well tolerated in felines and produced the predicted pharmacodynamic effect of histone acetylation. They also found that oral bioavailability of SAHA in their animals was generally poor. Therefore, they concluded that an alternative route of administration, such as subcutaneous injection (s.c.), may provide more optimal drug delivery in these animals. Our experiments documented that this dose of SAHA (10 mg/kg) was well tolerated and did not induce hematological toxicity or QT interval prolongation, which are both potential adverse effects that have been described previously (21, 31). In our study, QTc was shortened in banded animals treated with SAHA. This effect is likely related to the anti-hypertrophic effects of SAHA and the known relationship between LVH, QTc prolongation, and sudden cardiac death (32). In humans, reported adverse side effects of SAHA treatment include leuko- and thrombocytopenia, bone marrow suppression, and gastrointestinal symptoms (31). We found no evidence for any of these adverse effects in our studies. In addition, efficacious doses of HDAC inhibitors will likely be significantly lower for HF treatment than those required for cancer therapy (16).

We found that SAHA can blunt and reverse LVH caused by pressure overload. Consistent with our findings, pan-HDAC inhibitors in rodent HF models attenuated cardiac hypertrophy induced by infusion of angiotensin II (19) and isoproterenol (33). HDAC inhibition was also shown to reverse established cardiac hypertrophy after transverse aortic constriction in rodent models (19). A combination of chromatin and non-chromatin-based direct effects on protein activity have been proposed as important mechanisms by which HDAC inhibitors counteract pathological LV hypertrophy (16). In cultured myocytes, the transcription factor krüppel-like factor 4 (KLF4) increased after HDAC inhibition and blocked phenylephrine-induced hypertrophy (16). The anti-hypertrophic effect observed in the current study was still evident although the aortic band had not been removed and chronic pressure overload was present throughout the entire study. The notable decrease in LA size in the SAHA-treated cats is likely a consequence of the regression of LVH, improvement in diastolic function, and the resulting reduction in LVEDP. A decrease in LV filling pressures leads to reduced pressures within the LA, and thus, reduced atrial remodeling. Reduced LV filling pressures also explains the improvement of the pulmonary phenotype.

Patients with HFpEF have EFs within the normal range (34). In our study, LV EF did not differ between groups at any time point. EF is highly load dependent, making it a measure of LV capacitance rather than contractility, and EF is overestimated in the presence of LVH due to increased myocardial thickening (2). More sensitive imaging techniques, such as speckle-tracking based strain analysis, are powerful tools for exploring the idea that the HFpEF phenotype is accompanied by early systolic dysfunction, despite preserved LV EF (23). In line with these reports, we found a significant impairment in global radial strain prior to starting SAHA treatment at 2-months post-banding. Radial strain values gradually

deteriorated in veh-treated animals over time, whereas SAHA prevented this deterioration. In addition, the increase in $dP/dt_{\max}$ in response to dobutamine was significantly blunted in veh-treated versus sham animals and SAHA treatment significantly improved the response to dobutamine. Collectively, these findings document that animals with slow progressive pressure overload exhibit impaired systolic reserve, while their EF remained unchanged. An impaired systolic reserve also affects diastolic function because recoil and suction forces during early diastole are attenuated (35). These findings support the idea that SAHA improves systolic contractile reserve in this model.

Previous animal studies have tested pan-HDAC inhibitors in HF models with reduced ejection fraction (transverse aortic constriction, ischemia/reperfusion in rodents) (17–19). Our study tested HDAC inhibition in a large animal model exhibiting key clinical characteristics of HFpEF, including diastolic dysfunction and pulmonary impairment confirmed by invasive hemodynamics and state of the art pulmonary function testing. We showed that banded animals exhibited diastolic dysfunction (elevated LVEDP, decreased $t\text{-dia}/\tau$ ratio) before starting treatment, and that SAHA treatment significantly decreased LVEDP and improved isovolumic relaxation. A delay in LV isovolumic relaxation limits LV filling, impairs diastolic suction, and increases end-diastolic pressures because relaxation remains incomplete (36, 37). Increased LV filling pressures are the ultimate downstream expression of impaired LV diastolic function and provide sufficient evidence to secure a HFpEF diagnosis in symptomatic patients (38).

Although exercise intolerance is a hallmark clinical feature of HFpEF, full exercise stress testing was beyond the scope of the current study. As an alternative to controlled exercise, we used pharmacologic stress testing. Dobutamine infusion improved indices of diastolic function, which was expected since β -adrenoreceptor signaling accelerates relaxation and therefore counteracts acquired diastolic dysfunction (39). These findings are also consistent with a previously published hemodynamic study performed in patients with HFpEF in whom dobutamine infusion decreased LVEDP and τ and increased $dP/dt_{\min}$, while hand-grip exercises increased LVEDP and caused a prolongation of τ (40).

In a recent study, Jeong *et al.* (20) reported a non-genomic role for HDACs in the direct control of myofibril relaxation in murine models of diastolic dysfunction with preserved EF. The authors showed prolonged kinetics of the linear phase of relaxation in Dahl salt-sensitive (DSS) rats and aged mice. After a 10-week treatment period with the pan-HDAC inhibitor ITF2357, myofibril relaxation was significantly enhanced in DSS rats independent of myofibril composition, calcium sensitivity, titin function, and cardiac fibrosis. Similar findings were obtained in aged mice treated with ITF2357 for 11 months. Furthermore, this study demonstrated abnormal myofibril relaxation in samples collected from patients with restrictive cardiomyopathy, emphasizing the translational significance of this finding. In the present experiments, myofibril relaxation was significantly impaired in banded animals and this abnormality was reversed by SAHA treatment. Myofibril relaxation correlated with *in vivo* LVEDP and $t\text{-dia}/\tau$, a marker of active relaxation, but not with LV wall thickness, indicating a mechanism of action that is independent of the anti-hypertrophic effects of SAHA.

LV fibrosis is thought to be involved in HFpEF-related diastolic dysfunction, but neither biopsies from patients with HFpEF nor animal models strongly support this hypothesis (2, 20). SAHA and veh-treated animals had an increase in LV fibrotic area, which was more pronounced in the sub-endocardium compared to the sub-epicardium. This gradient could be related to a limited sub-endocardial coronary reserve (41), but this idea was not explored. Although HDAC inhibitors have been previously reported as having anti-fibrotic effects (17, 19, 29), we did not observe a significant reduction in fibrosis with SAHA treatment. Therefore, the improved LV diastolic function in the SAHA group was not strongly linked to collagen deposition. It is important to note that this model has a modest fibrotic phenotype, so SAHA treatment in a condition with a more robust presentation of fibrosis could produce an anti-fibrotic effect with functional significance.

The basis of pulmonary hypertension (PH) in HFpEF is still not well established, although it is a common and life-threatening complication associated with poor prognosis (42). We recently reported pulmonary arterial hypertension in animals with slow progressive pressure overload due to a passive backward transmission of elevated LV filling pressures (13). In the present study, mPAP was also significantly increased in banded animals compared to shams. SAHA-treatment normalized pulmonary arterial pressures, preserved lung structure, and improved lung compliance and function ($\text{PaO}_2/\text{F}_\text{I}\text{O}_2$, A-aDO_2 , intrapulmonary shunt). The beneficial effects of SAHA on lung function are best explained as a result of the reduction in left ventricular filling pressures, which subsequently lowers pulmonary pressures, thus preventing pulmonary remodeling and dysfunction. Veh-treated animals showed significant perivascular fluid cuff formation, which we previously reported for the first time in HFpEF (13). Perivascular fluid cuffs are suggestive of capillary endothelial disruption and impaired permeability due to elevated hydrostatic pressures (alveolar-capillary stress failure). In a lung injury model, fluid cuffs decreased lung compliance by increasing tissue resistance and impairing mechanical coupling between the lung parenchyma and bronchovascular bundle (43). The effects of SAHA that we observed in the present study are likely the downstream result of reduced pulmonary pressure. Since SAHA has been shown to have a direct beneficial effect on the lungs in rodent models of induced PH (44), it is possible that the improvements observed in our study are partially due to direct effects of SAHA on the lung.

The enhanced mitochondrial oxidative phosphorylation and increased percentage of type 1 skeletal muscle fibers in SAHA-treated animals could underlie the increase in in vivo O_2 consumption, decreased venous O_2 content, and smaller body weight. *Type 1 and 2a skeletal muscle fibers rely on oxidative metabolism as their primary source of energy, whereas type 2b fibers rely on glycolytic metabolism (45). There were no differences in the percentage of type 2a skeletal muscle fibers between groups.* Additionally, we found increased mRNA expression of genes responsible for mitochondrial biogenesis in skeletal muscle of SAHA-treated animals. SAHA had no effect on the Ca^{2+} content of the endoplasmic reticulum (ER) and did not induce ER Ca^{2+} leakage, indicating that SAHA does not affect the integrity of the ER. However, SAHA increased mitochondrial Ca^{2+} uptake, although the mitochondrial membrane potential and resting Ca^{2+} were reduced, which indicates that mitochondrial Ca^{2+} sequestration was not compromised. The underlying mechanism for increased mitochondrial Ca^{2+} uptake and respiratory activity in the presence of normal intracellular Ca^{2+} is unclear, but could potentially result from enhanced ER-mitochondria tethering or functional changes

to mitochondrial Ca^{2+} uptake machinery (46). SAHA may also affect mitochondrial fission machinery, reflected by elongated mitochondrial structures. Our in vitro results clearly show that SAHA treatment can directly modify mitochondria function, suggesting that modification of mitochondria function by SAHA could explain aspects of our in vivo findings.

Using mass spectrometry, we found that SAHA-induced changes in mitochondrial metabolism correlated with associated post-translational modifications. Succinate dehydrogenase A (SDHA), which is a subunit of complex II in the ETC, was three times less acetylated on lysine 547 in SAHA treated sham animals. This lysine residue is conserved in both mice and humans, and hyperacetylation of this site has previously been shown to negatively affect complex II activity (47). Thus, our finding of decreased acetylation at lysine 547 may lead to increased complex II activity. Consistent with our finding, Herr *et al.* (48) reported that selective HDAC1 inhibition led to a decrease in SDHA activity and subsequent ROS production in an ex vivo model of ischemia/reperfusion. Similarly, acetylation of lysine 259 of ATP synthase subunit beta (ATPB), the catalytic subunit of complex V, was reduced by two thirds. This site is conserved in both mammals and flies, and hyperacetylation has been shown to inhibit ATP synthase activity (49). Thus, ATP synthase activity may be modulated by SAHA treatment.

Hyperacetylation of mitochondrial aspartate aminotransferase (AATM) and malate dehydrogenase (MDHM) has been described to improve mutual interaction and activity between AATM and MDHM, resulting in increased transportation of NADH into mitochondria and enhanced ATP synthesis (50, 51). We found that acetylation of AATM was about 2000-fold higher on lysine 363 and about 500-fold higher on lysine 234 in SAHA treated sham animals. Acetylation was increased about 2000-fold on lysine 301 of MDHM. In contrast, acetylation of lysine 345 of AATM and lysine 73 of cytosolic malate dehydrogenase (MDHC) were reduced about 50% and 40%, respectively. AATM and MDHM lysine acetylation is altered by SAHA, but the effect of these changes on enzyme interaction and activity was beyond the scope of the study.

Consistent with our findings, Galmozzi *et al.* (52) reported that HDAC inhibition increased O_2 consumption and oxidative capacity in skeletal muscle in a mouse model of obesity and diabetes. HFpEF is associated with impaired mitochondrial oxidative capacity, rapid depletion of high-energy phosphate, increased formation of reactive oxygen species, and a reduction in the number of type 1 skeletal muscle fibers, leading to an imbalance in energy supply and demand. The observed effects of SAHA on mitochondrial function may help restore energy supply in heart failure, which is in contrast to current HF drugs that aim to reduce energy demand to match the reduced supply (24, 53).

Our study had some limitations. Invasive hemodynamics were performed in anesthetized cats in the supine position, which is an unusual posture for quadrupeds, and pharmacological stress testing was performed using dobutamine, which does not replicate physical exercise. Due to these factors, the results must be interpreted with caution. It is also worth noting that the pericardial sack was dissected during both the sham and aortic constriction procedures, which could have potentially resulted in an underestimation of the left-sided filling pressures

(54). Furthermore, we used only male animals for this study and did not address sex-related differences. RV function is known to be impaired in HFpEF but was not assessed in this study. Echo based imaging parameters (tricuspid annular plane systolic excursion, Strain) that are used to assess RV function in patients are not well established and validated in cats. *Only parasternal short-axis strain analysis is reported because acquiring the views for apical 4-chamber and parasternal long-axis views is inconsistent in cats, making it an unreliable parameter to assess subtle differences between treatment groups. The animals were young and did not have any comorbidities or other disease background, which is in contrast with the clinical presentation of many patients with HFpEF.* It is likely that in contrast to young animals, aged animals would have shown impairments in cardiovascular reserve. *No single animal model is likely to recapitulate the complex phenotype of human HFpEF, but the large animal model used in the present study could serve as a useful platform for testing therapies for select populations of patients.* Acetylation experiments were performed using only LV tissue from sham animals treated with SAHA in order to confirm that SAHA treatment induced the predicted pharmacodynamic effect of protein and histone acetylation without any other confounding influence from the aortic banding procedure. Performing the acetylation studies on other groups (i.e. b+SAHA or b+veh) was beyond the scope of the current study.

In summary, this study shows the efficacy of an FDA-approved HDAC inhibitor in a pre-clinical large animal model of diastolic dysfunction. We demonstrated that SAHA exerts robust anti-hypertrophic effects, improves diastolic function, reduces LVEDP, and ultimately improves lung compliance and pulmonary function. In addition, we showed that impaired myofibril relaxation is an HDAC-dependent mechanism for diastolic dysfunction, which can be restored by SAHA. Furthermore, SAHA treatment induced metabolic effects that could restore energy supply in the failing heart. Given the promising pre-clinical results and the unmet need for effective therapeutics to treat the growing HFpEF population, our results suggest that SAHA treatment could potentially improve cardiopulmonary dysfunction in patients with features of the model system we employed.

Materials and Methods

Study design

The aims of this study were to assess if HDAC inhibition could improve cardiopulmonary function and preserve cardiopulmonary structure in a feline model of diastolic dysfunction. State of the art invasive hemodynamics, echocardiography, immunohistochemistry, histology, tissue proteomics, mitochondrial function, and myofibril mechanic studies were performed. All animals were randomly assigned to treatment arms, and sample size was determined by power calculation ($\alpha=0.05$, $\beta=0.8$, effect size of 1.5 SD) to assess anti-hypertrophic effects of HDAC inhibition. All analyses were performed by investigators blinded to treatment or banding. All samples were given a code that was not linked to study group until analyses were completed. The number of animals included in each experimental parameter are listed in table S7. Primers for PCR analysis are listed in table S8. Please refer to the Materials and Methods section in the Online Supplemental Materials for a detailed

description of experimental methods and proteomic data (tables S4 and S5), as well as statistical analysis results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding:

This work was supported by the National Institute of Health [HL147558 to S.R.H. and T.A.M., HL33921 to S.R.H., HL116848 and HL127240 to T.A.M., R56-HL137850 and RO1-HL137850 to S.M.]; Department of Defense/Office of Naval Research [VA - N000141210810 to M.R.W.]; American Heart Association [16POST30960017 to Y.H.L., 16SFRN31400013 to T.A.M., SDG-15SDG25550038 to S.M.]; Colorado Clinical and Translational Science Institute [5KL2TR001080-02 to M.Y.J.]; Medical University of Graz - Start Funding Program [M.W.] and Stadt Graz [M.W.]; Austrian Science Fund [KLI425, KLI645, W1226 and F73 to R.B.].

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Fig. 1. Experimental study design and remodeling effects of SAHA.

(A) Baseline measurements were collected and felines then underwent the aortic banding or sham procedure. After 2 months, banded cats were assigned to either daily subcutaneous injection of vehicle or SAHA (10 mg/kg) for 2 months. At 4-months post-banding, invasive hemodynamic studies were performed. Conventional echocardiography data showing (B) left ventricular wall thickness, (C-D) LA size, (E) LA function, (F) LV diastolic function, (G) LV EF, and (H) LVEDD in sham, veh-treated, and SAHA-treated animals. (I) Speckle-tracking based strain analysis was quantified from sham, veh-treated, and SAHA-treated animals. (J) Parasternal long-axis images showing septal wall thickness and LA size. (K) Parasternal short-axis images with superimposed regional radial strain values. Data shown are means $\pm$ SEM. Statistical significance was determined by linear mixed-effects models. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ between b+veh and sham. § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$, §§§§ $P < 0.0001$ between b+SAHA and sham. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ between b+veh and b+SAHA.

0.01, ###P < 0.001, ####P < 0.0001 between b+SAHA and b+veh. NS, not significant. Ao=aorta, BGA=blood gas analysis, BL=baseline, E=peak early diastolic transmitral velocity, $\acute{e}$ =spectral tissue Doppler-derived peak early diastolic velocity, Echo=echocardiography, EF=ejection fraction, HFpEF=heart failure with preserved ejection fraction, LA=left atrium, LA EF=left atrial ejection fraction, LAV_{ES}=left atrial end-systolic volume, LV=left ventricle, LVEDD=left ventricular end-diastolic diameter, PFT=pulmonary function testing, SAHA=suberoylanilide hydroxamic acid. The number of animals included in each parameter is reported in table S7.

Fig. 2. SAHA improves diastolic function.

(A) Aortic band pressure gradient measured in banded animals treated with SAHA or vehicle. Invasive hemodynamics showing (B) cardiac output, (C) heart rate, (D) dp/dt_{max} , (E) dp/dt_{min} , (F) $t-dia/\tau$, (G) LVEDP, and (H) mPAP (3 banded cats added to b+veh) before and after dobutamine infusion. Data shown are means $\pm$ SEM. Statistical significance was determined by linear mixed-effects models. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between b+veh and sham. § $P < 0.05$, §§ $P < 0.01$ between b+SAHA and sham. # $P < 0.05$, ## $P < 0.01$ between b+SAHA and b+veh. NS, not significant. Dob-/+ = before and after dobutamine, dp/dt_{max} = maximum rate of pressure rise, dp/dt_{min} = maximum rate of pressure decay, LVEDP = left ventricular end-diastolic pressure, mPAP = mean arterial pulmonary pressure, τ = isovolumic relaxation constant, $t-dia$ = diastolic time interval. The number of animals included in each parameter is reported in table S7.

Fig. 3. SAHA improves myofibril relaxation and exerts anti-hypertrophic effects.

Ex vivo myofibril mechanical studies on LV tissue collected at 4-months post-banding showing (A) myofibril maximal tension and (B) linear relaxation duration. Correlations of ex vivo linear relaxation duration with in vivo (C) t-dia/tau, (D) LVEDP, and (E) LV wall thickness. LV hypertrophy was assessed by (F) HW/BW ratio and (G) LV cardiomyocyte cross-sectional area. (H) Confocal micrographs of LV tissue section stained for WGA (green) and DAPI (blue) show cardiomyocyte size. Scale bars=50 μm. Assessment of (I) LV fibrosis in sub-endocardial and sub-epicardial layers. Quantified plasma concentrations of (J) NT-proBNP. Data shown are means ± SEM. Statistical significance was determined by (A, B, F, G, I) Kruskal-Wallis tests with multiple comparisons using two-stage linear step-up procedure, (C-E) Pearson correlation, and (J) linear mixed-effects models. *P < 0.05, **P < 0.01, ***P < 0.001 between b+veh and sham. §P < 0.05 between b+SAHA and sham. #P < 0.05 between b+SAHA and b+veh. †P < 0.05 vs. sub-endo b+veh. @P < 0.05 vs. sub-

endo b+SAHA. NS, not significant. BW=body weight, CM=cardiomyocyte, CSA=cross-sectional area, HW=heart weight, NT-proBNP=N-terminal pro-brain natriuretic peptide, τ =isovolumic relaxation constant, t-dia=diastolic time interval. The number of animals included in each parameter is reported in table S7.

Fig. 4. SAHA improves lung mechanics, gas exchange, and attenuates lung morphological changes.

Lung function was assessed by measuring (A) lung compliance, (B) $\text{PaO}_2/\text{F}_i\text{O}_2$ ratio ($\text{F}_i\text{O}_2=1$), (C) A-aDO₂, and (D) intrapulmonary shunt-fraction. Correlation of lung compliance with (E) LV wall thickness, (F) LA size, and (G) $\text{PaO}_2/\text{F}_i\text{O}_2$. Quantification of (H) alveolar-capillary wall thickness, (I) alveolar area, (J) expansion index (the ratio of volume of gas exchange to parenchymal space), and (K) ratio of cuff/vessel area of extra-alveolar vessels. (L) Bright-field micrographs of H&E-stained lung sections. Scale bars=250 μm . Data shown are means $\pm$ SEM. Statistical significance was determined by (A) linear mixed-effects models, (B-D, H-K) Kruskal-Wallis tests with multiple comparisons using two-stage linear step-up procedure, and (E-G) Pearson correlation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between b+veh and sham groups. § $P < 0.05$ between b+SAHA and sham. # $P < 0.05$, ## $P < 0.01$ between b+SAHA and b+veh. Open symbols in (E) and (F) indicate baseline data points. A-aDO₂=alveolar-arterial oxygen difference, LA/Ao=left atria to aortic

root ratio, PaO_2 =partial pressure of oxygen in arterial blood, F_{IO_2} =fraction of inspired oxygen. *=bronchioles, †=extra-alveolar vessel, arrow=fluid cuff around extra-alveolar vessels. The number of animals included in each parameter is reported in table S7.

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Fig. 5. SAHA induces switch in skeletal muscle fiber composition and improves mitochondrial function.

In vivo measurements of (A) oxygen consumption. (B) mRNA expression of genes related to mitochondrial biogenesis (Pgc-1 α , Pgc-1 β , Tfam), glucose metabolism (Glut4), and TCA cycle (Idh3 α) in skeletal muscle. (C) Skeletal muscle fiber composition. Assessment of (D) mitochondrial membrane potential, (E) mitochondrial structure, and (F) mitochondrial calcium uptake before and after histamine administration in HeLa cells. Scale bars=3 μ m. (G) Oxygen consumption rate and extracellular acidification rate were measured using Seahorse Technology: basal (1) and maximal (3) mitochondria respiration, uncoupling (4), and proton leak (2) in HeLa cells. Data shown are means $\pm$ SEM. Statistical significance was determined by (A-C) Kruskal-Wallis tests with multiple comparisons using two-stage linear step-up procedure and (D, E, G) analysis of variance (ANOVA) followed by Bonferroni post hoc testing to compare mean values between groups. $\$P < 0.05$ between b+SAHA and sham.

#P < 0.05, ##P < 0.01 between b+SAHA and b+veh. The number of animals included in each parameter is reported in table S7.

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Fig. 6. SAHA mediates mitochondrial metabolism via post-translational modifications. Assessment of (A) Acetyl-CoA concentrations and (B-F) protein acetylation patterns measured using mass spectrometry of LV samples from sham and sham+SAHA animals. Acetylation patterns on (B) histone 3, proteins of the (C) electron transport chain (ETC), proteins of the (D) malate-aspartate shuttle, proteins of the (E) TCA cycle, and proteins involved in (F) mitochondrial fatty acid oxidation. Data shown are means $\pm$ SEM. Statistical significance was determined by two-tailed Student's *t* test with subsequent multiple testing correction by permutation-based FDR method ($P < 0.05$). NS, not significant. The number of animals included in each parameter is reported in table S7.