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Histone deacetylase inhibitors synergize with sildenafil to suppress purine metabolism and proliferation in pulmonary hypertension

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ABSTRACT

Rationale: Sildenafil, a well-known vasodilator known to interfere with purinergic signaling through effects on cGMP, is a mainstay in the treatment of pulmonary hypertension (PH). However, little is known regarding its effects on the metabolic reprogramming of vascular cells, which is a hallmark of PH. Purine metabolism, especially intracellular de novo purine biosynthesis is essential for vascular cell proliferation. Since adventitial fibroblasts are critical contributors to proliferative vascular remodeling in PH, in this study we aimed to investigate if sildenafil, beyond its well-known vasodilator role in smooth muscle cells, impacts intracellular purine metabolism and proliferation of fibroblasts derived from human PH patients.

Methods: Integrated omics approaches (plasma and cell metabolomics) and pharmacological inhibitor approaches were employed in plasma samples and cultured pulmonary artery fibroblasts from PH patients.

Measurements and main results: Plasma metabolome analysis of 27 PH patients before and after treatment with sildenafil, demonstrated a partial, but specific effect of sildenafil on purine metabolites, especially adenosine, adenine, and xanthine. However, circulating markers of cell stress, including lactate, succinate, and hypoxanthine were only decreased in a small subset of sildenafil-treated patients. To better understand potential effects of sildenafil on pathological changes in purine metabolism (especially purine synthesis) in PH, we performed studies on pulmonary fibroblasts from PAH patients (PH-Fibs) and corresponding controls (CO-Fibs), since these cells have previously been shown to demonstrate stable and marked PH associated phenotypic and metabolic changes. We found that PH-Fibs exhibited significantly increased purine synthesis. Treatment of PH-Fibs with sildenafil was insufficient to normalize cellular metabolic phenotype and only modestly attenuated the proliferation. However, we observed that treatments which have been shown to normalize glycolysis and mitochondrial abnormalities including a PKM2 activator (TEPP-46), and the histone deacetylase inhibitors (HDACi), SAHA and Apicidin, had significant inhibitory effects on purine synthesis. Importantly, combined treatment with HDACi and sildenafil exhibited synergistic inhibitory effects on proliferation and metabolic reprogramming in PH-Fibs.

Conclusions: While sildenafil alone partially rescues metabolic alterations associated with PH, treatment with HDACi, in combination with sildenafil, represent a promising and potentially more effective strategy for targeting vasoconstriction, metabolic derangement and pathological vascular remodeling in PH.

Abbreviations: PH, pulmonary hypertension; HDACi, histone deacetylase inhibitors.

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1. Introduction

Chronic Pulmonary Hypertension (PH) is an incurable disease with poor outcomes and a median survival of 5–7 years [1,2]. Pulmonary artery (PA) vasoconstriction and pathological vascular remodeling characterized by dysregulated proliferation, persistent inflammatory signaling and extracellular matrix synthesis and remodeling collectively contributes to pathogenic disease outcomes. Targeting both aspects would appear to be required for optimal treatment effect. Purinergic signaling plays a pivotal role in the control of vascular tone and remodeling [3–5] and thus has been proposed as a potential contributor to the pathogenesis of PH [6]. Adenosine triphosphate (ATP), released from endothelial cells in response to changes in blood flow and hypoxia, acts on P2 purinergic receptors on endothelial cells leading to the production of endothelium-derived hyperpolarizing factors including nitric oxide (NO) and prostaglandins to cause vasodilation [4]. On the other hand, $A_{2B}R$ activation and $P2X_7R$ as well as $P2Y_1R$ and $P2Y_{12}R$ activation can induce vascular remodeling [6]. Further, intracellular purines serve as building blocks for nucleic acids, cofactors and energy store molecules, the key molecules controlling bioenergetics and anabolism to sustain proliferation of smooth muscle cells (SMCs), endothelial cells (ECs) in the pulmonary vasculature. It has been shown that intracellular purine synthesis is increased in proliferative vascular SMCs and plays an important role in vascular proliferative remodeling [7]. However, whether intracellular purine metabolism, especially de novo purine synthesis is a druggable target in PH and other cardiovascular diseases remains unexplored.

Several therapeutic interventions in PH rely on the use of vasodilators interfering with purinergic signaling, such as the phosphodiesterase type 5 (PDE5) inhibitors sildenafil and tadalafil, which block the degradation of cyclic guanylate monophosphate (cGMP), providing the pharmacological basis for modulation of the NO/cGMP pathway in vascular ECs and SMCs [8–11]. It is known that sildenafil improves exercise capacity and quality of life in patients with symptomatic pulmonary arterial hypertension (PAH) [12] and is effective for cardiovascular and oxygenation endpoints in pediatric PH [13]. However, it is increasingly recognized that only some patients respond to vasodilator therapy with improved pulmonary hemodynamics and symptomatic status [14,15]. Reasons for lack of success include the presence of chronic vascular remodeling and inflammation, rather than simply active vasoconstriction [16–18] and the involvement of multiple cell types including adventitial fibroblasts and recruited monocytes, macrophages, and lymphocytes where the effects of vasodilating drugs such as sildenafil are less clear [19–23]. Thus, the observed proliferative vascular remodeling in PH patients raises questions about the potential metabolic changes, including purine metabolism in PH vascular cells, especially in response to sildenafil treatment.

In contrast to normal vascular cells, the cells of hypertensive subjects and particularly adventitial fibroblasts, exhibit complex metabolic reprogramming characterized by an increase in glucose uptake, glycolysis, pentose phosphate pathway (PPP) and biosynthetic processes to sustain increased cell growth and proliferation [19–21,23,24]. Purines are basic components of the nucleic acids DNA and RNA and also provide the necessary energy (purine nucleotides such as ATP and guanosine triphosphate (GTP)) and cofactors (such as nicotinamide adenine dinucleotide (NAD) and its phosphate, reduced counterpart (NADPH) – essential for almost all anabolic reactions - and coenzyme A) to promote cell growth [25,26]. Thus, alterations in purine metabolism can be associated with the progression of proliferative vascular responses in PH. Notably, altered purine metabolism is common in tumor cells. This discovery led to the development of the earliest antitumor drugs (purine antimetabolites) to treat cancers by blocking DNA and RNA synthesis and halting metastatic cell growth [27–30]. Importantly, de novo biosynthesis of purines requires the generation of 5-phosphoribose-1-pyrophosphate (PRPP) produced through the PPP parallel to glycolysis, which was recently observed in PH-Fibs [20,23].

In the current study, we investigated purine metabolism in plasma and cells derived from PH patients and controls and investigated the changes in response to sildenafil treatment. Given the role of glycolysis in fueling de novo purine synthesis, we also determined the effect of previously validated glycolysis inhibiting interventions (TEPP-46 and HDACi) on purine metabolism in PH-Fibs. Finally, we investigated the combined effect of sildenafil and HDACi on PH-Fibs' purine metabolism and proliferation. The results of our study provide evidence of the feasibility and promising efficacy of a novel treatment strategy for PH that combines the vasodilatory and anti-inflammatory effects of sildenafil, with treatments inhibiting metabolic reprogramming and de novo purine synthesis, such as HDACi.

2. Materials and methods

2.1. Plasma metabolomics from PH patients treated with sildenafil

Metabolomics analyses were performed on 20 ul of plasma from 27 PH patients (Age: 53.5 + 14.0; Sex: 24 female, 3 male; Ethnicity: 20 non-Hispanic white; 4 African American, 3 Hispanic Latino), prior to and after treatment with sildenafil. Detailed information in Supplementary Table 1 and Supplementary Fig. 1.

2.2. Cell isolation and culture

Human pulmonary artery fibroblasts were derived from patients with idiopathic PH ($n = 4$) undergoing lung transplantation at Papworth Hospital, Cambridge, UK or from control non-PH donors (n = 4) as previously described [22,23,31]. Cells were cultured under normoxic conditions. Cell phenotype was thoroughly characterized, and all experiments were performed on cells at passages 5–10. All patients provided written informed consent.

2.3. Proliferation assay

Cell proliferation was evaluated using CyQUANT proliferation analysis Kit (Thermo Fisher Scientific, REF# C7026) as previously described [20,23]. After various days in culture, plates were gently inverted to remove medium by blotting it onto paper towels and washed once with PBS. Cells in the plates were frozen and stored at −80 °C until samples to be assayed. Measurement was done following manufacture's instruction. Briefly, plates were thawed at room temperature, then 250 μl of the CyQUANT GR dye/cell-lysis buffer was added to each sample well. Mixed gently, incubated at room temperature for 5 min, protected from light, then fluorescence was measured using a fluorescence plate reader with filters at 480 nm excitation and 520 nm emission maxima.

2.4. Real-time RT-PCR

As described previously [22,23], extraction of total RNA was performed using RNeasy Mini Kit (Qiagen, Inc). Total RNA was reverse-transcribed to cDNA, using an iScript cDNA Synthesis Kit (Bio-Rad, Inc) for mRNA detection. The levels of mRNA were determined quantitatively using Quantitative-Reverse Transcriptase PCR (qRT-PCR). The iTaq Universal SYBR Green Supermix (Bio-Rad, Inc) and unique primer pair (HPRT forward primer: 5′- ACGTCTTGCTCGAGATGTGA −3′ and reverse primer: 5′- AATCCAGCAGGTCAGCAAAG -3′; ADA forward primer: 5′- CCTGGCCAAGTTTGACTACTAC–3′ and reverse primer: 5′-CCTCTTTGGCCTTCATCTCTAC -3′) were used to detect mRNAs. qRT-PCR product analysis was performed on CFX96 Real-Time System (Bio-Rad, Inc., Hercules, CA, USA). The data were normalized using the endogenous HPRT as control. Gene expression was calculated after normalization to control group using the delta-delta CT method.

2.5. Sildenafil, TEEP-46 and HDAC inhibitor treatment

The cultured cells (under normoxic conditions) were treated with sildenafil (50 nM, Pfizer) or TEPP-46 (100 μM, EMD Millipore Corp, Billerica, MA) or pan-HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA; 10 μM, ChemieTek, Indianapolis, IN) or class I HDAC inhibitor Apicidin (3 μM) (Enzo, New York, NY) as previously described [23]. The cells were harvested for Ultra-high Pressure Liquid Chromatography-Mass Spectrometry-Based Metabolomics Analyses after 48 h treatment.

2.6. Ultra-high pressure liquid chromatography-mass spectrometry-based metabolomics analyses

Steady state and U13C-glucose tracing metabolomics analyses were performed as previously reported [20,32]. Briefly, CO-Fibs and PH-Fibs ($n = 3$–4, each group) were either untreated or treated for 48 h with (i) sildenafil (50 nM); (ii) TEPP-46 (100 μM); (iii) HDACi (SAHA, 10 μM or Apicidin, 3 μM); (iv) sildenafil (50 nM) + Apicidin (3 μM). Cells were extracted (2 × 106 cells/ml ice-cold buffer methanol:acetonitrile:water 5:3:2). For tracing experiments, cells were incubated with U-13C-glucose (SIGMA) replaced DMEM media for 24 h. Extracts run on a C18 reversed phase column (Phenomenex, Torrance, CA) through an ultra-high performance chromatographic system (UHPLC – Vanquish, Themo Fisher) coupled online with a high resolution quadrupole orbi-trap instrument (Qexactive – Thermo Fisher) operated in both polarity modes at 70,000 resolution. Metabolite assignment, peak integration for relative quantitation and isotopologue distributions in tracing experiments were calculated through the software Maven (Princeton), against

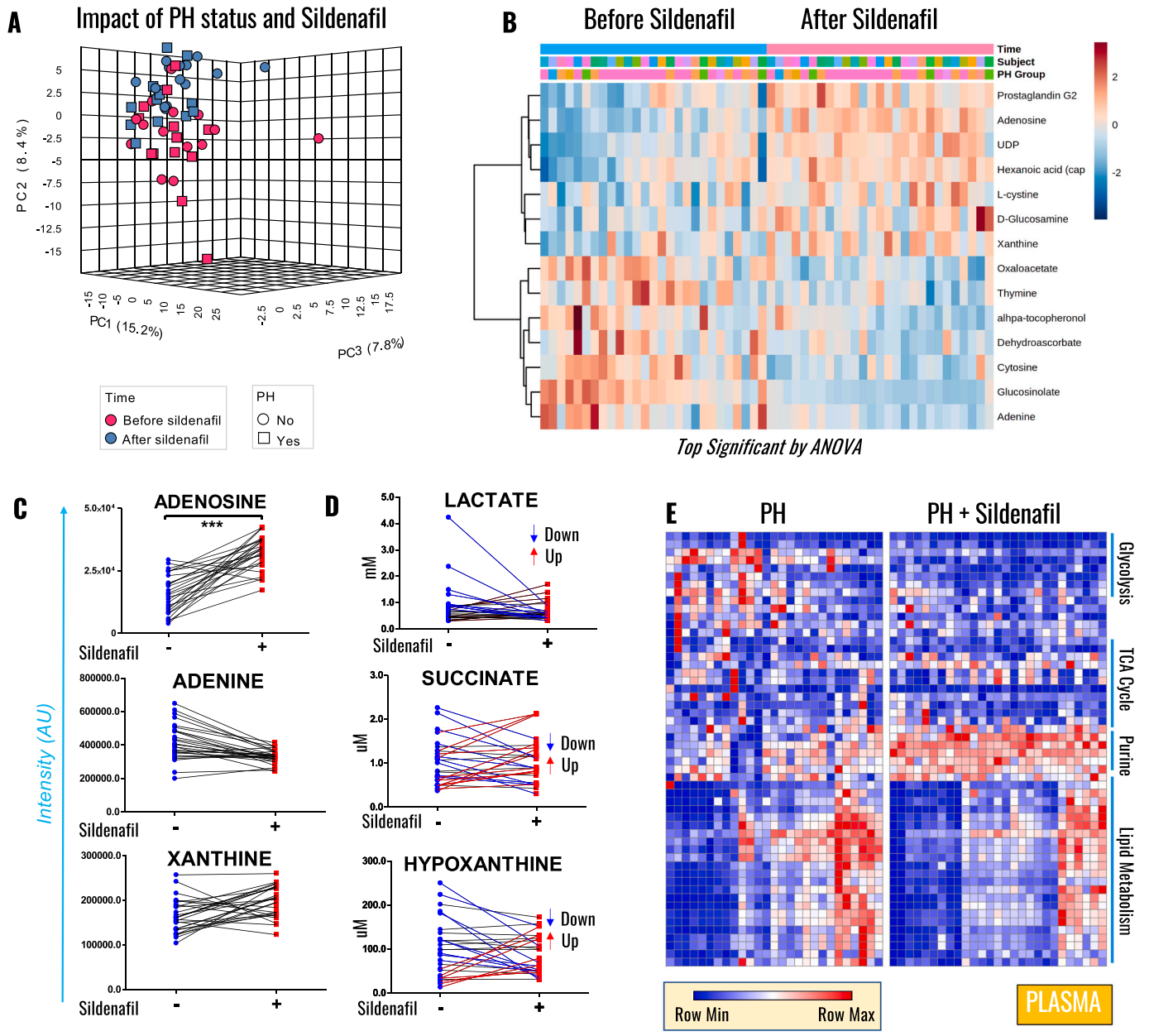

Fig. 1. Metabolomics analysis of plasma from 27 PH patients, before and after treatment with sildenafil. Sildenafil partially reverses plasma metabolic changes altered by PH as shown by the principal component analysis in A. Hierarchical clustering of the most significant metabolites by repeated measures ANOVA (B) shows a significant impact of sildenafil on the plasma levels of adenosine and several purines (C). Of note, mixed effects were observed in the population with respect to circulating markers of hypoxia, lactate, succinate and hypoxanthine (D). The heat map in E provides a more comprehensive of the impact (or lack thereof) of sildenafil on specific pathways, in particular lipid metabolism.

the KEGG pathway database and an in-house validated standard library (>800 compounds; SIGMA Aldrich; IROATech). Integrated peak areas were exported into Excel (Microsoft, Redmond, CA) for statistical analysis (*t*-test, ANOVA through the software GraphPad Prism, GENE E (Broad Institute) or Metaboanalyst 3.0 [33], for hierarchical clustering and partial-least square discriminant analysis (PLS-DA).

2.7. Statistical analysis

Unpaired Student t-test was used to compare two groups. For >2 groups with 1 variable, 1-way ANOVA was performed and for >2 groups with 2 independent variables, 2-way ANOVA followed by Tukey's multiple comparisons test was performed. The Kolmogorov- Smirnov, Shapiro-Wilk, and D'Agostino tests were used to assess for normality before applying parametric statistical tests. Nonparametric test was performed if data did not pass the parametric assumption. Statistical analysis for metabolomics analyses, including hierarchical clustering and partial-least square discriminant analysis (PLS-DA), were performed using GraphPad Prism, GENE E (Broad Institute), or Metaboanalyst 5.0. Data are presented as mean ± SEM. Differences with p values <0.05 were considered statistically significant.

3. Results

3.1. Sildenafil treatment in PH patients impacts plasma levels of purines with mixed effects on other metabolic pathways

Sildenafil was developed to mimic the purine ring of cGMP and thus inhibit the main enzyme that catalyzes its degradation, phosphodiesterase E5 (PDE5) [34]. While metabolic reprogramming is a hallmark of PH and sildenafil treatment is a mainstay in PH treatment, little is known about the impact of sildenafil on the plasma metabolome of PH patients in vivo. To bridge this knowledge gap, we evaluated the plasma metabolome of 27 patients with PH (including the patients with idiopathic pulmonary arterial hypertension (IPAH) and sickle cell PH), before and after treatment with sildenafil using UHPLC-MS (Fig. 1, Supplementary Fig. 1 and Supplementary Table 1).

Treatment with sildenafil had a significant effect on the plasma metabolome, as shown by the principal component analysis in Fig. 1. A. Significant effects were noted in the circulating levels of purines – including adenosine, adenine, and xanthine (Fig. 1. C) as well as several oxidant stress-related metabolites (prostaglandins, cystine, alpha-tocopherol, dehydroascorbate, glucosinolate – Fig. 1. B). However, circulating markers related to glycolysis/hypoxia including lactate, succinate and hypoxanthine were decreased only in a subset of patients following treatment with sildenafil (Fig. 1. D). Importantly, neither the carboxylic acid (TCA cycle) nor lipid metabolism was affected by sildenafil treatment (heat map in Fig. 1. E).

Clinical variables like 6-min walk distance (6MWD), body mass index (BMI), and age were recorded before and after sildenafil treatment and used to identify poor vs good responders to the treatment (Fig. 2. A). No correlation was observed between changes in 6MWD and circulating levels of sildenafil and its metabolite desmethylsildenafil, or the ratios of desmethylsildenafil/sildenafil (*data not shown*). BMI exhibited a significant negative correlation to 6MWD independently of sildenafil treatment and purine levels, especially adenosine and urate (Fig. 2. B). Improvements in 6MWD were positively associated with decreases in carboxylic acids (alpha-ketoglutarate), sulfur metabolites (taurine) and increases in arginine metabolism (arginine, guanidinoacetate - Fig. 2. A) and fatty acyl-carnitines. Patients' age had a significant negative correlation with 6MWD (Fig. 2. A) and plasma adenosine levels (Fig. 2. C).

Of note, we noticed divergent responses across the subjects enrolled in this study, with sildenafil either decreasing or increasing the circulating levels of metabolites, which may explain the diversity of responses of patients to sildenafil.

3.2. Metabolomics analyses reveal the up-regulation of purines and purine de novo synthesis in PH-Fibs cultured under normoxic conditions

To explore the role of purine metabolisms in the hyperproliferative state of vascular cells, human pulmonary artery fibroblasts derived from patients with IPAH (PH-Fibs) or from control donors (CO-Fibs) were used as in vitro model system. We have previously reported that these PH-Fibs, cultured under normoxic conditions, exhibit an imprinted proliferative and reprogrammed metabolic phenotype [20,23,35–37]. Herein we sought to examine the state of intracellular purine metabolism, especially de novo purine synthesis in CO- and PH-Fibs.

UHPLC-MS metabolomics revealed that PH-Fibs exhibited significant up-regulation of purines in comparison to corresponding controls as shown by the heatmap (Fig. 3. A). Purine levels successfully informed

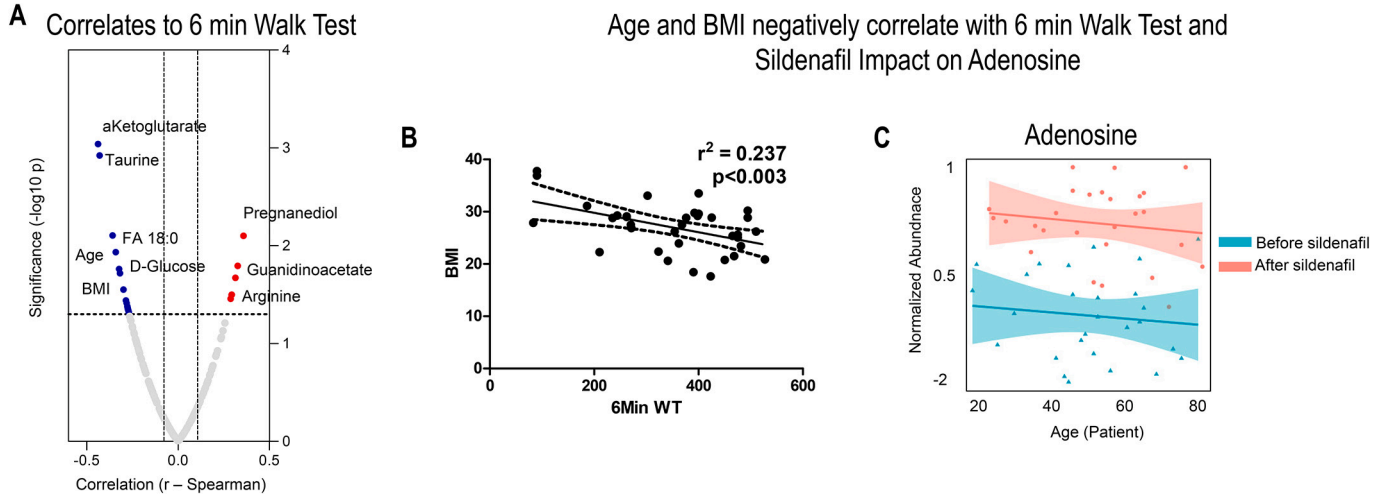

Fig. 2. Functional clinical measurements including 6-min walk test to determine the efficacy of the treatment with sildenafil were performed in 27 PH patients, before and after treatment with sildenafil. Top metabolic correlates to 6MWD included multiple metabolites in fatty acid and arginine metabolism, lipid oxidation products (A). However, patients' BMI and age were identified as critical covariates impacting such measurements and factors. Patients' BMI exhibited a significant negative correlation to 6MWD independently of sildenafil treatment and purine levels in B. Patients' age at least in part impacting the effect of sildenafil on purine metabolism (e.g., adenosine as a function of sildenafil treatment) in C. Adenosine/urate ranked among the top correlates to BMI independently from sildenafil treatment in Supplementary Table 1).

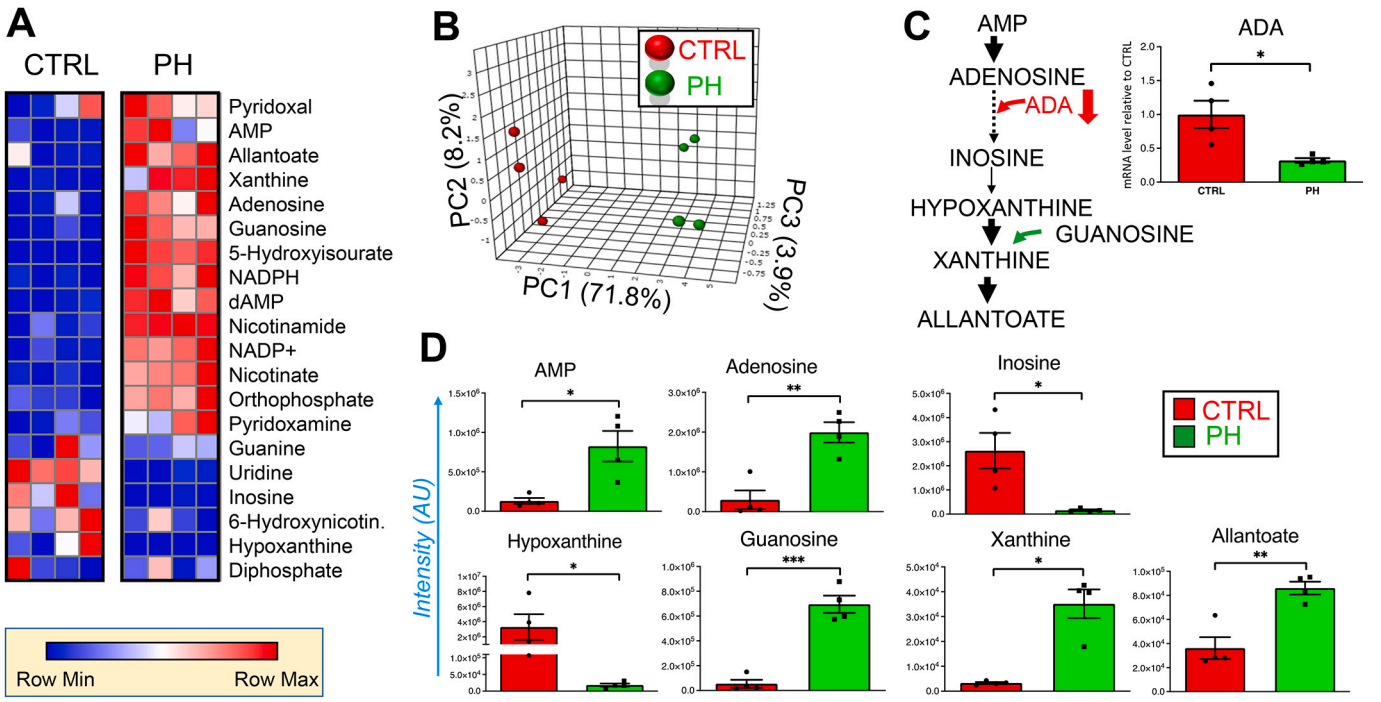

Fig. 3. PH-Fibs exhibit upregulation of purine metabolism. Deranged purine metabolism in fibroblasts derived from the pulmonary artery of health donors (CTRL) and PH patients (PH), as represented by heat maps (blue to red = low to high levels of Z score normalized metabolite abundances, $n = 4$) in (A). PLS-DA of CO-Fibs vs PH-Fibs is shown in B, based on purine metabolite levels. In C, an overview of purine metabolism and oxidation cascades. Metabolites from this pathway are highlighted in D. (*, **, *** $p < 0.05$, 0.01, 0.001, respectively; n = 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clustering of CO-Fibs vs PH-Fibs through PLS-DA analysis with PC1 explaining 71.8% of the total variance across samples (Fig. 3. B). An overview of purine metabolism and intermediates showing the most statistically significant changes between the two groups is provided in Fig. 3. C and D, respectively. We observed that PH-Fibs exhibited the elevated levels of nucleotides (e.g., AMP) and nucleosides (e.g., adenosine). Of note, significant decreases in the transcripts for adenosine deaminase (ADA) in PH-Fibs in comparison to CO-Fibs was accompanied by significant decreases in the levels of inosine (the byproduct of the ADA enzyme) and inosine-derived hypoxanthine in PH-Fibs. On the other hand, guanosine and downstream purine catabolism/oxidation byproducts xanthine and allantoate were increased significantly in PH-Fibs compared to CO-Fibs as illustrated by purine metabolism and oxidation cascades and purine metabolism intermediates (Fig. 3. C, D).

Significant increases in the steady state levels of purine nucleotides and nucleosides are suggestive of either decreased catabolism (e.g., by ADA) and/or increased anabolism, a necessary requirement for the biosynthesis of DNA and RNA in highly proliferating cells. Metabolic tracing experiments upon incubation of cells with U-^{13}C-glucose for 24 h were performed to test whether PH-Fibs engaged in increased nucleoside biosynthesis. An overview of the expected isotopologue distributions is presented in Fig. 4. A. Increased serine biosynthesis was observed, along with increased activation of the PPP (either at the oxidative or non-oxidative branch level) which resulted in the generation of $^{13}C_5$-ribose phosphate (Fig. 4. B). Since both pathways fuel de novo nucleoside synthesis (according to the scheme in Fig. 4. A), we identified significantly higher adenosine synthesis from both ribose and serine in PH-Fibs at 24 h from incubation with U-^{13}C-glucose (Fig. 4. B).

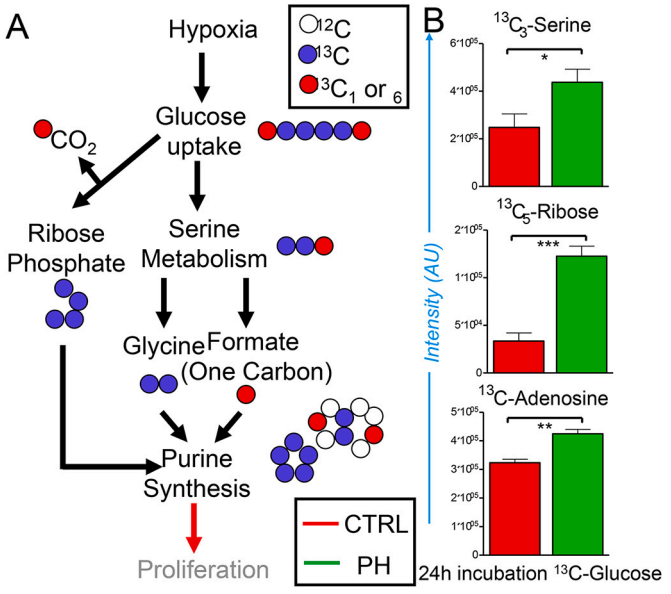

Fig. 4. Metabolic tracing of U-13C-glucose for 24 h reveals incorporation of heavy carbon atoms in de novo synthesized purines through activation of the pentose phosphate pathway (PPP) and serine metabolism in PH-Fibs. An overview of the expected isotopologue distributions is presented in A. Increased serine biosynthesis was observed, along with increased generation of 13C5-ribose phosphate and higher adenosine synthesis in PH-Fibs at 24 h from incubation with U-13C-glucose in B. (*, **, *** *t*-test $p < 0.05$, 0.01, 0.001, respectively; $n = 3$).

3.3. *Effects of sildenafil on metabolism and phenotype of PH-Fibs*

Next, we sought to determine the effect of sildenafil on the metabolic and proliferative state of human PH-Fibs. We treated PH-Fibs with Sildenafil (50 nM) and performed metabolomics analyses and proliferation assay. UHPLC-MS metabolomics revealed that sildenafil was insufficient to induce significant changes in the cellular levels of lactate and carboxylates including alpha-ketoglutarate, fumarate, and malate (Fig. 5.

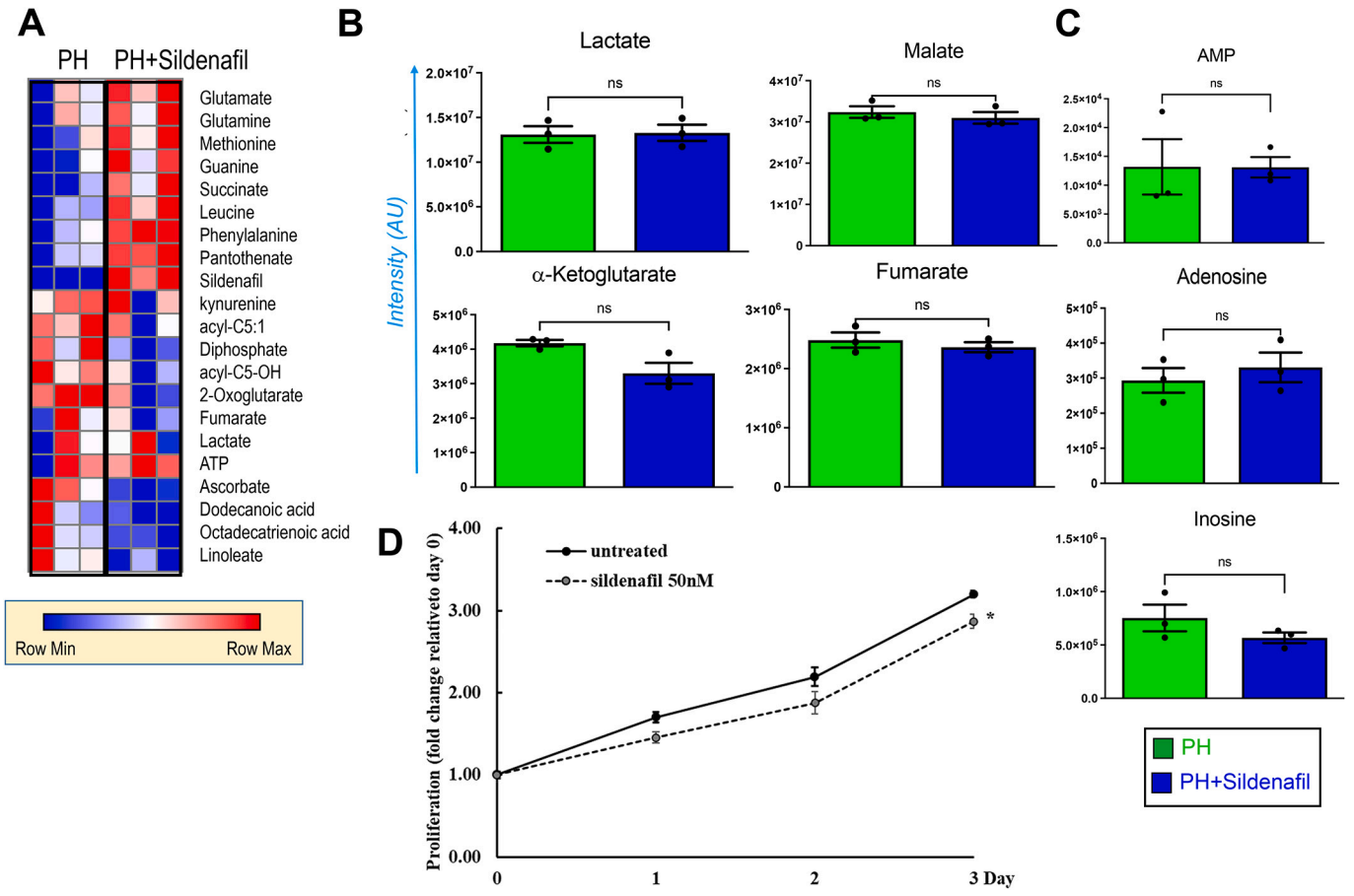

Fig. 5. Sildenafil was insufficient to correct metabolic and proliferative state in PH-Fibs. Metabolic analysis revealed that treatment with sildenafil did not decrease lactate generation and Krebs cycle aberrations as represented by heat maps (A) and plots (B), as well as purine metabolites (C) in PH-Fibs. ($n = 3$). (D) Proliferation assay was performed with CyQuant Kit in PH-Fibs with or without treatment of sildenafil (50 nM) up to 3 days (n = 3). Results were calculated as fold changes relative to day 0. *$p < 0.05$ compared to untreated.

A, B), which are the metabolites that have been proven significantly increased in PH-Fibs [20,23,36]. Further, we observed that sildenafil didn't significantly impact the levels the purine metabolites such as AMP, adenosine and inosine in PH-Fibs (Fig. 5. C). Importantly, we found sildenafil exhibited modest effect on inhibiting the proliferation of PH-Fibs (Fig. 5. D).

3.4. Promoting PKM activity through pharmacological treatment with TEPP-46 or HDACi decreases the subtracts of purine de novo synthesis (serine and ribose) and purines in PH-Fibs

Given the non-significant effect of sildenafil on the metabolism of PH-Fibs, then we have a reason for looking at other pathways. Next, we sought to examine whether suppression of serine metabolism and the PPP is sufficient to decrease purine synthesis. In other pathologies characterized by highly proliferative states such as cancer, increased serine biosynthesis is sustained by isoform switching of PKM and the up-regulation of the PKM2/PKM1 ratio [38]. Increases in this ratio result in total decreases in PKM activity and promote a metabolic bottleneck in late glycolysis fueling serine anabolism [39,40], which is required for purine synthesis as shown in Fig. 4.A. Recently, we noted that PH-Fibs undergo a similar transition towards up-regulation of the PKM2/PKM1 ratio by decreasing PKM1 expression, paralleled with glycolytic reprogramming, mitochondrial abnormalities and activation of serine metabolism and PPP [23,41]. Furthermore, we have shown that normalization of PKM activity by both PKM2 activator (TEPP-46) and HDAC inhibitors (HDACi) reverses the PH-Fibs phenotype by limiting glycolysis and promoting the normalization of mitochondrial metabolism [23]. Since purine synthesis requires ribose-5-phosphate from the PPP and formate from the serine synthesis pathway, here we hypothesized that normalization of PKM activity in PH-Fibs would limit purine biosynthesis by normalizing serine anabolism and PPP (Fig. 6. A), a prediction that we sought to validate through UHPLC-MS. We observed that both TEPP-46 (Fig. 6. B) and the HDACi SAHA and/or Apicidin (Fig. 6. C) decreased serine, ribose phosphate, and thus AMP significantly in PH-Fibs.

3.5. HDACi and sildenafil synergize to correct metabolic reprogramming and inhibit PH-Fibs proliferation

To further explore the potential treatment strategy of using an HDACi in combination with sildenafil to target both vasoconstriction and vascular remodeling, we treated PH-Fibs with sildenafil alone (50 nM), HDACi alone (Apicidin, 3 uM) or in combination. Metabolomics analyses revealed that the combination of Apicidin and sildenafil was more effective than either treatment alone in correcting the metabolic derangements of PH-Fibs (Fig. 7. A), especially with respect to hypoxic markers of carboxylates (two critical metabolites that contribute to the stabilization and regulate the degradation of hypoxia-inducible factor 1alpha – alpha-ketoglutarate and 2-hydroxyglutarate [42,43], the substrates of purine de novo synthesis (serine ($p = 0.069$) and ribose) and purine nucleotides (the building blocks of RNA and DNA, ATP and ADP) (Fig. 7. B). More importantly, we found that the combination of Apicidin and sildenafil suppressed the proliferation of PH-Fibs more effectively than either treatment alone (Fig. 7. C). It should be noted that Apicidin had a stronger effect on the inhibition of proliferation than sildenafil.

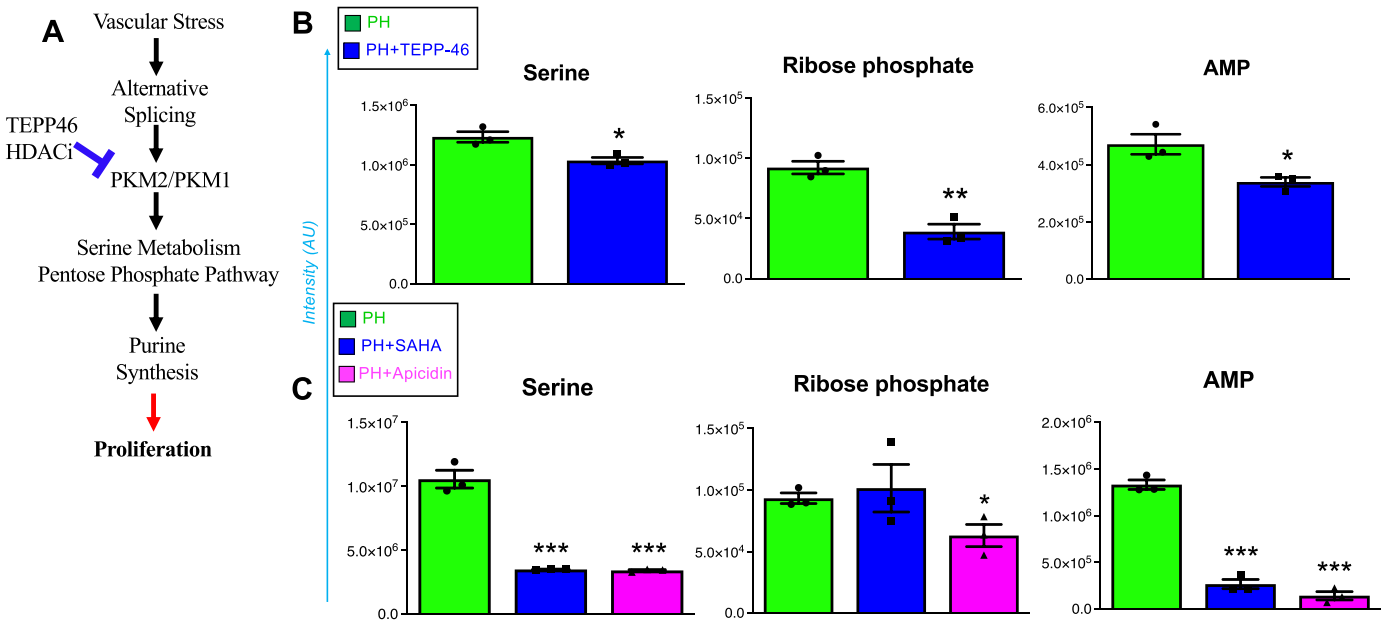

Fig. 6. TEEP-46 and HDACi decreased upstream to purine synthesis via inhibiting serine metabolism and pentose phosphate pathway (PPP) in PH-Fibs. The impaired PKM activity resulting from increased PKM2/PKM1 ratio can be rescued by TEPP46 and HDACi treatment (A). TEPP-46 (B) and HDACi (C) decreased the levels of serine, ribose phosphate and AMP in PH-Fibs. *, **, *** $p < 0.05$, $p < 0.01$, 0.001, compared to untreated PH-Fibs, n = 3).

4. Discussion

Advancements in our understanding of the etiological factors contributing to PH have fostered the introduction of drugs that specifically target PA vasoconstriction and potentially pathological vascular remodeling. Among these treatments, sildenafil showed significant promise as a strategy to inhibit PDE5 and thus prevent the degradation of cGMP, which in turn prolongs the smooth muscle relaxation mediated by NO production [34] and improves symptoms and hemodynamics in patients with symptomatic PAH [12]. However, conflicting data from currently available clinical trials showed general safety but overall inconsistent efficacy across patients, with some patients responding better than others [9]. These inconsistencies may be in part explained by considerations of sildenafil metabolism, which occurs primarily in the liver, where cytochrome P450 enzymes yield one active metabolite with a potency of approximately 50% of the parent drug. Sildenafil metabolism exerts its efficacy in some categories of patients, such as in patients older than 65, with creatinine clearance <30, and with hepatic cirrhosis, which are all characterized by reduced clearance of sildenafil [12,44]. Here, we performed metabolomics analyses on a clinical cohort of PH patients undergoing treatment with sildenafil. Results indicate that sildenafil alone only partially reversed the plasma metabolic derangements, which are commonly observed in PH patients [45,46]. Clinical (6MWD) and metabolic responses to sildenafil were found to be impacted by patients' age and BMI, suggesting that these patient characteristics may be leveraged in the future for targeted therapy in combination with sildenafil. On the other hand, circulating levels of sildenafil or desmethylsildenafil/sildenafil ratio – a marker of sildenafil metabolism by cytochrome P450 – were not predictive of in vivo responses in our clinical cohort.

Metabolic reprogramming of vascular cells is a critical hallmark of PH and is recognized as a potential target for PH treatment [19]. Hyperproliferative phenotypes of endothelial cells, smooth muscle cells, and fibroblasts have been associated with a Warburg-like metabolic reprogramming towards aerobic glycolysis in animal models or patients with PH [20,36]. Pulmonary artery fibroblasts play a critical role in PA pathological remodeling, however little is known about the effects of sildenafil on these cells. In the present study, we used human PA fibroblasts as an in vitro model to explore the effect of sildenafil on the metabolic reprogramming and the effect of combined treatment of sildenafil with HDACi. Using UHPLC-MS metabolomics and 13C tracing experiments, we documented that incomplete glucose oxidation in PH-Fibs feeds into purine nucleoside synthesis via serine synthesis and activation of PPP, which is similar to cancer cells [39]. Supportively, it was reported that the genetic abnormalities of the rate-limiting enzyme of the PPP, glucose 6-phosphate dehydrogenase, are protective against PH in sickle cell disease patients [47]. On the other hand, sildenafil is insufficient to reverse the incomplete glucose oxidation and the subsequently elevated purine synthesis which may partially be responsible for the inconsistencies of sildenafil's effect on PH patients.

The molecular structure of sildenafil is similar to that of cGMP, suggesting that as a purine compound, it may, through yet unexplored mechanisms, directly interact and/or be a cofactor for intracellular purine-converting enzymes involved in intracellular purine metabolism. In addition, intracellular cGMP is a second messenger, involved in the inhibition of proliferation in several cell types including SMC, endothelial, and tumor cells, suggesting its potential anti-proliferative effects in vascular cells [48,49]. Of note, we observed an increased guanine level in PH-Fibs after sildenafil treatment. The physiological role of increased intracellular guanine in vascular cells, except its function as a canonical nucleotide base in DNA and RNA, remains largely unknown. However, its ribosylated product, guanosine, has been shown to exhibit a protective role against neuroinflammation and oxidative stress and to exert trophic effects in neuronal and glial cells [50]. Moreover, guanine nucleotides, as GDP and GTP contribute to protein biosynthesis and signal transduction, also playing a role in cancer cell energy metabolism [51–53]. It cannot be excluded that in adventitial fibroblasts, de novo-produced GTP may serve as a substrate for guanylate cyclase-mediated cGMP production and thereby contribute to the suppression of cell proliferation.

Serine biosynthesis in cancer has been linked to increased purine synthesis, a phenomenon promoted by decreased PKM activity owing to increases in the ratios of PKM isoforms 2 to 1, the former being constitutively less active than the latter [38]. PKM activity is also allosterically regulated by early glycolytic intermediates (e.g., fructose 1,6-bisphosphate) and serine levels [40]. We previously proved that decreases in

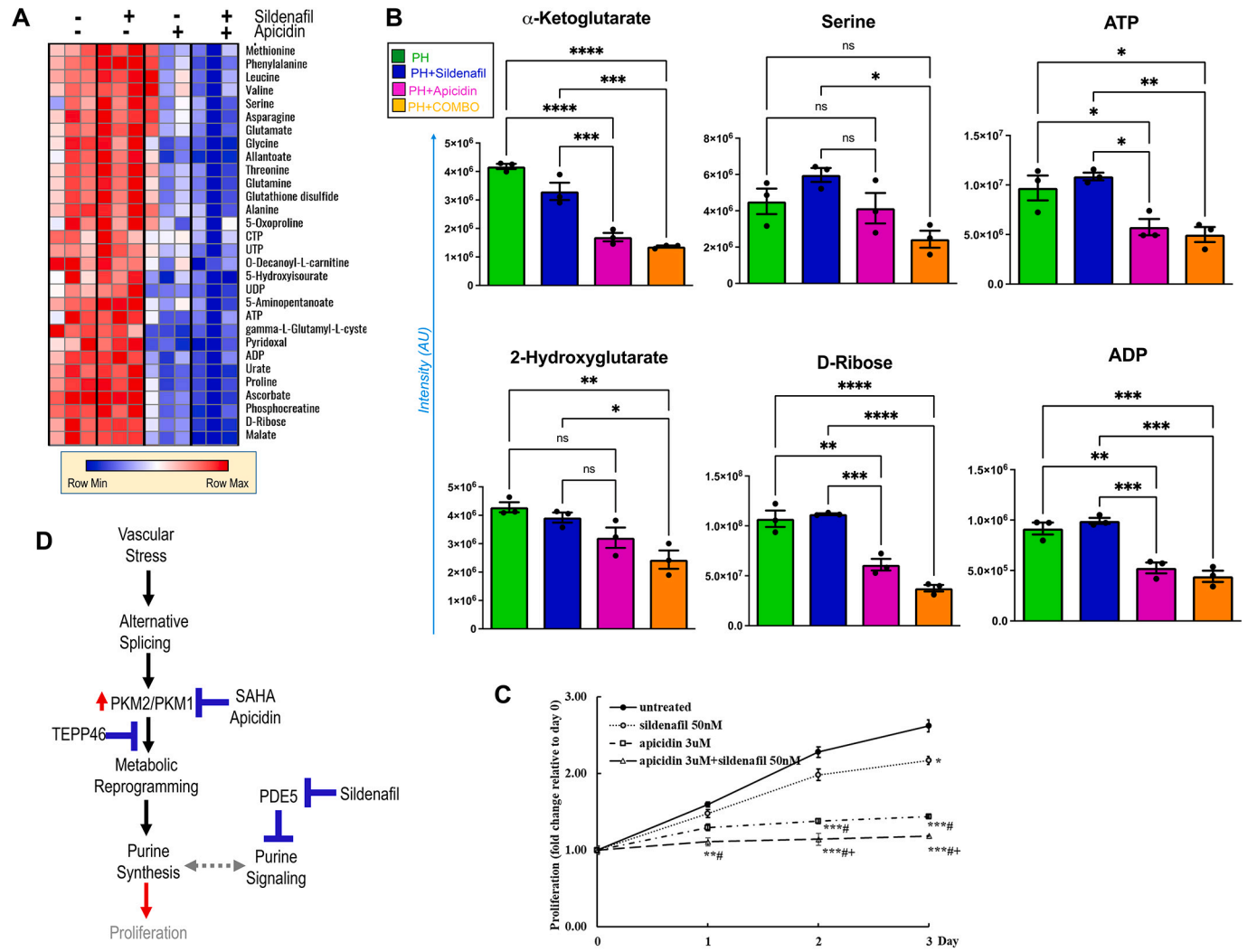

Fig. 7. Combined treatment with sildenafil and Apicidin is more effective than either treatment alone in correcting metabolism and arresting PH-Fibs proliferation. This effect was revealed by metabolic analysis in heatmap (A) and representative plots for metabolites associated with hypoxic metabolic reprogramming in the Krebs cycle (alpha-ketoglutarate and 2-hydroxyglutarate), the substrates of purine de novo synthesis (serine and ribose) and purine nucleotides (ATP and ADP) in (B), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 as indicated. (C), Proliferation assay was performed with CyQuant Kit in PH-Fibs with different treatment (sildenafil 50 nM or Apicidin 3uM, alone or the in combination) or left untreated up to 3 days (n = 3). Results were calculated as fold changes relative to day 0, *p < 0.05, **p < 0.01, ***p < 0.001 compared to untreated; #p < 0.05, compared to sildenafil; +p < 0.05, compared to Apicidin. (D), overview of the proposed strategies to target dysregulated purine metabolism and signaling in PH.

PKM1 expression in PH-Fibs underlie the increases in PKM2/PKM1 ratios and thus decreased PKM activity and contribute to the Warburg-like glycolytic phenotype of PH-Fibs. The pharmacological intervention with TEPP-46 treatment resulting in PKM tetramerization and increasing its activity and HDACi treatment resulting in decreasing the PKM2/PKM1 ratio (through miR-124/PTBP1/PKM axis by regulating PKM isoform alternative splicing), can reverse glycolytic reprogramming and highly proliferative phenotypes in these cells [23]. Here we show that serine, PPP, and purine levels are normalized in PH-Fibs treated with TEPP-46 and HDACi which both promote PKM activity. Furthermore, we found that combined treatment with HDACi (Apicidin) and sildenafil showed the synergistic effects on normalization of metabolism and inhibition of proliferation of PH-Fibs, paving the way for future studies on animal models and clinical trials exploiting combination therapeutic approaches.

In conclusion, HDAC inhibitors, alone or in combination with sildenafil, may represent a viable therapeutic strategy to target deranged (purine) metabolism in PH by influencing PKM activity (Fig. 7. D). The combined inhibitory effect of HDACi and sildenafil on pulmonary artery fibroblast proliferation reflect the existence of complex regulatory mechanisms that include coordinated action of epigenetic, signaling, and metabolic inputs. Further studies are necessary to evaluate the exact molecular mechanisms underlying the beneficial effect of sildenafil in combination with HDACi for the treatment of PAH.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2023.107157.

Author contributions

Hui Zhang, Angelo D'Alessandro and Kurt R. Stenmark contributed to the experimental design, results explanation, and manuscript preparation. Hui Zhang, Angelo D'Alessandro, Min Li, Julie A. Reisz and Suzette Riddle contributed to performed experiments, prepared samples and data analysis. Akshay Muralidhar and Todd Bull contributed to patients' data and blood sample collection. Lan Zhao and Evgenia Gerasimovskaya contributed to manuscript preparation.

Declaration of Competing Interest

AD is a founder of Omix Technologies Inc and Altis Biosciences LLC.

AD is a scientific Advisory Board member for Hemanext Inc and Macopharma Inc. All other authors disclose no conflicts of interest relevant to this study.

Data availability

Data will be made available on request.

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