PULMONARY ARTERIAL HYPERTENSION

Inhibition of pyruvate dehydrogenase kinase improves pulmonary arterial hypertension in genetically susceptible patients

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Pulmonary arterial hypertension (PAH) is a progressive vascular disease with a high mortality rate. It is characterized by an occlusive vascular remodeling due to a pro-proliferative and anti-apoptotic environment in the wall of resistance pulmonary arteries (PAs). Proliferating cells exhibit a cancer-like metabolic switch where mitochondrial glucose oxidation is suppressed, whereas glycolysis is up-regulated as the major source of adenosine triphosphate production. This multifactorial mitochondrial suppression leads to inhibition of apoptosis and downstream signaling promoting proliferation. We report an increase in pyruvate dehydrogenase kinase (PDK), an inhibitor of the mitochondrial enzyme pyruvate dehydrogenase (PDH, the gatekeeping enzyme of glucose oxidation) in the PAs of human PAH compared to healthy lungs. Treatment of explanted human PAH lungs with the PDK inhibitor dichloroacetate (DCA) ex vivo activated PDH and increased mitochondrial glucose oxidation in the PAs of human PAH compared to healthy lungs. In a 4-month, open-label study, DCA (3 to 6.25 mg/kg b.i.d.) administered to patients with idiopathic PAH (iPAH) already on approved iPAH therapies led to reduction in mean PA pressure and pulmonary vascular resistance and improvement in functional capacity, but with a range of individual responses. Lack of ex vivo and clinical response was associated with the presence of functional variants of SIRT3 and UCP2 that predict reduced protein function. Impaired function of these proteins causes PDK-independent mitochondrial suppression and pulmonary hypertension in mice. This first-in-human trial of a mitochondria-targeting drug in iPAH demonstrates that PDK is a druggable target and offers hemodynamic improvement in genetically susceptible patients, paving the way for novel precision medicine approaches in this disease.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a complex vascular disease leading to right ventricular (RV) failure and death from a proliferative vascular remodeling that obstructs the lumen of resistance pulmonary arteries (PAs) (1). Approved therapies (phosphodiesterase-5 inhibitors, endothelin receptor antagonists, soluble guanylate cyclase stimulators, and prostanoids) act mainly as vasodilators and improve symptoms but fail to arrest or reverse the disease (2). A cancer-like metabolic remodeling drives, at least in part, the structural changes in the pulmonary vascular wall. Specifically, a suppression of mitochondrial glucose oxidation in all cellular elements of the PAs [namely, PA smooth muscle cells (PASMCs), endothelial cells, and fibroblasts] leads to inhibition of mitochondria-dependent apoptosis and secondary up-regulation of glycolysis promoting pro-proliferative signaling and direction of carbon sources toward cellular building blocks rather than oxidation (3–9). Central to this is the inhibition of pyruvate dehydrogenase (PDH), the gatekeeping enzyme of glucose oxidation, which catalyzes the mitochondrial production of acetyl-coenzyme A from pyruvate, the end product of glycolysis, which in turn feeds the Krebs cycle to complete glucose oxidation.

Several potential mechanisms of PDH inhibition have been described. Induction of PDH kinase (PDK) leads to its complexing with, and tonic inhibition of, mitochondrial PDH by phosphorylation at serine-293. Whereas in healthy tissues PDK expression is low, the enzyme can be induced selectively in the pulmonary circulation by activation, even under normoxia, of hypoxia-inducible factor 1α (HIF1α), which is up-regulated in PAH (10). PDH can also be inhibited by tyrosine kinase–mediated phosphorylation at tyrosine-301 (11). Another mechanism is through inhibition of sirtuin 3 (SIRT3), the main mitochondrial deacetylase, which activates several mitochondrial enzymes including PDH; Sirt3 knockout mice develop spontaneous pulmonary hypertension, and SIRT3 activity is decreased in human PAH lungs (12). Last, inhibition of uncoupling protein 2 (UCP2), a mitochondrial protein that regulates calcium entry, leads to a decrease in mitochondrial calcium (13, 14). Many mitochondrial enzymes including PDH are calcium-dependent, and Ucp2 knockout mice develop spontaneous pulmonary hypertension (13). Thus, deficiency in SIRT3 and/or UCP2 inhibits PDH in a PDK-independent manner.

Two relatively common single-nucleotide polymorphisms (SNPs), rs11246020 for SIRT3 and rs659366 for UCP2, have been reported to decrease the activity and expression of these proteins, respectively (16–18). Both gene variants are relatively frequent in the general population and have been associated with metabolic syndrome in humans (16, 17, 19), which is highly prevalent in PAH patients (20).

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PDK is a druggable target that has been effectively inhibited by the small molecule dichloroacetate (DCA) in animal models and early-phase trials in cancer (21–25). DCA has also been clinically tested, with a good safety profile, in patients with congenital mitochondrial diseases, including PDH deficiency (26–29). Thus, we explored the role of PDK and DCA in human PAH with a series of experiments using archived human lungs, PAH lungs studied ex vivo immediately after removal at transplant surgery [ex vivo lung perfusion (EVLP)] and an explorative open-label phase 1 clinical trial in idiopathic PAH (iPAH) patients. We also explored whether the presence of loss-of-function gene variants for SIRT3 and UCP2 provides resistance to the ability of DCA to activate PDH and reverse mitochondrial suppression in human PAH.

RESULTS
PDK expression is increased in the PAs of human PAH lungs
We measured the expression of the two ubiquitously expressed PDK isoforms (1 and 2) (30) and PDH (Fig. 1A) in lung tissues from 10 patients (6 with PAH and 4 non-PAH controls) (Table 1) by confocal microscopy and immunoblotting. PDK protein was markedly increased in the wall of small muscularized PAs from PAH compared to non-PAH lungs (Fig. 1B and fig. S1). This up-regulation was strong in PASMCs co-stained with smooth muscle actin antibody but was also apparent throughout the vessel wall in other PA elements (for example, in endothelial cells, fibroblasts, and tissue inflammatory cells). This is consistent with animal studies that show that these cell types in PAH undergo a...
metabolic remodeling (suppressed glucose oxidation and up-regulated glycolysis) (7, 8) similar to that of PASMC (4, 5). Immunoblot from tissue samples taken from the periphery of lungs, which are enriched with small PAs, confirmed the up-regulation of both PDK isoforms in PAH (Fig. 1C). This was associated with phosphorylation of serine-293 on the PDH-E1α subunit, marking PDH inhibition. We concluded that PDK up-regulation is an active component of metabolic remodeling in human PAH and that the selectivity of this up-regulation to diseased tissues makes PDK a potential therapeutic target for PAH.

DCA increases PDH activity and mitochondrial function in human PAH lungs ex vivo
We then studied mitochondrial function in five human lungs (Table 1) explanted at transplant surgery using EVLP under conditions (pH, temperature, perfusion, and ventilation) that best mimic the in vivo environment. The lungs were perfused and ventilated upon removal (Fig. 2A), and biopsies were taken from peripheral sites of each lung, which are enriched with resistance PAs, as soon as baseline conditions were established at 37°C. After 1 hour of perfusion with DCA at a concentration similar to the trough concentrations reported in patients on oral DCA therapy, additional biopsies were taken and pre-post comparisons were made. PDH activity was measured with two standard techniques: a biochemical dipstick assay based on the PDH-driven production of NADH (reduced form of nicotinamide adenine dinucleotide) (Fig. 2B) and immunobLOTS measuring serine-293 PDH-E1α phosphorylation (Fig. 2C). We also measured mitochondrial respiration in these tissues using a Seahorse protocol (Fig. 2D). Variability in the response to DCA was observed. Lungs 1, 2, and 3 showed an increase in PDH activity and mitochondrial respiration, whereas lung 4 was resistant to DCA and showed a small decrease in respiration during DCA perfusion. Lung 5 (control) was perfused with vehicle and, like lung 4, exhibited no change in PDH activity and a small decrease in respiration during the same perfusion period. We speculated that non-PDK–dependent mechanisms, such as the presence of reduced SIRT3 and/or UCP2 activity, may impair the response to DCA.

Hemodynamics and functional capacity are improved in iPAH patients after chronic oral DCA administration
To investigate the therapeutic potential of PDK inhibition with DCA in vivo, a proof-of-concept 4-month, dose-finding trial of 3, 6.25, or 12.5 mg/kg by mouth twice daily [bis in die (b.i.d.)] was conducted in patients clinically stable on treatment with approved iPAH therapies (ClinicalTrials.gov NCT01083524). Of the 23 patients screened, 20 satisfied the inclusion/exclusion criteria and were enrolled in the study (Table 2). All patients were on sildenafil, 13 on dual therapy (sildenafil + endothelin receptor antagonist), and 1 on triple therapy (sildenafil, endothelin receptor antagonist, and parenteral prostanoid) in addition to background therapies. No patient had initiated PAH-approved therapies for a minimum period of 6 months before enrollment, and maintenance dose was stable during this time except for patient 12 (Table 2), who had an increase in the dose of an endothelial receptor antagonist 4.4 months before enrollment. This patient, like all others, was stable with no signs of clinical improvement or worsening of disease for at least 8 weeks before enrollment (an eligibility requirement). There was no clinically significant change in the QT interval of the electrocardiogram, cardiac rhythm, liver, bone marrow, or renal function. Dose-limiting toxicity, specifically paresthesia affecting the dorsum of the foot, toes, and fingers (grade II peripheral neuropathy),
consistent with a previously described reversible and dose-dependent, nondemyelinating peripheral neuropathy (26, 28), developed in all five patients taking the highest dose tested (12.5 mg/kg b.i.d.). Four patients withdrew from the study at that point (weeks 3 to 11), and one accepted a protocol-driven decrease in the dose to 6.25 mg/kg b.i.d., with improvement of symptoms within 1 to 3 months in all patients. Thus, all patients completing the protocol (n = 16) were taking 3 or 6.25 mg/kg b.i.d., and 6.25 mg/kg b.i.d. was established as the highest tolerated dose, consistent with previous studies in patients with genetic mitochondrial diseases and cancer (23–26). No patient deteriorated clinically or exhibited a decrease in the 6-min walk of more than 10% from baseline, and no patient required hospitalization during the study. There were no changes in PAH-approved and background therapies during the study.

Exposure to DCA led to an improvement in hemodynamic measurements at right heart catheterization [mean PA pressure (mPAP, P < 0.05), pulmonary vascular resistance (PVR, P < 0.05)] and functional capacity (6-min walk test, P < 0.05) in the cohort as a whole, but interindividual variability in response was apparent (Fig. 3A). Seven patients responded to DCA with a decrease in mPAP greater than 5 mmHg (mean decrease, 9.1 mmHg) and an increase or no change in cardiac output (highlighted box in Fig. 3B); mean PVR decreased by 118 ± 32 dynes·s·cm⁻⁵, and 6-min walk increased by 53 ± 18 m in this group. Considering that these patients were already treated with PAH therapies, this response is clinically meaningful and of a magnitude comparable to approved PAH therapies, which have shown a mean decrease in mPAP of less than 5 mmHg in landmark trials with previously untreated patients [epoprostenol, −4.5 mmHg (31); bosentan, −1.6 mmHg (32); sildenafil, −2.1 mmHg (33)].

To better understand the variation in response, we first looked at plasma DCA concentrations (Fig. 4). Mean trough concentration in both the 3 mg/kg b.i.d. (0.17 mM) and 6.25 mg/kg b.i.d. (0.59 mM) treatment groups demonstrated adequate exposure of PDK to DCA [the Ki (inhibition constant) of DCA for PDK2 is 0.2 mM (30)], and there was no dose-response relationship with respect to change in mPAP or PVR.
The mean trough DCA concentration in the seven patients with a good response to DCA was 0.22 mM compared to 0.54 mM in the "non-responder" group, excluding underexposure to DCA as an explanation of the lack of response.

**Differences in the response to DCA are associated with SIRT3 and UCP2 variants**

We speculated that the lack of response to DCA in both the clinical trial and the EVLP experiments has a genetically driven biochemical basis and is associated with the presence of functional variants in SIRT3 and UCP2. As discussed earlier, the presence of these variants may cause a PDH-independent inhibition of PDH, which would not be responsive to DCA.

The SIRT3 rs11246020 "A allele" is associated with a point mutation that causes a change of valine to isoleucine at residue 208 within the conserved catalytic deacetylase domain, causing a 34% decrease in SIRT3 activity compared to the "G allele" (17). The UCP2 rs659366 G allele affects the promoter region of the gene, associated with a decrease in transcription and decreased mRNA expression compared to the A allele (18, 19). Although SIRT3 and UCP2 are both on chromosome 11, they are not in linkage disequilibrium. There is evidence for a "gene dose-response" with both variants: Sirt3−/− mice show greater inhibition of enzymatic activity and worse pulmonary hypertension compared to Sirt3−/− mice (12), and UCP2 mRNA expression is associated with the presence of the G allele, with the GG, GA, and AA alleles associated with progressively increased mRNA expression (18). Thus, we developed an SNP score where 1 point is given for each loss-of-function variant present: An SNP score of 0 means that both the variants are absent, a score of 4 denotes that both variants are present in a homozygous manner, and scores in between represent the sum of points for each allelic risk variant present (table S1). A high SNP score predicts inhibition of PDH independent of PDK and greater resistance to DCA. Lung 4, which exhibited resistance to DCA, had an SNP score of 3, whereas lungs 1 to 3, which responded to DCA, had SNP scores of 0 or 1 (Fig. 2, B to D, and Table 1).

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*Dose decreased from 12.5 mg/kg.
Although the presence of each gene variant alone was inversely but weakly associated with a decrease in mPAP (fig. S2), their combined presence (fig. S3) exhibited a stronger relationship with the change in mPAP, suggesting an additive genetically driven resistance to DCA. A similar pattern characterized the response of RV function, the major determinant of morbidity and mortality in PAH. RV ejection fraction, measured by cardiac magnetic resonance imaging (MRI), improved in patients with an SNP score of 0 (P < 0.05 compared to their baseline), whereas the patients with SNP scores of 1 had variable responses and those with higher SNP scores showed no improvement (fig. S4).

**Exploratory imaging biomarkers support the clinical response to DCA**

We used two imaging biomarkers in patient subsets to assist with biological interpretation of the response to DCA. Using gadolinium lung perfusion imaging with MRI, we found that the responders with a decrease of mPAP >5 mmHg had a ~150% increase in lung perfusion compared to the nonresponder group (a mean decrease in transit time of 0.55 versus 0.22 s, respectively; fig. S5A). We also measured parenchymal lung glucose uptake by 18fluorodeoxyglucose positron emission tomography and computed tomography (18FDG-PET-CT). Consistent with increased glycolysis, animal models and patients with PAH exhibit higher lung glucose uptake as measured by lung18FDG-PET compared to no-PAH controls (8, 34). By increasing glucose oxidation, DCA is expected to reduce glycolysis and thus glucose uptake. The hemodynamic responders showed an overall decrease in 18FDG uptake, whereas the nonresponders showed an increase (fig. S5B). Consistent with the relationship between hemodynamic response to DCA and SNP score, a low SNP score was associated with a reduction in 18FDG uptake in response to DCA, whereas a high SNP score was
In a first attempt to target a mitochondrial enzyme to treat humans with vascular disease, we report that DCA in doses up to 6.25 mg/kg po (per os) b.i.d over 4 months are generally well tolerated and can result in a significant reduction in mPAP and PVR in iPAH. PDK inhibition by DCA increases PDH activity and oxygen consumption in isolated perfused/ventilated PAH lungs, consistent with improvement in mitochondrial function.

DCA has a very specific mechanism of action: Mimicking pyruvate’s structure, it competes with pyruvate for its binding “pocket” in PDK and thus mimics the “end-product inhibition” that results from pyruvate-PDK binding (35). This has been confirmed by crystallization studies of PDK with pyruvate or DCA in situ (36). In addition, molecular (small interfering RNA–induced) inhibition of PDK2 in cancer cells mimics DCA, which does not exert any additional effects in cells lacking PDK (21). PDK2 is induced in the lungs of PAH patients, and of all the PDK isoforms, PDK2 is the most sensitive to DCA (30).

Poor DCA responders were characterized by a biochemical resistance that was associated, both in the ex vivo experiments and the clinical trial, with the presence of variants in SIRT3 and UCP2 that predict reduced protein activity. We propose that PDH inhibition in these patients is less dependent on PDK. Specifically, DCA may inhibit PDK but fail to increase PDH activity and respiration in patients in whom PDH is also inhibited by increased acetylation and/or lack of mitochondrial calcium in vascular cells due to SIRT3 and UCP2 gene variants that inhibit SIRT3 activity and are associated with decreased UCP2 expression.

Fig. 4. Trough serum DCA concentrations. Top: DCA concentration (means ± SEM) in patients exposed to 6.25 mg/kg b.i.d. compared to 3 mg/kg b.i.d. Bottom: DCA concentration (means ± SEM) in responder patients (decrease in mPAP by more than 5 mmHg) compared to nonresponder patients.

associated with an increase in $^{18}$FDG uptake. Examples of two responders are shown in Fig. 5.

DISCUSSION

In a first attempt to target a mitochondrial enzyme to treat humans with vascular disease, we report that DCA in doses up to 6.25 mg/kg po (per os) b.i.d over 4 months are generally well tolerated and can result in a significant reduction in mPAP and PVR in iPAH. PDK inhibition by DCA increases PDH activity and oxygen consumption in isolated perfused/ventilated PAH lungs, consistent with improvement in mitochondrial function.

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Poor DCA responders were characterized by a biochemical resistance that was associated, both in the ex vivo experiments and the clinical trial, with the presence of variants in SIRT3 and UCP2 that predict reduced protein activity. We propose that PDH inhibition in these patients is less dependent on PDK. Specifically, DCA may inhibit PDK but fail to increase PDH activity and respiration in patients in whom PDH is also inhibited by increased acetylation and/or lack of mitochondrial calcium in vascular cells due to SIRT3 and UCP2 gene variants that inhibit SIRT3 activity and are associated with decreased UCP2 expression.

The correlation between the SNP score and clinical response to DCA was a post hoc analysis. However, unlike randomized trials where a post hoc exploration of a biomarker can introduce bias, this concern does not apply in studies of genetic variants with known effects that are not in linkage disequilibrium. The random distribution of the relevant variants during miosis reduces bias and “Mendelian randomization” is being increasingly used to dissect genotype-response relationships (37). Post hoc analysis of genetic factors causing resistance to a drug has been used successfully in cancer, leading to conclusions strong enough to change clinical practice, without evidence from prospective studies (38, 39). Our hypothesis-driven selection of these gene variants was based on strong preclinical studies, which directly link the function of these genes with PAH pathogenesis. Our sample size is too small to evaluate whether these variants are enriched in PAH compared to healthy populations. Nevertheless, a gene variant may not be enriched in a population with the disease but may still affect the response to therapy.

We used noninvasive imaging biomarkers, $^{18}$FDG-PET-CT and MRI lung perfusion, to aid the biological interpretation of the hemodynamic responses. These tools may not be necessary to assess response in future studies, but it would be of interest to know whether patients exhibiting high lung $^{18}$FDG uptake are more responsive to DCA in future trials. An increase in lung perfusion shown in the gadolinium-MRI studies, even in areas not perfused before DCA therapy (Fig. 5A), is consistent with potential regression of occlusive vascular remodeling.

Previous studies in animals and humans have described an inotropic effect of DCA on the right ventricle, perhaps due to a shift in the utilization of metabolic substrate (carbohydrates versus fatty acids) (40, 41). The combined effect of a decrease in RV afterload due to regression of pulmonary vascular remodeling and RV inotropy is clinically very attractive. The long-term direct effects of DCA on RV remodeling are not known but do not appear to be adverse, considering that none of our patients deteriorated clinically. We did detect an increase in RV ejection fraction in DCA responders but did not detect a decrease in RV hypertrophy (fig. S4). This is not necessarily a disadvantage. Given that the RV afterload is still high in DCA responders, the maintenance of a compensating RV state (hypertrophy) is beneficial compared to a potential transition to a decompensated state of RV dilatation and failure (42).

Activated immune cells play a paramount role in the pathogenesis of PAH (43), and activation of these cells is associated with a suppression of glucose oxidation and switch to glycolysis (44). Although the role of PDK in these cells is not known, it is possible that DCA may have effects
on these cells, limiting their activation and the inflammatory burden in PAH—a possibility that requires further studies.

PDK is induced by HIF, and this has been confirmed in both cancer and PAH models, where HIF activity is increased even under normoxia (10, 45). A primary mitochondrial suppression can lead to secondary activation of HIF, completing a positive feedback loop (Fig. 1A (12, 25, 46)). There are many potential mechanisms that can contribute to primary mitochondrial suppression in PAH (including the presence of SIRT3 and UCP2 variants), leading to a positive feedback loop between mitochondrial suppression and HIF activation, where PDK plays a central role (5). PDK inhibition can break this feedback loop and reverse the metabolic remodeling, promoting apoptosis and inhibiting proliferation within the PA wall.

Other molecular factors contributing to the metabolic reprogramming in PAH could contribute to a biochemical resistance to mitochondrial activators. In the case of DCA, these include a decrease in SIRT3 protein abundance by nongenetic mechanisms (12) or a potential decrease in the mitochondrial calcium uniporter (MCU), an important calcium entry regulator in mitochondria, recently found to be important in PAH (47). UCP2 has been shown to regulate the function of the MCU (14). However, the identification of these potential molecular causes of DCA resistance in patients requires lung biopsies, which are contraindicated in PAH patients. On the other hand, the gene variants that we studied here can be easily detected in many tissues, including blood, making them attractive tools for future precision medicine trial designs. Lastly, there is some evidence that SNPs in glutathione transferase ζ1/maleylacetoacetate isomerase lead to hyper- or hypometabolizers of DCA, at least in children (48). Although there does not appear to be a relation between the clinical response and DCA trough concentration in our small cohort (Fig. 4), we did not measure DCA metabolites, and the possibility that such SNPs may be relevant should be studied in future studies.

This work translates to humans the knowledge accumulated from multiple preclinical studies and supports the newly proposed metabolic theory of PAH (4, 5). The beneficial hemodynamic effects of DCA were observed in patients already taking licensed medications for PAH. These agents are not known to have any effect on PDH activity, and our observations suggest that the decreased PDH activity is an unexploited therapeutic target in clinical practice. A factor to consider in future clinical trials of DCA is combination with a tyrosine kinase inhibitor, because tyrosine kinases can both inhibit PDH by phosphorylating a site different than that of PDK (11) and activate PDK by phosphorylating PDK itself (49). We suggest that genotype should be considered as a basis for stratification in future clinical studies of DCA or other metabolic modulators, with a precision medicine design, either ensuring randomization of

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Fig. 5. Examples of functional imaging biomarkers in two DCA responders. 
(A) MR images showing gadolinium transit time before and after DCA and resolution of the D-shaped septum (arrow) in the heart of patient 3. Note the color scale of the gadolinium transit time: Blue indicates short transit time and increased perfusion; yellow/red indicates long transit time and little/no perfusion at baseline. 
(B) PA pressure recordings and 18FDG uptake pre- and post-DCA from right heart catheterization and 18FDG-PET-CT, respectively, of patient 10. A shift to the left of the distribution of the 18FDG uptake suggests a decrease in glucose uptake due to decreased glycolysis. Color heat maps show Patlak slope per gram of tissue (score) per voxel in lung sections, indicating the density of high-value voxels (colored in yellow and red, indicating areas of high glucose uptake), overlaid on corresponding CT images.
patients according to their SIRT3/UCP2 SNP score or excluding patients with an SNP score of >1 from the study, to maximize detection of a beneficial response.

MATERIALS AND METHODS

Study design
This work consists of a preclinical component, where the effects of DCA were tested on human PAH lungs ex vivo and the amount of its target enzyme (PDK) was measured in human PAH and control lungs, and a clinical component where a phase 1, dose discovery trial (NCT01083524) was conducted to measure the effects of DCA on several clinical parameters in patients with iPAH. The investigators performing the experiments were blinded for the preclinical data, but the clinical study was open-label.

Human tissues samples
The protocols for clinical and human tissue studies were approved by the human studies ethics committees at the University of Alberta (Edmonton, UK) and Imperial College/Hammersmith Hospital (London, UK). Human tissues were processed and studied under identical tissue handling and staining protocols (Table 1).

Mitochondrial respiration measurements
Lung biopsies were taken before and after treatment with DCA during EVLP for further analysis of oxygen consumption using the Seahorse XFe24 Analyzer (Agilent Technologies). Tissue was cut into segments weighing about 5 mg and immediately plated onto an XF24 Islet Capture Microplate, with islet capture screens to secure the tissue at the bottom of the well, and submerged in the EVLP perfusate. Oxygen consumption rate was measured and normalized to mass of tissue per well.

PDH activity analysis
PDH activity was measured with a commercially available MitoProfile Dipstick assay kit (MitoSciences) as previously described (50, 51). Protein (200 μg) was incubated with the dipstick containing the PDH complex antibody. With this method, the PDH complex is immunocaptured and the production of PDH-driven NADH is measured.

Immunoblots
Immunoblots were performed with SDS–polyacrylamide gel electrophoresis as previously described and analyzed using Image (National Institutes of Health) (12, 25). Antibodies used were as follows: PDK1 (Abcam, ab110025; detected at 49 kDa), PDK2 (Abcam, ab68164; detected at 46 kDa), PDH-E1α (Abcam, ab168379; detected at 43 kDa), phospho-Ser589–PDH-E1α (EMD Millipore, AP1062; detected at 44 kDa), and actin (Abcam, ab3280; detected at 42 kDa). The PDK-PDH antibodies have been validated in a recent publication (52).

Confocal imaging
Immunofluorescence staining was performed as previously described, and imaging was done using a Zeiss LSM-510 NLO model (Carl Zeiss) (12, 25, 52). Antibodies used were PDK1 (Abcam, ab110025), PDK2 (Santa Cruz Biotechnology, sc-14484), and α-smooth muscle actin (Abcam, ab5694).

SNP genotyping assay
Genomic DNA was extracted from buffy coat using AllPrep RNA/DNA kits (Qiagen). DNA samples were quantified with a NanoDrop spectrophotometer (ND-8000) and normalized to a concentration of 50 ng/μl (diluted in 10 mM tris/1 mM EDTA). Samples were genotyped by TaqMan SNP Genotyping Assays for rs11246020 and rs659366 (Applied Biosystems) and processed in triplicates according to standard protocol and read on the ABI Prism 7900 Sequence Detector (Applied Biosystems) (12).

Ex vivo lung perfusion
EVLP allowed the perfusion and ventilation of human lungs from donors and recipients at transplant surgery. The perfusate entered the PA via a tube, controlled by a pump in combination with pressure and flow-sensing catheters. The bronchi were connected with tubes to an intensive care unit ventilator. The efflux from the pulmonary veins entered a deoxygenator, returning deoxygenated blood into the PAs, mimicking in vivo conditions. Flow was initiated slowly in a retrograde fashion to remove air from the circuit. Anterograde flow started at 150 ml/min with an albumin-based solution supplemented with Na+ (138 mmol), K+ (6 mmol), Mg2+ (0.8 mmol), Cl− (142 mmol), SO4 2− (0.8 mmol), H2PO4 (0.8 mmol), and glucose (5 mmol). The perfusate temperature was increased to 37°C, and flow rate was gradually increased to 40 to 50% of the estimated patient cardiac output. A protective mode of ventilation was applied with a tidal volume of 7 ml/kg at 7 breaths/min, positive end-expiratory pressure of 5 cmH2O, and fraction of inspired oxygen of 21%. The lungs were periodically expanded with inspiratory holds to an airway pressure of 20 cmH2O. pH, partial pressure of CO2, electrolytes, and glucose were maintained at physiologic concentrations with frequent measurements. After baseline conditions were established, DCA (sodium salt, TCI America) (0.7 mg/ml) was added to the perfusate. Peripheral lung biopsies were obtained before and after DCA perfusion. The recipient’s lungs were obtained at the time of lung transplantation and transferred immediately (within 5 min) to the laboratory for EVLP, under a patient consent and protocol approved by the Institutional Review Board at the University of Alberta. EVLP details are shown in Fig. 2A.

Clinical protocol (NCT01083524)
Adult patients with iPAH diagnosed by recognized criteria (53) were treated with DCA for 4 months (Table 2). All were receiving background and approved PAH therapies and had to be clinically stable with no dosing changes for at least 8 weeks before enrollment (an eligibility requirement). Although initially, enrollment of patients in WHO functional classes III and IV was planned, the protocol was subsequently amended to also allow enrollment of patients in class II. Exclusion criteria included concomitant diabetes, chronic kidney and liver disease, and a history of neuropathy. There were three screen failures out of 23 patients screened. All patients signed informed consents approved by the local human ethics committees. Three doses of DCA were tested: 3, 6.25, and 12.5 mg/kg po b.i.d. Starting from the lowest dose, at least three patients in each site (Canada and UK) had to be treated without evidence of toxicity for 4 weeks before another patient could be enrolled to the higher dose. Because the main anticipated toxicity, peripheral neuropathy, was previously described to be a dose-dependent and reversible, nondemyelinating neuropathy, a decrease in the dose was allowed for the two higher doses if symptoms occurred. Pathogen-free DCA (sodium salt) was purchased from TCI America. Pharmacists placed the individual doses of DCA powder (according to patient weight) in individual 20-cm3 sterile light-proofed vials, and patients added water to each bottle and dissolved DCA, which is highly water soluble, before drinking it.
The primary end point was safety and tolerability, and secondary end points included change from baseline in (i) mPAP and PVR measured by standard right heart catheterization, (ii) functional capacity measured by the 6-min walk test, and (iii) RV mass and ejection fraction measured with standard cardiac MRI protocols. We also assessed two exploratory end points: (i) the biochemical response to DCA using lung 18FDG-PET-CT (London site) as a measure of glucose uptake in the lungs, because it was hypothesized that a DCA-induced activation of glucose oxidation would result in suppression of glycolysis and thus a decrease in glucose uptake; and (ii) MRI-measured lung perfusion, measuring the transit time of gadolinium through the pulmonary circulation, because it was hypothesized that a DCA-induced regression of pulmonary vascular remodeling would increase lung perfusion (decreasing gadolinium transit time) even in areas not perfused before DCA therapy (more than increasing the perfusion in already perfused areas). The safety visits, which included blood tests for renal, hepatic, and bone marrow toxicity, electrocardiography, physical examination, and 6-min walk distance, were performed monthly. Right heart catheterization, MRI, and PET studies were performed at baseline and at 4 months. There were no patients lost to follow-up. The protocol was terminated when the dose-limiting toxicity was clearly reached.

**MRI studies**

RV mass and volumes were measured using a standard approach by two readers blinded to the patient’s background or state (pre-DCA versus post-DCA). Lung perfusion was studied with three-dimensional dynamic contrast-enhanced MRI using 1.5T Siemens Sonata systems. Typical scan parameters included 0.54-ms echo time, 1.5-ms repetition time, 15° flip angle, 192 × 72 × 20 matrix with a 400 × 300 × 150-mm field of view (axial orientation), rate 2 parallel imaging [GRAPPA (Generalized Autocalibrating Partial Parallel Acquisition)], with a temporal resolution of 1.24 s per image, and a total of 30 acquired images. All subjects were injected with gadopentetate dimeglumine (0.1 mmol/kg; Magnevist, Bayer) at a rate of 5 ml/s followed by 20 ml of saline flush. Injections were timed to ensure five baseline (noncontrast) image acquisitions. Subjects were instructed to hold their breath at end-expiration. MRI studies were not possible on patient 16 for the RV mass and ejection fraction protocols (patient declined) and on patients 8, 11, and 15 for the perfusion studies because of motion artifacts (Table 2). The underlying theory and application of contrast-enhanced dynamic perfusion in the lungs has previously been described (54, 55). Briefly, signal intensity curves from the lung tissue and blood pool (main trunk of the PA, arterial input function) were first converted to contrast agent concentration using a Bloch-equation look-up table approach. The tissue time-intensity curves were subsequently used to estimate the mean transit time (MTT) of the contrast agent in the lung parenchyma, using an exponential deconvolution approach to account for the effects of the arterial input function. MTT values were calculated in each pixel, and the average values over the entire lung are reported, with exclusion of pixels with contributions from larger arteries or veins.

**PET-CT studies**

Lung 18FDG (120 megabecquerel) distribution was measured 1.5 hours after intravenous administration as previously described (34). Regional glucose metabolic rate was calculated from the PET image data and from blood samples taken during the scan. The lung region of interest was identified from the CT image, and the rate of change of activity was calculated for each voxel. Blood rate was plotted against image rate for each lung voxel to produce a plot, which tends to a straight line (Patlak plot), the slope of which gives rate of tissue uptake. The Patlak slope was calculated for every voxel in the lung region. A voxel score was then calculated by dividing by the CT-derived tissue density and scaling by 1000, giving an index of the glucose metabolic rate. Histograms of the voxel score distribution were plotted as “before-after” pairs, and a leftward shift in distribution indicates a reduction in 18FDG uptake (Fig. 5). The mode of the voxel score histogram was found by smoothing data with a Gaussian filter and searching for the peak. The PET-CT studies were only performed at the London site (n = 7).

**Trough DCA concentrations**

Plasma samples were prepared using dextran sulfate sodium (DSS) solution (0.5 mM) as an internal chemical shift reference. The samples themselves consisted of 250 μl of 90% plasma and 10% standard internal reference and were placed in Norell S-3-200 3-mm nuclear magnetic resonance (NMR) tubes. NMR data were collected on an Agilent 700 MHz (16.45T) NMR spectrometer equipped with a VNMR four-channel console as previously described (52). The system had a cryogenically cooled inverse triple-resonance (HCN) probe containing a Z-pulsed field gradient coil. An Agilent 7620 sample-handling robot was used. Samples were run at 25°C and individually tuned and matched via an Agilent ProTune module. Each spectral was automatically phased and manually corrected to reduce baseline distortion due to residual solvent signal. The spectra were referenced to DSS, and then rephased and re-referenced for each sample to confirm consistency. Regions for integration were 8.6364 to 6.5572 parts per million (ppm), 6.1000 to 6.000 ppm, 5.9682 to 5.6456 ppm, 5.4766 to 5.1386 ppm, 5.0259 to 0.5397 ppm, and 0.1200 to 0.1200 ppm. Regions not included in the integration were used to apply a standard VNMRJ baseline correction. The 6.1 to 6 and 0.12 to –0.12 integrated regions were subsequently used for DCA to DSS quantitation calculation, respectively.

**Statistical analysis**

Statistical analysis was performed using IBM SPSS, version 24.0 (IBM Analytics). Data are shown as means with error bars indicating SEM. All data undergoing statistical analysis were subjected to Shapiro-Wilk test of normality. Some groups in Fig. 1 did not follow a normal distribution. Thus, mean data from immunoblots (Fig. 1) were compared with the non-parametric Mann-Whitney U test. Mean differences (pre-post DCA) from clinical parameters (Fig. 3 and fig. S4) did not deviate from normality; therefore, mean differences were compared via the parametric paired t test. For correlation of SNP score to clinical parameters, the Spearman rank correlation was chosen given the small sample size in some SNP categories. Spearman rank correlation coefficient is shown (rS) with respective P values in Fig. 3 and fig. S4. Significance for all statistical testing was determined to be P < 0.05.

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/9/413/eaao4583/DC1

Fig. S1. Confocal immunohistochemistry of six PAH lungs and three non-PAH control lungs.

Fig. S2. Relationship between risk variants (SNP scores) and decrease in mPAP in patients treated with DCA.

Fig. S3. Relationship between the combined SNP score of both SIRT3 and UCP2 risk variants and the change in mPAP in patients treated with DCA.

Fig. S4. The effects of DCA on RV size and function.

Fig. S5. The effects of DCA on lung perfusion (MRI) and glucose uptake (18FDG-PET-CT).

Table S1. Gene variants score (SNPs) for DCA resistance.
REFERENCES AND NOTES


Inhibition of pyruvate dehydrogenase kinase improves pulmonary arterial hypertension in genetically susceptible patients


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Progress for PAH

In addition to thickening and occlusion of the pulmonary arteries, mitochondrial respiration is suppressed in pulmonary arterial hypertension (PAH). Michelakis et al. treated lungs from patients with PAH with dichloroacetate (DCA), a drug used to treat cancer and congenital mitochondrial disease that inhibits the mitochondrial enzyme pyruvate dehydrogenase kinase. DCA increased mitochondrial function; however, the response was variable, and this variable response was mirrored in a phase 1 trial, with some patients showing improved hemodynamics and functional capacity. The authors determined that patients with inactivating mutations in two genes encoding mitochondrial proteins were less responsive to DCA. This work highlights the importance of considering patient genotype in clinical trial design and identifies a drug target for PAH.