Targeting peptidyl-prolyl isomerase 1 in experimental pulmonary arterial hypertension

Nabham Rai1, Akylbek Sydykov1, Baktybek Kojonazarov1,2, Jochen Wilhelm1,2, Grégoire Manaud3, Swathi Veeroju1, Clemens Ruppert1,2, Frédéric Perros3, Hossein Ardeschir Ghofrani1, Norbert Weissmann1, Werner Seeger1,2,4, Ralph T. Schermuly1,5 and Tatyana Novoyatleva1,5

1Universities of Giessen and Marburg Lung Center (UGMLC), Excellence Cluster Cardio Pulmonary Institute (CPI), Member of the German Center for Lung Research (DZL), Justus-Liebig-University Giessen, Giessen, Germany. 2Institute for Lung Health, Giessen, Germany. 3Université Paris–Saclay, AP-HP, INSERM UMR_S 999, Service de Pneumologie et Soins Intensifs Respiratoires, Hôpital de Bicêtre, Le Kremlin Bicêtre, France. 4Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany. 5These co-senior authors contributed equally to this work.

Corresponding author: Tatyana Novoyatleva (tatyana.novoyatleva@innere.med.uni-giessen.de)

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Peptidyl-prolyl cis/trans isomerase, NIMA interacting 1 (Pin1) enzyme inhibition by Juglone administration reversed both hypoxia- and non-hypoxia-driven experimental PAH by improving pulmonary vascular remodelling and right ventricular function https://bit.ly/3zWqTvb

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Abstract

**Background** Pulmonary arterial hypertension (PAH) is a progressive disease characterised by pro-proliferative and anti-apoptotic phenotype in vascular cells, leading to pulmonary vascular remodelling and right heart failure. Peptidyl-prolyl cis/trans isomerase, NIMA interacting 1 (Pin1), a highly conserved enzyme, which binds to and catalyses the isomerisation of specific phosphorylated Ser/Thr-Pro motifs, acts as a molecular switch in multiple coordinated cellular processes. We hypothesised that Pin1 plays a substantial role in PAH, and its inhibition with a natural organic compound, Juglone, would reverse experimental pulmonary hypertension.

**Results** We demonstrated that the expression of Pin1 was markedly elevated in experimental pulmonary hypertension (i.e. hypoxia-induced mouse and Sugen/hypoxia-induced rat models) and pulmonary arterial smooth muscle cells of patients with clinical PAH. In vitro Pin1 inhibition by either Juglone treatment or short interfering RNA knockdown resulted in an induction of apoptosis and decrease in proliferation of human pulmonary vascular cells. Stimulation with growth factors induced Pin1 expression, while its inhibition reduced the activity of numerous PAH-related transcription factors, such as hypoxia-inducible factor (HIF)-α and signal transducer and activator of transcription (STAT). Juglone administration lowered pulmonary vascular resistance, enhanced right ventricular function, improved pulmonary vascular and cardiac remodelling in the Sugen/hypoxia rat model of PAH and the chronic hypoxia-induced pulmonary hypertension model in mice.

**Conclusion** Our study demonstrates that targeting of Pin1 with small molecule inhibitor, Juglone, might be an attractive future therapeutic strategy for PAH and right heart disease secondary to PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a fatal pulmonary vascular disease characterised by pulmonary vascular remodelling and increased pulmonary vascular resistance, culminating in right ventricular (RV) hypertrophy and failure [1]. The dynamic vasoconstriction of pulmonary arteries, their adverse structural remodelling, fibrosis and stiffening, are the main causes of increased pulmonary vascular resistance [2]. PAH shares some common features with cancer, such as the increase in cell proliferation and resistance to apoptosis [3]. Besides this, endothelial dysfunction, an increase in inflammation, dysregulated angiogenesis, metabolic disturbance, oxidative stress and mitochondrial dysregulation contribute to the progression of the disease [4]. PAH is a complex and multifactorial disease, the emergence of which has...
been explained by a concept known as the “multiple-hit hypothesis”, in which a combination of two or more hits is essential for triggering of key mechanisms that lead to vascular constriction [5–7].

The peptidyl-prolyl cis/trans isomerase, NIMA interacting 1 (Pin1), is a highly conserved enzyme, which belongs to the PPI-ase superfamily of proteins, comprising of cyclophilins, FK506-binding proteins (FKBPs) and parvulins [8]. Pin1 is the only known PPI-ase, which specifically and uniquely recognises phosphorylated Ser/Thr-Pro peptides and catalyses the cis/trans isomerisation of these pSer/Thr-Pro motifs [9, 10]. Post-phosphorylation cis/trans isomerisation, driven by Pin1, induces structural changes further affecting the phosphorylation/dephosphorylation status of target proteins [11–14]. As a consequence, conformational modifications of many target phosphoproteins affect their stability and activity, subcellular localisation and protein–protein interactions, triggering multiple cellular signalling pathways [14–16]. Thus, Pin1 serves as a molecular determinant of the fate of phosphoproteins [14], which adds a new layer of control in various signalling pathways [17]. Among Pin1 substrates are numerous cell cycle-regulatory proteins, such as cell division cycle 25 [18], cyclin D1 [19, 20], cyclin E [21] and polo-like kinase 1 [22, 23]; transcription factors, such as retinoblastoma protein [24] and neurogenic locus notch homologue protein 1 (Notch1) [24]; DNA-damage factors, such as CREB-binding protein/p300 [25] and homeodomain-interacting protein kinase 2; and apoptotic regulatory proteins, such as tumour suppressor p53 [26, 27] and survivin [28]. In association with this, a number of signalling pathways are markedly affected by Pin1, including cell cycle progression, gene transcription, tumour development, oxidative stress and apoptosis [17]; thus, regulation driven by Pin1 provides a new platform for assembly of multiple protein networks.

Here we investigated the expression and the role of Pin1 in experimental and clinical PAH by using Juglone, a specific and irreversible Pin1 inhibitor, established to covalently inactivate Pin1 [29, 30]. We show that inhibition of Pin1 by Juglone efficiently ameliorates both hypoxia (chronic hypoxia mouse model)- and non-hypoxia (Sugen5416 combined with chronic hypoxia rat model)-induced PAH.

Overall, our data indicate that Juglone provides beneficial outcomes on RV hypertrophy, RV systolic pressure and RV function in both hypoxia- and non-hypoxia-induced experimental pulmonary hypertension, suggesting that targeting of Pin1 activity offers a potential therapeutic option for pulmonary hypertension.

Methods

Animals

All in vivo procedures were approved by local and federal animal ethics committee authorities (approval number: GI 20/10 Nr G 82/2018).

Cell culture and reagents

Human pulmonary artery smooth muscle cells (hPASMCs) were either obtained from the Universities of Giessen and Marburg Lung Center Giessen Biobank, member of DZL Platform Biobanking or purchased from Lonza (Basel, Switzerland). Murine PASMCs were isolated directly from pre-capillary pulmonary arterial vessels using iron particles, as described previously [31, 32]. Human pulmonary artery endothelial cells (hPAECs) from healthy individuals were purchased from Lonza. Idiopathic pulmonary hypertension (IPAH) hPAECs were produced as described previously [33]. RV cardiac fibroblasts were isolated from adult mouse hearts, as described previously [34].

Proliferation and apoptosis assessment

hPASMCs and hPAECs were exposed to Juglone (5-hydroxy-1,4-naphthalenedione) (sc-202675; Santa Cruz Biotechnology, CA, USA) at concentrations between 1 and 10 µM. Proliferation of hPASMCs from non-PAH individuals and patients with IPAH, and hPAECs from healthy control individuals was assayed by monitoring the incorporation of 5-bromo-2-deoxyuridine (BrdU) into newly synthesised DNA (Cell Proliferation ELISA BrdU colorimetric kit; Roche, Basel, Switzerland). Apoptosis was assayed using the In Situ Cell Death Detection Kit, TMR red (Roche).

Echocardiography and haemodynamic measurements

Invasive methods to measure right ventricular systolic pressure (RVSP) and systemic arterial pressure were performed on all the animals in a blinded manner. Transthoracic echocardiographic examination was performed to assess cardiac function, as described previously [35].
Tissue preparation, histology, immunohistostaining

For histology, lungs were fixed with 10% neutral buffered formalin. The right lung was snap-frozen. The RV was separated from the left ventricle plus septum (LV+S), and the RV/(LV+S) ratio was calculated as an index of RV hypertrophy.

Statistical analysis

All data are presented as mean±SEM. For comparison of two groups, parametric t-test was applied; for comparisons involving more than two groups, one-way ANOVA with post hoc Newman–Keuls multiple comparisons test was applied. Values of p<0.05 were considered statistically significant.

The details on biospecimen collections and primary cell isolations, short interfering (si)RNA transfections, luciferase reporter assays, transcription factor array, reverse transcriptase quantitative (q)PCR, Western blot analyses, immunostaining and lung morphometry are provided in the supplementary material.

Results

Peptidyl-prolyl isomerase 1 activation in experimental and human PAH

The expression of Pin1 was examined in lung specimens of patients with IPAH and non-PAH donor individuals. The immune reactivity of Pin1 was evidently augmented in pulmonary vascular compartments of IPAH patients, in comparison to non-PAH controls (figure 1a). Co-immunostaining with α-smooth muscle actin established Pin1 localisation in the medial layer of the pulmonary arterial walls of IPAH lungs. Western blot analyses demonstrated remarkably higher Pin1 expression in IPAH hPASMCs, but not in IPAH hPAECs, as compared to their respective non-PAH control cells (figure 1b–e). Pin1 expression in IPAH hPASMCs exhibits a significant correlation with mean pulmonary arterial pressure. A weak nonsignificant correlation with pulmonary capillary wedge pressure, cardiac index and systolic pulmonary artery pressure of the IPAH patients was detected (figure 1f–i). Intriguingly, qPCR analyses of mRNA from the laser-assisted micro-dissected vessels and human lungs exhibited no significant difference of PIN1 between IPAH and non-PAH controls (supplementary figure S1a–c).

An accumulation of Pin1 protein in lung homogenates of Sugen5416/hypoxia (SuHx) rats and slight, but nonsignificant enhancement of Pin1 in lungs of mice exposed to chronic hypoxia (HOX), as compared to their respective controls, was noted (figure 1j–m). Hypoxia significantly elevated Pin1 protein accumulation in cultured mouse PASMCs (supplementary figure S1d). No significant alterations of Pin1 mRNA expression have been detected in lungs of SuHx and HOX experimental models of pulmonary hypertension (supplementary figure S1e, f). Pin1 expression is markedly elevated in pulmonary vessels of both experimental pulmonary hypertension models (supplementary figure S2a, b).

Pin1 blockage results in suppression of vascular cell proliferation and initiation of cell apoptosis in vitro

The effect of pharmacological inhibition of Pin1 by the small molecule inhibitor Juglone was first investigated on proliferation of hPASMCs. Juglone inhibited platelet-derived growth factor (PDGF)-BB-driven proliferation of both non-PAH hPASMCs and hPASMCs from IPAH patients (figure 2a). To further support the notion that Pin1 is critically important for PASMC proliferation, an siRNA approach targeting Pin1 was employed in vitro. The expression of Pin1 was strongly reduced in Pin1-silenced hPASMCs, as compared to control transfected cells (figure 2b, c). The ablation of Pin1 via siRNA or Juglone decreased the expression of proliferating cell nuclear antigen (PCNA) and Ki-67 in hPASMCs under basal conditions (figure 2b–d, f and supplementary figure S3a–c). Moreover, Pin1 blockage suppressed the proliferative response of hPASMCs under basal and stimulated (PDGF-BB) conditions (figure 2e), pointing to its role in cell cycle regulation. Interestingly, the proliferation of hPAECs challenged with fetal bovine serum was also markedly inhibited by Juglone and by a Pin1-siRNA approach both in control and diseased hPAECs (figure 2g, h). Blockage of Pin1 by Juglone caused the augmentation of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive control and diseased hPASMCs (figure 3a, i and supplementary figure S3d, e). Juglone also inhibited the resistance to apoptosis of both control and diseased hPASMCs, as indicated by upregulated expression of pro-apoptotic active cleaved forms of Caspase 3 and poly (ADP-ribose) polymerase (PARP-1) (figure 3b–d, j–l). Pin1 inhibition led to the resistance to apoptosis of hPAECs, as determined by elevated number of TUNEL-positive control and diseased hPAECs (figure 3e, o and supplementary figure S3f, g), increased expression of active Caspase 3 and PARP-1 apoptotic markers (figure 3f–h, p–r and supplementary figure S3g, h). A decrease in PCNA was detected in Juglone-treated IPAH hPASMCs (figure 3m, n), while no obvious change in IPAH hPAECs was observed (figure 3s, t). Taken together, our data suggest that Pin1 inhibition may have positive therapeutic impacts in PAH.
Pin1 controls the activity of a multitude of transcription factors

Next, we determined the effect of various pulmonary hypertension-inducing growth factors/pro-inflammatory cytokines on Pin1 expression in hPASMCs. Induction of Pin1 in hPASMCs was observed exclusively after stimulation with PDGF-BB, epidermal growth factor and growth medium, a mixture of growth factors, compared to untreated control cells (figure 4a). Interestingly, neither members of bone morphogenetic protein family nor pro-inflammatory cytokines (tumour necrosis factor-α, interleukin-6) recognisably modified Pin1 expression, indicative of a selective mode of Pin1 regulation in hPASMCs (supplementary figure S4a–d). As Pin1-driven isomerisation has been reported to play a significant role in the activity of various transcription factors facilitating multiple proliferation-supporting pathways [8, 36], we monitored the activity of a set of 96 transcription factors from Pin1-silenced and Juglone-treated hPASMCs. The depletion of Pin1 reduced the activity of growth-promoting and increased the activity of proliferation-restraining transcription factors (figure 4b). Several transcription factors and transcriptional co-activators implicated in pulmonary hypertension and RV dysfunction such as hypoxia-inducible factor (HIF), NF-κB, SMADs and signal transducers and activators of transcription.
Followed by densitometric analysis 24 h after Pin1 mRNA knockdown. Immunofluorescence staining for Ki-67+ cells in Pin1-silenced hPASMCs and hPAECs of donor control and IPAH patients in presence or absence of PDGF-BB and 10% FBS determined by 5-bromo-2-deoxyuridine (BrdU) incorporation. The rate of DNA synthesis for a, e, g and h was examined by measuring of BrdU incorporation [λ370nm]. Scr: scrambled; NS: nonsignificant. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post hoc test for multiple comparisons. **: p<0.01, ***: p<0.001, ****: p<0.0001 versus PDGF-BB or 10% FBS treated cells; a: p<0.05, ab: p<0.01, abc: p<0.001, abcd: p<0.0001 versus si scrambled or dimethyl sulfoxide (DMSO)-treated cells; b: p<0.05, bc: p<0.01, bcd: p<0.001, bce: p<0.0001 versus si scrambled or dimethyl sulfoxide (DMSO)-treated cells. (STATs) were dysregulated upon Pin1 inactivation either by knockdown of Pin1 or Juglone exposure (figure 4b and c). A remarkable decrease of HIF-1α in control and IPAH hPASMCs and upregulation of tumour suppressor C/EBPα in control hPASMCs was detected in Pin1-silenced hypoxia-treated hPASMCs (figure 4d–g). Hypoxia increased the hypoxia responsive element-luciferase activity that was strongly suppressed in Pin1-silenced hPASMCs (figure 4h).

**Juglone reduces pulmonary vascular remodelling and improves RV function in Sugen/hypoxia-induced PAH**

The effect of Pin1 inhibition was examined in the SuHx rat model of PAH (figure 5a), closely resembling some forms of human PAH [37]. Juglone administration (1.5 mg·kg⁻¹ body weight) from day 21 to day 35 significantly reduced RVSP in comparison to placebo-treated control rats (figure 5b). A decrease of RVSP was accompanied by diminished RV hypertrophy, as determined by the Fulton index (RV/LV+S).
Multiple comparisons. Data from three independent experiments are presented as mean±SEM. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post hoc test for multiple comparisons. Data from three independent experiments are presented as mean±SEM.

FIGURE 3 Peptidyl-prolyl cis/trans isomerase, NIMA interacting 1 (Pin1) blockage results in initiation of cell apoptosis in vitro. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay after 24 h treatment with increasing concentration of Juglone of a) control and i) idiopathic pulmonary arterial hypertension (IPAH) human pulmonary artery smooth muscle cells (hPASMCs), and of e) control and o) IPAH human pulmonary artery endothelial cells (hPAECs). b, j, m) Representative Western blots and c, d, k, l, n) subsequent densitometric analysis of control and IPAH hPASMCs after Juglone treatment.

g). Next, we determined the effect of Juglone on pulmonary vascular remodelling. Treatment of rats with Juglone reduced the number of h) control and p) IPAH hPASMCs, and of i) control and r) IPAH hPAECs. PARP: poly (ADP-ribose) polymerase; PCNA: proliferating cell nuclear antigen. *: p<0.05; **: p<0.01; ***: p<0.001 versus dimethyl sulfoxide (DMSO)-treated control cells. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post hoc test for multiple comparisons. Data from three independent experiments are presented as mean±SEM.

Compared with placebo-treated control rats (figure 5c). Administration of Juglone did not affect the systemic blood pressure (supplementary figure S4e). The decrease in RVSP and RV hypertrophy in Juglone-treated rats was followed by diminution in RV dilation (RV internal diameter), an increase in tricuspid annular plane systolic excursion (TAPSE), nonsignificant rise in cardiac index and a severe drop in total pulmonary vascular resistance index (PVRI) (figure 5d–g). Next, we determined the effect of Juglone treatment on pulmonary vascular remodelling. Treatment of rats with Juglone reduced the number
FIGURE 4 Peptidyl-prolyl cis/trans isomerase, NIMA interacting 1 (Pin1) controls the activity of multitude of transcription factors. a) Control and idiopathic pulmonary arterial hypertension (IPAH) human pulmonary artery smooth muscle cells (hPASMCs) after 24 h of serum starvation were subjected to platelet-derived growth factor (PDGF)-BB (50 ng·mL\(^{-1}\)), epidermal growth factor (EGF) (5 ng·mL\(^{-1}\)) and growth medium (GM) with 5% fetal bovine serum (FBS). Intracellular Pin1 levels were monitored by ELISA. *: \(p<0.05\), ****: \(p<0.0001\) versus control PASMCs; §§: \(p<0.01\), §§§: \(p<0.0001\) versus IPAH hPASMCs. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post hoc test for multiple comparisons. Data from three independent experiments are presented as mean±SEM. b) Pin1-silenced and Juglone-treated hPASMCs were stimulated with GM for 24 h and nuclear protein extracts were used for transcription factor activation profile array, presented as log-transformed signals in a volcano plot. c) Log-transformed scatter plot of combined transcription factor activation/inactivation in Pin1-silenced and Juglone-treated hPASMCs. Data from two independent experiments are presented. d, f) Western blots and e, g) subsequent densitometry analyses of hypoxia-inducible factor (HIF)-1α and C/EBPα transcription factors in Pin1-silenced control and IPAH hPASMCs subjected to hypoxia for 24 h. h) Hypoxia-responsive element (HRE) luciferase activity in Pin1-silenced hPASMCs after 24 h of hypoxia. Scr: scrambled; NS: nonsignificant. *: \(p<0.05\); ****: \(p<0.0001\) for normoxia (NOX) si Scr versus hypoxia (HOX) si Scr; §: \(p<0.05\); §§§: \(p<0.0001\) for HOX si Scr versus HOX si Pin1. Data from three independent experiments are presented as mean±SEM.
Juglone reduces pulmonary vascular remodelling and improves right ventricular (RV) function in Sugen/hypoxia-induced pulmonary arterial hypertension (PAH).

**a)** Schematics of the animal treatment. Echocardiography followed by physiological measurements was performed on Juglone-treated (1.5 mg·kg$^{-1}$ body weight), placebo-treated Sugen5416/hypoxia (SuHx) rats and healthy rats 35 days after initiation of SuHx treatment. **b)** Right ventricular systolic pressure (RVPsys), as measured by right heart catheterisation, **c)** ratio of RV mass to mass of left ventricle plus septum (RV/(LV+S)), **d)** right ventricle internal diameter (RVID), **e)** tricuspid annular plane systolic excursion (TAPSE) (normoxia (NOX) n=5, SuHx n=5, SuHx+Juglone n=9), **f)** cardiac index and **g)** pulmonary vascular resistance index (PVRI) of the rats as measured by echocardiography. NOX n=5, SuHx n=4, SuHx+Juglone n=4. Ex vivo analyses of lung tissues for reversal of remodelling and in vivo drug efficacy on fibrosis. **h)** The degree of muscularisation of small pulmonary arteries (diameter 20–50 μm) was determined by immunohistological stainings for von Willebrand factor and α-smooth muscle actin (SMA) antibodies of lung sections. M: fully muscularised; P: partially muscularised; N: nonmuscularised. **i)** Lung sections stained with Elastic von Gieson to determine medial wall thickness of vessels (%) and occlusion score (%) with a diameter of 20–50 μm. O: open; P: partial; C: closed. NOX n=5, SuHx n=5, SuHx+Juglone n=9. **j)** Quantitative analysis of proliferating cell nuclear antigen (PCNA)-positive cells per vessel in lungs (NOX n=4, SuHx n=4, SuHx+Juglone n=4). **k)** Effect of Juglone on RV fibrosis (NOX n=5, SuHx n=5, SuHx+Juglone n=7) and secreted collagen content in isolated cardiac fibroblasts. Data from three independent experiments are presented as mean±SEM. **l)** Western blot analyses followed by o) densitometry of lung homogenates of representative samples from all three experimental groups. NOX n=4, SuHx n=4, SuHx+Juglone n=4. s.c.: subcutaneous; i.p.: intraperitoneal; NS: nonsignificant. **: p<0.01; ****: p<0.0001 for SuHx versus NOX; $: p<0.05; $$: p<0.01; $$$$: p<0.001. §§§§: p<0.0001 for SuHx+Juglone versus SuHx. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post hoc test for multiple comparisons.

Homogenates demonstrated that Pin1 blockade enhanced cleaved Caspase 3 accumulation and resulted in a clear but insignificant reduction of STAT3 activation (figure 5n and o). In summary, our data show that Pin1 inhibition improved haemodynamics, RV function and pulmonary vascular and cardiac remodelling in the SuHx model of PAH.

**Juglone impairs the progression of pulmonary hypertension induced by chronic hypoxia**

Next, the impact of Pin1 inhibition in chronic-hypoxia-induced pulmonary hypertension was examined (figure 6a). Mice exposed to 35 days of chronic hypoxia developed pulmonary hypertension, indicated by an increase in RVSP and RV hypertrophy (figure 6b, c). Treatment with Juglone (3 mg·kg$^{-1}$ per day) from day 21 to day 35 resulted in a slight but nonsignificant reduction of both RVSP and Fulton index,
Juglone impairs the progression of pulmonary hypertension induced by chronic hypoxia in mice. Effect of Juglone on haemodynamics and right heart function in chronic hypoxia (HOX) mice. a) Schematics of the animal treatment. Echocardiography followed by physiological measurements was carried out on Juglone-treated (3 mg·kg⁻¹ body weight), placebo-treated HOX mice and healthy mice 35 days after initiation of hypoxia treatment. b) Right ventricular systolic pressure (RVSPsyst) as measured by right heart catheterisation, c) ratio of right ventricle mass to mass of left ventricle plus septum (RV/(LV+S)), d) right ventricle internal diameter (RVID) and e) tricuspid annular plane systolic excursion (TAPSE). Normoxia (NOX) n=5, HOX n=5, HOX+Juglone n=10. f) Cardiac index and g) pulmonary vascular resistance index (PVRI) of the mice as measured by echocardiography. NOX n=5, HOX n=5, HOX+Juglone n=9. Ex vivo analyses of lung tissues for reversal of remodelling. h, i) The degree of muscularisation of small pulmonary arteries (diameter 20–70 μm) was determined by immunohistology for von Willebrand factor and α-smooth muscle actin (SMA) of lung sections. M: fully muscularised, P: partially muscularised, N: nonmuscularised; lung sections stained with Elastica van Gieson were used to determine the medial wall thickness of vessels (%). j, k) Fluorescence molecular tomography using Annexin-Vivo fluorescence imaging of mice detected by fluorescence molecular tomography (FMT) using Annexin-Vivo 750 and representative images for all three groups. NOX n=5, HOX n=5, HOX+Juglone n=10. Apoptotic and proliferative indices determined by l) terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)+ (NOX n=4, HOX n=4, HOX+Juglone n=5) and m) Ki-67+ cells per vessel in lungs (NOX n=5, HOX n=5, HOX+Juglone n=5). i.p.: intraperitoneal; ns: nonsignificant. **: p<0.01, ***: p<0.001, ****: p<0.0001 for HOX versus NOX; §: p<0.05, §§: p<0.01, §§§: p<0.001, §§§§: p<0.0001 for HOX+Juglone versus HOX. Statistical analysis was performed using one-way ANOVA with Newman–Keuls post hoc test for multiple comparisons.
decrease in Ki-67+ vascular cells (figure 6j and supplementary figure S6d). Juglone did not affect the Pin1 protein expression in Juglone-treated mice (supplementary figure S6e). Taken together, our data demonstrate that Pin1 inhibition improves RV function and reverses pulmonary vascular remodelling in hypoxia-exposed mice.

Discussion

The emergence and development of PAH encompass complex pathological mechanisms, whereas successful therapeutic treatment of the disease currently remains a major challenge. Here we show a marked elevation in the expression of Pin1 in experimental pulmonary hypertension lungs and hPASMCs of patients with PAH. Pin1 abundance in PASMCs strongly correlates with the mean pulmonary artery pressure of IPAH patients. Inhibition of Pin1 by the naphthoquinone Juglone efficiently ameliorated both hypoxia and non-hypoxia-induced PAH, evidenced by improved haemodynamics, RV function and pulmonary vascular remodelling, verifying that inhibition of disease progression associated with Juglone-mediated effects on Pin1. These data are in correlation with in vitro results, in which Pin1 blockage diminished proliferation and declined the resistance to apoptosis of hPASMCs and hPAECs. Pin1 is an enzyme that catalyses the cis/trans conversion of its substrate upon binding to the pSer/Thr-Pro consensus motifs within the target [10, 38, 39]. Pin1-driven isomerisation of multiple substrates results in various biological outcomes [22, 40], providing an alternative path of control of abundant signalling cascades under different cellular conditions [14, 41]. Pin1 controls the activity of a range of transcription factors and could provide a pivotal switch in disease pathogenesis by regulating the gene expression of its transcriptional substrates [42]. Pin1 contributes to cell cycle control of various diseases [22, 40, 43, 44], partially by regulating the nucleo-cytoplasmic shuttling and activity of transcription factors, which control cell proliferation and inflammatory response. For example, Pin1-mediated nuclear shuttling and isomerisation of β-catenin leads to upregulation of its target genes, Cyclin D1 and c-Myc [45]. Pin1 promoted nuclear accumulation of NF-xB subunit RelA, via inhibition its binding to IxBo, enhances cell growth and inflammatory cytokine production [46]. Pin1 could also sequester the cytoplasmic shuttling of a tumour suppressor of forkhead family of transcription factor 4 (FOXO4), resulting in a decrease of its transcriptional activity toward its target genes, such as p27kip1 [47]. Pin1 can regulate the stability of plethora of transcription factors, key regulators of PAH emergence and progression, as FOXOM [48, 49]; peroxisome proliferator-activated receptor-γ [50, 51], HIF [52, 53], oestrogen receptor-α [54, 55] and transcriptional co-activators, such as Notch [56, 57]. Moreover, Pin1 has been reported to increase the stability and transcriptional activity of bromodomain-containing protein 4 (BRD4), one of the critical epigenetic drivers for PAH [58, 59]. In our study, transcription factor profiling arrays revealed that Pin1 efficiently controls the activity of key transcription factors, implicated in pulmonary hypertension emergence and development, suggesting that Pin1 is an important endogenous integrator in the context of PAH. Specifically, we show that siRNA-mediated or Juglone-treated depletion of Pin1 resulted in a strong decrease in transcriptional activity of crucial transcription factors, which have been implicated previously in cell proliferation and migration, chronic inflammation and tissue remodelling (STAT3, HIF, NF-xB and SMADs) [60–62]. This agrees with observations that Pin1 blockage resulted in a marked decrease in proliferative responses of hPASMCs and hPAECs, indicating that endogenous Pin1 contributes to the pseudomalignant phenotype of the disease. Anti-apoptotic resistance of vascular cells is another hallmark of PAH [3], and Pin1 has been implicated in cell death in various pathological conditions [63, 64]. Importantly, a strong and significant initiation of apoptosis and successive inhibition of vascular cell proliferation distinguished upon Pin1 deletion was tightly correlated with a substantial initiation of cell death in lungs of chronic-hypoxia-induced pulmonary hypertension. Knockdown of Pin1 in pulmonary vascular cells resulted in inhibition of proliferative and activation of pro-apoptotic responses, implicating the endogenous Pin1 for pulmonary hypertension development. Myocardial apoptosis has been associated with RV dysfunction and fibrosis [65]; thus, apoptosis may provide a detrimental impact on RV function. In our study, apoptosis initiation was exclusively presented in lung vascular cells, and more specifically in smooth muscle cells, while FMT did not reveal any apoptotic signal in the heart. Pin1 regulates the intensity and duration of cardiac hypertrophic response [66]. The inhibition of Pin1 alleviated cardiac damage and fibrosis in isoprenaline- and diabetes-induced myocardial fibrosis in rats [67, 68], verifying our data that Pin1 contributes to the development of cardiac remodelling in SuHx-administered rats. Furthermore, recently it was reported that Pin1 blockage reversed the PAH phenotype in PAH microvascular endothelial cells in vitro and in PAH rats in vivo [69]. Interestingly, our detailed expression analyses of Pin1 indicated inconsistency between mRNA and protein both in experimental and clinical PAH, suggesting that Pin1 protein might be stabilised in the course of the disease. Pik-1 (whose expression was recently shown to be significantly increased in distal pulmonary arteries and isolated PASMCs from PAH patients; Sébastien Bonnet, Pulmonary Hypertension and Vascular Biology Research Group, Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval, Department
of Medicine, Québec, Canada; personal communication) has been reported to stabilise Pin1 protein, which might explain the disparity in mRNA and protein levels of Pin1 in PAH [23].

Juglone, along with another naphthoquinone, Plumbagin, is intensively studied in cancer in vivo [70–73]. Plumbagin-inhibited PAH-hPASMC proliferation and resistance to apoptosis were associated with a decrease in pulmonary artery remodelling, mean pulmonary artery pressure and RV hypertrophy in experimental PAH rat models via the STAT3/NFAT axis [74]. The deletion of Pin1 in hPASMCs in our experiments also resulted in a strong and remarkable decline of both STAT3 and STAT5 activity, suggesting a common route of regulation for both naphthoquinones. One of the primary limitations of this study is that the control and PAH-hPAECs were obtained from different sources. The slight variations in the isolations and culturing procedures of these primary cells may impact the functional activity of these cells in response to Juglone. Many of naphthoquinones including Juglone are characterised by pro-oxidant properties. In this regard, Juglone acts not only as a redox-cycling agent, but also as producer of reactive oxygen species [75]. In addition to its pro-oxidant properties, Juglone also possess both the cytotoxic and the genotoxic properties [76], thus the damaging impact of Juglone on health cells cannot be completely ruled out. Pin1 serves as a linkage between various signalling mechanisms at multiple levels. A proposed Pin1 mechanism in pulmonary hypertension has been illustrated in figure 7.

FIGURE 7 A proposed signalling mechanism of peptidyl-prolyl cis/trans isomerase, NIMA interacting 1 (Pin1) in pulmonary hypertension. Aberrant growth factor signalling induces Pin1 leading to isomerisation of various target proteins and regulation of transcription factors, resulting in proliferation and survival of pulmonary vascular cells. Amplification of pro-proliferative and anti-apoptotic pathways by Pin1 leads to pulmonary vascular remodelling and right ventricular (RV) failure and Juglone, by inhibiting Pin1 response, could provide a potential prevention strategy for pulmonary hypertension. PDGF: platelet-derived growth factor; GF: growth factors; EGF: epidermal growth factor; HIF: hypoxia-inducible factor; STAT: signal transducers and activators of transcription; PCNA: proliferating cell nuclear antigen.

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Our data underscore the importance of Pin1 for controlling the activity of presently uninvestigated transcription factors in PAH. A deeper understanding of the molecular mechanisms driving Pin1 regulation and the molecular/cellular circuits driven by Pin1 in diseased vascular cells is essential for developing tailored therapeutic concepts focusing on Pin1 inhibition.

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References

1. Schermuly RT, Ghofrani HA, Wilkins MR, et al. Mechanisms of disease: pulmonary arterial hypertension. Nat Rev Cardiol 2011; 8: 443–455.
2. Thenappan T, Ormiston ML, Ryan JJ, et al. Pulmonary arterial hypertension: pathogenesis and clinical management. BMJ 2018; 360: j5492.
3. Guignabert C, Tu L, Le Hiess M, et al. Pathogenesis of pulmonary arterial hypertension: lessons from cancer. Eur Respir Rev 2013; 22: 543–551.
4. Vonk Noordegraaf A, Groeneveldt JA, Bogaard HJ. Pulmonary hypertension. Eur Respir Rev 2016; 25: 4–11.
5. Machado RD, James V, Southwood M, et al. Investigation of second genetic hits at the BMPR2 locus as a modulator of disease progression in familial pulmonary arterial hypertension. Circulation 2005; 111: 607–613.
6. McLoughlin VV, McGoon MD. Pulmonary arterial hypertension. Circulation 2006; 114: 1417–1431.
7. Yuan JX, Rubin LJ. Pathogenesis of pulmonary arterial hypertension: the need for multiple hits. Circulation 2005; 111: 534–538.
8. Zhou XZ, Lu KP. The isomerase PIN1 controls numerous cancer-driving pathways and is a unique drug target. Nat Rev Cancer 2016; 16: 463–478.
9. Lu KP, Zhou XZ. The prolyl isomerase Pin1: a pivotal new twist in phosphorylation signalling and disease. Nat Rev Mol Cell Biol 2007; 8: 904–916.
10. Lu PJ, Zhou XZ, Shen M, et al. Function of WW domains as phosphoserine- or phosphothreonine-binding modules. Science 1999; 283: 1325–1328.
11. Feng D, Yao J, Wang G, et al. Inhibition of p66Shc-mediated mitochondrial apoptosis via targeting prolyl-isomerase Pin1 attenuates intestinal ischemia/reperfusion injury in rats. Clin Sci 2017; 131: 759–773.
12. Paneni F, Costantino S, Castello L, et al. Targeting prolyl-isomerase Pin1 prevents mitochondrial oxidative stress and vascular dysfunction: insights in patients with diabetes. Eur Heart J 2015; 36: 817–828.
13. Zhou XZ, Kops O, Werner A, et al. Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. Mol Cell 2000; 6: 873–883.
14. Liou YC, Zhou XZ, Lu KP. Prolyl isomerase Pin1 as a molecular switch to determine the fate of phosphoproteins. Trends Biochem Sci 2011; 36: 501–514.
15. Chen Y, Wu YR, Yang HY, et al. Prolyl isomerase Pin1: a promoter of cancer and a target for therapy. Cell Death Dis 2018; 9: 883.
16. Perrucci GL, Gowran A, Zanobini M, et al. Peptidyl-prolyl isomerases: a full cast of critical actors in cardiovascular diseases. Cardiovasc Res 2015; 106: 353–364.
17. Zannini A, Rustighi A, Campaner E, et al. Oncogenic hijacking of the PIN1 signaling network. Front Oncol 2019; 9: 94.
18. Crenshaw DG, Yang J, Means AR, et al. The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Pxl1. EMBO J 1998; 17: 1315–1327.
19. Li H, Wang S, Zhu T, et al. Pin1 contributes to cervical tumorigenesis by regulating cyclin D1 expression. Oncol Rep 2006; 16: 491–496.
20. Liou YC, Ryo A, Huang HK, et al. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proc Natl Acad Sci USA 2002; 99: 1335–1340.
21. Yeh ES, Lew BO, Means AR. The loss of PIN1 deregulates cyclin E and sensitizes mouse embryo fibroblasts to genomic instability. J Biol Chem 2006; 281: 241–251.
22. Shen M, Stukenberg PT, Kirschner MW, et al. The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. Genes Dev 1998; 12: 706–720.
23. Eckerd F, Yuan J, Saxena K, et al. Polo-like kinase 1-mediated phosphorylation stabilizes Pin1 by inhibiting its ubiquitination in human cells. J Biol Chem 2005; 280: 36575–36583.
Kojonazarov B, Sydykov A, Pullamsetti SS, Bourgeois A, Lambert C, Habbout K, Kruiswijk F, Hasenfuss SC, Sivapatham R, Weissmann N, Dietrich A, Fuchs B, Novoyatleva T, Kojonazarov B, Owczarek A, Ryo A, Nakamura M, Wulf G, Han HJ, Kwon N, Choi MA, Shen ZJ, Esnault S, Malter JS. The peptidyl-prolyl isomerase Pin1 regulates the stability of Reineke EL, Lam M, Liu Q, Blaydes JP, Luciani MG, Pospisilova S, Pulikkan JA, Dengler V, Peer Zada AA, et al. Elevated PIN1 expression by C/EBPalpha-p30 blocks C/EBPalpha-induced granulocytic differentiation through c-Jun in AML. Leukemia 2010; 24: 914–923. Blaydes JP, Luciani MG, Pospisilova S, et al. Stoichiometric phosphorylation of human p53 at Ser315 stimulates p53-dependent transcription. J Biol Chem 2001; 276: 4699–4708. Wulf GM, Liu YC, Ryo A, et al. Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage. J Biol Chem 2002; 277: 47976–47979. Reineke EL, Lam M, Liu Q, et al. Degradation of the tumor suppressor PML by Pin1 contributes to the cancer phenotype of breast cancer MDA-MB-231 cells. Mol Cell Biol 2008; 28: 997–1006. Shen ZJ, Esnault S, Malter JS. The peptidyl-prolyl isomerase Pin1 regulates the stability of granulocyte-macrophage colony-stimulating factor mRNA in activated eosinophils. Nat Immunol 2005; 6: 1280–1287. Hennig L, Christner C, Kipping M, et al. Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerase by juglone. Biochemistry 1998; 37: 5953–5960. Weissmann N, Dietrich A, Fuchs B, et al. Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. Proc Natl Acad Sci USA 2006; 103: 19093–19098. Novoyatleva T, Kojonazarov B, Owczarek A, et al. Evidence for the fucoidan/P-selectin axis as a therapeutic target in hypoxia-induced pulmonary hypertension. Am J Respir Crit Care Med 2019; 199: 1407–1420. Perros F, Ranchoux B, Izikki M, et al. Nebivolol for improving endothelial dysfunction, pulmonary vascular remodeling, and right heart function in pulmonary hypertension. J Am Coll Cardiol 2015; 65: 668–680. Novoyatleva T, Schymura Y, Jansen W, et al. Deletion of Fnl14 receptor protects from right heart fibrosis and dysfunction. Basic Res Cardiol 2013; 108: 325. Kojonazarov B, Sydykov A, Pullamsetti SS, et al. Effects of multikinase inhibitors on pressure overload-induced right ventricular remodeling. Int J Cardiol 2013; 167: 2630–2637. Lu Z, Hunter T. Prolyl isomerase Pin1 in cancer. Cell Res 2014; 24: 1033–1049. Colvin KL, Yeager ME. Animal models of pulmonary hypertension: matching disease mechanisms to etiology of the human disease. J Pulm Respir Med 2014; 4: 198. Rotin D. WW (WWP) domains: from structure to function. Curr Top Microbiol Immunol 1998; 228: 115–133. Ingham RJ, Colwill K, Howard C, et al. WW domains provide a platform for the assembly of multiprotein networks. Mol Cell Biol 2005; 25: 7092–7106. Lu KP, Hanes SD, Hunter T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. Nature 1996; 380: 544–547. Lu KP, Liou YC, Zhou XZ. Pinning down proline-directed phosphorylation signaling. Trends Cell Biol 2002; 12: 164–172. Hu X, Chen LF. Pinning down the transcription: a role for peptidyl-prolyl cis-trans isomerase Pin1 in gene expression. Front Cell Dev Biol 2020; 8: 179. Cheng CW, Tse E. PIN1 in cell cycle control and cancer. Front Pharmacol 2018; 9: 1367. Yeh ES, Means AR. PIN1, the cell cycle and cancer. Nat Rev Cancer 2007; 7: 381–388. Ryo A, Nakamura M, Wulf G, et al. Pin1 regulates turnover and subcellular localization of β-catenin by inhibiting its interaction with APC. Nat Cell Biol 2001; 3: 793–801. Ryo A, Suizu F, Yoshida Y, et al. Regulation of NF-κB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. Mol Cell 2003; 12: 1413–1426. Brenkman AB, de Keizer PL, van den Broek NJ, et al. The peptidyl-isomerase Pin1 regulates p27kip1 expression through inhibition of Forkhead box O tumor suppressors. Cancer Res 2008; 68: 7597–7605. Kruiswijk F, Hasenfuss SC, Sivapatham R, et al. Targeted inhibition of metastatic melanoma through interference with Pin1-FoxM1 signaling. Oncogene 2016; 35: 2166–2177. Bourgeois A, Lambert C, Habbout K, et al. FOXM1 promotes pulmonary artery smooth muscle cell expansion in pulmonary arterial hypertension. J Mol Med 2018; 96: 223–235. Fujimoto Y, Shiraki T, Horiuchi Y, et al. Proline cis/trans-isomerase Pin1 regulates peroxisome proliferator-activated receptor γ activity through the direct binding to the activation function-1 domain. J Biol Chem 2010; 285: 3126–3132. Zhang D, Wang G, Han D, et al. Activation of PPAR-γ ameliorates pulmonary arterial hypertension via inducing heme oxygenase-1 and p21(WAF1): an in vivo study in rats. Life Sci 2014; 98: 39–43. Han HJ, Kwon N, Choi MA, et al. Peptidyl prolyl isomerase PIN1 directly binds to and stabilizes hypoxia-inducible factor-1α. PLoS One 2016; 11: e0147038. Pullamsetti SS, Mamazhakypov A, Weissmann N, et al. Hypoxia-inducible factor signaling in pulmonary hypertension. J Clin Invest 2020; 130: 5638–5651. Rajbhandari P, Ozers MS, Solodin NM, et al. Peptidylprolyl isomerase Pin1 directly enhances the DNA binding functions of estrogen receptor α. J Biol Chem 2015; 290: 13749–13762.
Frump AL, Albrecht M, Yakubov B, et al. 17β-Estradiol and estrogen receptor α protect right ventricular function in pulmonary hypertension via BMPR2 and apelin. J Clin Invest 2021; 131: e129433.

Rustighi A, Tiberi L, Soldano A, et al. The prolyl-isomerase Pin1 is a Notch1 target that enhances Notch1 activation in cancer. Nat Cell Biol 2009; 11: 133–142.

Dabral S, Tian X, Kojonazarov B, et al. Notch1 signalling regulates endothelial proliferation and apoptosis in pulmonary arterial hypertension. Eur Respir J 2016; 48: 1137–1149.

Hu X, Dong SH, Chen J, et al. Prolyl isomerase PIN1 regulates the stability, transcriptional activity and oncogenic potential of BRD4. Oncogene 2017; 36: 5177–5188.

Van der Feen DE, Kurakula K, Tremblay E, et al. Multicenter preclinical validation of BET inhibition for the treatment of pulmonary arterial hypertension. Am J Respir Crit Care Med 2019; 200: 910–920.

Pullamsetti SS, Perros F, Chelladurai P, et al. Transcription factors, transcriptional coregulators, and epigenetic modulation in the control of pulmonary vascular cell phenotype: therapeutic implications for pulmonary hypertension (2015 Grover Conference series). Pulm Circ 2016; 6: 448–464.

Sysol JR, Natarajan V, Machado RF. PDGF induces SphK1 expression via Egr-1 to promote pulmonary artery smooth muscle cell proliferation. Am J Physiol Cell Physiol 2016; 310: C983–C992.

Firth AL, Yao W, Remillard CV, et al. Upregulation of Oct-4 isoforms in pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 2010; 298: L548–L557.

Becker EB, Bonni A. Pin1 in neuronal apoptosis. Cell Cycle 2007; 6: 1332–1335.

Ryo A, Hirai A, Nishi M, et al. A suppressive role of the prolyl isomerase Pin1 in cellular apoptosis mediated by the death-associated protein Daxx. J Biol Chem 2007; 282: 36671-36681.

Zungu-Edmondson M, Shults NV, Wong CM, et al. Modulators of right ventricular apoptosis and contractility in a rat model of pulmonary hypertension. Cardiovasc Res 2016; 110: 30–39.

Toko H, Konstandin MH, Doroudgar S, et al. Regulation of cardiac hypertrophic signaling by prolyl isomerase Pin1. Circ Res 2013; 112: 1244–1252.

Wu X, Li M, Chen SQ, et al. Pin1 facilitates isoproterenol-induced cardiac fibrosis and collagen deposition by promoting oxidative stress and activating the MEK1/2-ERK1/2 signal transduction pathway in rats. Int J Mol Med 2018; 41: 1573–1583.

Liu X, Liang E, Song X, et al. Inhibition of Pin1 alleviates myocardial fibrosis and dysfunction in STZ-induced diabetic mice. Biochem Biophys Res Commun 2016; 479: 109–115.

Xue C, Sowden M, Berk BC. Correction to: Extracellular cyclophilin A, especially acetylated, causes pulmonary hypertension by stimulating endothelial apoptosis, redox stress, and inflammation. Arterioscler Thromb Vasc Biol 2017; 37: e68.

Sugie S, Okamoto K, Rahman KM, et al. Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats. Cancer Lett 1998; 127: 177–183.

Wang P, Zhang SD, Jiao J, et al. ROS-mediated p53 activation by juglone enhances apoptosis and autophagy in vivo and in vitro. Toxicol Appl Pharmacol 2019; 379: 114647.

Wu J, Zhang H, Xu Y, et al. Juglone induces apoptosis of tumor stem-like cells through ROS-p38 pathway in glioblastoma. BMC Neurol 2017; 17: 70.

Xu M, Cheung CC, Chow C, et al. Overexpression of PIN1 enhances cancer growth and aggressiveness with cyclin D1 induction in EBV-associated nasopharyngeal carcinoma. PLoS One 2016; 11: e0156833.

Courboulin A, Barrier M, Perreault T, et al. Plumbagin reverses proliferation and resistance to apoptosis in experimental PAH. Eur Respir J 2012; 40: 618–629.

Kappus H, Sies H. Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. EXPERIMENTA 1981; 37: 1233–1241.

Ahmad T, Suzuki YJ. Juglone in oxidative stress and cell signaling. Antioxidants 2019; 8: 91.