Plumbagin reverses proliferation and resistance to apoptosis in experimental PAH

One-sentence summaries: Plumbagin reverses pulmonary hypertension

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Abstract

Similarly to cancer, pulmonary arterial hypertension (PAH) is characterized by a pro-proliferative and anti-apoptotic phenotype. In PAH, pulmonary artery smooth muscle cells (PASMC) proliferation is enhanced and apoptosis suppressed. The sustainability of this phenotype requires the activation of pro-survival transcription factors like STAT3 and NFAT. Presently, clinically available drugs able to efficiently and safely inhibit this axis are not available. We hypothesized that Plumbagin (PLB), a natural vegetal organic compound known to block STAT3 in cancer cells, reverses experimental pulmonary hypertension. We demonstrated, in vitro, using human PAH-PASMC, that PLB inhibits the activation of a STAT3/NFAT axis, increasing voltage-gated K+ current, BMPR2 and decreasing [Ca^{2+}], ROCK1 and IL-6, contributing to the inhibition of PAH-PASMC proliferation and resistance to apoptosis (PCNA, TUNEL, Ki67, Anexine V). In vivo, PLB oral administration decreases distal PA remodeling, mean PA pressure and right ventricular hypertrophy without affecting systemic circulation in both monocrotaline- and Sugen/chronic hypoxia-induced PAH in rats.

This study demonstrates that STAT3/NFAT axis can be therapeutically targeted by PLB in human PAH-PASMC and experimental PAH rat models. Thus, PLB could be considered as a specific and attractive future therapeutic strategy for PAH.

Key words: Sugen, apoptosis, Plumbagin, pulmonary hypertension, vascular remodeling
Abbreviation list

**BMPR2**: Bone Morphologenetic Protein Receptor type II

**DMSO**: Dimethyl sulfoxide

**MCT**: Monocrotaline

**NFAT**: Nuclear Factor of Activated T-cells

**PAAT**: Pulmonary Artery Acceleration Time

**PAH**: Pulmonary arterial hypertension

**PASMC**: Pulmonary Artery Smooth Muscle Cells

**PCNA**: Proliferating Cell Nuclear Antigen

**PDGF**: Platelet-Derived Growth Factor

**PLB**: Plumbagin

**ROCK1**: Rho-associated coiled-coil containing protein kinase 1

**RV**: Right Ventricle

**STAT3**: Signal Transducer and Activator of Transcription 3

**TMRM**: Tetramethyl Rhodamine Methly Ester

**TUNEL**: Terminal deoxynucleotidyl transferase dUTP nick end labeling
Pulmonary arterial hypertension (PAH) is a devastating disease of the pulmonary vasculature defined by an increase in PA pressure due to a sustained elevation of the pulmonary vascular resistance, which will rapidly induce the failure of the right ventricle [1]. At the cellular level, PAH is characterized by enhanced inflammation [2], proliferation, and resistance to apoptosis of pulmonary artery smooth muscle cells (PASMC) [3, 4]. The sustainability of this phenotype is due in part to the activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) [5, 6]. This suggests that STAT3 inhibition could be of a great therapeutic interest for PAH. Similarly to cancer, STAT3 activation in PAH has been associated with the upregulation of the oncogene provirus integration site for Moloney murine leukemia virus (Pim-1), promoting the activation of the transcription factor nuclear factor of activated T-cells (NFATc2) [6]. NFATc2 activation has been shown to account for both proliferation and resistance to apoptosis in cancer and PAH [4, 7]. Indeed, by downregulating K+ channels like Kv1.5, NFATc2 leads to cell depolarization, increasing intracellular Ca2+ concentration ([Ca2+]i), and promoting cell proliferation, while by upregulating Bcl-2, NFATc2 activation leads to mitochondrial hyperpolarization and apoptosis resistance [4]. Moreover, STAT3 axis is implicated in tumoral upregulation of survivin [8, 9], described as an important protein in the pathogenesis of PAH [10]. Finally, STAT3 has also been associated with bone morphogenetic protein receptor 2 (BMPR2) downregulation, further promoting the proproliferative, antiapoptotic PAH phenotype [11]. For all these reasons, the STAT3/NFAT axis can be considered as a major signaling hub for PAH.

Taken together, these studies suggest that STAT3 axis inhibition might represent an attractive therapeutic strategy for PAH. Nonetheless, no clinically available drugs targeting
STAT3 axis is currently available.

Plumbagin (PLB) or 5-hydroxy-2-methyl-1,4-naphthoquinone is a natural product found in the plants of Plumbaginaceae, Droseraceae, Ancestrocladaceae, and Dioncophyllaceae families. PLB has been shown to exert anticancer activities against a wide variety of tumor cells, including breast cancer, lung cancer in which NFAT is activated [4, 12-15]. However, the mechanisms accounting for PLB anticancer effects remain unknown. Recent studies have showed that PLB downregulates the expression of survivin and growth factor receptor [16], which are implicated in PAH [9, 17] and modulated by STAT3 [18, 19] and NFAT [4]. More importantly, PLB was found to be an efficient STAT3 inhibitor in cancer cells [20], thus promoting apoptosis [21]. Hence, we hypothesized that inhibition of the STAT3/NFAT axis by PLB would decrease PAH-PASMC pro-proliferative and anti-apoptotic phenotype, thus preventing and reversing established PAH.

**Materials and methods:** All the experiments were performed with the approval of the Laval University Ethic and Biosafety Committee. This study was in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). It conforms to the principles outlined in the Declaration of Helsinki. All patients gave informed consent before the study (Ethic Committee protocol number 20142).

**Cell culture:** Human PAH-PASMC were isolated from ≈1,5 μm–diameter small pulmonary arteries from 3 PAH patients. Human healthy PASMC (n=5) were purchased (Cell application USA, #302K-05a). PASMC were grown in high-glucose DMEM supplemented with 10% FBS (Gibco, Invitrogen, Burlington, ON, Canada) and 1% antibiotic/antimyototic (Gibco,
Invitrogen, Burlington, ON, Canada) [7] and used until the fifth passage. Plumbagin (PLB) was purchased from (Sigma-Aldrich) and dissolved in <1% of DMSO. For all the experiments PAH-PASMC and PASMC were treated for 48h. PDGF (30 ng/mL), endothelin-1 (10 nM), angiotensin II (200 nM) were all from EMB Canada.

**Immunofluorescence:** Measurements of the mitochondrial membrane potential (ΔΨm) and [Ca^{2+}], in live PASMC (37°C) were performed using tetramethylrhodamine methyl-ester perchlorate (TMRM) and Fluo-3AM from Invitrogen (Branchburg, NJ, USA) at a final concentration of 5µM, as previously described [4, 22]. TMRM and Fluo3 fluorescence intensity were measured (n=20 to 50 cells/patients in 3 PAH and 5 healthy patients). PASMC apoptosis rates were measured using TUNEL and Annexin V (Millipore, Temecula, CA) after serum starvation (0.1% FBS during 48h) and proliferation using Ki67 and the proliferating cell nuclear antigen PCNA antibody (DAKO, Carpinteria, CA) according to the manufacturer’s instructions [4, 22]. Percentages of cells nuclei positive for TUNEL, Annexin V, PCNA or Ki67 were determined (n=20 to 50 cells/patient in 3 PAH and 5 healthy patients or n=5 arteries/animal in 5 animals). PY705-STAT3 (Cell Signaling, 1/250) and NFATc2 (Abcam, 1/250) staining were performed as previously described [4]. The number of cells (%) presenting a nuclear localization of the protein were measured in n=20 to 50 cells/patient in 3 PAH and 5 healthy patients or n=5 arteries/animal in 5 animals). Alexa Fluor 488 or 594 (Invitrogen, Branchburg, NJ, USA, 1/1000) were used as secondary antibodies.

**Quantitative RT-PCR:** To measure NFATc2 and BMPR2 expression (Taqman Gene expression Assay, Applied Biosystem, Foster, CA, USA) total mRNA was extracted from PAH-PASMC or control PASMC using trizol protocol, as previously described [4, 22]. 18S was used as housekeeping gene for qRT-PCR.
**Western Blot:** Total protein fraction was extracted from either PASMC or distal PA as indicated. PY705-STAT3, STAT3, (both Cell signaling, 1/1000) were quantified and normalized to the smooth muscle Actin (Santa Cruz, 1/400) as described (Bonnet et al., 2007). BMPR2 (Abcam, 1/1000), ROCK1 (BD, 1/1000) and PKCε (Santa Cruz, 1/100) were normalized to Ponceau Red. Evaluation for PY705-STAT3/STAT3 were obtained from the same gel after striping (30 min at 50°C).

**Electrophysiology:** Standard whole-cell patch-clamping was realized on voltage-clamped cells at a holding potential of -70mV, with solutions permitting potassium current [22]. Currents were evoked by 200ms test pulses from -70mV to +70mV, with 10mV steps, as previously described [22] (filtered at 1kHz and sampled at 2-4kHz). The results are shown with the current density as the observed cell current was normalized with its own cellular capacity. The results are also shown with 4-AP sensitive current, representing the amount of K⁺ current Kv channels dependent, as 4-AP is a Kv channels blocker. PAH-PASMC or healthy PASMC were chronically treated with PLB (48h; 1µM), and with 4-AP (1mM) during the patching-clamp.

**In vivo experiments:** Male Sprague-Dawley Rats were injected s.c. with 60 mg.Kg⁻¹ of monocrotaline (MCT) (Sigma). Plumbagin was given orally (gavage) at the concentration of 4mg.Kg⁻¹, either every day during 4 weeks following MCT injection, before PAH establishment (prevention protocol) (n=5), or every day during 2 weeks after PAH establishment (hemodynamic measurement), i.e 15 days after MCT injection (reversal protocol) (n=5).

Male Sprague Dawley Rats were injected s.c. 20 mg.Kg⁻¹ of Sugen (Sigma Aldrich #S8442-25MG), and maintained in Hypoxia (10% O₂) during 3 weeks. Two weeks after the end of hypoxia protocol, the rats were orally (gavage) treated with PLB at 4mg.Kg⁻¹ during 2 weeks.
**Hemodynamic measurements:** All rats underwent hemodynamic and echocardiography measurements as previously described [4]. Briefly, PAAT (pulmonary arterial acceleration time) known to decrease with the PAH severity in both rats and patients was measured using Echo-Doppler [4, 23], as well as right ventricle thickness, by echocardiography. Right catheterizations (closed chest) were performed using Science catheters.

**Histology measurements** PA media wall thickness was assessed as previously described [4]. Briefly, paraffin lung sections were stained with hematoxylin-eosyn, and PA media wall thickness was measured using Image ProPlus software (Media Cybernetics). Two measurements/artery in 5 animals for each group were performed.

**Data analysis:** Averaged data are presented as the mean±SEM. Normality of data was assessed by the Shapiro-Wilk normality test. All data were normally distributed. For comparison between two means, unpaired Student’s t test was used. One-way analysis of variance (ANOVA) followed by the Dunn’s post test was used for comparison between more than two means. For correlation, Pearson’s test was performed. Significances were represented as follow: (p<0.05) is indicated by (*), (p<0.01) by (**) and (p<0.001) by (***)

In cultured cell based experiment n indicates the number of experiments, while in vivo, it indicates either the number of cells measured in n animals or the number of animals only.

**Results**

**Plumbagin decreases PAH-PASMC proliferation and resistance to apoptosis**

To study Plumbagin effects on PASMC proliferation and apoptosis *in vitro*, human PAH-PASMC were exposed to 10% FBS (a condition that is known to promote proliferation) or 0.1% FBS (a “starvation” condition that promotes apoptosis) [4, 7, 24]. PAH-PASMC were treated with increasing doses (1nM, 10nM, 100nM, 1µM, 5µM, 10µM) of Plumbagin or its proper
vehicle (DMSO) (Fig. 1a). Compared to healthy PASMC, PAH-PASMC have a greater proliferation rate and were more resistant to starvation-induced apoptosis. PLB dose-dependently decreases proliferation and promotes apoptosis. Based on our dose/response effects, we decided to use PLB at 1µM (also corresponding to the dose previously used in other studies) [20] and able to significantly decrease both proliferation and resistance to apoptosis. At 1µM, PLB in “starved” PAH-PASMC (0.1% FBS) increases apoptosis measured by both TUNEL and annexin V when compared to vehicle treated PAH-PASMC (Fig. 1a, 1b). While in PAH-PASMC exposed to 10% FBS, PLB (1µM) decreases by ~ 2-fold proliferation measured by PCNA and Ki67 (Fig. 1a, 1b). Moreover, PLB effects on apoptosis were confirmed by TUNEL in staurosporine treated PASMC [25, 26] (Supplemental Fig. 1a).

Plumbagin effects on proliferation were further confirmed in healthy PASMC treated for 48h with PDGF (30 ng/mL), AngII (200 nM) and ET-1 (10 nM), all accepted STAT3 activators [27] and all known to be increased in PAH [28, 29] in presence and absence of PLB. As expected, PLB significantly decreases proliferation in Ang II, PDGF and ET-1 treated PASMC (Supplemental Fig. 1b).

**Plumbagin decreases the STAT3/NFAT axis activation in PAH-PASMC.**

We have previously shown that the pro-proliferative and anti-apoptotic phenotype seen in PAH-PASMC was mostly attributed to the activation of the STAT3/NFAT axis [4-6]. Thus, as PLB significantly decreases PAH-PASMC proliferation and resistance to apoptosis, we measured whether PLB affects STAT3 and NFATc2 activation in PAH-PASMC. STAT3 and NFATc2 activations were measured in human PASMC-isolated from control and non-familial PAH patient by qRT-PCR (NFATc2), immunoblot (PY705-STAT3/STAT3) and nuclear translocation assay
(confocal microscopy). Compared to control PASMC, PY705-STAT3/STAT3 ratio was increased by ~2-fold in PAH-PASMC (Fig. 2a), which was associated with a greater nuclear translocation (Fig. 2b). Similarly, a ~2-fold increase in NFATc2 expression (Fig. 2a) and nuclear translocation (Fig. 2b) was also observed in PAH-PASMC compared to control (Fig. 2). PLB (1µM for 48h) decreases both STAT3 and NFAT activation in PAH-PASMC. These findings suggest a significant activation of STAT3 and NFATc2 in PAH-PASMC compared to control-PASMC, which can be inhibited by PLB. (Fig. 2b).

**Plumbagin reverses the activation of pathophysiological pathways affected by the activation of the STAT3/NFAT axis.**

In PAH-PASMC, STAT3/NFAT-mediated proliferation [4, 30] has been linked to the downregulation of voltage gated K⁺ channels (Kv) [31, 32] resulting in membrane depolarization [31, 33], voltage-dependent calcium channels opening, and thereby increasing intracellular calcium concentrations ([Ca²⁺]ᵢ) [4, 30, 33]. Using whole cell patch clamping, we demonstrated that PLB (48h) restores the decrease in total K⁺ current density observed in PAH-PASMC (Fig. 3a, 3b). This increase in K⁺ current was attributed to the activation of the Kv channels as this increase was totally blocked by 4-AP, a specific Kv channels blocker [34]. (Fig. 3a, 3c). Note that PLB has no effects on healthy PASMC. These results are consistent with previously published studies [4, 35] demonstrating that STAT3/NFAT axis is implicated in Kv channel regulation such as Kv1.5.

Using Fluo-3AM technique, we investigated whether the increases in Kv current by PLB decreases [Ca²⁺]ᵢ. As expected, PLB decreases [Ca²⁺]ᵢ in PAH-PASMC to a level similar to ones seen in healthy-PASMC (Fig. 4a).
Because STAT3 and NFAT are implicated in mitochondrial membrane potential regulation [7, 36], the increase in apoptosis following PLB treatment might result from the activation of mitochondrial-dependent apoptosis. Since the mitochondria transition pore is voltage-dependent [37] the mitochondrial membrane potential ($\Delta \Psi m$) depolarization is a threshold index for mitochondrial-dependent apoptosis. In fact, apoptosis is associated with decreased $\Delta \Psi m$. To investigate the mechanism by which Plumbagin promotes apoptosis, we measured $\Delta \Psi m$ using tetramethylrhodamine methyl-ester (TMRM) in PAH-PASMC treated with PLB. PLB caused a significant $\Delta \Psi m$ depolarization (decreased TMRM red fluorescence), compared to vehicle treated PAH-PASMC. These data confirmed that Plumbagin affects the $\Delta \Psi m$ and therefore enhances mitochondrial-dependent apoptosis (Fig. 4a).

In addition to both the Kv/calcium axis and $\Delta \Psi m$, other pathways are known to be implicated in PAH-PASMC proliferation and resistance to apoptosis including BMPR2 protein and mRNA expression [38]; ROCK1 activation [39] and mRNA IL-6 expression [40]. Although the importance of each pathway in the etiology of PAH remains to be established (and is likely to be different among the different form of PAH), we provide evidences that PLB can affects all of them. Indeed, PLB significantly increases BMPR2 expression and decreases IL-6 and ROCK1 in PAH-PASMC (Fig. 4b). Although the exact molecular mechanism by which PLB affects these pathways remains unknown, the fact that all of them have been showed to be affected by STAT3 reinforce the concept demonstrating that STAT3/NFAT axis is an important integrative signal hub in PAH and that its inhibition might represent a novel and effective therapeutic strategies to improve PAH.

*Plumbagin reverses experimental PAH in rats.*
To determine the putative therapeutic potential of PLB, we determine *in vivo* whether PLB (4mg.Kg⁻¹/ day *per os*) can reverse establish PAH, in two accepted experimental rat PAH models: the monocrotaline (MCT) induced-PAH and the SUGEN/chronic hypoxia models. Longitudinal studies to assess the efficiency of our treatments were performed using non-invasive measurements (exercise capacity test; Doppler and Echocardiography) [4]. In prevention MCT model, PLB prevents changes in pulmonary hemodynamics, RV free wall thickness, general cardiac functions (exercise capacity on treadmill) seen in the vehicle treated rats. When given in rats with established PAH (2 weeks post MCT injection to reverse the disease), we showed that PLB increases PAAT (PA acceleration time: a Doppler parameter known to well correlate with PA pressure in both Humans and Rats; as the PA pressure rises, PAAT shortens) [4, 41]; decreases RV wall thickness and increases exercise capacity (Fig. 5a). These findings were invasively confirmed by direct PA pressure measurements in close chest animals (Fig. 5b). Interestingly the cardiac output was not affected by PLB treatment (Fig. 5b).

In order to determine whether PLB reduces PA remodeling in MCT-PAH rats, we measured PA media wall thickness, using H&E staining on lung histological sections. PLB given in prevention or in attempt to reverse established PAH in rats showed a significant reduction in the percentage medial thickness in small and medium-sized PA (Fig. 5c). This finding was associated with significant decrease in STAT3 and NFATc2 activation (Fig. 6a), and also in Src activation (Fig. 6b), which we previously shown to be the pathway activating STAT3 in PAH-PASMC [5], decreasing PASMC proliferation (Ki67) and resistance to apoptosis (TUNEL), *in vivo* (Fig. 6c, Suppl Fig. 2a).

Although MCT-induced PAH model is a very robust model of PAH, to further demonstrate the therapeutic effect of PLB, we tested whether PLB can improve PAH in rats injected (*s.c.*) with SUGEN, a type 2 VEGF receptor antagonist [42, 43] and exposed to chronic
hypoxia (10% O2) for 3 weeks, and placed back under normoxic conditions for an additional 4 weeks. PLB (4mg.Kg⁻¹/ day per os) has given once PAH was established (5 weeks post Sugen injection) for 2 weeks.

Although in a less efficient way than in the MCT-model, PLB significantly decreases mean PA pressure, RV hypertrophy and distal PA remodeling. (Fig. 7a). As expected, these effects were associated with decreased distal PA PASMC proliferation and increased apoptosis (Fig 7a, Suppl Fig 2b), associated with a significant decrease in STAT3 and NFATc2 activation measured by nuclear translocation assay in distal PA (Fig 7b) (<300μm), and also with a significant decrease in Src activation (Fig. 7c).

Discussion:

Here we provide, for the first time, evidences showing that Plumbagin (PLB) decreases the STAT3 constitutive activation seen in PAH. We showed that PLB-dependant STAT3 inhibition reverses PAH phenotype both in vitro and in vivo. Indeed, PLB restores most of the molecular and cellular abnormalities seen in human PAH-PASMC, including decreased activation of NFATc2, STAT3, IL-6 and ROCK1 and upregulation of BMPR2 (Fig. 2, 4). All these demonstrated pathways contribute to the decrease in human PAH-PASMC proliferation and resistance to apoptosis. In vivo, using 2 independent experimental models of PAH (MCT and Sugen/chronic hypoxia) we provide strong evidences that PLB is able to reverse PAH, highlighting its possible efficiency to treat this lethal pathology in PAH patients. Indeed, in both model Plumbagin significantly improves PA pressure, PA medial hypertrophy, and RV hypertrophy, without affecting systemic pressure and cardiac outputs (Supp Fig. 5, 6, 7).
Although previous studies have reported putative inotropic effect of PLB in isolated heart model, our in vivo study with PLB given orally did not affect significantly the global cardiac function such as cardiac output [44, 45]. Moreover, it significantly improves exercise tolerance in rats with PAH, without affecting systemic pressure (Fig 5b, 7a). Although several other measurements are needed to totally exclude long-term toxicity or adverse effects, our findings open new avenue of investigation and support a putative therapeutic role of PLB in PAH.

Indeed, we are the first group reporting an efficient improvement of PAH in the Sugen/chronic hypoxic rat model. In fact, the efficiency of PLB in 2 independent PAH model such as the monocrotaline and the Sugen models is true indication that PLB is of great therapeutic interest. Moreover, we demonstrated in both model that as in our human PAH-PASMC, both STAT3 and NFAT are up regulated in the distal PA and their inhibitions significantly improve PAH in the Sugen treated rats, this strongly reinforces the importance of this pathway in the etiology of PAH.

The activation of STAT3/NFAT axis that we described likely has a multifactorial etiology in PAH. Indeed, both STAT3 and NFAT might be critical integrators of multiple signaling pathways and their downstream effects might explain several and important features of PAH. This could explain why STAT3 inhibition might be so efficient in reversing PAH. In vivo, endothelial dysfunction and inflammation are recognized as ones of the earliest abnormalities in PAH, resulting in a well-recognized imbalance of endothelium-derived vasoactive factors; with increased vasoconstrictors (endothelin [46], thromboxane [47]), all of which lead to STAT3 activation and decreased vasodilators (like NO or prostacyclin [47]). In addition, increased circulating growth factor [48] and cytokines [49] have been reported in PAH, which also activate STAT3. Thus, circulating factors are likely implicated in STAT3 activation in PAH. Due to the presence of STAT3 binding sequence within NFATc2 promoter region [50], the increase in
NFATc2 expression seen in PAH-PASMC has been attributed to STAT3 activation [5, 6]. Moreover, STAT3 positively regulates the NFAT activator Pim1, which could explain the increase in NFAT activation [10]. Once activated, NFAT regulates multiple genes that might positively reinforce its own expression and activation. For example, the down-regulation of Kv1.5 leads to PASMCs depolarization, opening of L-type Ca$^{2+}$ channels and sustain the increase in [Ca$^{2+}$], (as shown in our study) and thus calcineurin-dependent NFAT activation [4]. NFAT is not the only mechanism affected by STAT3 in PAH phenotype. Indeed, it has been recently proposed that STAT3 activation upregulates microRNAs (miR-17/92) accounting for the downregulation of the receptor BMPR2 in PASMC [11]. Moreover, several STAT3 related proteins have been implicated in RhoA/ROCK activation [51] along with IL-6 expression [11]. This not only demonstrates the importance of the STAT3 axis in PAH, but also shows that STAT3 can be considered as an integrator of a multiple pathways implicated in PAH including NFAT activation, BMPR2 downregulation RhoA/ROCK activation and IL-6 expression [39, 52]. Thus, we believe that the Plumbagin-dependant inhibition of STAT3 could explains how a single drug such as Plumbagin can affect so many pathophysiological pathways and be efficient in several experimental models (Fig. 5, 6, 7).

We are not the first group reporting beneficial health effects of PLB. In fact, this is natural organic compound has been previously shown to have anti-proliferative and pro-apoptotic properties, (in part through STAT3 inhibition [20]) in cancer and vasoactive properties in bovine pulmonary arteries [53], all of which agreeing with a positive therapeutic effect in PAH. In this study, we demonstrated an inhibitory effect of PLB on STAT3 activation in human PASMC and showed that it also blocks NFAT expression and activation. Both effects induce mitochondrial membrane potential depolarization and decrease [Ca$^{2+}$], through the upregulation of Kv channels as previously described [4, 36]. These effects are likely responsible for the inhibition of PAH-
PASMC proliferation and resistance to apoptosis in vitro and in vivo in the reduction of distal PA remodeling processes. Indeed, we and others have previously reported that increasing Kv channels such as Kv1.5 in PAH-PASMC is sufficient to decrease $[\text{Ca}^{2+}]$, and PASMC proliferation [34, 54], while depolarizing mitochondria [9, 55] is sufficient to promote apoptosis in PAH-PASMC. As both mechanisms are controlled by STAT3 and NFAT [4, 6], the inhibition of the STAT3/NFAT axis by PLB should promote PAH-PASMC apoptosis and decrease proliferation. This is supported by our findings.

The mechanism of action of PLB remains elusive. While the mechanisms leading to NFAT inhibition by PLB most likely rely on STAT3 inhibition [5, 6], the mechanism accounting for STAT3 inhibition remains to be established. We recently showed that JAK2 (one of the most important STAT3 regulator [56]) is not implicated in PAH-PASMC [5], thus the effect of PLB is unlikely mediated by JAK2 inhibition. The second regulator of STAT3 activation is the Src pathway [57]. Src has been shown to be implicated in PAH [5, 30] and in the activation of STAT3 in human PAH-PASMC [5]. Although the implication of Src in STAT3 activation in human PAH-PASMC has not been reassessed in the present study, we showed that Src is activated in the distal PA’s of both MCT and Sugen rats, confirming our previous findings in humans. Moreover, we have evidences that Src activation is decreased in PLB treated animal (Fig. 6b, 7c). Thus, an inhibition of the Src pathway by Plumbagin as shown in cancer cells [20], could explain the STAT3 inhibition by Plumbagin seen in PAH.

STAT3 being integrator of multiple pathways implicated in PAH like NFAT, BMPR2 and ROCK; it is not surprising that its inhibition by Plumbagin is sufficient to improve PAH in both MCT-and SUGEN models. Although several studies have shown that PLB can also affect many other pathways including Akt or PKC epsilon, which are implicated in systemic vascular remodeling processes [22, 58], their implications in PAH remain nonetheless elusive. For
example, Akt is not activated in PAH [6], thus its putative inhibition by PLB should not affect PAH. The role of PKC epsilon in PAH has been suggested especially in the contractile response and endothelial cells physiology; for example PKC epsilon inhibition decreases the acute hypoxic vasoconstriction [59]. Nonetheless PKC epsilon knock out mice had a greater increase in right ventricular (RV) systolic pressure, and RV mass in response to chronic hypoxia than PKC-epsilon(+/−) mice [60]. Thus, if PLB effects were mediated by PKC epsilon inhibition, PLB should worsen PAH and not improve it as shown in our study (Suppl Fig 3). Although the implication of other pathways cannot be ruled out, we believe that since Plumbagin can affect most of the pathophysiological pathways implicated in PAH (STAT3, NFAT, BMPR2, ROCK, IL-6), it is already clinically very appealing.

Several other studies are needed to test the exact mechanism of action of Plumbagin along with putative toxicity effects (no side effects were observed in our treated animals). Nevertheless, our findings are strong evidences that such investigations are needed to eventually propose PLB as a new therapeutic tool for PAH. We believe that it will also lead to a better understanding of the regulation of apoptosis and proliferation by PLB, which will benefit many other human diseases like cancers.

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References


8. Lin L, Fuchs J, Li C, Olson V, Bekaii-Saab T, Lin J. STAT3 signaling pathway is necessary for cell survival and tumorsphere forming capacity in ALDH(+)/CD133(+) stem cell-like human colon cancer cells. *Biochem Biophys Res Commun*.


Figures legends:

Figure 1: Plumbagin (PLB) dose-dependently reduces PAH-PASMC proliferation and resistance to apoptosis. 

a) PCNA showed a significant increase in PAH-PASMC proliferation compared to control-PASMC; PLB (1 nM, 10 nM, 100 nM, 1 μM, 5 μM, 10 μM) dose-dependently decrease PAH-PASMC proliferation. TUNEL assay revealed a significant resistance to apoptosis in PAH-PASMC compared to healthy PASMC; which was dose-dependently decreased by PLB (1 nM, 10 nM, 100 nM, 1 μM, 5 μM, 10 μM) (n=100-150 cells/patient) (** p<0.01; ***p<0.001). Given these results, PLB concentration for the following experiments was chosen to be 1 μM. 

b) PLB (1 μM for 48h), reverses PAH-PASMC proliferation (Ki67) and resistance to apoptosis (Annexin V) (n=100-150 cells/patient) (** p<0.01; ***p<0.001).
Figure 2: Plumbagin (PLB) reverses STAT3/NFAT activation in human PAH-PASMC. a) NFATc2 expression was quantified by qRT-PCR in human healthy and PAH PASMC. mRNA expression was normalized to 18S. As shown, NFATc2 expression is significantly increased in PAH-PASMC compare to control-PASMC. PLB (1 μM for 48h) significantly decreases NFATc2 expression (n=3 experiments/patient; ** p<0.01). PY705-STAT3/STAT3 ratio, accounting for STAT3 activation, was assessed by immunobot. As shown, STAT3 is upregulated in PAH-PASMC compared to control-PASMC and PLB (1 μM for 48h) reverses this activation (n=3; * p<0.05). b) NFATc2 and STAT3 activation were further assessed in PASMC by nuclear translocation assay. As shown, the % of NFATc2 and P-STAT3...
translocated to the nucleus is significantly increased in PAH-PASMC compared to healthy. PLB (1 µM for 48h), significantly decreases both NFATc2 and STAT3 activation. (n=100-150 cells/patient) (** p<0.01; ***p<0.001).

Figure 2

**Plumbagin decreases NFATc2 expression and STAT3 activation in PAH-PASMC**

![Graph showing NFATc2 and STAT3 activation](image)

**STAT3 and NFATc2 activation decrease in PAH-PASMC treated with Plumbagin**

![Graph showing STAT3 and NFATc2 activation decrease](image)

Figure 3: Plumbagin reverses K⁺ current density decrease in PAH-PASMC throw the increase of 4-AP sensitive current density increase. a) Representative K⁺ currents with or without PLB (1 µM for 48h) and 4-AP (1 mM) in healthy and PAH-PASMC. b) PAH-PASMC have a significant decrease of K⁺ current density compared to vehicle treated PASMC. PLB (1µM for 48h) increases PAH-PASMC current density to a level similar to the one seen in
healthy PASMC (* p<0.05; ** p<0.01). c) The increased current density mainly rely on an upregulation of the voltage-gated K⁺ channels (Kᵥ) current as shown by a greater sensitivity to the Kᵥ channel blocker 4-AP at +70mV (* p<0.05).

Figure 3

a) Representative K⁺ currents with or without chronic PLB and 4-AP treatment in healthy and PAH-PASMC

b) Plumbagin reverses current density decreased in PAH-PASMC

c) Plumbagin reverses 4-AP sensitive current density decreased in PAH-PASMC

Figure 4: Plumbagin decreases [Ca²⁺]ᵢ in PAH-PASMC and the mitochondrial hyperpolarization; reverses BMPR2 and ROCK1 downregulation and IL-6 upregulation. a) PAH-PASMC have a significant increase in [Ca²⁺]ᵢ compared to healthy PASMC, which is reversed PLB (1 μM for 48h). Furthermore, PAH-PASMC display ΔΨᵢ hyperpolarization compared to healthy PASMC. PLB (1 μM for 48h) causes a significant ΔΨᵢ depolarization (decreased TMRM red fluorescence), compared to vehicle treated PAH-PASMC (*** p<0.001).
b) Compared to healthy PASMC (n=5 patients), PAH-PASMC (n=3 patients) have a significant decrease in BMPR2 expression (qRT-PCR and Western Blot n=3 experiments) and an upregulation in ROCK1 (Western Blot n=3), both of which are reversed by PLB (1 μM for 48h) (* p<0.05). Finally, PLB (1 μM for 48h) decreases significantly IL6 expression (qRT-PCR n=3) in PAH-PASMC (* p<0.05).

Figure 4

a  Plumbagin reverses the [Ca\textsuperscript{2+}]\textsubscript{i} increase (Fluo-3AM) and the mitochondrial hyperpolarization (TMRM) in PAH-PASMC.

b  BMPR2, IL-6 and ROCK1 expression is restored by PLB in PAH-PASMC.

Figure 5: Plumbagin prevents and reverses PAH in rats injected with monocrotaline.

The effects of orally delivered PLB (4mg/kg/day) in the prevention protocol (PLB treatment beginning the same day that MCT injection, before PAH establishment) for 4 weeks or to in the reversion protocol (PLB treatment beginning 2 weeks post MCT injection once PAH is
established) for 2 weeks were longitudinally assessed for 4 weeks. a) As shown, PLB prevents and reverses PAH (n=5 rats per group) assessed non-invasively by Doppler echocardiography (PAAT), M-mode ultrasound (RV hypertrophy), and exercise capacity (treadmill), (n=5 rats for each group; ** p<0.01; *** p<0.001). b) These findings were invasively confirmed by direct PA pressure measurements (n=5 rats for each group; * p<0.05; ** p<0.01; *** p<0.001). Note that PLB did not affect cardiac output. c) PLB treatment significantly prevents and reverses distal PA (<300 µm) remodeling measured by Hematoxylin/Eosin (H&E) coloration (n=10 arteries/rat with 5 rats per condition; * p<0.05; ** p<0.01; *** p<0.001).

Figure 5

a) Plumbagin reverses PAH development in MCT PAH rats

b) Plumbagin decreases mean PA pressure in MCT PAH rats

c) Plumbagin decreases PA wall thickness in MCT PAH rats
Figure 6: Plumbagin decreases STAT3, NFATc2 and Src activation, decreasing the associated PASMC proliferation and resistance to apoptosis seen in distal PA. a&b) PLB treated animals had a significant reduction in STAT3, NFATc2 and Src activation in distal PA’s measured by immunofluorescence compared to the distal PA of untreated rats (n=5 arteries/rat with 5 rats in each condition) (* p<0.05, *** p<0.001). c) This is associated with a decrease in PASMC proliferation (Ki67; n=5 arteries/rat in 5 rats; p<0.05) and resistance to apoptosis (TUNEL n=5 arteries/rat in 5 rats; p<0.05) both in prevention and reversion protocol.

Figure 7: In vivo, on Sugen-induced rats, Plumbagin administration reverse established PAH. The effects of orally delivered PLB (4mg/kg/day) was assessed in the
Sugen/chronic hypoxia rat model a) PLB reverses PAH (n=5 rats per group * p<0.05; ** p<0.01; *** p<0.001) assessed by direct PA pressure measurements, without significantly affecting systemic pressure or cardiac output. Furthermore, PLB reverses RV hypertrophy assessed invasively by the Fulton index (n=5 rats for each group; * p<0.05; ** p<0.01; *** p<0.001). The remodeling has also been assessed by Hematoxylin/Eosin coloration (n=10 arteries/rat with 5 rats per condition). PLB treatment significantly reverses distal PA (<300µm) remodeling. These results are associated with a PLB-dependant decrease in proliferation and increase in apoptosis in Sugen treated rats. b&c) At a protein level, PLB treatment is associated with a significant decrease in both STAT3, NFATc2 and Src activation (n=5 arteries/rat with 5 rats in each condition) (* p<0.05, *** p<0.001).

Figure 7

a Plumbagin reverses PAH development in experimental SUGEN-PAH rats

b Plumbagin reverses STAT3/NFATc2 activation in SUGEN PAH rats

c Plumbagin reverses P Src activation in SUGEN-PAH rats (IF)
Supplemental Figure 1: PLB promotes staurosporine induced-apoptosis in PAH-PASMC and decrease PASMC proliferation in growth factor/agonist stimulated healthy-PASMC. a) PAH-PASMC are resistant to staurosporine-induced apotosis, which is significantly reversed by PLB treatment (1 μM for 48h). b) Healthy PASMC display an increase in proliferation rate when stimulated with pro-proliferative factors (angiotensin II (AngII; 200 nM), Endothelin 1 (ET-1; 10nM) and PDGF (30 ng.mL-1) (n=50/patient in 5 healthy patients), which is significantly reduced with PLB treatment (1 μM for 48h) (*** P<0.001).

Supplemental Figure 2: In vivo, Plumbagin treatment decreases proliferation in pulmonary arteries. a) PASMC proliferation is increased in distal pulmonary arteries (<300μm) in MCTPAH rats compared to control rats. b) Similarly, PLB decreases proliferation (PCNA) and promotes apoptosis (TUNEL) in Sugen rats.

Supplemental Figure 3: PLB doesn’t affect PKCε expression in PAH-PASMC. PKCε expression was quantified by western blot and normalized by ponceau red. PLB doesn’t modulate the expression of PKCε in PAH-PASMC.