Role for DNA Damage Signaling in Pulmonary Arterial Hypertension

Running title: Meloche et al.; PARP inhibitors reverse pulmonary hypertension

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Abstract

Background—Pulmonary arterial hypertension (PAH) is associated with sustained inflammation known to promote DNA damage. Despite these unfavorable environmental conditions PAH pulmonary arterial smooth muscle cells (PASMC) exhibit, in contrast to healthy PASMC, a pro-proliferative and anti-apoptotic phenotype, sustained in time by the activation of miR-204, NFAT and HIF-1α. We hypothesized that PAH-PASMC have increased activation of Poly(ADP-ribose) polymerase-1 (PARP-1), a critical enzyme implicated in DNA repair, allowing proliferation despite the presence of DNA damaging insults, eventually leading to PAH.

Methods and Results—Human PAH distal pulmonary arteries and cultured PAH-PASMC exhibit increased DNA damage markers (53BP1 & γ-H2AX) as well as an overexpression of PARP-1 (immunoblot & activity assay), compared to healthy tissues/cells. Healthy PASMC treated with a clinically relevant dose of TNF-α harbored a similar phenotype, suggesting that inflammation induces DNA damage and PARP-1 activation in PAH. We also showed that PARP-1 activation accounts for miR-204 downregulation (qRT-PCR) and the subsequent activation of the transcription factors NFAT and HIF-1α in PAH-PASMC, previously shown to be critical for PAH in several models. These effects resulted in PASMC proliferation (Ki67, PCNA and WST1 assays) and resistance to apoptosis (TUNEL and AnnexinV assays). In vivo, the clinically available PARP inhibitor ABT-888 reversed PAH in two experimental rat models (Sugen/hypoxia and monocrotaline).

Conclusions—These results show for the first time that the DNA damage/PARP-1 signaling pathway is important for PAH development and provide a new therapeutic target for this deadly disease, with high translational potential.

Key words: pulmonary hypertension, animal model of human disease remodeling, PARP-1, DNA damage, miR-204, Sugen
Pulmonary arterial hypertension (PAH) is a disease with no known cure, characterized by obstructive vascular lesions of the distal pulmonary arteries (PA) due in part to excessive proliferation and resistance to apoptosis of the pulmonary artery smooth muscle cells (PASMC). This phenotype is often associated with a chronic inflammatory response characterized by sustained elevation of circulating pro-inflammatory molecules such as TNFα and IL-6. Although an environment of strong inflammation is known to be toxic for the cells and is known to induce DNA damage, PAH-PASMC apparently adapt to these unfavorable environmental conditions and are able to survive and proliferate. Indeed, PAH-PASMC share several features with cancer cells (which also proliferate under unfavorable conditions of accumulated genetic damage or hypoxia), including the expression of pro-survival proteins such as Pim-1 and survivin and a metabolic switch toward glycolysis even under normoxia (known as the Warburg effect). We have recently shown that all of these changes can be sustained in time by the downregulation of the microRNA miR-204, and the subsequent activation of the transcription factors NFAT and HIF-1α.

Nonetheless, a question remains unanswered: How can PAH-PASMC adapt to the DNA damage caused by sustained inflammation? We hypothesized that PAH is associated with a sustained activation of the DNA repair machinery that, in addition to DNA repair, may also directly promote PASMC proliferation.

Poly(ADP-ribose) polymerase-1 (PARP-1) is the best characterized and most abundant member of the PARP family, which currently comprises 6 members. Upon activation, by binding to DNA double-strand breaks (DSB), PARP-1 uses Nicotinamide adenine dinucleotide (NAD+) to generate large amounts of poly(ADP-ribose) (pADPr) along the break-site and therewith contributes actively to DNA repair. PARP-1 can trigger DNA repair and therewith
allow/promote cell survival (when limited stress leads to a light to moderate PARP-1 activation) or promote cell death when the stress and the subsequent PARP-1 activation and hence its NAD$^+$ consumption are excessive. PARP-1 can function as a cellular stress sensor. In addition to DNA repair, PARP-1 may be implicated in many other features seen in PAH. For example, PARP-1 is implicated in the transcriptional regulation of a number of genes involved in PAH including IL-6. Cells with activated PARP-1 produce high levels of IL-6, which can amplify inflammation and, by activating STAT3, can contribute to NFAT and HIF-1 activation which promotes the apoptosis resistance that characterizes PAH-PASMC. Using lung tissues and primary cultures of distal pulmonary artery PASMC from both humans and rodents, we demonstrate for the first time that PAH is associated with sustained DNA damage that leads to PARP-1 activation. PARP-1 activation promotes cell survival and proliferation by triggering a miR-204-dependent activation of NFATc2 and HIF-1. Most importantly, we show that the clinically available PARP inhibitor ABT-888 reverses the PAH in vivo in two PAH rat models.

Methods
All experiments were performed with Laval University and the IUCPQ Biosafety and Ethics Committee. The online-only Data Supplement provides more details on all methods, including the supplies and chemicals used.

Human tissue samples
Tissues were obtained from patients that had previously given signed consent. PAH and control small pulmonary arteries (<1000μm diameter) were freshly isolated from explanted lungs. Non-PAH lung tissues (controls) were obtained during lung resection for tumors from the non-cancerous segments. PAH and control tissues (lungs, brain, kidneys) were obtained from
Respiratory Health Network tissue bank (Table 1).

**Cell culture**

PAH-PASMC were isolated from <1000-µm-diameter small pulmonary arteries from 4 PAH patients, all confirmed by right catheterization (3 idiopathic PAH and 1 associated PAH (scleroderma)). PASMC phenotype was confirmed using α-smooth muscle actin staining. Control PASMC (n=5 cell lines) were purchased from Cell Application (San Diego, CA).

**PARP activity assay**

PARP activity was assayed using the Trevigen Universal chemiluminescent PARP assay kit with modifications.

**Proliferation and apoptosis measurements**

PASMC proliferation was measured using Ki67, PCNA and WST1 assay, and TUNEL and AnnexinV assessed apoptosis.

**DNA damage**

Immunofluorescence for γ-H2AX and immunohistochemistry for 53BP1 were performed to assess DNA damage.

**Animal models**

The Sugen-hypoxia model (Sugen) and the monocrotaline model (MCT) PAH rat models were used in the present study. Once PAH established (after 14 days for MCT and week 5 for Sugen), the PARP inhibitor (ABT-888, 6mg/kg⁻¹, Enzo life sciences) or vehicle (drinking water) was administrated *per os* every day for 2 weeks.

**Statistical Analysis**

Values are expressed as fold change or mean ± SEM, as they follow a normal distribution.

Unpaired Student’s *t* tests were used for comparisons between two groups and one-way ANOVA
followed by a Tukey-Kramer post-test was used for more than two groups. When same cells were used under different conditions, paired analyses were performed among treatments. Furthermore, if variance depends on mean of the data, statistical analyses were performed on log transformation of the data. Probability values less than 0.001 (***) 0.01 (**), and 0.05 (*) were considered as statistically significant.

**Results**

**Increased DNA damage and PARP-1 expression in human PAH**

We measured DNA damage in human distal pulmonary arteries (PA) from 6 PAH and 6 control patients (5 distal PA/patient), using the expression of the p53-binding protein 1 (53BP1) measured by immunohistochemistry, as marker of DNA damage. As shown in Figure 1a, DNA damage is significantly increased in PAH compared to normal tissue controls. Cells with positive nuclear staining were considered positive and this was divided by the total amount of cells (total nuclei) to calculate percent of positive cells. Immunohistochemistry negative control is provided in Supplemental Figure 1a. We then measured PARP-1 protein expression in human distal PA from 5 control and 5 PAH patients by immunoblot (Figure 1b) and found that PARP-1 expression is significantly increased in PAH compared to control distal PAs. Thus, we studied whether these changes were also present in primary cultured human PAH-PASMC. PAH-PASMC were freshly isolated from small PA of 4 PAH patients and 5 control PASMC cell lines were purchased. DNA damage and PARP-1 expression and activation were measured. Using γ-H2AX as DNA damage marker, we showed that, as in distal PAs, DNA damage is sustained in cultured PAH-PASMC compared to healthy PASMC. The number of γ-H2AX foci in PAH-PASMC are further increased by the addition of the PARP-1 inhibitor ABT-888 or AG14361 as
well as with PARP-siRNA (compared to siSCRM treated cells) (Figure 1c) suggesting that PARP-1 activation is important for the repair of these damages but not enough to eliminate them completely as a certain level of DNA damage persists in PAH-PASMC. This suggests that PARP-1 activation in PAH is mild, far from the level of activation that can be reached after $\gamma$-radiation for example$^{19}$. By western blot, we then demonstrated that PAH-PASMC have increased levels of PARP-1 and the further you increase DNA damage (by blocking the repair by ABT-888) the further PARP-1 expression is increased (Figure 1d). The same effect is also seen with AG14361 treatments (Supplemental Figure 1b). Moreover, as expected, although PARP-1 siRNA increases DNA damage, it did not increase PARP-1 expression demonstrating siRNA efficiency (Supplemental Figure 1b). Note that although ABT-888 increases PARP-1 expression it inhibits its activity$^{23}$ assayed using the chemiluminescent PARP assay kit (Figure 1e). We also confirmed that AG14361 blocks PARP-1 activity using the same assay (Figure 1e).

To evaluate DNA repair, we measured fold changes in DNA damage after DNA-damaging agent etoposide$^{24}$ treatment and a 2 hours recovery. Fold change were analyzed since PAH-PASMC have higher basal levels of DNA damage (Figure 1c). In brief, cells were treated with 1mM of etoposide for 1 h and after withdrawal of etoposide, cells were incubated in fresh medium for 2 hours to allow DNA repair, as previously described$^{25}$. The reminding DNA damage was then assessed (g-H2AX). For ABT-888 treatments, cells were incubated with 10mM ABT-888 for 48 hours prior to etoposide treatment and ABT-888 was kept in medium for the recovery period. As shown in Figure 2a, control PASMC treated with etoposide have increased DNA damage (3.5 fold increase). On the other hand, in PAH-PASMC, etoposide doesn’t significantly increase DNA damage, suggesting that increased PARP-1 expression in these cells provides a more efficient DNA repair in the 2-hour recovery period. Indeed, if PARP activity is
inhibited by ABT-888, etoposide treatment then shows increased DNA damage (2-fold increased compared to non treated PAH-PASMC) (Figure 2a).

In order to determine if the increased expression and activation of PARP-1 is restricted to the lungs, we analyzed other tissues from PAH patients and demonstrated that PARP-1 was not overexpressed (brain, kidney) (Supplemental Figure 2a). We also confirmed that PARP-1 expression is increased in both MCT- and Sugen-induced PAH lungs (Supplemental Figure 2b). Efficiency of treatment was measured, as shown in Supplemental Figure 2b. As in humans, PARP-1 is upregulated within distal PAs, but not other tissues such as left ventricle and liver (Supplemental Figure 2c).

The mechanism accounting for increased DNA damage and PARP-1 protein expression is likely to be multi-factorial, but the presence of sustained inflammatory signaling in PAH may play a major role. Hence, we measured whether TNF-α, an inflammatory cytokine increased and implicated in PAH pathogenesis, can trigger DNA damage and subsequent PARP-1 activation in PASMC. As shown in Figure 2b, control PASMC treated with TNF-α had an increased number of DNA damage foci (γ-H2AX) compared to vehicle-treated PASMC. Similar results were also found when PASMC were treated with IL-6 or PDGF (Supplemental Figure 1c). The increase in DNA damage was associated with a significant upregulation of PARP-1 protein expression measured by immunoblot and immunofluorescence (Figure 2c). As expected PASMC treated with both TNF-α and ABT-888, showed more DNA damage leading to a greater PARP-1 expression (Figure 2c).

**PARP inhibition decreases proliferation and promotes apoptosis in PAH-PASMC**

We next studied the implication of PARP-1 in PAH-PASMC survival and proliferation. We showed that PAH-PASMC have significantly greater proliferation rate (PCNA, Ki67 and WST1
assays) and a significant apoptosis-resistant phenotype (as indicated by AnnexinV and TUNEL assays), when compared to control-PASMC placed in the same conditions (Figure 3 and Supplemental Figure 3). PARP-1 inhibition by ABT-888 in PAH-PASMC restores a normal phenotype, thus decreasing proliferation and restoring apoptosis, to levels similar to those found in control cells. We further confirmed PARP-1 implication by the use of PARP-1 siRNA, showing similar findings to ABT-888 (Figure 3).

**PARP-1 inhibition reverses miR-204-dependent upregulation of NFAT and HIF-1α in PAH-PASMC**

We have previously described a critical role of the microRNA miR-204 in the etiology of PAH. We had demonstrated that through a STAT3-dependent mechanism the downregulation of miR-204 accounts for the upregulation of the transcription factors NFAT and HIF-1 in PAH-PASMC, which is contributing to their proliferation and resistance to apoptosis. Cells with activated PARP-1 produce high levels of IL-6, which by activating STAT3 contributes to miR-204 downregulation. We previously published that PAH-PASMC produce higher levels of IL-6 and have activated STAT3. Thus, by regulating IL-6 production, PARP-1 will downregulate miR-204. To determine whether PARP-1 can affect miR-204 expression, we performed qRT-PCR in PAH-PASMC in presence and absence of PARP-1 inhibitor ABT-888. We found that miR-204 downregulation in PAH-PASMC is reversed by ABT-888 (Figure 4a). As predicted, PARP-1 dependent downregulation of miR-204 triggers NFAT and HIF-1α activation measured by nuclear translocation (Figure 4b and 4c).

In PAH-PASMC, NFAT and HIF-1α mediated proliferation have been linked to the downregulation of K⁺ channels resulting in membrane depolarization, opening of the voltage-dependent calcium channels, thereby increasing intracellular calcium concentration.
Using Fluo-3AM, we measured the effect of PARP-1 inhibition on [Ca^{2+}]_i. The inhibition of PARP-1 (ABT-888) in PAH-PASMC decreased [Ca^{2+}]_i to the levels seen in control-PASMC (Figure 4d). Furthermore, resistance to apoptosis observed in PAH-PASMC has been linked to mitochondrial membrane potential (ΔΨ_m) hyperpolarization, blocking the release of pro-apoptotic mediators such as cytochrome c^{5,27,32}. Using tetramethylrhodamine methyl ester (TMRM), we observed that ABT-888 treatment in PAH-PASMC decreases ΔΨ_m to similar levels of control-PASMC (Figure 4d). To further investigate the implication of PARP-1, miR-204, NFAT and HIF-1α, we performed co-treatments of ABT-888 with miR-204 mimic, VIVIT (NFAT inhibitor) or siHIF-1α and measured the effect on calcium concentration (FLUO-3AM) and mitochondrial membrane potential (TMRM). Note that the VEET peptide was used as control for VIVIT and had no effects (not shown) as previously published by our group^{5}. As previously demonstrated, NFAT or HIF-1α inhibition^{5,33}, and miR-204 restoration^{13} in PAH-PASMC decrease intracellular [Ca^{2+}]_i and reduce ΔΨ_m to similar level to that seen in control-PASMC (n=3, p<0.001). We also observed that the ABT-888 effect is similar to those with the other treatments. Moreover, adding ABT-888 to other treatment doesn’t change [Ca^{2+}]_i nor ΔΨ_m (Figure 4d), demonstrating that there is no synergic effects showing that these targets act in the same molecular pathway.

**In vivo, ABT-888 treatment reverses both monocrotaline- and Sugen- induced PAH**

As in human distal PA, distal PAs of both Sugen-PAH and MCT-PAH rats had increased DNA damage, measured by g-H2AX lung expression and 53BP1 immunohistochemistry (Figure 5a and Suplemental Figure 4a) and increased PARP-1 expression (Supplemental Figure 2b and 2c). As suggested by our *in vitro* studies, inflammation could account for the increase in DNA damage inflammatory cytokines levels are increased (IL-6) in both Sugen-PAH and MCT-PAH
lungs. Furthermore, NFκB lung protein expression and H&E staining shows increased inflammation in PAH models and this seems to be decreased upon ABT-888 treatment (Supplemental Figure 4b). In order to test whether PARP-1 inhibition can reverse PAH in our established rat models, ABT-888 was given orally (6mg/kg/day) once PAH was established (5 weeks post Sugen injection or 14 days after MCT injection). We confirmed that ABT-888 administration per os decreases PARP-1 activity in the lungs, using the modified PARP-1 activity assay (Supplemental Figure 2b). We performed a 2-week longitudinal study to assess the efficacy of our treatment using non-invasive hemodynamic assessment with Doppler echocardiography. We observed PARP inhibition in both Sugen-PAH and MCT-PAH rats reduced PA pressure assessed by PA acceleration time (PAAT), a Doppler parameter linked to PA pressure (PAAT being inversely correlated to PA pressure) and also decreased RV hypertrophy (Supplemental Figure 5b; n=8 rats per group, p<0.05) (Supplemental Figure 5a). These findings were invasively confirmed by right heart catheterization (direct PA pressure) and RV/LVS (right ventricle/left ventricle with septum) weight ratio (Figure 5b). Also, ABT-888 significantly improved exercise capacity in both models, using standard treadmill exercise protocol, compatible with the improvement of the hemodynamic measurements (Supplemental Figure 5a). Furthermore, no kidney or liver toxicity was seen after ABT-888 treatment in both models, assessed by plasma creatinine and aspartate aminotransferase (AST) levels (Supplemental Figure 5b).

To determine whether ABT-888 reduced PA remodeling in Sugen-PAH and MCT-PAH animals, we measured medial wall thickness using H&E staining. We observed that animals treated with ABT-888 displayed a significant reduction in medial thickness of distal PAs (≤100µm) (Figure 5c). As in vitro, these findings were associated with a significant decrease in
PASMC proliferation (as assessed by Ki67) (Figure 6a) and resistance to apoptosis (TUNEL) (Figure 6b).

Finally, ABT-888 provides a therapeutic effect similar to the combination of both standard PAH therapies (Bosentan+Tadalafil) in MCT-PAH rats, while ABT888 given in addition to Bosentan and Tadalafil, provided a greater therapeutic effects compare to MCT-PAH rats treated with Bosentan+Tadalafil only. These findings suggest that ABT-888 could be used in human in combination with current therapies and would provide a more efficient treatment (Supplemental Figure 6).

Discussion

Using PAH lungs from both humans and rats we show for the first time that DNA damage may be etiologically implicated in PAH. Clinically, DNA damage has been suspected to be responsible for the occurrence of pulmonary hypertension in patients taking etoposide, an anti-cancer drug promoting DNA damage and PARP-1 activation34. We showed that sustained DNA damage correlated with PARP-1 expression (Figure 1 and Supplemental Figure 1), suggesting that it could modulate PARP-1 directly or indirectly. In addition to the DNA repair that may allow survival, PARP-1 appears to promote the activation of a pro-proliferative and anti-apoptotic program, characterized by miR-204 downregulation and the subsequent NFAT and HIF-1 upregulation; both of which have been shown to promote PAH-PASMC proliferation and resistance to apoptosis (Figure 3, 4 and Supplemental Figure 3). PARP-1 inhibition using the clinically available inhibitor ABT-888, reverses all these abnormalities in vitro (Figure 3 and 4) and reverses PAH in vivo in two widely used models of PAH (Figure 5 and Supplemental Figure 5 and 6). Schematic representation of our signaling pathway is presented in Figure 7.

Although not previously reported, the presence of increased DNA damage in distal PAs...
in PAH when compared to control patients is not surprising. PAH is characterized by sustained sterile inflammation and elevated circulating cytokines such as TNF and IL-6, both of which are known to promote DNA damage. We demonstrated that exposure of normal PASMC to pro-PAH factors, such as TNF-α, IL-6 or PDGF induces DNA damage (Figure 2a and Supplemental Figure 1c). Similar observations have been described in the systemic circulation within atherosclerotic lesions. Although not explored in the present study, other environmental stresses could also contribute to the increased level of DNA damage in PAH. For example, elevated levels of circulating S100A4, which is upregulated in PAH and is responsible for the advanced glycation end products receptor RAGE activation, is implicated in both DNA damage and PAH. It is possible that PARP-1 activation is a common denominator for diverse conditions that cause DNA damage in PAH, making it an attractive therapeutic target. Although our findings support the implication of DNA damage in PARP activation in PAH, inflammation itself may further contribute to PARP-1 activation (Figure 7).

PARP-1 may act as a sensor of intracellular stress. In the presence of extensive DNA damage (as seen after treatment with high doses with gamma radiation for example), PARP-1 is highly activated and promotes cell death, while in the presence of low to moderate DNA damage, as perhaps seen in PAH, PARP-1 triggers DNA repair by recruiting important repair factors to the DNA damage sites, allowing PASMC to survive and even proliferate.

Interestingly, recent studies have demonstrated that a mild to moderate PARP-1 activation can induce pro-proliferative responses by promoting the activation of NFAT, a transcription factor critical for PAH-PASMC proliferation. We previously reported that NFAT activation in PAH occurs downstream of the downregulation of miR-204 and the subsequent activation of STAT3 and Pim-1. In the present study, we report that PARP-1 inhibition is associated with a
complete reversal of miR-204 downregulation, limiting NFAT activation, which decreases PAH-PASMC proliferation. In line with our findings, studies in hematopoietic cells and neurons have shown a significant increase in Pim-1 in response to DNA damage contributing to cell survival\textsuperscript{43,44}. In addition to miR-204 downregulation, upregulation of Pim-1 in PAH\textsuperscript{10} could contribute to the PARP-1-dependent cell survival in PAH-PASMC. In addition, several recent studies have attributed numerous post-transcriptional functions to PARP proteins\textsuperscript{19} including the regulation of the microRNA binding protein Argonaute 2\textsuperscript{19}. These could contribute to the activation of other pathways implicated in PAH. For example Argonaute 2 is implicated in stem cells proliferation through modulation of voltage gated K\textsuperscript{+} channels including Kv1.5\textsuperscript{45}, which is implicated in PAH\textsuperscript{33}.

In addition to proliferation, resistance to apoptosis is another important feature of PAH-PASMC\textsuperscript{4,5}. Several reports have demonstrated that abnormal metabolic/mitochondrial function play a key role in this phenomenon, notably by promoting a normoxic activation of HIF-1\textalpha\textsuperscript{31,33}. Recent studies have established a direct link between PARP-1 and the transcription factor HIF-1\textalpha in cancer models\textsuperscript{46}. In fact, studies have presented PARP1 as a transcriptional co-activator of HIF-1\textalpha\textsuperscript{46}. Upon hypoxic induction of cells, PARP-1 was shown to interact with HIF1-\textalpha and to regulate the transcriptional activity of HIF-1\textalpha-dependent genes. In the present study we showed that PARP-1 inhibition significantly decreases HIF-1\textalpha activity resulting in a significant induction of apoptosis. We recently showed that the normoxic upregulation of HIF-1\textalpha in PAH-PASMC is attributed to Endoplasmic reticulum (ER) stress disrupting ER mitochondria units, impairing mitochondrial ROS production resulting in HIF-1\textalpha activation\textsuperscript{47}. Recently in neurons, PARP-1 activation has been shown to promote ER stress. This could explain why PARP-1 inhibition in PAH-PASMC decreases HIF-1\textalpha (Figure 4c) activation and restores mitochondria membrane
potential (Figure 4d).

Further experiments are required to understand the relative importance of the several mechanisms accounting for PARP-1 activation (DNA damage, inflammation, oxidative stress, etc.) in PAH and to elucidate the pathways implicated in the beneficial effect of ABT-888 treatment in experimental PAH. Although our data support a role for DNA damage in PARP-1 activation, previous studies reported that pro-inflammatory factors including TNF might also contribute to its activation48. Regarding the beneficial effects of PARP-1 inhibitors in experimental PAH, we provide evidence that PARP-1 inhibition upregulates miR-204 and subsequently reduces NFAT and HIF-1 activation in PAH-PASMC. Nonetheless, the implication of other pathways cannot be ruled out. In fact, the potential role of PARP-1 as a stress sensor in PASMC directly implies the involvement of multiple pathways as many signals may converge to PARP-1 and produce several downstream signals, which may vary according to experimental conditions or disease states. For example, PARP-1 activation can promote cytokine production48, suggesting that its inhibition may reduce the inflammatory response seen in PAH (as suggested in Supplemental Figure 4b) and thus improve vascular remodeling. This possibility will be addressed in future studies. Given the diversity of signaling mechanisms and protein expression between proximal and distal PAs, a limitation in our study is the use of human PASMC isolated from somewhat larger distal PAs (1000μm in diameter), as opposed to the less than 300-500μm vessels that are considered to be the typical resistance vessels. However, we managed to confirm our findings using immunofluorescence and immunohistochemistry measurements made in distal pulmonary arteries (≤ 100μm in diameter). In addition, there is emerging evidence that the proximal PAs may also be involved in PAH pathology, exhibiting remodeling that results in vascular stiffness, in contrast to earlier views suggesting that the primary signal for the disease is
restricted within the resistance PAs. Also, the inflammatory environment in PAH, which is increasingly being appreciated, implies that the more proximal PAs are equally exposed to circulating cytokines compared to the more distal arteries.

We focused our study on PASMC and not endothelial cells, which are also implicated in PAH. Although most investigators agree that PA endothelial cell apoptosis is one of the earliest events in the pathogenesis of PAH in which DNA damage might be implicated, less is known about endothelial cell biology as the disease progresses; many suspect that there is a switch toward a proliferative and apoptosis resistant phenotype. Although we did not study the possible PARP-1 effects in the biology of PAH-PAEC, it is possible that, as in PASMC, increased PARP-1 marks a proliferative endothelial cell with downregulated miR-204 and activated STAT3, NFAT and HIF-1a. Indeed, it has recently been shown that downregulated miR-204 and increased HIF-1a are found in plexiform lesions49, 50.

Although PARP-1 inhibition appears to be effective in terms of reversing established PAH in vivo, potential induction of apoptosis in other organs (following PARP-1 inhibition and thus accumulation of DNA damage) might be catastrophic and this needs to be monitored before clinical translation. On the other hand, it is expected that PARP-1 inhibitors (several of which are actively used clinically, including ABT-888, AZD 2281, and MK4827) will have effects preferentially in tissues where PARP-1 is activated. All of these compounds are already in Phase II or III clinical trials in cancer.

In conclusion this is the first study providing mechanistic evidence for the implication of DNA-damage and the resulting induction of PARP-1 in the etiology of human PAH. In addition to its implication in DNA repair, PARP-1 may account for many PAH features, including impaired microRNA regulation, cell proliferation (through STAT3 and NFAT) and resistance to
apoptosis (through HIF-1) (Figure 7). In that sense PARP-1 may be one the earliest factors contributing to the PAH phenotype. Finally, our findings support the translational potential of existing PARP-1 inhibitors as new therapies for PAH.

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Conflict of Interest Disclosures: None.

References:


Table 1. Clinical characteristics of PAH patients and controls

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</tr>
<tr>
<td>Medication [n (%)]</td>
<td></td>
<td></td>
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<tr>
<td>Endothelin receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antagonist</td>
<td>-</td>
<td>2 (28.57%)</td>
<td>1 (100%)</td>
<td>4 (44.44%)</td>
</tr>
<tr>
<td>PDE5 inhibitor</td>
<td>-</td>
<td>3 (48.86%)</td>
<td>1 (100%)</td>
<td>4 (44.44%)</td>
</tr>
<tr>
<td>Epoprostenol</td>
<td>-</td>
<td>2 (28.57%)</td>
<td>0</td>
<td>3 (33.33%)</td>
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</tbody>
</table>

Values are means ± SEM.
IPA: idiopathic pulmonary arterial hypertension; HPA: heritable pulmonary arterial hypertension; APAH: associated pulmonary arterial hypertension; CO: cardiac output; CI: cardiac index; mPAP: mean pulmonary arterial pressure; PVR: pulmonary vascular resistance; 6MWD: six-minute walk distance; PDE5: phosphodiesterase-5.
APAH include patients with connective tissue disease and pulmonary veno-occlusive disease associated pulmonary arterial hypertension. Please note that some patients take more than one type of medication.

Figure Legends:

Figure 1. DNA damage, PARP-1 expression and activation are increased in PAH distal PAs and PAH-PASMC. a) DNA damage (53BP1) was quantified in distal PAs in 5 control versus 6 PAH patients (5 PA/lung). b) PARP-1 expression was quantified in freshly isolated distal PAs from 5 control and 5 PAH patients. PARP-1 is increased in PAH distal PAs. c) PAH-PASMC are also
associated with increased DNA damage measured by γH2AX nuclear staining. As shown, PAH-PASMC have increased DNA damage, which is significantly increased by PARP-1 inhibition (by ABT-888, AG14361 or siPARP-1) (n=4). d) Consequently, PAH-PASMC have increased PARP-1 expression and this is further increased with ABT-888 treatment as demonstrated by western blot (n=4). e) Although PARP-1 expression is increased after ABT-888 treatment, we confirmed that it does block PARP-1 activity (measured by chemiluminescence assay) (n=3). *p<0.05; **p<0.01 and ***p<0.001

**Figure 2.** Inflammation triggers DNA damage and PARP-1 expression and activation. a) We measured fold changes in DNA damage after DNA-damaging agent etoposide treatment (1mM for 1h) and a 2 hours recovery, allowing DNA repair. The remaining DNA damage was then assessed (γ-H2AX) (n=3). b) Control cells (n=4) stimulated with TNFa (100ng/mL for 48 hours) have increased DNA damage as shown by γ-H2AX nuclear staining. This was further increased with ABT-888 treatment (n=3). c) This increase in DNA damage correlated with increased PARP-1 expression (measured by western blot (n=3). *p<0.05, **p<0.01

**Figure 3.** PARP inhibition decreases proliferation and increases apoptosis. a) PARP inhibition using ABT-888 (10μM) decreases PAH-PASMC proliferation (WST1 assay). The same effect is also seen with specific PARP-1 inhibitors (AG14361 and siPARP-1) (n=3). We also confirmed this by Ki67 assay. Again, pharmacological (ABT-888) or chemical (siPARP-1) inhibition of PARP-1 decreased the hyperproliferative phenotype seen in PAH-PASMC (n=3 to 5). b) Furthermore, we assessed resistance to apoptosis by TUNEL assay. PARP-1 inhibition promotes apoptosis in serum-starved cells to values similar to the one seen in control PASMC. Percent of
positive PASMC for Ki67 and TUNEL (nucleus localization) were determined and divided by the total amount of cells (total nuclei by DAPI). *p<0.05; **p<0.01 and ***p<0.001

**Figure 4.** ABT-888 in PAH-PASMC restores the miR-204/NFAT/HIF-1a axis. a) miR-204 levels were measured in control cells (n=5) and in PAH cells (n=4) with or without ABT-888 (10mM for 48 hours). PARP-1 inhibition increases miR-204 levels, as to the one seen in control cells. b) PARP-1 inhibition decreases NFATc2 activation measured by nuclear translocation assay (n=4 to 5). c) The same phenotype is also observed for HIF-1a, as its activation is increased in PAH-PASMC and decreased upon PARP-1 inhibition (n=4). d) PARP-1 effects are mediated by miR-204/NFAT/HIF-1a as adding ABT-888 to other treatment (miR-204 mimic, VIVIT, siHIF-1a) doesn’t change [Ca^{2+}]_i nor ΔΨ_m (for all the experiments: n=50 cells/experiments in 3 to 4 experiments). *p<0.05; **p<0.01 and ***p<0.001

**Figure 5.** Clinically available PARP inhibitor ABT-888 reverses MCT- and Sugen-induced PAH. a) DNA damage was assessed in lungs of PAH models with or without ABT-888 treatment (6mg/Kg/day administered *per os* for 2 weeks once PAH established). DNA damage, measured by g-H2AX (immunoblot, n=3), is increased in PAH rat models’ lungs and slightly increased with ABT-888 treatment. b) PARP-1 inhibition decreases PA pressure (measured right heart catheterization), RV hypertrophy (measured by Fulton index) and PA wall thickness (H&E staining) (n=6 to 10 rats per group). Since variance of these results with mean value (unequal variances), statistical analyses were performed on log transformation of the data. *p<0.05; **p<0.01 and ***p<0.001
**Figure 6.** ABT-888 decreases proliferation and apoptosis resistance in PAH-PASMC of distal PA. a) ABT-888 treatment decreases smooth muscle cell proliferation (Ki67) in distal PAs of both animal models (n=5 rats per group). b) Apoptosis was assessed using TUNEL assay and we showed that ABT-888 treatment increases apoptosis levels in distal PAs of both animal models. Only smooth muscle actin positive cells were considered in the calculation of positive cells. These experiments were performed in 5 distal PAs/rat in 5 rats per group. *p<0.05; **p<0.01 and ***p<0.001.

**Figure 7.** Proposed model. Inflammatory cytokines (TNFa, IL-6, PDGF) increase DNA damage inducing PARP-1 activation/expression. Inflammation can also trigger PARP-1 activation through a DNA damage-independent mechanism. This leads to decreased miR-204 expression and increased NFATc2 and HIF-1α expression/activation, factors known to induce proliferation.
**Figure 1**

**A** DNA damage is increased in PAH patients' lungs

**B** PARP-1 is increased in distal PAs of PAH patients

**C** DNA damage is increased in human PAH-PASMCs

**D** PARP-1 is increased in human PAH-PASMCs

**E** PARP-1 activity is increased in PAH-PASMCs
**Figure 2**

A. DNA repair capacity measured in control- and PAH-PASMCs

- Control
- Control+ABT-888
- Control+Etoposide
- Control+ABT-888+Etoposide

**B. Inflammation induces DNA damage**

- Control
- Control+TNF-α
- Control+TNF-α+ABT-888

γH2AX in green and DAPI in blue (63x)

**C. Inflammation triggers PARP-1 expression**

- PARP-1
- Amidoblack
- TNF-α: - + +
- ABT-888: - - +

PARP-1 protein expression (normalized to amidoblack)

* compared to non-treated cells
A) PARP-1 inhibition blocks the pro-proliferative phenotype

Ki67 in red and DAPI in blue (20x)

B) PARP-1 inhibition restores apoptosis levels

TUNEL in green and DAPI in blue (20x)
A PARP-1 inhibition reverses miR-204 expression in PAH-PASMC

B PARP-1 inhibition reverses NFAT activation in PAH-PASMC

C PARP-1 inhibition reverses HIF-1α activation in PAH-PASMC

D PARP-1 inhibition restores intracellular calcium concentration and mitochondrial membrane potential

Figure 4
A DNA damage is present in PAH animal model regardless of ABT-888 treatment

B PARP-1 inhibition by ABT-888 treatment reverses PAH
ABT-888 administration decreases proliferation in the pulmonary arterial wall

Control | Sugen-PAH | Sugen-PAH + ABT-888 | MCT-PAH | MCT-PAH + ABT-888

DAPI in blue, sm-actin in pink and Ki67 in green

B ABT-888 administration induces apoptosis in the pulmonary arterial wall

Control | Sugen-PAH | Sugen-PAH + ABT-888 | MCT-PAH | MCT-PAH + ABT-888

DAPI in blue, sm-actin in pink and TUNEL in green

Figure 6
Inflammation (TNF, IL-6, PDGF)

↑ DNA damage

ABT-888

PARP-1

miR-204/ NFATc2 / HIF-1α

DNA repair

↑ Proliferation

↓ Apoptosis

PAH

Figure 7
Role for DNA Damage Signaling in Pulmonary Arterial Hypertension

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell culture and treatments

PASMC were grown in high-glucose DMEM supplemented with 10% FBS (Gibco, Invitrogen, Burlington, ON, Canada) and 1% antibiotic/antimyotic (Gibco, Invitrogen, Burlington, ON, Canada). PARP was inhibited by a clinically relevant dose of ABT-888 (10 µM for 48h, Enzo life sciences). We also used a more specific PARP-1 inhibitor, AG14361 (5µM for 48h, Selleck Chemicals), to confirm the major implication of PARP-1. PASMC were transfected by CaPO4 precipitation with 20nM small interfering RNA (siRNA) oligonucleotides (siPARP-1 (Ambion), siHIF-1α (Thermo scientific) or their negative control siSCR (Ambion)). The Ca phosphate transfection method was also used to transflect miRIDAN miR-204 mimics (200nM for 48 h). NFATc2 was inhibited by a specific competitive peptide VIVIT 4 µM for 48 h (Calbiochem). Control PASMC were exposed to 100ng/mL TNF-α, 100µM IL-6 or 30ng/mL PDGF (all from EMB Canada) for 48 hours.

Quantitative RT-PCR and immunoblots

To measure PARP-1 mRNA expression, total mRNA was extracted from PASMC using a trizol protocol. Quantitative RT-PCR (qRT-PCR) was calculated with 18S as housekeeping gene (Taqman Gene expression Assay, Applied Biosystem, Foster, CA, USA) as previously described. All the experiments were performed in triplicate. To measure miR-204 expression, the mirVana kit (Applied Biosystems) was used to extract total RNA from PASMC. Stem-loop qRT-PCR for mature miRNAs was performed on a real-time PCR system (Applied Biosystems). For immunoblots, protein expression of PARP-1 (Santa Cruz, 1:500), γH2AX (Cell signaling and
Abcam, 1:500) and HIF-1α (Novus Biological, 1:1000) were quantified and normalized to amidoblack as previously described\textsuperscript{4}. All immunoblots were performed in triplicate.

**PARP activity assay**

PARP activity was assayed using the Trevigen Universal chemiluminescent PARP assay kit according to the manufacturer’s instruction, with modifications. Cell lysate (40 µg/well) was added to the wells containing PARP buffer and PARP cocktail. Activated DNA and PARP enzyme were not added to cell lysate, as previously described\textsuperscript{5}.

**Proliferation and apoptosis measurements**

To study the effect of PARP-1 on PASMC proliferation and apoptosis *in vitro*, cultured human PAH-PASMC were exposed to 10% FBS (a condition that is known to promote proliferation\textsuperscript{1, 4}) or 0.1% FBS (a starvation condition that promotes apoptosis\textsuperscript{1, 4}). PASMC proliferation was measured using Ki67 antibody (Millipore) and PCNA (Dako) and apoptosis by using Apoptag apoptosis detection kit (TUNEL; Millipore) and AnnexinV (Clontech). Percent of positive PASMC (Ki67, TUNEL) were determined and divided by the total amount of cells, calculated with DAPI (total nuclei). WST1 assay, also to assess proliferation, was used according to the manufacturer’s instruction with modifications. Briefly, 2000 cells/well were plated onto 24-well plates. After stabilization, cells were treated for 48 hours under different conditions. The reagent was added to the medium and after 2h incubation; the absorbance at 440 nm was measured using a microplate reader (Synergy H1, BioTek). Fluo-3AM (Invitrogen) and TMRM (Tetramethylrhodamine methyl ester perchlorate, Invitrogen) were measured in PASMC treated with different activators and inhibitors to evaluate the effect of modulating our studied pathway
on intracellular calcium and mitochondrial membrane potential (n=20 to 50 cells/experiment in 3 experiments).

**DNA damage**

Immunofluorescence for γ-H2AX and immunohistochemistry for 53BP1 were performed as previously reported in Rodrigue et al.\(^6\) using a monoclonal antibody directed against γ-H2AX (Cell Signaling Canada) and a polyclonal antibody against 53BP1 (Bethyl Canada).

**Confocal Microscopy/Immunofluorescence**

Sections of lungs and human PASMC were used for immunofluorescence staining. PASMC were fixed with 1% paraformaldehyde and permeabilized with 0.2% Triton X-100. For lung samples, only small and distal pulmonary arteries (<100 microns) were investigated. Rat lungs were fixed with 4% paraformaldehyde. Immunofluorescence was performed on 5µm lung slices. PARP-1 (Santa Cruz, 1:100), γ–H2AX (Cell signaling, 1:50), NFATc2 (Abcam; 1:250), HIF-1α (Novus Biologicals, 1:250), smooth muscle actin (Sigma, 1:250) primary antibodies were used and Alexa Fluor 488 and 594 were used as secondary antibodies. Co-localization studies were performed using the Zen system from Zeiss. Briefly, stack imaging (10 pictures taken at random on 4 micrometer thick slides) was analyzed and all co-localizations detected throughout the stack were shown in yellow. When measuring DNA damage *in vitro*, cells were classified based on the number of foci in the nucleus: cells with greater than 10 foci were counted as positive according to the standard procedure\(^2\) and graphs represent the percent of positive cells (n=20 to 50 cells/experiment in 3 experiments).
Animal models

Male Sprague-Dawley rats (250–350g) (strain 400, Charles River) were randomly distributed between the groups and all the hemodynamic measurements were performed blinded to the condition. For the Sugen-hypoxia model (Sugen), rats were injected with 20mg/kg of SU5416 (Sigma) and put in hypoxia (10% O₂) for 3 weeks. Chambers were opened twice a week for cleaning and replenishment of food and water. Oxygen concentrations were continuously monitored with blood gas analyzers. For the monocrotaline-induced PAH model (MCT), rats were injected s.c. with 60 mg/kg of crotaline (Sigma). PAH progression was monitored every week by non-invasive echocardiography measuring the pulmonary artery acceleration times (PAAT) and the right ventricular (RV) hypertrophy. At the end of the treatment period, all rats underwent right heart catheterizations (closed chest) using SciScence catheters to measure pulmonary arterial pressure, as previously described⁴,⁷. Histology measurements were performed as previously described³,⁸. Pulmonary artery wall thickness was measured using 2 measurements/artery in 10 arteries/animal, in at least 5 animals per group. We also performed experiments on MCT-induced PAH in rats to evaluate the therapeutic potential of ABT-888 (6mg/kg/day) compared to actual treatments used in PAH (Bosentan (100mg/kg/day) and Tadalafil (10mg/kg/day))⁹ and all these treatments started 2 weeks after MCT injection (when PAH was established) and were diluted in water to be administered per os. Drinking water was used in all conditions as the vehicle group.
Supplemental Figure Legends

Supplemental Figure 1: DNA damage and PARP-1 expression

a) We confirmed that no staining appeared on our immunohistochemistry negative control. b) In PAH-PASMC, AG14361 stimulates PARP-1 expression (as ABT-888 does). We also confirmed siPARP-1 (20nM) efficiency, as no PARP-1 expression is seen on the immunoblot. c) We demonstrated that other pro-PAH factors, IL-6 (100µM) and PDGF (30ng/mL), also induce DNA damage in control cells (measured by γ-H2AX staining; n=3, **p<0.001).

Supplemental Figure 2: PARP-1 upregulation is limited to the lungs

a) PARP-1 is overexpressed in human lung tissues (n=5 controls and 6 PAH patients), but not in human kidney or brain (n=6 controls and 4 PAH patients). b) PARP-1 expression is increased in lungs of both PAH models used: the monocrotaline (MCT) and the Sugen (with hypoxia)-induced PAH (n=5 rats per group). We confirmed that ABT-888 administration *per os* decreases PARP-1 activity in the lungs, using the modified PARP-1 activity assay (n=5 rats per group). c) This increased PARP-1 expression is located in the lungs, as no increase is observed in left ventricle or in the liver (n=5 rats per group). *p<0.05 and ***p<0.001

Supplemental Figure 3: PARP inhibition decreases proliferation and increases apoptosis

a) PARP-1 inhibition in PAH-PASMC with ABT-888 (10µM for 48 hours) decreases proliferation, measured by PCNA (n=4). b) Similarly, ABT-888 restores apoptosis levels in serum-starved media measured by AnnexinV (n=4). *p<0.05; **p<0.01 and ***p<0.001

Supplemental Figure 4: DNA damage and inflammation are present in PAH animal models
a) DNA damage (53BP1) was quantified in distal PAs (8 rats per group, 5 PAs/lung). Cells with positive nuclear staining were considered positive and this was divided by the total amount of cells (total nuclei). As in human PAH distal PAs express more DNA damage and, as in vitro, DNA damage is maintained with ABT-888 treatment. b) Both animal models show increased inflammation, measured by circulating IL-6 levels (a pro-inflammatory cytokine) and visualized in H&E staining. (n=3 per group). Furthermore, NFκB protein expression, an inflammatory marker, is increased in Sugen-PAH lungs and decreased when rats were treated with ABT-888. *p<0.05; **p<0.01 and ***p<0.001

Supplemental Figure 5: Clinically available PARP inhibitor ABT-888 reverses PAH in two experimental rat models

a) ABT-888 (6mg/Kg/day) per os given for 2 weeks once PAH established (2 weeks post MCT injection and 5 weeks post Sugen injection) efficiently decreases PA pressure (right heart catheterization) and RV hypertrophy (RV/LVS ratio) (n= a minimum of 6 rats per group). B) Echography and treadmill data taken at the end of protocols also demonstrate a beneficial effect of ABT-888 treatment of lung and heart function (echography) as well as exercise capacity (treadmill). b) Creatinine and aspartate aminotransferase (AST) levels were measured in plasma to assess liver and kidney toxicity (n=5 per group). Since variance of these results increases with mean value (unequal variances), statistical analyses were performed on log transformation of the data. *p<0.05; **p<0.01 and ***p<0.001

Supplemental Figure 6: Comparison between ABT-888 and actual therapies
Different treatments were administered starting at week 2 in the MCT-PAH model. ABT-888 (6mg/kg/day), Bosentan (100mg/kg/day) and Tadalafil (10mg/kg/day) were all diluted in water and administered *per os* (oral administration) on a daily basis. Right heart catheterization performed after two weeks of treatment shows that ABT-888 add to the beneficial effect of the current cocktail therapy. Since variance of these results with mean value (unequal variances), statistical analyses were performed on log transformation of the data. *p<0.05; **p<0.01 and ***p<0.001
Supplemental References


Supplemental Figure 1

A Immunohistochemistry negative control

53BP1 in brown and nuclei in blue (100X)

B AG14361 and siPARP-1 effect on PAH-PASMC

C Pro-PAH factors induce DNA damage

γH2AX in green and DAPI in blue (63x)
Supplemental Figure 2

A PARP-1 expression is not increased elsewhere than lungs

B PARP-1 expression is increased PAH lungs and ABT treatment decreases its activity

C PARP-1 expression is not increased elsewhere than lung tissues (MCT model)
Supplemental Figure 3

A. PARP-1 inhibition inhibits the pro-proliferative phenotype

PCNA in red and DAPI in blue (20x)

B. PARP-1 inhibition restores apoptosis levels

Annexin V in green and DAPI in blue (20x)
Supplemental Figure 4

A DNA damage is also present in PAH animal models regardless of ABT-888 treatment

53BP1 in brown and nuclei in blue (100X)

B Inflammation is present in both animal models

NFκB protein expression in lungs

Control | Sugen-PAH | Sugen-PAH + ABT-888
---|---|---
NFκB p65 65 kDa | | |
Stain free 45 kDa |
| |

Control | Sugen-PAH | Sugen-PAH + ABT-888
---|---|---
NFκB protein expression (normalized to stain free)

Control | Sugen-PAH | Sugen-PAH + ABT-888
---|---|---
| |

Control | Sugen-PAH | Sugen-PAH + ABT-888 | MCT-PAH | MCT-PAH + ABT-888
---|---|---|---|---
H&E (10X) | | | |

** Relative IL-6 concentration

** ** **
Supplemental Figure 5

A Follow-up measurements for both animal models

**Pulmonary artery acceleration time**

- PAAT (ms)

**RV free wall**

- RV thickness (mm)

**Treadmill**

- Distance (m)

B Indirect measurements of renal and liver health

**Creatinine**

- umol/L

**Aspartate aminotransferase (AST)**

- Units/L
A Adding ABT-888 to actual therapies reduces pulmonary pressures