

## 44. Pathogenesis and novel therapeutic targets in pulmonary hypertension

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### Prevention of pulmonary vascular and myocardial remodeling by the combined tyrosine and serine-/threonine kinase inhibitor, sorafenib, in pulmonary hypertension and right heart failure

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Inhibition of tyrosine kinases can reverse pulmonary hypertension but little is known about the role of serine-/threonine kinases in vascular and myocardial remodeling.

We investigated the effects of sorafenib, an inhibitor of the tyrosine kinases VEGFR, PDGFR and c-kit as well as the serine-/threonine kinase Raf-1, in pulmonary hypertension and right ventricular (RV) pressure overload.

In monocrotaline treated rats, sorafenib (10 mg/kg/d p.o.) reduced pulmonary arterial pressure, pulmonary artery muscularization and RV hypertrophy, and improved systemic hemodynamics (Table). Sorafenib prevented phosphorylation of Raf-1 and suppressed activation of downstream signaling pathways (Erk 1/2). After pulmonary banding, sorafenib, but not the PDGFR/c-KIT/ABL-inhibitor imatinib reduced RV mass and RV filling pressure significantly. Congruent with these results, sorafenib only prevented ERK phosphorylation and vasopressin induced hypertrophy of the cardiomyocyte cell line H9c2 dose dependently (IC50 = 300 nM).

**Conclusions:** Combined inhibition of tyrosine and serine-/threonine kinases by sorafenib prevents vascular and cardiac remodeling in pulmonary hypertension, which is partly mediated via inhibition of the Raf kinase pathway.

|                         | Control    | MCT + vehicle | MCT + Sorafenib |
|-------------------------|------------|---------------|-----------------|
| RVP, mmHg               | 27.0±0.5*  | 82.9±6.0      | 35.0±1.5*       |
| RV/(LV+S)               | 0.24±0.01* | 0.51±0.02     | 0.26±0.01*      |
| CO, ml/min              | 116±5 *    | 71±7          | 117±5 *         |
| PaO <sub>2</sub> , mmHg | 205±19*    | 108±14        | 200±10*         |

Mean ± SEM. N=12–14/group. \*, p<0.05 vs. vehicle, RVP=right ventricular pressure, CO=cardiac output

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### Peroxisome proliferator-activated receptor gamma (PPARγ) protects against pulmonary arterial hypertension (PAH)

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Bone morphogenetic protein 2 (BMP-2) counteracts PDGF-BB-induced smooth muscle cell (SMC) proliferation associated with PAH, but the mechanism is unknown. We hypothesized that PPARγ and its potential target apoE act downstream of BMP receptor (R) II since expression of all three is decreased in PAH lungs.

**Methods:** Using small hairpin (sh) BMP-RII pLenti 6 virus transfection, we established, by RT-qPCR, a 85% knock down of BMP-RII mRNA (shBMP-RIIi) vs. sh-control in human (H) PASMC. HPASMC, and murine wildtype (wt), apoE deficient (–/–), and PPARγ –/– PASMC were stimulated with BMP-2 (10ng/ml), PDGF-BB (20ng/ml), the PPARγ agonist rosiglitazone (Rosi 1μM), the PPARγ antagonist GW9662 (1μM) and apoE (10μg/ml). SMC proliferation was assessed by cell counts and MTT assay. We measured PPARγ DNA-binding, nuclear PPARγ and apoE secretion (immunoblot). We created a mouse with SMC deletion of PPARγ (SMC PPARγ –/–), and measured RV systolic pressure (RVSP) and RV hypertrophy (RVH).

**Results:** BMP-2, Rosi and apoE inhibited PDGF-BB-induced proliferation of control but not shBMP-RIIi or PPARγ antagonist-treated HPASMC, PPARγ –/– or apoE –/– PASMC. BMP-2 induced rapid nuclear shuttling and DNA-binding of PPARγ in HPASMC, whereas PDGF-BB had the opposite effect. BMP-2 and Rosi stimulated apoE secretion. BMP-2-induction of apoE was impaired in PPARγ –/– PASMC. SMC PPARγ –/– mice had PAH (RVSP 29.0 vs. 21.5mmHg) and RVH (p < 0.01 vs. controls).

**Conclusions:** Our study defines a novel BMP-2-PPARγ-apoE-axis necessary for the antiproliferative action of BMP-RII in HPASMC. We suggest that PPARγ agonists may reverse SMC proliferation in PAH patients with or without BMP-RII dysfunction.

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### In a model of genetic pulmonary hypertension, VIP attenuates pulmonary vascular remodeling and right ventricular hypertrophy

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We recently reported that targeted deletion of the vasoactive intestinal peptide (VIP) gene in mice results in a phenotype of moderate pulmonary arterial hypertension (PAH), pulmonary vascular remodeling, and right ventricular (RV) hypertrophy (Circulation, '07) We have now tested the hypothesis that VIP replacement in these mice would prevent, slow down, or attenuate the development of this phenotype.

**Methods:** Nine male VIP –/– mice, aged 4–12 weeks, were given VIP (15 μg, i.p., every other day, for 4 weeks, for a total of 14 injections. Another group of 9 male VIP –/– mice of a similar age received injections of buffer only, for the same duration. Following this treatment period, the mice were euthanized and the degrees of pulmonary vascular thickening and RV hypertrophy were evaluated.

**Results:** Mice that had been treated with VIP showed pulmonary arteries that were considerably less thickened than in the buffer-treated controls (mean medial area/total area ratio = 0.59±0.06, vs. 0.74±0.03, respectively, P=0.045). Similarly, there was markedly less RV hypertrophy [RV/(LV+ septum) ratio = 0.25±0.01] in the VIP-treated than in buffer-treated mice (0.34±0.01, n=9, P < 0.001).

**Conclusions:** The marked attenuation of pulmonary vascular thickening and RV hypertrophy by VIP: 1) establishes the direct causal relationship of PAH in this model to absence of the VIP gene; and 2) suggests that VIP is likely to prove effective in improving the key pathologic and pathophysiologic alterations in human PAH.

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### Combining bosentan and sildenafil improves mitochondrial capacity and restores right ventricular contractility in established pulmonary hypertension

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**Objective:** In Pulmonary Hypertension (PH) treatment effects of Bosentan (Bos) and Sildenafil (Sil) on right ventricular (RV) adaptation remain poorly understood. We studied the effects of Bos, Sil and their combination on RV contractility and cellular adaptation in monocrotaline (MCT) induced PH.

**Methods:** PH was induced in male Wistar rats by MCT (40 mg/kg s.c.). After 14 days MCT rats were treated for 10 days with Bos (100 mg/kg/day, n=7), Sil (1 mg/kg/day, n=7) or both (n=7). Healthy and MCT rats served as controls (both n=7). At day 25 RV systolic pressure (RVSP) was measured and fractional shortening (RVFS) was calculated using echocardiography. RV cardiomyocyte hypertrophy (cross sectional area, CSA) and mitochondrial capacity (succinate dehydrogenase activity, SDH<sub>act</sub>) were histochemically determined.

**Results:** MCT rats clearly developed PH: RVSP and CSA increased and RVFS decreased. Without affecting RVSP, both single treatments further reduced RVFS, whereas combination treatment returned RVFS to control. All treatments further increased CSA. Bos+Sil combined increased mitochondrial capacity (table 1).

**Conclusions:** The combination of Bos+Sil only, increased mitochondrial capacity resulting in a return of normal RV contractility. These data suggest direct effects of Bos+Sil on RV adaptation to PH.

Table 1

|         | RVSP (mmHg) | RVFS (%) | CSA (μm <sup>2</sup> ) | SDH <sub>act</sub> (a.u.) |
|---------|-------------|----------|------------------------|---------------------------|
| Control | 28±5*       | 57±3*    | 274±30*                | 0.14±0.03                 |
| MCT     | 62±5°       | 44±3°    | 412±23°                | 0.13±0.02                 |
| Bos     | 64±5°       | 33±3*°   | 522±19*°               | 0.13±0.01                 |
| Sil     | 55±6°       | 33±2*°   | 489±13*°               | 0.15±0.02                 |
| Bos+Sil | 55±10°      | 52±1*    | 511±22*°               | 0.18±0.01*°               |

\*p < 0.05 vs MCT; °p < 0.05 vs control

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### C-kit+ cell recruitment in remodelled pulmonary arteries of human PAH

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**Background:** Progressive obliteration of small pulmonary arteries is a hallmark of pulmonary arterial hypertension. In addition to the dogma that proliferation and migration of resident vascular wall cells are the sole contributors to vessel

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wall thickening, a nonresident source of cells within the adventitial compartment of vascular lesions is now assumed. Indeed, bone marrow-derived hematopoietic stem cells (BM-derived HSC) have been shown to differentiate into vascular cells that could participate in the development of vascular remodeling. A generally accepted marker for BM-derived HSC is c-kit.

**Methods:** BM-derived HSC localization was characterized by c-kit immunohistochemistry and c-kit gene expression in microdissected pulmonary arteries was measured by reverse transcription real time polymerase chain reaction (RT-PCR) in lung samples from 7 controls and 7 human IPAH.

**Results:** As compared to controls, morphometric analysis demonstrated increased numbers of c-kit+ cells in muscular pulmonary arteries in IPAH. In all human lung samples from IPAH patients, we identified c-kit+ cells in adventitial layers of pulmonary vasculature. Affected vessels in PAH lungs displayed c-kit+ cells infiltration into the adventitial and the medial layer. RT-PCR revealed an over four fold increase of c-kit expression in PAH-associated remodelled arteries (p < 0.001 versus controls). Moreover c-kit expression in microdissected pulmonary arteries was correlating with the extent of c-kit+ cells infiltration.

**Conclusion:** Our results support the concept that c-kit+ cells accumulate in remodelled pulmonary vessels and hence could be involved in the vascular remodelling of pulmonary hypertension.

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**The effect of bortezomib (Velcade®) on endothelial progenitor cell functions in vitro**

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Proteasome inhibition with bortezomib (Velcade®) is a novel therapeutic approach in preclinical development for lung cancer. It disrupts multiple intracellular pathways causing G2-M-phase arrest and apoptosis, and is proposed to have antiangiogenic properties. Endothelial progenitor cells (EPC) have been shown to be involved in the formation of new vessels in malignancies, tumour progression and metastasis. Consequently, we investigated the effect of bortezomib on biological functions of EPC.

Rat bone marrow-derived EPC were investigated for proliferation and angiogenic properties in the presence of different concentrations of bortezomib under different culture conditions. Cell viability was measured by MTT test and analysis of capillary tube formation was performed using a Matrigel Assay. Cultured EPC, using standard growth medium containing 0.5% FCS (minimal stimulation) or 10% FCS (maximal stimulation), were treated with various concentrations of bortezomib (ranging from 0.75 nmol/L to 20 nmol/L). Proliferation was assessed by MTT assay after 24 and 48 hours of incubation.

Cells cultivated in growth medium containing 0.5% FCS showed a concentration and time dependent inhibition of cell proliferation after treatment with bortezomib (IC50 of 20 nmol/L). In contrast, EPC stimulated with 10% FCS displayed higher viability in the presence of different concentrations of bortezomib (range: 0.75 nmol/L to 20 nmol/L). However, inhibition of capillary tube formation was comparable with the observed effect on mature endothelial cells.

The higher resistance of EPC to this agent when compared with mature endothelial cells should be considered in further clinical trials in lung cancer therapy.

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**Progression of monocrotaline-induced pulmonary hypertension in rats can be monitored by plasma activity of matrix metalloproteinase-2 and plasma levels of tenascin-C and proBNP**

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Progression of pulmonary arterial hypertension is determined by the degree of pulmonary vascular and cardiac remodeling. So far, plasma biomarkers are not available to monitor the activity of this disease non-invasively.

|                   | Control    | MCT + Vehicle | MCT + BR4946 |
|-------------------|------------|---------------|--------------|
| RVP, mmHg         | 27±1*      | 68±6          | 48±3*        |
| RV/(LV+S)         | 0.26±0.01* | 0.50±0.03     | 0.35±0.02*   |
| MMP2 ng/ml        | 8.0±0.9*   | 16.0±1.5      | 9.9±1.6*     |
| proBNP, pg/100µl  | 14.0±0.8*  | 41.1±4.9      | 27.1±4.7*    |
| Tenascin C, ng/ml | 52.2±2.6*  | 88.0±14.1     | 78.5±16.4    |

Mean ± SEM. N=12–14/group. \*, p < 0.05 vs. vehicle, RVP=right ventricular pressure

We investigated plasma levels of tenascin C and matrix metalloproteinase-2 (MMP2), both involved in extracellular matrix maintenance, as well as proBNP, a marker for myocardial hypertrophy, in pulmonary hypertension. Two weeks after injection of monocrotaline, male rats were treated for 14 days with the neutrophil elastase inhibitor BR4946 (50 mg/kg/bid p.o.). Compared to vehicle, BR4946

significantly reduced RV pressure, RV weight and plasma levels of MMP2 and proBNP (Table). Pulmonary pressure was linked to plasma levels of tenascin C (r=0.74), MMP2 (r=0.73), and proBNP (r=0.86, linear correlations, all p < 0.001). Similar relationships were observed between RV hypertrophy and the plasma markers.

**Conclusion:** Pulmonary arterial hypertension in rats is characterized by elevated plasma concentrations of MMP2, tenascin C and proBNP. Non-invasive measurement of these biomarkers may offer an option for monitoring the progression of pulmonary hypertension as well as response to treatment.

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**RNA expression pattern of peripheral blood B-lymphocytes in idiopathic pulmonary arterial hypertension**

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**Background:** Anti-endothelial cell antibodies have been described in patients with idiopathic pulmonary arterial hypertension (IPAH). We therefore analysed the RNA expression characteristics of peripheral blood B-cells in IPAH and healthy controls.

**Methods:** Patients were diagnosed with IPAH according to WHO. B-cells from peripheral blood of patients and controls were immediately separated via histopaque density gradient centrifugation followed by magnetic beads for CD19 and stored at -80°C in TRI-reagent. B-cell RNA was extracted using the RiboPureTM-Kit. The RNA was analysed by the use of a high sensitivity gene chip (Affymetrix HG-U133-Plus2) able to analyse 47,000 transcripts and variants of human genes. The array data was analysed by GeneChip® Operating Software (GCOS) and genesprings software (GSS).

**Results:** Highly purified B-cells (average 97% CD19+) of 5 patients with IPAH (mean pulmonary artery pressure 51±13 mmHg) and 5 controls (ctrl) were analysed. We found 225 genes which were at least 1.3fold up-regulated in IPAH vs Ctrl (1.3–30.7fold) by GCOS and 128 up-regulated genes by CSS (threshold 15 out of 25 comparison up-regulated). Combining both methods, 33 genes were found up-regulated in IPAH. In contrast, we found 244 and 1214 in down-regulated genes using GCOS and CSS, respectively. However, combining both methods did not retrieve any more down-regulated genes.

**Conclusion:** We found a distinct peripheral blood B-cell-RNA expression profile in IPAH compared to controls with distinct up-regulated genes using different analysing methods. The biological significance of these findings and if they result in altered protein expression has to be specified by further studies.