Online data supplement

Assessment of Inhaled Treprostinil Palmitil, Inhaled and Intravenous Treprostinil and Oral Selexipag in a Sugen/Hypoxia Rat Model of Pulmonary Arterial Hypertension

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Section Assignment: Cardiovascular

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Supplemental Methods and Results

A. Experimental study design

1. Acute inhaled hypoxia.

Rats were prepared with telemetry probes implanted in the right ventricle for the measurement of RVPP and descending aorta to measure the changes in RVPP and SAP that was induced by exposure to a 10% O₂ gas mixture, respectively. For each Hx challenge, RVPP and SAP were measured for 10 min before (Baseline), during and after (Post) Hx (Figure 1). The Hx challenges were performed on 3 separate occasions 24 h before the administration of TPIP, inhaled and IV TRE and oral selexipag, or their respective vehicles with data represented as the average from these 3 Hx challenges. The following day, test articles were administered with the Hx challenge performed at different times over a 24-48 h period. The study design for the experiments with acute inhaled Hx challenge in telemetered rats is illustrated in Figure 1.

Figure 1. Study design for acute hypoxia challenge in telemetered rats

2. Su/Hx challenged rats

One hundred and twenty (120) male Sprague Dawley rats, ranging in weight from 250 - 300 g at the beginning of the study, were separated into 10 cohorts that received either a SC
injection of Su (20 mg/kg, 2 mL/kg) dissolved in 100% DMSO followed by 3 weeks of daily exposure to an inhaled hypoxic gas mixture (10% O₂/balance N₂) or 100% DMSO (2 mL/kg) followed by 3 weeks of room air breathing for the Nx control group. Day 0 was defined as the day of the Su or DMSO injection with Day 21 defined as the transition from Hx to Nx. All rats were then switched to 5 weeks of room air breathing which was defined as Day 55, during which time the Su/Hx rats received daily administration of the test articles or their respective vehicles. The Nx control rats that received 100% DMSO, instead of Su/Hx with no treatment, were exposed to room air breathing for 8 weeks. Twenty-four hours after the last dose of TPIP which was defined as Day 56, inhaled TRE and oral selexipag, the rats were anesthetized and prepared for the collection of hemodynamic, lung and cardiac tissues for histology and blood samples for PK analysis. For studies with IV TRE, the infusion continued until after the hemodynamic data was collected on Day 56 at which time a blood sample was taken for PK analysis with cardiac and histological data collected thereafter. The overall study design for the experiments involving the Su/Hx challenge is illustrated in Figure 2 and details of the different treatments at their targeted and delivered drug doses are listed in Table 1.

Figure 2. Su/Hx challenge and drug administration in rats
Table 1. Treatment Group Assignment and Treatment Information

<table>
<thead>
<tr>
<th>Group</th>
<th>Group Description</th>
<th>Target Treatment Dose</th>
<th>Dosing Description</th>
<th>Route of Administration</th>
<th>Delivered Dose</th>
<th>Treatment Starting Day</th>
<th>Treatment Ending Day</th>
<th>Surgery Day</th>
<th>Group Size</th>
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<tr>
<td>1</td>
<td>Normoxic control</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>56</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Su-Hx + TPIP vehicle</td>
<td>n/a</td>
<td>170 mg at 1.0 V</td>
<td>Inhalation (QD)</td>
<td>n/a</td>
<td>21</td>
<td>55</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>Su-Hx + TPIP low dose</td>
<td>57 µg/kg</td>
<td>90 mg at 0.5 V</td>
<td>Inhalation (QD)</td>
<td>59 µg/kg</td>
<td>21</td>
<td>55</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>Su-Hx + TPIP high dose</td>
<td>138 µg/kg</td>
<td>170 mg at 1.0 V</td>
<td>Inhalation (QD)</td>
<td>117 µg/kg</td>
<td>21</td>
<td>55</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>Su-Hx + nebulized</td>
<td>n/a</td>
<td>6 mL</td>
<td>Inhalation (QID)</td>
<td>n/a</td>
<td>21</td>
<td>55</td>
<td>56</td>
<td>11</td>
</tr>
</tbody>
</table>
Abbreviations:  **BID**: Twice a day; **IV**: Intravenous; **MC**: methylcellulose; **n/a**: non applicable; **PBS**: phosphate buffered saline; **QD**: Once a day; **QID**: Four times a day; **Su-Hx**: Sugen-Hypoxia; **TRE**: Treprostinil; **V**: Volt.

† Additional animals were included in these groups because of some rats, initially included in the study, had disconnected IV catheters when the Alzet pumps were refilled on Day 21 of the infusion.

‡ Blood collected 24 hours after TPIP, inhaled TRE, and oral selexipag on Day 55 and immediately after the collection of hemodynamic data on Day 56 for IV TRE.

B. Pharmacokinetics with IV TRE infusions

Rats received an IV infusion of TRE using an implanted osmotic pump (ALZET pump) that was filled with 2 mL of TRE at 8.75 mg/mL at the start of the infusion on Day 21. The Alzet pump was replaced on the 19th day of the infusion that contained 2 mL a TRE solution at a concentration of 10.7 mg/mL. The higher TRE concentration was to
account for the increase in body weight from 450 to 550 g. Blood samples were collected on Days 22, 36-38 and 56 and analyzed for the concentration of TRE in the plasma using HPLC/MS/MS methods that have been previously described (Corboz et al., 2017).

When the Alzet pumps were refilled on the 19th day of the TRE infusion, some of the catheters were disconnected from the jugular vein. However, there was no difference in the plasma TRE concentrations in rats with “intact connected” (n = 6) and with “disconnected catheters (n = 7) (Figure 3) and on the basis of these results, all rats were used for studies involving IV TRE.

Figure 3. Concentration of TRE in the plasma with IV TRE infusion for 5 weeks

![Graph showing concentration of TRE in plasma](image-url)
Concentration of TRE in the plasma following IV TRE administration at 810 ng/kg/min. Values are mean ± SEM. Blood samples were collected during the drug infusion at day 22, 36-38 and 56 (start of the infusion on Day 21).

C. Proteomics in the right heart

The protein content in the right heart was measured using the SWATH technology (PhenoSwitch Bioscience, Sherbrooke, Qc, Canada) that uses mass spectrometry (MS/MS) to identify ion fragments of glycosolated peptide fragments from each protein. The ion library was generated with 10 peptide fragments from each protein from which the samples were combined to yield a value for each protein. Signal intensity of each peptide was log2 transformed and normalized with a R script using retention time-based loss and signal normalization. The normalized signal of the peptides from both green fluorescent protein (GFP) was summed for each protein and used to report an individual protein signal. Multivariate analysis, heatmap, volcano plot and gene ontology analysis were done using internal Python scripts. For gene ontology, pathways were fetched using orthologue human gene names using reactome plugging in Cytoscape. Statistically significant differences between the vehicle-treated Su/Hx controls and the TPIP (117 µg/kg)-treated rats were determined using a T-test in conjunction with false discovery rate (FDR) in multiple testing using the Benjamini/Hochberg method.

The results from this proteomic analysis on 1673 proteins found significant differences between the vehicle-treated Su/Hx control group and the TPIP-treated Su/Hx group for pathways involved with eicosanoid metabolism, extracellular matrix organization, oxidative stress induced gene expression, 5-hydroxytryptamine degradation, nicotinate and nicotinamide metabolism. Listed below are the proteins in some of these pathways that had statistically significant differences between TPIP vehicle and TPIP treatments.

**Extracellular matrix organization**
Numerous changes in proteomics occurred with proteins associated with the extracellular matrix organization in the heart, with 43 proteins identified. Of these 43 proteins, statistically significant ($P \leq 0.05$) differences were found in 16 proteins comparing the values in TPIP and TPIP-vehicle treated Su/Hx rats (Table 2). Representative examples with 5 of these proteins (protein disulfide isomerase, biglycan, lumican, versican, and dystroglycan 1) are also discussed and shown in the Figure 4.

Table 2: Differentially expressed proteins associated with extracellular matrix organization in the right ventricle myocardium of TPIP treated Su/Hx rats) as compared to vehicle treated Su/Hx animals.

<table>
<thead>
<tr>
<th>UniProt_id</th>
<th>Gene_name</th>
<th>Protein_name</th>
<th>Fold change</th>
</tr>
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<tbody>
<tr>
<td>P04785</td>
<td>P4HB</td>
<td>Protein disulfide-isomerase</td>
<td>-1.79 *</td>
</tr>
<tr>
<td>P47853</td>
<td>BGN</td>
<td>Biglycan</td>
<td>-1.87 *</td>
</tr>
<tr>
<td>P51886</td>
<td>LUM</td>
<td>Lumican</td>
<td>-1.01 *</td>
</tr>
<tr>
<td>Q01177</td>
<td>PLG</td>
<td>Plasminogen</td>
<td>-2.02 *</td>
</tr>
<tr>
<td>Q9ERB4</td>
<td>VCAN</td>
<td>Versican</td>
<td>-2.28 *</td>
</tr>
<tr>
<td>Q9QZA6</td>
<td>CD151</td>
<td>Ralph blood group</td>
<td>-1.04 *</td>
</tr>
<tr>
<td>Q9WVH8</td>
<td>FBLN5</td>
<td>Fibulin 5</td>
<td>-0.70 *</td>
</tr>
<tr>
<td>D4A917</td>
<td>LTBP4</td>
<td>Latent Transforming Growth Factor Beta Binding Protein 4</td>
<td>-4.85 *</td>
</tr>
<tr>
<td>F1LNY3</td>
<td>NCAM1</td>
<td>Neural Cell Adhesion Molecule 1</td>
<td>-4.24 *</td>
</tr>
<tr>
<td>F1LPD0</td>
<td>COL15A1</td>
<td>Collagen Type XV Alpha 1 Chain</td>
<td>0.71 *</td>
</tr>
<tr>
<td>F1LS29</td>
<td>CAPN1</td>
<td>Calpain 1</td>
<td>0.96 *</td>
</tr>
<tr>
<td>F1M8K0</td>
<td>DAG1</td>
<td>Dystroglycan 1</td>
<td>0.55 *</td>
</tr>
<tr>
<td>F1MAN8</td>
<td>LAMA5</td>
<td>Laminin Subunit Alpha 5</td>
<td>1.46 *</td>
</tr>
<tr>
<td>F1MAN8</td>
<td>CAPNS1</td>
<td>Calpain Small Subunit 1</td>
<td>1.59 *</td>
</tr>
<tr>
<td>Q6IN22</td>
<td>CTSB</td>
<td>Cathepsin B</td>
<td>-1.96 *</td>
</tr>
<tr>
<td>Q6PT6</td>
<td>CTSB</td>
<td>Cathepsin D</td>
<td>-0.73 *</td>
</tr>
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Fold change, expressed in log2 transformed data, was calculated by converting the average value of the TPIP treated Su/Hx group in log2 – the average value of the vehicle treated Su/Hx group in log2.

* indicates statistical significance ($p \leq 0.05$) between the vehicle treated Su/Hx group (n = 6) and the TPIP treated Su/Hx group (n = 5). $p$ was calculated using a student T-test in conjunction for false discovery rate (FDR) in multiple testing using the Benjamini/Hochberg method.

Positive fold change means that the proteins were upregulated by TPIP treatment when compared to the vehicle group and negative fold change means that the proteins were downregulated by TPIP treatment when compared to the vehicle group.

- **Protein disulfide isomerase** is a redox chaperone of the endoplasmic reticulum that is induced during stress and serves as a vital defense against general misfolding of
proteins that possess disulphide bonds. Protein disulfide isomerase is upregulated in the endoplasmic reticulum of cardiac tissue in both animals and humans with right and left heart failure (Vitello et al., 2012). In this study, protein disulfide isomerase was increased on average in Su/Hx rats and decreased in TPIP treated rats compared to both the Nx control and Su/Hx rats (Figure 4a).

- Biglycan is an important proteoglycan for matrix reorganization and interacts with collagen and binding to lipoprotein in blood vessels. Myocardial biglycan is induced in heart failure in rats (Ahmed et al., 2003) and was increased in our study in Su/Hx rats and reduced back to the Nx controls with TPIP (Figure 4b).

- Lumican is an extracellular matrix proteoglycan that binds to collagen and is involved with collagen fibril assembly. Lumican is involved with angiogenesis and is increased in experimental and clinical heart failure (Engebretsen et al., 2013). Lumican levels were increased by challenge with Su/Hx and reduced back to Nx values by treatment with TPIP (Figure 4c).

- Versican is another extracellular matrix proteoglycan that provides extracellular scaffold for inflammatory cells as they invade tissues from the circulation. Versican has been implicated in the pathology of a number of different cardiovascular and lung diseases and levels of this proteoglycan are increased in pressure-overloaded heart tissue (Vistnes et al., 2014). In our Su/Hx challenged rats, levels of versican increase over the Nx controls and were reduced by treatment with TPIP (Figure 4d).

**Dystroglycan** 1 is a component of the dystrophin-associated glycoprotein complex which bridges the inner cytoskeleton of the extracellular matrix (Ervasti et al., 1991). Deletion of the gene synthesizing dystrophin results in Duchenne muscular dystrophy, cardiomyopathy and a number of other disorders involving the extracellular matrix (Eklund et al., 2001) and loss of the dystroglycan function in cardiac mouse myocytes results in myocyte damage and progressive cardiomyopathy (Michele et al., 2009). In our study, the level of dystroglycan in the right heart was reduced following the Su/Hx challenge and improved after treatment with TPIP (Figure 4e), suggesting an effect of TPIP to maintain myofibril integrity due to an interaction with dystrophin synthesis.
**Figure 4.** Protein expression associated with extracellular matrix organization

a) Disulfide isomerase (PDIA1)

![Graph showing protein expression levels for a) Disulfide isomerase (PDIA1)](image)

b) PGS1 Biglycan

![Graph showing protein expression levels for b) PGS1 Biglycan](image)

c) Lumican

![Graph showing protein expression levels for c) Lumican](image)
d) CSPG2 Versican

e) Dystroglycan 1

Values of the y axis for Figures 4a-e refer to an area under the curve unit from the LC-MS/MS integration.
**5-hydroxytryptamine degradation**

Three proteins in the 5-hydroxytryptamine degradation pathway were quantified, and two of them identified from the proteomic analysis, aldehyde dehydrogenase 2 and retinaldehyde dehydrogenase 1, demonstrated statistically significant ($P \leq 0.05$) differences between TPIP treatment and the TPIP-vehicle control (Table 3).

**Table 3:** Aldehyde dehydrogenase and retinaldehyde dehydrogenase expression associated with 5-hydroxytryptamine in the right ventricle myocardium of TPIP treated Su/Hx rats as compared to vehicle treated Su/Hx animals.

<table>
<thead>
<tr>
<th>UniProt_id</th>
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<th>Protein_name</th>
<th>Fold change</th>
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</thead>
<tbody>
<tr>
<td>P11884</td>
<td>ALDH2</td>
<td>Aldehyde dehydrogenase, mitochondrial</td>
<td>-1.04*</td>
</tr>
<tr>
<td>A0A0H2UHP1</td>
<td>ALDH1A1</td>
<td>Retinaldehyde dehydrogenase Raldh1</td>
<td>-1.57*</td>
</tr>
</tbody>
</table>

Fold change, expressed in log2 transformed data, was calculated by converting the average value of the TPIP treated Su/Hx group in log2 – the average value of the vehicle treated Su/Hx group in log2.

* indicates statistical significance ($p \leq 0.05$) between the vehicle treated Su/Hx group ($n = 6$) and the TPIP treated Su/Hx group ($n = 5$). $p$ was calculated using a student T-test in conjunction for false discovery rate (FDR) in multiple testing using the Benjamini/Hochberg method.

Negative fold change means that the proteins were downregulated by TPIP treatment when compared to the vehicle group.

- **Aldehyde dehydrogenase 2 (ALDH2)** is an enzyme located in the mitochondria that metabolizes 5-hydroxyindole acetaldehyde, a product of serotonin degradation. Serotonin is a key mediator of PAH pathology and is a potent pulmonary vasoconstrictor. Furthermore, ALDH2 is an etiological factor of heart failure (Pang et al., 2017). ALDH2 levels were increased by Su/Hx and reversed below Nx control levels with TPIP (Figure 5a).

- **Retinaldehyde dehydrogenase 1 (Aldh1α1 also known as Raldh1)** is the other protein in the 5-HT degradation pathway changed by TPIP (Figure 5b) and an association of retinal dehydrogenase 1 in the development of embryonic heart muscle
and in cardiac remodelling in heart failure has been previously described (Dey et al., 2015).

**Figure 5.** Protein expression associated with 5-hydroxytryptamine degradation

a) Aldehyde dehydrogenase (ALDH2)

![Graph showing protein expression for ALDH2]

b) Retinal dehydrogenase (ALDH1α1)

![Graph showing protein expression for ALDH1α1]

Values of the y axis for Figures 5a-b refer to an area under the curve unit from the LC-MS/MS integration.

**Nictinate/nicotinamide metabolism**
The proteomic analysis identified ten proteins in the nicotinate/nicotinamide metabolic pathway and three of them demonstrated statistically significant (P ≤ 0.05) differences between the TPIP treatment and the TPIP-vehicle control groups (Table 4).

**Table 4:** Nicotinate phosphoribosyltransferase, NAD-dependent protein deacetylase and proton-translocating NAD(P)(+) transhydrogenase expression associated with Nicotinate/nicotinamide metabolism in the right ventricle myocardium of TPIP treated Su/Hx rats as compared to vehicle treated Su/Hx animals.

<table>
<thead>
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<th>Protein name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3V709</td>
<td>NAPRT</td>
<td>Nicotinate phosphoribosyltransferase</td>
<td>1.81*</td>
</tr>
<tr>
<td>C6ZII9</td>
<td>SIRT3</td>
<td>NAD-dependent protein deacetylase</td>
<td>0.76*</td>
</tr>
<tr>
<td>Q5BJZ3</td>
<td>NNT</td>
<td>Proton-translocating NAD(P)(+) transhydrogenase</td>
<td>1.20*</td>
</tr>
</tbody>
</table>

Fold change, expressed in log2 transformed data, was calculated by converting the average value of the TPIP treated Su/Hx group in log2 – the average value of the vehicle treated Su/Hx group in log2.

* indicates statistical significance (p ≤ 0.05) between the vehicle treated Su/Hx group (n = 6) and the TPIP treated Su/Hx group (n = 5). p was calculated using a student T-test in conjunction for false discovery rate (FDR) in multiple testing using the Benjamini/Hochberg method.

Positive fold change means that the proteins were upregulated by TPIP treatment when compared to the vehicle group.

Nicotinate phosphoribosyltransferase (NAPRT) has been identified as a damage-associated molecular pattern (DAMP) molecule by acting as a ligand for toll-like receptor 4 (TLR4) that is a critical mediator of inflammation (Manago et al., 2019). Its functional role in PAH pathology is not clear, but has been shown to have a protective role in lipopolysaccharide injury (Manago et al., 2019) and its mild elevation in the presence of TPIP may have protective effects in Su/Hx challenged heart (Figure 6a).

- The **NAD-dependent protein deacetylase** includes the sirtuin family of proteins and are critical regulators for a variety of cellular processes such as energy metabolism and stress responses. Sirtuins protect cardiac myocytes from oxidative stress, suppress cardiac hypertrophy and regulate apoptosis and stress responses in the heart (Matsushima et al., 2015). Levels of NAD-dependent protein deacetylase were reduced in Su/Hx rats and restored back to levels observed in Nx rats by TPIP (Figure 6b).
- **Proton-translocating NAD (P)(+) transhydrogenase** is present in the mitochondria and facilitates the transfer of protons across the mitochondrial membrane where it drives the formation of NADPH, a key defense against the presence of reactive oxygen species. This enzyme has been implicated in the pathological conditions observed in a number of diseases including hypertension and heart disease (Zhang et al., 2017). In Su/Hx rats, levels of this enzyme were decreased in the right ventricle, possibly leaving the cardiac tissue more susceptible to the pathology associated with oxidative stress, and levels were returned back to the levels seen in the Nx controls by treatment with TPIP (Figure 6c).

**Figure 6.** Protein expression associated with nictinate/nicotinamide metabolism

a) **Nicotinate phosphoribosyltransferase**

b) **NAD-dependent protein deacetylase**

c) **Proton-translocating NAD(P)(+) transhydrogenase**
Values of the y axis for Figures 6a-c refer to an area under the curve unit from the LC-MS/MS integration.

References


Engebretsen KV, Lunde IG, Strand ME, Waehre A, Sjaastad I, Marstein HS, Skrbic B,


