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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*Deceased December 2021.

Running Title Page: Inhaled Treprostinil Palmitil in the Sugen/Hypoxia Rat Model

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Abbreviations

\( \alpha \text{SMA} \): Alpha smooth muscle actin

\( \text{AUC}_{0-24h} \): Area under the concentration curve between time zero and 24-hrs

\( \text{AUC}_{0-\text{inf}} \): Area under the concentration curve extrapolated to infinity

\( \text{CCAC} \): Canadian Council on Animal Care

\( C_{\text{max}} \): Maximal concentration

\( \text{CO} \): Cardiac output

\( \Delta \text{RVPP} \): Increase in right ventricular pulse pressure

\( \text{DMSO} \): Dimethyl sulfoxide

\( \text{DP1} \): Prostaglandin D\(_2\) type 2 receptor

\( \text{DSPE-PEG-2000} \): 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000

\( \text{EP2} \): Prostaglandin E type 2 receptor

\( \text{EP4} \): Prostaglandin E type 4 receptor

\( \text{GLP} \): Good laboratory practice

\( \text{FDA} \): Food and drug administration

\( \text{H&E} \): Hematoxylin and Eosin

\( \text{HPLC/MS} \): High-performance liquid chromatography with mass spectrometry
HR: Heart rate
Hx: Hypoxia
ICH: International conference on harmonization
IP: Prostacyclin receptor
Intravenous: Intravenous
IRIC: Institute for Research in Immunology and Cancer
lambda z: Terminal elimination rate constant
MC: Methylcellulose
MHLW: Ministry of health, labour and welfare
mPAP: Mean pulmonary arterial blood pressure
mSAP: Mean systemic arterial blood pressure
NaCl: Sodium chloride
NBF: Neutral buffered formalin
Nx: Normoxic
OECD: Organization for economic co-operation and development
PAAT: Pulmonary artery acceleration time
PAH: Pulmonary arterial hypertension
PBS: Phosphate buffered saline
PK: Pharmacokinetics
PPAR: Peroxisome proliferator-activated receptor
PVR: Pulmonary vascular resistance
RVAWT: Right ventricular anterior wall thickness
RVPP: Right ventricular pulse pressure
RVSP: Right ventricular systolic pressure
SC: Subcutaneous
SpO₂: Pulse oximeter oxygen saturation
SV: Stroke volume
T½: Elimination half-time
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$T_{\text{max}}$</td>
<td>Time of maximal concentration</td>
</tr>
<tr>
<td>TP</td>
<td>Treprostinil palmitil</td>
</tr>
<tr>
<td>TPIA</td>
<td>Treprostinil palmitil inhalation aerosol</td>
</tr>
<tr>
<td>TPIP</td>
<td>Treprostinil palmitil inhalation powder</td>
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<tr>
<td>TPIS</td>
<td>Treprostinil palmitil inhalation suspension</td>
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<td>TRE</td>
<td>Treprostinil</td>
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<td>TREAeq</td>
<td>Molar equivalent for TRE</td>
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<tr>
<td>Su</td>
<td>Sugen5416</td>
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<tr>
<td>VAG</td>
<td>Vilnius aerosol generator</td>
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<td>vWF</td>
<td>Von Willebrand factor</td>
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Section Assignment: Cardiovascular

Author conflicts:

All authors are employees of the study sponsor, Insmed Incorporated.

No author has an actual or perceived conflict of Interest with the contents of this article.
Abstract

Treprostinil palmitil (TP), a long-acting inhaled pulmonary vasodilator prodrug of treprostinil (TRE), has beneficial effects in a Sugen5416/Hypoxia (Su/Hx) rat model of pulmonary arterial hypertension (PAH) that compare favorably to the oral PDE5 inhibitor, sildenafil. In this study in male Sprague Dawley rats, a dry powder formulation of TP (TPIP) was compared to inhaled and intravenous TRE and oral selexipag to evaluate inhibition of hemodynamic and pathological changes in the lungs and heart induced by Su/Hx challenge. Su (20 mg/kg) was injected subcutaneously followed by 3 weeks of Hx (10% O₂/balance N₂), then initiation of test article administration over 5 weeks with room air breathing. Hemodynamics and histopathology were measured at the end of the study. Su/Hx challenge approximately doubled the mean pulmonary arterial blood pressure (mPAP) and the Fulton index, decreased cardiac output (CO), doubled the wall thickness and muscularization of the small (10-50 μm) and medium (51-100 μm) sized pulmonary arteries and increased the percentage of obliterated pulmonary blood vessels. Even though inhaled TRE (65 μg/kg, QID), intravenous TRE (810 ng/kg/min) and oral selexipag (30 mg/kg, BID) provided some beneficial effects against the Su/Hx challenge, the overall benefit was generally greater with TPIP at high dose (117 μg/kg, QD). These results demonstrate that TPIP compares favorably to inhaled and intravenous TRE and oral selexipag with respect to inhibition of the pathophysiological changes induced by Su/Hx challenge in rats.

Word count: 230 (max 250)

Keywords:
Treprostinil palmitil
Pulmonary arterial hypertension
Sugen5416/Hypoxia rat model
Pulmonary vascular and cardiac remodeling
Inhaled and intravenous treprostinil

Selexipag

Significance Statement

Treprostinil palmitil (TP) is a long-acting pulmonary vasodilator prodrug of treprostinil (TRE) formulated for inhaled administration by dry powder (treprostinil palmitil inhalation powder, TPIP). Comparison of the activity of TPIP, inhaled and intravenous TRE and oral Selexipag in a Sugen5416/Hypoxia (Su/Hx) rat model of pulmonary arterial hypertension demonstrated that each of these drugs exert protection against the hemodynamic and histopathological changes induced by the Su/Hx challenge, with the greatest effect on these changes produced by TPIP.
Introduction

Treprostinil palmitil (TP) is an ester-linked prodrug of treprostinil (TRE) in development for the treatment of pulmonary arterial hypertension (PAH) and pulmonary hypertension associated with interstitial lung disease that has been formulated for inhaled delivery as a nebulized suspension (treprostinil palmitil inhalation suspension - TPIS), as a dry powder (treprostinil palmitil inhalation powder - TPIP) and as an aerosol for delivery with a metered dose inhaler (treprostinil palmitil inhalation aerosol - TPIA) (Corboz et al., 2017; Chapman et al., 2020; Chapman et al., 2021b; Plaunt et al., 2021). TP has many attributes that may prove to be beneficial, including long-acting pulmonary vasodilation (Corboz et al., 2017; Chapman et al., 2018), no evidence of tachyphylaxis with repeated administration (Chapman et al., 2021a), a reduced propensity to cause cough (Corboz et al., 2017; Chapman et al., 2020) and robust efficacy in a Sugen5416/Hypoxia (Su/Hx) rat model of PAH which compared favorably to results from the phosphodiesterase 5 (PDE5) inhibitor sildenafil (Corboz et al., 2021a).

Inhaled TP has several important features that may prove to be extremely important to treat PAH pathology. Administration by inhalation results in a locally high concentration of TP in the lung, and after a slow conversion of TP to TRE by the action of lung esterase (Leifer et al., 2018), leads to a greater duration of TRE exposure in the lungs resulting in beneficial effects such as long-acting pulmonary vasodilation (Sandifer et al., 2014; Chapman et al., 2018). TRE binds to the prostacyclin (IP), prostaglandin E type 2 (EP₂), prostaglandin E type 4 (EP₄), prostaglandin D₂ type 2 (DP₁) and the peroxisome proliferator-activated (PPAR) receptors (Ali et al., 2006; Clapp and Gurung, 2015; Corboz et al., 2017; Corboz et al., 2021b; Falcetti et al., 2007; Whittle et al., 2012), mostly found on structural, inflammatory and immune cells involved with PAH pathology, which discriminates it from oral selexipag that is a selective IP receptor agonist (Gatfield et al., 2017). Also, TP does not induce tachyphylaxis following inhalation for up to 32 consecutive days whereas tachyphylaxis and IP receptor desensitization are found when TRE is continuously infused by the intravenous (IV) route (Gatfield et al., 2017; Chapman et al., 2021a). Finally, inhaled TP inhibits much of
the pulmonary vascular remodeling that is induced by Su/Hx challenge in rats (Corboz et al., 2021a) that is a feature not observed for subcutaneously infused TRE (Chaudhary et al., 2018).

The present study was designed to compare the activity of TPIP to that produced by different drugs that act on the prostacyclin pathway that are currently used to treat PAH subjects including inhaled and IV TRE and oral selexipag. For this evaluation, we used a Su/Hx rat model of PAH as it recapitulates many of the important features of human PAH pathology such as increased pulmonary vascular resistance, pulmonary vascular remodeling, occlusion of small pulmonary blood vessels, an increase in right heart size, and reduced cardiac performance (Tarasevicius-Stewart 2001; de Raaf et al., 2014; Jiang et al., 2016; Toba et al., 2014; Bhat et al., 2017; Corboz et al., 2021a). The dose selection for the compounds tested was based upon a combination of published data (Chaudhary et al., 2018; Honda et al., 2020) and efficacy studies in healthy rats measuring the inhibition of pulmonary vasoconstriction induced by challenge with an inhaled hypoxic mixture (Corboz et al., 2021c).

**Word count:** 553 (max 750)
Material and Methods

Details of the methods and supporting data can be found in the on-line supplement.

Materials. TPIP was manufactured by Bend Research Inc. (Bend, OR, USA) as a dry powder for inhalation, composed of 68.50% mannitol, 29.25% leucine, 1.50% TP and 0.75% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG-2000). TRE and TP were obtained from Chirogate International (Taoyuan County, Taiwan, Republic of China). TRE was prepared for nebulization by dissolving 0.5 mM TRE in phosphate buffered saline (PBS). TRE for IV administration was prepared by dissolving 8.75 or 10.70 mg/mL of TRE with 3.0 mg/mL m-Cresol, 5.3 mg/mL sodium chloride (NaCl) and 6.3 mg/mL sodium citrate dihydrate. PBS was purchased from Mediatech (Manassas, VA, USA), m-Cresol and methylcellulose (MC) from Sigma-Aldrich (St Louis, MO, USA) and NaCl and sodium citrate dihydrate from Fisher (Waltham, MA, USA). Selexipag was suspended in 0.5% (w/v) MC and given by oral gavage in a volume of 10 mL/kg body weight. Selexipag was purchased from MedChemExpress (Monmouth Junction, NJ, USA). The vehicles for TPIP, inhaled and IV TRE and oral selexipag contained each excipient but no drug. Sugen 5416 (Su) was obtained from Adooq Bioscience (Irvine, CA, USA) and dissolved in 100% dimethyl sulfoxide (DMSO).

Animals. Experiments were performed in adult male Sprague-Dawley rats (Charles River Laboratories, St Constant, QC, Canada). The animals' weights, between 200 and 250 g at the beginning of the Su/Hx study, were measured weekly throughout the study. The rats were housed in temperature (21°C) and humidity-controlled conditions and were acclimated to the laboratory surroundings for 6 to 7 days before commencement of the study. All experimental procedures were performed in accordance with the Canadian Council on Animal Care (CCAC) guidelines and followed the principles of Good Laboratory Practice (GLP) Regulations of the United States Food and Drug...
Administration (FDA) (21 CFR Part 58) and current Organization for Economic Co-operation and Development (OECD)/Ministry of Health, Labour and Welfare (MHLW) and International Conference on Harmonization (ICH) guidelines.

**Inhaled Hypoxia Challenge.** Rats were prepared with telemetry probes inserted into the right ventricle and descending aorta to measure the right ventricular pulse pressure (RVPP) and mean systemic arterial blood pressure (mSAP) in response to an inhaled hypoxic gas mixture as described previously (Chapman et al., 2021a). The rats were placed in individual plexiglass chambers through which normoxic (Nx) air (21% O₂/balance N₂) from a compressed gas source was circulated at a flow rate of 8.75 L/min (Chapman et al., 2021a). After an equilibration period of 30 min, data was collected for the baseline Nx exposure followed by transition to a 10 min exposure of hypoxia (Hx) with hypoxic gas (10% O₂/90% N₂) also delivered from a compressed gas source, followed by a return to a 10 min period of room air breathing. The study design for the acute Hx challenge in telemetered rats is illustrated in Supp. Fig. 1. From these results, the increase in RVPP (ΔRVPP) due to Hx from the Nx values and change in mSAP were measured. Each rat was exposed to Hx on 3 separate occasions on the day before the administration of test articles with the baseline response representing the average of these three Hx exposures. The following day, the rats were treated with the test articles or vehicles with additional Hx challenges performed intermittently over the next 24 hours.

**Sugen/Hypoxia Challenge.** Rats received a subcutaneous (SC) injection of Su at 20 mg/kg followed by 3 weeks of exposure to an inhaled hypoxic gas mixture (10% O₂/90% N₂) and then a return to room air breathing for 5 weeks. The test articles (drugs or drug vehicles) were administered immediately after the Hx challenge and were dosed daily throughout the 5-week room air breathing period. A control group of rats received the vehicle for Su (100% DMSO) followed by 8 weeks of room air breathing. The study design for the acute Hx challenge in telemetered rats is illustrated in Supp. Fig. 2 and details of the transition from Nx to Hx and return to room air breathing and drug administrations are shown in Supp. Table 1. All animals were maintained on a light-dark...
(12 h/12 h) cycle receiving water and food *ad libitum*. Daily observations were made of the behavior and general health status of the animals and body weights were recorded weekly. For the test article administrations, the animals were randomized after the 3-week Hx period to the different treatment groups based on their body weight and transthoracic echocardiography measures. Animals within the same treatment group were pair-housed, except for the IV TRE groups implanted with an Alzet pump who were single-housed.

At the end of the study, the rats were anesthetized with a mixture of 2 to 2.5 % isoflurane in oxygen (Abbot Laboratories, Montreal, Canada), catheters were inserted into the trachea to facilitate artificial ventilation and the pulmonary artery and aorta were catheterized for the measurement of systolic, diastolic, mean pulmonary arterial blood pressure (mPAP) and mSAP as previously described (Corboz et al., 2021a). A pulse oximeter was placed on the paw for the measurement of heart rate (HR) and pulse oximeter oxygen saturation (SpO₂). Echocardiography was performed intermittently throughout the study using a GE Healthcare echocardiography system (Model Vivid 7, GE Healthcare, Chicago, Illinois, USA) to measure the pulmonary artery acceleration time (PAAT), right ventricular anterior wall thickness (RVAWT) and HR as previously described (Urboniene et al., 2010; Zhu et al., 2019). From these data, the cardiac output (CO) and stroke volume (SV) were calculated according to the relationships previously described (Lewis et al., 1984).

As a surrogate measure, pulmonary vascular resistance (PVR) was estimated using the following ratio (Wang et al., 2013):

\[
PVR = \frac{RVSP}{CO}
\]

with PVR: pulmonary vascular resistance (mmHg/mL.min⁻¹); RVSP: right ventricular systolic pressure (mmHg); CO: cardiac output (mL/min)

At the end of the hemodynamic recording, the rats were euthanized, and the heart and lungs removed for histological analysis and derivation of the Fulton index (Corboz et al., 2021a). The Fulton index was calculated from measurements of the right
ventricle and left ventricle plus septum weights as described previously (Fulton et al., 1952; Hangartner et al., 1985).

\[
\text{Fulton Index} = \frac{\text{right ventricle weight (g)}}{\text{(left ventricle weight+septum weight) (g)}}
\]

**Histological procedure of the lung**

For the histological evaluations on the lungs, tissues were embedded, sliced at 5 μm thickness and stained with Hematoxylin and Eosin (H&E), the pulmonary arteries/arterioles identified and categorized into small (10-50 μm), medium (51-100 μm) and large diameter vessels (>100 μm) and the percentage of these pulmonary blood vessels demonstrating the presence of a muscular (completely surrounded by a smooth muscle layer, > 90% circumference), semi-muscular (incompletely surrounded by a smooth muscle layer, 10-90% circumference) or a non-muscular (no apparent smooth muscle layer, < 10% circumference) appearance were quantified. The tissues were also stained with alpha smooth muscle actin (αSMA) to quantify the presence of smooth muscle present in the vascular wall of the pulmonary arteries (Corboz et al., 2021a).

To identify and quantify the occlusive lesions of the small pulmonary arteries, the tissues were embedded, sliced at 5 μm thickness and stained with Von Willebrand factor (vWF) and categorized into vessels having no evidence of neo-intimal formation (non-occluded), as partially obliterated with < 50% of luminal occlusion (semi-occluded), or mostly obliterated with ≥ 50% of luminal occlusion (mostly occluded).

Only intra-acinar vessels within the gas exchange regions of the lung (alveoli, alveolar ducts and respiratory bronchioles) were used in these analyses. All vessels associated with terminal bronchioles were excluded. For the histological analysis of cardiac tissue, the tissues were embedded, sliced at 5 μm thickness and stained with H&E for the assessment of morphology and with Masson’s trichrome stain to identify the presence of collagen (Corboz et al., 2021a).

**Histological Procedure of the Heart**

The hearts from each treatment were separated into two groups with half of the samples designated for the histology and stained with H&E or Masson’s trichrome. The
other half were used for measurement of the Fulton Index determination and proteomic parameters. The heart tissues harvested for histological analysis were fixed in 10% neutral buffered formalin (NBF) for 24h. A transversal section in the middle of the heart was cut and sent to the Institute for Research in Immunology and Cancer (IRIC, Montreal, Quebec, Canada) in 10% NBF embedded in paraffin, sliced at 5 µm thickness, mounted and stained with either H&E for overall assessment of the cardiomyocyte morphology or with Masson trichrome for collagen fiber visualization and quantification. Stained tissues were then scanned at 20X magnitude resolution for analysis. High resolution images were analyzed using NDP.view 2.7.25 Zoomer Digital Pathology (Hamamatsu) software for the general analysis and Infinity Analyze 5.0.3. for collagen quantification.

**Drug Administrations in the Acute Inhaled Hypoxia and Su/Hx Challenges.** Inhaled delivery of the test articles was performed using a 12-port nose-only inhalation chamber (CH Technologies, Westwood, NJ, USA) that was adapted for administration of either dry powder aerosols of TPIP or nebulized administration of TRE (Corboz et al., 2017; Chapman et al., 2020). Dry powder aerosol was generated using a Vilnius Aerosol Generator (VAG) (CH Technologies, Westwood, NJ, USA) and dispersed into the nose-only chamber with air from a compressed gas source at a flow rate of 7 L/min and nebulized aerosol was generated with an Aeroneb® Pro nebulizer (Aerogen, Galway, Ireland) that was dispersed into the nose-only chamber with compressed air at a flow rate of 6 L/min. To administer different inhaled doses of TPIP, the VAG was loaded with different amounts of material (25-170 mg) set at output values ranging from 0.125 to 1 Volt and continued until all of the powder had been aerosolized. Exposure of rats to TPIP at VAG outputs of 0.125, 0.25, 0.5 and 1 V resulted in total inhaled doses of 6, 23, 57 and 138 µg/kg, respectively, in the inhaled Hx study, and exposure of rats to TPIP at VAG outputs of 0.5 and 1 V resulted in total inhaled doses of 59 and 117 µg/kg, respectively, in the Su/Hx study. The aerosol concentration was maintained at the desired level with the aid of a portable aerosol monitor (Casella MicroDust Pro, Sterling, MA, USA) providing an auto-feedback circuit to the VAG. For the nebulization of TRE or
PBS vehicle, 6 mL of material was placed into the Aeronob® Pro nebulizer with the output set to the “FULLY ON” position until all the material had been nebulized. Nebulized TRE at concentrations of 0.125, 0.25, 0.50, 1 mM resulted in delivered doses of 15, 46, 110 and 215 μg/kg in the inhaled Hx challenge and nebulized TRE at concentrations of 0.50 mM resulted in delivered doses of 65 μg/kg in the Su/Hx challenge.

The time for aerosolization of the dry powder and nebulized test articles was recorded. A filter was connected to one of the outlet ports and attached to a vacuum pump from which a vacuum flow of 0.5 L/min was established for a 5 min period to collect the drug. The quantitation of drug deposited on the filter was performed using high-performance liquid chromatography with mass spectrometry (HPLC/MS Single Quad) and a charged aerosol detector as described previously (Corboz et al., 2017). The inhaled drug dose was calculated using the algorithm previously described with the deposition fraction established at 1.0 (Alexander et al., 2008) and incorporating the values for the concentration of drug sampled from the nose-only inhalation chamber, the duration of drug exposure, respiratory minute volume, and body weight (Table 1). A deposition factor of 0.1 was used for the derivation of the delivered pulmonary dose based upon the amount of drug deposited on the filter (Wolff and Dorato, 1993).

For the IV infusion of TRE and its vehicle, an Alzet pump (Alzet Osmotic Pumps, Cupertino, CA) was implanted subcutaneously in the neck region one day before the start of the infusions; details of the Alzet pump implantation and infusion of TRE have been previously described (Chapman et al., 2021a). For the TRE infusion, the Alzet pump was filled with 2 mL of 8.75 mg/mL TRE solution on day 21 and refilled on day 42 with a 10.7 mg/mL solution to account for the increase in body weight. It should be noted that after this surgical procedure on Day 21 of the IV infusions, some of the catheters were found to be disconnected from the jugular vein and had to be re-inserted to continue the IV infusions but no differences in plasma TRE concentrations were observed between rats with intact and disconnected catheters (Supp. Fig.3).

For oral selexipag or vehicle administrations, selexipag was suspended in 0.5% MC and administered by oral gavage to rats in a volume of 10 mL/kg. The
concentrations of selexipag ranged between 0.3 - 3 mg/mL to provide the appropriate testing dose of the drug, both in the acute hypoxia challenge studies and in the Su/Hx challenge experiments. The rats were given food and water throughout these studies.

Details of the different groups with treatments at targeted and delivered drug doses, and routes of administration are listed in Supp. Table 1.

**Pharmacokinetics.** Blood and lung tissue samples were intermittently collected, prepared for shipment to Insmed Incorporated (Bridgewater, NJ, USA) and analyzed for their drug concentrations by HPLC/MS/MS using techniques that have been previously described (Corboz et al., 2021a).

Administration of the drugs started at day 21 and ended at day 55. Blood samples were collected at: 1) day 22 (24 h after the first dose administered at day 21), day 38 (24 h after the dose administered at day 37) and day 56 (24 h after the last dose administered at day 55) for the TPIP groups (QD), 2) day 22 (12 h after the fourth dose administered at day 21), day 38 (12 h after the fourth dose administered at day 37) and day 56 (12 h after the last dose administered at day 55) for the inhaled TRE group (QID), 3) during the drug infusion at days 22, 38 and 56 for the IV TRE group, and 4) day 22 (16 h after the second dose administered at day 21), day 38 (16 h after the second dose administered at day 37) and day 56 (16 h after the last dose administered at day 55) for the oral selexipag group (BID).

Lung and heart were collected at day 56 for all groups, 24 h after the last TPIP dose administered at day 55 (QD), 12 h after the last inhaled TRE dose at day 55 (QID), immediately after the interruption of the continuous TRE injection, and 16 h after the last selexipag dose administered orally at day 55 (BID).

The following pharmacokinetics (PK) parameters were measured: lambda z (terminal elimination rate constant), T½ (elimination half-time), T_max (time of maximal concentration), C_max (maximal concentration), AUC_0-24h (area under the concentration curve between time zero and 24-hours) and AUC_0-inf (area under the concentration curve extrapolated to infinity) using the PKSolver program in Microsoft Excel (Zhang et al., 2010). For the conversion of TP concentrations into a molar equivalent for TRE
(TREeq), TP values were multiplied by a factor of 0.635 based upon the molecular weights of TP (614.9) and TRE (390.5).

**Data Analysis and Statistics.** All values are presented as the mean ± SEM. In the acute inhaled hypoxia experiments, a t-test with repeated measures was used to determine statistically significant differences between the baseline data and that obtained at different times after initiation of test articles. In the Su/Hx studies, the hemodynamic and echocardiography data of the different drug treatments were compared to their appropriate vehicles or to the Nx controls using a repeat ANOVA. A post-hoc analysis was performed with an unpaired Student’s t-test for repeated measures. For the histological evaluations, a repeat ANOVA was performed with post-hoc analysis performed with a Tukey’s test to determine statistically significant effects within the different treatment groups. A $P$ value of ≤ 0.05 was set to denote statistically significant effects.

The formula below was used to calculate the percentage inhibition produced by TPIP, inhaled and IV TRE and oral selexipag in each rat on mPAP, CO, Fulton index, wall thickness, percentage of muscularization and percentage of non-obliterated blood vessels after Su/Hx challenge. From these results, a combined average percentage inhibition was calculated for each drug.

$$\% \text{ inhibition} = \frac{(\text{Su/Hx} + \text{vehicle control}) - (\text{Su/Hx} + \text{drug})}{(\text{Su/Hx} + \text{vehicle}) - (\text{Nx control})} \times 100.$$  

Calculations for the inhaled drug dose of TPIP and TRE are shown in Table 1.
Results

**Inhaled Hypoxia Challenge.** Inhaled TPIP (6-138 µg/kg) inhibited the ∆RVPP due to hypoxia with the greatest inhibition occurring between 1 and 6 hours and was still significant ($P \leq 0.05$) by 12 hours and slowly returned to the baseline values by 24 hours at all doses (Figure 1a). At 24 hours, the highest TPIP dose (138 µg/kg) had 48% inhibition of the ∆RVPP response to hypoxia but failed to reach statistical significance ($P = 0.11$) as it could only be measured in 3 rats due to technical problems with the telemetry probe. In contrast, inhaled TRE had a relatively short duration of activity and significantly ($P \leq 0.05$) inhibited the ∆RVPP response to hypoxia only up to 1 hour at doses between 15-110 µg/kg that increased to 2 hours at the highest dose of 215 µg/kg (Figure 1b). Oral selexipag (30 mg/kg) significantly ($P \leq 0.05$) inhibited the ∆RVPP response to hypoxia up to 2 hours, but not at times beyond this and had no significant effects at a lower dose of 10 mg/kg (Figure 1c). Continuous infusion of IV TRE (810 ng/kg/min) significantly ($P \leq 0.05$) inhibited the ∆RVPP response to hypoxia on the first day of infusion (Day 0) but did not have a significant effect on days 4 through 16 (Figure 1d).

**Sugen/Hypoxia challenge.**

**Hemodynamics.** Su/Hx induced a statistically significant ($P \leq 0.05$) increase in mPAP and reduction in CO that resulted in a 3-fold increase in PVR compared to values in the Nx controls (Figure 2a-c). Echocardiography parameters of SV and PAAT were significantly ($P \leq 0.05$) reduced by the Su/Hx challenge with the greatest reductions occurring on Day 21, immediately after the inhaled hypoxia exposure (data not shown), and were still reduced on Day 56 at the end of the study (Figure 3a-b). Su/Hx also significantly ($P \leq 0.05$) increased the RVAWT (Figure 3c) with a significant ($P \leq 0.05$) increase in right heart size, measured by the Fulton index (Figure 4). There were no significant changes in mSAP or HR in the Su/Hx rats compared to the Nx controls (data not shown).
TPIP (59 and 117 µg/kg, QD) dose-dependently inhibited the increase in mPAP and PVR and the reduction of CO that was induced by the Su/Hx challenge (Figure 2a-c). Statistically significant ($P \leq 0.05$) effects on mPAP and PVR were observed for both doses of TPIP and on CO with the high TPIP dose. A dose-dependent inhibition by TPIP was also observed on the changes in SV, PAAT, and RVAWT induced by Su/Hx with significant ($P \leq 0.05$) effects on each parameter observed for the high TPIP dose (Figure 3a-c). In contrast, inhaled TRE (65 µg/kg, QID) had no significant effects on mPAP, CO, PVR, SV and RVAWT and had a small, but significant ($P \leq 0.05$) effect on PAAT induced by the Su/Hx challenge (Figures 2 and 3). Treatments with IV TRE (810 ng/kg/min) and oral selexipag (30 mg/kg, BID) also showed no significant improvement in the changes to CO, PAAT and RVAWT induced by Su/Hx and less effect on mPAP, PVR and SV than that observed with the high dose of TPIP (Figures 2 and 3).

The increase in Fulton index induced by Su/Hx was dose-dependently inhibited by treatment with TPIP and by oral selexipag (Figure 4). On the other hand, treatment with neither inhaled nor IV TRE significantly inhibited the increase in Fulton index induced by the Su/Hx challenge.

**Histopathology.** Su/Hx challenge significantly increased ($P \leq 0.05$) the wall thickness of the small and medium sized pulmonary arteries (Figure 5a) that was almost entirely due to an increase in the percentage of smooth muscle in the vascular wall evaluated by $\alpha$SMA (Figure 5b). There was a redistribution of the percentage of muscular, semi-muscular and non-muscular blood vessels in the pulmonary arteries with the majority of vessels having full muscularization after Su/Hx challenge (Figure 5c). Su/Hx challenge also increased the percentage of semi- and totally obliterated small diameter (10-50 µm) pulmonary blood vessels along with a parallel reduction in the percentage of non-obliterated blood vessels (Figure 5d).

Treatment of Su/Hx rats with TPIP (59 and 117 µg/kg, QD) dose-dependently inhibited the increased wall thickness, muscularization and obliteration of the small diameter pulmonary blood vessels (Figure 5a-d). The effects of TPIP were generally greater than those produced by inhaled TRE (65 µg/kg, QID), IV TRE (810 ng/kg/min) and oral selexipag (30 mg/kg, BID). Representative photomicrographs of the lungs
stained with αSMA (Figure 6a) and vWF (Figure 6b) for the different treatment groups illustrate the increased wall thickness, muscularization and obliteration of the pulmonary arteries.

In cardiac tissue, Su/Hx challenge produced an increase in RVAWT with the presence of collagen staining in most of the treatment groups (Figure 7a). The percentage of collagen in the right ventricle was not affected by TPIP, IV TRE or oral selexipag but was significantly reduced by inhaled TRE (data not shown). However, the vehicle group for inhaled TRE (nebulized PBS) unexpectedly showed a higher percentage of collagen compared to the other vehicle groups used in the study and there was no difference in the collagen content between the different treatment groups that received TPIP, IV TRE or oral selexipag.

Histological examination of cardiac tissues, showing an enlarged vessel with perivascular/interstitial fibrosis and cardiomyocyte hypertrophy after Su/Hx challenge, is displayed in Figure 7b. Right ventricles were stained with Masson’s Trichrome for collagen fiber visualization and severity of collagen deposition was depicted by the intensity and magnitude of the blue staining (Masson’s Trichrome). Fibrotic areas with increases of collagen deposition (high blue staining intensity) were observed in the cardiac perivascular region of the Su/Hx + TPIP vehicle group, relative to the normoxic control group. Perivascular and interstitial fibrosis were slightly reduced by treatment with 117 µg/Kg TPIP (Figure 7b).

**Overall comparison between TPIP, inhaled and IV TRE and oral selexipag.** The overall inhibition of the changes in mPAP, CO, Fulton index, wall thickness, muscularization and obliteration of the pulmonary arteries induced by Su/Hx was greatest for TPIP at high dose (117 µg/kg, QD) than for selexipag (30 mg/kg, BID), inhaled TRE (65 µg/kg, QID), low dose of TPIP (59 µg/kg, QD), and IV TRE (810 ng/kg/min) (Table 2). A multiparameter comparison graph illustrating these results is shown in Figure 8.
Pharmacokinetics. The concentration of TRE in the plasma, measured 24 h after the first administration of inhaled TPIP (59 and 117 µg/kg, QD) was below the level of detection (Table 3a) and was also very low 12 h after the fourth dose administrated the first day of dosing with inhaled TRE (65 µg/kg, QID) (Table 3a). There were variable increases in the plasma TRE concentrations over time with daily administration of both TPIP and inhaled TRE. On the other hand, the plasma TRE concentrations were much higher after IV TRE (810 ng/kg/min) where the samples were taken during the drug infusion with the highest concentration (39.35 ± 6.44 ng/mL) found 24 h after the beginning of infusion (Table 3a). The plasma concentrations of selexipag (BID) and its active metabolite, ACT-333679 were also highest 16 h after the second dose administered the first day and generally decreased with repeated dosing over 35 days (Table 3b).

The concentration of TRE in the lungs approximately doubled when the inhaled TPIP dose was doubled (59 and 117 µg/kg, QD) (Table 4a). The concentration of TRE in the lungs was approximately 50- to 100-fold greater after TPIP than after inhaled TRE even though the collection time was 24 h after TPIP compared to 12 h for inhaled TRE (Table 4a). A higher concentration of TRE in the lungs was found after IV TRE than inhaled TRE, but the samples were collected approximately 1 h at the end of the drug infusion (Table 4a). The concentration of ACT-333679 in the lung was approximately 39-fold higher than for selexipag (Table 4b).
Discussion

In this study, we compared the activity of TPIP, a long-acting prodrug of TRE, in a Su/Hx rat model of PAH to that of three other drugs that act on the prostacyclin pathway that have been approved for use in PAH subjects: inhaled and IV TRE and oral selexipag. The dose and frequency of administration with each compound for these Su/Hx studies was selected from experiments that measured their acute pulmonary vasodilator activity in rats challenged with Hx (Chapman et al., 2021a). Each of these four drugs offered some degree of protection against the hemodynamic and histopathological changes that were induced by the Su/Hx challenge, but the greatest and most consistent effect was produced by TPIP at high dose. Using a combined numerical average for the inhibition of the changes in mPAP, CO, Fulton index, wall thickness, muscularization, and obliteration of the pulmonary arteries induced by Su/Hx, the activity of TPIP at high dose (117 µg/kg, QD) showed advantageous effects, generally greater than for oral selexipag (30 mg/kg, BID), inhaled TRE (65 µg/kg, QID), and IV TRE (810 ng/kg/min) while the activity of TPIP at low dose (59 µg/kg, QD) was comparable to slightly favorable across most parameters tested. These results build upon the findings from a previous study that demonstrated effects of inhaled TP which compared favorably to those of the oral PDE5 inhibitor, sildenafil (Corboz et al., 2021a) and suggest that TPIP may offer additional benefits for the treatment of PAH compared to other drugs acting on the prostacyclin pathway that are currently approved for clinical use.

To select the dosing frequency of each compound for the Su/Hx studies, dose-response and duration of activity studies were performed in rats challenged with acute inhaled Hx that measured the inhibition of pulmonary vasoconstriction (Chapman et al., 2021a). As expected, TPIP produced a dose-dependent and long-acting pulmonary vasodilation over 12-24 h from which targeted doses of 57 and 138 µg/kg given QD were selected. The actual average delivered doses of TPIP in the Su/Hx studies, 59 and 117 µg/kg, were very close to these targeted doses. On the other hand, dose-response studies with inhaled TRE in the acute Hx challenged rats found a relatively short duration of action lasting between 1-2 hours. From these results, a targeted inhaled
TRE dose of 110 µg/kg given QID was selected, although the average delivered dose measured in the Su/Hx studies was 65 µg/kg due to variations such as room humidity, VAG conditions and flowability of dry powder. For IV TRE, we used a dose infusion rate of 810 ng/kg/min that was based upon data from a previous study in Su/Hx-challenged rats (Chaudhary et al., 2018) and demonstrated acute pulmonary vasodilation in our experiments. Furthermore, based upon previously published data in Su/Hx challenged rats, an oral dosing frequency of 30 mg/kg selexipag administered BID was selected (Honda et al., 2020) with pulmonary vasodilation at this dose observed up to 4 h in our acute Hx challenged rats.

In Su/Hx challenged rats, TPIP inhibited most of the hemodynamic and histopathological changes in the lungs and heart including effects on the increased mPAP and PVR, the reduction in CO, the increases in wall thickness, muscularization and obliteration of small diameter (10-50 µm) pulmonary arteries and the enlargement in right heart size measured by the increase in Fulton index and RVAWT. Furthermore, using glycoproteomic analysis on tissue from the right heart (Supp. Fig. 4-6 and Supp. Tables 2-4), TPIP inhibited the over- and under-expression of several proteins that are associated with cardiac and vascular diseases including heart failure, arrhythmias, vascular stenosis, endothelial dysfunction and hypertension (Ahmed et al., 2003; Vitello et al., 2012; Engebretsen et al., 2013; Vistnes et al., 2014; Dey et al., 2015; Gao and McNally 2015; Matsushima and Sadoshima, 2015; Perrucci et al., 2015; Pang et al., 2017; Zhang et al., 2017). In summary, TPIP demonstrated protective effects on nearly all of the hemodynamic and pathological changes induced by a Su/Hx challenge in rats. The same consistency of effect was not demonstrated by inhaled and IV TRE or oral selexipag.

For example, the increase in the Fulton index was not inhibited by inhaled or IV TRE and there were only modest effects of these drugs on the increases in mPAP, RVSP and PVR compared to the high dose of TPIP. Furthermore, while selexipag inhibited the increased Fulton index, mPAP, PVR, wall thickness and muscularization of the pulmonary arteries, it had no effect on the obliteration of small pulmonary arteries or on the reductions in cardiac performance such as CO and SV induced by the Su/Hx challenge. To numerically demonstrate the superiority of TPIP over the other
prostacyclin analogs used in our study, a percent inhibition was calculated for the changes in mPAP, Fulton index, CO, wall thickness, muscularization and obliteration of the pulmonary arteries in Su/Hx rats and represented as an average of these values (Figure 8). With this analysis, the effects of TPIP at high dose (117 µg/kg, QD) were greater than selexipag (30 mg/kg BID), inhaled TRE (65 µg/kg, QID), TPIP at low dose (59 µg/kg, QD), and IV TRE (810 ng/kg/min).

Several factors likely contribute to the beneficial effects of TPIP and discriminate it from the other prostacyclin analogs used in this study. First, TPIP was administered by inhalation which offers the advantage over systemically administered drugs like IV TRE and oral selexipag by delivering relatively high concentrations of the drug directly to the target organ. The presence of high TP and TRE concentrations in the lungs is extremely important to manifest the full biology with these drugs (Sandifer et al., 2005; Chapman et al., 2018). Second, TP is a prodrug of TRE that is slowly converted to TRE by the action of lung esterase (Leifer et al., 2018) that contributes to the prolonged TRE exposure in the lungs following inhalation (Corboz et al., 2017). This is in stark contrast to inhaled TRE that is rapidly eliminated from the lungs after inhalation and requires frequent administration to maintain lung concentrations above the threshold required to produce its biology (Kumar et al., 2016). Third, pulmonary vasodilator activity is maintained with repeat daily dosing with inhaled TP for up to 32 consecutive days whereas IV infused TRE loses pulmonary vasodilator activity that we speculate is due to desensitization of the IP receptor on the vascular endothelium (Nilius et al., 2000; Gatfied et al., 2017; Chapman et al., 2021a). And finally, TRE activates several prostanoid (IP, EP₂, DP₁ and EP₄) and PPAR receptors that provides TRE with a broad spectrum of activities on many different structural, inflammatory and immune cells in the lungs (Kolodsick et al., 2003; Foudi et al., 2008; Frumkin 2012; Benyahia et al., 2013; Zaslona and Peters-Golden, 2015; Lambers et al., 2018; Patel et al., 2018; Corboz et al., 2021a) and distinguishes it from drugs like selexipag that target only the IP receptor (Gatfield et al., 2017).

There are several limitations to the conclusions reached from these studies. First, the experiments were performed in Su/Hx challenged rats, and while this animal model is considered to be superior to other PAH models used for drug evaluations (Stenmark
et al., 2009; de Raaf et al., 2014), it does not recapitulate all of the PAH pathology in human subjects. Second, the doses of the drugs used for this evaluation are different from those used clinically and may not translate to clinically relevant doses in humans due to a number of factors such as differences in the method of drug administration, particularly inhalation, between rats and humans (Chapman et al., 2021a), differences in the metabolism and PK of these drugs (Kumar et al., 2016; Chaudhary et al., 2018; Ichikawa et al., 2018) and different binding affinity to the prostanoid and PPAR receptors between rats and humans. Third, pulmonary vasodilation was used to select the dosing frequency of the drugs and while this effect is largely mediated via IP receptor activation (Corboz et al., 2021b), a different dosing frequency may be required to ensure target engagement at other prostanoid and PPAR receptors.

In conclusion, TPIP had beneficial effects on the hemodynamic and pathological changes in the lungs induced by Su/Hx in rats with overall effects that compared favorably to the effects produced by inhaled and IV TRE and oral selexipag. Several factors may contribute to the beneficial effects of TPIP over these other prostacyclin analogs and include: 1) delivery by inhalation to maximize exposure in the target organ, 2) the slow release of TRE into the lung via the actions of lung esterase catalyzed hydrolysis of the prodrug bond of TP to produce TRE, 3) TRE has relatively high binding affinity to several prostanoid and PPAR receptors that provides a broader spectrum of activity to improve PAH pathophysiology compared to drugs like selexipag that target just the IP receptor.

**Word count:** 1462 (max 1500)

**Acknowledgements**

Tam Ngyuen, PhD, of Insmed Incorporated, conducted in vitro experiments with human primary smooth muscle and endothelial cells.
The authors gratefully acknowledge the scientists at IPS Therapeutique (Sherbrooke, Quebec, Canada) for their expertise in the design and completion of these experiments.

**Author Contributions**

Participated in research design: Corboz, Chapman  
Formulation Preparation: Plaunt, Malinin  
Performed PK data analysis: Li, Gauani, Chun  
Performed data analysis: Corboz, Chapman  
Wrote or contributed to the writing of the manuscript: Corboz, Chapman, Cipolla, Perkins  
Approved the manuscript for submission to the journal: All authors, with the exception of Dr. Chapman, who passed away in December 2021.
References


treprostinil provides pulmonary vasodilator activity at significantly lower plasma concentrations than infused treprostinil. *Pulmon Pharmacol & Ther* **49**: 104-111.


**Table 1**: Inhalation drug dose calculations for administration of TPIP and TRE in the 8-week Sugen 5416/Hypoxia study

<table>
<thead>
<tr>
<th>Drug</th>
<th>Aerosolization (mg)</th>
<th>Treatment</th>
<th>Duration (weeks)</th>
<th>Aerosol Concentration (µg/L)</th>
<th>RMV (L/min)</th>
<th>D (min)</th>
<th>BW (kg)</th>
<th>DF</th>
<th>TP Dose* Total (µg/kg)</th>
<th>DF</th>
<th>TP Dose** Pulmonary (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPIP</td>
<td>90</td>
<td>QD</td>
<td>5</td>
<td>2.6</td>
<td>0.32</td>
<td>33</td>
<td>0.48</td>
<td>1</td>
<td>58.5</td>
<td>0.1</td>
<td>5.8</td>
</tr>
<tr>
<td>TPIP</td>
<td>170</td>
<td>QD</td>
<td>5</td>
<td>4.7</td>
<td>0.32</td>
<td>37</td>
<td>0.47</td>
<td>1</td>
<td>116.8</td>
<td>0.1</td>
<td>11.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>Nebulizer Concentration (mM)</th>
<th>Treatment</th>
<th>Duration (weeks)</th>
<th>Aerosol Concentration (mg/L)</th>
<th>RMV (L/min)</th>
<th>D (min)</th>
<th>BW (kg)</th>
<th>DF</th>
<th>TRE Dose* Total (µg/kg)</th>
<th>DF</th>
<th>TRE Dose** Pulmonary (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRE</td>
<td>0.5</td>
<td>QID</td>
<td>5</td>
<td>6.1</td>
<td>0.3</td>
<td>15</td>
<td>0.44</td>
<td>1</td>
<td>64.9</td>
<td>0.1</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*TP Dose (µg/kg) = C (µg/L) X RMV (L/min) X D (min) X DF

BW (kg)

**TP Dose Pulmonary (µg/kg) = TP Dose Total (µg/kg) X DF

**Abbreviations:**

BW: body weight; D: duration of exposure; DF: deposition fraction assumed as being 100% for the calculation of total delivered dose and 10% for the calculation of pulmonary delivered dose; C: aerosol concentration QID: once-a-day; RMV: respiratory minute volume from the formula RMV = 0.608 X BW^{0.852} (Alexander et al., 2008); TP: treprostinil palmitil; TPIP: treprostinil palmitil inhalation powder; TRE: Treprostinal.
Table 2: Hemodynamics and airway remodeling in the 8-week Sugen 5416/Hypoxia study

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Parameter (% inhibition) †</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mPAP</td>
</tr>
<tr>
<td>TPIP (inhaled, QD)</td>
<td>59 µg/kg</td>
<td>53 ± 6</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>11</td>
</tr>
<tr>
<td>TPIP (inhaled, QD)</td>
<td>117 µg/kg</td>
<td>80 ± 8</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>10</td>
</tr>
<tr>
<td>TRE (Inhaled, QID)</td>
<td>65 µg/kg</td>
<td>34 ± 17</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>10</td>
</tr>
<tr>
<td>TRE (IV)</td>
<td>810 ng/kg/min</td>
<td>43 ± 10</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>13</td>
</tr>
<tr>
<td>Selexipag (Oral, BID)</td>
<td>30 mg/kg</td>
<td>59 ± 15</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>10</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM % inhibition due to drugs of the mPAP, CO, Fulton index, wall thickness, muscularization, percentage of non-obliterated pulmonary arteries or the combined total of these values in Su/Hx rats. n represents the number of determinations. The last column shows the average of all six individual parameters.

† % inhibition = (Su/Hx + vehicle) – (Su/Hx + drug)/ (Su/Hx + vehicle) – (Nx control) X 100.

**Abbreviations:**

CO: Cardiac output; BID: twice daily; IV: intravenous; mPAP: mean Pulmonary arterial pressure; QD: once daily; QID: four times daily; TPIP: Treprostinil palmitil inhalation powder; TRE: Treprostinil.
Table 3: Plasma pharmacokinetic in the 8-week Sugen 5416/Hypoxia study

**a)** Concentration of treprostinil (TRE) in the plasma following inhalation of TPIP at 59 and 117 μg/kg (QD), inhaled (inh) TRE at 65 mg/kg (QID), intravenous (IV) TRE at 810 ng/kg/min.

<table>
<thead>
<tr>
<th>Plasma TRE (ng/mL)</th>
<th>59 μg/kg TPIP (QD)</th>
<th>117 μg/kg TPIP (QD)</th>
<th>65 mg/kg Inh TRE (QID)</th>
<th>810 ng/kg/min IV TRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>22</td>
<td>38</td>
<td>56</td>
<td>22</td>
</tr>
<tr>
<td>Average</td>
<td>0.00</td>
<td>0.19</td>
<td>0.12</td>
<td>0.00</td>
</tr>
<tr>
<td>SEM</td>
<td>0.05</td>
<td>0.03</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>56</td>
<td>38</td>
<td>56</td>
</tr>
<tr>
<td>Average</td>
<td>0.14</td>
<td>0.08</td>
<td>0.09</td>
<td>0.22</td>
</tr>
<tr>
<td>SEM</td>
<td>0.03</td>
<td>0.02</td>
<td>0.03</td>
<td>0.04</td>
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<tr>
<td>n</td>
<td>11</td>
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<td>11</td>
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<td>38</td>
<td>56</td>
<td>22</td>
</tr>
<tr>
<td>Average</td>
<td>0.13</td>
<td>0.13</td>
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<td>39.35</td>
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<tr>
<td>SEM</td>
<td>0.03</td>
<td>6.44</td>
<td>1.36</td>
<td>0.93</td>
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<tr>
<td>n</td>
<td>13</td>
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**b)** Concentration of selexipag and its metabolite ACT-333679 in the plasma following oral administration of selexipag at 30 mg/kg.

<table>
<thead>
<tr>
<th>Plasma Selexipag and Metabolite (ng/mL)</th>
<th>30 mg/kg Selexipag (BID)</th>
<th>30 mg/kg Selexipag Metabolite (ACT-333679) (BID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>22</td>
<td>38</td>
</tr>
<tr>
<td>Average</td>
<td>11.37</td>
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</tr>
<tr>
<td>SEM</td>
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<td>n</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>56</td>
</tr>
<tr>
<td>Average</td>
<td>2512.94</td>
<td>817.58</td>
</tr>
<tr>
<td>SEM</td>
<td>268.61</td>
<td>147.25</td>
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<td>10</td>
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</tbody>
</table>

Abbreviations: **BID**: twice daily; **IV**: intravenous; **QD**: once daily; **QID**: four times daily; **TPIP**: Treprostinil palmitil inhalation powder; **TRE**: Treprostinil.

Blood samples for all groups were collected at day 22, day 38 and day 56. Details of the blood sample collection are described in the Material and Methods section.
Table 4: Lung pharmacokinetic in the 8-week Sugen 5416/Hypoxia study

a) Concentration of treprostinil (TRE) in the lung following inhalation of TPIP at 59 and 117 μg/kg (QD), inhaled (inh) TRE at 65 mg/kg (QID), intravenous (IV) TRE at 810 ng/kg/min.

<table>
<thead>
<tr>
<th></th>
<th>Lung TRE (ng/g)</th>
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<tbody>
<tr>
<td></td>
<td>59 mg/kg TPIP (QD)</td>
</tr>
<tr>
<td>Average</td>
<td>3.32</td>
</tr>
<tr>
<td>SEM</td>
<td>0.67</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
</tr>
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</table>

b) Concentration of selexipag and its metabolite ACT-333679 in the plasma following oral administration of selexipag at 30 mg/kg.

<table>
<thead>
<tr>
<th></th>
<th>Lung Selexipag and Metabolite (ng/g)</th>
</tr>
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<tr>
<td></td>
<td>30 mg/kg Selexipag (BID)</td>
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<tr>
<td>Average</td>
<td>7.02</td>
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<tr>
<td>SEM</td>
<td>2.05</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
</tr>
</tbody>
</table>

Abbreviations: BID: twice daily; IV: intravenous; QD: once daily; QID: four times daily; TPIP: Treprostinil palmitil inhalation powder; TRE: Treprostinil.
Funding:

Funding was provided by Insmed Incorporated (Bridgewater, NJ, USA), which included costs associated with experiments performed at Insmed Incorporated (Bridgewater, NJ 08807, USA) and IPS Therapeutique Incorporated (Sherbrooke, QC J1L2T9, Canada).
Figure Legends

**Figure 1**: Acute Inhaled Hypoxia Challenge. Right ventricular pulse pressure increase (\(\Delta RVPP\)) response to hypoxic challenge in rats exposed to: a) TPIP at 6 µg/kg, 23 µg/kg, 57 µg/kg and 138 µg/kg (n = 12 for baseline (BSL) and 3-4 for each time point after drug administration), BSL values before drug performed on 3 separate occasions in 4 rats, 1 day before drug administration, b) Inhaled TRE at 15 µg/kg, 46 µg/kg, 110 µg/kg, and 215 µg/kg (n = 10-23 for baseline (BSL) and 4-8 for each time point after drug administration), BSL values before drug administration performed on 3 separate occasions in 4-8 rats, 1 day before drug administration, c) oral Selexipag at 10 and 30 mg/kg, (n = 10-23 for baseline (BSL) and 4-8 for each time point after drug administration), BSL values before drug administration performed on 3 separate occasions in 4-8 rats, 1 day before drug administration, d) IV infused TRE at 810 ng/kg/min for 16 consecutive days (n = 21 for baseline (BSL) and 9-19 for each time point after drug administration), BSL values before drug administration performed on 3 separate occasions in 7 rats, 2 days before drug administration.

Values are the mean ± SEM. \(\Delta RVPP\): Change in right ventricular pulse pressure in response to hypoxia challenge. * P ≤ 0.05 compared to BSL.

**Figure 2**: Effect of TPIP, inhaled TRE, IV TRE, and oral selexipag on a) mean pulmonary arterial blood pressure (mPAP), b) cardiac output, c) pulmonary vascular resistance (PVR) in the 8-week Sugen 5416/Hypoxia (Su/Hx) study. Data represent mean ± SEM. * P ≤ 0.05 compared to Normoxic control group; # P ≤ 0.05 compared to Su/Hx + vehicle groups.

**Figure 3**: Effect of TPIP, inhaled TRE, IV TRE, and oral selexipag on a) stroke volume (SV), b) pulmonary artery acceleration time (PAAT), and c) right ventricle wall thickness (RVWT) in the 8-week Sugen 5416/Hypoxia (Su/Hx) study. Data represent mean ±
SEM. * P ≤ 0.05 compared to Normoxic control group; # P ≤ 0.05 compared to Su/Hx + vehicle groups.

**Figure 4:** Effect of TPIP, inhaled TRE, IV TRE, and oral selexipag on Fulton index in the 8-week Sugen 5416/Hypoxia (Su/Hx) study. Data represent mean ± SEM. * P ≤ 0.05 compared to Normoxic control group; # P ≤ 0.05 compared to Su/Hx + vehicle groups.

**Figure 5:** Effect of TPIP, inhaled TRE, IV TRE, and oral selexipag in the 8-week Sugen 5416/Hypoxia (Su/Hx) study on a) vascular wall thickness of pulmonary arteries stained with Hematoxylin and Eosin (H&E), b) vascular wall thickness of pulmonary arteries stained with alpha smooth muscle actin (αSMA), c) pulmonary vessel muscularization and d) pulmonary blood vessel obliteration in the 8 week Su/Hx study. Data represent mean ± SEM. * P ≤ 0.05 compared to Normoxic control group; # P ≤ 0.05 compared to Su/Hx + vehicle groups.

**Figure 6:** Representative photomicrograph of the effects of TPIP, inhaled TRE, IV TRE, and oral Selexipag on a) the remodeling of the smooth muscle cell of the left lung stained with α-smooth muscle actin (α-SMA), and on b) the histopathology of the pulmonary blood vessels of the left lung stained with von Willebrand Factor (vWF) in the 8-week Sugen 5416-Hypoxia (Su/Hx)-induced pulmonary arterial hypertension (PAH) in rats. Photomicrographs represent A) Normoxic control group, B) Su/Hx + TPIP vehicle (QD) group, C) Su/Hx + 59 μg/kg TPIP (QD) group, D) Su/Hx + 117 μg/kg TPIP (QD) group, E) Su/Hx + nebulized phosphate buffered saline (PBS) group, F) Su/Hx + 65 μg/kg nebulized TRE (QID) group, G) Su/Hx + IV TRE vehicle group, H) Su/Hx + 810 μg/kg IV TRE group, I) Su/Hx + oral methylcellulose (MC) group, J) Su/Hx + 30 mg/kg selexipag (BID) group, respectively. Scale bar = 100 μm.

a) Red arrows indicate increased muscularization of small arteries in vehicle or drug treatment groups, and black arrow indicates normal thin muscular small arteries. Lung
tissues were immunohistochemically stained with α-SMA that stained the smooth muscle cells.

b) Red arrows indicate occluded arteries in vehicle groups, black arrow indicates normal thin endothelial cells, yellow arrow the muscularized arteries and green arrow the non-muscularized arteries. Lung tissues were immunohistochemically stained with vWF that stained the endothelial cells responsible for the occlusion.

Figure 7: Representative cardiac photomicrographs of the effects of TPIP, inhaled TRE, IV TRE, and oral Selexipag on right ventricular cardiac tissue in the 8-week Sugen 5416-Hypoxia (Su/Hx)-induced pulmonary arterial hypertension (PAH) in rats.

Histological examination was performed on a cross-section of the right ventricle stained with Masson’s Trichrome from A) the Normoxic control group, B) the Su/Hx + TPIP vehicle (QD) group, C) the Su/Hx + 59 μg/kg TPIP (QD) group, D) the Su/Hx + 117 μg/kg TPIP (QD) group, E) the Su/Hx + nebulized phosphate buffered saline (PBS) group, F) the Su/Hx + 65 μg/kg inhaled TRE (QID) group, G) the Su/Hx + IV TRE vehicle group, H) the Su/Hx + 810 μg/kg IV TRE group, I) the Su/Hx + oral methylcellulose (MC) group, J) the Su/Hx + 30 mg/kg selexipag (BID) group, respectively. Scale bar = 400 µm.

Yellow arrows indicate fibrotic area (blue coloration). Staining with Masson’s Trichrome demonstrates increased fibrotic area in vehicle groups as shown by the blue stain in SuHx + TPIP vehicle (B), SuHx + nebulized PBS (E), SuHx + IV TRE vehicle (G), and SuHx + oral MC (I) groups as compared to Normoxic control (A), Su/Hx + 117 μg/kg TPIP (D), SuHx + 65 μg/kg inhaled TRE (F) and SuHx + 810 μg/kg IV TRE (H) groups.

b) Representative photomicrographs of cardiac tissue showing an enlarged vessel with perivascular/interstitial fibrosis and cardiomyocyte hypertrophy. The right ventricle was embedded in paraffin wax then stained with Masson’s trichrome for evaluation of collagen deposition. Higher intensity and magnitude of the blue staining (Masson’s trichrome), consistent with an increased and more pronounced collagen deposition in the perivascular region, was observed in the Su/Hx + TPIP vehicle group, relative to the
normoxic control group as represented by the yellow arrows. Black arrows show increased interstitial fibrosis, more apparent within cardiomyocyte cross-sectional area, in the Su/Hx groups. Treatment with 117 µg/Kg TPIP (QD) slightly reduced the appearance of perivascular and interstitial fibrosis.

**Figure 8:** Radar chart using a multiparameter scoring summary that includes the measurement of mean pulmonary arterial pressure (mPAP), Fulton index, cardiac output (CO) and the wall thickness, muscularization and obliteration of pulmonary arteries in the 8-week Sugan 5416/Hypoxia study.

Pulmonary Pressure: mPAP; Fulton Index = weight ratio Right Ventricle / (Left Ventricle + Septum); CO: volume of blood pumped by the heart per minute; Wall thickness: small vessel wall thickness; Muscularization: percentage of muscularized vessels; Obliteration: percentage of non-obliterated vessels.
Figure 1: Acute Inhaled Hypoxia Challenge

**Figure 1 a: TPIP**

- **BSL 0.5 hr 1 hr 2 hr 4 hr 6 hr 12 hr 24 hr**
- **Δ RVPP (mmHg)**
- Colors and values:
  - 6 μg/kg
  - 23 μg/kg
  - 57 μg/kg
  - 138 μg/kg

**Figure 1 b: Inhaled TRE**

- **BSL 0.5 hr 1 hr 2 hr 3 hr 4 hr 6 hr**
- **Δ RVPP (mmHg)**
- Colors and values:
  - 15 μg/kg
  - 46 μg/kg
  - 110 μg/kg
  - 215 μg/kg

**Figure 1 c: Oral Selexipag**

- **BSL 0.5 hr 1 hr 2 hr 4 hr 6 hr 12 hr 24 hr**
- **Δ RVPP (mmHg)**
- Colors and values:
  - 10 mg/kg
  - 30 mg/kg

**Figure 1 d: IV TRE (810 ng/kg/min)**

- **BSL Day 0 Day 4 Day 8 Day 12 Day 16**
- **Δ RVPP (mmHg)**
- Colors and values:
  - Δ
  - †
  - *
BID: twice daily; IV: intravenous; MC: methylcellulose; PBS: phosphate buffered saline; QD: once daily; QID: four times daily; TPIP: treprostinil palmitate inhalation powder; TRE: treprostinil.
Figure 3 a: SV

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* P ≤ 0.05 compared to Normoxic control
# P ≤ 0.05 compared to Su/Hx + Vehicle

Figure 3 b: PAAT

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* P ≤ 0.05 compared to Normoxic control
# P ≤ 0.05 compared to Su/Hx + Vehicle

Figure 3 c: RVAWT

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* P ≤ 0.05 compared to Normoxic control
# P ≤ 0.05 compared to Su/Hx + Vehicle
Figure 4: Fulton Index

*M P ≤ 0.05 compared to Normoxic control
** P ≤ 0.05 compared to Su/Hx + Vehicle

NORMOXIC control (n = 4)
TPII Vehicle (n = 5)
TPII 69 μg/kg (n = 5)
TPII 117 μg/kg (n = 5)
PBS (n = 3)
TRE 65 μg/kg (n = 5)
IV vehicle (n = 7)
TRE 810 ng/kg/min (n = 6)
IV vehicle 810 ng/kg/min (n = 6)
Su/Hx + nebulized (QID) (n = 3)
Su/Hx + IV (n = 5)
Su/Hx + oral (BID) (n = 4)

* P ≤ 0.05 compared to Normoxic control
# P ≤ 0.05 compared to Su/Hx + Vehicle
**Figure 5 a: Wall thickness (H&E)**

- Pulmonary Arterial Wall Thickness (%)
- Small
- Medium
- Large

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* P ≤ 0.05 compared to Normoxic control
# P ≤ 0.05 compared to Su/Hx + Vehicle

**Figure 5 b: Wall thickness (α-SMA)**

- Medial Layer Thickness (%)
- Small
- Medium
- Large

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* P ≤ 0.05 compared to Normoxic control
# P ≤ 0.05 compared to Su/Hx + Vehicle

**Figure 5 c: Muscularization**

- Pulmonary Arterial Muscularization (%)
- Non Muscular
- Semi-Muscular
- Muscular

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* P ≤ 0.05 compared to Normoxic control
# P ≤ 0.05 compared to Su/Hx + Vehicle

**Figure 5 d: Obliteration**

- Pulmonary Arterial Obliteration (%)
- Mostly Obliterated
- Semi-Obliterated
- Non Obliterated

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* P ≤ 0.05 compared to Normoxic control
# P ≤ 0.05 compared to Su/Hx + Vehicle
Figure 6

Figure 6 a: Left Lung (α-SMA)

A. Normoxic control
B. Su/Hx + TPiP vehicle, QD
C. Su/Hx + TPiP, 59 μg/kg QD
D. Su/Hx + TPiP, 117 μg/kg QD
E. Su/Hx + inhaled PBS, QID
F. Su/Hx + inhaled TRE, 65 μg/kg QID
G. Su/Hx + IV Vehicle
H. Su/Hx + IV Selexipag, 30 mg/kg BID
I. Su/Hx + oral MC, BID
J. Su/Hx + oral Selexipag, 30 mg/kg BID

Figure 6 b: Left Lung (vWF)

A. Normoxic control
B. Su/Hx + TPiP vehicle, QD
C. Su/Hx + TPiP, 59 μg/kg QD
D. Su/Hx + TPiP, 117 μg/kg QD
E. Su/Hx + inhaled PBS, QID
F. Su/Hx + inhaled TRE, 65 μg/kg QID
G. Su/Hx + IV Vehicle
H. Su/Hx + IV Selexipag, 810 ng/kg/min
I. Su/Hx + oral MC, BID
J. Su/Hx + oral Selexipag, 30 mg/kg BID
**Figure 7 a: Histology-Right Ventricle**

- **A**: Normoxic control
- **B**: Su/Hx + TPIP vehicle, QD
- **C**: Su/Hx + TPIP, 59 µg/kg QD
- **D**: Su/Hx + TPIP, 117 µg/kg QD
- **E**: Su/Hx + inhaled PBS, QID
- **F**: Su/Hx + inhaled TRE, 65 µg/kg QID
- **G**: Su/Hx + IV vehicle
- **H**: Su/Hx + IV TRE, 810 ng/kg/min
- **I**: Su/Hx + oral MC, BID
- **J**: Su/Hx + oral Selexipag, 30 mg/kg BID

**Figure 7 b: Cardiac Fibrosis**

- **A**: Normoxic control
- **B**: Su/Hx + TPIP vehicle, QD
- **C**: Su/Hx + TPIP, 117 µg/kg QD
Figure 8: Multiparameter Comparison of Treatment Groups
Assessment of Inhaled Treprostinil Palmitil, Inhaled and Intravenous Treprostinil and Oral Selexipag in a Sugen/Hypoxia Rat Model of Pulmonary Arterial Hypertension

Michel R. Corboz\textsuperscript{a}, Adam J. Plaunt\textsuperscript{a}, Vladimir Malinin\textsuperscript{a}, Zhili Li\textsuperscript{a}, Helena Gauani\textsuperscript{a}, Donald Chun\textsuperscript{a}, David Cipolla\textsuperscript{a}, Walter R. Perkins\textsuperscript{a}, Richard W. Chapman\textsuperscript{a}

\textsuperscript{a} Insmed Incorporated, 700 US Highway 202/206, Bridgewater, NJ 08807, USA.

Section Assignment: Cardiovascular
Paper ID: 84039503
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

![](image)

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% $O_2$/balance $N_2$) 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>
</tr>
</thead>
<tbody>
<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>
<tr>
<td></td>
<td>PBS</td>
<td>Su-Hx + nebulized TRE</td>
<td>110 µg/kg</td>
<td>6 mL of 0.5mM</td>
<td>Inhalation (QID)</td>
<td>65 µg/kg</td>
<td>21</td>
<td>55</td>
<td>56</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Su-Hx + IV vehicle†</td>
<td>n/a</td>
<td>2.5 µL/h</td>
<td>Continuous intravenous infusion</td>
<td>n/a</td>
<td>21</td>
<td>56</td>
<td>56</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>Su-Hx + IV TRE†</td>
<td>810 ng/kg/min</td>
<td>Continuous intravenous infusion</td>
<td>810 ng/kg/min</td>
<td>21</td>
<td>56</td>
<td>56</td>
<td>18</td>
<td>†</td>
</tr>
<tr>
<td>9</td>
<td>Su-Hx + oral MC</td>
<td>n/a</td>
<td>10 mL/kg</td>
<td>Oral (BID)</td>
<td>n/a</td>
<td>21</td>
<td>55</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>Su-Hx + Selexipag</td>
<td>30 mg/kg</td>
<td>10 mL/kg of 3mg/mL</td>
<td>Oral (BID)</td>
<td>30 mg/kg</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
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>
</thead>
<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>F1LN3</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>Q6P6T6</td>
<td>CTSB</td>
<td>Cathepsin D</td>
<td>-0.73 *</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.

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)

b) PGS1 Biglycan

c) Lumican
d) CSPG2 Versican

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 ≤ 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>
<th>Gene_name</th>
<th>Protein_name</th>
<th>Fold change</th>
</tr>
</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 ≤ 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)**

b) **Retinal dehydrogenase (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 Nictinate/nicotinamide metabolism 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>
</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.

**Nictinate 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**

![Nicotinate phosphoribosyltransferase graph](image)

b) **NAD-dependent protein deacetylase**

![NAD-dependent protein deacetylase graph](image)

c) **Proton-translocating NAD(P)(+) transhydrogenase**

![Proton-translocating NAD(P)(+) transhydrogenase graph](image)
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,


