Online Supplement

Inhaled Seralutinib Exhibits Potent Efficacy in Multiple Models of Pulmonary Arterial Hypertension

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Methods

Cell Culture

Human cell lines were obtained from commercial vendors and propagated under recommended culture conditions in a humidified incubator at 37°C, with 5% CO_2 in air: NCI-H1703 (ATCC, Manassas, VA, USA), Human Pulmonary Arterial Smooth Muscle Cells (HPASMC, Cell Applications, San Diego, CA, USA), Human Lung Fibroblasts (HLF; Cell Applications, San Diego, CA, USA), HEK-293T (ATCC), and Human Pulmonary Artery Endothelial Cells (HPAECs, Cell Applications, San Diego, CA, USA)

In Vitro **Characterization of Compounds**

Compound activity was assessed in platelet-derived growth factor receptor alpha (PDGFRα), PDGFR beta (PDGFRβ) and colony stimulating factor 1 receptor (CSF1R) enzymatic assays at Carna Biosciences Inc. (Kobe, Japan). A c-KIT enzymatic assay was performed at Gossamer Bio (San Diego, CA, USA) using the Promega c-KIT Kinase Enzyme System (Promega Corporation, Madison, WI, USA). Compounds (starting concentration 1.0E-06M) were titrated in 100% dimethyl sulfoxide (DMSO) then diluted in assay buffer supplements with 50 µM dithiothreitol (DTT) and 2 mM $MnCl_2$. One µL of 5x compound was pre-incubated with 2 µL containing 20 ng of c-KIT protein for 0 and 1 hour in a 384-well low volume plate. The adenosine triphosphate (ATP)/substrate mixture was diluted in assay buffer and 2 µL were added to the compound: enzyme complex with 50 µM final ATP and 1 µg/µL PolyE peptide concentrations as specified by the manufacturer. After two hours at room temperature, 5 µL of adenosine diphosphate (ADP) Glo reagent was added to the plate followed by a 40-minute incubation at room temperature. 10 µL of Kinase Detection Reagent was added and incubated for 30 minutes at room temperature.

Luminescence signal was then measured with the iD5 Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

H1703 Proliferation Assay

Cells were plated at 1,000 cells/well into a 384-well cell culture plate (Cat#3570, Corning Inc, Poway, CA, USA) and allowed to attach overnight prior to addition of compounds in dose response. Cells were incubated for 72 hours in the presence of treatment and proliferation was assessed by CellTiterGlo Luminescent Cell Viability assay according to manufacturer's directions (Promega Corporation, Madison, WI, USA).[1]

PDGF-BB-Induced Proliferation Assays in HLFs and HPASMCs

HLF cells were plated at a density of 4,000 cells per well in a 96-well cell culture plate (Cat#3904, Corning Inc., Poway, CA, USA) and allowed to attach overnight. Cells were then incubated in 0.1% FBS starvation media (Thermo Fisher Scientific, Waltham, MA, USA) for 24 hours and then compounds were added in dose response. One hour after compound addition, cells were stimulated with 10 ng/ml PDGF-BB (R&D Systems, Minneapolis, MN, USA). Impact on cell proliferation was assessed 48 hours later using CyQuant® Direct Cell Proliferation Assay (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's directions. The values for each concentration are normalized to PDGFB stimulation for day 3 (considered 100% proliferation) and no stimulation for day 3 (0% proliferation).

HPASMC cells were plated at a density of 1,000 cells per well in a Corning® 96-well clear flat bottom polystyrene tissue culture (TC)-Treated plate (Corning Inc., Poway, CA, USA) and attached overnight. One hour after compound addition, cells were stimulated with 25 ng/ml PDGF-BB

(R&D Systems). Impact on cell proliferation was assessed 72 hours later using the CellTiter-Glo®

Luminescent Cell Viability Assay (Promega Corporation, Madison, WI, USA).[1] Day 0 (D0)

represents the basal value at day 0 just prior to PDGFB stimulation.

M-CSF-Induced Activation of CSF1R-SRE Reporter in HEK293 Cells

CSF1R/SRE Reporter Kit (BPS Bioscience, San Diego, CA, USA) was used to assess impact of

compounds of M-CSF-induced CSF1R signaling. HEK-293T cells were seeded at a density of 0.5

million cells per 10-cm plate with complete growth media and incubated for 18 hours until cells

reached 70–90% confluency. HEK293T cells were transfected with the CSF1R expression vector

(~15 µg DNA) and the serum response element (SRE) luciferase reporter construct (~9 µg DNA)

according to the recommended protocol (BPS Bioscience, San Diego, CA, USA). Six hours post-

transfection, cells were re-suspended in growth media with 0.5% FBS, seeded into a 384-well assay

plate (Cat#3570, Corning Inc., Poway, CA, USA) at 10,000 cells per well and allowed to attach

overnight. The following day, cells were pre-incubated with compounds for 30 minutes, prior to

addition of recombinant M-CSF (10 ng/ml final concentration) (BPS Bioscience, San Diego, CA,

USA). Unstimulated control wells received assay medium without CSF1. Cells were incubated for 6

hours prior to lysis and luciferase readout using ONE-Step™ Luciferase Assay System (BPS

Bioscience, San Diego, CA, USA).

**Stem Cell Factor (SCF)-Induced Phosphorylation of c-KIT in Human Pulmonary Arterial
Endothelial Cells (HPAECs)**

HPAECs were plated at a density of 50,000 cells per well in a Corning® 96-well clear flat bottom

polystyrene tissue culture (TC)-Treated plate (Corning Inc., Poway, CA, USA) and attached

overnight. Cells were then incubated in starvation medium (Cell Applications, San Diego, CA,

USA) for 2 hours prior to addition of SCF. Cells were pre-incubated with compound in a 10-point dose response curve for 30 minutes, and then stimulated with 1000 ng/mL SCF (R&D Systems, Minneapolis, MN, USA) for 5 minutes at room temperature. After cell lysis, treatment impact on c-KIT phosphorylation was assessed using the PathScan® Phospho-c-KIT (Tyr719) Sandwich ELISA Kit (Cat# 7298C, Cell Signaling Technologies, Danvers, MA, USA). To detect total c-KIT, the detection antibody provided in the phospho-c-KIT ELISA was replaced with c-Kit Rabbit mAb (clone D3W6Y) at a 1:100 dilution (Cat #37805, Cell Signaling Technologies, Danvers, MA, USA). Data is shown as the percentage of c-KIT phosphorylation over total c-KIT expression.

Extracellular Regulated Kinase (ERK) Signaling Assays

HLF cells were plated at a density of 20,000 cells per well in a Corning® 96-well clear flat bottom polystyrene TC-Treated plate (Corning Inc, Poway, CA, USA), allowed to attach overnight and then incubated in serum-free media for 4 hours prior to addition of compound treatments in dose response. HPASMCs were plated at a density of 10,000 cells per well in a Corning® 96-well clear flat bottom polystyrene TC-Treated plate (Corning Inc, Poway, CA, USA), allowed to attach overnight and then incubated in serum-free media for 2 hours prior to addition of compound treatments in dose response. NCI-H1703 cells were plated at a density of 50,000 cells per well in a Corning® 96-well Clear Round Bottom TC-treated Microplate (Corning Inc, Poway, CA, USA) and allowed to attach for 3 hours prior to addition of compound treatments in dose response. For all cell lines, after a 30-minute compound pre-incubation, cells were stimulated with 25 ng/ml PDGF-BB (R&D Systems, Minneapolis, MN, USA) for 10 minutes. Inhibition of phospho-ERK signal was

then assessed using the advanced phospho-ERK homogeneous time resolved fluorescence (HTRF)

kit (PerkinElmer, Waltham, MA, USA) according to manufacturer's instructions.

Human Macrophage Assays

CD14+ monocytes isolated from human peripheral blood mononuclear cells (PBMCs) (StemExpress, San Diego, CA, USA) were seeded into 6-well tissue culture plates at a density of 500,000 cells per well in Differentiation Media (RPMI-1640, 1x Gentamicin, 1x non-essential amino acids (NEAA) and 10% heat inactivated FBS (Thermo Fisher Scientific) supplemented fresh with 10 ng/ml macrophage CSF (M-CSF) (PeproTech, Rocky Hill, NJ, USA). Media was replenished every 3–4 days. On day 8, Differentiation Media was further supplemented with 20 ng/ml recombinant human interleukin (IL)-4 and 20 ng/ml recombinant human IL-13 (PeproTech, Rocky Hill, NJ, USA) and cells were incubated for 24 hours prior to conditioned media collection for assessment of human monocyte chemoattractant protein-1 (MCP-1/CCL2) secretion using the MCP1 HTRF kit (Cisbio, Bedford, MA, USA).

To evaluate impact of seralutinib and imatinib on CSF1R phosphorylation in primary macrophages, monocytes were allowed to attach and differentiate into macrophages using Differentiation Media as described above. On day 8 of culture, cells were serum starved with compound for 2 hours, and stimulated with recombinant human M-CSF (50 ng/ml) (PeproTech, Rocky Hill, NJ, USA) for 5 minutes prior to lysis. Treatment impact on CSF1R phosphorylation was assessed using the PathScan® Phospho-M-CSF Receptor (panTyr) and the PathScan® Total M-CSF Receptor Sandwich ELISA Kits (Cell Signaling Technologies, Danvers, MA, USA).

Inhaled Delivery of Seralutinib

Seralutinib was formulated as a dry powder with aerosol properties designed to allow deposition in the deep lung. Placebo consisted of excipient without active ingredient. CH Technologies (Westwood, NJ, USA) system designed for passive inhalation drug delivery in rats with either a Rotating Brush Generator or Vilnius Dry Powder Generator was used for dosing.

Pharmacokinetic (PK) and Pharmacodynamic (PD) Assessments

To understand dose- and time-dependent PK/PD effects of seralutinib treatment compared to placebo, seralutinib was administered for 4 days via passive inhalation (120-minute treatment per day) at three dose levels: low (4.3 mg/kg/dose), mid (16.2 mg/kg/dose), and high (66.6 mg/kg/dose) to healthy 8–9-week-old Sprague Dawley male rats. Delivered dose levels were estimated using Alexander's formula.[2]

Immediately post-inhalation, a subset of animals were anesthetized and treated with either 300 µg rat PDGF-BB (GenScript Biotech, Nanjing, China) (PD study 1) or a cocktail of 150 µg rat PDGF-BB + 50 µg rat SCF (PeproTech, Rocky Hill, NJ, USA) (PD Study 2) reconstituted in 200 µl 0.1% bovine serum albumin (BSA) (prepared in sterile 0.9% NaCl) and delivered via pulmonary insufflation using a Penn-Century® Microsprayer (Penn-Century, Wyndmoor, PA, USA) 5 minutes prior to lung tissue collection to facilitate detection of PDGFR and cKIT autophosphorylation.

Rat lung lobes and plasma samples were collected for bioanalysis immediately at the end of inhalation dosing, 4, 8, 16, and 24 hours from the start of treatment and frozen in liquid nitrogen.

Seralutinib plasma and lung homogenate concentrations were determined against standard curves of known concentration using a protein precipitation extraction followed by separation and detection by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Lung Tissue Protein and Messenger RNA (mRNA) Extraction Procedures

To generate lung protein lysates, approximately 100 mg of the left lung lobe was weighed and placed in a centrifuge tube containing 2 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) and 1 ml of RIPA Lysis Buffer with phosphatase and protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Samples were then placed in a Mini-Beadbeater-96 (BioSpec Products, Bartlesville, OK, USA) and homogenized for 2 minutes at 2,500 rpm. The samples were cooled on ice for 10 minutes, and then centrifuged at 20,000 x g for 10 minutes. Lysate was pipetted into clean centrifuge tubes, while carefully avoiding the pelleted debris. The Rapid Gold BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA) was performed to quantify the protein concentration of each sample, and then diluted accordingly in Lysis Buffer to normalize each sample concentration to 10 mg/ml. Samples were stored at –80°C.

To extract lung tissue RNA, approximately 20–30 mg of lung tissue from the right upper lobe was disrupted and homogenized in Buffer RLT using the TissueLyser II (QIAGEN, Hilden, Germany). Lysates were processed on the QiaCube Connect using the QIAGEN RNeasy Mini Kit following the manufacturer's instructions, including the optional DNase I treatment (QIAGEN, Hilden, Germany). RNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), assessed for RNA integrity and quality using the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and converted into complementary DNA (cDNA) using the High-Capacity Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative Polymerase Chain Reaction (qPCR) Methods

MCP-1 (CCL2, Rn00580555_m1) and housekeeping control ACTB (Rn00667869_m1) mRNA expression levels were detected using the TaqMan™ Universal PCR Master Mix (no AmpErase™ UNG; Thermo Fisher Scientific, Waltham, MA, USA) on the QuantStudio5 (Thermo Fisher Scientific, Waltham, MA, USA). Data were analyzed using the QuantStudio Design & Analysis Software (Thermo Fisher Scientific, Waltham, MA, USA), with Ct thresholds being set in the linear range. Fold changes were calculated using the delta Ct (ΔΔCt) method, where Ct values were first normalized to ACTB, and then to the untreated control.

Assessment of Lung Protein Expression

Traditional Western blot analysis was performed to measure levels of phopsho-PDGFRβ Tyr1021, phospho-PDGFRα/β (Tyr849)/(Tyr857), total PDGFRβ, phospho-cKIT Tyr719 and β-actin. The following antibodies were used for protein expression: pPDGFRβ Y1021 at 1:500 (Cat# 2227), pPDGFRα/β (Tyr849)/(Tyr857) at 1:500 (Cat#3170), total PDGFRβ at 1:1,000 (Cat# 3169), phospho-cKIT Tyr719 at 1:500 (Cat# 3391) and β-Actin at 1:2,000 (Cat# 4970) (all from Cell Signaling Technologies, Danvers, MA, USA); total cKIT Ab at 1:500 (Cat# LS-C382844) from Lifespan Biosciences (Seattle, WA, USA). The immunoreactive bands were quantified by densitometry. Expression of phospho-proteins was normalized to respective total protein. Data are presented as percentage inhibition relative to control untreated samples.

The WES instrument (ProteinSimple, San Jose, CA, USA) was used to quantitate bone morphogenetic protein receptor type 2 (BMPR2) and phospho-SMAD1/5 protein level expression in rat lung lysates using the following antibodies: Anti-BMPR-II (RUO), Clone 18 (Cat# 612292, BD Biosciences), phospho-Smad1/5 (Ser463/465) (41D10) (Cat#9516, Cell Signaling

Technologies, Danvers, MA, USA) and β-actin (13E5) Mouse mAb (Cat #3700, Cell Signaling Technologies, Danvers, MA, USA). Area under the curve was calculated for each protein of interest and normalized to expression of β-actin. Data are shown as fold induction relative to control.

Rat Models of Pulmonary Arterial Hypertension (PAH)

For the monocrotaline pneumonectomy (MCTPN) model (Study 1) and the SU5416/H studies (Study 2 and 3) Guyton's formula[3] was used to estimate minute ventilation which was then used to estimate the average delivered dose for the animals.

SU5416/H Model (Study 1)

The purpose of this preclinical efficacy study was to determine efficacy of seralutinib when delivered as a dry powder in the rat SUGEN5416/hypoxia/normoxia model. Two cohorts were studied. Cohort 1 consisted of eight vehicle-treated animals and nine seralutinib-treated animals. Three of the vehicle animals had telemetry monitoring and all nine of the seralutinib-treated animals in cohort 1 had telemetry monitoring. In the telemetry subset of cohort 1, male Sprague Dawley rats underwent placement of DSI PAC40 telemetry devices (Data Sciences International) with catheter tip in the main pulmonary artery. After 10 days' recovery, the animals were administered SUGEN5416 (Sigma-Aldrich, St Louis, MO, USA) 20 mg/kg subcutaneous (SC) in carboxymethylcellulose/dimethyl sulfoxide (CMC/DMSO) suspension and placed in a COY hypoxia chamber with fraction of inspired oxygen (FiO_2) 10% (Cor Laboratory Products Inc, Grass Lake, MI, USA). The animals resided in the hypoxia chamber for 3 weeks. They were removed once a week for less than 10 minutes to monitor pulmonary artery (PA) pressures. After week 3 (day 21) they were removed from the hypoxia chamber permanently and PA pressures were monitored continuously for the first 24 hours. They were allowed to equilibrate to normoxia, and on

day 2 after removal from the chamber were assigned to inhaled placebo or inhaled seralutinib 2.5 mg/kg twice daily.

Cohort 2 consisted of six vehicle-treated rats and six rats treated with inhaled seralutinib. Rats were administered SU5416 20 mg/kg SC and placed in the hypoxia chamber for 3 weeks at 10% FiO_2. Treatment was started on day 2 after removal from hypoxia. In the cohort 2 active group, the average dose of seralutinib was 4.6 mg/kg twice daily. This cohort underwent end of study measurement of RV pressure, lumen/media analysis, and occlusive grading analysis of pulmonary arterioles.

Hemodynamics

Telemetry monitoring was recorded for 10 minutes of each hour for the study period. During dosing itself telemetry was not recorded due to logistical constraints of the telemetry system. RV pressure was also determined at the end of study.

End of Study Procedures

End of study procedures consisted of placement of a Scisense catheter (Transonic Systems) in the right ventricle for recording of RV hemodynamics. At the end of the study, the right upper or middle lobe of the lung was tied off, removed, and placed in liquid nitrogen. Then the heart and remaining lung were removed en bloc. The heart chambers (LV, IVS, RV) were dissected and weighed. The lung PA was perfused with heparinized saline then 10% formalin. 10% formalin was also used to inflate the lung via the trachea. The lung lobe snap frozen in liquid nitrogen was placed at minus 80°C for storage. After 2 days the formalin-infused organs were switched to 70% histology grade alcohol.

Histology and Morphometric Analysis

In cohort 2, H&E-stained sections of the lung were analyzed for pulmonary arteriole lumen/media ratio and grading of plexiform lesions. Sections were imaged with a standard bright field microscope with 10X and 40X objectives, and photomicrographs analyzed with ImageJ software (NIH, Bethesda, MD, USA). In one case lumen/media ratio was not determined. Immunohistochemistry was performed with a phospho-specific antibody against the PDGFRβ receptor (SC-12909) (Santa Cruz Biotechnologies, Dallas, TX, USA).

MCTPN Model (Study 2)

Telemetry

Study rats underwent implantation of a pulmonary artery (PA) monitoring telemetry catheter (DSI PAC40, Data Sciences International, St Paul, MN, USA) and left pneumonectomy with the exception of two animals in the seralutinib group which only underwent left pneumonectomy. Ten days after recovering from surgery all the pneumonectomized rats were given monocrotaline (Sigma-Aldrich, St. Louis, MO, USA) 50 mg/kg intraperitoneal (IP). Dosing of vehicle started, on average, 16 days after monocrotaline administration. Dosing of seralutinib started, on average, 20 days after monocrotaline administration. Seralutinib (2.5 mg/kg) dry powder or vehicle was administered by nose only inhalation 30 minutes (on average) twice daily to the rats via a Vilnius Dry Powder Generator (CH Technologies) connected to the Exposure tower (CH Technologies) for 11 days.

There were 12 animals in the vehicle group and 12 animals in the seralutinib-treated group. Pulmonary hypertension did not develop in one animal in the seralutinib group, and this animal was

excluded from the analysis. The DSI PAC40 malfunctioned in two other animals in the seralutinib group, and the telemetry data were not used from these two animals in the analysis. In the vehicle group, after 11 days of being administered vehicle, five of the animals were crossed over to seralutinib treatment and treatment was continued in these animals for up to 9 days. In the cross-over extension study, most of the animals in the vehicle group and some of the animals crossed over from vehicle to seralutinib required early euthanasia for distress. Data from end of study procedures, including measurement of right ventricular pressure, lumen to media ratio and heart weights for the vehicle group are reported for the vehicle animals that were not crossed over to seralutinib treatment and the primary seralutinib-treated group.

Plethysmography

Two chamber plethysmography was measured at end of study using an EMKA system and IOX software (EMKA Technologies, Montreal, Canada). Measurements included tidal volume, respiratory rate, minute ventilation, and airway resistance.

End of Study Procedures

At the end of the study rats underwent general anesthesia, intubation, left thoracotomy, and insertion of a Scisense catheter (Transonic Systems, Ithaca, NY, USA) to measure right ventricular pressures directly. The animals were heparinized, then euthanized by exsanguination under general anesthesia, and the heart and lungs excised. The pulmonary artery was perfused with heparinized saline followed by formalin, and the lung infused with 10% formalin via the trachea for a total volume of 1.5 ml. The heart was dissected, and the right ventricular free wall (RV), interventricular septum (IVS), and left ventricle (LV) were weighed separately. The ratio RV/(IVS+LV) was determined to evaluate right ventricular hypertrophy (RVH).

Measurement of Pulmonary Arteriole Lumen to Media Ratio

The lumen to media area ratio was determined on pulmonary arterioles from hematoxylin and eosin (H&E)-stained lung sections. Areas were traced with Image J software and pixel areas determined. The pulmonary arteriole media area was determined by subtracting the lumen area from the total vessel area encompassed by the outer media circumference. Taking the ratio of the lumen to media helps normalize for differences in blood vessel size. A vehicle-treated animal was found dead on the last day of dosing. The lungs were hemorrhagic and were not used for lumen/media analysis.

Grading of Neointimal Proliferation

Based on the method of Toba et al,[4] neointimal proliferation was graded according to the following system: Grade 0 (no significant obstruction), Grade 1 (<50% obstruction), Grade 2 (>50% obstruction of the vessel lumen). Picrosirius Red staining was performed to evaluate the extent of fibrosis.

SU5416/H Model (Study 3)

The purpose of this study was to directly compare efficacy of inhaled seralutinib to orally administered imatinib in the SU5416/H model. The model was developed as in Study 2 with a single injection of SU5416 20 mg/kg (AdooQ Bioscience, Irvine, CA, USA) followed by three weeks of hypoxia. In this study, echocardiography was performed to assure equal disease severity distribution between the treatment groups at the start of treatment and to assess the effect of treatment on cardiac performance non-invasively.

Echocardiography to monitor disease progression was carried out on days 0, 35, and 49. An echocardiograph (Model Q9, Chison Medical Imaging Co., Jiangsu, China) connected to a Chison

12.0 MHz probe was used to measure the pulmonary artery maximum velocity (Vmax), the Artery Pulmonary Velocity Time Integral (VTI), the pulmonary artery diameter, and heart rate. Randomization into treatment groups on day 35 was based on the following parameters: cardiac output (CO), pulmonary artery Vmax, and stroke volume (SV).

Treatment with inhaled seralutinib, oral gavage imatinib, or oral gavage vehicle control (40% PEG 300/60% Acetic Acid-acetate Buffer pH 4.6) was initiated on day 35, 2 weeks after animals were returned to normoxia. Seralutinib was administered twice daily by inhalation (60 minutes) at an estimated dose of 12.8 mg/kg/dose using a Vilnius Aerosol Generator connected to an inhalation tower (CH Technologies). Mercer-style cascade impactor (CH Technologies, Westwood, NJ, USA) was used to perform aerodynamic particle size distribution measurements. Imatinib 15 mg/kg was administered by oral gavage once a day. A set of healthy control rats were also included in the study. The selected dose was consistent with prior published studies, which evaluated imatinib doses of 5 to 50 mg/kg in rat PAH models.[5-7] Furthermore, imatinib activity was confirmed in the phospho-PDGFR rat PD model, demonstrating potent inhibition of PDGFR phosphorylation (**Figure S4**).

End of Study Procedures

On day 49, after 2 weeks of treatment, end of study procedures were performed: mean pulmonary arterial pressure (mPAP) and right ventricle systolic pressure (RVSP) were measured via an intra-arterial fluid-filled catheter (AD Instruments, Colorado Springs, CO, USA). Pulmonary vascular resistance index (PVRI) was calculated using the following formula: PVRI = mPAP / CI, where mPAP is mean pulmonary arterial pressure (mm Hg) and CI is cardiac index, defined as Cardiac

Output (ml/min) normalized to 100 g body weight. Stroke Volume Index (SVI) was defined as SV normalized to 100g body weight.

At the end of the hemodynamic measurements, right middle lung lobes were tied off with suture, excised, and snap frozen in liquid nitrogen for bioanalysis. The rest of the lung tissue was perfused with 10% formalin for histopathology analysis.

Plasma samples were collected at study termination for biomarker follow up. Circulating levels of NT-proBNP were assessed with the Rat NT-proBNP sandwich immunoassay kit (#K153JKD) as per manufacturer's protocol (Meso Scale Discovery, Rockville, MD, USA). Rat Pro-Inflammatory Panel 2 immunoassay kit (#K15059D, Meso Scale Discovery) and mouse/rat PDGF-BB Quantikine ELISA (MBB00, R&D Systems) were used to assess circulating plasma cytokines and PDGF-BB respectively. MCP-1 (CCL2) expression was determined using the Rat MCP-1 Ultrasensitive immunoassay kit (#K153AYC) as per manufacturer's protocol (Meso Scale Discovery).

Histopathology Methods

Left lung lobes were harvested, perfused, and fixed with 10% formalin before being sent to the Institute for Research in Immunology and Cancer in Montreal, Quebec, Canada, for embedding, sectioning, and staining with H&E. Scanned images were then analyzed by a blinded histopathologist. Fifty intra-acinar vessels associated with alveoli, alveolar ducts and respiratory bronchioles were analyzed. All vessels associated with terminal bronchioles and all larger airways were excluded. Each vessel was categorized as non-muscular (single elastic lamina for all of circumference), semi-muscular (10–90% smooth muscle layer circumference) or muscular (>90% smooth muscle layer circumference). NDP view 2.7.25 Zoomer Digital Pathology (Hamamatsu Photonics, Hamamatsu City, Japan) software was used for the analysis. A complementary analysis

of the small pulmonary arteries was performed using lung sections stained with α-smooth muscle actin (SMA; # M0851, Agilent, Santa Clara, CA): 30-50 Small vessels less than 100 microns and plexiform lesions where present were analyzed per sample using Aperio Imagescope software. Average Signal intensity was normalized to area analyzed. ANOVA followed by the Bonferroni correction was used for statistical analysis.

Rat microRNA analysis

miRNA Extraction

Ten to twenty mg of lung tissue from the right upper lobe (from SU5416 Study 3) were weighed and kept on dry ice in a 2.0 mL Eppendorf tube. As a recommended control, a master mix of 1 μL of RNA-Spike mix containing UniSp2, UniSp4, and UniSp5 was added for every 700 μL of QIAzol as a QC control. Immediately prior to lysing, one 5 mm stainless steel bead and 701 μL of QIAzol mix was added to each tube. The tubes were loaded onto the TissueLyser II and were homogenized for 2 minutes at 25.0 Hz. Once complete, the extraction followed the manufacturer's protocol by using the miRNeasy Mini Kit Protocol for animal tissue found on the QIAcube Connect. miRNA samples were then quantified by the NanoDrop2000. Leftover RNA was frozen in the -80 for long-term storage.

cDNA Preparation

The cDNA reaction was prepared using the miRCURY LNA RT Kit (QIAGEN, Hilden, Germany). UniSp6 was reconstituted in combination with cel-miR-39-3p as an additional spike-in control for the reverse transcription process. Twenty ng of template RNA was added to the reaction mix as per the manufacturer's protocol, and was prepared using the conditions described with the miRNome PCR Mouse/Rat Panel I.

qPCR Assay

miRCURY LNA miRNome Mouse/Rat PCR Panels intended for use with QuantStudio PCR Systems (Catalog # YAMR-301YE-4) were purchased from QIAGEN (Hilden, Germany). Prior to each run, one plate was thawed and spun down to pellet lyophilized primer pellets. The miRCURY LNA SYBR Green PCR Kit (Catalog# 339347) was purchased for use to prepare samples for qPCR assays. Master mix was prepared following the manufacturer's protocol with the low ROX dye concentration due to the thermal cycler being used. Plates were sealed, briefly vortexed, then spun down prior to loading on the instrument. The PCR cycling conditions used followed the protocol, with a 2-minute inactivation at 95°C, followed by 40 cycles of 10 second denaturation at 95°C and 60 second annealing/extension at 56°C. A melt curve was performed after the PCR as a QC check.

Data Analysis

Data was first analyzed by using Thermo Fisher's QuantStudio Design & Analysis software to ensure all thresholds were consistent across all different plates to produce Ct values. The Ct values were then normalized to ΔCt values using inter-plate calibrators followed by geNorm normalization as described by Vandesompele.[8] miRNA families predicted to target rat and human BMPR2 were downloaded from TargetScan (release 8.0).[9] Only miRNA predicted to target both rat and human BMPR2 were retained for differential expression analysis. P-values for pairwise comparisons among treatment groups were calculated using the limma eBayes method (version 3.46)[10] in R (version 4.1.2). miRNA with a p-value below 0.05 and absolute ΔΔCt change greater than or equal to 1 were considered differentially expressed. Fold change values for between-group comparisons are derived from the ΔΔCt values, using the formula $2^{\wedge}(-\Delta\Delta Ct)$.

Immunohistochemistry of PAH lung explants and control lungs

Human lung samples for immunohistochemistry were obtained from the PHBI tissue repository as

formalin fixed paraffin embedded specimens. Control samples consisted of donor lungs not used for

transplant. Ten PAH and ten controls samples were studied. The clinical data is shown in **Table S3**.

Antigen retrieval was performed with citrate solution (Vector Laboratories). Sections of formalin-

fixed paraffin-embedded (FFPE) lung tissues were stained with the following primary antibodies:

PDGF Receptor α (3174; 1;100, Cell Signaling Technologies, Danvers, MA, USA), PDGF

Receptor β (3169; 1:100; Cell Signaling Technologies, Danvers, MA, USA), CD117 (NCL-L-

CD117-032, pre-diluted, Leica Biosystem, Nussloch, Germany), CSF1R, (OAAB18690; 1:500,

Aviva Systems Biology, San Diego, CA, USA).

Antibody binding was visualized with DAKO polymer labeled, HRP-bound, secondary reagent

(DAKO Envision+ HRP rabbit (K4010) and HRP mouse (K4006). For counterstain, Light Green

(STLGCPT-StatLab Medical Products) was used. The slides were scanned by the Aperio system

(Leica Biosystems, Buffalo Grove, IL) and visualized in its ImageScope software. Random images

focused on pulmonary arteries were captured at 10x magnification as jpeg files and coded for

masked analyses. Coded images were uploaded into Metamorph (Molecular Devices, San Jose,

CA), thresholded based on positive control immunohistochemical signal and the signal quantified

using the region measurement application of the image software. This approach was used in our

prior publications.[11-13]

Anti-PDGFRα, PDGFRβ and CSF1R antibodies described above were rat-cross reactive and were

used for staining FFPE lung tissues from both the MCTPN and SU5416/H rats treated with vehicle

or seralutinib. The c-KIT antibody was not cross-reactive, and therefore c-KIT staining was not

evaluated in rat tissues.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) unless otherwise indicated. Statistical analysis was performed with XLSTAT (Addinsoft, Paris, France) or GraphPad Prism (GraphPad, La Jolla, CA, USA). For all studies, significance was set at $P<0.05$. For monocrotaline pneumonectomy model (MCTPN) Study 2 data, the unpaired t-test was used to compare pulmonary artery pressures, lumen to media ratio, respiratory parameters, and right ventricular hypertrophy (RVH), between the vehicle and seralutinib-treated groups. Analysis of neointimal proliferation grade was performed with the Mann-Whitney test. For the SU5416/H Study 1 analysis of variance (ANOVA) or repeated measures ANOVA was performed with the Bonferroni correction for multiple group comparisons. The unpaired t-test was used for 2 group comparisons. The Kruskal Wallis test was used for the graded occlusive lesion analysis. SU5416/H Study 3 statistics were calculated using a one-way ANOVA with Dunnett's or Tukey's Multiple Comparisons Test.

References

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Table S1. Seralutinib *In* Vitro Potency Versus Imatinib

Compound	Biochemical IC$_{50}$ (nM)				Cell-Based IC$_{50}$ (nM)							
	PDGFRα*	PDGFRβ*	CSF1R*	c-KIT[†]	H1703 (PDGFRα)[‡]	HLF (PDGFRβ >α)[§]	HPASMC (PDGFR α=β)[ll]	H1703 pERK**	HLF pERK**	[††]CSF1R pCSF1R	[†††]CSF1R Reporter assay	HPAECs pc-KIT[‡‡]
Seralutinib	7	14	92	20	32	29	33	70	60	14.4	8	7.8
Imatinib	8	75	1160	180	62	579	419	260	>10,000	500	1032	301

CSF1R = colony stimulating factor 1 receptor; HLF = human lung fibroblast; HPASMC = human pulmonary arterial smooth muscle cell; HPAEC = human pulmonary artery endothelial cells; PDGFR = Platelet-derived growth factor receptor; SCF = stem cell factor, M-CSF1= macrophage colony stimulating factor.

*Carna biochemical assay.
[†]c-KIT biochemical assay.
[‡]H1703 proliferation assay.
[§]PDGF-BB-induced HLF proliferation assay.
[ll]PDGF-BB-induced HPASMC proliferation assay.
**PDGF-BB induced pERK signaling assay in H1703 and HLF cells.
[††]M-CSF-induced pCSF1R in primary human macrophages.
[†††]M-CSF-induced activation of CSF1R-SRE reporter in HEK293.
[‡‡]SCF-induced phosphorylation of wild type KIT in HPAECs