Supplemental Methods:

Animal Studies

Pulmonary arterial isolation and cannulation

Arterial segments were mounted on cannulae and all side branches tied off. The exterior of the vessel was suffused with PSS from a water-jacketed reservoir at 37°C and aerated with 21% O_2, 5% CO_2 at pH 7.4. The arterial lumen was filled from a syringe containing PSS, which was aerated with the same gas mixture as the reservoir and connected to the vessel cannula with polyethylene tubing. Inflow pressure is adjusted by changing the height of the syringe. Pressure transducers are placed on the inflow side between the syringe and the vessel and at the outflow end of the system. Pressurization is achieved by occluding the outflow cannula. Vessels are discarded if the inflow and outflow pressures are not the same. The external diameter of the vessel is continuously observed with a stereomicroscope situated over the vessel chamber and connected to a video system containing a color camera (Panasonic Digital 5000) and television monitor (Sony PVM-1390). Vessel diameters are measured with a videoscaler (FORA IV 550). Lungs from a total of four pigs were included in these experiments, two vessels isolated from each pig and two conditions (AVE0991 and control compound).

RNASeq

For RNA, pig vessels were equilibrated in DMEM + 10% FBS, and treated for 6h with 10 µM AVE0991 or DMSO control. The working dose of AVE0991 was calculated based on vessel activity studies, and is higher than published to

optimize solubility. RNA-Seq was performed on an Illumina HiSeq system with a directional mRNA library prep, SR-50, with 30 million reads. TopHat was used to align RNA-Seq reads to consensus genome sequence using the ultra high-throughput short read aligner Bowtie2. Gene ontology analyses were performed with WebGestalt as previously reported(1, 2).

RNA-Seq data was validated in CCL2, CCL20, IL2RA and SOD2. Porcine pulmonary arteries were placed in DMEM10%+10% FBS with AVE0991 or vehicle for four hours. RNA was harvested and qPCR performed according to standard protocols. Lungs from one pig were included in this experiment with four total vessels isolated, 2 were controls and 2 were exposed to AVE0991.

Observational Studies of ACE2 in PAH Patients

Patient Characteristics

Inclusion criteria included heritable and idiopathic PAH, aged 18 years or older. Exclusion criteria included individuals receiving ACE inhibitors or angiotensin receptor blockers. Diagnosis, age, sex, weight, PAH-specific therapy, aldosterone antagonist use, NYHA class, blood pressure, biomarkers, and six-minute walk distance (6MWD) were recorded on day of blood acquisition. Right heart hemodynamic data from the most recent cardiac catheterization was obtained from medical records in PAH subjects.

RAS Peptide Measurement

Blood was drawn peripherally from subjects in the sitting position. 0.05 ml of protease inhibitor cocktail [0.44 mM 1,20 ortho-phenanthroline monohydrate

(Sigma; St. Louis MO.), 0.12 mM pepstatin (Peninsula Labs; Belmont CA), and 1 mM Na p-hydroxymercuribenzoate (Sigma; St. Louis MO)] per ml patient blood was added to two prechilled EDTA blood collection tubes. The samples were centrifuged under refrigeration for 10 minutes. Plasma was transferred into a prechilled polypropylene conical tube and centrifuged again for 10 minutes. Samples were frozen at -80° and mailed in dry ice to the Wake Forest Hypertension core where the plasma was extracted using Sep-Pak columns activated with 5 ml sequential washes of a mixture of ethanol:water:4% acetic acid (83:13:4), methanol, ultra pure water, and 4% acetic acid. After the sample was applied to the column, it was washed with ultrapure water and acetone and eluted with 3.5 ml washes of a mixture of ethanol:water:4% acetic acid. The sample was eluted, reconstituted, and Ang II was measured by radioimmunoassay (American Laboratory Products Company; Windham, NH). Recovery of radiolabeled Ang added to each sample and followed through the extraction was on average 92%. Samples were corrected for recovery within a given assay. The assay's lower detection limit is 0.9 fmol (0.8 pg)/tube for Ang II. The intra-assay coefficient of variation is 12% for Ang II. For Ang-(1-7), a TRIS buffer with 0.1% BSA was used. Recovery of radiolabeled Ang added to each sample and followed through the extraction was on average 92%. Samples were corrected for recovery within a given assay. Ang-(1-7) was measured using the antibody previously described(3, 4). The assay's lower detection limit was 1.39 fmol (2.5 pg)/tube for Ang-(1-7). Values at or below the minimum detectable

level of the assay were arbitrarily assigned that value for statistical analysis. The intra-assay coefficient of variation was 8% for Ang-(1-7).

SOD2 Protein Level by Aptamer Assay

Fasting plasma was drawn from 25 PAH patients and 26 matched controls as described above. Using the SomaScan aptamer-based platform (SomaLogic, Boulder, CO courtesy of Robert E. Gerszten, MD), protein abundance was quantitated and compared. The assay was performed as per the manufacturer's protocol.

Pilot Trial of GSK2586881

Inclusion and Exclusion Criteria Inclusion criteria: Heritable or idiopathic PAH defined according to standard criteria(5), WHO functional class I-III, no evidence of right heart failure, six minute walk distance (6MWD) >330m, stable diuretic does for 8 weeks with the exception of temporary increase of 3 days or fewer, 18 years of age and, if appropriate, using contraception. Exclusion criteria: previous treatment with any formulation of rhACE2, known hypersensitivity or allergy to GSK2586881, use of ACE inhibitor or angiotensin receptor blocker within 7 days of enrollment, systemic hypotension, GFR<60mL/min/1.73m^2, clinically significant liver disease, hospitalization for PAH within six months, additional medical condition that may significantly interfere with study compliance and follow-up activities. For this trial, given the low number of enrollees, an Independent Safety

Officer (Todd Rice, MD, MSCI) served as our Data Safety Monitor. All adverse events were reviewed directly with the Independent Safety Officer and full data were available to Dr. Rice at all times through an online, password protected database.

RAS peptide measurement

RAS peptides (Ang II and Ang-(1-7)) were performed in conjunction with GlaxoSmithKline using methodology adapted from Basu et al(6). For RAS peptide analysis, whole blood was collected in tubes containing an angiotensin-stabilizing protease inhibitor cocktail blocking angiotensin metabolism, containing broad spectrum inhibitors against metalloproteases (EDTA, 1,10-phenanthroline), aspartic proteases (pepstatin A), cysteine proteases (p-hydroxymercuribenzoic acid), serine proteases (AEBSF), and specific inhibitors for renin and aminopeptidases A and N to a final concentration of 5% v/v (Attoquant Diagnostics, Vienna, Austria). Tubes were thoroughly mixed and centrifuged at 3000 x g for 10 min at 4° C. Supernatant plasma was flash frozen in liquid nitrogen before being stored at -80° C for further analyses.

Stabilized protease inhibitor samples were spiked with stable isotope-labeled internal standards for each angiotensin metabolite, Ang II, Ang-(1–7) and Ang-(1–5), over the range of 2.5 to 250 pg/mL. Following C18-based solid-phase-extraction, samples were subjected to liquid chromatography-mass spectrometry/mass spectroscopy (LC-MS/MS) analysis using a reversed-phase analytical column operating in line with a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, Massachusetts). Internal standards were used to

correct for peptide recovery of the sample preparation procedure for each angiotensin metabolite in each individual sample. Endogenous Ang peptide concentrations were calculated from a standard curve using a surrogate analyte strategy where labeled Ang petides are spiked into human plasma. The lower limit of quantification of the circulating levels of the individual angiotensin peptides was 2.5 pg/mL for each of the three Ang peptides.

Superoxide dismutase 2 (SOD2) ELISA

A simple sandwich ELISA was performed to estimate the plasma SOD2, as explained in the supplier's manual (Abnova, Walnut, CA, USA). In brief, 100 uL of plasma (1:60 dilution) was incubated for two hours in presence of mouse monoclonal antibody specific to human SOD2 sandwiched in a 96 well-plate. Following multiple washings, the bound proteins were incubated for an hour in presence of biotinylated anti-human SOD2 antibody. Subsequently, Avidin conjugated horseradish peroxidase was added for 30' followed by the addition of substrate for 10'. On the addition of stop solution, the resultant yellow color produced was measured at 450 nm and the unknown concentration of SOD2 in the plasma samples were estimated against plotting a standard curve. All the incubations were performed at room temperature.

Cytokine Luminex Assay

A predesigned Human High Sensitivity T-Cell magnetic bead panel was utilized on Luminex platform in order to analyze the plasma cytokines (Millipore, Bellerica, MA, USA), as per supplier's instruction. Briefly, 25 uL of immobilized

magnetic bead antibody was added onto each well of a 96 well plate, following the addition of 25 uL of plasma (1:2 dilution) and 25 uL of assay buffer, incubated for an overnight at 4^{O}C. Following the overnight incubation, each well was washed thrice with the wash buffer, incubated with 50 uL of detection antibody for an hour and with 50 uL of streptavidin-phycoerythrin for 30'. Subsequently, 150 uL of drive fluid was added, which carry the sample to optics and the fluorescence emitted by each analyte were digitally processed on the Luminex platform using MAGPIX exponent software (version 2). Concentration of each analyte was estimated against plotting a standard curve for each cytokine, using Milliplex Analyst Software (Version 5.1).

Nitrotyrosine dot blot assay

In order to estimate the reactive nitrogen species, we performed the dot blot analysis of nitro tyrosine. Please see online supplement for full details. Briefly, a 7.1 X 8.5 cm sized, 0.2 um pore PVDF membrane (Bio-Rad, Hercules, CA, USA) presoaked in methanol for 15 sec, washed with deionized water was loaded with 2.5 uL of plasma samples. In order to avoid the sample spill over, 1 cm gap was maintained in between each dot (2.5 ul). Following an air dry, the membrane was soaked in methanol for 15 sec, washed with deionized water and blocked with 5% non-fat milk solution in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) for an hour. Subsequently, the membrane was probed with anti-nitrotyrosine antibody (1:1000), two days at 4^{O}C. After two days, the membrane was washed thrice in TBST, incubated with horseradish peroxidase conjugated goat anti-rabbit (1:5000) for an hour and detected with SuperSignal West Dura Extended

Duration Substrate (ThermoFisher Scientific) on a Chemidoc Touch Imaging System (Bio-Rad, Hercules, USA). Image J was utilized to analyze and calculate the arbitrary pixel density of the dot blot assay.

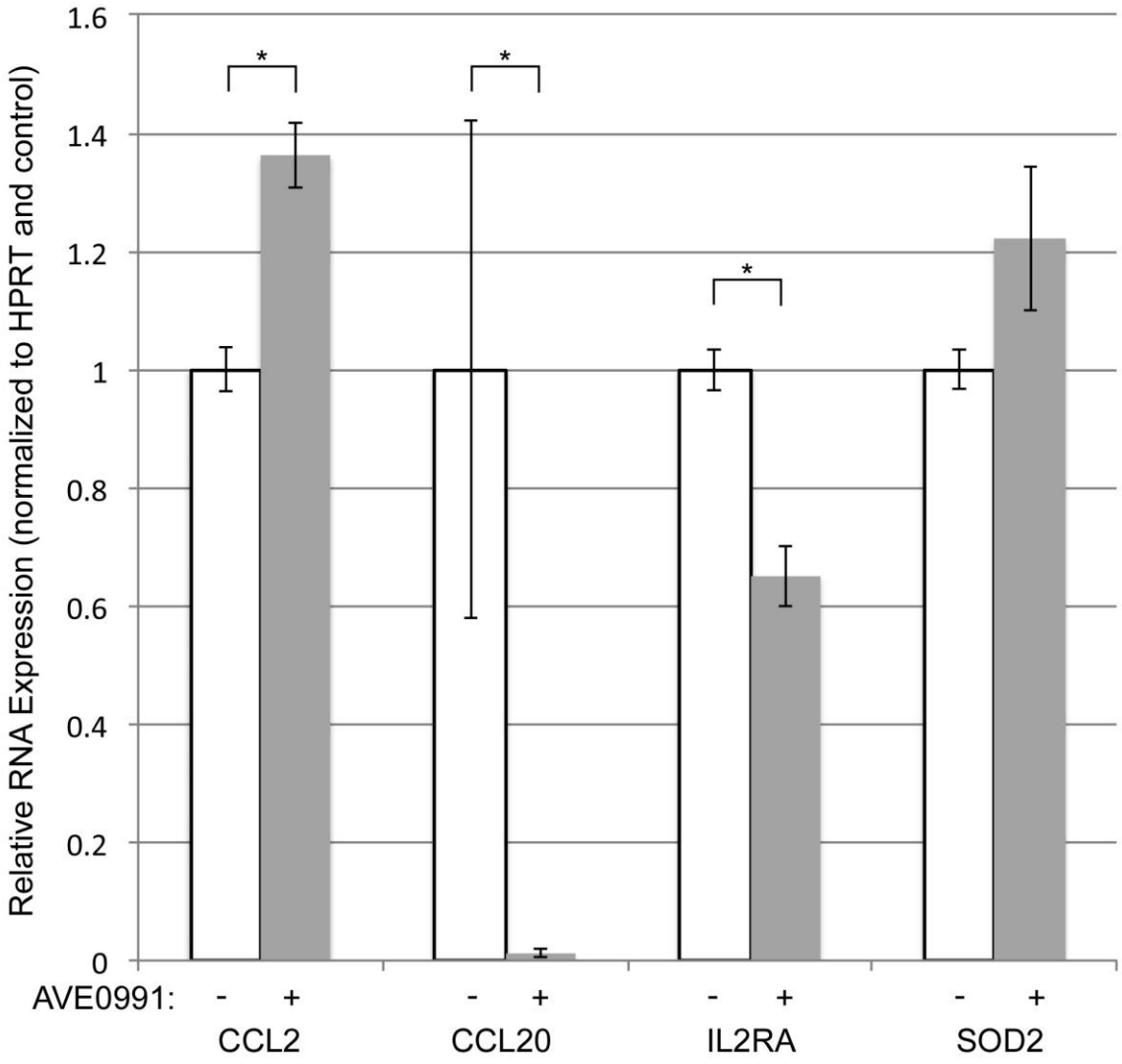
Isoprostane and Isofuran measurement

Blood was collected in EDTA tubes and placed immediately on ice. Plasma was isolated according to standard protocol and immediately snap frozen. F2-Isoprostances and Isofurans were measured by the VUMC Eicosanoid Core using high precision mass spectrometric assays.

Role of GSK in Study:

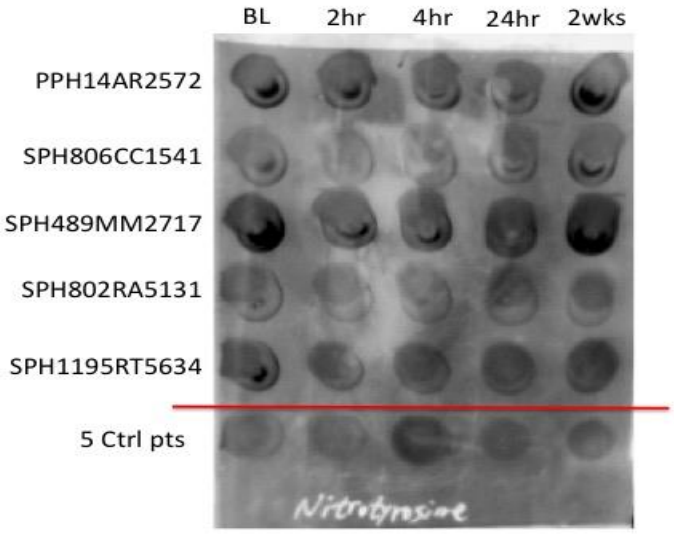
GSK was not involved in the development or implementation of the protocol nor was the company involved in data analysis. GSK provided study drug (GSK2586881) but did not participate in collection of data. GSK performed RAS peptide analysis given their expertise in this area. Finally, GSK performed PK/PD studies and immunogenicity studies. The remainder of the assays were performed by the study investigators. GSK did not provide funding for the study.

Supplemental Figure 1.



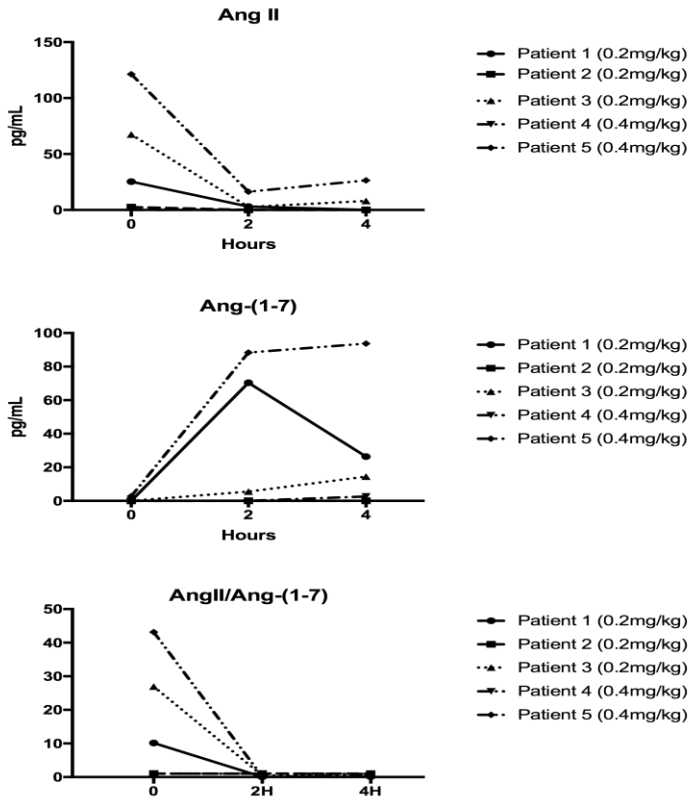
qPCR validation of RNASeq findings. Tissue is whole lung homogenate from mice with 2 lungs, 4 vessels per lung. *=p<0.05

Supplemental Figure 2.



Original unchanged nitrotyrosine dot blot (Figure 4C).

Supplemental Figure 3.



Patient level data on AngII, Ang-(1-7) and the AngII/Ang-(1-7) ratio just prior to rhACE2 infusion () time point, followed by measreument at 2 and 4 hours.

Supplemental Table 1. ACE2 Activity in PAH vs. Control Patient Demographics

	N=11
Age (years)	43 (12)
Sex (M/F)	2/9
PAH type (H/I)	4/7
6MWD (m)	390.7 (60.0)
PAH therapy (n)	
Parenteral prostaglandin	6
Phosphodiesterase type 5 inhibitor	4
Endothelin Receptor Antagonist	4
Aldosterone antagonist (n)	4

Data are presented as mean (SD) unless otherwise noted. PAH = pulmonary arterial hypertension, H= heritable PAH, I = idiopathic PAH, 6MWD=six minute walk distance.

Supplemental Table 2. Genes significantly changed in AVE0991 treated pig arteries

Gene Symbol	Gene Description	Fold Change	T-test
AASDHPPT	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	-1.2	0.047
AASS	aminoadipate-semialdehyde synthase	1.5	0.036
ABCC9	ATP binding cassette subfamily C member 9	1.3	0.047
ACE2	angiotensin I converting enzyme 2	-1.4	0.001
ACTC1	actin, alpha, cardiac muscle 1	-1.2	0.035
ADAM8	ADAM metallopeptidase domain 8	1.3	0.031
ADGRG6	adhesion G protein-coupled receptor G6	-1.4	0.005
ADHFE1	alcohol dehydrogenase, iron containing 1	1.2	0.027
AGT	angiotensinogen	1.2	0.028
AK5	adenylate kinase 5	1.3	0.039
ALOX15B	arachidonate 15-lipoxygenase, type B	1.5	0.035
AMIGO2	adhesion molecule with Ig like domain 2	1.2	0.039
ANAPC10	anaphase promoting complex subunit 10	-1.2	0.039
ANKRD29	ankyrin repeat domain 29	-1.2	0.019
ANO4	anoctamin 4	1.5	0.036
AR	androgen receptor	-1.2	0.022
ARHGAP42	Rho GTPase activating protein 42	1.3	0.035
ARNTL2	aryl hydrocarbon receptor nuclear translocator like 2	-1.4	0.042
ASIP	agouti signaling protein	1.3	0.016
ASPA	aspartoacylase	-1.2	0.009
BMP2K	BMP2 inducible kinase	1.2	0.021
CCBE1	collagen and calcium binding EGF domains 1	1.3	0.007
CCL20	C-C motif chemokine ligand 20	1.9	0.001
CCNB1IP1	cyclin B1 interacting protein 1	1.7	0.045
CCR9	C-C motif chemokine receptor 9	-1.2	0.014
CD244	CD244 molecule	-1.8	0.023
CDCA2	cell division cycle associated 2	1.3	0.004
CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1	1.6	0.036
CLMP	CXADR like membrane protein	1.2	0.038
CNIH3	cornichon family AMPA receptor auxiliary protein 3	-1.3	0.025
COA6	cytochrome c oxidase assembly factor 6	-1.3	0.042
CORIN	corin, serine peptidase	-1.2	0.010
CXCL16	C-X-C motif chemokine ligand 16	1.3	0.047
CXCL2	C-X-C motif chemokine ligand 2	1.6	0.002
CXCL2	C-X-C motif chemokine ligand 2	1.4	0.003
CXCL6	C-X-C motif chemokine ligand 6	2.0	0.012
CXorf58	chromosome X open reading frame 58	-1.2	0.003
CYP2U1	cytochrome P450 family 2 subfamily U member 1	-1.2	0.043

CYR61	cysteine rich angiogenic inducer 61	1.4	0.022
CYSLTR2	cysteinyl leukotriene receptor 2	1.3	0.037
DDX58	DExD/H-box helicase 58	1.4	0.013
DDX60	DExD/H-box helicase 60	1.3	0.006
DPT	dermatopontin	1.4	0.047
DSCC1	DNA replication and sister chromatid cohesion 1	-1.2	0.021
EFCAB5	EF-hand calcium binding domain 5	-1.4	0.006
EIF5B	eukaryotic translation initiation factor 5B	-1.2	0.018
EMB	embigin	-1.2	0.047
ENOX1	ecto-NOX disulfide-thiol exchanger 1	-1.4	0.013
ENOX2	ecto-NOX disulfide-thiol exchanger 2	-1.3	0.017
FAM13C	family with sequence similarity 13 member C	-1.4	0.013
FAM175A	family with sequence similarity 175 member A	-1.3	0.046
FANCM	Fanconi anemia complementation group M	-1.3	0.004
FCER1G	Fc fragment of IgE receptor Ig	1.2	0.016
FCGR1A	Fc fragment of IgG receptor Ia	-1.3	0.027
GFM2	G elongation factor mitochondrial 2	-1.2	0.030
GJA9	gap junction protein alpha 9	1.5	0.045
GKN1	gastrokine 1	-2.0	0.008
GLRX	glutaredoxin	1.4	0.029
GPATCH2	G-patch domain containing 2	-1.2	0.047
GPC3	glypican 3	1.3	0.037
GRHL3	grainyhead like transcription factor 3	1.6	0.021
GRIP2	glutamate receptor interacting protein 2	-1.2	0.031
IFIT3	interferon induced protein with tetratricopeptide repeats 3	1.5	0.046
IL2RA	interleukin 2 receptor subunit alpha	2.5	0.004
IL33	interleukin 33	-1.2	0.031
IL6	interleukin 6	1.7	0.040
INCENP	inner centromere protein	1.2	0.018
IQCB1	IQ motif containing B1	-1.5	0.047
ISLR	immunoglobulin superfamily containing leucine rich repeat	1.2	0.050
IYD	iodotyrosine deiodinase	-1.3	0.042
KITLG	KIT ligand	-1.3	0.030
KLHL15	kelch like family member 15	1.2	0.011
L2HGDH	L-2-hydroxyglutarate dehydrogenase	-1.2	0.049
LAMA3	laminin subunit alpha 3	1.8	0.029
LIPG	lipase G, endothelial type	1.5	0.049
LRRK2	leucine rich repeat kinase 2	-1.2	0.047
LYZ	lysozyme	-1.9	0.005
MAOB	monoamine oxidase B	-1.2	0.042
MAP7D3	MAP7 domain containing 3	-1.2	0.023
MAX	MYC associated factor X	1.2	0.006
MPEG1	macrophage expressed 1	-1.3	0.042
MSC	musculin	2.0	0.021

MSMO1	methylsterol monooxygenase 1	-1.2	0.005
MTHFR	methylenetetrahydrofolate reductase	1.2	0.045
MTMR7	myotubularin related protein 7	-1.3	0.001
MYBPC3	myosin binding protein C, cardiac	1.9	0.020
NECAB1	N-terminal EF-hand calcium binding protein 1	-1.4	0.020
NEK10	NIMA related kinase 10	1.3	0.005
NFIL3	nuclear factor, interleukin 3 regulated	1.2	0.014
NOSTRIN	nitric oxide synthase trafficking	-1.3	0.045
NREP	neuronal regeneration related protein	1.2	0.019
NSUN7	NOP2/Sun RNA methyltransferase family member 7	-1.2	0.001
OCIAD2	OCIA domain containing 2	-1.3	0.038
OPHN1	oligophrenin 1	-1.3	0.028
ORC3	origin recognition complex subunit 3	-1.2	0.046
PARD6B	par-6 family cell polarity regulator beta	1.3	0.008
PARP14	poly(ADP-ribose) polymerase family member 14	1.3	0.019
PCOLCE2	procollagen C-endopeptidase enhancer 2	1.2	0.025
PFDN4	prefoldin subunit 4	-1.2	0.026
PMS1	PMS1 homolog 1, mismatch repair system component	-1.3	0.033
PNP	purine nucleoside phosphorylase	1.3	0.001
POPDC2	popeye domain containing 2	-1.4	0.049
POSTN	periostin	1.2	0.047
PPIA	peptidylprolyl isomerase A	-1.3	0.031
PRSS35	protease, serine 35	1.5	0.011
PSMG4	proteasome assembly chaperone 4	1.3	0.022
PTGER2	prostaglandin E receptor 2	-1.4	0.016
RAB32	RAB32, member RAS oncogene family	1.2	0.029
RAB3C	RAB3C, member RAS oncogene family	-1.3	0.031
RBP4	retinol binding protein 4	1.6	0.039
RIMS2	regulating synaptic membrane exocytosis 2	-1.4	0.014
RNF170	ring finger protein 170	1.3	0.013
RPS16	ribosomal protein S16	1.2	0.041
SAA1	serum amyloid A1	2.0	0.015
SCARA5	scavenger receptor class A member 5	1.2	0.009
SCN1A	sodium voltage-gated channel alpha subunit 1	-1.5	0.035
SCN8A	sodium voltage-gated channel alpha subunit 8	1.2	0.006
SCN9A	sodium voltage-gated channel alpha subunit 9	1.3	0.046
SCUBE1	signal peptide, CUB domain and EGF like domain containing 1	1.4	0.006
SDHAF3	succinate dehydrogenase complex assembly factor 3	-1.5	0.035
SERPINB2	serpin family B member 2	1.5	0.027
SERPINB7	serpin family B member 7	-1.5	0.050

SEZ6L	seizure related 6 homolog like	-1.2	0.042
SLC13A5	solute carrier family 13 member 5	-1.4	0.037
SLC1A1	solute carrier family 1 member 1	1.6	0.023
SLCO5A1	solute carrier organic anion transporter family member 5A1	1.4	0.009
SMC4	structural maintenance of chromosomes 4	-1.2	0.010
SOD2	superoxide dismutase 2	1.6	0.004
STIM2	stromal interaction molecule 2	-1.2	0.031
TBXAS1	thromboxane A synthase 1	1.2	0.047
TENM1	teneurin transmembrane protein 1	-1.2	0.005
TFPI	tissue factor pathway inhibitor	-1.3	0.034
TGIF1	TGFB induced factor homeobox 1	1.2	0.003
THY1	Thy-1 cell surface antigen	1.5	0.045
TMEM144	transmembrane protein 144	-1.3	0.000
TPH1	tryptophan hydroxylase 1	-1.2	0.048
TTC32	tetratricopeptide repeat domain 32	-1.6	0.050
UBE3D	ubiquitin protein ligase E3D	1.2	0.003
UPF3B	UPF3 regulator of nonsense transcripts homolog B (yeast)	-1.2	0.021
WNT7B	Wnt family member 7B	2.3	0.029
ZDHHC15	zinc finger DHHC-type containing 15	-1.4	0.025
ZFAND4	zinc finger AN1-type containing 4	-1.2	0.047
ZFP14	ZFP14 zinc finger protein	-1.3	0.025
ZNHIT6	zinc finger HIT-type containing 6	-1.2	0.013

Supplemental Table 3. Schedule of Events

Assessment	Screen[a]	BL[a]	0	1 hr	2 hr	4 hr	6 hr	12 hr	24 hr	2 weeks
Informed consent	X									
Medical history	X*	X*								
Concomitant Medications	X**	X**								X
Physical examination	X*[b]	X*							X	X
Functional classification	X*	X*								X
6MWD	X*	X*								X
Blood pressure [c]	X	X	X	X	X	X		X	X	X
Pulse oximetry (HR, SO_2)[d]	X	X	X	X	X	X		X	X	X
Pregnancy test (urine)[e]		X								
Chemistry tests, troponin I	E (7 days)	E (7 days)							X	X
Hematology, BNP	E (7 days)	E (7 days)							X	X
Coagulation Panel	E (7 days)	E (7 days)							X	X
Serum Nitric Oxide	E (7 days)	E (7 days)	X		X	X			X	X
Plasma for SOD2 activity/isoprostanes/ isofurans	E (7 days)	E (7 days)	X		X	X			X	X
Pk measurements[l]	E (7 days)	E (7days)	X				X		X	
Immunogenicity Studies			X							X
Peripheral Ang II/Ang 1-7	E (7 days)	E (7 days)	X		X	X			X	
Transpulmonary Ang II/Ang 1-7			X			X				
Urinalysis	E (7 days)	E (7 days)							X	

Urine isoprostane and isofurane[f]			X			X			X	X
ECG		X				X			X	
Administration study drug			X							
Hemodynamic measurements [g]			X	X	X	X				
Adverse events [h]		X	X	X	X	X		X	X	X
Transthoracic echocardiography		X							X	

Screening (Screen) results must be available and baseline procedures performed before the first dose of study drug is administered. To avoid repeat tests, some screening values may be counted as baseline (BL) values if obtained within specified time window.

E (7 days): Can be performed at either visit, but at least one test must be performed no more than 7 days before study drug is administered. Results from this test must meet eligibility requirements and must be available before first study drug administration.

* Data will be recorded at screening and eligibility will be evaluated; at baseline, data will be reviewed and any changes since screening will be recorded and eligibility will be re-evaluated (as necessary).

** Concomitant medications for the 30 days prior (8 weeks prior for PAH medications) will be reviewed/recorded at screening to evaluate eligibility and changes will be recorded throughout the study.

Procedures are marked in table as follows:

[a] To facilitate scheduling, some screening procedures may occur up to 90 days prior to the first dose of study drug. Baseline procedures must be performed no more than 7 days prior to study drug.

[b] Physical examination includes weight and height measurement at screening.

[c] Measuring BP and Pulse Oximetry

BP will be measured after 5 minutes seated; and after dose administration through the 4 hour timepoint and sitting at the timepoints listed in the table above.

[d] Pulse oximetry and heart rate will be measured and recorded with each blood pressure assessment.

[e] In woman of childbearing potential only.

[f] First morning void, brought from home to study visit on ice, for urinary biomarkers. [g] Includes RAP, PWP, CO, CI, and PAsat

[h] At baseline, all SAEs and study-related AEs since signing of the Informed Consent Form will be recorded.

[I] PK measurements are to be taken at the following times: at the completion of the first infusion, six hours and 24 hours after the dose

Table 4. Effect of GSK2586881 on Six Minute Walk Distance

	Baseline (m)	24 hours (m)	2 weeks (m)
Patient 1	419	415	430
Patient 2	590	519	524
Patient 3	366	348	396
Patient 4	387	363	366
Patient 5	360	369	375
All patients, mean (SD)	424.2 (95.4)	402.8 (69.6)	418.1 (64.1)

P=0.2 baseline vs. 24 hours, p=1.0, baseline vs. 2 week.

Supplemental Table 5. Echocardiographic Effects of GSK2586881 Infusion in PAH

	Baseline	24 Hours	p value
RVSP	45 (31)	53 (18)	0.62
RAP	4 (2)	4 (2)	0.99
TAPSE	2.2 (0.6)	2.4 (0.5)	0.31
LVFS	34 (7)	35 (6)	0.56

RVSP = right ventricular systolic pressure, RAP = right atrial pressure, TAPSE = tripcuspid annular plane systolic excursion, LVFS = left ventricular fractional shortening.

Supplemental Table 6. Pharmacokinetic profile of GSK2586881

Patient Number		Unit			(ng/mL)
1	SCREEN		Plasma	GSK2586881	NQ
1	0	Hours	Plasma	GSK2586881	NQ
1	2	Hours	Plasma	GSK2586881	4390
1	4	Hours	Plasma	GSK2586881	3876
1	6	Hours	Plasma	GSK2586881	271
1	24	Hours	Plasma	GSK2586881	NQ
2	SCREEN		Plasma	GSK2586881	NS
2	0	Hours	Plasma	GSK2586881	NQ
2	2	Hours	Plasma	GSK2586881	2262
2	4	Hours	Plasma	GSK2586881	1681
2	24	Hours	Plasma	GSK2586881	249
3	SCREEN		Plasma	GSK2586881	NQ
3	0	Hours	Plasma	GSK2586881	NQ
3	2	Hours	Plasma	GSK2586881	3764
3	4	Hours	Plasma	GSK2586881	3378
3	24	Hours	Plasma	GSK2586881	546
4	SCREEN		Plasma	GSK2586881	NQ
4	0	Hours	Plasma	GSK2586881	NQ
4	2	Hours	Plasma	GSK2586881	4601
4	4	Hours	Plasma	GSK2586881	3760
4	24	Hours	Plasma	GSK2586881	NS
5	SCREEN		Plasma	GSK2586881	NQ
5	0	Hours	Plasma	GSK2586881	NQ
5	2	Hours	Plasma	GSK2586881	4845
5	4	Hours	Plasma	GSK2586881	3342
5	24	Hours	Plasma	GSK2586881	566

Patients 1, 2 and 3 received 0.2mg/kg and patient 4 and 5 received 0.4mg/kg of GSK2586881.

Supplemental Table 7. Hemodynamic Effect of GSK2586881 in PAH

	-1H	0	1H	2H	4H
Systolic BP	112.2 (12.1)	110 (9.7)	110 (11.2)	106.4 (10.2)	106.4 (23.2)
Diastolic BP	68.2 (10.8)	66.2 (5.2)	58.6 (14.6)	55 (5.1)	61.8 (12.6)
Heart Rate	83.6 (11.9)	70.2 (11.2)	76.6 (5.8)	79.4 (8.1)	78.2 (15.4)
sPAP	64.8 (29.5)	61.8 (24.4)	58.4 (18.7)	65.4 (26.7)	60.4 (27.6)
dPAP	29.4 (11.1)	30.2 (8.9)	26.6 (10.8)	27.0 (6.8)	29.8 (7.3)
mPAP	40.6 (13.7)	42.8 (13.8)	41.6 (15.4)	40.2 (13.7)	40.6 (11.7)
PWP	14.2 (2.8)	15.8 (5.0)	15.4 (8.1)	13.5 (10.4)	12 (4.3)
CO	4.2 (1.1)	5.3 (1.4)	5.8 (1.6)	6.1 (1.3)	6.3 (1.5)*
CI	2.66 (0.3)	2.8 (0.6)	3.23 (1.0)	3.4 (0.8)	3.4 (0.7)#
PVR	5.4 (1.9)	4.0 (1.3)	3.5 (1.1)	2.4 (1.9)	3.5 (0.8)

*p=0.008 vs. 0 and BL time points. #p=0.02 vs. O and BL time points

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A Phase 1b, Single Center, Open-label, Dose-escalation, and Multiple Dose Study to Evaluate the Safety of Recombinant Human Soluble Angiotensin Converting Enzyme 2 (rhACE2) in Subjects with Pulmonary Arterial Hypertension

(Short title: rhACE2 PAH Study)

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1.0 Background

Pulmonary arterial hypertension (PAH) is defined as mean pulmonary arterial pressure > 25 mm Hg at rest and a mean pulmonary wedge pressure of ≤ 15 mm Hg (Hoeper 2009, J Am Col Card). Patients may present with shortness of breath, swelling, fatigue, chest pain, and in advanced cases syncope. Women of childbearing age are most commonly affected, although PAH has been reported in children and older adults. Untreated PAH eventually leads to right-sided heart failure and death. PAH is a progressive disease, and mortality and morbidity remain high in spite of recent improvements in therapy.

The development of PAH is associated with connective tissue disease, liver disease, HIV infection, use of appetite suppressants, congenital systemic-to-pulmonary shunts (e.g., atrial septal defect), and portal hypertension. PAH may also occur with no known cause, in which case it is considered primary or idiopathic PAH (IPAH), and as an inherited disease, referred to as heritable PAH (HPAH). IPAH is a devastating disease which if untreated has a median life expectancy of 2.8 years (D'Alonzo 1991, Ann Intern Med). Outcome for PAH associated with other disorders such as scleroderma is even worse (Robbins, others).

At the histologic level, all forms of PAH are characterized by marked structural remodeling of small pulmonary arteries (<100 μ in diameter); specifically, vascular smooth muscle cell proliferation (medial hyperplasia), intimal fibrosis and hyperplasia, and in situ microthrombosis. In advanced disease, plexiform lesions, likely a disordered attempt at neovascularization, have been identified. These changes in the small pulmonary arteries lead to narrowing and obliteration of the vessel lumen, increased resistance to blood flow, and increased strain on the right ventricle. The molecular mechanisms underlying this vasculopathy remain poorly understood.

Management strategies for PAH include prevention of microthrombosis using anticoagulants such as warfarin and promoting vasodilation and regression of muscular hypertrophy and intimal fibrosis using endothelin receptor antagonists and phosphodiesterase (PDE) 5 inhibitors (Humbert 2004, N Engl J Med). In severe disease or in cases of treatment failure with oral therapy, prostanoids (inhaled, subcutaneous, or intravenous) are used. In selected patients not responsive to medical therapy, lung transplant is an option. Despite improvement in medical therapy, only 2/3 of patients are alive 3 years after treatment with most effective therapy available for PAH, continuous

intravenous epoprostenol (Sitbon 2002, J Am Coll Cardiol). The current therapeutic options remain unsatisfactory, and there remains an urgent need for novel therapies.

2.0 Rationale and Objectives

Rationale:

The ratio of Ang(1-7) to AngII, is reduced in both IPAH and HPAH patients. This difference is primarily driven by decreased Ang(1-7), rather than increased AngII, suggesting that ACE2 is suppressed in idiopathic and heritable PAH. Additionally, we have published therapeutic benefit of ACE2 in a transgenic mouse model of human heritable PAH. Because of this data showing decreased ACE2 in PAH patients, the demonstrated safety of ACE2 use in normal humans, and the strong efficacy data in mice, it would appear to be prudent and timely to test ACE2 in PAH patients. **The strategy is** to develop rhACE2 clinically for the treatment of pulmonary arterial hypertension; however, before pursuing a large scale treatment trial, a dose-finding, tolerability, and safety study in this patient population is warranted.

Objectives:

2.1 Primary Objective

The primary objective of the study is to determine safety of rhACE2 when administered as a single dose or multiple doses intravenously to subjects with PAH receiving background PAH-specific therapy.

2.2 Secondary Objectives

The secondary objective of the study is to evaluate changes in biomarkers of disease (BNP, AngII/Ang (1-7), serum soluble IDH, serum NO, cardiac troponin I, SOD2 activity, urine isoprostanes and isofuranes) in subjects with PAH receiving rhACE2. We will also evaluate changes in pulmonary and systemic hemodynamics and echocardiographic markers of right heart function in patients receiving rhACE2.

3.0 Animal Studies and Previous Human Studies (IB)

We have used an animal model of heritable PAH and tested the effects of a six week infusion of rhACE2. We found marked reduction in pulmonary hypertension with microarray data from lung showing improvement in cytoskeletal function (Johnson J et al, AJP Lung 2012). In human studies, we have looked for alterations in the Ang II/Ang (1-7) ratio and found reduced plasma Ang (1-7) and reduced Ang II/Ang (1-7),

suggesting reduced function of ACE2 in pulmonary arterial hypertension. Other groups have had similar findings in rodent models (Shenoy V et al, Hypertension 2014, Shenoy et al, AJRCCM 2013). Aperion Pharmaceuticals (who owned the compound prior to GSK) has previously conducted safety trials in healthy subjects: 5 subjects at 0.1 mg/kg, and 4 each at 0.2 mg/kg, 0.4 mg/kg, and 0.8 mg/kg, then three repeated doses, then six repeated doses, at 0.4 mg/kg in three subjects. There were no clinically significant abnormal labs or vital signs in any patient. There were 4 adverse events (AEs), 3 of which occurred in the two lowest doses; all were mild and resolved without a need for intervention and without any sequelae. GSK has an ongoing trial of ACE2 administration in acute respiratory distress syndrome with three days of twice daily infusions. While the trial is ongoing and efficacy data is not available, they have not found any significant or severe adverse events related to study drug administration (personal communication 11/24/14, ARH).

4.0 Study Design:

This is a Phase 1, single center, open-label, dose-escalation in subjects with PAH whose symptoms have been clinically stable for 8 weeks prior to enrollment and have had no change in PAH-specific therapy 12 weeks prior to enrollment. Eligible subjects will undergo baseline assessments before beginning treatment with rhACE2.

PHASE A: Single Dose Escalation:

The dose escalation will follow the traditional 3+3 design[56]. Briefly, the first cohort of 3 subjects will be treated at dose 0.2mg/kg. If none of the 3 subjects experiences a dose limiting toxicity (DLT), another three subjects will be treated at the next higher dose. Since ACE2 has been administered to human subjects at a lower dose and we plan to start at a safe dose of 0.2mg/kg, we will not follow the Fibonacci sequence to avoid a rapid increase of dose[57].

Instead, we fix the increment at 0.2-0.4mg/kg, i.e. the dose will be 0.2, 0.4, 0.8, to a maximum of 1.2. If one of the first 3 subjects experiences a DLT, three more subjects will be treated at the same dose level. The dose escalation continues until at least two subjects of 3 to 6 subjects experience DLT. The dose right below this toxic dose level will be considered the maximum tolerated dose (MTD). If we reach 1.2 mg/kg and there is no identified toxicity, which is quite possible given the prior experience, we will assess for 50% increase in Ang(1-7):AngII ratio to ensure biochemical efficacy.

During the treatment period, the planned rhACE2 dose-escalation regimen for the initial 12 subjects is as follows:

- First 3 subjects will receive rhACE2 intravenously at 0.2 mg/kg, if no dose limiting toxicities are noted:
- Next 3 subjects will receive rhACE2 intravenously at 0.4 mg/kg, if no dose limiting toxicities are noted:
- Next 3 subjects will receive rhACE2 intravenously at 0.8 mg/kg, if no dose limiting toxicities are noted:
- Next 3 subjects will receive rhACE2 intravenously at 1.2 mg/kg, if no dose limiting toxicities are noted:

Study design will follow the Up-and-down design. If **0** of **3** patients experiences a dose-limiting toxicity (DLT), the dose is **escalated**. If **1** of **3** patients experiences a DLT, **3 additional patients** are treated at the same dose. If **none** of the additional patients develops a DLT, the dose is escalated; otherwise escalation ceases. If $\geq$ 2 **of 3** or $\geq$ **2 of 6** patients experience a DLT, the maximum tolerated dose (MTD) has been exceeded. Dose limiting toxicities are defined as toxicities that, due to their severity or duration, are considered unacceptable, and limit further dose escalation. Examples of DLT's:

- Severe headache unrelieved by NSAIDS
- Nausea/Vomiting
- Hypotension as defined by mean arterial pressure <60mmHg or a drop in systolic blood pressure of 25%
- Changes in echocardiogram suggesting worsening right ventricular function

rhACE2 will be administered in the Cardiac Catheterization lab or the Cath lab holding area where the participant will be monitored for 4 hours following dosing with pulmonary arterial catheter in place. After four hours, the catheter will be removed. Transpulmonary blood will be collected from the mixed venous position and the wedge position at baseline and four hours after drug administration prior to removal of the catheter. Study participants will then be admitted to the Clinical Research Center (CRC) for further monitoring for a total of 24 hours post dosing. Hemodynamics will be measured at time points described in the schedule of events. After 24 hours from drug administration, patients will be discharged from the CRC. For each dosing group, an acceptable safety profile at each dose level will be required prior to dose escalation. In addition, the safety profile of the planned dose-escalation regimen will be evaluated throughout the study. Subjects will return after a two-week washout period for follow-up assessments.

Enrollment will continue until 12 subjects have completed the washout period and follow-up assessments.

If no safety concerns have been identified in the dose escalation phase, the multiple phase dosing will proceed as described below using one dose below the maximal tolerated dose of the single dose phase or at 1.2mg/kg if no MTD is reached.

5.0 Inclusion/Exclusion Criteria

Inclusion Criteria:

- Documented diagnosis of PAH, defined as mPAP > 25 mm Hg and PWP ≤ 15 mm Hg,
- IPAH, HPAH, or PAH associated with collagen vascular disease, repaired congenital heart disease, or appetite suppressant use.
- World Health Organization (WHO) functional class I, II, or III, stable for at least 8 weeks prior to enrollment.
- Hemodynamically stable without evidence of right heart failure. Hemodynamic stability will be defined as having a normal right atrial pressure (≤10 mm Hg) and cardiac index (≥2.5 l/min) with no more than mildly impaired right ventricular function on echocardiogram.
- 6MW distance, as performed at screening or within 12 weeks prior to screening, of > 330 meters
- Mean blood pressure of >60 mmHg
- Receiving stable doses of one or more medications that are approved for treatment of PAH, *including endothelin receptor antagonists, phosphodiesterase 5 inhibitors, and prostanoids*, for a minimum of 12 consecutive weeks before enrollment. *Note: Anticoagulant therapy can be adjusted according to target INR,*
- Diuretic dose stable for 8 weeks.
- 18 years of age or older
- Willing and able to complete an informed consent form.
- Sexually active subjects must be willing to use an acceptable method of contraception while participating in the study.
- Females of childbearing potential must have a negative pregnancy test at screening, using accepted birth control, and be willing to have additional pregnancy tests during the study.

Exclusion Criteria

- Previous treatment with any formulation of rhACE2.
- Known allergy or hypersensitivity to rhACE2.
- History of systemic hypotension, defined as systolic BP < 90 mm Hg and/or diastolic BP <60 mm Hg.
- Hospitalization for PAH associated deterioration in the previous 6 months
- Use of any investigational product or device within 30 days prior to dosing, or known requirement for any investigational agent prior to completion of all scheduled study assessments.
- Known to be positive for human immunodeficiency virus (HIV), Hep B, or Hep C or with cirrhosis from any cause.
- Any additional medical condition, serious intercurrent illness, or other extenuating circumstance that, in the opinion of the Investigator, may significantly interfere with study compliance, including all prescribed evaluations and follow-up activities. Concurrent disease or condition that may interfere with study participation or safety include bleeding disorders, arrhythmia, organ transplant, organ failure, current neoplasm, poorly controlled diabetes mellitus, and serious neurological disorders.
- Complex congenital heart disease
- Estimated GFR <60mL/min/1.73m^2 or hepatic enzyme levels more than 2 times the upper limit of normal.
- Pregnant or lactating at screening, or planning to become pregnant (self or partner) at any time during study.
- Weight >75kg.
- Any bleeding concerns as evidenced by INR > 1.5 (in patients not receiving anticoagulation therapy) or platelet count < 80,000
- Hematocrit < 30%
- Any use of an ACE inhibitor or angiotensin receptor blocker within 7 days of enrollment

6.0 Enrollment/Randomization

Participants will be recruited from the Pulmonary Hypertension Clinic at Vanderbilt Medical Center. All patients seen at our clinic meeting eligibility requirements will be approached for participation.

7.0 Study Procedures

7.1 Physical Examination and Medical History

Physical examination will be performed to ensure suitability according to the inclusion and exclusion criteria at screening and baseline and to document the health status at the time points specified in the Schedule of Events (Appendix 1). The physical examination comprises measurement of height and body weight (at screening only), vital signs including blood pressure, heart rate, respiratory rate, and oxygen saturation, and a routine medical examination. Functional Class will also be assessed at this time.

A medical history will be recorded at screening only. The medical history will elicit information concerning existing medical conditions, major illnesses, and related surgical procedures. Any prescribed or over-the-counter medications that the subject received within the past 30 days should be recorded on the case report form (CRF). Medication prescribed for the treatment of PAH for the 12 weeks prior to enrollment should be recorded on the CRF. Subjects will be instructed to notify the primary investigator or study coordinator before beginning new prescribed or over-the-counter medications.

7.2 Blood Pressure

Systolic and diastolic systemic BP will be measured by means of either a standard manual or an automatic BP measuring device (cuff method). For each subject, the same method should be used during the entire study, and the type of device used should be recorded on the CRF. The same arm will be used for each measurement of BP, and BP will be measured after 5 minutes seated.

7.3 Pulse Oximetry (Oxygen Saturation) and Heart Rate

Heart rate (HR) and oxygen saturation will be measured by pulse oximetry after the subject has been at rest for at least 5 minutes. Pulse oximetry provides a non-invasive method of monitoring the percentage of hemoglobin, which is saturated with oxygen. The pulse oximeter consists of a probe attached to the subject's finger or ear lobe, which is linked to a computerized unit. The unit displays the percentage of hemoglobin saturated with oxygen and the calculated HR and has an audible pulse signal and user-programmable, audible alarms. An oximeter detects hypoxia before clinical signs of cyanosis can be detected.

7.4 Six-Minute Walk Test and Borg Dyspnea Scoring

The 6MW test is a submaximal exercise test that is widely used as an outcome and safety measure in clinical trials and cardiopulmonary rehabilitation (American Thoracic

Society 2002, Am J Respir Crit Care Med) (Hoeper 2004, J Am Coll Cardiol). The test is simple and inexpensive, could likely be applied to the elderly, the frail, and those with walking difficulties. Furthermore, the test might reflect more accurately the activities undertaken in normal life than physiological measurements at peak exercise. During this test the subject is asked to walk along a prescribed path (typically 30 meters long) as far as possible during a 6-minute interval. The subject may walk at whatever pace is comfortable, with the goal of walking the longest distance possible. The subject may rest as needed.

Borg scales are used in practice and in clinical studies as a reliable way to help assess the rate of exertion as perceived by the patient or subject (Grant 1999, Chest) (Mador 1995, Chest). The scale consists of a vertical scale labeled 1 through 10, with text descriptors that express correspondingly increased intensity of effort at fixed points along the scale. In this study, the Borg scale will be used to assess the degree of discomfort evoked by breathing and the degree of effort required to breathe, as reported by the subject after the 6MW test.

7.5 Electrocardiogram

In subjects with PAH, the electrocardiogram (ECG) typically reveals right axis deviation and right ventricular (RV) hypertrophy. The standard 12-lead ECG will be recorded while the subject is resting in supine position. The following parameters will be assessed: HR, RR, PQ, QRS, QT and QTc. Additionally, the occurrence of de- or repolarization disorders, arrhythmic disorders or other abnormalities will be assessed and obvious changes of ECG parameters compared to the baseline record will be commented upon. ECGs will be interpreted locally. Changes to the ECG will be coded by the Investigator as clinically significant or not clinically significant. Clinically significant changes will be reported as AEs. Original printouts will be archived within the subject file and ECG recording results will be noted in the subject's CRF.

7.6 Echocardiogrophy

Two-dimensional (2D) and Doppler echocardiography will be performed using standard techniques on commercially available equipment with a predefined imaging protocol. Images will be obtained with the subjects in the left lateral position. The maximal tricuspid regurgitant jet (TRJ) velocity will be assessed by determining the peak regurgitant velocity (v) in the continuous-wave Doppler flow profile obtained from the cardiac apex. The tricuspid valve pressure gradient (TPG) (i.e., the pressure difference

between the right ventricle and right atrium during systole) will be calculated by applying the modified Bernoulli equation. Pulmonary arterial systolic pressure will be calculated using the formula $4v^2$ + right atrial pressure. Right atrial pressure will be assessed by clinical examination of the jugular venous pressure, and echocardiographic diameter of the inferior vena cava and its change with inspiration.

The right atrial volume is calculated at end systole from the apical four chamber view using the diameter and the long axis length of the atrium according to the equation: Volume = (7(pi x D^2 L)/6, where D = minor axis (cm), L = major or long axis (cm) (Wang 1984, Chest). The eccentricity index, a measure of the degree of septal displacement, will be measured at end diastole and end systole from parasternal short-axis projections of the left ventricle. It is calculated as the ratio of the minor axis of the left ventricle parallel to the septum at the level of the chordae (a), divided by the minor axis perpendicular to and bisecting the septum at the same section (b) (eccentricity index = a/b). The Doppler-derived RV MPI is calculated as described previously by Tei et al (Tei 1996, J.Am.Soc.Echocardiogr.) using the length of two time intervals in the formula (a-b)/b, where a equals the time between the onset of QRS complex and onset of tricuspid inflow and b equals the ejection time of RV outflow. TAPSE will be measured as per protocol (Forfia et al, AJRCCM, 2006).

7.7 Pregnancy Test

Female subjects of childbearing potential will have a pregnancy test (urine) performed at screening and at timepoints specified in the Schedule of Events (Appendix 1). Female subjects with a positive pregnancy test at screening may not be enrolled in the study. Female subjects already enrolled in the study must be terminated from the study if a positive pregnancy test result is obtained at any visit after screening.

7.8 Laboratory Assessments

Routine clinical laboratory parameters (hematology, chemistry, coagulation panel, and urinalysis) as well as the biomarkers BNP and troponin I will be analyzed by the local hospital laboratory.

Ang II/(1-7) ratios in peripheral blood will be measured for analysis. In Phase A only, the transpulmonary Ang II/(1-7) ratio will be measured. Ratio will be analyzed by Wake Forest or GSK.

Urine isoprostanes and isofuranes will be processed in the central lab.

All samples, other than routine labs (SOD2 activity, IDH activity), will be stored at -80˚C, batched and analyzed at the conclusion of each dose escalation.

Clinical laboratory tests include the following:

Hematology	white blood cell count with differential, red blood cell count, platelet count, hemoglobin, and hematocrit
Standard chemistry	
Substrates	albumin, total bilirubin, total protein, BUN, creatinine, glucose
Electrolytes	calcium, chloride, sodium, phosphorous, potassium
Enzymes	aspartate aminotransferase/glutamate oxalo-acetate transaminase (ASAT/GOT); alanine aminotransferase/glutamate pyruvate transaminase (ALAT/GPT); alkaline phosphatase (AP); creatine kinase (CK)
Coagulation panel	international normalized ratio (INR)/prothrombin time (PT), PTT
Biomarkers Array analysis	Collection of blood for RNA expression (PAXgene tube) and cultured lymphocytes (yellow top tube)
Ang II/Ang (1-7)	Blood samples for Angiotensin II/ Ang (1-7) ratios: Protease inhibitor cocktail at 0.05ml/1ml whole blood will be added to EDTA tubes. Two pink-top 6ml tubes will be collected at baseline and at 2 hours. The samples will be mixed and centrifuged at 3000g for 10 minutes. The plasma will be placed into labeled tubes and frozen at -80°C until shipment to Wake Forest Hypertension Laboratory or GSK.
SOD2 activity assay	Plasma activity assay
Serum, local lab	BNP, isoprostanes, isofurans, troponin I, inflammatory biomarkers (TGFb, IL2).
Urine, central lab	8-isoprostanes and isofuranes Two frozen urine samples of 10 mL <u>each</u> will be stored in a specially assigned freezer at -80^{o}C until shipment.
Standard urinalysis	appearance, color, pH, specific gravity, ketones, protein, glucose, bilirubin, nitrite, urobilinogen, sodium, pregnancy (where appropriate)
Immunogenicity studies Pharmacokinetic profile	To be analyzed by GSK.

7.9 Hemodynamic Assessments

Pulmonary arterial catheters for Phase A will only be placed in patients undergoing standard clinically-indicated right heart catheterization. A pulmonary artery catheter (PAC) will be placed under fluoroscopy in the VU Cath Lab using sterile technique. The PAC is inserted percutaneously into a major vein (jugular, subclavian, femoral) via an introducer sheath.

The measurements that will be assessed include: right atrial pressure (RAP), pulmonary arterial pressure (PAP), pulmonary wedge pressure (PWP), cardiac output (CO), cardiac index (CI), pulmonary vascular resistance (PVR), and pulmonary artery saturation (PAsat).

Hemodynamics measurements will be obtained at baseline in the cardiac catheterization lab has been performed for comparator values.

Blood will be drawn from the mixed venous position and from the wedge position to measure transpulmonary gradient of AngII/(1-7) ratio.

The patient will then be moved to the recovery area. Repeat baseline values will be obtained and rhACE2 will be administered over 30 minutes. Hemodynamic measurements will be repeated and at 30 minutes, 1, 2, 3, and 4 hours post dosing. Transpulmonary gradient blood will be collected at baseline prior to drug administration and at four hours after administration prior to removal of the catheter. The catheter will be removed prior to the subject leaving the cardiac catheterization lab for the CRC. The subject will then be monitored for 20 hours in the CRC.

Variables that will be recorded from the right heart catheterization include right atrial pressure, right ventricular systolic, diastolic and end diastolic pressures, pulmonary artery systolic and diastolic pressure, pulmonary wedge pressure, pulmonary arterial saturation, systemic systolic and diastolic blood pressure, heart rate, systemic arterial saturation measured by pulse oximetry, cardiac output and index measured by either thermodilution or assumed Fick.

7.10. Volume of Blood Collection

The approximate blood volume collected from each individual during the study will be as follows:

Phase A Assessment	**Number of Collections***	**mL/Collection**	**Total (mL)**
Chemistry	3 or 4	5	20ml
BNP	3 or 4	5	20 ml
Hematology	3 or 4	5	20ml
Coagulation	3 or 4	3	12ml
AngII/Ang 1-7 SOD2 Activity Serum nitric oxide Isoprostanes/Isofurans	6 5 5 5	105 2 (see SOD2)	6025 10
Pk	3	5	15
immunogenicity	2	5	10
Total blood volume:			≈192

*Number of collections depends upon whether samples are taken both at screening and baseline.

Phase B Assessment	**Number of Collections***	**ml/collection**	**Total (ml)**
Chemistry	6 or 7	5	35
BNP	6 or 7	5	35
Hematology	6 or 7	5	35
Coagulation	6 or 7	5	35
Serum nitric oxide	7 or 8	2	16
SOD2/Cytokines/ isoprostanes/isofurans	7 or 8 Cytokines (4)	5	40
AngII/Ang 1-7	7 or 8	10	80
RNA	1	5	5
Lymphocytes	1	8	8
pK	3	5	15
	Total blood volume		304

Number of collections depends upon whether samples are taken both at screening and baseline.

7.111. Prior and Concomitant Medications

Medications (prescription, over-the-counter, and herbal) and nutritional supplements taken during the 30 days prior to dosing and medication taken for the treatment of PAH during the 12 weeks prior to dosing will be reviewed and recorded at screening. At the first Baseline visit, current medications will be recorded. At each subsequent Treatment, or Early Discontinuation visit (if applicable), the change in medications since the previous visit will be recorded.

7.12 Screening and Baseline

7.12.1 Screening

Screening procedures (unless otherwise specified below) must be performed no more than 90 days prior to the first dose of study drug. Results from some screening procedures which are performed no more than 7 days prior to the first dose of study drug, as specified below and in Appendix 1 can also be used as baseline values. In Phase B, there must be a minimum of 10 days and a maximum of 18 days between screening/baseline visit and date of first study drug administration.

Prior to performing any study-related assessments, the Investigator will inform the patient, orally and in writing, about the potential benefit and any risks associated with the participation in this trial.

After having given their informed consent in writing, subjects will undergo the following screening evaluations:

- General medical and drug history, including recording of demographic data; concomitant diseases; review of medication(s) or investigational products taken or investigational devices used in the past 30 days and medications taken for the treatment of PAH in the 12 weeks prior to enrollment; and complaints/symptoms for baseline safety evaluation.
- Physical examination (including body weight and height)
- WHO classification
- BP (systemic BP is taken after 5 minutes seated and again after 5 minutes standing)
- Pulse oximetry and heart rate
- Pregnancy test (urine) in females of childbearing potential

- Blood samples taken for standard clinical laboratory tests (hematology, chemistry, and coagulation panel)
- Urinalysis
- If the subject has not had a 6MW test with documented results within three months (12 weeks) prior to screening, administer the 6MW test, followed by Borg dyspnea scoring. If the 6MW test and Borg dyspnea score is performed at screening, the results can also be used for baseline assessments and that test will not have to be repeated at baseline..
- Provide instructions to fast for 2 hours prior to the baseline visit and bring in first morning urine void on ice for urine assessment of biomarkers.

7.12.2 Baseline

The following baseline assessments must be performed prior to the first dose of study drug unless otherwise specified below.

- Change since screening in medical history or physical exam, including concomitant diseases; medication(s) or investigational products taken or investigational devices used; WHO classification; and complaints/symptoms for baseline safety evaluation.
- BP (systemic BP is taken after 5 minutes seated and again after 5 minutes standing)
- Pulse oximetry and heart rate
- Pregnancy test (urine) in females of childbearing potential*
- Urine and blood samples taken for standard clinical laboratory tests (hematology, chemistry, urinalysis, and coagulation panel*
- Urine (first morning void, on ice) and blood samples taken for biomarker assessments as outlined in Appendix 1 and 2
- ECG
- Echocardiogram
- Assessment and recording of any SAEs and study-related issues (if any) since signing of the Informed Consent Form

- Recording of current concomitant medications
- Instruct subjects to bring in first morning void to follow-up visit*** in two weeks.

*** Note:** Results obtained at screening for the indicated assessments may be counted as baseline values if assessment was performed no more than 7 days prior to the first dose of study drug.

Results of standard laboratory tests must be available and reviewed before start of treatment.

8.0 Study Treatment

rhACE2 will be administered intravenously at a slow infusion at a rate of 1ml/minute for approximately 30 minutes.
The individual amount of rhACE2 and corresponding volume will be calculated as:

- Volume rhACE2 (ml) = Body weight (kg) X dose (mg/kg)/ concentration (5.2 mg/ml)
- Add 30ml-calculated volume rhACE2 to physiological solution of NaCl to reach 30ml infusion solution.

rhACE2 is produced by Glaxo Smith Kline. The drug is stored in a controlled environment and will be shipped with a certificate of analysis. Temperature will be recorded until delivery to the clinical center. Shipment and long term storage at <-20 °C.

9.0 Risks

9.1 Risks of Study Drug:

It is possible that rhACE2 treatment alone, or when combined with other medications used to treat PAH, will lower systemic BP in pulmonary arterial hypertension patients. The first participants (initial single- dose subjects) will be admitted for study drug administration and monitored for 24 hours after drug administration. Subsequent multi-dose subjects will be admitted for the entire 6 day dosing course and will be monitored for 5 hours after their last dose of rhACE2.

9.2 Risks of Study Procedures:

9.2.1 Risks of Blood Draw: Pain, redness, soreness, bruising, or infection may occur at the needle stick site. Rarely some people faint.

9.2.2 Pulmonary arterial catheters will only be placed for clinical indications, thus there is no increased risk associated with this procedure compared to usual clinical care

9.2.3. Risks of echocardiography: some patients experience minor discomfort with the procedure for proper placement of the ultrasound probe.

10 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

10.1 Adverse Event Terminology

10.1.1 Adverse Event

An AE is "any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment". An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not considered related to the medicinal (investigational) product.

AEs include any of the following:

- Worsening (change in nature, severity or frequency) of conditions present at the onset of the trial
- Subject deterioration due to the primary illness
- Intercurrent illnesses
- Drug interactions
- Events related or possibly related to concomitant medications
- Abnormal laboratory values or changes of vital signs, as well as significant shifts from baseline within the range of normal, which the Investigator considers to be clinically significant

10.1.2 Adverse Drug Reaction

In the pre-approval clinical experience with a new medicinal product or its new usage, particularly as the therapeutic dose(s) may not be established, an adverse drug reaction is defined as:

All noxious and unintended responses to a medicinal product related to any dose should be considered adverse drug reactions (ADR).

10.1.2.1 Unexpected Adverse Drug Reaction

An unexpected ADR is:

An adverse drug reaction, the nature or severity of which is not consistent with the applicable product information, also known as reference safety information. For rhACE2, an investigational medicinal product, the reference safety information is the Investigator's Brochure**.

10.1.3 Serious Adverse Event/Serious Adverse Drug Experience

During clinical investigations, serious AEs may occur. If the event is suspected to be drug-related, the event may be significant enough to lead to important changes in the way the medicinal product is developed (e.g. change in dose, population, needed monitoring, consent forms). This is particularly true for reactions, which, in their most severe forms, threaten life or function.

A serious AE (SAE) or serious adverse drug experience (SADE) is any untoward medical occurrence that:

- Results in death.
- Is life-threatening. 'Life-threatening" refers to an event in which the subject is at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe (ICH E6).
- Requires inpatient hospitalization or prolongation of existing hospitalization.
- Results in persistent or significant disability/ incapacity (as per reporter's opinion).
- Results in a congenital anomaly/birth defect.
- Causes another medically important condition. Important medical conditions that may not result in death, be life-threatening or require hospitalization may be considered as SAEs or SADEs when, based upon appropriate medical judgment, they may jeopardize the subject or may require intervention to prevent one of the outcomes listed in the definition above. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of

drug dependency or drug abuse [Code of Federal Regulations Title 21, Volume 5, 21CFR312.32, revised April 1, 2006].

Please note: Serious is not synonymous with severe. An event may be severe (e.g., severe headache) but still be of minor medical significance. Serious refers to an event that poses a threat to the subject's life or functioning.

10.1.4 Assigning Severity to an Adverse Event

Mild: Causing no limitation of usual activities;
the subject may experience slight discomfort.

Moderate: Causing some limitation of usual activities;
the subject may experience annoying discomfort.

Severe: Causing inability to carry out usual activities;
the subject may experience intolerable discomfort or pain.

10.1.5 Assigning Relationship of Adverse Event to Study Drug (Causality)

The Investigator will determine the relationship of each AE to study drug (i.e., causality) by using the classification criteria 'not related', 'possibly related', or 'probably related'. Descriptions of the three classification categories are as follows:

10.1.6 Not Related

Exposure to study drug has not occurred; administration of study drug and the adverse event are not reasonably related in time; or the AE is considered by the Investigator to be due to a pre-existing condition, a known manifestation of the target disease, a recurrent condition, or is likely explained by environmental or diagnostic therapeutic factors or was pre-existing and did not deteriorate.

10.1.7 Possibly Related

The AE occurred during or within a reasonable period of time after administration of the study drug, or a pre-existing event worsened within an appropriate period of time after administration of study drug, but the AE could be explained equally well by factors or causes other than exposure to the study drug.

This category will also be used if there is a lack of information, or insufficient or conflicting evidence exists for classifying the causality of the AE.

10.1.8 Probably Related

The AE occurred during or within a reasonable period of time after administration of the study drug or a pre-existing event worsened within an appropriate period of time after administration of study drug, and at least one of the following criteria is applicable:

- the event could not be explained by the clinical condition or history of the subject, environmental or toxic factors, or other diagnostic or therapeutic measures;
- the event was an expected ADR associated with study treatment or a class-labeled drug effect;
- the AE subsided or disappeared after withdrawal or dose reduction of study treatment; or
- the AE recurred after re-exposure to study treatment.

10.2 Adverse Event Recording and Reporting

10.2.1 Adverse Event Recording

At baseline, all SAEs and study-related AEs since signing of the Informed Consent Form will be recorded. After initial study drug administration, all AEs will be recorded.

Each AE occurring to a subject, either spontaneously revealed by the subject or observed by the Investigator, whether believed by the Investigator to be related or unrelated to the study drug, must be recorded on the AE Case Report Form and on the subject's file.

Type and severity of AEs will be reported by the subjects without being given a list of fixed symptoms beforehand.

The Investigator will also determine the relationship of any AE to study drug (causality) and record it on the appropriate section of the AE CRF as well as their severity, time of onset, duration, and the precautions carried out, and whether or not the event meets one or more of the definitions of an SAE.

Laboratory results will be recorded on the case report form and the investigator will indicate whether abnormal results (high or low) are clinical or not clinically significant. Clinically significant laboratory abnormalities will be entered on the 'Adverse Event Form' of the CRF. Clinically significant changes in vital signs (e.g., tachycardia) or other clinically significant changes observed by the physician will be entered in the appropriate CRF. These AEs will also be noted on the 'Adverse Event Form' of the CRF as well as in the subject file.

10.2.2 Serious Adverse Event Reporting

All SAEs must be reported promptly to both investigators and to Glaxo Smith Kline. SAEs should be followed to resolution, even if this is after the study reporting period.

10.3 Data and Safety Monitoring

The Data and Safety Monitor (DSM) is an independent individual that will act in an advisory capacity and will monitor subject safety and the efficacy of rhACE2 in subjects that participate in this study. The roles and responsibilities of the DSM will be outlined in a separate charter agreed to by the DSM. The DSM has the following responsibilities:

- Review the study protocol, informed consent form, and plans for collecting safety data and monitoring subject safety.
- Evaluate the progress of the trial; the quality and timeliness of study data collected; subject recruitment, accrual, and retention; subjects' risk versus benefit; and other factors that could affect the study outcome.
- Consider relevant information that may have an impact on the safety of the subjects or the ethics of the study.
- Review CRFs after completion of the dose-escalation cohort and determine multiple dose dosage.
- Protect the safety of the study subjects in accordance with the study protocol.
- Make recommendations to the Investigators concerning continuation, termination, or other modifications of the study based on their observations of the study.
- If needed, conduct an interim analysis of safety.
- Review all SAEs.

11 Study Withdrawal/Discontinuation

At any time, subjects may withdraw from the study (i.e., withdraw consent to participate) at their own request. A subject's participation in the study may be discontinued at any time at the discretion of the Investigator and in accordance with his/her clinical judgment. No disadvantage will arise for any subject who withdraws consent for participation at any time or who is withdrawn from the study by the Investigator.

Reasons for discontinuation of study treatment will be recorded on the appropriate page of the CRF in any case and may include the following:

- Subject's request for withdrawal
- Investigator's decision that discontinuation is in the best interest of the subject
- Non-compliance with the regimen and timing that might result in dropping out from the study
- Development of an intolerable AE due to study participation as determined by the Investigator, subject, or both.
- Development of an intercurrent illness, condition, or procedural complication, which would interfere with the subject's continued participation.
- Subject is lost to follow-up

Subjects who discontinue treatment will be asked to complete a follow-up examination prior to leaving the study, if possible. Any subject who discontinues treatment for medical reasons, e.g. because of adverse events (AEs) or clinical laboratory abnormalities, should be followed up at medically appropriate intervals in order to evaluate the course and to ensure reversibility or stabilization of the abnormality or event. If a subject fails to return for a scheduled visit, a documented effort must be made to determine the reason. This information should be recorded in the study records.

12 Statistical Considerations

Descriptive statistics will be used to characterize patients at study entry. Dose analysis will be approached using the design below to determine the dose limiting toxicity (DLT). No statistics will be performed specifically on tolerability data. Instead, AEs will be described in their entirety and evaluated by descriptive statistical methods. Despite sample size limitations that hamper statistical inference, analysis of secondary endpoints will be conducted using mixed-effects regression modeling techniques, supported by conditional estimation methods with interaction. For example, the change in pulmonary and systemic hemodynamic measurements according to echocardiogram from baseline to end of study, the change in six minute walk distance (6MWD) from baseline to end of study, and the change in biochemical variables (BNP, NO and SOD2) from baseline to Day 6 and end of study will be assessed to determine the delta in paired comparisons over time.

13 Privacy/Confidentiality Issues

The Principal investigator will collect data and enter it into password protected computer in a locked office. Each patient will have a unique identifier number, with the key to the patient's medical record number kept in a locked cabinet in the office. Only research associates or those individuals directly involved with the study will have access to data. Information is for research purposes only and when used for publication purposes, all participants will have their names concealed. Access to identified patient information will be limited to the investigators listed within the IRB application. De-identified information with HIPAA identifiers removed will be available to other investigators following IRB approval. Confidentiality and security will be maintained for the database. The database is stored behind a firewall (in addition to the institutional firewall) with the highest level of protection, i.e. the same level of protection as the on-line hospital information system at Vanderbilt. This means that users must logon to a web server that sits between the institutional firewall and the firewall to the database, and only this application server is allowed to query the database. Only users approved through our institutional review board will be allowed access to patient identifiers. Other levels of authorization may exist for future approved users following IRB approval, e.g. access to de-identified data.
Data is initially collected in the medical record for each individual study participant. The information will be extracted from the patient's medical record and then transferred into the Case Report Form (CRF). The study data will be kept on site and in a securely locked room to protect patient confidentiality.
The CRFs do not include personal identifiers for any participant. Numbers and initials are assigned for each participant and these become the identifying information for each study participant. A master list is kept separately that identifies which names go with which numbers and initials.
Study personnel (PI and co-investigators) and government regulatory agencies have access to all research records as required by law. Others (such as law enforcement agencies) may have access to records as defined by law.

14 Follow-up and Record Retention

The PI must retain all study records by the applicable regulations in a secure and safe facility. The institution must consult with the PI before disposal of any study records, and must notify the PI of any change in the location, disposition or custody of the study files. The PI/institution must take measures to prevent accidental or premature destruction of

essential documents, that is, documents that individually and collectively permit evaluation of the conduct of a study and the quality of the data produced, including paper copies of study records (e.g., subject charts) as well as any original source documents that are electronic as required by applicable regulatory requirements.

All study records must be retained for at least 2 years after the last approval of a marketing application in the U.S. or an ICH region and until (1) there are no pending or contemplated marketing applications in the U.S. or an ICH region or (2) at least 2 years have elapsed since the formal discontinuation of clinical development of the IP. The PI/institution should retain subject identifiers for at least 15 years after the completion or discontinuation of the study. Subject files and other source data must be kept for the maximum period of time permitted by the hospital, institution or private practice, but not less than 15 years. These documents should be retained for a longer period, however, if required by the applicable regulatory requirements. PI must be notified and will assist with retention should institution be unable to continue maintenance of subject files for the full 15 years. It is the responsibility of PI to inform the institution as to when these documents no longer need to be retained.

If an Investigator moves, withdraws from an investigation, retires, requests to move records to another location or to assign these records to another party or (e.g. other Investigator) who will accept the responsibility, written notice of this transfer must be made to and agreed upon by each party.

The originals of the protocol, and the Medication Accountability List will be archived by the Investigator.

Appendix 1

Modified NYHA (WHO) Classification:

Functional assessment of PAH will be made according to the modified New York Heart Association (NYHA) (WHO) classification system (World Symposium on Primary Pulmonary Hypertension, 1998, Evian, France, sponsored by the World Health Organization).

Class I: Patients with PAH without limitation of physical activity. Ordinary physical activity does not cause increased dyspnea or fatigue, chest pain, or near syncope.

Class II: Patients with PAH resulting in slight limitation of physical activity. No discomfort at rest. Normal physical activity causes increased dyspnea or fatigue, chest pain, or near syncope.

Class III: Patients with PAH resulting in marked limitation of physical activity. There is no discomfort at rest. Less than ordinary activity causes increased dyspnea or fatigue, chest pain, or near syncope.

Class IV: Patients with PAH with inability to carry out any physical activity without discomfort. Indications of manifest right heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by the least physical activity.

Appendix 2: Single Dose Schedule of Events

Assessment	Screening[a]	Baseline[a]	0	1 hr	2 hr	4 hr	6 hr	12 hr	24 hr	2 weeks
Informed consent	X									
Medical history	X*	X*								
Concomitant Medications	X**	X**								X
Physical examination	X*[b]	X*							X	X
Functional classification	X*	X*								X
6MWD	X*	X*								X
Blood pressure [c]	X	X	X	X	X	X		X	X	X
Pulse oximetry (HR, SO_2)[d]	X	X	X	X	X	X		X	X	X
Pregnancy test (urine)[e]		X								
Chemistry tests, troponin I	E (7 days)	E (7 days)							X	X
Hematology, BNP	E (7 days)	E (7 days)							X	X
Coagulation Panel	E (7 days)	E (7 days)							X	X
Serum Nitric Oxide			X		X	X			X	X
Plasma for SOD2 activity/isoprostanes/ isofurans			X		X	X			X	X
Pk measurements[i]			X				X		X	
Immunogenicity Studies			X							X
Peripheral Ang II/Ang 1-7			X		X	X			X	
Transpulmonary Ang II/Ang 1-7			X			X				
Urinalysis	E (7 days)	E (7 days)							X	
Urine isoprostane and isofurane[f]			X			X			X	X
ECG		X				X			X	
Administration study drug			X							
Hemodynamic measurements [g]			X	X	X	X				
Adverse events [h]		X	X	X	X	X		X	X	X
Transthoracic		X							X	

echocardiography										

Screening results must be available and baseline procedures performed before the first dose of study drug is administered.

To avoid repeat tests, some screening values may be counted as baseline values if obtained within specified time window.

E (7 days): Can be performed at either visit, but at least one test must be performed no more than 7 days before study drug is administered. Results from this test must meet eligibility requirements and must be available before first study drug administration.

* Data will be recorded at screening and eligibility will be evaluated; at baseline, data will be reviewed and any changes since screening will be recorded and eligibility will be re-evaluated (as necessary).

** Concomitant medications for the 30 days prior (8 weeks prior for PAH medications) will be reviewed/recorded at screening to evaluate eligibility and changes will be recorded throughout the study.

Procedures are marked in table as follows:

[a] To facilitate scheduling, some screening procedures may occur up to 90 days prior to the first dose of study drug. Baseline procedures must be performed no more than 7 days prior to study drug.

[b] Physical examination includes weight and height measurement at screening.

[c] Measuring BP and Pulse Oximetry

BP will be measured after 5 minutes seated; and after dose administration through the 4 hour timepoint and sitting at the following timepoints.

[d] Pulse oximetry and heart rate will be measured and recorded with each blood pressure assessment.

[e] In woman of childbearing potential only.

[f] First morning void, brought from home to study visit on ice, for urinary biomarkers. [g] Includes RAP, PWP, CO, CI, and PAsat

[h] At baseline, all SAEs and study-related AEs since signing of the Informed Consent Form will be recorded.

[I] PK measurements are to be taken at the following times: at the completion of the first infusion, six hours and 24 hours after the dose