Testing of HIV Protease Inhibitors to suppress inflammation and improve cardio pulmonary hemodynamics in subjects with pulmonary arterial hypertension

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PROTOCOL SYNOPSIS

Protocol Title:	Testing of HIV Protease Inhibitors to suppress inflammation and improve cardio pulmonary hemodynamics in subjects with idiopathic pulmonary arterial hypertension(IPAH)
Version # and Date:	February 01st 2014
Clinical Phasetype:	An exploratory clinical study
Investigational Drug:	Saquinavir plus Ritonavir
Trial Site:	Single-Center Trial
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Manufacturer:	Roche, Abbott Laboratories
Study Rationale:	There is recent evidence that HIV protease inhibitors (HIV-PI) can improve pulmonary hemodynamics in experimental models of pulmonary arterial hypertension (PAH). There is also experimental evidence that both TLR4 and high mobility group box 1 (HMGB1) participate in the pathogenesis of experimental pulmonary hypertension. A recent high throughput screen for inhibitors of HMGB1 induced macrophage activation yielded HIV-protease inhibitors (PIs) as potent inhibitors of HMGB1 induced cytokine production. Based on the experimental evidence

we propose a trial to determine whether HIV-PIs will alter the pathobiology of PAH.

Study Objectives:

The main objective of this study is to determine whether saquinavir and ritonavir (SQV+RIT) which have a well-characterized safety profile in humans will reduce bio markers of inflammation and pulmonary artery pressures in patients with IPAH.

Study Hypothesis:

- We hypothesize that the HIV-PI, SQV+RIT, will reduce circulating parameters of inflammation including HMGB1, IL1-beta, IL-6, TNF-alpha and CRP. Our end points will be changes in these parameters from baseline over the duration of the study.
- We hypothesize that treatment with SQV+RIT will reduce pulmonary artery (PA) pressure of patients with PAH as measured by echocardiography.
- We hypothesize that treatment with SQV+RIT will improve NYHA/WHO functional class and Brog dyspnea scale index from baseline at 14 days.

Study Aims:

- To determine if short-term use of SQV+RIT reduces parameters of chronic inflammation and PA pressure of IPAH based on echocardiographic parameters.
- To determine if short-term use of SQV+RIT improve the symptoms of IPAH patients.
- Safety issue also evaluated at the same time.

Study Design:

This is a single center open label exploratory study to evaluate the effect of SQV +RIT in patients with IPAH. Subjects with IPAH (N=20) will be enrolled into a study, which will be divided into 3 cohorts and entail the administration of HIV protease inhibitors in three doses. The first cohort (n=3) will receive a starting dose of SQV 0.3 mg/kg daily in combination with RIT 0.03 mg/kg daily. If the first dose is well-tolerated, the second cohort (n= 3) with IPAH will be given doses of SQV 3 mg/kg and RIT 0.3 mg/kg daily. If the second dose is well-tolerated, the last cohort (n= 14) with IPAH will be given doses of SQV 15 mg/kg and RIT 1.5 mg/kg daily.

Data from standard diagnostic approaches will be obtained

to confirm that the patient has PAH. Patients currently on oral background PAH therapy will be instructed to continue to take the regular regiment throughout the study with the possible need to modify doses of PDE5 inhibitors and endothelin receptor antagonists. Baseline markers of inflammation known to be associated with IPAH including HMGB1, TNF-alpha, IL-6, -1 beta, as well as C-reactive protein will be obtained at baseline. NT-Pro BNP will be measured as a biomarker of increased pulmonary arterial pressures. Echocardiography will be measured at baseline and day 14. Subjects will be tested and estimated for the changes in PA pressure with treatment. NYHA/WHO functional class and Brog dyspnea scale index will be recorded.

Subjects will receive the SQV+RIT for up to 2 weeks and blood draws will take place at baseline x 2 (Day -1,Day 0), day 1, 13, 14 and 28.

Patients will be monitored carefully for occurrence of adverse events; laboratory tests abnormalities and changes in their clinical condition. Adverse experiences will be evaluated according to the criteria outlined in the NCI common terminology criteria for adverse events (CTCAE) version 4.0.

Planned Sample Size: Male and female patients with IPAH (N = 20)

Duration of Treatment: 2 weeks

Inclusion Criteria:

- Age: 18-60
- Idiopathic pulmonary arterial hypertension (IPAH)
- Personally signed and dated informed consent document indicating that the subject (or a legally acceptable representative) has been informed of all pertinent aspects of the study
- Subject willing and able to comply with scheduled visits, treatment plans, laboratory tests, and other study procedures
- Diagnosis of PH confirmed by cardiac catheterization: mean pulmonary artery pressure (mPAP) $\geqslant$ 25 mm Hg (at rest), pulmonary capillary wedge pressure equal or less than 15mmHg, and normal or reduced cardiac output
- Stable PAH therapy for at least three months

Exclusion Criteria:

- Baseline systemic hypotension, defined as MAP less than 50 mmHg
- Requires intravenous inotropes within 30 days prior to study participation
- Has uncontrolled systemic hypertension as evidenced by sitting systolic blood pressure >160 mm Hg or sitting diastolic blood pressure >100 mm Hg at screening
- History of portal hypertension or chronic liver disease, including cirrhosis, chronic alcoholism, hepatitis B and/or hepatitis C (with evidence of recent infection and/or active virus replication) defined as moderate to severe hepatic impairment (Child-Pugh Class B-C)
- Chronic renal insufficiency as defined by serum creatinine >2.5 mg/dL at screening or requires dialysis support
- Hemoglobin concentration <9 g/dL at Screening
- History of atrial septostomy
- Repaired or unrepaired congenital heart disease
- Pericardial constriction
- Restrictive or congestive cardiomyopathy
- Left ventricular ejection fraction 40% as shown by multiple gated acquisition scan (MUGA), angiography, or echocardiography
- Symptomatic coronary disease with demonstrable ischemia
- Other severe acute or chronic medical or laboratory abnormality that may increase the risk associated with study participation or investigational product administration or may interfere with the interpretation of study results and, in the judgment of the investigator, would make the subject inappropriate for entry into this study
- Psychiatric, addictive, or other disorder that compromises the ability to give informed consent for participating in this study. This includes recent history of alcohol or illicit drug abuse 30 days prior to study screening (day 1) and for the duration of the study
- Poorly-controlled asthma, defined by active wheezing and/or cough with FEV1 < 70% predicted, responsive to inhaled BD (>15% increase in FEV1 with BD)
- Clinically significant intercurrent illness (including lower respiratory tract infection) or clinically significant

surgery within four weeks before the administration of study drug
- History of hypersensitivity or idiosyncratic reaction to drugs from multiple drug classes
- Receipt of an investigational product or device, or participation in a drug research study within a period of 15 days (or five half-lives of the drug, whichever is longer) before the first dose of study drug
- Blood loss or blood donation >550mL within 90 days or plasma donation >500 mL within 14 days before administration of study drug
- Patients with a QTc interval > 450 msec
- Diabetes mellitus as defined by symptoms of hyperglycemia and serum fasting plasma glucose level≥7.0mmol/l or casual plasma glucose≥11.1mmol/l at screen
- Hyperlipidemia as TC≥6.22 mmol/L, LDL-C≥4.14 mmol/L or TG≥2.26 mmol/L
- History of Crohn's disease, ulcerative colitis, inflammatory bowel disease, etc.
- Unwilling to take contraceptive measures during the study
- Use of certain other medications will need to be evaluated for possible exclusion based on the potential for adverse drug interactions

Study Endpoints:

Primary endpoint:
The primary measure of efficacy will be change in HMGB1 level from baseline at 14 days, measured using an ELISA kit.

Secondary endpoints:
- The changes in the parameters of TNF-α, IL-1β, IL-6, N-terminal pro-brain natriuretic peptide (NT-proBNP), and C-reactive protein (CRP), across the 2 weeks treatment.
- The changes in PA pressure and total right heart function measured by echocardiography from baseline to 14 days.
- NYHA/WHO functional class and Brog dyspnea scale index from baseline to 14 days.

The safety endpoints:

- Vital signs, including blood pressure
- Change in physical examination
- Urinalysis
- CBC
- Reticulocyte count
- Peripheral oxygen saturation by pulse oximetry
- AE monitoring
- ECG

Monitoring for potential Drug interaction

The following medicines may increase blood levels and side effects of SQV+RIT

- delavirdine (RESCRIPTOR®)
- atazanavir (REYATAZ®)
- omeprazole (PRILOSEC®)
- clarithromycin (BIAXIN®)
- indinavir (CRIXIVAN®)

SQV+RIT may not work as well when taken together with the following medicines, herbal products, or dietary supplements:

- efavirenz (SUSTIVA®)
- nevirapine (VIRAMUNE®)
- Anticonvulsants such as carbamazepine (CARBATROL®, TEGRETOL®), phenobarbital, and phenytoin (DILANTIN®)
- dexamethasone
- Garlic capsules, an herbal product sold as a dietary supplement
- the herbal supplement St. John's wort (Hypericum perforatum) or products containing St. John's wort

More close monitoring may be needed if HIV-PIs are taken with the following medicines:

- medicines for erectile problems, such as tadalafil (CIALIS®), vardenafil (LEVITRA®), or sildenafil citrate (VIAGRA®)
- Antidepressants such as trazodone (DESYREL®), (amitriptyline (ELAVIL®), or imipramine (TORFRANIL®)
- Benzodiazepines used as sedatives or sleeping pills such as alprazolam (XANAX®), clorazepate (TRANXENE®), diazepam (VALIUM®), and flurazepam (DALMANE®)

- atorvastatin (LIPITOR®) used for lowering cholesterol
- Calcium channel blockers used for treatment of high blood pressure or heart disease, such as diltiazem (CARDIZEM®, CARTIA XT®, DILACOR XR®, DILTZAC®, TAZTIA XT®, TIAZAC®), felodipine (PLENDIL®), nifedipine (PROCARDIA®), nicardipine (CARDENE®), nimodipine (NIMOTOP®), verapamil-containing medications (such as CALAN®, VERELAN®), amlodipine-containing medications (such as CADUET®, NORVASC®), nisoldipine (SULAR®), and isradipine (DYNACIRC®)
- ketoconazole (NIZORAL®) and itraconazole (SPORANOX®) used to treat fungal infections
- Medicines to prevent organ transplant rejection: cyclosporine SANDIMMUNE®), cyclosporine (NEORAL®), sirolimus (RAPAMUNE®), or tacrolimus (PROGRAF®)
- fluticasone propionate (FLONASE®, FLOVENT®, ADVAIR®), given by nose or inhaled to treat allergic symptoms or asthma
- digoxin (LANOXIN®) used to treat of heart rhythm problems or other heart conditions
- bosentan (TRACLEER®) and tadalafil (ADCIRCA®) used to treat pulmonary arterial hypertension
- medicines for gout, such as colchicine (COLCRYS®)
- Oral contraceptives containing ethinyl estradiol used for preventing pregnancy
- Methadone
- rifabutin (MYCOBUTIN®)

1. OBJECTIVE, SPECIFIC AIMS, BACKGROUND, AND SIGNIFICANCE

1.1 OBJECTIVE

The main objective of this study is to determine whether SQV+RIT which have a well-characterized safety profile in humans will reduce bio markers of inflammation and pulmonary artery pressures in patients with IPAH.

1.2 SPECIFIC AIMS

Hypothesis:

- We hypothesize that the HIV-PI, SQV+RIT, will reduce circulating parameters of inflammation including HMGB1, IL1-beta, IL-6, TNF-alpha and CRP. Our end points will be changes in these parameters from baseline over the duration of the study.
- We hypothesize that treatment with SQV+RIT will reduce pulmonary artery (PA) pressure of patients with PAH as measured by echocardiography.

Specific Aims:

- To determine if SQV+RIT reduces parameters of chronic inflammation in patients with IPAH.
- To assess if short-term use of SQV+RIT reduces PA pressure of IPAH based on echocardiographic parameters.

1.3 BACKGROUND

1.3.1. Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a relatively rare and devastating illness characterized by high morbidity and mortality rates[2]. The 1993 American College of Chest Physicians consensus statement reports that the incidence ranges from one to two cases per million[3]. The median age at diagnosis is 36. In the NIH registry, the diagnosis of pulmonary hypertension required a mean pulmonary artery pressure greater than 25 mm Hg at rest or greater than 30 mm Hg with exercise. PAH may occur in either sporadic or familial forms. Familial PPH is inherited as an autosomal dominant disorder with incomplete penetrance, and has been mapped to a locus designated PPH1 on chromosome 2q33 that produces loss of function mutations in Bone morphogenetic protein type II receptor (BMPR2)[4].Identified defects of this receptor are predicted to disrupt ligand binding, kinase activity, and heteromeric dimer formation – cellular processes that may ultimately be important in the maintenance of blood vessel integrity.

Familial and sporadic PAH appear to be phenotypically identical. The median survival for all subjects in the NIH registry was 2.8 years; the one, three, and five year survivals were 68%, 48%, and 34%, respectively.[2] With the advent of epoprostenol therapy, the one, three, and five year survivals for PAH subjects have improved to 87%, 63%, and 54%, respectively.[5] Despite these improvements in

outcome, mortality remains unacceptably high. The lack of a routine screening test for PAH, and the fact that early symptoms are nonspecific ensures that subjects typically present with advanced disease.

1.3.2 Pulmonary Arterial Hypertension as an inflammatory disease

Pulmonary hypertension is characterized by high pulmonary artery pressure and a high resistance to blood flow. The mechanisms underlying idiopathic PAH (IPAH) are still poorly understood, but inflammation and immunity alteration are increasingly recognized features of pulmonary arterial hypertension (PAH) as suggested by infiltration of inflammatory cells, including macrophages and T and B lymphocytes, and dendritic cells in pulmonary perivascular spaces and around the plexiform lesions in PAH[6, 7]. In addition, increased cytokine and growth factor expression in remodeled pulmonary vessels, and the presence of circulating chemokines, cytokines and autoantibodies[8-14]. As a cascade of pathological vascular events occur and inflammatory continuous which eventually lead to vasoconstriction and vascular remodeling. Preclinical trials targeting specific inflammatory pathways have shown promising results in animal models[15-17].

The Toll like receptors (TLRs) play a key role in innate immune responses by initiating specific anti-bacterial and anti-viral defenses in recognition of signature molecular motifs on the surface of invading pathogens. Some TLRs (e.g. TLR4) can also be activated by endogenous molecules (e.g. HMGB1) released by stressed or damaged tissue. Ligation of TLRs initiates signaling cascades that result in the expression of inflammatory mediators.[18] There are experimental evidences that both TLR4[19, 20] and one of its ligands, high mobility group box 1 (HMGB1) [14], participate in the pathogenesis of experimental pulmonary hypertension. Bauer, et al have shown that neutralizing HMGB1 or deleting TLR4 both protect in model of experimental PAH. These authors also demonstrated increased HMGB1 expression in pulmonary arteries from patients with PAH(Figure 1A). Our preliminary data also revealed that elevated HMGB1 level in blood stream from PAH(Figure 1B).

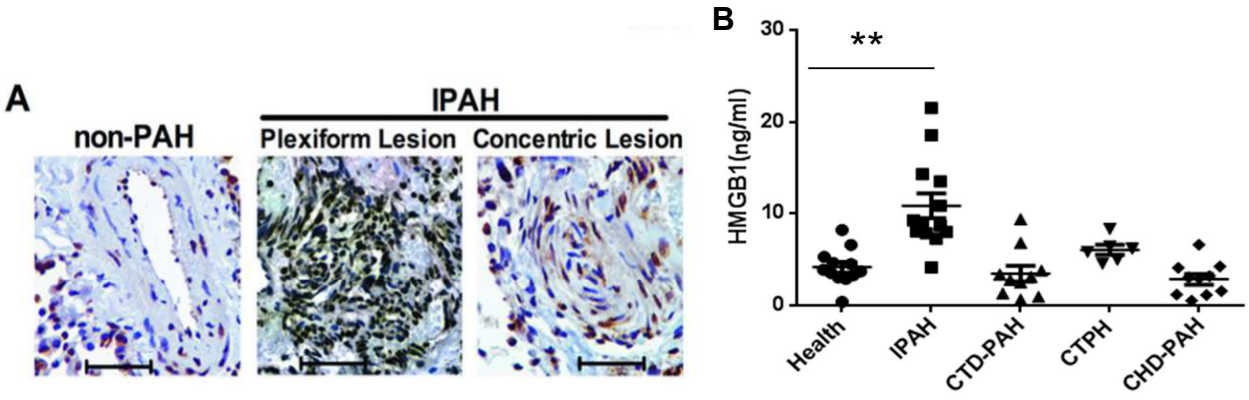


Figure 1: Extranuclear HMGB1 in Human. (A) Localization of HMGB1 in lung sections of patients with and without IPAH by immunohistochemistry (Brown=HMGB1, Blue=Hematoxylin, scale bar=50μm). (B) Expression of HMGB1 in serum of patients with different type of PAH. CTD-PAH: Connective tissue disease-PAH, CTPH: Chronic Thromboembolic Pulmonary Hypertension; CHD-PAH: Congenital heart disease-PAH. **: $P<0.01$.

HMGB1 is passively released during cell injury and necrosis, or actively secreted during immune cell activation and cell stress, positioning it at the intersection of sterile and infection-associated

inflammation. HMGB1 shows inflammatory cytokine activity by binding to TLR4 on macrophages and stimulating TNF-α and other cytokine release.[21] TNF-a is a key cytokine that is largely produced by activated macrophages but, importantly, is also released by vascular smooth muscle cells[22] and endothelial cells[23]. In clinical studies of patients with PAH, serum levels of TNF-a and other pro-inflammatory cytokines IL-1b, IL-2, IL-4 IL-6, IL-8, IL-10 and IL-12p70 are elevated as compared to healthy controls and these cytokine level had a significant impact on survival [1]. Inflammatory processes appear to play an important role in the vascular remodeling characteristic of PAH and TLR4 signaling pathway might be important target for PAH therapy. Therefore, a therapy that blocks TLR4 signaling could reduce the inflammatory response during PAH and improve the clinical course of PAH patients.

1.3.3 Investigational Drug

There is recent evidence that HIV protease inhibitors (HIV-PI) can improve pulmonary hemodynamics in experimental models of pulmonary arterial hypertension (PAH)[1]. A recent high throughput screen for inhibitors of HMGB1-induced macrophage activation yielded first generation HIV-protease inhibitors (PIs) as potent inhibitors of HMGB1 induced cytokine production. The most potent inhibitor of macrophage activation via TLR4 is saquinavir (SQV) (Figure 2).

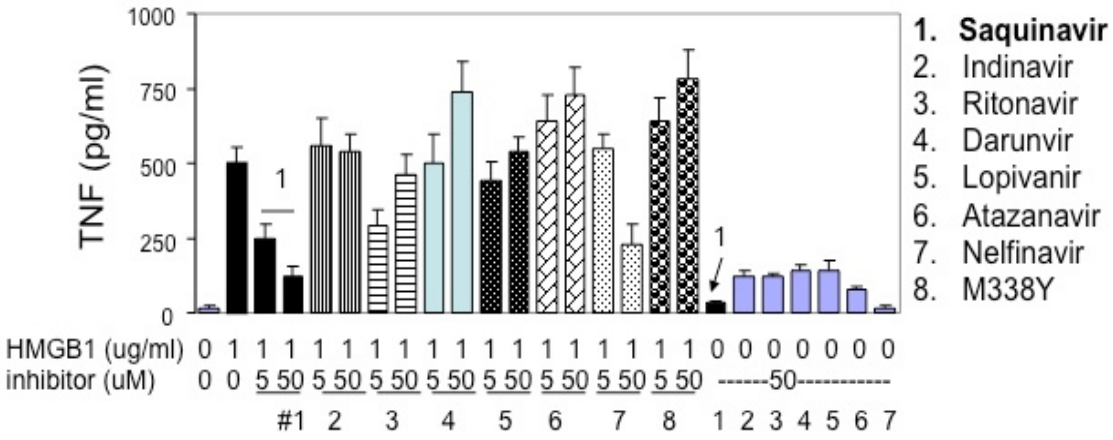


Figure 2: SQV dose-dependently inhibits HMGB1-induced TNF release from human macrophages

SQV was the first HIV-PI approved by the Food and Drug Administration (FDA). SQV has been shown to be safe even when give chronically in humans. SQV is typically administered with ritonavir (RIT) which increases the bioavailability of SQV[24, 25]. The FDA approved dosage of SQV+ RIT for HIV infection is SQV1000-mg twice daily (5 x 200-mg capsules or 2 x 500-mg tablets) in combination with RIT 100-mg twice daily. RIT should be taken at the same time as SQV and within 2 hours after a meal. Abundant of clinical studies have demonstrated this regimen was well tolerated and safe in HIV-infected participants[24-28].

SQV acts primarily as peptidomimetic inhibiting the HIV protease. However, it is now clear that SQV inhibits some mammalian protease. SQV has been shown to inhibit activity of matrix metalloproteinases 2 (MMP2), 20S and 26S proteasome, PI3K/Akt signaling, NF**k**B and TNF-s[29-

34]. These drugs are commonly used for prolonged periods without severe side effects in HIV seropositive subjects of any age. Recently, a six month course of SQV was shown to improve steroid resistant nephritic syndrome in humans[32]. Based on the experimental evidence we propose a trial to determine whether HIV-PIs(SQV+ RIT) will alter the pathobiology of PAH. If successful, this approved and safe drug combination could be used for a new purpose to treat a devastating disease.

2. STUDY DESIGN AND METHORD

2.1 CLASSIFICATION AND METHODOLOGICAL DESIGN

The main objective of this study is to determine whether SQV+RIT which have a well-characterized safety profile in humans will reduce biomarkers of inflammation and pulmonary artery pressures in patients with PAH.

2.2 STUDY DESIGN AND METHODS

2.2.1. Study Design

Patients with idiopathic pulmonary arterial hypertension(IPAH) (N=20) with a confirmed diagnosis of pulmonary hypertension and meeting all inclusion/exclusion criteria will be enrolled in the study, which will be divided into 3 cohorts and entail the administration of HIV protease inhibitors in three doses. Patients will be involved into 3 cohorts and receive the SQV+ RIT in micro (SQV 0.3mg/kg + RIT 0.03mg/kg), low (SQV 3mg/kg + RIT 0. 3mg/kg) and standard doses (SQV 15mg/kg + RIT 1.5mg/kg) twice daily respectively. Trials will continue only when the does is well–tolerated.

Recruitment:

Potential participants will be identified and screened based on the clinical data collected during their routine outpatient appointment at Xiangya hostipal. Potential subjects will be first identified by the physician investigator who is also the primary care or treating physician. The physician investigator who already has knowledge of and access to subjects' information will review the subjects' records to identify potential research subjects for the study. After identifying potentially eligible subjects, the physician investigator will then approach these subjects to discuss the research opportunity.

20 Patients who are eligible to participate in the study will sign the informed consent according to the sequence of appointment time. The first three patients will entry into the micro dose group and next 3 patients will entry into the low dose group. After 1 month wash-out period, those 6 patients may entry into the standard dose group under their willing will.

Screening :

The potential study subjects are followed on a routine basis in the Department of Cardiology of Xiangya Hospital and are well known to the study investigator. Initial screening evaluations including physical examination, electrocardiograph(ECG) for detection of abnormal heart rhythms, echocardiography(Echo) with documentation of tricuspid regurgitant velocity (TRV) for estimation of pulmnory artery pressures and right heart function, medical and clinical laboratory assessments will be

conducted to determine study eligibilities during a routine clinic visit at Department of Cardiology in Xiangya Hospital. Subjects who meet the inclusion criteria and none of the exclusion criteria will be entered into the study.

Experimental Procedures :

The study visit will occur on two days before subjects are scheduled for SQV+RIT treatment. Subjects will be instructed to take their normal PAH therapy (if applicable) during the study. However, in standard dose group, if administered concomitantly with sildenafil should reduce doses of 25 mg every 48 hours with increased monitoring of adverse events. If administered concomitantly with bosentan should discontinue use at least 36 hours prior to initiation of SQV+RIT. After at least 10 days following the initiation of SQV+RIT, resume bosentan at 62.5 mg once daily or every other day based upon individual tolerability.

Subjects will be evaluated for additional medical history since screening. They will also participate in a physical examination, baseline laboratory testing, ECG, Echo as part of their routine clinical care.

Blood samples will be obtained at time-points as follow: baseline (including day-1 and day 0), day1, day 13, day 14. After the last dose, blood will also be collected at day 28.

Subjects will be monitored carefully in the ward of Department of Cardiology in Xiangya Hospital for adverse events, laboratory test abnormalities, and changes in vital signs. Adverse experiences will be evaluated according to criteria outlined in the NCI Common Terminology Criteria for Adverse Events (CTCAE), version 4.0.

Following the study treatment, subjects will be followed as an in-patient during the first 3 days (day 0,1 and 2) and last 2 days (day 13 and 14). An additional follow-up assessment will occur on Day 28.

2.3 STUDY TREATMENT

Patients with idiopathic pulmonary arterial hypertension(IPAH) (N=20) with a confirmed diagnosis of pulmonary hypertension and meeting all inclusion/exclusion criteria will be enrolled in the study, which will be divided into 3 cohorts and entail the administration of HIV protease inhibitors in three doses. Patients will be involved into 3 cohorts and receive the SQV+ RIT in micro (SQV 0.3mg/kg + RIT 0.03mg/kg), low (SQV 3mg/kg + RIT 0. 3mg/kg) and standard doses (SQV 15mg/kg + RIT 1.5mg/kg) twice daily respectively. Trials will continue only when the does is well–tolerated. Baseline blood collections and echocardiography will be performed.

Baseline markers of inflammation known to be associated with IPAH including HMGB1, IL1-beta, IL-6, TNF-alpha and CRP will be obtained at baseline. NT-Pro BNP will be measured as a biomarker of increased pulmonary arterial pressures. Whole blood assays will be employed to measure the change in endotoxin- induced/ HMGB1-induced cytokine response after systematically exposure to SQV+RIT.

Patients currently on oral background PAH therapy will be instructed to continue to take the regular regiment throughout the study with the possible need to modify doses of PDE5 inhibitors and

endothelin receptor antagonists in standard dose group. Subjects will receive the SQV+RIT for up to 2 weeks and blood draws will take place at baseline (Day -1, Day 0), day1, day13, day14 and day28.

Table 3 Dose Limiting Toxicity Criteria

Sign or Symptom	Moderate[1]	Severe[2]
Bronchospasm/ wheezing	Symptomatic but not requiring therapy	Symptomatic and requiring therapy
SBP[3]	*Supine* ≥20 but <30 mmHg drop from pre-dose baseline on same day AND symptomatic, or SBP <80 mmHg AND symptomatic	*Supine* ≥30 m Hg drop from pre-dose baseline on same day AND symptomatic *or* requiring fluid replacement or other therapy
DBP[3]	*Supine* ≥10 but <20 mmHg drop from pre-dose baseline on same day AND symptomatic, or DBP <45 mmHg AND symptomatic	*Supine or* ≥20 mmHg drop from pre-dose baseline on same day AND symptomatic *or* requiring fluid replacement or other therapy
Dyspnea	Causes more discomfort (than a mild, easily tolerated AE) and interrupts the subject's usual daily activities	Incapacitating and causes considerable interference with the subject's usual daily activities
Cough	Causes more discomfort (than a mild, easily tolerated AE) and interrupts the subject's usual daily activities	Incapacitating and causes considerable interference with the subject's usual daily activities
Hypoxemia[4]	SaO_2 ≤93% but >90%	SaO_2 ≤90%
Other drug-related signs or symptoms[7]	Causes more discomfort (than a mild, easily tolerated AE) and interrupts the subject's usual daily activities OR CTCAE v.4[7] Grade 2 toxicity	Incapacitating and causes considerable interference with the subject's usual daily activities OR CTCAE v.4[7] Grade 3 or 4 toxicity

AE = adverse event; CTCAE = Common Toxicity Criteria for Adverse Events; DBP = diastolic blood pressure; DLT = dose limiting toxicity; FEV_1 = forced expiratory volume in one second; FVC = forced vital capacity; MTD = maximum tolerated dose; SaO_2 = oxygen saturation (hemoglobin); SBP = supine blood pressure

[1] Moderate DLTs which in a single subject will result in no further dose escalation for that subject, and occurring in more than 3 subjects in a cohort will result in re-evaluation and diminution of planned escalation in subsequent subjects in the same cohort or future cohorts.

[2] Severe DLT seen in one subject which, after review, is deemed to be study drug-related will preclude further dosing with AIR001 for that subject.

[3] Blood pressure changes should be confirmed by the mean of triplicate measurements taken within 5 minutes of each other. Symptomatic refers to dizziness or fainting. Discontinuation of the dosing will be based the mean of these repeated measurements.

[4] SaO_2 should be confirmed by measuring pulse oximetry at a second site, with 3 measurements 2 minutes approximately apart; in addition, the pulse rate should correspond to palpated radial or carotid pulse.

[5] Common Terminology Criteria for Adverse Events v. 4.0 (CTCAE). CTCAE criteria will be applied if considered related to study drug and confirmed on repeat testing.

Table 4 Toxicity Criteria

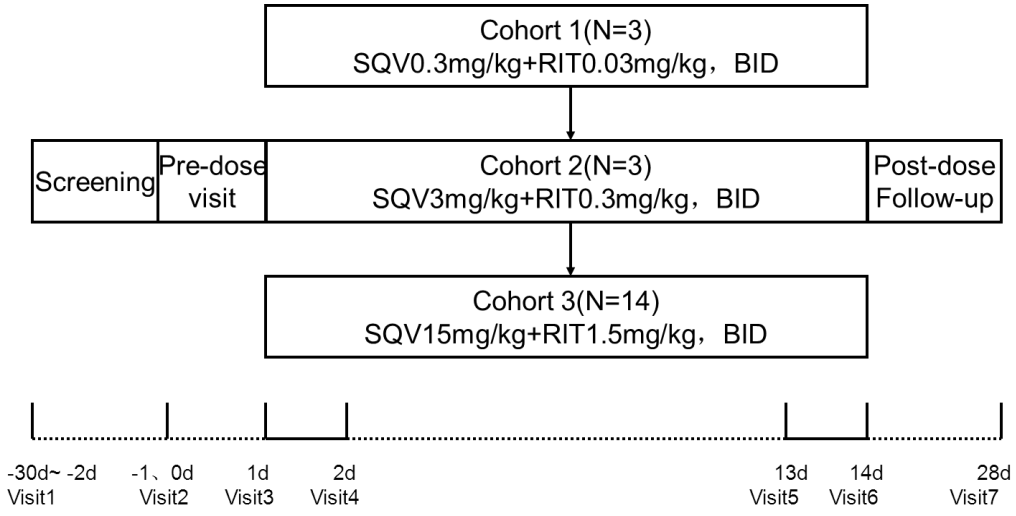
Parameter	Definition
Serious adverse event	Serious adverse event
Increased liver transaminases[a]	Serum AST and ALT are both increased to >5 × ULN (i.e., CTCAE Grade ≥3)[b]
Decreased total leukocyte count[a]	Leukopenia to <3000/μL [<3.0 × 10^9/L] (i.e., CTCAE ≥Grade 2)
Decreased total neutrophil count[a]	Neutropenia to <1500/μL [<1.5 × 10^9/L] (i.e., CTCAE ≥Grade 2)
Decreased lymphocyte count[a]	Lymphopenia to <500/μL [<0.5 × 10^9/L] (i.e., CTCAE ≥Grade 3)
Decreased platelet count[a]	Thrombocytopenia to <100,000/μL [<100 × 10^9/L]
Increased serum creatinine[a]	Serum creatinine to ≥ 1.5 × ULN (i.e., CTCAE Grade ≥2)
Increased blood sugar[a]	Fasting glucose value >8.9 - 13.9 mmol/L(i.e., CTCAE Grade ≥2)
Increased diarrhea[a]	Increase of 4 - 6 stools per day over baseline (i.e., CTCAE Grade ≥2)
Increased Cholesterol level[a]	Cholesterol to >7.75 -10.34 mmol/L(i.e., CTCAE Grade ≥2)
Prolongation of QT_cF interval[a]	QT_cF >500 msec (i.e., CTCAE Grade ≥3) or increase from baseline of ≥60 msec (i.e., CTCAE Grade ≥2)
Unspecified	If considered appropriate by the Medical Monitor and Investigator (i.e., CTCAE Grade ≥2)

CTCAE = Common Toxicity Criteria for Adverse Events; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ULN = upper limit of normal reference range of the clinical laboratory; QT_cF = QT interval (Fridericia's correction)

a. To be confirmed by a repeat test.

b. If elevations in transaminases are associated with hyperbilirubinemia, a lower threshold of AST and/or ALT of >3 × ULN (i.e., CTCAE Grade ≥2) will be used

2.4. STUDY DESIGN SCHEMATIC



2.5. DURATION OF FOLLOW-UP

All subjects enrolled in the study will be followed for 28 days for study. Subjects removed from study for unacceptable adverse events will be followed until resolution or stabilization of the adverse event.

2.6. SAFETY MONITORING

PR and QT Interval Prolongation

The changes to the electrical activity of the heart possibly associated with these drugs, known as prolonged QT or PR intervals, can be seen on an electrocardiogram (ECG). A prolonged QT interval can increase the risk for abnormal heart rhythms, including a serious abnormal rhythm called torsades de pointes. A prolonged PR interval can cause the electrical signal responsible for generating a heart beat to slow or even stop; this is known as heart block and can affect how fast the heart is able to beat.

SQV and RIT are antiviral medications given together to treat HIV infection. RIT is given at a low dose with SQV in order to increase the level of SQV in the body. This is a process known as "boosting." FDA's analysis of these data is ongoing. However, healthcare professionals should be aware of this potential risk for changes to the electrical activity of the heart. SQV and RIT should not be used in patients already taking medications known to cause QT interval prolongation such as Class IA (such as quinidine,) or Class III (such as amiodarone) antiarrhythmic drugs; or in patients with a history of QT interval prolongation.

QT_cF >500 msec or increase from baseline of ≥60 msec will result in a discontinuation of the study treatment.

Diabetes Mellitus and Hyperglycemia

New onset diabetes mellitus, exacerbation of preexisting diabetes mellitus and hyperglycemia have been reported during postmarketing surveillance in HIV-1-infected patients receiving protease-inhibitor therapy. Some patients required either initiation or dose adjustments of insulin or oral hypoglycemic agents for the treatment of these events. In some cases diabetic ketoacidosis has occurred. In those patients who discontinued protease-inhibitor therapy, hyperglycemia persisted in some cases. Because these events have been reported voluntarily during clinical practice, estimates of frequency cannot be made and a causal relationship between protease-inhibitor therapy and these events has not been established.

Hepatotoxicity

In patients with underlying hepatitis B or C, cirrhosis, chronic alcoholism and/or other underlying liver abnormalities, there have been reports of worsening liver disease.

Hemophilia

There have been reports of spontaneous bleeding in patients with hemophilia A and B treated with protease inhibitors. In some patients additional factor VIII was required. In the majority of reported cases treatment with protease inhibitors was continued or restarted. A causal relationship between protease inhibitor therapy and these episodes has not been established.

Hyperlipidemia

Elevated cholesterol and/or triglyceride levels have been observed in some patients taking SQV in combination with RIT Marked elevation in triglyceride levels is a risk factor for development of pancreatitis. Cholesterol and triglyceride levels should be monitored prior to initiating combination dosing regimen of SQV and RIT, and at periodic intervals while on such therapy. In these patients, lipid disorders should be managed as clinically appropriate.

Lactose Intolerance

Each capsule contains lactose (anhydrous) 63.3 mg. This quantity should not induce specific symptoms of intolerance.

Fat Redistribution

Redistribution/accumulation of body fat including central obesity, dorsocervical fat enlargement (buffalo hump), facial wasting, peripheral wasting, breast enlargement, and “cushingoid appearance” have been observed in patients receiving antiretroviral therapy. A causal relationship between protease-inhibitor therapy and these events has not been established and the long-term consequences are currently unknown.

Potentiate drug interaction and management in PAH

Concomitant Drugs	Potential Clinical Effects	Management
PDE5 Inhibitors	Increased potential for sildenafil-associated adverse events (which include visual disturbances, hypotension, prolonged erection, and syncope). A safe and effective dose has not been established when used with Saquinavir /ritonavir.	Use sildenafil with caution at reduced doses of 25 mg every 48 hours with increased monitoring of adverse events when administered concomitantly with Saquinavir /ritonavir.
HMG-CoA Reductase Inhibitors	Potential for myopathy including rhabdomyolysis.	Titrate atorvastatin dose carefully and use the lowest dose necessary; do not exceed atorvastatin 20 mg/day.
Endothelin receptor antagonists: Bosentan	Increases in serum Bosentan concentration when Bosentan was coadministered with Saquinavir /ritonavir. Saquinavir Effect on is Bosentan not well established	Discontinue use of bosentan at least 36 hours prior to initiation of Saquinavir /ritonavir. After at least 10 days following the initiation of Saquinavir /ritonavir, resume bosentan at 62.5 mg once daily or every other day based upon individual tolerability.

Anticoagulant: Warfarin	Increased warfarin effects (eg, increased INR and risk of bleeding)	Concentrations of warfarin may be affected. It is recommended that INR (international normalized ratio) be monitored.
Calcium channel blockers	The impact on the PR interval of co-administration of Saquinavir /ritonavir with other drugs that prolong the PR interval	Use with caution. Titrate CCB dose and monitor closely. ECG monitoring is recommended when CCB used with Saquinavir /ritonavir.
Antiplatelete: aspirin	Saquinavir Effect on aspirin is not well established	
Prostacyclin analogue: iloprost	Saquinavir Effect on iloprost is not well established	

Following study drug treatment, subjects will be evaluated as an outpatient at the clinic on Day 28. Subjects will be followed for evidence of acute or delayed adverse effects from the study treatment and to assess their clinical status. All adverse events experienced by subjects will be collected from the time of dosing, through the study and until the final study visit. Subjects continuing to experience toxicity at the off study visit may be contacted for additional assessments until the toxicity has resolved or is deemed irreversible. If possible, symptoms should be managed symptomatically. In the case of toxicity, appropriate medical treatment should be used.

In addition to these formal evaluations, subjects will be encouraged to immediately contact the study investigator and/or the study coordinator with questions, concerns, or to report new symptoms that occur during their study participation. If appropriate, based upon the evaluation, medical treatments will be provided to subjects, including appropriate referral to physicians at the Center of Clinical Pharmacology of the Third Xiangya Hospital.

2.7. STOPPING RULES

In addition to the dose limiting toxicity as outlined in Tables 3 and 4 above, study treatment will be discontinued if any of the following occurs:

- Systemic hypotension (MAP ≤ 50 mm Hg or 20% below baseline if initial MAP <55)
- Desaturation (10% below baseline)
- Decreased cardiac index (20% below baseline)
- Any serious adverse event thought to be possibly related to the study treatment

2.8. DRUG SUPPLIES

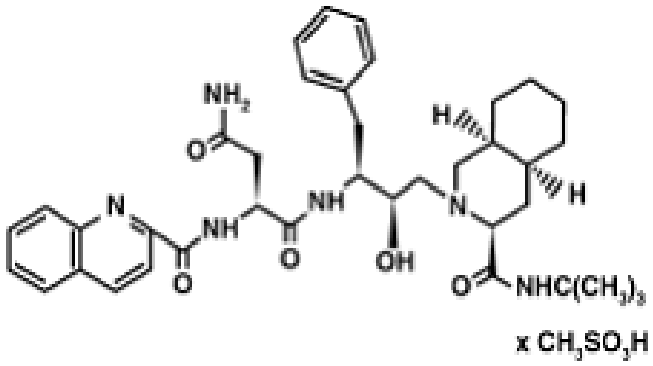
2.8.1. Formulation and Packaging

Formulation:

The chemical name for saquinavir mesylate is N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[[N-(2quinolylcarbonyl)-L-asparaginyl]amino]butyl]-(4aS,8aS)-isoquinoline-3(S)-carboxamide methanesulfonate with a molecular formula $C_{38}H_{50}N_6O_5 \bullet CH_4O_3S$ and a molecular weight of 766.96.

The molecular weight of the free base is 670.86. Saquinavir mesylate is a white to off-white, very fine powder with an aqueous solubility of 2.22mg/mL at 25°C.
Saquinavir 200-mg capsules are light brown and green opaque capsules with ROCHE and 0245 imprinted on the capsule shell—bottles of 270 (NDC 0004-0245-15).
Saquinavir 500-mg film-coated tablets are light orange to greyish- or brownish-orange, oval cylindrical, biconvex tablets with ROCHE and SQV 500 imprinted on the tablet face—bottles of 120 (NDC 0004-024451).

Saquinavir mesylate has the following structural formula:



Packaging:

All capsules are labeled, packaged and distributed by Roche Pharmaceuticals, Inc. will provide bulk drug supplies to the investigational site in vial-specific shelf packs, each containing 12 single-use vials. Each shelf pack and each vial will be packaged and labeled in English, in accordance with FDA requirements. All drugs will directly transported and repacked in Fangsheng Pharmaceuticals Company in Changsha,China.

2.8.2. Availability

Saquinavir mesylate is a FDA- approved drug and will be obtained from Roche Pharmaceuticals.

2.8.3 Preparing and Dispensing

The study drug will only be dispensed by the research pharmacist once a subject has provided written informed consent and the subject has been identified by the Sponsor-Investigator and has met all eligibility criteria for entry into the study on Day 0, following completion of all clinical assessment.

2.9 Drug Administration

Saquinavir must be used in combination with ritonavir, because ritonavir significantly inhibits saquinavir's metabolism to provide increased plasma saquinavir levels.
Adults (Over the Age of 16 Years)

- Saquinavir ***7.5mg/kg twice daily*** (about 500mg twice daily; 1 x 500-mg tablet) in combination with ritonavir ***0.75 mg/kg twice daily***(about 50mg twice daily; 1/2 x 100-mg capsule).
- Saquinavir 15***mg/kg*** daily (about 1000mg twice daily; 2 x 500-mg tablets) in combination with ritonavir ***1.5mg/kg*** twice daily(about 100mg twice daily; 1 x 100-mg capsule)..
- Ritonavir should be taken at the same time as saquinavir.
- Saquinavir and ritonavir should be taken within 2 hours after a meal.

2.10 DRUG STORAGE

The investigational drug will be kept in its original packaging and stored in a locked, secure area at 25°C (77°F); excursions permitted to 15° to 30°C (59° to 86°F) in tightly closed bottles. The temperature of the storage area must be monitored to ensure compliance with required temperatures. A temperature log will be maintained to make certain that the drug supplies are stored at the correct temperature at all times. Access to and administration of the investigational drug will be limited to the study investigators and authorized research staff. Study drugs may only be dispensed to subjects enrolled in this study.

2.11. DRUG ACCOUNTABILITY

The study investigators or the study coordinator will document the amount of study drugs dispensed and/or administered to subjects. The study drugs accountability records will be maintained throughout the course of the clinical trial. Any discrepancies in drug supplies will be noted and explained.

2.12. STUDY PROCEDURES

2.12.1 Screening

Screening evaluations to determine eligibility will take place within 3 weeks prior to receiving study treatment. The potential study subjects are followed on a routine basis in the ward of Department of Cardiology in Xiangya Hospital are well known to the study investigators. During a routine clinic visit, patients who express interest in participation will undergo the screening assessment to determine that all inclusion/exclusion criteria are met prior to receiving the study treatment.

If the patient has had any of the tests done within the 3 months prior to the screening period, they will not be repeated for study purposes. The results collected from these tests and procedure will serve as a baseline for comparison of the subject's overall condition following study treatment.

Screening-- Outpatient Clinic Assessment

- Obtain written informed consent.
- Medical history review and demographics.
- Complete history and physical examination to include vital signs, blood pressure, body weight, and height.
- Assessment of oxygen saturation

- Clinical laboratory evaluations including complete blood count with differential, electrolytes, glucose, BUN, serum creatinine, calcium, magnesium, phosphorus, liver function tests, LDH, PT/PTT, and albumin.
- Assessment of NYHA(See Appendix 10.1) /WHO functional class.
- ECG
- Echocardiogram with documentation of TRV for estimation of pulmanory artery pressures and right heart function. All echocardiograms should be performed at least rest for one hour.
- Adverse event monitoring
- Concomitant medications
- Compliance evaluation

2.12.2 Experimental procedures

Subjects who meet the Inclusion Criteria and none of the Exclusion Criteria will receive the study treatment of SQV.

Pre-Dose: Baseline (Day, -1 and 0)-- Inpatient Clinic Assessment

- Vital signs
- Assessment of oxygen saturation
- Clinical laboratory evaluations
- Venous blood will be obtained for HMGB1, IL1-beta, IL-6, TNF-alpha, CRP, and NT-pro Brain Natriuretic Peptide levels. Note: All these biomarkers should be assessed on each day of day, -1 and 0.
- ECG
- Echocardiogram
- Whole blood assays for evaluating the effect of SQV + RIT reduces in-vitro endotoxin-induced/ or HMGB1-induced cytokinemia using blood sampling. Note: whole blood assays should be done on day -1.
- Assessment of NYHA/WHO functional classAdverse event monitoring
- Concomitant medications
- Compliance evaluation

Treatment: Day 1 and 2 -- Inpatient Clinic Assessment

- Vital signs
- Assessment of oxygen saturation
- Venous blood will be obtained for HMGB1, IL1-beta, IL-6, TNF-alpha and CRP. Note: All these biomarkers should be assessed on each day of day 1 and 2.
- ECG
- Adverse event monitoring
- Concomitant medications
- Compliance evaluation

Post-treatment: Day 13 -- Inpatient Clinic Assessment

- Vital signs

- Assessment of oxygen saturation
- Clinical laboratory evaluations
- Venous blood will be obtained for HMGB1, IL1-beta, IL-6, TNF-alpha and CRP.
- Adverse event monitoring
- Concomitant medications
- Compliance evaluation

Final Measurement: Day 14 -- Inpatient Clinic Assessment

- Physical Exam
- Vital signs
- Assessment of oxygen saturation
- Clinical laboratory evaluations
- Venous blood will be obtained for HMGB1, IL1-beta, IL-6, TNF-alpha, CRP, and NT-pro Brain Natriuretic Peptide levels.
- ECG
- Echocardiogram
- Whole blood assays for evaluating the effect of SQV + RIT reduces in-vitro endotoxin-induced/ or HMGB1-induced cytokinemia using blood sampling.
- Adverse event monitoring
- Concomitant medications
- Compliance evaluation

Post-treatment Follow-up monitor: Day 28

Outpatient visits should be completed as close to the scheduled visit dates as possible. However, a variance of 5 days will be allowed for the follow-up monitoring to facilitate scheduling or to account for weekends or holidays.

- Physical Exam
- Vital signs
- Assessment of oxygen saturation
- Venous blood will be obtained for HMGB1, IL1-beta, IL-6, TNF-alpha, CRP, and NT-pro Brain Natriuretic Peptide levels.
- Echocardiogram
- Assessment of NYHA/WHO functional class.
- Adverse event monitoring
- Concomitant medications

In addition to these formal evaluations, subjects will be encouraged to immediately contact the study coordinator and/or the physician investigators with questions, concerns, or to report new symptoms that occur during their study participation. If appropriate, based upon the evaluation, medical treatments will be provided to subjects, including appropriate referral to physicians at the Center of Clinical Pharmacology of the Third Xiangya Hospital.

Any clinically significant adverse event, laboratory test or physical examination observed during final assessments will be followed as medically appropriate until resolved or explained.

2.12. SCHEDULE OF ACTIVITIES (STUDY ASSESSMENT TABLE)

The table below summarizes the protocol procedures that will be performed at screening, during the study treatment, and at follow-up.

Study Phase	Screening	Pre-Treatment	Treatment				
Visit Type	Outpatient Clinic	Inpatient assessment					Outpatient Clinic
Days on Intervention							Day 28
Study Procedures		Baseline (Day -1 & 0)	Day 1	Day 2	Day 13	Day 14	
Informed Consent	x						
Medical History and Demographics	x						
I/E Criteria	x						
Physical Exam	x	x				x	x
6MWD		x				x	x
Vital Signs	x	x	x	x	x	x	x
Oxygen Saturation	x	x	x	x	x	x	x
Laboratory Tests	x	x				x	x
Pro-BNP、ANP		x				x	x
Inflammatory Biomarker		x	x	x	x	x	x
ECG	x	x	x	x		x	
Echocardiogram	x	x				x	x
NYHA/WHO functional class assessment	x	x		x		x	x
Compliance evaluations		x	x	x	x	x	
AE Assessment		x	x	x	x	x	x
Concomitant medications	x	x	x	x	x	x	x

2.13. DESCRIPTION OF STUDY PROCEDURES

2.13.1. Physical Examination and Vital Signs

A physical examination and set of vital signs will be assessed at each clinic visit. Vital sign measurements include weight, diastolic and systolic blood pressure, heart rate, and temperature. To determine whether the subject's blood pressure qualifies him/her for the intervention, have the subject rest for at least 5 minutes prior to the assessment. Up to 3 blood pressures may be taken. Once an assessment qualifies on BOTH the systolic and diastolic, no further assessments are needed, and the subject qualifies for the study per exclusion criterion. The intent is to exclude patients who have uncontrolled systemic hypertension, rather than to exclude patients in a temporary/transitory state with a one time measurement out of normal range (e.g., "white coat hypertension"). Therefore, if the clinician believes, based on experience with the subject, that the blood pressure assessment is not reflective of the subject's actual level of systemic blood pressure, the assessment may be repeated.

2.13.2. Medical History

At screening, the subject's medical history will be recorded including: previous treatment for PAH, previous ACS events, previous strokes, pain crises frequency and usual management, history of priapism, previous transfusions and reactions to transfusions and to medications.

2.13.3. Whole blood asssays

Collection of blood and whole blood assay. Blood 10ml from IPAH patients was drawn into heparinized syringes (20 U/ml; endotoxin contamination ,5 pg/ml heparin according to the limulus amoebocyte lysate assay). Bring the blood sample under the hood in the small tissue culture room, remove the rubber cap and empty into a 50mL conical.

1. Sonicate LPS for at least 30 minutes.
2. Pipette 500µL into 12 tubes pre-labeled.
   a. i.e.: ID number and letter A-L for LPS concentration (A-C is 0ng/ml, D-F is 0.1ng/ml, G-I is 1ng/ml, and J-L is 10ng/ml).
3. Make your LPS dilutions in pre-labeled tubes A, B, C, D.
   b. Add 50µL of stock (1 mg/ml) to 950µL of saline to make 50µg/ml (A)
   c. Add 10µL of A into 990µL of saline to make 500ng/ml (B = 10ng in blood)
   d. Add 100µL of B into 900µL of saline to make 50ng/ml (C = 1ng in blood)
   e. Add 100µL of C into 900µL of saline to make 5ng/ml (D = 0.1ng in blood)
   i. Vortex thoroughly between dilutions.
4. Pipette 10µL of saline into tubes A-C. Pipette 10µL of (D) into tubes D-F. Pipette 10µL of (C) into tubes G-I. Pipette 10µL of (B) into tubes J-L. Be sure to vortex and mix the LPS solutions in between additions to the tubes.
5. Cap the tubes and invert to mix by wrapping a paper towel around the rack and taping it down to hold the tubes in place.
6. Place the rack on its side on a rocker and secure with tape inside the incubator (at 37°C in a 5% CO2 atmosphere). Incubate for 4 hrs.
7. After 4 hrs, take samples out of the incubator and centrifuge tubes at 6000 RPM for 5 minutes.

8. Transfer plasma supernatant via pipette to appropriately labeled tubes (labeled the same as the first tube) ensuring no cells are transferred. Expected volume is about 200µl.
9. Freeze the samples for analysis with human TNF ELISA.

For HMGB1 stimulation, we used similar protocol (4 hr treatment) with 5 ug/ml HMGB1 and measured IL-8 release[35].

2.13.4. Echocardiography

Transthoracic echocardiography will be performed according to the guidelines of the American Society of Echocardiography. Transmitral flow, Doppler determinations of the severity of valvular regurgitation, and left ventricular stroke volume will be assessed and graded. Peak velocities of the E wave and A wave, the ratio of the E wave to the A wave, and the deceleration time will be measured. Isovolumic relaxation time will be measured as the time from aortic valve closure to the start of mitral inflow. (See Appendix10.2) for Echo checklist. Tricuspid regurgitation will be assessed in the parasternal right ventricular inflow, parasternal short-axis, and apical four-chamber views, and a minimum of five sequential complexes will be recorded. Continuous-wave Doppler sampling of the peak regurgitant jet velocity will be used to estimate the right-ventricular-to-right-atrial systolic pressure gradient with the use of the modified Bernoulli equation (4 x [tricuspid regurgitate jet velocity]2). Pulmonary-artery systolic pressure will be quantitated by adding the Bernoulli-derived pressure gradient to the estimated mean right atrial pressure. The mean right atrial pressure will be calculated according to the degree of collapse of the inferior vena cava with inspiration: 5 mm Hg for a collapse of at least 50 percent and 15 mm Hg for a collapse of less than 50 percent. During follow-up visits echocardiography should be performed on 1-2 hours after study medication intake. All echocardiograms will be de-identified (removal of all of the patient's personal information) prior to them being sent for review and quality control.

2.13.5. Laboratory Testing

Peripheral blood samples will be obtained from all subjects for hemoglobin measurement and clinical laboratory testing at screening, prior to dosing on Day 1 and Day 30.

Basic Laboratory Profiles

Clinical laboratory tests (including the pregnancy test, if applicable) will be performed on blood samples collected at screening and all clinic visits for the interventional Trial.
Urinalysis laboratory tests will be performed on urine samples collected at screening and certain clinic visits indicating in study assessment table. The following laboratory tests will be performed:

- Liver function tests (AST, ALT, albumin, total protein, total bilirubin, alkaline
- phosphatase)
- Renal function tests (blood urea nitrogen (BUN), and creatinine)
- Mineral panel tests (potassium, sodium, chloride, magnesium, phosphate, calcium)
- CBC (Hemoglobin, hematocrit, RBC, MCHC, mean cell volume (MCV), reticulocyte counts, white blood cell (WBC) count, platelet count, and ANC)
- INR values for subjects who are on vitamin K antagonists (e.g. warfarin)
- Activated Partial Thromboplastin Time (aPTT) for subjects who are on therapeutic doses of unfractionated heparin

2.13.6. Biomarker Analyses

All subjects will provide blood for the biomarkers studies. Approximately 30 mL of blood will be needed for the set of assays on a given day (listed on Study Assessment Table). The baseline sample will be drawn at study entry before study drug is administered. Samples will be processed and assayed in central laboratory of Center of Clinical Pharmacology of the Third Xiangya Hospital for evaluation of HMGB1, IL1-beta, IL-6, IL-8, IL-10, TNF-alpha, CRP, and NT-pro Brain Natriuretic Peptide levels.

2.13.7. Specimen collection and management

Specimen Collection / Documentation

Blood samples will be accurately recorded for the actual sample collection time. Covariate information, e.g., age, body weight, height, disease type, concomitant medications, laboratory values, etc., will be obtained at each time point. This is expected to yield an information-rich data set through dense sampling, enabling better characterization of the biomarkers in this patient population.

Specimen Handling and Labeling (De-Identification)

Specimens collected will be properly labeled. All research biological specimens and all records associated with the samples will be labeled only with a unique code that contains no personal identifiers. The information linking these code numbers to the corresponding subject's identity will be kept in a secure location in the PI's office, and will not be available to staff managing samples at the research laboratories.

Immediately upon receipt of the biological specimens, all attempts will be made to process, isolate, collect, and store the specimens. The code number and date on which the specimen is frozen, all other information about the specimen, and subsequent processing will be entered on the specimen processing worksheet.

Specimen Management and Storage

Specimens in excess of immediate assay requirements may be stored indefinitely in a locked freezer under the control of the principal investigator.

The blood samples will be stored after appropriate coding to remove patient identifiers. The coding information linking patient identifiers to the stored samples will be maintained in a locked, secure area that will be accessible only to the study investigator. Subjects may request to have their samples destroyed at any time. These samples will be destroyed immediately upon receipt of the subjects' written request to do so. Identification of which samples to destroy will be available from the coding information linking patient identifiers to the stored samples as described earlier in this paragraph.

Restrictions to Direct Access of Specimens

Specimens will be kept in the responsible study investigators' laboratories indefinitely and will be under the control of the principal investigator. Investigators or other personnel not involved with the management or operations of the study are not permitted direct access to the specimens.

2.14. ENDPOINTS

2.14.1. Primary Endpoints

The primary measure of efficacy will be change in HMGB1 level from baseline at 14 days, measured using an ELISA kit.

2.14.2. Secondary Endpoints

- The changes in the parameters of TNF-α, IL-1β, IL-6, N-terminal pro-brain natriuretic peptide (NT-proBNP), and C-reactive protein (CRP), across the 2 weeks treatment.
- The changes in PA pressure and total right heart function measured by echocardiography from baseline to 14 days.
- NYHA/WHO functional class and Brog dyspnea scale index from baseline to 14 days.

2.15. SUBJECT WITHDRAWAL

A subject may voluntarily discontinue participation in this study at any time. The investigator may also, at his or her discretion, discontinue a subject from this study at any time. Every effort should be made by the investigator to keep the subject in the study.

Subjects may be withdrawn from the study prior to completion if any of the following criteria are observed:

- Intercurrent illness or an unexpected fatal or life-threatening adverse event, which requires discontinuation of study treatment
- Subject reached protocol-defined stopping criteria
- Request by the subject to withdraw from the study
- Protocol violations
- Persistent non-compliance
- Lost to follow-up
- Investigator discretion
- Study closed/terminated

2.15.1. Dropouts and withdrawals

To be considered complete, a subject must complete all study visits as specified in the protocol without violations of the protocol so significant as to obscure the response to study treatment.

Subjects who fail to complete all study required visits will not be considered complete and may not enrolled at a later date and will not be replaced. A record will be kept of all subjects who fail to complete all study visits and their primary reasons for discontinuation.

In the event of subject withdrawal, subjects will be encouraged to continue all follow-up visits for safety monitoring or to continue follow up as directed by their personal primary physicians ,unless the subject withdraws consent at any time (without having to justify the decision). All available data from subjects who discontinued during the study, for whatever reason, will be included in the safety analysis.

2.16 STATISTICAL ANALYSIS

2.16.1 Sample Size and Power

This is an exploratory study and the sample size was chosen according to the principle of FDA Phase 0 guidelines (less than 15 patients) in the first and second cohorts, and with respect to safety as well. We expect that six patients in the first and second cohorts will give us safety information and an effective idea of the change in inflammatory factors.

In the standard dose cohort, we hypothesize that treatment with SQV+RIT in IPAH patients will result in a 50% reduction in HMGB1. In our preliminary data, the average levels of HMGB1 were 4.16±1.96ng/ml, 10.85±4.88ng/ml, 3.46±2.73ng/ml, 6.06±1.33ng/ml, and 2.85±1.84ng/ml in healthy volunteers and IPAH, connective tissue disease-PAH, chronic thromboembolic pulmonary hypertension, and congenital heart disease-PAH patients, respectively. These levels were significantly raised in the IPAH group compared with healthy control subjects ($P<0.01$), but no significant difference was observed in patients with other type of PAHs compared with healthy control subjects. Setting power=0.90, α=0.05, using a two sided test and calculated using $n = [\frac{(u_\alpha + u_\beta)}{\delta / \sigma}]^2 + \frac{1}{2} u_\alpha{}^2$, the number of patients that should be recruited is 12. An additional 15% increase in sample size is under consideration due to possible incomplete data, measurement variation, and drop out. Therefore, the total number is 14 in the standard dose group. After a one month wash-out period, patients in cohorts 1 and 2 will be able to join the last cohort under their own will, so the total number is 14 to 20. We expect that a sample size of 14 in the standard dose group will be sufficient to detect meaningful changes in inflammation biomarkers. We also expect to see trends in cardiopulmonary hemodynamics and symptom improvement in this group.

2.16.2 Statistical Analysis

General Approach

Both patients and physicians will know the type and the dosing of the research drugs. The statistician analyzing the results will be blinded to the study cohort. All causes of missing data will be collected and reviewed to determine the type of missing outcome data. All data will be analyzed at the Center of Clinical Pharmacology at the Third Xiangya Hospital with the single blind analysis method. T-test will be performed to determine difference of mean values for outcome variables at baseline and day 14.

Safety Analysis

Adverse events (AEs) will be grouped by body system. AEs occurring pre-treatment, during active treatment and post-treatment will be summarized separately. The number and percentage of subject experiencing at least one AE of any type, AEs within each body system and AEs within each preferred term will be tabulated and listed. Separate summaries will be provided for all AEs, AEs by maximum severity, drug related AEs, severe adverse events (SAEs), and for AEs leading to withdrawal.

Handling of missing data:
Every effort will be made to collect complete data on each study day. With respect to safety evaluation, it is not planned to impute missing data.

3. HUMAN SUBJECTS

3.1 SUBJECT POPULATION

The racial, gender and ethnic characteristics of the proposed subject population in this research protocol shall reflect the demographics of the population of Hunan and the surrounding area. We shall attempt to recruit subjects in proportion to these demographics. No exclusion criteria shall be based on race, ethnicity or gender.
Every effort will be made to keep subjects in the study until they complete all study procedures.

3.2 INCLUSION CRITERIA

Potential study subjects must satisfy the following criteria to be enrolled in the study:

- Age 18-60
- Idiopathic pulmonary arterial hypertension (IPAH)
- Personally signed and dated informed consent document indicating that the subject (or a legally acceptable representative) has been informed of all pertinent aspects of the study
- Subject willing and able to comply with scheduled visits, treatment plans, laboratory tests, and other study procedures
- Diagnosis of PH confirmed by cardiac catheterization: mean pulmonary artery pressure (mPAP) $\geq$ 25 mm Hg (at rest), pulmonary capillary wedge pressure equal or less than 15mmHg, and normal or reduced cardiac output
- Stable PAH therapy for at least three months

3.3 EXCLUSION CRITERIA

Subjects meeting any of the exclusion criteria at baseline will be excluded from participating in study.

- Baseline systemic hypotension, defined as MAP less than 50 mmHg
- Requires intravenous inotropes within 30 days prior to study participation
- Has uncontrolled systemic hypertension as evidenced by sitting systolic blood pressure >160 mm Hg or sitting diastolic blood pressure >100 mm Hg at screening
- History of portal hypertension or chronic liver disease, including cirrhosis, chronic alcoholism, hepatitis B and/or hepatitis C (with evidence of recent infection and/or active virus replication) defined as moderate to severe hepatic impairment (Child-Pugh Class B-C)

- Chronic renal insufficiency as defined by serum creatinine >2.5 mg/dL at screening or requires dialysis support
- Hemoglobin concentration <9 g/dL at Screening
- History of atrial septostomy
- Repaired or unrepaired congenital heart disease
- Pericardial constriction
- Restrictive or congestive cardiomyopathy
- Left ventricular ejection fraction 40% as shown by multiple gated acquisition scan (MUGA), angiography, or echocardiography
- Symptomatic coronary disease with demonstrable ischemia
- Other severe acute or chronic medical or laboratory abnormality that may increase the risk associated with study participation or investigational product administration or may interfere with the interpretation of study results and, in the judgment of the investigator, would make the subject inappropriate for entry into this study
- Psychiatric, addictive, or other disorder that compromises the ability to give informed consent for participating in this study. This includes recent history of alcohol or illicit drug abuse 30 days prior to study screening (day 1) and for the duration of the study
- Poorly-controlled asthma, defined by active wheezing and/or cough with FEV1 < 70% predicted, responsive to inhaled BD (>15% increase in FEV1 with BD)
- Clinically significant intercurrent illness (including lower respiratory tract infection) or clinically significant surgery within four weeks before the administration of study drug
- History of hypersensitivity or idiosyncratic reaction to drugs from multiple drug classes
- Receipt of an investigational product or device, or participation in a drug research study within a period of 15 days (or five half-lives of the drug, whichever is longer) before the first dose of study drug
- Blood loss or blood donation >550mL within 90 days or plasma donation >500 mL within 14 days before administration of study drug
- Patients with a QTc interval > 450 msec
- Diabetes mellitus as defined by symptoms of hyperglycemia and serum fasting plasma glucose level≥7.0mmol/l or casual plasma glucose≥11.1mmol/l at screen
- Hyperlipidemia as TC≥6.22 mmol/L, LDL-C≥4.14 mmol/L or TG≥2.26 mmol/L
- History of Crohn's disease, ulcerative colitis, inflammatory bowel disease, etc.
- Unwilling to take contraceptive measures during the study
- Use of certain other medications will need to be evaluated for possible exclusion based on the potential for adverse drug interactions

4. RECRUITMENT AND INFORMED CONSENT PROCEDURES

4.1 RECRUIMENT METHODS

The potential study subjects will be recruited from three Xiangya Hospital affiliated with Central South University. Potential subjects will be first identified by the physician investigator who is also the primary care or treating physician. The physician investigator who already has knowledge of and access to subjects' information will review the subjects' records to identify potential research subjects for the study. After identifying potentially eligible subjects, the physician investigator will then approach these subjects to discuss the research opportunity.

To minimize the possibility that subjects will feel obligated to participate, investigators will reinforce with their subjects that participation is voluntary, that they do not have to participate, and the decision not to participate will not affect their care, now or in the future. The physician investigator will also allow subjects to make further inquiries if they are interested.

4.2 INFORMED CONSENT PROCEDURES

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continuing throughout the individual's study participation. Extensive discussion of risks and possible benefits of participation in this study will be provided to the subjects and their families. Consent forms describing in detail the study procedures and risks are given to the subject and written documentation of informed consent is required prior to enrolling in the study. Consent forms will be IRB approved and the subject will be asked to read and review the document. Upon reviewing the document, the investigator will explain the research study to the subject and answer any questions that may arise. The subjects will sign the informed consent document prior to being enrolled in the study. The subjects should have the opportunity to discuss the study with their surrogates or think about it prior to agreeing to participate. The subjects may withdraw consent at any time throughout the course of the study. A copy of the informed consent document will be given to the subjects for their records. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

Prior to performing any of the research study procedures or interventions, subjects must provide informed consent. The physician investigator will verbally explain the study to the potential subject in a language understandable to subjects, providing all pertinent information (purpose, procedures, risks, benefits, alternatives to participation, etc.), and will allow potential subjects ample opportunity to ask questions to elicit a better understanding of the study. Following this verbal explanation, potential subjects will be provided with a local IRB approved consent form and will be asked to read and review the document. Upon reviewing the document, the physician investigator will provide adequate opportunity for the subject to consider all options, answer any additional questions the potential subject may have. Every effort will be made to ensure that subjects have comprehended the study information prior to obtaining subject's voluntary agreement to participate.

In addition, older potential participants whose competency to consent is in question will be tested for sufficient comprehension and recall of the information presented. Prospective subjects who do not remember the important facts about participation in the research study after repeated testing will not be included in the study. The investigators will also assess whether a participant understands

experimental procedures over time, including assessment throughout the full duration of participation in the study.

The subjects may withdraw consent at any time throughout the course of the study. A copy of the informed consent document will be given to the subjects for their records. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

5. POTENTIAL RISKS AND BENEFITS

5.1 POTENTIAL RISKS

As with any experimental procedure, there may be adverse events or side effects that are currently unknown, and certain of these unknown risks could be permanent, severe or life threatening. Every attempt will be taken to minimize these risks.

Risks of taking saquinavir:
Short term SQV+RIT is well tolerated. Risks are mainly unexpected allergic reactions which are very rare. A history of allergic reactions to HIV protease inhibitors will exclude the subject from the trial.

Potential side effects of taking saquinavir
Medicines and their possible side effects can affect individual people in different ways. The following are some of the side effects that are known to be associated with this medicine. Just because a side effect is stated here, it does not mean that all people using this medicine will experience that or any side effect.

- Disturbances of the gut such as diarrhea, constipation, nausea, vomiting, indigestion or abdominal pain.
- Headache.
- Difficulty in sleeping (insomnia).
- Depression.
- Anxiety.
- Fatigue.
- Chest pain.
- Changes in sex drive.
- Alteration in taste.
- Fever (pyrexia).
- Mouth ulcers.
- Skin reactions such as rash and itch.
- Dizziness.
- Pins and needles (paraesthesia).
- Disorder of the peripheral nerves causing weakness and numbness (peripheral neuropathy).
- Weakness or loss of strength (asthenia).

- Pain.
- Liver disorders.
- Inflammation of the pancreas (pancreatitis).
- Kidney stones.

The side effects listed above may not include all of the side effects reported by the medicine's manufacturer. For more information about any other possible risks associated with this medicine, please read the information provided with the medicine or consult your doctor or pharmacist.

Risks of Echocardiography:
This is a noninvasive procedure. There is no known risk associated with this procedure, although rarely patients may experience mild discomfort from the ultrasound transducer.

Blood Draws:
Some subjects may experience localized bruising at the site where blood is drawn. Routine blood drawing protocol will be followed to minimize this risk.

Risks of Breach of Confidentiality:
Participation in this research study does involve the potential risks of a breach of confidentiality of the medical record information and associated privacy of the participants. This research study will result in identifiable information that will be placed into the subject's medical records held at the Center of Clinical Pharmacology of the Third Xiangya Hospital. The nature of the identifiable information resulting from participation in this research study that will be recorded in the medical record includes laboratory test results. This potential for breach of confidentiality could impact future insurability, employability, or have other personal consequences for the subjects.

5.2. PROTECTIONS AGAINST RISK

Protection Against Patient Risks Related to the Study Drug
The study has been designed with a focus on protecting subjects against risk from the medication including:

- Specific exclusion criteria to provide a stable population of subjects, and non-enrollment of subjects with significant co-morbidities that might place them at excess risk (see exclusion criteria above).
- Continuous monitoring by the DSMB.
- Involvement by trained staff / investigators with experience in the administration of inhaled nitrite.
- Specific holding criteria related to study adverse events.
- Frequent monitoring the cardiopulmonary hemodynamics for over the duration of the study.

Protection Against Patient Risks Related to the Study Radiographic Procedures
Woman of child bearing potential will be advised regarding the potential risks associated with radiation exposure. These subjects must undergo a pregnancy test prior to enrollment in the clinical investigation. The radiographic studies will be conducted in the hospital environment with trained staff familiar experienced in the proper performance of these procedures.

Protection Against General Risks of Study Procedures

All research interventions/activities will be conducted in private patient care areas. The collection of sensitive information about subjects is limited to the amount necessary to achieve the aims of the research, so that no unneeded sensitive information is being collected.

All demographic and clinical information about the subject will be stored on an electronic password-guarded study database under the supervision of the PIs for this protocol. The data will be stripped of individual identifiers and stored anonymously with a subject number. Information linking subject identifiers with the coded subject number will be stored under password protection on computers in locked areas, with access only to the database manager. Maintaining records in locked files in locked offices will protect confidentiality of subjects. All staff will sign confidentiality statements. Access to the database will be limited to the data manager and staff under the supervision of the PIs.

To prevent excessive blood sampling, a single withdrawal of blood for a combination of clinically indicated and this research study will not exceed 5% of the circulating blood volume, and the cumulative withdrawal over 1 month will not exceed 10% of the circulating blood volume. In addition, careful attention will be made to cardiovascular status and hemoglobin evaluation.

Specimens will be stripped of subject identifiers and stored securely according to a similar coding protocol as described above. These specimens will be stored safely in the custody of the Principal Investigator responsible for the individual assays. These Investigators will limit future access to any remaining sample to only those investigators with prior IRB approval for their studies.

All staff involved in this study are properly credentialed and instructed in the areas of testing, confidentiality, and safety. To minimize the risks associated with the study procedures and/or collection of specimens, trained staff or experienced physician investigators will perform the study procedures.

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5.3 ALTERNATIVE TREATMENTS

If subjects choose not to participate in this study**,** they are to continue their medical care under the direction of their primary physicians.

5.4 POTENTIAL BENEFITS

There will be no direct benefit to the subjects participating in this study, but the society at large may benefit from the increased knowledge gained from this study that will lead to new treatment for individuals diagnosed with PAH in the future.

5.5 DATA SAFETY MONITORING PLAN

5.5.1 Data Safety Monitoring Board

The local Data Safety Monitoring Board (DSMB) chaired by Dr.Yuan Hong, the PI of this clinical trial, is comprised of members including senior experts in pulmonary medicine, clinical research, and clinical trial design, biostatistics, and research ethics. A Data and Safety Monitoring Board (iDSMB) independent of the study investigators will also monitor this clinical trial for additional measure of subject protection. The iDSMB consists of clinicians completely independent of the investigators who have no financial, scientific, or other conflict of interest with the trial.

The DSMB will conduct interim monitoring of accumulating data from research activities to assure the continue safety of human subjects, relevance and appropriateness of the study, and the integrity of research data.

5.5.2 Data Safety Monitoring Plan

Assuring patient safety is an essential component of this protocol. The study Principal Investigator has primary responsibility for the oversight of the data and safety monitoring. The study investigators will evaluate all adverse events. All subjects who have AEs, whether considered associated with the use of the study medication or not, must be monitored to determine the outcome. The clinical course of the AE will be followed up according to accepted standards of medical practice, even after the end of the period of observation, until a satisfactory explanation is found or the Principal Investigator considers it medically justifiable to terminate follow-up.

All untoward medical occurrences observed in subjects receiving the study drug will be recorded on the participants' adverse event case report forms (CRF) by the study coordinator under the supervision of the principal investigator. The CRFs will then be reviewed for completeness and internal consistency. In addition to internal safeguards built into a computerized system, external safeguards will be put in place to ensure that access to the computerized system and to the data is restricted to authorized personnel. Training conducted by qualified individuals on a continuing basis will be provided to individuals in the specific operations with regard to computerized systems that they are to perform during the course of the study.

***Stopping Rule:**

For safety reasons, we propose to discontinue this study treatment if any of the following occurs:

<u>Dose Limiting Toxicity</u>

Study treatment will be discontinued in individual subjects if any of the following occurs:

- Systemic hypotension (MAP ≤ 55 mm Hg or 20% below baseline if initial MAP <55)
- Desaturation (systemic oxygen saturation 10% below baseline)
- Decreased cardiac index (20% below baseline)
- Any serious adverse event thought to be possibly related to the study treatment

<u>Intolerable Dose Criteria</u>

A dose level will be considered intolerable if any of the following occurs:

- Systemic hypotension (MAP ≤ 55 mm Hg or 20% below baseline if initial MAP <55) resulting in a dose discontinuation in 3 or more subjects on active study treatment.
- Desaturation (systemic oxygen saturation 10% below baseline) resulting in a dose discontinuation in 3 or more subjects on active study treatment.
- Decreased cardiac index (20% below baseline) resulting in a dose discontinuation in 3 or more subjects on active study treatment.
- Coughing or dyspnea which prevents administration of study drug within a 20 minute time frame in 3 or more subjects on active study treatment.
- Dose limiting toxicity criteria in 3 or more subjects on active study treatment.

If an intolerable dose is identified, no further doses at that or higher doses will be administered. The dosing scheme will be changed to the lower tolerated dose for the remaining subjects.

In addition, if first 3 of the 10 subjects enrolled in the study experience any unexpected fatal or life-threatening events that can be attributed to the study drug, the study will be halted, until data review by investigators and the Data Safety Monitoring Board has rendered a final recommendation about study continuation.

The PI, will work with the reporting investigators to prepare a detailed written summary of serious, unexpected, and treatment related adverse events, and will compare, and contrast the event with prior events. The detailed written summary will be provided to the DSMB and the IRB.

In addition, the DSMB Report addressed the following information will be submitted to the IRB at the time of continuing review annually or more often as required:

- A list of the research personnel who participated in the data and safety monitoring.

- The frequency of monitoring that took place during the renewal intervals and/or the dates that data and safety monitoring was conducted.

- A summary of cumulative data related to unanticipated problems (including adverse events) including a determination of causality and whether the risk to benefit assessment has changed.

- If appropriate, a summary of pertinent scientific literature reports, therapeutic developments, or results of related studies that may have an impact on the safety of study participants or the ethics of the research study.

- A summary of the outcome of reviews conducted to ensure subject privacy and research data confidentiality.

- Final conclusions regarding changes to the anticipated benefit-to-risk assessment of the study participation and final recommendations related to continuing, changing, or terminating the study.

5.5.3 Parameters to be Monitored

The following progress will be monitored throughout the course of the research to ensure the safety of subjects as well as the integrity and confidentiality of their data.

- An evaluation of the progress of the research study, including subject recruitment and retention, and an assessment of the timeliness and quality of the data.

- A review of collected data (including adverse events, unanticipated problems, and subject withdrawals) to determine whether there is a change to the anticipated benefit-to-risk assessment of study participation and whether the study should continue as originally designed, should be changed, or should be terminated.

- An assessment of external factors or relevant information (eg. Pertinent scientific literature reports or therapeutic development, results of related studies) that may have an impact on the safety and study participants or the ethics of the research study.

- A review of study procedures designed to protect the privacy of the research subjects and the confidentiality of their research data.

The severity of adverse changes in physical signs or symptoms will be classified as follows:

- Grade 1 (Mild): asymptomatic or mild symptoms; clinical or diagnostic observation only; intervention not indicated.

- Grade 2 (Moderate): minimal, local or noninvasive intervention indicated; limiting age-appreciate instrumental ADL (Activities of Daily Living).

- Grade 3 (Severe): medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL.

- Grade 4 (Life-threatening): consequences; urgent intervention indicated.

- Grade 5 (Death): event is a direct cause of death.

5.5.4 Frequency of Monitoring

The principal investigator will review subject safety data as it generated. The principal investigator, co-investigators, and the research staff will meet on a two week interval to re-evaluate study goals, subject recruitment, data coding and retention, documentation and identification of adverse events, complaints and confidentiality of subjects. There will be an evaluation of the progress of the research study, including assessments of data quality, time lines, participant recruitment, accrual, and retention. The principal investigator will also review the outcome and adverse event data to determine whether there is any change to the anticipated benefit-to-risk ratio of study participation and whether the study

should continue as originally designed or should it be re-evaluated and changed.

The DSMB is expected to meet at least two times a year at the call of the Chairperson to review the progression of the study including patient enrollment, protocol compliance, and adverse event reports. An emergency meeting of the DSMB may be called at any time by the Chair should participant safety questions or other unanticipated problems arise.

5.5.5. Adverse Event Reporting:
The study investigators will be responsible for detecting, documenting and reporting events that meet the following definition of an adverse event.

5.5.5.1. Adverse event definitions
Adverse event. Any untoward medical occurrence in a clinical study; regardless of the causal relationship of the event with the investigational drug or study treatment(s).

Associated with the use of the investigational drug or study treatment(s). There is a reasonable possibility that the adverse event may have been caused by the investigational drug or study treatment(s).

Disability. A substantial disruption of a person's ability to conduct normal life functions.

Life-threatening adverse event. Any adverse event that places the patient or subject, in the view of the investigator, at immediate risk of death from the event as it occurred (i.e., does not include an adverse event that, had it actually occurred in a more severe form, might have caused death).

Serious adverse event. Any adverse event occurring at any dose that results in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect.

- Hospitalization shall include any initial admission (even if less than 24 hours) to a healthcare facility as a result of a precipitating clinical adverse event; to include transfer within the hospital to an intensive care unit. Hospitalization or prolongation of hospitalization in the absence of a precipitating, clinical adverse event (e.g., for a preexisting condition not associated with a new adverse event or with a worsening of the preexisting condition; admission for a protocol-specified procedure) is not, in itself, a serious adverse event.

Unexpected adverse event. Any adverse event, the frequency, specificity or severity of which is not consistent with the risk information described in the clinical protocol(s) or elsewhere in the current IND application, as amended.

5.5.5.2. Recording/Reporting requirements
Eliciting adverse event information
Clinical study subjects will be routinely questioned about adverse events at study visits.

Recording requirements

All observed or volunteered adverse drug events (serious or non-serious) and abnormal test findings, regardless of treatment group or suspected causal relationship to the investigational drug or study treatment(s) will be recorded in the subjects' case histories. For all adverse events, sufficient information will be pursued and/or obtained so as to permit 1) an adequate determination of the outcome of the event (i.e., whether the event should be classified as a serious adverse event) and; 2) an assessment of the casual relationship between the adverse event and the investigational drug or study treatment(s).

Adverse events or abnormal test findings felt to be associated with the investigational drug or study treatment(s) will be followed until the event (or its sequelae) or the abnormal test finding resolves or stabilizes at a level acceptable to the investigator-sponsor.

Abnormal test findings

An abnormal test finding will be classified as an adverse event if one or more of the following criteria are met:

- The test finding is accompanied by clinical symptoms.
- The test finding necessitates additional diagnostic evaluation(s) or medical/surgical intervention; including significant additional concomitant drug treatment or other therapy
    - Note: simply repeating a test finding, in the absence of any of the other listed criteria, does not constitute an adverse event.
- The test finding leads to a change in study dosing or discontinuation of subject participation in the clinical study
- The test finding is considered an adverse event by the investigator-sponsor of the IND application

Causality and severity assessment

The investigator-sponsor will promptly review documented adverse events and abnormal test findings to determine 1) if the abnormal test finding should be classified as an adverse event; 2) if there is a reasonable possibility that the adverse event was caused by the investigational drug or study treatment(s); and 3) if the adverse event meets the criteria for a serious adverse event.

If the investigator-sponsor's final determination of causality is "unknown and of questionable relationship to the investigational drug or study treatment(s)", the adverse event will be classified as associated with the use of the investigational drug or study treatment(s) for reporting purposes. If the investigator-sponsor's final determination of causality is "unknown but not related to the investigational drug or study treatment(s)", this determination and the rationale for the determination will be documented in the respective subject's case history.

5.5.6. Reporting of adverse events

5.5.6.1. Reporting of adverse events to the FDA

Written Safety Reports

5.5.7. Reporting adverse events to the responsible IRB

In accordance with applicable policies of the Xiangya Third Hosptial Institutional Review Board (IRB), the investigator-sponsor will report, to the IRB, any observed or volunteered adverse event that is determined to be 1) associated with the investigational drug or study treatment(s); 2) serious; and 3) unexpected. Adverse event reports will be submitted to the IRB in accordance with the respective IRB procedures.

Applicable adverse events will be reported to the IRB as soon as possible and, in no event, later than 10 calendar days following the investigator-sponsor's receipt of the respective information. Adverse events which are 1) associated with the investigational drug or study treatment(s); 2) fatal or life-threatening; and 3) unexpected will be reported to the IRB within 24 hours of the investigator-sponsor's receipt of the respective information.

Follow-up information to reported adverse event will be submitted to the IRB as soon as the relevant information is available. If the results of the sponsor-investigator's follow-up investigation show that an adverse event that was initially determined to not require reporting to the IRB does, in fact, meet the requirements for reporting; the investigator-sponsor will report the adverse event to the IRB as soon as possible, but in no event later than 10 calendar days, after the determination was made.

6. STUDY ADMINSTRATION

6.1. QUALITY CONTROL AND QUALITY ASSURANCE

Independent monitoring of the clinical study for protocol and GCP compliance will be conducted periodically (i.e., at a minimum of annually) by qualified staff of the Education and Compliance Office – Human Subject Research, Research Conduct and Compliance Office, the Third Xiangya Hospital.

The investigator-sponsor and the The Center of Clinical Pharmacology of the Third Xiangya Hospital will permit direct access of the study monitors and appropriate regulatory authorities to the study data and to the corresponding source data and documents to verify the accuracy of this data.

6.2. DATA HANDLING AND RECORD-KEEPING

6.2.1 Data recording/Case Report Forms

A Case Report Form (CRF, see Appendix 1) will be completed for each subject enrolled into the clinical study. The investigator-sponsor will review, approve and sign/date each completed CRF; the investigator-sponsor's signature serving as attestation of the investigator-sponsor's responsibility for ensuring that all clinical and laboratory data entered on the CRF are complete, accurate and authentic.

Source Data are the clinical findings and observations, laboratory and test data, and other information contained in *Source Documents*. *Source Documents* are the original records (and certified copies of original records); including, but not limited to, hospital medical records, physician or office charts, physician or nursing notes, subject diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, x-rays, etc. Information recorded on the CRF must be consistent with the *Source Data* recorded on the *Source Documents* or discrepancies must be explained.

The CRFs must be kept current to reflect subject status at each phase during the course of the trial. In all cases, subjects must not be identified on the CRF by name. Appropriate coded identifications (i.e. Subject ID number) will be used. Every effort will be made to collect complete data for each study visit. Causes of *missing data* will be fully documented. With respect to safety evaluation, it is not planned to impute missing data.

6.2.2 Record maintenance and retention

The investigator-sponsor will maintain records in accordance with Good Clinical Practice guidelines; to include:

- IRB correspondence (including approval notifications) related to the clinical protocol; including copies of adverse event reports and annual or interim reports
- Current and past versions of the IRB-approved clinical protocol and corresponding IRB-approved consent form(s) and, if applicable, subject recruitment advertisements

- Financial disclosure information (investigator-sponsor and clinical protocol sub-investigators)
- Curriculum vitae (investigator-sponsor and clinical protocol sub-investigators)
- Certificates of required training (e.g., human subject protections, Good Clinical Practice, etc.) for investigator-sponsor and listed sub-investigators
- Listing of printed names/signatures of investigator-sponsor and listed sub-investigators
- Normal value(s)/range(s) for medical/laboratory/technical procedures or tests included in the clinical protocol
- Laboratory certification information
- Instructions for on-site preparation and handling of the investigational drug(s), study treatment(s), and other study-related materials (i.e., if not addressed in the clinical protocol)

- Signed informed consent forms
- Completed Case Report Forms; signed and dated by investigator-sponsor
- Source Documents or certified copies of Source Documents

- Monitoring visit reports
- Copies of investigator-sponsor correspondence to sub-investigators, including notifications of safety information
- Subject screening and enrollment logs
- Subject identification code list
- Investigational drug accountability records, including documentation of drug disposal.
- Retained biological specimen log
- Interim data analysis report(s)
- Final clinical study report

Subject-specific data and Case Report Forms will be coded and the subject identification code list will be stored so as to protect the subjects' confidentiality. Subject names or other directly identifiable information will not appear on any reports, publications, or other disclosures of clinical study outcomes.

The investigator-sponsor will retain the specified records and reports for up to 2 years after the marketing application is approved for the investigational drug; or, if a marketing application is not submitted or approved for the investigational drug, until 2 years after investigations on this drug for this indication have discontinued.

6.3. ETHICS

Institutional Review Board (IRB) Approval

The Investigator will obtain, from the Xiangya Third Hospital Institutional Review Board (IRB), prospective approval of the clinical protocol and corresponding informed consent form(s); modifications to the clinical protocol and corresponding informed consent forms, and brochures (i.e., directed at potential research subjects and clinical faculty/staff) for study recruitment.

The only circumstance in which a deviation from the current IRB-approved clinical protocol/consent form(s) may be initiated in the absence of prospective IRB approval is to eliminate an apparent immediate hazard to the research subject(s). In such circumstances, the investigator-sponsor will promptly notify the Xiangya Third Hospital IRB of the deviation.

Ethical and scientific conduct of the clinical study

The clinical study will be conducted in accordance with the current IRB-approved clinical protocol; ICH Guidelines on GCP; and relevant policies, requirements, and regulations of the Central South University.

The investigator-sponsor will make certain that appropriate processes and procedures are in place to ensure that ongoing questions and concerns of enrolled subjects are adequately addressed and that the subjects are informed of any new information that may affect their decision to continue

participation in the clinical study. In the event of substantial changes to the clinical study or the risk-to-benefit ratio of study participation, the investigator-sponsor will obtain the informed consent of enrolled subjects for continued participation in the clinical study.

8. QUALIFICATIONS AND SOURCES OF SUPPORT

8.1 QUALIFICATIONS OF THE INVESTIGATORS

Principal Investigator:

Hong Yuan, MD, PhD

Co-Investigators:

Zai-xin, MD, PhD

8.2 SOURCE OF SUPPORT

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10. Appendix

10.1 Modified NYHA Functional Classification

Class I Patients with pulmonary hypertension but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnea or fatigue, chest pain, or near syncope.

Class II Patients with pulmonary hypertension resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnea or fatigue, chest pain, or near syncope.

Class III Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary physical activity causes undue dyspnea or fatigue, chest pain, or near syncope.

Class IV Patients with pulmonary hypertension resulting in the inability to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnea and/or fatigue may be present at rest, and discomfort is increased by any physical activity.

10.2 Echocardiographic parameters

LV Structure and Function	LVED size (mm) LVES size(mm) Septal thickness (mm) PW thickness (mm) LVMI (m/h) LVEF (%)
LV Diastolic	Left atrial area (cm2) Function Max LA vol/BSA (ml/m2) Peak E velocity (m/s) Peak A velocity (m/s) E/A ratio Deceleration time (msec) Isovolumic relaxation time (msec) Pulmonary vein flow Tissue Doppler of the septum and lateral walls
Right Heart Size and Function	Right atrial area (cm2) RV end diastolic area (cm2) RV end systolic area (cm2) RV area change (%) RV:LV diastolic area ratio Tricuspid regurgitant jet velocity (m/s)
Valvular Function	