

## 1.) TITLE PAGE

# **PHARMACOLOGICAL CHARACTERIZATION OF IW-1973, A NOVEL SOLUBLE GUANYLATE CYCLASE STIMULATOR WITH EXTENSIVE TISSUE DISTRIBUTION, ANTI-HYPERTENSIVE, ANTI-INFLAMMATORY, AND ANTI- FIBROTIC EFFECTS IN PRECLINICAL MODELS OF DISEASE**

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## **2.) RUNNING TITLE PAGE**

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#### **d) NONSTANDARD ABBREVIATIONS**

sGC – soluble guanylate cyclase

NO – nitric oxide

PKG – protein kinase G

PDE – phosphodiesterase

eNOS – endothelial nitric oxide synthase

VASP – vasodilator-stimulated phosphoprotein

pVASP – phosphorylated VASP

GC – guanylate cyclase

PK – pharmacokinetic

DETA-NONOate – diethylenetriamine NONOate

DMSO – dimethylsulfoxide

HTRF – homogeneous time resolved fluorescence

CI – Confidence Interval

FBS – fetal bovine serum

HBSS – Hank’s Balanced Salt Solution with calcium and magnesium

IBMX – 3-isobutyl-1-methylxanthine

LC-MS/MS – liquid chromatography with tandem mass spectrometry

PBS – phosphate buffered saline

PEG – polyethylene glycol

U – unit

ACh – acetylcholine

CCRC – cumulative concentration response curve

SNP – sodium nitroprusside

DSI – Data Sciences International

SHR – spontaneously hypertensive rat

HPMC – hydroxypropyl methylcellulose

MAP – mean arterial pressure

HR – heart rate

AOC – area over the curve

ANOVA – analysis of variance

DSS – Dahl Salt Sensitive

NS – normal salt

HS – high salt

LPS – lipopolysaccharide

CBA – Cytometric Bead Array

FCAP – flow cytometric analysis program

UUO – unilateral ureteral obstruction

MFI – median fluorescence intensity

T<sub>max</sub> – time to reach maximum concentration

C<sub>max</sub> – maximum concentration

V<sub>ss</sub> – steady state volume of distribution

QD – quaque die, once-daily

QWBA – quantitative whole-body autoradiography

mmHg – millimeters mercury

IL-6 – interleukin 6

TGFβ1 – tumor growth factor-β1

Col1α1 – collagen 1α1

TNFα – tumor necrosis factor-α

IL-10 – interleukin 10

ROS – reactive oxygen species

#### **e) SECTION ASSIGNMENT**

Cardiovascular

## ABSTRACT

Soluble guanylate cyclase (sGC), a key signal-transduction enzyme, increases the conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine-3',5'-monophosphate (cGMP) upon binding of nitric oxide (NO). Endothelial dysfunction and/or reduced NO signaling have been implicated in cardiovascular disease pathogenesis and complications of diabetes and have been associated with other disease states and aging. sGC stimulators are small-molecule drugs that bind sGC and enhance NO-mediated cGMP signaling. The pharmacological characterization of IW-1973, a novel clinical-stage sGC stimulator under clinical investigation for treatment of heart failure with preserved ejection fraction and diabetic nephropathy, is described. In the presence of NO, IW1973 stimulated sGC in a human purified enzyme assay and a HEK-293 whole cell assay. sGC stimulation by IW-1973 in cells was associated with increased phosphorylation of vasodilator-stimulated phosphoprotein (VASP). IW-1973 at doses of 1-10 mg/kg significantly lowered blood pressure in normotensive and spontaneously hypertensive rats. In a Dahl salt-sensitive hypertension model, IW-1973 significantly reduced blood pressure, inflammatory cytokine levels and renal disease markers, including proteinuria and renal fibrotic gene expression, results that were affirmed in mouse LPS-induced inflammation and rat unilateral ureteral obstruction renal fibrosis models. A quantitative whole-body autoradiography study of IW-1973 revealed extensive tissue distribution. Pharmacokinetic studies showed a large volume of distribution and a profile consistent with predicted once-a-day dosing in humans. In summary, IW-1973 is a potent, orally available sGC stimulator that exhibited renoprotective, anti-inflammatory, and antifibrotic effects in nonclinical models.

## INTRODUCTION

Soluble guanylate cyclase (sGC) is the major receptor for nitric oxide (NO) and a key signal-transduction enzyme in the NO-cyclic guanosine 3',5'-monophosphate (cGMP) signaling pathway. NO is a transient, locally-acting signaling molecule that binds to the heme prosthetic group of sGC to induce a conformational change, resulting in the conversion of guanosine triphosphate (GTP) to cGMP. cGMP in turn binds to and modulates the activity of downstream targets including protein kinase G (PKG), cyclic nucleotide-gated ion channels, and phosphodiesterases (PDEs) (Derbyshire and Marletta, 2012).

sGC is expressed in many cells and tissues (Budworth, 1999), but its role in vascular smooth muscle is the best understood. In response to vascular shear stress, endothelial nitric oxide synthase (eNOS) produces NO, which activates sGC present in neighboring vascular smooth muscle cells (Moncada and Higgs, 2006). This results in vasodilation and a concomitant increase in local blood flow (Moncada and Higgs, 2006). Several risk factors (e.g., age, smoking) and disease states (e.g., diabetes, heart failure, hypertension, and pulmonary hypertension) are associated with impaired NO signaling and endothelial dysfunction (Munzel, 2008). These associations as well as genetic studies of the NO-sGC-cGMP pathway suggest that long-term reduction in NO-sGC-cGMP signaling may contribute to hypertension; shortness-of-breath symptoms in heart failure and pulmonary hypertension; diabetic complications including nephropathy, and increased incidence of myocardial infarction, stroke, and death. A pharmacologic mechanism that enhances NO signaling and elevates cGMP levels may restore endothelial function and improve the course of disease.



The NO-sGC-cGMP pathway has a long history as a pharmacologic target beginning with the discovery of the NO donor nitroglycerin as a treatment for angina pectoris in the late-1800s (Murrell, 1879). NO released from donors such as nitroglycerin activate sGC, but do not preserve the precise spatiotemporal control of endogenous NO signaling. Although NO donors are potent vasodilators, tolerance to these agents readily develops. In the 1990s, the first inhibitors of the cGMP phosphodiesterase PDE5, which prevent cGMP breakdown, were approved for the treatment of erectile dysfunction, and later for the treatment of pulmonary arterial hypertension (Hrometz and Shields, 2006). However, endogenous cGMP production is a prerequisite for their pharmacological action, and PDE5 inhibitors are not specific to the sGC pathway because PDE5 modulates cGMP levels not only in cells containing sGC, but also in cells containing the particulate guanylate cyclases. Indeed, the PDE5 inhibitor sildenafil was shown to augment vasodilatory activity of cGMP pools generated by particulate guanylate cyclases (GCs) (Baliga, 2008).

sGC stimulators provide a new approach to modulating the NO-sGC-cGMP pathway by directly binding to sGC to enhance NO signaling. sGC stimulators are heme-dependent and, importantly, act in synergy with NO to increase cGMP production (Follmann, 2013). In this way, sGC stimulators preserve spatiotemporal control of endogenous NO signaling. The mechanism of action of sGC stimulators contrasts with sGC “activators”, which are heme-independent and do not act in synergy with NO to increase cGMP (Follmann, 2013). sGC activators indiscriminately activate sGC and do not enhance endogenous NO signaling. To date, the clinical development of sGC activators has been limited by unacceptable hypotension (Erdmann, 2013).

The first sGC stimulator described was YC-1, a compound shown to increase platelet cGMP in vitro (Ko, 1994). More recently, newer sGC stimulators with improved pharmacologic properties have demonstrated efficacy in many preclinical disease models, including models of systemic and pulmonary hypertension, heart failure, nephropathy, and fibrotic diseases (Gheorghiade, 2013; Sandner and Stasch, 2017; Stasch, 2011; Stasch, 2015; Glynos, 2015). In addition, sGC stimulators have demonstrated anti-inflammatory (Glynos, 2015), antifibrotic (Sandner and Stasch, 2017) and beneficial metabolic effects (Hoffmann, 2015a) in preclinical models. The sGC stimulator riociguat is approved in several countries for the treatment of pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension (Conole and Scott, 2013). There is increasing understanding of the extensive physiological role of NO-sGC-cGMP signaling, and likewise a growing appreciation of the multidimensional pharmacology of sGC stimulators.

Based on the broad therapeutic potential of sGC stimulation, a medicinal chemistry effort was conducted to synthesize and characterize novel sGC stimulators with suitable pharmacological, pharmacokinetic (PK), and pharmaceutical properties for clinical investigation. Herein, we describe the pharmacology of IW-1973, a novel sGC stimulator in clinical development with potential as a treatment for a broad range of diseases.

## MATERIALS AND METHODS

### Compounds

IW-1973 was synthesized at Ironwood Pharmaceuticals. Losartan (losartan potassium) was purchased from Tecoland Corporation (Irvine, CA). Dexamethasone was purchased from Sigma-Aldrich (St. Louis, MO).

### Animals

All animals used in the studies were housed in AAALAC-accredited animal facilities; all animal-use protocols were reviewed and approved by the Institutional Animal Care and Use Committee prior to commencement.

For the mouse study, animals were housed under controlled conditions (temperature 71.6 - 77° F, relative humidity 30-70%) and exposed to a 12-h light-dark cycle. All animals were acclimated to the facility for 7 days prior to study start and allowed free access to water and standard rodent chow.

For rat studies, all animals were housed under controlled conditions (temperature 72 ± 8°F and relative humidity 30-70%) and exposed to a 12-h light-dark cycle (6AM:6PM).

All animals were allowed free access to water and standard rodent chow (PicoLab Rodent Diet 20) or special chow. All animals were acclimated to the facility for at least 2-3 days prior to study.

## Human Tissues

Human subcutaneous resistance arteries for use in experiments studying human vascular tissue relaxation were obtained with proper authorization and full ethical approval from three donors (ReproCELL Europe Ltd, Beltsville, MD).

## Formulations

Various formulations were employed to study both the acute and chronic effects of IW-1973 in a range of animal models. Formulations were chosen for suitability to a particular dosing regimen and animal model. IW-1973 formulation for each assay is described within the methods. Experimental outcomes were benchmarked using the plasma exposure of IW-1973 to make comparisons across studies.

## sGC Enzyme Assay

Purified human recombinant  $\alpha 1\beta 1$  sGC was purchased from Enzo Life Sciences (Farmingdale, NY, #ALX-201-177). A solution containing 12.5 ng/mL human sGC, 30  $\mu$ M diethylenetriamine NONOate (DETA-NONOate, Enzo Life Sciences, Farmingdale, NY), 100 mM Tris (pH7.4), 4 mM  $MgCl_2$ , 2 mM dithiothreitol and 0.05% bovine serum albumin was allowed to equilibrate to 37°C for 10 min in a prewarmed assay plate. For each test concentration, IW-1973 was diluted in dimethyl sulfoxide (DMSO) to 33.3 $\times$  its assay concentration, then added to the assay plate. In some experiments Tween 20 or 1H-[1,2, 4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ) was added to this stock. Removal of the heme group by the non-ionic detergent Tween-20 is known to render sGC insensitive to activation by NO and heme-dependent sGC stimulators, while leaving

basal enzyme activity intact (Stasch, 2002). GTP was added to the assay plate to a final concentration of 300  $\mu$ M. Assay plates were incubated at 37°C for 20 min with shaking. The enzyme reaction was stopped with an equal volume of ice-cold 20% acetic acid. cGMP concentrations were determined using cGMP homogeneous time resolved fluorescence (HTRF) (Cisbio, Bedford, MA) per manufacturer's instructions. A cGMP standard curve was fit using a 4-parameter equation [log(inhibitor) vs. response – variable slope] with a 95% confidence interval (CI) using GraphPad Prism software v.6. Samples were diluted appropriately to ensure that all values fell within the linear range of the standard curve. Concentration response data were fit using a 4-parameter fit [log(agonist) vs. response – variable slope] using GraphPad Prism v.6. The EC<sub>50</sub>, defined as the concentration at which IW-1973 elicits 50% of its maximal response, was interpolated from the curve fit.

### **sGC Whole Cell Assay**

HEK-293 cells (ATCC, Manassas, VA, #CRL-1573) were grown and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. For sGC activity assays, cells were seeded in 384-well poly-D-lysine-coated flat-bottom plates (Fisher Scientific, Pittsburgh, PA) in 50  $\mu$ L medium at a density of  $1.5 \times 10^4$  cells/well. Cells were incubated 24 h at 37°C in a humidified chamber supplemented with 5% CO<sub>2</sub>. Medium was removed, and cells were washed once with 40  $\mu$ L of Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium. Cells were then incubated with 40  $\mu$ L of a solution containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in HBSS for 15 min at 37°C. 10  $\mu$ L of 5x the indicated final concentration of IW-1973 (diluted from a 10 mM DMSO stock) with or

without DETA-NONOate was added to the cells, which were then incubated for 20 min at 37°C. Assay buffer was then removed and 50 µL of ice-cold 10% acetic acid + 150 ng/mL internal standard cyclic <sup>13,15</sup>N<sub>2</sub>-guanosine 3',5'-monophosphate (+3 cGMP) was added to each well. Samples were incubated on ice for 30 min. Following centrifugation at 4°C for 5 min at 1000 x g to pellet cell debris, the supernatant was transferred to a clean plate and the samples were analyzed for cGMP content by reverse-phase liquid chromatography with tandem mass spectrometry (LC-MS/MS). cGMP concentrations were determined using a standard curve (supplemental Fig. 1A and B). Concentration-response data were fit using a 4-parameter fit [log (agonist) vs. response – variable slope] with a 95% CI using GraphPad Prism v.5. The EC<sub>50</sub> was interpolated from the curve fit.

### **cGMP and Vasodilator-Stimulated Phosphoprotein (VASP) Phosphorylation**

HEK-293 cells expressing GloSensor™ 40F cGMP (Promega, #CS182801) were maintained in Dulbecco's Modification of Eagle's Medium (DMEM, Corning Cellgrowth, #10-013-CV) supplemented with 10% fetal bovine serum (Hyclone, #SH30066.03) and 200 µg/mL hygromycin (Invitrogen, #10687.010). Cells were plated in culture medium in a 100 µL volume at a density of 50,000 cells/well in a poly-D-lysine coated 96-well flat bottom plate (BD, #354461). Cells were incubated overnight at 37°C in a humidified chamber with 5% CO<sub>2</sub>. Medium was removed, and cells were washed twice with HBSS with calcium and magnesium. Cells were then incubated with 90 µL of a solution containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in HBSS for 15 min at 37°C followed by the addition of 10 µL of 10x the indicated final concentration of IW-1973

(diluted from a 10 mM DMSO stock) in the presence of varying concentrations of DETA-NONOate and incubated for an additional 20 min at 37°C. For detection of cGMP formation, cells were lysed with 50  $\mu$ L per well of cGMP HTRF lysis buffer (CisBio, #62CL1FDD) and then assayed following the protocol of HTRF cGMP assay kit (CisBio, #62GM2PEC). For detection of total VASP and pVASP formation, cells were lysed with 50  $\mu$ L per well of HTRF lysis buffer (CisBio, #63ADK000ULB2) and then assayed following the protocol of the total VASP (CisBio, #63ADK067PEH) and pVASP Ser239 (CisBio, #63ADK065PEG) HTRF assay kits. All pVASP levels were normalized to total VASP, and data were normalized to the maximum response for cGMP and pVASP, respectively, determined with 1  $\mu$ M DETA-NONOate + 100  $\mu$ M IW-1973. Maximum cGMP and maximum pVASP were determined from a series of concentration responses of IW-1973 combined with varying levels of DETA-NONOate. Both cGMP and pVASP were found to plateau at the highest concentration of IW-1973 (100  $\mu$ M) + DETA-NONOate (1  $\mu$ M).

## Pharmacokinetics

Male Sprague-Dawley rats (275 – 300 g, Harlan Laboratories, Indianapolis, IN) with indwelling jugular vein cannulae were fasted overnight prior to dosing. Animals were dosed either intravenously (i.v.) via bolus injection through a percutaneous catheter in the tail or orally (p.o.) via gavage. IW-1973 was formulated in 60% polyethylene glycol (PEG) 400/ 40% water at 0.3 mg/ml (0.3 mg/kg) for i.v. dosing, and at 1 mg/ml (3 mg/kg) in 100% PEG 400 for oral dosing. Chow was returned to animals 4 h post-dose. Blood from rats dosed via i.v. injection was collected at 15 timepoints ranging from 0.033 to 48 h post-dose. Blood from p.o dosed rats was collected at 15 timepoints

ranging from 0.25 to 48 h post-dose. Samples were collected into K<sub>2</sub>EDTA separator tubes and processed to plasma by centrifugation (3500 rpm for 10 min). Plasma samples were prepared by protein precipitation and analyzed using LC-MS/MS.

### **Tissue Distribution**

Wistar rats (male, 250-275 g, n=6; Harlan Laboratories, Indianapolis, IN) were administered IW-1973 at 10 mg/kg once a day for 5 days via oral gavage. IW-1973 was formulated at 2 mg/ml (10 mg/kg) in 0.5% (weight/volume) methylcellulose added to 1% hydroxypropyl methylcellulose (HPMC) with 0.2% Tween 80 in Milli-Q water. At 2 h post-dose on day 5, plasma was collected, animals were perfused with PBS containing 1mM IBMX and 1 unit (U)/mL heparin. Liver, heart, kidney, and lung samples were collected and homogenized. Plasma and tissue samples were analyzed for IW-1973 levels using LC-MS/MS.

### **Human Vascular Tissue Relaxation**

To determine the relaxation effect of IW-1973 on human blood vessels, human subcutaneous resistance arteries were obtained from three donors, females ages 27 -51 undergoing elective surgeries. Arteries were mounted in myograph baths by means of a 40  $\mu$ m wire running through the lumen of the artery. Changes in tension were recorded using a Danish Myotech isometric transducer (Aarhus, Denmark). The arteries were pre-contracted with the thromboxane mimetic, U46619 at a final concentration of 100 nM. Upon stabilization of U46619 contractile response, a single addition of acetylcholine (ACh) was made for a final concentration of 10  $\mu$ M to confirm functionality of the vascular endothelium including the presence of endogenous NO. Tissues that did not



reach  $\geq 50\%$  relaxation in response to ACh were excluded from the study. Tissues were then washed and contracted with U46619 at a final concentration of 100 nM, and a IW-1973 cumulative concentration-response curve (CCRC; 0.1 nM to 10  $\mu$ M) was generated. At the end of the CCRC, sodium nitroprusside (SNP) was added for a final bath concentration of 100  $\mu$ M to determine maximal tissue relaxation response. IW-1973-induced relaxation at each concentration was calculated as a percentage of the SNP-mediated maximum relaxation of U46619-induced contraction. A sigmoidal dose-response (variable slope) curve was fit with bottom values constrained to zero, and an  $EC_{50}$  value was estimated from this fitted curve using GraphPad Prism v.6.

## Blood Pressure

The Dataquest A.R.T.<sup>TM</sup> acquisition and analysis system (Data Sciences International [DSI], St. Paul, MN) to monitor and analyze hemodynamic data from conscious, freely moving rats surgically implanted with a telemetry pressure transmitter (PA-C40).

Telemetry transmitter implantation was performed on rats under sterile conditions.

Briefly, spontaneously hypertensive rats (SHRs) (male, 230-250 g, 14 weeks of age; Charles River Laboratories, Wilmington, MA) and Wistar rats (male, 230-250 g, 12 weeks of age; Charles River Laboratories, Wilmington, MA) were anesthetized with isoflurane and body temperature was maintained with a heating pad during surgery. A laparotomy was performed to expose the abdominal aorta. The catheter tip of the telemetry transmitter was inserted into the abdominal aorta and secured with a 5-0 silk suture (Ethicon, Inc., Somerville, NJ). The abdominal incision was closed with uninterrupted suture (4-0 silk, Ethicon, Inc., Somerville, NJ) and the body of the telemetry transmitter was placed in the abdominal cavity and secured to the abdominal

wall. Approximately 100  $\mu$ L of 0.25% marcaine was applied directly to the closed abdominal wall, and the skin was then closed with suture (4-0 Vicryl® absorbable, Ethicon, Inc., Somerville, NJ). Buprenorphine (0.05 mg/kg/day, s.c.) was administered immediately after the surgery for postoperative pain relief. After recovery from anesthesia, rats were returned to their home cages, placed on DSI receivers, and allowed a 5- to 14-day recovery period. IW-1973 was formulated in 0.5% (weight/volume) methylcellulose added to 1% hydroxypropyl methylcellulose (HPMC) with 0.2% Tween 80 in Milli-Q water at concentrations of 0.06, 0.2, 0.6, and 2.0 mg/ml. Each week, animals received a single concentration via oral gavage for 4 days followed by a 3-day washout period; the same group of animals received the next concentration the following week.

Maximum change from baseline of mean arterial pressure (MAP) was calculated using the 24-h pre-1<sup>st</sup> dose average subtracted from the lowest MAP value measured within 2 h of dosing. Maximum change from baseline of heart rate (HR) was calculated using the 3-h pre-1<sup>st</sup> dose average subtracted from the 1-h postdose average. Area over the curve (AOC) was calculated using GraphPad Prism v.5. The data are expressed as mean  $\pm$  standard error of the mean (SEM). To determine the significant difference of the means across the independent strains, maximum change from baseline in MAP at day 3, maximum change from baseline in HR at days 1 and 3, AOC at day 1, and total AOC were analyzed by two-way analysis of variance (ANOVA), and significance was determined by a Dunnett's post-hoc analysis. Statistical significance was indicated by a *P*-value less than 0.05. The average of 60 min of data derived from the real time raw data was used for analysis.

### **Dahl Salt-Sensitive Rat Model (DSS)**

Telemetry transmitter implantations and data monitoring, acquisition, and analysis were performed as described above using Dahl Salt-Sensitive rats (male, 230-270 g; Harlan Laboratories, Indianapolis, IN). Normotensive control animals were maintained on 0.3% NaCl (normal-salt [NS]) diet for the 8-week study period. The vehicle control and treated animals were placed on 8% NaCl (high-salt [HS]) diet for 2 weeks followed by 6 weeks of HS diet plus their respective treatments of vehicle, IW-1973 (1, 3, or 10 mg/kg/day) or, as a reference control, losartan (30 mg/kg/day equivalent administered in the water). IW-1973 was formulated at 8.3, 25, and 83 mg/kg standard chow with 8% NaCl at Research Diets, Inc. (New Brunswick, NJ). At study end, animals were anesthetized with ketamine/xylazine (75/10 mg/kg, i.p.), and whole blood was collected for serum and plasma analyses. Following blood collection, the heart was perfused with heparin (1 U/mL of PBS) containing 1 mM IBMX. Kidneys and other organs were removed. Right and left kidneys were weighed separately, cut on the sagittal plane and one half of each was snap frozen in liquid nitrogen and the other half was fixed with 10% neutral buffered formalin for histological evaluation. At week 8, 24-h urine samples were collected, centrifuged to remove debris, and stored at -80°C until analysis. Serum samples were obtained at necropsy and stored at -80°C until analysis. Urine and serum samples were analyzed using the Randox Daytona Clinical Chemistry Analyzer (Randox, Kearneysville, WV). All measurements were performed in accordance with manufacturer's instructions.

For gene expression analysis, the left kidney was pulverized and powder (5-10 mg) from the tissue was homogenized and processed using a QuantiGene sample processing kit in accordance with manufacturer's instructions (Affymetrix, Fremont, CA).

Gene expression in the tissue homogenates was measured using a QuantiGene 2.0 Plex Assay (Affymetrix / Life Technology, Santa Clara, CA) following the user's manual. Analytes were measured using Luminex MAGPIX™ (Bio-Rad, Hercules, CA). Median fluorescence intensity (MFI) was generated for each gene target and normalized to the geometric mean expression of housekeeping genes (hprt1 and ppib), which were chosen to match the target transcript abundance.

MAP was averaged over each 24-h period. Urinary protein was determined using urine protein and urine creatinine. Analyses of individual serum samples for IL-6 was conducted using a multi array assay kit with a proinflammatory panel (Meso Scale Discovery, Gaithersburg, MD). Data were interpolated from standard curve values using GraphPad Prism v.6. To determine the significant difference of the means across multiple groups a one-way ANOVA was conducted, followed by a Dunnett's multiple comparison test versus the HS group. Statistical significance was indicated by a *P*-value less than 0.05.

### **Lipopolysaccharide (LPS)-Induced Inflammation Model**

C57BL/6 mice (female, 8-13 weeks; Taconic, Hudson, NY) were weighed and assigned to groups to achieve similar average weights across groups. Animals were dosed with vehicle (p.o.), dexamethasone (5 mg/kg, i.p.), or IW-1973 (1 or 10 mg/kg, p.o.). IW-1973 was formulated in 0.5% methylcellulose added to 1% HPMC with 0.2% Tween 80 in Milli-Q water and dosed at 5 mL/kg.

One hour postdose, animals received 100 ng of LPS in 0.2 mL PBS via i.v. injection. At 2 h post-LPS dose, mice were euthanized via CO<sub>2</sub> inhalation and blood was collected via cardiac puncture. Serum was prepared and stored at -80°C. Cytokine levels were determined using Cytometric Bead Array (CBA) analysis kits [BD, Franklin Lakes, NJ] in accordance with the manufacturer's protocol. A single analysis was performed for each sample. Data from CBA analysis kits were analyzed using Flow Cytometric Analysis Program (FCAP) array software. Data for individual samples were interpolated from standard curve values for each of the cytokines tested. Data were graphed and analyzed for statistical significance using GraphPad Prism, v. 6. All data are expressed as mean  $\pm$  SEM. To account for variance between groups, significance was determined by one-way ANOVA followed by a Fisher's Least Squares Difference test versus the vehicle group. Statistical significance was indicated by a *P*-value of less than 0.05.

### **Unilateral Ureteral Obstruction (UUO) Model**

UUO surgeries were performed on male Sprague-Dawley rats (male, 230-270 g; Harlan Laboratories, Indianapolis, IN) under sterile conditions. Briefly, animals were anesthetized with isoflurane and body temperature was maintained with a heating pad during surgery. The left kidney and ureter were exposed via a midline incision (2-3 cm). The ureter was ligated at two points proximal to kidney with 4-0 silk suture (Ethicon, Inc., Somerville, NJ). The ureter was then cut between the ligatures to prevent retrograde urinary tract infection. Rats in the control group underwent a sham laparotomy with ureteric manipulation through a midline incision. The abdominal incision was closed with uninterrupted suture (4-0 silk). Approximately 100  $\mu$ L of 0.25% marcaine was applied directly to the closed abdominal wall, and the skin was closed

with a 4-0 Vicryl synthetic absorbable suture (Ethicon, Inc., Somerville, NJ). Long-acting buprenorphine (1.0 mg/kg s.c.) was given for pain relief. To achieve a dose level of 10 mg/kg/day, IW-1973 was formulated at 83 mg/kg in open standard diet chow (Research Diets Inc., New Brunswick, NJ). All treatments were initiated immediately after animals recovered from anesthesia. Body weights were obtained prior to surgery and at 1 and 2 weeks postsurgery. Plasma, serum, and 24-h urine samples were collected at 1 and 2 weeks post-surgery. At the end of the study, animals were euthanized with CO<sub>2</sub> and the kidneys were removed. The right and left kidneys were weighed separately and cut sagittally with half snap frozen in liquid nitrogen and the other half fixed with 10% neutral buffered formalin. Urine samples were collected over 24 hours at 1 and 2 weeks post-surgery. Urine samples were centrifuged to remove debris and stored at -80°C until analysis. Whole blood was collected, and serum and plasma samples were prepared and stored at -80°C until analysis.

The hydroxyproline content of the left kidney (obstructed) was assayed using the Hydroxyproline Assay Kit (Sigma-Aldrich, St. Louis, MO). Briefly, 100 mg tissue was homogenized in 1 mL of water and transferred to a pressure-tight glass vial. HCl (37%) at a volume of 1 mL was then added to the glass vial. The tissue samples were hydrolyzed at 120°C for 20 h. Hydrolyzed samples and hydroxyproline standards (0-1.6 µg/well) were added to a 96-well plate, 30 µL per well. The 96-well plate was placed in a 60°C oven until all wells were evaporated to dryness. Chloramine T/Oxidation buffer mixture (100 µL) was added to each sample and standard well, and the plate was incubated at room temperature for 20 min. Diluted DMAB reagent was then added at a volume of 100 µL to each sample and standard well, and incubated at 60°C for 60 min.

The absorbance was measured at 560 nm.

Gene expression in the left kidney was measured according to the methods described in the DSS model above. Median fluorescence intensity (MFI) was generated for each gene target and normalized to the geometric mean expression of housekeeping genes (pol2ra and ppib), which were chosen to match the target transcript abundance. All data are expressed as mean  $\pm$  SEM. To determine the significant difference of the means across multiple groups a one-way ANOVA was conducted, followed by a Dunnett's multiple comparison test compared to the UO control. Statistical significance was indicated by a *P*-value of less than 0.05.

## RESULTS

### IW-1973 is an sGC stimulator that enhances NO signaling

IW-1973 (1,1,1,3,3,3-hexafluoro-2-(((5-fluoro-2-(1-(2-fluorobenzyl)-5-(isoxazol-3-yl)-1Hpyrazol-3-yl) pyrimidin-4-yl)amino)methyl)propan-2-ol), is an sGC stimulator from a novel pyrazolopyrimidine heterocyclic structural class (Fig. 1 A). IW-1973 stimulation of sGC as measured by cGMP production was determined using purified human recombinant  $\alpha 1\beta 1$  sGC (Fig. 1B). At a concentration of 30  $\mu$ M, IW-1973 alone increased cGMP 6-fold from baseline to a mean of 303 nM (95% CI, 196 - 469, n=5). In the presence of the NO donor DETA-NONOate (30  $\mu$ M), IW-1973 stimulated a concentration-dependent increase in the production of cGMP (geometric mean of EC<sub>50</sub> 267 nM [8 separate experiments]; 95% CI, 152.1 nM - 467.9 nM), with 30  $\mu$ M raising

cGMP levels to 3001 nM (95% CI, 2567-3510, n=5), a 10-fold increase relative to 30  $\mu$ M IW-1973 without NO (n=5) and 60-fold relative to baseline (without NO or IW-1973).

IW-1973 did not increase guanylate cyclase activity in heme-free sGC, generated through incubation of the recombinant enzyme with Tween 20. Furthermore, oxidation of sGC heme by treatment with ODQ reduced IW-1973-stimulated activity by approximately 60% (Figure 1C.) In contrast, the heme-independent sGC activator cinaciguat potently increased cGMP production under heme-free conditions (Fig. 1C).

The ability of IW-1973 to stimulate sGC and act in synergy with NO was further assessed in HEK-293 cells, which endogenously express sGC (Fig. 2A). In the absence of NO, IW-1973 stimulated a concentration-responsive increase in cGMP. With increasing concentrations of the NO donor DETA-NONOate, 30  $\mu$ M IW-1973 stimulated greater increases in cGMP production (96 nM cGMP for IW-1973 alone vs 431 nM cGMP for IW-1973 with 10  $\mu$ M DETA-NONOate), and progressively left-shifted the IW1973 concentration response EC<sub>50</sub> (by >54-fold with 10  $\mu$ M DETA-NONOate). In the presence of 10  $\mu$ M DETA-NONOate, IW-1973 stimulated sGC with an EC<sub>50</sub> of 197 nM (geometric mean of 11 independent experiments, 95% confidence interval of 94.4 nM to 411 nM).

NO-sGC-cGMP signaling induced by IW-1973 was further characterized in HEK-293derived GloSensor<sup>TM</sup> 40F cGMP cells by monitoring phosphorylation of VASP, a target of PKG. cGMP and pVASP were measured following treatment with IW-1973 alone and in combination with DETA-NONOate. The cGMP and pVASP response to IW-1973 (alone, no DETA-NONOate) is shown in Fig. 2B. Although both cGMP and pVASP



demonstrated a concentration response to IW-1973, near-complete phosphorylation of VASP (78%) was associated with effects on cGMP that were small (2%) relative to the maximal cGMP effect (determined with 1  $\mu$ M DETA-NONOate + 100  $\mu$ M IW-1973, data not shown).

### **IW-1973 pharmacokinetics and tissue distribution in rats**

The PK profile of IW-1973 was assessed in male and Sprague Dawley rats via i.v. (Fig. 3A) and p.o. routes (Fig. 3B). The median time to reach maximum concentration ( $T_{max}$ ) for IW-1973 was 8 h with a maximum concentration ( $C_{max}$ ) of 254 ng/mL; the estimated oral half-life was 9.2 h, with an estimated oral bioavailability of 102 %. Systemic clearance as determined from i.v. PK was 13.8 mL/min/kg and the steady state volume of distribution ( $V_{ss}$ ) was 10.5 L/kg.

Tissue distribution was assessed in the liver, heart, kidneys, and lungs of animals administered oral IW-1973 at 10 mg/kg once daily (QD) for 5 days. Tissue levels in the organs analyzed were greater than plasma levels, and the highest levels were observed in the liver (Table 1). These results were further verified by quantitative whole-body autoradiography (QWBA) (Banijamali, 2017).

### **IW-1973 reduced blood pressure in normotensive and hypertensive rats**

The effect of IW-1973 on systemic hemodynamics was tested in vivo in telemetered normotensive and spontaneously hypertensive rats. When administered orally QD for 4 days at doses of 0.3, 1, 3, and 10 mg/kg, IW-1973 invoked a dose-dependent reduction in MAP that was sustained for 6 h in both normotensive (Fig. 4A) and hypertensive (Fig. 4B) rats. Maximum changes in MAP were observed between 2 and 6 h after dosing. On

day 3 of dosing, significant effects on maximum change from baseline in MAP were observed at the 1, 3, and 10 mg/kg doses in both normotensive ( $n=6$  per group,  $p \leq 0.01$  for all doses) and hypertensive rats ( $n=6$  per group,  $p \leq 0.01$ ,  $p \leq 0.001$ , and  $p \leq 0.001$  respectively) compared to their respective vehicle controls (Fig. 4E). Heart rate was significantly increased versus controls on day 3 at the 0.3, 3, and 10 mg/kg doses in normotensive ( $p \leq 0.05$ ,  $p \leq 0.01$ , and  $p \leq 0.05$  respectively) rats, and at the 10 mg/kg dose in hypertensive ( $p \leq 0.05$ ) rat (Fig. 4F). The maximum absolute change in MAP was greater in SHR than in normotensive rats ( $25.2 \pm 0.8$  vs.  $11.9 \pm 0.9$  mm Hg at 10 mg/kg on day 3), while the maximum absolute change in HR was less in SHR compared to normotensive rats ( $71.4 \pm 11.4$  vs.  $39.1 \pm 13.7$  mm Hg at 10 mg/kg on day 3).

### **IW-1973 relaxes endothelium intact human resistance arteries**

The mechanism for blood pressure reduction was explored with human vascular rings in vitro. IW-1973 effected a concentration-dependent relaxation of endothelium intact human subcutaneous resistance arteries pre-contracted with U-46619 (contractions ranging from 8 to 22 g) with EC<sub>50</sub> values ranging from 4.9 to 402.7 nM and a maximum relaxation response of 93.2% to 96.8% (Supplemental Fig. 2) as calculated from the SNP-mediated maximum relaxation that ranged from 97 to 99% for each of the tissues tested.

### **Chronic effects of IW-1973 on hemodynamics, inflammation, proteinuria and renal fibrosis in the DSS model**

The DSS rat model of hypertension and heart failure (Geschka, 2011) was used to assess the effects of chronic IW-1973 treatment on body weight, hemodynamics, inflammation, proteinuria, and renal fibrosis. Animals on a high salt diet had a lower rate

of weight gain throughout the course of the study. There was no significant difference between the HS control group and IW-1973 or losartan treated animals until the 6<sup>th</sup> and final week of treatment (Supplemental Fig. 3)

Prior to the start of treatment, MAP was higher in rats in the HS control group than in the normal salt (NS) group ( $145.5 \pm 4.5$  millimeters mercury (mmHg) vs.  $113.25 \pm 1.75$  mmHg). Treatment with IW-1973 induced a dose-dependent decrease in MAP that began during the first week of dosing and was sustained for the 6 weeks of treatment (Fig. 5). The effect of IW-1973 at 3 mg/kg/day on MAP was comparable to the effect of losartan at 30 mg/kg/day throughout the study. HR remained unchanged throughout the course of the study (Supplemental Fig. 4).

After 6 weeks of treatment with IW-1973 or controls, proinflammatory cytokines were assessed in the serum of all groups. Levels of serum interleukin-6 (IL-6) were significantly greater in animals in the HS vehicle group compared to animals on a normal salt diet (Fig. 6A). Compared to the HS vehicle group, rats treated with 10 mg/kg/day IW-1973 had reduced levels of IL-6 ( $p \leq 0.01$  vs HS vehicle), that were similar to IL-6 levels in animals on the normal salt diet as well as those receiving losartan at 30 mg/kg day ( $p \leq 0.05$  vs HS vehicle).

Urinary protein was higher in animals receiving a HS diet than in those on a normal salt diet ( $p \leq 0.001$ ). Urinary protein was lower in both IW-1973-treated animals at 10 mg/kg/day and losartan-treated animals at 30 mg/kg/day than in HS vehicle animals ( $p \leq 0.05$  respectively) (Fig. 6B).

Renal gene expression of the fibrosis markers tumor growth factor- $\beta$ 1 (TGF $\beta$ 1) and collagen 1 $\alpha$ 1 (Col1 $\alpha$ 1) were higher in the HS vehicle group relative to the normal salt group ( $p \leq 0.001$ ) and expression of these genes were lower in the kidneys of rats treated with 3 and 10 mg/kg/day IW-1973 compared to the HS vehicle rats ( $p \leq 0.001$ ) as shown in Fig. 6B. At 3 mg/kg, IW-1973 was comparable to losartan which significantly decreased the expression of both genes ( $p \leq 0.001$  vs HS).

### **Effect of IW-1973 in LPS-induced inflammation model**

To further characterize effects on inflammation, IW-1973 was assessed in an acute mouse model of LPS-induced inflammation. Dexamethasone (5 mg/kg) was used as a positive control. At the 10 mg/kg IW-1973 dose, levels of pro-inflammatory cytokines IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were lower ( $p \leq 0.01$ ) as compared to vehicle treated mice. Levels of the anti-inflammatory cytokine interleukin-10 (IL-10) were increased, but not significantly so, compared to vehicle (Fig. 7).

### **Effects of IW-1973 in a rat UUO model of renal fibrosis**

The effects of IW-1973 were also examined in a non-hypertensive model of renal fibrosis. In this rat model, the ureter to the left kidney was ligated, resulting in fibrosis of the left kidney. In vehicle treated rats, UUO increased renal levels of the collagen protein marker hydroxyproline ( $p \leq 0.001$ ) and Col1 $\alpha$ 1 mRNA expression ( $p \leq 0.001$ ) and histopathological interstitial fibrosis ( $p \leq 0.001$ ). In rats treated with 10 mg/kg/day IW-1973 for 14 days following UUO, increases in hydroxyproline protein levels ( $p \leq 0.001$ ) and col1 $\alpha$ 1 mRNA expression ( $p \leq 0.05$ ) associated with UUO were attenuated (Fig. 8).

## DISCUSSION

IW-1973 is a clinical-stage small molecule sGC stimulator from a novel pyrazolopyrimidine heterocyclic structural class (Fig. 1). In vitro, IW-1973 demonstrated concentration-dependent stimulation of cGMP production by sGC in purified enzyme and whole-cell assays. Consistent with the definition of an sGC stimulator (Follmann, 2013), IW-1973 acted only on the reduced, heme-containing form of the enzyme, and demonstrated NO-independent stimulation of sGC and synergistic action with NO.

IW-1973 alone and in combination with an NO donor stimulated cGMP production in whole cells and triggered phosphorylation of VASP, a downstream target in the NO-sGC-cGMP-PKG signaling pathway. Interestingly, comparatively low levels of sGC stimulation were sufficient to induce near-maximal pVASP levels. Ex vivo, IW-1973 relaxed human subcutaneous resistance arteries, demonstrating that IW-1973 can act in concert with endogenous NO to elicit smooth muscle relaxation in systems with a functional endothelium. Together these results suggest that IW-1973 can act in systems with low and normal NO levels and that downstream signaling pathways can be activated even at relatively low levels of sGC stimulation.

In vivo, NO-sGC-cGMP signaling plays a central role in regulating vascular tone. Orally administered IW-1973 reduced blood pressure in normotensive rats and in two rat models of hypertension (SHR and DSS). The magnitude of the blood pressure effect was greater in hypertensive rats than in normotensive rats. Robust and dose-dependent blood pressure reduction in the model of salt-sensitive hypertension, a disease associated with endothelial dysfunction and impaired NO signaling (Bragulat and de las Sierra, 2002; Hoffmann, 2015b), suggests that sGC stimulators will provide benefit in

diseases with NO impairment. At a dose of 3 mg/kg/day IW-1973 produced sustained blood pressure reduction over 6 weeks that was comparable to losartan over the same time period. These results suggest that, like losartan, IW-1973 has a durable effect on blood pressure that is not attenuated by the development of tolerance, which limits chronic use of NO donor drugs.

Several lines of evidence suggest that NO signaling through sGC plays a role in suppressing inflammation. For example, leukocyte rolling and adhesion were increased 6-fold in eNOS<sup>-/-</sup> mice relative to WT controls, and both the sGC stimulator Bay 41-2272 and the NO donor DETA-NO restored leukocyte rolling and adhesion to WT levels (Ahluwalia, 2004). Furthermore, reduced NO has been shown to contribute to inflammation in adipose tissue in models of induced obesity (Handa, 2011). In the DSS model, IW-1973 treatment reduced serum levels of the pro-inflammatory cytokine IL-6. This finding, which is consistent with anti-inflammatory effects reported for sGC agonists in the DSS model (Geschka, 2011; Hoffmann, 2015b), prompted us to explore anti-inflammatory effects of IW-1973 in the LPS model, an acute inflammation model in normotensive mice. In this model, IW-1973 significantly reduced the pro-inflammatory cytokines IL-6 and TNF $\alpha$ , and though not significant, increased the anti-inflammatory cytokine IL-10. Our findings in the LPS model add to mounting evidence (Glynos, 2015; Ahluwalia, 2004; Handa, 2011) that sGC stimulators like IW-1973 have direct anti-inflammatory effects.

Consistent with other sGC agonists (Stasch, 2015), IW-1973 showed renoprotective effects in kidney disease models of different etiologies. In the DSS rat model, orally administered IW-1973 reduced proteinuria and renal expression of the pro-fibrotic

cytokine TGF $\beta$  and Col1 $\alpha$ 1, the major component of Type I collagen. Renal histological studies in the DSS model revealed renoprotective effects of IW-1973 at doses as low as 1 mg/kg, including effects on glomerulosclerosis, interstitial fibrosis, interstitial inflammation, and tubular damage (Shea, 2015). The renoprotective effects of IW-1973 were also explored in the UUO model of renal fibrosis. Compared to vehicle-treated animals, IW-1973-treated animals had significantly reduced kidney levels of Col1 $\alpha$ 1 gene expression and hydroxyproline. These results are consistent with antifibrotic effects reported for other sGC agonists as well as for other effectors of the cGMP pathway in the kidney and other organs (Sandner and Stasch, 2017). In addition to IW-1973's effects in the kidney, we have recently shown that IW-1973 has anti-inflammatory and anti-fibrotic activity in the liver in a model of non-alcoholic steatohepatitis at doses that do not affect blood pressure (Flores, 2017).

IW-1973 exhibited a PK profile in the rat that includes high oral bioavailability (>90%), a large volume of distribution and moderate clearance. Clinical studies suggest that predictions of once daily dosing from preclinical models translates to the humans (Hanrahan, 2017). Mass balance studies performed in rats revealed that IW-1973 was primarily cleared by the liver (Zimmer, 2017). IW-1973 extensively distributed to tissues, with high levels observed in the liver, kidney, heart, and lung relative to the vascular compartment. A whole-body autoradiography study in rats also revealed high levels of IW-1973 in adipose tissue and skeletal muscle (Banijamali, 2017) The differential in large tissue distribution coupled with the high protein binding of IW-1973 leads to increased extravascular tissue access and sGC stimulation while plasma levels remain at low levels. The potential clinical significance of higher levels of compound in target

tissues like liver and kidney may be to maximize the extravascular effects on inflammation and fibrosis relative to vascular, blood pressure effects.

Reduced NO signaling resulting from increased generation of reactive oxygen species, endothelial damage, and/or NOS impairment can lead to endothelial dysfunction, fibrosis, and or inflammation. sGC stimulators are a relatively new class of NO-sGC-cGMP pathway modulators. Because sGC stimulators bind directly to sGC to amplify NO signaling and increase local cGMP production, they offer a unique opportunity to treat hemodynamic dysregulation, inflammation, and fibrosis by restoring physiological function (Figure 9). We have shown that IW-1973 can positively impact each of these pathologies in animal models. The capacity of IW-1973 to restore normal hemodynamic function, and suppress inflammation and fibrosis suggests that its pharmacologic utility may not be limited only to diseases associated with reduced NO signaling, but that it has potential for treating or preventing diseases independent of NO. Indeed, the extensive tissue distribution of IW-1973 may afford an opportunity to exert pharmacological effects on inflammatory cells such as macrophages, neutrophils, and lymphocytes, pro-fibrotic fibroblasts and hepatic stellate cells, in target organs including the kidney.

In summary, IW-1973 is an orally active sGC stimulator that reduces blood pressure and exhibits antifibrotic, anti-inflammatory, and renoprotective effects in animal models of hypertension, inflammation, and kidney disease. These animal models suggest that IW-1973's extensive distribution to tissues may allow for beneficial effects on organ fibrosis, inflammation, and function at doses associated with little to no effects on blood pressure. IW-1973's with high oral bioavailability and a long pharmacokinetic half-life



that translated into PK consistent with once-daily dosing in humans. Indeed, in a Phase 1 study in healthy volunteers, QD dosing of IW-1973 elicited not only dose-related increases in plasma cGMP but also reductions in blood pressure that were sustained through 24 hours postdose (Hanrahan, 2017). Clinical studies will be required to determine whether IW-1973's ability to enhance NO-sGC-cGMP signaling offers the potential to treat diseases associated with impaired NO signaling as well as diseases involving inflammation and fibrosis. The preclinical pharmacology of IW-1973 supports clinical studies in patients with diabetes and hypertension and its current clinical investigation for treatment of heart failure with preserved ejection fraction and diabetic nephropathy.

### **Authorship Contribution**

Participated in research design: DZ, KT, ATP, GTM, MGC, JLM

Conducted experiments: JVT, CS, PG, SGB, GL, KL, JM, SR, SJ, KS, JW, RS, ARB

Contributed new reagents analytic tools: G-YJI, JS, JDM

Performed Data Analysis: JVT, DZ, CS, PG, SGB, GL, KL, JM, SR, SJ, KS, JW, RS, ARB

Wrote or contributed to the writing of the manuscript: JVT, DZ, CS, PG, SGB, GL, JM, SJ, KS, JW, RS, ARB, ATP, GTM, JLM

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## Footnotes

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## FIGURE LEGENDS

**Figure 1.** (A) Chemical structure of IW-1973, an sGC stimulator from a novel pyrazolopyrimidine heterocyclic class of compounds. (B) Representative curve showing stimulation of human recombinant  $\alpha 1\beta 1$  sGC with IW-1973 in the absence and presence of the NO donor DETA-NONOate (30  $\mu$ M). Data are presented as mean + SEM, n=5 per group. (C) cGMP production of IW-1973 and cinaciguat using heme-dependent and independent sGC enzyme, data represented as mean of % cGMP response  $\pm$  SEM, n = 3-5.

**Figure 2.** (A) Stimulation of endogenous sGC in HEK-293 cells by IW-1973 (30 pM-30  $\mu$ M) in the absence of the NO donor DETA-NONOate and in the presence of varying concentrations of DETA-NONOate (0.12 – 10  $\mu$ M). Data are presented as mean  $\pm$  SEM, n=2 per group. (B) cGMP and pVASP concentration response relationship to IW-1973 (without DETA-NONOate) in HEK-293 GloSensor™ 40F cGMP cells. cGMP levels and pVASP(Ser239)/VASP levels were determined by CisBio HTRF assays. For plotted cGMP and pVASP, each data point represents three independent cellular assays.

**Figure 3.** Pharmacokinetic profile of IW-1973 in male Sprague-Dawley rats following a single IV administration of IW-1973 (0.3mg/kg in 60% PEG 400/ 40% water) (A) and an oral administration of IW-1973 (3 mg/kg in PEG 400) (B). Data are presented as mean  $\pm$  95% CI, n = 6.

**Table 1.** Tissue-to-plasma ratios (mean  $\pm$  SEM) of IW-1973 were determined from whole organ analysis performed 2 h after final dose in male Sprague-Dawley rats



administered oral IW-1973 QD for 5 days. Data are presented as tissue / plasma ratio  $\pm$  SEM, n=6.

**Figure 4.** Mean arterial pressure and heart rate in normotensive Wistar (4A and C respectively) and SHR (4B and D respectively) measured by telemetry for 1 day before dosing (baseline) and continuing for 3 days of QD administration of vehicle or IW-1973 (0.3 – 10 mg/kg/day) by oral gavage for a total of 4 days. Data are plotted as mean + SEM over time, n= 6 per group. (4E) Data from 4A and 4B are plotted as peak change from 24-hr baseline in MAP  $\pm$  SEM following the day 3 dose (48-72 h). (4F) Data from 4C and 4D are plotted as peak change from 24-hr baseline in HR  $\pm$  SEM following the day 3 dose. Significant changes in MAP and HR compared to the vehicle for the respective rat strains was determined by two-way ANOVA followed by a Dunnett's post-test, \*p  $\leq$  0.05, \*\*p  $\leq$  0.01, \*\*\*p  $\leq$  0.001.

**Figure 5.** MAP in DSS rats throughout 6 weeks of treatment with vehicle, IW-1973 (1, 3, and 10 mg/kg, in chow), or losartan (30 mg/kg, in drinking water), shown as average MAP  $\pm$  SEM (mmHg), n= 8 per group. Rats were provided normal-salt diet or high-salt diet for 2 weeks before initiation of and during treatment with vehicle, IW-1973, or losartan. Baseline measures were determined 1-3 days before initiation of treatment.

**Figure 6.** Serum IL-6 (A) and urinary protein (protein (g)/creatinine (g)) and fibrotic gene expression (normalized to geometric mean of housekeeping genes hprt1, ppib) in kidneys (B) in the DSS rat model. All data are plotted as mean + SEM. N = 8 / group. All measures were taken after 6 weeks of treatment with IW-1973 or controls,

significance relative to the HS vehicle group was determined by one-way ANOVA followed by Dunnett's post-test, \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .

**Figure 7.** Effect of IW-1973 on levels of the pro-inflammatory cytokines, IL-6 and TNF $\alpha$ , and on the anti-inflammatory cytokine, IL-10. C57Bl/6 male mice were dosed with vehicle, dexamethasone, or IW-1973 followed by LPS challenge to induce inflammation. Serum was collected 2 h post-induction, and samples were analyzed for cytokine concentrations. Data are presented as concentration (mean + SEM) of cytokine in pg/ml,  $n = 10$  per group, significance was determined one-way ANOVA followed Fisher's least square difference post-hoc analysis, compared to vehicle, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ .

**Figure 8.** Effects of IW-1973 (10 mg/kg/day in chow) on renal markers of fibrosis in the rat UUO model. Hydroxyproline levels (mean + SEM,  $n=8$ ) are reported as  $\mu\text{g/ml}$  of tissue, and col1 $\alpha$ 1 mRNA expression data are reported as ratio of Col1 $\alpha$ 1 to the housekeeping genes polr2a and ppib. Significance relative to vehicle control UUO animals was determined one-way ANOVA followed by Dunnett's post-test, \* $p \leq 0.05$ , \*\*\*  $p \leq 0.001$ .

### **Figure 9. Model for mechanism of action of IW-1973**

During healthy function, NO binds to sGC, triggering the conversion of GTP to cGMP. The cGMP-PKG signaling pathway maintains normal physiological function primarily through regulation of vascular tone and control of local blood flow. In diseases associated with oxidative stress (from increased reactive oxygen species [ROS]) and endothelial dysfunction, NO-sGC-cGMP signaling is disrupted. Consequently, local

regulation of blood flow is impaired, and fibrosis, inflammation, vascular leakage, leukocyte infiltration, and abnormal vascular proliferation may be found. IW-1973 binds to sGC and acts synergistically with available NO to amplify NO-cGMP signaling and restore physiological function. Even when NO levels are normal, sGC stimulation by IW1973 may be useful for treating diseases involving reduced peripheral blood flow, inflammation, and/or fibrosis.

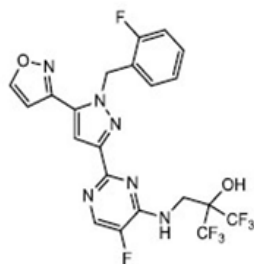
## TABLES

**Table 1. Tissue to Plasma Ratios of IW-1973**

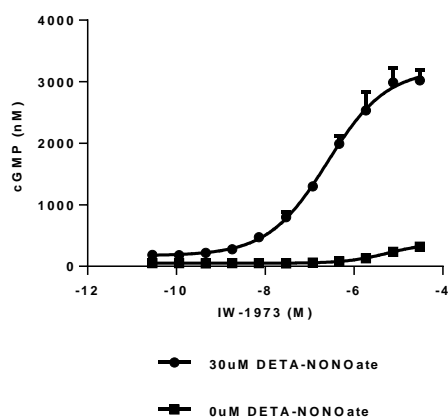
	Liver	Heart	Kidney	Lung
IW-1973 tissue/plasma	19.9 ± 1.3	4.7 ± 0.5	5.9 ± 2.0	7.6 ± 0.7

## Figures

A.



B.



C.

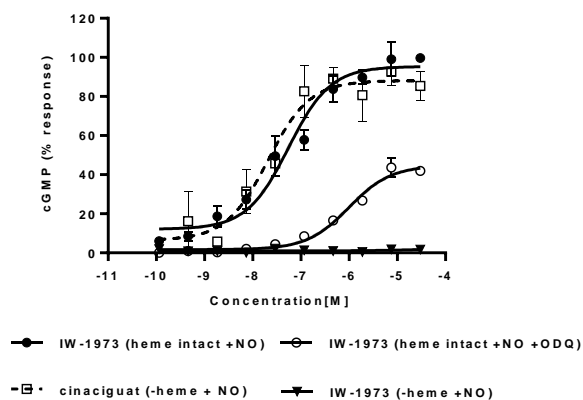
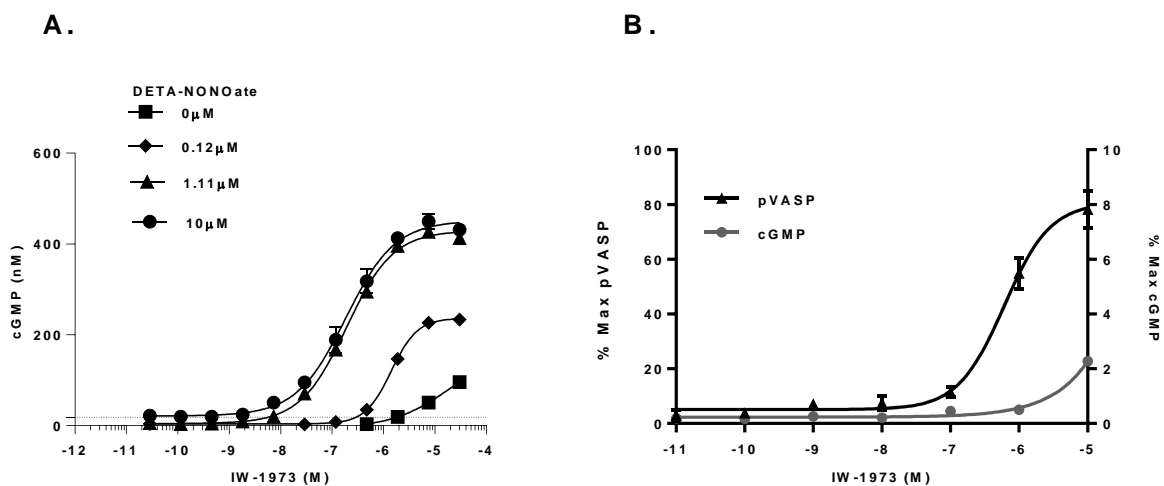
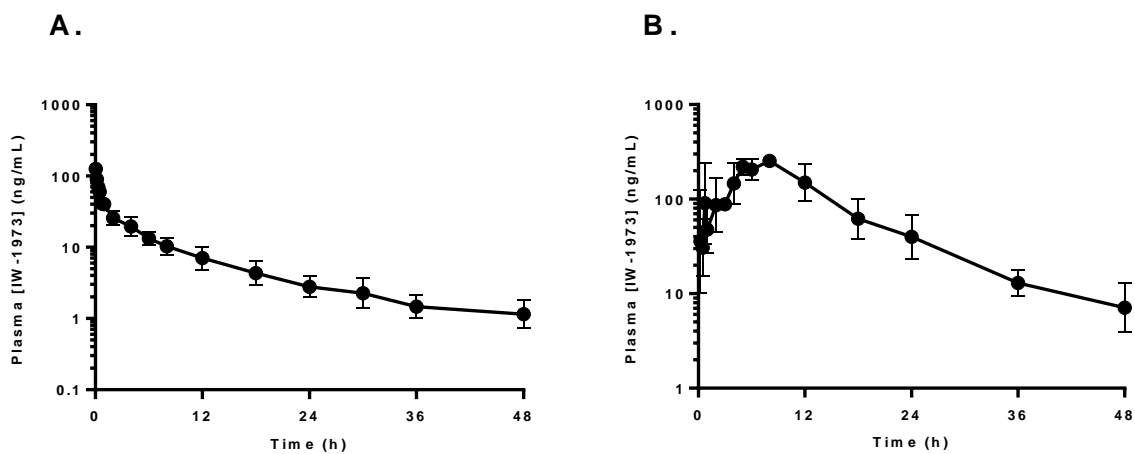


Figure 1



**Figure 2.**



**Figure 3.**

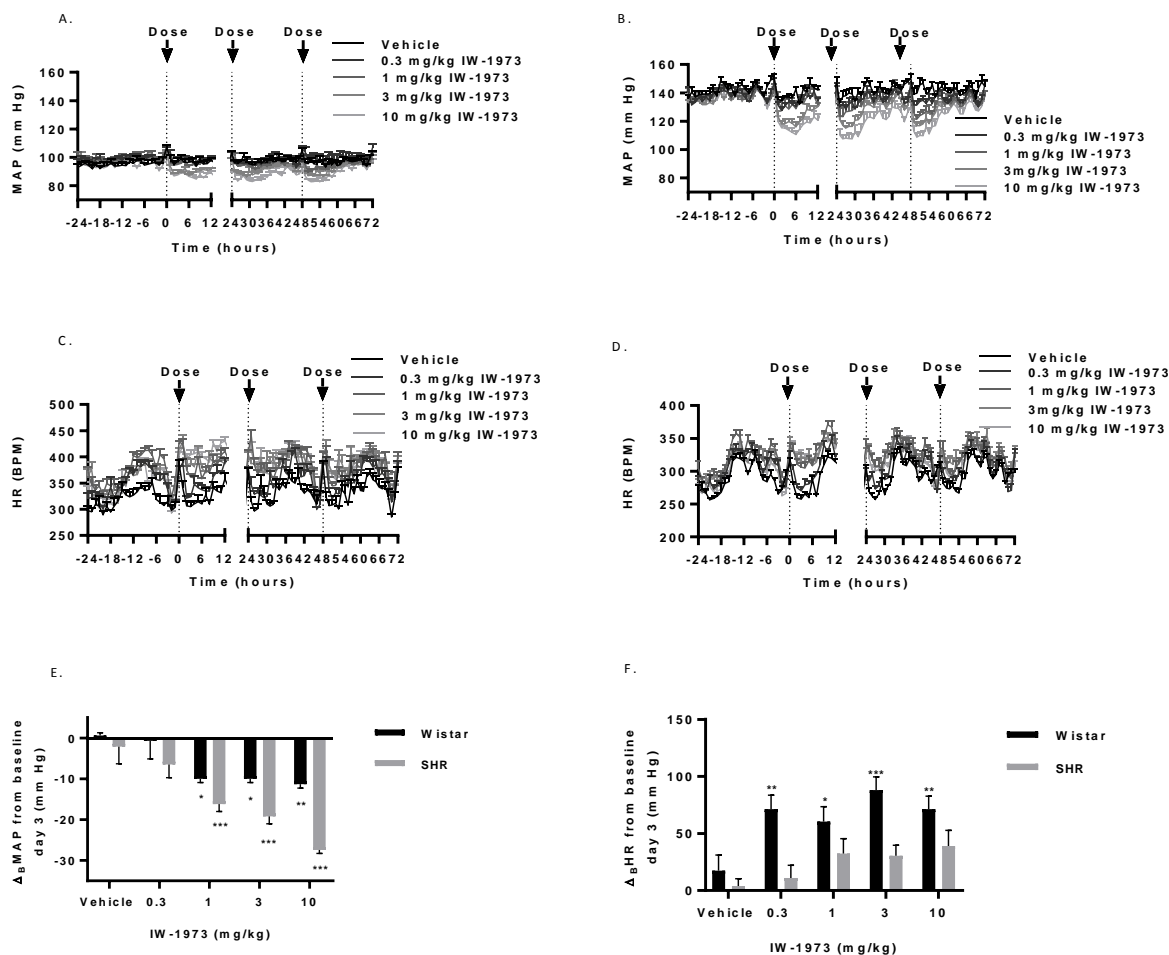


Figure 4.



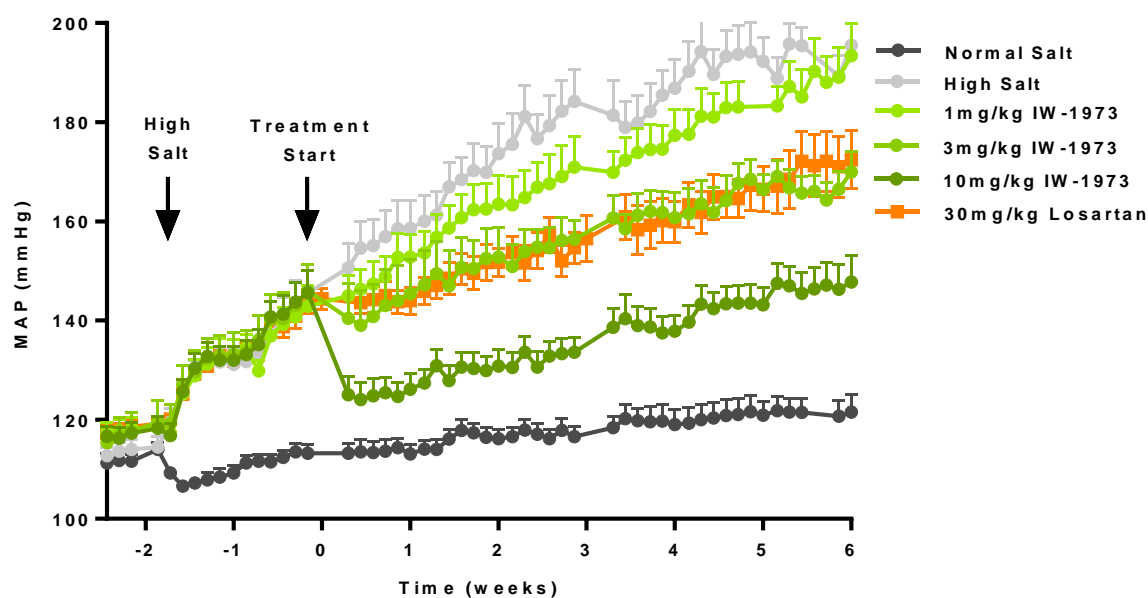
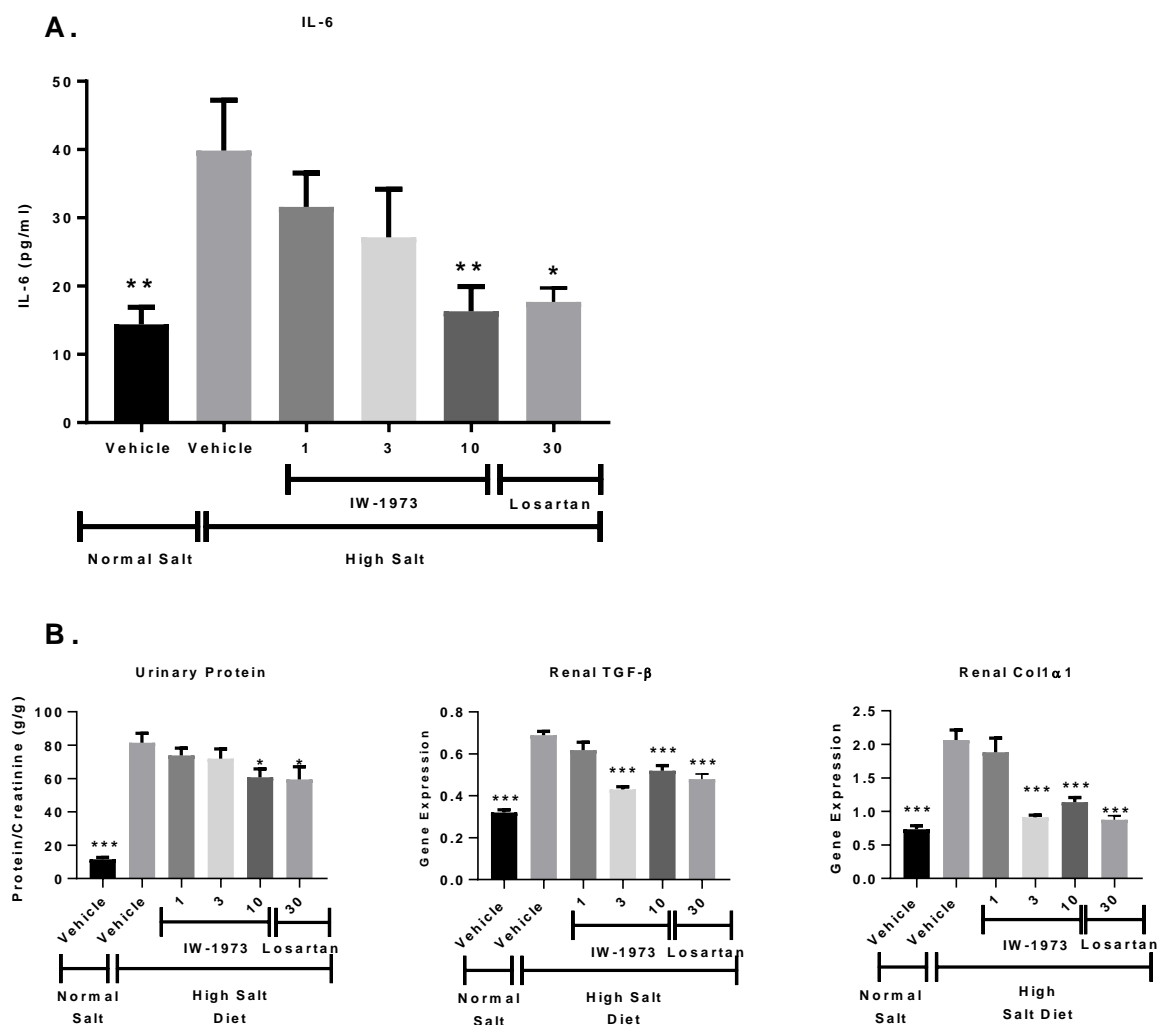
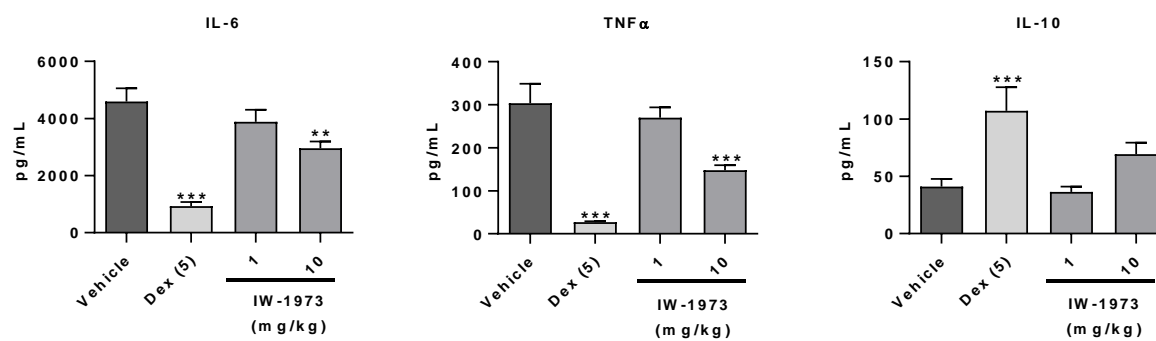


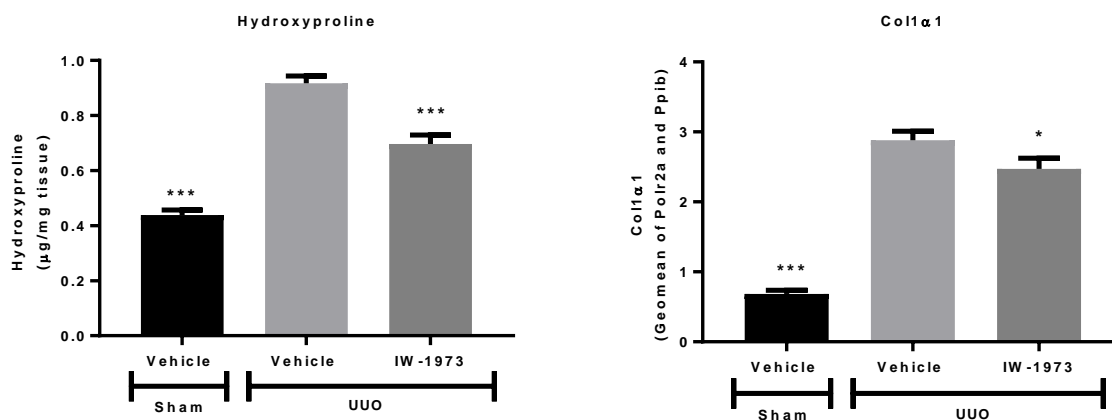
Figure 5.



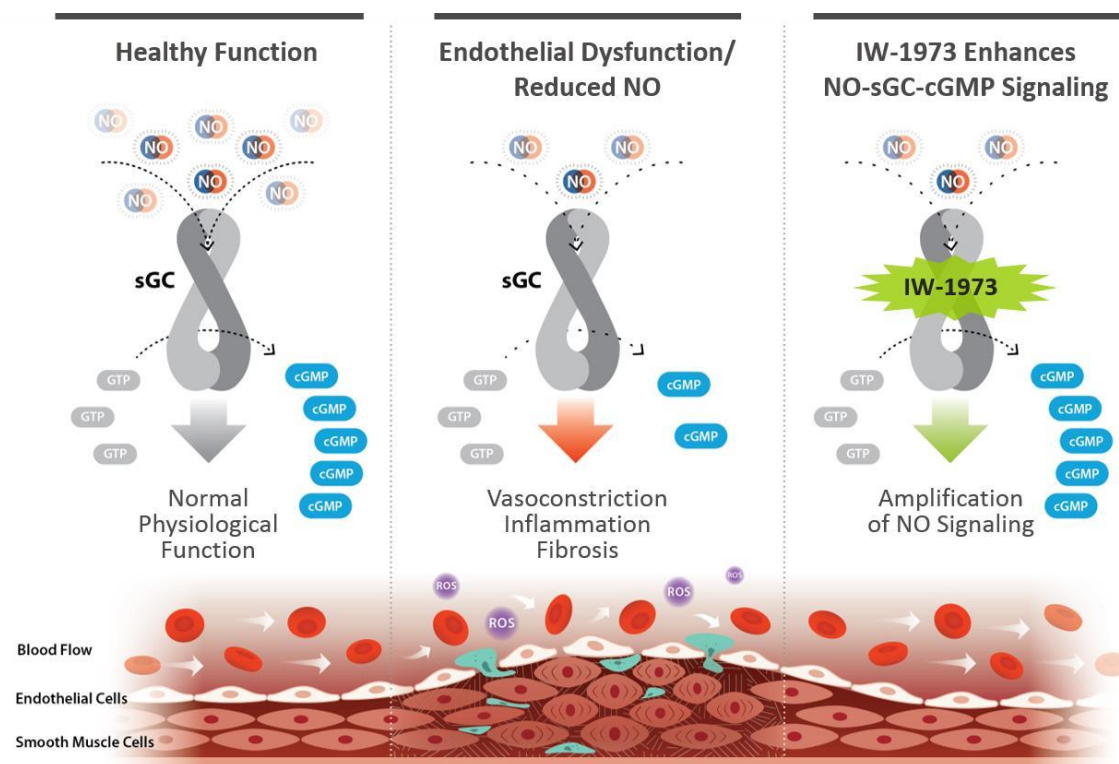
**Figure 6.**



**Figure 7.**



**Figure 8.**



**Figure 9.**

**1.) TITLE PAGE**

**PHARMACOLOGICAL CHARACTERIZATION OF IW-1973, A NOVEL SOLUBLE  
GUANYLATE CYCLASE STIMULATOR WITH EXTENSIVE TISSUE  
DISTRIBUTION, ANTI-HYPERTENSIVE, ANTI-INFLAMMATORY, AND ANTI-  
FIBROTIC EFFECTS IN PRECLINICAL MODELS OF DISEASE**

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Guang Liu, Kim Long, Joy Miyashiro, Sheila Ranganath, Sarah Jacobson, Kim Tang, G-  
Yoon Jamie Im, James Sheppeck II, Joel D. Moore, Kristine Sykes, James Wakefield,  
Renee Sarno, Ali R. Banijamali, Albert T. Profy, G. Todd Milne, Mark G. Currie, and  
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PG, SGB, GL, JM, SJ, G-YJI, JS, KS, JW, RS, ARB, ATP, GTM, MGC, JLM

**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental figure 1.** (A) LC-MS/MS trace of the cGMP measurement with internal standard, ion mass (m/z) for cGMP internal standard were 346 > 152 and 349 > 155 respectively with a retention time of 0.62 m for both (B) representative cGMP standard curve representing duplicate measurements from the beginning and end of the LC-MS/MS run.

**Supplemental figure 2.** IW-1973 induces relaxation of human subcutaneous resistance arteries, each data point represents the concentration dependent % relaxation of one of three tissues from human donors. The dashed line represents the nonlinear curve fit of the average data from which an EC<sub>50</sub> can be derived.

**Supplemental figure 3.** Body weight in DSS rats throughout 6 weeks of treatment with vehicle, IW-1973 (1, 3, and 10 mg/kg, in chow), or losartan (30 mg/kg, in drinking water), shown as change in weight  $\pm$  SEM (g) from start of treatment at week 0, n= 8 per group. Rats were provided normal-salt diet or high-salt diet for 2 weeks before initiation of and during treatment with vehicle, IW-1973, or losartan. Significance was determined by two-way ANOVA followed by a Tukey's post-hoc test for multiple comparisons as compared to the high salt group, \*p  $\leq$  0.05, \*\*p  $\leq$  0.01, and \*\*\*p  $\leq$  0.001.

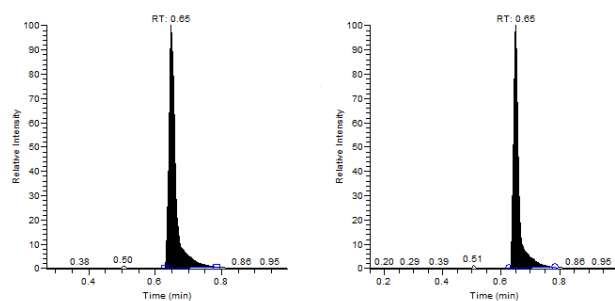
**Supplemental figure 4.** HR in DSS rats throughout 6 weeks of treatment with vehicle, IW-1973 (1, 3, and 10 mg/kg, in chow), or losartan (30 mg/kg, in drinking water), shown as average HR $\pm$  SEM (BPM), n= 8 per group. Rats were provided normal-salt diet or

high-salt diet for 2 weeks before initiation of and during treatment with vehicle, IW-1973, or losartan. Baseline measures were determined 1-3 days before initiation of treatment.

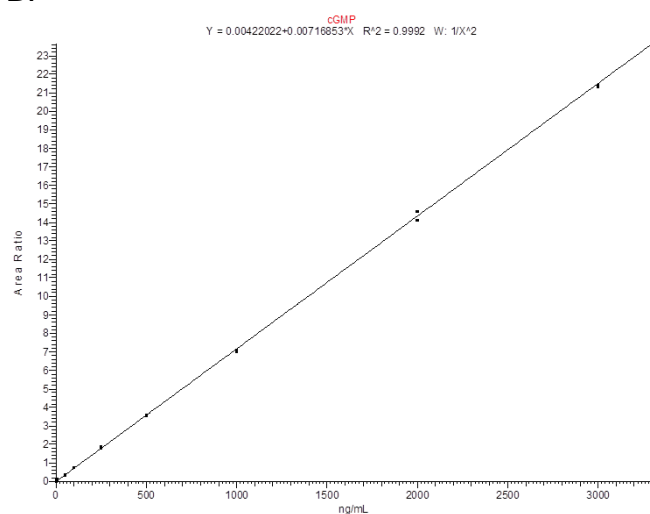


## Supplemental Figures

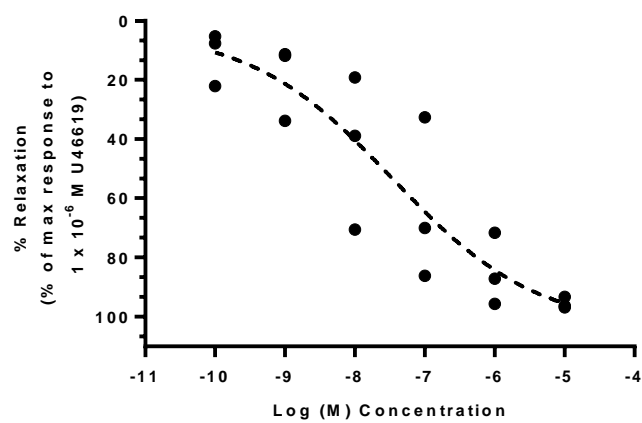
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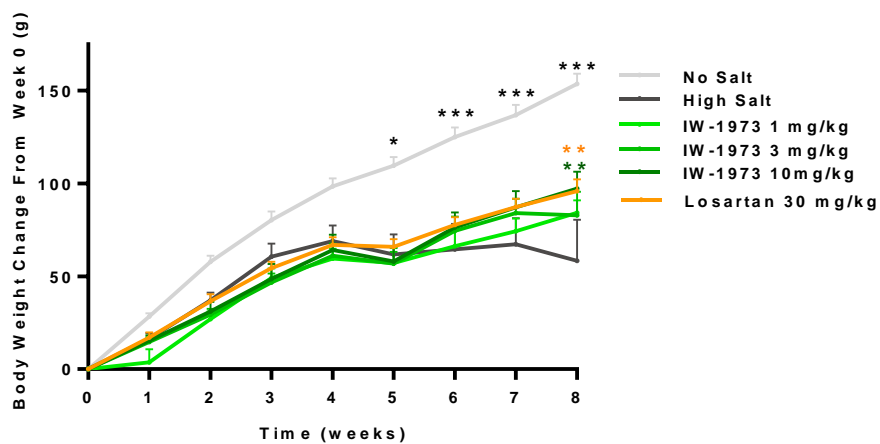
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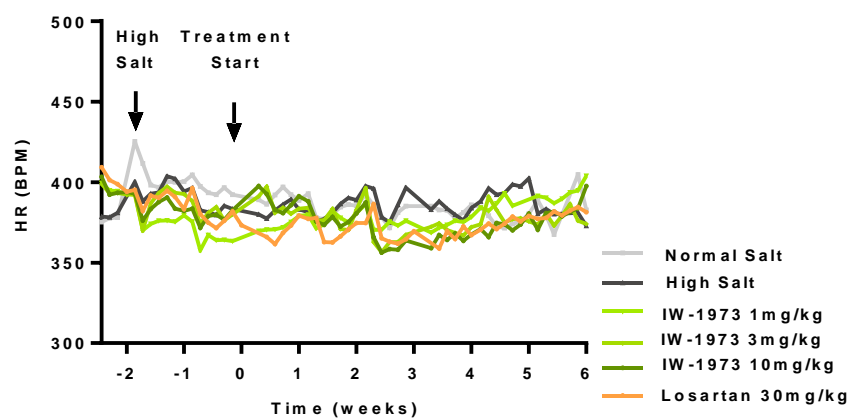
Supplemental Figure 1



**Supplemental Figure 2.**



Supplemental Figure 3.



**Supplemental Figure 4.**