

Pharmacological Characterization of IW-1973, a Novel Soluble Guanylate Cyclase Stimulator with Extensive Tissue Distribution, Antihypertensive, Anti-Inflammatory, and Antifibrotic Effects in Preclinical Models of Disease[§]

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Received December 21, 2017; accepted March 20, 2018

ABSTRACT

Soluble guanylate cyclase (sGC), a key signal-transduction enzyme, increases the conversion of guanosine-5'-triphosphate to 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. Soluble guanylate cyclase (sGC) stimulators are small-molecule drugs that bind sGC and enhance NO-mediated cGMP signaling. The pharmacological characterization of IW-1973 [1,1,1,3,3,3-hexafluoro-2-(((5-fluoro-2-(1-(2-fluorobenzyl)-5-(isoxazol-3-yl)-1H-pyrazol-3-yl) pyrimidin-4-yl)amino)methyl)propan-2-ol], 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, IW-1973 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. 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. The results were affirmed in mouse lipopolysaccharide-induced inflammation and rat unilateral ureteral obstruction renal fibrosis models. A quantitative whole-body autoradiography study of IW-1973 revealed extensive tissue distribution and 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 exhibits 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-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-5'-triphosphate to cGMP. cGMP in

turn binds to and modulates the activity of downstream targets including protein kinase G, cyclic nucleotide-gated ion channels, and phosphodiesterases (PDEs) (Derbyshire and Marletta, 2012).

sGC is expressed in many cells and tissues (Budworth et al., 1999), but its role in vascular smooth muscle is the best understood. In response to vascular shear stress, endothelial NO synthase 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 and smoking) and disease states

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<https://doi.org/10.1124/jpet.117.247429>.

[§] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: ANOVA, analysis of variance; CI, confidence interval; DETA-NONOate, diethylenetriamine NONOate; DSS, Dahl salt sensitive; HBSS, Hanks' balanced salt solution; HR, heart rate; HS, high salt; HTRF, homogeneous time-resolved fluorescence; IBMX, 3-isobutyl-1-methylxanthine; IL-6, interleukin 6; IW-1973, 1,1,1,3,3,3-hexafluoro-2-(((5-fluoro-2-(1-(2-fluorobenzyl)-5-(isoxazol-3-yl)-1H-pyrazol-3-yl) pyrimidin-4-yl)amino)methyl)propan-2-ol; LPS, lipopolysaccharide; MAP, mean arterial pressure; NO, nitric oxide; NS, normal salt; PDE, phosphodiesterase; PK, pharmacokinetic; pVASP, phosphorylated vasodilator-stimulated phosphoprotein; secondGC, second guanylate cyclase; sGC, soluble guanylate cyclase; SHR, spontaneously hypertensive rat; UUO, unilateral ureteral obstruction; VASP, vasodilator-stimulated phosphoprotein.

(e.g., diabetes, heart failure, hypertension, and pulmonary hypertension) are associated with impaired NO signaling and endothelial dysfunction (Münzel et al., 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 of 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 (Baliga et al., 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 et al., 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 et al., 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 et al., 2013).

The first sGC stimulator described was YC-1, a compound shown to increase platelet cGMP in vitro (Ko et al., 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 (Stasch et al., 2011, 2015; Gheorghide et al., 2013; Glynos et al., 2015; Sandner and Stasch, 2017). In addition, sGC stimulators have demonstrated anti-inflammatory (Glynos et al., 2015), antifibrotic (Sandner and Stasch, 2017), and beneficial metabolic effects (Hoffmann et al., 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 physiologic 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 [1,1,1,3,3,3-hexafluoro-2-((5-fluoro-2-(1-(2-fluorobenzyl)-5-(isoxazol-3-yl)-1H-pyrazol-3-yl)pyrimidin-4-yl)amino)methyl)propan-2-ol], a novel sGC stimulator in clinical development with potential as a treatment of 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 Association for Assessment and Accreditation of Laboratory Animal Care-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-hour 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-hour light-dark cycle (6 AM to 6 PM). All animals were allowed free access to water and standard rodent chow (PicoLab Rodent Diet 20, LabDiet, St. Louis MO) or special chow. All animals were acclimated to the facility for at least 2 to 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 subsequently. 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). A solution containing 12.5 ng/ml human sGC, 30 μ M diethylenetriamine NON-Oate (DETA-NONOate) (Enzo Life Sciences), 100 mM Tris (pH 7.4), 4 mM $MgCl_2$, 2 mM dithiothreitol, and 0.05% bovine serum albumin was allowed to equilibrate to 37°C for 10 minutes in a prewarmed assay plate. For each test concentration, IW-1973 was diluted in dimethylsulfoxide to 33.3 \times its assay concentration, and then added to the assay plate. In some experiments, Tween 20 or 1H-[1,2, 4]-oxadiazolo[4,3,-a]quinoxalin-1-one was added to this stock. Removal of the heme group by the nonionic 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 et al., 2002). Guanosine-5'-triphosphate was added to the assay plate to a final concentration of 300 μ M. Assay plates were incubated at 37°C for 20 minutes 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 the manufacturer's instructions.

A cGMP standard curve was fit using a four-parameter equation [log(inhibitor) vs. response – variable slope] with a 95% confidence interval (CI) using GraphPad Prism software version 6 (Graphpad Software, San Diego, CA). 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 four-parameter fit [log(agonist) vs. response – variable slope] and GraphPad Prism version 6. The EC₅₀, 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) were grown and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/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 µl medium at a density of 1.5×10^4 cells/well. Cells were incubated for 24 hours at 37°C in a humidified chamber supplemented with 5% CO₂. Medium was removed, and cells were washed once with 40 µl of Hanks' balanced salt solution (HBSS) with calcium and magnesium. Cells were then incubated with 40 µl of a solution containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in HBSS with calcium and magnesium for 15 minutes at 37°C. Then, 10 µl of 5× the indicated final concentration of IW-1973 (diluted from a 10 mM dimethylsulfoxide stock) with or without DETA-NONOate was added to the cells, which were then incubated for 20 minutes at 37°C. Assay buffer was then removed and 50 µl of ice-cold 10% acetic acid + 150 ng/ml internal standard cyclic ^{13,15}N₂-guanosine 3',5'-monophosphate (+3 cGMP) was added to each well. Samples were incubated on ice for 30 minutes. Following centrifugation at 4°C for 5 minutes at 1000g 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–tandem mass spectrometry. cGMP concentrations were determined using a standard curve (Supplemental Fig. 1, A and B). Concentration-response data were fit using a four-parameter fit [log(agonist) vs. response – variable slope] with a 95% CI using GraphPad Prism version 5. The EC₅₀ was interpolated from the curve fit.

cGMP and Vasodilator-Stimulated Phosphoprotein (VASP) Phosphorylation. HEK-293 cells expressing GloSensor 40F cGMP (Promega, Madison, WI) were maintained in Dulbecco's modified of Eagle's medium (Corning, Corning, NY) supplemented with 10% fetal bovine serum (HycloneThermoFisher Scientific, Waltham, MA) and 200 µg/ml hygromycin (Invitrogen, Carlsbad, CA). 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, Franklin Lakes, NJ). Cells were incubated overnight at 37°C in a humidified chamber with 5% CO₂. 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 IBMX in HBSS with calcium and magnesium for 15 minutes at 37°C, followed by the addition of 10 µl of 10× the indicated final concentration of IW-1973 (diluted from a 10 mM dimethylsulfoxide stock) in the presence of varying concentrations of DETA-NONOate, and then incubated for an additional 20 minutes at 37°C. For detection of cGMP formation, cells were lysed with 50 µl per well of cGMP HTRF lysis buffer (Cisbio), and then assayed following the protocol of the HTRF cGMP assay kit (Cisbio). For detection of total VASP and phosphorylated VASP (pVASP) formation, cells were lysed with 50 µl per well of HTRF lysis buffer (Cisbio), and then assayed following the protocol of the total VASP (Cisbio) and pVASP Ser239 (Cisbio) 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 µM DETA-NONOate + 100 µM 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 µM) + DETA-NONOate (1 µM).

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 via bolus injection through a percutaneous

catheter in the tail or orally via gavage. IW-1973 was formulated in 60% polyethylene glycol 400/40% water at 0.3 mg/ml (0.3 mg/kg) for intravenous dosing and at 1 mg/ml (3 mg/kg) in 100% polyethylene glycol 400 for oral dosing. Chow was returned to animals 4 hours postdose.

Blood from rats dosed via intravenous injection was collected at 15 time points ranging from 0.033 to 48 hours postdose. Blood from orally dosed rats was collected at 15 time points ranging from 0.25 to 48 hours postdose. Samples were collected into K₂EDTA separator tubes and processed to plasma by centrifugation (3500 rpm for 10 minutes). Plasma samples were prepared by protein precipitation and analyzed using liquid chromatography–tandem mass spectrometry.

Tissue Distribution. Wistar rats (male, 250–275 g, *n* = 6; Harlan Laboratories) 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 with 0.2% Tween 80 in Milli-Q water. (Millipore Sigma, Burlington, MA) At 2 hours postdose on day 5, plasma was collected and animals were perfused with phosphate-buffered saline containing 1 mM IBMX and 1 U/ml heparin. Liver, heart, kidney, and lung samples were collected and homogenized. Plasma and tissue samples were analyzed for IW-1973 levels using liquid chromatography–tandem mass spectrometry.

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 µm wire running through the lumen of the artery. Changes in tension were recorded using an isometric transducer (Danish Myo Technology A/S, Aarhus, Denmark). The arteries were precontracted with the thromboxane mimetic, U46619, at a final concentration of 100 nM. Upon stabilization of U46619 contractile response, a single addition of acetylcholine was made for a final concentration of 10 µM to confirm functionality of the vascular endothelium including the presence of endogenous NO. Tissues that did not reach ≥50% relaxation in response to acetylcholine were excluded from the study. Tissues were then washed and contracted with U46619 at a final concentration of 100 nM, and an IW-1973 cumulative concentration-response curve (0.1 nM to 10 µM) was generated. At the end of the cumulative concentration-response curve, sodium nitroprusside was added for a final bath concentration of 100 µM to determine maximal tissue relaxation response. IW-1973-induced relaxation at each concentration was calculated as a percentage of the sodium nitroprusside-mediated maximum relaxation of U46619-induced contraction. A sigmoidal dose-response (variable slope) curve was fit with bottom values constrained to zero, and an EC₅₀ value was estimated from this fitted curve using GraphPad Prism version 6.

Blood Pressure. The Dataquest A.R.T. acquisition and analysis system (Data Sciences International, St. Paul, MN) was used to monitor and analyze hemodynamic data from conscious, freely moving rats surgically implanted with a telemetry pressure transmitter (PA-C40, Data Sciences International). 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) 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.) and the body of the telemetry transmitter was placed in the abdominal cavity and secured to the abdominal wall. Approximately 100 µl 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.). Buprenorphine (0.05 mg/kg/d, 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 Data Sciences International 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 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-hour pre-first dose average subtracted from the lowest MAP value measured within 2 hours of dosing. Maximum change from baseline of heart rate (HR) was calculated using the 3-hour pre-first dose average subtracted from the 1-hour postdose average. Area over the curve was calculated using GraphPad Prism version 5. The data are expressed as mean \pm S.E.M. To determine the significant difference of the mean values across the independent strains, maximum change from baseline in MAP at day 3, maximum change from baseline in HR at days 1 and 3, area over the curve at day 1, and total area over the curve were analyzed by two-way analysis of variance (ANOVA), and significance was determined by Dunnett's post-hoc analysis. Statistical significance was indicated by a *P* value of less than 0.05. The average of 60 minutes of data derived from the real-time raw data was used for analysis.

Dahl Salt-Sensitive (DSS) Rat Model. Telemetry transmitter implantations and data monitoring, acquisition, and analysis were performed as described previously using DSS rats (male, 230–270 g; Harlan Laboratories). 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/d) or, as a reference control, losartan (30 mg/kg/d equivalent administered in 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 phosphate-buffered saline) 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 histologic evaluation. At week 8, 24-hour 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 the 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 the 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 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-hour period. Urinary protein was determined using urine protein and urine creatinine. Analyses of individual serum samples for interleukin 6 (IL-6) were conducted using a multiarray assay kit with a proinflammatory panel (Meso Scale Discovery, Gaithersburg, MD), and data were interpolated from standard curve values using GraphPad Prism version 6. To determine

significant differences of mean values across multiple groups a one-way ANOVA was conducted, followed by Dunnett's multiple comparisons test versus the HS group. Statistical significance was indicated by a *P* value of 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 (by mouth), dexamethasone (5 mg/kg, i.p.), or IW-1973 (1 or 10 mg/kg, by mouth). IW-1973 was formulated in 0.5% methylcellulose added to 1% hydroxypropyl methylcellulose 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 phosphate-buffered saline via intravenous injection. At 2 hours post-LPS dose, mice were euthanized via CO_2 inhalation and blood was collected via cardiac puncture. Serum was prepared and stored at -80°C . Cytokine levels were determined using cytometric bead array analysis kits (BD) in accordance with the manufacturer's protocol. A single analysis was performed for each sample. Data from cytometric bead array analysis kits were analyzed using flow cytometric analysis program 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, version 6. All data are expressed as mean \pm S.E.M. To account for variance between groups, significance was determined by one-way ANOVA followed by 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 (UO) Model. UO surgeries were performed on male Sprague-Dawley rats (male, 230–270 g; Harlan Laboratories) 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 to 3 cm).

The ureter was ligated at two points proximal to kidney with 4-0 silk suture (Ethicon, Inc.). 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 μl 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.). Long-acting buprenorphine (1.0 mg/kg, s.c.) was given for pain relief. To achieve a dose level of 10 mg/kg/d, IW-1973 was formulated at 83 mg/kg in open standard diet chow (Research Diets Inc.). 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-hour urine samples were collected at 1 and 2 weeks postsurgery. At the end of the study, animals were euthanized with CO_2 and the kidneys were removed. The right and left kidneys were weighed separately and cut sagittally, with one-half snap frozen in liquid nitrogen and one-half fixed with 10% neutral buffered formalin. Urine samples were collected over 24 hours at 1 and 2 weeks postsurgery. 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). 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 hours. Hydrolyzed samples and hydroxyproline standards (0–1.6 $\mu\text{g}/\text{well}$) 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. A 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 minutes. Diluted

p-dimethylaminobenzaldehyde (DMAB) reagent was then added at a volume of 100 μ l to each sample and standard well, and incubated at 60°C for 60 minutes. The absorbance was measured at 560 nm.

Gene expression in the left kidney was measured according to the methods described previously for the DSS model. Median fluorescence intensity 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 S.E.M. To determine the significant difference of the mean values across multiple groups a one-way ANOVA was conducted, followed by Dunnett's multiple comparisons test compared with the UUO control. Statistical significance was indicated by a *P* value of less than 0.05.

Results

IW-1973 is a sGC Stimulator that Enhances NO Signaling. IW-1973 is a sGC stimulator from a novel pyrazolopyrimidine heterocyclic structural class (Fig. 1A). IW-1973 stimulation of sGC as measured by cGMP production was determined using purified human recombinant α 1 β 1 secondGC (Fig. 1B). At a concentration of 30 μ 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 μ M), IW-1973 stimulated a concentration-dependent increase in the production of cGMP

[geometric mean of EC₅₀ 267 nM (eight separate experiments); 95% CI, 152.1–467.9 nM], with 30 μ M raising cGMP levels to 3001 nM (95% CI, 2567–3510, *n* = 5), a 10-fold increase relative to 30 μ 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 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one reduced IW-1973-stimulated activity by approximately 60% (Fig. 1C.) In contrast, the heme-independent sGC activator cinaciguat potentially 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 μ 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 μ M DETA-NONOate), and progressively left shifted the IW-1973 concentration-response EC₅₀ (by >54-fold with 10 μ M DETA-NONOate). In the presence of 10 μ M DETA-NONOate, IW-1973 stimulated sGC with an EC₅₀ of 197 nM (geometric mean of 11 independent experiments, 95% CI of 94.4–411 nM).

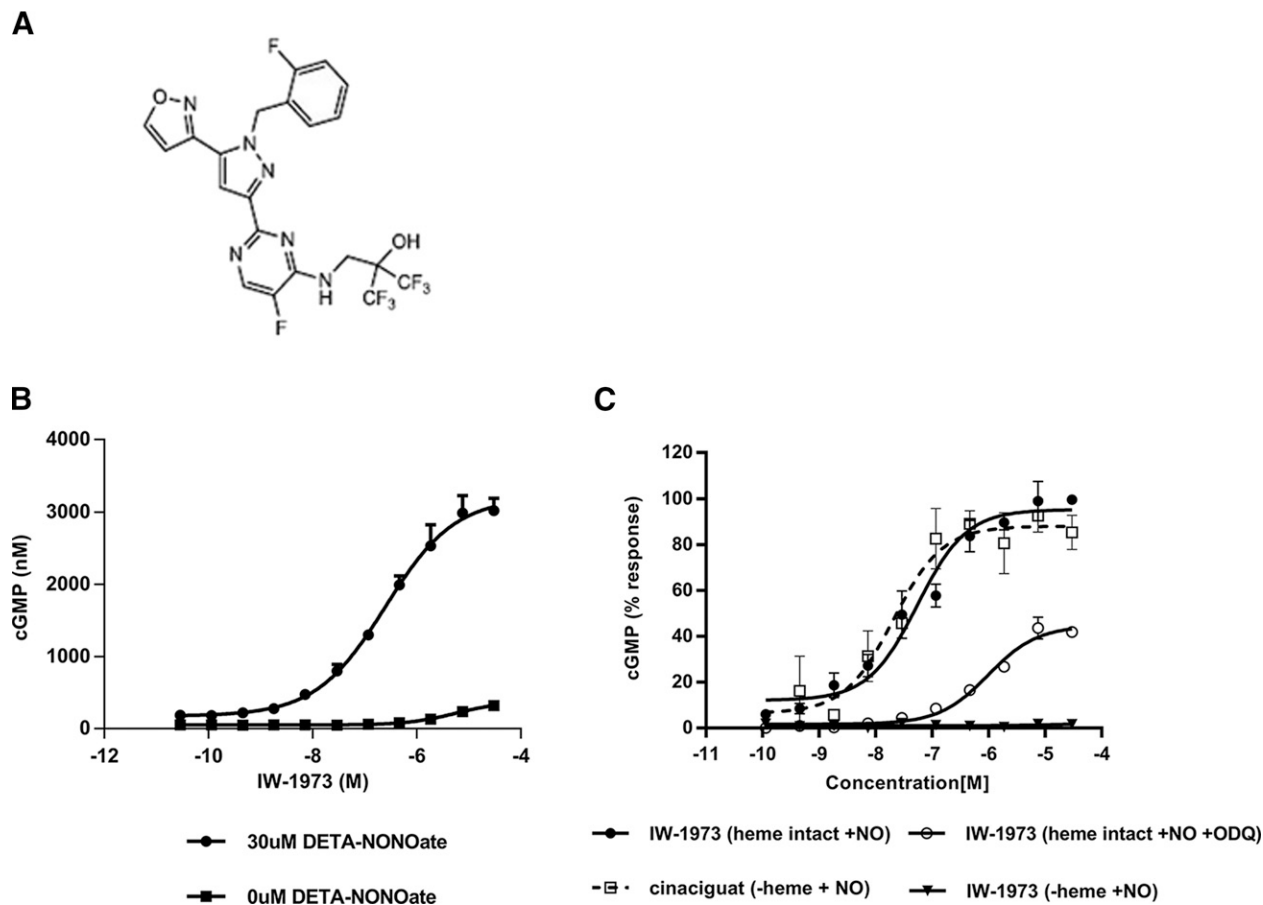


Fig. 1. (A) Chemical structure of IW-1973, a sGC stimulator from a novel pyrazolopyrimidine heterocyclic class of compounds. (B) Representative curve showing stimulation of human recombinant α 1 β 1 sGC with IW-1973 in the absence and presence of the NO donor DETA-NONOate (30 μ M). Data are presented as mean \pm S.E.M., *n* = 5 per group. (C) cGMP production of IW-1973 and cinaciguat using heme-dependent and independent sGC enzyme, data represent mean of % cGMP response \pm S.E.M., *n* = 3–5.

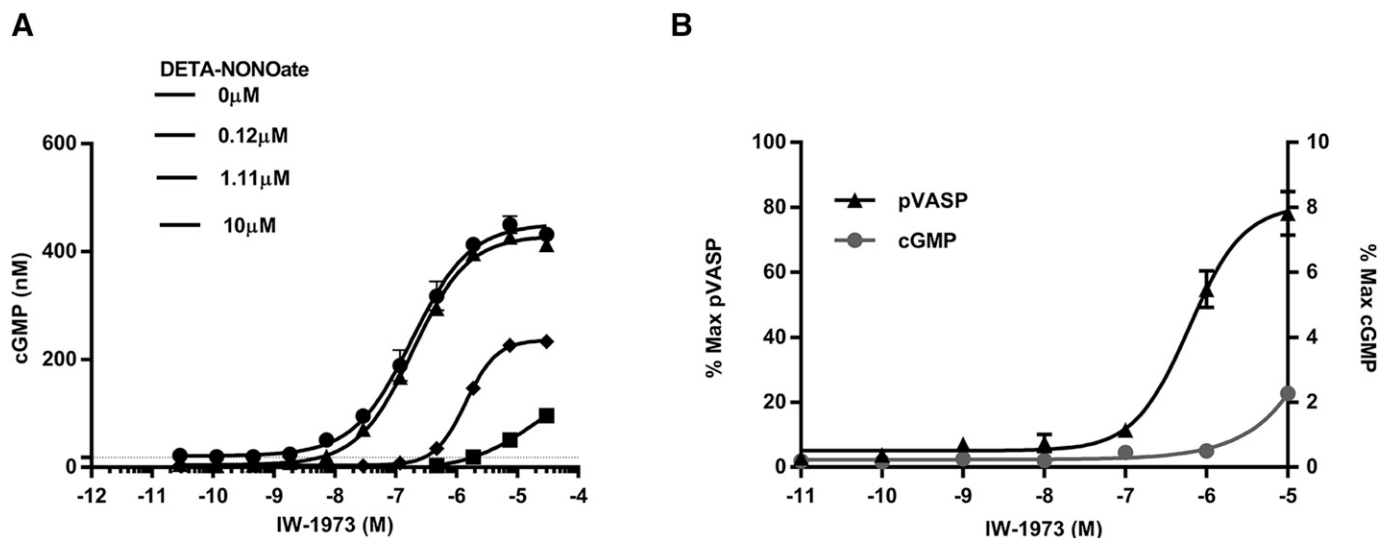


Fig. 2. (A) Stimulation of endogenous sGC in HEK-293 cells by IW-1973 (30 pM to 30 μ M) in the absence of the NO donor DETA-NONOate and in the presence of varying concentrations of DETA-NONOate (0.12–10 μ M). Data are presented as mean \pm S.E.M., $n = 2$ per group. (B) cGMP and pVASP concentration response 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.

NO-sGC-cGMP signaling induced by IW-1973 was further characterized in HEK-293-derived GloSensor 40F cGMP cells by monitoring phosphorylation of VASP, a target of protein kinase G. 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 μ M DETA-NONOate + 100 μ M IW-1973, data not shown).

IW-1973 Pharmacokinetics and Tissue Distribution in Rats. The PK profile of IW-1973 was assessed in male Sprague-Dawley rats via intravenous (Fig. 3A) and oral routes (Fig. 3B). The median time to reach maximum concentration for IW-1973 was 8 hours with a C_{max} of 254 ng/ml; the

estimated oral half-life was 9.2 hours, with an estimated oral bioavailability of 102%. Systemic clearance as determined from intravenous PK was 13.8 ml/min/kg and the steady-state volume of distribution 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 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 (Banijamali et al., 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 SHR. When administered orally once daily 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 hours in

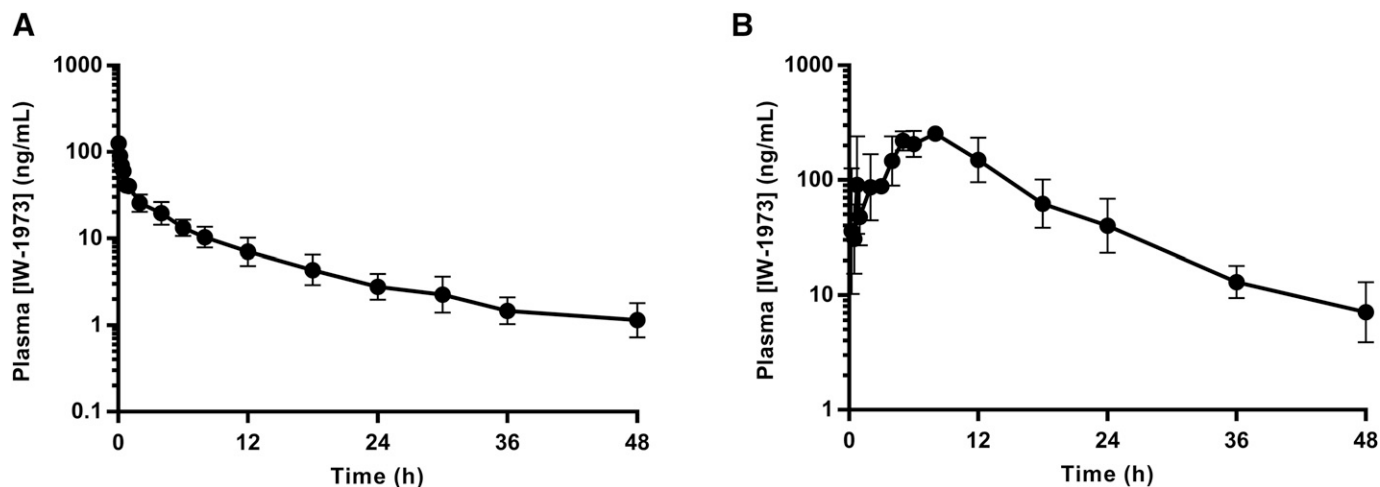


Fig. 3. Pharmacokinetic profile of IW-1973 in male Sprague-Dawley rats following single intravenous administration of IW-1973 [0.3 mg/kg in 60% polyethylene glycol (PEG) 400/40% water] (A) and oral administration of IW-1973 (3 mg/kg in PEG 400) (B). Data are presented as mean \pm 95% CI, $n = 6$.

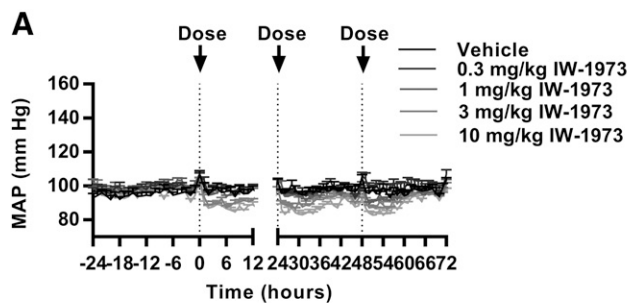
TABLE 1

Tissue-to-plasma ratios of IW-1973

Tissue-to-plasma ratios (mean \pm S.E.M.) of IW-1973 were determined from whole organ analysis performed 2 hours after final dose in male Sprague-Dawley rats administered oral IW-1973 once daily for 5 days. Data are presented as tissue/plasma ratio \pm S.E.M., $n = 6$.

IW-1973	Liver	Heart	Kidney	Lung
Tissue/plasma ratio	19.9 \pm 1.3	4.7 \pm 0.5	5.9 \pm 2.0	7.6 \pm 0.7

both normotensive (Fig. 4A) and hypertensive (Fig. 4B) rats. Maximum changes in MAP were observed between 2 and 6 hours 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 with 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 ± 0.8 mm Hg vs. 11.9 ± 0.9 mm Hg at 10 mg/kg on day 3), while the maximum absolute change in HR was less in SHR compared with normotensive rats (71.4 ± 11.4 mm Hg vs. 39.1 ± 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 precontracted with U-46619 (contractions ranging from 8 to 22 g) with EC₅₀ values ranging from 4.9 to 402.7 nM and a maximum relaxation response of 93.2%–96.8% (Supplemental Fig. 2) as calculated from the sodium nitroprusside-mediated maximum

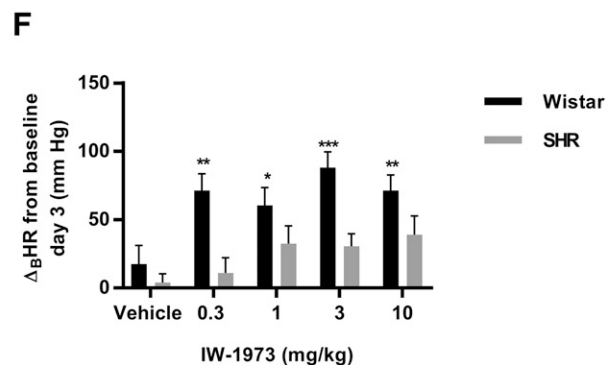
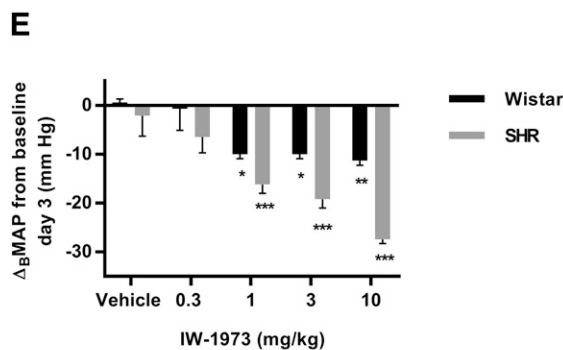
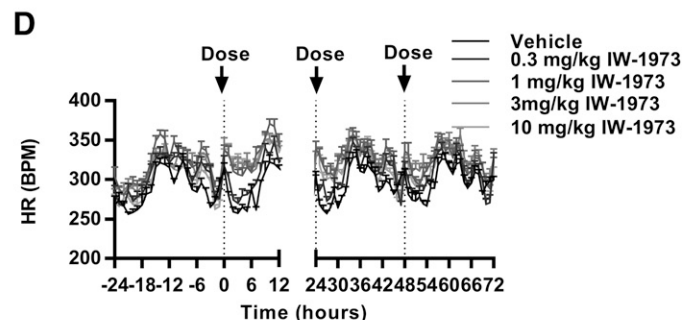
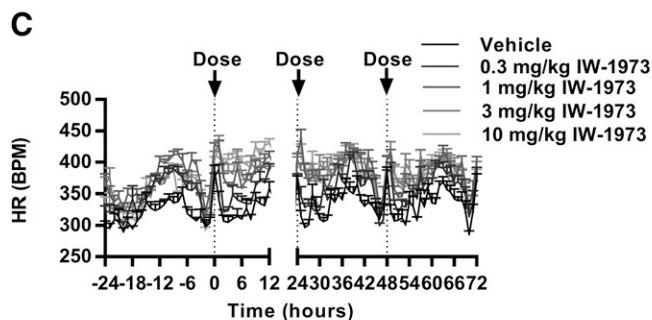
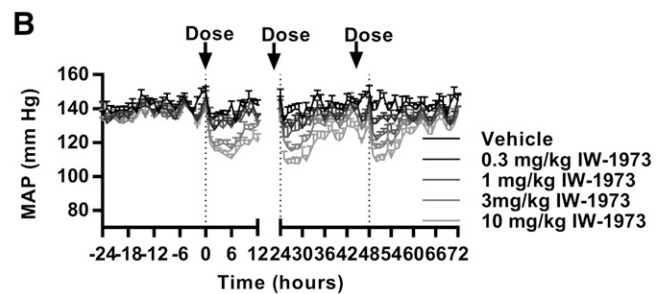


Fig. 4. MAP and HR in normotensive Wistar (A and C, respectively) and SHR (B and D, respectively) measured by telemetry for 1 day before dosing (baseline) and continuing for 3 days of once daily administration of vehicle or IW-1973 (0.3–10 mg/kg/d) by oral gavage for a total of 4 days. Data are plotted as mean \pm S.E.M. over time, $n = 6$ per group. (E) Data from (A and B) are plotted as peak change from 24-hour baseline in MAP \pm S.E.M. following the day 3 dose (48–72 hours). (F) Data from (C and D) are plotted as peak change from 24-hour baseline in HR \pm S.E.M. following the day 3 dose. Significant changes in MAP and HR compared with the vehicle for the respective rat strains was determined by two-way ANOVA followed by Dunnett's post-test, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

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 et al., 2011) was used to assess the effects of chronic IW-1973 treatment on body weight, hemodynamics, inflammation, proteinuria, and renal fibrosis. Animals on a HS 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 sixth 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 NS group (145.5 ± 4.5 mm Hg vs. 113.25 ± 1.75 mm Hg). 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/d on MAP was comparable to the effect of losartan at 30 mg/kg/d 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 IL-6 were significantly greater in animals in the HS vehicle group compared with animals on a NS diet (Fig. 6A). Compared with the HS vehicle group, rats treated with 10 mg/kg/d 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 NS diet as well as those receiving losartan at 30 mg/kg/d ($P \leq 0.05$ vs. HS vehicle).

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

Renal gene expression of the fibrosis markers TGF β 1 and Coll α 1 were higher in the HS vehicle group relative to the NS group ($P \leq 0.001$) and expression of these genes were lower in the kidneys of rats treated with 3 and 10 mg/kg/d IW-1973 compared with 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 proinflammatory cytokines IL-6 and TNF α were lower ($P \leq 0.01$) compared with vehicle-treated mice. Levels of the anti-inflammatory cytokine interleukin 10 were increased, but not significantly, compared with vehicle (Fig. 7).

Effects of IW-1973 in a Rat UO Model of Renal Fibrosis. The effects of IW-1973 were also examined in a nonhypertensive 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, UO increased renal levels of the collagen protein marker hydroxyproline ($P \leq 0.001$) and Coll α 1 mRNA expression ($P \leq 0.001$) and histopathological interstitial fibrosis ($P \leq 0.001$). In rats treated with 10 mg/kg/d IW-1973 for 14 days following UO, increases in hydroxyproline protein levels ($P \leq 0.001$) and col1 α 1 mRNA expression ($P \leq 0.05$) associated with UO 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

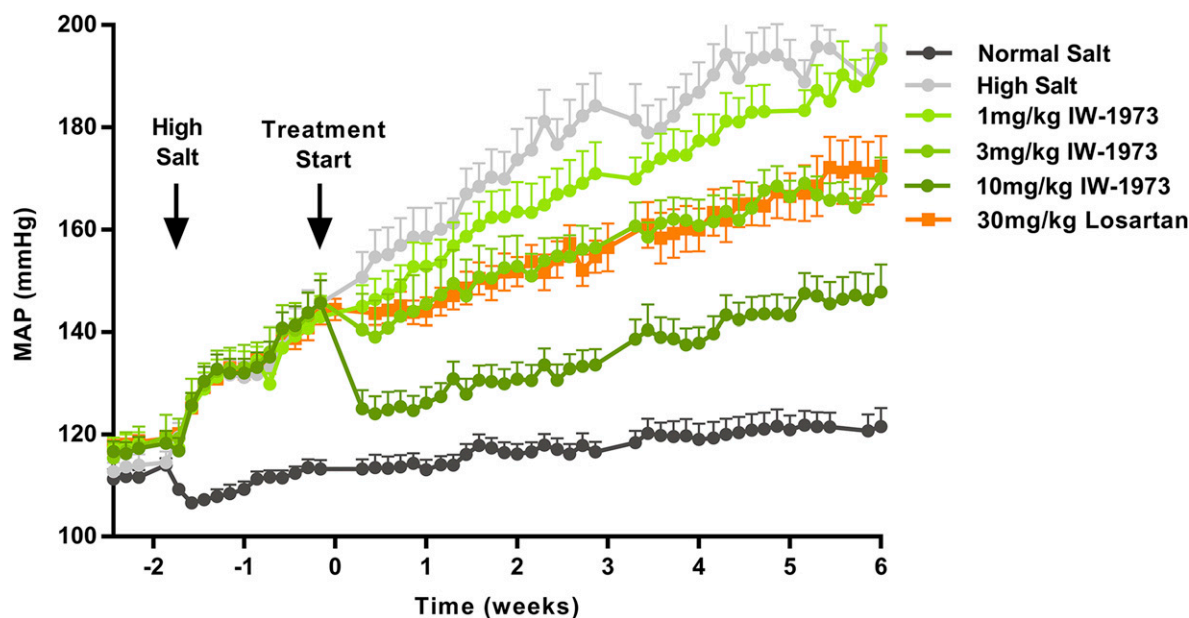


Fig. 5. MAP in DSS rats throughout 6 weeks of treatment with vehicle, IW-1973 (1, 3, and 10 mg/kg, in chow), or losartan (30 mg/kg, in drinking water), shown as average MAP \pm S.E.M. (mm Hg), $n = 8$ per group. Rats were provided NS or HS diet for 2 weeks before initiation of treatment and during treatment with vehicle, IW-1973, or losartan. Baseline measures were determined 1–3 days before initiation of treatment.

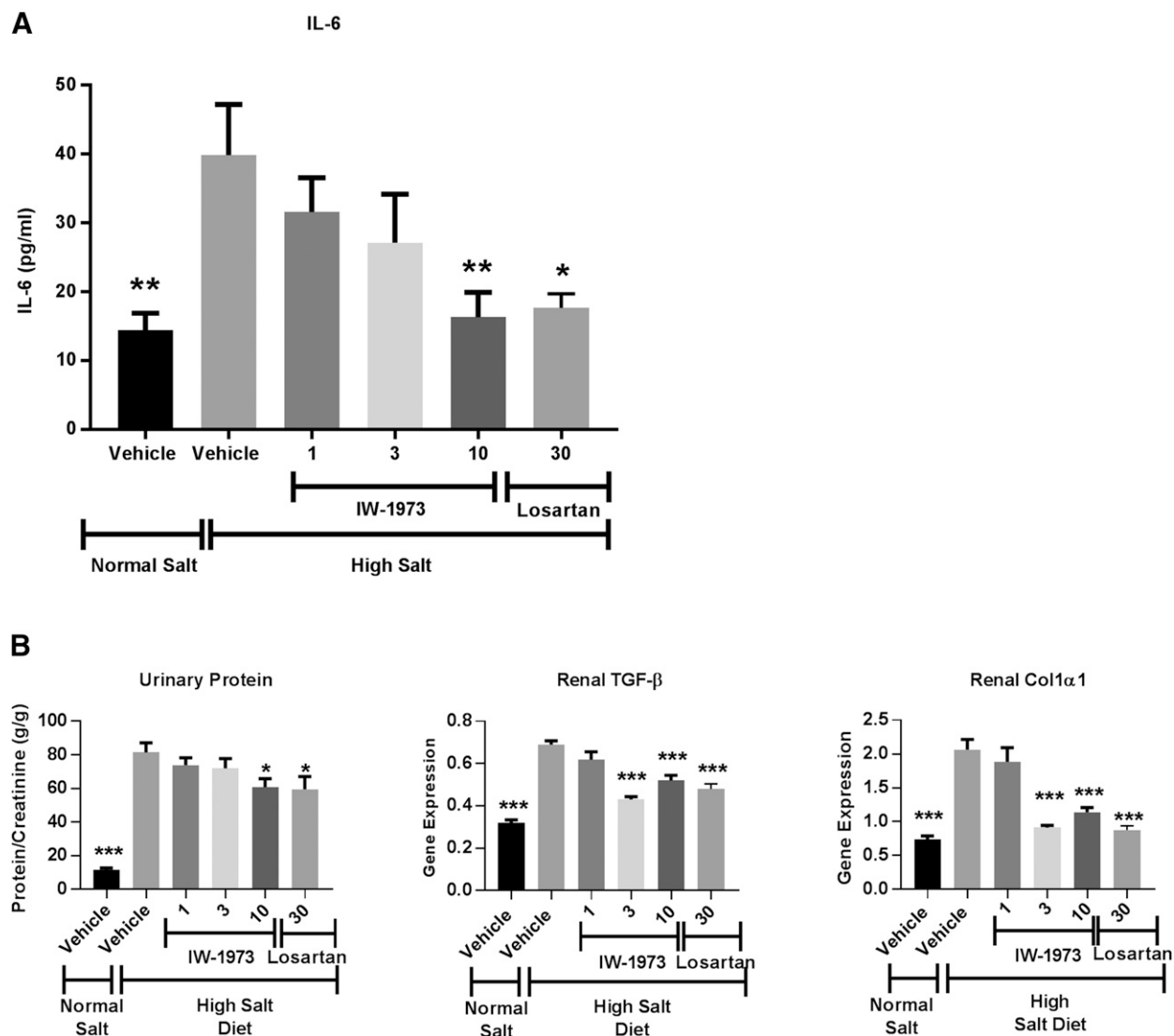


Fig. 6. Serum IL-6 (A) and urinary protein [protein (gram)/creatinine (gram)] and fibrotic gene expression (normalized to geometric mean of housekeeping genes *hprt1*, *ppib*) in kidneys (B) in the DSS rat model. All data are plotted as mean + S.E.M., $N = 8$ /group. All measures were taken after 6 weeks 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, $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

enzyme and whole cell assays. Consistent with the definition of a sGC stimulator (Follmann et al., 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 a NO donor stimulated cGMP production in whole cells and triggered phosphorylation of VASP, a downstream target in the NO-sGC-cGMP-protein kinase G 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 la Sierra, 2002; Hoffmann et al., 2015b), suggests that sGC stimulators will provide benefit in diseases with NO impairment. At a dose of 3 mg/kg/d 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,

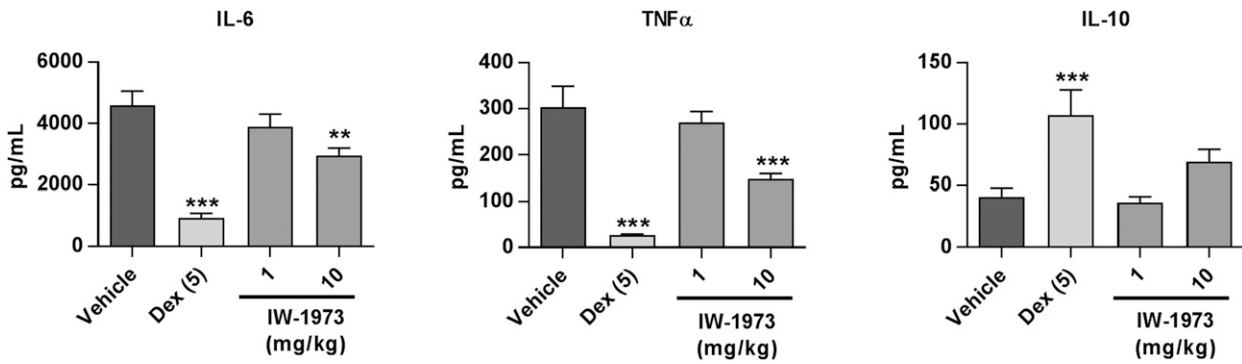


Fig. 7. Effect of IW-1973 on levels of the proinflammatory cytokines, IL-6 and TNF α , and on the anti-inflammatory cytokine, interleukin 10 (IL-10). C57Bl/6 male mice were dosed with vehicle, dexamethasone, or IW-1973 followed by LPS challenge to induce inflammation. Serum was collected 2-hour postinduction,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

leukocyte rolling and adhesion were increased 6-fold in eNOS^{-/-} mice relative to wild-type controls, and both the sGC stimulator Bay 41-2272 and the NO donor DETA-NO restored leukocyte rolling and adhesion to wild-type levels (Ahluwalia et al., 2004). Furthermore, reduced NO has been shown to contribute to inflammation in adipose tissue in models of induced obesity (Handa et al., 2011). In the DSS model, IW-1973 treatment reduced serum levels of the proinflammatory cytokine IL-6. This finding, which is consistent with anti-inflammatory effects reported for sGC agonists in the DSS model (Geschka et al., 2011; Hoffmann et al., 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 proinflammatory cytokines IL-6 and TNF α , and although not significant, increased the anti-inflammatory cytokine interleukin 10. Our findings in the LPS model add to mounting evidence (Ahluwalia et al., 2004; Handa et al., 2011; Glynos et al., 2015) that sGC stimulators like IW-1973 have direct anti-inflammatory effects.

Consistent with other sGC agonists (Stasch et al., 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 profibrotic cytokine TGF β and Col1 α 1, the major component of type I collagen. Renal histologic 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 et al., 2015). The renoprotective effects of IW-1973 were also explored in the UO model of renal fibrosis. Compared with vehicle-treated animals, IW-1973-treated animals had significantly reduced kidney levels of Col1 α 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 antifibrotic activity in the liver in a model of nonalcoholic steatohepatitis at doses that do not affect blood pressure (Flores-Costa et al., 2018).

IW-1973 exhibited a PK profile in the rat that includes high oral bioavailability (>90%), a large volume of distribution, and

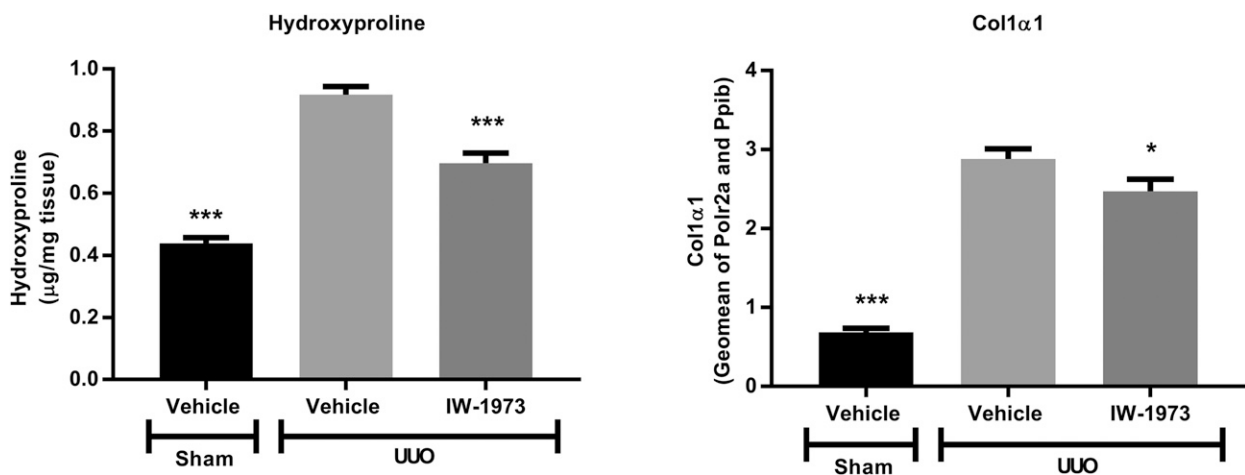


Fig. 8. Effects of IW-1973 (10 mg/kg/d in chow) on renal markers of fibrosis in the rat UUO model. Hydroxyproline levels (mean + S.E.M., $n = 8$) are reported as micrograms per milliliter of tissue, and Col1 α 1 mRNA expression data are reported as ratio of Col1 α 1 to the housekeeping genes polr2a and ppib. Significance relative to vehicle control UUO animals was determined by one-way ANOVA followed by Dunnett's post-test, * $P \leq 0.05$; *** $P \leq 0.001$.

moderate clearance. Clinical studies suggest that predictions of once daily dosing from preclinical models translate to humans (Hanrahan et al., 2017). Mass balance studies performed in rats revealed that IW-1973 was primarily cleared by the liver (Zimmer et al., 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 et al., 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 NO synthase 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 physiologic function (Fig. 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, profibrotic 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 the extensive distribution of IW-1973 to tissues may allow for beneficial effects on organ fibrosis,

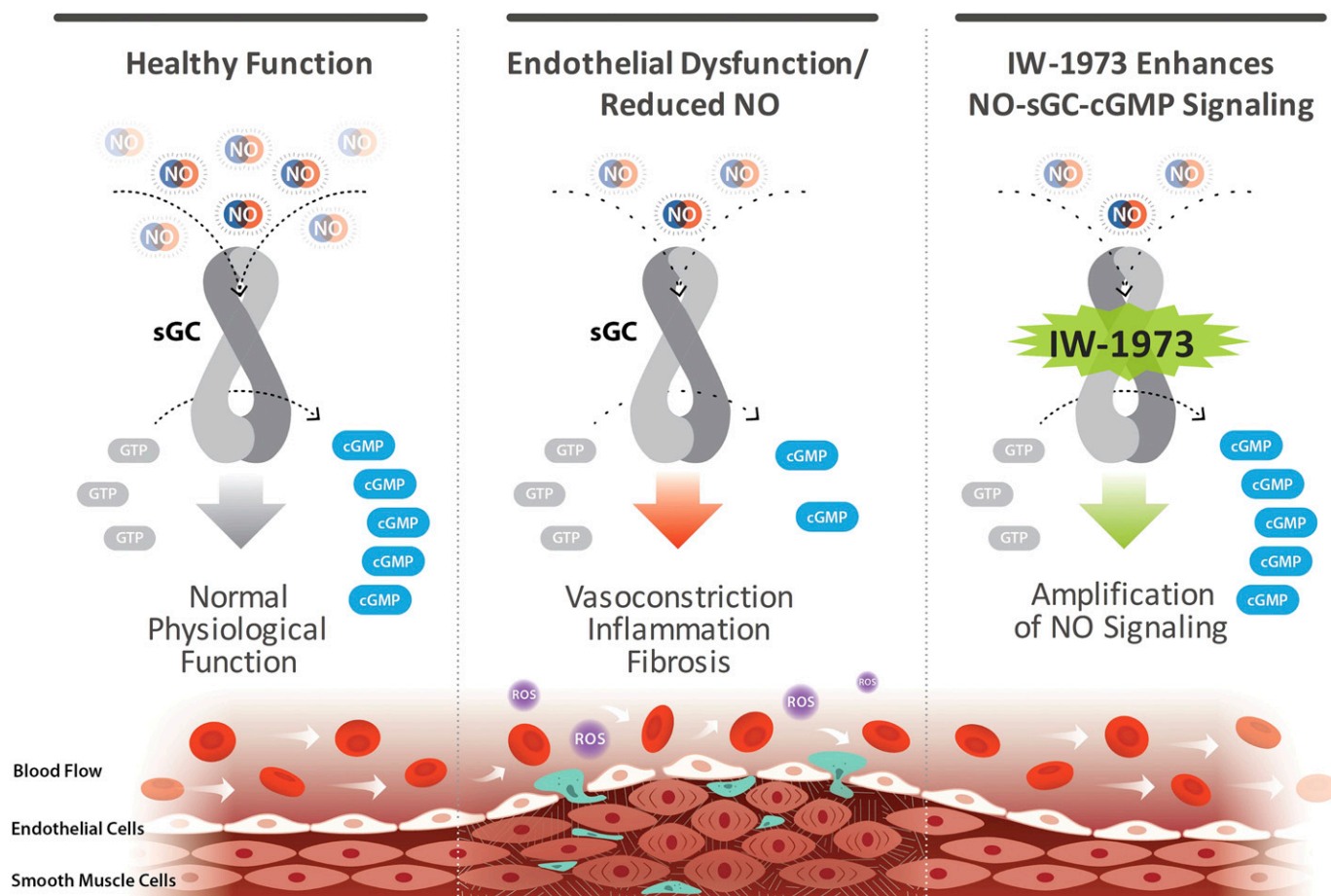


Fig. 9. Model for mechanism of action of IW-1973. During healthy function, NO binds to sGC, triggering the conversion of guanosine-5'-triphosphate (GTP) to cGMP. The cGMP-protein kinase G signaling pathway maintains normal physiologic function primarily through regulation of vascular tone and control 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 physiologic function. Even when NO levels are normal, sGC stimulation by IW-1973 may be useful for treating diseases involving reduced peripheral blood flow, inflammation, and/or fibrosis.

inflammation, and function at doses associated with little to no effects on blood pressure. IW-1973 has high oral bioavailability and a long PK half-life, which translated into PK consistent with once daily dosing in humans. Indeed, in a phase 1 study in healthy volunteers, once daily 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 et al., 2017). Clinical studies will be required to determine whether the ability of IW-1973 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 Contributions

Participated in research design: Zimmer, Tang, Profy, Milne, Currie, Masferrer.

Conducted experiments: Tobin, Shea, Germano, Bernier, Liu, Long, Miyashiro, Ranganath, Jacobson, Sykes, Wakefield, Sarno, Banijamali.

Contributed new reagents or analytic tools: Im, Sheppeck, Moore.

Performed data analysis: Tobin, Zimmer, Shea, Germano, Bernier, Liu, Long, Miyashiro, Ranganath, Jacobson, Sykes, Wakefield, Sarno, Banijamali.

Wrote or contributed to the writing of the manuscript: Tobin, Zimmer, Shea, Germano, Bernier, Liu, Miyashiro, Jacobson, Sykes, Wakefield, Sarno, Banijamali, Profy, Milne, Masferrer.

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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
Jaime L. Masferrer

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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 EC₅₀ can be derived.

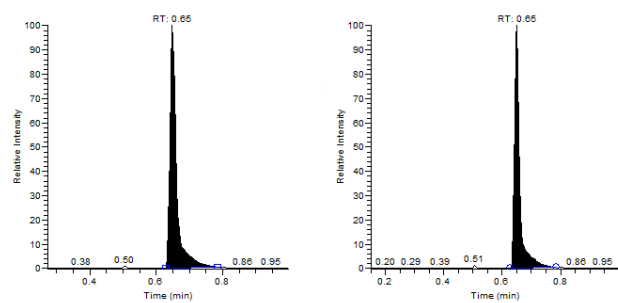
Supplemental figure 3. Body weight in DSS rats throughout 6 weeks of treatment with vehicle, IW-1973 (1, 3, and 10 mg/kg, in chow), or losartan (30 mg/kg, in drinking water), shown as change in weight ± SEM (g) from start of treatment at week 0, n= 8 per group. Rats were provided normal-salt diet or 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 ≤ 0.05, **p ≤ 0.01, and ***p ≤ 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± SEM (BPM), n= 8 per group. Rats were provided normal-salt diet or

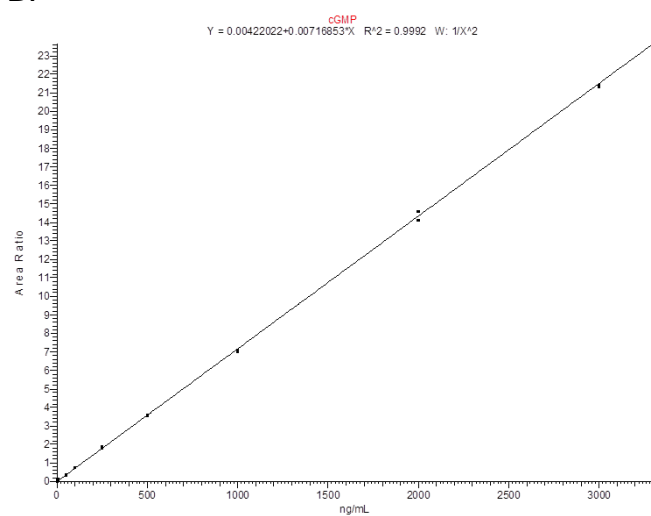
high-salt diet for 2 weeks before initiation of and during treatment with vehicle, IW-1973, or losartan. Baseline measures were determined 1-3 days before initiation of treatment.

Supplemental Figures

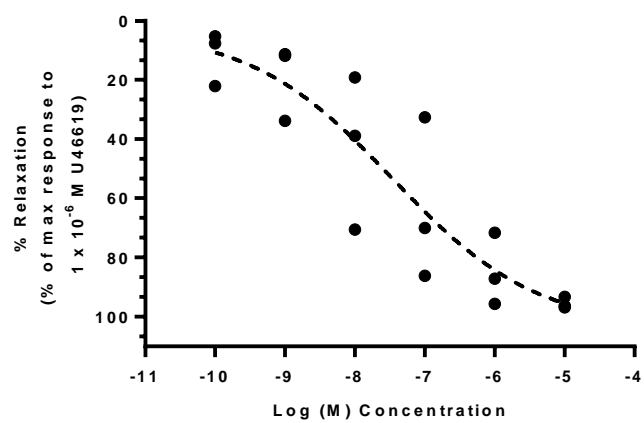
A.



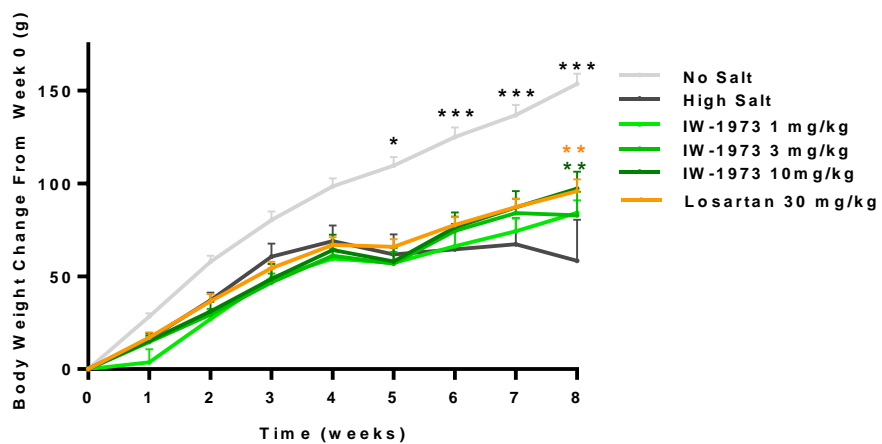
B.



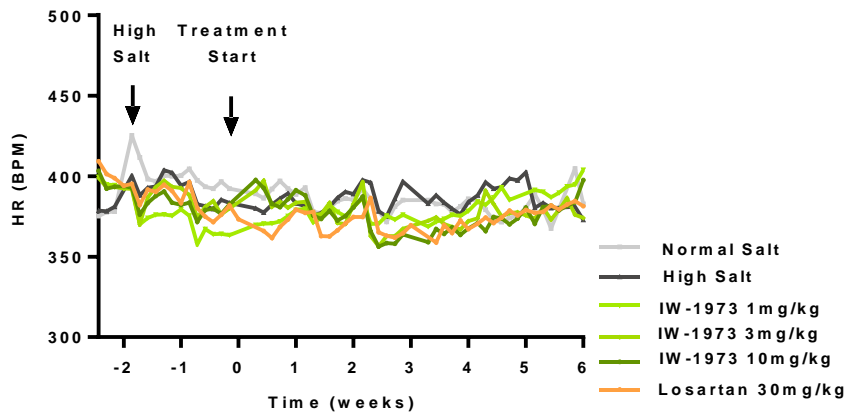
Supplemental Figure 1



Supplemental Figure 2.



Supplemental Figure 3.



Supplemental Figure 4.