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Title Page

A Soluble Guanylate Cyclase Activator Inhibits the Progression of Diabetic Nephropathy in the ZSF1 rat

Authors:

Carine M. Boustany-Kari, Paul C. Harrison, Xongxing Chen , Kathleen Lincoln , Hu Sheng Qian, Holly Clifford, Hong Wang, Xiaomei Zhang, Kristina Gueneva-Boucheva, Todd Bosanac, Diane Wong, Ryan M. Fryer, Jeremy G. Richman, Chris Sarko , Steven S. Pullen

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Departments of Cardiometabolic Diseases Research (CMBK, PCH, XC, KL, HSQ, HC, HW, XZ, RMF, JGR, SSP) and Small Molecule Discovery Research (KGB, TB, DW, CS) Boehringer Ingelheim Pharmaceuticals, Inc, Ridgefield, CT

Running Title Page

A sGC Activator Inhibits Progression of DN in the ZSF1 Rat

Corresponding Author:

Carine M. Boustany-Kari

900 Ridgebury Road, PO Box 368

Ridgefield, CT 06877

203-798-4971

carine.boustany@boehringer-ingelheim.com

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Non-standard abbreviations:

α-SMA	**alpha smooth muscle actin**
ACE	**angiotensin converting enzyme**
ARB	**angiotensin receptor blocker**
BI 703704	**WO 2013/025425 example 89**
bpm	**beats per minute**
CKD	**chronic kidney disease**
eNOS	**endothelial nitric oxide synthase**
ESRD	**end stage renal disease**
H&E	**hematoxylin and eosin**
HR	**heart rate**
KIM-1	**kidney injury molecule 1**
LC/MS/MS	**liquid chromatography/mass spectrometry/mass spectrometry`**
MAP	**mean arterial pressure**
mmHg	**millimeters mercury**

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NO	**nitric oxide**
PAM	**periodic acid methenamine**
PBS	**phosphate buffered saline**
sGC	**soluble guanylate cyclase**
T2DM	**type 2 diabetes mellitus**
UPE	**urinary protein excretion**

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Abstract

Therapies which restore renal cyclic GMP (cGMP) levels are hypothesized to slow the progression of diabetic nephropathy. We investigated the effect of BI 703704, a soluble guanylate cyclase (sGC) activator, on disease progression in obese ZSF1 rats. BI 703704 was administered at doses of 0.3, 1, 3 and 10 mg/kg/day to male ZSF1 rats for 15 weeks, during which mean arterial pressure (MAP), heart rate (HR), and urinary protein excretion (UPE) were determined. Histological assessment of glomerular and interstitial lesions was also performed. Renal cGMP levels were quantified as an indicator of target modulation. BI 703704 resulted in sGC activation as evidenced by dose-dependent increases in renal cGMP levels. After 15 weeks of treatment, sGC activation resulted in dose-dependent decreases in UPE (from 463±58 mg/day in vehicle controls to 328±55, 348±23, 283±45 and 108±23 mg/day in BI 703704 treated rats at 0.3, 1, 3 and 10 mg/kg, respectively). These effects were accompanied by a significant reduction in the incidence of glomerulosclerosis and interstitial lesions. Decreases in MAP and increases in HR were only observed at the high dose of BI 703704. These results are the first demonstration of renal protection with sGC activation in a nephropathy model induced by type 2 diabetes. Importantly, beneficial effects were observed at doses that did not significantly alter MAP and HR.

Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease (ESRD) in Western societies and is characterized by a progressive decline in renal function accompanied by mesangial expansion, glomerular basement membrane thickening, and tubulointerstitial damage (Saran, 2015 and Terveart, 2010). Diabetic nephropathy patients receiving current standard of care (SoC) treatment, namely angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB), continue to progress to ESRD, indicating a clear unmet medical need for new therapies that will slow or arrest renal function decline in these patients (Saran 2015).

Endothelial dysfunction has been linked in humans and pre-clinically with the progression of diabetic nephropathy. Increased plasma levels of endothelial dysfunction markers, namely von Willebrand factor, soluble vascular cell adhesion molecule-1, and soluble intercellular cell adhesion molecule-1, have been associated with disease progression in diabetic nephropathy patients (Persson, 2008). Furthermore, asymmetric dimethyl arginine, an endogenous inhibitor of endothelial nitric oxide synthase (eNOS), is associated with the development and progression of diabetic nephropathy in patients (Hanai, 2009, Shibata, 2008 and Lajer, 2007). Consistent with these studies, eNOS polymorphisms resulting in reduced enzyme expression or function have been associated with diabetic nephropathy (Dellamea, 2014). In an analysis of pre-clinical models of disease, it was demonstrated that deficiency of eNOS in db/db diabetic mice produced a renal gene expression profile that more closely resembled human diabetic nephropathy, compared to db/db mice with intact eNOS (Hodgin, 2013). Taken together, these studies

highlight a role for impaired nitric oxide (NO) production/signaling in the progression of diabetic nephropathy.

A key messenger for NO signaling is cGMP, the product of GTP catalysis by soluble guanylate cyclase (sGC) (Lewicki, 1982). Under conditions of oxidative stress such as those found in diabetes, the heme prosthetic group of sGC becomes oxidized rendering it non-responsive to NO (Stasch, 2006). This finding has led to the generation of pharmacological compounds aimed at restoring cGMP levels, thereby repairing endothelial dysfunction. As such, a class of synthetic compounds termed sGC activators has been demonstrated to bypass NO signaling impairment by displacing the defective heme prosthetic group thereby stabilizing the enzyme in an activated conformation and restoring cGMP generation (Stasch, 2002). Another class of compounds termed sGC stimulators is also able to increase cGMP production by sGC, but not when the heme prosthetic group has been oxidized (Stasch, 2001).

Given that diabetic nephropathy is associated with endothelial dysfunction and increased oxidative stress, we sought to investigate the effect of sGC activation on the progression of the disease in the ZSF1 rat. The ZSF1 (*Lep*^{fa}/*Lepr*^{cp}) obese rat, a hybrid cross between ZDF♀ (*Lep*^{fa}) and SHHF♂ (*Lepr*^{cp}), was selected for these studies as it exhibits many of the traits of human diabetic nephropathy including proteinuria ,renal lesions, hyperglycemia, dyslipidemia, mild hypertension, oxidative stress, and obesity (Tofovic, 2003, Prabhakar, 2007 and Boerrigter, 2007). Our findings indicate a pronounced and dose-dependent effect of BI 703704 (Figure 1; Berry, 2013), a sGC activator, to reduce progression of renal damage in the ZSF1 rat, and highlight the potential of sGC activation as an effective therapy for diabetic nephropathy.

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Materials and Methods

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and under an IACUC approved study protocol at Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT 06877.

Animals. Male ZSF1 obese rats were obtained from Charles River Labs (Kingston, NY) at 10-11 weeks of age following surgical implantation of telemetry transmitters (Data Sciences International, Inc., PA11TA-C40) in the abdominal artery to allow for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR). Rats were acclimated to metabolism cages (Lab Products, Inc., Seaford, DE) for 1 week prior to baseline collections, and remained in these cages throughout the study to allow for weekly collection of urine. Baseline measurements were collected one week prior to start of treatment.

Experimental Design

Fifty eight male ZSF1 rats (12-13 weeks of age) were randomly assigned based on their average baseline urinary protein excretion (UPE), MAP and body weight to receive either enalapril (3 mg/kg/day in the drinking water, n=10/group) or one of four doses of BI 703704 (0.3, 1, 3, or 10 mg/kg/day, n=9-10/group) incorporated into the diet (Purina Formulab 5008, WF Fisher & Son, Inc., Somerville, NJ). Dietary concentrations of BI 703704 were estimated based on historical data of daily food intake (33 g/day) and body weight of ZSF1 rats, and were adjusted every five weeks. Rats were subsequently monitored for 15 weeks during which body weight was recorded once per week, and food intake and water intake thrice per week. Urine was collected chilled

once weekly over a 24 hour period for measurement of protein contents. Blood samples were collected at baseline, following 5 and 10 weeks of treatment, and at study termination for pharmacokinetic, clinical chemistry, plasma glucose and HbA1c measurements. Hemodynamic parameters were recorded intermittently (1 minute of continuous data every 10 minutes) and weekly 24-hour averages were calculated. At study end, rats were sacrificed by exsanguination through cardiac puncture under isoflurane anesthesia,and kidneys were collected for histological analysis and determination of drug concentrations and cGMP levels.

Biochemical and Pharmacokinetic Measurements

Following centrifugation (930 rcf for 10 min), urine samples were diluted in 0.1 N NaOH and protein concentration was determined using Coomassie Plus Reagent (Thermo Fisher, Waltham, MA, #23238).

The quantitation of enalaprilat, and BI 703704 in heparin plasma and kidney cortex was performed using liquid chromatography and mass spectro

metry (LC/MS/MS). An API 5000 triple quadruple mass spectrometer with Turbo V Ion Source (Applied Biosytems, Toronto, Canada), set to electrospray positive ionization mode, and Analyst 1.4.2 operating software was used. Ion transitions used for quantification were: enalaprilat (349.0-206.0 m/z, CE 25, DP 80V) and BI 703704 (560.2-216.2 m/z, CE 40, DP 80V). The liquid chromatography system was an Agilent 1200 Series pump and column oven (Fullerton, CA, USA) with a LEAP Technologies HTS-PAL Autosampler (Carrboro, NC, US). The analytical column was a Phenomenex Synergi Polar RP, 2.1 x 50 mm, 4 µm (Torrance, CA, US), and the mobile phase consisted of 10mM Ammonium Acetate with 0.1% formic acid in water (A) and Acetonitrile (B). Gradient was maintained at 5%B for 0.1 minute, increased to 95% B in

1.8 minutes, held at 95%B for 0.5 minute and decreased to 5% B for 0.2 minute and held at 5% B for a total time of 3 minutes at a flow rate of 0.5 ml/min. The total run time per injection was 3 minutes.

Sample Preparation: Enalaprilat and BI 703704 standard stock solutions containing 1 mg/mL in methanol were serially diluted in rat plasma to prepare an 8- point standard curve ranging from 1 to 5000 ng/mL. 20 µLs of plasma or homogenized kidney samples, calibration standards and blank plasma samples were deproteinized by precipitation with 180 µL of Internal Standard (100 ng/mL; proprietary small molecule) diluted in acetonitrile: water (85:15). Samples were mixed for 1 min, filtered through AcroPrep Multi-well filter plates (Pall Corporation, Ann Arbor, MI) using Sciclone ALH 3000 Workstation (Caliper Life Sciences, Hopkinton, MA, USA) and transferred into 96-well injection plate. 5 µL of the solution was then injected into the LC/MS/MS system for analysis.

Cyclic GMP levels were determined in frozen kidney cortex sections. Tissues were pulverized using a ceramic mortar and transferred into pre-chilled tubes containing MP lysing matrix homogenizing beads (MP Biomedicals Life Sciences, Newport Beach, CA) for further disruption. Following acidification and acetylation, cGMP levels were determined using a cGMP ELISA assay kit (EnZO Life Sciences Direct, Farmingdale, NY).

Protein levels were measured in urine collected weekly over a 24 hour period. Protein contents were quantified utilizing a Bradford assay (Coomassie Plus, Thermo Fisher Scientific, Waltham, MA). HbA1c was measured by HPLC (DTI Laboratories Inc, Thomasville, GA). Fasting plasma glucose was measured utilizing a glucose meter (Bayer Breeze2, Bayer HealthCare LLC, Mishawaka, IN).

Tissue Collection and Processing

For histological assessment, kidneys were removed, weighed and a mid-organ transverse section of the left kidney was collected and immediately fixed by immersion in 10% phosphate-buffered formalin for 48 hours. Subsequently, formalin-fixed tissues were washed in phosphate buffer, dehydrated through a graded series of ethanol and xylene, embedded in paraffin, and sectioned at 4 µm.

General Assessment of Renal Histopathology

Kidney tissue sections (4 µm) were stained with periodic acid methenamine silver (PAM) for the general assessment of incidence of glomerulosclerosis and hematoxylin–eosin (H&E) for the general assessment of total number of foci of interstitial lesions. For the glomerular lesions, the number of glomeruli showing mild to severe glomerulosclerosis were counted in each kidney section and expressed as a percent of the total glomerular population. Tubulointerstitial lesions were assessed as the number of foci showing obvious renal damage, which included marked hypercellularity (inflammation or resident myofibroblast increases), fibrosis, tubular hyperplasia, casts, or necrosis. Both assessments were performed under blinded conditions at 10X magnification.

Immunohistochemistry and Image Analysis

Four micrometer kidney sections were air dried overnight at 37°C, dewaxed and rehydrated in graded ethanol to phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by ImmPRESS (Vector Laboratories, Burlingame, CA) for 20 min at ambient temperature. Sections were then washed and incubated with the primary antibody for α-smooth muscle actin (α-SMA; A5228, Sigma, St. Louis, MO) at a dilution of 1:3000 and kidney injury molecule-1

(KIM-1; AF3689, R&D Systems, Minneapolis, MN) at a dilution of 1:200 and were subsequently incubated with the respective secondary antibodies (ImmPress Reagent Kit, Vector Laboratory, Burlingame, CA) for 30 minutes in a hydration chamber. Immunoperoxidase detection was performed using the avidin-biotin complex method (Vector Laboratories, Burlingame, CA) using 3, 3′-diaminobenzidine tetra hydrochloride as substrate.

Quantitative image analysis of α-SMA and KIM-1 staining was performed under blinded conditions. Ten random digital images were captured at 10X objective magnification, with each field representing approximately 0.6 mm^2 for a total area of 6.0 mm^2 being assessed. The percentage of positive staining area per field in the cortex region was measured using computer assisted image analysis with Image-Pro imaging software.

Statistical Analysis

Data collected repetitively over time were analyzed using a 2-way ANOVA with repeated measures and a Bonferroni post-hoc analysis. Data obtained through single measurements (cGMP, histological endpoints) were analyzed using a 1-way ANOVA with Tukey's post-hoc analysis. Significance was achieved at $p<0.05$. Statistical analysis was performed using GraphPad Prism Version 6.01 (GraphPad Software, Inc., La Jolla, CA).

Pharmacokinetics/Pharmacodynamics (PK/PD) modeling

Renal cGMP changes were correlated with BI 703704 individual mean plasma concentrations using an E_{max} model. Additionally, changes from baseline in UPE were correlated with BI 703704 individual mean plasma concentrations using an I_{max} model. All analyses were performed using Phoenix WinNonlin Version 6.1 (Pharsight, Cary, NC).

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Results

Pharmacokinetics of BI 703704 in Plasma and Kidney

Administration of BI 703704 at 0.3, 1, 3, and 10 mg/kg/day yielded plasma concentrations that were dose-dependent throughout the study (Table 1). Following 15 weeks of treatment, BI 703704 renal concentrations were also dose-dependent, demonstrating presence of compound in the target organ (Table 1). Plasma concentrations of enalaprilat, the active metabolite of enalapril, were consistent across the study (Table 1).

Effect of sGC Activation on cGMP Content in the Kidney

Renal content of cGMP, a biomarker of sGC activation, was determined in the kidney cortex at study termination. BI 703704 treatment resulted in a dose-dependent increase in renal cGMP content (16.75 ± 1.86, 23.85 ± 2.65, 24.84 ± 2.08, 28.02 ± 2.55 pmol/g kidney tissue at doses of 0.3, 1, 3, and 10 mg/kg respectively, $p<0.05$ vs. vehicle) indicating that the compound had resulted in an activation of sGC *in vivo* within the target organ. At the dose of 10 mg/kg, cGMP content was significantly greater than seen in the vehicle (18.01 ± 1.55 pmol/g tissue) and enalapril (14.98 ± 1.17 pmol/g tissue) groups. To derive BI 703704 *in vivo* potency in activating sGC, renal cGMP content was associated with plasma and renal drug concentrations utilizing an E_{max} model. The plasma concentration of BI 703704 was correlated with renal cGMP content (Figure 2) and the plasma concentration necessary to elicit a 50% increase in renal cGMP content (EC_{50}) was estimated to be 420 nM (108% CV). Similarly, BI 703704 renal concentrations were correlated with renal cGMP content and the EC_{50} was estimated to be 105 nM (93% CV). These results demonstrate that increasing concentrations of BI 703704 in the

systemic circulation as well as in the target organ lead to production of renal cGMP, thereby validating that BI 730704 effectively modulates sGC in a concentration-dependent manner.

Effect of sGC Activation on Metabolic Measurements

At baseline, there was no significant difference in body weight, food intake or water intake between study groups (Table 2). Following BI 703704 administration there was a significant, dose-dependent increase in body weight gain vs. vehicle, despite a decrease in food and water intake at the doses of 3 and 10 mg/kg. In contrast, enalapril did not significantly affect body weight or food and water intake compared to vehicle. Concommittantly with the reductions in food intake, HbA1c levels were significantly decreased by the end of the study at the doses of 3 and 10 mg/kg of BI 703704 (change from baseline in HbA1c %: Vehicle: 1.1±0.2, enalapril: 1.1±0.2, BI 703704 0.3 mg/kg: 0.5±0.2, 1 mg/kg: 0.5±0.2, 3 mg/kg: 0.3±0.1*, 10 mg/kg: -0.9 ±0.1 *$^{\#}$; *p< 0.05 vs. vehicle, $^{\#}$p<0.05 vs. enalapril). Non-fasting plasma glucose was not significantly different between groups by week 15 (change from baseline in plasma glucose: Vehicle:106.8±52.9, enalapril: 119.9±34.6, BI 703704 0.3 mg/kg: 66.11±41.4, 1 mg/kg: -53.22±36.6, 3 mg/kg: -17.11±35.35, 10 mg/kg: 10.78±29.3 mg/dl; p>0.05).

Effect of sGC Activation on Hemodynamic Measurements6.8

At baseline, there was no significant difference in MAP or HR between study groups (MAP: 119±3, 117±1, 119±1, 119±2, 117±2 and 118±1 mmHg and HR: 316±5, 305±10, 320±4, 323±3, 318±5 and 315±4 bpm for vehicle, enalapril and BI 703704 at 0.3, 1, 3 and 10 mg/kg, respectively; p>0.05). Administration of BI 703704, at the dose of 10 mg/kg, resulted in a significant decrease in MAP starting from week 2 onwards (Figure 3). The doses of 0.3, 1 and 3

mg/kg did not significantly affect MAP compared to vehicle. In comparison, enalapril, tested at 3 mg/kg, resulted in a significant reduction in MAP vs. vehicle from week 11 onwards..

Heart rate decreased over time in all study groups including vehicle. At the highest dose tested, BI 703704 resulted in a significant increase in heart rate vs. vehicle and enalapril (Week 15 heart rate; vehicle: 265±5 bpm and BI 703704 at 10 mg/kg: 297±5 bpm; Figure 4). The doses of 0.3, 1 and 3 mg/kg did not elicit a significant effect on heart rate. Similarly, enalapril did not significantly alter heart rate.

Effect of sGC Activation on Renal Parameters

At baseline there was no significant difference in proteinuria between study groups. BI 703704 treatment resulted in dose-dependent decreases in proteinuria (Week 15 UPE; vehicle: 463±58 mg/day and BI 703704: 328±55, 348±23, 283±45 and 108±23 mg/day at 0.3, 1, 3 and 10 mg/kg, respectively; Figure 5). The effects of BI 703704 were significantly different from vehicle at doses of 1, 3, and 10 mg/kg/day, and from enalapril at doses of 3 and 10 mg/kg/day. To determine BI 703704 *in vivo* potency in reducing UPE, plasma concentrations of BI 703704 were correlated with UPE (as a change from baseline) utilizing an I_{max} model (Figure 6). The plasma concentration of BI 703704 necessary to elicit a 50% decrease in UPE (IC_{50}) was estimated to be 788 nM (36% CV), consistent with its potency in activating renal cGMP production.

Following 15 weeks of treatment with BI 703704, rats were sacrificed and kidney weights were determined. BI 703704 resulted in a dose-dependent reduction in both left and right kidney weights normalized to body weight (average kidney weight/body weight ratio: Vehicle: 0.392±0.019 and BI 703704: 0.364±0.023, 0.333±0.014, 0.316±0.017 and 0.258±0.006 at 0.3, 1, 3 and 10 mg/kg, respectively). Reductions were significantly different from vehicle at the doses

of 3 and 10 mg/kg (reductions of 19% and 34% for left kidney, $p<0.05$). Enalapril (3 mg/kg/day) had no effect on kidney to body weight ratio (0.388±0.013) compared to vehicle control (0.392±0.019).

Enalapril at the dose tested reduced modestly but not significantly the incidence of glomerulosclerosis (20% decrease vs. vehicle; $p>0.05$). In comparison, BI 703704 resulted in a significant, dose-dependent decrease in glomerulosclerosis compared to vehicle (44, 48 and 71% vs. vehicle at the doses of 1, 3, and 10 mg/kg, respectively; Figures 7 and 8 A). Additionally, at the highest dose tested, BI 703704 was significantly different from enalapril. Tubulointerstitial lesions were also dose-dependently decreased by BI 703704 at 0.3, 1, 3, and 10 mg/kg compared to vehicle (14, 21, 33 and 67%, respectively), however this was only significant in the group receiving BI 703704 at 10 mg/kg (Figure 8 B). Enalapril reduced tubulointerstitial lesions (24% vs.vehicle) modestly, but not significantly.

Markers of renal damage including α-SMA, a marker of myofibroblast activation, and KIM-1, a marker for renal tubular injury, were quantified in kidney cortex tissue by immunohistochemistry. BI 703704 resulted in a significant reduction in α-SMA expression in kidney cortex at all doses tested compared to vehicle (Table 3). Enalapril (3 mg/kg) modestly reduced α-SMA expression in this study, albeit not significantly. Likewise, BI 703704 significantly reduced KIM-1 expression at 1, 3 and 10 mg/kg compared to vehicle and at 10 mg/kg compared to enalapril (3 mg/kg) (Table 3). Enalapril resulted in a modest reduction in KIM-1 expression that was not significantly different from vehicle (Table 3).

Discussion

We investigated the effect of sGC activation in the ZSF1 rat model of type 2 diabetes mellitus (T2DM)-induced nephropathy. The present study demonstrates that BI 703704, a potent sGC activator, is highly efficacious in preventing the progression of diabetic nephropathy in a pre-clinical model that displays many of the clinical manifestations of diabetic nephropathy in patients including obesity, hyperglycemia, dyslipidemia, mild hypertension, proteinuria, and renal lesions. Importantly, ZSF1 rats exhibit markers of oxidative stress as evidenced by increased levels of urinary 8-hydroxy-2-deoxyguanosine (Prabhakar, 2007). Given that oxidative stress can result in an inactivation of sGC through oxidation of its heme prosthetic group, it was important to test the effectiveness of a sGC activator in such a setting. Our data demonstrate that BI 703704 is effective in elevating renal cGMP levels in a dose-dependent manner and in slowing the progression of diabetic nephropathy in an oxidative stress milieu.

Given that the ZSF1 rat does not exhibit a decline in GFR by 27 weeks of age, proteinuria and renal histological measures of glomerulosclerosis and tubulointerstitial lesions were assessed as surrogate measures of renal function. Proteinuria has been demonstrated to be a strong risk factor for renal functional decline (de Zeeuw, 2004). Furthermore, reductions in proteinuria with ARBs have been shown to be predictive of renal functional outcome in patients with diabetic nephropathy (Lewis, 2001 and Brenner, 2001). The sGC activator BI 703704 resulted in significant, dose-dependent reductions in UPE in ZSF1 rats within 15 weeks of treatment. Notably, this was achieved at doses of BI 703704 (0.3, 1, and 3 mg/kg) that did not significantly reduce MAP. This is consistent with a previous report indicating that treatment with the sGC

activator HMR1766 can reduce urinary albumin excretion in the rat 5/6 nephrectomy model of chronic renal failure without affecting blood pressure (Benz, 2007). Thus, although BI 703704 is capable of reducing blood pressure, significant hemodynamic effects are not required to induce robust proteinuria reductions in ZSF1 rats. Moreover, the potency of BI 703704 to reduce proteinuria was consistent with the potency of the molecule to elevate renal cGMP, thereby demonstrating that these anti-proteinuric effects are due to sGC activation. Thus, our results confirm the mechanism of action of BI 703704 *in vivo*.

A hallmark of diabetic nephropathy is the development of glomerulosclerosis. Severity of glomerular lesions has been shown to be associated with progression to ESRD and doubling of serum creatinine in T2DM patients with biopsy proven diabetic nephropathy (An, 2014). BI 703704 produced a dose-dependent reduction in the incidence of glomerulosclerosis in ZSF1 rats when compared to vehicle. Similar to effects on proteinuria, reductions in glomerulosclerosis were observed at doses of BI 703704 (1 and 3 mg/kg) that did not significantly affect blood pressure. These results confirm the efficacy of BI 703704, a sGC activator, in preventing glomerular remodeling, thereby inhibiting disease progression.

Tubulointerstitial remodeling, including interstitial fibrosis and volume, has been shown to correlate with serum creatinine concentrations in diabetic nephropathy patients (Bader, 1980). In this study, we assessed tubulointerstitial lesions by measuring the number of foci showing mild to severe lesions indicative of an inflammatory and pro-fibrotic milieu. Treatment with BI 703704 dose-dependently reduced the number of interstitial foci with a significant effect observed at the highest dose compared to vehicle. This was accompanied with a decrease in α-SMA staining, a marker of myofibroblast activation; thereby supporting an anti-fibrotic effect of BI 703704. Furthermore, BI 703704 effectiveness in preventing tubulointerstitial damage was

supported by significant reductions in KIM-1 staining, a marker of tubular injury. Taken together, these results demonstrated the efficacy of a sGC activator in preventing tubulointerstitial damage in a model of T2DM-induced nephropathy.

Currently, approved therapies for the treatment of diabetic nephropathy consist of ACE inhibitors and ARBs (Lewis, 1993, Lewis, 2001 and Brenner, 2001). In our study, enalapril, used as a positive control, significantly reduced proteinuria, but failed to significantly prevent glomerulosclerosis and interstitial lesions. Importantly, the magnitude of enalapril anti-hypertensive and anti-proteinuric effects were consistent with that seen in clinical trials (Lewis, 2001, Brenner, 2001 and The SOLVD investigators, 1991), indicative that the dose selected (3 mg/kg) was clinically relevant. It is noteworthy that BI 703704 produced similar or lesser blood pressure reductions than enalapril in our study at all but the highest dose, yet provided greater renal protection as evident by significant reductions in glomerular and interstitial lesions. This suggests that sGC activation provides renal protection independent of blood pressure effects, and that ACE inhibitors and sGC activators may affect different molecular pathways.

The metabolic effect of the sGC-cGMP pathway are emerging. A hyperinsulinemic euglycemic clamp performed in high fat diet fed mice indicated that treatment with a PDE5 inhibitor and NO-donor improved significantly insulin sensitivity compared to control (Ayala, 2007). In addition, recent studies demonstrate protection from diet-induced obesity with a sGC stimulator. This effect was accompanied with browning of fat mass, improved glucose tolerance, increased food intake, and increased energy expenditure (Hoffman, 2015). Consistent with these beneficial effects on glycemic control, our data indicate a reduction in HbA1c at the doses of 3 and 10 mg/kg of BI 703704. Importantly, these findings cannot be solely attributed to increased cGMP as HbA1c lowering occurred in this study concomitantly with a reduction in food intake, an

effect that may be resulting from taste aversion. Despite the reduction in food intake, BI 703704 treated rats exhibited an increase in body weight which may be driven by a reduction in energy expenditure. Nonetheless, the beneficial effects on glycemia raise the possibility that renal protection may in part be the indirect result of such an improvement. However, given that significant effects on proteinuria and renal histological measures were observed at the dose of 1 mg/kg, a dose that did not significantly affect HbA1c, our data are also supportive of glucose-independent effects of sGC activation on the kidney. Moreover, as the ZSF1 rat is leptin receptor deficient, the metabolic effects BI 703704 on body weight and food intake cannot be adequately interpreted in this model. Future studies will further investigate these effects utilizing suitable animal models for such measures such as diet-induced obesity rodents.

Few published reports have explored the utility of sGC activators for the treatment of chronic kidney disease. Both BAY 58-2667 and HMR1766 provided renal protection in the rat 5/6 nephrectomy model of chronic renal failure (Benz, 2007 and Kalk, 2006). This model is characterized by surgically-induced impaired renal function but lacks the diabetic milieu and its impact on renal molecular pathways. BAY 58-2667 also reduced serum creatinine, blood urea nitrogen levels and improved survival in hypertensive transgenic renin rats [TG(mRen2)27 rats] treated with a nitric oxide inhibitor, supporting the persistent efficacy of a sGC activator in the presence of oxidative stress (Stasch, 2006). Additionally, sGC stimulators have been investigated for their effects on renal function in models of kidney disease (Stasch, 2015). Notably, the effect of the sGC stimulator riociguat was tested alone and in combination with telmisartan, an ARB, in the streptozotocin –treated eNOS deficient mouse, a model of type 1 diabetes and endothelial dysfunction (Ott, 2012). While monotherapy with riociguat produced a significant decrease (~9 mmHg) in systolic blood pressure, there was no effect on albuminuria or

glomerulosclerosis with 11 weeks of treatment, possibly due to sGC heme oxidation which renders the enzyme non-responsive to stimulators. In contrast, BI 703704 significantly reduced proteinuria in the ZSF1 rat with no notable effects on blood pressure, supporting a greater efficacy of sGC activators compared to stimulators in the presence of oxidative stress. Future studies directly comparing sGC activators and stimulators in diabetes-induced nephropathy models will provide further insights into the potential utility of these two modalities for treating diabetic nephropathy. Furthermore, studies aimed at investigating the renoprotective effects of sGC activators when combined with the standard of care (ACE inhibitors, ARBs) are warranted.

To our knowledge, this is the first report of renal beneficial effects, including histological improvements, of a sGC activator in a model of type 2 diabetes-induced nephropathy. Importantly, we demonstrate beneficial effects of BI 703704 in the absence of significant hemodynamic effects. Therefore, we propose sGC activation as a novel pharmacological approach for the treatment of diabetic nephropathy.

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Authorship Contributions

Participated in research design: Boustany-Kari, Harrison, Chen, Lincoln, Qian, Clifford, Wong, Gueneva-Boucheva, Fryer, Richman, Sarko and Pullen.

Conducted experiments: Harrison, Chen, Lincoln, Clifford, Wang and Zhang.

Contributed new reagents: Bosanac and Sarko.

Performed data analysis: Chen, Wang, Wong and Gueneva-Boucheva.

Wrote or contributed to writing of the manuscript: Boustany-Kari, Harrison, Qian, Wong, Gueneva-Boucheva, Fryer, Sarko and Pullen.

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Figure Legends

Figure 1. Structure of BI 703704

Figure 2. Correlation of BI 703704 plasma concentrations with kidney cGMP contents

Using a simple E_{max} model, individual mean plasma concentrations (nM; mean of three time points) of BI 703704 were associated with respective kidney cGMP contents (pmol/g tissue) measured after 15 weeks of treatment. Increasing kidney cGMP contents correlated with increasing BI 703704 concentrations and an EC_{50} of 420 nM (108% CV) was calculated as the BI 703704 plasma concentration associated with a 50% increase in renal cGMP.

Figure 3. Effect of sGC activation on weekly MAP

Beginning at week 2, BI 703704 at 10 mg/kg resulted in a significant reduction in MAP compared to vehicle. There were no relevant significant effects of BI 703704 at 0.3, 1, and 3 mg/kg on MAP. Enalapril at 3 mg/kg showed a significant reduction in MAP from week 11 onwards. * and # indicate statistical significance compared to vehicle and enalapril (3 mg/kg), respectively ($p<0.05$, 2-way ANOVA with repeated measures and Bonferrroni post-hoc analysis).

Figure 4. Effect of sGC activation on weekly mean HR

BI 703704 at 10 mg/kg resulted in a significant increase in HR beginning on week 4. There was no significant effect on HR of BI 703704 at 0.3, 1 and 3 mg/kg, or enalapril at 3 mg/kg. * signifies statistical significance compared to vehicle ($p<0.05$, 2-way ANOVA with repeated measures and Bonferrroni post-hoc analysis).

Figure 5. Effect of sGC activation on weekly UPE

BI 703704 at 1, 3 and 10 mg/kg significantly reduced UPE levels from weeks 14, 7 and 6 onwards, respectively. Enalapril at 3 mg/kg significantly reduced UPE levels from week 13 onwards. * and # signifies statistical significance compared to vehicle and enalapril, respectively ($p<0.05$, 2-way ANOVA with repeated measures and Bonferroni post-hoc analysis).

Figure 6. Correlation of BI 703704 plasma concentrations with UPE

Using a simple Imax model, BI 703704 individual mean plasma concentrations on weeks 5, 10 and 15 were associated with the overall change from baseline UPE levels (mg/day) for individual animals. Decreasing UPE levels correlated with increasing BI 703704 plasma concentrations and an IC_{50} of 788 nM (36% CV) was calculated as the BI 703704 plasma concentration associated with a 50% reduction in UPE.

Figure 7. Effect of sGC activation on glomerular lesions

Representative glomerulosclerosis from A-vehicle, B-enalapril at 3 mg/kg and C-F-BI 703704 at 0.3, 1, 3 and 10 mg/kg, respectively. Glomerular semi-quantitative scoring performed with PAM on 25 random glomeruli and scored as: 1+ showing segmental condensation and structural collapse of approximately one quarter of the glomerular area, 2+ showing segmental condensation and structural collapse of half of the glomerular area and 3+ showing condensation and structural collapse of most of the glomerular area. Red arrows indicate areas of advanced glomerulosclerosis in the vehicle control tissue. BI 703704 resulted in a dose-dependent reduction in glomerulosclerosis.

Figure 8. Effect of sGC activation on incidence of glomerulosclerosis (A) and tubulointerstitial lesions (B)

BI 703704 at 1, 3 and 10 mg/kg dose-dependently and significantly reduced the incidence of glomerulosclerosis (A, glomerulosclerosis %) and interstitial lesions (B, the number of foci/kidney section). * and # signifies statistical significance compared to vehicle and enalapril (3 mg/kg), respectively ($p<0.05$, 1-way ANOVA with Tukey's post-hoc analysis).

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Tables

Table 1: Mean plasma and intra-renal concentrations of BI 703704 and enalaprilat

Compound	Dose (mg/kg/day)	Mean Concentration			
		Plasma			Kidney
		Week 5	Week 10	Week 15	Week 15
BI 703704	0.3	101 ± 9	78 ± 4	94 ± 19	25 ± 2
	1	524 ± 100	319 ± 22	496 ± 114	83 ± 5
	3	1129 ± 80	1082 ± 79	1358 ± 247	243 ± 15
	10	3455 ± 455	2815 ± 165	3175 ± 130	1509 ±114
Enalaprilat	3	120 ± 21	141 ± 21	212 ± 23	124 ± 13

Plasma levels reported as mean ± SEM in nM for BI 703704 and enalaprilat, the active metabolite of enalapril, at weeks 5, 10 and 15 of the study. Kidney concentrations reported in nM for BI 703704 and enalaprilat at week 15.

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Table 2: Effect of BI 703704 and enalapril on metabolic parameters

Treatment	Body Weight (g)		Food Intake (g)		Water Intake (g)	
	Baseline (Day-0)	Weight Gain (through week 15)	Baseline (Day-0)	Cumulative (through week 15)	Baseline (Day-0)	Cumulative (through week 15)
Vehicle	501 ± 7	211 ± 10	34 ± 1	3527 ± 148	55 ± 8	8452 ± 916
Enalapril 3 mg/kg	504 ± 10	178 ± 9	32 ± 1	3294 ± 89	50 ± 6	7507 ± 682
BI 703704 0.3 mg/kg	502 ± 10	220 ± 13	34 ± 2	3181 ± 165	46 ± 4	7471 ± 1440
BI 703704 1 mg/kg	507 ± 11	276 ± 26*#	33 ± 1	3127 ± 67	53 ± 5	5745 ± 315
BI 703704 3 mg/kg	488 ± 8	273 ± 9*#	35 ± 1	2957 ± 48*	54 ± 5	4671 ± 245*
BI 703704 10 mg/kg	500 ± 5	313 ± 8*#	31± 1	3030 ± 91*	38 ± 5	4378 ± 170*#

Metabolic parameters of body weight, food intake and water intake shown at baseline (Day 0) and following 15 weeks of treatment. * and # significant $p<0.05$ vs. vehicle and enalapril, respectively, 1-way ANOVA.

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Table 3. Effect of sGC activation on markers of renal damage

Treatment	α-SMA		KIM-1	
	% Positive Expression	Percent Change	% Positive Expression	Percent Change
Vehicle	6.08±0.70		3.15±0.45	
Enalapril 3 mg/kg	4.45±0.49	-26.7	2.28±0.31	-27.5
BI 703704 0.3 mg/kg	2.09±0.36	-65.6*#	1.94±0.22	-38.4
BI 703704 1 mg/kg	1.65±0.39	-72.9*#	1.29±0.26	-59.0*
BI 703704 3 mg/kg	1.92±0.47	-68.4*#	1.54±0.30	-51.2*
BI 703704 10 mg/kg	1.97±0.30	-67.6*#	0.89±0.20	-71.8*#

Markers of renal damage, α-SMA and KIM-1, were quantified by immunohistochemistry and expressed as mean ± SEM percent positive expression area/glomeruli and as a percent change from vehicle control. *and # significant $p<0.05$ vs. vehicle and enalapril, respectively using 1-way ANOVA.

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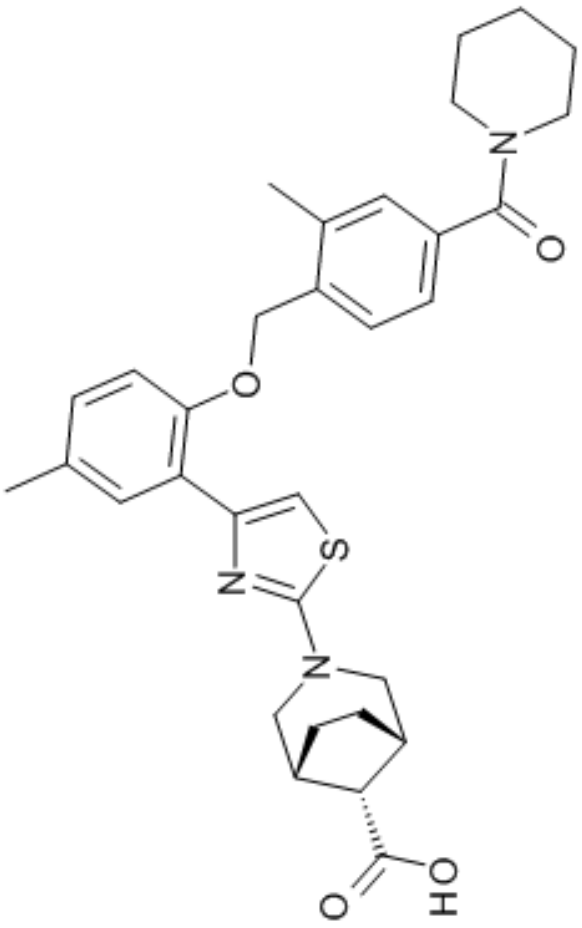
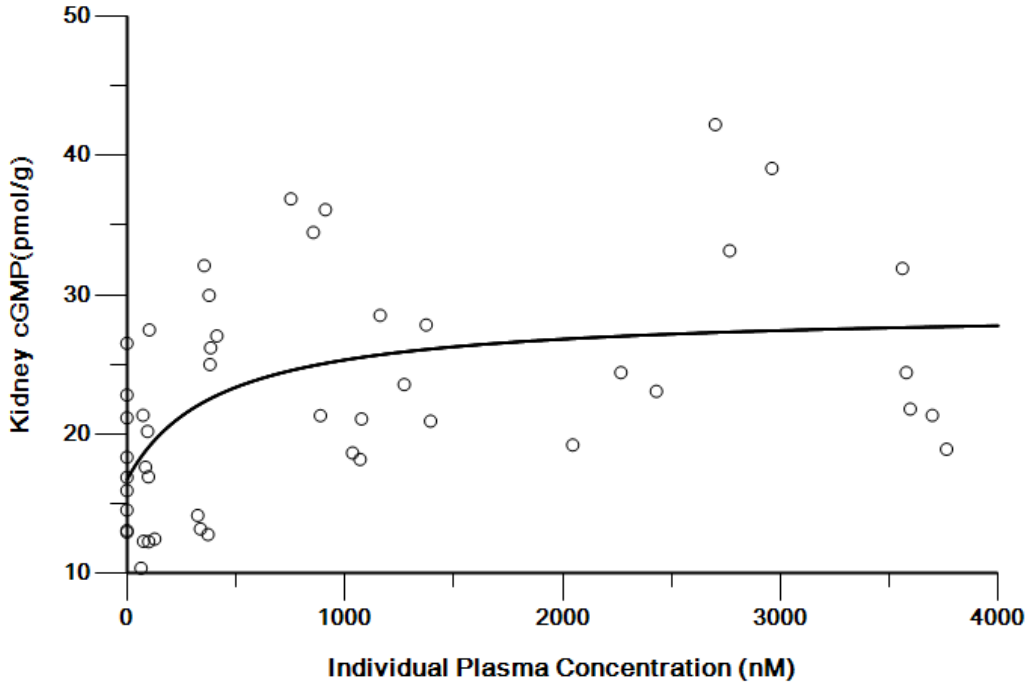


Figure 1

Figure 2



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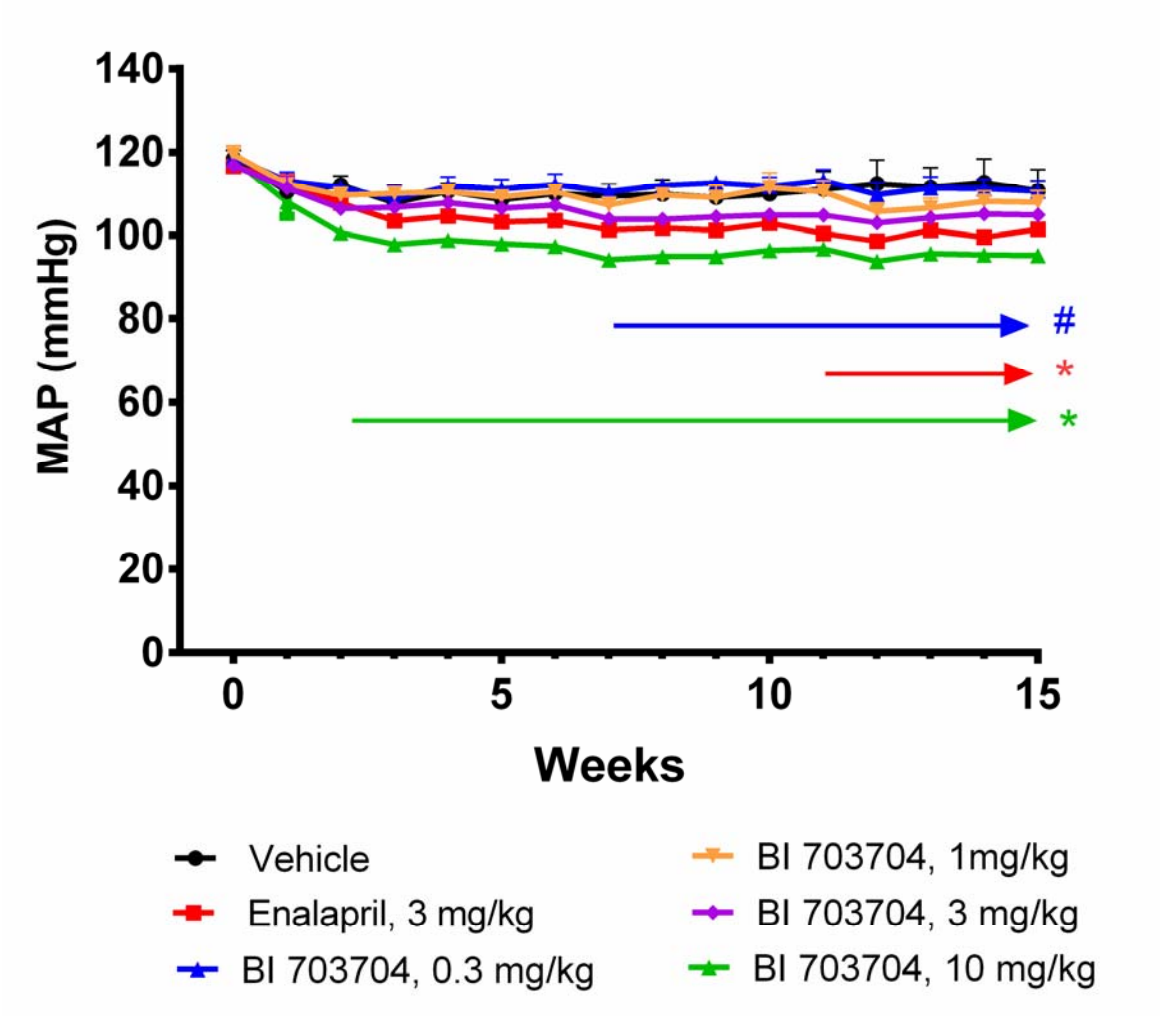


Figure 4

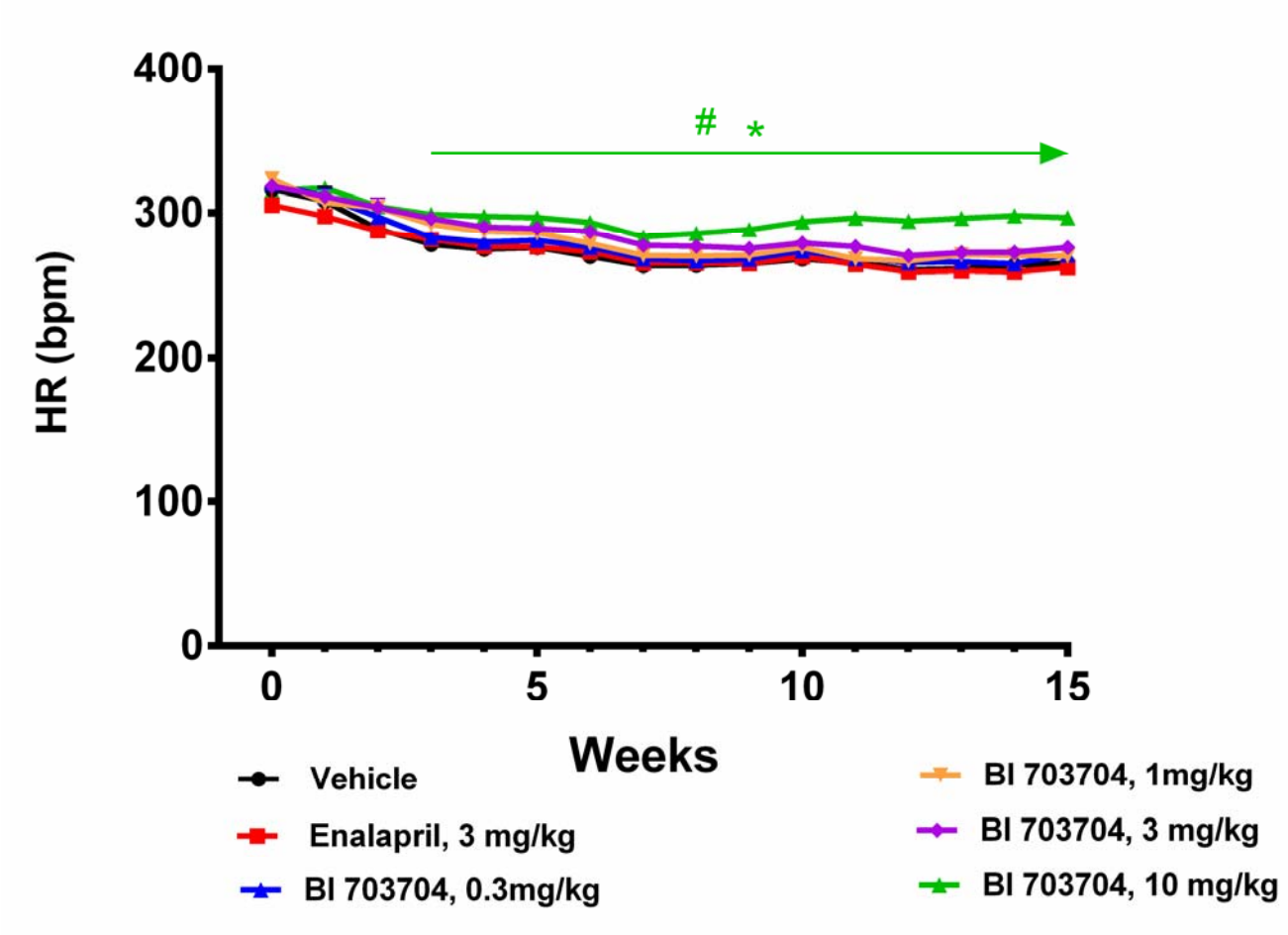


Figure 5

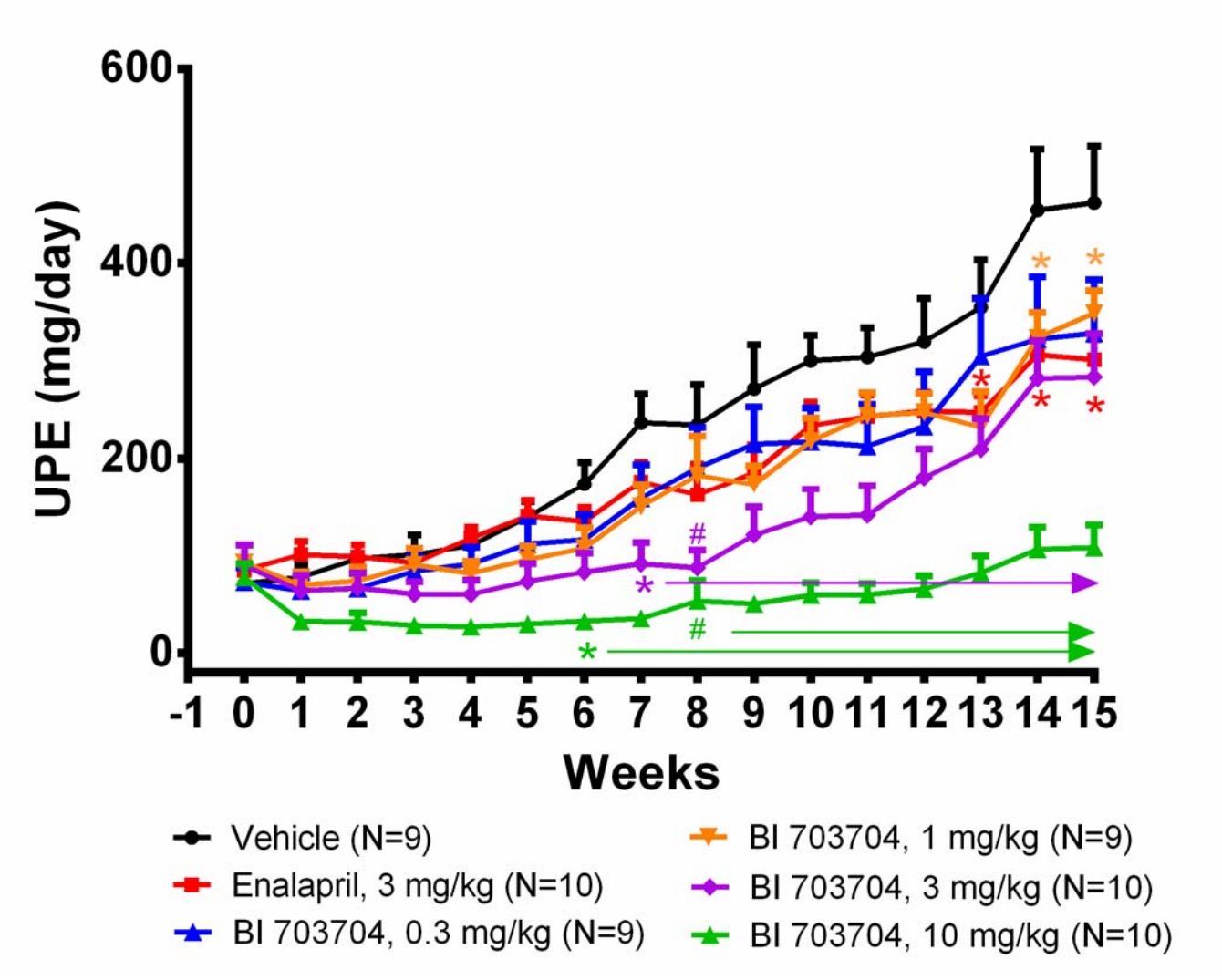
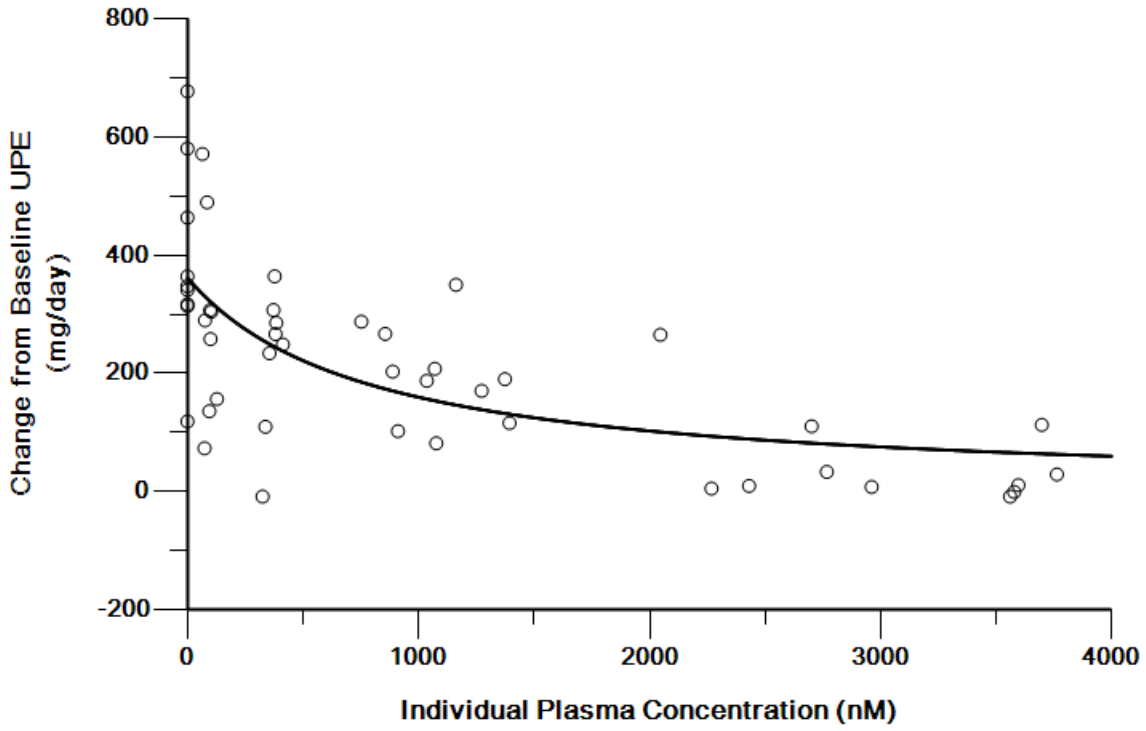


Figure 6



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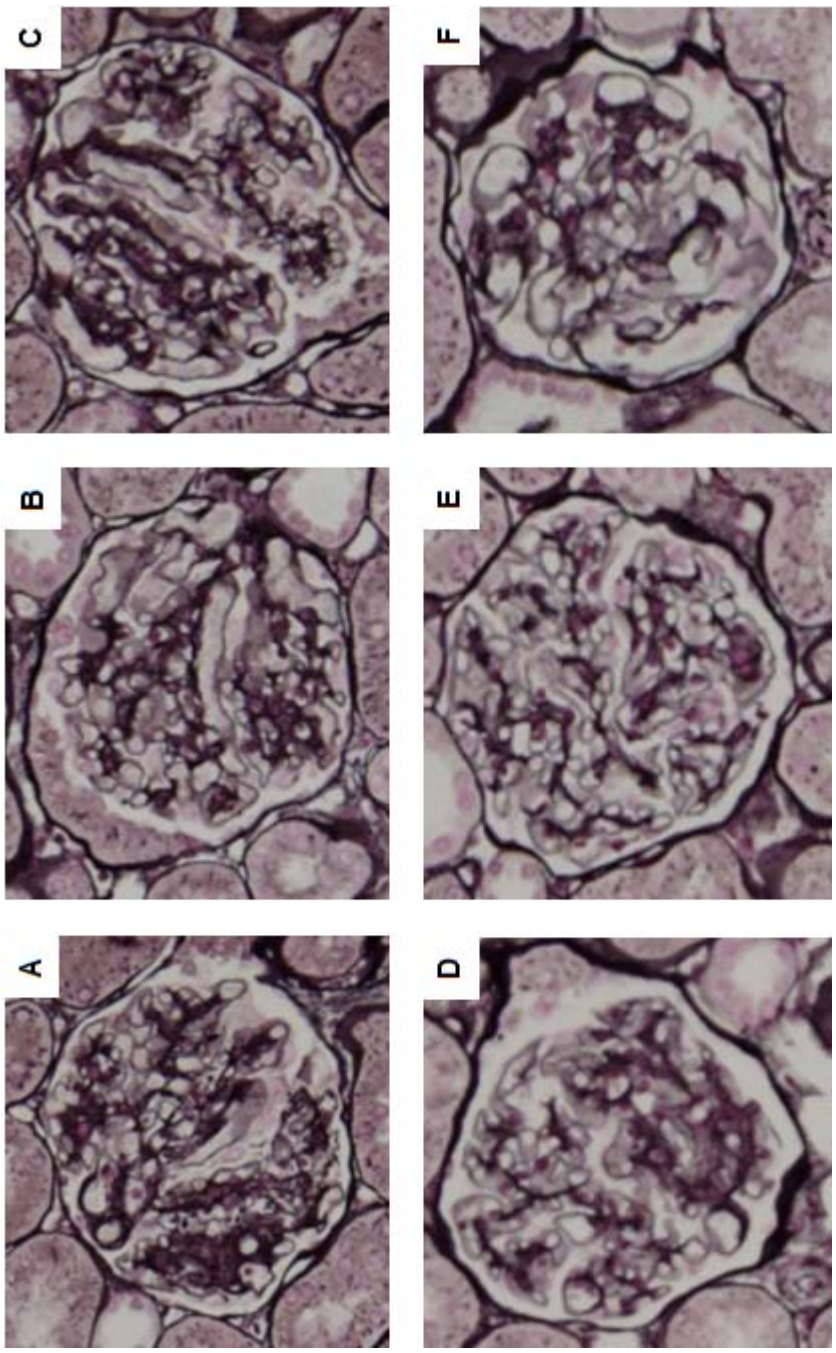

Figure 7

Figure 8