Selonsertib Enhances Kidney Protection Beyond Standard of Care in a Hypertensive, Secondary Glomerulosclerosis CKD Model

DOI: 10.34067/KID.0001032022

Shawn Badal, Tareq Al Tuhaifi, Ya-Fen Yu, David Lopez, Craig Plato, Kristin Joly, David Breckenridge, Hai-Chun Yang, John Liles, and Agnes Fogo

Key Points:
* Selonsertib (SEL) a selective apoptosis signal-regulating kinase 1 inhibitor, targets an oxidative stress pathway in chronic kidney disease.
* SEL plus enalapril reduces glomerulosclerosis, attenuates kidney function decline, and reduces podocyte loss more than either agent alone.
* SEL has a non-hemodynamic mechanism and has additional effects on apoptosis and fibrosis in the kidney when combined with standard of care.

Abstract:
Background: Despite widespread use of renin-aldosterone-angiotensin system inhibitors and the benefits of lowering glomerular pressure in patients with chronic kidney disease (CKD), there remains a major unmet need for therapies targeting underlying causes of CKD progression. Apoptosis signal-regulating kinase 1 (ASK1) promotes apoptosis and glomerulosclerosis, and is implicated in the progression of diabetic kidney disease (DKD), a major cause of CKD. Selonsertib is a selective ASK1 inhibitor currently in clinical development for the treatment of DKD. We examined the added benefits of selonsertib on existing glomerulosclerosis and related molecular pathways in the non-diabetic 5/6 nephrectomy (5/6 Nx) rat model in combination with the angiotensin-converting enzyme inhibitor (ACEI) enalapril. Methods: Male Sprague Dawley rats underwent 5/6 Nx with kidney biopsy 8 weeks later for assessment of glomerulosclerosis, and were randomized to four treatment groups with equal glomerulosclerosis: selonsertib, enalapril, combination (selonsertib+enalapril), and untreated controls. Serum creatinine, systolic blood pressure (SBP) and urinary albumin were measured at intervals. Animals were sacrificed at week 12 for histological, biochemical, and molecular analyses. Results: All rats developed hypertension, albuminuria, and glomerulosclerosis by week 8. Kidney function further declined, and glomerulosclerosis and albuminuria progressively increased in controls from week 8 to 12. Enalapril treatment alone from week 8-12 reduced SBP versus controls, decreased albuminuria and resulted in numerically lower glomerulosclerosis. Selonsertib alone had no effect on SBP but preserved kidney function. Combined treatment significantly reduced glomerulosclerosis, with more regression than either monotherapy. Enalapril treatment resulted in fewer interstitial macrophages, while selonsertib treatment reduced apoptosis and podocyte loss. RNA-Seq revealed that combined treatment impacted pathways related to extracellular matrix and wound-healing. Conclusions: Selonsertib targets a novel, non-hemodynamic pathway in CKD. Our data suggest that ASK1 inhibition when combined with ACEI has additive effects to reduce progression of glomerulosclerosis, attenuate kidney function decline, and reduce podocyte loss.
Disclosures: S. Badal reports the following: Employer: Gilead Sciences, Inc.; and Ownership Interest: Gilead Sciences, Inc. T. Al Tuhaifi reports the following: Ownership Interest: MERCK & CO INC; BUTTERFLY NETWORK INC. D. Lopez reports the following: Employer: Gilead Sciences; and Ownership Interest: Gilead Sciences. C. Plato reports the following: Employer: Plato BioPharma, Inc. (PBI); Craig Plato is the Founder and President of PBI.; Inotiv Inc. acquired PBI on October 4th, 2021. Craig Plato is now Senior Vice President, Physiological Sciences; Ownership Interest: Plato BioPharma, Inc. was acquired October 4th, 2021, that ownership interest no longer exists.; Craig Plato acquired Restricted Stock (not yet unrestricted) in Inotiv, Inc. as part of the acquisition of PBI. Craig Plato was granted stock options in Inotiv Inc. as part of an employment offer with Inotiv, Inc. Those stock options have not yet vested in any way.; Research Funding: Plato BioPharma, Inc. (PBI) is a leading preclinical drug discovery contract research organization (CRO). PBI was compensated for providing research services resulting in generation of data supporting submitted abstracts and potential publications.; Inotiv, Inc. acquired PBI on Oct 4th, 2021 and is a CRO, thus compensated for providing research services resulting in generation of data supporting submitted abstracts and potential publications.; and Advisory or Leadership Role: Plato BioPharma, Inc. (PBI); As Founder and President of PBI, Craig Plato served on PBI's Board of Directors. Inotiv, Inc. acquired PBI on Oct 4th, 2021, thus Craig Plato is no longer an officer or Board Member of any company. K. Joly reports the following: Employer: Plato BioPharma, Inc.; Ownership Interest: Plato BioPharma, Inc.; and Research Funding: Plato BioPharma, Inc. (PBI) is a leading preclinical drug discovery contract research organization (CRO). ; As such, PBI was compensated for providing research services resulting in the generation of data supporting submitted abstracts and potential full publications. D. Breckenridge reports the following: Employer: Actio Biosciences; Ownership Interest: Actio Biosciences; Gilead Sciences Inc.; and Advisory or Leadership Role: Actio Biosciences. J. Liles reports the following: Employer: Gilead Sciences, Inc.; Ownership Interest: Gilead Sciences, Inc.; and Research Funding: Gilead Sciences, Inc. A. Fogo reports the following: Consultancy: Novartis; Research Funding: Gilead, Bayer, Novartis; Honoraria: Novartis, Amgen, GSK; Advisory or Leadership Role: Advisory Committee Bayer, Noncompany: Editorial Board for Human Pathology and JASN, AM J Physiol (Renal), AM J Path; Associate Editor Lab Invest and Kidney International, Subject Editor NDT, Current opinion nephrology and hypertension guest editor for yearly pathology focus; and Other Interests or Relationships: Noncompany: Editorial Board for Human Pathology and JASN and AM J Physiol (Renal), Associate Editor Lab Invest, Subject Editor NDT, speaker at various national nephrology meetings, AE KI; President Elect ISN (president as of April 19, 2021). The remaining authors have nothing to disclose.


Author Contributions: Shawn Badal: Conceptualization; Formal analysis; Investigation; Writing - original draft; Writing - review and editing Tareq Al Tuhaifi: Investigation; Writing - review and editing Ya-Fen Yu: Investigation; Writing - review and editing David Lopez: Formal analysis; Writing - review and editing Craig Plato: Investigation; Writing - review and editing Kristin Joly: Investigation; Writing - review and editing David Breckenridge: Conceptualization; Formal analysis; Methodology; Writing - original draft; Writing - review and editing Hai-Chun Yang: Conceptualization; Formal analysis; Investigation; Writing - original draft; Writing - review and editing John Liles: Conceptualization; Formal analysis; Methodology; Writing - original draft; Writing - review and editing Agnes Fogo: Conceptualization; Formal analysis; Methodology; Writing - original draft; Writing - review and editing

Data Sharing Statement: All data is included in the manuscript and/or supporting information.

Clinical Trials Registration:

Registration Number:

Registration Date:

The information on this cover page is based on the most recent submission data from the authors. It may vary from the final published article. Any fields remaining blank are not applicable for this manuscript.
Selonsertib Enhances Kidney Protection Beyond Standard of Care in a Hypertensive, Secondary Glomerulosclerosis CKD Model

Shawn S. Badal,*1 Tareq Al Tuhaifi,2 Ya-Fen Yu,2,3 David Lopez,1 Craig T. Plato,4 Kristin Joly,4 David G. Breckenridge,1 Hai-Chun Yang,2 John T. Liles,1 and Agnes B. Fogo2

1Gilead Sciences, Inc., Foster City, CA, USA

2Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA

3Fourth Hospital, Wuxi, Anhui, China

4PlatoBiopharma, Inc., Westminster, CO, USA

*These authors contributed equally to this work.

Corresponding author: Agnes B. Fogo, M.D.

MCN C3310

Department of Pathology, Microbiology, and Immunology

Vanderbilt University Medical Center

Nashville, TN, 37232, USA

E-mail: agnes.fogo@vanderbilt.edu

Phone: 615-322-3114

Fax: 615-343-7023
Key points:

- Selonsertib (SEL), a selective apoptosis signal-regulating kinase 1 inhibitor, targets an oxidative stress pathway in chronic kidney disease.
- SEL plus enalapril reduces glomerulosclerosis, attenuates kidney function decline, and reduces podocyte loss more than either agent alone.
- SEL has a non-hemodynamic mechanism and has additional effects on apoptosis and fibrosis in the kidney when combined with standard of care.
Abstract

Background: Despite widespread use of renin–aldosterone–angiotensin system inhibitors and the benefits of lowering glomerular pressure in patients with chronic kidney disease (CKD), there remains a major unmet need for therapies targeting underlying causes of CKD progression. Apoptosis signal-regulating kinase 1 (ASK1) promotes apoptosis and glomerulosclerosis, and is implicated in the progression of diabetic kidney disease (DKD), a major cause of CKD. Selonsertib is a selective ASK1 inhibitor currently in clinical development for the treatment of DKD. We examined the added benefits of selonsertib on existing glomerulosclerosis and related molecular pathways in the non-diabetic 5/6 nephrectomy (5/6 Nx) rat model in combination with the angiotensin-converting enzyme inhibitor (ACEI) enalapril.

Methods: Male Sprague Dawley rats underwent 5/6 Nx with kidney biopsy 8 weeks later for assessment of glomerulosclerosis, and were randomized to four treatment groups with equal glomerulosclerosis: selonsertib, enalapril, combination (selonsertib+enalapril), and untreated controls. Serum creatinine, systolic blood pressure (SBP) and urinary albumin were measured at intervals. Animals were sacrificed at week 12 for histological, biochemical, and molecular analyses.

Results: All rats developed hypertension, albuminuria, and glomerulosclerosis by week 8. Kidney function further declined, and glomerulosclerosis and albuminuria progressively increased in controls from week 8 to 12. Enalapril treatment alone from week 8–12 reduced SBP versus controls, decreased albuminuria and resulted in numerically lower glomerulosclerosis. Selonsertib alone had no effect on SBP but preserved kidney function. Combined treatment significantly reduced glomerulosclerosis, with more regression than either monotherapy. Enalapril treatment resulted in fewer interstitial macrophages, while
selonsertib treatment reduced apoptosis and podocyte loss. RNA-Seq revealed that combined treatment impacted pathways related to extracellular matrix and wound-healing.

**Conclusions:** Selonsertib targets a novel, non-hemodynamic pathway in CKD. Our data suggest that ASK1 inhibition when combined with ACEI has additive effects to reduce progression of glomerulosclerosis, attenuate kidney function decline, and reduce podocyte loss.
**Introduction**

Chronic kidney disease (CKD) often leads to end-stage kidney disease and remains a growing public health problem despite treatment options designed to reduce both systemic and glomerular hypertension. A hallmark of late-stage CKD and diabetic kidney disease (DKD) is the significant and progressive loss of functional nephrons, a pathologic sequela that can be studied preclinically in rodents following 5/6 nephrectomy (5/6 Nx). The removal of renal mass in this model causes compensatory derangements within the remaining nephrons, including hypertrophy and proteinuria, triggering hypertension and the subsequent development of glomerulosclerosis lesions (1). Previous studies in this model have helped to elucidate the mechanisms underlying the benefit of standard CKD therapies, including angiotensin-converting enzyme inhibitors (ACEI), angiotensin type 1 receptor blockers (ARB) and aldosterone inhibitors (2). We have previously demonstrated that high-dose ACEI or ARB therapy ameliorated the progression of glomerulosclerosis, and even induced regression of existing glomerulosclerosis, in about two-thirds of rats treated from week 8 to week 12 after 5/6 Nx (3). However, monotherapy with renin–aldosterone–angiotensin system (RAAS) blockade alone is insufficient to induce long-term regression of glomerulosclerosis (4). This suggests multipronged therapeutic approaches that go beyond hemodynamic effects will be necessary for the optimal treatment of CKD.

In progressive CKD, reactive oxygen species (ROS) in the kidney can drive cellular injury, apoptosis, inflammation, and fibrosis. ROS influence the activation of multiple cellular processes, including the activation of apoptosis signal-regulating kinase 1 (ASK1), a redox-sensitive mitogen-activated protein kinase kinase kinase (MAPKKK), which activates downstream terminal MAPK kinases p38 and c-Jun N-terminal kinase (JNK) (5). The JNK/p38 pathway stimulates production of inflammatory cytokines/chemokines, promotes
cell apoptosis, and contributes to metabolic perturbations, and induces fibrosis (6,7). Increased kidney cortical and glomerular p38 and JNK activation has been observed in several kidney diseases, including DKD, mesangial proliferative glomerulonephritis, and anti-glomerular basement membrane glomerulonephritis, in both humans and experimental models (8-10). Previous studies indicate that global knockout or pharmacological inhibition of ASK1 can attenuate the activation of JNK and p38, reduce progressive podocyte apoptosis, improve podocyte morphology, attenuate renal fibrosis, and halt declining glomerular filtration rate (GFR) in multiple rodent models of CKD (11-14).

The ASK1 inhibitor selonsertib is currently under clinical investigation as a potential treatment in DKD (ClinicalTrials.gov identifiers: NCT02177786, NCT04026165) (15). Selonsertib initiation is associated with an acute decrease in estimated GFR (eGFR) that is hypothesized to be due to inhibition of tubular secretion of creatinine and not due to a hemodynamic mechanism (15). We therefore assessed the acute impact of selonsertib on renal hemodynamics in the setting of CKD. In a chronic 5/6 Nx model, we evaluated the renal benefits of selonsertib treatment when combined with an ACEI, the latter at a dose established to normalize glomerular hemodynamics.

**Materials and Methods**

**Animals**

Adult male Sprague Dawley rats (8–9 weeks old; 250–350g; Charles River, Nashville, TN, USA) were housed under normal conditions with a 12-hour light/dark cycle at 70 °F with 40% humidity and 12 air exchanges/hour. All studies were approved by the Institutional Animal Care and Use Committee.
For chronic studies of CKD, glomerulosclerosis was induced by removing one kidney and ablation of two-thirds of the contralateral kidney by ligating branches of the renal arteries, as previously described (1). All rats underwent open shave renal biopsy at 8 weeks to assess the severity of glomerulosclerosis, as previously described (3). On average, 15 glomeruli (range 10–30 glomeruli) were available in these biopsies. Rats were randomized at 8 weeks into four groups with similar levels of systemic blood pressure, urinary protein levels and glomerulosclerosis: control group (n = 11) received normal rat chow and water ad libitum (“5001” diet, Purina Laboratory Rodent diet, 23.4% protein, 4.5% fat, 6.0% fiber, 0.40% sodium); selonsertib group (n = 12; ASK-1 inhibitor, selonsertib, from Gilead Sciences Inc., Foster City, CA, USA) received chow containing 0.009% selonsertib; enalapril group (n = 12) received normal rat chow and enalapril 50 mg/l in drinking water (antihypertensive dose); and the combination group (n = 12) received both selonsertib and enalapril treatment. Doses were based on efficacy in previous rodent studies (3). Selonsertib was provided at a dose designed to achieve >90% inhibition of ASK1 function (Supplemental Figure 1), similar to the 18 mg oral, once daily dose in humans (15). Animals were anesthetized for surgeries with sodium pentobarbital (Nembutal, Abbott laboratories, North Chicago, IL, USA; 25–30 mg/kg body weight, intraperitoneal). Animals were then sacrificed at week 12 after 5/6 Nx.

In addition, a separate group of male Sprague Dawley rats (6–7 weeks old, 176–200g; Charles River Laboratories, Wilmington, MA, USA) were used for acute studies of selonsertib in an ablation, non-ligation 5/6 Nx model. These rats were acclimatized under standard conditions at Plato Biopharma Inc. (Westminster, CO, USA) with a 12-hour light/dark cycle and fed a standard chow diet (22% protein, 0.4% sodium; “8640” diet, Envigo, Indianapolis, IN, USA) for at least 7 days before the study. Rats (n = 56) underwent
right unilateral nephrectomy via a retroperitoneal approach, and a group of weight-matched controls (n = 16) underwent sham surgery. One week later (day −7), the uninephrectomized rats underwent subtotal left nephrectomy by targeted renal ablation via pole resection using a retroperitoneal approach, and the control rats underwent a second sham surgery before recovery for 1 week in standard housing conditions. After 7 weeks (day 49), kidney function was assessed and rats with disease induction, defined as plasma blood urea nitrogen (BUN) >30 mg/dl and plasma creatinine >0.65 mg/dl, were entered into further intervention studies. Starting on day 53, the 5/6 Nx rats were treated orally with selonsertib 10 mg/kg or vehicle 5 ml/kg (0.5% carboxymethyl cellulose sodium, medium viscosity, 0.25% polysorbate-80 in pH 2.0 deionized H2O) twice daily for seven consecutive doses over 4 days. Clearance measurements were taken on day 56, 2 hours after the final morning dose of selonsertib. The same dose of selonsertib (10 mg/kg, twice daily) was used based on pharmacokinetic/pharmacodynamic data in rats (Supplemental Figure 1) demonstrating comparable profile to a selonsertib 18 mg dose in humans (15). Control rats (sham surgery) received vehicle only.

**Hemodynamics in Resection 5/6 Nx Rats**

Kidney hemodynamic measurements were done by steady-state bladder-catheterized urinary clearances of 3H-inulin and 14C-para-aminohippurate (PAH; PerkinElmer, Hopkinton, MA, USA). Renal blood flow was calculated as effective renal plasma flow (eRPF)/(1-Hct), and eRPF was calculated as the urinary clearance of PAH. Blood pressure and pulse were measured using an arterial catheter and transducer.

**Renal Function in Chronic Ligation 5/6 Nx Rats**

Systolic blood pressure (SBP), albuminuria, serum creatinine, and creatinine clearance (Ccr) were assessed at weeks 0, 8, and 12. SBP was measured using tail-cuff
plethysmography in conscious, trained rats (IITC; Life Science Inc., Woodland Hills, CA, USA). Animals were placed in metabolic cages for 24 hours for urine collection. Urine albumin was measured by enzyme-linked immunosorbent assay, and serum and urine creatinine were measured by VitrosCREA slides (Johnson & Johnson Clinical Diagnostics Inc., Rochester, NY, USA). Ccr was calculated as urinary creatinine × urine volume/serum creatinine, and was expressed as μl/min/body weight (1). Blood counts (reticulocyte, hemoglobin, hematocrit, and red blood cells) were assessed at sacrifice (Supplemental Table 1).

**Morphologic Assessment**

Kidney tissue from biopsy and autopsy was immersion-fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) solution and routinely processed, and 3 μm sections were cut and stained with periodic acid-Schiff. A semi-quantitative score was used to evaluate the degree of glomerulosclerosis. Sclerosis was defined as obliteration of glomerular capillary loops by extracellular matrix. The severity of sclerosis in each glomerulus was graded from 0 to 4+ as follows: 0, no lesion, 1+ sclerosis of <25%, 2+ sclerosis of 25–50%, 3+ sclerosis of >50–75%, 4+ sclerosis of >75% of the glomerulus, respectively. A whole kidney average sclerosis index for each rat was obtained by averaging scores from all glomeruli (range 10–30) of biopsy slides and all glomeruli on the section (n ≥ 60) of autopsy slides. All sections were examined without knowledge of the treatment. Change of sclerosis from biopsy to autopsy was calculated for each animal as previously described (3).

**Immunohistochemistry**

For assessment of Wilms tumor-1 (WT1) and ED1 positivity, sections were microwaved in citrate buffer (pH 6.0) for 5 min. Endogenous peroxidase was quenched with
3% hydrogen peroxidase for 10 min, and slides were then exposed to Power Block (BioGenex Laboratories, San Ramon, CA, USA) for 45 min. The primary antibodies used were rabbit anti-rat WT1 (1:800; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and mouse anti-rat ED1 (1:50; Dako North America, Carpinteria, CA, USA), incubated overnight at 4 °C. Immunoperoxidase staining was performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), with diaminobenzidine as a chromogen. Hematoxylin was used as a counterstain. WT1+ cell number per glomerulus area and percentage of ED1+ area in glomeruli or cortex were assessed. Positive and negative controls showed appropriate staining.

**TUNEL Staining**

The TUNEL staining technique was used for detecting DNA breaks using ApopTag Peroxidase In Situ Apoptosis Detection kit (Intergen Co, Purchase, NY, USA) (16). In brief, 3 μm sections from paraffin-embedded tissue were pretreated by incubation with proteinase K (2 μg/ml) for 15 min at room temperature. Endogenous peroxidase was inhibited by 3% H2O2 in PBS for 5 min, and sections were rinsed with PBS, immersed in terminal deoxynucleotidyl transferase (TdT) equilibration buffer, and then incubated with TdT and digoxigenin-dUTP at 37 °C for 60 min. The reaction was blocked with buffer, and anti-digoxigenin peroxidase conjugate was applied and incubated for 30 min. The slides were developed by using 3,3’-diaminobenzidine (DAB) substrate. Apoptosis was assessed by scoring TUNEL+ cell number per glomerulus or tubulointerstitial area. All sections were examined without knowledge of the treatment. Positive and negative controls showed appropriate staining.

**Western Blot**
Frozen kidney tissue samples were transferred in radio immunoprecipitation assay (RIPA) buffer containing 1:100 phosphatase inhibitor cocktail I, 1:100 phosphatase inhibitor cocktail II (Sigma-Aldrich, St. Louis, MO, USA), and 1:100 proteinase inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Samples were homogenized and centrifuged, and the protein concentration was measured using the DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Connective tissue growth factor (CTGF; 1:400; Abcam PLC, Cambridge, MA, USA), plasminogen activator inhibitor 1 (PAI-1; 1:400; R&D Systems, Minneapolis, MN, USA), p-JNK (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), p-p38 (1:1000; Cell Signaling Technology Inc.) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:500; Cell Signaling Technology, Inc.) were detected by incubating with the corresponding antibody overnight at 4 °C. Horseradish peroxidase-labeled anti-goat or anti-rabbit IgG secondary antibody (1:2500 in 5% milk TBS-Tween20) was added and incubated at room temperature for 45 min. Protein bands on western blots were visualized by ECL Plus (Amersham, Arlington Heights, IL, USA) according to the manufacturer’s instructions and were developed on film. Analysis was shown as p-JNK and p-p38 compared with total JNK (1:1000; Cell Signaling Technology, Inc.) and p38 (1:1000; Cell Signaling Technology, Inc.), respectively, in all groups.

**Statistical Analysis**

Data are expressed as means ± standard error, and as change in each rat from start of intervention until sacrifice. Differences among groups were examined by one-way ANOVA followed by Bonferroni’s correction for multiple comparisons. Nonparametric data were compared by Mann–Whitney U test. Differences among groups were examined by Kruskal–Wallis followed by Dunns’s correction for multiple comparisons. All *P* values below 0.05
were considered significant. Continuous variables were then tested with paired \( t \)-test to compare week 8 with week 12. For data that were not normally distributed, the Wilcoxon matched-pairs signed rank test was used for comparisons of week 8 and week 12 outcomes.

**RNA Sample Preparation and Sequencing**

Up to six sections of 10 µM-thick formalin-fixed and paraffin-embedded (FFPE) scrolls were processed for RNA according to the manufacturer’s instructions (RNeasy FFPE Kit, Qiagen, MD, USA). RNA quality was determined on an Agilent Bioanalyzer for RNA integrity and quantitated by Nanodrop. RNA samples were converted into cDNA libraries using the Illumina TruSeq Stranded Total RNA sample preparation kit (RS-122-2303; Illumina, CA, USA). Briefly, total RNA samples were concentration normalized, and ribosomal RNA (rRNA) was removed using biotinylated probes that selectively bind rRNA species. The resulting rRNA-depleted RNA was fragmented using heat in the presence of divalent cations. Fragmented RNA was converted into double-stranded cDNA, with dUTP utilized in place of dTTP in the second strand master mix. Final libraries were quantified, normalized and pooled. Sequencing was performed using an Illumina sequencing platform resulting in a median number of 92.6 million reads across samples after removal of sequencing artifacts.

**RNA-seq Data Analysis**

Low quality reads, bases and adapter sequences were removed using fastq-mcf (http://expressionanalysis.github.io/ea-utils/). Trimmed and filtered sequencing data was aligned to the rat reference genome RGSC Rnor_6.0 using STAR (version 2.4) and quantified using RSE (version 1.2.14) accounting for reads which align to multiple genes and/or isoforms. Gene expression profiles were normalized using edgeR (version 3.24.3) using trimmed mean of M-values (TMM) normalization. Differential gene expression
analysis was performed using the limma-voom package (version 3.38.3) and differential expression was defined as fold change (FC) \( >1.5\times \) and FC \( <0.67\times \) for upregulation and downregulation, respectively, at 5% false discovery rate in comparison to the control group. Longitudinal comparisons were defined as the changes in a respective treatment group over time (i.e., autopsy vs biopsy) relative to the expression differences in the vehicle-treated group over time. Gene ontology and pathway enrichment analysis was performed using Metascape (https://metascape.org) with differentially expressed genes to identify selonsertib, enalapril and combination specific transcriptional effects.

**Results**

**Selonsertib Does Not Affect Renal Hemodynamics in the 5/6 Nx Model**

We first assessed if selonsertib had acute effects on systemic or kidney hemodynamic parameters. We used classic radiolabeled clearance techniques for precise measurements of GFR, renal blood flow, filtration fraction, and systemic blood pressure in 5/6 Nx rats at week 8 after kidney mass resection (17). For these acute studies, we used a resection ablation method of 5/6 Nx where the additional renal mass was surgically removed via polar excision 1 week after uninephrectomy; this approach causes similar impairment in renal function but induces only modest hypertension compared with the ligation method used for the chronic studies, and was therefore less likely to mask any potential hemodynamic effects of selonsertib (18). At 7 weeks after 5/6 Nx by resection these rats had elevated plasma creatinine (0.80 mg/dl versus 0.39 mg/dl in controls) and plasma BUN concentrations (32.56 mg/dl versus 15.08 mg/dl in controls). After 4 days of treatment with selonsertib or vehicle, plasma levels of creatinine or BUN did not change in either group (**Figure 1, A and B**). Selonsertib did not cause any changes in GFR, systemic blood pressure, or heart rate
(Figure 1, C–F). Additionally, renal blood flow, renal vascular resistance, and filtration fraction, as measured by clearance of radiolabeled inulin and PAH, were also unchanged following selonsertib treatment (Figure 1, G–I), confirming that selonsertib does not change systemic or renal hemodynamics.

Selonsertib and Enalapril Combined Further Attenuate Kidney Function Decline

We next investigated the efficacy of selonsertib and the ACEI enalapril alone and in combination in the chronic ligation 5/6 Nx model. At 8 weeks after 5/6 Nx, blood pressure was significantly increased, as expected (SBP 202.6 mmHg compared with baseline of 123.8 mmHg). Selonsertib was well tolerated, evidenced by the fact that animals in all selonsertib treatment groups gained weight during the duration of treatment (Table 1). Serum creatinine increase from biopsy at week 8 to autopsy at week 12 was attenuated in animals treated with selonsertib (+19.1 ± 17.5%), or the combination (+22.3 ± 17.7%), but not with enalapril alone, compared with controls (+97.8 ± 25.8%, \( P < 0.05 \)) (Figure 2A, Table 1). The selonsertib and combination groups maintained \( \text{Ccr} \) (selonsertib, +11.9 ± 28.6%, \( P < 0.05 \) versus control; combination, +0.2 ± 19.6%, \( P = 0.07 \) versus control), whereas \( \text{Ccr} \) decreased from week 8 to week 12 in the control (−49.1 ± 7.7%) and enalapril (−35.2 ± 10.8%) groups (Figure 2B, Table 1). Albuminuria, measured by urine albumin to creatinine ratio (ACR), was similar by study design among groups at week 8 prior to treatment initiation. In non-treated, disease control rats, ACR increased by 252.8 ± 75.5% from week 8 to week 12. Selonsertib alone showed a similar increase in proteinuria (+181.0 ± 47.3%), while both enalapril alone and the combination of selonsertib plus enalapril resulted in significantly attenuated albuminuria increases (enalapril, +57.8 ± 24.4%; combination, +50.2 ± 27.5%; both \( P < 0.05 \) versus control) (Figure 2C, Table 2). Enalapril significantly reduced SBP at 12
weeks compared with control. Selonsertib had no significant effect on SBP and combined treatment did not further reduce SBP versus enalapril alone (Table 1).

**Selonsertib and Enalapril Combination Reduced Glomerulosclerosis Progression and Attenuated Podocyte Cell Loss**

In untreated disease controls, glomerulosclerosis increased by 56.6 ± 35.39% (Figure 2, D and E, and Table 2) from biopsy at 8 weeks to autopsy at 12 weeks. Enalapril or selonsertib only numerically attenuated progression of glomerulosclerosis (enalapril, +9.1 ± 12.50%; selonsertib, +27.3 ± 14.4%) versus week 8. However, combined treatment not only halted the progressive increase in glomerulosclerosis, but even showed less sclerosis on average at end of study than at biopsy (combination, −21.5 ± 31.25%). Specifically, 7 of 12 rats in the combination group demonstrated less sclerosis at autopsy versus biopsy, compared with only 2 of 12 in the controls, 5 of 12 in the enalapril group and 4 of 12 in the selonsertib group (Figure 2F, Table 2).

The density of WT1⁺ glomerular cells, a marker of mature podocytes, decreased from biopsy to autopsy in control 5/6 Nx rats, with minimal numerical improvement with enalapril or selonsertib monotherapy. By contrast, the combination group had higher WT1⁺ cell density at 12 weeks (6.59 ± 0.33 × 10⁻³/µm²) than controls (4.21 ± 0.55 × 10⁻³/µm²). Higher WT1⁺ cell density from biopsy was significantly enhanced (P<0.05) at 12 weeks with combination treatment versus control or selonsertib alone (Figure 2G, Table 2).

**Combination of Selonsertib and Enalapril Modifies Gene Expression**

We next assessed effects of the combination of selonsertib and enalapril on molecular signatures versus enalapril or selonsertib alone by RNA-Seq analysis. We compared biopsy (week 8) to autopsy (week 12) samples in the same rats, and also compared changes in gene expression over time across groups (longitudinal comparisons within each paired animal
across groups) (Figure 3, A and B). In longitudinal comparisons, 172 genes were specifically modified by selonsertib alone, 190 genes by enalapril alone, and 114 genes by the combination of both agents alone, suggesting different protection mechanisms by these interventions (Figure 3C). We observed a significant number of downregulated genes in the combination group over time (Figure 3D, left panel). The top 10 significantly ($P<0.05$) differentially regulated genes from this analysis are shown in Figure 3D, right panel. The most downregulated gene following combination treatment was for haptoglobin (Hp), which has been previously proposed to be a biomarker of DKD progression (19,20). In the combination group, significantly affected pathways included genes related to regulation of apoptotic processes, Wnt signaling, and general inflammation (Figure 3E). However, the comparison between treated and control groups at 12 weeks (end-of-study) revealed fewer statistically significant changes in the treatment groups (Supplemental Figures 2 and 3). The longitudinal comparisons suggest that interventions predominantly modified the trajectory of gene expression over time, as opposed to resulting in significant changes across groups at the end of study. To understand expression patterns of genes significantly modulated by selonsertib and enalapril combination, we examined publicly available single-cell sequencing data from the Kidney Precision Medicine Atlas (Supplementary Figures 4 and 5), which revealed that the affected genes were broadly expressed in different cell types and not enriched for one particular cell type.

Different Effects of Selonsertib, Enalapril, and Combination on Apoptosis, MAPK, Inflammation, and Fibrosis

To further investigate the effects of selonsertib versus enalapril on apoptosis, MAPK, inflammation, and fibrosis, we measured related protein expression by western blot and immunostaining. Both selonsertib alone and combination therapy, but not enalapril alone,
decreased the density of apoptotic cells in the kidney, mainly in the tubulointerstitial area (selonsertib, $1.25 \pm 0.22 \times 10^{-5}/\mu m^2$; combination, $1.28 \pm 0.2 \times 10^{-5}/\mu m^2$; both $P<0.05$ vs controls) compared with controls ($2.07 \pm 0.21 \times 10^{-5}/\mu m^2$) and enalapril alone ($2.74 \pm 0.37 \times 10^{-5}/\mu m^2$) (Figure 4A). Significant inhibition (>90%) of p-JNK and p-p38 (both downstream targets of ASK1) was observed by western blot of the kidney cortex following selonsertib treatment, confirming activity of the ASK1 inhibitor (Figure 4B).

Glomerular macrophage infiltration, assessed as the percentage of glomerular area with ED1 positivity, increased from biopsy to autopsy in control animals ($0.68 \pm 0.13\%$ vs $1.07 \pm 0.16\%$, respectively) and in animals treated with selonsertib alone ($0.49 \pm 0.18\%$ vs $1.22 \pm 0.44\%$, respectively), whereas only minor changes were observed in animals treated with enalapril alone ($0.56 \pm 0.13\%$ vs $0.41 \pm 0.07\%$, respectively) or the combination ($0.58 \pm 0.21\%$ vs $0.64 \pm 0.11\%$, respectively) (Figure 4C and Table 2).

Transforming growth factor β (TGF-β) is a major profibrotic molecule linked with progression of glomerulosclerosis and tubulointerstitial fibrosis. Persistent activation of TGF-β is associated with kidney scarring. CTGF is induced by TGF-β and mediates many of its fibrotic effects (21). CTGF protein levels relative to β-actin were only numerically reduced by monotherapy with enalapril ($0.50 \pm 0.11$) or selonsertib ($0.49 \pm 0.08$) versus control ($0.64 \pm 0.13$). Combined treatment with selonsertib and enalapril significantly decreased CTGF expression ($0.32 \pm 0.04$, $P<0.05$) versus control (Figure 4D). Increased PAI-1 expression is also associated with inhibition of matrix degradation. Combination treatment only numerically decreased PAI-1 expression relative to β-actin ($0.05 \pm 0.01$) versus control ($0.11 \pm 0.05$) (Figure 4D).
Discussion

In the current study, selonsertib in combination with the ACEI enalapril attenuated glomerulosclerosis progression and renal function decline when treatment was started when renal injury and decreased GFR were already established, mimicking the clinical setting. These beneficial effects were greater than those observed with each single agent alone, and were not attributable to additional hemodynamic effects by selonsertib. Importantly, selonsertib did not influence systemic blood pressure or albuminuria in this chronic 5/6 Nx model, demonstrating an independent, but complimentary, mechanism to RAAS inhibition. Selonsertib was also well tolerated in the chronic study. Our acute studies in a 5/6 Nx model further showed that selonsertib does not acutely change GFR, renal blood flow, blood pressure or heart rate. Of note, both ACEI and sodium-glucose cotransporter-2 (SGLT2) inhibitors are known to cause acute decreases in eGFR (‘eGFR dip’) due to acute lowering of systemic and/or glomerular hemodynamics, whereas selonsertib has also been shown to cause acute decreases in eGFR which are attributed to the inhibition of the tubular creatinine transporters MATE1/2K (22,23). Although these transporters do not have a prominent role in regulating plasma creatinine in rodents (24), the current and published studies demonstrating no effect on hemodynamics with selonsertib or ASK1 inhibition are consistent with the blockade of creatinine secretion as a cause for eGFR changes; however, more clinical studies are needed to fully address this. Our data are thus consistent with previously published findings in ASK1 knockout mice and from studies with ASK1 inhibitors in rodent models of renal dysfunction in which no changes in hemodynamics were detected (25). This non-hemodynamic mechanism differentiates selonsertib from current CKD therapies such as ACEI, ARB, and the novel SGLT2 inhibitors, which reduce intraglomerular pressure, among other actions (26). ACEI have well-defined beneficial
hemodynamic, anti-inflammatory, and anti-fibrotic effects in CKD/DKD, and the current results highlight the potential benefits of combining a glomerular pressure-lowering therapy with an agent that targets glomerular inflammation and apoptosis (13,27).

In normal physiological conditions, ASK1 is maintained in an inactive state by binding to the redox-sensitive protein thioredoxin and is only activated via auto-phosphorylation of the activation loop upon stimulation by factors such as ROS, hypoxia, proinflammatory cytokines, and angiotensin II (27,28). ASK1 affects downstream MAPK signaling through p38 and JNK, both of which play roles in renal inflammation, apoptosis, and fibrosis (11,29). In both human and experimental CKD/DKD, ASK1 pathway activation is increased, as evidenced by higher levels of p-ASK1 and p-p38 (11). ASK1−/− mice are well characterized and do not have altered cardiac or systemic hemodynamics compared with wild-type mice, but are protected from heart failure and cardiac injury (30,31). In previous models of CKD, treatment with small-molecule inhibitors of ASK1 improved GFR without an associated effect on blood pressure, similar to the current rat studies (11). Importantly, this study further demonstrates that ASK1 inhibition has additive effects on reducing glomerulosclerosis, which translate to less functional decline over ACEI alone.

Combination treatment with enalapril and seleosertib also resulted in a greater preservation of podocytes, which have limited, if any, regenerative potential after injury. ACEI can reduce intraglomerular pressure and thereby reduce mechanical stress on the podocyte; we observed significant podocyte protection, as measured by WT1+ staining density, in animals treated with the combination of enalapril and seleosertib. The loss of podocytes is strongly associated with progressive kidney disease. Additional studies from our group and others have reported that ASK1 inhibition can play a role to preserve
podocyte numbers in the setting of kidney injury (11,12). Interestingly, single agents previously showed efficacy on podocyte loss in milder models of CKD, including the mild diabetic nephropathy model of db/db eNOS−/− mice. In contrast, a single agent alone initiated after disease is established is not sufficient to reduce podocyte loss in a more severe, hypertensive model of glomerulosclerosis, like the 5/6 nephrectomy model, highlighting the need for a combination of therapeutic agents to attenuate podocyte loss in most human CKD settings. Additionally, our results show significant reductions in overall kidney apoptotic cells, including tubular epithelial cells, as measured by TUNEL staining, following selonsertib or combination treatments, but not with enalapril alone. This suggests the effect of selonsertib on apoptosis in the 5/6 Nx model is not limited to glomerular cells and that the effect on apoptosis with combination therapy is primarily driven by selonsertib. While selonsertib treatment induced an accumulation in ED1+ cells, there may be shifts in macrophage subtypes between pro- and anti-inflammatory subsets (i.e. M1 vs M2). Future studies are required to understand whether such population shifts occur in this setting.

In addition to demonstrating a combination effect on glomerulosclerosis and kidney function, unbiased transcriptomic analysis revealed several pathways modulated by enalapril and selonsertib alone or in combination. Both enalapril and selonsertib affected a similar number of genes when analyzed over time (longitudinal comparison between autopsy and biopsy); however, the combination treatment affected additional genes and pathways that may underly the current efficacy. Pathways affected by the combination include those involved in regulating apoptosis, Wnt signaling, and immunity/inflammation. Several of these genes, including *Hp*, *CD163*, *Col14a1*, and *Fgb* have been linked to kidney injury, inflammation, and fibrosis, and to kidney disease progression in various CKDs (19,20,32-
this subset of genes and pathways may explain, in part, the beneficial effects of combination treatment, although further evidence would be needed to confirm.

A limitation of the current study is that comparison between biopsy and autopsy RNA-Seq data was challenging given that histopathology scores at biopsy, not gene expression profiles, were used to normalize study groups prior to treatment, introducing an additional level of variability into the end-of-study comparison, which may impact statistical power. However, these data still serve as an initial starting point to provide insight and hypothesis generation for further investigations. Additionally, the RNA-Seq was performed on bulk tissue which may confound the heterogeneity of cell types detected. However, single-cell RNA-Seq data from public domain databanks confirmed that there was not one specific cell type enriched for the genes that were significantly modulated by the selonsertib and enalapril combination.

In summary, our results demonstrating an additive effect of selonsertib with enalapril to reduce glomerulosclerosis progression and functional decline were observed without any additional hemodynamic effects beyond those of ACEI alone, coupled with an unbiased examination of molecular pathways affected by combination therapy. The current data advance understanding of how ASK1 inhibition contributes to kidney protection and support previous observations in humans, demonstrating potential for selonsertib in reducing eGFR slope decline in patients with DKD who are receiving ACEI therapies (15). These findings provide further rationale for the continued investigation of selonsertib for the treatment of kidney disease when added to current standard of care.

Disclosures
S. Badal reports the following: Employer: Gilead Sciences, Inc.; and Ownership Interest: Gilead Sciences, Inc. T. Al Tuhaifi reports the following: Ownership Interest: MERCK & CO INC; BUTTERFLY NETWORK INC. D. Lopez reports the following: Employer: Gilead Sciences; and Ownership Interest: Gilead Sciences. C. Plato reports the following: Employer: Plato BioPharma, Inc. (PBI); Craig Plato is the Founder and President of PBI.; Inotiv Inc. acquired PBI on October 4th, 2021. Craig Plato is now Senior Vice President, Physiological Sciences; Ownership Interest: Plato BioPharma, Inc. was acquired October 4th, 2021, that ownership interest no longer exists.; Craig Plato acquired Restricted Stock (not yet unrestricted) in Inotiv, Inc. as part of the acquisition of PBI. Craig Plato was granted stock options in Inotiv Inc. as part of an employment offer with Inotiv, Inc. Those stock options have not yet vested in any way.; Research Funding: Plato BioPharma, Inc. (PBI) is a leading preclinical drug discovery contract research organization (CRO). PBI was compensated for providing research services resulting in generation of data supporting submitted abstracts and potential publications.; Inotiv, Inc. acquired PBI on Oct 4th, 2021 and is a CRO, thus compensated for providing research services resulting in generation of data supporting submitted abstracts and potential publications.; and Advisory or Leadership Role: Plato BioPharma, Inc. (PBI); As Founder and President of PBI, Craig Plato served on PBI’s Board of Directors. Inotiv, Inc. acquired PBI on Oct 4th, 2021, thus Craig Plato is no longer an officer or Board Member of any company. K. Joly reports the following: Employer: Plato BioPharma, Inc.; Ownership Interest: Plato BioPharma, Inc.; and Research Funding: Plato BioPharma, Inc. (PBI) is a leading preclinical drug discovery contract research organization (CRO); As such, PBI was compensated for providing research services resulting in the generation of data supporting submitted abstracts and potential full publications. D. Breckenridge reports the following: Employer: Actio Biosciences;
Ownership Interest: Actio Biosciences; Gilead Sciences Inc.; and Advisory or Leadership Role: Actio Biosciences. J. Liles reports the following: Employer: Gilead Sciences, Inc.; Ownership Interest: Gilead Sciences, Inc.; and Research Funding: Gilead Sciences, Inc. A. Fogo reports the following: Consultancy: Novartis; Research Funding: Gilead, Bayer, Novartis; Honoraria: Novartis, Amgen, GSK; Advisory or Leadership Role: Advisory Committee Bayer, Noncompany: Editorial Board for Human Pathology and JASN, AM J Physiol (Renal), AM J Path; Associate Editor Lab Invest and Kidney International, Subject Editor NDT, Current opinion nephrology and hypertension guest editor for yearly pathology focus; and Other Interests or Relationships: Noncompany: Editorial Board for Human Pathology and JASN and AM J Physiol (Renal), Associate Editor Lab Invest, Subject Editor NDT, speaker at various national nephrology meetings, AE KI; President Elect ISN (president as of April 19 2021). The remaining authors have nothing to disclose.

Funding

This work was supported in part by a grant from Gilead Sciences, Inc. and by a grant from NIDDK DK 56942 (ABF).

Acknowledgments

Hadramout Establishment for Human Development (HEHD) is thanked for sponsoring TAT’s research fellowship program at Vanderbilt University. The authors would like to thank Swati Kaushik for bioinformatics support. Editorial support was provided by Sarah Graham of PharmaGenesis London, London, UK, and funded by Gilead Sciences, Inc.

Author Contributions
SSB, JTL, DGB, ABF, HCY contributed to the design, interpretation, analysis and writing of the manuscript. JTL, DGB, and ABF conceptualized the experimental plan. HCY, YFY, CTP and KJ performed portions of animal experiments. TAT assisted with performance of animal experiments. SSB and HCY performed in vitro experiments and molecular analysis. DL performed bioinformatics analysis and interpreted the results. All authors contributed to data interpretation and critical review of the manuscript. All authors approved the final version of the manuscript.

**Data Sharing**

All data is included in the manuscript and/or supporting information.

**Supplemental Material**

Supplemental Table 1. Hematocrit and hemoglobin at week 12

Supplemental Figure 1. Selonsertib PK/PD relationship in a rat model of fibrosis

Supplemental Figure 2. Differentially expressed genes with selonsertib and ACEI treatment from longitudinal comparisons

Supplemental Figure 3. Differentially expressed genes with selonsertib, ACEI or combination treatment from end-of-study comparisons

Supplemental Figure 4. Single-Cell RNA-Seq violin plots of genes down-regulated in selonsertib and enalapril combination longitudinal comparisons.

Supplemental Figure 4. Single-Cell RNA-Seq violin plots of genes up-regulated in selonsertib and enalapril combination longitudinal comparisons.
References


https://doi.org/10.1038/ki.2008.519
### Table 1. Functional parameters at week 0 and week 12

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 8</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ACEI</td>
<td>ACEI+SEL</td>
</tr>
<tr>
<td><strong>SBP, mmHg</strong></td>
<td>123.8±3.8</td>
<td>118.9±3.5</td>
<td>122.1±2.3</td>
</tr>
<tr>
<td><strong>BW, g</strong></td>
<td>329.1±2.5</td>
<td>340.4±4.9</td>
<td>328.0±5.3</td>
</tr>
<tr>
<td><strong>UACR, µg/mg</strong></td>
<td>4.3±0.7</td>
<td>3.3±0.6</td>
<td>4.5±0.9</td>
</tr>
<tr>
<td><strong>Scr, mg/dl</strong></td>
<td>0.24±0.02</td>
<td>0.23±0.03</td>
<td>0.25±0.03</td>
</tr>
<tr>
<td><strong>Ccr, ml/min/100g BW</strong></td>
<td>0.70±0.07</td>
<td>0.86±0.12</td>
<td>0.81±0.13</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM. n = 9–12 animals per group. 

*P<0.05 vs control at week 12. †P<0.05 vs at baseline (week 0), ‡P<0.05 vs ACEI, §P<0.05 vs ACEI+SEL and ¶P<0.05 vs SEL at week 8. ACEI, angiotensin converting enzyme inhibitor; UACR, urine albumin creatinine ratio; BW, body weight; Ccr, creatinine clearance; SEL, selonsertib; SEM, standard error of the mean; SBP, systolic blood pressure; Scr, serum creatinine.
Table 2. Structural parameters at week 8 and 12

<table>
<thead>
<tr>
<th></th>
<th>W8</th>
<th>W12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control ACEI ACEI+SEL SEL</td>
<td>Control ACEI ACEI+SEL SEL</td>
</tr>
<tr>
<td>Sclerosis index (0-4 scale)</td>
<td>0.95±0.15 0.95±0.13 0.98±0.16 0.90±0.15</td>
<td>1.24±0.17† 0.94±0.09 0.77±0.11*¥ 0.99±0.14</td>
</tr>
<tr>
<td>Glomerular WT1⁺ cell density, ×10⁻³/µm²</td>
<td>6.33±0.64 5.79±0.62 6.05±0.55 5.59±0.45</td>
<td>4.12±0.61† 4.38±0.57 6.59±0.33*¢ 3.51±0.45¢</td>
</tr>
<tr>
<td>ED1⁺ area/gglomerular area, %</td>
<td>0.68±0.13 0.56±0.13 0.58±0.21 0.49±0.18</td>
<td>1.07±0.16† 0.41±0.08* 0.64±0.11 1.22±0.44¢</td>
</tr>
<tr>
<td>ED1⁺ area/tubulointerstitial area, %</td>
<td>0.27±0.05 0.17±0.03 0.27±0.07 0.47±0.13</td>
<td>1.23±0.21† 0.67±0.11*† 0.94±0.25 1.24±0.16¢</td>
</tr>
<tr>
<td>TUNEL⁺ cell number/tubulointerstitial area, 10⁻³/µm²</td>
<td>0.92±0.11 1.03±0.13 0.98±0.09 0.77±0.07</td>
<td>2.07±0.21† 2.74±0.37† 1.28±0.20* 1.25±0.22*¢</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM. n = 9–12 animals per group. *P<0.05 vs control, †P<0.05 vs ACEI and ¥P<0.05 vs SEL at week 12. ‡P<0.05 vs baseline (week 0), §P<0.05 vs ACEI, ¶P<0.05 vs ACEI+SEL and ¥P<0.05 vs SEL at week 8. ACEI, angiotensin converting enzyme inhibitor; SEL, selonsertib; SEM, standard error of the mean; WT1, Wilms tumor-1.
Figure Legends

**Figure 1. Selonsertib does not have a hemodynamic effect in renal disease.** Renal function parameters in 5/6 Nx rats treated with selonsertib or vehicle for 4 days at week 8 after surgery (n = 11–16 animals per group). (A) Plasma creatinine concentration. (B) Plasma blood urea nitrogen (BUN) concentration. (C) GFR represented as an average of four clearance periods. (D) GFR over time during four clearance periods. (E and F) Mean arterial pressure (MAP) and heart rate (HR), respectively, shown as an average of four clearance periods. (G) Renal blood flow (RBF). (H) Renal vascular resistance (RVR). (I) filtration fraction (FF). Data are presented as individual data points per animal (horizontal bar shows mean), except for D (mean [SEM]). *P<0.05 vs control. 5/6 Nx, 5/6 nephrectomy; GFR, glomerular filtration rate; SEL, selonsertib; SEM, standard error of the mean.

**Figure 2. Combination of selonsertib and enalapril prevents decline of kidney function and progression of glomerulosclerosis.** 5/6 Nx rats were biopsied and randomized to treatment groups (n = 9–12 animals per group.) at week 8 and sacrificed at week 12. (A) Change in serum creatinine concentration. (B) Change in creatinine clearance (Ccr). (C) Change in urine albumin to creatinine ratio (ACR). (D) Glomerulosclerosis at biopsy and autopsy, assessed using a semi-quantitative 0–4+ score. (E and F) Change in glomerulosclerosis. (G) Change in density of WT1+ glomerular cells. Data are presented as individual data points per animal (horizontal bar shows mean). *P<0.05 vs control. 5/6 Nx, 5/6 nephrectomized; ACEI, angiotensin-converting enzyme inhibitor; SEL, selonsertib; WT1, Wilms tumor-1.
Figure 3. **Differential gene ontology in CKD progression in response to treatment with selonsertib, enalapril, or the combination.** 5/6 Nx rats were biopsied and randomized to treatment groups (n = 9–12 animals per group) at week 8 and sacrificed at week 12. (A) Preparation of samples for RNA-Seq analysis. (B) Longitudinal comparisons. (C) Number of genes modified by each treatment. (D) Genes specifically modified by the combination treatment. (E) Significantly affected molecular pathways. 5/6 Nx, 5/6 nephrectomy; ACEI, angiotensin-converting enzyme inhibitor; CKD, chronic kidney disease; FC, fold change; FFPE, formalin-fixed paraffin-embedded; SEL, selonsertib.

Figure 4. **Different effects of treatment with selonsertib, enalapril, or the combination on apoptosis, MAPK, inflammation, and fibrosis.** 5/6 Nx rats were biopsied and randomized to treatment groups (n = 8–12 animals per group.) at week 8 and sacrificed at week 12. (A) Density of apoptotic cells. (B) Relative expression of activated (p-) and total (t-) p38 and JNK at week 12. (C) Glomerular ED1 positivity. (D). Expression of connective tissue growth factor (CTGF) and plasminogen activator inhibitor-1 (PAI-1) at week 12. Data are presented as individual data points per animal (horizontal bar shows mean). *P<0.05 vs control. 5/6 Nx, 5/6 nephrectomy; ACEI, angiotensin-converting enzyme inhibitor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; SEL, selonsertib.
Figure 1
Figure 2

A. Serum creatinine change from biopsy to autopsy (%)

B. Ccr change from biopsy to autopsy (%)

C. ACR change from biopsy to autopsy (%)

D. Sclerosis change from biopsy to autopsy (%)

E. Sclerosis at biopsy and autopsy

F. Sclerotic cell density change from biopsy to autopsy (%)

G. WTI+ cell density change from biopsy to autopsy (%)

Biopsy (week 8)

Autopsy (week 12)
Paired biopsy (week 8) and autopsy (week 12) FFPE sections

FFPE RNA isolation

Library prep

RNA-Seq (PE, 56bp HiSeq2000, Illumina)

Longitudinal comparisons

Compared biopsy to autopsy for:
- Control
- ACEI
- ACEI+SEL
- SEL

Enriched biological processes

> 1.5 FC, P<0.05 (X genes – 12 up, 12 down)

- GO:2000351: regulation of endothelial cell apoptotic process
- GO:0098532: histone H3-K27 trimethylation
- GO:0030178: negative regulation of Wnt signaling pathway
- R-RNO-3371571: HSF1-dependent transactivation
- WP1310: selenium micronutrient network
- GO:0051289: protein homotrimerization
- R-RNO-2980736: peptide hormone metabolism
- GO:0038083: peptidyl-tyrosine autophosphorylation
- GO:0002709: regulation of T cell mediated immunity
- GO:0007009: plasma membrane organization
- GO:0007568: aging
- ko000096: sulfur amino acid metabolic process
- GO:0009415: response to water
- ko05322: systemic lupus erythematosus
- GO:0051251: positive regulation of lymphocyte activation
- GO:2000573: positive regulation of DNA biosynthetic process
- ko00590: arachidonic acid metabolism
Supplemental Material

Selonsertib Enhances Kidney Protection Beyond Standard of Care in a Hypertensive, Secondary Glomerulosclerosis CKD Model

Shawn S. Badal et al.

Table of Contents

Supplemental Table 1. Hematocrit and hemoglobin at week 12
Supplemental Figure 1. Selonsertib PK/PD relationship in a rat model of fibrosis
Supplemental Figure 2. Differentially expressed genes with selonsertib and ACEI treatment from longitudinal comparisons
Supplemental Figure 3. Differentially expressed genes with selonsertib, ACEI or combination treatment from end-of-study comparisons
Supplemental Figure 4. Single-Cell RNA-Seq violin plots of genes down-regulated in selonsertib and enalapril combination longitudinal comparisons.
Supplemental Figure 4. Single-Cell RNA-Seq violin plots of genes up-regulated in selonsertib and enalapril combination longitudinal comparisons.
Supplemental Table 1. Hematocrit and hemoglobin at week 12

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ACEI</th>
<th>ACEI+SEL</th>
<th>SEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct, %</td>
<td>39.49±2.26</td>
<td>35.85±1.09</td>
<td>42.34±2.15</td>
<td>36.38±2.85</td>
</tr>
<tr>
<td>RBC, 10⁶/mm³</td>
<td>7.60±0.41</td>
<td>6.88±0.19</td>
<td>8.27±0.47</td>
<td>6.96±0.53</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>13.84±0.79</td>
<td>12.44±0.45</td>
<td>14.87±0.83</td>
<td>12.68±1.04</td>
</tr>
<tr>
<td>Retic, %</td>
<td>0.07±0.03</td>
<td>0.06±0.03</td>
<td>0.04±0.02</td>
<td>0.14±0.07</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM. n = 9–12 animals per group. ACEI, angiotensin converting enzyme inhibitor; Hb, hemoglobin; Hct, hematocrit; RETIC, reticulocyte count; RBC, red blood cells; SEL, selonsertib; SEM, standard error of the mean.
Supplemental Figure 1. Selonsertib PK/PD relationship in a rat model of tubulointerstitial fibrosis. Selonsertib (1–30 mg/kg, orally, twice daily) was administered to Sprague-Dawley rats for 7 days following unilateral ureteral obstruction (UUO) surgery. Quantification of western blot signals demonstrated significant decreases in (A) p-ASK1, (B) p-p38 and (C) p-JNK in kidney cortex as compared to UUO animals treated with vehicle (n = 6–10). Data are presented as individual data points per animal (horizontal bar shows mean). *P<0.05 vs control (sham surgery), †P<0.05 vs vehicle; ANOVA followed by Newman-Keuls multiple comparison test. (D) Correlation between the level of p-p38 in the kidney cortex and the plasma concentration of selonsertib in individual animals at trough on day 7. p-p38 was measured by an ELISA. Data were analyzed by GraphPad Prism 5 software using nonlinear regression curve fitting with variable slope to determine the EC95 value. ANOVA, analysis of variance; ASK1, apoptosis signal-regulating kinase 1; EC95, effective concentration required to inhibit 95%; ELISA, enzyme-linked immunosorbent assay; JNK, c-Jun N-terminal kinase; PD, pharmacodynamic; PK, pharmacokinetic; RLU, relative light unit; SD, standard deviation; SEL, selonsertib.
Supplemental Figure 2. Differentially expressed genes with selonsertib and ACEI treatment from longitudinal comparisons. (A) Significantly affected molecular pathways (B) Genes specifically modified by each treatment. ACEI, angiotensin-converting enzyme inhibitor; FC, fold change; SEL, selonsertib.
Supplemental Figure 3. Differentially expressed genes with selonsertib, ACEI or combination treatment from end-of-study comparisons. (A) Comparisons across groups. (B) Overlap of differentially expressed genes (B) Genes specifically modified by each treatment. ACEI, angiotensin-converting enzyme inhibitor; FC, fold change; SEL, selonsertib.
Supplemental Figure 4. Single-Cell RNA-Seq violin plots of genes down-regulated in selonsertib and enalapril combination longitudinal comparisons. Data obtained from Kidney Precision Medicine Project Atlas (www.kpmp.org/atlas).
Supplemental Figure 5. Single-Cell RNA-Seq violin plots of genes up-regulated in selonsertib and enalapril combination longitudinal comparisons. Data obtained from Kidney Precision Medicine Project Atlas (www.kpmp.org/atlas).
Supplemental Material

Selonsertib Enhances Kidney Protection Beyond Standard of Care in a Hypertensive, Secondary Glomerulosclerosis CKD Model

Shawn S. Badal et al.

Table of Contents

Supplemental Table 1. Hematocrit and hemoglobin at week 12

Supplemental Figure 1. Selonsertib PK/PD relationship in a rat model of fibrosis

Supplemental Figure 2. Differentially expressed genes with selonsertib and ACEI treatment from longitudinal comparisons

Supplemental Figure 3. Differentially expressed genes with selonsertib, ACEI or combination treatment from end-of-study comparisons

Supplemental Figure 4. Single-Cell RNA-Seq violin plots of genes down-regulated in selonsertib and enalapril combination longitudinal comparisons.

Supplemental Figure 4. Single-Cell RNA-Seq violin plots of genes up-regulated in selonsertib and enalapril combination longitudinal comparisons.
Supplemental Table 1. Hematocrit and hemoglobin at week 12

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ACEI</th>
<th>ACEI+SEL</th>
<th>SEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct, %</td>
<td>39.49±2.26</td>
<td>35.85±1.09</td>
<td>42.34±2.15</td>
<td>36.38±2.85</td>
</tr>
<tr>
<td>RBC, 10^6/mm^3</td>
<td>7.60±0.41</td>
<td>6.88±0.19</td>
<td>8.27±0.47</td>
<td>6.96±0.53</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>13.84±0.79</td>
<td>12.44±0.45</td>
<td>14.87±0.83</td>
<td>12.68±1.04</td>
</tr>
<tr>
<td>Retic, %</td>
<td>0.07±0.03</td>
<td>0.06±0.03</td>
<td>0.04±0.02</td>
<td>0.14±0.07</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM. n = 9–12 animals per group. ACEI, angiotensin converting enzyme inhibitor; Hb, hemoglobin; Hct, hematocrit; RETIC, reticulocyte count; RBC, red blood cells; SEL, selonsertib; SEM, standard error of the mean.
Supplemental Figure 1. Selonsertib PK/PD relationship in a rat model of tubulointerstitial fibrosis. Selonsertib (1–30 mg/kg, orally, twice daily) was administered to Sprague-Dawley rats for 7 days following unilateral ureteral obstruction (UUO) surgery. Quantification of western blot signals demonstrated significant decreases in (A) p-ASK1, (B) p-p38 and (C) p-JNK in kidney cortex as compared to UUO animals treated with vehicle (n = 6–10). Data are presented as individual data points per animal (horizontal bar shows mean). *P<0.05 vs control (sham surgery), †P<0.05 vs vehicle; ANOVA followed by Newman-Keuls multiple comparison test. (D) Correlation between the level of p-p38 in the kidney cortex and the plasma concentration of selonsertib in individual animals at trough on day 7. p-p38 was measured by an ELISA. Data were analyzed by GraphPad Prism 5 software using nonlinear regression curve fitting with variable slope to determine the EC95 value. ANOVA, analysis of variance; ASK1, apoptosis signal-regulating kinase 1; EC95, effective concentration required to inhibit 95%; ELISA, enzyme-linked immunosorbent assay; JNK, c-Jun N-terminal kinase; PD, pharmacodynamic; PK, pharmacokinetic; RLU, relative light unit; SD, standard deviation; SEL, selonsertib.
Supplemental Figure 2. Differentially expressed genes with selonsertib and ACEI treatment from longitudinal comparisons. (A) Significantly affected molecular pathways (B) Genes specifically modified by each treatment. ACEI, angiotensin-converting enzyme inhibitor; FC, fold change; SEL, selonsertib.
Supplemental Figure 3. Differentially expressed genes with selonsertib, ACEI or combination treatment from end-of-study comparisons. (A) Comparisons across groups. (B) Overlap of differentially expressed genes (B) Genes specifically modified by each treatment. ACEI, angiotensin-converting enzyme inhibitor; FC, fold change; SEL, selonsertib.
Supplemental Figure 4. Single-Cell RNA-Seq violin plots of genes down-regulated in selonsertib and enalapril combination longitudinal comparisons. Data obtained from Kidney Precision Medicine Project Atlas (www.kpmp.org/atlas).
Supplemental Figure 5. Single-Cell RNA-Seq violin plots of genes up-regulated in selonsertib and enalapril combination longitudinal comparisons. Data obtained from Kidney Precision Medicine Project Atlas (www.kpmp.org/atlas).