Podocyte injury and loss are key drivers of primary and secondary glomerular diseases, such as focal segmental glomerulosclerosis (FSGS) and diabetic kidney disease (DKD). We previously demonstrated the renoprotective role of protein S (PS) and its cognate tyrosine-protein kinase receptor, TYRO3, in models of FSGS and DKD and that their signaling exerts anti-apoptotic and anti-inflammatory effects to confer protection against podocyte loss. Among the three TAM receptors (TYRO3, AXL, and MER), only TYRO3 expression is largely restricted to podocytes, and glomerular TYRO3 mRNA expression negatively correlates with human glomerular disease progression. We, therefore, posited that the agonism PS-TYRO3 signaling could serve as a potential therapeutic approach to attenuate glomerular disease progression. As PS function is not limited to TYRO3-mediated signal transduction but includes its anticoagulant activity, we focused on the development of TYRO3 agonist as an optimal therapeutic approach to glomerular disease. Among the small molecule TYRO3 agonist compounds screened, compound-10 (C-10) showed a select activation of TYRO3 without any effects on AXL or MER. We also confirmed that C-10 directly binds to TYRO3, but not the other receptors. In vivo, C-10 attenuated proteinuria, glomerular injury, and podocyte loss in mouse models of adriamycin-induced nephropathy and db/db model of type 2 diabetes. Moreover, these renoprotective effects of C-10 are lost in Tyro3 knockout mice, indicating that C-10 is a select […]
TYRO3 AGONIST AS A NOVEL THERAPY FOR GLOMERULAR DISEASE

Fang Zhong¹*, Hong Cai¹,²*, Jia Fu¹, Zeguo Sun¹, Zhengzhe Li¹, David Bauman¹, Lois Wang¹, Bhaskar Das³, Kyung Lee¹, and John Cijiang He¹,⁴

¹ Department of Medicine/Nephrology, Icahn School of Medicine at Mount Sinai, New York, NY
² Department of Nephrology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, China
³ Arnold and Marie Schwartz College of Pharmacy and Health Sciences, Long Island University, Brooklyn, NY
⁴ Renal Section, James J Peters Veterans Affair Medical Center, Bronx, NY

Running title: Protective role of TYRO3 in glomerular disease

Keywords: Diabetic kidney disease, glomerular disease, podocytes, TYRO3, and apoptosis

*These authors contributed equally to the study

Corresponding authors:
John Cijiang He, MD/PhD, Kyung Lee, PhD, or Bhaskar Das, PhD (for chemistry part)
Division of Nephrology, Box 1243
Icahn School of Medicine at Mount Sinai
One Gustave L. Levy Place
New York NY 10029
Tel: 212-659-1703, Fax: 212-987-0389
Email: cijiang.he@mssm.edu, kim.lee@mssm.edu, or bhaskar.das@liu.edu
ABSTRACT

Podocyte injury and loss are key drivers of primary and secondary glomerular diseases, such as focal segmental glomerulosclerosis (FSGS) and diabetic kidney disease (DKD). We previously demonstrated the renoprotective role of protein S (PS) and its cognate tyrosine-protein kinase receptor, TYRO3, in models of FSGS and DKD and that their signaling exerts anti-apoptotic and anti-inflammatory effects to confer protection against podocyte loss. Among the three TAM receptors (TYRO3, AXL, and MER), only TYRO3 expression is largely restricted to podocytes, and glomerular TYRO3 mRNA expression negatively correlates with human glomerular disease progression. We, therefore, posited that the agonism PS-TYRO3 signaling could serve as a potential therapeutic approach to attenuate glomerular disease progression. As PS function is not limited to TYRO3-mediated signal transduction but includes its anticoagulant activity, we focused on the development of TYRO3 agonist as an optimal therapeutic approach to glomerular disease. Among the small molecule TYRO3 agonist compounds screened, compound-10 (C-10) showed a select activation of TYRO3 without any effects on AXL or MER. We also confirmed that C-10 directly binds to TYRO3, but not the other receptors. In vivo, C-10 attenuated proteinuria, glomerular injury, and podocyte loss in mouse models of adriamycin-induced nephropathy and db/db model of type 2 diabetes. Moreover, these renoprotective effects of C-10 are lost in Tyro3 knockout mice, indicating that C-10 is a select agonist of TYRO3 activity that mitigates podocyte injury and glomerular disease. Therefore, C-10, a novel TYRO3 agonist, could be potentially developed as a new therapy for glomerular disease.
INTRODUCTION

Primary and secondary glomerular diseases are responsible for almost two third of end-stage kidney disease (ESKD) (1), and primary glomerular disease, such as focal segmental glomerulosclerosis (FSGS), is a leading cause of ESKD (1). Diabetic kidney disease (DKD), a secondary glomerular disease, is the most common cause of ESKD in the US and globally (1). The current treatment regimens for both primary and secondary glomerular diseases are limited. Since podocyte injury and loss is a critical event leading to the development and progression of DKD and FSGS (2-5), treatments targeting specifically podocyte injury and loss would be an important approach to prevent albuminuria and to slow the glomerular disease progression. To develop a potential therapy, we recently focused on identifications of molecules that may mediate renoprotection against podocyte injury in experimental models of glomerular disease, and established protein S (PS) as one of these molecules (6). We showed that PS expression is significantly increased in the podocytes during the early stages, but decreased at the late stages of DKD, and that the loss of PS exacerbates podocyte injury and DKD progression of DKD in mice. In contrast, the podocyte-specific overexpression of PS attenuates kidney injury in DKD, supporting a protective role of PS glomerular disease (6).

PS shares structural similarities with its homolog GAS6, and both PS and GAS6 bind to TAM tyrosine kinase receptors (TYRO3, AXL, and MER receptors) that regulate various biological processes including cell survival and inflammation (7). Although structurally similar, PS and GAS6 have disparate binding affinities to individual TAM receptors (8, 9), leading to divergent functions (10). GAS6 binds with the strongest affinity to AXL, whereas PS does so to TYRO3 (7) (11). GAS6/AXL signaling induces mesangial cell proliferation and glomerular hypertrophy in early DKD through the activation of Akt/mTOR pathway (12, 13), thereby promoting diabetic glomerulopathy. In contrast, PS/TYRO3 reduces cellular injury by reducing the inflammatory responses (14-16). Consistent with these observations, we showed that PS has anti-inflammatory and anti-apoptotic effects through activation of TYRO3 in cultured podocytes (6).

However, independent of TYRO3 activation, PS plays a significant role in the anti-coagulation system, and would not be an optimal target for the treatment of glomerular disease. Similar to PS knockout, we previously showed that TYRO3 aggravates podocyte injury, and conversely, the induction of TYRO3 in podocytes attenuates injury in the animal models of glomerular disease (17). Therefore, as a potential therapeutic target in glomerular disease, we focused on the agonism of TYRO3. Using medicinal chemistry and in vivo experimental approaches, the present study demonstrates that select TYRO3 agonism markedly improves kidney function and attenuates podocyte injury in experimental models of DKD and FSGS and that TYRO3 small molecular agonists such as C-10 can be further developed as specific therapy for glomerular disease.
RESULTS

TYRO3 expression is found almost exclusively in podocytes of mouse and human kidneys

We first determined kidney cell-specific mRNA expression of TYRO3 using the recently published single-cell RNA-sequencing (scRNAseq) data of the mouse and human kidneys. The mouse glomerular scRNAseq dataset (18) showed that while all three TAM receptors were expressed in podocytes (Fig. S1A), TYRO3 expression was limited to podocytes as compared to the broader expressions of Mer and Axl. In another recently published mouse kidney scRNAseq dataset, which contains all major kidney cell subtypes but without significant representation of glomerular cells (19), TYRO3 expression was absent in non-glomerular cells, while Mer was broadly expressed and Axl was expressed in kidney macrophages (Fig. S1B). We further examined a scRNAseq dataset of CD45-enriched kidney immune cells (20), which similarly showed a minimal expression of Tyro3, but varying degrees of expression of Mer and Axl in immune cell subsets (Fig. S1C). We also examined the expression of TYRO3 in human kidneys using the single-nuclear RNAseq datasets of control subjects and DKD patients (21) (http://humphreyslab.com/SingleCell). TYRO3 expression was limited to podocytes in both control and diabetic kidneys (Fig. S1D) and consistent with our earlier observation, TYRO3 expression in early DKD was not decreased in comparison to control kidneys. These data indicate that kidney expression of TYRO3 is largely restricted to podocytes, unlike the other two TAM receptors.

Small molecule C-10 is a specific TYRO3 agonist

For the development of a small molecule agonist of TYRO3, we undertook two approaches of in silico homology modeling, in the absence of a known crystal structure TYRO3, by taking advantage of the specificity of ligand-receptor interactions between PS/TYRO3 and GAS6/AXL. In the first approach, by comparison of PS structure with that of GAS6, which binds TYRO3 with weak affinity, we deduced the potential allosteric interaction sites between PS to the extracellular region of TYRO3. In the second approach, we compared the tyrosine autophosphorylation sites of TAM receptors in the intracellular region (TYRO3-Y804, AXL-Y821, and MER-Y872) that are surrounded by unique neighboring sequences using the PDBsum database (22). Based on these two approaches, we designed 12 compounds with 3 different pharmacophore groups, shown in Fig. S2A. Fig. S2B shows the examples of synthesis reactions of the two select compounds. We first screened the 12 compounds based on their effects on TYRO3 phosphorylation, as a surrogate for its increased signaling activity, in cultured human podocytes. As shown in Figure 1A, compound 8 (C-8) and compound 10 (C-10) showed robust phosphorylation of TYRO3 among the screened compounds. Because C-10 and C-8 shared structural similarities (Fig. S2A-B), but the effect of C-10 was superior to C-8, we focused on further characterization of C-10 in cultured human podocytes. Treatment of C-10 led to dosage-dependent phosphorylation of TYRO3 and activation of its downstream molecule, AKT (Figure 1B). We previously demonstrated that PS/TYRO3 signaling attenuated the NF-κB-mediated inflammatory response in podocytes (6, 17). Therefore, we also examined the effects
of C-10 in TNF-α-treated podocytes. Real-time PCR analysis of NF-κB target genes, IL6 and CCL2, showed a significant attenuation of their expression in C10 dose-dependent manner in cultured podocytes (Figure 1C, Figure S3). These results indicate that C-10 is a potent agonist of TYRO3. Moreover, no cellular cytotoxicity was observed in cultured podocytes at these effective dosages (Figure S4). Because increased expression of TYRO3 has been observed in several types of cancers, including gastric cancer, we also tested whether C-10 might influence their proliferation. As shown in Figure S5, C-10 had no obvious effects on the proliferative ability of gastric cancer cells, even when incubated with 500nM of C-10, a dose nearly 10 fold higher than the dose range that activates TYRO3 in cultured podocytes.

To test for the specificity of C-10 for TYRO3 among the TAM receptors, we next examined the effects of C-10 on the phosphorylation of the endogenous TAM receptors in podocytes. Cultured human podocytes were treated with varying doses of C-10 for 30 minutes, western blot was performed using an antibody that recognizes all phosphorylated TAM receptors. C-10 significantly induced TYRO3 phosphorylation in a dose-dependent manner, but it had no effect on AXL and MER activation (Figure 2A). We additionally examined the effects of C-10 in podocytes overexpressing FLAG-tagged TYRO3, AXL, or MER (Fig. 2B). C-10 led to a strong induction of AKT phosphorylation only in TYRO3 overexpressing cells (Fig. 2C, Figure S3). To confirm that C-10 exerts its specific effects on TYRO3 by direct interaction, we utilized the drug affinity responsive target stability (DARTS) method (23-26), which is used to infer a direct binding of a small molecule compound to a protein. It utilizes the change in the target protein conformation resulting from a direct interaction with the small molecule, thereby altering its susceptibility to proteolysis. As shown in Figure 2D, increasing concentrations of C-10 conferred resistance to pronase-mediated degradation of TYRO3 (Fig. 2D, Figure S3), but it had no effects on pronase-mediated AXL or MER degradation (data not shown). Together, these results strongly indicate that C-10 is a potent and select agonist of TYRO3.

C-10 attenuates glomerular injury in mice with adriamycin-induced nephropathy

We next tested the efficacy of TYRO3 agonism by C-10 in vivo using the experimental model of FSGS, Adriamycin-induced nephropathy (ADRN). ADRN was induced in the 10-week old male Balb/c mice by Adriamycin injection (8mg/kg body weight). Mice were treated with either vehicle control or C-10 (2.5mg/kg body weight) by daily intraperitoneal injection, after 3 days post-adriamycin administration. Kidneys were examined after 4 weeks post-adriamycin administration. C-10 significantly reduced albuminuria in ADRN mice by both spot-collection and 12-hour collection of urine samples (Fig. 3A and 3B). There was also a significant attenuation in glomerulosclerosis development with C-10 treatment (Fig 3C and 3D). C-10 also restored podocyte number in ADRN mice, as assessed by WT-1 staining (Fig. 4A-D), and reduced ADR-induced podocyte foot process effacement (Fig. S6). No obvious toxicity has been observed in these mice during the treatment period.

C-10 attenuated glomerular injury in mice with DKD
We next examined the effects of C-10 in DKD. Male db/db mice in the BKS background were treated with C-10 at a dose of 2.5 mg/k by daily intraperitoneal injection starting at 10 weeks of age when db/db mice developed significant albuminuria for a total of 8 weeks. The mice were sacrificed at age of 18 weeks. We found that C-10 significantly reduced albuminuria in these diabetic mice as measured by both spot-collection and 12-hour collection of urine samples (Fig. 5A). C10 treatment led to a significant improvement in glomerular volume and mesangial expansion in db/db mice (Fig 5B-D). C-10 also restored podocyte number, as assessed by WT-1 staining (Fig. 6A-D), and reduced podocyte foot process effacement in diabetic kidneys of db/db mice (Fig. S7). No obvious toxicity was observed in these mice during the treatment period.

C-10 failed to attenuate glomerular injury in Tyro3-knockout mice

To further confirm the role of C-10 is through binding to Tyro3 receptor, we treated Tyro3 knockout mice with C-10 after induction of ADRN. Briefly, ADRN was induced in the Tyro3 knockout mice by injection of adriamycin at a dose of 18mg/kg at the age of 10 weeks. The mice were fed with either C-10 at a dose of 2.5mg/kg body weight or vehicle by daily intraperitoneal injection started on the day 3 after they received an injection of adriamycin and ended in 4 weeks when the mice were sacrificed. We found that C-10 did not reduced albuminuria in these knockout mice with ADRN as measured by albuminuria/creatinine ratio and timely collection of urine samples (12 hours) (Fig. 7A-B). There was no significant improvement of glomerulosclerosis score (Fig. 7C-D) or podocyte foot process effacement with C-10 treatment (Fig. S8) in ADRN mice. Taken together, the in vivo data indicate that C-10 confers renoprotection in glomerular disease by specific agonism of TYRO3 in podocytes.

DISCUSSION

Podocyte injury by either apoptosis or detachment or other mechanisms leads to the progression of glomerular disease (2, 3, 27). The reduction in podocyte density is a strong predictor of progressive DKD (5, 28), and the extent of podocyte reduction correlates with the magnitude of proteinuria (29, 30). Many studies have attempted to identify specific treatments to prevent podocyte injury. Retinoic acid and vitamin D3 have been shown to attenuate podocyte injury in glomerular disease (31) (32), but whether these are effective therapy has not been shown in clinical settings. Several immunosuppressive medications used for the treatment of primary glomerular disease have been shown to have direct protective effects against podocyte injury such as calcineurin inhibitors, steroids, and rituximab (33-35). However, these medications have many other effects than podocyte protection. Therefore, there is an urgent need to develop more specific and better podocyte-protective drugs for the treatment of glomerular disease.

Our previous study showed that PS/ TYRO3 signal transduction protects podocytes from injury in the settings of glomerular disease (6) (17). Our previous and current data thus indicate that TYRO3
agonism may offer a new therapeutic avenue, based on these key findings: 1) TYRO3 expression negatively correlates with the progression of human glomerular disease, supporting a critical role of TYRO3 in kidney disease in humans (17); 2) single-cell transcriptomic data indicates that TYRO3 expression in the kidney is limited to podocytes, with relatively low expression in macrophages when compared to AXL and MER. Therefore, the effects of select TYRO3 agonists would be podocyte-specific; and 3) we have strong in vivo data supporting a protective role of PS-TYRO3 signaling axis against podocyte injury in glomerular disease; this is in contrast to the disease-promoting effects of AXL/MER signaling in mesangial cells in DKD (12, 17). Moreover, as PS exerts additional anti-coagulant effect, TYRO3 agonist, rather than that of PS, would be more optimal as a therapeutic approach.

It is well acknowledged that the designing of agonists is generally more challenging than the designing of antagonists. In addition, the three-dimensional crystal structure of TYRO3 is not yet resolved, posing an additional challenge. To achieve the specificity of select TYRO3 agonism, we leveraged the homology modeling approach by looking for amino acid sequences at the allosteric interaction points. Since we know that two known ligands of TAM receptors PS and GAS6 binds to TAM receptors with different affinities (i.e., PS binds mostly with TYRO3, while GAS6 binds mostly to AXL/MER), we compared their amino acid sequences to identify the specific sequence for PS-TYRO3 interaction. In addition, we also focused on the tyrosine autophosphorylation site of TAM receptors, which is required for dimerization of receptor and its activation and found that tyrosine autophosphorylation site of Tyro3 has a unique sequence, which is different from AXL and Mer. By using these approaches, we were able to develop specific TYRO3 agonists and selected C-10 and C-8 for further studies based on their effects on AKT phosphorylation (cell survival) and NF-κB suppression (anti-inflammatory effects). Both C-8 and C-10 was derived from the former approach of deducing the PS-TYRO3 interaction points, and share structural similarities. Because C-10 was more potent than C-8, we further characterized its effects in cultured podocytes. Indeed, the C-10 treatment of cultured podocytes resulted in the activation of endogenous TYRO3 in absence of additional PS treatment, without affecting AXL and MER activation. The activation of TYRO3 led to a dual effects of promoting podocyte survival and dampening of inflammation in injury settings. Interestingly, the key modulator of inflammation, NF-κB, has been shown to act as a ‘double-edged sword’ in the pathogenesis of podocytopathy (36), in that the broad-range inhibition of NF-κB mitigates the inflammatory response of podocytes, but does so at the expense of sensitized cell death (36). As C-10 treatment resulted in anti-inflammatory effects while promoting cell survival effects in injured podocytes, it is plausible that C-10’s actions may ‘fine-tune’ the NF-κB activity to simultaneously mitigate inflammatory response and promote cell survival (36).

Importantly, similar to what was observed previously with PS/TYRO3 overexpression models, we confirmed that C-10 administration attenuated podocyte injury in vivo in two mouse models of glomerular diseases, ADRN as an FSGS model and diabetic db/db model. However, C-10 did not have any effects in Tyro3 knockout mice, further supporting that the renoprotective effects of C-10 are mediated through direct
agonism of TYRO3. In addition, C-10 did not cause any cell toxicity in cultured podocytes, and mice fed with C-10 for 8 weeks did not exhibit any abnormal phenotype such as weight loss or behavior changes. Therefore, we believe that C-10 could be a potential lead compound that will be further optimized for its druggable properties in future studies.

One major concern of using Tyro3 agonists to treat glomerular disease is its potential tumorigenesis and suppression of anti-tumor immunity (37). While the roles of Mer and Axl in these cancer processes have been well-described, less is known about the roles of Tyro3. In addition, many studies have assessed the effects of Tyro3 inhibition in conjunction with inhibition of Mer and/or Axl in an effort to evaluate overlapping functions and have not evaluated the contributions of the individual family members. Similarly, studies describing the effects of TAM kinase ligands in biological systems do not always evaluate the contributions of individual family members. However, studies suggest that Tyro3 expression is increased in several types of cancers (38) although its baseline expression in the normal tissues is relatively low. Tyro3 mutations have been also noted in human malignancies, but the function of these mutations has not been proven to elucidate the potential significance of these mutations in cancer (38). Increased Tyro3 expression may promote tumorigenesis through the activation of AKT pathway and affect anti-tumor immunity through its anti-inflammatory effects. However, these cell survival and anti-inflammatory effects are beneficial for podocytes in glomerular disease. Phosphorylation of AKT has been shown to be a pro-survival pathway in podocytes in early DKD (39, 40). A similar observation was reported for other molecules such as Yes-associated protein (YAP). YAP, a downstream gene of Hippo kinase is a cell survival protein for podocytes but may promote tumorigenesis (41). The question to resolve in future studies is how we could avoid potential tumorigenesis, while we treat glomerular disease with Tyro3 agonists. We found that C10-did not induce cell proliferation of a gastric cancer cell line at 500nM, which is 5-10 fold higher than the dose used to induce Tyro3 phosphorylation in cultured podocytes. We also found that mice treated with C-10 for 2 months did not develop any cancer. We need to further examine whether treatment with C-10 for longer time period will develop any cancer. We will also assess whether the low dose of Tyro3 agonist (2.5mg/kg/day) used for the treatment of glomerular disease will affect tumorigenesis in vitro and in vivo. Although it is still challenging, several approaches have been developed to target drugs directly into podocytes or glomeruli, which will help us to avoid potential systemic side effects (42) (43) (44) (45). Another concern is that we used male mice in the study due to the high susceptibility to kidney injury in these animal models (46). However, we will validate the effects of C-10 in female mice in future studies.

In conclusion, we developed a novel Tyro3-specific agonist (C-10) which was shown to attenuate proteinuria and podocyte injury in two animal models of glomerular disease. Our data further support the protective role of Tyro3 in podocytes and Tyro3 agonists could be a potential new therapy for glomerular disease.
EXPERIMENTAL PROCEDURES

Synthesis of the compounds:
The detailed synthetic scheme (Fig. 2B) for our hit molecules (C-8 and C-10) based on their biological activity is described below. In an inert atmosphere, the corresponding carbonitrile 1 and 3 (1 eq.) was treated with the corresponding α-mercaptocarboxylic acid 2 (1 eq.) and pyridine (20 mol %). The mixture was stirred for 24 h at 120 °C. After completion of the reaction, the crude solid obtained was further purified by silica gel column chromatography and chemical structures were elucidated using analytical tools (NMR and Mass spectroscopy). For C-8: 2-(4-bromo-3-methoxyphenyl)-5-methylthiazol-4-ol (8). A yellow solid (0.065 g, 43%). 1H NMR (DMSO, 300 MHz) δ 13.38 (s, 1H), 8.00 (s, 1H), 7.74 (d, J = 9 Hz, 1H), 7.06 (d, J = 9 Hz, 1H), 3.91 (s, 3H), 2.25 (s, 3H) ppm; 13C NMR (DMSO, 75 MHz) δ 174.4, 149.2, 140.0, 138.5, 133.0, 129.6, 128.7, 128.2, 122.4, 74.9, 60.5, 15.4 ppm. HRMS (EI) Calcd. for C_{10}H_{5}ClO_{4}[M+H]^+ requires 299.9694, found 299.9707. For C-10: (5-(4-hydroxy-5-methylthiazol-2-yl)thiophen-2-yl)boronic acid (10). A yellow solid (0.061 g, 46%). 1H NMR (600 MHz, MeOH-d4), δ: 10.30 (s, 1H), 7.57 (dd, J = 7.87, 1.49 Hz, 1H), 7.25 - 7.22 (m, 1H), 6.90 (d, J = 8.26 Hz, 1H), 6.87 - 6.84 (m, 1H), 2.26 (s, 3H) ppm. 13C NMR (150 MHz, MeOH-d4), δ: 174.6, 156.5, 133.3, 119.9, 113.6, 106.8, 16.0 ppm. HRMS (EI) Calcd. for C_{10}H_{5}ClO_{4}[M+Na]^+ requires 263.9936, found 263.9991.

Cell culture
Human podocytes were obtained from Dr. Moin Saleem and cultured as described (47). Cells were serum starved in 1% serum containing medium for 12 hours. The cells were stimulated with tumor necrosis factor-α (10 ng/mL) for an additional 24 hours.

Overexpression and knockdown TAM receptors with shRNA-lentivirus
Lentiviral vectors for overexpression or shRNA-mediated knockdown of TYRO3 were used as described previously (17). Overexpression plasmids of Mer and Axl (Origene Technologics, Inc. # RC600074 and # RC206431, respectively) were subcloned into a lentivector construct, similarly as described for TYRO3 (17), and podocytes with stable expression of each TAM receptor were selected using blasticidin.

Luciferase reporter assays
To measure the effects of compounds on TNFα-induced NF-κB activation in podocytes, cells were transfected with NF-κB luciferase reporter plasmid (#219077, Stratagene, La Jolla, CA) for 2 days and incubated with or without of TNFα with or without compounds for an additional 24 hours. To normalize transfection efficiency, the renilla luciferase plasmid was co-transfected. Reporter activity was detected by using a luciferase assay system (#E1910, Promega Corp., Madison, WI).

Western blot
Cells were homogenized in lysis buffer containing protease inhibitor cocktail. Equal amounts of protein samples were separated on SDS polyacrylamide gel, transferred to PVDF membranes (Millipore) and probed with primary antibodies: anti-TYRO3 (Abcam, #ab109231); anti-MER, anti-AXL, Anti-GAPDH, anti-pAKT and anti-total AKT (Cell Signaling, #4694, #8661, #2118, #4060, and #9272); anti-FLAG (Sigma, F2555). Then, membranes were incubated with a horseradish peroxidase conjugated secondary antibodies to mouse IgG or to rabbit IgG (Promega, # W401B, #W402B). Blots were developed with the enhanced chemiluminescence system.

DARTS assay
DARTS assay was conducted as described previously (23-26). Briefly, cultured human podocytes were lysed in M-PER mammalian protein extraction buffer (#78501, ThermoFisher) with protease inhibitors (#11836,#153001, Roche) and phosphatase inhibitors (50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM Na3VO4). 10X TNC buffer (500 mM Tris–HCl pH 8.0, 500 mM NaCl, 100 mM CaCl2) was added to the cell lysate and protein concentration was measured using the Bradford assay (Bio-rad, #500–0006). The cell lysates were incubated with C-10 at indicated doses at room temperature for 1 h. After incubation, cell lysates were subject to proteolysis with pronase (Roche, #10165921001) at indicated dilution at room temperature for 20 min.

Mouse models
Male db/db mice in BKS background were purchased from The Jackson Laboratory (Bar Habor, ME). Sex- and age-matched db/m littermates were used as controls. C-10 dissolved in 5% DMSO was administered in 10-week old mice by daily intraperitoneal injection (2.5mg/kg body weight/day) for 8 weeks. Vehicle-treated mice were used as controls. Adriamycin-induced nephropathy was induced by injection of adriamycin at 8mg/kg for Balb/c mice and 18mg/kg for Tyro3 knockout mice via tail vein as described previously (48). C-10 (2.5mg/kg body weight) and vehicle were administered intraperitoneally daily starting 3 days post-adriamycin injection for 28 days. Proteinuria was monitored every week, and the mice were sacrificed at 28 days post-injection. Global Tyro3 knockout mice were purchased from The Jackson lab and used as described in our previous study (17).

Measurement of Urine Albumin and Creatinine
Urine albumin was quantified by ELISA using a kit from Bethyl Laboratories, Inc. (#E99-134). Urine creatinine levels were measured in the same samples using QuantiChrom™ creatinine assay kit (DICT-500, BioAssay Systems) according to the manufacturer's instructions. The urine albumin excretion rate was expressed as the ratio of albumin to creatinine. 12-hour urine collections in the metabolic cages were also used for the determination of urinary albumin excretion.
Kidney histology

Kidneys were removed and fixed with 4% paraformaldehyde 16 hours at 4 °C. The 4μm sections were cut from paraffin-embedded kidney tissues. Sections were stained with periodic acid–Schiff (PAS) for histologic analysis. Assessment of the mesangial and glomerular cross-sectional areas was performed by pixel counts on a minimum of 10 glomeruli per section in a blinded fashion, under 400x magnification (Zeiss AX10 microscope, Carl Zeiss Canada Ltd, Toronto, ON, Canada). Renal histological abnormalities were scored as previously described (49). The glomerulosclerosis was graded on a semiquantitative scale (0–3+): 0 (absent), 1+ (involving 1–25% of all glomeruli sampled), 2+ (involving 26–50% of glomeruli), and 3+ (involving >50% of glomeruli) as described. An average of 50 glomeruli was sampled per animal.

Electron Microscopy

Tissues were fixed in 2.5% glutaraldehyde with 0.1M sodium cacodylate (pH 7.4) for 72 hr at 4°C. Samples were further incubated with 2% osmium tetroxide and 0.1M sodium cacodylate (pH 7.4) for 1 hr at room temperature. Ultrathin sections were stained with lead citrate and uranyl acetate and viewed on a Hitachi H7650 microscope. Briefly, negatives were digitized, and images with a final magnitude of up to X10,000 were obtained. ImageJ 1.26t software (National Institutes of Health, rsb.info.nih.gov) was used to measure the length of the peripheral GBM, and the number of slit pores overlying this GBM length was counted. The arithmetic mean of the foot process width ($W_{FP}$) was calculated as shown below:

$$W_{FP} = \frac{\pi}{4} \times \frac{\sum GBM\ LENGTH}{\sum slits}$$

where $\Sigma_{slits}$ indicates the total number of slits counted; $\Sigma_{GBM\ LENGTH}$ indicates the total GBM length measured in one glomerulus, and $\pi/4$ is the correction factor for the random orientation by which the foot processes were sectioned (50).

Isolation of mouse glomeruli

Mouse glomeruli were isolated as described (51). Briefly, animals were perfused with Hanks' buffered salt solution containing 2.5mg/ml iron oxide and 1% bovine serum albumin. At the end of perfusion, kidneys were removed, decapsulated, minced into 1mm³ pieces, and digested in Hanks' buffered salt solution containing 1mg/ml collagenase A and 100units/ml deoxyribonuclease I. Digested tissue was then passed through a 100μm cell strainer and collected by centrifugation. The pellet was resuspended in 2 ml of Hanks’ buffered salt solution, and glomeruli were collected using a magnet. The purity of glomerular was verified under microscopy. Total RNA was isolated from kidney glomeruli of mice using TRIzol (Invitrogen, #15596026).

Real-time PCR
Total RNA was extracted by using TRIzol (Invitrogen, #15596026). First strand cDNA was prepared from total RNA (2.0 μg) using the Superscript™ III first strand synthesis kit (Invitrogen) and cDNA (1 μl) was amplified in triplicate using SYBR GreenER qPCR Supermix on an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA). Light Cycler analysis software was used to determine crossing points using the second derivative method. Data were normalized to housekeeping genes (GAPDH) and presented as a fold change compared with control group using the $2^{-\Delta\Delta CT}$ method. Following primers were used: Wt-1 (forward, 5’ -GAGAGCCAGCCTACCATCC-3’; reverse 5’ -GGGTCTCGTGTTTGAAGGAA-3’), Il-6 (forward, 5’-CCAGCTATGAACTCCTTCTC - 3’; reverse 5’-GCTTGTTCCTACATCTCTC - 3’), and Ccl2 (forward, 5’ –AGGTGACTGGGGCATCTGAT-3’; reverse 5’-GCCTCCAGCATGAAAGTCTC-3’).

Immunofluorescence
Kidney sections from mice were prepared accordingly. Immunostaining was performed using anti-WT1 antibodies (Santa Cruz Biotechnology, Cat # sc-192). After washing, sections were incubated with a fluorophore-linked secondary antibody (Alexa Fluor 488 anti-rabbit IgG, #A32731 and Alexa Fluor 568 anti-mouse IgG #A-11004 from Invitrogen). After staining, slides were mounted in Aqua Poly Mount (Polysciences Inc., #18606-5) and imaged with AxioVision Ile microscope with a digital camera.

Statistical Analysis
Data are expressed as mean ± SD. Unpaired t-test was used to analyze data between two groups. The analysis of variance with Bonferroni post-hoc test was used when more than two groups were present. All experiments were repeated at least three times, and representative experiments are shown. Statistical significance was achieved when p< 0.05.

Study Approval
All animal protocols were approved by the Institutional Animal Care and Use Committee at Icahn School of Medicine at Mount Sinai (#PROTO202000020).

ACKNOWLEDGMENTS:
JCH is supported by VA Merit Award I01BX000345 and NIH/NIDDK R01DK109683, R01DK122980, R01DK129467, and P01DK56492. KL is supported by NIH/NIDDK R01DK117913-01, R01DK129467, and 1R01DK133912. J.F. is supported by NIH/NIDDK K01DK125614-01A1.

AUTHOR CONTRIBUTIONS
J.C.H., K.L, F.Z. designed the research project; F.Z, H.C, D.B., and L.W. performed the experiments; B.D designed and synthesized the compounds; Z.L designed and made the plasmid expression constructs; J.F and Z.S performed and analyzed single-cell transcriptomic data; J.C.H, K.L., F.Z., and H.C. analyzed the data; J.C.H., K.L., and F.Z. drafted and revised the manuscript. All authors approved the final version of
the manuscript.

**CONFLICT OF INTEREST:**
The authors declare that they have no competing financial interests.
FIGURE LEGENDS:

**Figure 1:** C-10 is a novel TYRO3 agonist. (A) Cultured human podocytes were treated with control vehicle (C) or TYRO3 agonists (compounds 1-12) for 30 minutes. Cell lysates were probed for phosphorylated or total TYRO3 (p-TYRO3 or t-TYRO3). GAPDH was used as a loading control. (B) Cultured human podocytes were stimulated with compound 10 (C-10) for 30 minutes at different doses as indicated, and cell lysates were probed for phosphorylated or total TYRO3 (p-TYRO3 or t-TYRO3) and for phospho- or total AKT (p-AKT or t-AKT). GAPDH was used as a loading control. (C) Cultured podocytes were treated with vehicle control (C), 10µM TNF-α alone (T) or TNF-α with TYRO3 agonists (compounds 1-12) for 2 hours. Real-time PCR analysis was performed for NF-κB target genes, IL-6 and CCL2 expression. N=3, *p<0.05 or **p<0.01 when compared to TNF-α treated by 1-way ANOVA with Bonferroni correction.

**Figure 2:** C-10 is a select agonist of TYRO3. (A) Cultured human podocytes were stimulated with C-10 at different doses for 30 minutes. Cell lysate was probed with the antibody recognizing the phosphorylated form of all three TAM receptors. Phosphorylated MER, AXL, and TYRO3 receptors are differentiated by their corresponding molecular weight. (B) HEK293 cells were transfected with either control vector, or FLAG-tagged expression vector for individual TAM receptors (AXL-FLAG, TYRO3-FLAG, or MER-FLAG). Lysates from transfected cells were probed for FLAG-tagged protein expression. (C) Transfected HEK293 cells in (B) were treated with 100nM C-10 for 30 minutes, and lysates were probed for phosphorylated or total AKT (p-AKT or t-AKT) and FLAG proteins. GAPDH was used as a loading control. The representative blots of three independent experiments are shown. (D) Drug Affinity Responsive Target Stability (DARTS) assay was performed to test the direct binding of C-10 to TYRO3. Podocyte lysates were pre-incubated with various concentrations of C-10 as indicated at 25 °C for 1 h prior to digestion with pronase (0 or 1:1000 dilution) for 20 min. Lysates were then probed for TYRO3 expression, with GAPDH as a loading control.

**Figure 3:** C-10 ameliorates albuminuria and glomerulosclerosis in mice with ADRN. (A) Urinary albumin/creatinine ratio in control or ADRN mice treated with vehicle or C-10. (B) Twelve-hour albumin excretion in control and ADRN mice. (C) Periodic acid-Schiff–stained kidney images at 200x (top) and 400x (bottom) magnifications. Scale bar: 50 μm. (D) Glomerulosclerosis scoring in control and ADRN mice. n=6 mice per group; ***P<0.001, and ****P<0.0001 when compared with control group; ###P < 0.001, and ####P < 0.0001 when compared with ADR+Vehicle mice by 1-way ANOVA with Bonferroni correction.

**Figure 4:** C-10 ameliorates podocyte loss in mice with ADRN. (A) Representative images of WT1 staining in glomeruli are shown (n=6, original magnification x400, scale bar: 50μm). (B-C) WT1-positive cell number per 1000 µm2 glomerular tuft area (B) and per glomerular cross-section (C) were quantified and expressed as relative fold changes to Control mice (60 glomeruli quantified per group). (D) mRNA levels of Wt1 were determined using real-time PCR. n=6 mice per group. *P<0.05, ***P<0.001, and ****P<0.0001 when compared with control group; #P<0.05, and ##P<0.01 when compared with ADR+Vehicle mice by 1-way ANOVA with Bonferroni correction.

**Figure 5:** C-10 ameliorates albuminuria and glomerulosclerosis in diabetic mice. Type 2 diabetic db/db mice were treated with C-10 (2.5 mg/kg) for 8 weeks, starting at 10 weeks of age. (A) Urinary albumin/creatinine ratio (left panel) and albuminuria in12-hour urine collection (right panel) were measured in control and diabetic mice treated with vehicle or C-10. (B) Periodic acid-Schiff–stained kidney images at
200x (top) and 400x (bottom) magnifications. Scale bars: 50μm. (C-D) Glomerular area (C) and mesangial matrix fraction (D) in control and diabetic mice. n=6 mice per group; *P<0.05, **P<0.01, and ****P <0.0001 when compared with control db/m mice; #P < 0.05, ##P < 0.01, ###P < 0.001, and ####P < 0.0001 when compared with vehicle-treated db/db mice by 1-way ANOVA with Bonferroni correction.

Figure 6: C-10 ameliorates podocyte loss in diabetic mice. (A) Representative images of WT1 staining in glomeruli are shown (n=6, original magnification x400, scale bar: 50μm). (B-C) WT1-positive cell number per 1000 μm² glomerular tuft area (B) and per glomerular cross section (C) were quantified and expressed as relative fold changes to control db/m mice (60 glomeruli quantified per group). (C) mRNA levels of Wt1 were determined using real-time PCR. n=6 mice per group. **P<0.01, ***P<0.001, and ****P<0.0001 when compared with control db/m group; #P<0.05, and ##P<0.01 when compared with db/db+Vehicle mice by 1-way ANOVA with Bonferroni correction.

Figure 7: C-10 fails to protect against ADRN in Tyro3-knockout mice. (A) Urinary albumin/creatinine ratio in Tyro3 wildtype (WT) and knockout (KO) mice with ADRN treated with vehicle or C-10. (B) Twelve-hour albumin excretion in control and ADRN mice. (C) Periodic acid-Schiff–stained kidney images at 200x (top) and 400x (bottom) magnifications. Scale bar: 50μm. (D) Glomerulosclerosis scoring in control and ADRN mice. n=6 mice per group; ***P<0.001, and ****P<0.0001 when compared with WT ADR+Vehicle group; #P<0.05, and ####P<0.0001 when compared with Tyro3 KO ADR+C-10 mice by 1-way ANOVA with Bonferroni correction.

REFERENCES


10. Studer RA, Opperdoes FR, Nicolaes GA, Mulder AB, and Mulder R. Understanding the functional difference


Figure 1: C-10 is a novel TYRO3 agonist. (A) Cultured human podocytes were treated with control vehicle (C) or TYRO3 agonists (compounds 1-12) for 30 minutes. Cell lysates were probed for phosphorylated or total TYRO3 (p-TYRO3 or t-TYRO3). GAPDH was used as a loading control. (B) Cultured human podocytes were stimulated with compound 10 (C-10) for 30 minutes at different doses as indicated, and cell lysates were probed for phosphorylated or total TYRO3 (p-TYRO3 or t-TYRO3) and for phospho- or total AKT (p-AKT or t-AKT). GAPDH was used as a loading control. (C) Cultured podocytes were treated with vehicle control (C), 10µM TNF-α alone (T) or TNF-α with TYRO3 agonists (compounds 1-12) for 2 hours. Real-time PCR analysis was performed for NF-kB target genes, IL-6 and CCL2 expression. N=3, *p<0.05 or **p<0.01 when compared to +TNF-α+vehicle group by 1-way ANOVA with Bonferroni correction.
Figure 2: C-10 is a select agonist of TYRO3. (A) Cultured human podocytes were stimulated with C-10 at different doses for 30 minutes. Cell lysate was probed with the antibody recognizing the phosphorylated form of all three TAM receptors. Phosphorylated MER, AXL, and TYRO3 receptors are differentiated by their corresponding molecular weight. (B) HEK293 cells were transfected with either control vector, or FLAG-tagged expression vector for individual TAM receptors (AXL-FLAG, TYRO3-FLAG, or MER-FLAG). Lysates from transfected cells were probed for FLAG-tagged protein expression. (C) Transfected HEK293 cells in (B) were treated with 100nM C-10 for 30 minutes, and lysates were probed for phosphorylated or total AKT (p-AKT or t-AKT) and FLAG proteins. GAPDH was used as a loading control. The representative blots of three independent experiments are shown. (D) Drug Affinity Responsive Target Stability (DARTS) assay was performed to test the direct binding of C-10 to TYRO3. Podocyte lysates were pre-incubated with various concentrations of C-10 as indicated at 25 °C for 1 h prior to digestion with pronase (0 or 1:1000 dilution) for 20 min. Lysates were then probed for TYRO3 expression, with GAPDH as a loading control.
Figure 3: C-10 ameliorates albuminuria and glomerulosclerosis in mice with ADRN. (A) Urinary albumin/creatinine ratio in control or ADRN mice treated with vehicle or C-10. (B) Twelve-hour total urinary albumin excretion (UAE) in control and ADRN mice. (C) Periodic acid-Schiff–stained kidney images at 200x (top) and 400x (bottom) magnifications. Scale bar: 50μm. (D) Glomerulosclerosis scoring in control and ADRN mice. n=6 mice per group; ***P<0.001, and ****P<0.0001 when compared with control group; #P< 0.01, and ####P < 0.0001 when compared with ADR+Vehicle mice by 1-way ANOVA with Bonferroni correction.
Figure 4: C-10 ameliorates podocyte loss in mice with ADRN. (A) Representative images of WT1 staining in glomeruli are shown (n=6, original magnification x400, scale bar: 50μm). (B-C) WT1-positive cell number per 1000 μm² glomerular tuft area (B) and per glomerular cross section (C) were quantified and expressed as relative fold changes to Control mice (60 glomeruli quantified per group). (D) mRNA levels of Wt1 were determined using real-time PCR. n=6 mice per group. *P<0.05, ***P<0.001, and ****P<0.0001 when compared with control group; #P<0.05, and ##P<0.01 when compared with ADR+Vehicle mice by 1-way ANOVA with Bonferroni correction.
Figure 5: C-10 ameliorates albuminuria and glomerulosclerosis in diabetic mice. Type 2 diabetic db/db mice were treated with C-10 (2.5 mg/kg) for 8 weeks, starting at 10 weeks of age. (A) Urinary albumin/creatinine ratio (left panel) and albuminuria in 12-hour urine collection (right panel) were measured in control and diabetic mice treated with vehicle or C-10. (B) Periodic acid-Schiff–stained kidney images at 200x (top) and 400x (bottom) magnifications. Scale bars: 50μm. (C-D) Glomerular area (C) and mesangial matrix fraction (D) in control and diabetic mice. n=6 mice per group; *P<0.05, **P<0.01, and ****P<0.0001 when compared with control db/m mice; #P<0.05, ##P<0.01, ###P<0.001, and ####P<0.0001 when compared with vehicle-treated db/db mice by 1-way ANOVA with Bonferroni correction.
Figure 6: C-10 ameliorates podocyte loss in diabetic mice. (A) Representative images of WT1 staining in glomeruli are shown (n=6, original magnification x400, scale bar: 50μm). (B-C) WT1-positive cell number per 1000 μm² glomerular tuft area (B) and per glomerular cross section (C) were quantified and expressed as relative fold changes to control db/m mice (60 glomeruli quantified per group). (C) mRNA levels of Wt1 were determined using real-time PCR. n=6 mice per group. **P<0.01, ***P<0.001, and ****P<0.0001 when compared with control db/m group; *P<0.05, and ##P<0.01 when compared with db/db+Vehicle mice by 1-way ANOVA with Bonferroni correction.
Figure 7: C-10 fails to protect against ADRN in Tyro3-knockout mice. (A) Urinary albumin/creatinine ratio in Tyro3 wildtype (WT) and knockout (KO) mice with ADRN treated with vehicle or C-10. (B) Twelve-hour albumin excretion in control and ADRN mice. (C) Periodic acid-Schiff–stained kidney images at 200x (top) and 400x (bottom) magnifications. Scale bar: 50μm. (D) Glomerulosclerosis scoring in control and ADRN mice. n=6 mice per group; ***P<0.001, and ****P <0.0001 when compared with WT ADR+Vehicle group; #P < 0.05 and ####P < 0.0001 when compared with Tyro3 KO ADR+C-10 mice by 1-way ANOVA with Bonferroni correction.