Inhibition of Thrombin-Activated Fibrinolysis Inhibitor Increases Survival in Experimental Kidney Fibrosis

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ABSTRACT

Uncontrolled diabetes, inflammation, and hypertension are key contributors to progressive renal fibrosis and subsequent loss of renal function. Reduced fibrinolysis appears to be a feature of ESRD, but its contribution to the fibrotic program has not been extensively studied. Here, we show that in patients with CKD, the activity levels of serum thrombin-activated fibrinolysis inhibitor and plasmin strongly correlated with the degree of renal function impairment. We made similar observations in rats after subtotal nephrectomy and tested whether pharmacologic inhibition of thrombin-activated fibrinolysis inhibitor with UK-396082 could reduce renal fibrosis and improve renal function. Compared with untreated animals, UK-396082–treated animals had reduced glomerular and tubulointerstitial fibrosis after subtotal nephrectomy. Renal function, as measured by an increase in creatinine clearance, was maintained and the rate of increase in proteinuria was reduced in UK-396082–treated animals. Furthermore, cumulative survival improved from 16% to 80% with inhibition of thrombin-activated fibrinolysis inhibitor. Taken together, these data support the importance of the fibrinolytic axis in regulating renal fibrosis and point to a potentially important therapeutic role for suppression of thrombin-activated fibrinolysis inhibitor activity.


Renal fibrosis is the common final pathway in CKD, leading to ESRD. Increasing clinical evidence has identified CKD as an independent risk factor for coronary artery disease and cardiovascular events. The scarring is characterized by expansion of both glomerular and tubular basement membranes,1,2 with normal extracellular matrix (ECM) components being replaced by excessive interstitial collagens.3–6 Several pathways have been implicated in this process of scar tissue formation, including those regulated by TGF-β1,7–9 connective tissue growth factor,10,11 transglutaminase-2,12,13 and plasmin activator inhibitor 1 (PAI-1).14,15 While PAI-1 and plasmin are most often associated with the regulation of fibrinolytic system, recent studies have indicated an additional contribution to renal impairment and mortality.16–18 Furthermore, increasing evidence also suggests that members of the fibrinolytic system, such as fibrinogen,19,20 plasmin,15 tissue plasmin activator (tPA),21 urokinase plasmin activator (uPA)22 and thrombin-activated fibrinolysis inhibitor activity (TAFI)23–25 are important players in the fibrotic response to cellular injury.

A balance between ECM degradation and deposition is a fundamental requisite for maintaining the structural and functional integrity of multiple organs, including the kidney. The pathways involved in normal ECM homeostasis and the transition to overt fibrosis following tissue injury are poorly understood, but
decreased local activation of ECM-degrading enzymes may play a critical role. A potentially important emerging physiologic activator of matrix degradation in the kidneys is the plasminogen-plasmin system. While plasmin is a serine protease that degrades fibrin, it also activates matrix metalloproteinases to break down elastin and collagens, and can degrade laminin, fibronectin as well as other ECM proteins directly. At sites of vascular injury and thrombosis, intraglomerular fibrin formation contributes to renal scarring by serving as a matrix on which fibroblasts can proliferate and secrete ECM. Studies in patients with acute coronary syndrome have highlighted the increased propensity of fibrin clot formation, impaired fibrinolysis, and higher PAI-1 levels in patients with renal impairment. Few experimental studies have examined the levels in patients with renal impairment. Plasmin may also additionally protect against renal fibrosis. For instance, transgenic mice overexpressing PAI-1 are associated with more severe forms of GN compared with mice with physiologic levels of plasmin. In contrast, fibrinogen heterozygous mice subjected to ischemia-reperfusion injury or unilateral ureteral obstruction, or TAFI knockout mice subjected to immune-mediated GN, are protected from renal injury and fibrosis. These observations extend into the clinical space as serum and urine PAI-1 levels have been reportedly elevated in patients with diabetic nephropathy. Few experimental studies have examined the effect of the modulation of the fibrinolytic system beyond knockout and transgenic model systems, and in some instances the data are controversial, perhaps reflecting developmental effects.

TAFI is a circulating zymogen that, when activated by thrombin or plasmin, prevents fibrin clot from being prematurely broken down by plasmin by removing exposed C-terminal lysine residues from fibrin. This downregulates tPA-mediated plasmin generation, thus lowering plasmin activity. We have previously demonstrated that inhibition of TAFI can reduce in vitro ECM accumulation in response to high glucose. Here, we sought to determine whether levels of plasmin and TAFI were altered in patients with CKD compared with healthy individuals and to test whether pharmacologic blockade of TAFI could reduce kidney fibrosis and improve kidney function when dosed to rats subjected to 5/6 subtotal nephrectomy (SNx).

RESULTS

Serum TAFI and Plasmin Activity Are Altered in Patients with Renal Impairment and in Rats Following SNx
To assess whether TAFI and plasmin activity are altered in patients with CKD, archival serum samples collected from individuals with a spectrum of noncystic disease presentation and renal impairment (Table 1) were analyzed. Serum plasmin activity was reduced from 76.8 μg/ml in individuals with normal renal function to 35.3 μg/ml in those with renal impairment (Figure 1A). TAFI activity was similarly affected; normal activity in humans was 23.3 μg/ml, increasing to 45.7 μg/ml in patients with CKD stages 4–5 (Figure 1B). There was a strong correlation between the levels of TAFI and plasmin with eGFR (r = −0.793 and r = 0.742, respectively) (Figure 1).

To determine whether this observation could be experimentally mimicked, the effects of 5/6 SNx on plasmin and TAFI activity in the rat were studied. Rats subjected to SNx had a 30% reduction in plasmin activity compared with baseline or control animals when measured over time after SNx (Figure 2A). In contrast, TAFI activity increased approximately 4-fold by 21 days after SNx (Figure 2B). In both instances, the elevation in TAFI activity, or reduction in plasmin activity, was sustained to 60 days after SNx. Taken together, these data suggested a good correlation between the observations of altered plasmin activity in patients with CKD and the rat SNx model, making SNx a relevant system to test the contribution of the plasmin-TAFI axis to renal fibrosis and function.

Inhibition of TAFI Activity with UK-396082 Alters the Activity of Components of the Fibrinolytic Pathway in the SNx Rat
UK-396082 is a potent and selective small molecule TAFI inhibitor previously shown to block high glucose–induced ECM deposition in in vitro cell assays and exhibit antithrombotic efficacy in a rabbit model of venous thrombosis. We first tested escalating doses of UK-396082 for serum TAFI activity.

### Table 1. Patient demographic characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>CKD Stage</th>
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<tbody>
<tr>
<td></td>
<td>1 (eGFR≥90 ml/min per 1.73 m²)</td>
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<tr>
<td>Patients (n)</td>
<td>5</td>
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<tr>
<td>Mean proteinuria (mg/ml)</td>
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<tr>
<td>Median age (range) (yr)</td>
<td>49 (31–68)</td>
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<tr>
<td>Gender ratio (M:F)</td>
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<tr>
<td>Race (%)</td>
<td>White: 100</td>
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<td></td>
<td>Afro-Caribbean: 50</td>
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Patients were selected from a range of nonpapillary CKD conditions, including diabetic nephropathy and various forms of GN (diabetic nephropathy, 23%; GN, 29%; hypertension, 9%; other, 40%). None of the patients with CKD stage 5 were receiving RRT at the time of sampling.
inhibition in normal rats to set a dose level for study in SNx rats. A dose of 60 mg/kg per day was selected because it maximally inhibited TAFI activity in normal animals over the dosing period (Supplemental Figure 1). Administration of UK-396082 lowered TAFI activity at all time points, with levels not statistically higher than untreated sham animals at any time point except day 60 after SNx (Figure 2B). The effects of UK-396082 were studied under prophylactic dosing (day 14 after SNx) and therapeutic dosing (day 35 after SNx) paradigms. When treated from day 35 after SNx, a similar reduction in TAFI activity occurred by day 42 and remained lower than activity in untreated SNx animals (Figure 2B). In the untreated SNx animals, serum plasmin activity significantly decreased (change, −19%) compared with values in sham animals at days 42 and 60 (Figure 2B). UK-396082 did not elevate plasmin activity in normal animals (Figure 2A); however, in SNx animals, UK-396082 led to an increase by day 35 (21%) that progressively increased by day 60 (52%) in both treatment groups. As UK-396082 is renally cleared, we confirmed the terminal serum exposures (Supplemental Figure 2) that correlated with the extent of renal impairment and inhibition of TAFI activity.

Terminal renal tissue samples were examined for evidence of modulation of the fibrinolytic system, especially plasmin, TAFI, uPA, and tPA. Sham-operated animals had renal TAFI activity (600 μg/ml) that remained stable throughout the time course, with a 33% elevation at day 60 in SNx animals (Figure 3A). UK-396082 treatment of both sham and SNx animals reduced tissue TAFI activity and increased plasmin activity (Figure 3B). The uPA activity was elevated following SNx, and this was not affected by UK-396082 treatment (Figure 3C). In contrast, tPA activity was slightly elevated following SNx, and this
increased further (by 30%) following UK-396082 treatment (Figure 3D).

**Inhibition of TAFI Activity with UK-396082 Improves Renal Function Following SNx**

SNx caused an elevation in serum creatinine (SCr), BUN, systolic BP, and proteinuria by day 35 after SNx (Figure 4). In animals treated with UK-396082 from day 14 or day 35, SCr levels were maintained at day-35 levels or lower (Figure 4A). This improvement was mirrored by a reduced rate of decline in creatinine clearance (Figure 4B) and a lowering in the rate of proteinuria development (Figure 4C) in each treatment group compared with vehicle control. UK-396082 treatment had no effect on BP in sham-operated or SNx animals (Figure 4D), indicating the effects of UK-396082 on renal function were independent of a hemodynamic effect. Treatment of SNx animals with UK-396082 also significantly lowered terminal BUN compared with vehicle-treated animals (Figure 4E).

**Inhibition of TAFI Activity with UK-396082 Reduces Renal Fibrosis Following SNx and Prevents Progression When Dosed Therapeutically**

Kidney sections were stained with Masson's trichrome to visualize the changes in tissue fibrosis in both glomerular and tubulointerstitial compartments (Figure 5). Tubulointerstitial fibrosis progressively increased in SNx animals at days 35 and 60 after surgery. This was associated with extensive epithelial cell flattening, tubular atrophy, and a large interstitial infiltrate. There was extensive glomerulosclerosis in SNx animals with both glomerular basement membrane (GBM) thickening and mesangial matrix expansion. Scarring was significantly reduced in both glomerular and tubulointerstitial compartments in animals treated with UK-396082 from day 14 or 35 after SNx compared with their relevant control group (Figures 5A and 6A). When quantified by multiphase image analysis, sections showed a greater than 4-fold reduction in interstitial collagen in the tubulointerstitium and 50% reduction in the
glomeruli for both treatment regimens compared with the day-60 untreated SNx animals. Progression of ECM expansion was halted in tubules and glomeruli with treatment from day 35. Total kidney collagen (hydroxyproline) was halved in animals treated with UK-396082 from days 14 and 35 after SNx (Figure 5B and Figure 6, B and C) compared with untreated day-60 SNx animals, and was prevented from increasing with treatment from day 35.

Figure 4. Renal function is improved with UK-396082. Blood and urine (metabolic cage) were collected at days 0, 35, 42, and 60 after SNx, and SCr (A), creatinine clearance (B), and proteinuria (C) measured. BP was measured using a Model 229 Blood Pressure Monitoring System (IITC) 1 day before termination (D). Serum urea was determined at termination (E). Data represent mean ± SEM. *P < 0.05 and **P < 0.01 (t test with Bonferroni corrections) indicate significance against sham-operated animals or indicated comparisons. †P < 0.05 and ††P < 0.01 (t test with Bonferroni corrections) indicate significance against untreated SNx animals. UK, UK-396082 treatment from day 14 (D14) or day 35 (D35). All animals were culled at day 60 from those culled at day 35 (sham/SNx D35).
Changes in individual collagens (collagen I, III, and IV) (Figure 7), as well as laminin and fibronectin (Supplemental Figure 3), were determined by immunofluorescence and multi-phase image analysis (Supplemental Figure 5). In sham-operated animals, sparse collagen I staining was localized to the tubular basement membrane (TBM) with faint glomerular staining in the mesangial matrix (Figures 7 and 8). These animals also had a peritubular distribution of collagen III that increased slightly as the animals aged unless treated with UK-396082 (Figure 8, C and D). Collagen IV displayed fine TBM and GBM staining in sham-operated animals, which was unaltered by UK-396082 (Figure 8, E and 8F). In contrast, vehicle control SNx animals showed a small, but significant, increase in glomerular collagen I at day 35 (predominantly GBM), with a larger increase in the TBM (Figures 7 and 8). Untreated SNx animals also showed increased levels of collagen III and IV in both glomerular and tubular compartments by day 35, with a significant increase between this time point and the termination of the study at d60 only seen in glomerular collagen IV (Figure 8). UK-396082–treated SNx animals had significantly reduced glomerular and interstitial collagen I and IV when dosed prophylactically and did not increase from day-35 levels when dosed therapeutically (Figures 7 and 8), with similar findings in fibronectin (Supplemental Figure 4). The effects of UK-396082 on collagen III and laminin deposition were mostly observed in the tubules (Figure 8, Supplemental Figures 4 and 5).

**Effect of Inhibition of TAFI Activity with UK-396082 on Urine Biomarkers Following SNx**

Tubular injury biomarkers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) (Figure 9, A and B) plus surrogate fibrosis biomarkers tissue

**Figure 5.** Collagen accumulation is reduced with UK-396082. Following SNx, animals were culled at day 60 or day 35; kidneys were recovered, quartered, and fixed in neutral buffered formalin and stained with Masson’s trichrome to assess kidney fibrosis. Representative staining in the tubulointerstitium (top panel, A) and glomeruli (bottom panel, B). Sham-operated animals (left, upper and lower) and untreated SNx animals analyzed at day 35 (middle upper) and day 60 (right upper). UK-396082 (60 mg/kg per day)–treated animals analyzed on day 60; sham-treated from day 14 (left, lower) and SNx from day 35 (middle, lower) and day 14 (right, lower). Glomeruli are at ×400 magnification; tubulointerstitium at ×200 magnification.
inhibitor of metalloproteinase-1 (TIMP-1) and transglutaminase (TG2) (Figure 9, C and D), were measured. Urine NGAL, KIM-1, TG2, and TIMP-1 levels were low in sham-operated animals but increased following SNx surgery. Treatment of SNx animals with UK-396082 from day 14 or 35 after surgery reduced urinary KIM-1 and NGAL levels compared with levels in control SNx animals at the same time point and reduced TG2 levels when dosed prophylactically. In contrast, TIMP-1 levels increased in UK-396082–treated animals compared with control SNx animals.

UK-396082 Improves Cumulative Survival
All sham-operated animals survived for the 60-day study period. Untreated SNx animals began to show moribund signs from day 20. Animals were culled when they showed signs of ESRF (SCr >4 μg/dl). In the prophylactic treatment group, only 16% of untreated SNx animals survived to day 60, compared with 80% of those treated from day 14 with UK-396082 (P=0.00167, log-rank test) (Figure 10A). At day 35, when treatment with UK-396082 commenced in the therapeutic group, 60% of untreated SNx animals were alive. Of the animals left untreated from day 35 onward, only 27% survived to day 60, in contrast to 75% of animals that survived when treated with UK-396082 from day 35 (P=0.022, log-rank test) (Figure 10B). Taken together these data suggest that animals treated with UK-396082 had a survival advantage compared with untreated animals after SNx.

DISCUSSION
Interest and awareness of the potential role of the fibrinolytic system in cardiorenal impairment are increasing. We have confirmed and extended these initial observations by demonstrating a strong reciprocal correlation between serum TAFI and plasmin activity with loss of renal function in clinical specimens. We wanted to understand the potential significance of this in an experimental setting and selected the rat 5/6 SNx model on the basis of similar observations of impaired plasmin and elevated TAFI activity with the development of renal function decline in this model.

Our previous in vitro studies had demonstrated that blockade of TAFI activity was associated with reduced ECM accumulation. Several studies in transgenic and knockout mice have also indicated that modulating the fibrinolytic system can alter the physiologic response to injury, reducing renal fibrosis or improving renal function. Data examining the effects of renal injury in TAFI knockout mice are somewhat conflicting. In a model of immune complex-mediated GN, genetic ablation reduced proteinuria, BUN, and renal fibrosis. However, data from the same group on the effect of ureteral obstruction suggested a worse outcome, with elevated collagen deposition and proinflammatory cytokines compared with wild-type operated animals. While these opposing observations could be due to the different profibrotic forces in each model, the possibility of developmental effects or compensation by other pathways also cannot be ruled out. Given the observations of elevated TAFI activity in clinical and preclinical specimens with renal function impairment, we sought to test whether pharmacologic blockade could restore renal function.

The SNx model is characterized by initial glomerular hypertrophy and hypertension, which proceeds to proteinuria, and then to tubulointerstitial fibrosis. Our observa...
mesangial matrix expansion, glomerular and tubulointerstitial fibrosis leading to uremia. As a hypertensive model, it is relevant to human disease because it follows a pathogenesis not dissimilar to that of FSGS in humans. Likewise therapeutic treatment held the tubulointerstitial measures of collagens I, III, and IV at levels consistent with or slightly lower than at the start of therapy. When taken together, the observations from early and late intervention data suggest that the approach was effective even in more advanced disease.

Uremia progressed more rapidly than our experience with this model and the study had to be shortened from 90 to 60 days. Signs of renal failure appeared in some animals as early as 20 days after SNx and by day 35, 40% of the untreated SNx animals had highly elevated SCr consistent with renal failure, with extensive glomerulosclerosis and interstitial fibrosis. This more rapid progression allowed us to collect cumulative survival data. There was a strong correlation between protection of renal function and histology with progression to renal failure in UK-396082–treated animals compared with untreated SNx rats.

We additionally tracked the effects of UK-396082 with changes in urinary tubular injury markers, NGAL and KIM-1, and the surrogate fibrosis markers, TIMP-1 and TG2. We observed elevation of the tubular injury markers with disease progression and a marked decrease in the levels with UK-396082. Renal TG2 also appears to be highly correlated with the progression of fibrosis in patients with CKD, and urinary TG2 has recently been proposed as a possible biomarker for CKD progression. Like the tubular injury markers, UK-396082 reduced urinary levels of TG2 compared with vehicle control, supporting the notion that one of the beneficial effects of TAFI blockade was on ECM turnover. Elevated serum and urinary TIMP-1 has also been associated with the progression of CKD and renal injury, and indeed urine TIMP-1 increased after SNx. However, in contrast to TG2, KIM-1, and NGAL, TIMP-1 increased with UK-396082 therapy. While this observation is somewhat counterintuitive to observations of elevated TIMP expression in CKD tissues, urinary TIMP-1 mRNA is reduced in patients with DN and loss of TIMP-3 appears to be associated with a worse renal outcome in preclinical models suggesting a more complex relationship between matrix metalloproteinase/TIMP activity and renal disease.
In this hypertensive model, angiotensin-converting enzyme inhibitors,\textsuperscript{47,48} angiotensin receptor blockers,\textsuperscript{49} and endothelin receptor antagonists\textsuperscript{50} almost completely prevent disease, in substantial part due to a reduction in systemic and glomerular pressure. UK-396082 treatment was without effect on systolic BP, underwriting a direct effect of TAFI inhibition on protection of ECM turnover and renal function decline. While we did not study the potential additional benefit of angiotensin inhibition, due to the sensitivity of this axis in this model, it is tantalizing to consider the possibility that compounds like UK-396082 could provide benefit when given with antihypertensive therapy in the clinical situation.

On commencement of this study, we hypothesized whether the restoration of renal plasmin activity would underlie any beneficial effect. Serum plasmin activity decreased after SNx, but kidney plasmin activity was not significantly reduced. UK-396082 treatment, however, raised plasmin levels above baseline levels in kidney and serum, suggesting that this might be a component to the improvement in outcome,\textsuperscript{25} consistent with other observations. Levels of PAI-1 appear to be elevated in patients with CKD, and knockout of PAI-1 appears to protect the kidney from fibrosis;\textsuperscript{15} however, the same group also demonstrated that the benefit of PAI-1 was not through plasmin, but potentially through tPA,\textsuperscript{15} as had been suggested before.\textsuperscript{51} It remains unclear how tPA may be beneficial because this is not through elevated plasmin activity. In this study, tPA in the kidney was also raised by UK-396082, suggesting that both increased tPA and plasmin activity may play a role.

In conclusion, this study showed a beneficial effect of UK-396082 in the prevention and treatment of kidney fibrosis induced by SNx. It was effective in slowing the development of CKD when given before fibrosis developed and in treating more advanced disease. The observations of impaired plasmin activity and fibrinolysis, which might contribute to cardiovascular events in ESRD, may not be merely an independent bystander response but may actively contribute to renal function decline. The correlation between our clinical observations of elevated TAFI activity and renal function impairment and the same experimental observations in the SNx rat point to a significant mechanism whereby modulation of fibrinolytic activity through TAFI inhibition could be an important treatment for established kidney fibrosis.

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**Figure 8.** Collagens I, III, and IV are reduced with UK-396082. The level of collagen I staining from Figure 7 in the tubulointerstitium (A) and glomeruli (B), along with collagen III (C and D) and collagen IV (E and F) was assessed by multiphase image analysis using Cell F software. Ten glomeruli (original magnification, $\times 400$) or 10 cortical fields (original magnification, $\times 200$) were analyzed for each animal. $^*P<0.05$ and $^{**}P<0.01$ ($t$ test with Bonferroni corrections) indicate significance against sham-operated animals or indicated comparisons. Sham D35 and SNx D35 indicate values determined from animals culled early, at day 35. UK, UK-396082 treatment from day 14 (D14) or day 35 (D35).
Rat 5/6 SNx Model of Kidney Fibrosis

Male Wistar rats (225–250 g) were subjected to SNx by upper and lower ligation and resection of the right kidney, followed by removal of the left kidney 7 days later under isoflurane anesthesia. The removed tissue was weighed to ensure 5/6 removal. Sham operations were used as controls. Two groups of 12 SNx animals were fed normal rat chow (Harlan Teklad) and euthanized at day 35 or 60. Two additional groups were fed rat chow containing UK-396082 (Research Diets) at a concentration such that the animal received 60 mg UK396082/kg body weight per day from day 14 or day 35. Rats were maintained at 20°C and 45% humidity on a 12-hour light/dark cycle and allowed free access to food and water. All procedures were carried out under license according to regulations laid down by Her Majesty’s Government, UK (Animals Scientific Procedures Act 1986). Any animal showing signs of renal failure had the serum creatinine measured and were culled according to Home Office and University Animal Welfare Guidelines.

**Kidney Function and Damage**

Urinary creatinine was measured in 24-hour urine samples. Urine creatinine was measured using the standard auto analyzer technique (Clinical Chemistry Department, Royal Hallamshire Hospital, UK). Serum creatinine analysis was performed using a serum creatinine kit (Arbor Assays) as per the manufacturer’s instructions. Proteinuria was determined in rat urine using a modification of the Lowry method. TIMP-1 was measured using a rat TIMP-1 Quantikine ELISA Kit (R&D Systems) on urine diluted 1:20 with reaction buffer (R&D Systems). NGAL and KIM-1 were measured using a rat NGAL or KIM-1 DuoSet (R&D Systems) on urine diluted 1:20 with 1% BSA in PBS. TG2 was measured using a sandwich ELISA on urine diluted 1:25 with 1% BSA in PBS. The coating antibody was goat anti-TG2 antibody (ab421; Abcam, Inc.) diluted 1:1000 in 50 mM carbonate buffer (pH, 9.6). The primary antibody was CUB7402 monoclonal anti-TG2 antibody (Abcam, Inc.) diluted 1:1000 in 3% BSA in PBS, and the secondary was biotinylated goat anti mouse IgG (H+L) antibody (Abcam, Inc.).

**Plasmin Activity Assay**

Serum samples (100 μl) or 10% (w/v) renal homogenates were mixed with D-Val-Leu-Lys-pNa substrate (100 μl to a final concentration of 3 mM) in 10 mM potassium phosphate, 70 mM sodium phosphate, and 100 mM lysine buffer (pH, 7.5) and pipetted into a 96-well plate (Iwaki, Japan). The plate was incubated at 37°C in the dark, with absorbance read at 405 nm at 0, 30, 60, 90 and 120 minutes on the Multiskan Ascent plate reader (Thermo Lab-systems, Cheshire, UK). Plasmin activity as related to recombinant protein was then determined using a standard curve of recombinant plasmin.

**TAFI Activity Assay**

TAFI activity as related to recombinant protein was measured using a proprietary TAFI assay kit (Pentapharm) according to the manufacturer’s instructions.

**tPA and uPA Activity Assay**

Triplicate samples of serum (100 μl) or 10% (w/v) renal homogenate were assayed as related to recombinant protein in the same manner as used for plasmin but with S-2222 substrate (Chromogenix, ...
Carmarthenshire, UK) for tPA and S-2244 (Chromogenix) substrate for uPA with absorbance at 405 nm measured at 0, 30, 60, 90 and 120 minutes.

Tissue Cytochemistry and Immunofluorescence
Analysis of renal scarring was determined on 4-μm-thick formalin-fixed, paraffin-embedded sections stained using a Masson’s trichrome staining kit (Sigma-Aldrich, Bromborough, UK) as per the manufacturer’s instructions. The degree of scarring was carried out using multiphase image analysis with CellF software (Olympus, Milton Keynes, UK).

Immunofluorescence for collagens I, III, and IV was performed on paraffin-embedded sections using goat anti–collagen III (Southern Biotech), mouse anti–collagen I (Abcam, Inc.), rabbit anti–collagen IV (MP Biomedicals), rabbit antilaminin (Sigma-Aldrich), and rabbit antifibronecit (Sigma) with secondary antibodies (goat anti-rabbit FITC [Sigma-Aldrich], goat anti-mouse FITC [Sigma-Aldrich], and donkey anti–goat FITC [Santa Cruz Biotechnology]) using previously published protocols.33

Multiphase Image Analysis
Ten fields of cortex (×100) or glomeruli (×400) were acquired using a CC-12 digital camera (Soft Imaging Systems, Muenster, Germany), and staining was assessed using a three-phase analysis approach with Cell F imaging software (Olympus) ensuring total coverage of 95%, as previously described.25

BP Measurements
BP was measured using a noninvasive tail-cuff method with a Model 229 BP monitoring system (IITC, Norfolk, UK).

Serum UK-396082
Circulating levels of UK-396082 were measured by York Bioanalytical Solutions LTD (UK) in serum by HPLC/tandem mass spectrometry analysis.

Hydroxyproline
Hydroxyproline was measured on 10% renal homogenate using a Biochrom 30 amino acid analyzer (Biochrom, UK), as previously described.53

Human Serum Samples
Blood was collected into a 5-ml Vacutainer containing clotting agent (Becton Dickenson), allowed to clot for 30 minutes, spun at 400 g for 10 minutes to separate serum. The serum was immediately frozen and stored at −80°C till analysis. Blood was collected under National Health Service R&D protocols STH15020 (diabetic) and STH 15004 (nondiabetic) under United Kingdom ethical approvals 08/H1308/234 and 08/H1305/64, respectively. Patients were selected with noncystic kidney disease to span the full range of kidney function impairment (CKD stages 1–5). No patients were receiving RRT.

Statistical Analyses
Statistical analyses were performed with Minitab and Prism, and Bonferroni corrections were performed for multiple comparisons applied by the software. Significance has been reported at the unadjusted level (*P=0.05, **P=0.01) but confirmed at the adjusted level (*P=[0.05/n comparisons], **P=[0.01/n comparisons]).

Ethics
All animal studies were approved by the University of Sheffield review board. Written consent was obtained from all participants in the human serum study.
ACKNOWLEDGMENTS

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DISCLOSURES

N.P. is an employee of Pfizer PGDR.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014030303/-/DCSupplemental.
Supplementary Figure 1: Dose Optimisation of UK-396082

Rats were subjected to 5/6 subtotal nephrectomy and treated with increasing doses of UK-396082 incorporated into the rat chow. Blood (0.3 mL) was taken from the rat tail vein under anaesthesia after 4 weeks of UK-396082 treatment. Blood was left to clot for 30 mins and then spun (400g, 15 mins) to obtain serum. Serum TAFI activity was calculated using the Pentapharm TAFI activity kit. Data represent mean activity equivalent to recombinant human TAFI in µg/mL ±SEM, n=5, **p<0.01 (t-test) indicated significance against untreated
Supplementary Figure 2: Terminal UK-396082 serum concentration

Blood (0.3 mL) was taken from the rat tail vein under anaesthesia at the time of termination. Blood was clotted and spun (400g, 15 mins) to obtain serum. Serum UK-396082 was determined by Pfizer, UK, by HPLC-MS/MS. Data represents mean serum UK-396082 concentrations in ng/mL ±SEM. **p<0.01 (t-test) indicates significance against sham operated rats dosed with UK-396082 (n=5)
Following subtotal nephrectomy, animals were culled at day 60 or day 35, kidneys recovered, quartered and fixed in neutral buffered formalin and stained with an anti-Laminin antibody using an FITC secondary antibody. Representative laminin staining in tubulointerstitium (top panel) and glomeruli (bottom panel). Sham operated animals (A) untreated SNx at day 35 (B) and Day 60 (C). UK-396082 treatment at 60 mg/kg/day in sham operated animals from day 14 (D), and SNx from day 35 (E) and Day 14(F). Glomeruli are at 400x magnification and tubulointerstitium at 200x magnification.
Supplementary Figure 4: Fibronectin Immunofluorescence in the rat subtotal nephrectomy model of chronic kidney disease treated with UK-396082

Following subtotal nephrectomy, animals were culled at day 60 or day 35, kidneys recovered, quartered and fixed in neutral buffered formalin and stained with an anti-fibronectin antibody using an FITC secondary antibody. Representative fibronectin staining in tubulointerstitium (top panel) and Glomeruli (bottom panel). Sham operated animals (A) untreated SNx at day 35 (B) and Day 60 (C). UK-396082 treatment at 60 mg/kg/day in sham operated animals from day 14 (D), and SNx from day 35 (E) and Day 14(F). Glomeruli are at 400x magnification and tubulointerstitium at 200x magnification.
Supplementary Figure 5: Laminin and Fibronectin Quantification

The level of laminin (A&B) and fibronectin (C&D) staining in tubulointerstitial fibrosis and glomerulosclerosis was assessed by multiphase image analysis. 10 Glomeruli (400x) or 10 cortical fields (200x) were analysed for each animal. **p<0.01, *p<0.05 (t-test) indicates significance against sham operated animals. ††p<0.01, †p<0.05 (t-test) indicates significance against untreated SNx animals.