Nilotinib Attenuates Renal Injury and Prolongs Survival in Chronic Kidney Disease

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ABSTRACT
The tyrosine kinase inhibitor imatinib is beneficial in experimental renal diseases, but the effect of the new tyrosine kinase inhibitor nilotinib on the progression of renal failure is unknown. We administered either nilotinib or vehicle to Sprague–Dawley rats beginning 2 weeks after 5/6 nephrectomy (Nx) or laparotomy and continuing for 8 weeks. Serum creatinine levels were significantly lower in the nilotinib group after 6 and 8 weeks of treatment. Furthermore, nilotinib-treated rats had less proteinuria, attenuated glomerulosclerosis and tubulointerstitial damage, and reduced macrophage infiltration into the tubulointerstitium. Treatment with nilotinib also significantly decreased renal cortical expression of profibrogenic genes, such as IL-1β and monocyte chemotactic protein-1, which correlated closely with the tubulointerstitial damage score and ED1-positive macrophages score. In addition, nilotinib treatment significantly prolonged survival. Taken together, these results suggest that nilotinib may limit the progression of chronic kidney disease.


Imatinib (Gleevec, Novartis Pharmaceuticals, Co., Basel, Switzerland) is a selective tyrosine kinase inhibitor that inhibits the tyrosine kinase activity of Bcr-Abl, c-Kit, and PDGF receptors (PDGFRs) and has been demonstrated to be highly active in patients with chronic myeloid leukemia and gastrointestinal stromal tumors. The therapeutic benefit of imatinib in animal models of kidney diseases (e.g., mesangial proliferative glomerulonephritis, chronic allograft nephropathy, diabetic nephropathy, lupus nephritis, and unilateral obstructive nephropathy) has been reported. In aggregate, these studies have shown that the beneficial effects of imatinib therapy are the result of its inhibitory action on PDGFRs, leading to reductions of glomerular cell proliferation and extracellular matrix accumulation. In addition, we recently demonstrated that its immunosuppressive actions on B cells and macrophages resulted in amelioration of cryoglobulinemic membranoproliferative glomerulonephritis in mice and nephrotoxic serum nephritis in rats, respectively.

Nilotinib (Novartis Pharmaceuticals, Co.) is a new phenylaminopyrimidine with enhanced activity and selectivity against the Bcr-Abl tyrosine kinase compared with imatinib, whereas the cellular and biochemical IC50 values of nilotinib and imatinib for PDGFRs inhibition are similar; nilotinib promises to provide improved treatment of chronic myeloid leukemia and imatinib-resistant disease. Thus, it is presumable that nilotinib has beneficial effects in renal diseases comparable to imatinib. Although nilotinib has shown efficacy in models of liver fibrosis and oxidative stress in a thioacetamide rat model compared with imatinib, the effects of nilotinib on experimental models of renal disease have not yet been reported.

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The pathogenesis of chronic kidney disease (CKD) involves a complex interaction between hemodynamic abnormalities (represented mainly by systemic and glomerular hypertension) and inflammatory events (e.g., macrophages infiltration) as well as exaggerated production of extracellular matrix. The interaction between all of these phenomena to promote CKD is heavily dependent on the production of cytokines, chemokines, and growth factors, including PDGF and TGF-β. According to this, it is plausible that nilotinib could attenuate the progression of CKD by inactivation of PDGFRs as well as through its immunomodulatory effects.

In vivo studies

(1) Experimental 1 (therapeutic study)

(2) Experimental 2 (survival study)

Figure 1. Schematic presentation of the experimental protocol.

The objectives were to determine whether nilotinib treatment of rats with 5/6 nephrectomy (Nx), a commonly used model of CKD, would provide renal protection comparable to other experimental models of kidney disease treated by imatinib. Whether such renal protection would be reflected in the survival rate was also investigated.

RESULTS

Effects of Nilotinib on Biochemical Parameters in 5/6 Nx Rats

The experimental design is described in Figure 1. The 5/6-Nx-vehicle and 5/6-Nx-nilotinib rats had higher systolic BP and lower body weights (BWs) than the Sham rats throughout the study period. There were no significant changes in BP and BW in the 5/6-Nx-vehicle and 5/6-Nx-nilotinib rats throughout the study period (Figure 2, a and b). Figure 2, c and d, show the results of urinary total protein and the serum creatinine (Cr) level for each group. Urinary total protein and serum Cr levels were significantly higher in 5/6-Nx-vehicle rats than in Sham rats from 1 week after treatment and throughout the study period, respectively. The increased urinary total protein excretion in 5/6-Nx-vehicle rats was significantly reduced by nilotinib treatment from 1 week after treatment to the end of the study (Figure 2c). After 6 and 8 weeks of treatment, serum Cr levels were significantly lower in the 5/6-Nx-nilotinib rats than in the 5/6-Nx-vehicle rats (Figure 2d). Ni-
loatinib treatment also decreased remnant kidney hypertrophy (2.09 ± 0.06 g versus 1.90 ± 0.08 g, *P* < 0.05).

**Effects of Nilotinib on Renal Histologic Findings in 5/6 Nx Rats**

Figure 3 shows the representative periodic acid–Schiff (PAS), silver, and Masson trichrome stainings of the kidneys from the study groups. Renal histologic findings in 5/6-Nx-vehicle rats were characterized by hypertrophy, glomerular sclerosis, and tubulointerstitial fibrosis. The quantitative analysis of the glomerular tuft area is presented in Table 1. Nilotinib treatment significantly reduced glomerular hypertrophy in rats with remnant kidney (*P* < 0.05). The glomerular sclerosis score increased significantly in 5/6-Nx-vehicle rats compared with Sham rats (*P* < 0.001), and this increase was significantly reduced by nilotinib treatment (*P* < 0.01) (Table 1). The tubulointerstitial damage score was greater in 5/6-Nx-vehicle rats than in Sham rats (*P* < 0.001). Nilotinib treatment significantly improved the tubulointerstitial damage in 5/6 Nx rats (*P* < 0.01) (Table 1).

**Effects of Nilotinib on Profibrogenic Genes and Proteins in 5/6 Nx Rats**

The gene expression level of collagen type I was much higher in 5/6-Nx-vehicle than in Sham rats, as assessed by real-time reverse transcriptase (RT)-PCR. Nilotinib treatment significantly decreased collagen type I gene expression in 5/6 Nx rats (Figure 4a). There was an increase in fibronectin and plasminogen activator inhibitor-1 (PAI-1) gene expressions in 5/6-Nx-vehicle rats compared with Sham rats. Fibronectin and PAI-1 gene expressions in the kidneys of 5/6 Nx rats were attenuated by nilotinib treatment (Figure 4, b and c). As shown in Figure 4, d and e, there was a significant increase in PDGF-B and TGF-β gene expressions in the kidneys of 5/6-Nx-vehicle rats compared with Sham rats that was significantly reversed by nilotinib treatment. For examination

![Figure 3](image)

**Table 1.** Morphologic evaluation of glomerular size, glomerular sclerosis, and tubulointerstitial damage at the end of study

<table>
<thead>
<tr>
<th></th>
<th>5/6-Nx-Vehicle</th>
<th>5/6-Nx-Nilotinib</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular size (μm²)</td>
<td>12,494.75 ± 729.15</td>
<td>10,894.50 ± 501.24*</td>
<td>7032.33 ± 274.44</td>
</tr>
<tr>
<td>Glomerular sclerosis score (0 to 4)</td>
<td>1.56 ± 0.30</td>
<td>0.60 ± 0.20*</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Tubulointerstitial damage score (0 to 5)</td>
<td>3.20 ± 0.42</td>
<td>1.57 ± 0.22*</td>
<td>0</td>
</tr>
</tbody>
</table>

*P* < 0.01, 5/6-Nx-vehicle versus 5/6-Nx-nilotinib.
of the effect of nilotinib on TGF-β1 protein synthesis in 5/6 Nx rats, kidney tissue homogenate was measured using the TGF-β1 ELISA kit (Figure 5). The TGF-β1 level was significantly higher in 5/6 Nx rats than in Sham rats (P < 0.05). There was a 26% (P < 0.05) reduction of TGF-β1 by nilotinib treatment in 5/6 Nx rats (Figure 5). To verify the decrease of collagen gene expressions by nilotinib treatment, immunohistochemical staining for collagen type IV was performed. As shown in Figure 6,

![Figure 4](image1.jpg)

**Figure 4.** Nilotinib decreases profibrogenic gene expression levels in 5/6-nephrectomized rats. Real-time RT-PCR for (a) collagen type I, (b) fibronectin, (c) PAI-1, (d) PDGF-B, and (e) TGF-β in each group. The horizontal dotted lines show the expression levels in Sham rats. Data are expressed as means ± SEM. The values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GADPH) values and then expressed as relative quantification. *P < 0.05, **P < 0.01 versus 5/6-Nx-vehicle.

![Figure 5](image2.jpg)

**Figure 5.** Nilotinib decreases renal cortical TGF-β1 protein level in 5/6-nephrectomized rats. Renal cortical homogenate TGF-β1 protein levels of each group measured by ELISA. Data are expressed as means ± SEM. Mann–Whitney test: *P < 0.05: 5/6-Nx-vehicle versus 5/6-Nx-nilotinib; #P < 0.05: 5/6-Nx-Nx-vehicle versus Sham.

![Figure 6](image3.jpg)

**Figure 6.** Nilotinib decreases urinary collagen level in 5/6-nephrectomized rats. Urinary collagen levels of each group measured as described in Concise Methods. Data are expressed as means ± SEM. Mann–Whitney test: **P < 0.01: 5/6-Nx-vehicle versus 5/6-Nx-nilotinib; ###P < 0.01: 5/6-Nx-vehicle versus Sham.
Nilotinib treatment was associated with a significant decrease in the glomerular extracellular matrix as assessed by the percentage of the glomerular tuft area occupied by the collagen type IV-stained matrix ($P < 0.05$).

**Effects of Nilotinib on Tubulointerstitial Macrophage Infiltration in 5/6Nx Rats**

Quantitative evaluation of tubulointerstitial macrophage infiltration was performed by measurement of ED1-positive cells in 5/6-Nx-vehicle rats and Sham rats. There was a 4.27-fold increase in ED1-positive macrophages in the tubulointerstitium in 5/6-Nx-vehicle rats compared with Sham rats (Figure 7). The increase in ED1-positive macrophages in the tubulointerstitium in 5/6-Nx-vehicle rats was reduced to 57% by nilotinib treatment (Table 2). Immunostaining for ED1 in glomeruli was faint in each study group (data not shown).

**Effects of Nilotinib on Proinflammatory Genes in 5/6 Nx Rats**

Because macrophage-derived proinflammatory cytokines are fundamental in the pathogenesis of progressive CKD, they were examined by real-time RT-PCR. The gene expression levels of IL-6, IFN-γ, IL-1β, TNF-α, and monocyte chemotactic protein-1 (MCP-1), which are known as macrophage-associated proinflammatory cytokines, were much higher in 5/6-Nx-vehicle than in Sham rats. Consistent with the reduction of macrophage infiltration, nilotinib treatment significantly decreased gene expression levels of IL-6 ($P < 0.01$), IFN-γ ($P < 0.05$), IL-1β ($P < 0.05$), TNF-α ($P < 0.05$), and MCP-1 ($P < 0.05$) in 5/6 Nx rats (Figure 8). The gene expression levels of IL-1β in the 5/6 Nx rats correlated closely with the ED1-positive macrophages score ($r = 0.65$) and the tubulointerstitial damage score ($r = 0.70$). Similarly, the gene expression levels of MCP-1 in the 5/6 Nx rats were significantly correlated with the ED1-positive macrophages score ($r = 0.60$) and the tubulointerstitial damage score ($r = 0.61$) (Figure 9). Double immunostaining of ED1-positive macrophages with IL-1β and MCP-1 was performed to confirm that a significant reduction of IL-1β and MCP-1 in the kidneys is associated with inhibition of macrophage accumulation by nilotinib treatment. Many ED1-positive macrophages showed double staining for IL-1β and MCP-1 in 5/6-Nx-vehicle rats (Figure 10). In 5/6-Nx-nilotinib rats, there was a significant reduction of macrophage accumulation in the tubulointerstitium, along with inhibition of IL-1β and MCP-1 protein expressions that was co-localized with ED1-positive macrophages (Figure 10).

**Effects of Nilotinib on the Survival Rate in 5/6 Nx Rats**

The survival rate was analyzed at 18 weeks after the start of nilotinib treatment. Kaplan–Meier survival analysis showed that nilotinib treatment significantly prolonged the survival time of 5/6 Nx rats ($P < 0.05$) (Figure 11). Eight of 13 5/6-Nx-vehicle rats and 3 of 13 5/6-Nx-nilotinib rats died during 18 weeks of treatment, mainly in the fourth month of follow-up. Mortality was associated with lower BW, high BP, and marked renal insufficiency including increased proteinuria, high serum Cr levels, and histologic injury. Thus, nilotinib treatment attenuated renal insufficiency, and that resulted in improving survival rate.

**Effects of Nilotinib on PDGFRβ Phosphorylation Induced by Angiotensin II or PDGF-BB in Cultured Mesangial Cells and Renal Fibroblasts**

Whether PDGFRβ phosphorylation is increased in mesangial cells and renal fi-
broblasts stimulated with angiotensin II (AT-II) or PDGF-BB was investigated. Stimulation with PDGF-BB for 5 minutes increased PDGFRα/H9252 phosphorylation, which was significantly inhibited by pretreatment with nilotinib in cultured mesangial cells and renal fibroblasts. On the other hand, the transactivation of PDGFRα with AT-II stimulation was faint on Western blot analysis (Figure 12).

**DISCUSSION**

This study demonstrated that nilotinib, a clinically available, second-generation, selective tyrosine kinase inhibitor, attenuated renal disease progression and prolonged survival in rats with remnant kidney through its effects against fibrosis and inflammation.

The major finding of the study presented here is that nilotinib treatment had a significant protective effect against CKD progression, with reductions in proteinuria and renal dysfunction and amelioration of histologic changes in remnant kidney. These effects occurred independently of any change in BP, which was consistent with previous reports in which imatinib was administered in hypertensive rats.21,22 There was also no toxicity in this model, and there was no difference in BW or behavior between the nilotinib- and vehicle-treated rats. The renal pathology of remnant kidney is characterized by progressive glomerulosclerosis and interstitial fibrosis, which were significantly ameliorated by nilotinib treatment in this study. Furthermore, nilotinib reduced the upregulation of fibrosis-related genes (e.g., collagen type I, fibronectin, PAI-1, TGF-β, and PDGF-B) in remnant kidneys. This was reflected in the protein levels of total collagen in urine and TGF-β in kidney homogenate. Several lines of evidence show that imatinib has therapeutic benefits in animal models of renal disease via in-

![Cytokines/GAPDH mRNA ratios](image)

**Figure 8.** Nilotinib decreases proinflammatory gene expression levels in 5/6-nephrectomized rats. Real-time RT-PCR for IL-6, IFN-γ, IL-1β, and TNF-α in the study groups. Data are expressed as means ± SEM. The values were normalized to the GADPH values and then expressed as relative quantification. *P < 0.05, **P < 0.01: 5/6-Nx-vehicle versus 5/6-Nx-nilotinib.

![Proinflammatory genes correlated with tubulointerstitial macrophage infiltration and damage](image)

**Figure 9.** Proinflammatory genes correlated with tubulointerstitial macrophage infiltration and damage. The gene expression levels of IL-1β in the 5/6 Nx rats are closely correlated with (a) the ED1 score and (b) the tubulointerstitial damage score. Similarly, the gene expression levels of MCP-1 in the 5/6 Nx rats are significantly correlated with (c) the ED1 score and (d) the tubulointerstitial damage score.
The activity of nilotinib against the PDGFRs is similar to that of imatinib. Indeed, we verified that nilotinib suppressed PDGFR phosphorylation in PDGF-BB-stimulated glomerular mesangial cells and renal fibroblasts in vitro. The involvement of PDGF-B/PDGFR signaling in the development of glomerulosclerosis and tubulointerstitial fibrosis in 5/6 Nx rats has been reported. Floege et al. reported that the proliferation of renal intrinsic glomerular cells precedes glomerulosclerosis and that it may be sustained by PDGF released from intrinsic glomerular cells. Kliem et al. reported that tubular and interstitial PDGF-B/PDGFR signaling might play a role in mediating fibroblast migration and/or proliferation in tubulointerstitial injury. Thus, the inhibitory effect of nilotinib against PDGFR in glomerular mesangial cells and renal fibroblasts may potentially contribute to the observed attenuation of renal injury.

On the other hand, AT-II is known to play a crucial role in the pathogenesis of this experimental model of progressive CKD. AT-II transactivates receptor tyrosine kinases, including PDGFRβ and EGF receptor. PDGFRβ transactivation by AT-II has been reported in cardiac fibroblasts and vascular smooth muscle cells, and it was inhibited by imatinib treatment. However, the AT-II-PDGFR pathway in renal intrinsic cells has rarely been reported. In the study presented here, significant AT-II-mediated PDGFRβ transactivation could not be detected in mesangial cells or renal fibroblasts in vitro, which indicates that the renoprotective effect of nilotinib in remnant kidney was independent of the interruption of the AT-II-PDGFR pathway. Similarly, Escano et al. showed the differences in AT-II-mediated signaling between thoracic aorta smooth muscle cells and renal microvascular smooth muscle cells: AT-II activates extracellular signal-regulated kinase in thoracic aorta smooth muscle cells, but not renal microvascular smooth muscle cells, through transactivation of EGF and PDGFRs.

It has been reported that one of the possible mechanisms by which imatinib attenuates renal disease is related to its effects on inflammatory cells. We recently reported that renal and systemic injuries in thymic stromal lymphopoietin transgenic mice, a model of cryoglobulinemia and cryoglobulin-associated membranoproliferative glomerulonephritis, are dramatically attenuated by imatinib treatment. This protective effect seems to be largely due to the effects of imatinib on B cell development, which, in turn, diminishes cryoglobulin production. We also reported that imatinib treatment had renal preventive and therapeutic effects in rats with nephrotoxic nephritis, with a dramatic reduction of glomerular macrophage infiltration, possibly by M-CSF/c-fms signaling inactivation. Reductions of serum IgG levels, anti-dsDNA antibody, lymph node swelling, and immune complex deposition in lupus model mice suggest that imatinib has inhibitory effects on T cells and/or B cells. Indeed, several in vitro studies have reported that imatinib suppresses various kinds of inflammatory cells, including T cells, B cells, macrophages, dendritic cells, and mast cells. These lines of evidence suggest that nilotinib would have some effects on inflammatory cells in rats with remnant kidney in which monocytic/macrophage infiltration is involved in disease progression. In this study, nilotinib attenuated the tubulointerstitial infiltration of macrophages in remnant kidney. This was accompanied by reduction of proinflammatory cytokine gene expression in the renal cortex, including IL-6, IFN-γ, IL-1β, TNF-α, and MCP-1, in addition to protein levels of IL-1β and MCP-1, which were co-localized with ED1-positive macrophages, as indicated by double immunostaining. IL-1β and MCP-1 are two of the most important cytokines for renal fibrosis and are expressed in the remnant kidney model.

**Figure 10.** Many ED1-positive macrophages in the tubulointerstitium are double stained by IL-1β and MCP-1. Double immunostaining for ED1 with IL-1β and MCP-1 in 5/6 Nx rats. Kidney sections were stained using two-color immunohistochemistry with ED1 stained red and (a and b) the IL-1β and (c and d) MCP-1 stained brown in (a and c) a 5/6-Nx-vehicle rat and (b and d) a 5/6-Nx-nilotinib rat. Original magnifications, ×200.

**Figure 11.** Nilotinib treatment significantly prolongs survival compared with vehicle treatment in rats with renal ablation (P < 0.05).
TGF-β than imatinib. c-Abl is known as a noncanonical (non-Smad) affinity to the Abl family, including the abelson nonreceptor study. In addition to Bcr-Abl, nilotinib has stronger binding nilotinib in progressive renal failure was not verified in this tubulointerstitial damage score.

Significantly correlated with the ED1-positive macrophage score and the hypothesis, gene expression levels of MCP-1 and IL-1 proinflammatory cytokines in remnant kidney. Consistent with this macrophage recruitment and concomitant downregulation of these interstitial fibrosis by nilotinib may be in part due to the suppression of AT-II (1 mol/L), PDGF-BB (20 ng/ml), or medium alone for 5 minutes. Cell lysates were separated by gel electrophoresis and blotted with antibodies against phosphorylated PDGFRβ and PDGFRβ. The results presented are representative of four independent experiments.

Dissociation of IL-1β and MCP-1 has also been found to ameliorate progressive renal fibrosis. Therefore, the attenuation of tubulointerstitial fibrosis by nilotinib may be in part due to the suppression of macrophage recruitment and concomitant downregulation of these proinflammatory cytokines in remnant kidney. Consistent with this hypothesis, gene expression levels of MCP-1 and IL-1β were significantly correlated with the ED1-positive macrophage score and the tubulointerstitial damage score.

A difference in the therapeutic effects between imatinib and nilotinib in progressive renal failure was not verified in this study. In addition to Bcr-Abl, nilotinib has stronger binding affinity to the Abl family, including the abelson nonreceptor tyrosine kinase (c-Abl) and v-Abl (a mutated form of c-Abl), than imatinib. c-Abl is known as a noncanonical (non-Smad) TGF-β signaling pathway in mesenchymal cells, specifically in fibroblasts but less in epithelial cells. Wang et al. reported that imatinib effectively blocks c-Abl in the kidney of obstructive nephropathy and diminishes the number of interstitial fibroblasts and myofibroblasts and the interstitial accumulation of extracellular matrix proteins. Thus, it is likely that potent c-Abl inactivation by nilotinib might have also contributed to attenuating fibrosis in remnant kidney.

In conclusion, nilotinib treatment significantly attenuates renal injury and decreases mortality after subtotal renal ablation in rats. The effects may be mediated by inactivation of PDGFRs and inhibition of macrophage accumulation and subsequent cytokine production, resulting in less vigorous fibrotic and inflammatory responses. The results presented here suggest that nilotinib may prove useful in limiting the progression of CKD to end-stage renal failure.

**CONCISE METHODS**

**Experimental Protocol**

The experimental protocol for this study was reviewed and approved by the Animal Care Committee of Showa University in Tokyo. Ten-week-old male Sprague-Dawley rats weighing 270 to 320 g were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) and used in all of the experiments. The animals were housed in the animal care facility of Showa University (25°C, 50% humidity, 12-hour dark/light cycle) with free access to food and water. Fifty-six rats were subjected to 5/6 Nx (right Nx with surgical resection of the lower and upper thirds of the left kidney). Sham-operated rats (Sham Corporation, n = 9) underwent the same procedure without surgical reduction of the kidney. In experiment 1 (therapeutic study) (Figure 1), 30 rats with 5/6 Nx were then administered either nilotinib (45 mg/kg, Novartis Pharmaceuticals) (5/6-Nx-nilotinib, n = 15) or vehicle (5/6-Nx-vehicle, n = 15) via daily oral gavage from 2 weeks after surgery and for a period of 8 weeks. Vehicle-treated groups received an equal volume of sterile water. At the end of the study, the rats were anesthetized, their blood was collected by cardiac puncture, and their remnant kidneys were collected. Renal tissue was divided; some portions were snap-frozen in liquid nitrogen and some portions were fixed in 2% paraformaldehyde/PBS for later use. In experiment 2 (survival study) (Figure 1), 26 rats with 5/6 Nx were then administered either nilotinib (45 mg/kg, Novartis Pharmaceuticals) (5/6-Nx-nilotinib, n = 13; 5/6-Nx-vehicle, n = 13) were used. Animals were carefully monitored, and deaths were recorded every day. Survival rates were compared between the groups at 18 weeks after the start of drug treatment.

**Proteinuria and Cr Determination**

For the analysis of proteinuria, rats were housed individually in metabolic cages for 24-hour urine collection. Urine samples were collected on the day before sacrifice. Urinary protein was determined using the Biuret method. Serum and urinary Cr levels were measured using an automated analyzer (Hitachi Corporation, Tokyo, Japan) according to the manufacturer’s instructions.

**Measurement of Urinary Total Collagen Levels**

Urinary total collagen levels were determined by analysis of urinary hydroxyproline content as described by Kivirikko et al. Hydroxyproline values were converted to collagen content by multiplying by a factor of 6.94 (because hydroxyproline represents approximately 14.4% of the amino acid composition of collagen) and expressed further as a proportion of the urinary Cr levels (µmol of collagen levels/mg of urinary Cr levels).

**Light Microscopic Study**

Tissues fixed in 2% paraformaldehyde/PBS were embedded in paraffin using routine protocols. Paraffin-embedded materials were sec-
tioned at 4 μm for routine staining with PAS and Masson trichrome. Sections (2 μm thick) were used for periodic acid-methenamine silver stains (silver). The glomerular tuft area was quantified in 50 full-sized glomeruli (PAS stain) using WinROOF image processing software (Mitani Corporation, Tokyo, Japan). Glomerulosclerosis was assessed in 50 glomeruli on silver-stained sections under ×400 magnification using a semiquantitative score from 0 to 4 (0, no sclerosis; 1, sclerosis up to 25% of glomeruli; 2, sclerosis from 25% to 50% of glomeruli; 3, sclerosis from 50% to 75% of glomeruli; 4, sclerosis >75% of glomeruli), and the results were averaged. For evaluating tubulointerstitial damage, 15 fields for each section (Mason trichrome stain) were evaluated at ×200 magnification using WinROOF image processing software (Mitani Corporation). The extent of tubulointerstitial damage was evaluated by counting the percentage of areas with tubular dilation, interstitial infiltration, and fibrosis per field of cortex. Scores from 0 to 5 were used (0, normal interstitium; 1, <10% of areas injured; 2, 11% to 25% of areas injured; 3, 26% to 50% of areas injured; 4, 51% to 75% of areas injured; 5, >75% of areas injured), and the results were averaged. All histologic analyses were performed by two investigators without knowledge of the origin of the slides, and the mean values were calculated.

Immunohistochemistry
The mouse anti-rat ED1 monoclonal antibody, a macrophage marker, was purchased from BMA (Augst, Switzerland). Biotinylated rabbit anti-mouse IgG and peroxidase-conjugated streptavidin (LSAB 2 kit/horseradish peroxidase [HRP]) were purchased from Dako (Glostrup, Denmark). Immunohistochemical staining for ED1 was performed as follows. The paraffin sections of renal tissues were dewaxed, washed in PBS, drained, and incubated with the anti-ED1 antibody as the primary antibodies overnight at 4°C. For antigen retrieval, sections were treated twice for 5 minutes in a conventional household microwave (500 W; Sharp, Osaka, Japan) using citrate buffer. The sections were incubated with LSAB 2 kit/HRP and developed using diaminobenzidine (DAKO) as the substrate to produce a brown stain. The quantification of ED1-positive cells in the tubulointerstitium was performed by grading on a 5-point scale (0, absent; 1, weak; 2, moderate; 3, severe; 4, very severe) in 15 consecutive renal cortical fields at ×200 magnification. The mean score was then calculated as the ED1 staining score. Two-color immunostaining for IL-1β/ED1 and MCP-1/ED1 was performed as described previously.9

Cell Culture Studies
Normal human mesangial cells in primary culture were purchased from Lonza (Basel, Switzerland). Normal rat kidney fibroblast cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM containing 5% FBS, 1% streptomycin-penicillin mixture, 44 mM sodium bicarbonate, and 14 mM HEPES in an atmosphere of 5% carbon dioxide and 95% air at 37°C in a humidified incubator.

Western Blot Analysis
Cells were preincubated with nilotinib or medium for 30 minutes, followed by stimulation with PDGF-BB, AT-II, or medium for 5 minutes. Cells were then washed with PBS, and total protein was extracted in M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) containing 1% (vol/vol) protease inhibitor cocktail (Sigma) and 1% (vol/vol) phosphatase inhibitor cocktail (Sigma). Harvested lysates were then centrifuged for 10 minutes at 4°C to remove cellular debris. The supernatants were collected and stored at −80°C. Protein concentration was measured using the BCA protein assay reagent kit (Pierce Biotechnology). For Western blotting, 10 μg of protein from each sample were then separated on a 4% to 20% gradient gel (Invitrogen) using SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The blots were blocked with TBST buffer (20 mM Tris-HCl [pH 7.4], 140 mM sodium chloride, and 0.05% Tween 20) containing 5% skim milk at room temperature for 1 hour, washed 3 times in TBST buffer, and incubated with primary antibody (phosphorylated-PDGFRβ and total-PDGFRβ; 1:1000 [Cell Signaling Technology, Danvers, MA]) overnight at 4°C. The membranes were then incubated with secondary antibody (HRP-conjugated anti-rabbit IgG antibody, 1:3000; Cell Signaling Technology) at room temperature for 1 hour. The reaction products were detected using the enhanced chemiluminescence detection system.

Real-Time RT-PCR
Gene expressions of rat collagen type I, fibronectin, PAI-1, PDGF-B, TGF-β, IL-6, IFN-γ, IL-1β, TNF-α, MCP-1, and glyceraldehyde-3-phosphate dehydrogenase were analyzed using real-time RT-PCR. Briefly, kidney tissues (cortex) were homogenized using a TissueLyser (Qiagen, Hilden, Germany), and total RNA was isolated using an RNeasy fibrous tissue mini kit (Qiagen) in accordance with the manufacturer’s instructions. cDNA synthesis was carried out using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Predesigned TaqMan probe sets for the targets indicated above were purchased from Applied Biosystems (Foster City, CA). Each probe has a fluorescence reporter dye (FAM) linked to its 5’ end and a downstream quencher dye (TAMRA) linked to its 3’ end. Each reaction consisted of 25 μl containing 2× Universal Master Mix (Applied Biosystems), primers, labeled probes, and cDNA. The amplification conditions consisted of 40 cycles of 95°C for 15 seconds and 60°C for 1 minute after incubation at 95°C for 10 minutes. Amplification and fluorescence measurements were performed using the MicroAmp optical 96-well reaction plate on the ABI PRISM 7700 sequence detection system (Applied Biosystems). mRNA expressions were normalized using glyceraldehyde-3-phosphate dehydrogenase as an endogenous control to correct for the differences in the amount of total RNA added to each reaction.

Homogenization of Kidney Tissues
Kidney tissues (cortex) were homogenized with T-PER mammalian protein extraction reagent (20 ml/g renal tissues; Pierce Biotechnology) containing 1% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich) using a TissueLyser (Qiagen). Harvested lysates were then centrifuged for 10 minutes at 4°C to remove the cellular debris. The supernatants were collected and stored at −80°C. Pro-
tein concentration was measured using the BCA protein assay reagent kit (Pierce Biotechnology).

Measurement of TGF-β1 Protein Levels in Kidney Tissue Homogenate

Total TGF-β1 protein levels were measured in kidney tissue homogenates from each sample using the TGF-β1 ELISA kit (R&D Systems, Abingdon, Oxfordshire, United Kingdom) following the manufacturer’s instructions. To control for the difference between samples, the concentration was corrected based on the amount of total tissue protein.

Statistical Analysis

Data were recorded as means ± SEM. The Mann–Whitney test was performed, and values of $P < 0.05$ were considered significant. Data were analyzed using the Kaplan–Meier life table method for survival curves.

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DISCLOSURES

None.

REFERENCES


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