Treatment of Hypertension and Renal Injury Induced by the Angiogenesis Inhibitor Sunitinib
Preclinical Study


Abstract—Common adverse effects of angiogenesis inhibition are hypertension and renal injury. To determine the most optimal way to prevent these adverse effects and to explore their interdependency, the following drugs were investigated in unrestrained Wistar Kyoto rats exposed to the angiogenesis inhibitor sunitinib: the dual endothelin receptor antagonist macitentan; the calcium channel blocker amlodipine; the angiotensin-converting enzyme inhibitor captopril; and the phosphodiesterase type 5 inhibitor sildenafil. Mean arterial pressure was monitored telemetrically. After 8 days, rats were euthanized and blood samples and kidneys were collected. In addition, 24-hour urine samples were collected. After sunitinib start, mean arterial pressure increased rapidly by ≈30 mm Hg. Coadministration of macitentan or amlodipine largely prevented this rise, whereas captopril or sildenafil did not. Macitentan, captopril, and sildenafil diminished the sunitinib-induced proteinuria and endothelinuria and glomerular intraepithelial protein deposition, whereas amlodipine did not. Changes in proteinuria and endothelinuria were unrelated. We conclude that in our experimental model, dual endothelin receptor antagonism and calcium channel blockade are suitable to prevent angiogenesis inhibition–induced hypertension, whereas dual endothelin receptor antagonism, angiotensin-converting enzyme inhibitor, and phosphodiesterase type 5 inhibition can prevent angiogenesis inhibition–induced proteinuria. Moreover, the variable response of hypertension and renal injury to different antihypertensive agents suggests that these side effects are, at least in part, unrelated.

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Key Words: acute renal injury ■ endothelin-1 ■ hypertension ■ sunitinib ■ therapeutics ■ vascular endothelial growth factor A

Angiogenesis, the formation of new vessels from pre-existing vasculature, is critical to solid tumor growth as well as to the development of metastasis. This process is regulated by numerous factors among which vascular endothelial growth factor (VEGF) plays a dominant role. Angiogenesis inhibition, by targeting VEGF or its receptors, has become an established treatment for various forms of cancer but is featured by adverse effects including hypertension and renal injury. Hypertension has been reported in ≤36% of patients treated with bevacizumab, a monoclonal antibody against VEGF, and in ≤60% of patients treated with sunitinib, an orally active multitarget VEGF receptor tyrosine kinase inhibitor (RTKI). Renal toxicity, mainly proteinuria, has been reported in 41% to 63% of patients treated with bevacizumab. In a recent phase 3 randomized trial performed in patients with metastatic clear cell renal cell carcinoma, proteinuria occurred in 18% for patients randomized for pazopanib and in 14% for patients randomized for sunitinib.

The development of hypertension and renal injury may compromise the use of VEGF inhibition in patients with cancer who develop these side effects. Hence, exploration of therapeutic approaches to counteract these side effects is important, but clinical studies comparing different agents to manage these particular side effects are lacking and unlikely will be performed in this category of patients. In previous studies we have demonstrated that activation of the endothelin-axis is involved in sunitinib-induced hypertension and renal injury. Moreover, we observed that concurrent administration of the dual endothelin (ET)\_A/ET\_B receptor blocker macitentan in a sunitinib hypertensive rat model could to a large extent prevent the sunitinib-induced rise of blood pressure (BP) and proteinuria. Because endothelin receptor...
blocks are not approved for the treatment of systemic hypertension and renal injury, we explored here to what extent angiotensin-converting enzyme (ACE) inhibition, calcium channel blockade (CCB), and phosphodiesterase 5 inhibition were able to prevent hypertension and renal injury in our animal model.

**Materials and Methods**

**In Vivo Study**

Male Wistar Kyoto rats (280–300 g), obtained from Charles River, were housed in individual cages and maintained on a 12-hour light/dark cycle, having access to standard laboratory rat chow and water ad libitum. Intra-aortic BP recordings were performed by radiotelemetry (Data Sciences International) and the sunitinib and vehicle solution were prepared and administered by oral gavage as described previously. Before and after implantation of the telemetry transmitters (Data Sciences International TA11PA-C40) using 2% isoflurane anesthesia, rats received analgesic treatment using Temgesic subcutaneously (0.05 mg/kg; RB Pharmaceuticals Limited) for 2 days. At the end of each experiment, rats were euthanized with 60 mg/kg pentobarbital IP and blood was sampled for measurement of serum ET-1, serum creatinine, VEGF, and sunitib levels, and kidneys were rapidly excised. Five experiments were performed. In the first experiment, rats were randomly administered sunitinib (26.7 mg/kg per day of sunitinib-l-malate; Sutent, Pfizer; n=10) or vehicle (n=10) by oral gavage (0.5 mL) for 8 days. The dose of sunitinib was based on initial experimental studies, investigating its effectiveness in a rat breast cancer model. In the second experiment, rats (n=8) were orally administered the combination of sunitinib and macitentan (ACT-064992, kindly provided by Actelion) 30 mg/kg per day for 8 days. In the third experiment, rats were administrated the combination of sunitinib and amlodipine 3 mg/kg per day by oral gavage for 8 days. In the fourth experiment, rats (n=9) were administrated the combination of sunitinib by oral gavage and captopril at 3 or 12 mg/kg per day (C0402, Sigma-Aldrich) using osmotic minipumps (Alzet 2ml2) for 8 days. In the final experiment, rats (n=6) were administrated the combination of sunitinib and sildenafil 1.5 mg/kg per day (Revatio; Pfizer). In all experiments, 6 days before (baseline) and 6 days after administration of the various agents, rats were housed in metabolic cages for 48 hours with free access to food and water; the first day to acclimatize and the second day to collect 24-hour urine samples for the determination of protein, ET-1, NO metabolites (NO2+NO3 [NOx]), and cGMP. BP was not monitored when rats were housed in metabolic cages because of the absence of telemetry receivers. Urine was collected on antibiotics (Antibiotic Antimycotic Solution, A5955, Sigma-Aldrich) to prevent formation of NO metabolites. Macitentan was dissolved in vehicle containing 0.5% methylcellulose aqueous solution and 0.05% Tween 80. Amlodipine besylate (Bioconnect, Huissen, The Netherlands) was suspended in 1% tragacanth gum solution. Sildenafil was suspended in 0.5% carboxymethylcellulose. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

**Renal Histology**

Details of the light and electron microscopy in this study are available in the online-only Data Supplement. Briefly, transversely sliced kidney sections were stained for hematoxylin–eosin and periodic acid Schiff. Periodic acid Schiff–stained sections were blindly evaluated by a pathologist (F.M.M.S.) for the presence (1) or absence (0) of endothelial cell and epithelial cell swelling in 50 glomeruli, as well as scored for the presence of ischemia and intraepithelial protein. For electron microscopy, 2 glomeruli in each biopsy section were examined. The presence of glomerular endotheliosis and podocyte morphology were registered. Both reflection contrast and electron micrographs were obtained from reproccessed paraffin-embedded tissue.

**Biochemical Measurements**

ET-1 and VEGF were assessed using a chemiluminescent ELISA QuantiGlo, R&D Systems) and Quantikine Immunoassay (R&D Systems), respectively. Urine NO2 concentration was determined by fluorimetric quantification of nitrite content (Cayman Chemicals, Ann Arbor, MI). To investigate the systemic and local effects of sildenafil treatment, cGMP levels were determined in serum and urine, respectively, using an ELISA kit (Enzo Life Sciences, Farmingdale, NY). Serum creatinine and urinary protein concentrations (Cobas c502 and c702, CREP2 and TP2/IPUC3, Roche Diagnostics) were measured at the clinical chemical laboratory of the Erasmus MC. Sunitinib levels were measured by a validated ultrasensitiveie liquid chromatography/tandem mass spectrometry system.

**Statistical Analysis**

Data are presented as mean±SEM. Statistical analysis between groups was performed by unpaired t testing or by repeated measures ANOVA followed by Newman–Keuls or Dunnett multiple comparison testing. For correlation analysis the Pearson r correlation coefficient was used. GraphPad Prism version 5.0 was used for all statistical analysis.

**Results**

**In Vivo Study**

Baseline mean arterial pressure (MAP) and heart rate were 99.1±4.1 mm Hg and 357.9±5.8 bpm. Administration of sunitinib by oral gavage was associated with a rapid rise of intraarterial BP (ΔMAP, 31.6±0.9 mm Hg), whereas BP remained unchanged during administration of vehicle (Figure 1). The sunitinib-induced rise in BP was associated with a decrease in heart rate. Coadministration of macitentan (ΔMAP, 12.3±1.5 mm Hg) or amlodipine (ΔMAP, 11.4±1.7 mm Hg) attenuated the sunitinib-induced rise in BP by 73% (P<0.001) and 63% (P<0.001), respectively, whereas coadministration of both dosages of captopril and sildenafil had no BP-lowering effect. The sunitinib-induced decrease in heart rate was not prevented by each of the 4 compounds and even aggravated by the low dose of captopril (Figure 1).

Sunitinib administration was associated with a 3-fold rise in serum creatinine concentration when compared with vehicle. This rise was not prevented by each of the 4 compounds (Figure 2A). Proteinuria was below the limit of detection during vehicle administration and increased to ≈30 mg per day during sunitinib administration. The rise in proteinuria was attenuated by macitentan (P<0.01), both dosages of captopril (P<0.001) and sildenafil (P<0.01). Conversely, proteinuria tended to increase further with amlodipine (Figure 2B).

Circulating ET-1 concentration was 0.61±0.08 pg/mL during vehicle administration and increased during sunitinib administration (P<0.01). Amlodipine, captopril, and sildenafil did not influence this rise. Because of a decrease in clearance caused by blockade of the ETB receptor, ET-1 rose further during coadministration of macitentan (Figure 2C). Sunitinib administration was also associated with a rise in 24-hour urinary ET-1 excretion (Figure 2D). This rise was prevented by macitentan, captopril, and sildenafil but not by amlodipine (Figure 2D). Proteinuria and endothelinuria did not correlate (r=0.17; P>0.05).

Urinary excretion of NOx per 100 g body weight was 2.2±0.2 μmol per day during vehicle administration.
Sunitinib administration was associated with a decrease in urinary NOx excretion ($P<0.01$). This decrease was not affected by macitentan or amlodipine and weakly attenuated by captopril (Figure 3). Changes in NOx excretion and endothelinuria were not correlated ($r=0.14; P>0.05$). Urinary cGMP decreased during sunitinib administration from 4563±190 pmol/mL per day ($P<0.05$). This decrease was prevented by concurrent administration of sildenafil. Circulating cGMP concentration was not affected either by sunitinib administration or by the combination of sunitinib and sildenafil (data not shown).

Mean serum concentration of sunitinib was 376±87 ng/mL. This concentration increased during treatment with sildenafil and by both dosages of captopril, to 800±151, 679±17, and 1082±238 ng/mL, respectively ($P<0.05$), confirming that their renoprotective effects were not because of a suppression of sunitinib bioavailability. Circulating VEGF increased from 4.9±2.3 pg/mL during vehicle to 947.8±45.1 pg/mL during sunitinib administration. This rise was unaffected by sildenafil and the low dose of captopril (959±132 and 905±141 pg/mL) and attenuated by the high dose of captopril (689±67 pg/mL; $P<0.05$) versus sunitinib alone. Unfortunately, plasma to measure sunitinib and VEGF during treatment with amlodipine or macitentan was no longer available.

**Renal Histology**

Sunitinib administration was associated with a rise in kidney weight/body weight ratio from 3.0±0.04 to 3.5±0.04 g/kg ($P<0.001$). This rise was not changed by any of the 4 agents.

Periodic acid Schiff–stained kidney sections showed marked glomerular changes, including intraepithelial droplets and epithelial and endothelial cell swelling with narrowing of the capillary lumina after administration of sunitinib (Table). Neither macitentan nor the low dose of captopril reversed the sunitinib-induced glomerular ischemia or endothelial and epithelial cell swelling. Only a high dose of captopril and sildenafil was more renoprotective. However, consistent with the decrease in proteinuria, glomerular...
intraepithelial protein deposition diminished during coadministration of macitentan and captopril (Figure S1 in the online-only Data Supplement; Table). In rats coadministered sunitinib and amlodipine, glomerular ischemia and endothelial and epithelial cell swelling were more prominent compared with rats exposed to sunitinib alone, as was the glomerular intraepithelial protein deposition (Table). Reflection contrast and electron microscopy showed intraepithelial resorption droplets and severe glomerular endotheliosis during administration of sunitinib. This was partly prevented by macitentan and reversed with a high dose of captopril and sildenafil (Figure S2).

Discussion

Administration of the multitarget VEGF receptor blocker sunitinib is accompanied by a rise in BP, renal injury and proteinuria, activation of the ET-1 axis, and renin suppression.\(^{6,7,17}\) Previously, we found that the rise in BP and proteinuria could largely be prevented by the dual ETA/B receptor antagonist macitentan, indicating that activation of the ET-1 axis is critical for the development of these side effects. Here, we explored whether alternative antihypertensive agents attenuate the occurrence of these side effects and to what extent they are interrelated.

Coadministration of amlodipine with sunitinib was associated with a marked attenuation of the rise in BP, comparable with the degree observed with macitentan. However, renal injury could not be prevented, while proteinuria even
The beneficial effects of sildenafil and high dose of captopril on proteinuria occurred independently of a BP-lowering effect. A BP-independent beneficial effect of sildenafil on renal function has also been reported in deoxycorticosterone acetate-salt hypertensive rats. This nephroprotective effect of sildenafil seems to be associated with anti-inflammatory, antifibrotic, and antiapoptotic effects with downregulation of sildenafil. Antifibrotic, and antiapoptotic effects with downregulation of sildenafil seems to be associated with anti-inflammatory,:

**Table.** Light Microscopic Evaluation of Kidney Sections Obtained From Rats Exposed to Sunitinib and Coadministration of Macitentan, Amlodipine, Captopril, and Sildenafil for 8 days Compared With Controls

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Glomerular Ischemia (Percentage of Glomeruli)</th>
<th>Endothelial Cell Swelling (Percentage of Glomeruli)</th>
<th>Epithelial Cell Swelling (Percentage of Glomeruli)</th>
<th>Intraepithelial Protein (Percentage of Glomeruli)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>17±2*</td>
<td>78±3*</td>
<td>63±5*</td>
<td>99±1*</td>
</tr>
<tr>
<td>Sunitinib+macitentan</td>
<td>15±8*</td>
<td>85±3*</td>
<td>77±6*</td>
<td>1±1</td>
</tr>
<tr>
<td>Sunitinib+amlodipine</td>
<td>12±3*</td>
<td>53±10*†</td>
<td>16±4*†</td>
<td>29±4*</td>
</tr>
<tr>
<td>Sunitinib+captopril 3 mg</td>
<td>4±2*</td>
<td>54±4*†</td>
<td>23±6*</td>
<td>48±2*</td>
</tr>
<tr>
<td>Sunitinib+captopril 12 mg</td>
<td>73±3†</td>
<td>73±7†</td>
<td>46±6*†</td>
<td>23±4*†</td>
</tr>
<tr>
<td>Sunitinib+sildenafil</td>
<td>4±2*</td>
<td>4±2*†</td>
<td>77±5*†</td>
<td>7±2†</td>
</tr>
</tbody>
</table>

Data of macitentan from Kappers et al. n.a. indicates not applicable. P<0.05 vs control; †P<0.05 vs sunitinib.

In rats exposed to the RTKI sorafenib (20 mg/kg per day) for 4 weeks, a rise in systolic BP of ≈60 mm Hg and marked albuminuria has been reported. In this study, concurrent administration of captopril was associated with a marked reduction in both albuminuria and renal histological abnormalities and also with a 50% reduction of the rise in BP. Captopril in that study was given orally in a daily oral dose of 40 mg. Captopril in our study (3 and 12 mg/kg per day) was given subcutaneously by means of osmotic minipumps. The doses selected were based on previous studies performed in our laboratory in spontaneously hypertensive rats. These studies showed that subcutaneously administered captopril at doses of 3 and 6 mg/kg per day for 1 week lowered MAP by 14 and 28 mm Hg, respectively. In addition, this BP reduction was associated with an ≈10-fold rise in renin. This rise is of comparable magnitude as that observed in young hypertensive patients exposed to ACE inhibition or a change from a high to a low salt intake. Based on these findings, we are confident that the maximal dose of captopril of 12 mg/kg per day was sufficient to induce pronounced blockade of the renin angiotensin system (RAS) with beneficial renal, but not with BP-lowering effects. Interestingly, Curwen et al observed in rats exposed to a relatively low dose of the RTKI cediranib, resulting in a BP rise of ≈10 mm Hg, that captopril (30 mg/kg orally) could completely reverse this rise in BP. In contrast, the same dose of captopril was without any effect in rats exposed to a relatively high dose of cediranib, resulting in a 40 mm Hg BP rise. These findings indicate that other factors than RAS activation are instrumental for the development of hypertension when higher doses of a RTKI are administered and that the RAS is likely downregulated in an attempt to attenuate the development of severe hypertension. This probably also was the case in our rat model, where...
sunitinib administration caused a rise in MAP of 30 mmHg. This rise in BP related to the relatively high dose of sunitinib (resulting in plasma levels that were 5–10× higher than those in humans) was considerably larger than observed in our clinical study. In that study, MAP rose by 12 mmHg, but interestingly, this moderate rise in BP was also already associated with >60% renin suppression, indeed suggesting that the BP elevation in response to RTKI treatment in humans is not RAS dependent and, consequently, less responsive to anti-RAS agents. Renin suppression during angiogenic treatment might also be caused by an increase in aldosterone production. Only a limited number of studies has looked at the effect of RTKIs on aldosterone. In these studies no increase in circulating aldosterone levels or urinary aldosterone excretion has been observed. Recently, VEGF-stimulated aldosterone release has been reported. Therefore, reduced rather than increased aldosterone production during antiangiogenic treatment is to be expected.

As reflected by the decrease in urinary NO metabolites and cGMP, antiangiogenic treatment is associated with a decrease in the activity of the NO system. Using the human forearm model it has been reported recently that intra-arterial infusion of bevacizumab inhibits the local vasodilator response to acetylcholine, but not to sodium nitroprusside, implying impairment of endothelium-dependent vasodilation. This decreased activity of the NO system can contribute to the development of hypertension as well as renal injury. There are sporadic reports that an exogenous NO donor can lower BP in patients who develop hypertension during angiogenesis inhibition. In the current study, the phosphodiesterase 5 inhibitor sildenafil was used to increase NO responsiveness. With this agent the sunitinib-induced reduction in urinary cGMP excretion was completely prevented, but this was not associated with any BP-lowering effect. Sildenafil was used in a daily dose of 1.5 mg/kg. This dose is equivalent to a daily dose of 100 mg in patients and has been shown to prevent the rise in systolic BP from 129±8 mm Hg in control rats to 183±6 mm Hg in rats exposed to the NO synthase inhibitor Nω-nitro-L-arginine methyl ester. The rise in BP induced by sunitinib could largely be prevented by the calcium channel blocker amlodipine, which is in line with an experimental study, demonstrating that nifedipine could completely reverse the rise in BP induced by the RTKI cediranib.

As reported previously by our group, angiogenesis inhibition by sunitinib is associated with activation of the endothelin system. During sunitinib administration, both the circulating ET-1 concentration and the 24-hour urinary excretion of ET-1 were increased. ET-1 within the kidney is produced by glomerular endothelial, mesangial cells, as well as renal tubular cells, and urinary ET-1 excretion is considered to reflect the degree of renal ET-1 production. Recently, it has been shown that ET-1 produced by endothelial cells induces nephrin shedding from podocytes, which could be prevented by ETₐ receptor antagonism. Because mutations in the gene encoding for nephrin are associated with severe forms of the nephrotic syndrome, it has been speculated that increased glomerular endothelial cell production of ET-1 is one of the mediators of proteinuria. Based on these data not only loss of the protective effect of VEGF on glomerular endothelial cells but also the activation of the ET-1 system observed during angiogenesis inhibition may contribute to the development of proteinuria. To obtain further insight in this mechanism, we explored whether endothelinuria and proteinuria were correlated. This seemed not to be the case.

Randomized controlled trials concerning the optimal treatment of angiogenesis inhibition–induced hypertension are lacking, therefore no clear recommendation for a particular antihypertensive agent or class of antihypertensive agents can be given. Based on the present observations together with other experimental and clinical studies, showing that the hypertension induced by sunitinib is associated with renin suppression, dihydropyridine calcium channel blockers rather than anti-RAS agents are probably more effective for the treatment of hypertension. Given their beneficial effects on the occurrence of proteinuria, an anti-RAS agent can be combined with a calcium channel blocker in case of the development of renal injury. In addition, it has been shown that an anti-RAS agent can enhance the effect of sunitinib in a murine xenograft tumor model. Although NO donors such as nitrates or phosphodiesterase 5 inhibitors may also be beneficial, it has been suggested that these agents may potentially compromise the antiangiogenic effect and therefore they can best be avoided. The knowledge that activation of the endothelin-axis is involved in the hypertension induced by angiogenesis inhibition may also favor the use of endothelin receptor blockers in angiogenesis inhibition–induced hypertension. ETₐ receptor stimulation has been shown to be mitogenic in cancer cells through activation of the mitogen-activated protein kinase pathway. Thus, besides lowering BP, endothelin receptor antagonism may exert antitumor effects. However, because of adverse effects, single endothelin receptor blockers are currently not being marketed for the treatment of systemic hypertension, but only for pulmonary hypertension. In addition, because macitentan is a strong inducer of the CYP3A4 enzyme, whereas sunitinib is metabolized by this enzyme, this combination should be avoided because of unwanted pharmacokinetic interaction.

**Perspectives**

Antiangiogenic treatment targeting the VEGF–VEGF receptor pathway is complicated by the development of hypertension and renal injury. Occurrence of these particular side effects may compromise anticancer treatment, but the most optimal way to treat these remains to be identified. Given the lack of large clinical studies, this study provides further insight into the mechanisms underlying sunitinib-induced hypertension and proteinuria as well as ways to counteract these adverse events. Translation of these findings to the clinic strongly suggests that dependent on the toxicity encountered, different classes of antihypertensive agents should preferably be used, that is, a calcium channel blocker in the case of hypertension and a RAS blocker in the case of renal injury. Of interest, both captopril and sildenafil increased the steady-state sunitinib concentrations. The underlying pharmacokinetic interaction is currently unknown but warrants further investigation in humans, not
only because it may enhance the anticancer effectiveness of sunitinib but also its side-effect profile.

Disclosures

None.

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**Novelty and Significance**

**What Is New?**
- This is the first study that provides information about treatment of hypertension and renal injury during antiangiogenic treatment by comparing different antihypertensive agents.

**What Is Relevant?**
- Our findings show that the effect of different antihypertensives to counteract hypertension and renal injury is variable, suggesting that different pathogenetic pathways underlie these side effects and that they require a dedicated treatment approach.

**Summary**
In a rat model of sunitinib-induced hypertension and renal injury, we found evidence for beneficial antihypertensive and renoprotective effects with calcium channel blockade and angiotensin-converting enzyme or phosphodiesterase inhibition, respectively, thereby providing a rational basis for optimal treatment of the reno-cardiovascular side effects associated with angiogenesis inhibition.
Treatment of Hypertension and Renal Injury Induced by the Angiogenesis Inhibitor Sunitinib: Preclinical Study


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TREATMENT OF HYPERTENSION AND RENAL INJURY INDUCED BY THE ANGIOGENESIS INHIBITOR SUNITINIB: A PRECLINICAL STUDY

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Short title: Angiogenesis-induced hypertension and proteinuria
EXPANDED METHODS

Light microscopy

The left kidney was rapidly excised from euthanized rats, decapsulated, weighed and sliced transversely into 2-mm thick sections. Slices were fixed in a 3.5-4% formaldehyde solution for light microscopic evaluation. After fixation in the formaldehyde solution, tissue was dehydrated and paraffin-embedded. Deparaffinized 2-µm thick sections were stained for haematoxilin-eosine (HE) and periodic acid Schiff (PAS). PAS-stained sections were blindly evaluated by a pathologist (F.M.M.S) for the presence (score 1) or absence (score 0) of endothelial cell and epithelial cell swelling. Glomerular ischemia was scored semiquantitatively and defined as the degree of open glomerular capillaries, wrinkling of the glomerular basement membrane and filling of Bowmans space. Wide open glomerular capillaries filling Bowman's space entirely corresponded with no ischemia. Partially open glomerular capillaries with mild wrinkling of the glomerular basement membrane and Bowman's glomerular space largely filled was classed as moderate ischemia. Totally collapsed glomeruli and extensive wrinkling of the glomerular basement membrane and only partial filling of Bowman's space corresponded with severe ischemia. Furthermore, the presence of glomerular intra-epithelial protein deposition was evaluated using a semiquantitative scale: 0 (no protein), 1 (protein present in 1-50% of the epithelial cells), 2 (protein present in >50% of the epithelial cells). Fifty glomeruli per kidney section (PAS staining) were evaluated. All images were obtained using a Reichert microscope and Leica DFC420 camera (40x objective) and Leica LAS software.

Electron microscopy

Formalin fixed paraffin embedded renal tissue was reprocessed for reflection contrast microscopy and electron microscopy. In brief, 1mm³ tissue blocks were deparaffinized, rehydrated, and post fixed with 1.5% glutaraldehyde, followed by incubation in 1% osmium tetroxide (OsO₄) in 0.1 M sodium cacodylate for 1 h. After each step of the fixation, the fish were rinsed twice with 0.1 M sodium cacodylate and finally dehydrated in a series of 70%, 80%, 90% and 3× 100% ethanol, prior to immersion in a 1:1 epon:propylene oxide solution for 1 h. The samples were washed afterwards with pure epon, embedded in pure epon LX112 and polymerized at 60 °C for 2 days. Sequential 100 um sections were placed on glass slides for reflection contrast microscopy or on grids for electron microscopy. The preparations were examined under a Leitz Orthoplan microscope (Leitz, Wetzlar, Germany) equipped for epillumination, which was adapted for reflection contrast microscopy as described (1). The slides were examined under a 100× objective lens. A JEOL JEM-1011 electron microscope equipped with a MegaView III digital camera was used for ultra-structural analysis.
FIGURE S1.

Kidney sections from WKY rats administered vehicle (A), sunitinib (B), macitentan (C), amlopidine (D), captopril (E/F) or sildenafil (G), stained with PAS stain (magnification x500). After administration of sunitinib for 8 days marked glomerular changes could be observed, including intra-epithelial droplets (arrowhead). Consistent with the decrease in proteinuria, glomerular intra-epithelial protein deposition diminished during co-administration of macitentan, captopril and sildenafil.
FIGURE S2.

Reflection contrast and transmission electron micrographs of representative kidney sections from rats administered vehicle (A1; magnification x1500, A2; magnification x5000, A3; magnification 12000), sunitinib (B1; magnification x1500, B2; magnification x5000, B3; magnification x12000) or in combination with macitentan (C1; magnification x1500, C2; magnification x5000, C3; magnification x12000), amlodipine (D1; magnification x1500, D2; magnification x5000, D3; magnification x12000), low (E1; magnification x1500, E2; magnification x5000, E3; magnification x12000) and high dosage of captopril (F1; magnification x1500, F2; magnification x5000, F3; magnification x12000), or sildenafil (G1; magnification x1500, G2; magnification x5000, G3; magnification x12000). After sunitinib administration for 8 days intra-epithelial resorption droplets (white arrows) and glomerular endotheliosis (endothelial cell swelling; asterix) were observed. None of the abnormalities above could be observed in control kidney sections (A1-3) and they were partly prevented by macitentan, and reversed with a high dose of captopril and sildenafil.