Greater Sensitivity of Blood Pressure Than Renal Toxicity to Tyrosine Kinase Receptor Inhibition With Sunitinib


Abstract—Hypertension and renal injury are off-target effects of sunitinib, a tyrosine kinase receptor inhibitor used for the treatment of various tumor types. Importantly, these untoward effects are accompanied by activation of the endothelin system. Here, we set up a study to explore the dose dependency of these side effects. Normotensive Wistar Kyoto rats were exposed to 3 different doses of sunitinib or vehicle. After 8 days, rats were euthanized. Telemetrically measured blood pressure rose dose dependently, from 13 to 30 mm Hg. Proteinuria was present at all doses, but a rise in cystatin C occurred only at the intermediate and high doses. Compared with vehicle circulating endothelin-1 increased dose dependently, whereas 24-hour urinary endothelin excretion decreased. Light and electron microscopy revealed glomerular endotheliosis and ischemia with the intermediate and high doses of sunitinib but completely absent histological abnormalities with the low dose. Podocyte number per glomerular circumference did not change. Glomerular nephrin, Nep1, podocin, and endothelin-converting enzyme gene expression were downregulated in a dose-dependent manner. We conclude that the sunitinib-induced rise in blood pressure requires lower doses than its induction of renal function impairment and that functional changes in glomerular filtration barrier contribute to the occurrence of proteinuria, given the lack of histopathologic changes with the low dose of sunitinib. (Hypertension. 2015;66:543-549. DOI: 10.1161/HYPERTENSIONAHA.115.05435.) ● Online Data Supplement

Key Words: endothelin-1 ■ hypertension ■ proteinuria ■ sunitinib ■ vascular endothelial growth factor A

Neoangiogenesis, the formation of vessels from pre-existing vasculature, is critical to solid tumor growth and metastasis. Vascular endothelial growth factor (VEGF) plays a dominant role in this process. Therefore, angiogenesis inhibition, by targeting VEGF or its receptors, has become an established treatment for several tumor types. Sunitinib is an orally active angiogenesis inhibitor that blocks the VEGF receptors (1–3) and other tyrosine kinase receptors, including platelet-derived growth factor and c-Kit receptors. Off-target effects of sunitinib and other antiangiogenic agents, sometimes necessitating discontinuation of treatment, are hypertension, proteinuria, and renal failure.

In the kidney, glomerular endotheliosis and thrombotic microangiopathy are the most frequently observed histological abnormalities seen during treatment with sunitinib. These abnormalities are comparable with those observed in preeclampsia likely due to the fact that both conditions share the same pathogenetic mechanism, that is, disruption of the VEGF signaling pathway, which in preeclampsia is caused by increased placental production of the soluble Flt-1 (sFlt-1). In previous clinical and experimental studies, we observed that activation of the endothelin system is involved in the rise in blood pressure (BP) during sunitinib treatment. Recent studies have shown that endothelin-1 (ET-1) induces podocyte injury mediated by activation of the endothelin receptor type A on podocytes. It may be hypothesized, therefore, that the renal injury occurring during antiangiogenic treatment is mediated both by a direct effect related to interruption of VEGF signaling and by an indirect effect related to activation of the ET-1 system. In our previous studies, we used a sunitinib dose of 26.7 mg/kg per day by oral gavage. This high dose was associated with a rapid development of severe hypertension, proteinuria, and irreversible glomerular renal injury, making it difficult to infer to what extent the renal injury is BP-independent. In addition, the extent of

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sunitinib-mediated side effects in this particular model at this dose is much more severe than seen in patients, rendering it questionable whether this dose adequately reflected the off-target effects of sunitinib in clinical practice. Here, we tested the dose dependency of these side effects, aiming to find a sunitinib dose that, with regard to hemodynamic and renal side effects, including ET-1 elevation, better resembles the dose applied in patients. With this approach, we also wanted to unravel whether the proteinuria observed during antiangiogenic treatment occurs irrespective of the presence of glomerular histological changes.

Materials and Methods

Animal Study
Normotensive, 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, and sunitinib (SU11248; Sutent, Pfizer) and vehicle solution were prepared and administered by oral gavage as described previously. After implantation of the telemetry transmitters, 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 IP of pentobarbital and blood was sampled for measurement of circulating ET-1, cystatin C levels, VEGF, and sunitinib levels, and kidneys were rapidly excised. Rats were randomly administered a low (7 mg/kg per day) or intermediate (14 mg/kg per day) dose of sunitinib-

Biochemical Measurements
ET-1 and VEGF were assessed using a chemiluminescent ELISA (QuantiGlo, R&D Systems; range 0.34–250 pg/mL) and Solid Phase Sandwich ELISA (Quantikine, R&D Systems; range, 31.2–2000 pg/mL), respectively. cGMP levels were determined in urine, using an ELISA kit (Enzo Life Sciences, Farmingdale, NY). Serum cystatin C and urinary protein concentrations (Cobas c502 and c702, CYSC and TP2/TPUC3; Roche Diagnostics) were measured at the clinical chemical laboratory of the Erasmus MC. Sunitinib levels were measured by a validated ultra-performance liquid chromatography/tandem mass spectrometry system, at the laboratory of Translational Pharmacology of the Erasmus MC Cancer Institute.

Renal Histology
Details of the light microscopy and electron microscopy in this study are available in the online-only Data Supplement.

Podocyte and Glomerular Quantification
Immunohistochemical staining for Wilms tumor protein, a podocyte-specific transcription factor, was performed to identify podocytes. In brief, 3-μm paraffin sections were deparaffinized, rehydrated, and used for immunostaining after antigen retrieval procedure. Endogenous peroxidase activity was blocked for 15 minutes in 0.1% H2O2 in water. After washing with PBS, sections were incubated with mouse anti–Wilms tumor protein monoclonal antibodies (WLM04, Abcam) diluted in 1% bovine serum albumin in PBS for 2 hours, 1-hour incubation with antiamouse envision (DAKO), and the slides were developed with diaminobenzidine. Slides were counterstained with hematoxylin, dehydrated, and mounted. The number of positive nuclei per glomerulus in 25 glomeruli was counted, and the glomerular surface area was measured with the Philips Image Management System 2.3 software (Philips Medical Systems Nederland B.V., The Netherlands).

Intercellular adhesion molecule-1 staining was performed on frozen sections using an antirat CD54 monoclonal antibody (clone 1A29). A horseradish peroxidase–labeled anti–mouse IgG (H+L) antibody (KPL, Gaithersburg, MD) was used as a secondary antibody.

mRNA Expression
Quantitative polymerase chain reaction was performed to quantify mRNA expression. The RNA was isolated using the TRIzol method and reversed to cDNA using an AMV cDNA synthesis kit (Roche, Indianapolis, IN). For the polymerase chain reaction, iQ SYBR Green supermix (BioRad) was used. Relative transcription levels for Nep1, Nphs1, and Nphs2 were determined and corrected to the podocyte-specific gene Wt1 using the CFX manager software (BioRad) with primer sequences as described earlier. The Edn1, Ece1, and Vegfa mRNA levels were measured and corrected to a general housekeeping gene hypoxanthine phosphoribosyltransferase-1 (Hprt1). The primer sequences used are shown in Table S1 in the online-only Data Supplement.

Statistical Analysis
Data are presented as mean±SEM. Statistical analysis between groups was performed by unpaired t test or by repeated-measures ANOVA followed by Newman–Keuls or Dunnett multiple comparison test. Correlations were performed by Pearson test. GraphPad Prism version 5.0 was used for all statistical analysis.

Results

Plasma Concentrations of Sunitinib
Plasma concentrations of sunitinib increased dose dependently with the 3 oral doses of sunitinib (Figure 1). The plasma concentration of sunitinib at the lowest dose was comparable with the systemic concentrations reached in patients treated with a standard daily dose of sunitinib of 50 mg. Sunitinib is metabolized by cytochrome P450 3A4 to the active compound N-desethyl sunitinib. Even at the lowest dose of sunitinib, the plasma concentration of this compound in rats was much higher than in patients during standard dosing. The ratio of metabolite/sunitinib was 5.1±0.7 compared with an average of 0.3 in humans (data not shown), implying a higher rate of N-de-ethylation in rodents.

Dose Dependency of BP Rise, Renal Function Impairment, and Proteinuria in Sunitinib-Exposed Rats
Mean arterial BP (MAP) increased dose dependently, ranging from 13.4±3.3 mm Hg at the lowest to 31.1±9.9 mm Hg at the highest dose versus vehicle (Figure 2A). With all 3 doses, the rise in MAP dose dependently was accompanied by a decrease in heart rate (Figure 2B).

Compared with vehicle, cystatin C increased with the high and intermediate doses, but it was identical to that in vehicle-treated rats with the low dose of sunitinib (Figure 2C). Proteinuria markedly increased with the high dose of sunitinib, whereas the intermediate and low doses of sunitinib were associated with modest proteinuria (Figure 2D). Cystatin C and proteinuria were not related to MAP or ET-1.
Dose-Dependent Changes in cGMP, ET-1, and VEGF

Sunitinib was associated with dose-dependent decreases in urinary cGMP excretion (Figure 3A). In previous clinical and preclinical studies, we have shown that sunitinib administration is associated with activation of the ET-1 axis. At the different doses of sunitinib, circulating ET-1 increased dose dependently (Figure 3B). Changes in urinary cGMP excretion and in ET-1 were unrelated (r=0.18; P>0.05).

Figure 1. Trough plasma concentrations of sunitinib (A) and its active metabolite, N-desethyl sunitinib or SU12662, (B) in rats in response to administration of a low (7 mg/kg per day), an intermediate (14 mg/kg per day), and a high dose of sunitinib (26.7 mg/kg per day). *P<0.05 vs sunitinib (26.7 mg); #P<0.05 vs sunitinib (14 mg).

Figure 2. Time course of changes in mean arterial pressure (MAP; A) and heart rate (HR; B) in response to administration of vehicle, a low (7 mg/kg per day), an intermediate (14 mg/kg per day), and a high dose of sunitinib (26.7 mg/kg per day). **Bottom**, Areas under the curve (AUC). C and D, Serum cystatin C and change in proteinuria (before and after treatment), respectively, in rats after administration of vehicle, a low (7 mg/kg per day), an intermediate (14 mg/kg per day), and a high dose of sunitinib (26.7 mg/kg per day). *P<0.05 vs sunitinib (26.7 mg); †P<0.05 vs vehicle; #P<0.05 vs sunitinib (14 mg).
Circulating ET-1 and MAP were related ($r=0.49$; $P<0.05$).
Urinary ET-1 excretion at baseline was 9.1±0.5 pg per day. This excretion slightly decreased by 2.3±0.7 pg per day with vehicle and by 5.4±1.9 pg per day at the low-, 4.1±1.1 pg per day at the intermediate-, and 4.1±1.1 pg per day at the high-sunitinib dose ($P<0.05$ sunitinib versus vehicle).

Plasma VEGF dose dependently and markedly increased in response to sunitinib administration (Figure 3C). Plasma VEGF was related to MAP, sunitinib, and N-desethyl sunitinib ($r=0.61$, $r=0.72$, and $r=0.83$ respectively; $P<0.01$). The rise in circulating VEGF was not accompanied by a rise in the renal expression of the gene encoding VEGF-A (Figure 4A). The expression of the gene encoding for ET-1 did not change with any of the doses, whereas the gene expression of endothelin-converting enzyme decreased (Figure 4B and 4C).

Renal Histopathology

Kidney weight/body weight ratio was 3.0±0.04 g/kg in vehicle-treated rats. This ratio increased to 3.3±0.2, 3.4±0.1, and 3.5±0.04 g/kg, respectively, at the low, intermediate, and high doses of sunitinib ($P<0.05$ for all). Light microscopy of kidney sections revealed that the intermediate and high doses of sunitinib were associated with increases in endothelial cell swelling (Figure S1C and S1D). No endothelial cell swelling was present at the low dose of sunitinib (Figure S1B). Phosphotungstic acid hematoxylin staining showed fibrin deposits in glomerular capillaries (Figure S1E) and in small arteries at the high dose of sunitinib only (Figure S1F).

Quantification of renal histopathology based on light microscopy images is displayed in Figure S2. The glomerular ischemia score in rats exposed to vehicle and low-dose sunitinib was identical, whereas this score increased dose dependently with the intermediate and high dose of sunitinib. Comparable dose-dependent findings were obtained for the deposition of intraepithelial droplets. Peritubular capillary density or the presence of interstitial fibrosis and tubular atrophy between high dose of sunitinib and controls did not differ (data not shown).

**Figure 3.** Change in urinary cyclic GMP (cGMP) excretion (A), circulating endothelin-1 (ET-1) levels (B), and circulating vascular endothelial growth factor (VEGF) levels (C) in rats after administration of vehicle, a low (7 mg/kg per day), an intermediate (14 mg/kg per day), and a high dose of sunitinib (26.7 mg/kg per day). *P<0.05 vs sunitinib (26.7 mg); †P<0.05 vs vehicle; ‡P<0.05 vs sunitinib (14 mg).

**Figure 4.** Relative renal mRNA expression level of vascular endothelial growth factor (VEGF-A; A), endothelin-1 (ET-1; B) and endothelin-converting enzyme (ECE; C) after correction for the housekeeping gene Hprt. Relative renal mRNA expression of Neph1 (D), nephrin (Nphs1; E), and podocin (Nphs2; F). mRNA levels were corrected for the podocyte specific gene Wt1. †P<0.05 vs vehicle.
Transmission electron microscopy of kidney sections showed normal foot processes and cytoplasmic morphology at the low dose of sunitinib (Figure S4B). At the high dose of sunitinib, endothelial cell swelling with luminal obliteration and loss of intercellular fenestrations occurred (Figure S4C), whereas multiple intraepithelial droplets were present at the intermediate and high doses of sunitinib (Figure S4D).

Glomerular Circumference, Podocyte Number, and Gene Expression

Glomerular circumference tended to increase with the intermediate and high doses of sunitinib, but the number of podocytes per glomerular circumference remained unchanged (data not shown). The relative expression of the slit-diaphragm mRNAs of genes encoding Nep1, nephrin, and podocin decreased at the high and intermediate doses of sunitinib compared with vehicle (Figure 4D–4F). Glomerular endothelium intercellular adhesion molecule-1 expression was increased during sunitinib treatment (Figure S3).

Discussion

Off-target effects of angiogenesis inhibitors that interfere with VEGF signaling are the development of hypertension and renal injury.\(^3\)\(^,\)\(^6\) By applying different doses of sunitinib, we explored the dose dependency of these side effects and their interrelation. Our findings indicate that the severity of hypertension and the development of renal injury are dose-related. They also show a lower threshold dose for developing hypertension and proteinuria than for developing renal function impairment, as reflected by the rise in cystatin C concentration and the severity of glomerular ischemia. These findings concur well with clinical studies reporting a higher incidence of hypertension in patients treated with angiogenesis inhibitors.\(^3\)\(^,\)\(^7\) With the lowest dose of sunitinib applied, the BP rise of ≈10 mm Hg is of similar magnitude as the BP rise we have observed in patients after 2 and after 4 weeks of administration of sunitinib, stressing the potential relevance of our low-dose model for the clinical situation.\(^9\) Of note, the plasma sunitinib concentration with the low dose of sunitinib was comparable with the concentration measured in patients treated with a standard dose of sunitinib of 50 mg per day.\(^15\) However, the concentration of its active metabolite N-desethyl sunitinib was markedly higher, indicating increased metabolism of the parent compound in rats, as reported previously.\(^18\)

Glomerular endotheliosis, sometimes accompanied by thrombi, is a hallmark of angiogenesis inhibition–induced renal injury.\(^1\) In this study, glomerular endotheliosis with almost complete obliteration of glomerular capillaries was observed at the highest dose of sunitinib, whereas endotheliosis was less severe with the intermediate and absent with the low dose of sunitinib. Using fibrin staining, fibrin clots in glomerular capillaries and small arteries, indicating thrombotic microangiopathy, were present at the high but not at the low and intermediate doses of sunitinib. Glomerular thrombotic microangiopathy has been reported in patients treated with antiangiogenic treatment and can also occur in preeclampsia.\(^5\)\(^,\)\(^6\)\(^,\)\(^19\) Exposure to sunitinib was not associated with a decrease in renal peritubular capillaries or interstitial fibrosis, indicating that the adverse renal effects are restricted to the glomeruli.

Genetic depletion studies in mice have shown that podocyte-specific heterozygosity for VEGF-A results in renal disease by 2.5 weeks of age characterized by proteinuria and endotheliosis.\(^20\) Also rats or mice injected with a sFlt-1 or overexpressing sFlt-1 by virus injection develop endotheliosis and proteinuria.\(^21\)\(^,\)\(^22\) Collectively, these findings indicate that interference with the VEGF-A signaling pathway, either genetically or by a soluble receptor trapping VEGF, or by a tyrosine kinase directly targeting VEGF receptors, results in a similar glomerular phenotype.

In the kidney, VEGF is abundantly expressed in podocytes, whereas glomerular capillary endothelial cells preferentially express VEGF receptors.\(^23\) VEGF produced by podocytes is essential for the maintenance of the integrity of glomerular endothelial cells.\(^20\)\(^,\)\(^24\) In addition, VEGF is also required for the maintenance of podocyte function and slit-diaphragm proteins, implying that podocyte-derived VEGF exerts both paracrine and autocrine effects.\(^25\) In preeclamptic patients and mice exposed to a VEGF antibody or sFlt-1, nephrin expression decreases.\(^22\)\(^,\)\(^26\) Moreover, in rats with progressive glomerulonephritis increased expression of sFlt-1 resulted in massive proteinuria and downregulated nephrin expression.\(^23\) More recently, the interaction of the cytoplasmatic domains of nephrin and VEGF-R2 has been demonstrated in both in vivo and in vitro studies.\(^27\) On VEGF binding, this nephrin–VEGFR2 interaction diminishes, resulting in a change in shape and size of cultured podocytes.\(^27\) Sunitinib administration in this study was also associated with a dose-dependent decreased expression of the gene encoding nephrin and a decrease in the genes expressing Nep1 and podocin, encoding for slit-diaphragm proteins that bind to nephrin (Figure 4).\(^29\) Given that null mutations in the genes encoding nephrin, Nep1, and podocin are all associated with severe forms of congenital nephrotic syndrome, it seems reasonable to conclude that the diminished expression of these genes is involved in the development of proteinuria during treatment with sunitinib and treatment with other agents interfering with VEGF signaling.\(^28\)\(^,\)\(^29\)

In line with our previous observations, sunitinib treatment was associated with activation of the circulating endothelin system, which was already present at the low dose of sunitinib.\(^3\)\(^,\)\(^9\) The observed rise in ET-1 may be a direct consequence of VEGF inhibition, as shown in human lung microvascular endothelial cells.\(^30\) The rise may also be related to the activation of the endothelium in response to VEGF inhibition. This, however, was not supported by the present finding that despite activation of glomerular endothelial cells as reflected by the presence of glomerular endotheliosis and increased glomerular expression of intercellular adhesion molecule-1, expression of gene encoding for ET-1 within the kidney was not increased. In addition to a direct adverse effect of VEGF inhibition on podocyte function and expression of slit-diaphragm proteins, ET-1 can exert negative effects on podocyte function, which, additionally to the disruption of VEGF pathway, may contribute to glomerular injury and proteinuria.\(^9\)\(^,\)\(^10\)\(^,\)\(^11\)\(^,\)\(^12\) For instance, a subpressor dose of ET-1 administered to Sprague-Dawley rats was found to increase glomerular permeability and inflammation, as well as proteinuria, effects that could be blocked by an endothelin receptor type A antagonist.\(^31\) In...
cultured podocytes, ET-1 through activation of endothelin receptor type A induces nephrin shedding concomitant with a redistribution of the podocyte’s cytoskeleton. Recently, Buelli et al have shown activation of the β-arrestin-1 signaling pathway by ET-1, resulting in transition of podocytes from an epithelial to mesenchymal cell type. Given the present observation that urinary ET-1 excretion was not increased during sunitinib administration or even decreased, the possibility that activation of ET-1 axis has contributed to the proteinuria in our rat model remains questionable. If ET-1 is involved it should, based on the present findings and a previous study, reach the podocytes by ultrafiltration.

Some limitations of our study should be mentioned. The duration of exposure to sunitinib was only 8 days. It would be interesting to see whether the rise in BP and renal pathology is progressive during more prolonged exposure. With the lowest dose of sunitinib, the serum concentration of the active metabolite was considerably higher than observed in patients, yet the rise in BP was comparable. Finally, although in this study we have focused on the renal effects of sunitinib, it is well known that VEGF inhibition can also negatively affect other organs, especially the myocardium and endocrine organs. In more long-term studies, it would be interesting to see whether the lowest or even a still lower dose of sunitinib as used in this study still preferentially affects the kidney or also has adverse effects on the mentioned organs when administrated for prolonged periods.

Perspectives

This study shows activation of the circulating, but not of the renal ET-axis, already at a relatively low dose of sunitinib. This activation likely contributes to the off-target effects of sunitinib. This study still preferentially affects the kidney or also has adverse effects on the mentioned organs when administrated for prolonged periods.

Acknowledgments

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Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

- This is the first study with the tyrosine kinase inhibitor sunitinib that explores the dose dependency of hypertension and renal injury, aiming to find a sunitinib dose that, with regard to side effects, closely mimics the human situation.

**What Is Relevant?**

- Our findings indicate that sunitinib-induced hypertension requires a lower dose than its induction of renal function impairment.

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**Summary**

This study shows activation of the systemic endothelin axis, already at a low dose of sunitinib. This activation may not only contribute to the rise in blood pressure but also to the proteinuria and renal damage occurring during antiangiogenic treatment.
Greater Sensitivity of Blood Pressure Than Renal Toxicity to Tyrosine Kinase Receptor Inhibition With Sunitinib

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GREATER SENSITIVITY OF BLOOD PRESSURE THAN RENAL TOXICITY TO TYROSINE-KINASE RECEPTOR INHIBITION WITH SUNITINIB

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Expanded methods

Light microscopy

The left kidney was rapidly excised from euthanized rats, decapsulated, weighed and sliced transversely. 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 or absence of glomerular ischemia and intra-epithelial protein deposition in 50 glomeruli. 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. Phosphotungstic acid-haematoxylin (PTAH) staining was used to identify fibrin deposits.

To assess the number of peritubular capillaries (PTC), 4µm formalin fixed paraffin-embedded tissue sections were cut and stained with CD31. Staining was performed following routine diagnostic procedures on the Benchmark Ultra stainer (Venatana Discovery), using buffers provided by Ventana that are validated for diagnostics. Antibody against CD31 (1:50 dilution, ab28364, Abcam, Cambridge, UK) were used to detect ptc’s. The tissue sections were stained simultaneously to reduce inter-staining variation. Incubation with antibody was done for 60 minutes, after antigen retrieval of 64min at pH8.4. After staining, sections were blindly evaluated at 40x objective by a pathologist (M.C.vG.) selecting 10 random, non-overlapping fields. Images were acquired using a Canon EOS 1100D camera and an Olympus BX40 microscope. The medulla and the subcapsular cortex with a width of 5 tubuli were excluded when images were acquired. Per image field, the number of PTC’s and the number of tubuli were counted, and a ratio was made (ptc:tubuli). Evaluation of the staining has been adapted from Steegh et al.\textsuperscript{1}

In addition, in order to assess the presence of interstitial fibrosis (ci) and tubulus atrophy (ct), 3µm sections were PAS stained following routine diagnostic procedures. Sections were subsequently analyzed (M.C.vG.) using a 20x objective and scored for ci and ct according to the Banff classification of renal allograft rejection.\textsuperscript{2}
Electron microscopy

Kidney samples were processed for transmission EM as previously described. Immediately after resection, cortical renal tissue was cut into blocks and immersed in Karnovsky’s fixative containing 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After fixation, 1 mm$^3$ pieces were cut and postfixed in 1% OsO$_4$ in 0.1 M cacodylate buffer for 1 h. The specimens, were hereafter dehydrated in ethanol, immersed in acetone and embedded in Epon 812 R (Merck). Survey sections (2 µm) were stained with toluidine blue. Ultrathin (50-70 nm) sections with 2-3 randomly selected glomeruli were cut, mounted on copper grids and contrasted with uranyl acetate and lead citrate. Serial sections (8-10 sections per grid and 4-5 neighboring grids) were examined using a Philips Morgagni 261 EM microscope. Sections were blindly evaluated by a renal pathologist (A.H) for the occurrence of glomerular endotheliosis (endothelial cell swelling, encroachment of the capillary spaces and loss of endothelial fenestration) and podocyte morphology as previously defined.

References


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**Figure S1.** Kidney sections from WKY rats administered vehicle (A) or sunitinib; including a low dose (B), intermediate dose (C) or high dose (D), stained with HE stain (magnification x500). After administration of a high dose of sunitinib for 8 days marked glomerular changes could be observed, including intra-epithelial droplets and reduction in the number of capillaries. PTAH staining shows fibrin deposits in glomerular capillaries (E) and small arteries (F) with high dose of sunitinib (n=1).
Figure S2. Light microscopic evaluation of kidney sections obtained from rats exposed to a low, an intermediate and a high dose of sunitinib for 8 days compared to controls. All evaluations were performed in 50 glomeruli of a PAS stained section and the numbers of glomeruli with each score were counted. *p<0.05 vs sunitinib (26.7 mg); †p<0.05 vs vehicle; #p<0.05 vs. sunitinib (14mg).

Figure S3. Kidney sections from WKY rats administered vehicle (A) or sunitinib (B). ICAM-1 staining shows increased glomerular endothelium staining, suggesting endothelium activation at all dosages of sunitinib (n=1).
**Figure S4.** Transmission electron micrographs of representative kidney sections from Wistar Kyoto (WKY) rats treated with vehicle (A) or sunitinib (B-D). (A) Normal capillary lumina (asterix) and endothelial cells with preserved fenestration (arrows) in rat receiving vehicle. (B) Podocytes showing preserved foot processes (arrows) and normal cytoplasmic morphology (asterix) in low dose sunitinib-treated rat. (C) Glomerular endotheliosis with endothelial swelling (ES), loss of fenestration (arrows) and maximal luminal obliteration in high dose sunitinib-treated rat. (D) Podocyte containing multiple intracellular droplets (arrow) in intermediate dose sunitinib-treated rat. Scale bars = 2 µm.