The Vascular Endothelial Growth Factor Receptor Inhibitor Sunitinib Causes a Preeclampsia-Like Syndrome With Activation of the Endothelin System

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Abstract—Angiogenesis inhibition is an established treatment for several tumor types. Unfortunately, this therapy is associated with adverse effects, including hypertension and renal toxicity, referred to as “preeclampsia.” Recently, we demonstrated in patients and in rats that the multitarget tyrosine kinase inhibitor sunitinib induces a rise in blood pressure (BP), renal dysfunction, and proteinuria associated with activation of the endothelin system. In the current study we investigated the effects of sunitinib on rat renal histology, including the resemblance with preeclampsia, as well as the roles of endothelin 1, decreased nitric oxide (NO) bioavailability, and increased oxidative stress in the development of sunitinib-induced hypertension and renal toxicity. In rats on sunitinib, light and electron microscopic examination revealed marked glomerular endotheliosis, a characteristic histological feature of preeclampsia, which was partly reversible after sunitinib discontinuation. The histological abnormalities were accompanied by an increase in urinary excretion of endothelin 1 and diminished NO metabolite excretion. In rats on sunitinib alone, BP increased (ΔBP: 31.6±0.9 mm Hg). This rise could largely be prevented with the endothelin receptor antagonist macitentan (ΔBP: 12.3±1.5 mm Hg) and only mildly with Tempol, a superoxide dismutase mimetic (ΔBP: 25.9±2.3 mm Hg). Both compounds could not prevent the sunitinib-induced rise in serum creatinine or renal histological abnormalities and had no effect on urine nitrates but decreased proteinuria and urinary endothelin 1 excretion. Our findings indicate that both the endothelin system and oxidative stress play important roles in the development of sunitinib-induced proteinuria and that the endothelin system rather than oxidative stress is important for the development of sunitinib-induced hypertension. (Hypertension. 2011;58:295-302.) ● Online Data Supplement

Key Words: angiogenesis inhibition ■ renal toxicity ■ preeclampsia ■ endothelin 1

Angiogenesis, the formation of new capillaries from an existing vasculature, is critical to tumor growth as well as metastasis. Vascular endothelial growth factor (VEGF) and its corresponding receptors play key roles in the regulation of this process. Angiogenesis inhibition, by targeting VEGF or its receptors, has become an established treatment of several tumor types. Common adverse effects of angiogenesis inhibition are hypertension and renal toxicity.1 Hypertension has been reported in up to 36% of patients treated with bevacizumab, a monoclonal antibody against VEGF, and in up to 60% of patients treated with sunitinib, an orally active multitarget VEGF receptor tyrosine kinase inhibitor used in the first-line treatment of metastatic renal cell carcinoma or imatinib-resistant gastrointestinal stromal tumors.1 Renal toxicity, mainly proteinuria, has been reported in 41% to 63% of patients treated with bevacizumab.2 The incidence of proteinuria in patients treated with receptor tyrosine kinase inhibitors is less well known, because in the initial studies patients were not routinely screened for this adverse effect.

It has been suggested that inhibition of the VEGF pathway reduces nitric oxide (NO) bioavailability, leading to a disturbed balance between NO and endothelin 1 (ET-1), and thus promotes the development of hypertension.3 Indeed, we reported recently that the rise in blood pressure (BP), renal dysfunction, and proteinuria induced by the receptor tyrosine kinase inhibitor sunitinib was associated with a 2- to 3-fold rise in circulating ET-1 levels in both patients and rats.3 Whether activation of the endothelin system plays a pathophysiological role in sunitinib-induced hypertension and renal toxicity remains to be established.

There is abundant evidence that oxidative stress is involved in the development of renal injury and that decreased levels of VEGF may contribute to oxidative stress-induced endothelial...
dysfunction.\textsuperscript{4–7} In addition, in various hypertensive rat models, administration of Tempol, a superoxide dismutase mimetic, which metabolizes superoxide and other reactive oxygen species, has been shown to reduce BP.\textsuperscript{8} Whether increased reactive oxygen species production is involved in sunitinib-induced hypertension and renal toxicity is unknown.

Hypertension and proteinuria during angiogenesis inhibition are also referred to as a preeclampsia-like syndrome.\textsuperscript{9} Preeclampsia is a pregnancy-related disorder characterized by proteinuria and hypertension, as well as increased circulating ET-1 levels.\textsuperscript{10} One of the underlying pathophysiological mechanisms is thought to be an increased placental production of soluble fms-like tyrosine kinase 1 (sFlt-1).\textsuperscript{11,12} Within the maternal circulation, sFlt-1 binds VEGF, thereby abrogating the VEGF-VEGF receptor axis activity and thus creating a condition similar to that induced by VEGF receptor inhibitors.

The aim of our current studies was to explore the effects of sunitinib on renal histology, including the resemblance with preeclampsia, and the roles of ET-1, decreased NO bioavailability, and reactive oxygen species in the development of sunitinib-induced hypertension and renal adverse effects.

Methods

\textbf{In Vivo Study}

Male Wistar Kyoto rats (280 to 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 the sunitinib and vehicle solutions were prepared and administered by oral gavage as described previously.\textsuperscript{3} Macitentan (ACT-064992), a dual ET\textsubscript{A}/ET\textsubscript{B} receptor antagonist kindly provided by Actelion, was dissolved in vehicle containing 0.5% methylcellulose aqueous solution and 0.05% Tween 80. Tempol (4-hydroxy-tempo, 97%; Sigma-Aldrich) was dissolved in 0.9% saline. Four separate experiments were performed. At the end of each experiment, rats were euthanized with 60 mg/kg of pentobarbital i.p., and blood was sampled for measurement of plasma ET-1, as well as serum creatinine levels, and kidneys were rapidly excised. In the first experiment, rats were randomly administered sunitinib (26.7 mg/kg per day of sunitinib i-maleate; n=10) or vehicle (n=10) by oral gavage (0.5 mL) for 8 days. In the second experiment, rats (n=7) were administered sunitinib at the same dose for 8 days followed by an 11-day recovery period, after which they were euthanized. In the third experiment, rats (n=8) were orally administered the combination of sunitinib and macitentan at 30 mg/kg per day for 8 days. At the end of this treatment period, 4 rats were anesthetized using isoflurane to monitor the BP response to bolus injections of ET-1 to test the degree of macitentan-induced ET-1 receptor blockade, as described in the online Data Supplement (please see http://hyper.ahajournals.org). In the fourth experiment, rats were administered the combination of sunitinib by oral gavage and Tempol, 200 mmol/kg per minute s.c., using osmotic mini-pumps (ALZET 2ML2) for 8 days (n=6).\textsuperscript{14} In all of the experiments, 6 days before (baseline) and 6 days after treatment initiation, 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 determination of protein, ET-1, NO metabolites (NO\textsubscript{2}+NO\textsubscript{3}), and thiobarbituric acid reactive substances. Urine was collected on antibiotics (A9555, Sigma) to prevent formation of NO metabolites. In the second experiment, rats were also housed in metabolic cages 1 week after treatment discontinuation.

Male spontaneously hypertensive rats (SHR; 280 to 300 g, 8 to 9 weeks old; mean arterial pressure: 146±3 mm Hg; n=2), obtained from Charles River (Germany), were used as hypertensive controls to compare renal histology obtained from Wistar Kyoto rats exposed to sunitinib with that of SHRs.\textsuperscript{15} All of the experiments were performed under the regulation and permission of the Erasmus MC Animal Care Committee.

\textbf{In Vitro Studies and Renal Histology}

\textbf{Langendorff Studies}

Langendorff studies were performed as described in the online Data Supplement. Dose-response flow curves to bolus injections of bradykinin and angiotensin II were constructed, after which the maximum coronary flow was determined by injecting sodium nitroprusside (10 mmol/L).

\textbf{Light Microscopy}

Details of the light microscopy in this study are available in the online Data Supplement. Briefly, transversely sliced kidney sections were stained for hematoxylin-eosin, periodic acid Schiff, and Jones silver. Periodic acid Schiff–stained sections were blindly evaluated by a pathologist for the presence or absence of endothelial cell and epithelial cell swelling in 50 glomeruli and were semiquantitatively scored for the presence of ischemia and intraepithelial protein.

\textbf{Electron Microscopy}

One glomerulus in each biopsy section was examined by electron microscopy, and the occurrence of glomerular endotheliosis (endothelial cell swelling, encroachment of the capillary spaces, and loss of endothelial fenestration) and podocyte morphology was registered.

\textbf{Determination of Prepro–ET-1 mRNA Levels in Renal Cortex and Medulla}

Immediately after euthanization of the rats, the kidneys were harvested and renal cortex and medulla separated. All of the tissues were quickly frozen in nitrogen and stored at −80°C. Total RNA was isolated from kidneys using the TRizol reagent (Gibco-BRL) and reverse transcribed. The resulting cDNA was amplified in 40 cycles (denaturation at 95°C for 10 minutes; thermal cycling at 95°C for 15 seconds; annealing/extension at 60°C for 1 minute) with a Step-One cycler (NYSE, Applied Biosystems) using the SYBR Green Q-PCR core kit (Applied Biosystem). Primer of rat prepro–ET1 (forward: AGGGAACAGATGCCAGTGTGCT; reverse: TG- CATGTGACTTTGGGCTCGGA) was from Invitrogen. The comparative cycle time method was used for relative quantification of gene expression. Messenger RNA expression was normalized versus actin and expressed as the ratio of target:control value.\textsuperscript{16}

\textbf{Biochemical Measurements}

ET-1 was assessed using a chemiluminescent ELISA (QuantiGlo, R&D Systems), urine albumin by enzyme immunoassay (Spi-Bio, Montigny-Le-Bretonneux, France), and tumor necrosis factor-α by colorimetric sandwich ELISA (R&D Systems). Urine NO\textsubscript{2}+NO\textsubscript{3} concentration was determined by fluorometric quantification of nitrite content (Cayman Chemicals, Ann Arbor, MI) and lipid peroxidation in urine by measurement of thiobarbituric acid reactive substances.\textsuperscript{17} Serum creatinine and urinary protein were measured at the clinical chemical laboratory of the Erasmus MC.

\textbf{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. P<0.05 was considered significant. GraphPad Prism version 4.03 was used for all of the statistical analysis.

\textbf{Results}

\textbf{In Vivo Study}

BP dose-response curves to ET-1 bolus injections in macitentan-treated rats showed effective blockade of the ET-1–induced rise compared with control rats (Figure S1, available in the online
Data Supplement at http://hyper.ahajournals.org). The sunitinib-induced rise in BP was largely prevented by concomitant administration of macitentan and mildly attenuated by Tempol (Figure 1A), whereas the sunitinib-induced decrease in heart rate was not affected by either compound (Figure 1B). The previously reported sunitinib-induced loss of circadian BP rhythm was not reversed by either macitentan or Tempol (data not shown).3

Kidney weight:body weight ratio, proteinuria, albuminuria, and urinary ET-1 excretion increased during sunitinib administration (Table 1), in parallel with the previously described increase in BP, serum creatinine, and circulating ET-1 levels.3 All of the parameters, with the exception of serum creatinine and urinary ET-1 excretion, returned to baseline after discontinuation of sunitinib administration (Table 1). Sunitinib-induced changes in urinary ET-1 excretion and BP were not correlated (r = −0.50; P < 0.05; n = 7), and neither were changes in urinary ET-1 excretion and proteinuria (r = 0.47; P < 0.20; n = 7). Circulating tumor necrosis factor-α levels were mostly below the lowest detection limit in both the vehicle- and sunitinib-administered groups (data not shown). Urine nitrates decreased during sunitinib administration (Figure 2) and partly returned after sunitinib withdrawal. Urine thiobarbituric acid reactive substances did not change (0.80 ± 0.19 μmol/kg of body weight per 24 hours at baseline versus 0.87 ± 0.08 μmol/kg of body weight per 24 hours after 8 days of treatment; P = 0.69) during treatment with sunitinib. The sunitinib-induced decrease in urinary nitrate excretion was not reversed by macitentan or Tempol (data not shown). Macitentan did not change kidney weight:body weight ratio compared with sunitinib alone (data not shown), nor did it change serum creatinine, but it significantly decreased proteinuria and urinary ET-1 excretion, whereas circulating ET-1 levels increased (Figure 3). Treatment with Tempol also did not change kidney weight:body weight ratio compared with sunitinib alone (data not shown), nor did it change serum creatinine or circulating ET-1 levels, although it did decrease proteinuria and urinary ET-1 excretion (Figure 3).

Table 1. Parameters in Rats After 8 Days of Treatment With Sunitinib (n=10) and After Treatment Discontinuation (n=7) Compared With Baseline Values or Control Rats (n=6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control/Baseline</th>
<th>Sunitinib</th>
<th>Stop</th>
<th>P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>94 ± 3</td>
<td>124 ± 1</td>
<td>&lt;0.001</td>
<td>92 ± 8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Left KW:BW ratio, g/kg</td>
<td>3.0 ± 0.04</td>
<td>3.4 ± 0.05</td>
<td>&lt;0.001</td>
<td>3.3 ± 0.05</td>
<td>0.001</td>
</tr>
<tr>
<td>Right KW:BW ratio, g/kg</td>
<td>3.0 ± 0.04</td>
<td>3.5 ± 0.04</td>
<td>&lt;0.001</td>
<td>3.3 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum creatinine, μmol/L</td>
<td>8.0 ± 2.7</td>
<td>28.8 ± 6.4</td>
<td>0.03</td>
<td>25.1 ± 1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma endothelin 1, pg/mL</td>
<td>0.6 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>0.003</td>
<td>0.7 ± 0.1</td>
<td>0.40</td>
</tr>
<tr>
<td>Urinary endothelin 1, pg/d</td>
<td>3.6 ± 0.8</td>
<td>7.3 ± 1.0</td>
<td>0.007</td>
<td>7.6 ± 0.6</td>
<td>0.007</td>
</tr>
<tr>
<td>Proteinuria, mg/d</td>
<td>9.1 ± 1.4</td>
<td>39.2 ± 5.2</td>
<td>&lt;0.001</td>
<td>12.4 ± 0.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Albuminuria, mg/d</td>
<td>0.2 ± 0.02</td>
<td>13.1 ± 3.1</td>
<td>&lt;0.001</td>
<td>0.6 ± 0.2</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

MAP indicates mean arterial pressure; KW, kidney weight; BW, body weight; NA, not applicable. Data are shown as mean ± SEM. P value was compared with control/baseline. Serum creatinine, plasma endothelin 1, and proteinuria are from Kappers et al.3
In Vitro Studies and Renal Histology

Langendorff Studies

In the Langendorff preparation, coronary flow responses to bradykinin, angiotensin II, or sodium nitroprusside were not significantly changed after coadministration of sunitinib and macitentan or Tempol compared with sunitinib alone (Figure S2).

Renal Histology

Light microscopic examination showed marked abnormalities, including periodic acid Schiff-positive intraepithelial protein droplets and epithelial, as well as endothelial cell swelling in glomeruli from rats exposed to sunitinib compared with control rats (Figure 4). In addition, these glomeruli were more shrunken with narrowed capillary lumina contain-
ing less erythrocytes (ischemia) compared with those of control rats (Figure 4). The semiquantitative scores of the renal abnormalities are provided in Table 2. The percentage of glomeruli in renal biopsy sections with a score 2 for intraepithelial protein was not correlated with the change in proteinuria \( r = 0.45; P = 0.38; n = 5 \), the change in protein: creatinine ratio \( r = 0.45; P = 0.19; n = 10 \), or circulating ET-1 levels at the end of treatment \( r = 0.14; P = 0.71; n = 10 \). In SHR, used as hypertensive controls, none of the mentioned renal changes were present (Figure 4). All of the renal abnormalities observed on light microscopic examination completely reversed after sunitinib withdrawal. Remarkably, on light microscopic examination, all of the evaluated sections showed subcortical dilatation of proximal tubules with swelling and vacuolization of epithelial cells and also some collapse of subcapsular glomeruli, suggestive of agonal ischemic changes. These changes can probably be attributed to short-term ischemia, which develops between euthanizing the rats and processing the kidneys.

Electron microscopic examination revealed intraepithelial resorption droplets, glomerular endotheliosis (endothelial cell swelling with encroachment of the capillary spaces and loss of endothelial fenestration), effacement, and fusion of podocyte foot processes, as well as narrowing of the slit pores in renal sections from rats exposed to sunitinib, whereas none of these changes could be demonstrated in sections of control rats or of SHR (Figure 5). The basement membrane was normal in sunitinib-administered rats. Changes were only partly and locally reversible after sunitinib discontinuation (Figure 5).

In macitentan-administered rats, moderate ischemia was less observed compared with the rats administered sunitinib alone (Table 2). In addition, macitentan tended to decrease intraepithelial protein deposits, whereas no significant effects

### Table 2. Light Microscopic Evaluation of Kidney Sections Obtained From Rats Exposed to Sunitinib, Sunitinib and Macitentan, or Sunitinib and Tempol for 8 Days (n=10) Compared With Controls (n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomerular Ischemia, % Glomeruli</th>
<th>Endothelial Cell Swelling, % Glomeruli</th>
<th>Epithelial Cell Swelling, % Glomeruli</th>
<th>Intraepithelial Protein, % Glomeruli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Moderate</td>
<td>Severe</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>60±13</td>
<td>32±10</td>
<td>8±4</td>
<td>0</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>17±2*</td>
<td>54±4*</td>
<td>29±5*</td>
<td>63±5*</td>
</tr>
<tr>
<td>Sunitinib + macitentan</td>
<td>15±6*</td>
<td>35±2†</td>
<td>51±4†</td>
<td>85±3*</td>
</tr>
<tr>
<td>Sunitinib + Tempol</td>
<td>16±2*</td>
<td>41±4</td>
<td>44±3*</td>
<td>84±4*</td>
</tr>
</tbody>
</table>

All of the evaluations were performed in 50 glomeruli of a periodic acid Schiff–stained section, and the numbers of glomeruli in each section were counted. Endothelial and epithelial cell swelling were scored as present (1) or absent (0) in each glomerulus. The presence of intraepithelial protein was evaluated using a semiquantitative scale: 0 (no protein in the epithelial cells of a glomerulus), 1 (protein present in 1% to 50% of the epithelial cells of a glomerulus), and 2 (protein present in >50% of the epithelial cells of a glomerulus).

* \( P < 0.05 \) vs control.
† \( P < 0.05 \) vs sunitinib.
on endothelial and epithelial cell swelling were observed (Table 2). Tempol had no effect on either of the mentioned sunitinib-induced renal abnormalities (Table 2).

**Prepro-ET-1 mRNA Levels in Renal Cortex and Medulla**

Prepro-ET-1 mRNA levels in renal cortex (0.5±1.0- versus 1.3±0.4-fold change; n=6 and n=10; P=0.06) and medulla (1.04±0.09- versus 1.01±0.08-fold change; n=4 and n=10; P=0.88) were not different between sunitinib-administered and control rats.

**Discussion**

Recently we have reported in both patients and rats that the multitarget VEGF receptor tyrosine kinase inhibitor sunitinib induces a rise in BP, loss of circadian BP rhythm, renal dysfunction, and proteinuria that is associated with a 2- to 3-fold rise in circulating ET-1 levels. Our current study shows that sunitinib administration is also associated with marked renal histopathological changes, especially glomerular endothelialitis. This renal toxicity was accompanied by increased urinary excretion of ET-1. Importantly, renal histopathology and urinary ET-1 excretion were not or were only partly reversible after 11 days of sunitinib withdrawal, although BP had already returned to baseline at that time.

Because sunitinib-induced hypertension and renal toxicity are associated with activation of the endothelin system, we evaluated whether these adverse effects could be prevented by the dual ET<sub>A</sub> and ET<sub>B</sub> receptor antagonist macitentan. Compared with sunitinib alone, coadministration of macitentan diminished renal injury, as reflected by a decrease in proteinuria, urinary ET-1 excretion, and severe glomerular intraepithelial protein deposition, but the sunitinib-induced rise in serum creatinine was not prevented. Initially, coadministration of macitentan completely blocked the sunitinib-induced rise in BP, but after 4 days, a secondary rise in BP was observed, although it was less pronounced than with sunitinib alone. Because the BP response to ET-1 bolus injections was abolished with the dose of macitentan applied, this secondary rise in BP cannot be explained by ineffective ET receptor blockade, indicating that, apart from activation of the endothelin system, other factors are likely to be involved in the sunitinib-induced hypertension. Of note, in contrast to the current findings, hypertension induced by the multitarget tyrosine kinase inhibitor ABT-869 could be completely prevented by the selective ET<sub>A</sub> receptor blocker atrasentan.

Whether selective ET<sub>A</sub> receptor blockade could also prevent renal toxicity has not been evaluated in that study.

Although, ET<sub>B</sub> receptor–dependent systemic vasodilation has been observed in healthy volunteers, this effect is lost in pathological conditions, such as atherosclerosis and type 2 diabetes mellitus, possibly because of upregulation of contractile smooth muscle cell ET<sub>B</sub> receptors. Thus, in pathological conditions, dual ET<sub>A</sub>/ET<sub>B</sub> receptor blockade may be more favorable than selective ET<sub>B</sub> receptor blockade. However, chronic kidney disease might be an exception, because in this condition, ET<sub>B</sub> receptor–mediated vascular constriction has been reported to be less important than ET<sub>B</sub> receptor–mediated vasodilation. If, in chronic kidney disease, renal histopathological changes are mainly ET<sub>A</sub> receptor mediated, selective ET<sub>A</sub> receptor blockade might be preferred. Indeed, in young hypertensive Ren-2 transgenic rats on a high-salt diet immediately after weaning, chronic administration of the selective ET<sub>A</sub> receptor blocker atrasentan diminished proteinuria and renal injury to a greater extent than the dual ET receptor antagonist bosentan. However, in adult hypertensive Ren-2 transgenic rats on a high-salt diet, no differences regarding proteinuria and renal histology between both compounds were found. Furthermore, the dual ET<sub>A</sub>/ET<sub>B</sub> receptor blocker macitentan that was used in the present study reduced renal injury in the streptozotocin-induced diabetic rat. Taken together, these findings provide strong evidence that dual ET<sub>A</sub>/ET<sub>B</sub> receptor blockade, like selective ET<sub>A</sub> antagonism, exerts renal protective effects in various models of renal injury.

Coadministration of sunitinib and macitentan did not lower serum creatinine and had only modest effects on renal histology but did reduce urinary ET-1 excretion and proteinuria. How can we explain this discrepancy? Because urinary ET-1 excretion is kidney derived and reflects renal injury when increased, a decrease in this parameter indicates improvement of renal injury. Apparently, this decrease in urinary ET-1 excretion is not necessarily accompanied by reversal of the renal histological abnormalities or normalization of serum creatinine concentration, suggesting that increased urinary ET-1 excretion is a very sensitive marker of renal injury. ET-1 is known to increase glomerular permeability to albumin. This effect is BP independent and is mediated by a direct effect on the cytoskeleton of podocytes. Thus, the observed decrease in proteinuria with macitentan might be a direct consequence of renal ET receptor blockade. In addition, BP decreased by 60% during administration of macitentan. This decrease in BP might have contributed to the decrease in proteinuria by lowering glomerular filtration pressure.

Oxidative stress is a common pathway for the development of renal injury and has been shown to be involved in the development of hypertension in several animal models. Therefore, we explored whether the superoxide-dismutase mimetic Tempol had a beneficial effect on sunitinib-induced renal toxicity and hypertension. Although coadministration of Tempol had only a small effect on the development of hypertension induced by sunitinib and no effect on renal histological abnormalities, proteinuria and urinary ET-1 excretion were markedly reduced, thus indicating that oxidative stress is more important for sunitinib-induced renal functional toxicity than for sunitinib-induced hypertension. Urinary excretion of thiobarbituric acid reactive substances did not increase in response to sunitinib administration, suggesting that this is a measure of global rather than of renal oxidative stress.

Urinary excretion of nitrates as a measure of NO availability decreased during sunitinib administration. This is in agreement with observations that NO synthesis is activated by VEGF through Akt-dependent phosphorylation of endothelial NO synthase. Moreover, administration of a specific antibody against the VEGF2 receptor was associated with a reduced expression of endothelial and neuronal NO synthase in the mouse kidney. Using the Langendorff coronary...
perfusion model, we reported impaired coronary flow responses to the endothelium-dependent vasodilator bradykinin in the hearts obtained from rats exposed to sunitinib. However, in this model, responses to the endothelium-independent vasodilator sodium nitroprusside and the vasoconstrictor angiotensin II were also impaired, indicating generalized microvascular dysfunction rather than selective endothelial dysfunction in response to sunitinib administration. Coadministration of macitentan or Tempol did not normalize the impaired microvascular function induced by sunitinib, although the vasodilator response to sodium nitroprusside tended to improve.

Because the hypertension, proteinuria, renal function impairment, and activated endothelin system induced by sunitinib closely resemble the features of preeclampsia, we explored whether there is also resemblance with regard to renal histopathology in this disease. Indeed, kidneys of sunitinib-exposed rats showed pronounced glomerular endotheliosis, a characteristic renal abnormality in preeclamptic women (Figure S3). Sunitinib-associated toxicity not only resembles preeclampsia with regard to the mentioned clinical and histopathological features but also with regard to the underlying pathophysiology. Preeclampsia is associated with increased placental production of sFlt-1, a soluble VEGF binding receptor with antiangiogenic properties. Furthermore, injection of recombinant adenovirus encoding the murine sFlt-1 gene product in pregnant rats induced a rise in BP and proteinuria, as well as glomerular endotheliosis, intraepithelial protein resorption droplets, and foot-process effacement, all changes fully identical to the abnormalities found in our rats. Increased oxidative stress is also likely to play a role in the development of preeclampsia, as demonstrated by decreased symptoms of preeclampsia in sFlt-1–injected rats administered Tempol during pregnancy.

Recently, Murphy et al showed that infusion of sFlt-1 in healthy pregnant rats was accompanied by a 3-fold increased expression of prepro–ET-1 mRNA in the renal cortex, whereas the expression in the aorta and placenta was not increased. In contrast to these findings, no increased expression of prepro–ET1 mRNA levels in renal cortex or medulla was found in our rat model, although we did observe an increase in circulating ET-1 levels and urinary ET-1 excretion. This rise may, therefore, be attributed to other mechanisms, for example, an increase in endothelin-converting enzyme activity and/or a reduction in ETB receptor number. Blockade of the latter (clearance) receptor by macitentan explains why the circulating ET-1 levels increased even further during coadministration of sunitinib and macitentan. However, urinary ET-1 excretion decreased during coadministration of macitentan. Given clinical observations that urinary ET-1 excretion is a marker for renal injury (vide supra), this most likely reflects the beneficial renal effects of macitentan. A dissociation between circulating and renal ET systems has also been described in patients with systemic inflammatory disease and active renal involvement. Furthermore, our findings showing normalization of circulating ET-1 levels after sunitinib withdrawal, although the increased urinary ET-1 excretion, as well as renal functional and histological abnormalities, did not (completely) normalize, are also in agreement with the concept of separate mechanisms regulating systemic and renal ET-1 levels.

Because sunitinib administration is associated with a marked rise in BP, the sunitinib-induced renal toxicity might be secondary to the BP rise. Nonetheless, the rise in BP and renal toxicity associated with sunitinib are more likely to be independent adverse effects. First, the rise in BP occurred as soon as after 1 day of administration of sunitinib. Second, despite BP normalization after sunitinib withdrawal, renal histological abnormalities were still present. Third, it has been demonstrated that conditional gene targeting to selectively delete VEGF from renal podocytes in adult mice results in profound glomerular injury that precedes the development of hypertension. VEGF is produced by glomerular podocytes and is necessary for maintaining a healthy fenestrated glomerular endothelium by interacting with endothelial cell VEGF receptors. Fourth, the independence of the sunitinib-induced hypertension and renal toxicity is also supported by our observations in 8- to 9-week–old SHR rats. Although these rats were hypertensive since birth, with BPs as high as 146±3 mm Hg, glomerular injury was absent. Lastly, as mentioned previously, ET-1 has been reported to increase glomerular permeability independent of BP.

Perspectives
Angiogenesis inhibition with sunitinib induces hypertension and marked renal abnormalities associated with activation of the endothelin system that are partly reversible after sunitinib withdrawal. Endothelin receptor antagonism with macitentan can, to a large extent, prevent the sunitinib-induced rise in BP, whereas Tempol only mildly reduces this rise in BP. However, both compounds reduce sunitinib-induced proteinuria and urinary ET-1 excretion, with little effect on sunitinib-induced renal histological changes. Therefore, oxidative stress appears to be mainly important for the development of sunitinib-induced proteinuria and urinary ET-1 excretion, whereas the ET-1 system plays a role in the sunitinib-induced hypertension, as well as urinary excretion of protein and ET-1. Considering these findings and taking into account that ET-1 has also been shown to promote angiogenesis in cancer, ET-1 receptor antagonists appear to be logical candidates for treatment of sunitinib-induced cardiovascular and renal adverse effects and may even provide complementary therapeutic antiangiogenic effects. However, further studies in patients are warranted. Our current findings and the previously reported cases of severe renal injury in patients treated with an angiogenesis inhibitor support our recommendation to closely monitor renal function and BP in patients subjected to angiogenesis inhibition.

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Disclosures
None.

References
The Vascular Endothelial Growth Factor Receptor Inhibitor Sunitinib Causes a Preeclampsia-Like Syndrome With Activation of the Endothelin System
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THE VEGF-RECEPTOR TYROSINE KINASE INHIBITOR SUNITINIB CAUSES A PREECLAMPSIA-LIKE SYNDROME WITH ACTIVATION OF THE ENDOTHELIN-SYSTEM

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Expanded methods

*ET-1 challenge study*
After 8 days of administration of sunitinib and macitentan, in 4 rats the carotid artery and jugular vein were canulated (canule 0.58x0.99, SciComInc) under isoflurane anesthesia. Blood pressure was measured (ADinstruments, Powerlab, Labchart) in response to increasing intravenous bolus injections (100, 300, 600, 800 and 1000 pmol/kg) of endothelin-1 (ET-1) and compared to the responses in control rats (n=3). At the end of the experiment, rats were sacrificed.

*In vitro studies and renal histology*

*Langendorff studies*
Hearts were rapidly excised from euthanized rats and perfused according to Langendorff.\(^1\) Coronary flow (CF) was measured with a flow probe (Transonic systems). After a stabilization period of 30 minutes, baseline values of CF were obtained. Next, bolus injections (100 \(\mu\)L) of Tyrode’s buffer were applied three times to determine injection-induced changes in CF. Dose-response curves to bradykinin and Ang II were constructed by bolus injections, after which the maximum CF was determined by injecting sodium nitroprusside (SNP; 10 mmol/L).

*Light microscopy*
The left kidney was rapidly excised from euthanized rats, decapsulated, weighed and sliced transversely. One slice was fixed in a 3.5-4% formaldehyde solution for light microscopic and another slice was fixed in 2% glutaraldehyde for electron microscopic evaluation. After fixation in the formaldehyde solution, tissue was dehydrated and paraffin-embedded. Deparaffinized 2-\(\mu\)m thick sections were stained for Haematoxilin-eosine (HE), PAS and Jones silver. Sections were blindly evaluated by a pathologist for the presence (score 1) or absence (score 0) of endothelial cell and epithelial cell swelling 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 intra-epithelial protein was evaluated using a semiquantitative scale: 0 (no protein in the glomerular epithelium), 1 (protein present in 1-50% of the epithelial cells of a glomerulus), 2 (protein present in >50% of the epithelial cells of a glomerulus). Fifty glomeruli per kidney section (PAS staining) were evaluated.

References
Figure S1.
Change of mean arterial pressure (MAP; A) and heart rate (HR; B) compared to baseline in response to bolus injections of endothelin-1 (ET-1) in rats administered sunitinib and macitentan (n=4) for 8 days and in control rats administered vehicle (n=3).
Coronary flow responses to bradykinin (BK; A), ang II (B) and a single injection to sodium nitroprusside (SNP; 10 mmol/L); C) in isolated rat hearts after administration of vehicle (n=9), sunitinib and vehicle (n=10), sunitinib and macitentan (n=4), and sunitinib and tempol (n=6) for 8 days. The x-axis in A and B displays the concentration in the injection fluid. T indicates Tyrode’s buffer. *P<0.05 vs sunitinib + vehicle; †P<0.05 vs vehicle.
Figure S3.
A. Electron micrograph of a normal glomerulus in a healthy pregnant woman (magnification ×4000). Note the open capillary lumen, endothelial fenestrations (white arrowheads) and normal podocytes (white asterix) with open slit pores (white arrow). B, C. Electron micrographic overviews of glomeruli in patients with preeclampsia (magnifications ×2500 and ×5000 respectively). Note the narrow or completely occluded capillary lumen (white asterix) due to extreme swelling of endothelial cells. The glomerular basement membrane is only slightly irregular but mostly within normal limits. The cytoplasm of the endothelial cells shows some degenerative vacuolization (white arrow). The epithelial cells are swollen and show intra-cytoplasmatic resorption droplets (black asterix). Furthermore, there is extensive fusion of the podocyte foot processes with narrowing of the slit pores (white arrowheads).