Cardiovascular, renal and thyroid toxicity during angiogenesis inhibition:

A translational approach

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Cardiovascular, Renal and Thyroid Toxicity During Angiogenesis Inhibition: a Translational Approach

Cardiovasculaire, nefro- en schildkliertoxiciteit tijdens angiogeneseremming: een translationele benadering

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To the memory of my father

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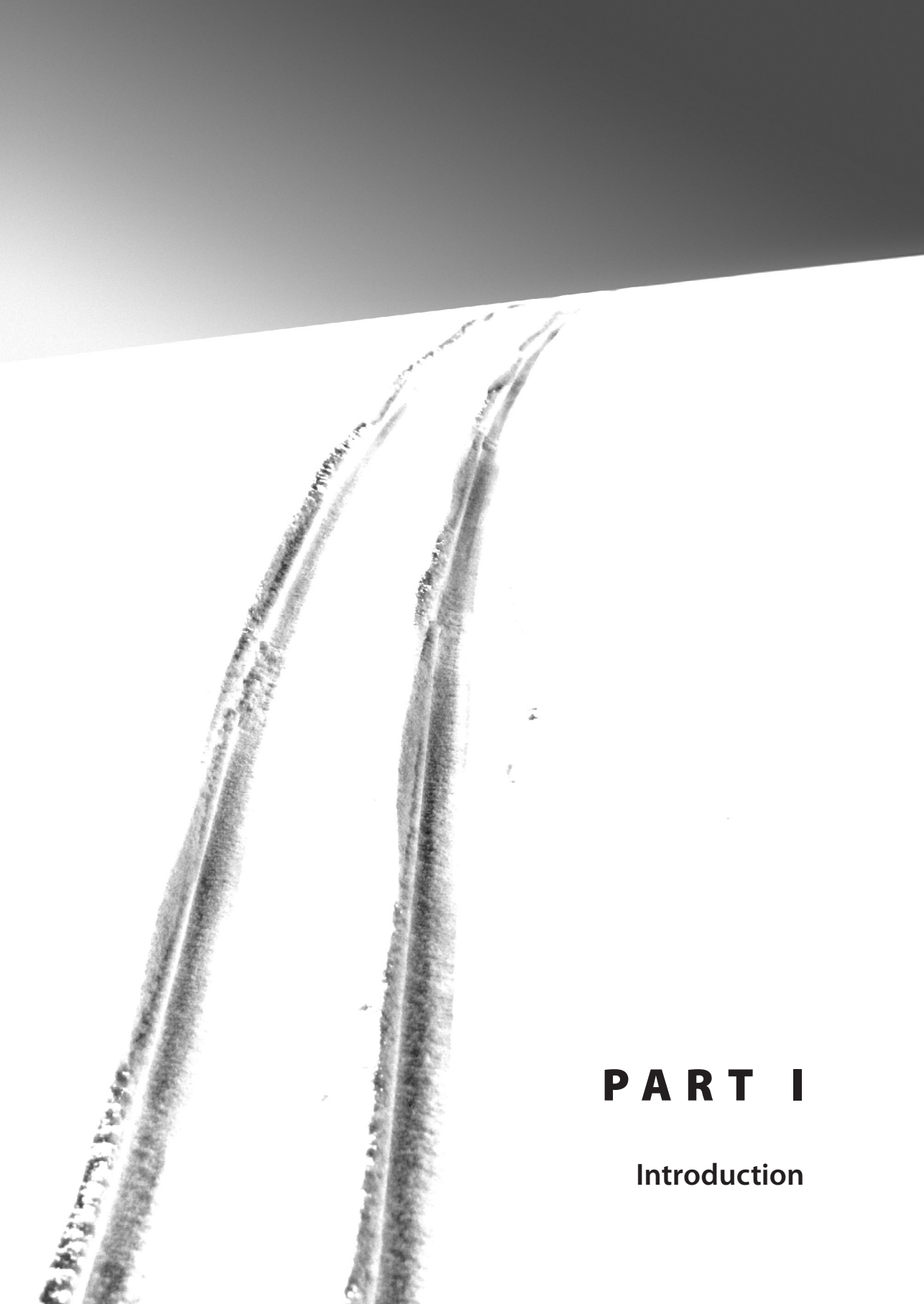

PART I

Introduction

1

Cardiovascular and renal toxicity during angiogenesis inhibition: clinical and mechanistic aspects

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ABSTRACT

Inhibition of angiogenesis with humanized monoclonal antibodies to vascular endothelial growth factor (VEGF) or with tyrosine kinase inhibitors targeting VEGF receptors has become an established treatment for various tumor types. Contrary to expectations, angiogenesis inhibition by blocking VEGF-mediated signaling is associated with serious side effects including hypertension and renal and cardiac toxicity in a substantial proportion of patients. Fortunately, most of these side effects as discussed in this paper seem to be manageable, but likely become more problematic when survival increases. Although several hypotheses regarding the etiology of angiogenesis inhibition-related cardiovascular and renal side effects have been postulated, many of the underlying pathophysiological mechanisms remain to be elucidated. This may lead to the development of more specific angiogenesis inhibitors, better management of their side effects and may potentially provide new insights into the pathogenesis of cardiovascular disease in general.

INTRODUCTION

Angiogenesis, the formation of new capillaries from an existing vasculature, is critical to tumor growth as well as metastasis. This process is regulated by several growth factors and their receptors among which vascular endothelial growth factor (VEGF) and its corresponding receptors play a key role. Angiogenesis inhibition as a therapeutic strategy against malignancies was first proposed by Folkman in 1971.[1] Meanwhile a variety of drugs, that target VEGF or its receptors, have been developed for the treatment of different tumor types and the expectation is that a number of new agents will be introduced within coming years. VEGF receptors (VEGFRs) are mainly expressed on endothelial cells. As over 99% of endothelial cells is quiescent under physiological conditions, it was expected that angiogenesis inhibition would have minimal side effects.[2] However, clinical experience has revealed that inhibition of VEGF induces several side effects including hypertension and renal and cardiac toxicity. Insight into the pathophysiological mechanisms of these side effects likely contributes to improved management of the toxicities associated with VEGF inhibition. Furthermore, the cardiovascular side effects observed with angiogenesis inhibition may provide new insights into the pathogenesis of cardiovascular disease in general. In this review we focus on the physiology of VEGF, its receptors and the signal transduction involved after VEGFR stimulation, the various forms of VEGF inhibition currently available, the vascular, renal and cardiac side effects of VEGF inhibition and potential pathophysiological mechanisms and proposals for the management of side effects, in particular angiogenesis inhibition-associated hypertension.

VEGF AND ITS RECEPTORS

VEGF, a 45 kDA glycoprotein, is an angiogenic growth factor normally produced by endothelial cells, podocytes, macrophages, fibroblasts and in malignancies by tumor cells or adjacent stroma.[3] Alternative splicing of the VEGF gene results in six different isoforms constituting of respectively 121, 145, 165, 183, 189 and 206 amino acids.[4,5] All isoforms express identical biological activity, but different binding to heparin and extracellular matrix. Loss of the heparin-binding domain of VEGF results in a reduction of its angiogenic activity. $VEGF_{165}$ (VEGF-A) is the predominant, most biologically active isoform and will be referred to as VEGF in the review. As depicted in Figure 1, the expression of VEGF is stimulated and regulated by multiple factors. Among these factors, hypoxia is the main stimulator of VEGF transcription mediated through the hypoxia inducible factor 1 (HIF-1).[3,4] Transcription of the VEGF gene is inhibited by tumor necrosis factor alpha (TNF-α). VEGF upregulates the expression of endothelial nitric oxide synthase (eNOS)

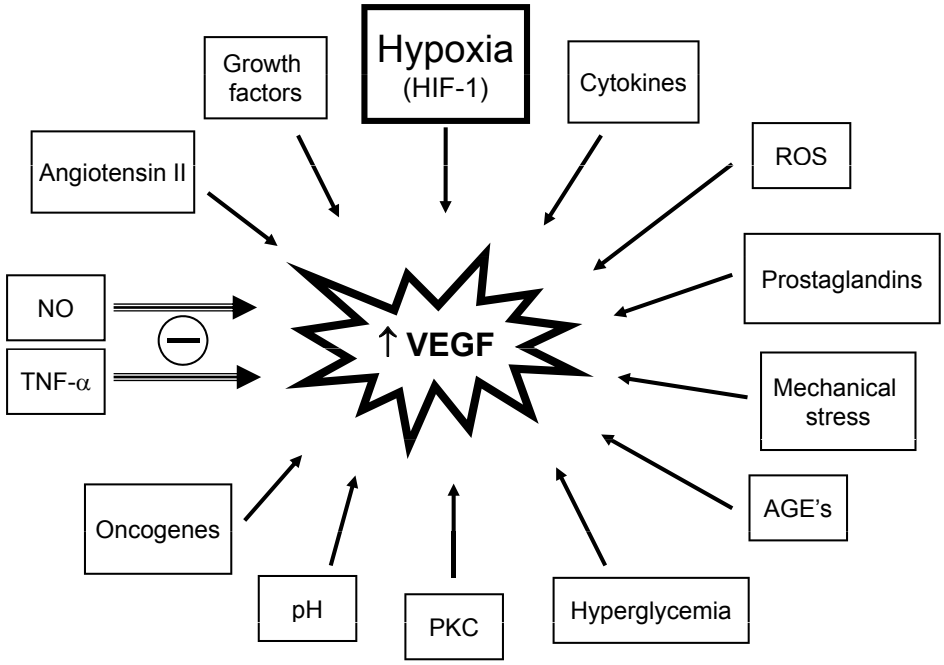

Figure 1. Regulation of VEGF expression. AGE's, advanced glycation endproducts; HIF-1, hypoxia inducible factor 1; NO, nitric oxide; PKC, protein kinase C; ROS, reactive oxygen species; TNF-α, tumour necrosis factor α, VEGF, vascular endothelial growth factor.

and increases nitric oxide production. Nitric oxide on the contrary may down-regulate VEGF expression via a negative feedback mechanism.[6] Tumor suppressor genes and oncogenes have also been found to play an important role in regulating VEGF gene expression. Loss or inactivation of tumor suppressor genes, such as von Hippel-Lindau (VHL), p53, p73, Phosphatase and Tensin homolog (PTEN) and p16, as well as activated forms of oncogenes, such as Ras, Src, human epidermal growth factor receptor 2 (HER2/neu) and Breakpoint cluster region/Abelson (Bcr/Abl), increase VEGF gene expression.[7]

VEGF binds two tyrosine kinase receptors, VEGF receptor 1 [VEGFR-1 or fms-like tyrosine kinase (Flt-1) murine homologue] and VEGF receptor 2 [VEGFR-2 or kinase domain region (KDR) human homologue or Flk-1 murine homologue]. Both receptors contain an extracellular region consisting of seven immunoglobulin-like domains, a hydrophobic transmembrane domain and a cytoplasmatic bipartite tyrosine kinase domain. VEGFR-1 and VEFGR-2 are expressed on endothelial cells of most blood vessels, including those of preglomerular, glomerular and peritubular vessels. Furthermore, these receptors are present on hematopoietic stem cells, circulating endothelial progenitor cells, dendritic cells, trophoblasts, monocytes, retinal progenitor cells and certain types of tumor cells.[3,8] Most of the biologically relevant VEGF signaling in endothelial cells is mediated by

VEGFR-2, activated by ligand-stimulated receptor dimerization and trans- (auto-) phosphorylation of the tyrosine residues in the cytoplasmatic domain.[9]

The extracellular domain of Flt-1 is also present as a soluble protein (sFlt-1) that inhibits angiogenesis by forming an inactive complex with circulating VEGF. Soluble Flt-1 can act as a physiological VEGF inhibitor by binding and inactivating membrane-bound VEGFR-1 and VEGFR-2 via receptor homodimerization or heterodimerization.[8] VEGFR-1 has a 10-fold higher affinity for binding VEGF than VEGFR-2 but autophosphorylation of the tyrosine residues of the VEGFR-1 in response to VEGF binding is weak.[8] VEGFR-1, like sFlt-1, may rather perform a decoy function by sequestering VEGF and leaving less VEGF available for VEGFR-2, than mediating a mitogenic response. VEGFR-1 also binds placental growth factor (PlGF), which might be of importance for the reported synergism between VEGF and PlGF in vivo. In contrast to sFlt-1, PlGF potentiates the action of VEGF by displacing VEGF from VEGFR-1, thereby directing VEGF towards the VEGFR-2.[3]

VEGFR-3 (fms-like-tyrosine kinase (Flt)-4) is also a member of the receptor tyrosine kinases, but only responds to VEGF-C or VEGF-D and not to VEGF-A. This receptor is mainly found on lymphatic endothelium and is important for lymphangiogenesis. VEGF also interacts with neuropilin-1 and -2 receptors. The interaction of neuropilin-1 and VEGFRs facilitates VEGF binding to the VEGFR.[3,7]

VASCULAR ENDOTHELIAL GROWTH FACTOR SIGNAL TRANSDUCTION AND BIOLOGICAL EFFECTS

General aspects

VEGF exerts a variety of biological activities. Its original name, vascular permeability factor, indicates that it enhances permeability. Furthermore, VEGF plays a key role in the mobilization of endothelial progenitor cells from the bone marrow, endothelial cell proliferation, migration, survival and tube formation. It is a potent stimulator of angiogenesis during embryogenesis, menstrual cycle, wound healing and tumor growth. In addition, it inhibits antigen-presenting dendritic cells and stimulates monocyte chemotaxis and the expression of adhesion molecules. Finally, it induces vasodilation through activation of the nitric oxide pathway.[3] The mentioned activities of VEGF are effectuated via several pathways including activation of the PI3K/Akt (protein kinase B)/mTOR pathway, partly mediating VEGF-induced nitric oxide production via eNOS phosphorylation (Fig. 2). Other actions include the activation of phospholipase C-γ (PLC-γ), protein kinase C (PKC), Raf-1, extracellular-signal-regulated protein kinase (ERK1/2), fatal adhesion kinase (Fak) and mitogen-activated protein kinase (MEK1/2) pathways.

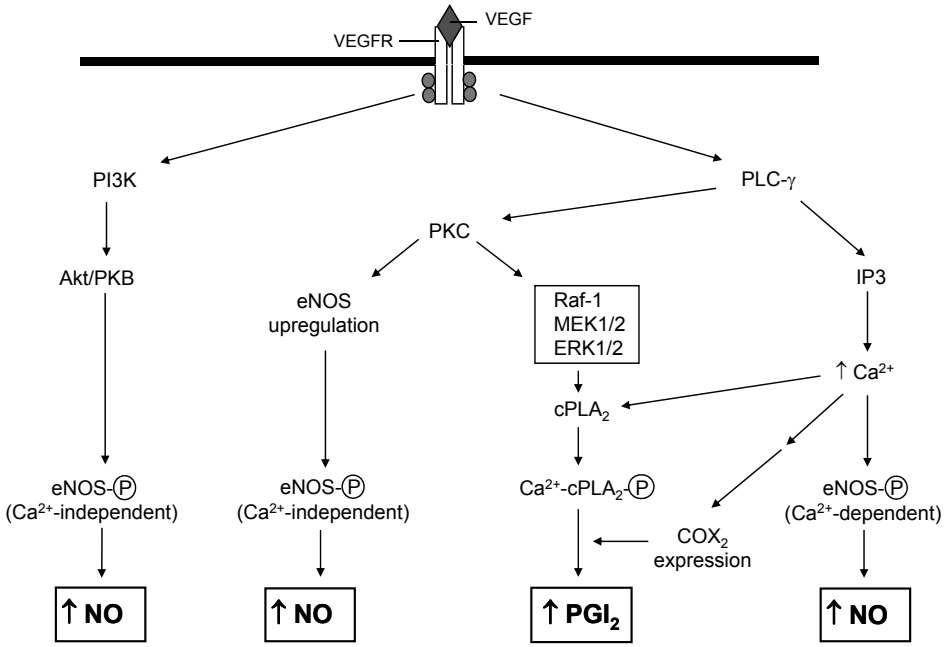

Figure 2. NO and PGI_2 synthesis through VEGF-mediated signaling. Akt/PKB, anti-apoptotic kinase / protein kinase B; Ca^{2+}, calcium; COX_2, cyclo-oxygenase 2; $cPLA_2$, cytosolic phospholipase A_2; $cPLA_2$-P, phosphorylated $cPLA_2$; eNOS, endothelial nitric oxide synthase; e-NOS-P, phosphorylated eNOS; ERK1/2, extracellular-signal-regulated protein kinase; IP_3, 1,4,5-triphosphate; MEK1/2, mitogen-activated protein kinase; NO, nitric oxide; PGI_2, prostacyclin; PKC, proteinkinase C; PIK3, phosphoinositide 3-kinase; PLC-γ, phospholipase C; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Like the PI3K/Akt/mTOR pathway, the PLC-γ pathway also plays a crucial role in the production of nitric oxide and prostacyclin (PGI_2) by endothelial cells.[9-11] Calcium mobilization is essential for VEGF-induced PGI_2 production. In addition, calcium signalling also mediates short-term nitric oxide production through activation of the constitutive eNOS isoform. Calcium-independent nitric oxide production mediated via the PI3K/Akt/mTOR pathway may also involve VEGF-induced up-regulation of eNOS mRNA, whereas long-term prostanoid production is mediated through VEGF-induced expression of the inducible cyclo-oxygenase (COX)-2 isoform (Fig. 2).[9] In human umbilical vein endothelial cells, VEGF stimulates a biphasic, dose-dependent and time-dependent release of nitric oxide: an initial acute phase (1 h) regulated by calcium-spiking and a secondary chronic phase (> 24 h) caused by an increase in eNOS mRNA transcription. The VEGF-induced upregulation of eNOS mRNA and protein levels is dose-dependent.[6]

Part of the VEGF signaling occurs in a paracrine way, which is essential for the proliferation, survival, permeability responses and endothelial differentiation of the angiogenic cascade. An autocrine signaling loop (cell-autonomous) for VEGF is also required for

survival of blood vessels. Both paracrine and autocrine activation are mediated by VEGFR-2.[12]

Vascular effects induced by vascular endothelial growth factor

VEGF has shown to induce endothelium-dependent vasorelaxation in arteries of different sizes of various species including human vessels.[13-17] This vasodilatation appears to be mainly mediated via nitric oxide, as it is attenuated in the presence of N-nitro-L-arginine (L-NNA), an eNOS inhibitor.[13-15] In the presence of both L-NNA and HbO, a nitric oxide scavenger, the VEGF-induced vasodilatation is further decreased and it is almost completely abolished after removal of the endothelium, indicating a crucial role of the endothelium in the VEGF-mediated vasorelaxation.[16-18] This endothelium-dependent vasodilation is mainly due to VEGFR-2 stimulation.[17] In internal mammary arteries obtained from patients with severe coronary artery atherosclerosis, both PGI_2 and nitric oxide appeared to contribute to the VEGF-mediated vasorelaxation.[15] VEGF-induced vasorelaxation is impaired in spontaneously hypertensive rats (SHR) compared with normotensive Wistar Kyoto (WKY) rats, possibly related to endothelial dysfunction in these rodents.[13] In porcine vessels VEGF dilates the small-sized arterioles and venules and has no vasorelaxing effect on medium-sized arteries and veins.[14]

In addition to the observed vasorelaxation *in vitro*, *in vivo* experiments showed that intravenous injection of VEGF in conscious male Sprague-Dawley rats resulted in a dose-dependent decrease in mean arterial pressure and an increase in heart rate almost immediately after VEGF injection.[17] An intravenous bolus injection of VEGF in anesthesized rats caused an acute reduction in diastolic blood pressure of more than 20 mmHg, whereas a second injection of VEGF 40 min later had no effect, indicating that VEGF-related vasodilatation is subject to tachyphylaxis.[19] In the VEGF in Ischemia for Vascular Angiogenesis (VIVA) trial performed in patients with coronary artery disease both intracoronary and intravenous infusions of recombinant human VEGF produced dose-related falls in systolic blood pressure of up to 22% with the highest dose and facial flushing.[20] In summary, VEGF induces hypotension through vasorelaxation, which appears to be endothelium-dependent and in part mediated by nitric oxide.

VASCULAR ENDOTHELIAL GROWTH FACTOR INHIBITION (ANTI-ANGIOGENESIS)

As diseases relying on angiogenesis such as cancer are often partially driven by VEGF, inhibition of angiogenesis as a therapeutic strategy against malignancies was proposed by Folkman already in 1971.[1] Meanwhile a variety of drugs, which target VEGF or its

receptors, have been developed for the treatment of cancer including monoclonal antibodies to VEGF, small receptor tyrosine kinase inhibitors (RTKIs) and circulating VEGF receptors to trap VEGF ('VEGF-Trap'). Bevacizumab (Avastin; Genentech, South San Francisco, California, USA) is a humanized monoclonal antibody that selectively binds VEGF and was the first FDA-approved VEGF inhibitor for systemic use in various forms of cancer including colorectal, breast, renal and nonsquamous, non-small cell lung cancer.[21-24] Bevacizumab has to be administered intravenously in contrast to the RTKIs such as sunitinib (SU011248, Sutent; Pfizer, New York, New York, USA) and sorafenib (Bay 43-9006, Nexavar; Bayer Pharmaceuticals, West Haven, Connecticut, USA and Onyx Pharmaceuticals, Richmond, California, USA), which are suitable for oral administration. These agents are not selective, as they target a number of tyrosine kinases. For instance, sunitinib inhibits VEGFR-1, VEGFR-2, VEGFR-3, platelet derived growth factor (PDGFR)-α and PDGFR-β, c-KIT, fms-like tyrosine kinase-3 (Flt3), colony stimulating factor receptor type 1 and the glial cell line-derived neutrophic factor receptor RET (rearranged during transfection). Sunitinib is approved for the treatment of imatinib-resistant metastatic gastrointestinal stromal tumors (GIST) and first-line treatment of metastatic renal cell carcinoma.[24,25] Sorafenib is approved for the treatment of advanced renal cell carcinoma after failure of interleukin-2 or interferon-α treatment.[24,26] The RTKIs are administered in cycles according to a 4 weeks "ON" and 2 weeks "OFF" (wash-out period) scheme. VEGF-Trap (Regeneraon Pharmaceuticals, Tarrytown, New York, USA) is a protein consisting of portions of the extracellular domains of VEGFR-1 and VEGFR-2, fused to the Fc-portion of human immunoglobulin γ1. It binds and thereby inactivates VEGF in the circulation and tissues. The clinical effectiveness of this drug has still to be determined.[26]

Anti-angiogenic therapy rarely produces complete tumor regression and resistance to treatment develops after a median of 6-12 months of therapy. Mechanisms responsible for tumor cell resistance may include incomplete inactivation of the VEGF pathway, activation of other hypoxia-inducible factor-driven genes or selection of tumor cell populations able to survive in the presence of VEGFR-2 blockade for example through activation of VEGF-independent pathways such as fibroblast growth factor and interleukin-8.[27]

SIDE EFFECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR INHIBITION

As over 99% of endothelial cells is quiescent under physiological conditions, side effects during angiogenesis inhibition were expected to be minimal.[2] Meanwhile, it has been well established that inhibition of VEGF is associated with the development of several untoward effects (table 1).[24,28,29] In particular RTKIs, which also inhibit numerous

Table 1. Clinical adverse effects of VEGF-signaling inhibitors

Constitutional	Fatigue, fever
Gastrointestinal	Diarrhea, nausea, mucositis / stomatitis, vomiting, constipation, abdominal pain, gastrointestinal perforation
Cardiovascular	Hypertension, left ventricular dysfunction, cardiac failure, cardiac ischemia or infarction
Renal	Proteinuria, glomerulonephritis, thrombotic micorangiopathy
Haemorrhage and thrombosis	Epistaxis, bleeding at all sites, venous and arterial thrombosis
Endocrine dysfunction	Thyroid dysfunction (hypo- and hyperthyroidism)
Haematology	Leucopenia, neutropenia, anemia, thrombopenia, lymphopenia
Dermatology	Hand-foot syndrome, skin discoloration, rash
Metabolism / nutrition	Anorexia, asthenia
Respiratory	Dyspnea, cough
Neurology	Altered taste, headache
Musculoskeletal	Arthalgia, back pain, myalgia
Impaired wound healing	

Table based on information from [24,28,29].

receptors other than the VEGFR, are characterized by a broad spectrum of toxicities. Of these, hypertension and renal toxicity are also observed when using monoclonal antibodies directed towards VEGF. As these antibodies specifically inhibit VEGF and do not directly affect other factors, hypertension and renal toxicity can be considered truly VEGF-inhibition-dependent side effects. Given the relationship of hypertension and renal toxicity with cardiac events, the latter will be addressed as well. Cardiac toxicity has been reported less frequently with the use of bevacizumab than with RTKIs, suggesting that this side effect is in part independent of VEGF inhibition *per se*. However, this side effect was not routinely monitored for and could therefore have been underreported.

Hypertension

Hypertension has been reported in up to 36% of patients during treatment with the humanized VEGF antibody bevacizumab with blood pressure normalization after treatment cessation.[21,30,31] Initial reported incidences of sunitinib-induced hypertension varied from 16-23%, but more recent studies reported an incidence of up to 47% (Table2).[32-41] This difference in reported incidence of hypertension might be related to greater awareness of the development of this side effect and to the fact patients with poorly controlled hypertension were excluded from participation in the initial trials. Moreover, according to the Common Toxicity Criteria for Adverse Events, hypertension in earlier studies was diagnosed when blood pressure was more than 150/100 mmHg or diastolic blood pressure had increased by more than 20 mmHg.[42] These threshold values are considerably higher than those recommended by the ESH/ISH. With more complete

Table 2. Incidence of hypertension in patients treated with sunitinib

Reference	Disease	Sunitinib dose (mg/day)	Treatment duration	No. of patients	Hypertension (%)
Azizi *et al.*[34]	mRCC	50	12 weeks (2 cycles)	7[a]	100[a]
Zhu *et al.*[35] (unpublished data)	NA	NA	NA	NA	22.5
Chu *et al.*[33]	GIST[b]	50	24 weeks	75	47
Rixe *et al.*[36]	mRCC	50	12 weeks (2 cycles)	40	22.5
Motzer *et al.*[37]	mRCC	50	6 months	375	24
Motzer *et al.*[38]	mRCC	50	7 months	106	16
Faivre *et al.*[39]	Solid malignancy[c]	50-150	3-4 weeks	28	18
Motzer *et al.*[40]	mRCC	25-75[d]	9 months	63	5
Demetri *et al.*[41]	GIST[b]	50	12 weeks (2 cycles)	207	11
Maki *et al.*[32]	GIST[b]	50	1 year	97	16

GIST, gastro-intestinal stromal tumor; mRCC, metastatic renal cell carcinoma; NA, not available. [a] Home blood pressure monitoring in 7 normotensive patients treated with sunitinib; after 4 weeks of treatment all 7 patients had become hypertensive. [b] imatinib-resistant metastatic GIST. [c] advanced solid malignancy for which no other therapy was possible. [d] intra-patient dose escalation up to 75 mg/day was permitted in the absence of treatment-related toxicity, dose reduction was allowed to 25 mg/day.

angiogenesis inhibition the incidence of hypertension appears to increase further. For instance the incidence of hypertension was 67% with the combined treatment of bevacizumab and sorafenib and 92% with the combination of bevacizumab and sunitinib in patients with advanced solid tumors or renal cell carcinoma respectively.[43,44]

The rise in blood pressure after initiation of angiogenesis inhibition is relatively fast and shows an "ON"/"OFF" effect similar to the treatment scheme as demonstrated by a study using home blood pressure monitoring.[34] After 4 weeks of treatment with sunitinib, blood pressure in patients who were normotensive at baseline had increased by 22 ± 6 mmHg systolic and 17 ± 6 mmHg diastolic and returned to baseline values during the 2 weeks off-treatment period.[34] Likewise, during treatment with the RTKI pazopanib (GW786034) hypertension developed during the first 4 weeks of treatment in almost all patients.[45] The reported severity of hypertension is variable, with in rare cases development of malignant hypertension complicated by the reversible posterior leukoencephalopathy syndrome or hemorrhagic stroke.[46-49]

The clinical finding of hypertension due to VEGF inhibition has been verified in experimental studies. Single oral doses of the RTKI cediranib (AZD2171, Recentin, Astra-

Zeneca) administered to Wistar-Kyoto rats was associated with a 15 mmHg rise in blood pressure, whereas daily administration of higher doses for 4 days was associated with a rise in blood pressure of 35-50 mmHg.[19]

Mechanism of hypertension

Although the mechanism underlying the development of hypertension induced by angiogenesis inhibition still remains to be elucidated, decreased nitric oxide bioavailability is thought to be a critical factor (Fig. 3). Because eNOS is upregulated by VEGF, inhibition of VEGF by neutralizing antibodies or a VEGFR blocker will lead to a decrease in nitric oxide production by endothelial cells that may account for the development of hypertension.[24] Clinical evidence for the presence of endothelial dysfunction induced by VEGF inhibition is still very limited. Hovens *et al.* measured flow-mediated and nitroglycerin-mediated dilatation of the brachial artery as respective measures of endothelial-dependent and independent vasodilatation in patients treated with the experimental RTKI telatinib (Bay 57-9352).[50] After 5 weeks of treatment systolic and diastolic blood pressure were increased by respectively 6.6 and 4.7 mmHg. This rise in blood pressure was associated with a small decrease in flow-mediated dilatation of 2.1% and a decrease in nitroglycerin-mediated dilatation of 5.1%. Although reduced nitric oxide availability might have caused the decrease in flow-mediated dilatation in this study, it cannot be ruled out that the development of hypertension itself had caused this reduction.[50] Of note, impaired nitric oxide production may not only cause a generalized vasoconstrictor response but may also affect renal sodium handling, contributing to the maintenance of hypertension in the longer run.[51]

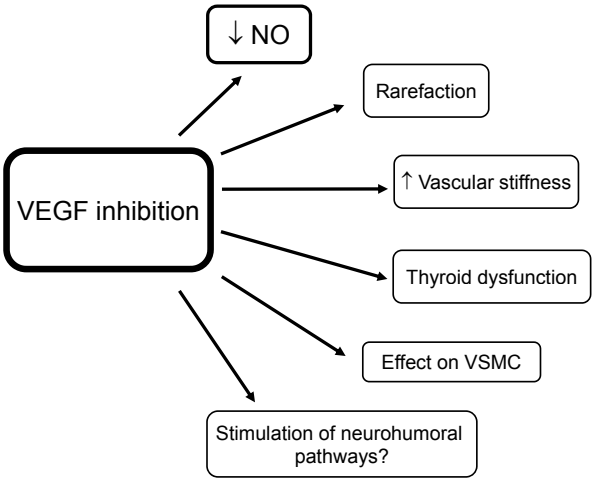

Figure 3. Possible mechanisms involved in hypertension due to vascular endothelial growth factor inhibition. NO, nitric oxide; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.

Due to the anti-angiogenic properties, another potential mechanism involved in the development of hypertension during angiogenesis inhibition is functional (a decrease in perfused microvessels) or structural (a reduction in capillary density) rarefaction.[52,53] Using intravital video-microscopy to measure dermal capillary densities in the dorsum of the fingers a significant decrease in functional and structural capillary densities after 6 months of treatment with bevacizumab compared with baseline was observed in patients, whereas blood pressure had increased by 16mmHg systolic and 8 mmHg diastolic.[54] In addition, a reduction of capillaries in the mucosa of the mouth in patients enrolled in a phase 1 study with telatinib has been reported.[53] Despite these observations, rarefaction as a cause of hypertension, at least in the initial phase of angiogenesis inhibition seems not very likely, considering the short interval at which hypertension can develop after initiation and the almost immediate reversibility after cessation of anti-angiogenic therapy. The observed rarefaction in the studies mentioned might be a consequence rather than a cause of hypertension. The same reasoning, that is, consequence rather than cause, might be true for the rise in large artery stiffness reported during angiogenesis inhibition.[50,55]

To further explore potential mechanisms involved in the development of hypertension during angiogenesis inhibition, Veronese *et al.* have measured plasma levels of catecholamines, renin, aldosterone, urotensin II and endothelin-1 (ET-1) in patients treated with sorafenib.[55] After a 3-week treatment period systolic blood pressure had increased by 20 mmHg in 60% of patients. This rise in blood pressure was initially accompanied by a decrease in heart rate. More interestingly, there was an inverse correlation between change in heart rate and change in systolic blood pressure after 3 weeks of treatment. Despite the rise in blood pressure, plasma concentrations of the previous mentioned parameters remained unchanged, suggesting that neither the renin-angiotensin system nor the sympathetic nervous system is involved in the development of hypertension.[55] In contrast to the study reported by Veronese *et al*, we found a marked decrease in plasma renin activity after a 4-week treatment period with sunitinib, whereas blood pressure at that time had increased by 15 mmHg systolic and 14 mmHg diastolic (n = 12) [unpublished data]. With respect to ET-1, blockade of VEGF signaling may disturb the balance between nitric oxide and ET-1, favoring vasoconstriction.[2] In a recently published study in telemetry-instrumented rats, the rise in blood pressure caused by an experimental TKI could be prevented by an ET_A receptor blocker.[56] As administration of this ET_A receptor blocker to untreated rats also lowered blood pressure, definite conclusions about the role of the endothelin system in the rise in blood pressure during angiogenesis inhibition cannot be drawn at this moment.

Thyroid dysfunction might be another factor that contributes to the development of hypertension during RTKI administration. Hypothyroidism or transient elevation of TSH levels have been reported in up to 46-85% of patients treated with sunitinib and hyperthyroidism in up to 24% of patients.[57-59] Since both hyperthyroidism and hypothyroidism have been reported to be associated with hypertension, such a mechanism might sometimes be involved in angiogenesis-inhibition associated hypertension. The mean time to develop hypothyroidism during treatment with sunitinib generally is 50 weeks, although it has been reported to occur as soon as after 12 weeks (cycle 2) of the treatment course. Therefore, hypothyroidism as a cause of hypertension, at least in the initial phase of angiogenesis inhibition, seems unlikely.[57,58]

Patients with renal cell carcinoma may be more susceptible to develop hypertension due to previous nephrectomy and renal dysfunction. In a meta-analysis of Wu *et al.* on the incidence and risk of hypertension associated with the use of sorafenib, no significant difference was detected between patients with or without a renal cell carcinoma.[60] It should be remarked that in this meta-analysis the incidence of hypertension was compared in relation to the presence of one or two kidneys rather than in relation to the actual renal function.

Renal toxicity

Another frequently observed side effect of angiogenesis inhibition is renal toxicity, predominantly reflected by proteinuria. According to the Common Terminology Criteria for Adverse Events version 3.0 proteinuria is classified in four different grades: grade 1 (0.15-1.0 g/24hrs corresponding with dipstick 1+), grade 2 (>1.0-3.5 g/24hrs corresponding with dipstick 2+ to 3+), grade 3 (>3.5 g/24hrs corresponding with dipstick 4+) and grade 4, nephrotic syndrome range proteinuria.[42] Table 3 provides an overview of the incidence of proteinuria in response to various angiogenesis inhibiting treatments.[30,31,61-66] Proteinuria related to treatment with bevacizumab is clearly a dose-dependent side effect and has been reported in up to 41-63% of patients.[35] Although patients with renal cell carcinoma may especially be prone to develop proteinuria because of the prior nephrectomy causing impaired renal function or because of the presence of the primary tumor in situ, the risk of proteinuria and hypertension related to bevacizumab treatment did not alter after exclusion of patients with renal cell cancer in a meta-analysis.[35]

Although bevacizumab-associated proteinuria is usually not severe, proteinuria more than 3.5 g/day has been reported in up to 6.5% of patients.[30] The incidence of proteinuria associated with RTKIs is not well known, because screening for this condition was not routinely performed in the initial studies. In a phase 1 trial of the investigational RTKI KRN951, 14 of 15 patients developed hypertension and three (20%) dose-limiting

Table 3. Incidence of proteinuria during VEGF inhibition

Reference	Disease	Regimen	No. of patients	Proteinuria (%)
Kabbinavar *et al.*[61]	mCRC	bevacizumab	67	25.3
Kabbinavar *et al.*[62]	mCRC	bevacizumab	244	13.1
Hurwitz *et al.*[21]	mCRC	bevacizumab	402	26.5
Yang *et al.*[30]	mRCC	bevacizumab	76	
		low-dose[a]		40.5
		high-dose[a]		64.1
Miller *et al.*[31]	mBC	bevacizumab	232	22
Johnson *et al.*[63]	NSCLC	bevacizumab	66	31.8
Nguyen *et al.*[64]	ARMD	VEGF-Trap	19	31.6
Rugo *et al.*[65]	Various cancers	AG013736[b]	36	8
Patel *et al.*[66]	GIST[c]	sunitinib / sorafenib	298	2.3[d]

ARMD, age-related macula degeneration; GIST, gastro intestinal stromal tumor; mBC, metastatic breast cancer; mCRC, metastatic colorectal cancer; mRCC, metastatic renal cell carcinoma; NSCLC, non-small cell lung carcinoma; VEGF, vascular endothelial growth factor. [a] low-dose bevacizumab: 3mg/kg body weight i.v. every two weeks, high-dose bevacizumab: 10mg/kg body weight i.v. every two weeks. [b] AG013736 (Axitinib, Pfizer Inc, New York, NY), an oral tyrosine kinase inhibitor of all VEGFRs, PDGFR-β and c-KIT. [c] case series of 7 patients with proteinuria were selected from the total population of patients treated with sunitinib or sorafenib, 5 patients with GIST, 1 patient with epitheloid hemangioendothelioma, 1 patient with teratocarcinosarcoma. [d]The true prevalence of sunitinib- or sorafenib-associated proteinuria is probably higher because patients were not routinely and systematically screened for the development of proteinuria.

proteinuria.[67] In contrast, in a report of Patel *et al*. only 2.3% of 298 patients treated with sunitinib or sorafenib developed substantial proteinuria (average level of 3.8 g/day) with a peak occurring after a median treatment period of 24 weeks.[66] The relationship between antiangiogenic treatment and proteinuria seems to be causal, since proteinuria diminished or disappeared after reduction or discontinuation of this treatment.[66,68]

The proteinuric effect of VEGF inhibition is reported to be increased in patients using non-steroidal anti-inflammatory drugs and bisphosphonates.[31] An apparent association between hypertension and proteinuria has been noted in patients with metastatic breast cancer treated with the combination of bevacizumab plus the cytotoxic drug capecitabine.[31] Patients who did develop proteinuria were more likely to have hypertension (47.1%) than those who did not (16.9%).

Renal biopsy specimens of patients treated with VEGF inhibitors who developed proteinuria have revealed various pathological abnormalities such as endotheliolysis, mesangiolysis, endothelial swelling, red cell fragments and thrombi.[68] Glomerular thrombotic microangiopathy is a commonly reported finding that reverses when the antiangiogenic drug is withdrawn.[68] The type and course of these renal lesions resemble those observed

during preeclampsia in which increased levels of s-Flt-1, produced by the placenta, bind and inactivate VEGF and PlGF. After delivery of the placenta, preeclampsia, including the renal lesions, resolves.[68] Other reported renal pathologies include cryoglobulinemic, collapsing, membranoproliferative, and ischemic glomerulopathies as well as interstitial nephritis.[69]

Mechanism of renal toxicity

In the human kidney, VEGF-expression is most prominent in glomerular podocytes and tubular epithelial cells, whereas VEGFR-1 and VEGFR-2 are mainly found on preglomerular, glomerular and peritubular endothelial cells.[5] Eremina *et al.* showed, by conditional gene targeting (deleting a single VEGF allele from renal podocytes), that local reduction of VEGF within the kidney of adult mice resulted in profound thrombotic glomerular injury.[68] This injury preceded the development of hypertension making hypertension as the cause of proteinuria unlikely. In this context it is interesting to note that disrupted glomerular VEGF signalling has also been implicated in the pathogenesis of preeclampsia.[70,71]

In a rodent model of crescentic glomerulonephritis, administration of the soluble VEGF receptor accelerated proteinuria with the development of massive ascites. Renal morphology revealed glomerulosclerosis and interstitial fibrosis associated with the loss of nephrin and endothelium.[72] Single intravenous infusions of anti-VEGF antibodies or s-Flt-1 into normal healthy mice resulted in excessive proteinuria via glomerular endothelial cell detachment and a decrease in the expression of nephrin.[73] Nephrin is a protein exclusively expressed by glomerular podocytes and is critical for the maintenance of the glomerular filtration barrier by preventing podocyte apoptosis.[72] It has been suggested that VEGF maintains podocyte function and survival by regulation of nephrin expression.[72] Given these findings, inhibition of glomerular VEGF signalling appears to be a plausible mechanism that underlies the renal toxic effects of angiogenesis inhibition. Remarkably, not only underexpression, but also VEGF overexpression may induce renal toxicity. VEGF overexpression by podocytes in mice led to development of a collapsing glomerulopathy.[74] In contrast, injection of VEGF in normal rats had no effect on kidney function and glomerular morphology, did not induce proteinuria and did not affect glomerular cell proliferation and the number of endothelial fenestrations.[5]

Cardiac toxicity

In addition to hypertension and renal toxicity, angiogenesis inhibition might also lead to cardiac toxicity. An overview of the incidence of cardiac toxicity in patients treated with sunitinib is provided in Table 4.[33,37,40,41,75] In a retrospective study impaired cardiac function has been observed in up to 28% of patients treated with sunitinib, indicated by

Table 4. Incidence of cardiac toxicity in patients treated with sunitinib

Reference	Disease	Sunitinib dose (mg/day)	Treatment duration[a]	No. of patients	Decrease in LVEF (% of patients)
Motzer *et al.*[37]	mRCC	50	6 months	375	10
Motzer *et al.*[40]	mRCC	50	9 months	63	11
Demetri *et al.*[41]	mGIST[b]	50	8 weeks	207	0
Chu *et al.*[33]	mGIST[b]	50	24 weeks	75	28
Khakoo *et al.*[75]	Various cancers	25-50	4 – 44 days	224	2.7
Schmidinger *et al.*[76]	mRCC	50[c]	8 weeks	74	12

LVEF, left ventricular ejection fraction; mGIST, metastatic gastrointestinal stromal tumor; mRCC, metastatic renal cell carcinoma. [a] Treatment duration after which a decrease in LVEF was reported. [b] Imatinib-resistent GIST. [c] Patients received either sunitinib 50 mg/day (schedule:'4 weeks ON / 2 weeks OFF') or sorafenib 800mg/day (continuously).

a decrease in left ventricular ejection fraction (LVEF) of 10-15% or more.[33] The median time to onset of congestive heart failure was 33 weeks (range 5 to 74 weeks). Multivariate analysis in sunitinib-treated patients revealed that coronary artery disease was the only independent predictor of congestive heart failure. A reduction in left ventricular ejection fraction (LVEF) was present in most of the patients treated with sunitinib and was related with the duration of treatment. Six percent (2/36) of the patients had LVEF reductions of at least 20% and 19% (7/36) of at least 25%, whereas 8% (3/36) of patients developed New York Heart Association class III–IV heart failure. A decrease in LVEF has also been reported in patients treated with sorafenib (19%) or bevacizumab (5-11%).[76,77] The cardiac toxicity seems to be non-reversible, although it should be noted that long-term follow-up after discontinuation of the angiogenesis inhibitors is lacking.[75] Apart from a reduction in LVEF, other abnormalities, varying from an increase in biomarkers (e.g. creatine kinase, creatine kinase MB, cardiac troponin T, brain natriuretic peptide) and ECG changes only to mild or life-threatening clinical symptoms (e.g. angina, dyspnea, treatment in ICU) have also been reported.[76]

Mechanism of cardiac toxicity

Several hypotheses concerning the mechanism of cardiac toxicity due to angiogenesis inhibition can be postulated. Endomyocardial biopsy samples obtained from patients developing impaired cardiac function following treatment with sunitinib revealed cardiomyocyte hypertrophy on light microscopy. Electron microscopy showed myocyte hypertrophy and changes in mitochondrial structure without inflammatory or fibrotic changes.[33] It should be noted that all of the patients in this study had previously been treated with imatinib and some also with anthracyclines, agents known to induce cardiotoxicity. Hence, the possibility that this pre-treatment had contributed to the cardiac dysfunction observed during sunitinib treatment cannot be excluded.[33] In accordance

with the histopathological changes in cardiomyocytes observed in human individuals, mitochondrial swelling and degenerative changes of cardiomyocytes after 12 days of sunitinib administration were also found in mice.[33] The contractile ventricular dysfunction observed with RTKIs could therefore be due to impaired adenosinetriphosphate (ATP) generation secondary to mitochondrial dysfunction. However, only sorafenib has been shown to impair mitochondrial function at clinically relevant concentrations in isolated rat heart mitochondria, whereas concentrations above clinical values were needed for sunitinib, imatinib and dasatinib.[78] In cultured rat myocytes, sunitinib has shown to induce cytochrome C release into the cytosol and activation of caspase 9, leading to activation of mitochondrial apoptotic pathways.[33] In contrast, no apoptosis or fibrosis was observed in hearts of mice treated with sunitinib for 12 days, unless the mice were also exposed to phenylephrine, the latter to induce hypertension.[33] Another possible explanation for the cardiotoxic effect of sunitinib might be found in the inhibition of KIT, the receptor for stem cell factor, expressed on hemangioblasts.[79] Hemangioblasts are the precursors of hematopoietic stem cells and endothelial progenitor cells and inhibition of KIT on these cells may prevent the beneficial homing of progenitor endothelial cells to areas of injury in the heart.[79]

Inhibition of VEGF-VEGFR signaling in the heart might be of particular concern in situations of poorly controlled hypertension. In mice, disruption of VEGF-VEGFR signaling by means of adenoviral delivery of a decoy VEGFR was associated with a reduction in myocardial capillary density, contractile dysfunction, fibrosis and heart failure during imposition of pressure load, whereas in the absence of a pressure load angiogenesis inhibition was without adverse cardiac effects.[79-81] The preservation of myocardial capillary density during angiogenesis inhibition in absence of pressure load is supported by the findings of Kamba *et al*.[81] These authors found that capillaries of pancreatic islets, thyroid, adrenal cortex and pituitary do depend on VEGF for survival, but in the heart only a few of these capillaries are present.

In addition to inhibiting several growth factor receptors, sorafenib also inhibits Raf1, which may affect cardiac function.[79] Deletion of Raf1 in the heart of mice led to a dilated, hypocontractile heart with enhanced cardiomyocyte apoptosis and fibrosis.[79] Raf1 kinase activity does not seem to be important in the absence of pressure load, whereas it provides protection in the pressure-loaded heart.[79] As sorafenib inhibits Raf1 and induces hypertension, it can be expected that sorafenib, like sunitinib, induces cardiac dysfunction.

Patients treated with angiogenesis inhibitors will be predominantly older individuals (e.g. renal cell carcinoma occurs predominantly in patients over the age of 50), some-

times with a history of coronary artery disease and the presence of various cardiac risk factors. Due to these unfavorable characteristics they are likely more prone to develop left ventricular dysfunction and congestive heart failure. Finally, RTKI-induced hypothyroidism can exacerbate or even cause left ventricular dysfunction.

Thromboembolic complications

Angiogenesis inhibition, like chemotherapy, is associated with an increased risk of venous and arterial thrombosis.[82,83] In a recently published meta-analysis, combination therapy with a chemotherapeutic agent and bevacizumab was associated with a 33% increased risk to develop venous thromboembolic events (VTE) compared with chemotherapy alone. This risk appeared to be dose-independent. It should be noted that the progression-free survival times in the combination groups were longer than in the control groups. Related to this increased survival, exposure to the combination therapy was also longer, which in turn might have contributed to the increased risk. As some smaller studies evaluating the role of bevacizumab as monotherapy did not report significantly higher incidences of VTE, the increased risk of VTE might be a consequence of bevacizumab-combination therapy rather than of bevacizumab itself.[83] Combination therapy of the RTKI samaxibin (SU5416) and chemotherapy also resulted in a marked increase in VTE rate with evidence of endothelial injury as reflected by increased plasma levels of E-selectin, von Willebrand factor and tissue factor.[84] The incidence of thromboembolic complications associated with sunitinib and sorafenib appears to be low, but sporadic cases of arterial thromboembolism, particularly myocardial infarction have been reported.[82]

Mechanism of thromboembolic complications

A variety of factors may account for the increased thromboembolic risk associated with angiogenesis inhibition. First of all, VEGF is necessary for the regeneration of endothelial cells. During VEGF inhibition this regeneration may be impaired, leading to an increased exposure of platelets and coagulation factors to subendothelial-located procoagulant phospholipids. Second, VEGF is involved in the production of PGI_2 and nitric oxide by the endothelium. PGI_2 and nitric oxide have both antiplatelet activity, and a decrease in their production may promote thrombosis. Third, due to increased production of erythropoietin, VEGF inhibition can increase hematocrit and blood viscosity, which may contribute to a prothrombotic state. Fourth, anti-angiogenic therapy may lead to an increased release of procoagulant factors from the tumor and may also induce increased production of proinflammatory cytokines, thereby increasing the risk of thrombosis. On the other hand, the reported increased incidence of thrombotic events may also result from the prolonged survival time associated with the use of angiogenesis inhibitors. Finally, the etiology of a thromboembolic event in most instances is multifactorial because factors

as age, mobility, functional status, concurrent chemotherapy, stage and histology of the malignant tumor can all contribute to its development.[83]

At this moment it remains difficult to determine whether patients who are treated with anti-angiogenic agents should be treated preventively with anticoagulant or antiplatelet drugs. This decision is complicated by the knowledge that anti-angiogenic treatment is associated with a higher risk of bleeding, although to date no increased prevalence of hemorrhage has been reported in bevacizumab-treated patients who were already using antiplatelet drugs or warfarin for thrombotic events.[82]

Angiogenesis inhibition in retinal disorders

VEGF has been demonstrated in the vitreous fluid of many ocular neovascularizing syndromes such as age-related macular degeneration, proliferative diabetic retinopathy and diabetic macular edema and is thought to play an important role in the abnormal vessel proliferation that is prominent in these disorders.[85] Anti-VEGF agents such as bevacizumab have shown to be beneficial in the treatment of proliferative ocular diseases. As systemic administration of bevacizumab was commonly associated with hypertension, intravitreal administration of bevacizumab as an alternative to minimize systemic effects was proposed by Rosenfeld *et al*.[86,87] The safety of intravitreal administration of either 1.25 mg or 2.5 mg of bevacizumab in patients with a variety of retinal disorders was retrospectively investigated in the uncontrolled PACORES (Pan-American Collaborative Retina Study Group) study. In the 1173 treated patients (on average 3.3 injections per eye) with 1 year of follow-up, the most common systemic side effect was a transient elevation in blood pressure (0.6%). The blood pressure rise was generally mild and occurred from 7 h to 2 weeks following injection. The most common ocular complication was subconjunctival hemorrhage, which was seen in 838 patients. Routine cardiac examination was not performed and therefore the rate of cardiac adverse events might have been underestimated. On the basis of PACORES' findings, intravitreal injection of bevacizumab appeared to be safe and well tolerated, at least during the first year after injection.[85]

Management of hypertension induced by vascular endothelial growth factor inhibition

The recognition that anti-angiogenic therapy is associated with the development of de novo hypertension, worsening of pre-existent hypertension, proteinuria, and renal and cardiac function impairment implies that patients subjected to such a treatment should be closely monitored for the occurrence of these side effects. For optimal management of angiogenesis inhibition-induced side effects close collaboration between oncologists, cardiologists, hypertensive specialists and nephrologists is mandatory. As hypertension can occur at any moment after initiation of angiogenesis inhibition, blood

pressure should be measured at least during every standard visit in the outpatient clinic, and even weekly monitoring during the first 6-8 weeks after initiation of angiogenesis inhibition has been advocated.[88] Home blood pressure measurements and telemonitoring may be advantageous by making it possible to timely initiate and, when necessary, to discontinue antihypertensive therapy. Especially when antihypertensive treatment has been initiated, blood pressure can drop with the risk of hypotension during the 2 week 'OFF'-period. As cancer usually occurs at advanced age, a substantial proportion of patients eligible for anti-angiogenic therapy may already have hypertension. In these patients care should be taken that their blood pressure is controlled before the anti-angiogenic therapy is initiated.

Because of the lack of controlled studies favoring another approach, management of patients with angiogenesis-inhibitor induced hypertension should be in accordance to standard clinical practice. According to the ESH/ESC guidelines of 2007 blood pressure should be reduced to at least below140/90 mmHg in all hypertensive patients and to at least below 130/80 mmHg in high-risk patients with clinical conditions such as renal dysfunction, proteinuria, a history of cardiovascular disease or diabetes mellitus.[89] Whether these strict targets should also apply for patients with non-curable cancer and a decreased life expectancy is questionable. Nevertheless, timely starting of antihypertensive treatment is recommended in order to prevent the development of serious complications such as hypertensive encephalopathy, hemorrhagic strokes and heart failure, conditions that all have been reported to associate with the use of antiangiogenic treatment.[33,46-48] As is true for the management of hypertensive patients in general, comorbid conditions are an important guide for the selection of the most appropriate antihypertensive therapy in an individual patient.[89]

In various studies all major classes of antihypertensive agents have been successfully used to treat anti-angiogenesis-induced hypertension.[88] On the basis of pathophysiological insights, drugs acting to increase nitric oxide, such as nitrates or phosphodiesterase inhibitors, are likely to be effective, but as these agents are not registered for the treatment of hypertension they are not recommended as first-line agents. There is limited evidence that long-acting oral nitrates can restore blood pressure to baseline values in patients with hypertension refractory to combination treatment with an ACE-inhibitor and calcium channel blocker (CCB).[90] The CCB nifedipine effectively reduced high blood pressure induced by the RTKI cediranib in rats.[19] As nifedipine has shown to induce VEGF secretion thereby possibly promoting angiogenesis, other CCBs might be preferred.[60] Most of the clinically approved RTKIs are metabolized in the liver by the CYP3A4 system. As the non-dihydropyridine CCBs verapamil and diltiazem inhibit this system they can increase the plasma concentration of RTKIs and therefore are contraindicated.[60,88]

Angiotensin-converting-enzyme inhibitors (ACE-inhibitors) and angiotensin receptor blockers (ARBs) also seem appropriate in the treatment of hypertension induced by angiogenesis inhibition considering their renal protective effect and absence of pharmacokinetic interaction with RTKIs. ACE-inhibition has shown to restore podocyte nephrin expression in diabetic nephropathy.[91,92] As nephrin underexpression is one of the potential mechanisms of proteinuria induced by anti-angiogenic therapy, treatment with ACE-inhibitors seems a logical choice, especially in the presence of both hypertension and proteinuria.[69] It should be remarked that in a clinical study performed at our medical center a marked decrease in plasma renin activity was observed during treatment with sunitinib [unpublished data]. This renin-suppression may impair the blood pressure lowering effect of ACE-inhibitors or ARBs. Both ACE-inhibitors and ARBs might exert antineoplastic effects due to a reduction of angiotensin II synthesis or blockade of its effects. Angiotensin II exerts mitogenic activities and promotes VEGF-induced tube formation *in vitro*.[93] Furthermore, angiotensin II regulates apoptotic pathways and both ACE-inhibitors and ARBs can inhibit myocardial angiogenesis induced by VEGF.[60,88] On the other hand, bradykinin, which increases during ACE-inhibition, might stimulate angiogenesis via activation of the B2 receptor, inducing nitric oxide and prostacyclin synthesis.[10] Finally, it has been reported that hypertension induced by angiogenesis inhibitors can successfully be treated with diuretics and β-blockers. As non-vasodilating β-blockers can further increase peripheral vascular resistance and may disturb cardiac conduction or modify QT- intervals they should in our view not be considered as first-choice agents for the treatment of anti-angiogenesis-associated hypertension.[76] Nebivolol might theoretically be a good choice as this agent exerts a vasodilating effect mediated by the L-arginine nitric oxide pathway and lowers peripheral resistance.[88] Diuretics should be used with caution, especially in the presence of bone metastasis and hypercalcemia, because of the risk of inducing or aggravating hypercalcemia.

Prophylactic antihypertensive treatment instead of starting antihypertensive treatment after hypertension has developed might be an alternative strategy to minimize dose reductions or dose interruptions of anti-angiogenic therapy. This has been explored in a recent study with the investigational RTKI cediranib.[94] Patients (n = 126) were randomized for antihypertensive prophylaxis with a low dose of a CCB 3-7 days before initiation of treatment with the angiogenesis inhibitor cediranib or no antihypertensive prophylaxis. Although antihypertensive prophylaxis markedly reduced the development of severe hypertension, it did not result in less dose reductions or dose interruptions of cediranib as compared with the non-prophylaxis group. Hence, prophylactic antihypertensive treatment does not seem to provide much advantage.[94]

In summary, we propose the following recommendations considering the treatment of hypertension induced by angiogenesis inhibition:

1. Blood pressure should be measured before the initiation of an angiogenesis inhibitor and thereafter on a regularly basis, at least during every standard visit in the outpatient clinic. Home blood pressure measurements and telemonitoring may be especially advantageous.
2. In patients with hypertension, blood pressure should be controlled before anti-angiogenic therapy is initiated.
3. Prophylactic treatment with antihypertensive drugs in patients without hypertension is not recommended as such a strategy appears to have no effect on the number of dose interruptions or dose reductions of the angiogenesis inhibitor.
4. Antihypertensive treatment should be started in accordance with standard clinical practice. To prevent *acute* hypertensive complications a target blood pressure of less than 140/90 mmHg is advised for all patients treated with anti-angiogenesis inhibitors.
5. Preferential antihypertensive drugs include CCBs, ACE-inhibitors or ARBs chosen on an individual basis (e.g. in patients with proteinuria ACE-inhibitors or ARBs are preferred). Non-dihydropyridine CCBs should be avoided during treatment with TKIs because of pharmacokinetic interaction.
6. In case of uncontrolled hypertension despite adequate antihypertensive treatment, discontinuation of the angiogenesis inhibitor, either temporarily or permanently, should be considered.

Conclusion

Angiogenesis is a critical factor in primary tumor growth and metastasis. Decades ago, angiogenesis inhibition has been proposed as a therapeutic strategy for cancer therapy, which in the mean time has proven to be highly effective. Unfortunately, VEGF inhibition, either with monoclonal antibodies to VEGF or with RTKIs involves unwanted side effects including hypertension and renal and cardiac toxicity. Although the exact underlying pathophysiological mechanisms remain to be elucidated, the literature discussed earlier provides several possible explanations. Fortunately, most of the side effects seem manageable but likely become more problematic as survival increases. In addition, in daily practice, treated patient populations include mainly older individuals with co-morbidity, often not meeting the strict inclusion criteria applied in clinical studies. As a consequence, a higher incidence of cardiovascular side effects than reported in clinical studies is likely. Both clinical trials as well as experimental studies are needed to further explore the pathophysiological mechanisms of cardiovascular side effects due to angiogenesis inhibition. This may lead to the development of more specific angiogenesis inhibitors, better management of the side effects and may possibly provide new insights into the pathogenesis of cardiovascular disease in general.

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2

Thyroid dysfunction during angiogenesis inhibition

Angiogenesis inhibition induced by multitarget RTKIs, such as sunitinib, is not only associated with cardiovascular and renal toxicity, but also with thyroid dysfunction, mainly hypothyroidism (Table 1).[1,3,4,5,7,8,9,10,11] The rise in thyroid stimulating hormone (TSH) levels occurs early, generally within 4 weeks of treatment, increases progressively during consecutive treatment cycles and fluctuates according to the 'ON/OFF' treatment regimen of sunitinib.[1-3] These observations suggest that the condition is reversible once therapy has been discontinued and that the risk of hypothyroidism increases with increasing duration of sunitinib therapy. Whether the elevated TSH levels also represent overt clinical hypothyroidism remains unclear since differentiation between clinical symptoms related to hypothyroidism from those related to malignancy can be difficult. For instance, fatigue, one of the major symptoms of hypothyroidism, is also a common feature of cancer or cancer therapies and has been reported in patients treated with sunitinib in up to 50% (Table 1). Moreover, in one study 50% of patients with sunitinib-induced hypothyroidism did not show symptomatic improvement with levothyroxine (LT_4) replacement.[4] These findings suggest that a proportion of patients with abnormal thyroid function tests on sunitinib therapy has no clinically overt hypothyroidism.

Sunitinib-associated hyperthyroidism, although to a lesser extent, has also been reported, often preceding the hypothyroidism.[5] Grossmann *et al*. have reported thyroid dysfunction in 48% of patients with mRCC treated with sunitinib. Of these patients 24% developed hyperthyroidism, eventually resulting in permanent hypothyroidism in 32% of patients.[5]

Thyroid dysfunction has also been reported during treatment with sorafenib, another RTKI, although the incidence of hypothyroidism associated with this drug is much lower

Table 1. Reported incidence in literature of sunitinib-associated altered thyroid function tests and related clinical symptoms.

Reference	No. of patients	Tumor type	Altered TFTs (% patients)	Related symptoms (% patients)
Rini *et al*.[4]	66	mRCC	85	84
Desai *et al*.[7]	42	mGIST	62	NR
Mannavola *et al*.[3]	24	mGIST	46-71	NR
Demetri *et al*.[8]	207	mGIST	4	NR
Schöffski *et al*.[9]	33	mRCC/mGIST	37-57	NR
Shaheen *et al*.[10]	62	mRCC	65	53
Grossmann *et al*.[5]	25	mRCC	48	NR
Wong *et al*.[11]	40	various	53	57
Wolter *et al*.[1]	59	mRCC/mGIST	61	NR

mGIST, metastatic gastrointestinal stromal tumor; mRCC, metastatic renal cell carcinoma; NR, not reported; TFT, thyroid function test.

than that associated with sunitinib. Riesenbeck *et al.* reported in a prospective study of 66 patients with mRCC that 31.8% developed hypothyroidism. Of these patients 38.1% was treated with sorafenib and 61.9% with sunitinib.[2] Although both sunitinib and sorafenib are RTKIs of the VEGFR-1, and -2, they also target a number of other RTKs or target different receptors. Therefore, the lower incidence of thyroid dysfunction observed in patients on sorafenib could be related to the degree of inhibition of these receptors by these two drugs. Interestingly, thus far no alterations in thyroid function tests have been observed during treatment with bevacizumab, an anti-VEGF monoclonal antibody, suggesting that the thyroid dysfunction during angiogenesis inhibition with RTKI is due to an 'off-target' effect of these drugs rather than due to inhibition of the VEGF signaling itself.[6]

Mechanism of thyroid dysfunction

Since the expression of VEGF and VEGF receptor mRNA and protein, mediated in part by TSH, have been demonstrated in normal thyroid follicular cells, it seems logical that VEGF inhibition affects thyroid function and histology.[4] Several mechanisms for the sunitinib-induced thyroid dysfunction have been hypothesized. Firstly, it has been demonstrated that capillaries of the thyroid are extremely susceptible to VEGF inhibition. This capillary regression is reversible after withdrawal of the angiogenesis inhibitor.[12] In line with this report, sunitinib might induce thyroidal capillary regression ('capillary regression hypothesis') leading to thyroid tissue injury and dysfunction.[4,13] Indeed, in some patients no thyroid tissue could be visualized on ultrasound imaging during treatment with sunitinib suggesting atrophy of the thyroid induced by capillary regression.[7] In addition, Mannovola *et al.* found significant decreases in ^{123}I-uptake in patients with sunitinib-induced hypothyroidism, suggesting that the underlying mechanism might be impaired iodine uptake.[3] These data are contradicted by thyroid cell culture studies showing that sunitinib does not inhibit iodide uptake. Moreover, in these cells sunitinib caused a slight increase in intracellular iodide and did not reduce iodide efflux or sodium-iodide symporter (NIS) gene expression. NIS is a plasma membrane glycoprotein that mediates active iodide transport into the thyroid follicular cells, the first step in thyroid hormone synthesis.[14] Because of the critical role of the TSH receptor in NIS expression, and therefore in the production of triiodothyronine (T_3) and thyroxine (T_4), it has also been studied whether sunitinib impairs TSH receptor function. No such effect was observed.[14] Secondly, in a small proportion of patients sunitinib might induce a thyroiditis-like syndrome with transient thyrotoxicosis and subsequent hypothyroidism.[5] In agreement with such a mechanism lymphocytic infiltration has been reported in one patient during treatment with sunitinib.[5] On the other hand, no changes in antithyroid peroxidase (TPO) antibody levels have been observed, making an autoimmune-mediated mechanism less likely.[13] Thirdly, a 'metabolic hypothesis' has been postulated,

based on a case-report of a patient with a history of thyroidectomy in whom an increase in LT_4 dose was required during treatment with sunitinib, suggesting interference with the T_4/T_3 hormone metabolism.[15] This metabolic hypothesis is further supported by the findings of Wong *et al.*, who demonstrated, using various *in vitro* assay systems, that sunitinib has anti-peroxidase activity similar to that of propylthiouracil (PTU), an effective peroxidase inhibitor, thereby reducing thyroid hormone synthesis. Since the antiperoxidase activity of sunitinib is much weaker (20-30%) than that of PTU not all patients will develop hypothyroidism.[11] Fourthly, also the iodinated contrast used in CT-scanning during follow-up of the malignancy might interfere with thyroid function.[13]

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AIMS OF THE THESIS

Angiogenesis plays a critical role in tumor growth. During this process, the cells in the center will become hypoxic and pro-angiogenic signals such as vascular endothelial growth factor (VEGF) will be expressed. VEGF, a key mediator in the angiogenic process, binds two receptors, the VEGF receptor (VEGFR)-1 and -2. Biologically, the latter is the most important. Binding of VEGF to this receptor leads to an increase in nitric oxide (NO) production. VEGFRs contain two tyrosine kinase domains. Angiogenesis inhibition by means of blocking these tyrosine kinases with sunitinib has become an important strategy for the treatment of several tumor types, including metastatic renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumors. Unfortunately, treatment with sunitinib is associated with several side effects: hypertension, renal and cardiac toxicity as well as thyroid dysfunction. Since the occurrence of side effects can be a reason to lower the sunitinib dose or even to stop therapy, thereby compromising its potential efficacy, it is from a clinical point of view important to unravel the mechanisms underlying these toxicities. The findings of the studies presented in this thesis may help to develop therapies to combat these adverse effects.

Sunitinib-induced cardiovascular and renal toxicity

Hypertension and renal toxicity

Hypertension during angiogenesis inhibition with sunitinib has been reported in up to 47% of patients. The incidence of sunitinib-induced renal toxicity, mainly proteinuria, has not been well documented since patients were not routinely screened for this condition in the initial studies. It has been postulated that inhibition of the VEGF-pathway reduces NO bioavailability, which may result in vasoconstriction and contributes directly to the development of hypertension. Additionally, decreased NO bioavailability may lead to a disturbed balance between NO and the vasoconstrictor endothelin-1 (ET-1), favoring ET-1 production, thereby inducing vasoconstriction and contributing to the development of hypertension. Furthermore, oxidative stress is involved in the pathogenesis of hypertension, cardiovascular disease as well as renal injury, and decreased levels of VEGF may contribute to oxidative stress-induced endothelial dysfunction. Since VEGF plays a role in podocyte survival and function, it is indirectly involved in the maintenance of the glomerular filtration barrier. As a consequence, inhibition of VEGF signaling might affect the integrity of this barrier.

In the current studies we aimed to obtain more insight into the mechanisms underlying sunitinib-induced hypertension and renal toxicity. Firstly, the effect of sunitinib and its withdrawal on blood pressure and renal function and histology was investigated in

patients and rats (Chapter 3). Secondly, in rats and swine the roles of ET-1, NO-bioavailability and oxidative stress in the development of sunitinib-associated hypertension and renal toxicity were explored (Chapter 4, 5, 6).

Cardiac toxicity

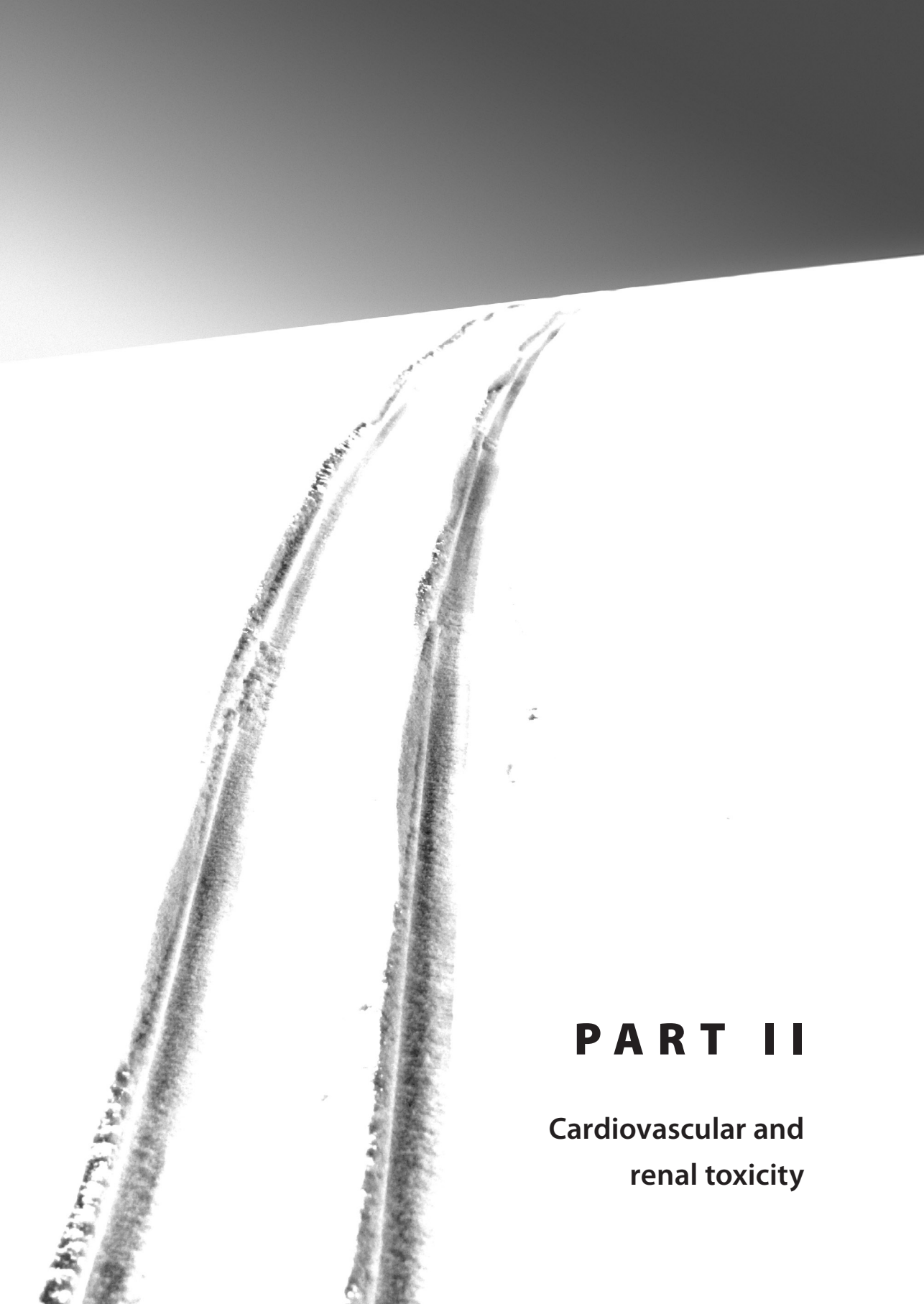
Sunitinib-induced cardiac toxicity has been observed in patients. A decrease in LVEF of 10-15% occurs in up to 28% of patients treated with sunitinib. In addition, angina pectoris and increases in biomarkers of cardiac damage have been observed. Since cardiac mitochondrial structural changes have been observed in these patients, it has been postulated that impaired ATP generation secondary to mitochondrial dysfunction could underlie the contractile ventricular dysfunction, but the exact mechanism underlying sunitinib-induced cardiac damage remains unknown.

In the studies presented in this thesis the effect of sunitinib on *ex vivo* mitochondrial function in cardiomyocytes obtained from sunitinib-administered rats was studied (Chapter 3). Additionally, in swine we investigated the effect of sunitinib on cardiac function as well as on coronary and pulmonary hemodynamics focusing on the potential activation of the endothelin system, decreased NO-bioavailability and increased oxidative stress as underlying mechanisms (Chapter 5).

Sunitinib-induced thyroid dysfunction

Sunitinib-induced thyroid dysfunction, mainly hypothyroidism, has been reported in up to 53-85% of patients in retrospective and in up to 36-46% of patients in prospective studies. Since in some patients, already on thyroid hormone replacement therapy before sunitinib initiation, a dose-increase was required during sunitinib treatment, it has been hypothesized that sunitinib interferes with thyroid hormone metabolism ('metabolic hypothesis'). In mice, administration of an experimental VEGF receptor inhibitor or a soluble VEGF receptor, binding circulating VEGF, was associated with a marked increase in TSH levels and pronounced regression of capillaries in the thyroid gland ('capillary regression hypothesis').

In the current study we aimed to investigate the incidence of sunitinib-induced thyroid dysfunction, its time-course and pathophysiological mechanisms involved, including thyroid hormone metabolism and changes in capillary density, in patients and in rats (Chapter 8).



PART II

Cardiovascular and renal toxicity

3

Hypertension induced by the tyrosine kinase inhibitor sunitinib is associated with increased circulating endothelin-1 levels

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ABSTRACT

Angiogenesis inhibition with sunitinib, a multitarget tyrosine kinase inhibitor of the vascular endothelial growth factor receptor, is associated with hypertension and cardiac toxicity of which the underlying pathophysiological mechanism is unknown. We investigated the effects of sunitinib on blood pressure, its circadian rhythm and potential mechanisms involved, including the endothelin-1 system, in 15 patients with metastatic renal cell carcinoma or gastrointestinal stromal tumors. In addition, we investigated in rats the effect of sunitinib on blood pressure, serum endothelin-1 levels, coronary microvascular function, cardiac structure and cardiac mitochondrial function. In patients, blood pressure increased by almost 15 mm Hg while heart rate decreased after 4 weeks of treatment. Furthermore, the nocturnal dipping of blood pressure diminished. Plasma endothelin-1 concentration increased twofold ($P<0.05$), plasma renin decreased ($P<0.05$), whereas plasma catecholamines and renal function remained unchanged. In rats, 8 days of sunitinib-administration induced an almost 30 mmHg-rise in blood pressure, an attenuation of the circadian blood pressure rhythm and a threefold rise in serum endothelin-1 and creatinine, which all but the rise in creatinine reversed after sunitinib-withdrawal. Coronary microvascular function studies after 8 days of sunitinib-administration showed decreased responses to bradykinin, angiotensin II and sodium nitroprusside, all normalizing after sunitinib-withdrawal. Cardiac structure and cardiac mitochondrial function did not change. In conclusion, sunitinib induces a reversible rise in blood pressure in patients and in rats associated with activation of the endothelin-1 system, suppression of the renin-angiotensin system and generalized microvascular dysfunction.

INTRODUCTION

Angiogenesis, the formation of new capillaries from an existing vasculature, is critical to tumor growth as well as metastasis. This process is regulated by numerous growth factors and their receptors among which vascular endothelial growth factor (VEGF) and its corresponding receptors play a key role. Angiogenesis inhibition as a therapeutic strategy against malignancies was first proposed by Folkman in 1971.[1] Meanwhile a variety of drugs, targeting VEGF or its receptors, have been approved for the treatment of several tumor types. Unfortunately, angiogenesis inhibition is associated with side effects, in particular hypertension, which has been reported in up to 60% of patients treated with sunitinib, an orally active multitarget VEGF receptor tyrosine kinase inhibitor (RTKI) and one of the most commonly used angiogenesis inhibitors.[2] Decreased nitric oxide bioavailability might underlie this phenomenon.[3,4]

VEGF inhibition with sunitinib is also associated with cardiac toxicity, evidenced by a decrease in left ventricular ejection fraction (LVEF) in up to 28% of patients.[5] Given the sunitinib-induced changes in cardiac mitochondrial structure, this could relate to impaired adenosine triphosphate (ATP) generation secondary to mitochondrial dysfunction.[5] However, sunitinib, at clinically relevant concentrations, did not impair mitochondrial function in a rat myoblast cell line.[6]

The occurrence of the mentioned side effects can be a reason to lower the sunitinib dose and sometimes even to stop therapy, thereby compromising its potential efficacy. For optimal management, it is important to improve insight into the mechanisms underlying the sunitinib-induced toxicities. Our research, involving both patients and rats, was aimed to elucidate the pathophysiological mechanism(s) involved in the development of hypertension during sunitinib treatment. Simultaneously, we addressed the question whether and to what extent sunitinib affects cardiac structure and cardiac mitochondrial function.

METHODS

Clinical study

Between January 2008 and January 2009, patients with either metastatic renal cell carcinoma (mRCC) or imatinib-resistent gastrointestinal stromal tumor (GIST), who were eligible for treatment with sunitinib, were invited to participate in our study. Patients were followed for 10 weeks during treatment with sunitinib which was taken according to a 4 weeks 'on', 2 weeks 'off' regimen with a starting dose of 50 mg/day. According to

the judgment of the patient's physician this dose could be adjusted. If blood pressure (BP) increased to stage II hypertension or above, antihypertensive treatment was initiated with a calcium channel blocker, an ACE-inhibitor or an angiotensin (Ang) II type 1 receptor blocker and a diuretic as a first, second and third choice. Nocturnal dipping of BP was defined as a more than 10% decrease in systolic, diastolic or mean arterial pressure during sleep. Patients visited the outpatient clinic every 2 weeks. At baseline and at the end of the first and second treatment cycle, 24-hour ambulatory BP measurements (ABPM) were performed (Suntech Oscar 2 ABP monitor and AccuwinPro soft). During the visits, sitting BP was measured during 30 minutes at 5 minutes intervals using an automated device (Dynamap, Critikon Inc, model 8101) in a private room. Blood samples for laboratory measurements were obtained from an intravenous line at baseline and at the end of the first and second treatment cycle, after a 30-minutes rest period. 24-Hour urine samples for measurement of protein, creatinine and sodium were collected at baseline and at weeks 4, 6 and 10. The study was approved by the Institutional Review Board and Ethical Committee of the Erasmus MC in Rotterdam. Written informed consent was obtained from each patient.

Rat study

Male Wistar-Kyoto rats (280-300 grams), obtained from Charles River, were housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow and water ad libitum. Intra-aortic BP recordings were performed by radiotelemetry for which a transmitter (TA11PA-C40, Datascience Inc.) was implanted into the abdominal cavity.[7] Telemetric data were recorded and digitalized using the Dataquest Acquisition & Analysis system (DQ ART 3.1 Silver, Datascience Inc.). Each animal was sampled for 10 seconds at 10-minute intervals. All recordings were averaged for the day and night period. Baseline values of mean arterial pressure (MAP) and heart rate (HR) were averages of three day recordings before treatment was started. One week after recovery the rats were acclimatized to drug administration by oral gavage, by administering water for 8 days. The content of sunitinib L-malate capsules, obtained from patients who discontinued treatment, was dissolved in HCl (0.1 mol/L), containing 0.5% polysorbate and 10% polyethylene glycol, after which NaOH (0.1 mol/L) was added to adjust pH to 3.5. Two separate experiments were performed. In the first experiment rats were randomly administered sunitinib (26.7 mg/kg/day of sunitinib L-malate; n=10) or vehicle (see above; n=10) by oral gavage (0.5 mL) for 8 days and were euthanized with 60 mg/kg pentobarbital i.p. at the end of this period, at which time blood was sampled and the heart and kidneys were rapidly excised. 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 sacrificed. Six days before (baseline) and 6 days after treatment initiation, rats were housed in metabolic cages for 48 hours; the first day

to acclimatize and the second day for collection of 24-hour urine for protein measurement. In the second experiment rats were also housed in metabolic cages one week after treatment discontinuation. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

In vitro studies and histology

Hearts were rapidly excised from euthanized rats and perfused according to Langendorff.[8] 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 µL) 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).

Next, the hearts were collected, and the heart weight was determined after removal of the atria and large vessels to determine the heart weight/body weight (HW/BW) ratio. The apex was removed to isolate cardiomyocyte mitochondria.[9] The ventricles were cut into 3 transversal slices and fixed in a 3.5-4% formaldehyde solution. After fixation, the slices were dehydrated and paraffin-embedded. Gomori's silver staining was applied to deparaffinized 5-µm thick sections to visualize individual cardiomyocytes.[10] Only transversally cut cells showing a nucleus were used to determine cardiomyocyte area.

Kidneys were collected directly after euthanization and sliced into 2-mm thick transverse sections. After fixation in 3.5-4% formaldehyde solution for 12 hours, the slices were routinely processed to paraffin blocks from which 2-µm thick sections were cut and stained with Jones silver stain to allow microscopic examination.

Cell culture study

Human umbilical vein endothelial cells (HUVECs) were obtained and cultured as described before.[11] Cells (passage 5-6) were seeded at 5000 cells/cm^2 in 6-well plates, and upon reaching confluency exposed to 10 nmol/L sunitinib (a concentration which has been reported to inhibit tyrosine phosphorylation of the VEGF receptor 2) or culture medium only.[12] After 72 hours, medium and cells were collected and stored at -80^{o}C for the determination of endothelin-1 (ET-1).

Biochemical measurements

Plasma renin activity (PRA) was measured by an in house assay as described before.[13] Plasma renin concentration (PRC) was measured by an immunoradiometricassay (Cisbio), aldosterone by radioimmunoassay (Coat-A-Count®, Siemens), ET-1 by chemiluminescent

ELISA (QuantiGlo®, R&D Systems), VEGF by enzyme immunoassay (Quantikine®, R&D Systems) human N-terminal pro-brain natriuretic peptide (NT-proBNP) by radioimmunoassay (Phoenix Pharmaceuticals, Inc.), rat B-type natriuretic peptide-45 (BNP-45) by enzyme immunoassay (Phoenix Pharmaceuticals Inc.), and total protein by colorimetric detection (Pierce® Protein Assay Kit). Catecholamines were measured by electrochemical detection after separation by high-performance liquid chromatography.[14,15] Von Willebrand factor (vWf) antigen was determined with an in-house ELISA assay, using polyclonal rabbit anti-human vWF and horseradish peroxidase conjugated anti-human vWF (DakoCytomation). Serum creatinine and urinary protein were measured at the clinical chemical laboratory of the Erasmus MC.

Cardiac mitochondrial respiratory activity, complex I- and II-dependent respiration, mitochondrial ATP production and mitochondrial swelling were measured as described in the Supplement.

Data analysis

Data are presented as mean±SEM or geometric mean and 95% confidence intervals. Data obtained with the Langendorff preparation were recorded and digitalized as previously described.[8] Statistical analysis between groups was performed by repeated-measures ANOVA followed by Newman-Keuls multiple comparison testing, or two-way ANOVA. VEGF- and NT-proBNP-levels were analyzed by Wilcoxon rank sum test. 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 statistical analysis.

RESULTS

Clinical study

Fifteen patients (10 male, 5 female), mean age of 59.8 ± 6.1 years, with mRCC (n=13) or GIST (n=2) were included. Five patients had pre-existent hypertension. Twelve patients had a history of smoking, 3 were still smoking and 3 had a history of cardiovascular disease. Treatment with sunitinib was associated with a rise in BP showing a clear on/off-effect according to the on/off-treatment regimen, and was accompanied by opposite changes in heart rate (Figure 1). ABPM also showed a rise in day and night-time BP that sustained during the second treatment cycle (Figure 1). The rise in BP was accompanied by an attenuation of the circadian BP rhythm (Figure 1). Initiation or adjustment of antihypertensive therapy was indicated in 6 and 2 patients respectively.

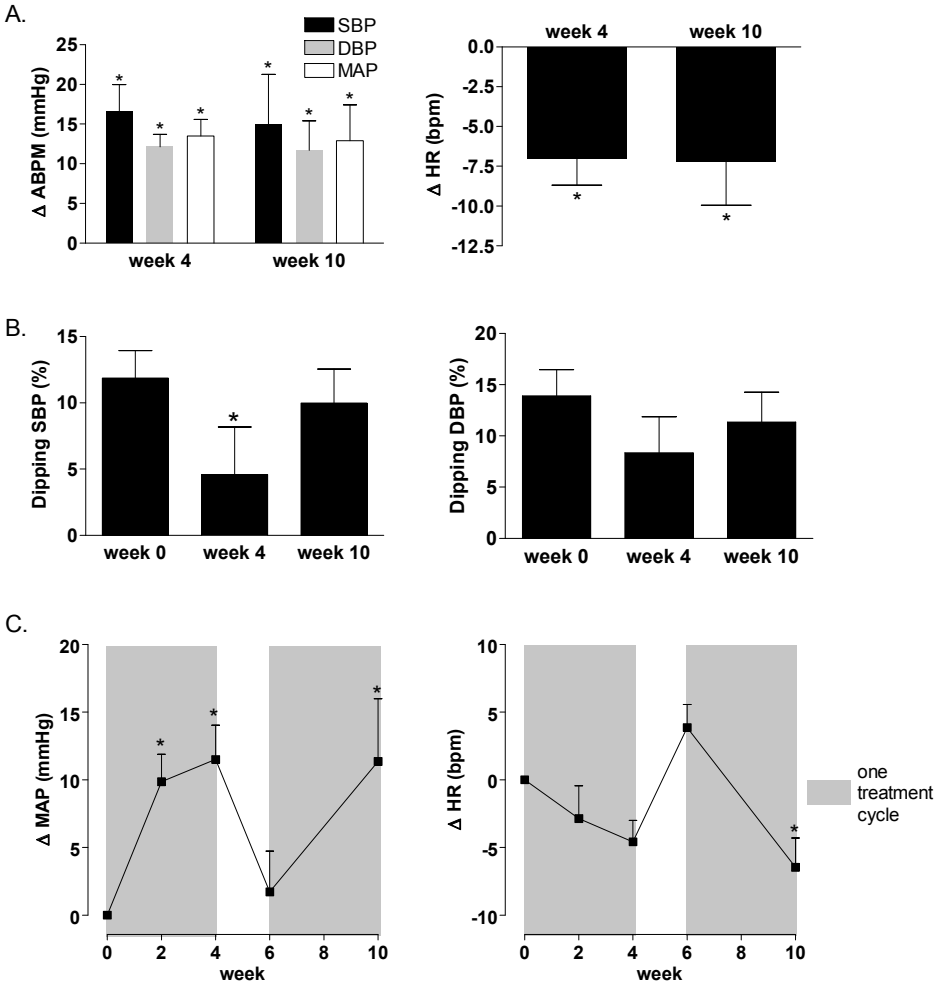

Figure 1. Change in blood pressure and heart rate, and nocturnal dipping of blood pressure in response to treatment with sunitinib. A. Changes in 24-hour ambulatory systolic, diastolic and mean arterial pressure (SBP, DBP and MAP) and heart rate (HR) at baseline and after 4 and 10 weeks of sunitinib treatment. B. Nocturnal dipping of SBP and DBP at baseline and after 4 and 10 weeks of sunitinib treatment. C. Change in MAP and HR measured with an automated device for 30 minutes at baseline, during the first treatment cycle with sunitinib (week 2 and 4), during the 'off'-treatment period (week 6) and at the end of the second treatment cycle (week 10). *P<0.05 compared to baseline.

Body weight decreased from 80±3 kg at baseline to 78±4 kg (P=0.31) at week 4 and to 73±4 kg (P=0.02) at week 10. Serum creatinine (77±6 μmol/L) did not change. Proteinuria increased from 0.19±0.02 to 0.44±0.16 g/24h (P=0.14) at week 4, decreased to 0.20±0.04 g/24h (P=0.97) at week 6 and increased again to 0.35±0.13 g/24h (P=0.19) at week 10.

Treatment with sunitinib was associated with a fall in PRC and PRA and an increase in plasma ET-1 and VEGF concentration, whereas plasma concentrations of aldosterone, norepinephrine, epinephrine, NT-proBNP and vWf did not change (Table 1). Changes in plasma ET-1 and renin concentration did not correlate with the changes in BP.

Rat study

Daily administration of sunitinib to WKY-rats induced a rise in BP within 1-2 days, reaching a plateau after 6 days. At that time, MAP had increased by almost 30 mmHg whereas heart rate had decreased (Figure 2A). The rise in BP was accompanied by an inversion of the normally-occurring decrease in BP during sleep (Figure 2A). In control rats BP and HR remained stable (Figure 2A). Both the rise in BP and the attenuation of its circadian rhythm were reversible after sunitinib-withdrawal (Figure 2B).

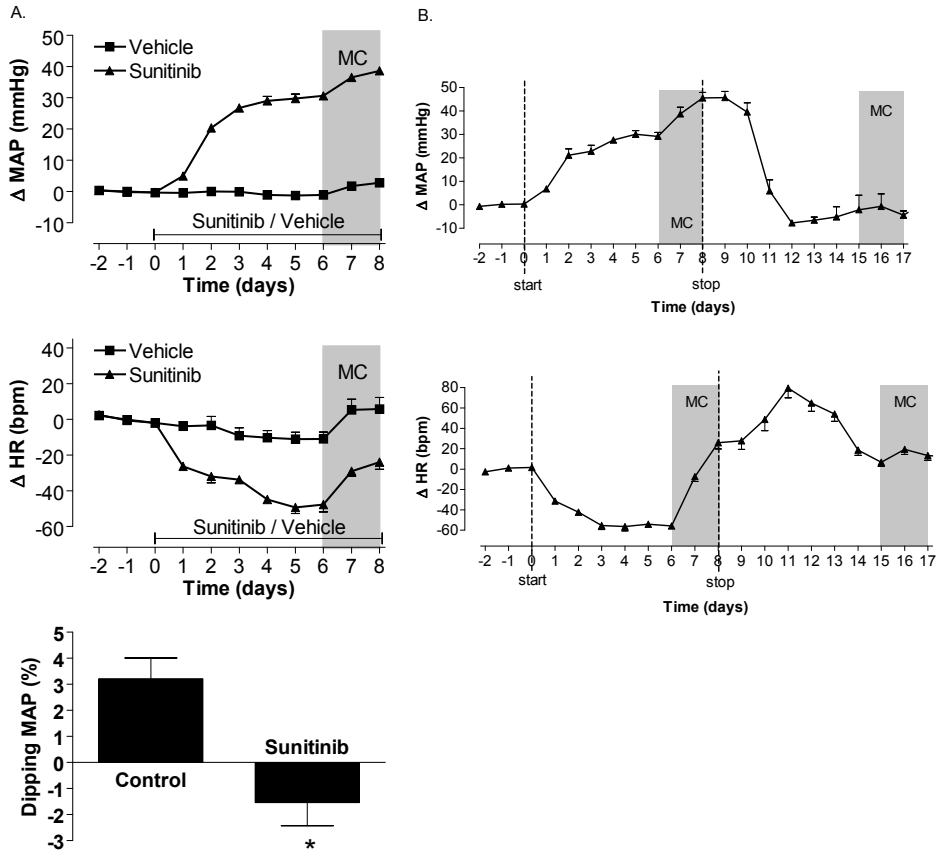

Figure 2. A. Changes in mean arterial pressure (MAP), heart rate (HR) and dipping of MAP during sleep (day 6) in response to sunitinib (n=10) or vehicle treatment in rats (n=6). B. Changes in MAP and HR during administration and after discontinuation of sunitinib (n=7). MC, metabolic cage. *P<0.05.

Table 1. Plasma concentrations of neurohormones, von Willebrand factor antigen (vWf-Ag) and vascular endothelial growth factor (VEGF), at baseline and at the end of the first (week 4) and second (week 10) treatment cycle of sunitinib in patients.

Plasma parameters	Week 0	Week 4	P-value	Week 10	P-value
PRC (pg/ml)	9.9±2.1	3.6±0.8	0.02	5.2±1.9	0.16
PRA (pmol Ang I/ml/h)	1.3±0.3	0.5±0.1	0.001	0.75±0.2	0.28
Aldosterone (pg/ml)	44.5±13.1	36.8±8.6	0.34	55.2±16.6	0.98
Endothelin-1 (pg/ml)	3.0±0.4	5.2±0.8	0.004	6.0±1.2	0.008
Norepinephrine (pg/ml)	265.5±23.3	258.3±37.4	0.95	351.3±83.5	0.38
Epinephrine (pg/ml)	31.7±8.9	25.8±4.9	0.51	41.2±14.7	0.59
vWf-Ag (U/ml)	1.6±0.2	1.7±0.2	0.55	1.4±0.2	0.79
NT-proBNP (pg/ml)	850.5 (648.1-1116)	885.6 (637.4-1230)	0.54	729.2 (521.2-1020)	0.76
VEGF (pg/ml)	35.5 (22.9-55.2)	153.6 (83.9-280.9)	0.002	150.6 (63.0-360.1)	0.005

PRA, plasma renin activity; PRC, plasma renin concentration. Data shown as mean±SEM. VEGF and NT-proBNP values are shown as geometric mean and 95% CIs. *P* value compared with baseline (week 0).

The increase in body weight (from 368±3 to 382±3 g) was lower (P<0.001) in rats on sunitinib than in control rats (from 383±3 to 406±3 g). Serum creatinine increased during administration of sunitinib and remained elevated after its discontinuation (Figure 3A). Administration of sunitinib was associated with proteinuria that normalized after sunitinib withdrawal (Figure 3B). Histological evaluation of the kidney using light microscopy showed marked glomerular changes following sunitinib treatment (Figure S1, Supplement). Comparable to findings in patients, administration of sunitinib was associated with an almost 3-fold increase in plasma ET-1 concentration, which normalized after its discontinuation (Figure 3C).

Circulating BNP-45 levels were almost two-fold higher in the sunitinib-administered group compared to the control group (164±34 vs 93±15 ng/ml; P=0.03) and normalized after sunitinib-withdrawal (93±9 ng/ml; P=0.03). Heart weight-to-body weight ratio (3.0±0.04 vs 3.1±0.06 g/kg; P=0.18) and cardiomyocyte area (513±37 vs 552±42 μm^2; P=0.51) were not different between the sunitinib and control group.

In the Langendorff preparation, CF responses to bradykinin, Ang II and SNP were markedly attenuated in sunitinib-administered rats (Figure 4; P<0.05), and all responses normalized after discontinuation of sunitinib.

Complex I- and II-dependent basal respiratory activity (state 2), as well as the ADP-stimulated (state 3) respiration were not different between groups (Figure S2A, B, Supple-

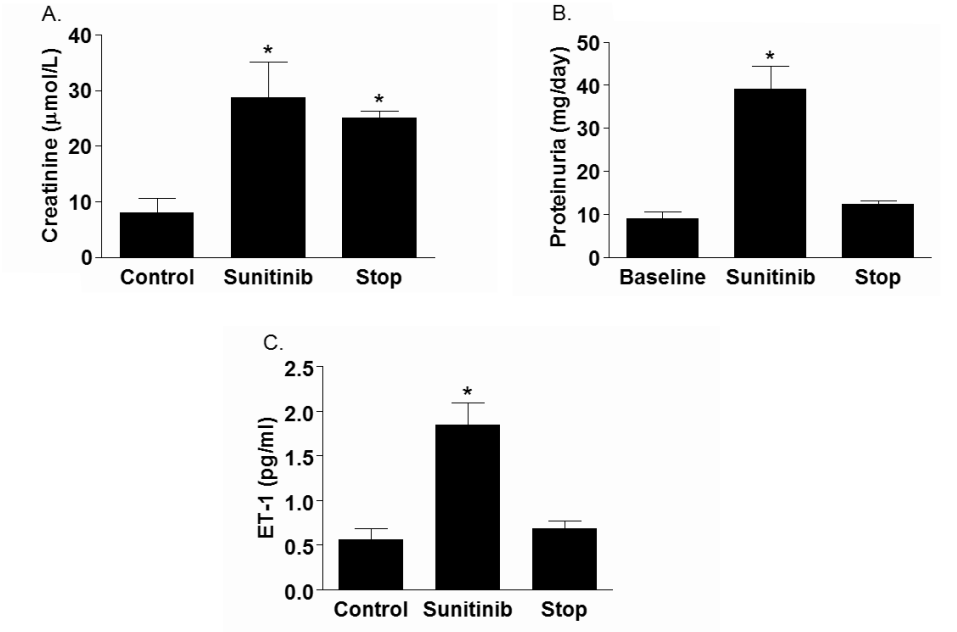

Figure 3. Serum creatinine (panel A), proteinuria (panel B) and serum endothelin-1 (ET-1, panel C) in rats after administration of sunitinib (n=10-13), vehicle (control; n=6) or sunitinib withdrawal (stop; n=7). *P<0.05 compared to baseline/control.

ment). The RCI's did not change either (data not shown). Complex I- and II-dependent ATP-production were not different in the sunitinib compared to the control group or after sunitinib withdrawal (Figure S2C, Supplement). The calcium-induced mitochondrial swelling was lower in the sunitinib than in the control group and remained low after sunitinib withdrawal (Figure S2D, Supplement).

Cell culture study

ET-1 production by sunitinib-treated HUVECs was identical to that in control cells (22±5 vs 20±5 ng/µg protein; P=0.73, n=9).

DISCUSSION

Hypertension is a frequent and sometimes severe adverse effect of anti-angiogenic therapy. With our clinical and experimental studies we aimed to obtain more insight into the mechanisms underlying the development of hypertension. In agreement with previous reports we found that the RTKI sunitinib induced a substantial rise in BP.[5,16] Evaluation of the time course of the BP rise in our experimental study revealed that this already

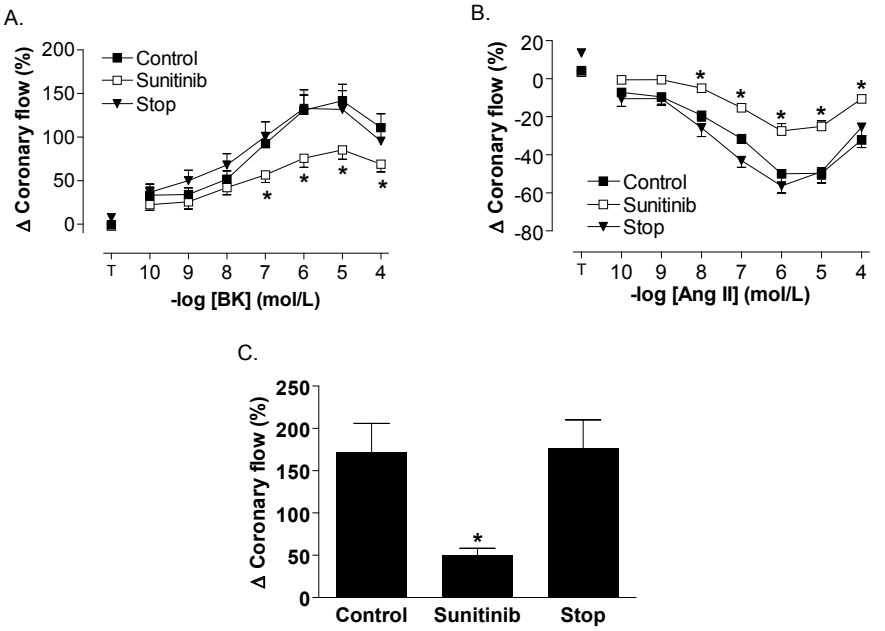

Figure 4. Coronary flow responses to bradykinin (BK, n=6-10; panel A), angiotensin (Ang) II (n=7-9; panel B) and a single injection of sodium nitroprusside (SNP; 10 mmol/L; n=6-10; panel C) in isolated rat hearts after administration of sunitinib, vehicle (control) or sunitinib withdrawal (stop). The x-axis in panel A and B displays the concentration in the injection fluid. T, Tyrode's buffer. *P<0.05 compared to control.

occurred one day after its administration. Furthermore, both in humans and in rats the BP response showed an on/off-effect that paralleled the on/off-administration regimen. In patients the rise in BP was accompanied by attenuation and in rats by reversal of the circadian BP variation and in both species it was accompanied by a decline in heart rate. Remarkably, both in our clinical and experimental study sunitinib administration was associated with a substantial rise in circulating ET-1 levels. To our knowledge, increased plasma ET-1 levels in response to sunitinib have not been reported before. Although ET-1 is predominantly produced by endothelial cells, sunitinib did not affect ET-1 release from HUVECs. This suggests that the ET-1 increase has a non-endothelial origin.

The question whether activation of the endothelin-system is involved in the sunitinib-induced rise in BP cannot be answered at this moment. In a recent study performed in telemetry-instrumented rats, pre-treatment with the selective ET_A-receptor antagonist atrasentan completely prevented the rise in BP induced by a RTKI.[17] Since the same ET_A-receptor blocker also lowered BP in control rats, definite conclusions about the role of the endothelin-system, in particular activation of ET_A-receptors, in the rise of BP associated with the use of anti-angiogenic therapy cannot be drawn. Notably, increased plasma ET-1 levels have also been described in patients with preeclampsia.[18] There is

accumulating evidence that increased placental production of soluble fms-like tyrosine kinase 1 (sFlt-1), a VEGF-binding factor, is involved in the pathogenesis of preeclampsia.[19,20] Indeed, recently Murphy et al reported the development of hypertension in pregnant rats during infusion of sFlt-1 for 6 days.[21] Such infusion was accompanied by a 3-fold increased expression of pre-proendothelin mRNA in the renal cortex, but not by increased expression in the renal medulla or aorta. ET_A-receptor blockade in this model completely prevented the BP rise. As sFlt-1 impairs VEGF signalling, its infusion may therefore create a condition comparable to that induced by sunitinib and the increased ET-1 levels in our studies might be of renal origin as well.

Since the rise in BP was associated with a decrease in renin, involvement of the renin-angiotensin system could be excluded as an underlying mechanism. Despite the decrease in renin, aldosterone levels did not change. The possibility that mineralocorticoid-receptor activation has played a role in the development of hypertension can therefore not be excluded. The sunitinib-induced rise in blood pressure was not associated with an increase in plasma catecholamine levels, indicating that sympathetic nervous system activation was not involved in the BP elevation. This is supported by the observation that the rise in BP was accompanied by a decrease in heart rate. To some extent, our findings disagree with those reported by Veronese et al, as these authors did not report an increase in plasma ET-1 concentration or a decrease in PRA during treatment with the RTKI sorafenib.[22] Sorafenib has partly different receptor targets compared to sunitinib which might account for the different findings in both studies. Furthermore, in the study of Veronese et al, baseline blood samples were taken in the first two weeks after treatment initiation and changes in hormone levels might therefore have been missed.

Renal function impairment, apart from the development of hypertension, is also a well-documented side effect of angiogenesis inhibition.[23-25] In our clinical study no change in serum creatinine concentration was observed, but proteinuria tended to increase. In contrast, in our experimental study sunitinib was associated with a marked impairment of renal function, development of pronounced proteinuria as well as glomerular changes. For several reasons it is unlikely that impairment of renal function accounted for the rise in BP. First, and in accordance with other clinical and experimental studies, the rise in BP already occurred within 1-2 days after administration of sunitinib.[17,26] Second, after sunitinib withdrawal in rats, BP completely normalized, whereas renal function did not. Third, in our clinical study the BP elevation was not accompanied by any deterioration in renal function. Since body weight or NT-proBNP-levels did not increase in our patients, fluid retention is also unlikely to be involved in the rise in BP.

VEGF has been reported to increase the expression of eNOS. Inhibition of VEGF-signalling may therefore result in impaired NO-production, leading to hypertension.[2,3] We used the Langendorff model to investigate whether sunitinib administration impaired endothelium-dependent vasodilation. We found that not only the response to the endothelium-dependent vasodilator bradykinin, but also the responses to the endothelium-independent vasodilator sodium-nitroprusside, as well as the vasoconstrictor Ang II were all considerably impaired in sunitinib-administered rats. These findings indicate that exposure to sunitinib is associated with a generalized impaired function of the vascular smooth muscle cell. From our studies we cannot conclude whether this is related to inhibition of VEGF-signalling and/or interaction of sunitinib with other target receptors or a consequence of the elevated BP.

Not only hypertension, but also left ventricular dysfunction in patients and cardiomyocyte hypertrophy and changes in mitochondrial structure in endomyocardial biopsy samples of patients have been observed during sunitinib treatment.[5] Impaired ATP-generation secondary to mitochondrial dysfunction has been proposed as an underlying mechanism for the development of cardiac dysfunction.[27] However, studies performed in isolated rat heart mitochondria have shown that sunitinib, contrary to sorafenib, impairs mitochondrial function only at supra-therapeutic concentrations.[6] In accordance with these findings in our ex-vivo studies no evidence was found that sunitinib administration, at a dose that markedly increased BP, affected mitochondrial ATP production. Due to the large inter-animal variation in cardiac mitochondrial ATP production and the necessity to perform the studies in different groups of animals, we cannot completely exclude the possibility that an effect of sunitinib on ATP production has been missed. In our current opinion the observed increase in plasma BNP levels in the rats is more likely a consequence of elevated BP as well as a decreased renal clearance rather than reflecting direct cardiac damage by sunitinib.

Some limitations of our studies should be addressed. First, we did not perform a randomized controlled trial. Since sunitinib is a first-line treatment of patients with mRCC or imatinib-resistent GIST it is not possible to perform a placebo-controlled trial in these patients. Second, the number of included patients in our study was rather limited. Despite this limited number we still found relevant changes in blood pressure and various neurohormones because the responses of these parameters among patients were quite uniform. Third, because of our extensive experience with this cell-line, we have used HUVECs to investigate the effect of sunitinib on ET-1 release. Since no increased ET-1 release in response to sunitinib could be demonstrated, it would be worthwhile to study the effect of sunitinib on ET-1 release in other endothelial cell-lines to ascertain the uniformity of this finding.

PERSPECTIVES

Inhibition of VEGF signaling with the multitarget RTKI sunitinib is associated with a substantial rise in BP according to an on/ off-mechanism in both humans and rats, and is accompanied by a rise in circulating ET-1 concentration. Whether this activation of the endothelin-system is instrumental for the rise in BP cannot be concluded at this moment and requires further investigation. Experimental evidence that the endothelin-system plays a role in the development of hypertension during administration of anti-angiogenic agents is emerging.[17,21] If such a mechanism can also be established in patients, antihypertensive treatment with an endothelin-receptor blocker would be a logical choice. Finally, in our clinical study the development of hypertension was associated with renin suppression. The implication of this finding could be that agents interfering with the renin-angiotensin system are less effective in reducing blood pressure in anti-angiogenic therapy-induced hypertension and that calcium-channel blockers or, as discussed earlier, endothelin-receptor blockers may be preferable instead. Since the use of anti-angiogenic agents is expected to increase, future clinical and experimental studies have to be performed to establish which antihypertensive therapy is most effective for the management of hypertension associated with these drugs.

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SUPPLEMENT

Expanded methods

Cardiac mitochondrial function assessment

Mitochondrial respiratory activity, measured as the oxygen consumption rate (flux in pmol oxygen/second/mg mitochondrial protein) was assessed at 37°C by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments). Complex I- and II-dependent respiration were measured in state 2 (respectively, in the presence of 2 mmol/L malate and 10 mmol/L glutamate, and in the presence of 10 mmol/L succinate) and state 3 (in the presence of substrates and 0.25 mmol/L ADP).[1] To prevent retrograde flux of electrons via complex I, complex II-dependent respiration was measured in the presence of 0.5 µmol/L of the complex I inhibitor rotenone. The respiratory adenylate control index (RCI) was calculated by dividing the oxygen flux in state 3 by the flux in state 2. Mitochondrial ATP production was determined by incubating mitochondrial protein (1, 10 and 100 ng/mL) in medium as described by Korsten et al, but without digitonin, in 96-well black microplates (Optiplate; PerkinElmer).[2] Mitochondrial swelling was measured as described by Wang et al.[3] Pore opening was induced by 2 µmol/L $CaCl_2$ with or without 30 nmol/L cyclosporine A. Swelling was measured as a change in light (520 nm) absorption/min 5 minutes after addition of $CaCl_2$.

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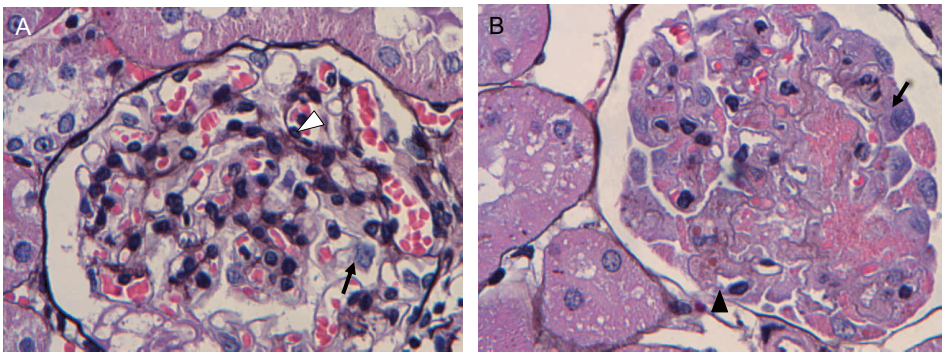

Figure S1. Kidney sections from WKY-rats treated with vehicle (A) and sunitinib (B) stained with Jones silverstaining (magnification ×800). The glomerulus of the vehicle-treated rat is nicely unfolded showing wide capillary lumina, filled with numerous erythrocytes, normal-sized endothelial cells (white arrowhead) and epithelial cells (arrow). After administration of sunitinib, the glomerulus appears more shrinked with narrowing of the capillary lumina and swelling of endothelial (arrowhead) and epithelial cells (arrow).

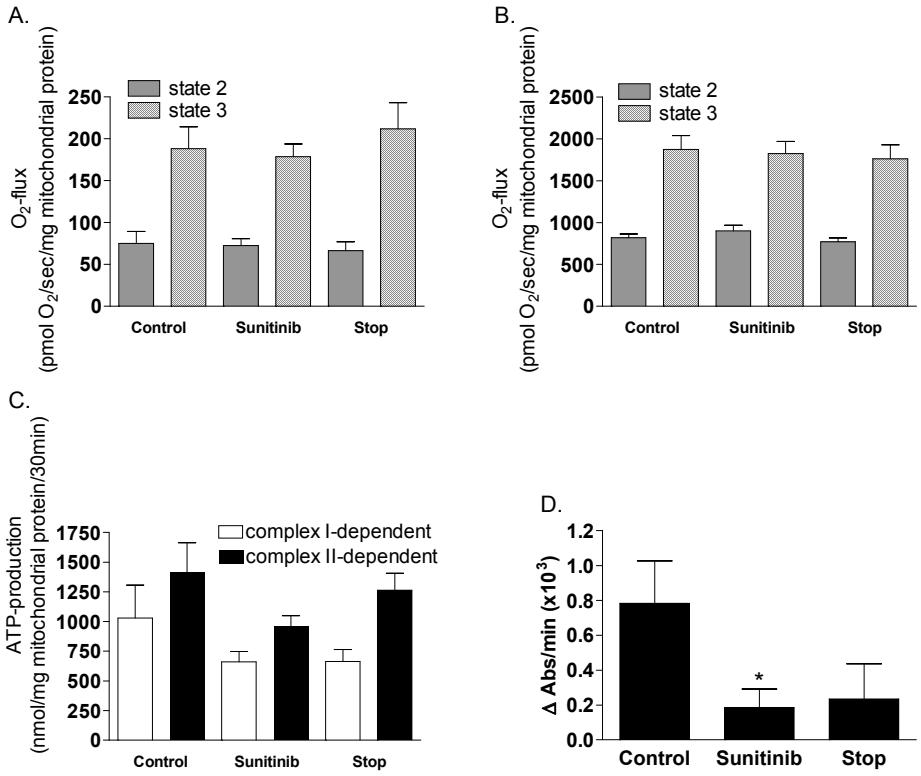

Figure S2. Complex I-dependent (panel A), complex II-dependent (panel B) state 2 and state 3 mitochondrial respiratory activity (measured as oxygen consumption or O_2-flux), ATP production (panel C) and swelling (panel D) in mitochondria isolated from cardiomyocytes of rats after administration of sunitinib (n=6-13), vehicle (control; n=7) or sunitinib withdrawal (stop; n=7). *P<0.05 compared to control.

4

The VEGF-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 side 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, and the role of endothelin-1, decreased nitric oxide-bioavailability as well as of 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 nitric oxide-metabolite excretion. In rats on sunitinib alone, BP increased (ΔBP 31.6±0.9 mmHg). This rise could largely be prevented with the endothelin receptor antagonist macitentan (ΔBP 12.3±1.5 mmHg) and only mildly with tempol, a superoxide dismutase mimetic (ΔBP 25.9±2.3 mmHg). 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.

INTRODUCTION

Angiogenesis, the formation of new capillaries from an existing vasculature, is critical to tumor growth as well as to 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 (RTKI) used in the first-line treatment of metastatic renal cell carcinoma or imatinib-resistent gastrointestinal stromal tumors.[1] Renal toxicity, mainly proteinuria, has been reported in 41-63% of patients treated with bevacizumab.[2] The incidence of proteinuria in patients treated with RTKIs is less well known, as 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 so promotes the development of hypertension.[1] Indeed, we recently reported that the rise in blood pressure (BP), renal dysfunction and proteinuria induced by the RTKI sunitinib was associated with a two-to-three-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.[4-7] In addition, in various hypertensive rat models administration of tempol, a superoxide-dismutase mimetic, which metabolizes superoxide and other reactive oxygen species (ROS), has been shown to reduce BP.[8] Whether increased ROS 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.[9] Preeclampsia is a pregnancy-related disorder characterized by proteinuria and hypertension as well as increased circulating ET-1 levels.[10] One of the underlying pathophysiological mechanisms is thought to be an increased placental production of soluble fms-like tyrosine kinase 1 (sFlt-1).[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 role of ET-1, decreased NO-bioavailability as well as of ROS in the development of sunitinib-induced hypertension and renal side effects.

METHODS

In vivo study

Male Wistar Kyoto rats (WKY, 280-300 gram) obtained from Charles River, were housed in individual cages and maintained on a 12-h 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 solution prepared and administered by oral gavage as described previously.[3] Macitentan (ACT-064992), a dual ET_A / ET_B receptor antagonist kindly provided by Actelion, was dissolved in vehicle containing 0.5% methylcellulose aqueous solution and 0.05% Tween80. 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 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/day of sunitinib L-malate; 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 sacrificed. In the third experiment, rats (n=8) were orally administered the combination of sunitinib and macitentan 30mg/kg/day, for 8 days.[13] 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 Data Supplement. In the fourth experiment, rats were administered the combination of sunitinib by oral gavage and Tempol 200 nmol/kg/min s.c. using osmotic minipumps (Alzet 2ml2) for 8 days (n=6).[14] In all 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, nitric oxide (NO) metabolites (NO_2+NO_3 [NO_x]) and thiobarbituric acid reactive substances (TBARS). Urine was collected on antibiotics (A5955, Sigma) to prevent formation of NO metabolites. In the second experiment, rats were also housed in metabolic cages one week after treatment discontinuation.

Male spontaneous hypertensive rats (SHR, 280-300 gram, 8-9 weeks old; MAP 146±3mmHg; n=2), obtained from Charles River (Germany), were used as hypertensive controls to compare renal histology obtained from WKY-rats exposed to sunitinib to that of SHR.[15] All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

In vitro studies and renal histology

Langendorff studies

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

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 Haematoxylin-eosin (HE), periodic acid Schiff (PAS) and Jones silver. PAS-stained sections were blindly evaluated by a pathologist for the presence or absence of endothelial cell and epithelial cell swelling in 50 glomeruli, as well as semi-quantitatively scored for the presence of ischemia and intra-epithelial protein.

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 were registered.

Determination of preproET-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 tissues were quickly frozen in nitrogen and stored at -80ºC. 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 min; thermal cycling at 95°C for 15 sec, annealing/extension at 60°C for 1 min) with a Step-One cycler (NYSE, Applied Biosystems) using the SYBR Green Q-PCR core KIT (Applied Biosystem). Primer of rat preproET1 (forward AGGGAACAGATGCCAGTGTGCT, reverse TGCATGGTACTTTGGGCTCGGA) was from Invitrogen. The comparative cycle time method (ΔΔCT) was used for relative quantification of gene expression. Messenger RNA

expression was normalized versus actin and expressed as the ratio of target to control value.[16]

Biochemical measurements

ET-1 was assessed using a chemiluminescent ELISA (QuantiGlo®, R&D Systems), urine albumin by enzyme immunoassay (Spi-Bio, France) and TNF-alpha by colorimetric sandwich ELISA (R&D Systems). Urine NOx concentration was determined by fluorimetric quantification of nitrite content (Cayman Chemicals, Ann Arbor, MI) and lipid peroxidation in urine by measurement of TBARS.[17] Serum creatinine and urinary protein were measured at the clinical chemical laboratory of the Erasmus MC.

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's 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 statistical analysis.

RESULTS

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 to control rats (Figure S1, Supplement). 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 (HR) 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-to-body weight (KW/BW)-ratio, proteinuria, albuminuria and urinary ET-1 excretion increased during sunitinib administration (Table 1), in parallel to the previously described increase in BP, serum creatinine and circulating ET-1 levels.[3] All 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 ($r = -0.50$; $p=0.25$; $N=7$), neither were changes in urinary ET-1 excretion and proteinuria ($r = 0.47$; $p=0.20$; $N=7$). Circulating TNF-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

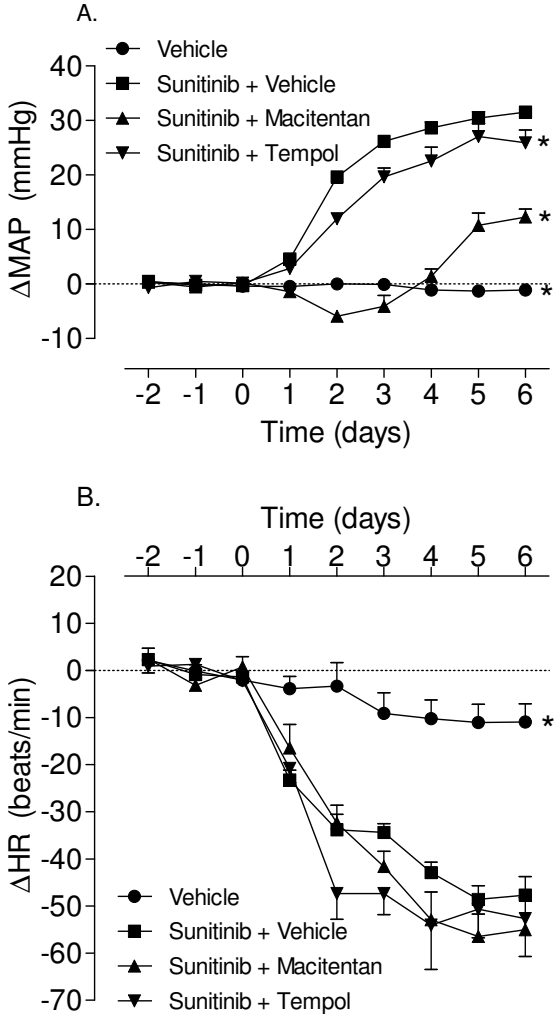

Figure 1. Changes in MAP (A) and HR (B) in response to administration of vehicle (n=6), sunitinib and vehicle (n=12), sunitinib and macitentan (n=8), or sunitinib and tempol (n=6) in rats.
*p<0.05 vs sunitinib + vehicle.

administration (Figure 2) and partly returned after sunitinib withdrawal. Urine TBARS did not change (0.80±0.19 µmol/kg BW/24h at baseline versus 0.87±0.08 µmol/kg BW/24h 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 KW/BW-ratio compared to 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 3A, B, C, D). Treatment with tempol did also not change KW/BW-ratio compared to 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 3A, B, C, D).

Table 1. Parameters in rats after 8 days of treatment with sunitinib (N=10) and after treatment discontinuation (N=7) compared to baseline values or vehicle-treated rats (N=6).

Parameters	Control / Baseline	Sunitinib	P-value	Stop	P-value
MAP (mmHg)	94±3	124±1	<0.001	92±8	>0.05
Left KW/BW-ratio (g/kg)	3.0±0.04	3.4±0.05	<0.001	3.3±0.05	0.001
Right KW/BW-ratio (g/kg)	3.0±0.04	3.5±0.04	<0.001	3.3±0.03	<0.001
Serum creatinine (µmol/L)	8.0±2.7	28.8±6.4	0.03	25.1±1.2	<0.001
Plasma endothelin-1 (pg/ml)	0.6±0.1	1.8±0.2	0.003	0.7±0.1	0.40
Urinary endothelin-1 (pg/day)	3.6±0.8	7.3±1.0	0.007	7.6±0.6	0.007
Proteinuria (mg/day)	9.1±1.4	39.2±5.2	<0.001	12.4±0.7	>0.05
Albuminuria (mg/day)	0.2±0.02	13.1±3.1	<0.001	0.6±0.2	>0.05

Mean arterial pressure (MAP), serum creatinine, plasma endothelin-1 and proteinuria are from Kappers et al.[3] KW, kidney weight; BW, body weight; NA, not applicable. Data are shown as mean±SEM. P-value compared to control/baseline.

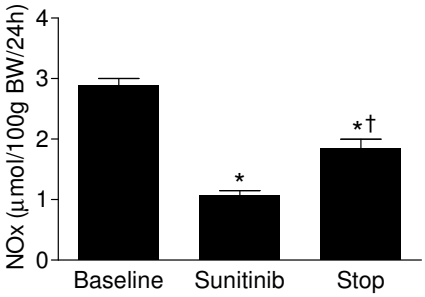

Figure 2. Nitric oxide metabolites (NOx) excretion in urine of WKY rats (n=4) at baseline, after 8 days of administration of sunitinib and after sunitinib withdrawal. * p<0.05 vs baseline, † p<0.05 vs sunitinib.

In vitro studies and renal histology

Langendorff studies

In the Langendorff preparation, coronary flow (CF) responses to bradykinin, Ang II or SNP were not significantly changed after co-administration of sunitinib and macitentan or tempol compared to sunitinib alone (Figure S2A-C, Supplement).

Renal histology

Light microscopic examination showed marked abnormalities including periodic acid Schiff (PAS)-positive intra-epithelial protein droplets and epithelial, as well as endothe-

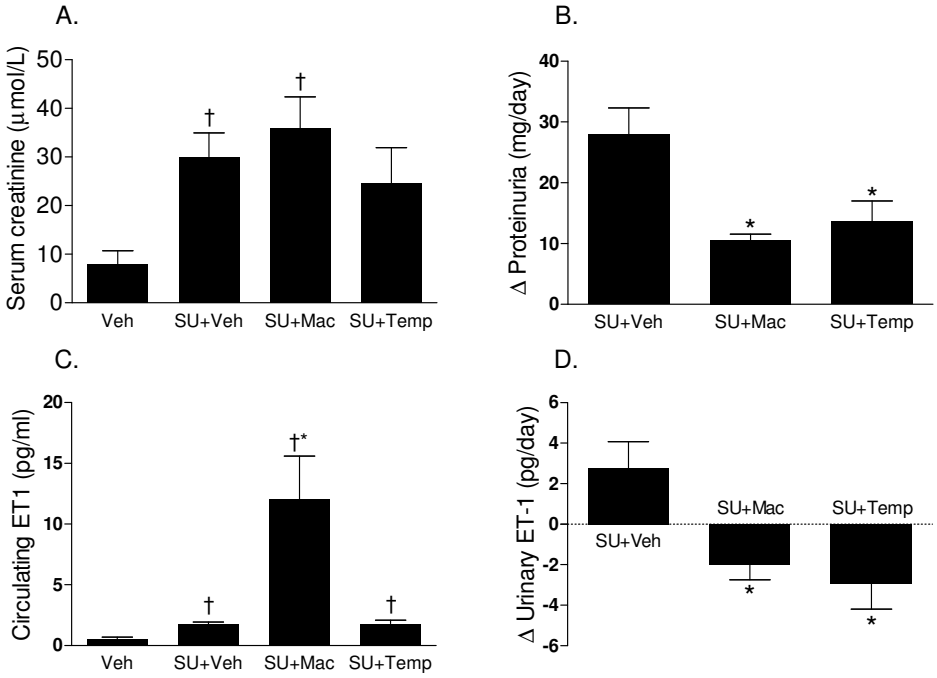

Figure 3. Serum creatinine (A), change in proteinuria (B), circulating ET-1 levels (C) and change in urinary ET-1 excretion (D) in rats after administration of vehicle (n=6), sunitinib and vehicle (n=16), sunitinib and macitentan (n=4-6), or sunitinib and tempol (n=4-6) for 8 days.
*p<0.05 vs sunitinib + vehicle; †p<0.05 vs vehicle.

lial cell swelling in glomeruli from rats exposed to sunitinib compared to control rats (Figure 4). In addition, these glomeruli were more shrunken with narrowed capillary lumina containing less erythrocytes (ischemia) compared to those of control rats (Figure 4). The semi-quantative scores of the renal abnormalities are provided in Table 2. The percentage of glomeruli in renal biopsy sections with a score 2 for intra-epithelial protein was not correlated with the change in proteinuria (r = 0.51; 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 renal abnormalities observed on light microscopic examination completely reversed after sunitinib withdrawal. Remarkably, on light microscopic examination all 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 sacrificing the rats and processing the kidneys.

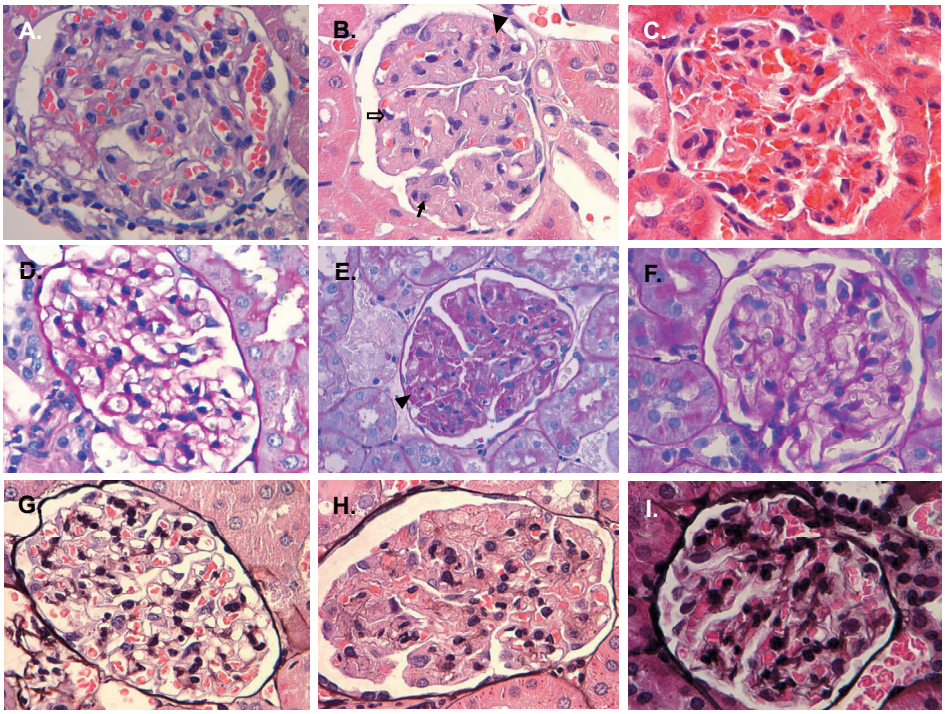

Figure 4. Kidney sections from WKY rats administered vehicle (A, D, G) or sunitinib (B, E, H) compared to SHR rats (C, F, I), stained with HE-, PAS- and Jones silver stain (magnification ×800). After administration of sunitinib for 8 days marked glomerular changes could be observed, including intra-epithelial protein droplets (arrowhead), epithelial cell swelling (arrow), endothelial cell swelling (open arrow) with narrowing of the capillary lumina. None of these abnormalities were observed in kidney sections of 9 weeks-old SHR rats.

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 to controls (N=6).

Group	Glomerular ischemia (% glomeruli)			Endothelial cell swelling (% glomeruli)		Epithelial cell swelling (% glomeruli)		Intra-epithelial protein (% glomeruli)		
Score	None	Moderate	Severe	0	1	0	1	0	1	2
Control	60±13	32±10	8±4	100	0	100	0	99±1	1±1	0
Sunitinib	17±2*	54±4*	29±5*	78±3*	22±3*	63±5*	37±5*	29±4*	48±2*	23±4*
Sunitinib + Macitentan	15±6*	35±2†	51±4*†	85±3*	15±3*	77±6*	23±6*	33±8*	59±7*	8±3*†
Sunitinib + Tempol	16±2*	41±4	44±3*	84±4*	16±4*	56±5*	44±5*	30±11*	50±10*	20±14*

All evaluations were performed in 50 glomeruli of a PAS-stained section and the numbers of glomeruli with each score were counted. Endothelial and epithelial cell swelling were scored as present (1) or absent (0) in each glomerulus. The presence of intra-epithelial protein was evaluated using a semiquantative scale: 0 (no protein in the epithelial cells of a glomerulus), 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). * $p<0.05$ vs control; † $p<0.05$ vs sunitinib.

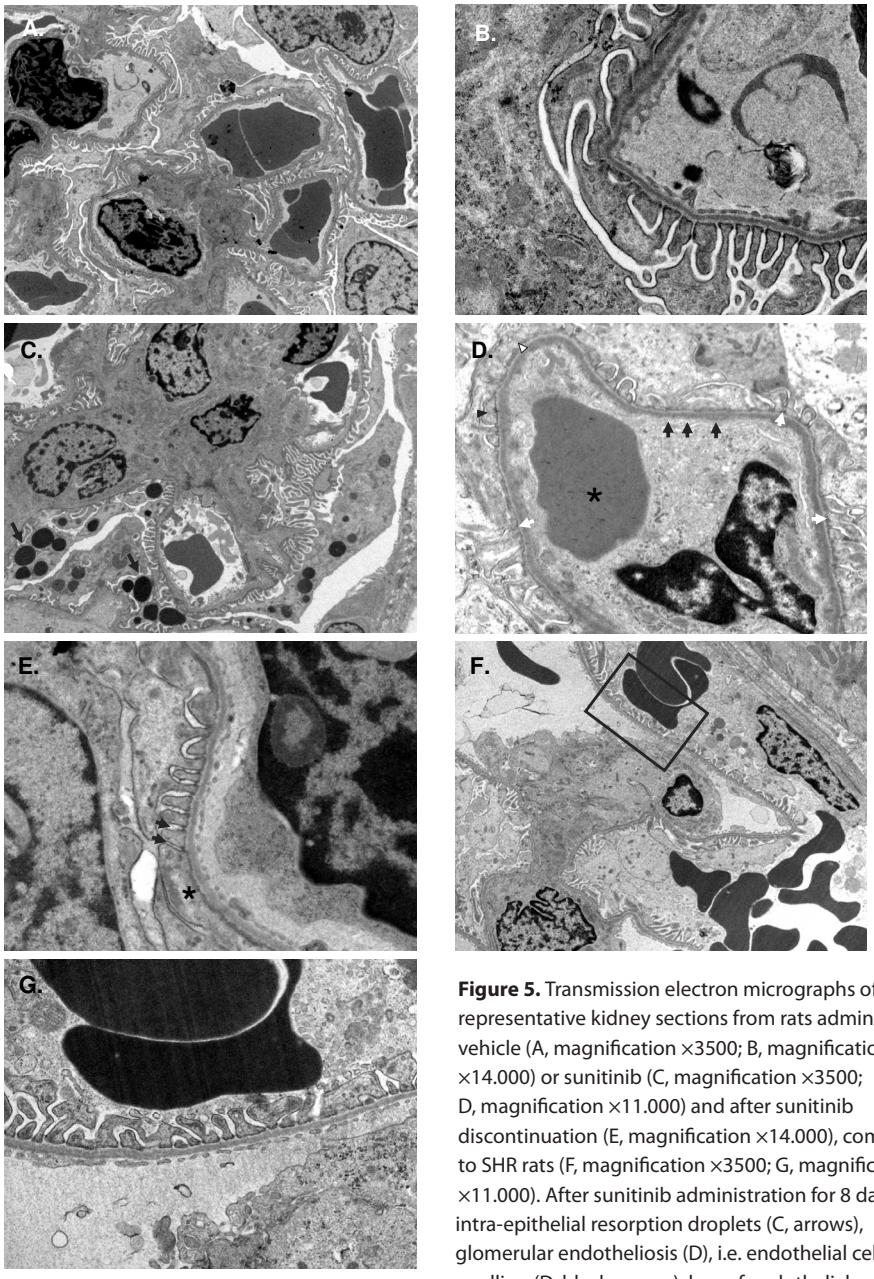

Figure 5. Transmission electron micrographs of representative kidney sections from rats administered vehicle (A, magnification ×3500; B, magnification ×14.000) or sunitinib (C, magnification ×3500; D, magnification ×11.000) and after sunitinib discontinuation (E, magnification ×14.000), compared to SHR rats (F, magnification ×3500; G, magnification ×11.000). After sunitinib administration for 8 days intra-epithelial resorption droplets (C, arrows), glomerular endotheliosis (D), i.e. endothelial cell swelling (D, black arrows), loss of endothelial fenestration (black arrows) and obliteration of capillary lumen (D, asterix), effacement (D, black arrowhead) and fusion of podocyte foot processes (D, white arrowhead) and narrowing of the slit pores (D, white arrows) could be observed. These changes were only partly reversible after sunitinib discontinuation with persistent fusion of podocyte foot processes (E, asterix), persistent narrowing of slit pores (E, arrows) and only local reappearance of endothelial fenestrations. None of the abnormalities mentioned above could be observed in kidney sections from 8-9 weeks-old SHR rats (F, G).

Electron microscopic examination revealed intra-epithelial 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 to the rats administered sunitinib alone (Table 2). In addition, macitentan tended to decrease intra-epithelial protein deposits whereas no significant effects 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).

PreproET-1 mRNA levels in renal cortex and medulla

PreproET-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 two-to-three-fold rise in circulating ET-1 levels.[3] Our current study shows that sunitinib administration is also associated with marked renal histopathological changes, especially glomerular endotheliosis. This renal toxicity was accompanied by increased urinary excretion of ET-1. Importantly, renal histopathology and urinary ET-excretion were not or only partly reversible after 11 days of sunitinib-withdrawal, although BP had already returned to baseline at that time.[3]

Since 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_A- and ET_B-receptor antagonist macitentan. Compared to sunitinib alone, co-administration of macitentan diminished renal injury as reflected by a decrease in proteinuria, urinary ET-1 excretion and severe glomerular intra-epithelial protein deposition, but the sunitinib-induced rise in serum creatinine was not prevented. Initially, co-administration of macitentan completely blocked the sunitinib-induced rise in BP, but after 4 days a secondary rise in BP was observed, although less pronounced than with

sunitinib alone. Since 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 completely be prevented by the selective ET_A-receptor blocker atrasentan.[18] Whether selective ET_A-receptor blockade could also prevent renal toxicity has not been evaluated in that study.

Although, ET_B-receptor-dependent systemic vasodilation has been observed in healthy volunteers, this effect is lost in pathological conditions such as atherosclerosis and type II diabetes, possibly due to upregulation of contractile smooth muscle cell ET_B receptors.[13,19,20,21] Thus, in pathological conditions dual ET_A/ET_B receptor blockade may be more favorable than selective ET_A receptor blockade. However, chronic kidney disease (CKD) might be an exception because in this condition ET_B receptor-mediated vasoconstriction has been reported to be less important than ET_B receptor-mediated vasodilation. [22] If in CKD renal histopathological changes are mainly ET_A-receptor-mediated, selective ET_A receptor blockade might be preferred.[23] Indeed, in young hypertensive Ren-2 transgenic rats on a high salt diet immediately after weaning, chronic administration of the selective ET_A receptor blocker atrasentan diminished proteinuria and renal injury to a greater extent than the dual ET receptor antagonist bosentan.[24] 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.[25] Furthermore, the dual ET_A/ET_B receptor blocker macitentan that was used in the present study reduced renal injury in the streptozotocin-induced diabetic rat.[13] Taken together, these findings provide strong evidence that dual ET_A/ET_B receptor blockade, like selective ET_A antagonism, exerts renal protective effects in various models of renal injury.

Co-administration 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 to explain this discrepancy? Since urinary ET-1 excretion is kidney-derived and reflects renal injury when increased, a decrease in this parameter indicates improvement of renal injury.[26] 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.[27,28] 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.[4,5] Therefore we explored whether the superoxide-dismutase mimetic tempol had a beneficial effect on sunitinib-induced renal toxicity and hypertension. Although co-administration 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 TBARS 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 (NOS).[29] Moreover, administration of a specific antibody against the VEGF2-receptor was associated with a reduced expression of endothelial and neuronal NOS in the mouse kidney.[30] 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.[3] 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.[3] Co-administration of macitentan or tempol did not normalize the impaired microvascular function induced by sunitinib, although the vasodilator response to SNP 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.[10] Indeed, kidneys of sunitinib-exposed rats showed pronounced glomerular endotheliosis, a characteristic renal abnormality in preeclamptic women (Figure S3, Supplement).[31] 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 anti-angiogenic properties.[11,12] 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, intra-epithelial protein resorption droplets and foot-process effacement, all changes fully identical to the abnormalities found in our rats.[12] 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.[6]

Recently, Murphy et al showed that infusion of sFlt-1 in healthy-pregnant rats was accompanied by a 3-fold increased expression of preproET-1 mRNA in the renal cortex, whereas the expression in the aorta and placenta was not increased.[32] In contrast to these findings, no increased expression of preproET1 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 due to other mechanisms, e.g. an increase in endothelin-converting enzyme activity and/or a reduction in ET_B receptor number. Blockade of the latter (clearance) receptor by macitentan explains why the circulating ET-1 levels increased even further during co-administration of sunitinib and macitentan. Yet, urinary ET-1 excretion decreased during co-administration 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.[26] A dissociation between circulating and renal ET systems has also been described in patients with systemic inflammatory disease and active renal involvement.[33] Furthermore, our findings showing normalization of circulating ET-1 levels after sunitinib-withdrawal, whereas 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.

Since 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 side effects. Firstly, the rise in blood pressure occurred as soon as after 1 day of administration of sunitinib. Secondly, despite BP normalization after sunitinib withdrawal renal histological abnormalities were still present. Thirdly, it has been demonstrated that conditional gene targeting to delete selectively VEGF from renal podocytes in adult mice results in profound glomerular injury that precedes the development of hypertension.[34] VEGF is produced by glomerular podocytes and is necessary for maintaining a healthy fenestrated glomerular endothelium by interacting with endothelial cell VEFGRs. Fourthly, the independency of the sunitinib-induced hypertension and renal toxicity is also supported by our observations in 8-9 week-old SHR rats. Although these rats were hypertensive since birth with BPs as high as 146±3mmHg, glomerular injury was absent.[15]

Lastly, as previously mentioned, ET-1 has been reported to increase glomerular permeability independent of BP.[27]

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 seem logical candidates for treatment of sunitinib-induced cardiovascular and renal adverse effects and may even provide complementary therapeutic anti-angiogenic effects.[35,36] 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 blood pressure in patients subjected to angiogenesis inhibition.[34]

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SUPPLEMENT

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 µL) 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-µm 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 semiquantative 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.

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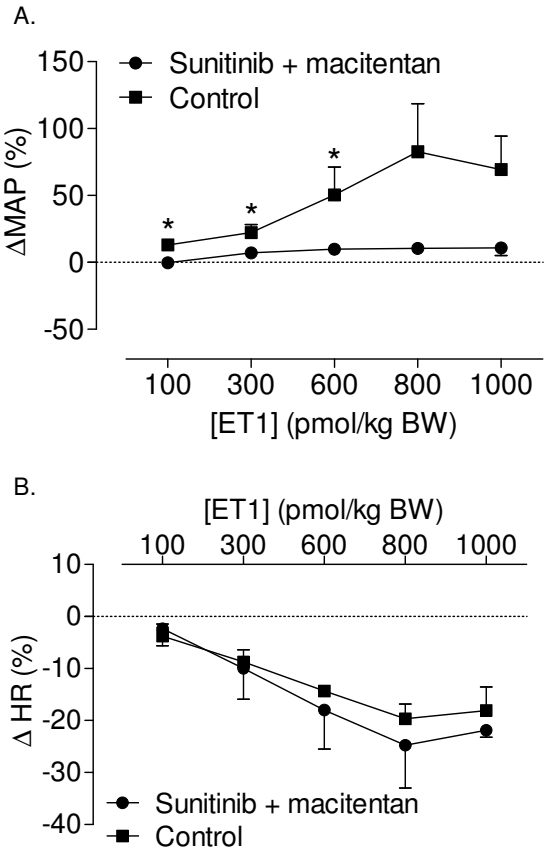

Figure S1. Change of mean arterial pressure (MAP; A) and heart rate (HR; B) compared to baseline in response to bolus injections of endothelin-1 (ET-1) in rats administered sunitinib and macitentan (n=4) for 8 days and in control rats administered vehicle (n=3).

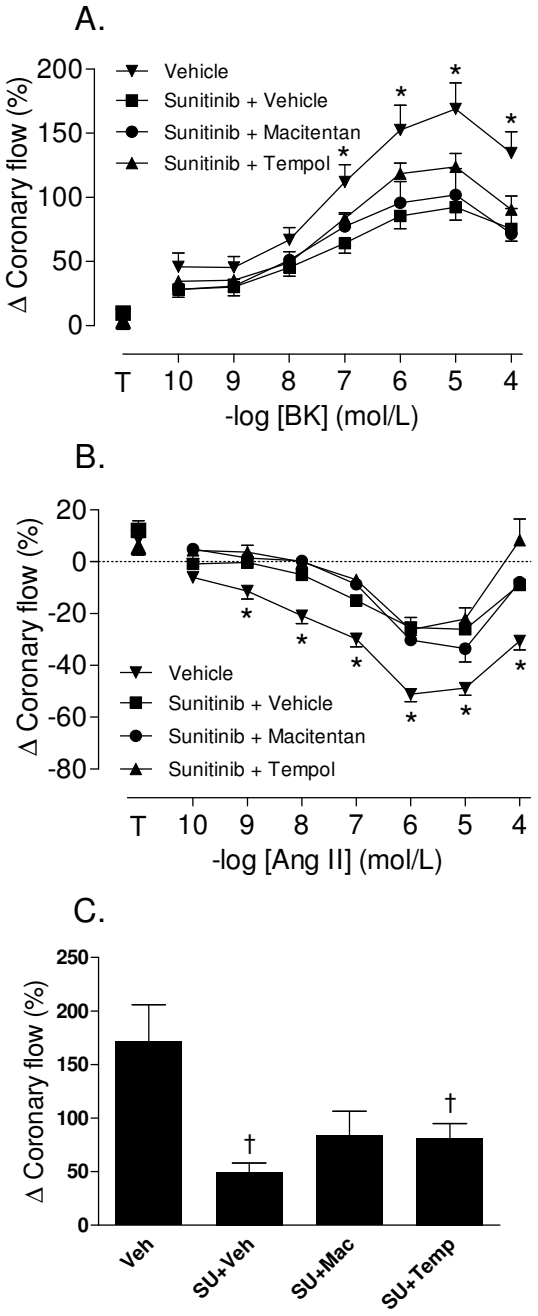

Figure S2. Coronary flow responses to bradykinin (BK; A), ang II (B) and a single injection to sodium nitroprusside (SNP; 10 mmol/L); C) in isolated rat hearts after administration of vehicle (n=9), sunitinib and vehicle (n=10), sunitinib and macitentan (n=4), and sunitinib and tempol (n=6) for 8 days. 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.

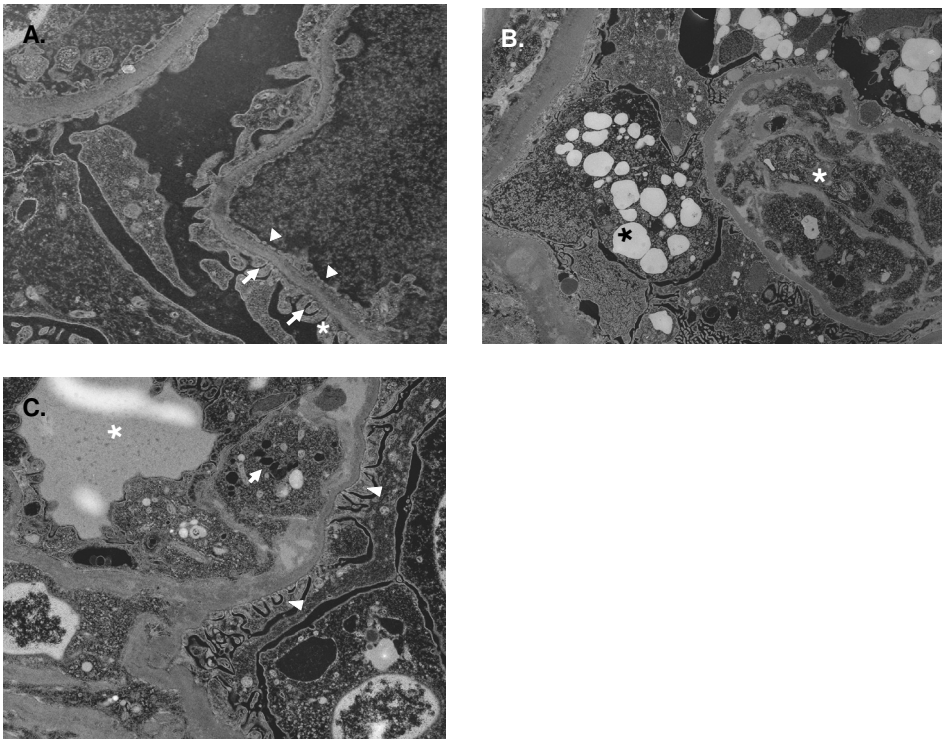

Figure S3. A. Electron micrograph of a normal glomerulus in a healthy pregnant women (magnification ×4000). Note the open capillary lumen, endothelial fenestrations (white arrowheads) and normal podocytes (white asterix) with open slit pores (white arrow). B, C. Electron micrographic overviews of glomeruli 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).

5

Sunitinib induced systemic vasoconstriction is endothelin-mediated and does not involve decreased NO availability or enhanced oxidative stress

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Hypertension, provisionally accepted

ABSTRACT

Angiogenesis inhibition with agents targeting tyrosine kinases of vascular endothelial growth factor receptors (VEGFR) is an established anticancer treatment, but is, unfortunately, frequently accompanied by systemic hypertension and cardiac toxicity. Whether VEGFR-antagonism also has adverse effects on the pulmonary and coronary circulations is presently unknown. In chronically instrumented awake swine, the effects of the VEGFR-antagonist sunitinib on the systemic, pulmonary and coronary circulation were studied. One week after sunitinib (50 mg p.o. daily) mean aortic blood pressure (MABP) had increased from 83±5 at baseline to 97±6 mmHg ($P<0.05$) due to a 57±20% increase in systemic vascular resistance as cardiac output decreased. In contrast, sunitinib had no discernible effects on pulmonary and coronary hemodynamics or cardiac function. We subsequently investigated the mechanisms underlying the sunitinib-induced systemic hypertension. Intravenous administration of NO-synthase inhibitor N^{ω}-nitro-L-arginine increased MABP by 24±1 mmHg under baseline conditions, while it increased MABP even further following sunitinib administration (32±3 mmHg,$P<0.05$). Reactive oxygen species scavenging with a cocktail of antioxidants lowered MABP by 13±2 mmHg before, but only by 5±2 mmHg ($P<0.05$) after sunitinib administration. However, intravenous administration of the dual ET_A/ET_B receptor blocker tezosentan, which did not lower MABP at baseline, completely reversed MABP to pre-sunitinib values. These findings indicate that sunitinib produces vasoconstriction selectively in the systemic vascular bed, without affecting pulmonary or coronary circulations. The sunitinib-mediated systemic hypertension is principally due to an increased vasoconstrictor influence of endothelin-1, with no apparent contributions of a loss of NO-bioavailability or increased oxidative stress.

INTRODUCTION

Angiogenesis inhibition, by targeting the tyrosine kinases of the vascular endothelial growth factor (VEGF) receptors (VEGFRs), has become an established treatment of several tumor types. This therapy is associated with adverse effects including the development of hypertension and cardiac and renal toxicity.[1] Hypertension has been reported in up to 60% of patients treated with sunitinib, an orally active multitarget receptor tyrosine kinase inhibitor (RTKI), targeting amongst others the VEGFR-1 and -2, that is used as first-line treatment of metastatic renal cell carcinoma or imatinib-resistant gastrointestinal stromal tumors.[1] In addition, impaired cardiac function, as reflected by a decrease in left ventricular ejection fraction (LVEF) of 10-15%, has been observed in up to 28% of patients treated with sunitinib.[2] Angina pectoris and increased levels of biomarkers reflecting ischemic myocardial damage may also occur during sunitinib treatment.[3] Thus far, clinical and experimental studies on the cardiovascular side effects of angiogenesis inhibition only focused on the systemic vasculature. Whether adverse effects of angiogenesis inhibition with sunitinib also occur in the pulmonary and/or coronary circulation is unknown. It should be mentioned that pulmonary arterial hypertension has recently been reported during treatment with dasatinib, a RTKI used for the treatment of chronic myeloid leukemia.[4]

VEGF, through activation of VEGFR-2, stimulates endothelial NO synthase (eNOS) resulting in enhanced NO production and vasodilation.[5,6] It has therefore been suggested that NO bioavailability is reduced during inhibition of the VEGF-pathway, resulting in vasoconstriction and the development of hypertension. Indeed, in mice administration of an anti-VEGFR-2 antibody, attacking the same receptor as RTKIs, caused a rapid increase in mean aortic blood pressure (MABP) and a marked reduction in the expression of endothelial and neuronal NOS in the kidney.[7] Furthermore, prior administration of the eNOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) in mice abolished the difference in MABP between vehicle and anti-VEGFR-2 treated groups, suggesting that decreased NO-bioavailability in response to anti-angiogenic agents is one of the mechanisms causing hypertension.[7] However, clinical studies, using flow-mediated dilatation (FMD) as an index of NO-bioavailability, do not unequivocally support the hypothesis that a decrease in NO-bioavailability underlies the increase in BP, as decreases in both endothelium-dependent and endothelium-independent vasodilatation have been reported in patients treated with RTKIs.[8,9] In previous clinical and experimental studies we and others have shown that activation of the endothelin system is involved in the sunitinib induced rise in MABP.[10-12] Apart from inducing vasoconstriction, ET-1 activates vascular NADPH-oxidase, leading to an increase in oxidative stress through enhanced reactive oxygen species (ROS) production.[13,14] Since oxidative stress is generally increased

in hypertension and plays a pathogenic role in the development and progression of cardiovascular disease, we hypothesized that enhanced formation of ROS, contributes to the sunitinib-induced cardiovascular side effects.[15]

To further explore the cardiovascular side effects of sunitinib we performed detailed studies in chronically instrumented awake swine. Firstly, we studied the effects of angiogenesis inhibition on the systemic, pulmonary and coronary circulations, as well as on cardiac performance under resting conditions and during exercise. Secondly, we explored to what extent alterations in NO-mediated vasodilator tone, ET-1-mediated vasoconstriction and oxidative stress contribute to the cardiovascular side effects of angiogenesis inhibition.

METHODS

Animals

Studies were performed in accordance with the American Physiological Society's "Guiding Principles in the Care and Use of Laboratory Animals" and with approval of the Animal Care Committee of Erasmus MC. Six crossbred Yorkshire x Landrace swine of either sex (2 to 3 months old; 21 ± 1 kg at the time of surgery) were entered into the study.

Surgical Procedures

Swine were sedated (20 mg/kg ketamine i.m. + 1 mg/kg midazolam i.m.), anesthetized (thiopental sodium 15 mg/kg iv), intubated and ventilated with a mixture of O_2 and N_2 (1:2).[16] Anesthesia was maintained with midazolam (2 mg/kg + 1 mg kg^{-1} h^{-1} i.v.) and fentanyl (10 µg $kg^{-1}h^{-1}$, i.v.). Subsequently, animals were instrumented under sterile conditions as previously described.[17,18] Briefly, a thoracotomy was performed through the left fourth intercostal space. Subsequently, a polyvinylchloride catheter was inserted into the aortic arch, for the measurement of aortic pressure and blood sampling for the determination of PO_2, PCO_2, pH, O_2 saturation and hemoglobin concentration (ABL800, Radiometer). A high fidelity Konigsberg pressure transducer was inserted into the left ventricle (LV) via the apex for measurement of LV pressure and maximum rate of rise and fall of LV pressure ($LVdP/dt_{max}$, $LVdP/dt_{min}$) as indices for contractility and relaxation. Fluid-filled catheters were implanted for measurement of blood pressures in the LV, left atrium (LA) and pulmonary artery (PA). Flow probes were placed around the proximal left anterior descending coronary artery (LAD, 2.5-3 mm, Transonic Systems) for measurement of coronary blood flow (CBF) and around the aorta (16 mm, Transonic Systemic) for measurement of cardiac output and stroke volume (SV).[17,18] Electrical wires and catheters were tunneled subcutaneously to the back. The chest was closed and the animals were

allowed to recover. Animals received analgesia (0.3 mg buprenorphine i.m.) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamycin i.v.) for 5 days.

Experimental protocols

Sunitinib

Studies were performed 1-4 weeks after surgery with swine resting and/or exercising on a motor-driven treadmill. Excellent reproducibility of exercise trials has been reported previously.[17,18] After obtaining hemodynamic measurements with swine lying quietly on a treadmill, a five-stage (1-5 km/h) treadmill exercise protocol was started with each exercise stage lasting 2-3 min. Hemodynamic variables were continuously recorded and both arterial and mixed venous blood samples were collected during the last 60 seconds of each exercise stage for the determination of body oxygen consumption.[18] Sunitinib L-malate was given in a daily oral dose of 50 mg, i.e. the same dose as used in patients. Sunitinib was mixed with food and administered early in the morning. Resting hemodynamics were measured 4 hours after the first dose of sunitinib and after 1 week of daily sunitinib administration. After 1 week of sunitinib administration the exercise protocol was repeated to assess the effects of sunitinib on exercise-induced hemodynamic responses.

Acute pharmacological interventions

After measuring systemic, pulmonary and coronary hemodynamics at rest, acute intravenous doses of an endothelial NO-synthase (NOS) inhibitor, a dual ET_A and ET_B receptor ($ET_A/_B$) antagonist or a ROS scavenger cocktail were given on separate days in random order and cardiovascular effects were assessed. Subsequently, swine were exposed to sunitinib for 7-10 days and the pharmacological interventions were repeated, in random order on separate days, to assess the roles of NO, ET-1 and ROS in sunitinib-induced cardiovascular effects.

Pharmacological agents

The eNOS inhibitor N^{ω}-nitro-L-arginine (LNNA [Sigma]) was given as a single intravenous dose of 20 mg/kg.[19] The dual ET_A and ET_B receptor (ET_A/ET_B) antagonist tezosentan (a kind gift from Dr Clozel, Actelion Pharmaceuticals Ltd.) was intravenously administered over 10 min in a dose of 3 mg/kg, followed by a continuous infusion of 6 $mg \cdot kg^{-1} \cdot h^{-1}$ iv.[20] For ROS scavenging, the superoxide dismutase (SOD)-mimetic 4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL; 30 mg/kg iv), in combination with N-Acetylcysteine (NAC, 150 mg/kg iv), and N-Mercaptoproprionylglycine (MPG, 1 mg/kg/min iv) were infused in 10 minutes prior to the measurements.[21-24]

Data acquisition and analysis

Digital recording and off-line analysis of hemodynamic data and computation of body O_2 consumption (BVO_2) have been described in detail elsewhere.[18] To correct for growth of swine, cardiac index (CI) and stroke volume index (SVi) were calculated as cardiac output and stroke volume, respectively, divided by body weight. Systemic vascular resistance index (SVRi) was computed as MABP divided by CI. Pulmonary vascular resistance index (PVRi) was computed as mean pulmonary artery pressure (MPAP) minus mean left atrial pressure (MLAP) divided by CI. Finally, coronary vascular resistance (CVR) was calculated as MABP divided by CBF. Statistical analysis was performed using regression analysis with each animal as a dummy variable and with BVO_2, heart rate, as well as sunitinib as independent variables. Statistical significance was accepted at $P \leq 0.05$. Data are presented as mean ± SEM.

RESULTS

Effect of sunitinib on hemodynamic parameters at rest

Oral administration of the first dose of sunitinib resulted in an increase in MABP within 4 hours in 5 out of 6 animals (Table 1). The increase in MABP was associated with an

Table 1. Resting hemodynamic responses prior to and 4 hours and 7 days after sunitinib administration.

Parameters	Baseline	4h Sunitinib	1wk Sunitinib
HR (beats/min)	112 ± 6	111 ± 10	105 ± 7*
MABP (mmHg)	83 ± 5	93 ± 5*	97 ± 6*
CI (ml min^{-1} kg^{-1})	155 ± 11	162 ± 17	132 ± 12*
SVRi (mmHg L^{-1} min kg)	547 ± 45	607 ± 69	780 ± 108*
LVSP (mmHg)	110 ± 6	115 ± 8	116 ± 10
SVi (ml kg^{-1})	1.38 ± 0.06	1.46 ± 0.06*	1.26 ± 0.10
LV dP/dt_{max} (mmHg s^{-1})	2800 ± 460	2870 ± 260	2620 ± 250
LV dP/dt_{min} (mmHg s^{-1})	-2220 ± 160	-2280 ± 130	-2320 ± 190
MPAP (mmHg)	15 ± 2	17 ± 2	14 ± 2
MLAP (mmHg)	5 ± 2	7 ± 2	4 ± 1
PVRi (mmHg L^{-1} min kg)	64 ± 12	59 ± 5	68 ± 6
CBF (ml/min)	48 ± 2	44 ± 2	47 ± 3
CVR (mmHg ml^{-1} min)	1.64 ± 0.14	1.90 ± 0.23	2.00 ± 0.17*

Values are mean ± SE. CBF, coronary bloodflow; CI, cardiac index; CVR, coronary vascular resistance; HR, heart rate; LV dP/dt, left ventricular rate of rise in pressure; LVSP, left ventricular systolic pressure; MABP, mean aortic blood pressure; MLAP, mean left atrial pressure; MPAP, mean pulmonary artery pressure; PVRi, pulmonary vascular resistance index; SVi, stroke volume index; SVRi, systemic vascular resistance index.
*P <0.05 vs baseline.

increase in LVSP, but $LVdP/dt_{max}$, $LVdP/dt_{min}$ and SVi did not change (Table 1). Also, no change in CBF occurred, while CVR slightly increased, likely reflecting an autoregulatory increase in coronary vasomotor tone in response to the increase in MABP. The first dose of sunitinib had no effect on either PAP or PVRi (Table 1).

Seven days of daily sunitinib administration resulted in sustained systemic vasoconstriction as evidenced by an increase in SVRi and MABP in all animals (Table 1). The increase in MABP was accompanied by a decrease in heart rate and CI and an increase in LVSP, while $LVdP/dt_{max}$, $LVdP/dt_{min}$ and SVi were unchanged. The first dose or repeated doses of sunitinib had no effect on the coronary or the pulmonary vasculature, as CBF, CVR, MPAP and PVRi were similar to values before sunitinib administration (Table 1).

Effect of sunitinib on hemodynamic parameters during exercise

Sunitinib did not influence the exercise-induced increase in heart rate or CI, while the sunitinib-induced increase in MABP and SVRi decreased with increasing exercise intensity (Figure 1), indicating that the vasoconstrictor effect of sunitinib diminished with increasing exercise intensity. Sunitinib also had no effect on MPAP, PVRi or CBF during exercise, while the sunitinib-induced increase in CVR decreased with exercise-intensity (Figure 1), reflecting the waning effect of sunitinib on MABP during exercise.

Effect of sunitinib on hemodynamic parameters during NO, ET and ROS inhibition

Administration of LNNA resulted in systemic vasoconstriction as evidenced by an increase in SVRi that resulted in increases in MABP and LVSP (Figure 2). The increase in MABP was associated with a, likely baroreflex-mediated decrease in heart rate and CI (Table 2). The effect of LNNA on systemic hemodynamics was slightly more pronounced after 1 week of sunitinib treatment (Figure 2). Under baseline conditions, LNNA also increased PVRi, reflecting pulmonary vasoconstriction, while the effect of LNNA on PVRi after sunitinib treatment failed to reach statistical significance (P=0.13, Figure 2). LNNA had no effect on CBF, but CVR increased. Sunitinib did not affect the LNNA-induced increase in CVR (Figure 2).

Prior to sunitinib, $ET_{A/B}$ receptor blockade with tezosentan had no effect on MABP or SVRi (Figure 2). In contrast, in the presence of sunitinib, MABP and SVRi decreased in response to tezosentan administration (Figure 2). The tezosentan-induced decrease in SVRi was identical to the increase in SVRi induced by sunitinib. In the absence of sunitinib, administration of tezosentan resulted in a decrease in PVR. This tezosentan-induced pulmonary vasodilation was not altered by administration of sunitinib (Figure 2). Tezosentan had no effect on the coronary vasculature (Figure 2) or on LVSP, LVdP/

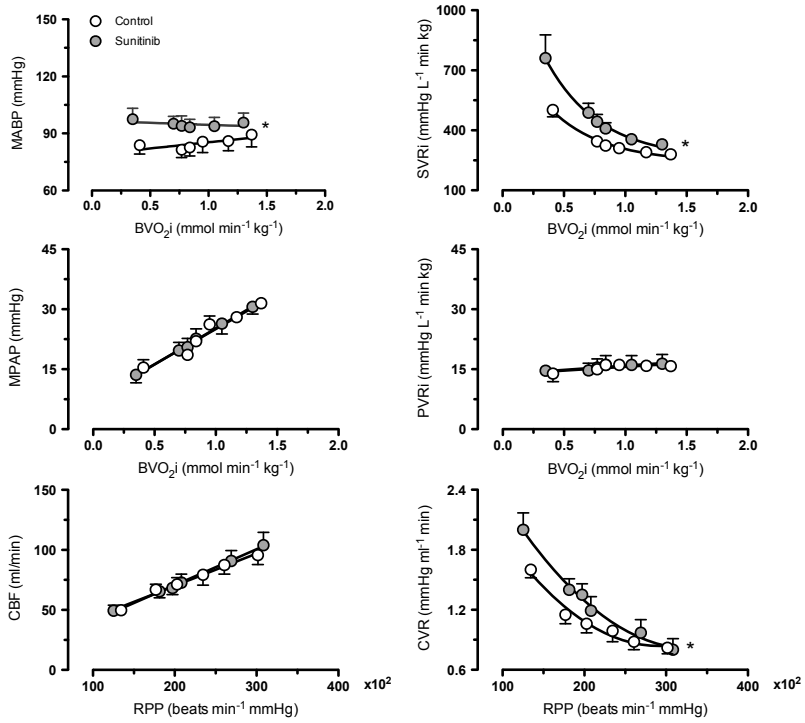

Figure 1. Effect of sunitinib on systemic, pulmonary and coronary hemodynamic parameters at rest and during graded treadmill exercise.
BVO_2i: Body oxygen consumption; MABP: mean aortic blood pressure; SVRi: systemic vascular resistance; MPAP: mean pulmonary artery pressure; PVR: pulmonary vascular resistance; RPP: rate pressure product; CBF: coronary blood flow; CVR: coronary vascular resistance; *P < 0.05 sunitinib vs control; ** P<0.06 vs control.

dt_{max}, $LVdP/dt_{min}$ and SVi in the absence of sunitinib, while SVi increased slightly during sunitinib administration (Table 2).

Prior to sunitinib, ROS scavenging resulted in systemic vasodilation as evidenced by the decreases in MABP and SVRi (Figure 2). This effect was reduced following sunitinib (Figure 2). In the pulmonary circulation, ROS scavenging resulted in a decrease in PAP prior to sunitinib, while it had no effect on PAP in the presence of sunitinib. Moreover, ROS scavenging did not affect PVRi either in the absence or presence of sunitinib (Figure 2). ROS scavenging had no effect on CBF either before or after sunitinib. Finally, ROS scavenging did not affect LVSP, dP/dt_{max}, dP/dt_{min} or Svi, either before or after sunitinib (Table 2).

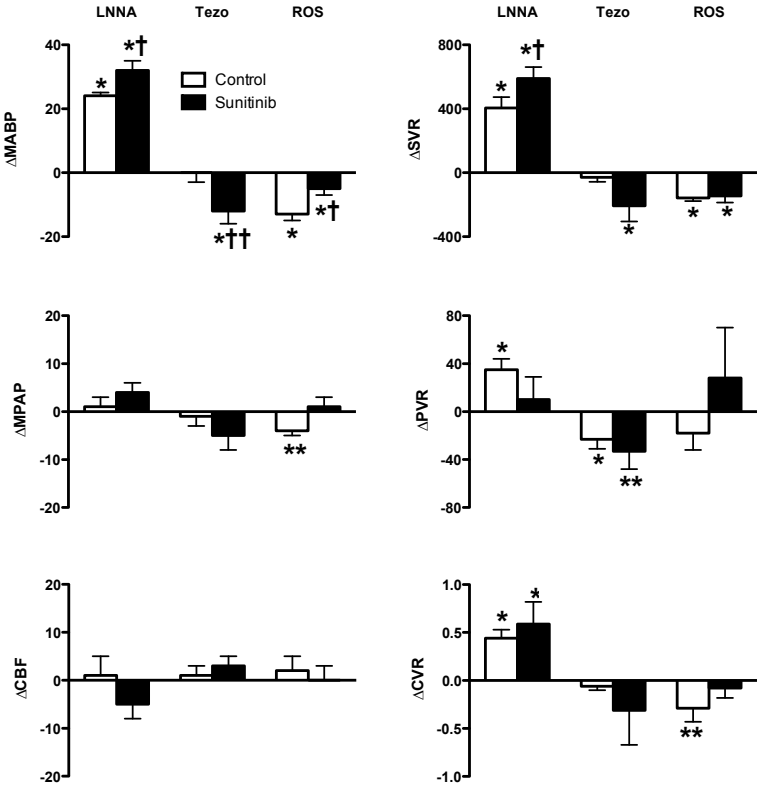

Figure 2. Effect of inhibition of eNOS with LNNA, $ET_{A/B}$ receptor blockade with tezosentan (tezo) and ROS scavenging on systemic, pulmonary and coronary hemodynamic parameters in the absence and presence of sunitinib. Data are shown as changes form corresponding baseline. MABP: mean aortic blood pressure; SVRi: systemic vascular resistance; MPAP: mean pulmonary artery pressure; PVR: pulmonary vascular resistance; CBF: coronary blood flow; CVR: coronary vascular resistance; *P < 0.05 vs control; **P<0.10 vs control; †P<0.05, ††P<0.10, pre- vs postsunitinib.

DISCUSSION

The main findings of the present study in chronically instrumented awake swine are (*i*) administration of the RTKI sunitinib induces a rapid rise in systemic BP due to an increase in systemic vascular resistance, without affecting the pulmonary or coronary circulation; (*ii*) the vasoconstrictor effect of sunitinib on the systemic circulation waned with increasing exercise intensities; (*iii*) sunitinib had no adverse effects on cardiac function either at rest or during exercise; (*iv*) the systemic hemodynamic effects of sunitinib were fully reversed upon administration of the ET_A/ET_B receptor blocker tezosentan, confirming previous findings that an increase in endogenous ET-mediated vasoconstrictor tone is involved in the sunitinib-induced rise in MABP; and finally, (*v*) no evidence was found

Table 2. Changes in hemodynamic parameters as a result of eNOS inhibition, $ET_{A/B}$ blockade and ROS scavenging in the absence and presence of sunitinib.

		LNNA	Tezosentan	ROS
ΔHR	Control	-11 ± 7	6 ± 3	22 ± 8*
(beats/min)	Sunitinib	-24 ± 3*†	0 ± 5	22 ± 7*
ΔMABP	Control	24 ± 1*	0 ± 3	-13 ± 2*
(mmHg)	Sunitinib	32 ± 3*†	-12 ± 4*	-5 ± 2*†
ΔCI	Control	42 ± 8*	12 ± 5	35 ± 6*
(ml min^{-1} kg^{-1})	Sunitinib	47 ± 8*	13 ± 4*	22 ± 4*
ΔLVSP	Control	18 ± 5*	4 ± 3	-5 ± 5
(mmHg)	Sunitinib	24 ± 7*	-9 ± 6	-10 ± 3*
ΔSVi	Control	-0.23 ± 0.10	0.02 ± 0.04	0.05 ± 0.06
(ml/kg)	Sunitinib	-0.16 ± 0.07	0.10 ± 0.03*	-0.03 ± 0.06
ΔLV dP/dt_{max}	Control	-123 ± 216	-20 ± 123	158 ± 152
(mmHg/s)	Sunitinib	-580 ± 342	217 ± 69*	118 ± 129
ΔLV dP/dt_{min}	Control	-185 ± 46*	-27 ± 135	22 ± 78
(mmHg/s)	Sunitinib	-202 ± 70*	82 ± 170	156 ± 69†

Abbreviations as in table 1. * P<0.05 effect of LNNA, Tezosentan or ROS scavenging vs corresponding baseline;
†P<0.05 effect of LNNA, Tezosentan or ROS scavenging altered as a result of sunitinib.

that a decrease in NO-bioavailability or an increase in oxidative stress contributed to the systemic vasoconstriction produced by sunitinib.[10-12]

In accordance with data in both rats and patients, administration of sunitinib induced a rapid and sustained increase in systemic MABP in swine.[10-12] Since VEGF is not only important for the formation, but also for the maintenance of blood vessels, a sunitinib-induced decrease in microvessel density per unit of volume has been proposed as a potential mechanism underlying the MABP rise during anti-angiogenic therapy.[5] Indeed, prolonged treatment with the RTKIs sunitinib and telatinib has been reported to be associated with capillary rarefaction.[9,25,26] Yet, the observation that the main increase in MABP occurred within 4 h after administration of the first dose of a RTKI in the present study, and within 24 h in studies in rats, makes rarefaction a less likely cause of the increase in vascular resistance and MABP.[10,11] Moreover, a hemodynamic model based on the cheek pouch circulation of the hamster indicates that 40% rarefaction of fourth

order vessels is required to increase vascular resistance in that particular vascular bed by 5%.[27] It is inconceivable that such an extensive degree of systemic rarefaction can occur within 4 h after initiation of angiogenesis inhibition. In view of the rapid increase in MABP and the knowledge that dramatic rarefaction is required to increase vascular resistance, we consider vasoconstriction the most important mechanism involved in the MABP rise during anti-angiogenesis therapy. In addition, the current observations that the sunitinib-induced systemic vasoconstriction diminished with increasing levels of exercise and the immediate normalization of MABP and SVRi with subsequent ET_A/ET_B blockade, further argue against rarefaction as a mechanism underlying the BP rise associated with angiogenesis inhibition (Fig. 2).

To explore potential mechanisms underlying the sunitinib-induced rise in vascular resistance and MABP we investigated the acute effects of ET_A/ET_B receptor blockade, eNOS inhibition and ROS scavenging prior and following one week of sunitinib treatment. In previous studies we have shown that administration of sunitinib in patients and rats is associated with an increase in circulating ET-1 levels.[10] In addition, we and others have demonstrated in rats that co-administration of an ET-1 receptor antagonist with a RTKI can largely or completely prevent the rise in MABP, suggesting that activation of the endothelin pathway plays an important role in the sunitinib-induced MABP rise.[11,12] These observations in rats are fully supported by the present findings in swine, as the sunitinib-induced increase in SVRi and MABP completely reversed to pre-sunitinib values in response to acute administration of $ET_{A/B}$ receptor blocker tezosentan.

ET-1 exerts part of its vasoconstrictor effect through activation of NADPH-oxidase and generation of ROS.[28-30] In addition, hypertension has been reported to be associated with an increase in ROS.[15] Since ROS can be derived from multiple sources within the circulation, including NADPH-oxidase, mitochondrial respiration, and neutrophils, we used a cocktail of antioxidants, including the SOD-mimetic TEMPOL, to achieve extensive ROS scavenging.[24,31,32,33] Using this approach, no evidence for an increase in sunitinib-induced ROS-mediated vasoconstriction was found. On the contrary, the effect of ROS-scavenging on MABP was reduced, while the effect on SVRi was unchanged following 1 week of sunitinib treatment. Of note, activation of NADPH-oxidase is also a critical step in the VEGF-receptor signaling cascade.[34] If this pathway is blocked following sunitinib administration, it is possible that activation of NADPH-oxidase via the ET-1 pathway is offset by a decreased activation of NADPH-oxidase due to VEGF-receptor inhibition.

Since VEGF is known to stimulate eNOS via the phosphatidylinositol 3-kinase-AKT pathway through activation of the VEGFR2 receptor, the enhanced vasoconstrictor response to acute eNOS inhibition after sunitinib administration observed in the present

study was unexpected.[6,35,36] This finding implies that the sunitinib-induced rise in MABP is accompanied by an increase in the NO-dependent vasodilator tone. This contrasts with observations that sunitinib treatment for 8 days was accompanied by a decreased urinary nitrate excretion in rats and in patients receiving various inhibitors of the VEGF-pathway, as well as with the observation that the RTKI vandetanib decreases systemic plasma nitrate/nitrite levels in treated patients.[8,12,37] Results of clinical studies using FMD of the brachial artery as an index of NO bioavailability are conflicting. In one study, administration of the RTKI telatinib was associated with a decrease in FMD from 6.0% to 3.9%, while in another study, administration of the RTKI vandetanib had no effect on FMD (12.0% before and 13.8% after vandetanib).[8,9] In a study by Steeghs *et al*., the vasodilation to nitroglycerin was also reduced (from 17.0% to 9.7%) to a comparable extent as the decrease in FMD, indicating a diminished response of the vascular smooth muscle cells to NO rather than a selective decrease in NO-bioavailability.[9] In the coronary microcirculation of rats exposed to sunitinib for 8 days we found, using the Langendorff heart model, that the vasodilator responses to both bradykinin (endothelium-dependent) and sodium-nitroprusside (endothelium-independent) were impaired.[10] Remarkably, in this model we also found an attenuated vasoconstrictor response to angiotensin II. These findings suggest generalized impairment of vascular smooth muscle function during sunitinib administration that is still unexplained. Altogether, until now evidence is lacking to conclude that a decrease in NO-bioavailability is a predominant factor in RTKI-induced vasoconstriction. Given the complex interaction between NO and ET, it is possible that the enhanced NO-mediated vasodilator tone is due to increased ET_B stimulation while simultaneously limiting ET-induced vasoconstriction.

Our study is the first to investigate potential adverse effects of the RTKI sunitinib on the pulmonary vasculature. Contrary to its vasoconstrictor effect in the systemic circulation, sunitinib did not cause pulmonary vasoconstriction either at rest or during exercise. Variation in expression and function of ET_A and ET_B receptors between these two circulations may provide an explanation for this difference. The ET_A receptor predominates in the systemic and coronary vasculature, whereas the ET_B receptor predominates in the pulmonary microcirculation, where it is present on both the endothelium and the vascular smooth muscle cells and also functions as a clearance receptor.[38] In a previous study, using the same animal model as in the present study, we found that the dose of ET-1 required to induce vasoconstriction in the systemic circulation is lower than the dose to induce pulmonary vasoconstriction.[20] Apparently, the sunitinib-induced activation of the endothelin-pathway was not sufficient to induce pulmonary vasoconstriction thereby precluding adverse effects of RTKI on the pulmonary circulation.

In the present study, administration of sunitinib was without adverse effects on stroke volume or $LVdP/dt_{max}$ and $LVdP/dt_{min}$, indices of left ventricular systolic and diastolic function, either at rest or during exercise. Moreover, no evidence was found for coronary vasoconstriction as CBF did not change during sunitinib administration. In contrast, sunitinib treatment of patients with imatinib-resistant, metastatic gastrointestinal stromal tumors was associated with the development of congestive heart failure in 8% of patients and a decrease in LVEF of at least 10% in 28% of patients.[2] This discrepancy might in part be explained by the difference in exposure time to sunitinib as well as the fact that our intervention was performed in young and healthy animals, whereas in the clinical study pre-existent coronary artery disease remained as the only statistically significant predictor for congestive heart failure in a multivariate logistic regression model.

PERSPECTIVES

Angiogenesis inhibition with sunitinib induces a marked rise in MABP, which is independent of changes in endogenous ROS and NO production, but completely reversed by endothelin receptor antagonism with tezosentan. These findings support previous observations that sunitinib-induced hypertension is endothelin-mediated, whereas decreased NO-bioavailability or enhanced oxidative stress do not appear to be involved. Since we recently showed that endothelin receptor antagonism could also prevent sunitinib-induced proteinuria and urinary ET-1 excretion (markers of renal injury), endothelin receptor antagonists appear logical candidates to counteract the sunitinib-induced cardiovascular and renal adverse effects.[12] In addition, ET-1 has been shown to promote angiogenesis in cancer and therefore ET-1 receptor antagonism may have complementary therapeutic effects.[39,40] However, carefully conducted clinical trials are required to demonstrate whether indeed endothelin receptor antagonists may be the preferred agents to treat the cardiovascular and renal side effects associated with anti-angiogenesis therapy in patients with cancer.

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6

Blood pressure independent renal toxicity induced by the VEGF receptor inhibitor sunitinib: role of endothelin-1

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Submitted

ABSTRACT

Angiogenesis inhibition, an established treatment for several tumor types, is associated with hypertension and renal toxicity. In patients and in rats we demonstrated that the multitarget tyrosine kinase inhibitor sunitinib induces a rise in blood pressure (BP), renal dysfunction, proteinuria, and marked renal histological abnormalities associated with activation of the circulating and renal endothelin system. Moreover, these side effects could partly be prevented by the dual ET_A/ET_B endothelin receptor blocker macitentan. In the current study we investigated whether the macitentan-induced decrease in BP accounts for the decrease in proteinuria and urinary ET-1 excretion or whether the renal toxicity is BP-independent due to activation of the renal endothelin system. Normotensive Wistar Kyoto rats were administered sunitinib alone, sunitinib and macitentan or sunitinib and the calcium channel blocker amlodipine for 8 days, at which time point they were sacrificed and blood and kidneys collected. At baseline and at the end of treatment 24 h urine samples were collected. In rats on sunitinib alone, BP increased (ΔBP 31.6±0.9 mmHg). Co-administration of macitentan (ΔBP 12.3±1.5 mmHg) or amlodipine (ΔBP 11.4±1.7 mmHg) largely and equally prevented this rise. Both compounds could not prevent the sunitinib-induced rise in serum creatinine. Macitentan diminished sunitinib-induced proteinuria, urinary ET-1 excretion and glomerular intra-epithelial protein deposition, whereas amlodipine did not, despite a similar decrease in BP. In conclusion, hypertension and proteinuria are independent side effects of sunitinib treatment and are due, at least in part, to activation of the circulating and renal endothelin system.

INTRODUCTION

Angiogenesis, the formation of new capillaries from an existing vasculature, is critical to tumor growth as well as to metastasis and is regulated by numerous factors among which vascular endothelial growth factor (VEGF) and its corresponding receptors. 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 (RTKI) 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 41-63% of patients treated with bevacizumab.[2] The incidence of proteinuria in patients treated with RTKIs is less well defined, as 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), favoring ET-1-mediated vasoconstriction and thus promoting the development of hypertension.[1] This is in accordance with our recent findings, showing that the rise in blood pressure (BP), renal dysfunction and proteinuria induced by the RTKI sunitinib were associated with a two-to-three-fold rise in circulating ET-1 levels in both patients and rats.[3] Moreover, the sunitinib-induced proteinuria and increased urinary ET-1 excretion were largely diminished during co-administration of the dual ET_A/ET_B-receptor blocker macitentan.[4] Since macitentan also diminished the sunitinib-induced rise in BP by 60%, the question whether the decrease in BP *per se* rather than blockade of the sunitinib-activated renal endothelin system mainly accounted for the decrease in proteinuria could not be answered. To address this point we initiated the current study in which we compared the effect of the calcium channel blocker amlodipine and macitentan on sunitinib-induced hypertension, renal injury and microvascular function when given at doses that lowered blood pressure identically.

METHODS

In vivo study

Male Wistar Kyoto rats (WKY, 280-300 gram) obtained from Charles River, were housed in individual cages and maintained on a 12-h 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 solution were prepared and administered by oral gavage as described previously.[3] Macitentan (ACT-064992), a dual ET_A / ET_B receptor antagonist kindly provided by Actelion, 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. Three separate experiments were performed. At the end of each experiment, rats were euthanized with 60 mg/kg 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/day of sunitinib L-malate; n=10) or vehicle (n=10) by oral gavage (0.5 mL) for 8 days. In the second experiment, rats (n=8) were orally administered the combination of sunitinib and macitentan 30mg/kg/day, for 8 days.[5] In the third experiment, rats were administered the combination of sunitinib and amlodipine 3mg/kg/day by oral gavage for 8 days.[6,7] 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 determination of protein, ET-1, nitric oxide (NO) metabolites (NO_2+NO_3 [NO_x]) and thiobarbituric acid reactive substances (TBARS). Urine was collected on antibiotics (A5955, Sigma) to prevent formation of NO metabolites. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

In vitro studies

Microvascular function

To assess coronary microvascular function, hearts were rapidly excised from euthanized rats and perfused according to Langendorff.[8] 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 µL) of Tyrode's buffer were applied three times to determine injection-induced changes in CF. Dose-response curves to the endothelium-dependent vasodilator bradykinin and the vasoconstrictor Ang II were constructed by bolus injections. In addition, endothelium-independent maximal vasodilatation was assessed by a single bolus injection of sodium nitroprusside (SNP; 10 mmol/L).

Renal histology

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-µm thick sections were stained for Haematoxilin-eosine (HE), PAS and Jones silver. PAS-stained 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 glomerular intra-epithelial protein deposition was evaluated using a semiquantative 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.

Biochemical measurements

ET-1 was assessed using a chemiluminescent ELISA (QuantiGlo®, R&D Systems) and urine albumin by enzyme immunoassay (Spi-Bio, France). Urine NOx concentration was determined by fluorimetric quantification of nitrite content (Cayman Chemicals, Ann Arbor, MI) and lipid peroxidation in urine by measurement of TBARS.[9] Serum creatinine and urinary protein concentrations were measured at the clinical chemical laboratory of the Erasmus MC.

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's multiple comparison testing. GraphPad Prism version 4.03 was used for all statistical analysis.

RESULTS

In vivo study

Co-administration of macitentan or amlodipine comparably diminished the sunitinib-induced rise in BP, whereas the sunitinib-induced decrease in heart rate (HR) was not affected by both compounds (Figure 1).

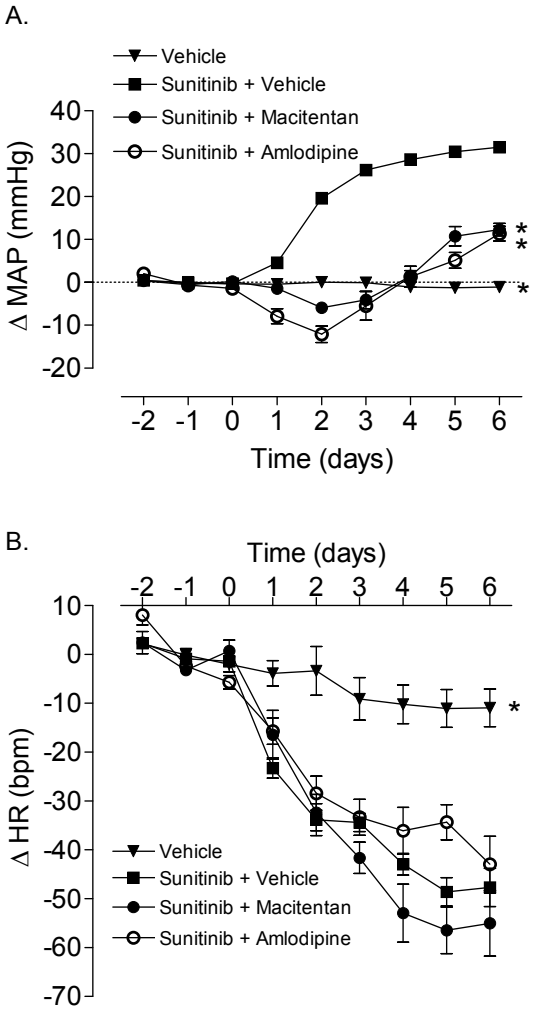

Figure 1. Changes in mean arterial pressure (MAP, A) and heart rate (HR, B) in response to administration of vehicle (n=6), sunitinib and vehicle (n=12), sunitinib and macitentan (n=8), or sunitinib and amlodipine (n=8). Vehicle, sunitinib and vehicle, and sunitinib and macitentan are from Kappers et al.[4] *$p<0.05$ vs sunitinib + vehicle.

Macitentan did not change the sunitinib-induced increases in kidney weight-to-body weight-ratio (KW/BW-ratio) (data not shown) and serum creatinine, but it decreased proteinuria and urinary ET-1 excretion, whereas circulating ET-1 levels increased (Figure 2).[4] Treatment with amlodipine did also not diminish the sunitinib-induced increase in KW/BW-ratio (data not shown), nor did it decrease the rise in serum creatinine, circulating ET-1 levels or urinary ET-1 excretion, whereas proteinuria tended to increase (Figure 2). The sunitinib-induced decrease in urinary nitrate excretion was not reversed by either macitentan or amlodipine (Figure 3).[4]

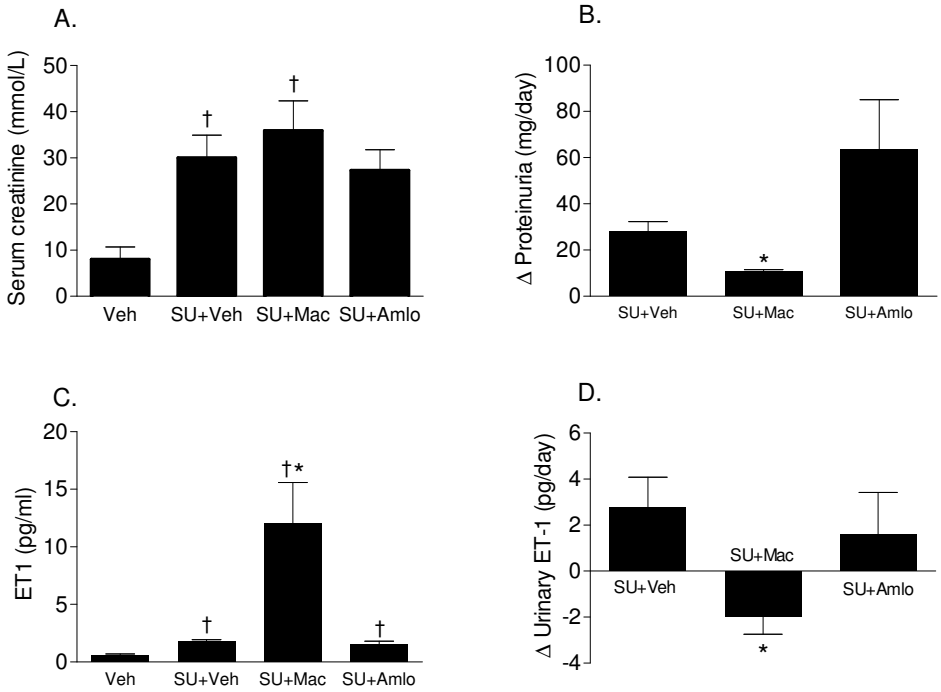

Figure 2. Serum creatinine (A), change in proteinuria (B), circulating ET-1 levels (C) and change in urinary ET-1 excretion (D) in rats after administration of vehicle (n=6), sunitinib and vehicle (n=16), sunitinib and macitentan (n=4-6), or sunitinib and amlodipine (n=8) for 8 days. Vehicle, sunitinib and vehicle, and sunitinib and macitentan are from Kappers et al.[4]
*p<0.05 vs sunitinib + vehicle; †p<0.05 vs vehicle.

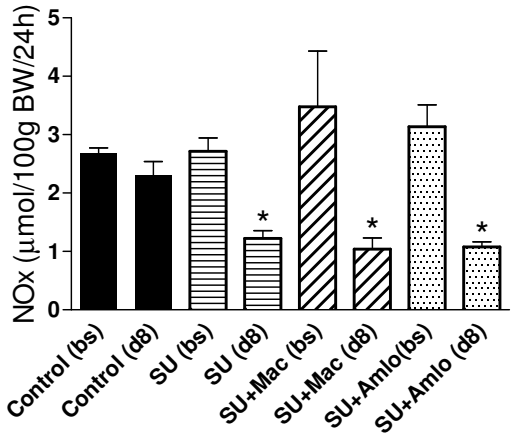

Figure 3. Nitric oxide metabolites (NOx) excretion in urine of WKY rats at baseline and after an 8-days administration of vehicle (n=4), sunitinib (n=9), sunitinib and macitentan (n=7) or sunitinib and amlodipine (n=8). Vehicle and sunitinib are from Kappers et al.[4]
* p<0.05 vs baseline, † p<0.05 vs sunitinib.

In vitro studies

Microvascular function

The bradykinin and SNP-induced increase in CF was markedly diminished in rats exposed to sunitinib, as was the Ang II-induced decrease in CF (Figure 4). The sunitinib-induced alterations of the responses to bradykinin and Ang II were not reversed by macitentan or amlodipine. In contrast, the sunitinib-induced diminished response to SNP slightly reversed with amlodipine but not with macitentan (Figure 4).

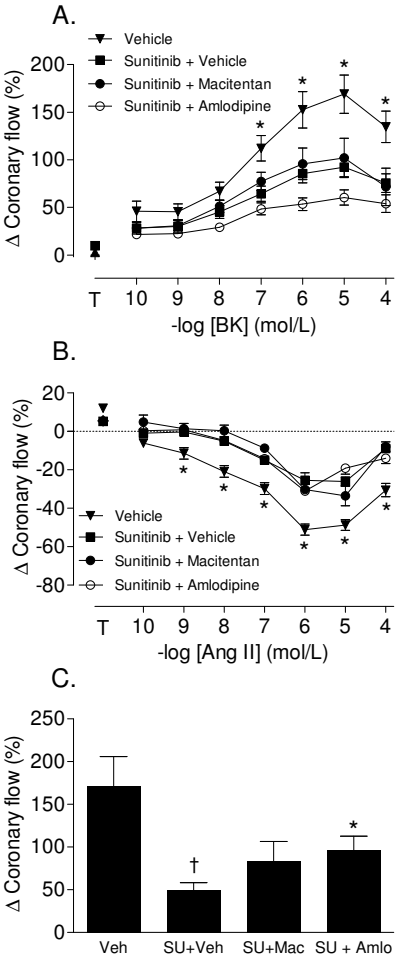

Figure 4. Coronary flow responses to bradykinin (BK; A), angiotensin II (AngII, B) and a single injection to sodium nitroprusside (SNP 10 mmol/L, C) in isolated rat hearts after administration of vehicle (n=9), sunitinib and vehicle (n=10), sunitinib and macitentan (n=4), and sunitinib and amlodipine (n=8) for 8 days. The x-axis in A and B displays the concentration in the injection fluid. T indicates Tyrode's buffer. Vehicle, sunitinib and vehicle, and sunitinib and macitentan are from Kappers et al.[4] *P<0.05 vs sunitinib + vehicle; †P<0.05 vs vehicle.

Renal histology

Light microscopic examination of PAS-stained kidney sections showed that macitentan did not reverse the previously observed sunitinib-induced glomerular ischemia or endothelial and epithelial cell swelling. However, consistent with the decrease in proteinuria, glomerular intra-epithelial protein deposition diminished during co-administration of macitentan (Table 1). In rats co-administered sunitinib and amlodipine, glomerular ischemia and endothelial and epithelial cell swelling were more prominent compared to rats exposed to sunitinib alone, as was the glomerular intra-epithelial protein deposition (Table 1).

Table 1. Light microscopic evaluation of kidney sections obtained from rats exposed to sunitinib, sunitinib and macitentan or sunitinib and amlodipine for 8 days compared to controls.

	Glomerular ischemia (% glomeruli)			Endothelial cell swelling (% glomeruli)		Epithelial cell swelling (% glomeruli)		Intra-epithelial protein (% glomeruli)		
Group	None	Moderate	Severe	0	1	0	1	0	1	2
Control	60±13	32±10	8±4	100	0	100	0	99±1	1±1	0
Sunitinib	17±2*	54±4*	29±5*	78±3*	22±3*	63±5*	37±5*	29±4*	48±2*	23±4*
Sunitinib + Macitentan	15±6*	35±2†	51±4*†	85±3*	15±3*	77±6*	23±6*	33±8*	59±7*	8±3*†
Sunitinib + Amlodipine	12±3*	40±4†	47±6*†	53±10*†	47±10*†	16±4*†	84±4*†	7±2*†	18±4*†	75±5*†

All evaluations were performed in 50 glomeruli of a PAS-stained section and the numbers of glomeruli with each score were counted. Endothelial and epithelial cell swelling were scored as present (1) or absent (0) in each glomerulus. The presence of intra-epithelial protein was evaluated using a semiquantative scale: 0 (no protein in the epithelial cells of a glomerulus), 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). Control, sunitinib, and sunitinib and macitentan are from Kappers et al.[4] * $p<0.05$ vs control; † $p<0.05$ vs sunitinib.

DISCUSSION

Recently, we have reported in both patients and rats that the multitarget VEGF-receptor tyrosine kinase inhibitor sunitinib induces a rise in BP, a loss of circadian BP rhythm and renal toxicity, including a rise in serum creatinine, proteinuria and urinary ET-1 excretion as well as histopathological changes, all associated with a rise in circulating ET-1 levels.[3,4] Furthermore, co-administration of the dual ET_A/ET_B-receptor blocker macitentan in rats diminished the sunitinib-induced rise in BP almost completely, indicating that this rise is largely ET-1-mediated. Co-administration of macitentan also decreased the sunitinib-induced increase in proteinuria and urinary ET-1 excretion, both markers of renal injury, suggesting that the sunitinib-induced renal toxicity is ET-1-mediated as

well.[4] Since the decrease in BP with macitentan might have contributed to the decrease in proteinuria by lowering glomerular filtration pressure, the possibility that renal toxicity observed during angiogenesis inhibition with sunitinib is mainly BP-dependent could not be excluded in our previous study. To further explore the separate roles of BP and the endothelin-system in the development of renal toxicity during angiogenesis inhibition we administered rats the combination of sunitinib and amlodipine to induce a decrease in BP comparable that induced by macitentan. Contrary to macitentan, amlodipine could not prevent the sunitinib-induced rise in proteinuria and urinary ET-1 excretion despite an identical decrease in BP, strongly suggesting that these aspects of sunitinib-induced renal toxicity are ET-1-mediated and BP-independent, which is in line with studies showing that ET-1 increases glomerular permeability to albumin by a direct effect on the podocyte cytoskeleton.[10,11] Furthermore, conditional gene targeting to selectively delete VEGF from renal podocytes in adult mice has been shown to result in profound glomerular injury that precedes the development of hypertension, additionally supporting our observation that hypertension and renal toxicity are two independent sunitinib-associated adverse effects.[12]

Our studies revealed that proteinuria was not only diminished, but even tended to increase during co-administration of sunitinib and amlodipine, and concomitant with this increase, glomerular intra-epithelial protein deposition was also more prominent. Dihydropyridine calcium antagonists, such as amlodipine, diminish glomerular afferent vasoconstrictor tone and thereby can cause an increase in renal blood flow and glomerular filtration pressure.[13] These renal haemodynamic effects of amlodipine might explain why despite a decrease in BP renal histological abnormalities, proteinuria and increased urinary ET-1 excretion did not improve but even deteriorated during amlodipine co-administration. Of note circulating ET-1 levels did also not decrease during amlodipine co-administration, whereas these levels, due to blockade of the ET_B clearance receptor, increased during co-administration of macitentan.

It is difficult to explain why macitentan diminished the sunitinib-induced proteinuria and urinary ET-1 excretion, but had no effect on elevated serum creatinine levels and only small effects on renal histopathology. In this regard it would be interesting to investigate whether serum creatinine concentration and renal histological changes completely normalize when macitentan is administrated for an extended period after sunitinib withdrawal. In a previous study we showed that serum creatinine concentration was still increased 11 days after sunitinib withdrawal.[3] Urinary ET-1 has been reported to be completely kidney-derived and its increased urinary excretion reflects renal injury.[14] Consequently, reversal of the increased urinary ET-1 excretion indicates remission of renal injury. Interestingly, the current findings show that the decrease in

urinary ET-1 excretion observed during co-administration of macitentan 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 possibly a sensitive but incomplete marker of renal injury.

Since binding of VEGF to its receptors on endothelial cells is important for their survival and increases NO-production through Akt-dependent phosphorylation of endothelial NO synthase, it has been postulated that VEGF-inhibition leads to endothelial dysfunction and decreased NO-bioavailability.[15] However, hypertension itself can also induce endothelial dysfunction and a decrease in NO-bioavailablity.[16-18] Recently we reported that urinary excretion of nitrates (a measure of NO availability) decreased during sunitinib administration.[4] However, neither macitentan nor amlodipine could reverse the decreased nitrate excretion despite a marked decrease in BP. These findings suggest that the decreased NO-bioavailability during sunitinib administration is directly related to its interaction with the VEGF-pathway. This is supported by the findings in the Langendorff model. In this model, endothelium-dependent vasodilation induced by bradykinin did not improve during co-administration of macitentan or amlodipine. In contrast, amlodipine did improve endothelium-independent vasodilation induced by sodium nitroprusside, indicating improved vascular smooth muscle cell vasodilatory function.

In conclusion, angiogenesis inhibition with the tyrosine kinase receptor inhibitor sunitinib induces hypertension and proteinuria, a rise in urinary ET-1 excretion and renal histological abnormalities that are independent side effects, in part due to activation of the endothelin system. The endothelin receptor blocker and the calcium channel blocker in the dosages used were equally effective in preventing the sunitinib-induced rise in BP and therefore can both be used to treat sunitinib-induced hypertension. Considering our finding that endothelin receptor blockade also diminished proteinuria and elevated urinary ET-1 excretion, whereas these two parameters tended to increase with the calcium channel blocker, endothelin receptor blockade might be preferred in patients who develop hypertension and proteinuria during treatment with sunitinib. Finally, although not the focus of the present study, it would be worthwhile to explore the effect of antihypertensive drugs with a renal protective profile such as ACE-inhibitors or Ang II type 1-receptor blockers on sunitinib-induced hypertension and renal toxicity in our rat model.

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7

Hypertension as a biomarker of efficacy in patients with metastatic renal cell carcinoma treated with sunitinib

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Rini *et al*. reported a large retrospective analysis that showed that patients with metastatic renal cell carcinoma who developed hypertension when treated with the tyrosine kinase inhibitor sunitinib have improved overall survival and progression-free survival compared with patients who remained free of hypertension.[1] The authors concluded that the development of hypertension is a potential valuable biomarker for the prediction of treatment response. However, we wonder whether a categorical division of patients is the most appropriate way to look at the data. Hypertension is defined as a systolic blood pressure of 140 mm Hg or greater and/or a diastolic blood pressure of 90 mm Hg or greater and this definition was also used in the analysis by Rini *et al*.[1] However, it is well known that within the so-called normotensive range a higher blood pressure is associated with an increased cardiovascular risk, indicating that the cut-off values of 140 mm Hg and 90 mm Hg are arbitrary. If a patient has a baseline blood pressure of 138 mm Hg systolic, an only 2 mm Hg increase in blood pressure will classify him or her as being hypertensive, whereas a patient with a baseline systolic blood pressure of 110 mm Hg experiencing a 28 mm Hg increase would not. According to this reasoning we think that the patient's absolute or relative blood pressure increase rather than the presence or absence of hypertension is a more appropriate way of analyzing the data. Perhaps the authors can perform an analysis based on blood pressure increases rather than on the subdivision in hypertension and normotension.

With regard to the mechanism leading to hypertension, the authors mentioned several possibilities. One of these is rarefaction, i.e. presence of less perfused microvessels and/or a diminished number of microvessels. Indeed rarefaction of skin capillaries during sunitinib treatment in patients with metastatic renal cell carcinoma has been reported.[2] In that study, an inverse association between the degree of rarefaction and the increase in blood pressure was observed. This association does not imply causality. The rarefaction could be a consequence of the blood pressure rise. Rarefaction is present in patients with untreated hypertension, but is absent when the elevated blood pressure is normalized with an antihypertensive treatment.[3] Another point to consider is how much rarefaction is needed to cause blood pressure to increase. A mathematical model based on the hamster cheek pouch microcirculation indicates that 42% rarefaction of the fourth order arterioles is necessary to increase resistance in that particular vascular bed by 5%.[4] This estimate suggests that a considerable amount of rarefaction in various vascular beds is required to induce an increase in vascular resistance and hence in blood pressure, assuming that cardiac output remains unchanged. Furthermore, in unrestrained rats in which blood pressure was continuously recorded by telemonitoring, we observed an increase in blood pressure within 1 day of sunitinib administration by oral gavage.[5] From this result, it appears that it is highly unlikely that rarefaction is an initial cause of sunitinib-induced hypertension.

One mechanism that was not mentioned by Rini *et al*. but could potentially underlie the occurrence of hypertension by sunitinib is activation of the endothelin-1 pathway. In the experimental model just described and in patients treated with sunitinib we found that the rise in blood pressure is accompanied by an activation of endothelin-1 pathway.[1,5] Because renin was suppressed in these patients and we observed no evidence of activation of the sympathetic nervous system or fluid retention, we think that activation of the endothelin-1 pathway is an important mediator of the blood pressure increase induced by sunitinib. This conclusion fits well with the observation that the rise in blood pressure induced by a VEGF receptor tyrosine kinase inhibitor can be largely prevented by endothelin-receptor antagonism.[6,7] The mechanism by which sunitinib activates the endothelin-1 pathway is still not clear.

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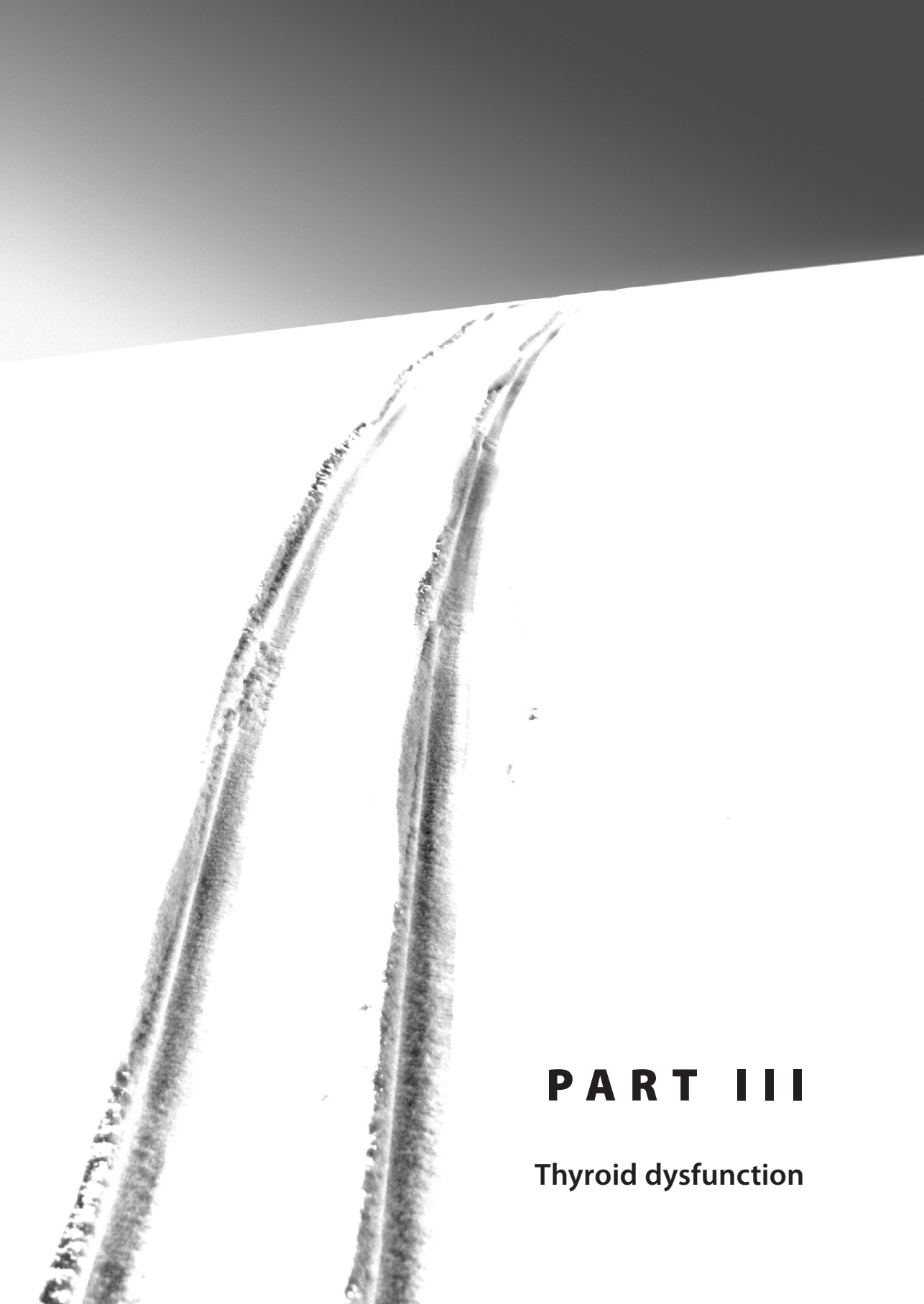

PART III

Thyroid dysfunction

8

Sunitinib-induced hypothyroidism is due to induction of type 3 deiodinase activity and thyroidal capillary regression

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ABSTRACT

Context – Anticancer treatment with the tyrosine kinase inhibitor sunitinib causes thyroid dysfunction.

Objective – Our objective was to investigate the time course and underlying mechanisms of sunitinib-induced thyroid dysfunction.

Design – Thyroid function tests of 83 patients on sunitinib were collected retrospectively for their total treatment duration between January 2006 and November 2009, and prospectively in 15 patients on sunitinib for 10 wk. Additionally, thyroid function and histology were assessed in rats on sunitinib (8 d; n=10) and after sunitinib withdrawal (11 d; n=7), and compared to controls (n=7).

Setting – Patients were seen at a university outpatient oncology clinic.

Patients / animals – Patients with metastatic renal cell carcinoma or gastrointestinal stromal tumors participated in the clinical study and Wistar Kyoto rats were used in the rat study.

Intervention – Sunitinib was taken according to a 4 wk 'on', two wk 'off' treatment regimen. Blood samples for measurement of thyroid function were collected at baseline, and at wk 4 and 10. In rats, blood, liver and thyroid were collected to assess thyroid hormones, deiodinase activity and thyroid histology.

Main Outcome Measure(s) – TSH and free T_4 levels, deiodinase activity, and thyroid histology were assessed.

Results – Forty-two percent of patients in the retrospective study developed elevated TSH levels. Prospective analysis showed increased TSH levels within 10 wk of treatment, accompanied by a decreased T_3/rT_3 ratio. In rats, serum T_4 and T_3 decreased, hepatic type 3 deiodinase activity increased, and thyroid histology showed marked capillary regression, which all but thyroid hormones reversed after sunitinib withdrawal.

Conclusion – Sunitinib induces hypothyroidism due to alterations in T_4/T_3 metabolism as well as thyroid capillary regression.

INTRODUCTION

Inhibition of angiogenesis by means of blocking vascular endothelial growth factor (VEGF)-mediated signaling has become an established treatment for various malignancies. Sunitinib, an oral multitarget tyrosine kinase inhibitor of among others the VEGF receptor, is approved for the treatment of metastatic renal cell carcinoma (mRCC) and imatinib-resistent metastatic gastrointestinal stromal tumors (GIST).[1-2] A frequently observed side effect of sunitinib treatment is thyroid dysfunction, mainly hypothyroidism, with a reported incidence of 53-85% in retrospective and 36-46% in prospective studies.[3] Sporadically, suppression of TSH preceded the development of hypothyroidism compatible with transient thyroid hormone (TH) overproduction.[4] Thyroid dysfunction not only is associated with sunitinib treatment but has also been reported during treatment with other receptor tyrosine kinase inhibitors (RTKIs) such as nilotinib, dasatinib and sorafenib.[5-7] The precise mechanism underlying sunitinib and other RTKI-induced thyroid dysfunction has not been elucidated yet. In a patient with a history of thyroidectomy for which he was on levothyroxine (LT_4) replacement therapy, an increased dose of LT_4 was required during treatment with sunitinib, suggesting induction of TH metabolism by sunitinib ('metabolic hypothesis').[8]

In mice, administration of an experimental VEGF receptor inhibitor or soluble VEGF receptor was associated with pronounced regression of capillaries in the thyroid gland and a marked increase in serum TSH levels ('capillary regression hypothesis').[9] A possibly correlated clinical finding is the observed reduction in thyroid volume in patients treated with sunitinib.[10,11] In addition, impaired uptake of radiolabeled iodine during sunitinib treatment has also been reported.[12]

Our research, using a clinical and experimental approach, was aimed at further investigating the incidence of sunitinib-induced thyroid dysfunction, its time-course and the pathophysiological mechanisms involved. Our focus was on T_4/T_3 metabolism and on capillary density of the thyroid gland during sunitinib administration and after its withdrawal.

METHODS

Retrospective patient study

Between January 2006 and November 2009, patients with either mRCC or imatinib-resistant GIST receiving sunitinib at the Department of Medical Oncology of the Erasmus Medical Center (Rotterdam, The Netherlands) were evaluated retrospectively for the

development of thyroid dysfunction. Sunitinib was taken according to a 4 wk 'on', 2 wk 'off' regimen with a starting dose of 50 mg/d. During treatment this dose was adjusted according to the judgment of the patient's physician. Only patients with thyroid function tests (TFT) available on at least two consecutive time points were included. Patients with abnormal TFT before initiation of sunitinib or with previous TH replacement therapy due to underlying thyroid disease were excluded. Serum TSH levels >5.0 mU/liter or <0.4 mU/liter and free T_4 (FT_4) levels above 25 or below 11 pmol/liter were considered to be abnormal. TH replacement therapy was initiated according to the judgment of the patient's physician, which was mainly based on the degree of both elevated TSH levels and accompanying decreased FT_4 levels with or without aggravation of clinical symptoms such as fatigue.

Prospective patient study

Between January 2008 and January 2009, patients who were eligible for treatment with sunitinib, were invited to participate in a prospective study in which they were followed for 10 wk. Sunitinib was taken according to the above-mentioned treatment regimen. Blood samples for measurement of TSH, FT_4, T_3, rT_3 and antibodies against thyroid peroxidase (TPO) were obtained at baseline and at the end of the first and second treatment cycle. The study was approved by the Institutional Review Board and Ethical Committee of the Erasmus MC in Rotterdam. Written informed consent was obtained from each patient.

Rat study

Male Wistar-Kyoto rats (280-300 grams), obtained from Charles River Laboratories (Köln, Germany), were housed in individual cages and maintained on a 12-h light, 12-h dark cycle, having access to standard laboratory rat chow and water *ad libitum*. The sunitinib and vehicle solutions were prepared and administered by oral gavage as previously described.[13] Three separate experiments were performed. In the first experiment rats were randomly administered sunitinib (26.7 mg/kg/d of sunitinib L-malate; n=10) or vehicle (n=7) by oral gavage (0.5 ml) for 8 d and were euthanized with 60 mg/kg pentobarbital i.p. at the end of this period, at which time blood was sampled for measurement of T_3, T_4 and TSH. Furthermore, sections of liver tissue for measurement of type 1 (D1) and type 3 (D3) deiodinase activities were collected, snap frozen and stored at -80°C. Both thyroid lobes were also collected and fixed in a 4% formaldehyde solution for histological examination. In the second experiment, rats (n=7) were administered sunitinib at the same dose for 8 d followed by an 11-d recovery period, after which they were sacrificed. Six days before (baseline) and 6 d after treatment initiation, rats were housed in metabolic cages for 48 h with free access to food and water; the first day to acclimatize and the second day for collection of 24-h urine for protein and T_3 and T_4 measurement.

In the second experiment rats were also housed in metabolic cages 1 wk after sunitinib withdrawal. In the third experiment, rats (n=8) were treated with the combination of sunitinib and macitentan (ACT-064992) 30 mg/kg/d for 8 d. Macitentan, a dual type A and type B endothelin (ET) receptor antagonist which was kindly provided by Actelion, was dissolved in vehicle containing 0.5% methylcellulose aqueous solution and 0.05% Tween 80. All experiments were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC.

Ex vivo studies and histology

Liver tissue was homogenized in 0.1 M phosphate, 2 mM EDTA, 10 mM dithiothreitol (PED10), and hepatic D1 and D3 activities were measured essentially as previously described.[13] D1 assay mixtures contained 0.1 μM [^{125}I]rT_3 and 0.1 mg/ml homogenate protein in PED10, and were incubated for 30 min at 37 °C. D3 assay mixtures contained 1 nM [^{125}I]T_3 without or with 0.5 μM unlabeled T3 and 1.5 mg/ml homogenate protein in PED10, and were incubated for 2 h at 37 °C. D3 but not D1 is saturated at 0.5 μM T_3. Therefore, difference in percentage conversion at low (1 nM) and high (0.5 μM) T_3 concentration represents D3 activity. Assay mixtures were processed and reaction products were analyzed by HPLC as previously described.[14]

After fixation in a 4% buffered formaldehyde solution, the thyroids were dehydrated and paraffin-embedded. Hematoxilin-eosine (HE) staining and actin staining were applied to deparaffinized 2-μm-thick sections. In each section in five consecutive fields (magnification, ×400) the total number of follicles and vessels was counted and the vessel-to-follicle-ratio calculated.

Biochemical measurements

Human serum TSH, FT_4 and T_3 were measured by chemoluminescence assays (Vitros ECi Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). Serum rT_3 was measured by radioimmunoassay. Anti-TPO antibodies were assessed using the immunoCAP method (Phadia® 250, Uppsala, Sweden). Rat serum and urinary T_4 and T_3 levels were measured by radioimmunoassay.[14]

Statistics

Data are presented as mean±SEM or median and interquartile ranges. Statistical analysis between groups was performed by unpaired *t*-testing or repeated-measures ANOVA followed by Newman-Keuls multiple comparison testing. $P<0.05$ was considered significant. GraphPad Prism version 5.02 was used for all statistical analyses.

RESULTS

Retrospective patient study

In total, 83 of the 108 patients were eligible for evaluation. Twenty-five patients were excluded: in 17 patients no TFTs were available on two consecutive time points; three patients had a history of thyroid dysfunction; and in five patients initial measurements showed abnormal TFTs. Demographics, disease, treatment duration and TFTs are presented in Table 1. Thirty-five (42%) of the 83 patients developed elevated TSH values during treatment with sunitinib. In four patients no baseline TFTs were performed; however, the first TFTs available during treatment were within the normal range and patients were therefore included in the study. In 5 patients (14%), the elevation of TSH was preceded by suppressed TSH values (Table 2).

Table 1. Characteristics and TFTs of patients developing TSH above 5.0 mU/L during treatment with sunitinib.

Characteristic	Result
No. of patients [n (%)][a]	35 (42)
Male [n (%)]	18 (51)
Female [n (%)]	17 (49)
Age at baseline (yr)	61.4±1.0
Malignancy	
mRCC [n (%)]	32 (91)
GIST [n (%)]	3 (9)
Time to onset [d (range)]	90 (14-856)
Baseline TSH (mU/L)	2.43±0.19
Maximum TSH (mU/L)	31.39±6.66
Time to onset [d (range)]	221 (20-1033)
Initiation of LT4-Rx [n (%)]	7 (20)
TSH>10 mU/L[b]	
No. of patients [n (%)][c]	20 (57)
Time to onset	221 (34-1021)

Patients with TSH above 5.0 mU/L at initial measurement or a history with hypothyroidism were excluded; FT4 reference range is 11-25pmol/L; TSH reference range is 0.4-4.3mU/L; LT4-Rx, TH replacement therapy with LT4. [a]percentage of total number of patients (83) with TFTs available. [b] highest TSH level measured in a patient was 150mU/L. [c]percentage of the number of patients (35) with TSH above 5.0mU/L

Prospective patient study

Fifteen patients (10 male, 5 female), mean age 59.8 ± 6.1 years, with mRCC (n=13) or GIST (n=2) were included. One patient with a history of hypothyroidism (Hashimoto's disease) required an increased LT_4 replacement dose during sunitinib therapy. Mean serum TSH values increased 2-fold after 10 wk of treatment with sunitinib. Although no statistically

Table 2. Characteristics and biochemical data of patients who developed hypothyroidism with preceding suppressed serum TSH-levels during treatment with sunitinib.

Patient no.	Sex	Age (years)	Malignancy	TSH T_0 (mU/L)	FT4 T_0 (pmol/L)	TSH Lo (mU/L)	FT4 (pmol/L)	Time to onset Lo (days)	TSH Hi (mU/L)	FT4 (pmol/L)	Time to onset Hi (days)
1	F	51	mRCC	2.58	NA	0.080	24.2	352	26.0	11.9	587
2	F	58	mRCC	3.31	18.6	0.014	26.2	200	133.0	6.9	317
3	M	60	mRCC	1.42	NA	0.010	49.5	201	31.5	16.8	345
4[a]	M	71	mRCC	2.36	15.6	0.008	40.0	82	11.4	15.9	296
5	F	62	mRCC	2.89	18.0	0.026	33.2	152	108	7.8	305

F, female; M, male; NA, not available; T_0, basal level of TSH and FT4 before initiation of sunitinib treatment; TSH Hi, highest level of TSH and its corresponding FT4 level during treatment with sunitinib; TSH Lo, lowest level of TSH and its corresponding FT4 level during treatment with sunitinib

[a]In this patient antibodies against TPO and the TSH receptor (TR) were assessed: TPO antibodies were negative, TR antibodies were 0.50 IU/L.

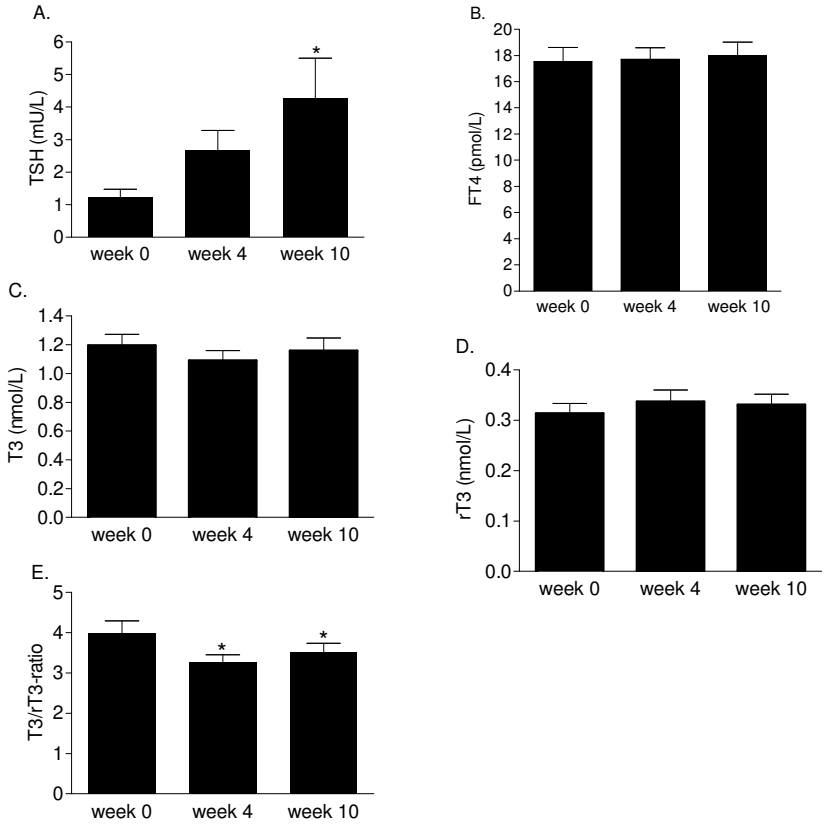

Figure 1. Thyroid function tests at baseline and after 4 and 10 weeks of treatment in patients treated with sunitinib according to a 4 week 'on' and 2 weeks 'off' treatment regimen (N=15). Plasma TSH levels increased twofold during 2 treatment cycles (panel A) whereas no changes in plasma FT4 (panel B), T3 (panel C) and rT3 (panel D) were observed. T3/rT3-ratio decreased during treatment with sunitinib (panel E). * P<0.05

different changes in FT_4, T_3 and rT_3 were observed, the T_3/rT_3 ratio significantly decreased during treatment with sunitinib (Fig. 1). Baseline circulating anti-TPO levels were low [1.6 iU/ml (0.5-5.5 iU/ml)] and did not change during sunitinib treatment.

Rat study

The increase in body weight in the sunitinib group (from 368±3 to 384±3 grams) was smaller than in the control group (from 383±3 to 406±3 grams, p=0.0002).
Serum T_4 and T_3 levels decreased during sunitinib administration and did not return to control levels after its withdrawal (Fig. 2A). Urinary excretions of T_3 and T_4 were very low at baseline (below detection limit and 0.02±0.01nmol/d, respectively) and did not change during sunitinib administration. During co-administration of sunitinib and macitentan, serum T_3 levels tended to be higher (control, 0.8±0.1 nmol; sunitinib, 0.4±0.1 nmol; sunitinib + macitentan, 0.6±0.1 nmol; n=3; p=0.06 for sunitinib + macitentan

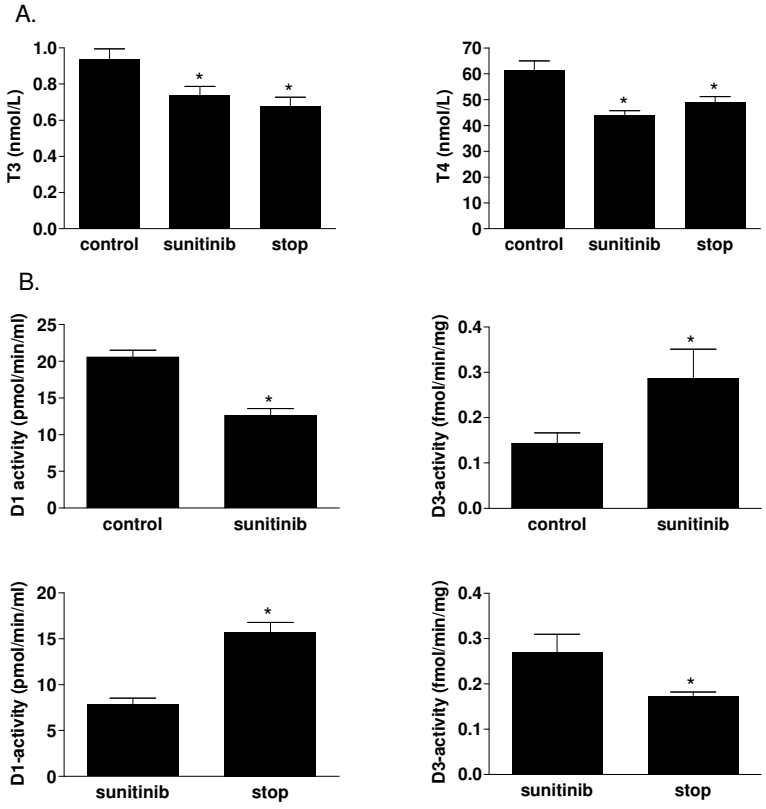

Figure 2. A. Circulating thyroid hormone levels in rats after administration of sunitinib (n=9), vehicle (n=6) and discontinuation of sunitinib (n=7) B. Deiodinase activities in isolated rat liver after administration of sunitinib (n=5), vehicle (n=5) or sunitinib withdrawal (n=7).
* P<0.05 (in panel B sunitinib and stop vs control)

vs. sunitinib) compared to administration of sunitinib alone, whereas serum T_4 levels were identical (control, 46.3±4.3 nmol; sunitinib, 31.3±1.9 nmol; sunitinib + macitentan, 33.8±3.3 nmol; n=3; p=0.59 for sunitinib + macitentan *vs*. sunitinib).

Ex vivo studies and histology

Measurement of hepatic deiodinase activities showed a significant induction of D3 activity corresponding with the observed decreases in serum T_4 and T_3 levels (Fig. 2). Also a decrease in D1 activity after sunitinib administration was found (Fig. 2B). Changes in both deiodinase activities were reversible after sunitinib discontinuation.

Histological examination of the thyroid sections stained with HE showed a marked decrease in the total number of vessels as well as a decreased vessel-to-follicle ratio after administration of sunitinib (Fig. 3, 4). Staining of thyroid sections for actin, which marks large vessels containing smooth muscle cells rather than small capillaries stained by HE, showed no changes in the number of actin-positive vessels or vessel-to-follicle ratio after sunitinib administration compared to controls (18±2 *vs.* 20±4; p=0.64 and

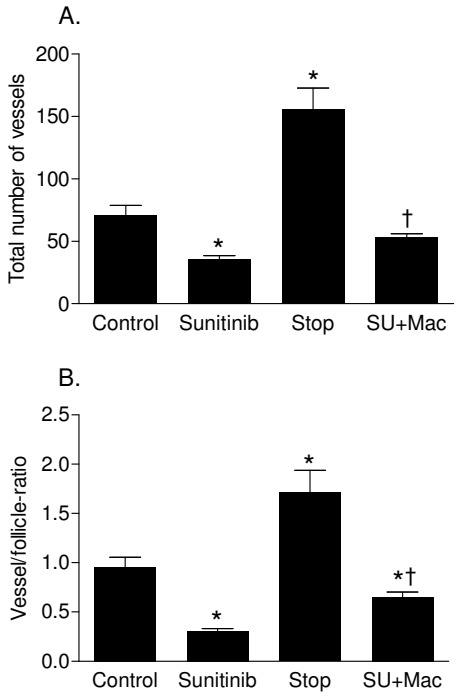

Figure 3. Total number of vessels (A) and vessel-to-follicle ratio (B) counted in 5 high power fields (magnification ×400) of thyroid sections stained with HE obtained from WKY rats administered vehicle (control, n=7), sunitinib (n=9), sunitinib and macitentan (SU+Mac, n=8) or after sunitinib withdrawal (stop, n=4). * P<0.05 compared to control, † P<0.05 compared to sunitinib.

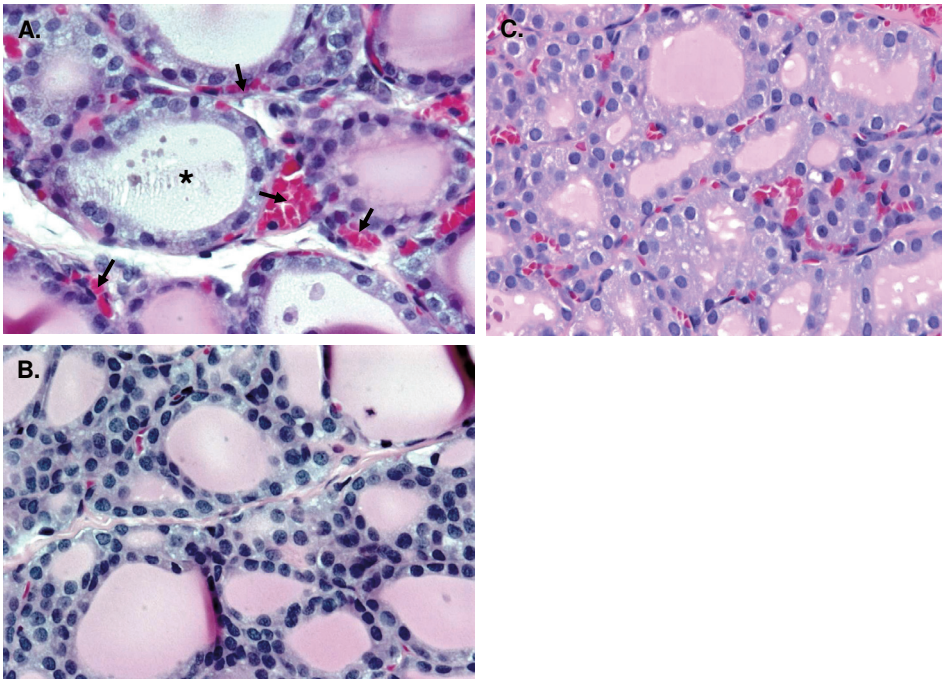

Figure 4. Thyroid sections stained with HE (magnification ×400) of a control rat (A), after 8 days of sunitinib administration (B) and after 11 days of sunitinib withdrawal (C). Note in A the numerous small capillaries (arrows) around most of the thyroid follicles (asterix), whereas in B the number of capillaries is markedly decreased. In rats after sunitinib withdrawal the number of capillaries is even higher compared to that of control rats (C).

0.14±0.07 *vs.* 0.27±0.02; p=0.14, respectively). Inflammation or epithelial cell destruction were not observed. Intra-epithelial vacuoles and colloidal resorption vacuoles appeared to be more prominent in HE-stained sections of control rats than in sections of sunitinib-administered rats (Fig. 4). After withdrawal of sunitinib for 11 d the histological abnormalities in the thyroid observed with HE-staining completely reversed. The number of actin-positive vessels (16.8±3.5) or vessel-to-follicle ratio (0.21±0.04) after sunitinib withdrawal were not statistically different from those of controls (p=0.79 and p=0.81, respectively) or sunitinib-administered rats (p=0.26 and p=0.15, respectively). In rats treated with the combination of sunitinib and macitentan, the total number of HE-stained vessels and the vessel-to-follicle ratio was significantly higher than in rats administered sunitinib alone (Figure 3).

DISCUSSION

Thyroid dysfunction, mainly hypothyroidism, is a frequently reported adverse effect of sunitinib treatment.[4,10,12,15-20] With our clinical and experimental studies we aimed to obtain more insight into the time course, incidence and mechanisms involved in sunitinib-associated thyroid dysfunction. In agreement with previous reports we found that sunitinib-induced hypothyroidism, as reflected by increased serum TSH values, develops in a substantial proportion of patients.[3] Prospective evaluation of the time course of ensuing thyroid dysfunction showed an increase in TSH levels already within 10 wk of treatment, although values at this time were still within the normal reference range. Retrospective analysis showed a wide range in time to onset of elevated TSH levels: in some patients, thyroid dysfunction developed as soon as after 4 wk of treatment, in others, only after years. Because TFT were not performed at fixed time points, the number of patients with an early onset of thyroid dysfunction is probably underestimated. Whether the rise in TSH levels in our retrospective study was accompanied by clinical symptoms was not routinely documented. Differentiation between clinical symptoms related to hypothyroidism from those related to malignancy can be difficult. For instance, fatigue, one of the major symptoms of hypothyroidism, is also a common feature of cancer and of cancer therapies, while weight gain, another symptom of hypothyroidism, might be masked by malignancy- or treatment-induced anorexia.

The observation in our retrospective study that in a small proportion of patients the development of elevated TSH levels was preceded by depressed TSH levels and elevated FT_4 levels may be indicative of a thyroiditis-like mechanism underlying the development of sunitinib-induced hypothyroidism. For several reasons this is probably not autoimmune-mediated. Firstly, in our prospective study as well as in other studies the rise in TSH was not accompanied by increased circulating anti-TPO antibodies.[4,12,15] Secondly, the incidence of autoimmune-mediated thyroiditis is much higher in women than in men. The observation that the incidence of transiently elevated TSH levels was similar in women and men in our study, makes an auto-immune-mediated mechanism less likely. Thirdly, histological examination of the thyroid of rats exposed to sunitinib did not show an inflammatory reaction that would have been suggestive for immune-mediated damage.

Sunitinib may also cause thyroid tissue injury, eventually leading to thyroid tissue destruction, by inducing capillary regression (rarefaction). Indeed, ultrasound and computed tomographic evaluations have reported a reduction in thyroidal volume during sunitinib treatment.[10,11] In addition, impaired uptake of radiolabeled iodine during sunitinib treatment has been reported.[12] Because sunitinib did not inhibit iodide uptake

in cultured rat FRTL-5 thyroid cells, the impaired iodide uptake during sunitinib treatment probably also reflects the decreased number of (functional and / or structural) capillaries necessary for the delivery of iodide to the thyroid.[12,21] Indeed, in the rats exposed to sunitinib for 8 d, we observed a marked decrease in the total number of thyroid vessels as well as a decreased vessel-to-follicle ratio upon histological examination. Interestingly, this appeared to be limited to the capillaries as the total number of larger vessels containing actin-positive smooth muscle cells or the vessel-to-follicle ratio was not reduced. Dose-dependent regression of capillaries has also been reported in mice exposed to soluble VEGF-receptors or a small-molecule VEGF-RTKI for 1-3 wk.[9] Of the various endocrine organs examined in these mice, capillary regression (up to 68%) was most pronounced in the thyroid.[9] The capillary regression was reversible after anti-VEGF treatment was stopped for 1 wk. Also in our rat study thyroid capillary regression reversed after sunitinib had been withdrawn for 11 d and even resulted in a, possibly compensatory, overshoot. To explore whether functional rarefaction (a decrease in perfused microvessels) contributed to the observed decrease in microvessels we treated rats with the combination of sunitinib and the endothelin receptor blocker macitentan, as we have demonstrated that administration of sunitinib is associated with a rise in plasma ET-1 levels.[13] ET-1 is a potent vasoconstrictor and could have induced functional rarefaction.[22] In rats exposed to the combination of sunitinib and macitentan the decrease in vessel-to-follicle ratio was less pronounced compared to rats administered sunitinib alone. These findings are compatible with the hypothesis that, apart from structural rarefaction (a reduction in capillary density), also functional rarefaction contributes to the decrease in thyroidal microvessels observed during sunitinib exposure. Since co-administration of macitentan only resulted in maintenance of T_3 but not of T_4 secretion, macitentan probably does not completely prevent the effects of sunitinib on the thyroid. In normal rats, as much as 50% of circulating T_3 may be derived directly from thyroidal secretion.[23] In a relatively iodine-deficient gland this may be even more. Fenestrated capillaries with a high expression of VEGF-receptors are especially present in endocrine organs and depend on VEGF for their survival.[9] The repeated periods of capillary regression in the thyroid related to the 'on' and 'off' treatment cycles with sunitinib in patients may cause destructive thyroiditis. In a small proportion of patients this may be accompanied by a transient increase in TH release.[10] It is also possible that the mere decreased number of capillaries into which TH can be secreted is involved in the development of hypothyroidism.

Apart from capillary regression, interference with T_4/T_3 metabolism seems to be a second mechanism involved in sunitinib-induced hypothyroidism. In our patients a decrease in T_3/rT_3 ratio was observed, reflecting enhanced T_4/T_3 metabolism. These clinical findings are supported by our experimental data showing increased hepatic D3

activity and decreased D1 activity in rats exposed to sunitinib for 8 d. The changes in deiodinase activities were accompanied by corresponding decreases in serum T_3 and T_4 hormone levels. It has previously been reported that hypoxia-inducible factor 1 (HIF-1) is a positive modulator of the gene encoding D3.[24] Although the expression of HIF-1 is not increased by sunitinib, HIF-1 might be up-regulated indirectly because of sunitinib's anti-angiogenic effect.[25] Interestingly, after sunitinib withdrawal, deiodinase activities reversed, whereas TH levels remained low. Although capillary regression was also reversible after sunitinib discontinuation, apparent restoration of capillaries is not necessarily paralleled by simultaneous normalization of TH synthesis and secretion. Thus, it would be interesting in this respect to measure TH stores in the thyroid gland during and after sunitinib treatment in future studies.

Chronic conditions, such as cancer and fasting can be associated with low T_3 levels and increased rT_3 levels, also referred to as non-thyroidal illness syndrome (NTIS).[26,27] Although we did not measure rT_3 levels in our retrospective study, and in the prospective study, rT_3 levels did not change to support NTIS, this condition cannot be completely ruled out. However, the increase in TSH levels after initiation of sunitinib treatment, makes the involvement of NTIS in sunitinib-induced hypothyroidism less likely as NTIS is usually associated with suppressed TSH levels. Of note, Wong et al have demonstrated that sunitinib, like propylthiouracil and methimazole, inhibits thyroid peroxidase activity at concentrations in the micromolar range, which may further contribute to the development of sunitinib-induced hypothyroidism.[20]

Our study has some limitations. Firstly, part of the human data were collected using a retrospective design, resulting in the sporadic assessment of FT_4 levels, at least initially, and the lack of symptom recording related to thyroid dysfunction, as well as the lack of FT_3 and rT_3 assessment. Secondly, although no changes in circulating anti-TPO antibodies were observed making an autoimmune-mediated destructive mechanism less likely, assessment of thyroglobulin antibodies could have further supported this conclusion.

In conclusion, inhibition of VEGF signaling with the receptor tyrosine kinase inhibitor sunitinib induces thyroid dysfunction in both patients and rats and is due to capillary regression in the thyroid and alterations in the T_4/T_3 metabolism. Considering the treatment duration of patients, who are treated with sunitinib for months to years, it seems likely that in patients the sunitinib-induced rarefaction induces thyroid tissue injury and eventually tissue destruction. Since sunitinib-induced thyroid dysfunction occurs in a significant proportion of patients and has been observed as soon as after 34 d of treatment, routine monitoring of thyroid function during treatment with sunitinib is recommended. Whether all patients with abnormal TFTs should be prescribed TH

remains unclear. Even in patients reporting fatigue in association with sunitinib and developing abnormal TFTs, symptomatic improvement was not observed in nearly 50% of the patients with LT_4 replacement therapy.[15] As suggested by the American Thyroid Association, TH should be withheld in asymptomatic patients with only mild elevation of TSH (5-10 mIU).[28] In patients with TSH levels above 10 mIU and low FT_4 levels, or TSH levels above 10 mIU and symptoms of hypothyroidism, treatment with a low-dose of LT_4 should be considered.[29] In patients already on TH replacement, extra attention should be paid to the thyroid function, and an earlier increase in the LT_4 dose during treatment with sunitinib might be considered as sunitinib is associated with enhanced TH metabolism.

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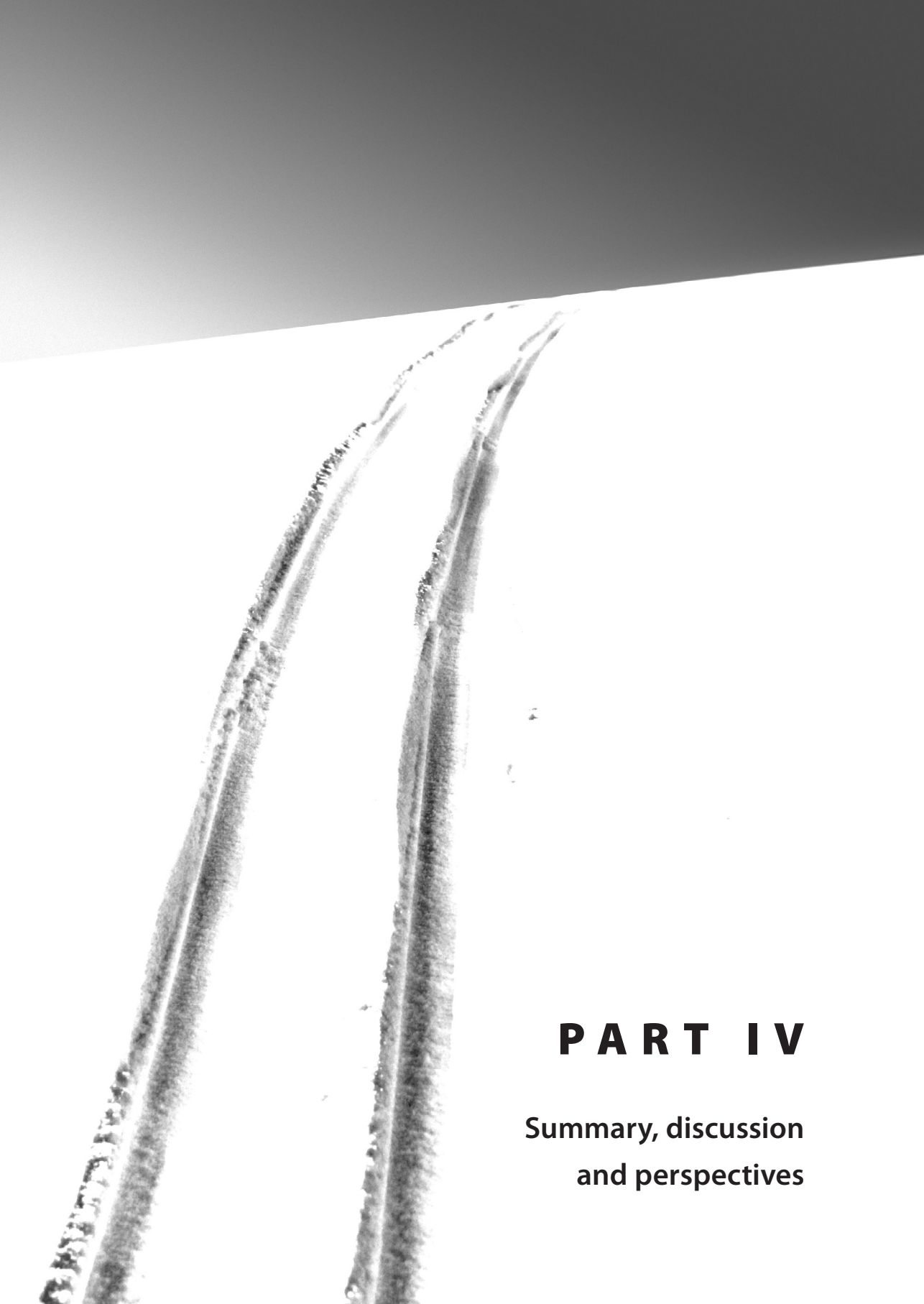

PART IV

Summary, discussion and perspectives

SUMMARY

Angiogenesis is critical to tumor growth as well as to metastasis. Vascular endothelial growth factor (VEGF) and its receptors play key roles in the regulation of this process. Inhibition of VEGF signaling by means of blocking the VEGF receptor tyrosine kinases has become an established treatment for various tumor types. Sunitinib, an oral VEGF receptor tyrosine kinase inhibitor, is currently approved for the treatment of metastatic renal cell carcinoma and imatinib-resistent gastrointestinal stromal tumors. Unfortunately, this therapy is associated with several side effects among which hypertension, renal toxicity, cardiac toxicity and thyroid dysfunction in a substantial proportion of patients. The studies presented in this thesis have unravelled pathophysiological mechanisms that are involved in the development of these adverse effects and provide directions for their most appropriate clinical management, thereby optimizing the potential efficacy of angiogenesis inhibition as anticancer treatment.

Sunitinib-induced cardiovascular and renal toxicity

Hypertension

Sunitinib-induced hypertension has been reported in up to 47% of patients **(Chapter 1)**. Since experimental data indicate that binding of VEGF to its receptors increases endothelial nitric oxide synthase (eNOS) and thus nitric oxide (NO) bioavailability, it has been suggested that inhibition of the VEGF-pathway reduces NO bioavailability resulting in vasoconstriction and a rise in blood pressure (BP). Additionally, decreased NO bioavailability disturbs the balance between the vasodilator NO and the vasoconstrictor endothelin-1 (ET-1), favoring ET-1 production, thereby inducing further vasoconstriction and an additional rise in BP **(Chapter 1)**.[1] Apart from inducing vasoconstriction, ET-1, by activating NADPH-oxidase, increases oxidative stress through an increase in reactive oxygen species (ROS) production.[2,3] Hypertension is generally associated with an increase in oxidative stress. Moreover, TEMPOL, a superoxide-dismutase mimetic, that metabolizes superoxide and other ROS, has been shown to reduce BP in several experimental hypertension models.[4] The effect of sunitinib on BP in patients, rats and swine, as well as the roles of ET-1, decreased NO-bioavailability and/or enhanced oxidative stress in its effect were explored in this thesis.

In patients, sunitinib treatment, when given according to a 4 weeks 'on', two weeks 'off' regimen (one cycle), induced a rise in mean BP of almost 15 mmHg and a decrease in HR of 8 beats/min after 4 weeks of treatment. Both hemodynamic effects were reversible during the 2-weeks off-treatment period. The normally occurring decrease in BP during sleep ('dipping' ≥ 10%) was diminished after 4 weeks of sunitinib treatment. The BP

increase was associated with a rise in plasma ET-1 levels and a decrease in plasma renin levels, whereas plasma catecholamine levels remained unchanged, indicating that activation of the endothelin system, but not the renin-angiotensin system or sympathetic nervous system, is involved in the sunitinib-induced rise in BP **(Chapter 3)**.

In rats, 8 days of sunitinib administration induced an increase in BP of 30 mmHg that was accompanied by a decrease in HR and a loss of circadian BP rhythm. These changes disappeared after sunitinib withdrawal and were associated with a reversible rise in circulating ET-1 levels. Moreover, the sunitinib-induced rise in BP in rats could largely be prevented by concomitant administration of the dual ET_A/ET_B-receptor antagonist macitentan, confirming a crucial role for the ET system in the development of sunitinib-induced hypertension. Urinary excretion of nitric oxide metabolites, which is regarded a measure of global NO-bioavailability, decreased during administration of sunitinib, but did not normalize after sunitinib withdrawal. This indicates that administration of sunitinib is associated with a long-term decrease in NO-bioavailability. The ROS-scavenger TEMPOL only modestly prevented the sunitinib-induced rise in BP, indicating that oxidative stress does not play an important role in the development of sunitinib-induced hypertension **(Chapter 3, 4)**.

In chronically instrumented awake swine, sunitinib induced a rise in BP of 10±5 mmHg within 4 hours following sunitinib administration reaching a maximum of 14±5 mmHg after one week of daily administration, whereas HR decreased. The rise in BP was due to an increase in systemic vascular resistance. Administration of the dual ET_A/ET_B receptor blocker tezosentan in sunitinib-treated swine, induced a larger decrease in systemic vascular resistance and BP compared to control conditions, indicating enhanced ET-1-mediated sunitinib-induced vasoconstriction. Thus, also in swine activation of the endothelin system is involved in the development of sunitinib-induced rise in BP. The vasoconstrictor effect of the eNOS inhibitor LNNA was increased after treatment with sunitinib, indicating an increased NO-mediated vasodilator tone during sunitinib administration, likely counteracting the ET-1-mediated vasoconstriction. Administration of a cocktail of ROS scavengers, including TEMPOL, provided no evidence that the sunitinib-induced vasoconstriction is due to enhanced oxidative stress. Thus, neither decreased NO bioavailability nor increased production of ROS are involved in the sunitinib-induced rise in BP in this swine model **(Chapter 5)**.

Renal toxicity

The incidence of sunitinib-induced renal toxicity, mainly reported as proteinuria, is not well known because in the initial studies patients were not routinely screened for this condition. In a phase I trial of the investigational receptor tyrosine kinase inhibitor

KRN951 3 (20%) of the 15 patients developed dose-limiting proteinuria **(Chapter 1)**. VEGF is important for the maintenance of podocyte function and survival via regulation of nephrin expression, a protein that prevents podocyte apoptosis. Therefore, VEGF is important for the maintenance of the glomerular filtration barrier. Inhibition of the VEGF signaling pathway might affect the integrity of this filtration barrier leading to proteinuria **(Chapter 1)**. In chronic kidney disease, renal histopathological changes are thought to be ET-1-mediated, mainly via stimulation of the ET_A-receptor and ET-1 has shown to increase glomerular permeability, independent of BP.[5] Furthermore, abundant evidence indicates that oxidative stress is involved in the development of renal injury in general.

The effect of sunitinib on renal function and histology in patients and rats, as well as the roles of ET-1 and oxidative stress in mediating this effect, were further explored in this thesis.

In patients, serum creatinine did not change after 10 weeks of treatment with sunitinib, but urinary creatinine clearance, as an estimate of glomerular filtration rate, decreased within 4 weeks of treatment with sunitinib (Kappers *et al*., unpublished results) whereas proteinuria tended to increase **(Chapter 3)**.

In rats, sunitinib induced a rise in serum creatinine, proteinuria, albuminuria and urinary ET-1 levels. All parameters, except for serum creatinine and urinary ET-1 levels reversed after sunitinib withdrawal. Renal histological examination, using light and electron microscopy, showed that sunitinib induced marked abnormalities, including intra-epithelial protein droplets, epithelial and endothelial cell swelling leading to obliteration of the capillary lumen, loss of endothelial fenestration as well as podocyte foot process effacement. The combination of endothelial cell swelling, loss of fenestration and obliteration of the capillary lumen is also referred to as glomerular endotheliosis. Interestingly, preeclampsia, a condition occurring in the third trimester of pregnancy, is also characterized by hypertension, proteinuria, increased circulating ET-1 levels and glomerular endotheliosis. One of the underlying pathophysiological mechanisms is thought to be an increased placental production of soluble fms-like tyrosine kinase (sFlt-1), the extracellular domain of the VEGF receptor (VEGFR)-1, which binds circulating VEGF, thereby abrogating the VEGF-VEGFR signaling pathway and thus creating a condition similar to that induced by sunitinib. Therefore, hypertension and proteinuria during angiogenesis inhibition with sunitinib are often referred to as a preeclampsia-like syndrome **(Chapter 4)**.

To further explore the independent roles of ET-1 and BP and the role of oxidative stress in the development of sunitinib-induced renal toxicity, rats on sunitinib were concomitantly administered the dual ET_A/ET_B-receptor antagonist macitentan, amlodipine or the ROS scavenger TEMPOL. Both macitentan and amlodipine equally diminished the

sunitinib-induced rise in BP. Macitentan diminished proteinuria and urinary ET-1 excretion whereas the elevated serum creatinine concentration remained unchanged. In contrast, amlodipine at an equipotent BP-lowering dose as macitentan, did not reduce sunitinib-induced proteinuria and the increase in urinary ET-1 excretion **(Chapter 6)**. Administration of the ROS scavenger TEMPOL also decreased proteinuria and urinary ET-1 excretion while it only marginally reduced BP. Neither macitentan, nor amlodipine or TEMPOL diminished the sunitinib-induced renal histological abnormalities, although intra-epithelial protein deposition was diminished with macitentan **(Chapter 4)**. From these findings it can be concluded that sunitinib-induced renal toxicity is ET-1-mediated and occurs independently of BP. Moreover, oxidative stress appears not to be involved in the sunitinib-induced rise in BP, but plays an important role in sunitinib-induced renal toxicity.

Cardiac toxicity

Impaired cardiac function has been observed in up to 28% of patients treated with sunitinib. In addition, angina pectoris and increases in biomarkers for cardiac damage have been reported **(Chapter 1)**. In patients developing cardiac dysfunction during treatment with sunitinib, myocardial biopsy specimens showed structural changes in mitochondria of cardiomyocytes. Impaired ATP generation secondary to mitochondrial dysfunction could therefore underlie the contractile ventricular dysfunction occurring in some patients on sunitinib treatment. Additionally, oxidative stress is generally increased in hypertension and plays a pathogenic role in the development and progression of cardiovascular disease. Therefore, enhanced formation of ROS may also be involved in the sunitinib-induced cardiovascular side effects. Apart from the effects on the systemic circulation, no studies on the effects of angiogenesis inhibition on the coronary and pulmonary circulation have been reported.

The effects of sunitinib on cardiac mitochondrial function and coronary microvascular function in rats as well as its effect on the pulmonary and coronary circulation and cardiac function in swine, and the possible roles of ET-1, NO-bioavailability and oxidative stress were further explored in this thesis.

Ex vivo studies in rat cardiac mitochondria demonstrated that sunitinib did not affect ATP production, suggesting that impaired cardiac mitochondrial ATP-production is not involved in the sunitinib-induced decrease in cardiac contractile function **(Chapter 3)**. Coronary microvascular function studies in hearts obtained from sunitinib-administered rats using the Langendorff model demonstrated that, not only coronary flow responses to the endothelium-dependent vasodilator bradykinin, but also responses to the endothelium-independent vasodilator sodium nitroprusside and the vasoconstrictor angiotensin II were diminished. These findings indicate that sunitinib induces general

coronary vascular smooth muscle cell dysfunction that, in part, may also account for the observed decrease in coronary endothelial dysfunction as represented by the diminished response to bradykinin **(Chapter 3)**. The mechanism behind this generalized impaired microvascular smooth muscle cell function remains to be clarified.

In swine, administration of sunitinib had no effect on the pulmonary vasculature and coronary blood flow. Acute administration of the ET_A/ET_B receptor blocker tezosentan reversed the systemic hemodynamic effects of sunitinib, supporting previous observations that an increase in endogenous ET-mediated vasoconstrictor tone underlies the sunitinib-induced rise in BP **(Chapter 3, 4)**. No evidence was found that the systemic vasoconstriction induced by sunitinib was caused by a decrease in NO-mediated vasodilator tone or an increase in oxidative stress. Furthermore, although sunitinib has been shown to induce a decline in left ventricular ejection fraction in patients, in swine it had no effect on LVdP/dtmax or LVdP/dtmin, as respective indices of left ventricular systolic and diastolic function, or on stroke volume **(Chapter 5)**. It should be noted that the exposure time to sunitinib in swine was short. Therefore, prolonged exposure to sunitinib might also be associated with left ventricular dysfunction in swine, although differentiation between BP-dependent and BP-independent effects remains difficult.

Sunitinib-induced thyroid dysfunction

Thyroid dysfunction, mainly hypothyroidism, has retrospectively been reported in up to 53-85% of patients and prospectively in up to 36-46% of patients treated with sunitinib **(Chapter 2)**.[6] Since especially the capillary bed of the thyroid is susceptible to VEGF inhibition, it has been hypothesized that capillary regression is involved in sunitinib-associated thyroid dysfunction ('capillary regression hypothesis').[7] In addition, in patients with a history of hypothyroidism already on levothyroxine (LT_4) replacement therapy, LT_4 dose increments were needed during treatment with sunitinib, indicating interference of sunitinib with thyroxine 4 (T_4) and triiodothyronine (T_3) metabolism ('metabolic hypothesis') **(Chapter 2)**.

The effect of sunitinib on thyroid function and the potential mechanisms involved in its effect, including thyroid vasculature and thyroid hormone metabolism, in patients and in rats were further explored in this thesis.

Retrospective analysis in 108 patients demonstrated that 42% of patients developed elevated thyroid stimulating hormone (TSH) levels (TSH > 5.0 mU/l), which in 5 patients was preceded by TSH suppression. Prospective analysis in 15 patients showed a 4-fold increase in TSH levels, within 10 weeks of treatment, whereas the thyroid hormone levels FT_4 and T_3 remained unchanged. Nonetheless, this was accompanied by a decrease in

T_3/rT_3-ratio, indicating enhanced thyroid hormone metabolism. No changes in thyroid peroxidase antibodies were observed **(Chapter 8)**.

In rats, sunitinib administration was associated with increased type 3 deiodinase activity, converting T_3 and T_4 into the less active metabolites T_2 and rT_3, and decreased type 1 deiodinase activity, converting T_4 and rT_3 into T_3 and T_2. Correspondingly, circulating thyroid hormone levels decreased. Both deiodinase activities reversed after sunitinib discontinuation. Histological examination of the thyroid showed marked capillary regression after sunitinib administration, which resulted in a, probably compensatory, capillary overshoot after sunitinib withdrawal. Interestingly, in rats on both sunitinib and the dual ET_A/ET_B receptor blocker macitentan, that induces vasodilation in conditions with an activated endothelin system, capillary regression was less pronounced compared to rats on sunitinib alone **(Chapter 8)**. This finding indicates that sunitinib induces not only structural but also functional rarefaction within the thyroid. In conclusion, our results demonstrate that sunitinib-induced hypothyroidism is due to a combination of alterations in deiodinase activity affecting the T_4/T_3 metabolism and capillary regression within the thyroid.

DISCUSSION

Sunitinib-induced cardiovascular and renal toxicity

Hypertension and renal toxicity

As can be concluded from the findings in this thesis, the sunitinib-induced rise in BP and renal toxicity are mainly due to activation of the endothelin system. Although blockade of ET-1 signaling can to a large extent prevent these toxicities, the exact mechanism causing this activation of the endothelin system is currently unknown and several mechanisms can be proposed. Firstly, VEGF plays an important role in endothelial cell survival and function. VEGF receptor inhibition with sunitinib might thus induce endothelial cell activation leading to increased production of ET-1 by these cells. We investigated the effect of sunitinib on ET-1 production by human umbilical vascular endothelial cells (HUVECs) and demonstrated that sunitinib did not increase the ET-1 production by these cells **(Chapter 3)**. However, this might be a cell type-specific finding and studies using other endothelial cell lines, for example human coronary endothelial cells, still have to be performed. Secondly, although others have demonstrated increased expression of preproET-1 mRNA in the renal cortex obtained from rats that were administered sFlt-1, a soluble VEGF-receptor that binds circulating VEGF, we observed no change in preproET-1 mRNA expression in rat renal cortex and medulla after 8 days of sunitinib

administration.[8] This finding makes enhanced ET-1 gene transcription as a cause of increased circulating ET-1 levels in our model unlikely. Thirdly, an increase in ET-1 converting enzyme activity might account for the observed elevated circulating ET-1 levels, but this was not explored in the current thesis. Fourthly, binding of VEGF to its receptors leads to an increase in NO production and, as a consequence, VEGF receptor inhibition with sunitinib might induce a decrease in NO bioavailability, thereby disturbing the balance between NO and ET-1, favoring the production of ET-1 **(Chapter 1)**. Indeed, a decrease in urinary NO metabolites excretion, considered a global measure of (systemic) NO-bioavailability, was observed in our rats on sunitinib **(Chapter 4)**. In contrast, in swine, the vasoconstriction induced by administration of the eNOS inhibitor LNNA was increased after sunitinib exposure, indicating an increased NO-mediated vasodilator tone during sunitinib administration **(Chapter 5)**. This increase in NO-mediated vasodilator tone might be secondary to the rise in BP, counteracting the ET-1 mediated vasoconstriction. With respect to the observed decrease in urinary excretion of nitrates in rats on sunitinib, our findings indicate that this is not equivalent to a diminished basal NO-mediated vasodilator tone or, alternatively, that urinary NO-metabolites excretion is more likely to be a reflection of local renal NO-bioavailability rather than of systemic NO-bioavailability.

ET-1 is known to activate vascular NADPH oxidase, one of the major sources of ROS, leading to an increase in oxidative stress.[2,3] Since oxidative stress is generally increased in hypertension and has been implicated in the development and progression of cardiovascular disease, we investigated whether oxidative stress was involved in sunitinib-induced hypertension and renal toxicity. We demonstrated that enhanced oxidative stress was mainly involved in sunitinib-induced renal toxicity, but not in the sunitinib-induced rise in BP **(Chapter 4, 5)**. Of note, the effects of ROS on vascular tone as well as on angiogenesis are complex. Activation of NADPH oxidase is also a critical step in the VEGF-receptor signaling cascade, which is blocked following sunitinib administration.[9] Therefore, the increased activation of NADPH oxidase by increased activity of the endothelin system might be offset by a decreased activation of NADPH oxidase by VEGF inhibition, resulting in a net decrease rather than an increase in oxidative stress, limiting rather than enhancing the vasodilator effect of ROS scavenging on the systemic vasculature.

Since microvessels (arterioles and capillaries) constitute the main determinant of vascular resistance, functional (a decrease in perfused microvessels) or structural (a reduction in capillary density) rarefaction might be involved in the rise in BP during angiogenesis inhibition. Using nailfold capillary microscopy in patients on sunitinib, a 12% decrease in structural capillary density was reported after 14 days and a 6% decrease after 28

days of treatment, whereas MAP at that time had increased by 7%.[10] In these patients no significant changes in functional rarefaction were observed. Whether the decrease in capillary density accounts for observed rise in BP during treatment with sunitinib is questionable. A mathematical model based on the hamster cheek pouch microcirculation indicates that 42% rarefaction of the 4th order arterioles is needed to increase resistance in that particular vascular bed by 5%.[11] This indicates that a considerable amount of rarefaction in various vascular beds is required to induce an increase in vascular resistance and hence in BP, assuming that cardiac output remains unchanged. Based on this knowledge, it seems highly unlikely that rarefaction is an important determinant in the rise in BP during angiogenesis inhibition. Additionally, considering the short time interval at which hypertension develops (within 4 hours after administration of the first dose in swine and within 1 day in rats and patients), structural rarefaction as a cause of sunitinib-induced rise in BP is highly unlikely **(Chapter 3, 5)**.[12] Finally, a decrease in capillary density might be a consequence rather than the cause of the rise in BP as rarefaction is present in patients with hypertension not yet treated, but absent when the elevated BP is normalized with antihypertensive treatment.[13] Once hypertension occurs, rarefaction develops or worsens over a relatively short time interval.[14]

The combination of hypertension and renal toxicity during angiogenesis inhibition is often referred to as a preeclampsia-like syndrome. Preeclampsia, a condition occurring after 20 weeks of pregnancy, is characterized by hypertension, proteinuria, elevated circulating ET-1 levels as well as glomerular endotheliosis. Indeed, we demonstrated that angiogenesis inhibition with sunitinib in rats induces similar clinical and renal histopathological characteristics **(Chapter 4)**. Interestingly, both conditions also share common underlying pathophysiological mechanisms. Preeclampsia is associated with increased placental production of sFlt-1, a soluble VEGF-binding receptor with anti-angiogenic 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 depositions and foot-process effacement, changes which are all fully identical to the abnormalities found in our rats exposed to sunitinib.[15] 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.[16] Likewise, in our rats exposed to sunitinib TEMPOL diminished proteinuria and urinary ET-1 excretion although it had no effect on the elevated BP.

Hypertension has been suggested as a biomarker of therapeutic efficacy in patients with mRCC treated with sunitinib. In a large retrospective analysis it has recently been shown that patients with mRCC who develop hypertension when treated with sunitinib have

an improved overall and progression free survival compared to patients who remained free of hypertension.[17] However, in this study, patients were categorical divided into those developing hypertension or not, using the hypertension definition of a systolic BP ≥ 140 mmHg and/or a diastolic BP ≥ 90 mmHg. It is well known that also within the so-called normotensive range a higher BP is associated with an increased cardiovascular risk, indicating that the cutoff value of 140/90 mmHg is arbitrary. If a patient has a baseline BP of 138 mmHg systolic, an only 2 mmHg increase in BP will classify him or her as being hypertensive, whereas a patient with a baseline systolic BP of 110 mmHg experiencing a 28 mmHg increase would not. In our patient study we demonstrated that after 4 weeks of sunitinib treatment BP had increased (mean rise of 13.5 ±2.1 mmHg) in all patients, independently of the baseline BP, but that not all patients met the criteria of hypertension at that time. According to this reasoning the patient's absolute or relative BP increase rather than the presence or absence of hypertension would be a more sensitive biomarker. It further underscores the necessity for repeated standardized BP measurements in patients treated with angiogenesis inhibition.

Cardiac toxicity

Although in patients sunitinib treatment has been reported to be associated with the development of congestive heart failure, in swine administered sunitinib for 1 week, no effects on left ventricular systolic and diastolic function or on stroke volume were observed **(Chapter 5)**.[18] This discrepancy in observations might be due to the difference in exposure time to sunitinib and to the fact that the swine were young and healthy, whereas in patients pre-existent coronary artery disease remained as the only statistically significant predictor of congestive heart failure. The BP elevation induced by sunitinib was due to an increase in peripheral vascular resistance. We investigated whether this vasoconstrictor effect was also present in the pulmonary and coronary vasculature. If so, the sunitinib-induced deterioration of cardiac function could be partially explained by an increase in right ventricular afterload through pulmonary vasoconstriction and/or a decrease in myocardial perfusion resulting from coronary vasoconstriction. No such effects were observed. Although myocardial biopsy specimens of patients developing cardiac dysfunction during treatment with sunitinib, showed cardiac mitochondrial structural changes suggesting impaired cardiac mitochondrial function, we found no evidence of such impaired function that could underlie the sunitinib-induced cardiac toxicity in rats. Instead, the cardiac toxicity might be due to 'off-target' effects of sunitinib such as inhibition of the ribosomal S6 kinase (RSK), which signals survival in the heart. Indeed, inhibitor-binding studies have shown that inhibition of RSKs by sunitinib occurs at clinically relevant concentrations.[19,20]

Sunitinib-induced thyroid dysfunction

Sunitinib-induced thyroid dysfunction is manifested mainly as hypothyroidism and is due to a combination of capillary regression within the thyroid and enhanced peripheral thyroid hormone metabolism as a consequence of induction of type 3 deiodinase activity that inactivates T_3 and T_4 **(Chapter 8)**. The capillary regression appears to be both functional, due to ET-1-mediated vasoconstriction, as well as structural, directly related to sunitinib-induced angiogenesis inhibition **(Chapter 8)**. As a consequence, delivery of iodide to the thyroid is reduced as reflected by impaired uptake of radiolabeled iodine during sunitinib treatment reported by others.[21] Furthermore, capillary regression might indirectly induce thyroid tissue injury, eventually leading to thyroid tissue destruction. Since hypothyroidism frequently occurs in patients treated with sunitinib, regular monitoring of thyroid function during this treatment is recommended.

With respect to the previously mentioned resemblance of sunitinib-induced hypertension and renal toxicity with preeclampsia, it is also interesting to note that preeclampsia is associated with a higher risk of subclinical hypothyroidism during pregnancy and with a higher risk of developing hypothyroidism many years after the occurrence of preeclampsia.[22] Thus, also with regard to the sunitinib-induced thyroid toxicity there appears to be a resemblance with preeclampsia.

PERSPECTIVES

Although increasing evidence demonstrates that the development of hypertension during treatment with sunitinib is associated with an improved survival, this does not imply that antihypertensive treatment in these patients should be withheld. Indeed, the improved survival related to antiangiogenic therapy and the knowledge that hypertension may accelerate and is frequently accompanied by renal damage dictate in our view aggressive antihypertensive treatment when this condition develops. Since activation of the endothelin system plays an important role in both the sunitinib-induced hypertension as well as in renal toxicity, endothelin receptor blockers seem logical candidates to combat these adverse effects. Endothelin receptor blockers are effective in patients with hypertension but are associated with several side effects. For instance, the selective ET_A receptor blocker darusentan markedly reduced BP (17/10±15/9 mmHg with the lowest dose) in patients with resistant hypertension, but was associated with a high incidence of peripheral edema and fluid retention.[23] These side effects limit the use of darusentan and this agent can currently not be recommended for the treatment of angiogenesis-inhibition induced hypertension. With the knowledge that calcium channel blockers are

effective, safe and well-tolerated vasodilating antihypertensive agents, and of which the effectiveness in sunitinib-induced hypertension has been demonstrated in our experimental studies, these drugs might be preferred. In case of concomitant proteinuria, angiotensin-converting enzyme inhibitors (ACEi) or angiotensin II type 1 receptor blockers (ARBs) might be added, since these agents stimulate podocyte nephrin expression and therefore may exert a beneficial effect on the integrity of the glomerular filtration barrier. Since plasma renin activity is decreased in response to sunitinib treatment, ACEi and ARBs might be less effective in lowering BP in patients treated with these agents. Further experimental studies have to be performed to investigate whether administration of anti- renin-angiotensin system (anti-RAS) agents can prevent the development of renal damage in response to sunitinib administration, because, notwithstanding effective BP reduction, renal damage could not be prevented by the calcium-channel blocker amlodipine in our studies **(Chapter 6)**. It should be noted that anti-RAS agents have pro- as well as anti-angiogenic effects and theoretically might affect the anticancer potential of angiogenesis inhibitors.[24,25] With respect to future clinical research, it might be worthwhile to explore whether genetic or other factors may predict the development of sunitinib-induced hypertension in view of the growing evidence that the development of hypertension is associated with improved survival.

Clinical and experimental studies described in this thesis have demonstrated that activation of endothelin plays a critical role in the development of hypertension and renal toxicity during sunitinib exposure, however, the mechanism behind this activation is unknown. As an initial step we investigated the effect of sunitinib on ET-1 production by HUVECs. In these cells sunitinib did not increase the ET-1 production. To exclude a cell-type specific finding, future experiments are needed to investigate whether sunitinib can increase ET-1 production in other endothelial cell cultures or in HUVECs under different experimental conditions. If so, the molecular mechanisms underlying the sunitinib-induced activation of the endothelin system can be further explored.

Notwithstanding the major role for ET-1 in the sunitinib-induced hypertension and renal toxicity, the potential involvement of other mechanisms needs to be considered. Since both clinical and experimental data on NO-bioavailability during angiogenesis inhibition are conflicting, the enhanced NO-mediated vasodilator tone observed in sunitinib-administered swine, needs to be confirmed in our rat model. Furthermore, as suggested previously, this enhanced NO-mediated vasodilator tone may be a protective mechanism to counteract the adverse effects of ET-1. If so, it can be expected that the adverse effects of sunitinib are more pronounced in conditions where the vasodilator NO system is attenuated or switched off. To further investigate this hypothesis, studies in our rat model in which the NO-system is clamped by the combined administration of the

eNOS inhibitor L-NAME and the exogenous NO-donor sodium-nitroprusside, need to be performed. Additionally, the effects of sunitinib administration on BP and renal function can be explored in eNOS knock-out mice.

The observation that sunitinib impairs renal function and that this renal dysfunction appears in part to be independent from the sunitinib-induced rise in BP raises the question to what extent renal function impairment contributes to the rise in BP. Although we did not observe sodium retention during sunitinib (no higher increase in body weight compared to controls) as a potential mechanism for the increase in BP in our experimental studies this does not exclude the possibility that it might occur in situations where sodium intake is increased. To further explore the role of sodium retention in the sunitinib-induced rise in BP, studies in rats on a low (normal) and high salt diet have to be performed.

In the Langendorff heart model we demonstrated that responses to the endothelin-dependent vasodilator bradykinin, the endothelium-independent vasodilator sodium-nitroprusside as well as to the vasoconstrictor angiotensin II were diminished in rats exposed to sunitinib **(Chapter 3)**. These observations imply a generalized sunitinib-induced impaired vascular smooth muscle function of which the underlying mechanism remains to be clarified.

Structural rarefaction might be involved, although the exercise studies in swine are not supportive for such a mechanism. Furthermore, a direct effect of sunitinib on vascular smooth muscle function or impaired vascular smooth muscle function due to prolonged activation of the endothelin system might also be considered. However, the observation that the diminished responses to the vasodilator and vasoconstrictor substances did not normalize during co-administration of the endothelin receptor antagonist macitentan makes the latter possibility less likely **(Chapter 4)**.

Similarly to what has been reported for the development of hypertension, the occurrence of hypothyroidism during treatment with sunitinib is associated with a longer progression free survival.[26] Whether patients developing sunitinib-induced hypothyroidism should be treated with thyroid hormone replacement therapy is still under debate. Firstly, although TSH levels increase during prolonged treatment with sunitinib in almost all patients, it remains difficult to determine if all such patients develop clinically overt hypothyroidism as differentiation between clinical symptoms related to hypothyroidism or to malignancy is difficult. This is supported by the observation that 50% of the patients who developed sunitinib-induced hypothyroidism and were treated with levothyroxine did not report symptomatic improvement.[27] Secondly, accelerated growth of solid tumors has been observed after thyroid hormone replacement.[28] Furthermore, a

hypothyroid state may benefit the management of various cancers as exemplified by the observation that patients with mRCC and a history of hypothyroidism seem to do better on anticancer therapy than those without such history.[29] On the other hand, T_3 is also known to stimulate cell differentiation and to inhibit cell growth and therefore thyroid hormone replacement in patients with sunitinib-induced hypothyroidism might have beneficial effects.[26] Since until now thyroid hormone replacement has not shown to influence survival in patients treated with sunitinib, this therapy should not be withheld in patients developing TSH levels above 10 mU/L and clinical symptoms or developing overt hypothyroidism.[26]

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Nederlandse samenvatting

SAMENVATTING

Angiogenese, de vorming van nieuwe bloedvaten, speelt een belangrijke rol bij de groei van tumoren. Vasculair endotheliale groeifactor (VEGF) en bijbehorende receptoren spelen een sleutelrol bij de regulatie van dit proces. Remming van VEGF-signaaloverdracht via het blokkeren van de tyrosine kinases van deze receptoren is inmiddels een belangrijke behandelingsmodaliteit van verschillende vormen van kanker. Zo wordt sunitinib, een orale VEGF receptor tyrosine kinase remmer, als eerstelijns middel gebruikt in de behandeling van het gemetastaseerde niercelcarcinoom en imatinib-resistente gastrointestinale tumoren. Helaas gaat deze behandeling gepaard met bijwerkingen waaronder hypertensie, renale en cardiale toxiciteit, en schildklierdisfunctie bij een groot aantal van de behandelde patiënten. De onderzoeken beschreven in dit proefschrift hebben mechanismen die zijn betrokken bij het ontstaan van deze bijwerkingen ontrafeld en vormen daarmee een aanknopingspunt voor de ontwikkeling van nieuwe behandelingsstrategieën van deze bijwerkingen, waardoor de potentiële effectiviteit van anti-angiogenese behandeling verder geoptimaliseerd kan worden.

Sunitinib-geïnduceerde cardiovasculaire toxiciteit en nefrotoxiciteit

Hypertensie

Sunitinib-geïnduceerde hypertensie treedt bij ongeveer 47% van de patiënten op **(Hoofdstuk 1)**. Binding van VEGF aan de VEGF receptoren stimuleert het endotheliale stikstofmono-oxidesynthase (eNOS) waardoor de biologische beschikbaarheid van de vaatverwijder stikstofmono-oxide (NO) toeneemt. Verondersteld wordt daarom dat blokkade van de VEGF signaaloverdracht leidt tot een afname in NO-beschikbaarheid, hetgeen resulteert in vasoconstrictie en een bloeddrukstijging. Daarnaast kan een afname van NO de balans tussen de vaatverwijder NO en de vaatvernauwer endotheline-1 (ET-1) verstoren met als resultaat een toename van ET-1-geïnduceerde vasoconstrictie en een verdere bloeddrukstijging **(Hoofdstuk 1)**. ET-1 induceert niet alleen vasoconstrictie, maar veroorzaakt, via activatie van het NADPH-oxidase en een verhoogde productie van zuurstofradicalen ("reactive oxygen species" ROS), een toename van oxidatieve stress. Bij hypertensie is de oxidatieve stress vaak toegenomen, hetgeen wordt bevestigd door studies die hebben laten zien dat TEMPOL, een superoxide-dismutasemimeticum met anti-oxidatieve werking, de bloeddruk in verschillende hypertensieve diermodellen verlaagt.

De effecten van sunitinib op de bloeddruk in patiënten, ratten en varkens en de onderliggende rol van ET-1, NO-beschikbaarheid en oxidatieve stress zijn in de studies beschreven in dit proefschrift onderzocht.

In patiënten was al na 4 weken behandeling met sunitinib de bloeddruk met 15 mmHg gestegen, terwijl de hartslag afnam. Daarnaast werd de fysiologische bloeddrukdaling die tijdens de nacht optreedt ('dipping' ≥ 10%) niet meer waargenomen. Genoemde hemodynamische veranderingen verdwenen na het staken van sunitinib. Laboratoriumonderzoek liet een stijging zien van de plasma ET-1-spiegel tijdens behandeling met sunitinib, terwijl de plasma reninespiegel daalde en de plasma catecholaminespiegels onveranderd bleven. Deze bevindingen wijzen erop dat activatie van het endotheline systeem, maar niet van het renine-angiotensine systeem of een toegenomen sympathicustonus, een rol speelt bij het ontstaan van hypertensie tijdens behandeling met sunitinib **(Hoofdstuk 3)**.

In ratten leidde toediening van sunitinib tot een reversibele bloeddrukstijging, een afname in hartslag, het verdwijnen van het circadiane bloeddrukritme en een stijging van de plasma ET-1-spiegel. Gelijktijdige behandeling met de duale ET_A/ET_B receptorblokker macitentan kon de bloeddrukstijging grotendeels voorkomen, wijzend op een cruciale rol van het endotheline-systeem bij het ontstaan van sunitinib-geïnduceerde hypertensie. De uitscheiding van NO-metabolieten in de urine, een maat voor globale NO-beschikbaarheid, nam af onder invloed van sunitinib en herstelde niet na het stoppen van dit middel. Gelijktijdige toediening van de anti-oxidant TEMPOL had een minimaal effect op de sunitinib-geïnduceerde bloeddrukstijging, erop wijzend dat oxidatieve stress geen belangrijke rol speelt in de pathogenese van sunitinib-geïnduceerde hypertensie **(Hoofdstuk 3, 4)**.

In varkens leidde toediening van sunitinib al binnen 4 uur na de eerste dosis tot een bloeddrukstijging van 10±5 mmHg. Deze stijging bereikte een maximum van 14±5 mmHg na dagelijkse toediening van sunitinib gedurende 1 week en ging gepaard met een daling van de hartslag. De bloeddrukstijging werd veroorzaakt door een toename van de perifere vaatweerstand. Toediening van de duale ET_A/ET_B receptorblokker tezosentan in varkens die vooraf waren blootgesteld aan sunitinib leidde tot een sterkere daling van de systemische vaatweerstand en bloeddruk dan bij de controle dieren, hetgeen wijst op een toegenomen ET-1- gemedieerde vasoconstrictie tijdens sunitinib. Toediening van de L-arginine analoog LNNA, een eNOS-remmer, leidde tot een sterkere bloeddrukstijging in de met sunitinib-behandelde dieren dan in de controle dieren, wijzend op een toegenomen NO-gemedieerde vaatverwijdende tonus tijdens sunitinib. Wij veronderstellen dat deze toegenomen NO-gemedieerde vaattonus een tegenspeler is van de ET-1 gemedieerde vasoconstrictie. Toediening van een cocktail van anti-oxidanten, waaronder TEMPOL, liet geen aanwijzingen zien voor een toename van oxidatieve stress als oorzaak van de sunitinib-geïnduceerde vasoconstrictie. Dus noch een afname in NO-beschikbaarheid, noch een toename in oxidatieve stress bleken

in dit varkensmodel de sunitinib-geïnduceerde bloeddrukstijging te mediëren **(Hoofdstuk 5)**.

Nefrotoxiciteit

Sunitinib-geïnduceerde nefrotoxiciteit manifesteert zich vooral als proteïnurie. Aangezien in de allereerste klinische studies met sunitinib niet routinematig werd gescreend op proteïnurie is de precieze incidentie van deze bijwerking onbekend. VEGF speelt een belangrijke rol in de functie en overleving van podocyten in de glomerulus via regulatie van de expressie van nefrine, een eiwit dat de podocytenapoptosis tegengaat. VEGF is daarom belangrijk voor het behoud van de glomerulaire filtratiebarrière **(Hoofdstuk 1)**. Remming van VEGF- signaaloverdracht zou de integriteit van deze barrière kunnen aantasten, leidend tot proteïnurie. Er wordt verondersteld dat ET-1, via stimulatie van voornamelijk de ET_A receptor, bij chronische nieraandoeningen een belangrijke rol speelt bij het ontstaan van de histopathologische nierafwijkingen, en tevens de glomerulaire permeabiliteit verhoogt, onafhankelijk van de bloeddruk. Ook is er veel literatuur waarin wordt aangetoond dat oxidatieve stress een rol speelt bij het ontstaan van nierschade in het algemeen.

Het effect van sunitinib op nierfunctie en – histologie bij patiënten en ratten, alsmede de rol van ET-1 en oxidatieve stress hierbij, werd onderzocht in de studies beschreven in dit proefschrift.

In patiënten trad er geen verandering op in het serum kreatinine na 10 weken behandeling met sunitinib, maar de urine kreatinineklaring, een schatting van de glomerulaire filtratie snelheid, daalde tijdens sunitinib behandeling, terwijl de proteïnurie toenam **(Hoofdstuk 3)**.

In ratten leidde toediening van sunitinib gedurende 8 dagen tot een stijging van het serum kreatinine, alsmede tot proteïnurie, albuminurie en een stijging van de urine ET-1 excretie. Al deze parameters normaliseerden na staken van sunitinib, met uitzondering van het serum kreatinine en de urine ET-1 excretie. Licht- en electronenmicroscopisch onderzoek van de nier toonde forse afwijkingen, waaronder intra-epitheliale eiwitdeposities, epitheel- en endotheelcelzwelling leidend tot obliteratie van het capillaire lumen, verlies van endotheliale fenestratie en afvlakking van de podocytenvoetjes. De combinatie van endotheelcelzwelling, verlies van fenestratie en obliteratie van het capillaire lumen wordt ook wel glomerulaire endotheliose genoemd. Interessant hierbij is dat pre-eclampsie, een aandoening die zich kan voordoen bij vrouwen vanaf de 20ste zwangerschapsweek, ook wordt gekenmerkt door hypertensie, proteïnurie, een stijging in circulerende ET-1 spiegels en glomerulaire endotheliose. Er wordt gedacht dat een verhoogde placentaire produktie van het vrij circulerende fms-achtige tyrosinekinase-1

(sFlt-1), het extracellulaire deel van de VEGF receptor (VEGFR)-1, hierbij een rol speelt. sFlt-1 bindt VEGF en onderbreekt zo de VEGF-VEGFR signaaloverdracht waardoor er een situatie ontstaat die vergelijkbaar is met die tijdens de behandeling met sunitinib. Daarom wordt de combinatie van hypertensie en proteïnurie die tijdens behandeling met sunitinib optreedt ook wel een pre-eclampsie-achtig syndroom genoemd **(Hoofdstuk 4)**.

Om verder te onderzoeken wat de rol is van ET-1, de bloeddruk en oxidatieve stress bij het ontstaan van sunitinib-geïnduceerde nefrotoxiciteit, werden de ratten tijdens toediening van sunitinib gelijktijdig behandeld met de duale ET_A/ET_B receptor antagonist macitentan, amlodipine of de ROS-scavenger TEMPOL. Macitentan en amlodipine in de gebruikte doseringen verlaagden de sunitinib-geïnduceerde bloeddrukstijging even effectief. Macitentan verminderde ook de proteïnurie en urine ET-1 excretie, maar had geen effect op het serum kreatinine. Amlodipine daarentegen, had geen effect op de sunitinib-geïnduceerde proteïnurie en toegenomen urine ET-1 excretie, hoewel het even effectief als macitentan de bloeddrukstijging verminderde **(Hoofdstuk 6)**. Toediening van de antioxidant TEMPOL verminderde ook de proteïnurie en urine ET-1 excretie, maar had slechts een marginaal effect op de bloeddruk. Noch macitentan, noch amlodipine, noch TEMPOL verminderde de sunitinib-geïnduceerde histologische nierafwijkingen, hoewel tijdens behandeling met macitentan de glomerulaire intra-epitheliale eiwitdeposities afnamen **(Hoofdstuk 4)**. Op grond van deze bevindingen kan worden geconcludeerd dat sunitinib-geïnduceerde nefrotoxiciteit gemedieerd wordt door ET-1, maar onafhankelijk is van de bloeddrukstijging. Oxidatieve stress blijkt vrijwel geen rol te spelen bij de sunitinib-geïnduceerde bloeddrukstijging, maar speelt wel een rol bij het ontstaan van sunitinib-geïnduceerde nefrotoxiciteit.

Cardiale toxiciteit

Bij 28% van de patiënten die wordt behandeld met sunitinib is een verminderde hartfunctie waargenomen. Daarnaast zijn angina pectoris en een stijging in biomarkers van cardiale schade gerapporteerd **(Hoofdstuk 1)**. Hartspierbiopten van patiënten die een verminderde hartfunctie ontwikkelden tijdens behandeling met sunitinib, tonen structurele veranderingen in de mitochondria van de cardiomyocyten. Daarom is er gesuggereerd dat een verminderde ATP-productie, secundair aan mitochondriale disfunctie, ten grondslag zou kunnen liggen aan de verminderde linkerventrikelfunctie die bij sommige patiënten ten tijde van behandeling met sunitinib wordt waargenomen. Tevens is bekend dat oxidatieve stress vaak verhoogd is bij hypertensie en dat dit een pathogenetische rol speelt in de ontwikkeling en progressie van hart- en vaatziekten. Een toegenomen oxidatieve stress zou dus ook een rol kunnen spelen in het ontstaan van de sunitinib-geassocieerde cardiovasculaire bijwerkingen. Hoewel de effecten van sunitinib op de systemische circulatie enigszins bekend zijn, zijn er geen studies gerap-

porteerd die het effect van sunitinib op de coronaire en pulmonale circulatie hebben bestudeerd.

De effecten van sunitinib op de cardiale mitochondriale functie en de coronaire microvasculaire functie in ratten, alsmede het effect op de coronaire en pulmonale circulatie en op de cardiale functie in varkens, en de mogelijke rol van ET-1, NO-beschikbaarheid en oxidatieve stress werden verder onderzocht in de studies beschreven in dit proefschrift.

Ex vivo onderzoek van mitochondria van cardiomyocyten afkomstig van de rat toonde dat sunitinib geen invloed had op de ATP-productie, suggerend dat verminderde cardiale mitochondriale ATP-productie geen rol speelt in de sunitinib-geïnduceerde afname in linkerventrikelfunctie **(Hoofdstuk 3)**. Coronaire microvasculaire functie studies in het Langendorff hartmodel van ratten behandeld met sunitinib, toonden dat de coronaire flow niet alleen verminderde in respons op de endotheel-afhankelijke vaatverwijder bradykinine, maar ook in respons op de endotheel-onafhankelijke vaatverwijder natrium-nitroprusside en de vaatvenauwer angiotensine II. Deze resultaten wijzen erop dat sunitinib gegeneraliseerde gladde spierceldisfunctie induceert, hetgeen ook, tenminste ten dele, de verminderde respons op bradykinine kan verklaren **(Hoofdstuk 3)**. Het mechanisme achter deze gegeneraliseerde microvasculaire gladde spierceldisfunctie moet nog worden opgehelderd.

In varkens had toediening van sunitinib geen effect op de pulmonale circulatie of coronaire bloeddoorstroming. Acute toediening van de duale ET_A/ET_B receptorblokker tezosentan maakte de systemische hemodynamische effecten van sunitinib ongedaan. Dit ondersteunt onze eerdere observatie in ratten dat een toename in ET-1-gemedieerde vasoconstrictoire tonus ten grondslag ligt aan de sunitinib-geïnduceerde bloeddrukstijging **(Hoofdstuk 3, 4)**. Er werden geen aanwijzingen gevonden voor een afname in NO-gemedieerde vasodilatatoire tonus of een toename in oxidatieve stress die een rol zouden kunnen spelen in de systemische sunitinib-geïnduceerde vasoconstrictie. Hoewel beschreven is dat sunitinib in patiënten een afname in linkerventrikelejectiefractie kan veroorzaken, was dit niet aantoonbaar in de varkens **(Hoofdstuk 5)**. Hierbij dient wel te worden opgemerkt dat de tijd van blootstelling aan sunitinib kort was. Mogelijk dat een langere blootstellingsduur ook in onze varkens leidt tot een afname in linkerventrikelfunctie, hoewel, zeker bij langdurige blootstelling aan sunitinib, het lastig is om te differentiëren tussen bloeddrukafhankelijke en -onafhankelijke effecten.

Sunitinib-geïnduceerde schildkliertoxiciteit

Schildklierdisfunctie, met name hypothyreoïdie, wordt in retrospectieve studies beschreven bij 53-85% en in prospectieve studies bij 36-46% van de patiënten behandeld

met sunitinib **(Hoofdstuk 2)**. Aangezien met name het capillaire bed van de schildklier gevoelig is voor VEGF-remming, is gesuggereerd dat capillaire regressie een rol speelt bij het ontstaan van sunitinib-geassocieerde schildklierdisfunctie ('capillaire regressie hypothese'). Tevens is beschreven dat bij patiënten met een voorgeschiedenis van hypothyreoïdie die al werden behandeld met levothyroxine (LT_4), een dosisverhoging van LT_4 was geïndiceerd tijdens behandeling met sunitinib. Dit wijst erop dat sunitinib interfereert met het thyroxine (T_4) en triiodothyronine (T_3) metabolisme ('metabolic hypothesis') **(Hoofdstuk 2)**.

Het effect van sunitinib op de schildklierfunctie en de mogelijke mechanismen die hierbij betrokken zouden kunnen zijn, inclusief de schildkliervasculatuur en het schildklierhormoonmetabolisme werd verder onderzocht in de studies beschreven in dit proefschrift.

Retrospectieve analyse in 108 patiënten liet een stijging in TSH-spiegels (TSH > 5.0 mU/l) zien bij 42% van de patiënten, die in 5 patiënten vooraf werd gegaan door een onderdrukking van het TSH. Prospectieve analyse in 15 patiënten toonde een 4-voudige stijging van de TSH-spiegels, binnen 10 weken na het starten van de behandeling, terwijl de FT_4 en T_3 spiegels onveranderd bleven. Desalniettemin trad er wel een daling op in de T_3/rT_3-ratio, wijzend op een versneld schildklierhormoonmetabolisme. De schildklierperoxidase-antistoftiter veranderde niet **(Hoofdstuk 8)**.

In ratten was toediening van sunitinib geassocieerd met een toename van dejodase type 3 activiteit, dat T_3 en T_4 omzet in de minder actieve metabolieten T_2 en rT_3, en een afname in dejodase type 1 activiteit, dat T_4 en rT_3 omzet in T_3 en T_2. De schildklierhormoonspiegels daalden overeenkomstig. Beide dejodaseactiviteiten normaliseerden na staken van sunitinib. Histologische beoordeling van schildklierweefsel toonde uitgesproken capillaire regressie in schildklierweefsel afkomstig van ratten behandeld met sunitinib. Na het stoppen van sunitinib toediening normaliseerde niet alleen het aantal capillairen, maar onstond er zelfs een capillaire overshoot. Ratten die naast sunitinib ook de duale ET_A/ET_B receptor antagonist macitentan kregen toegediend, een middel dat vasodilatatie induceert in situaties waar het endotheline-systeem is geactiveerd, toonden een minder uitgesproken capillaire regressie in het schildklierweefsel dan de ratten die alleen sunitinib kregen toegediend **(Hoofdstuk 8)**. Dit wijst erop dat sunitinib in de schildklier niet alleen structurele, maar ook functionele rarefactie veroorzaakt. Concluderend laten onze resultaten zien dat sunitinib-geïnduceerde hypothyreoïdie wordt veroorzaakt door een combinatie van veranderingen in dejodaseactiviteit, leidend tot verandering in het T_4/T_3 metabolisme, en uitgesproken capillaire regressie in de schildklier.

ABBREVIATIONS

ABPM	Ambulatory Blood Pressure Measurement
ACE-I	Angiotensin Converting Enzyme Inhibitor
ADP	Adenosine DiPhosphate
Ang II	Angiotensin II
ARB	Angiotensin Receptor Blocker
ATP	AdenosineTriPhosphate
BK	Bradykinin
BNP	B-type Natriuretic Peptide
BP	Blood Pressure
BVO2	Body oxygen consumption
BW	Body Weight
CBF	Coronary Blood Flow
CF	Coronary Flow
CO	Cardiac Output
CVR	Coronary Vascular Resistance
D1	type 1 Deiodinase
DBP	Diastolic Blood Pressure
eNOS	endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
FT4	Free Thyroxine
GIST	GastroIntestinal Stromal Tumor
HIF-1	Hypoxia Inducible Factor 1
HR	Heart Rate
HUVEC	Human Umbilical Vein Endothelial Cell
HW	Heart Weight
KW	Kidney Weight
LAP	Left Atrial Pressure
L-NNA	Nω-nitro-L-arginine
LT4	Levothyroxine
LVdP/dt	change in Left Ventricular Pressure
LVEF	Left Ventricular Ejection Fraction
LVSP	Left Ventricular Systolic Pressure
MAP	Mean Arterial Pressure
mRCC	metastatic Renal Cell Carcinoma
NADPH-oxidase	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
NO	Nitric Oxide
PAP	Pulmonary Artery Pressure

PVR	Pulmonary Vascular Resistance
rT3	reverse T3
ROS	Reactive Oxygen Species
RTKI	Receptor Tyrosine Kinase Inhibitor
SBP	Systolic Blood Pressure
sFlt-1	soluble Fms-Like Tyrosine kinase 1
SHR	Spontaneous Hypertensive Rat
SNP	Sodium NitroPrusside
SVR	Systemic Vascular Resistance
T3	Triiodothyronine
T4	Thyroxine
TBARS	ThioBarbituric Acid Reactive Substances
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl
TFT	Thyroid Function Test
TH	Thyroid Hormone
TPO-Ab	Thyroid PerOxidase antibodies
TSH	Thyroid Stimulating Hormone
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
vWf	von Willebrand factor
WKY	Wistar Kyoto rat

CURRICULUM VITAE

Mariëtte Kappers werd geboren op 9 oktober 1975 in Groningen. In 1993 voltooide ze het Gymnasium-β aan de Christelijke Scholengemeenschap Comenius in Capelle a/d IJssel. In 1993 begon ze met de studie geneeskunde aan de Rijksuniversiteit Antwerpen, België. In 1997 behaalde ze haar kandidaatsdiploma met onderscheiding. Het 'eerste doctoraal', het vierde studiejaar, werd in 1998 met onderscheiding voltooid aan de Universiteit van Wenen. Daarna vervolgde zij de studie geneeskunde aan de Erasmus Universiteit te Rotterdam alwaar ze in 2002 het artsenexamen behaalde. In 2003 begon zij aan de opleiding tot internist. In de laatste twee jaar van deze opleiding volgde zij het aandachtsgebied vasculaire geneeskunde. Van januari 2009 tot augustus 2011 is zij werkzaam geweest als internist-vasculair geneeskundige in het Erasmus MC te Rotterdam. Daarnaast heeft zij, na het verkrijgen van een grant, het onderzoek opgezet dat is beschreven in het huidige proefschrift. Sinds 1 augustus 2011 is zij werkzaam als internist-vasculair geneeskundige in het Amphia ziekenhuis te Breda.

PUBLICATIONS

Publications on angiogenesis inhibition

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PhD PORTFOLIO

Cardiovascular, renal and thyroid toxicity during angiogenesis inhibition: a translational approach

M.H.W. Kappers

Erasmus MC Department:	Internal Medicine
Research School:	Cardiovascular Research School Erasmus University Rotterdam (COEUR), Department of Vascular Pharmacology
Promotor:	Prof.dr. A.H.J. Danser
Supervisor:	Dr. A.H. van den Meiracker
PhD period:	2009-2011

Oral and poster presentations

2011
- 21th annual scientific meeting of the European Society of Hypertension (ESH), Milan, Italy
  '*Hypertension and renal functional toxicity during VEGF-receptor inhibition with sunitinib: focus on endothelin-1 and oxidative stress*'
- Nederlandse Internistendagen, Maastricht
  '*Sunitinib-induced hypothyroidism is associated with induction of deiodinase type 3 activity and capillary regression*'
- Cardiovasculaire Conferentie, Noorwijkerhout
  '*Hypertension and renal toxicity induced by sunitinib, an oral angiogenesis inhibitor, are associated with activation of the endothelin system*'

2010
- High Blood Pressure Research Conference (HBPRC) of the American Heart Association, Washington, United States:
  'The VEGF receptor inhibitor sunitinib causes a preeclampsia-like syndrome in rats'†
- Nederlandse federatie voor Nefrologie, najaarssymposium, Utrecht
  'Hypertension, renal toxicity and activation of the endothelin-system with sunitinib, an oral angiogenesis inhibitor'
- 20th annual scientific meeting of the European Society of Hypertension (ESH), Oslo, Norway
  'Administration of sunitinib, an oral angiogenesis inhibitor, is associated with hypertension, but not with structural cardiac changes or cardiomyocyte mitochondrial dysfunction'†
  'The VEGF-inhibitor sunitinib causes a preeclampsia-like syndrome in rats'

- Nederlandse Internistendagen, Maastricht
  'Hypertension, loss of circadian blood pressure rhythm, renal toxicity and activation of the endothelin-system with sunitinib, an oral angiogenesis inhibitor'

2009
- FIGON Dutch Medicines Days 2009, Lunteren
  'VEGF-blockade with sunitinib induces hypertension: insights from studies in humans and rats'
- Joint Meeting of the Dutch Society of Hypertension and the Belgian Hypertension Committee, Maastricht
  'Hypertension, loss of circadian blood pressure rhythm and decrease in coronary microvascular function with sunitinib, an oral tyrosine kinase inhibitor, in normotensive rats'
  'Development of hypertension with sunitinib, an angiogenesis inhibitor, is associated with a rise in plasma endothelin-1 concentration'
- High Blood Pressure Research Conference of the American Heart Association, Chicago, United States
  'Development of hypertension with sunitinib, an angiogenesis inhibitor, is associated with a rise in plasma endothelin-1 concentration'
  'Hypertension, loss of circadian blood pressure rhythm and decrease in coronary microvascular function with sunitinib, an oral tyrosine kinase inhibitor, in normotensive rats'
- Internistensymposium: 'Op het grensvlak van de vasculaire geneeskunde en nefrologie', Rotterdam
  'Angiogeneseremming: klinische en pathofysiologische aspecten'
- European Society of Hypertension, Milan, Italy
  'Hypertension and loss of circadian blood pressure rhythm due to treatment with sunitinib, an oral angiogenesis inhibitor'†

Grants, prizes

2009
- 'New Investigator Award for European Citizens'
  High Blood Pressure Research Conference of the American Heart Association, Chicago, United States

2008
- The Netherlands Foundation for Cardiovascular Excellence (€92.000)

† poster presentation

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