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Selective ET_A versus dual $ET_{A/B}$ receptor blockade for the prevention of angiogenesis inhibitor-induced hypertension and albuminuria

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Abstract

While effective in preventing tumor growth, angiogenesis inhibitors cause off-target effects including cardiovascular toxicity and renal injury, most likely via endothelin-1 (ET-1) upregulation. ET-1 via stimulation of the ET_A receptor has pro-hypertensive actions whereas stimulation of the ET_B receptor can elicit both pro- or anti-hypertensive effects. In this study, our aim was to determine the efficacy of selective ET_A versus dual $ET_{A/B}$ receptor blockade for the prevention of angiogenesis inhibitor-induced hypertension and albuminuria. Male WKY rats were treated with vehicle, sunitinib (angiogenesis inhibitor; 14 mg/kg/day) alone or in combination with macitentan ($ET_{A/B}$ receptor antagonist; 30 mg/kg/day) or sitaxentan (selective ET_A receptor antagonist; 30 or 100 mg/kg/day) for 8 days. Compared to vehicle, sunitinib treatment caused a rapid and sustained increase in mean arterial pressure of ~25 mmHg. Co-treatment with macitentan or sitaxentan abolished the pressor response to sunitinib. Sunitinib did not induce endothelial dysfunction. However, it was associated with increased aortic, mesenteric and renal oxidative stress, an effect that was absent in mesenteric arteries of the macitentan and sitaxentan co-treated groups. Albuminuria was greater in the sunitinib- than vehicle-treated group. Co-treatment with sitaxentan, but not macitentan, prevented this increase in albuminuria. Sunitinib treatment increased circulating and urinary prostacyclin levels and had no effect on thromboxane levels. These increases in prostacyclin were blunted by co-treatment with sitaxentan. Our results demonstrate that ET_A receptor antagonism is sufficient to block the consequences of the rise in ET-1 during sunitinib treatment, and uncover a role for prostacyclin in the development of these effects.

Key words

Angiogenesis inhibition, Hypertension, Renal injury, Oxidative stress, Endothelin-1, Endothelin receptor antagonists

Introduction

Angiogenesis inhibitors, which target the vascular endothelial growth factor (VEGF) pathway are a modern form of cancer therapy and are especially effective for some cancers with previously limited treatment options.[1] The most common angiogenesis inhibitors used clinically are parenteral treatment with monoclonal antibodies against VEGF-A and oral treatment with agents that inhibit the three VEGF receptors, also called receptor tyrosine kinase inhibitors (RTKIs). Sunitinib is a widely used RTKI, particularly for the treatment of metastatic renal cell carcinoma.[1] VEGF inhibition can induce cardiovascular toxicity, most frequently hypertension, and also kidney damage characterized by proteinuria.[2] The severity of these side effects may require dose reduction and/or early termination of treatment, which compromises the efficacy of VEGF inhibitor therapy and patient survival. Understanding the mechanisms leading to VEGF inhibitor-induced hypertension and kidney damage is vital for the development of appropriate interventions to prevent these unwanted side effects.

Activation of the endothelin (ET) system has emerged as a key pathway in the development of hypertension during VEGF inhibition and possibly in the development of renal injury.[3-7] Plasma ET-1 levels are elevated 2-3-fold in patients and animals treated with VEGF inhibitors,[5] with the increase in ET-1 being dose-dependent during VEGF inhibition.[6] We previously demonstrated in rodents and

swine that dual $ET_{A/B}$ receptor antagonism can prevent sunitinib-induced hypertension.[3, 4] More recently we reported that microparticles from VEGF inhibitor-treated cancer patients mediate endothelial cell dysfunction through ET-1-dependent mechanisms.[8] ET-1 plays an important role in microparticles. ET-1 is a highly potent vasoconstrictor that is mainly produced by endothelial cells. Stimulation of the ET_B receptor on endothelial cells induces nitric oxide (NO)- and prostaglandin (PGI_2)-mediated vasodilation, while stimulation of ET_A and ET_B receptors on vascular smooth muscle cells leads to prolonged vasoconstriction. Under pathological conditions such as hypertension, endothelial ET_B receptor-dependent vasodilation can be lost.[9-11] Whether this is the case during treatment with VEGF inhibitors is not known.

Oxidative stress and reductions in the vasodilators NO and PGI_2, leading to endothelial dysfunction and enhanced vasoconstriction, are also implicated in the pathogenesis of VEGF inhibitor-induced hypertension.[12] This may occur as a direct consequence of VEGF inhibition since second messenger pathways linked to VEGF signalling induce an upregulation in NO and PGI_2. Or alternatively, via activation of the ET system, since ET-1 induces the generation of reactive oxygen species (ROS) and an upregulation in cyclooxygenase (COX)-2-dependent prostanoid production.[13] While several studies have investigated the contribution of ROS and NO deficiency in the development of VEGF inhibitor-induced hypertension,[3, 4, 14] there is a dearth of knowledge about the role of prostanoids (PGI_2, thromboxane (TXA_2), prostaglandin (PG)E_2, PGD_2 or $PGF_{2\alpha}$). Moreover, to our knowledge, no study has investigated if the contribution of these factors to the development of VEGF inhibitor-induced hypertension is dependent on activation of the ET system.

In the present study, our aims were to determine the efficacy of selective ET_A receptor blockade versus dual $ET_{A/B}$ receptor blockade for the prevention of hypertension and albuminuria during VEGF inhibitor treatment with sunitinib and whether endothelial dysfunction, oxidative stress and alterations in prostanoid biosynthesis during sunitinib treatment is dependent on ET_A and/or ET_B receptor activation. To address these aims, normotensive Wistar Kyoto (WKY) rats were treated with vehicle, sunitinib alone or in combination with the dual $ET_{A/B}$ receptor antagonist, macitentan or the selective ET_A receptor antagonist, sitaxentan.

Methods

Detailed methods are available in the online supplement (please see http://hyper.ahajournals.org). Experiments were performed in accordance with the Principles of Laboratory Animal Care and Guidelines, after obtaining approval from the Erasmus Medical Centre Animal Ethics Committee (protocol number 118-16-01). Male WKY rats were obtained at 10 weeks of age from Charles River, Germany and due to the temporary unavailability of the WKY strain at Charles River, from Janvier Labs, France. Animals were housed in an experimental room with temperature maintained at 21–22°C and a 12 h light-dark cycle. Rats had *ad libitum* access to normal rodent chow and water.

In vivo study

Mean arterial pressure (MAP) and heart rate were measured by radiotelemetry. After establishing baseline MAP and heart rate, rats were randomly assigned to one of 5 treatment groups (n=6-7/group): vehicle, sunitinib (14 mg/kg/day), sunitinib in the

presence of dual $ET_{A/B}$ receptor blockade (macitentan 30 mg/kg/day) or sunitinib in the presence of a low or high dose of selective ET_A receptor blockade (sitaxentan 30 mg/kg/day or 100 mg/kg/day, respectively) for 8 days. MAP and heart rate were measured on days 1-6 of treatment. On days 7-8 of treatment, rats were placed into metabolic cages to collect a 24 h urine sample.

Ex vivo vascular studies

At the end of the treatment period, vascular function was assessed in response to the vasodilator acetylcholine (ACh) or the vasoconstrictors ET-1 or phenylephrine (PE) in isolated iliac artery segments. In additional segments, ACh experiments were performed in the presence of NO-synthase inhibition with L^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), endothelium-derived hyperpolarising factor inhibition with the intermediate conductance Ca^{2+}-activated K^+ channel inhibitor TRAM34 or the small conductance Ca^{2+}-activated K^+ channel inhibitor apamin or their combination and for ET-1, in the presence of ET_A receptor (BQ123) or ET_B receptor (BQ788) blockade.

Circulating and urinary biochemical measurements

Circulating and urinary ET-1, $PGF_{2\alpha}$ (measured as 8-iso-$PGF_{2\alpha}$) and PGI_2 and TXA_2 levels (via the measurement of their stable metabolites, 6-keto-$PGF_{1\alpha}$ and TXB_2, respectively), were assessed via enzyme-linked immunosorbent assay (ELISA). Albuminuria and the urinary excretion of neutrophil gelatinase-associated lipocalin (NGAL), PGE_2 and its metabolite, PGE-M, were all measured via ELISA.

Renal mRNA expression

Quantitative polymerase chain reaction (qPCR) was performed to determine renal mRNA expression of VEGF, endothelin converting enzyme (ECE), ET-1, ET_A receptor, ET_B receptor, COX-1, COX-2, PGI_2 synthase and TXA_2 synthase.

Oxidative stress

Aortic and renal superoxide anion (O_2^-) levels were measured by lucigenin chemiluminescence assay. Mesenteric arteries O_2^- production was measured by electron paramagnetic resonance (EPR). Aortic, cardiac and renal hydrogen peroxide (H_2O_2) levels were measured by Amplex Red assay. Aortic mRNA expression of NADPH oxidase (Nox) isoforms (Nox1, 2 and 4) and antioxidant enzymes (SOD1, catalase, GXP1, HO1, Trdx1 and Prdx1) were determined by qPCR.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software Inc, San Diego CA). Data are presented as mean ± SEM. Data were analyzed using a one-way analysis of variance (ANOVA) followed by post-hoc t-tests with Holm-Sidak correction to reduce the risk of Type-1 error associated with multiple comparisons. Correlations were analyzed using Pearson's correlation. $P<0.05$ was considered statistically significant.

Results

Vascular effects

Treatment with sunitinib resulted in a rapid increase in MAP which peaked at ~25 mmHg (Figure 1A). This increase in MAP was accompanied by a decrease in heart

rate (Figure 1B). Co-treatment with macitentan (dual $ET_{A/B}$ receptor antagonist) and both doses of sitaxentan (selective ET_A receptor antagonist) completely prevented the sunitinib-induced rise in blood pressure (Figures 1A and 1C). To ensure that the ET_A receptor was adequately blocked two doses of sitaxentan were tested. Results with the low and high dose of sitaxentan were essentially the same, so that in future studies the 30 mg/kg of sitaxentan seems sufficient. The sunitinib-induced decrease in heart rate was prevented by macitentan and unaffected by co-treatment with sitaxentan (Figures 1B and 1D).

Circulating ET-1 was higher in the sunitinib-treated groups than vehicle-treated, yet this only reached statistical significance in the macitentan co-treated group (4.8 ± 1.0 versus 1.4 ± 0.1 pg/ml in vehicle treated, P=0.01; Figure 2A). Circulating 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, was higher following treatment with sunitinib alone or in combination with macitentan or low dose sitaxentan (all $P<0.05$ versus vehicle treated; Figure 2B). Circulating 6-keto-$PGF_{1\alpha}$ was 30% lower in the sunitinib plus high-dose sitaxentan group as compared to sunitinib treatment alone. Circulating TXB_2, the stable metabolite of TXA_2, was not affected by treatment (Figure 2C). There were no significant differences in the PGI_2/TXA_2 ratio between the groups (Figure 2D). $PGF_{2\alpha}$, measured as 8-iso-$PGF_{2\alpha}$ that is produced by COX dependent and independent pathways, was similar among the groups (Figure 2F).

Vascular function was studied *ex vivo* to determine if sunitinib treatment induced endothelial dysfunction as well as the possible prevention of this effect by macitentan or sitaxentan. The vasodilator response to ACh was identical between the groups (Figure 3A, Table 1). Further, while the vasodilator response to ACh was reduced in

presence of L-NAME alone (Figure 3B, Table 1) or in combination with TRAM34 and apamin (Table 1) this effect was similar between the treatment groups. In the presence of TRAM34 and apamin alone, the vasodilator response to ACh was reduced in the sunitinib and macitentan co-treatment group only (Table 1). The vasoconstrictor responses to ET-1 and the alpha-adrenergic agonist PE were not enhanced by sunitinib alone or sunitinib in combination with macitentan or sitaxentan (Figures 3C and 3D, Table 1). While the sensitivity to ET-1 was reduced in the presence of the ET_A receptor antagonist, BQ123, the maximum vasoconstrictor responses to ET-1 in the absence or presence of BQ123 or BQ788 were not different (Table 1), demonstrating that ET_B-mediated vasoconstriction is not enhanced with sunitinib treatment.

Renal effects

Sunitinib treatment increased albuminuria (Figure 4A), but the urinary excretion of NGAL, a biomarker of (acute) tubular damage, did not increase (Figure 4B). Co-treatment with low or high dose of the selective ET_A receptor blocker sitaxentan, but not the dual $ET_{A/B}$ receptor blocker macitentan, inhibited the sunitinib-induced increase in albuminuria (Figure 4A). There was no difference in the urinary excretion of ET-1 between the groups (Figure 4C). The urinary excretion of 6-keto-$PGF_{1\alpha}$ increased 12-fold during sunitinib treatment as compared to vehicle treatment (602 ± 100 versus 45 ± 10 ng/24h, respectively, P=0.003; Figure 4D), while the urinary excretion of TXB_2 did not change (Figures 4E). ET receptor blockade inhibited the excretion of 6-keto-$PGF_{1\alpha}$, but had no effect on the excretion of TXB_2. The PGI_2/TXA_2 ratio was greater in the sunitinib alone and sunitinib plus macitentan treated groups as compared to vehicle treatment (Figure 4F). There was no effect of

sunitinib alone or in combination with macitentan or sitaxentan on the urinary excretion of 6-iso-$PGF_{2\alpha}$, PGE_2 or PGE-M (Figures S1A-C).

There were no significant differences in the renal mRNA expression of the genes encoding VEGF, ECE, ET-1, ET_A receptor, ET_B receptor, COX-1, COX-2, PGI_2 synthase or TXA_2 synthase between the groups (Figures S2A-I). However, there were positive correlations between the renal mRNA expression of COX-2 and ET-1 (r^2=0.16, P=0.02; Figure 5A) and PGI_2 synthase and ECE (r^2=0.17, P=0.01; Figure 5B).

Oxidative stress

Sunitinib treatment increased vascular and renal O_2^- production, while vascular, cardiac and renal H_2O_2 production did not change (Figures 6A-F and S3). Co-treatment with macitentan or sitaxentan prevented the sunitinib-induced increase in mesenteric O_2^- production (Figure 6B). In rodents, NADPH oxidase (Nox)1 and Nox2 generate O_2^- and Nox4 mainly generates H_2O_2. Sunitinib treatment increased aortic mRNA expression of Nox1 (P=0.03 versus vehicle treated; Figure S4A), but did not affect mRNA expression of Nox2 and Nox4 (Figures S4B-C). There were no significant differences in expression of the antioxidant genes catalase, SOD1, catalase, GPX1, HO1, Prdx or Trdx following treatment with sunitinib (Figures S4D-I). However, co-treatment with the higher dose of sitaxentan induced a 5-fold increase in SOD1, 7-fold increase in catalase, 5-fold increase in GPX1, 2-fold increase in HO1 and 4-fold increase in Prdx1 as compared to vehicle treated (all P<0.05 versus vehicle and sunitinib treated groups; Figures S4D-H).

Discussion

In preclinical and clinical studies, we and others have shown that the rise in blood pressure during VEGF inhibition is accompanied by activation of the ET system.[3-7, 15] Given the predominant abluminal release of ET-1, a rise in circulating ET-1 is often difficult to detect.[13] Yet, the findings of the present study unequivocally demonstrate that such activation is responsible for the rise in blood pressure during VEGF inhibition, since the increase in blood pressure during treatment with the RTKI, sunitinib, can be completely prevented by the simultaneous administration of an ET receptor antagonist. Moreover, our study shows that the increase in blood pressure is exclusively mediated by activation of the ET_A receptor since sunitinib induced-hypertension was prevented just as well with the selective ET_A receptor blocker, sitaxentan, as with the dual $ET_{A/B}$ receptor blocker, macitentan.

In addition to hypertension, glomerular kidney damage is a side effect of VEGF inhibition. Previous studies have demonstrated that sunitinib treatment results in glomerular endotheliosis and albuminuria associated with decreased mRNA expression of the gene encoding nephrin, a marker of podocyte injury.[3, 6] In the present study sunitinib-induced albuminuria was prevented by selective ET_A receptor antagonism with sitaxentan but not by dual $ET_{A/B}$ receptor blockade with macitentan. This finding was unexpected since in a previous study sunitinib-induced albuminuria was inhibited by macitentan.[7] Although the reason for the discrepancy between these studies is not clear, the development of albuminuria seems to be dependent on the ET_A receptor rather than the ET_B receptor. For example, endogenous and exogenous ET-1 via the ET_A receptor, disrupts the podocyte actin cytoskeleton.[16-19] This effect was independent of whether the ET_B receptor is blocked or not.[20] It has

also been demonstrated that ET-1 may lead to podocyte loss of nephrin and that this effect is prevented by a selective ET_A receptor antagonist.[21] Importantly, our present data confirm that albuminuria is independent of sunitinib-induced hypertension,[6] as both selective ET_A and dual $ET_{A/B}$ receptor blockade completely inhibited the sunitinib-induced increase in MAP, whereas albuminuria was only inhibited by selective ET_A receptor blockade.

VEGF stimulates endothelial NO production by, among other factors, increased expression of the gene encoding the endothelial NO synthase. In line with this, previous studies have shown that VEGF inhibition is associated with reduced NO production.[6, 22] Decreased NO production may therefore contribute to the rise in blood pressure during VEGF inhibition and lead to endothelial dysfunction. In our experiments in segments of isolated iliac vessels we found no evidence for endothelial dysfunction. For example, neither the vasodilator response to ACh (stimulation of NO production) nor the vasoconstrictive response to the NO synthase inhibitor L-NAME (size for basal NO production) was reduced. These findings are consistent with preclinical and clinical data which suggest that NO bioavailability, and hence endothelial function, may be preserved during VEGF inhibitor therapy.[1] However, we cannot exclude the possibility that endothelial dysfunction may be detected in other vascular beds such as the mesenteric arteries which are resistance vessels. Neves et al., recently reported that 2 weeks of treatment with the VEGF inhibitor vatalanib induced mesenteric endothelial dysfunction in mice albeit this occurred in the absence of a VEGF inhibitor-induced rise in blood pressure.[14] Furthermore, a previous study suggested that VEGF inhibition is not only associated with ET-1 activation, as is often reflected by elevated ET-1 levels, but also with an

enhanced vasoconstrictor response to ET-1.[10] This could not be confirmed in the current study. Nor was the response to the alpha-adrenergic receptor agonist PE enhanced. These findings are consistent with the absence of endothelial dysfunction.

The bioavailability of NO is influenced by ROS.[23] Increased oxidative stress is a suggested mechanism that can contribute to vascular damage and the rise in blood pressure during VEGF inhibition.[14] On the other hand, hypertension itself can lead to an increase in oxidative stress.[24] Whether oxidative stress is a cause or consequence of VEGF inhibitor-induced hypertension is not clear. In the current study, we found increased O_2^- levels in the vasculature and kidney but no change in H_2O_2 levels during VEGF inhibition with sunitinib. The elevated O_2^- levels did not fully normalize during concomitant ET receptor blockade. Since the VEGF inhibitor-induced rise in blood pressure was completely prevented during concomitant ET receptor blockade, it is unlikely that oxidative stress plays a major role in the pathogenesis of hypertension during VEGF inhibition. This is in line with our previous research that showed that inhibitors of oxidative stress do not reduce the VEGF inhibitor-induced rise in blood pressure.[3, 4] However, the sunitinib-induced proteinuria was decreased by tempol, a SOD mimetic, suggesting that oxidative stress plays a role in VEGF inhibitor-induced kidney damage.[3]

The hypertension, proteinuria and glomerular damage that occurs during treatment with VEGF inhibitors is similar to that observed during preeclampsia and accordingly, these effects of VEGF inhibition have been called a “preeclampsia-like” syndrome.[25] Furthermore, in preeclampsia, the levels of soluble-like tyrosine kinase (sFlt-1), a naturally occurring antagonist of VEGF are elevated before the clinical onset of

preeclampisa[26] and majority of studies report that plasma ET-1 levels are elevated 1.5 to 2-fold in preeclamptic women as compared with normotensive pregnant women.[27] Given the similarities between preeclampsia and VEGF inhibitor-induced hypertension and kidney damage, the underlying mechanisms are thought to be the same. VEGF can stimulate the production of PGI_2, which via stimulation of its own receptors, the prostacyclin (IP) receptors, elicits vasodilation. Moreover, PGI_2 is the main prostanoid generated by COX-2. VEGF inhibitor therapy is thought to lead to a reduction in PGI_2 production, thereby contributing to the rise in blood pressure and increased thrombotic tendency during VEGF inhibitor therapy.[12, 28] Consistent with this hypothesis, preeclampsia is characterized by a reduction in PGI_2 levels,[29-31] such that the PGI_2/TXA_2 ratio is skewed towards TXA_2.[32] This shift towards vasoconstriction is further potentiated by altered $PGF_{2\alpha}$ and PGE_2 production during preeclampsia.[33-36] In contrast to prediction, in the present study we observed an increase rather than a decrease in circulating and urinary PGI_2 levels following sunitinib treatment, while TXA_2, $PGF_{2\alpha}$ and PGE_2 levels remained unchanged. Vascular relaxation to PGI_2 is associated with smooth muscle cell hyperpolarization, such that PGI_2 acts as an endothelium-derived hyperpolarizing factor (EDHF).[37] However, in pathological situations such as hypertension, PGI_2 can elicit vasoconstriction via TXA_2 receptor stimulation, thereby acting as an endothelium derived contracting factor (EDCF).[37-42]

There are indications that ET-1 can stimulate the production of PGI_2, mediated via the ET_A receptor.[4] Indeed, we found that the increase in circulating and urinary levels of PGI_2 was inhibited by the selective ET_A receptor antagonist, sitaxentan. In mice treated with sFlt-1, pressor responsiveness to ET-1 is enhanced in isolated carotid

artery segments and this effect is abrogated by indomethacin, a non-specific COX inhibitor.[10] While *in vivo*, sFlt-1-induced hypertension is abolished by high-dose aspirin which inhibits both COX-1 and COX-2.[10] In the present study, we found positive correlations between ET-1 and COX-2 and also between PGI_2 synthase, which converts the common prostanoid precursor PGH_2 into PGI_2, and ECE. Stimulation of COX-2 production is potentially a negative effect of VEGF inhibition because it can stimulate tumor angiogenesis. The therapeutic effect of the combination of a VEGF inhibitor with a COX-2 inhibitor is being investigated in experimental oncology.[43] The fact that the reduction in PGI_2 was not seen with macitentan, may point to a negative counter-regulatory effect of ET_B receptors on PGI_2 release.

Perspectives

The ET system plays a key role in the pathogenesis of VEGFi-induced hypertension and renal injury. Here we demonstrate that this is primarily mediated via the ET_A receptor and that this involves an increase in PGI_2 production. Selective ET_A receptor antagonism may therefore be the optimal approach to prevent the rise in blood pressure and renal injury during angiogenesis inhibitor therapy. Moreover, compared to dual $ET_{A/B}$ receptor blockade, which may overwhelm the reduction in PGI_2 due to concomitant ET_B receptor blockade, selective ET_A receptor blockade will potentially have a more specific side effect profile. Since ET receptor antagonists are not currently approved for the treatment of systemic hypertension or renal injury, an alternative approach might be to target downstream ET-1 signaling such as the increase in PGI_2. Further studies are now warranted to investigate the effects of

COX inhibitors such as aspirin on the development of VEGF inhibitor-induced hypertension and renal injury.

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Conflict(s) of Interest/Disclosure(s)

The authors declare that they have nothing to disclose.

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Novelty and Significance

1) What is new?

- This study directly compared the effects of selective ET_A versus dual $ET_{A/B}$ receptor antagonism on the development of hypertension and renal injury during angiogenesis inhibitor treatment
- This is the first study to investigate if changes in the prostanoid biosynthesis pathway contributes to the development of angiogenesis inhibitor-induced hypertension and renal injury and whether these effects are dependent on the ET system

2) What is relevant?

- Selective ET_A and $ET_{A/B}$ receptor blockade were equally as effective in preventing the rise in blood pressure during treatment with the angiogenesis inhibitor sunitinib

- Sunitinib-induced albuminuria was prevented selective ET_A but not dual $ET_{A/B}$ receptor antagonism
- PGI_2 was increased, rather than decreased during VEGF inhibition with sunitinib and this effect was abolished by concomitant ET_A receptor blockade
- TXA_2, PGE_2 and $PGF_{2\alpha}$ levels were unchanged during sunitinib treatment

Summary

We found that the development of sunitinib-induced hypertension and renal injury is solely dependent on the ET_A receptor and that an upregulation in PGI_2 plays a previously unidentified role in the deleterious effects of angiogenesis inhibitors. Targeting the ET_A receptor or downstream pathways such as the increase in PGI_2 may be the optimal treatment for cardiovascular and renal side effects associated with angiogenesis inhibition.

Tables

Table 1. pEC_{50} and E_{max} values for acetylcholine (ACh), endothelin-1 (ET-1) and phenylephrine (PE) in iliac arteries following 8 days treatment with vehicle, sunitinib (14 mg/kg/day; SU), sunitinib plus the dual $ET_{A/B}$ receptor blocker macitentan (30 mg/kg/day; SU+M) or sunitinib plus a low or high dose of the ET_A receptor antagonist sitaxentan (30 mg/kg/day, SU+S30; 100 mg/kg/day, SU+S100) in the absence or presence of L-NAME, BQ123, BQ788, TRAM34 (T) and/or apamin (A).

	Vehicle	SU	SU+M	SU+S30	SU+S100
pEC_{50}					
ACh	6.7 ± 0.2	6.6 ± 0.1	6.3 ± 0.3	6.3 ± 0.2	5.8 ± 0.3
ACh+L-NAME	6.9 ± 0.4	7.7 ± 0.6	6.3 ± 0.3	6.6 ± 0.3	6.8 ± 0.3
ACh+T+A	6.5 ± 0.5	6.6 ± 0.3	6.3 ± 0.5	5.8 ± 0.5	7.4 ± 0.5*
ACh+L-NAME+T+A	7.1 ± 0.5	7.4 ± 0.3	7.1 ± 0.2	7.1 ± 0.6	7.3 ± 0.4*
ET-1	8.5 ± 0.2	8.3 ± 0.2	8.1 ± 0.1	8.2 ± 0.2	8.2 ± 0.2
ET-1+BQ123	7.5 ± 0.2***	7.2 ± 0.3****	7.1 ± 0.2****	7.2 ± 0.2****	7.4 ± 0.2**
ET-1+BQ788	8.4 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	8.3 ± 0.1	8.5 ± 0.1
PE	5.1 ± 0.2	5.0 ± 0.1	4.7 ± 0.3	4.9 ± 0.1	4.7 ± 0.1
E_{max}					
ACh	91 ± 10	77 ± 6	81 ± 10	73 ± 12	77 ± 14
ACh+L-NAME	39 ± 13**	36 ± 16*	33 ± 13**	23 ± 9**	34 ± 9*
ACh+T+A	65 ± 18	46 ± 14	11 ± 7***	76 ± 9	49 ± 18
ACh+L-NAME+T+A	24 ± 4***	37 ± 13*	19 ± 10***	22 ± 8**	12 ± 5**
ET-1	145 ± 23	132 ± 31	136 ± 21	170 ± 25	199 ± 16
ET-1+BQ123	147 ± 37	173 ± 42	158 ± 22	149 ± 25	110 ± 33
ET-1+BQ788	149 ± 31	125 ± 35	166 ± 24	167 ± 26	196 ± 13
PE	90 ± 39	129 ± 42	171 ± 49	40 ± 5	160 ± 70

Data are presented as mean ± SEM (n=5-7/group). *P<0.5, **P<0.01, ***P<0.001, ****P<0.0001 versus control segment.

Figure legends

Figure 1. Changes in mean arterial pressure (MAP) and heart rate (HR) in response to treatment with vehicle, sunitinib (14 mg/kg/day; SU) alone or during dual $ET_{A/R}$ receptor blockade with macitentan (30 mgkg/day; SU+M) or selective ET_A receptor blockade with sitaxentan (30 mg/kg/day, SU+S30; 100 mg/kg/day, SU+S100). Time course of changes in **(A)** MAP and **(B)** HR. Areas under the curve (AUC) for the cumulative change in **(C)** MAP and **(D)** HR. Data are presented as mean ± SEM (n=6-7/group). *P<0.5, **P<0.01, ****P<.0001.

Figure 2. Circulating **(A**) ET-1 and PGI_2 and TXA_2 levels, as measured by their stable metabolites, **(B)** 6-keto-$PGF_{1\alpha}$ and **(C)** TXB_2, respectively, **(D)** the PGI_2/TXA_2 ratio and **(E)** $PGF_{2\alpha}$ measured as 6-keto-$PGF_{2\alpha}$, following treatment with vehicle, sunitinib (14 mg/kg/day; SU) alone or during dual $ET_{A/R}$ receptor blockade with macitentan (30 mg/kg/day; SU+M) or selective ET_A receptor blockade with sitaxentan (30 mg/kg/day, SU+S30; 100 mg/kg/day, SU+S100). Data are presented as mean ± SEM (n=6-7/group). *P<0.05.

Figure 3. Relaxations/constrictions of iliac arteries following treatment with vehicle, sunitinib (14 mg/kg/day; SU) alone or during dual $ET_{A/R}$ receptor blockade with macitentan (30 mg/kg/day; SU+M) or selective ET_A receptor blockade with sitaxentan (30 mg/kg/day, SU+S30; 100 mg/kg/day, SU+S100) to acetylcholine (ACh) in the **(A)** absence or **(B)** presence of L-NAME (100 µmol/l), **(C)** endothelin-1 (ET-1) and **(D)** phenylephrine (PE). Data are presented as mean ± SEM (n=5-7/group). Relaxation data are expressed as a percentage of the response to U46619 (0.1-0.3 µmol/l). See Table 1 for statistical information.

Figure 4. (A) Albuminuria and urinary excretion of **(B)** neutrophil gelatinase-associated lipocalin (NGAL), **(C)** ET-1 and **(D)** PGI_2 and **(E)** TXA_2 levels, as measured by their stable metabolites, 6-keto-$PGF_{1\alpha}$ and TXB_2, respectively, and **(F)** the PGI_2/TXA_2 ratio following treatment with vehicle, sunitinib (14 mg/kg/day; SU) alone or during dual $ET_{A/R}$ receptor blockade with macitentan (30 mg/kg/day; SU+M) or selective ET_A receptor blockade with sitaxentan (30 mg/kg/day, SU+S30; 100 mg/kg/day, SU+S100). Data are presented as mean ± SEM (n=6-7/group). *P<0.05, **P<0.01, ***P<0.001.

Figure 5. Positive correlation between renal mRNA expression of **(A)** COX-2 and ET-1 and **(B)** PGI_2 synthase and endothelin converting enzyme (ECE) following treatment with vehicle, sunitinib (14 mg/kg/day; SU) alone or during dual $ET_{A/R}$ receptor blockade with macitentan (30 mg/kg/day; SU+M) or selective ET_A receptor blockade with sitaxentan (30 mg/kg/day, SU+S30; 100 mg/kg/day, SU+S100).

Figure 6. Oxidative stress following treatment with vehicle, sunitinib (14 mg/kg/day; SU) alone or during dual $ET_{A/R}$ receptor blockade with macitentan (30 mg/kg/day; SU+M) or selective ET_A receptor blockade with sitaxentan (30 mg/kg/day, SU+S30; 100 mg/kg/day, SU+S100). Superoxide (O_2^-) generation in **(A)** aortic and **(B)** mesenteric vessels and **(C)** kidney. Hydrogen peroxide (H_2O_2) production in **(D)** aortic and **(E)** mesenteric vessels and **(F)** kidney. Data are normalized to protein content and are expressed relative to the vehicle treated group. Data are presented as mean ± SEM (n=6-7/group). *P<0.05, ***P<0.001, ****P<0.0001.