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Role of endothelin ET_A receptors in the hypertension induced by the VEGFR-2 kinase inhibitors axitinib and lenvatinib in conscious freely-moving rats

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ABSTRACT

Receptor tyrosine kinase inhibitors (RTKIs) suppress tumour growth by targeting vascular endothelial growth factor receptor 2 (VEGFR-2) which is an important mediator of angiogenesis. Here, we demonstrate that two potent RTKIs, axitinib and lenvatinib, are associated with hypertensive side effects. Doppler flowmetry was used to evaluate regional haemodynamic profiles of axitinib and lenvatinib. Male Sprague Dawley rats (350–500 g) were instrumented with Doppler flow probes (renal and mesenteric arteries and descending abdominal aorta) and catheters (jugular vein and distal abdominal aorta, via the caudal artery). Rats were dosed daily with axitinib (3 or 6 $mg.kg^{-1}$) or lenvatinib (1 or 3 $mg.kg^{-1}$) and regional haemodynamics were recorded over a maximum of 4 days. Both RTKIs caused significant ($p < 0.05$) increases in mean arterial pressure (MAP), which was accompanied by significant ($p < 0.05$) vasoconstriction in both the mesenteric and hindquarters vascular beds. To gain insight into the involvement of endothelin-1 (ET-1) in RTKI-mediated hypertension, we also monitored heart rate (HR) and MAP in response to axitinib or lenvatinib in animals treated with the ET_A receptor selective antagonist sitaxentan (5 $mg.kg^{-1}$) or the mixed ET_A/ET_B receptor antagonist bosentan (15 $mg.kg^{-1}$) over two days. Co-treatment with bosentan or sitaxentan markedly reduced the MAP effects mediated by both RTKIs ($p < 0.05$). Bosentan, but not sitaxentan, also attenuated ET-1 mediated increases in HR. These data suggest that selective antagonists of ET_A receptors may be appropriate to alleviate the hypertensive effects of axitinib and lenvatinib.

1. Introduction

Vascular Endothelial Growth Factor (VEGF) is a key signalling molecule in angiogenesis [1–5]. One of its three receptors, VEGFR-2, has been shown to be the dominant mediator of VEGF-A-induced cellular responses involved in the formation of new vasculature to support cancer growth and is therefore an important target in oncology [6–10]. Growth of tumours beyond 1–2 mm^3 depends on cancer angiogenesis to meet their high demand for nutrients and oxygen [1,7,10]. Most human tumours overexpress VEGF messenger ribonucleic acid (mRNA), and many tumour cell lines have an increased expression of VEGF receptors, emphasizing the crucial role of VEGF as a mediator in tumour angiogenesis [1,7,10]. To inhibit angiogenesis in cancer, a number of therapeutic agents that interfere with VEGF signalling have been developed [1,10,11]. These include small molecule receptor tyrosine kinase inhibitors (RTKIs) that target the intracellular adenosine triphosphate (ATP)-binding site of the VEGFR-2 kinase domain and inhibit its downstream signalling pathways [8,11].

Abbreviations: DMEM, Dulbecco's Modified Eagle's Media; DMSO, dimethyl sulfoxide; ET-1, endothelin 1; ET_A, endothelin receptor type A; ET_B, endothelin receptor type B; FCS, foetal calf serum; HPβCD, hydroxypropyl β-cyclodextrin; HR, heart rate; HVC, hindquarters vascular conductance; i.v., intravenous; MAP, mean arterial pressure; MVC, mesenteric vascular conductance; NFAT, nuclear factor of activated T-cells; NFAT-RE, NFAT response element; PBS, phosphate buffered saline; RTKI, receptor tyrosine kinase inhibitor; RVC, renal vascular conductance; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2.

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Two clinically-used RTKIs that most potently inhibit VEGFR-2 signalling are axitinib and lenvatinib [12,13]. Axitinib is used in the treatment of adult patients with cytokine-refractory metastatic renal-cell cancer [14,15], while lenvatinib is used for thyroid cancers, endometrial carcinoma and other solid tumours [16–18]. Axitinib binds to the kinase ATP-binding site and a neighbouring region and adopts an asparatate-phenylanine-glycine (DFG) "out" conformation [19,20]. In contrast, lenvatinib also binds to both the kinase ATP-binding site and a neighbouring region but adopts a DFG "in" conformation [19,21,22]. With all VEGF-A targeting therapies, however, hypertension and proteinuria are frequently reported side effects [10,23,24]. Generally, drugs inhibiting VEGFR-2 with a higher relative potency compared to other tyrosine kinases, result in higher rates of elevated blood pressure than less potent VEGFR-2 inhibitors [23,25,26].

Hypertension is one of the most common complications reported in cancer patients treated with axitinib (40.4 %), with 15.3 % developing severe (grade 3 or higher) hypertension, defined as systolic blood pressure $\geq$ 180 mmHg and/or diastolic blood pressure $\geq$ 120 mmHg [27–29]. Hypertension is also reported in 42 % of patients with metastatic renal cell carcinoma treated with lenvatinib [30] and the use of lenvatinib in advanced or metastatic thymic cancer patients resulted in 64 % developing grade-3 hypertension [31]. However, the mechanisms underlying these effects are largely unknown.

One proposed mechanism for the hypertension induced by RTKIs involves the endothelin-1 (ET-1) axis [32,33]. Clinical studies have shown that the hypertensive response due to the administration of the RTKI sunitinib is associated with increased plasma levels of ET-1 [34,35]. ET-1 is a potent vasoconstrictor peptide, whose biological activity is mediated by its binding to two G protein-coupled receptors, ET_A (endothelin receptor type A) and ET_B (endothelin receptor type B), which are both expressed on vascular smooth muscle cells [36]. ET_Bs are also expressed on endothelial cells, where the interaction with ET-1 promotes nitric oxide synthesis and prostacyclin production [36]. In conscious telemetry-instrumented rats, pre-treatment with the selective ET_A antagonist atrasentan was able to abolish the hypertensive response due to the administration of linifanib [37]. Likewise, radiotelemetry studies in rats showed that macitentan also attenuated the rise in blood pressure induced by sunitinib [38].

Agents which antagonise the ET system, however, range in selectivity from those exhibiting dual ET_A/ET_B antagonism (e.g., bosentan with a pA_2 of 7.2 for ET_A and 6.0 for ET_B; [39]) to those producing a highly selective ET_A blockade (e.g., sitaxentan; pIC_{50} 8.9 for for ET_AR and 5.0 for ET_B; [40]). In this context, we have previously shown that bosentan did not prevent the rise in mean arterial pressure (MAP) induced by cediranib in conscious freely moving rats [23], suggesting that concomitant antagonism of ET_Bs may not be beneficial.

In the present study, we have used the selective ET_A antagonist sitaxentan and the non-selective ET_A/ET_B antagonist bosentan to investigate their effect on VEGFR-2 signalling and MAP induced by two of the most potent VEGFR-2 inhibitors, axitinib and lenvantinib, in conscious, freely-moving rats.

2. Materials and methods

2.1. Drugs, chemical reagents and other materials

The nuclear factor of activated T-cells (NFAT) -RE-luc2P HEK293 cell line, a clonal derivative of HEK293 cells expressing the firefly luciferase reporter gene (luc2P) under the control of NFAT response elements (NFAT-REs) and stably transfected with the expression vector containing the *KDR* gene (human gene encoding for VEGFR-2) (Promega pGL4.30, E8481), was purchased from Promega Corporation (Madison, USA), and is referred as VEGFR-2/NFAT-ReLuc2P HEK293 cells hereafter. The cell line was used between passage 10 and 30. No further authentication of cell lines was performed and HEK293 cell lines are not listed in the ICLAL register of commonly misidentified cell lines; www.iclac.org). Dulbecco's Modified Eagle's Media (DMEM), Dulbecco's phosphate buffered saline (PBS), foetal calf serum (FCS), dimethyl sulfoxide (DMSO), trypsin (0.25 % w/v in EDTA solution) and Poly-D-lysine hydrobromide were purchased from Sigma-Aldrich (Gillingham, UK). Axitinib, erlotinib, lenvatinib, linifanib, SU-14813, bosentan, sitaxentan and vatalanib were purchased from Stratech Scientific Ltd (Ely, UK). ONE-Glo™ Luciferase Assay System was purchased from Promega Corporation (Madison, USA). $VEGF_{165}$a was provided by R&D (Abingdon, UK). Other tissue culture reagents were purchased from Sigma-Aldrich (Gillingham, UK). Cell culture consumables were purchased from Fischer Scientific (Loughborough, UK). Hydroxypropyl β-cyclodextrin (HPβCD) and ET-1 were purchased from Sigma-Aldrich (Gillingham, UK). Fentanyl citrate was purchased from Martindale Pharmaceuticals (Essex, UK). Medetomidine Hydrochloride (Sedastart), Atipamezole Hydrochloride (Sedastop) and Buprenorphine (Buprecare) were purchased from Animalcare Ltd. (York, UK). Meloxicam (Metacam) was purchased from Boehringer Ingelheim Animal Health UK (Berkshire, UK) and heparin sodium was purchased from Wockhardt (Wrexham, UK). Pentobarbitone (Dolethal) was purchased from Alstoe Animal Health, (York, UK).

2.2. NFAT luciferase reporter gene assay

VEGFR-2/NFAT-ReLuc2P HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Gillingham, UK), supplemented with 10 % fetal calf serum (FCS, Sigma Aldrich, Gillingham, UK) at 37 °C/5% CO_2. Cells passaging was performed at 80 – 90 % confluency using PBS and trypsin (0.25 % w/v in versene). Cells were seeded 48 h prior to experimentation at 20,000 cells/well in DMEM/10 % FCS in white 96 well plates, coated with 0.1 $mg.mL^{-1}$ poly-D-lysine in PBS. After 24 h, medium was replaced by 100 µL serum-free DMEM and cells were incubated for a further 24 h. On the day of the experiment, medium was replaced by serum-free DMEM/0.1 % BSA. In the appropriate wells, cells were pre-treated with increasing concentrations of inhibitor in triplicate for 1 h. $VEGF_{165}$a was then added to the wells in a total volume of 100 µL/well and plates were incubated for 5 h. Finally, medium was replaced by 50 µL/well serum-free DMEM/0.1 % BSA and 50 µL/well ONE-Glo luciferase reagent. Following a 5-minute delay, luminescence was measured by a TopCount plate reader (Perkin Elmer, UK).

2.3. Animals and surgery

Experiments were carried out on male Sprague-Dawley rats (Charles River Laboratories, UK; weights 350 to 500 g). Animals were group-housed in a temperature-controlled (21–23 °C) environment with a 12-h light–dark cycle (lights on at 6:00 am) with free access to food (18 % Protein Rodent Diet; Envigo, Madison WI, USA) and water for a minimum of 7 days prior to any surgical intervention. All procedures were approved by the University of Nottingham Animal Welfare and Ethical Review Board and performed in line with the Animals (Scientific Procedures) Act (1986), under UK Home Office approved Project Licence and Personal License authority. All animal studies are reported in compliance with the ARRIVE guidelines [41].

2.4. Surgery

Under general anaesthesia (fentanyl and medetomidine, 300 $\mu g.kg^{-1}$ each, i.p., supplemented as required), miniature pulsed Doppler flow probes were implanted around the left renal and superior mesenteric arteries and the descending abdominal aorta (providing blood flow to the hindquarters) to monitor Doppler shift, an index of blood flow [23]. The probe wires were secured to the abdominal wall and led subcutaneously to the posterior of the neck. The wires were then secured with suture and sterile tape to the nape of the neck. Reversal of anaesthesia and postoperative analgesia was provided by atipamezole hydrochloride

(1.0 mg.kg^{-1}, s.c.) and buprenorphine (30 μg.kg^{-1}, s.c.). A second dose of analgesia (buprenorphine 15 μg.kg^{-1}, s.c.) was given 4 h post-surgery. Additional analgesia (meloxicam, 1.0 mg.kg^{-1}.day^{-1}, s.c.) was given before the start of the surgical procedure, and also daily for a further three days post-operation.

At least 10 days after probe implantation and after a satisfactory inspection from the Named Veterinary Surgeon, catheter implantation was performed under anaesthesia (fentanyl and medetomidine, 300 μg.kg^{-1} each, i.p., supplemented as required). The catheters were filled with heparinised saline (15 U.ml^{-1}) and were inserted into the distal abdominal aorta via the ventral caudal artery [positioned to monitor arterial blood pressure and heart rate (HR)]. Up to three intravenous (i.v.) catheters were implanted into the right jugular vein for drug administration [23]. All catheters were led subcutaneously to the nape of the neck. The probe wires were released from the nape of the neck, soldered into a miniature plug (Omnetic connector corporation, USA) and mounted onto a custom-designed harness worn by the rat. Secured to the harness was a spring which the catheters and probe wires ran through for protection. A counterbalanced pivot system supported this whole assembly to allow the free movement of the animal. Reversal of anaesthetic and analgesia was administered (as described above). The arterial catheter was infused with heparinised (15 U.ml^{-1}) saline overnight to maintain patency at a rate of 0.4 ml.h^{-1}.

Experiments began 24 h after surgery for catheter implantation, with animals fully conscious and unrestrained in home cages, with access to food and water *ad libitum*.

2.5. Cardiovascular recordings

During the cardiovascular monitoring periods, rats were connected to the customised data-acquisition software, described below, via a tether system. Recordings were made for at least 30 min prior to the administration of any interventions and continuously for a minimum of 4 h thereafter. HR, arterial blood pressure, and renal, mesenteric and hindquarters Doppler shifts were measured by a transducer amplifier (13-4615-50; Gould, Cleveland, OH, USA), a Doppler flowmeter (Crystal Biotech, Holliston, MA, USA), and a VF-1 mainframe (pulse repetition frequency 125 kHz) fitted with high-velocity (HVPD-20) modules. These measurements were recorded by customised computer software (IdeeQ; Maastricht Instruments, Maastricht, The Netherlands). Raw data were sampled by IdeeQ every 2 ms, averaged, and stored to disc every cardiac cycle. Changes in renal vascular conductance (VC), mesenteric VC, and hindquarter VC, were calculated from the changes in MAP and Doppler shift.

2.6. Experimental protocol

In Series 1, experiments were run for 4 days; the contemporaneous control group was administered vehicle (40 % HPβCD in sterile saline). Experiments were run with treatment groups of 8 to 10 rats. Two groups of rats were used to assess the cardiovascular responses to axitinib. On day 1 of the experiment, after a period of baseline recordings, rats were randomised to receive either axitinib (3 (low) mg.kg^{-1} or 6 (high) mg.kg^{-1}) as an i.v. bolus (0.2 ml administered over 10 s) followed by a 1 h i.v. infusion (0.4 ml.h^{-1}) at the same dose. Two groups of rats were also used to evaluate the cardiovascular changes induced by lenvatinib. On day 1 of the experiment, after a period of baseline recordings, rats were randomised to receive lenvatinib (1 (low) mg.kg^{-1} or 3 (high) mg.kg^{-1}) as an i.v. bolus (0.2 ml administered over 10 s) followed by a 1 h i.v. infusion (0.4 ml.h^{-1}) at the same dose. The contemporaneous control group received vehicle as an i.v. bolus (0.2 ml administered over 10 s) followed by a 1 h i.v. infusion (0.4 ml.h^{-1}). Haemodynamic recordings were continued for a further 5 h after completion of the axitinib, lenvatinib or vehicle i.v. infusion period. The same treatment regimen was followed on days 2–4 after a baseline recording period on each day.

In series 2, experiments were run for 2 days. Experiments were run with treatment groups of 7 to 8 rats. Three groups of rats were used for each RTKI, namely axitinib and lenvatinib, to assess the role of endothelin receptor antagonism in the prevention of the cardiovascular responses associated with these agents. Rats were randomly assigned to one of three treatment groups: (i) axitinib or lenvatinib in the presence of vehicle (40 % HPβCD in sterile saline); (ii) axitinib or lenvatinib in the presence of a dual ET_A and ET_B antagonist (bosentan) or (iii) axitinib or lenvatinib in the presence of a selective ET_A/ET_B antagonist (sitaxentan). On day 1 of the experiment, after a period of baseline recordings, rats were dosed with bosentan (15 mg.kg^{-1}) or sitaxentan (5 mg.kg^{-1}) as an i.v. bolus (0.2 ml administered over 10 s) followed by a 5 h (for axitinib groups) or 6 h (for lenvatinib groups) i.v. infusion (0.4 ml.h^{-1}) at the same dose. The contemporaneous control group received vehicle (40 % HPβCD in sterile saline) as an i.v. bolus (0.2 ml administered over 10 s) followed by a 5 h (for axitinib groups) or 6 h (for lenvatinib groups) i.v. infusion (0.4 ml.h^{-1}). After 1-h infusion of the endothelin antagonists or vehicle, axitinib (3 mg.kg^{-1}) or lenvatinib (1 mg.kg^{-1}) was administered to all animals as an i.v. bolus (0.2 ml administered over 10 s) followed by a 1 h i.v. infusion (0.4 ml.h^{-1}) at the same dose. The same treatment regimen was followed on day 2 after a baseline recording period. At the end of day 2, all groups received ET-1 (administered as 0.1 ml bolus in 1 % bovine serum albumin/saline) at the following concentrations: 0.1 μM, 0.3 μM and 1 μM, 3 min per concentration. It is to be noted that ET-1, given systemically, may not mimic the locally formed compound, and ET_B receptor activation is likely to predominate. Here we used this approach to monitor the extent to which ET_B receptor activation was inhibited by the two ET-1 receptor antagonists.

2.7. Data analysis

Power calculations were carried out to estimate the sample size required to measure clinically relevant changes in blood pressure (>10 mmHg) and in regional blood flows (>10 %) (80 %, $p = 0.05$, effect size of 10 mmHg, SD = 8.48, paired *t*-test), as well as to minimise the number of animals used. Combining previous experience with the results of the NC3R's Experimental Design Assistant tool, eight animals per group were found to be appropriate. All *in vivo* data were collected and analysed using IdeeQ software (Maastricht Instruments, Maastricht University, NL). For all experiments, the experimental unit for analysis was a single animal. Time-averaged data are shown as changes from baseline [HR (beats.min^{-1}); MAP (mmHg); VC (%)]. Statistical comparisons between groups of animals were performed on the integrated changes over specified time periods. Due to loss of Doppler signal or blockage of arterial catheter, some animals were excluded from subsequent analysis. This was most apparent after day 2. A Friedman's test, which is a nonparametric, repeated-measures analysis of variance was used for within-group comparisons to baseline; this data analysis was performed on the first 2 days of the experimental period due to changes in sample size (n) after day 2. A Mann-Whitney *U* test for integrated area under or above curve analysis was used for comparisons between groups, again restricted to the first 2 days of the experimental period. The Friedman's test and Mann-Whitney *U* test for area under or over the curve analysis was performed using BIO-medical software 3.4 (Medical Physics, University of Nottingham, UK). A Mann-Whitney *U* test was also performed for comparisons between groups at a specific time point. Vascular conductances were calculated from the MAP and Doppler shift (flow) data (Prism 10.0, Graphpad software, San Diego, CA, USA).

In vitro $VEGF_{165}$a concentration–response data were normalized to responses to 10 nM $VEGF_{165}$a and fitted to a non-linear regression with the following equation:

$$Response = \frac{E_{max} \times [A]^n}{[A]^n + EC_{50}}$$

where E_{max} is the maximal response, [A] is the concentration of $VEGF_{165}$a, EC_{50} is the concentration of $VEGF_{165}$a required to generate

Table 1
IC_{50} values for inhibition of 1 nM $VEGF_{165}$a-stimulated NFAT responses. Values are mean ± SEM from n independent experiments. #Data taken from Van Daele et al. [42].

Receptor tyrosine kinase inhibitor	pIC_{50} for inhibition of $VEGF_{165}$a-induced NFAT response	*n*
Axitinib	9.28 ± 0.13	5
Lenvatinib	8.88 ± 0.13	4
Linifanib	8.34 ± 0.19	5
SU-14813	8.00 ± 0.23	5
Vatalanib	7.36 ± 0.26	6
Erlotinib	NI	6
Sunitinib	7.90 ± 0.12#	5
Cediranib	8.73 ± 0.02#	5

50 % of the E_{max} and n is the Hill coefficient. All inhibition data obtained from NFAT luciferase reporter gene assays were normalised to the control response to 1 nM $VEGF_{165}$a, then fitted with a non-linear regression using the following equation:

$$\%\ of\ response\ to\ VEGF_{165}a = \frac{100 \times IC_{50}}{[I] + IC_{50}}$$

Where IC_{50} is the molar concentration of RTKI required to inhibit 50 % of the response to VEGF and [I] is the concentration of RTKI.

3. Results

3.1. Effect of axitinib and lenvatinib on $VEGF_{165}$a-induced NFAT responses

The impact of RTKIs on VEGFR-2 receptor-mediated signalling can be monitored using an NFAT luciferase reporter gene assay [42]. Here, we have used this assay to compare the effect of a range of RTKIs on $VEGF_{165}$a-induced responses (Table 1). In HEK293 cells stably expressing VEGFR-2 and the NFAT-RE-luc2P reporter, $VEGF_{165}$a produced a concentration-dependent increase (pEC_{50} 9.73 ± 0.11, $n = 5$) in NFAT-luciferase production (Fig. 1a). The pEC_{50} obtained was in close agreement with that reported previously (pEC_{50} 9.85; [42]). Both axitinib and lenvatinib were the most potent inhibitors of the NFAT-luciferase response to 1 nM $VEGF_{165}$a (Fig. 1b, c; Table 1). As a control, we also showed that the epidermal growth factor receptor-selective RTKI, erlotinib, was devoid of inhibitor activity at concentrations up to 1 μM (data not shown; n = 6). This finding is consistent with the low binding affinity of erlotinib for VEGFR-2 [43].

3.2. In vivo haemodynamic profile of lenvatinib

To characterise the haemodynamic impact of axitinib and lenvatinib on cardiovascular parameters, pulsed Doppler flowmetry was used in conscious and freely-moving rats, allowing the simultaneous evaluation of their effects on HR, MAP and regional VC [renal VC (RVC), mesenteric VC (MVC) and hindquarters VC (HVC)]. Baseline cardiovascular

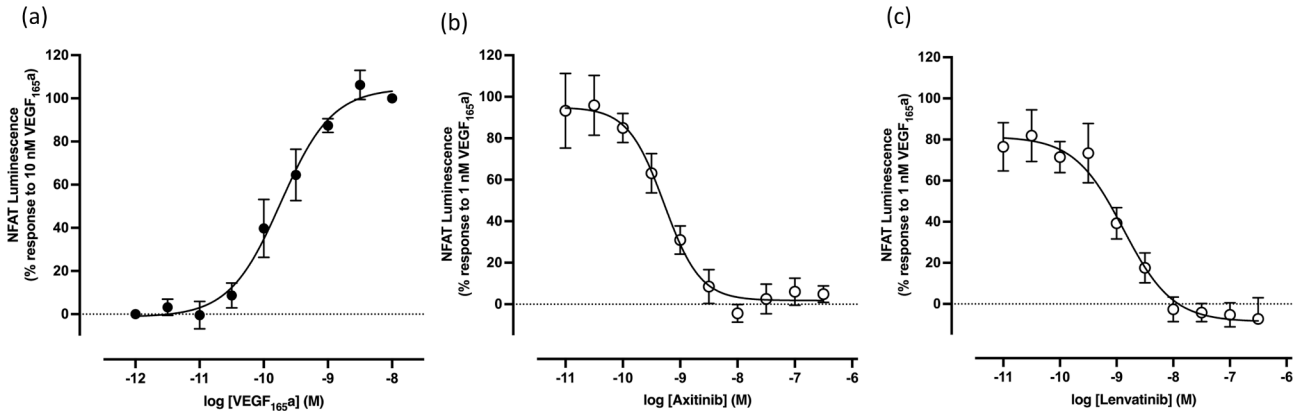

Fig. 1. The effect of $VEGF_{165}$a and RTKI inhibitors on NFAT-mediated gene transcription in VEGFR-2/NFAT-ReLuc2P HEK293 cells. (a) Agonist responses to $VEGF_{165}$a. Data are from five independent experiments expressed as a percentage (mean ± SEM) of the response to 10 nM $VEGF_{165}$a. Each individual experiment was performed in triplicate. (b-c) Effect of (b) axitinib and (c) lenvatinib on the response to 1 nM $VEGF_{165}$a. Data are expressed as a percentage of the response to 1 nM $VEGF_{165}$a in the absence of inhibitor, with basal levels set at 0 %. Data are shown as mean ± SEM of 4–6 independent experiments (actual number of experiments given in Table 1). Each experiment was performed using triplicate wells.

Table 2
Baseline cardiovascular variables prior to administration of vehicle or RTKIs. Values are mean ± SEM. Units of vascular conductance (VC) are kHz. $mmHg^{-1}$ x 10^3. U, units.

	Vehicle		Axitinib 3 $mg.kg^{-1}$		Axitinib 6 $mg.kg^{-1}$		Lenvatinib 1 $mg.kg^{-1}$		Lenvatinib 3 $mg.kg^{-1}$	
	Mean ± SEM	*n*	*Mean ± SEM*	*n*	*Mean ± SEM*	*n*	*Mean ± SEM*	*n*	*Mean ± SEM*	*n*
HR ($beats.min^{-1}$)	344 ± 9	9	346 ± 8	10	357 ± 13	8	342 ± 12	8	340 ± 14	9
MAP (mmHg)	103 ± 3	9	101 ± 4	10	99 ± 6	8	98 ± 3	8	96 ± 3	9
DBP (mmHg)	84 ± 3	9	84 ± 3	10	82 ± 5	8	83 ± 3	8	79 ± 2	9
SBP (mmHg)	142 ± 4	9	136 ± 6	10	135 ± 8	8	135 ± 4	8	136 ± 4	9
RVC (U)	92 ± 6	7	94 ± 5	10	67 ± 5	8	97 ± 7	8	74 ± 12	8
MVC (U)	98 ± 9	9	84 ± 10	10	109 ± 11	8	85 ± 7	8	104 ± 11	8
HVC (U)	55 ± 4	9	51 ± 10	10	52 ± 7	8	53 ± 8	8	59 ± 8	8

Figure 2

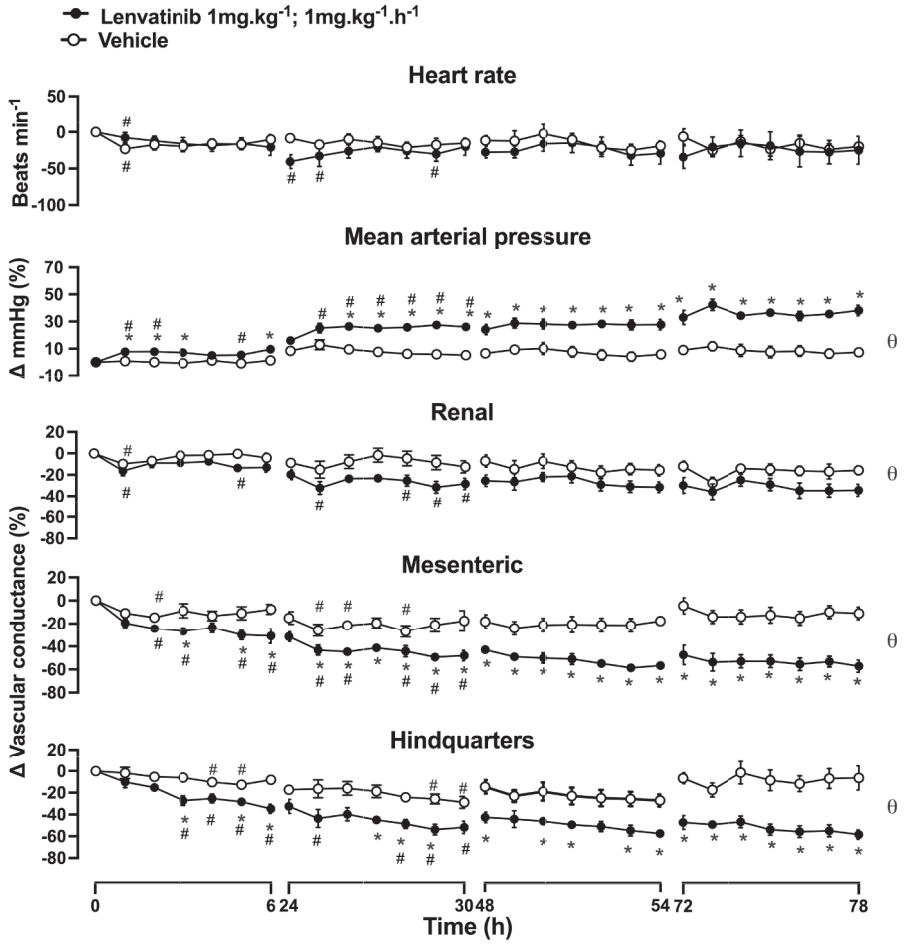

Fig. 2. Cardiovascular responses to lenvatinib in conscious, freely moving rats. Animals were dosed with 1 mg.kg^{-1} lenvatinib i.v. (initial bolus followed by 1 mg.kg^{-1}.h^{-1}, i.v. infusion for 1 h). Vehicle controls were administered 40 % HPβCD in sterile saline as described in Methods. The same dose regimen was repeated on day 2, 3 and 4. Data points are means; vertical bars represent SEM. Friedman's test was performed on the first 2 days of the experimental period (# $p < 0.05$ vs. baseline). A Mann-Whitney *U* test was conducted between treated and vehicle control groups for a comparison of area under/over the curve (θ $p < 0.05$, 0–30 h) and to determine differences at each individual time point (* $p < 0.05$, 0 – 78 h; Mann-Whitney *U* test). 9 animals were used in the vehicle group and 8 in the lenvatinib-treat group. Only 7 and 6 animals were incorporated into the analysis on days 3 and 4 respectively because of blocked catheters or loss of the Doppler signal in one of the probes. The Friedman test and the area under the curve analyses were therefore confined to the first two days of treatment.

parameters taken before the administration of axitinib, lenvatinib or vehicle control are shown in Table 2. Administration of vehicle did not elicit consistent significant cardiovascular effects, despite some sporadic reduction in MVC and HVC at the end of day 2 (# $p < 0.05$, Friedman's test; Figs. 2 and 3).

Lenvatinib, at 1 mg.kg^{-1} or 3 mg.kg^{-1} significantly increased MAP when compared to baseline values (# $p < 0.05$, Friedman's test; Figs. 2 and 3), without consistently affecting HR. The low and high doses of lenvatinib were associated with maximal increases in MAP of 32 % and 38 % on day 4 when compared to baseline (Figs. 2 and 3). The lenvatinib-induced hypertensive effect was accompanied by significant reductions in MVC and HVC, which were observed with both doses of the compound (# $p < 0.05$, Friedman's test; Figs. 2 and 3). MVC was maximally reduced by 59 % (on day 3 of low-dose lenvatinib) and 69 % (on day 4 of high-dose lenvatinib) (Figs. 2 and 3).

Comparing the integrated responses over the first two days of the recording period, the pressor effects and the vascular responses observed in lenvatinib-receiving animals were significantly different from the vehicle group (θ $p < 0.05$, Mann-Whitney *U* test, integrated area under curve, 0–30 h; Figs. 2 and 3). Comparison of each individual time point showed a significant difference in MAP, MVC and HVC between the lenvatinib-treated groups and vehicle-receiving group for almost all time points of the analysis (*$p < 0.05$, Mann-Whitney *U* test; Figs. 2 and 3).

3.3. *In vivo haemodynamic profile of axitinib*

Axitinib, at both 3 mg.kg^{-1} and 6 mg.kg^{-1}, evoked a consistent increase of MAP, which was evident from day 1 of the experimental period (#$p < 0.05$, Friedman's test; Figs. 4 and 5), with no consistent corresponding alteration in HR. Treatment with low or high dose of axitinib led to maximal increases in MAP on day 4 of 29 % and 37 %, respectively (Figs. 4 and 5). This hypertensive response was associated with a significant vasoconstriction in the mesenteric and hindquarters vasculature at both doses used (#$p < 0.05$, Friedman's test; Figs. 4 and 5). A maximal reduction of 50 % and 62 % in MVC occurred on day 4 of

Figure 3

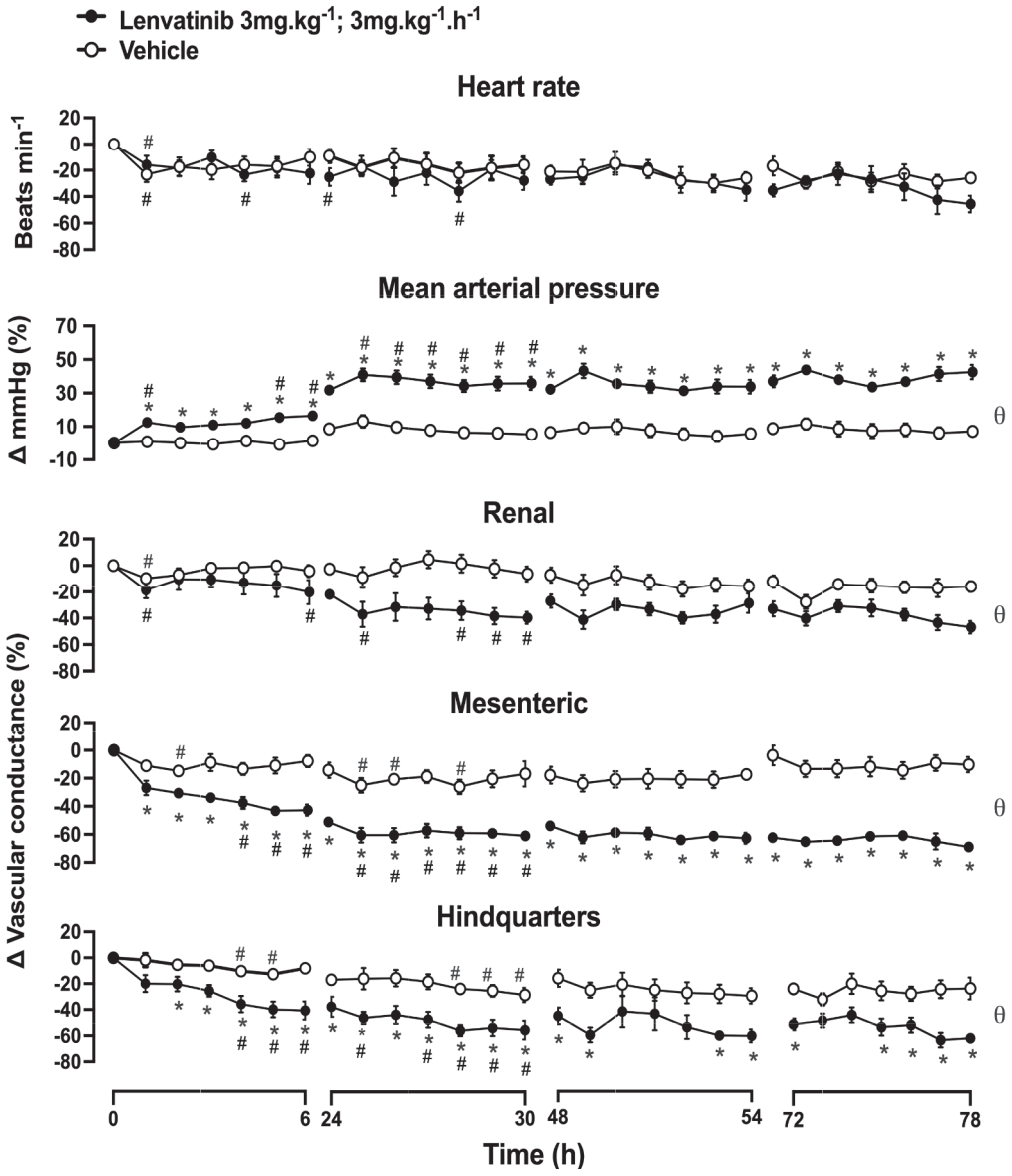

Fig. 3. Cardiovascular responses to lenvatinib in conscious, freely moving rats. Animals were dosed with 3 mg.kg^{-1} lenvatinib i.v. (initial bolus followed by 3 mg.kg^{-1}.h^{-1}, i.v. infusion for 1 h). Vehicle controls were administered 40 % HPβCD in sterile saline as described in Methods. The same dose regimen was repeated on day 2, 3 and 4. Data points are means; vertical bars represent SEM. Friedman's test was performed on the first 2 days of the experimental period (# $p < 0.05$ vs. baseline). A Mann-Whitney *U* test was conducted between treated and vehicle control groups for a comparison of area under/over the curve (θ $p < 0.05$, 0–30 h) and to determine differences at each individual time point (* $p < 0.05$, 0 – 78 h; Mann-Whitney *U* test). 9 animals were used in the vehicle group and 8 in the lenvatinib-treated group. Only 6 and 5 animals were incorporated into the analysis on days 3 and 4 respectively because of blocked catheters or loss of the Doppler signal in one of the probes. The Friedman test and the area under the curve analyses were therefore confined to the first two days of treatment.

treatment with low or high dose of the drug, respectively (Figs. 4 and 5). HVC was maximally reduced on day 4 by 61 % (following the administration of low-dose axitinib) and 69 % (following the administration of high-dose axitinib) (Figs. 4 and 5). However, a sustained renal vasoconstriction was only observed with the low and the high dose of axitinib on day 2. This was more marked in the group receiving the 6 mg.kg^{-1} dose (Fig. 5).

Considerable differences in blood pressure and vascular responses were observed between the axitinib-dosed groups and vehicle group over the first two days of the recording period (θ $p < 0.05$, Mann-Whitney *U* test, integrated area under curve, 0–30 h; Figs. 4 and 5). An additional Mann-Whitney test was conducted between treated and control groups at each individual time point to determine the time of onset of the cardiovascular effects (*$p < 0.05$, Mann-Whitney *U* test; Figs. 4 and 5). In both the low dose- and high dose-treated groups, the pressor effect and the vascular changes, particularly in the mesentery

Figure 4

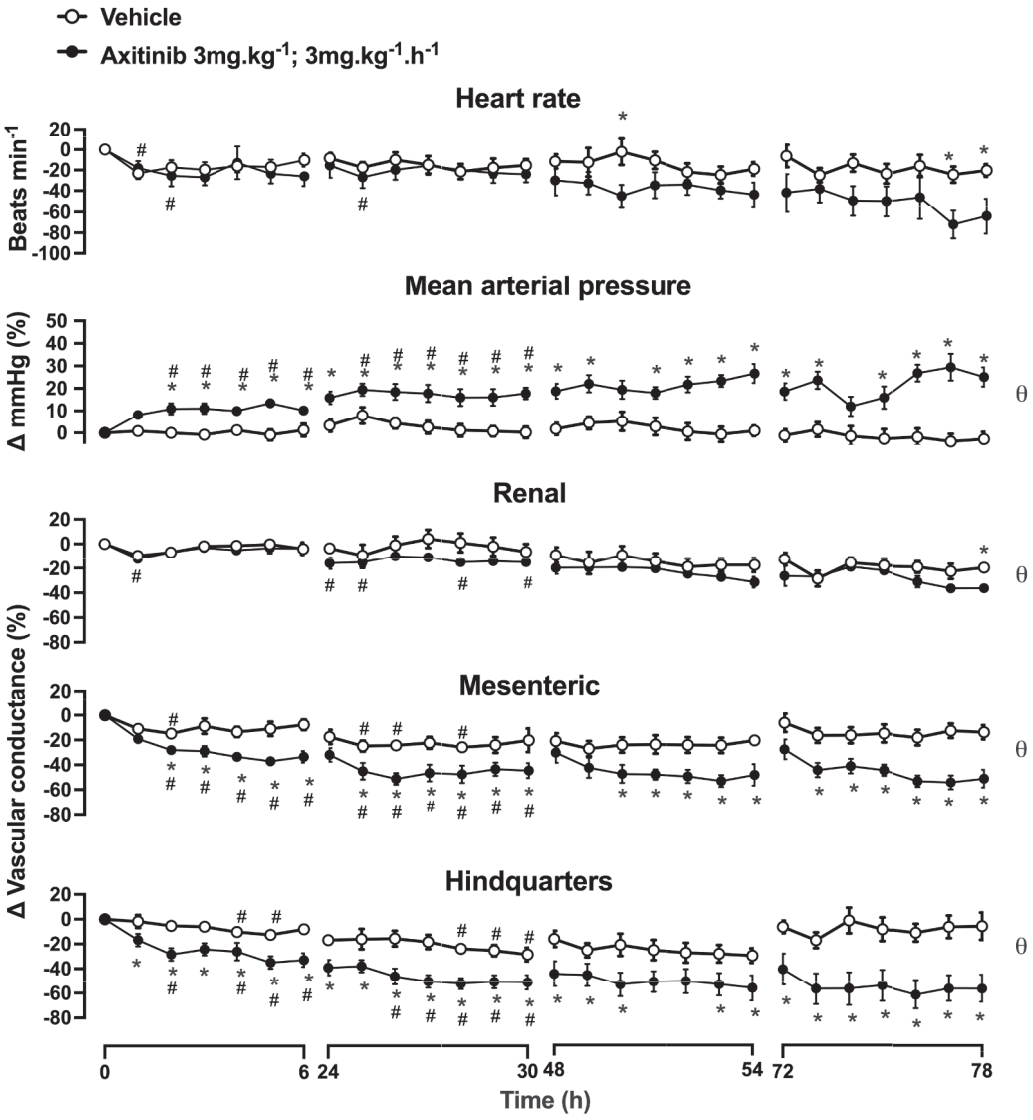

Fig. 4. Cardiovascular responses to axitinib in conscious, freely moving rats. Animals were dosed with 3 mg.kg^{-1} axitinib i.v. (initial bolus followed by 3 mg.kg^{-1}.h^{-1}, i.v. infusion for 1 h). Vehicle controls were administered 40 % HPβCD in sterile saline as described in Methods. The same dose regimen was repeated on day 2, 3 and 4. Data points are means; vertical bars represent SEM. Friedman's test was performed on the first 2 days of the experimental period (# $p < 0.05$ vs. baseline). A Mann-Whitney *U* test was conducted between treated and vehicle control groups for a comparison of area under/over the curve (θ $p < 0.05$, 0–30 h) and to determine differences at each individual time point (* $p < 0.05$, 0 – 78 h; Mann-Whitney *U* test). 9 animals were used in the vehicle group and 10 in the axitinib-treated group. Only 9 and 5 animals were incorporated into the analysis on days 3 and 4, respectively, because of blocked catheters or loss of the Doppler signal in one of the probes. The Friedman test and the area under the curve analyses were therefore confined to the first two days of treatment.

and hindquarters, showed a striking difference from the vehicle group at all time points (*$p < 0.05$, Mann-Whitney *U* test; Figs. 4 and 5).

3.4. Impact of ET-1 receptor antagonists on the changes in HR and MAP induced by lenvatinib

In this series of experiments, lenvatinib evoked a significant elevation in MAP on day 2 in the presence of the ET-1 receptor antagonist vehicle, producing a maximal increase of 24 % (#$p < 0.05$, Friedman's test), accompanied by a small alteration of HR (Figs. 6 and 7). The co-treatment with bosentan significantly reduced the overall lenvatinib-induced HR and hypertensive effects (θ $p < 0.05$, Mann-Whitney *U* test, integrated area under curve, 0–29 h; Fig. 6). At the end of the 29 h treatment period, ET-1 was administered at 0.1 μM, 0.3 μM and 1 μM (3 min per concentration). In animals co-treated with vehicle and lenvatinib, ET-1 produced a significant dose-dependent increase in HR and a decrease in MAP (# $p < 0.05$, Friedman's test; Fig. 6) that was completely prevented in animals treated with bosentan (Fig. 6).

Figure 5

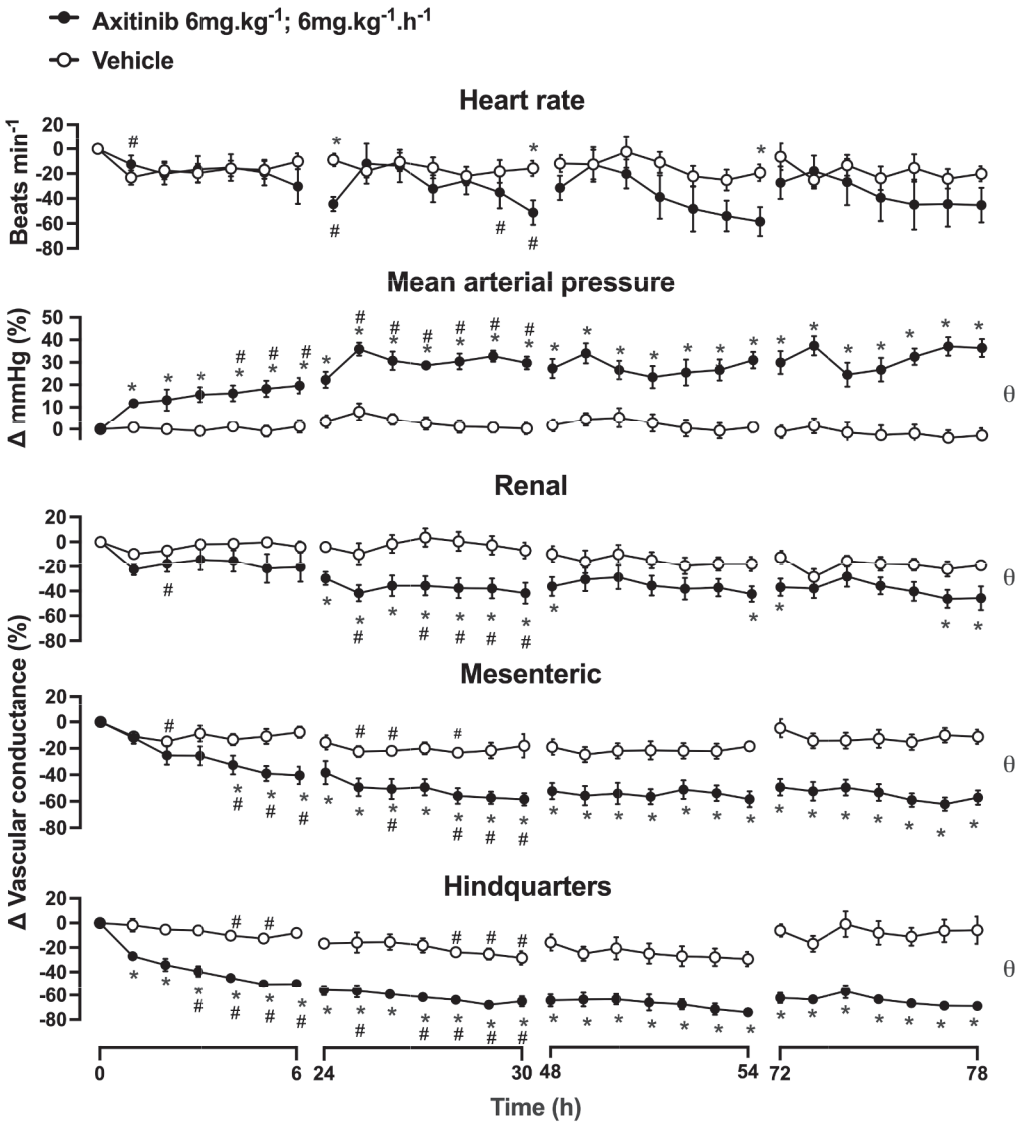

Fig. 5. Cardiovascular responses to axitinib in conscious, freely moving rats. Animals were dosed with 6 mg.kg^{-1} axitinib i.v. (initial bolus followed by 6 mg.kg^{-1}.h^{-1}, i.v. infusion for 1 h). Vehicle controls were administered 40 % HPβCD in sterile saline as described in Methods. The same dose regimen was repeated on day 2, 3 and 4. Data points are means; vertical bars represent SEM. Friedman's test was performed on the first 2 days of the experimental period (#$p < 0.05$ vs. baseline). A Mann-Whitney *U* test was conducted between treated and vehicle control groups for a comparison of area under/over the curve (θ $p < 0.05$, 0–30 h) and to determine differences at each individual time point (* $p < 0.05$, 0 – 78 h; Mann-Whitney *U* test). 9 animals were used in the vehicle group and 8 in the axitinib-treat group. The Friedman test and the area under the curve analyses were confined to the first two days of treatment to be consistent with Figs. 2-4.

Sitaxentan completely attenuated the lenvatinib-induced hypertensive effect (θ $p < 0.05$, Mann-Whitney *U* test, integrated area under curve, 0–29 h; *$p < 0.05$, Mann-Whitney *U* test to determine differences at each time point; Fig. 7). Co-treatment with sitaxentan did not, however, reduce the significant effect of lenvatinib on HR (#$p < 0.05$, Friedman's test; Fig. 7), and no difference was observed when compared to the control group. Administration of ET-1 at the end of the experiment revealed that sitaxentan did not significantly inhibit the increased HR response to ET-1 (Fig. 7). The effect on MAP was, however, significantly attenuated (Fig. 7).

3.5. Impact of ET-1 receptor antagonists on the changes in HR and MAP induced by axitinib

Administration of axitinib in animals pre-treated with vehicle caused a significant rise in MAP, resulting in a maximal increase of 23 % on day 2 (#$p < 0.05$, Friedman's test; Figs. 8 and 9). The pressor effect of axitinib was not accompanied by consistent changes in HR (Figs. 8 and 9). Co-treatment with bosentan completely prevented the axitinib-induced increase in MAP (θ$p < 0.05$, Mann-Whitney *U* test, integrated area under curve, 0–28 h; *$p < 0.05$, Mann-Whitney *U* test to determine differences at each time point; Fig. 8. Similar to the effect observed in animals co-treated with vehicle and lenvatinib (Fig. 6), ET-1 produced a

Figure 6

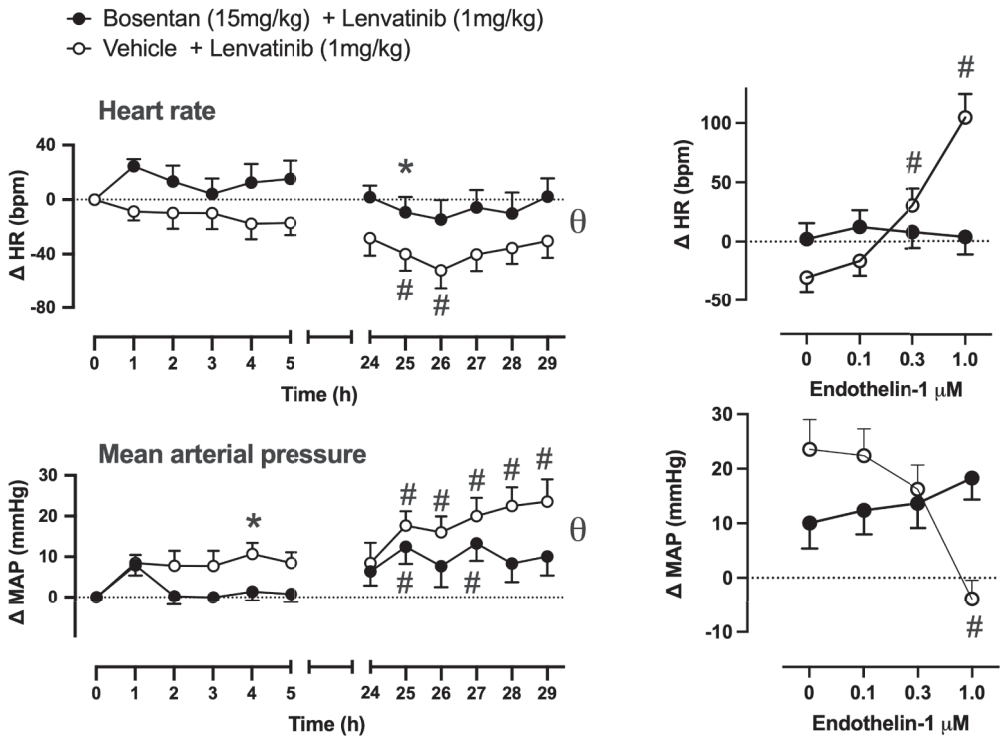

Fig. 6. Effect of the dual ET_A/ET_B receptor antagonist (bosentan) on lenvatinib-induced cardiovascular responses in conscious, freely moving rats. Animals were dosed with bosentan (15 $mg.kg^{-1}$) or vehicle as an i.v. bolus followed by a 6 h i.v. infusion. One hour after commencement of the endothelin receptor antagonist or vehicle infusion, lenvatinib (1 $mg.kg^{-1}$) was administered to all animals as an i.v. bolus followed by a 1 h i.v. infusion. The same dose regimen was repeated on day 2, after which ET-1 was administered as a bolus at increasing concentrations. Data points are means; vertical bars represent SEM. Friedman's test was performed for within-group comparisons to baseline (0 h time point was used as a baseline for the entire 29 h period prior to the administration of ET-1, while 29 h time point was used as a baseline for the ET-1 dose–response (# $p < 0.05$ vs. baseline). Mann-Whitney *U* test was conducted between treated and vehicle control groups for a comparison of area under/over the curve (θ $p < 0.05$) and to determine differences at each time point (* $p < 0.05$, Mann-Whitney *U* test). 8 animals were treated with vehicle and 7 with bosentan.

significant dose-dependent increase in HR and a decrease in MAP (# $p < 0.05$, Friedman's test; Fig. 8) in animals treated with axitinib and vehicle that was completely prevented in animals treated with bosentan (Fig. 8).

Sitaxentan completely attenuated the axitinib-induced hypertensive effect (θ $p < 0.05$, Mann-Whitney *U* test, integrated area under curve, 0–28 h; *$p < 0.05$, Mann-Whitney *U* test to determine differences at each time point; Fig. 9). Co-treatment with sitaxentan did not, however, reduce the significant effect of lenvatinib on HR (#$p < 0.05$, Friedman's test; Fig. 9), and no difference was observed when compared to the control group. Administration of ET-1 at the end of the experiment revealed that sitaxentan did not significantly inhibit the increased HR response to ET-1 (Fig. 9) but did prevent the reduction in MAP (Fig. 9).

Baseline cardiovascular variables obtained prior to administration of vehicle, bosentan or sitaxentan in axitinib-treated rats are shown in Table 3.

4. Discussion

Among the RTKIs evaluated in the present study as inhibitors of $VEGF_{165}$a-stimulated NFAT responses, axitinib and lenvatinib showed the highest relative potency for inhibition of VEGFR-2 signalling. These findings are consistent with the high rates of hypertensive side effects reported for cancer patients treated with these two RTKIs [27–31]. Here, we have also demonstrated that both axitinib and lenvatinib elicited a striking elevation in MAP within 24 h of commencement of treatment, which became even more marked on the second day of treatment, when the pressure values essentially reached a plateau. A similar trend has also been reported previously for cediranib, sorafenib, pazopanib and vandetanib, indicating a similar timeline for the onset of hypertension following treatment with RTKIs [23].

In addition to the rise in blood pressure induced by these anti-angiogenic agents, there were parallel and regionally-selective changes in vascular flow. There was a significant reduction in the hindquarters and mesenteric VC following treatment with lenvatinib or axitinib that was indicative of vasocontriction. For both axitinib and lenvatinib, renal vascular blood flow was least affected, and this occurred mainly at the highest doses of each RTKI used. These findings are consistent with the hypertensive and vascular effects previously observed with the same experimental model for other RTKIs targeting VEGFR-2 [23]. Cediranib, sorafenib, pazopanib and vandetanib have previously been shown to produce a hypertensive effect that occurred concurrently with hindquarters vasoconstriction [23]. Similar to the results presented here, the renal vasculature conductance showed the least susceptibility to treatment with these other RTKIs [23]. This may be indicative of a compensatory adaptation of the renal vasculature in response to hypertensive conditions. Taken together, our findings suggest that vasoconstrictions observed in the hindquarters and mesenteric vascular beds are the major causal factors in the development of hypertension

Figure 7

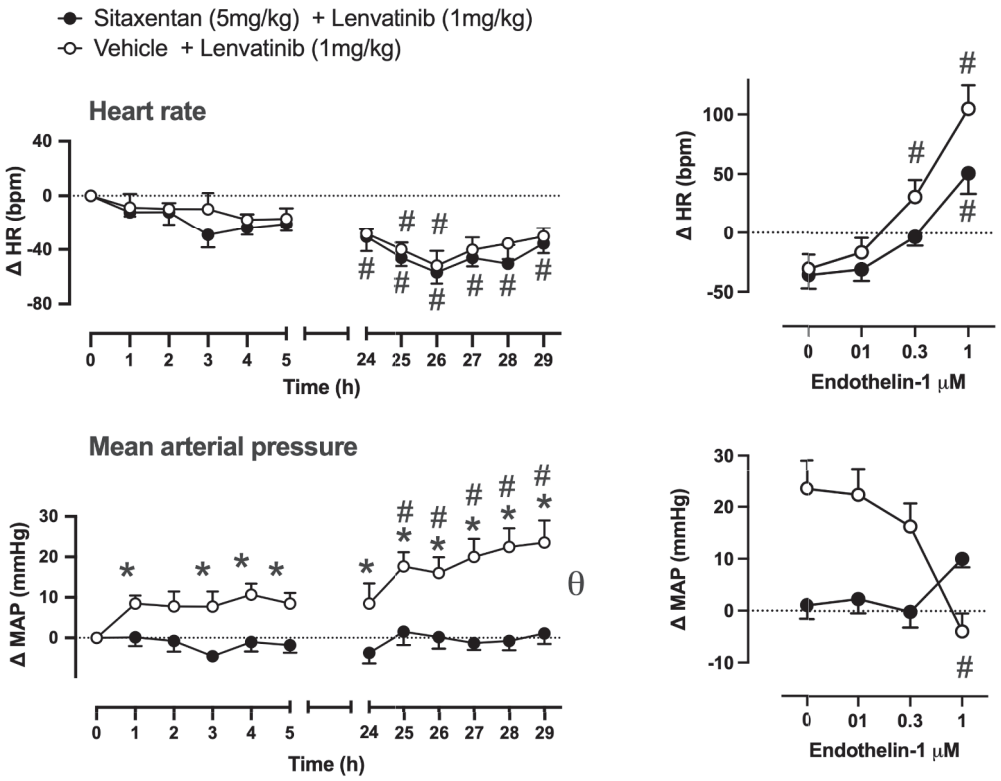

Fig. 7. Role of the selective ET_A receptor antagonist (sitaxentan) on lenvatinib-induced cardiovascular responses in conscious, freely moving rats. Animals were dosed with sitaxentan (5 $mg.kg^{-1}$) or vehicle as an i.v. bolus followed by a 6 h i.v. infusion. One hour after commencement of the endothelin receptor antagonist or vehicle i.v., lenvatinib (1 $mg.kg^{-1}$) was administered to all animals as an i.v. bolus followed by a 1 h i.v. infusion. The same dose regimen was repeated on day 2, after which ET-1 was administered as a bolus at increasing concentrations. Data points are means; vertical bars represent SEM. Friedman's test was performed for within-group comparisons to baseline (0 h time point was used as a baseline for the entire 29 h period prior to the administration of ET-1, while 29 h time point was used as a baseline for ET-1 dose–response (# $p < 0.05$ vs. baseline). Mann-Whitney *U* test was conducted between treated and vehicle control groups for a comparison of area under/over the curve (θ $p < 0.05$) and to determine differences at each time point (* $p < 0.05$, Mann-Whitney *U* test). 8 animals were treated with vehicle and 7 with sitaxentan.

produced by axitinib and lenvatinib.

Angiogenetic pathways have a crucial role in the pathogenesis of renal cell carcinoma [43], where the loss of the tumour suppressor function of von Hippel-Lindau protein promotes a hypoxic status and the consequent overexpression of VEGF, resulting in uncontrolled angiogenesis, tumour progression and metastatic dissemination [43,44]. As a result, RTKIs that block the kinase activity of VEGFR-2 have provided a useful therapeutic approach for patients with metastatic renal cell carcinoma, for which both axitinib and lenvatinib represent the recommended second-line treatment options [45,46]. Despite an overall improvement of clinical outcomes, however, safety concerns have arisen from the use of these drugs, particularly in terms of the hypertensive response associated with these agents [47,48].

Activation of the endothelin axis has been previously suggested as a mechanism for the hypertensive effect associated with antiangiogenic therapies [34,37,38,49]. Clinical studies have shown that the hypertensive response due to the administration of the RTKI sunitinib is associated with increased plasma levels of ET-1 [34,35]. Furthermore, in animal studies treated with sunitinib there was a dose-dependent rise in both HR and circulating ET-1 levels [50]. The present study confirms a major role for ET_A activation in the increase in MAP induced by both axitinib and lenvatinib. Thus, our results demonstrate that the increase in blood pressure induced by both axitinib and lenvatinib is completely attenuated by the ET_A selective antagonist sitaxentan. Interestingly, although the mixed ET_A/ET_B antagonist bosentan abolished the axitinib-induced hypertension it did not completely inhibit the lenvatinib-induced pressor effect. It is notable that bosentan only has a ten-fold higher affinity for ET_A (pA_2 of 7.2) over that for ET_B (pA_2 of 6.0) [39]). Interestingly, we have previously reported that bosentan did not significantly suppress the hypertensive response to cediranib [23]. These data suggest that blockade of vasodilator ET_B on endothelial cells may offset to some extent the beneficial impact of ET_A antagonism for some RTKIs.

It was notable that sitaxentan completely prevented the hypertension induced by both axitinib and lenvatinib. In addition to increasing ET-1, a number of other mediators have been implicated in the cardiovascular side-effects of RTKIs including reduced nitric oxide generation, changes in prostaglandin production and alterations in redox balance [33]. The results obtained in the present study suggest that in this rat model at least, ET-1 is the major mediator. In contrast, bosentan attenuated rather than fully blocked the increase in MAP induced by lenvatinib. It is possible that this lower affinity antagonist of ET_A did not reach sufficiently high receptor occupancies to achieve full antagonism. However, if we assume a plasma volume of *circa* 20 ml [51] for a 350–500 g rat and plasma binding levels of 98 % for bosentan [52] or 99.5 % for sitaxentan [53] then the estimated initial free plasma levels obtained in the present

Figure 8

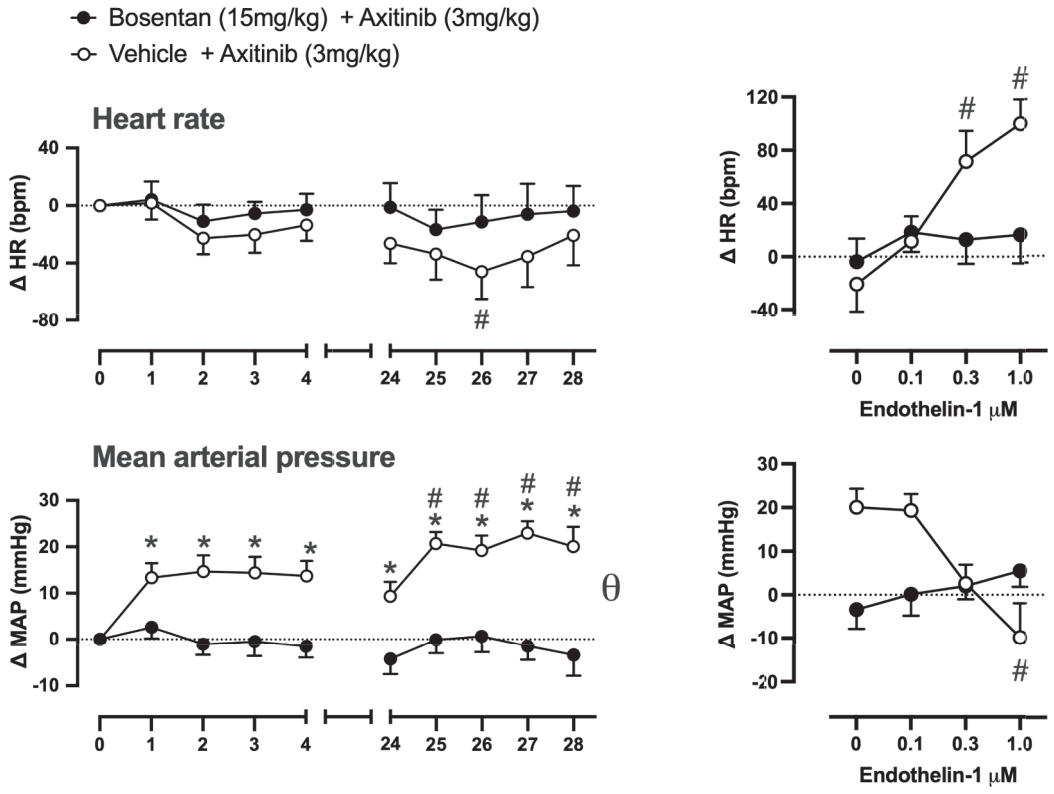

Fig. 8. Effect of the dual ET_A/ET_B receptor antagonist (bosentan) on axitinib-induced cardiovascular responses in conscious, freely moving rats. Animals were dosed with bosentan (15 $mg.kg^{-1}$) or vehicle as an i.v. bolus followed by a 5 h i.v. infusion. One hour after commencement of the endothelin receptor antagonist or vehicle infusion, axitinib (3 $mg.kg^{-1}$) was administered to all animals as an i.v. bolus followed by a 1 h i.v. infusion. The same dose regimen was repeated on day 2 and at the end of this ET-1 was administered as a bolus at increasing concentrations. Data points are means; vertical bars represent SEM. Friedman's test was performed for within-group comparisons to baseline (0 h time point was used as a baseline for the entire 28 h period prior to the administration of ET-1, while 28 h time point was used as a baseline for ET-1 dose–response (# $p < 0.05$ vs. baseline). Mann-Whitney *U* test was conducted between treated and vehicle control groups for a comparison of area under/over the curve (θ $p < 0.05$) and to determine differences at each time point (* $p < 0.05$, Mann-Whitney *U* test). 8 animals were treated with vehicle and 7 with bosentan.

study would be approximately 10 μM and 1 μM for bosentan and sitaxentan respectively. This leads to predicted receptor occupancies of 99.4 % and 99.1 % for bosentan at the ET_A and ET_B receptors respectively. Similarly, the predicted receptor occupancies for sitaxentan are 99.9 % for ET_A and 9.5 % for ET_B respectively. These data suggest that significant blockage of ET_B receptors by bosentan is contributing to the reduced effect on MAP for some RTKIs.

Following co-treatment of rats with endothelin receptor antagonists and either axitinib or lenvatinib, the increase in HR induced by subsequent i.v. infusion of ET-1 at the end of the experiment was completely attenuated by bosentan ($p < 0.05$) but not significantly affected by the ET_A selective antagonist sitaxentan. These data suggest that the tachycardia induced by ET-1 is a secondary reflex response to the vasodilator effects of ET_B activation on endothelial cells (which are the likely targets for i.v. infused ET-1) and furthermore indicate that there is antagonism of ET_B at the dose of bosentan used. Interestingly, the effect of ET-1 on MAP depended on the blood pressure at the start of the ET-1 infusion. Thus, in rats treated only with axitinib or lenvatinib, ET-1 predominantly caused a reduction in MAP (perhaps again indicative of an ET_B-mediated vasodilation) from the level elevated by the presence of the RTKI. In marked contrast, in animals treated with either bosentan or sitaxentan (where the MAP had been significantly lowered) there was no further reduction in MAP by application of ET-1.

In summary, the present study has confirmed that two potent inhibitors of VEGFR-2-signalling (axitinib and lenvatinib) can induce a significant increase in MAP that is accompanied by significant vasoconstriction within the mesenteric and hindquarters vascular beds. RVC was much less affected by these two RTKIs which may be a consequence of compensatory changes in the renal vasculature in response to the hypertensive conditions. These compensatory changes might be beneficial in order to maintain circulation to the kidney and delivery of drugs to renal cancer cells [45,46]. Data obtained with ET-1 receptor

Figure 9

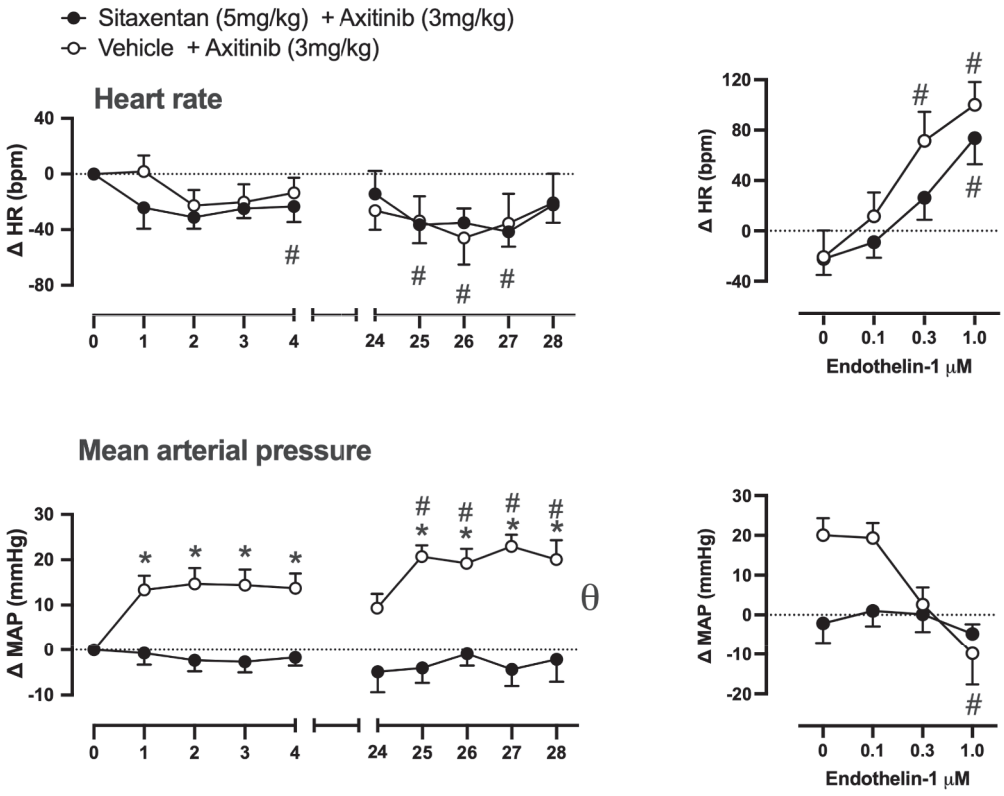

Fig. 9. Role of the selective ET_A receptor antagonist (sitaxentan) on axitinib-induced cardiovascular responses in conscious, freely moving rats. Animals were dosed with sitaxentan (5 $mg.kg^{-1}$) or vehicle as an i.v. bolus followed by a 5 h i.v. infusion. One hour after commencement of the endothelin receptor antagonist or vehicle infusion, axitinib (3 $mg.kg^{-1}$) was administered to all animals as an i.v. bolus followed by a 1 h i.v. infusion. The same dose regimen was repeated on day 2, after which ET-1 was administered as a bolus at increasing concentrations. Data points are means; vertical bars represent SEM. Friedman's test was performed for within-group comparisons to baseline (0 h time point was used as a baseline for the entire 28 h period prior to the administration of ET-1, while 28 h time point was used as a baseline for ET-1 dose–response (# $p < 0.05$ vs. baseline). Mann-Whitney *U* test was conducted between treated and vehicle control groups for a comparison of area under/over the curve (θ $p < 0.05$) and to determine differences at each time point (* $p < 0.05$, Mann-Whitney *U* test) 8 animals were treated with vehicle and 7 with sitaxentan.

Table 3
Baseline cardiovascular variables prior to administration of vehicle or dual ET_A and ET_B receptor antagonist (bosentan) or selective ET_A receptor antagonist (sitaxentan) in axitinib-treated rats. Values are mean ± SEM.

	Vehicle		Bosentan		Sitaxentan	
	Mean ± SEM	*n*	*Mean ± SEM*	*n*	*Mean ± SEM*	*n*
HR (beats.min^{-1})	358 ± 15	8	364 ± 17	7	372 ± 12	7
MAP (mmHg)	112 ± 5	8	116 ± 5	7	109 ± 6	7

antagonists have also shown that the hypertensive response to both axitinib and lenvatinib can be prevented by selective ET_A antagonism. Taken together, the results obtained in the present study indicate that treatment of patients with selective ET_A antagonists might prevent the development of the drug-induced hypertension that is a common side effect of both axitinib and lenvatinib. Although sitaxentan was removed from clinical use in 2010, highly selective antagonists for ET_A receptors such as clazosentan have been recently approved [54,55].

Author contributions

Conceived the study: Hill, Woolard.
Participated in research design: Pannucci, Woolard, Hill.
Conducted experiments: Pannucci, Wragg, Cooper, March, Groenen, Van Daele, Woolard.
Performed data analysis: Pannucci, Cooper, Woolard, Hill.
Wrote or contributed to the writing of the manuscript: All authors.

CRediT authorship contribution statement

Patrizia Pannucci: Writing – review & editing, Methodology, Investigation, Formal analysis. **Marieke Van Daele:** Writing – review & editing, Methodology, Investigation. **Samantha L. Cooper:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Edward S. Wragg:** Writing – review & editing, Methodology, Investigation. **Julie March:** Writing – review & editing, Resources, Methodology. **Marleen Groenen:** Writing – review & editing, Methodology. **Stephen J. Hill:** . **Jeanette Woolard:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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