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RESEARCH PAPER

Stimulation of angiotensin AT_2 receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats

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Background and purpose: Angiotensin type 2 receptor (AT_2 receptor) stimulation evokes vasodilator effects *in vitro* and *in vivo* that oppose the vasoconstrictor effects of angiotensin type 1 receptors (AT_1 receptors). Recently, a novel non-peptide AT_2 receptor agonist, Compound 21, was described, which exhibited high AT_2 receptor selectivity.
Experimental approach: Functional cardiovascular effects of the drug candidate Compound 21 were assessed, using mouse isolated aorta and rat mesenteric arteries *in vitro* and in conscious spontaneously hypertensive rats (SHR).
Key results: Compound 21 evoked dose-dependent vasorelaxations in aortic and mesenteric vessels, abolished by the AT_2 receptor antagonist, PD123319. *In vivo*, Compound 21 administered alone, at doses ranging from 50 to 1000 $ng{\cdot}kg^{-1}{\cdot}min^{-1}$ over 4 h did not decrease blood pressure in conscious normotensive Wistar-Kyoto rats or SHR. However, when given in combination with the AT_1 receptor antagonist, candesartan, Compound 21 (300 $ng{\cdot}kg^{-1}{\cdot}min^{-1}$) lowered blood pressure in SHR only. Further analysis in separate groups of conscious SHR revealed that, at a sixfold lower dose, Compound 21 (50 $ng{\cdot}kg^{-1}{\cdot}min^{-1}$) still evoked a significant depressor response in adult SHR (~30 mmHg) when combined with different doses of candesartan (0.01 or 0.1 $mg{\cdot}kg^{-1}$). Moreover, the Compound 21-evoked depressor effect was abolished when co-infused (50 $\mu g{\cdot}kg^{-1}{\cdot}min^{-1}$ for 2 h) with the AT_2 receptor antagonist PD123319.
Conclusion and implications: Collectively, our results indicate that acute administration of Compound 21 evoked blood pressure reductions via AT_2 receptor stimulation. Thus Compound 21 can be considered an excellent drug candidate for further study of AT_2 receptor function in cardiovascular disease.
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Keywords: Compound 21; angiotensin; angiotensin AT_2 receptor; spontaneously hypertensive rats; blood pressure; vascular function

Abbreviations: Ang II, angiotensin II; AT_1 receptor, angiotensin type 1 receptor; AT_2 receptor, angiotensin type 2 receptor; MAP, mean arterial pressure; SHR, spontaneously hypertensive rat; WKY rat, Wistar-Kyoto rat

Introduction

The octapeptide angiotensin II (Ang II) is the main biologically active mediator of the renin-angiotensin system and plays an important role in cardiovascular function by influencing vascular tone, structure, fluid and electrolyte balance via direct effects on endothelial and smooth muscle cells (Widdop *et al.*, 2003; Jones *et al.*, 2008). Two main receptor subtypes have been identified as binding sites for Ang II: angiotensin type 1 receptor (AT_1 recepter) and type 2 receptor (AT_2 receptor) (de Gasparo *et al.*, 2000); nomenclature follows Alexander *et al.*, 2008). Ang II has similar affinity for both AT_1 receptors and AT_2 receptors, whereas CGP42112 and PD123319 are the prototypical examples of an agonist and antagonist, respectively, at the AT_2 receptor subtype. On the other hand, compounds such as candesartan and losartan are selective AT_1 receptor antagonists that are used clinically for the treatment of hypertension. It is well established that most of the cardiovascular effects induced by Ang II, such as vasoconstriction, water and salt retention, are mediated via AT_1

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receptor (de Gasparo *et al*., 2000; Carey, 2005). In contrast, it has been suggested that the function of the AT_2 receptor is to counter-regulate AT_1 receptor-mediated actions, mainly based on experiments in which AT_2 receptor function was deduced from the effects of AT_2 receptor blockade, or altered responses in genetically modified animal models of AT_2 receptor over-expression or deletion. However, demonstration of AT_2 receptor-mediated effects, particularly in an *in vivo* setting, has been hampered by a lack of non-peptide AT_2 receptor selective agonists and antagonists that exhibit oral bioavailability.

In this context, Wan *et al*. (2004b) have recently described the first non-peptide, selective AT_2 receptor agonist, Compound 21 (*N*-butyloxycarbonyl-3-(4—imidazol-1-ylmethylphenyl)-5-isobutylthiophene-2-sulphonamide). Compound 21 was derived from a medicinal chemistry programme aimed at transforming the drug-like but non-selective AT_1 and AT_2 receptor agonist L-162313 (Wan *et al*., 2004a) into a selective AT_2 receptor agonist. Compound 21 exhibits a K_i value of 0.4 nM for the AT_2 receptor and a $K_i > 10\ \mu M$ for the AT_1 receptor. In addition, due to the non-peptide nature of the drug, Compound 21 has an estimated oral bioavailability of 20–30% and 4 h half-life in rats (Wan *et al*., 2004b). Compound 21 has been shown to induce neurite outgrowth in cell culture and to increase duodenal mucosal alkalinization in the rat via stimulation of MAPK and NO/cGMP signalling pathways (Wan *et al*., 2004b). Furthermore, Compound 21 decreased mean arterial blood pressure (MAP) in anaesthetized spontaneously hypertensive rats (SHR), although detailed and systematic evaluation of haemodynamic responses to Compound 21 were not performed in this earlier study.

AT_2 receptor-mediated relaxation is a well-established effect in isolated resistance vessels (Matrougui *et al*., 1999; Dimitropoulou *et al*., 2001; Widdop *et al*., 2002); conversely, there is less consensus regarding the influence of AT_2 receptors on blood pressure regulation *in vivo*. Studies using AT_2 receptor knockout mice support a role for AT_2 receptors in haemodynamic control, as these animals exhibit elevated basal blood pressure and enhanced sensitivity to the vasopressor effects of Ang II (Hein *et al*., 1995; Ichiki *et al*., 1995). Conversely, over-expression of AT_2 receptors in vasculature did not alter basal blood pressure, but markedly impaired Ang II-induced pressor activity (Tsutsumi *et al*., 1999). In conscious SHR, Ang II-mediated vasodilatation during AT_1 receptor blockade was not observed (Gohlke *et al*., 1998), presumably because the hypotensive effect of AT_2 receptor stimulation was masked by the concomitant, dominant AT_1 receptor-mediated pressor action during Ang II infusion. In order to avoid such confounding influences of AT_1 receptor stimulation on potential AT_2 receptor vasodilator function, we and others have also assessed the effect of selective AT_2 receptor agonists and antagonists during AT_1 receptor blockade. Using this approach, selective stimulation of AT_2 receptors by CGP42112 lowered blood pressure, provided that there was a background of AT_1 receptor blockade in conscious SHR (Wistar-Kyoto rat, WKY) (Barber *et al*., 1999), and Sprague-Dawley rats (Carey *et al*., 2001) in a PD123319-reversible manner. Furthermore, this blood pressure-lowering response to AT_2 receptor stimulation was shown to be associated with increased blood flow in renal, mesenteric and hindquarter circulations in conscious SHR suggesting widespread vasodilatation (Li and Widdop, 2004).

Therefore, in the current study we determined the effects of Compound 21 on blood pressure in conscious SHR and WKY rats, as well as in isolated vasculature. In addition, AT_2 receptor selectivity of Compound 21 was determined by simultaneous administration of the selective AT_2 receptor antagonist, PD123319, to determine whether or not these effects were AT_2 receptor-mediated.

Methods

Animals

All animal care and experimental procedures were approved by the Monash University Animal Ethics Committee and performed according to the guidelines of the National Health and Medical Research Council of Australia for animal experimentation.

Male 16- to 18-week-old SHR and WKY rats, weighing approximately 300 to 350 g and male 16-week-old FVB/N mice, weighing approximately 25–30 g were obtained from the Animal Resource Centre (Perth, WA, USA). Animals were maintained on a 12 h day/night cycle with standard laboratory rat or mice chow and water available *ad libitum*.

In vitro *reactivity*

Mice were killed by isoflurane inhalation followed by decapitation. The thoracic aorta was removed and cut transversely into ring segments for organ bath studies. Two stainless steel wires were threaded through the lumen of each aortic ring and the rings were then mounted and suspended in vertical 10 mL organ baths containing Krebs bicarbonate solution [composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.7; pH 7.4], which was maintained at 37°C and continuously bubbled with carbogen (95% oxygen and 5% CO_2). Isometric tension was continuously measured via a force transducer (Grass FT03) interfaced to a MacLab data acquisition device (ADInstruments, Sydney, Australia) displayed on a Macintosh computer. Aortic rings were set to 0.5 g resting tension and allowed to equilibrate for 90 min, during which time Krebs bicarbonate solution was changed every 15 min. After equilibration, 0.3 μM of the thromboxane A_2 receptor agonist, U46619, was used to obtain the maximum contractile response. Once a maximum response was determined, tissues were then washed with Krebs' solution and allowed to equilibrate for 30 min or until baseline had been reached.

Tissues were pre-contracted with U46619 to attain 30–40% of the maximum contractile response. In the first series of experiments, all vessels were pretreated with the AT_1 receptor antagonist, losartan (0.1 μM). Cumulative dose–response curves at log intervals to Compound 21 (1 pM to 1 μM) were performed in absence or presence of the AT_2 receptor antagonist PD123319 (0.1 μM). In a further series, AT_1 receptor blockade was omitted and cumulative dose-response curves to Compound 21 were performed in the absence and presence of either the NOS inhibitor, L-NAME (10 μM) or PD123319. A

parallel tissue served as a time control in which only U46619 was given. At the end of the experiment, 10 μM of the endothelium-independent vasodilator, sodium nitroprusside was added to the organ bath to test the integrity of the vascular smooth muscle cells.

In analogous studies, thoracic aortic rings from male SHR were set up at 2 g resting tension and a maximum response was obtained to 124 mM K+. After washing, tissues were pre-contracted with the α_1-adrenoceptor agonist phenylephrine to 30–40% of maximum K+ response and cumulative dose–response curves to either Ang II (in the presence of 0.1 μM candesartan) or Compound 21 in the presence or absence of candesartan were obtained.

Mesenteric artery

Male WKY rats, approximately 16 weeks of age, were killed by isoflurane inhalation followed by decapitation and the gut was removed in order to dissect 3 to 5 mm long sections of the third order branch from mesenteric artery. The arterial sections were cannulated at both ends and mounted in a video-monitored perfusion system (Living Systems Instrumentation, Burlington, VT, USA), as previously described (Matrougui *et al.*, 1999; Loufrani *et al.*, 2001; Widdop *et al.*, 2002). Mesenteric artery sections were bathed in 20 mL organ baths that contained Krebs solution to which the AT_1 receptor antagonist, candesartan (1 μM) was added. The solution was bubbled with carbogen (95% O_2 and 5% CO_2), with temperature maintained at 37°C and the pH at 7.4. The arterial sections were superfused at a rate of 4 mL·min^{-1} and perfused at a rate of 100 μL·min^{-1}. The intraluminal pressure was set at 75 mmHg. The diameter of the arterial sections was constantly measured and recorded with a video-monitoring system. Following an equilibration period of approximately 30 min, phenylephrine was added to achieve 20–30% of the maximum contractile response. Once the plateau was reached, a concentration response curve to Compound 21 (0.1 nM to 1 μM) was constructed. Analogous experiments were performed in which the AT_2 receptor antagonist, PD123319 (1 μM) was added 30 min before Compound 21. An additional tissue was set up that served as a time control and was only pre-contracted with phenylephrine.

In vivo *procedures*

Rats were anaesthetized (ketamine and xylazine; 75 mg·kg^{-1} and 10 mg·kg^{-1}, i.p., respectively; supplemented as required). Two catheters were inserted into the right jugular for i.v. drug administration. A catheter was inserted into the right carotid artery for direct blood pressure measurement as described previously (Barber *et al.*, 1999; Li and Widdop, 2004; Walters *et al.*, 2005). Rats were housed in individual cages and allowed free access to food and water while maintained on 12 h day/night cycle. The arterial catheter was infused overnight with heparinized saline using an infusion pump.

Twenty-four hours after the surgery, the arterial catheter was attached to a pressure transducer (Gould Inc., Eichstetten, Germany), connected to a MacLab-8 data acquisition system (ADInstruments) and interfaced to a Macintosh computer. Mean arterial pressure (MAP) and heart rate were computed from the phasic blood pressure signal.

Experimental protocol

Rats received drug combinations in a randomized fashion over a 4 or 5 day protocol, as described previously (Barber *et al.*, 1999; Walters *et al.*, 2005). Doses of candesartan and PD123319 were chosen on the basis of previous studies (Barber *et al.*, 1999; Walters *et al.*, 2005). Animals in group 1 (WKY; dose-ranging) and group 2 (SHR; dose-ranging) were randomized to receive following treatments: (i) a 4 h Compound 21 infusion (50, 100 or 300 ng·kg^{-1}·min^{-1} in WKY rats or 100, 300 and 1000 ng·kg^{-1}·min^{-1} in SHR); and (ii) a 4 h Compound 21 infusion (50 and 300 ng·kg^{-1}·min^{-1} in WKY rats or 300 and 1000 ng·kg^{-1}·min^{-1} in SHR) given simultaneously with candesartan (0.1 mg·kg^{-1} i.v.).

Based on the dose-ranging results, additional SHR (group 3) received the following treatments in randomized fashion: (i) candesartan (0.1 mg·kg^{-1} i.v.); (ii) Compound 21 infusion (50 ng·kg^{-1}·min^{-1} for 4 h); (iii) a 4 h Compound 21 infusion together with candesartan; and (iv) a 4 h Compound 21 infusion in the presence of candesartan and PD123319 infusion (50 μg·kg^{-1}·min^{-1} for 2 h). The dose of PD123319 was based on our previous experience using this compound in similar *in vivo* experiments (Barber *et al.*, 1999; Li and Widdop, 2004). In analogous experiments in separate SHR (group 4), an identical protocol was repeated to that of group 3 but a 10-fold lower dose of candesartan (0.01 mg·kg^{-1} i.v.) was used. We have previously shown that basal BP recordings are stable over these time periods. Nevertheless, in this latter group, SHR also received a 4 h infusion (0.1 mL·kg^{-1}·h^{-1} i.v.) of saline (0.9% NaCl) to confirm a lack of effect on MAP.

Statistical analysis

All data are presented as mean responses ± standard error of the mean (SEM). Differences in vasorelaxation or MAP between treatments were analysed using a two-way repeated measure, ANOVA. Statistical analysis was performed using GraphPad Prism (Version 5.0). *P*-values <0.05 were considered statistically significant.

Materials

Compound 21 was provided by A Hallberg, Department of Medicinal Chemistry, Uppsala University; PD123319 and candesartan were kind gifts from Pfizer and AstraZeneca respectively. All other chemicals were purchased from commercial sources: L-NAME (Sigma), sodium nitroprusside (Sigma, Sydney, Australia), ketamine (Troy Laboratories, Sydney, Australia), xylazine (Troy Laboratories), phenylephrine (Sigma), isoflurane (Baxter, Deerfield, IL, USA) and U46619 (Saphire Bioscience, Sydney, Australia).

Results

In vitro *relaxation evoked by Compound 21*

Compound 21 caused a dose-dependent relaxation of mouse aorta in the presence of AT_1 receptor blockade, which was

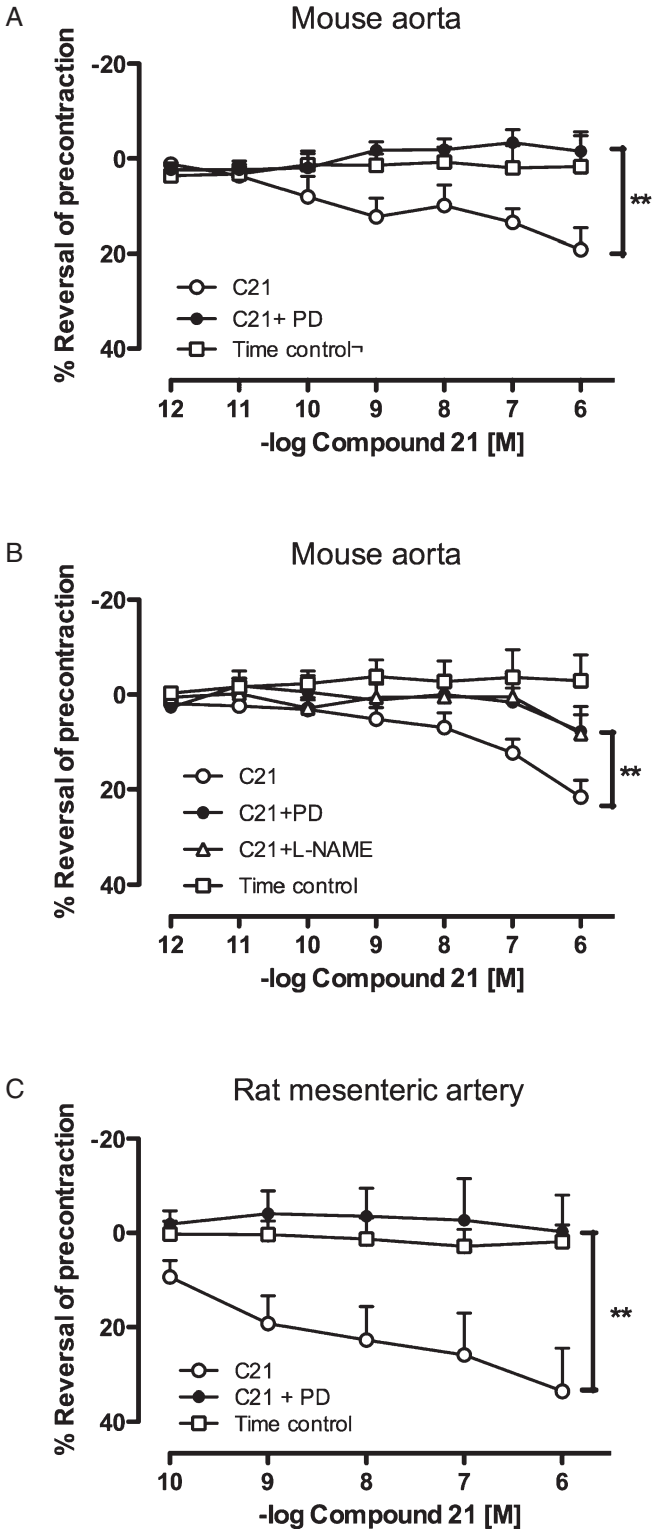

Figure 1 Dose–response curves to Compound 21 (C21) performed in (A) mouse aorta and (C) third order perfused rat mesenteric artery in the absence (n = 5 and 9 for aorta and mesenteric artery respectively) and presence (n = 5 and 3 for aorta and mesenteric artery respectively) of PD123319 (PD); all experiments in Figure 1A and C performed in the presence of angiotensin type 1 receptor (AT_1 receptor) blockade, as described in the *Methods*. (B) Effect of Compound 21 in mouse aorta in the absence of AT_1 receptor blockade and in the presence of PD123319 or L-NAME (n = 10 for each). Values represent mean ± SEM. $^{**}P < 0.01$ for treatment effect between Compound 21 and Compound 21 + PD123319 or Compound 21 + L-NAME (two-way repeated measures ANOVA).

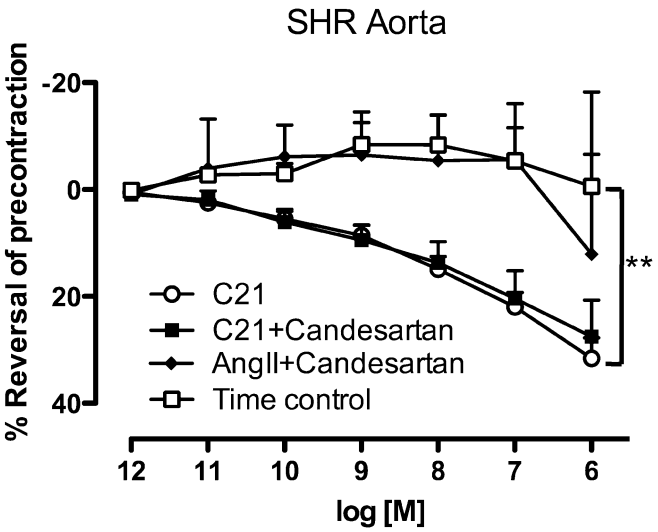

Figure 2 Dose–response curves to either angiotensin II (Ang II) or Compound 21 (C21) performed in aorta obtained from naive spontaneously hypertensive rat (SHR) in the absence (Compound 21) and presence (Ang II & Compound 21) of candesartan (0.1 μM) (n = 5 for each). Values represent mean ± SEM. $^{**}P < 0.01$ for treatment effect between Compound 21 and time control (two-way repeated measures ANOVA).

markedly inhibited by PD123319 (Figure 1A). Furthermore, Compound 21-evoked relaxation was also evident in the absence of AT_1 receptor blockade and was abolished by L-NAME (Figure 1B). In addition, Compound 21 caused vasodilatation in third order perfused rat mesenteric artery, which was also blocked by PD123319 (Figure 1C).

Experiments were also performed using aortic rings obtained from naive SHR, a preparation that is reported to be unresponsive to the AT_2 receptor-mediated vasorelaxant effects of Ang II (Cosentino *et al.*, 2005). Indeed, Ang II did not cause relaxation; on the other hand, Compound 21 evoked dose-dependent relaxation that was similar in the presence or absence of AT_1 receptor blockade (Figure 2).

In vivo effect of Compound 21 in conscious rats

Basal MAP of SHR over the four or five experimental days for each group are listed in the Table 1. There was no significant difference between resting MAP over the experimental period for any of the treatment groups, suggesting that none of the acute treatments had effects that lasted more than 24 h, and therefore did not influence baseline MAP on subsequent days.

In both WKY (Figure 3) and SHR (Figure 4), infusion of Compound 21 alone, at doses ranging from 50 to 300 $ng \cdot kg^{-1} \cdot min^{-1}$, had no significant effect on MAP. Combined administration of Compound 21 (100 and 300 $ng \cdot kg^{-1} \cdot min^{-1}$) and candesartan (0.1 $mg \cdot kg^{-1}$) also had no effect on MAP in WKY rats (Figure 3); however, the

Table 1 Resting mean arterial pressure (MAP) of spontaneously hypertensive rat recorded on separate days before drug treatments, as indicated

Treatment	*MAP (mmHg)*
Group 2 (n = 5)	
Compound 21 (100 ng·kg^{-1}·min^{-1})	174 ± 8
Compound 21 (300 ng·kg^{-1}·min^{-1})	177 ± 8
Compound 21 (1000 ng·kg^{-1}·min^{-1})	165 ± 5
Compound 21 (300 ng·kg^{-1}·min^{-1}) & candesartan (0.1 mg·kg^{-1})	164 ± 8
Compound 21 (1000 ng·kg^{-1}·min^{-1}) & candesartan (0.1 mg·kg^{-1})	163 ± 5
Group 3 (n = 7)	
Compound 21 (50 ng·kg^{-1}·min^{-1})	178 ± 6
Candesartan (0.1 mg·kg^{-1})	191 ± 6
Compound 21 & candesartan	190 ± 7
Compound 21, candesartan & PD123319 (50 µg·kg^{-1}·min^{-1})	177 ± 7
Group 4 (n = 7)	
Saline	175 ± 6
Compound 21 (50 ng·kg^{-1}·min^{-1})	179 ± 7
Candesartan (0.01 mg·kg^{-1})	184 ± 8
Compound 21 & candesartan	190 ± 7
Compound 21, candesartan & PD123319 (50 µg·kg^{-1}·min^{-1})	170 ± 10

The values shown in the Table are means ± SEM. n = 5–7 per group.

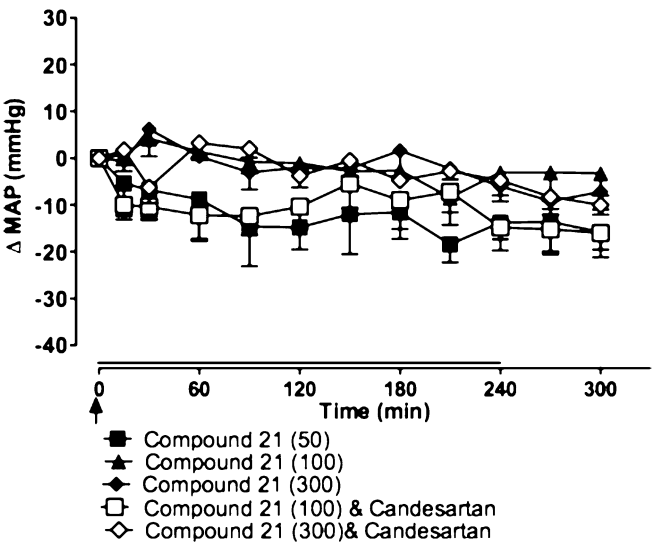

Figure 3 Effects of Compound 21 (50, 100 and 300 ng·kg^{-1}·min^{-1}), administered as a 4 h infusion (shown by horizontal line), on mean arterial pressure (MAP) in Wistar-Kyoto rats (n = 5). Compound 21 was given in the presence or absence of the angiotensin type 1 receptor antagonist, candesartan (0.1 mg·kg^{-1} i.v. bolus; shown by an arrow). Values represent mean ± SEM.

combination of Compound 21 (300 ng·kg^{-1}·min^{-1}) and candesartan, significantly decreased MAP in SHR compared with Compound 21 alone (P < 0.001) (Figure 4). Interestingly, at the highest dose tested (1000 ng·kg^{-1}·min^{-1}), Compound 21 alone caused an increase in MAP in SHR (P < 0.05), suggesting a lack of selectivity of Compound 21 at this dose. This Compound 21-mediated pressor effect was attenuated by simultaneous AT_1 receptor blockade (Figure 4).

Given the lack of response to Compound 21 in WKY rats, further examination of the effect of Compound 21 on MAP

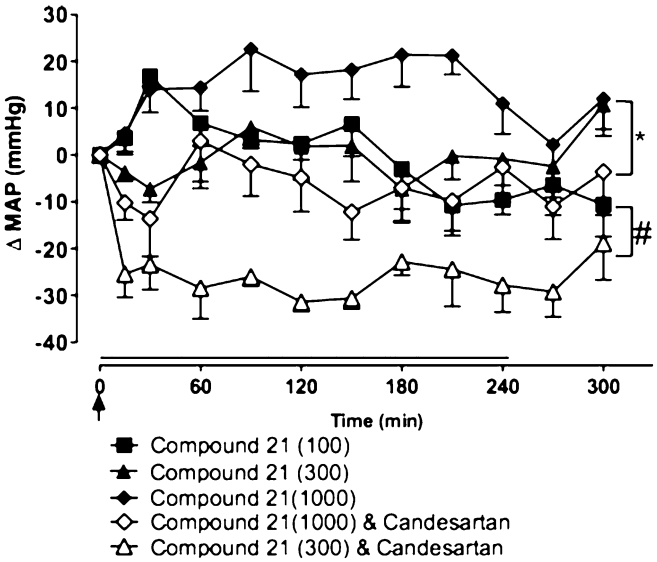

Figure 4 Effects of Compound 21 (100, 300 and 1000 ng·kg^{-1}·min^{-1}), administered as a 4 h infusion (shown by horizontal line), on mean arterial pressure (MAP) in spontaneously hypertensive rats (n = 5). Compound 21 was given in the presence or absence of the angiotensin type 1 receptor agonist, candesartan (0.1 mg·kg^{-1} i.v. bolus, shown by an arrow). Values represent mean ± SEM. #P < 0.001 for treatment effect between Compound 21 (300 ng·kg^{-1}·min^{-1}) + candesartan and Compound 21(300 ng·kg^{-1}·min^{-1}) (two-way repeated measures ANOVA); *P < 0.05 for treatment effect between Compound 21(1000 ng·kg^{-1}·min^{-1}) + candesartan and Compound 21(1000 ng·kg^{-1}·min^{-1}) (two-way repeated measures ANOVA).

was performed in separate groups of SHR, to determine the effects of lower dose of Compound 21 in combination with AT_1 receptor block. As had been previously determined, Compound 21 infusion alone (50 ng·kg^{-1}·min^{-1}) had no effect on MAP in SHR (Figure 5). However, when combined with either high-dose (0.1 mg·kg^{-1}; Figure 5) or low-dose (0.01 mg·kg^{-1}; Figure 6) candesartan, Compound 21 caused a significant reduction in MAP in SHR (P < 0.001). Importantly, when the AT_2 receptor antagonist, PD123319 (50 µg·kg^{-1}·min^{-1}), was co-infused for 2 h with Compound 21 and candesartan, this blood pressure-lowering effect was abolished, indicating AT_2 receptor selectivity of Compound 21 (P < 0.001). Furthermore, infusion of saline had no effect on blood pressure (Figure 6).

Discussion

The main finding of the current study was that the novel non-peptide AT_2 receptor agonist, Compound 21, evoked vasorelaxation *in vitro*, which translated into vasodepressor responses in conscious SHR against a background of AT_1 receptor blockade. To our knowledge, this study represents the first systematic study of the vascular effects of Compound 21, particularly in hypertension.

Compound 21 exhibits a similar binding profile at AT_2 receptors to that of Ang II and CGP42112 but with little affinity for AT_1 receptors. In mouse aortic rings, Compound 21, in the presence of AT_1 receptor antagonists, caused concentration-dependent vasorelaxation that was inhibited

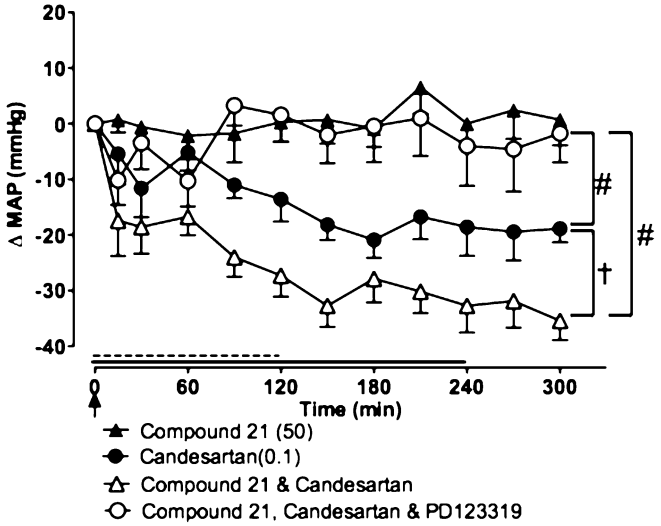

Figure 5 Effect of Compound 21 (50 ng·kg^{-1}·min^{-1}; 4 h infusion shown by full line), high-dose candesartan (0.1 mg·kg^{-1} bolus i.v.; shown by an arrow), Compound 21 + candesartan and Compound 21 + candesartan + PD123319 (50 μg·kg^{-1}·min^{-1} for 2 h; (dashed line), on mean arterial pressure (MAP) in spontaneously hypertensive rat ($n = 7$). Values represent mean ± SEM. #$P < 0.001$ for overall effect of treatment versus Compound 21 (two-way repeated measures ANOVA); †$P < 0.01$ for treatment effect between Compound 21 + candesartan and candesartan (two-way repeated measures ANOVA).

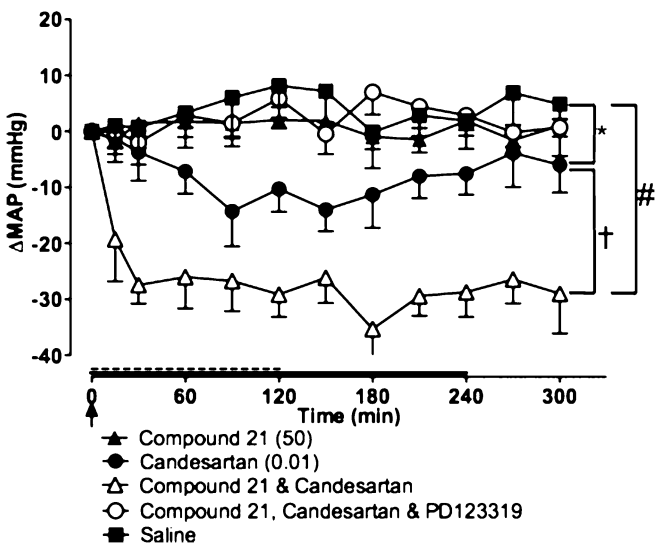

Figure 6 Effect of Compound 21 (50 ng·kg^{-1}·min^{-1}; 4 h infusion shown by full line), low-dose candesartan (0.01 mg·kg^{-1} bolus i.v.; shown by an arrow), Compound 21 + candesartan and Compound 21 + candesartan + PD123319 (50 μg·kg^{-1}·min^{-1} for 2 h; shown by dashed line), on mean arterial pressure (MAP) in spontaneously hypertensive rat ($n = 7$). Values represent mean ± SEM. *$P < 0.05$ for treatment effect between Compound 21 and candesartan; #$P < 0.001$ for overall effect of individual treatment versus Compound 21 (two-way repeated measures ANOVA); †$P < 0.01$ for treatment effect between Compound 21 + candesartan and candesartan alone (two-way repeated measures ANOVA).

by the AT_2 receptor antagonist, PD123319. This *in vitro* effect was not dependent on background AT_1 receptor blockade, as it was *in vivo*, and was also inhibited by L-NAME. Thus, Compound 21 elicited classical AT_2 receptor-mediated NO signalling, a hallmark of AT_2 receptors in vascular tissue (Widdop *et al.*, 2003; Jones *et al.*, 2008). This compound was also tested using rat isolated mesenteric arteries, a preparation well recognized as exhibiting AT_2 receptor-mediated vasorelaxation (Matrougui *et al.*, 1999; Henrion *et al.*, 2001; Widdop *et al.*, 2002). Indeed in these resistance-like vessels, Compound 21 caused vasorelaxation that was inhibited by PD123319, confirming selective AT_2 receptor-mediated actions of the compound.

In the present study, dose–response analysis was performed in conscious SHR and WKY rats in which multiple doses were tested in the same animals on different days, allowing within-animal analysis. When tested over a wide dose range, Compound 21 alone did not reduce MAP in SHR or WKY rats except during AT_1 receptor blockade in SHR, as seen previously with CGP42112 (Barber *et al.*, 1999; Li and Widdop, 2004). It is likely that these results can be explained by the fact that circulating endogenous Ang II itself exerts tonic AT_1 receptor-mediated vasoconstriction that, once removed, allows AT_2 receptor-mediated vasodilatation to be manifest. These data are consistent with previous studies in conscious SHR in which Ang II did not cause vasodilatation during AT_1 receptor blockade (Gohlke *et al.*, 1998), presumably because the hypotensive effect of AT_2 receptor stimulation was masked by the concomitant, dominant AT_1 receptor-mediated pressor action during Ang II infusion. A differential effect on vascular tone to AT_2 receptor stimulation has also been noted by others using SHR and WKY rats (Savoia *et al.*, 2005), and is consistent with a lack of effect of Compound 21 on blood pressure in anaesthetized normotensive rats (Wan *et al.*, 2004b). This lack of effect of Compound 21 on BP in normotensive animals may relate to the fact that subtle AT_2 receptor-mediated depressor responses are more easily observed from a higher basal blood pressure. Alternatively, it may represent strain-dependent differences in sensitivity to AT_2 receptor stimulation or drug-induced changes in vascular AT_2 receptor expression. In this context, aortic AT_2 receptor expression is higher in adult SHR compared with age-matched WKY, whereas mesenteric AT_2 receptor expression is increased in young SHR but decreased in adult SHR (see Widdop *et al.*, 2008).

Limited *in vivo* studies in anaesthetized SHR implied a role of this compound on vascular tone as, when given as bolus i.v. injections to anaesthetized SHR, Compound 21 lowered BP (Wan *et al.*, 2004b). The discrepancy between the two studies most likely reflects the different experimental designs between the current and previous studies (Wan *et al.*, 2004b). The highest effective dose (0.05 mg·kg^{-1}) of Compound 21 previously tested (Wan *et al.*, 2004b) was probably higher than our maximally effective depressor dose achieved during a 4 h infusion (i.e. 300 ng·kg^{-1}·min^{-1} ~ 0.072 mg·kg^{-1} total; during AT_1 receptor blockade), once half-life and pharmacokinetic considerations are taken into account, although it should be noted that a higher dose of Compound 21 (1000 ng·kg^{-1}·min^{-1}) alone also did not decrease blood pressure. Another important difference is that the previous study (Wan *et al.*, 2004b) tested barbiturate-anaesthetized SHR, which are less physiological than consciously instrumented SHR that would be more able to buffer potential blood pressure reductions by homeostatic reflex mechanisms. Moreover,

we were keen to be able to make direct comparisons with previous studies that also reported AT_2 receptor-mediated depressor effects of the selective agonist CGP42112 during AT_1 receptor blockade (Barber *et al.*, 1999; Li and Widdop, 2004).

Curiously, AT_2 receptor stimulation does not generally cause vasorelaxation in vessels isolated from SHR strains (Matrougui *et al.*, 2000; Cosentino *et al.*, 2005; Savoia *et al.*, 2005; You *et al.*, 2005). Chronic treatment with AT_1 receptor antagonists is associated with increased AT_2 receptor expression in aortae from SHR (Cosentino *et al.*, 2005; Savoia *et al.*, 2005), and human subcutaneous gluteal arteries (Savoia *et al.*, 2007), as well as in mesenteric arteries from SHR, which normally exhibited decreased AT_2 receptor expression under basal conditions (You *et al.*, 2005). Indeed, sartan-induced up-regulation of AT_2 receptors unmasked *ex vivo* AT_2 receptor-mediated vasorelaxation in otherwise unresponsive vessels (Yayama *et al.*, 2004; Cosentino *et al.*, 2005; Savoia *et al.*, 2005; Savoia *et al.*, 2007). Therefore, it is conceivable that enhanced *in vivo* sensitivity of untreated SHR, compared with WKY rats, to Compound 21 was due to higher AT_2 receptor expression, although elevated MAP *per se* could still contribute to AT_2 receptor-mediated depressor activity *in vivo*. In any case, whether or not *ex vivo* AT_2 receptor-mediated relaxation evoked by Compound 21 is more manifest following chronic treatment with an AT_1 receptor antagonist awaits further investigation. However, we did test the *in vitro* effects of Compound 21 acutely in naive SHR. As expected, Ang II did not evoke vasorelaxation whereas, strikingly, Compound 21 relaxed aortae. Thus, in aortic tissue known to be refractory to acute AT_2 receptor-mediated effects of Ang II (Cosentino *et al.*, 2005; Savoia *et al.*, 2005), Compound 21 caused vasorelaxation, in SHR, both *in vitro* and *in vivo*, at least in the presence of AT_1 receptor blockade. Thus, the current study highlights the importance of using subtype selective compounds.

At the highest dose tested (1000 $ng \cdot kg^{-1} \cdot min^{-1}$), Compound 21 alone actually increased MAP in SHR, most likely representing a lack of AT_2 receptor selectivity at this concentration. Although the sensitivity of this pressor effect of Compound 21 to blockade by PD123319 was not tested in the current study, simultaneous AT_1 receptor inhibition restored MAP responses to baseline. This finding could indicate that Compound 21 caused AT_1 receptor stimulation at higher doses, which may relate to the higher AT_1/AT_2 receptor ratio in vasculature, or that the hypotensive effect of candesartan offset Compound 21-mediated vasoconstriction via other mechanisms. The fact that a lower dose of Compound 21 (300 $ng \cdot kg^{-1} \cdot min^{-1}$), in combination with candesartan, significantly lowered MAP in these same SHR makes it likely that a more selective AT_2 receptor vasodilator effect was manifest at doses <1000 $ng \cdot kg^{-1} \cdot min^{-1}$. Similarly, pressor doses of Ang II infused in the presence of AT_1 receptor blockade do not always reduce blood pressure (Gohlke *et al.*, 1998), most likely for the same reasons of opposing vascular effects of AT_1 and AT_2 receptor stimulation (Barber *et al.*, 1999; Li and Widdop, 2004).

When infused at a sixfold lower dose, the depressor effect of Compound 21 (50 $ng \cdot kg^{-1} \cdot min^{-1}$) was similar in groups concomitantly administered either high- or low-dose candesartan, suggesting that the maximum achievable fall in MAP had been reached. By contrast, using CGP42112, we did not find additional depressor effects when combined with high-dose candesartan (Barber *et al.*, 1999), which may reflect a difference in metabolic fate of these two compounds as Compound 21 is a non-peptide compound. The additive effect of Compound 21 and candesartan may also reflect the fact that sartan-induced elevation in Ang II levels was probably not maximal. In any case, we have found that AT_2 receptors do not functionally desensitize even in the face of raised Ang II levels (Widdop *et al.*, 2002), therefore the non-peptide AT_2 receptor agonist may exert a prolonged effect. Importantly, in these same animals, we also tested PD123319, which completely abolished the depressor effect of combined Compound 21 and candesartan; consistent with the AT_2 receptor selectivity demonstrated by both the current *in vitro* data and radioligand binding assays performed with this compound (Wan *et al.*, 2004b). Interestingly, although tested in separate animal groups, it also appeared that there was no difference in the maximal Compound 21-mediated depressor effect using either 50 or 300 $ng \cdot kg^{-1} \cdot min^{-1}$. In this context, a bell-shaped dose–response relationship for the effect of Compound 21 on BP was also reported by Wan *et al.* (2004b), who found that the depressor effect of bolus Compound 21 administration in anaesthetized SHR was present at lower doses, but lost at higher doses (>0.05 $mg \cdot kg^{-1}$).

Collectively, these data implicate the AT_2 receptor as a potential target for the treatment of hypertension, although until now, there have been no drug-like candidates available to directly test this premise. In this context, the effects of Compound 21 have recently been reported in the setting of myocardial infarction. In that study, Compound 21, given for 7 days after myocardial infarction, improved systolic and diastolic function and reduced infarct size (Kaschina *et al.*, 2008), thus illustrating the potential use of this non-peptide compound in a number of cardiovascular settings. Indeed, our current findings suggest additive effects of AT_1 receptor blockade and AT_2 receptor stimulation would be beneficial for BP reduction, and fit with clinical findings of increased vascular AT_2 receptor expression after long-term sartan treatment (Savoia *et al.*, 2007), highlighting the need for future determination of the chronic effects of Compound 21 in hypertensive settings.

In conclusion, we have established that Compound 21 evoked vasorelaxation in mouse and SHR aortae or rat mesenteric arteries, and vasodepressor responses in conscious SHR, via AT_2 receptor stimulation. The BP-lowering effect of Compound 21 was additive to candesartan when the latter compound was given at a dose that itself lowered BP. Further studies are warranted on the chronic effects of Compound 21, alone and in combination with AT_1 receptor antagonists, in hypertensive-related diseases. These studies implicate the AT_2 receptor as a potential therapeutic target in the setting of hypertension and related cardiovascular diseases.

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Conflicts of interest

None.

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