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Impact of the AT_2 Receptor Agonist C21 on Blood Pressure and Beyond

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Abstract It is now widely accepted that the angiotensin AT_2 receptor (AT_2R) plays an important protective role during pathophysiologic conditions, acting as a repair system. The development of the first selective nonpeptide AT_2R agonist C21 accelerated our understanding of AT_2R-mediated protective signaling and actions. This article reviews the impact of C21 on blood pressure in normotensive and hypertensive animal models. Although C21 does not act as a classical antihypertensive drug, it could be useful in preventing hypertension-induced vascular and other end organ damages *via* anti-apoptotic, anti-fibrotic and anti-inflammatory actions. In particular, a strong body of evidence started to emerge around its anti-inflammatory feature. This property should be further investigated for potential clinical indications in cardiovascular diseases and beyond.

Keywords Hypertension · Blood pressure · Angiotensin · AT_2R · Renin-angiotensin system · RAS · Compound 21 · C21 · Inflammation · Antihypertensive drug therapy

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Introduction

The renin angiotensin system (RAS) plays a key role in blood pressure regulation. Its actions have been described as mostly dependent on stimulation of the Ang II receptor type 1 (AT_1R). The development of AT_1R blockers (ARB) and other receptor subtype-specific ligands in the 1990s revealed a second Ang II receptor subtype, the angiotensin AT_2 receptor (AT_2R) [1]. Since this discovery, several research groups have contributed to improving our understanding of this 'enigmatic' receptor [2]. It is now widely recognized that the AT_2R receptor exerts actions opposing those of the AT_1R, such as vasodilation, anti-proliferation, cell differentiation and anti-inflammation [3–7]. Although all mechanisms are still not fully deciphered, a picture of AT_2R signaling has emerged with three major transduction mechanisms: (1) activation of the NO/cGMP pathway, (2) activation of a series of protein phosphatases and (3) activation of phospholipase A2 inducing the release of arachidonic acid [8]. All or some of these signaling cascades seem to be initiated by various proteins binding to the C-terminal end of the AT_2-R such as the "AT_2-R binding proteins" (ATIP or ATBP, [9, 10•]), the homeodomain enhancer protein and Zfhep, and the transcription factor PLZF (promyelocytic leukemia zinc finger, [11, 12]). These pathways have to be further investigated, especially with regard to their importance in anti- or pro-proliferative and cellular differentiation actions of the AT_2R (see [6, 13•] for review of AT_2R signaling).

The AT_1R-opposed actions of the AT_2R are not only determined by specific signaling pathways but also by the levels of AT_2R expression in a given tissue. Early evidence for this was provided by Gohlke et al. in 1998 [14] and Pees et al. [15]. These authors demonstrated that AT_2R stimulation in the aorta of adult SHR-SP was responsible for activation of the bradykinin/NO/GMPc pathway [14]. In contrast, they did not find any evidence for such

AT_2R-mediated NO production in the vascular wall of WKY rats [15]. What seems to be a discordance between these two rat strains can indeed be explained by a significantly higher expression level of AT_2R in SHR compared to WKY [16]. In fact, the AT_1R/AT_2R ratio seems to be modified according to a given pathophysiologic state [17, 18]. In particular, AT_2R expression is upregulated in tissue injury [19, 20], suggesting this could constitute a protective system during pathophysiologic processes.

In this context, exploiting the therapeutic potential of the 'protective arm' of the RAS, to which the AT_2R as well as ACE2 and the Ang1-7/Mas receptor system belong [21], became a focus of interest with respect to drug development. The AT_2R thus became a potential therapeutic target, and synthesis of compounds stimulating the AT_2R was initiated. Compound 21 (C21) evolved from these efforts as the first selective nonpeptide AT_2R agonist [22].

Before the advent of C21, research on AT_2R functions was conducted either *via* indirect stimulation with Ang II in the presence of an ARB, *via* blockade of AT_2R function with an AT_2R antagonist or by elimination of AT_2R function using AT_2R knockout animals. The only early compound to directly stimulate the AT_2R was the peptide CGP42112A. Unfortunately, due to its peptidic nature, its use *in vivo* was limited. Moreover, CGP42112A also features antagonistic properties at low concentrations [4]. The synthetic AT_2R agonist, C21, thus constitutes the first pharmacokinetically unproblematic tool for the direct study of AT_2R functions and the first AT_2R agonist with drug-like properties. As a result, several experimental studies using C21 have been performed. With the background provided by preclinical studies since 2004 [22] and a currently ongoing toxicological program, it is anticipated that C21 is soon going to enter a clinical phase I study. In this article, we will summarize the important properties of C21 identified up to now.

Compound 21 and Blood Pressure

Since stimulation of AT_2R induces vasodilation in isolated vessels and production of NO, one might reasonably think that administration of C21 would generate a decrease in blood pressure (BP). However, as summarized in Table 1, stimulation of AT_2R *in vivo* does not yield a hypotensive effect – at least not acutely.

In fact, C21 alone did not decrease blood pressure when administered in normotensive animals [22, 23••, 24, 25]. Moreover, in animal models of hypertension, regardless of the type of hypertension (genetic or induced), C21 did not provide any antihypertensive action either [23••, 26•, 27•, 28•, 29]. A decrease in BP was only described in the original publication about C21 synthesis and design [22]. In this study, a decrease in BP was observed following an acute i.v. infusion of C21 in SHR, but this was observed in anesthetized animals in which anesthesia may have hampered baroreflex control of BP. Thus, current evidence does not reveal any acute antihypertensive action of C21 in conscious animals, despite the diversity of treatment dosage, duration and route of administration tested (Table 1).

However, a potential antihypertensive effect of C21 alone may not be easily detectable *in vivo* because of a predominant AT_1R-dependent angiotensinergic tone. Thus, in order to observe effects resulting from AT_2R stimulation, it may be necessary to block AT_1R. In fact, when combined with a low-dose ARB that does not modify BP, C21 exerted an antihypertensive action [23••]. This is in agreement with previous results obtained with CGP42112A [30–32]. When C21 was combined with a high dose of an ARB, which induces an antihypertensive effect, no additive effect of C21 on BP was observed [27•, 28•].

Although stimulation of AT_2R with the AT_2 agonist C21 does not engender direct, acute antihypertensive effects, a secondary reduction of BP may occur because of favorable effects of chronic C21 administration on vascular remodeling and kidney function. A trend towards a reduction of BP has indeed been observed in models of renal hypertension [26•], L-NAME induced hypertension [28•] and diabetic SHR-SP (unpublished observation), but these effects did not reach statistical significance.

Since the decrease in BP by AT_2R-stimulation is only minor or in some studies not detectable at all, favorable effects of C21 in models of hypertensive end organ damage can be regarded as mainly or entirely BP independent. For example, Rehman et al. highlighted a reduction in vascular stiffness by C21 treatment in the aorta but also in mesenteric resistance arteries from hypertensive rats (SHR-SP) independently of any BP reduction [27•]. Moreover, our group has recently demonstrated that C21 treatment prevents the development of hypertension-induced aortic remodeling and accelerated pulse-wave velocity in L-NAME hypertensive rats without significantly changing BP [28•]. The C21-induced reduction of stiffness of mesenteric resistance arteries observed in the study by Rehman et al. as well as attenuated aortic stiffness in our study could in both cases be explained by a decrease in extracellular matrix components, collagen content and fibronectin. This may limit the increase in vascular resistance and, subsequently, the progression of hypertension as well as end-organ damage concomitant with chronic hypertension. Furthermore, at the kidney level, AT_2R stimulation with C21 produced vasodilatory and natriuretic effects and may therefore also improve renal function [24]. Despite the absence of direct antihypertensive action, C21 could thus be a useful additional tool in the long-term management of hypertension.

Table 1 Effect of C21 on blood pressure. Effect of C21 alone, associated with a high antihypertensive dose or a low non-antihypertensive dose of angiotensin receptor blocker (ARB) on blood pressure of normotensive, hypertensive or other pathologic animal models. C21 dosage, route and treatment duration are detailed

Animal model	C21 dosage	Route	Duration	Anesthetized animal	Effect on BP	Ref.
C21 alone						
S-D	0.05 mg/kg	i.v.	acute	Yes	No	[22]
SHR	0.05 mg/kg	i.v.	acute	Yes	↓	[22]
WKY or SHR, 16-18 w.o.	50 to 1000 ng/kg/min	i.v.	4 h	No	No	[23••]
S-D, 11-12 w.o.	100 to 300 ng/kg/min	i.v.	acute	Yes	No	[24]
S-D, 2K1C	0.3 mg/kg/day	i.p.	4 days	No	No	[26•]
Wistar rats with myocardial infarction	0.01, 0.03, or 0.3 mg/kg	i.p.	6 days	Yes	No	[33••]
C57BL/6 mice, 10-12 w.o.	1, 3, and 10 mg/kg/day	i.p.	2 weeks	No	No	[25]
SHR-SP, 6 w.o.	1 mg/kg/day	*per os*	6 weeks	No	No	[27•]
Wistar, 10 w.o. + L-NAME	0.3 mg/kg/day	*per os*	6 weeks	No	No	[28•]
SHR-SP, 4-5 w.o. + 1 % NaCl	0.75, 5 or 10 mg/kg/day	*per os*	until death	No	No	[29]
C21 associated with a high-dose ARB						
SHR-SP, 6 w.o. + losartan (10 mg/kg/day *per os*)	1 mg/kg/day	*per os*	6 weeks	No	No	[27•]
Wistar, 10 w.o. + L-NAME + olmesartan medoxomil (10 mg/kg/day *per os*)	0.3 mg/kg/day	*per os*	6 weeks	No	No	[28•]
C21 associated with a low-dose ARB						
SHR, 16-18 w.o. + candesartan (0.1 mg/kg bolus i.v.)	50 ng/kg/min	i.v.	4 h	No	↓	[23••]

Compound 21 and Anti-inflammation

Recent investigations, reviewed in the following, have revealed other beneficial effects beyond those for hypertension provided by C21.

In a model of myocardial infarction (MI) performed in Wistar rats, treatment with C21 improved cardiac function and decreased scar size after 7 days of treatment [33••]. The underlying mechanisms may include the strong anti-inflammatory effects of C21. Several inflammatory markers, increased following MI, were indeed lowered by C21, such as plasma monocyte chemoattractant protein-1 (MCP-1) and several proinflammatory cytokines. Moreover, in the peri-infarct zone, C21 attenuated the rise of apoptosis markers. These effects were blocked by the AT_2R antagonist PD123319, supporting that specific AT_2R stimulation by C21 exerted anti-inflammatory and anti-apoptotic actions in the context of MI.

In contrast, in a recent study with MI induced in mice, the authors did not observe a reduction of left ventricular remodeling following AT_2R stimulation with C21 [34]. A potential reason for the lack of benefit of C21 in this study was the fact that the same dose of C21 used successfully as a bolus injection in a MI model in rats [33••] was applied in this study as a continuous infusion by minipumps, thus−considering a plasma half-life of C21 of 4 h−presumably not reaching effective plasma levels. Moreover, the authors themselves discussed that the larger infarct sizes obtained in their study could have masked beneficial effects of C21.

In a model of hypertension-induced renal dysfunction (salt-loaded SHR-SP), C21 delayed the occurrence of brain damage and reduced proteinuria [29]. These beneficial effects were specifically related to AT_2R stimulation since they were abolished by PD123319. The authors observed an attenuation of inflammatory and fibrotic processes in the kidneys, pointing again to the anti-inflammatory properties of C21. In a two-kidney-one-clip (2K1C) rat model of hypertension, in which the inflammatory status is upregulated as highlighted by the increase of TNF-α tumor necrosis factor-α), IL-6 (interleukin 6) and TGF-β1 expression in the clipped kidneys, C21 significantly decreased these inflammatory markers [26•]. However, these effects were not completely blocked by PD123319. One possible explanation may rely on differences between these two AT_2R ligands concerning the choice of their administration route or their affinity for AT_2R [26•].

These recent studies highlighted that C21 affords anti-inflammatory properties *via* direct stimulation of AT_2R. Underlying mechanisms were investigated by Rompe et al. [35••]. Human primary dermal fibroblasts were incubated with TNF-α in order to induce IL-6 expression. C21 treatment decreased IL-6, MCP-1 and TNF-α expressions, thus arguing for its anti-inflammatory action. This effect was also reported in endothelial cells from the human umbilical vein. Pre-incubation of cells with PD123319 abolished this effect,

providing the evidence that these actions were AT_2R-dependent. The authors further observed that the inhibitory effect of C21 on IL-6 expression was suppressed under inhibition of serine/threonine or tyrosine phosphatases, thus demonstrating that the anti-inflammatory cascade below AT_2R stimulation implies stimulation of phosphatases. Moreover, the effect of C21 on IL-6 generation was also suppressed when cells were preincubated with a selective inhibitor of arachidonate epoxygenation, indicating that the arachidonic acid metabolite 11,12- epoxyeicosatrienoic acid (EET) constitutes a second messenger in the AT_2R-dependent anti-inflammation pathway. This is in agreement with EET acting as an anti-inflammatory mediator in vascular inflammation [36]. Rompe et al. also investigated whether the changes in IL-6 expression by C21 were related to a change in NF-κB (nuclear factor-κB) activity as IL-6 transcription is under the control of this transcription factor. This was achieved by monitoring nuclear translocation of the NF-κB p50 subunit and also by measuring NF-κB–dependent IL-6 promoter transcriptional activity *via* a luciferase reporter assay. C21 indeed reduced NF-κB activity and translocation. Moreover, in a mouse model of cutaneous inflammation, in which IL-6, MCP-1 and TNF-α mRNA are upregulated, C21 induced a significant reduction of these inflammatory markers, providing further *in vivo* evidence of C21's anti-inflammatory properties. Thus, AT_2R-dependent anti-inflammation seems to involve activation of protein phosphatases, epoxidation of arachidonic acid to EETs and inhibition of NF-κB reducing pro-inflammatory cytokines (Fig. 1) [35••]. It is important to note that, on the IL-6 promoter level, the NF-κB inhibition in this study was comparable in strength to inhibition provided by hydrocortisone, thus supporting the idea that C21 should be studied as a potential drug candidate in inflammatory diseases [35••].

Finally, as inflammatory processes also contribute to the pathophysiology of hypertension, their inhibition may potentiate the benefit of blood pressure reduction [37]. The AT_2R agonist C21, partly *via* its anti-inflammatory properties, may thus be the ideal partner of ARBs in the context of hypertension and related cardiovascular complications (Fig. 1).

Perspectives

Although stimulation of AT_2R by C21 seems to generate numerous actions such as anti-apoptosis and anti-fibrosis [33••], several recent studies reviewed above highlight anti-inflammation as a major function of C21. Therapeutically, C21 could therefore be used as a tool in order to counteract pathologic inflammatory processes. Beyond cardiovascular diseases, in which inflammation is only a part of the pathophysiological process, C21 could enlarge

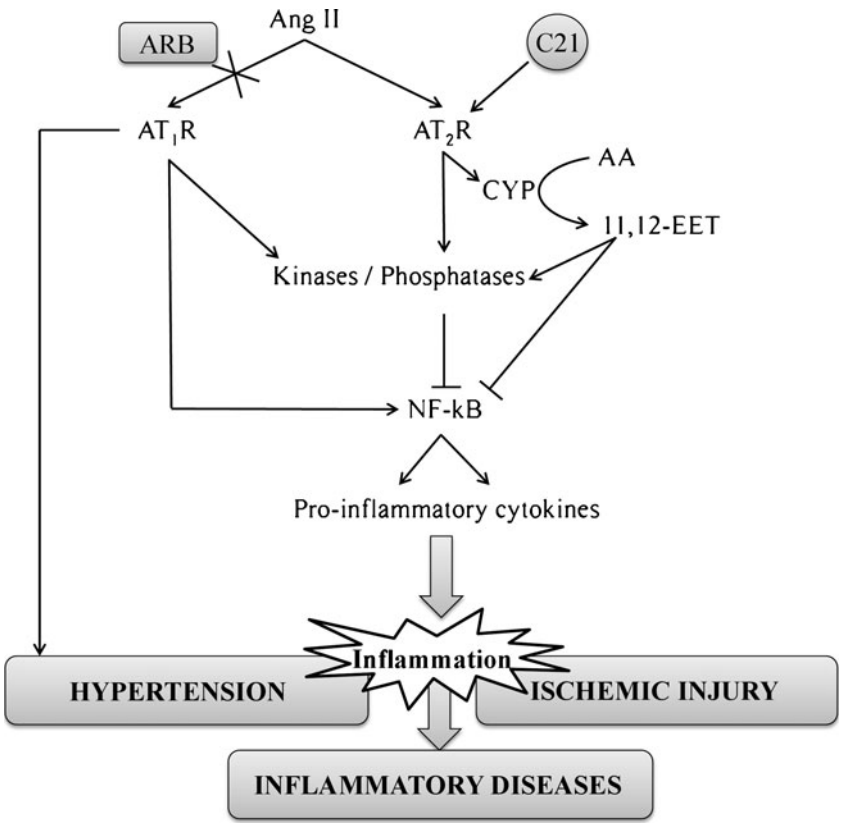

Fig. 1 Schematic overview of the AT_2R-dependent anti-inflammation signaling pathway, counteracting the proinflammatory action of AT_1R activation. Due to its anti-inflammatory properties, the AT_2R agonist C21 may be the ideal partner of ARBs in the context of hypertension and related cardiovascular complications. C21 could also enlarge its therapeutic perspectives to non-cardiovascular inflammatory diseases. *Ang II*, angiotensin II; *AT_1R*, angiotensin II receptor subtype 1; *AT_2R*, angiotensin II receptor subtype 2; *ARB*, angiotensin receptor blocker; *C21*, compound 21; *CYP*, cytochrome P450; *AA*, arachidonic acid; *11, 12-EET*, 11, 12-epoxyeicosatrienoic acid; *NF-kB*, nuclear factor κB

its therapeutic perspectives to non-cardiovascular inflammatory diseases.

Beyond classical treatments, stem cells constitute promising candidates for future therapies [38]. Moreover, there is a growing body of evidence suggesting that RAS, and AT_2R in particular, are implied in the proliferation and differentiation of hematopoietic and mesenchymal stem cells (see [39•] for review). In their recent review, Durik et al. addressed the impact of RAS modulation, and in particular AT_2R stimulation, on tissue regeneration by progenitor cells [39•]. Indirect evidence suggests that AT_2R stimulation may improve the therapeutic effects of MSC grafts in myocardial infarction [40] and brain ischemia [41].

The cellular mechanisms involved in the cardioprotective role of AT_2R have been further investigated following MI. The expression of AT_2R has been indeed observed in human cardiac stem cells as well as in CD8-positive T cells ($CD8^+$ AT_2R^+ T cells) in the peri-infarct area [42••, 43]. AT_2Rs are increased in human cardiac stem cells after MI, and their stimulation with C21 attenuated apoptosis of cardiomyocytes [43]. Besides, AT_2Rs are also expressed in a fraction of CD8-positive T cells in the peri-infarct area [42••]. Contrary to $CD8^+$ AT_2R^- T cells, $CD8^+$ AT_2R^+ T cells did not induce cytotoxicity to cardiomyocytes and exhibited a decreased expression of proinflammatory cytokines. Intramyocardial transplantation of these cells after MI reduced the infarct size, thus providing *in vivo* evidence of cardioprotection *via* $CD8^+$ AT_2R^+ T cells. These studies highlighted an AT_2R-mediated cellular mechanism protecting the heart from injury at least in part *via* anti-apoptotic and anti-inflammatory actions. This could contribute to the beneficial effects observed in post-MI following an acute [33••] or a chronic C21 treatment [44].

Otherwise, the neuroprotective action of AT_2R has in particular been previously investigated after transient cerebral ischemia by unilateral middle cerebral artery occlusion in the rat [19]. In this model, AT_2Rs were upregulated in neuronal cells of the peri-ischemic area, and this was associated with cerebroprotective actions. In order to explore the underlying mechanisms, the authors focused on the role of AT_2Rs in primary neuronal cells. They highlighted that AT_2R stimulation promotes neurite outgrowth and neuronal survival [19]. This may support the AT_2R-dependent neuroprotection provided by MSC grafts during brain ischemia [41]. Stimulation of AT_2R of progenitor cells seems to improve the effects of cell therapy treatments in the context of cardiovascular and neural injury, acting as a repair system [39•].

Conclusion

Although the AT_2R agonist C21 does not act as a classical antihypertensive drug, it could be useful in preventing hypertension-induced organ damage. Moreover, a body of evidence emerges around its anti-inflammatory feature: this should be further investigated for a potential clinical indication.

Considering the AT_2R expression levels in healthy (low expression) and injured tissues (upregulated expression), direct AT_2R stimulation with C21 could constitute a selective repair therapy directed at the injury site, with a limited occurrence of adverse events. AT_2R agonists are the first agonists of the RAS developed with a therapeutic goal. Up to now, the therapeutic goal of interfering with the RAS was slowing down the renin/ACE/AT_1 axis. In contrast to this approach, AT_2R agonists, with the lead compound C21, may afford new therapeutic options *via* promotion of the "protective RAS."

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