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The angiotensin AT_2-receptor agonist compound 21 is an antagonist for the thromboxane TP-receptor – Implications for preclinical studies and future clinical use☆

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ABSTRACT

Since the AT_2-receptor (AT_2R) agonist C21 has structural similarity to the AT_1-receptor antagonists Irbesartan and Losartan, which are antagonists not only at the AT_1R, but also at thromboxane TP-receptors, we tested the hypothesis that C21 has TP-receptor antagonistic properties as well. Isolated mouse mesenteric arteries from C57BL/6 J and AT_2R-knockout mice ($AT_2R^{-/y}$) were mounted in wire myographs, contracted with either phenylephrine or the thromboxane A_2 (TXA_2) analogue U46619, and the relaxing effect of C21 (0.1 nM - 10 μM) was investigated. The effect of C21 on U46619-induced platelet aggregation was measured by an impedance aggregometer. Direct interaction of C21 with TP-receptors was determined by an β-arrestin biosensor assay. C21 caused significant, concentration-dependent relaxations in phenylephrine- and U46619-contracted mesenteric arteries from C57BL/6 J mice. The relaxing effect of C21 was absent in phenylephrine-contracted arteries from $AT_2R^{-/y}$ mice, whereas it was unchanged in U46619-contracted arteries from $AT_2R^{-/y}$ mice. C21 inhibited U46619-stimulated aggregation of human platelets, which was not inhibited by the AT_2R-antagonist PD123319. C21 reduced U46619-induced recruitment of β-arrestin to human thromboxane TP-receptors with a calculated K_i of 3.74 μM. We conclude that in addition to AT_2R-agonistic properties, C21 also acts as low-affinity TP-receptor antagonist, and that – depending on the constrictor – both mechanisms can be responsible for C21-induced vasorelaxation. Furthermore, by acting as a TP-receptor antagonist, C21 inhibits platelet aggregation. These findings are important for understanding potential off-target effects of C21 in the preclinical and clinical context and for the interpretation of C21-related myography data in assays with TXA_2-analogues as constrictor.

1. Introduction

Angiotensin II (Ang II), the main effector hormone of the renin-angiotensin system (RAS), acts via two receptor subtypes, angiotensin AT_1 (AT_1R) and angiotensin AT_2 receptors (AT_2R) [1,2]. AT_1Rs and AT_2Rs generally have opposing actions such as vasoconstriction mediated by the AT_1R, and vasodilation mediated by the AT_2R.

With the synthesis of Compound 21 (C21) in 2004, a potent non-peptide AT_2R agonist became available with high affinity at the AT_2R (K_i 0.4 nM), high selectivity over the AT_1R (> 10000), oral bioavailability of 20–30% and a plasma half-life of 4–6 h [3]. Due to its favourable pharmacokinetic properties compared to available peptide

Abbreviations: ACEI, angiotensin converting enzyme inhibitor; Ang II, angiotensin II; ARB, AT_1R antagonist; AT_1R, angiotensin AT_1-receptor; AT_2R, angiotensin AT_2-receptor; AUC, area under the curve; β-Gal, β-galactosidase; C21, Compound 21 (AT_2R agonist); eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PD123319, S-(+)− 1-[(4-(Dimethylamino)− 3-methylphenyl)methyl]− 5-(diphenylacetyl)− 4,5,6,7-tetrahydro-1 H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate (AT_2R antagonist); PE, phenylephrine; TP, thromboxane prostanoid; U46619, (5Z)− 7-[(1R,4 S,5 S,6 R)− 6-[(1E,3 S)− 3-Hydroxy-1-octenyl]− 2-oxabicyclo[2.2.1]hept-5-yl]− 5-heptenoic acid (thromboxane analogue).

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agonists, C21 significantly facilitated AT_2R research, and it was the first AT_2R-agonist to be forwarded into a drug development program.

Stimulation of AT_2Rs in isolated blood vessels causes vasorelaxation which is mediated by activation of endothelial nitric oxide synthase (eNOS), NO production and formation of cGMP [4–6]. This vasorelaxant effect has been shown by several independent research groups using vessels from different vascular beds and various vasoconstrictors such as the α-adrenergic receptor agonist phenylephrine (PE) or the thromboxane (TP) receptor agonist U46619 [7–13]. During our own studies using these two vasoconstrictors, we noticed that the vasorelaxant effect of C21 was much stronger in U46619-preconstricted vessels compared to phenylephrine-preconstricted vessels. Since C21 is structurally related to the AT_1R-antagonists losartan and irbesartan [14,15], and since it is known that losartan and irbesartan have antagonistic properties at TP-receptors [16–18], we reasoned that C21 may have off-target, antagonistic properties at TP-receptors as well. In the present study, we tested this hypothesis by investigating the vasorelaxant effect of C21 in mouse mesenteric arteries derived from wildtype and AT_2R--knockout ($AT_2R^{-/y}$) mice and preconstricted in a TP-receptor dependent or independent way. Moreover, we explored the effect of C21 on U46619-induced β-arrestin recruitment to TP-receptors and the effect of C21 on U46619-induced platelet aggregation.

2. Materials and methods

2.1. Chemicals

Compound 21 was a kind gift from Vicore Pharma AB (Gothenburg, Sweden). All other chemicals were purchased from commercial sources: U46619 (Tocris Bioscience, United Kingdom), valsartan, PD123319, and phenylephrine (PE) (from Sigma-Aldrich, USA). All chemicals were dissolved in ddH_2O except for U46619, which was dissolved in 100% ethanol.

2.2. Animals

Mesenteric arteries were isolated from male C57BL/6 J (7–8 weeks, 25 ± 3 g, n = 12; Taconic Farms Inc., Ejby, Denmark) or male AT_2R-deficient ($AT_2R^{-/y}$) mice on a C57BL/6 J background (6 weeks, 21 ± 2 g, n = 12). $AT_2R^{-/y}$ mice originated from a strain generated by L. Hein [19]. $AT_2R^{-/y}$ mice were twice backcrossed over nine generations on a C57BL/6 J background. Mice were housed in The Biomedical Laboratory's Animal Housing Facilities in a temperature-controlled room with 12-hours light/dark cycle and fed ad libitum standard chow and water. Mice were euthanized with 80% CO_2 inhalation, and 1st order mesenteric artery side branches were isolated. Mesenteric arteries were used from 6 C57BL/6 J mice (22 arterial segments) with an average diameter of 187 ± 5 μm and a maximal contraction of 2.03 ± 0.15 N/m, and from 9 $AT_2R^{-/y}$ mice (36 arterial segments) with an average diameter of 175 ± 5 μm and a maximal contraction of 1.86 ± 0.13 N/m. Euthanasia of animals was approved by the Danish Animal Experiments Inspectorate under the Danish Ministry of Justice, and all animal care followed the guidelines of the National Institutes of Health.

2.3. Recording of vasomotor responses

Mesenteric resistance arteries from mice were used immediately after isolation of the tissue. Arterial segments were studied in wire myographs (DMT, Aarhus, Denmark) as previously described [20]. The 5 mL organ baths contained physiological salt solution (PSS, concentrations in mM: NaCl 115, $NaHCO_3$ 25, K_2HPO_4 2.5, $MgSO_4$ 1.2, HEPES 10, glucose 5.5, $CaCl_2$ 1.3), aerated with 5% CO_2 in air and maintained at 37 °C.

Mouse mesenteric resistance arteries were distended to 90% of a diameter and passive wall tension that according to the law of Laplace correspond to a transmural pressure of 100 mmHg. A viability test was performed by inducing contraction with 32 mM K^+ followed by 10 μM acetylcholine to prove presence of functional endothelium.

In order to investigate potential relaxing effects of C21, submaximal (~ 50% of maximal contraction), amplitude-matched contractions were induced as proposed by others [7] using 0.1 μM U46619 or 1 μM PE as contractile stimulus.

Cumulative concentration-response curves with half-logarithmic intervals were constructed with C21 (0.1 nM - 10 μM) in separate arterial segments in the absence or presence of the AT_1R antagonist valsartan (3 nM). Increasing concentrations of C21 were added in intervals of about 2 min. Incubation with valsartan started 30 min prior to addition of C21. An additional arterial segment (time control), which did not receive C21, was used to detect and normalise for spontaneous changes in contractile tension over time. A cocktail of three different contractile agonists (16 nM ET-1, 1 μM U46619 and 32 mM K^+) was used at the end of each experiment to determine maximal contraction.

2.4. Interaction of C21 at thromboxane TP-receptors

Interaction of C21 with human TP-receptors was determined using the Arrestin Biosensor Assay (DiscoveRx, Fremont, USA). The technology uses β-galactosidase (β-Gal) as the functional reporter, which becomes activated when β-arrestin is recruited to the receptor of interest. In the non-activated state, the mutant β-Gal is split into two inactive complementary parts, where one is fused to β-arrestin and the other part to the G protein coupled receptor of interest, in this case the TP-receptor. Upon receptor stimulation and concomitant β-arrestin recruitment to the receptor, the two β-Gal fragments form a complex and the emitted light intensity can be quantified [21].

The protocol was performed according to the manufacturers' instructions. In brief, PathHunter® cells were plated into 384-well microplates. In order to determine, whether C21 directly interferes with U46619-induced β-arrestin recruitment to TP-receptors, cells were pre-incubated for 30 min with C21 (0.5 nM to 10 μM) followed by stimulation with U46619 (514 nM, which corresponds to the EC_{80} for the TP-receptor) for 90 min. A 1-hour incubation with 15 μL of PathHunter detection reagent cocktail elicited the chemiluminescent signal, which was analysed with a PerkinElmer Envision™ device. A concentration-response curve was created, and fitting performed using GraphPad Prism (GraphPad Software Inc., San Diego, USA). The K_i was calculated using the following equation:

$$K_i = \frac{IC_{50}}{\frac{[A]}{EC_{50}}+1}$$

IC_{50}: half maximal inhibitory concentration of tested compound at TP-receptor; [A]: concentration of agonist; EC_{50}: concentration of agonist that results in half maximal activation of the receptor.

2.5. Platelet aggregation

Whole blood was collected by venous puncture from healthy volunteers shortly before the start of the experiments and drawn into vacutainer tubes containing 0.129 M sodium citrate. Volunteers were free from aspirin and any other medication that could influence platelet activity. Informed consent was obtained, the study approved by the internal Hospital Ethical Committee and performed according to the Declaration of Helsinki.

300 μL whole blood and 300 μL 0.9% NaCl were added to the cuvette of an impedance aggregometer (Multiplate® Analyser, Roche, Switzerland). Whole blood samples were pre-incubated for 10 min at 37 °C under continuous stirring with different concentrations of C21 (0.1–10 μM) in the absence or presence of the AT_2R antagonist PD123319 (10 μM). Platelet aggregation was induced by addition of 20 μL of 0.3 μM U46619 (final concentrations 10 nM). 20 μL of 0.9% NaCl served as control. 10 nM U46619 was selected on the basis of existing literature [18]. Aggregation was recorded during a 6-minute time period. Curves of aggregation over time were drawn by a

computer-based system and the area under the curve (AUC) was calculated.

Furthermore, whole blood was incubated with C21 or PD123319 (10 μM each) without addition of U46619 in order to exclude any stimulatory effect of these compounds on platelet aggregation. At least 3 independent experiments were performed. Studies were finalised within 3 h from their initiation.

2.6. Statistical analysis

All data are shown as mean ± SEM. C21-induced relaxations are shown as percentage change from the pre-contracted state. E_{max} was calculated as mean ± SEM of maximal contraction/relaxation. Statistical analyses were performed with GraphPad Prism 6.04 (GraphPad Software Inc, San Diego, CA, USA) using two-way ANOVA with Bonferroni's post-hoc test. P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Vasomotor responses to Compound 21

3.1.1. α_1-adrenergic contraction

In mouse (C57BL/6 J) mesenteric arteries, C21 (1 nM – 10 μM) significantly and concentration-dependently relaxed contractions induced by the α_1-adrenergic receptor agonist PE (1 μM) (E_{max}: −52 ± 10%, n = 6, $P < 0.01$, Fig. 1A). Blockade of the AT_1R by 3 nM valsartan in order to unmask potential AT_2R effects that might have been overruled by AT_1R endogenous activation tended to increase the maximal response to C21 (E_{max}: −64 ± 10%, n = 5), but this increase was not statistically significant. Importantly, C21 had no effect in PE-contracted mesenteric arteries from AT_2R-deficient mice ($AT_2R^{-/y}$) (Fig. 1B), indicating that the relaxation of α_1-adrenergic contractions by C21 was indeed mediated by AT_2Rs and not an off-target effect. The (non-significant) increase in vascular contraction at the highest doses of C21 (5×10^{-6} M and 10^{-5} M) is likely an off-target agonistic effect at the AT_1R, which has been described before (Fig. 1B). This assumption is supported by the fact that this additional contraction did not occur in vessels with concomitant AT_1R blockade by valsartan.

Collectively, our data support that C21 relaxes α_1-adrenergic contractions in mouse mesenteric arteries by an AT_2R-specific mechanism.

3.1.2. TP-receptor-mediated contraction

In mouse mesenteric arteries contracted by TP-receptor stimulation with U46619, C21 (1 nM – 10 μM) caused marked, statistically significant, concentration-dependent relaxations (E_{max}: −93 ± 4%, n = 6) (Fig. 2A). The relaxing effect of C21 was fully preserved in mesenteric arteries from $AT_2R^{-/y}$ (E_{max}: −95 ± 1%, n = 9) (Fig. 2B). These data support that C21 relaxes TP-receptor mediated contractions in mouse mesenteric arteries by an AT_2R-independent, off-target mechanism.

3.2. Inhibition constant of C21 at the thromboxane-receptor

Since the off-target, relaxing effect of C21 in U46619-contracted arteries could point to an antagonistic effect of C21 at TP-receptors, and since compounds with similar structure as C21 such as the AT_1R-antagonists losartan and irbesartan are known to have TP-receptor antagonistic properties (Fig. 3) [16–18], we tested whether C21 interferes with β-arrestin recruitment to activated TP-receptors. This is an accepted method for determining compound/receptor interaction avoiding the use of radioactive material in radioligand binding studies. Using the CHO "PathHunter" biosensor assay, we could show that C21 concentration-dependently inhibited U46619 (514 nM = EC_{80} concentration) induced β-arrestin recruitment to the TP-receptor (Fig. 4A). A K_i of 3.74 μM for C21 at TP-receptors was calculated from these data indicating that C21 is a low affinity antagonist at human TP-receptors.

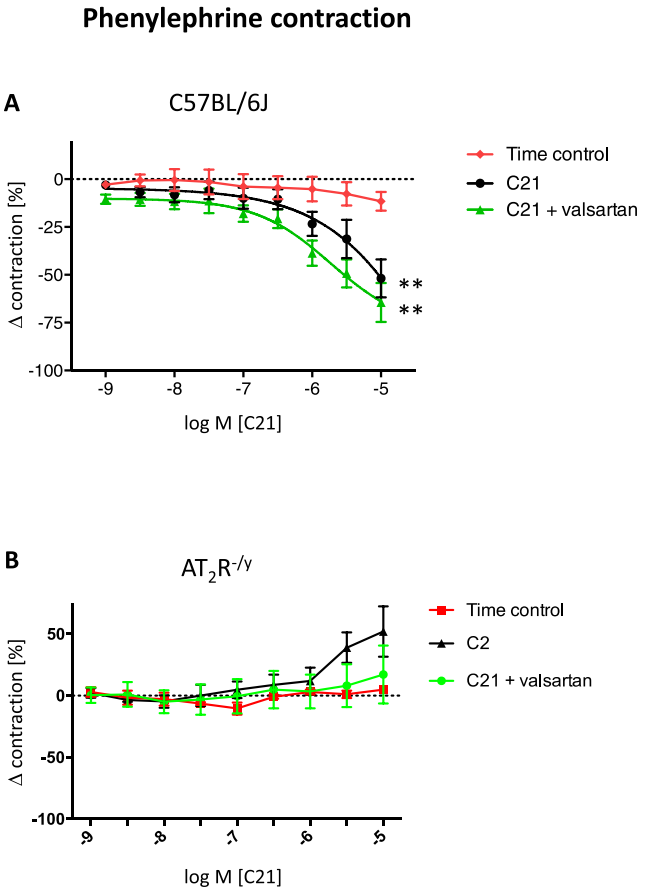

Fig. 1. Effect of Compound 21 on phenylephrine-contracted mouse mesenteric arteries.
Effect of C21 on mesenteric arteries from **A)** C57BL/6 J (n = 6) and **B)** $AT_2R^{-/y}$ (n = 3) mice. Arteries were pre-contracted with phenylephrine (1 μM) and treated with increasing concentrations of C21 (1 nM to 10 μM) in the absence or presence of valsartan (3 nM). C21 induced a statistically significant relaxation of arteries from C57BL/6 J, but not from $AT_2R^{-/y}$ mice. * * $P < 0.01$ (Two-way ANOVA, compared to time control).

The TP-receptor antagonist ramatroban [22], which was used as internal control, inhibited U46619-induced arrestin recruitment concentration-dependently and with high efficacy (IC_{50}: 9 nM) (Fig. 4B).

3.3. Effect of C21 on platelet aggregation

C21 inhibited platelet aggregation induced by 0.3 μM U46619 in a concentration-dependent manner (Fig. 5). At concentrations ≥ 1 μM, this effect was statistically significant. C21 had no effect on platelet aggregation when applied alone (Fig. 5). The effect of 1 μM C21 was not inhibited by the AT_2R antagonist PD123319 (10 μM), indicating that the effect of C21 on platelet aggregation was AT_2R-independent.

4. Discussion

This study provides evidence that the AT_2R agonist C21 is also a low-affinity thromboxane-(TP)-receptor antagonist. This conclusion is based on three experimental findings:

(i) C21 interferes with β-arrestin recruitment to TP-receptors (induced by the agonist U46619) in an inhibitory way. A Ki of 3.74 μM for C21 at TP-receptors was calculated from these experiments; (ii) C21 inhibits arterial contraction induced by U46619 in an AT_2R-independent way; (iii) C21 inhibits U46619-induced platelet aggregation in an AT_2R-independent way.

The first observation in this study that made us suspect an off-target

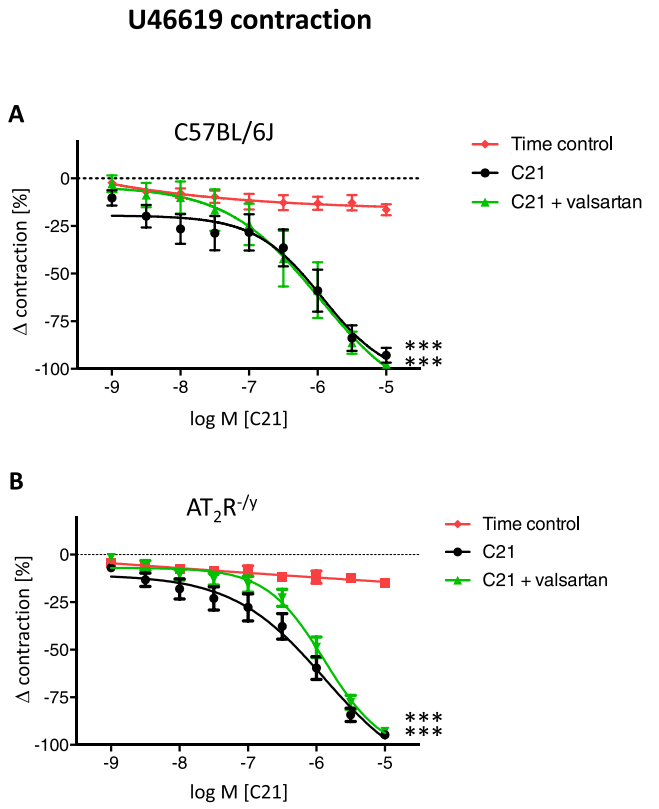

Fig. 2. Effect of Compound 21 on U46619-contracted mouse mesenteric arteries. Effect of C21 (1 nM to 10 μM) on U46619-induced (0.1 μM) contractions in mesenteric arteries from A) C57BL/6 J (n = 6) and B) $AT_2R^{-/y}$ (n = 9) mice in the absence or presence of valsartan (3 nM). C21 induced a statistically significant relaxation of arteries from C57BL/6 J and from $AT_2R^{-/y}$ mice. *** $P < 0.001$ (Two-way ANOVA, compared to time control).

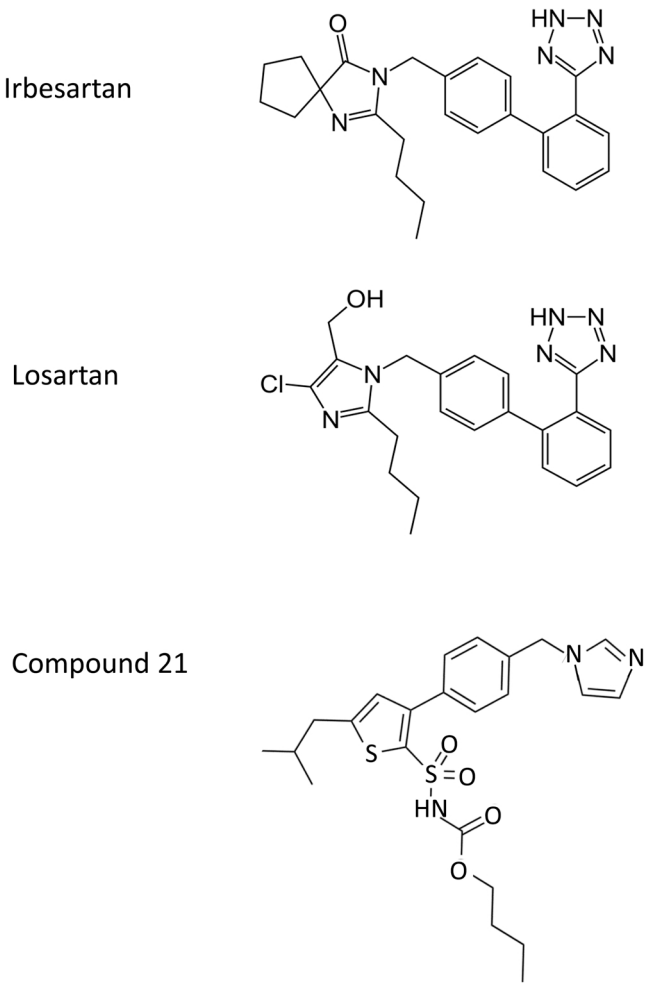

Fig. 3. Chemical Structures of Irbesartan, Losartan and Compound 21.

effect of C21 was that resistance arteries from mouse mesentery, which were precontracted with the TXA_2 analogue U46619, responded to C21 with an unusually strong relaxation. Moreover, and importantly, this response was preserved in arteries derived from $AT_2R^{-/y}$ mice, i.e. in arteries devoid of AT_2Rs. The most straightforward explanation for the AT_2R-independent, inhibitory effect of C21 on TP-receptor mediated contractions was an antagonistic, off-target effect of C21 at TP-receptors by binding to these receptors without intrinsic activity.

We therefore determined interaction of C21 with TP-receptors using a biosensor assay based on agonist-stimulated β-arrestin recruitment. C21 concentration-dependently inhibited U46619-induced β-arrestin recruitment for which a K_i of 3.74 μM was calculated, indicating a low affinity effect. The antagonistic effect of C21 at TP-receptors was further supported by an attenuating effect on the prototypical TXA_2 action, namely platelet aggregation. Of note, the effect of C21 on platelet aggregation was not inhibited by an AT_2R antagonist, thus again supporting an off-target effect. The AT_1R-antagonists losartan and irbesartan, which are structurally similar to C21 and which also possess low-affinity TP-receptor antagonistic properties, have also been shown in previous studies to inhibit U46619-induced platelet aggregation [17,18]. The antagonistic effect of C21 on U46619-induced vasoconstriction in mouse mesenteric arteries was stronger (almost 100% inhibition) than the effect on U46619-induced human platelet aggregation (28% inhibition). Exploring the reason for this difference was out of the scope of this study. However, such a phenomenon has been described before for the TP-receptor antagonist S-145. In this case, the discrepancy was due to a difference in the association kinetics between the drug and the receptor in platelets versus vascular smooth muscle cells [23]. Potential other reasons may be a different affinity or efficacy of C21 at mouse versus human TP-receptors, or the existence of two TP-receptors isoforms coupled to different signaling mechanisms in humans, but not in mice [24,25].

Our observations are of importance, since C21 has become the standard agonist in AT_2R research, and contraction by U46619 is a standard approach in studies on arterial vasomotor tone. Therefore, it is crucial for AT_2R researchers to know and be aware that in U46619-preconstricted vessels, any potential effect by C21 would likely be a result of TP-receptor antagonism and not AT_2R agonism. Without this knowledge, C21 effects in such an experimental setting would be regarded as AT_2R-mediated effects and therefore misinterpreted. A literature search revealed that there are indeed a number of studies, in which C21 effects on vasorelaxation have been studied in U46619-preconstricted vessels and which, therefore, need to be re-evaluated [7,10,12]. These studies also include the publication by Verdonk et al., which – based on the finding that C21-induced vasorelaxation in U46619-constricted mouse and rat arteries was not blocked by an AT_2R-antagonist or by AT_2R-knockdown - claimed that vasorelaxant effects of C21 are generally off-target [12]. This conclusion is now challenged by our data, which suggest that in case vascular constriction is achieved by TP-receptor stimulation (usually by U46619), vasorelaxation by C21 in mainly due to TP-receptor antagonism, whereas if vessels are constricted in a TP-receptor independent way (e.g. by phenylephrine), C21-induced relaxation is due to an AT_2R-mediated effect. However, to draw a final conclusion on this topic, further studies are needed in other vascular beds and other species but man and mouse (e.g. in rats).

Whether the TP-receptor antagonistic effect – though low-affinity -

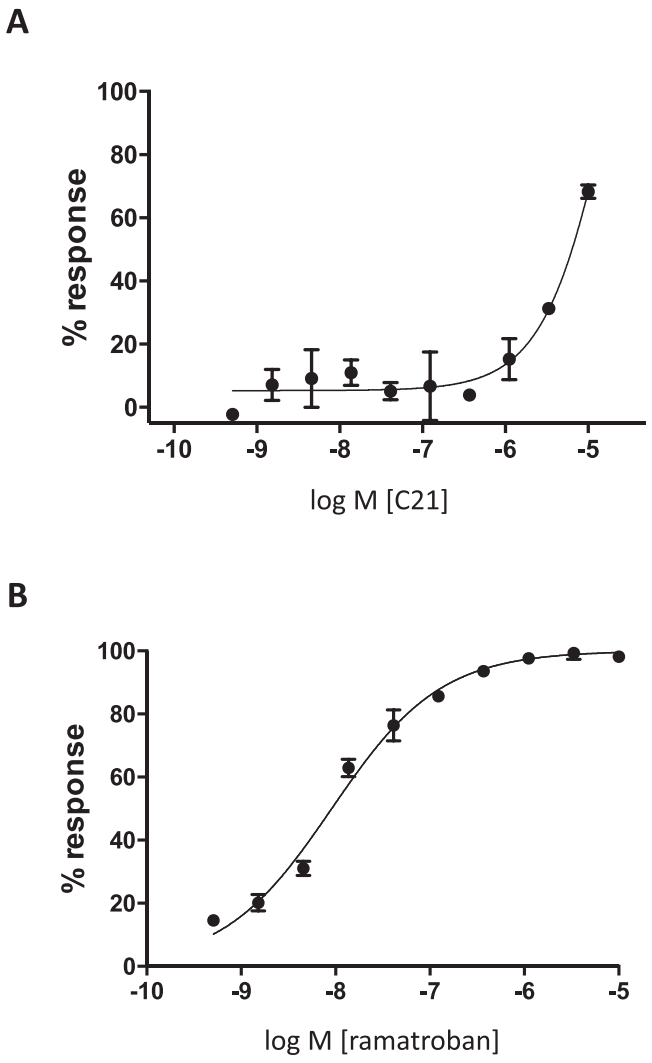

Fig. 4. Effect of Compound 21 on U46619-induced β-arrestin recruitment to human TP-receptors. Inhibition of U46619-induced β-arrestin recruitment to human TP-receptors by **A**) C21 (0.5 nM to 10 μM) and **B**) the TP-receptor antagonist ramatroban (positive control; 0.5 nM to 10 μM). C21 inhibits U46619-induced β-arrestin recruitment, but only at micromolar concentrations.

has clinical relevance is currently hard to decide, since clinical data with C21 are still sparse. However, it is well conceivable that the antithrombotic effect of C21 could provide additional benefits in patients with cardiovascular disease. The fact that dosage of C21 in current clinical trials is very high (2×100 mg/day p.o., which is the human equivalent dose to 2 ×8.8 mg/kg in rats) suggests that the low-affinity effect at TP-receptors needs to be taken in consideration when interpreting the data. Bleeding complications do not seem to constitute a significant problem, since no such side effects have been reported in the various clinical trials with C21 so far [26,27], but this potential side effect should still be monitored carefully.

Our observations strongly suggest that apart from being a high affinity AT_2R agonist, C21 is also a low-affinity TP-receptor antagonist resulting in inhibition of vasoconstriction and platelet aggregation induced by TP-receptor stimulation.

CRediT authorship contribution statement

Maise Fredgart: Investigation, data analysis, **Thomas Leurgans**: Investigation, Methodology, **Martin Stenelo**: Investigation, **Mads Nybo**: Methodology, Supervision, **Maria Bloksgaard**: Methodology,

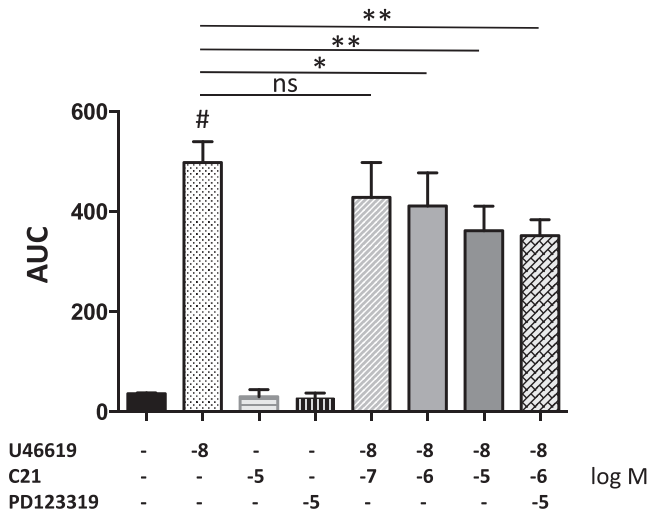

Fig. 5. Effect of Compound 21 on aggregation of human platelets. Effect of C21 (0.1, 1 or 10 μM) on U46619-induced (0.3 μM) aggregation of human platelets in the absence or presence of 10 μM PD123319. C21 concentration-dependently inhibited U46619-induced platelet aggregation. This effect was not inhibited by the AT_2R-antagonist PD123319. $^{\#}P < 0.05$ vs control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs U46619 alone (Two-way ANOVA; n = 3 independent experiments). AUC: area under the curve; All concentrations shown as $-Log_{10}$ M.

Supervision, **Lena Lindblad**: Investigation, Jo **G. R. De Mey**: Conceptualization, funding acquisition, supervision, project administration, data analysis, original draft, **U. Muscha Steckelings**: Conceptualization, funding acquisition, supervision, project administration, data analysis, original draft, revision.

Conflicts of interest

Lena Lindblad was an employee of Vicore Pharma, Gothenburg, Sweden, by the time of the study.

Data Availability

Data will be made available on request.

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