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Functional assay for assessment of agonistic or antagonistic activity of angiotensin AT_2 receptor ligands reveals that EMA401 and PD123319 have agonistic properties

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ABSTRACT

With the discovery of the protective arm of the renin-angiotensin system (RAS), interest has grown in protective RAS-related receptors such as the angiotensin AT_2-receptor [AT_2R] as potential new drug targets. While it is known that AT_2R couple to Gi, it is also apparent that they do not signal via inhibition of adenylyl cyclase/decrease in cAMP, as do many Gi-coupled receptors. Thus, standard commercially-available assays cannot be applied to test for agonistic or antagonistic properties of AT_2R ligands. This lack of standard assays has hampered the development of new drugs targeting the AT_2R.

Therefore, we aimed at developing a reliable, technically easy assay for the determination of intrinsic activity of AT_2R ligands, primarily for distinguishing between AT_2R agonists and antagonists. We found that measurement of NO release by DAF-FM fluorescence in primary human aortic endothelial cells (HAEC) or in AT_2R-transfected CHO cells is a reliable assay for the characterization of AT_2R ligands. While testing the assay, we made several novel findings, including: a) C21 is a full agonist at the AT_2R (with the same efficacy as angiotensin II); b) C21 has no intrinsic activity at the receptor Mas; c) AT_2R-transfected HEK-293 cells are unresponsive to AT_2R stimulation; d) EMA401 and PD123319, which are commonly regarded as AT_2R antagonists, are partial agonists at the AT_2R.

Collectively, we have developed and tested an assay based on the measurement and quantification of NO release in HAEC or in AT_2R-CHO cells that is suitable for the characterisation of novel and established AT_2R ligands.

Abbreviations: Ang II, Angiotensin II; Ang-(1–7), Angiotensin-(1–7); ANOVA, Analysis of variance; AT_1R, Angiotensin AT1 receptor; AT_2R, Angiotensin AT2 receptor; C21, Compound 21; CHO, Chinese hamster ovary; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; EC_{50}, Half maximal effective concentration; EGM-2, Endothelial growth medium; FITC, Fluorescein isothiocyanate; GPCR, G protein-coupled receptor; HAEC, Human aortic endothelial cell; HBSS, Hanks' balanced salt solution; HEK-293, Human embryonic kidney 293 cells; HUVEC, Human umbilical vein endothelial cell; IL-6, Interleukin 6; Mas, Mas receptor; NO, Nitric oxide; PBS, Phosphate-buffered saline; RAS, Renin-angiotensin system; RM-ANOVA, Repeated measures ANOVA; RT-PCR, Reverse transcription polymerase chain reaction; SEM, Standard error of the mean; TNF-α, Tumour necrosis factor alpha.

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1. Introduction

The renin-angiotensin system (RAS) is one of the most commonly targeted biological systems in cardiovascular and renal medicine [1]. Angiotensin II (Ang II) mainly signals through two receptor subtypes: the AT_1 receptor (AT_1R) and the AT_2 receptor (AT_2R) [2,3]. While the AT_1R has been a common drug target in cardiovascular medicine for many years, interest in the AT_2R as a pharmacological target has only arisen more recently. Despite this interest, only two small-molecule drugs targeting the AT_2R have progressed to advanced stages of drug development programs: the AT_2R agonist Compound 21 (C21: Vicore Pharma, Sweden; currently in Phase II for idiopathic pulmonary fibrosis) and the AT_2R antagonist EMA401/Olodanrigan (Novartis, Switzerland; successfully tested in Phase II for postherpetic neuropathic pain [4], but discontinued due to drug-related - not class-related - safety issues [5]).

A major reason for the low number of drug development programs targeting the AT_2R is the lack of reliable assays in which agonistic or antagonistic properties of novel AT_2R ligands can be tested. Standard and commercial assays for G-protein coupled receptors – e.g. assays based on cyclic AMP (cAMP) levels, β-arrestin recruitment or calcium release - target signaling mechanisms, which are not applicable to the AT_2R. C21 has been mainly tested in assays of neurite outgrowth, which are hard to quantify, time-consuming and technically challenging [6]. A second assay for C21 has been to measure inhibition of TNFα-induced IL-6 expression in primary fibroblasts by RT-PCR [7]. However, due to the requirement of primary cells and the need of pre-stimulation with TNF-α for measurement of an inhibitory, AT_2R-mediated effect, this assay is quite complex. Other researchers have used *ex vivo* myography assays or *in vivo* blood pressure measurements to test intrinsic activity of AT_2R ligands [6,8]. All of these assays are clearly not suitable for large-scale testing. Tests that have been performed in AT_2R knockout animals for a member of the EMA401 family, EMA 300, provided information on whether the tested drug acts through the AT_2R, but did not distinguish between agonists and antagonists [9].

In this study, we aimed at establishing an *in vitro* assay for testing AT_2R ligands for their agonistic or antagonistic properties, respectively. Ideally, such an assay should work in a cell line (instead of primary cells) and it should be technically straightforward and highly reproducible.

Here we report real-time measurement of nitric oxide (NO) release from primary endothelial cells or AT_2R-transfected Chinese Hamster Ovary (CHO) cells by fluorescence microscopy as a novel approach for assessing the characteristics of AT_2R ligands. Using the compound DAF-FM, which upon binding to NO emits fluorescent light, this is a quantifiable, highly reproducible assay, which allows for distinguishing between AT_2R agonists and antagonists. With this assay at hand, we further clarified some essential, still open questions related to the AT_2R, which were: (i) Is C21 a full agonist? (ii) Is C21 AT_2R-selective or is it also an agonist for the receptor Mas? (iii) Are EMA401 and PD123319 AT_2R antagonists?

2. Materials and methods

2.1. Cells, cell transfection and culture

Non-transfected Chinese hamster ovary (CHO) cells carrying an Flp Recombination Target site (Invitrogen, USA) were stably transfected with the human AT_2R or with the human receptor Mas. For this purpose, $8x10^4$ CHO cells were seeded in each well of a 6-well plate (Nunc, USA) and incubated at 37 °C with 5% CO_2 for 24 h, resulting in a cell confluence between 60 and 80%. Cells were washed twice with PBS 1x (Invitrogen, USA) and 700 μl of serum-free and phenol red-free Opti-MEM (Invitrogen, USA) added to each well. Plasmids containing full-length DNA encoding the AT_2R or Mas (ImaGenes, Germany) and Lipofectamine 2000 (Invitrogen, USA) were separately diluted in Opti-MEM media and subsequently mixed 1:1 and incubated for 5 min. The plasmid/lipofectamine mix was added to the cells resulting in a final amount of 2.5 μg DNA and 5 μl of Lipofectamine 2000 reagent per well. Subsequently, cells were incubated at 37 °C with 5% CO_2 for 48 h. After the transfection period, the media in each well was replaced by 1 mL of fresh DMEM-F12 (Invitrogen, USA) supplemented with 2% fetal bovine serum (FBS; Invitrogen, USA). G418 was used as resistance antibiotic for selection of transfected cells. This selection process continued for one week, with media replacement every 2–3 days. AT_2R-transfected (CHO-AT_2R) and Mas-transfected (CHO-Mas) cell clones were selected through a serial dilution method in 96-well plates (Nunc, USA) using G418 resistance antibiotic. After 8 days of culture, single, isolated colonies were selected, further expanded and receptor expression verified by PCR.

Non-transfected human embryonic kidney cells 293 (HEK-293) and HEK-293 cells stably transfected with the rat AT_2R (HEK-293-AT_2R) were kindly provided by Prof. Wally Thomas, University of Queensland, Australia [10]. All non-primary cells were grown in 25 cm^2 culture flasks (TPP, USA) in DMEM-F12 (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA) and 1% penicillin/streptomycin (Invitrogen, USA). All cells were maintained at 37 °C with 5% CO_2.

Primary human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC) were from Lonza (Switzerland) and grown in 25 cm^2 culture dishes (Thermo, USA) using the supplemented medium Clonetics EGM-2 BulletKit (Lonza, Switzerland). Primary cells were used for experiments in passages 5–7.

2.2. Nitric oxide release measurement

Nitric oxide (NO) release was measured real-time by a fluorescence-based assay in alive cells over a period of 10 min [11]. This duration of measurements was determined in preparatory experiments (data not shown), which revealed that after around 10 min of measurements with a fluorescence excitation every 30 s, a significant proportion of the maximum effect is reached, whereas at later time points the photobleaching effect gets so strong that the quality of data becomes uncertain.

For NO measurements, cells were cultured on sterile glass coverslips of 10 mm diameter (0.13——0.16 mm thickness) (Thermo, USA), Prior to seeding the cells, the coverslips were placed into 24-well plates (Nunc, USA) and coated with poly-L-lysine (Sigma, USA). For this purpose, 100 μl of poly-L-lysine was pipetted onto each coverslip and the non-adherent portion immediately removed again. The coated coverslips were kept at 37 °C with 5% CO_2 for 24 h until they were completely dried. All cell types were seeded on coated glass coverslips at a density of 5000 cells/well (CHO, HEK-293) or 6000 cells/well (HAEC, HUVEC), respectively. All cells were cultivated in their standard maintenance medium for at least 48 h for full cell adherence to the coverslips and for reaching subconfluence (not more than 80%). Subsequently, medium was removed, and cells were treated with 1 μM of the cell-permeable fluorescent NO indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM, excitation at 495 nM, emission at 515 nM; Life Technologies, USA) diluted in serum-free and phenol red-free medium for exactly 30 min. The DAF-FM media was removed and the coverslips were washed with Phosphate Buffered Saline (PBS) (for all CHO and HEK-293 cell lines) or Hank's Balanced Salt Solution (HBSS) (for HAEC and HUVEC) for 15 min at 37 °C for de-esterification of the intracellular diacetates. After this washing step, PBS/HBSS was replaced by fresh PBS/HBBS containing the following drugs: Compound 21 (10 nM–10 μM) (C21; Vicore Pharma, Sweden), angiotensin II (1 μM) (Ang II; Sigma, USA), CGP42112A (1 μM) (Sigma, USA), EMA401 (1 and 10 μM) (kindly provided by M. Hallberg, University of Uppsala, Sweden), PD123319 (1 and 10 μM) (Abcam, UK) or angiotensin-(1–7) (0.1 and 1 μM) [Ang-(1–7); Bachem, Switzerland]. The standard concentration for molecules used as agonists was 1 μM [except for Ang-(1–7) (0.1 μM)], while the standard concentration of molecules used as antagonists was 10 μM, i.e. ten times higher than the agonists' dose in order to potently

inhibit the agonists' effect. Antagonists were applied to the cells 10 min prior to agonists. Cells treated with PBS or HBSS (vehicle) were used as controls and also underwent the 10 min recording.

At the beginning of each experimental day, cells were first tested for proper functionality by making sure that they reacted with NO release to the positive control, C21 (1 μM), but not to the addition of vehicle. In case they failed one of these tests, the entire batch of cells was discarded.

Recordings of fluorescence signals were started at the same time as agonists were added (t_0). During the preincubation with antagonists, fluorescence was not recorded to avoid early initiation of photobleaching. Fluorescence signals were recorded and pictures taken every thirty seconds over a period of 10 min under an excitation wavelength of 495 nm and an emission wavelength of 515 nm (200 ms laser exposure) using an Olympus IX71 inverted phase/fluorescence microscope (Olympus, UK) equipped with 20x objective lens. The intensity of fluorescence was quantified from the recorded pictures for individual cells (from one visual field per coverslip) using Xcellence software (Olympus, UK). Detached and dead cells or cells out of focus were excluded and all suitable cells marked manually as region of interest (ROI) on the picture taken at T_0 - the software then recognizes the ROIs on all subsequent pictures. Selection of cells was done blinded to the treatment. This way, 12–40 cells per coverslip were analyzed in 2–4 independent experiments. From these raw data, the relative change in fluorescence intensity ($\Delta F = (F/F_0)$ was calculated for each cell. In order to eliminate any possible photobleaching effect, data are displayed as the difference from control (set to 100% for each time point) in percent. Statistical analyses were performed on the original data.

The entire workflow of the assay is depicted in Fig. 1.

2.3. Statistical analysis

All data were analyzed with GraphPad Prism 9.0. Data are presented as mean ± standard error of the mean (SEM). One-way ANOVA analysis was used for comparison between groups in Fig. 9. Repeated measures (RM) two-way ANOVA was used for analyzing effects of time and treatment in Figs. 2 to 8. Šídák test was used for Post-hoc analysis. Statistical significance was set as $p \leq 0.05$. Significance indicated in graphs refers to the interaction between treatment and time. To make graphs not too busy, non-significance is usually not indicated.

3. Results

3.1. Tests of various cell types for suitability in the NO release assay

3.1.1. Primary cells: HAEC and HUVEC

We first tested whether primary cells, which naturally express functional AT_2Rs, namely primary human aortic endothelial cells (HAEC) and primary human umbilical vein endothelial cells (HUVEC) [12,13], are suitable for the DAF-FM NO release assay.

In HAEC, we observed an increase in DAF-FM fluorescence intensity

Figure 1

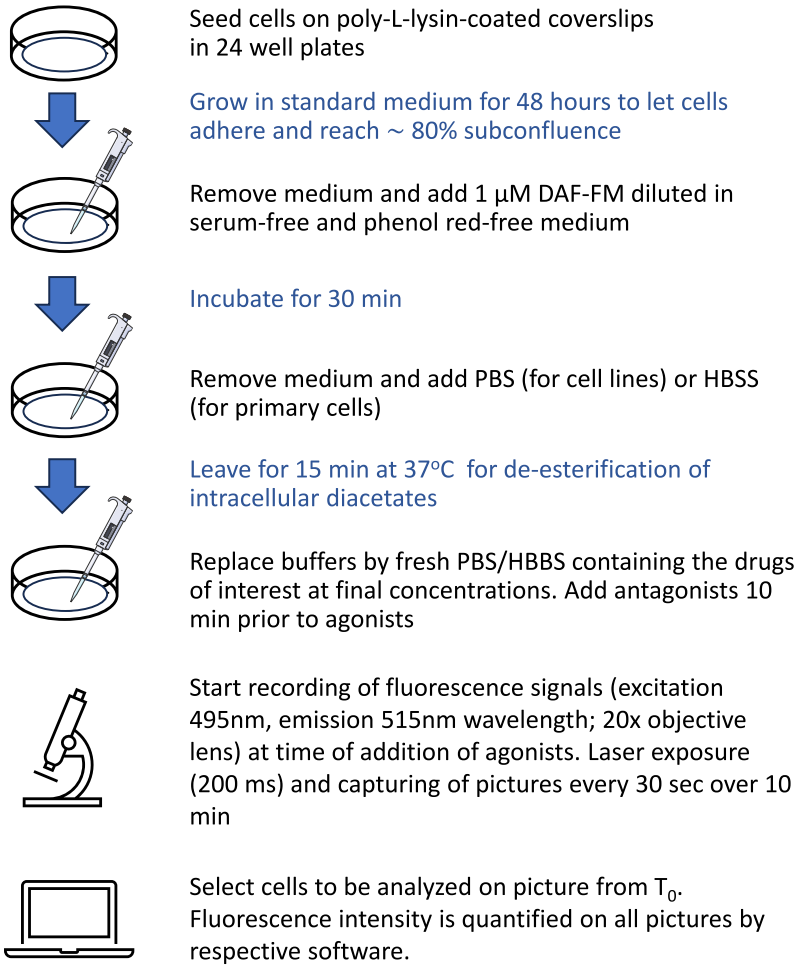

Fig. 1. Flow-chart illustrating the protocol of the NO release assay.

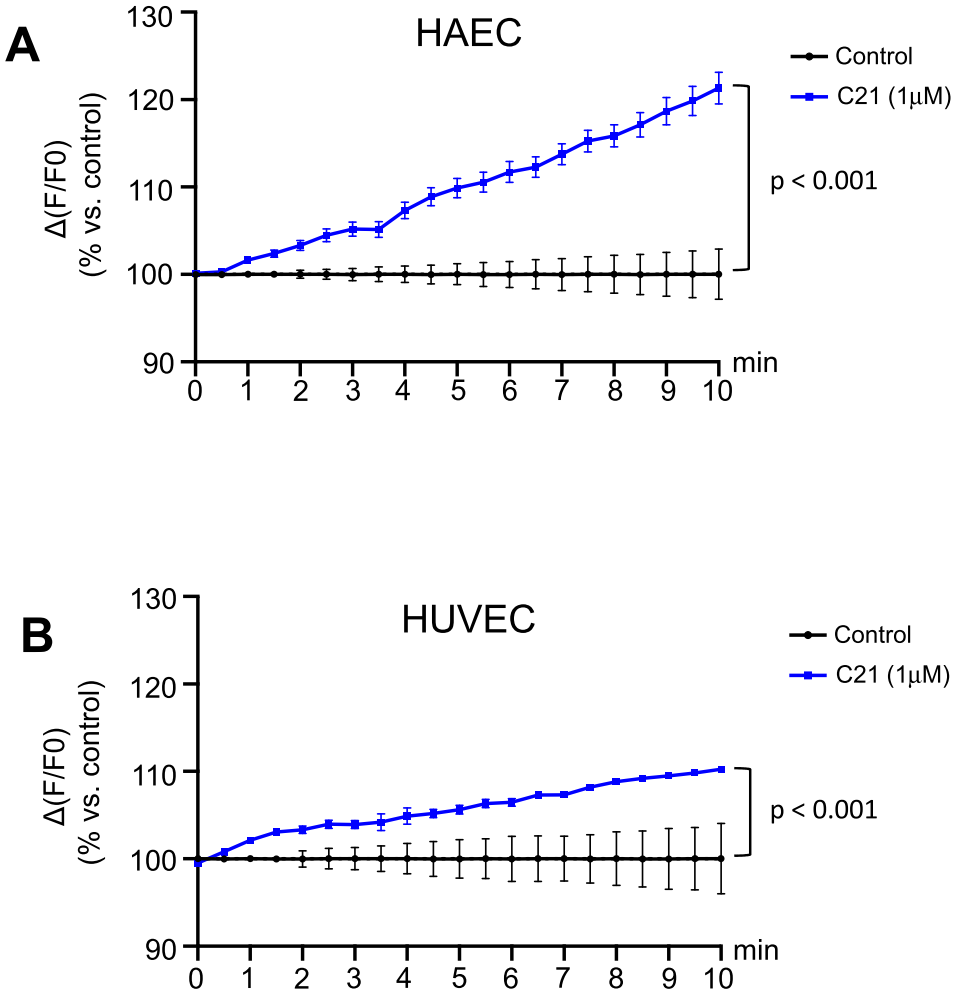

Fig. 2. Effect of AT_2R stimulation by C21 on NO release in primary endothelial cells. (A) Stimulation of HAEC with C21 (1 μM) elicited a statistically significant increase in DAF-FM fluorescence indicating a significant increase in NO release. Two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. PBS control. (B) Stimulation of HUVEC with C21 (1 μM) elicited a statistically significant increase in DAF-FM fluorescence (two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. PBS control), which was weaker than the effect in HAEC.All results shown in Fig. 2 are expressed as the mean ± SEM of 2 to 4 independent experiments, with 12–30 cells per experiment.

after AT_2R stimulation with C21 (1 μM) when compared to PBS-treated controls which was statistically significant in terms of treatment, time and interaction of the two (Fig. 2A, B). In HUVEC, there was an increase in fluorescence intensity of about 10% in response to C21, which was much weaker than in HAEC. This effect was statistically significant in terms of interaction of time and treatment, but not for time or treatment alone.

3.1.2. Cell lines: Stably transfected CHO and HEK-293

Since cell lines are usually easier to handle (i.e. growing faster, not requiring costly, specialized media and suitable for use over many passages), we tested AT_2R-transfected CHO (CHO-AT_2R) and AT_2R-transfected HEK-293 cells (HEK-293-AT_2R) in our assay (Fig. 3). Non-transfected CHO and HEK-293 served as controls.

Stimulation of CHO-AT_2R cells with C21 (1 μM) elicited a statistically significant (treatment, time, interaction) increase in NO release (Fig. 3A), which was comparable in strength with (if not stronger than) the effect in HAEC (Fig. 2A). There was no effect of C21 in non-transfected CHO (Fig. 3B).

AT_2R-transfected HEK-293 cells were unresponsive to stimulation with C21 (1 μM) (Fig. 3C), as were non-transfected HEK-293 (Fig. 3D).

Based on these results, we continued our experiments using CHO-AT_2R cells.

3.2. Test of agonistic properties of C21 at the receptor Mas

As it had not previously been determined whether the AT_2R agonist C21 is also an agonist for the receptor Mas, we tested the effect of C21 (1 μM) on NO release in Mas-transfected CHO cells (CHO-Mas). While the endogenous Mas agonist, Ang-(1–7) at 0.1 μM, which served as positive control, elicited a statistically significant (treatment, time, interaction) increase in NO release from CHO-Mas cells, C21 (1 μM) had no statistically significant effect on the release of NO in these cells (Fig. 4A), indicating that C21 is selective for the AT_2R over the receptor Mas.

3.3. Test of agonistic properties of Ang-(1–7) at the AT_2R

There is evidence that Ang-(1–7) may act as an agonist at the AT_2R [14], and so we tested the effect of the Mas-agonist Ang-(1–7) (0.1 and 1 μM) on NO release from CHO-AT_2R.

Ang-(1–7) at the standard dose of 0.1 μM had no effect on NO release from CHO-AT_2R (Fig. 4B). However, 1 μM Ang-(1–7) induced a statistically significant (treatment, time, interaction) increase in NO release that was similar in magnitude to that elicited by the AT_2R agonist C21 (1 μM) (Fig. 4B). There was no statistically significant difference between the effects of C21 (1 μM) and Ang-(1–7)(1 mM).

Importantly, in non-transfected CHO cells, neither the AT_2R agonist C21 (1 μM) (Fig. 3B) nor the Mas-agonist Ang-(1–7) (0.1 μM) (Fig. 4C; controls are the same as in Fig. 3B) had any effect on the release of NO.

3.4. Concentration-response curve with C21 to determine sensitivity of the assay

In order to test the sensitivity of the assay and whether it is suitable for determining concentration–response relationships of AT_2R agonists, we stimulated CHO-AT_2R with increasing concentrations of C21 (10 and

Figure 3

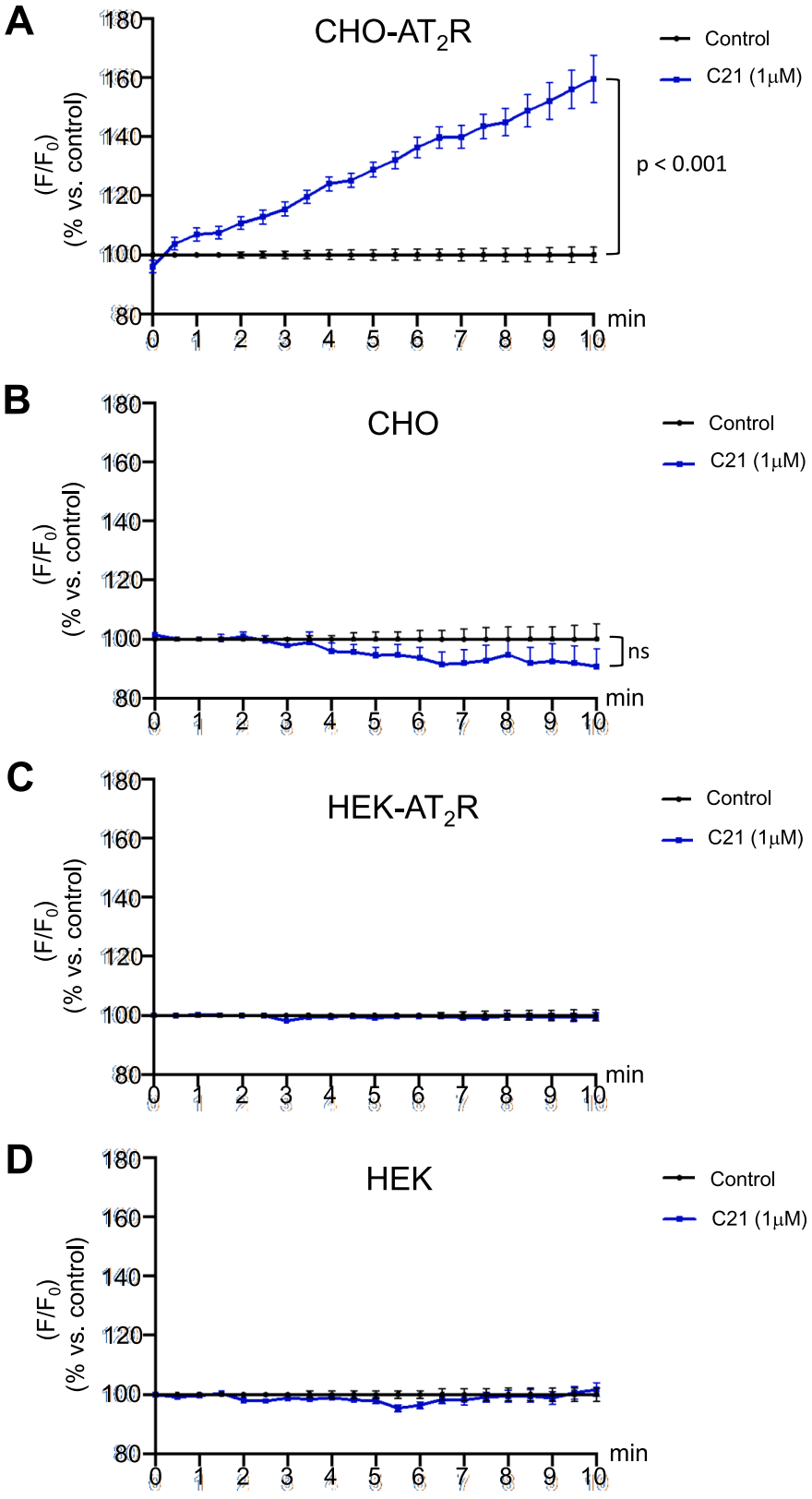

Fig. 3. Effect of AT_2R stimulation by C21 on NO release in AT_2R-transfected and non-transfected cell lines. (A) Stimulation of AT_2R-transfected CHO cells with C21 (1 μM) elicited a statistically significant increase in NO release compared to PBS-treated control cells. Two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. control. (B) C21 had no effect on NO release in non-transfected CHO cells. (C) Stimulation of AT_2R-transfected HEK-293 cells or (D) non-transfected HEK-293 cells with C21 (1 μM) did not increase NO release compared to PBS controls. Results are expressed as the mean ± SEM of 2 to 4 independent experiments, with 15–30 cells per experiment.

Figure 4

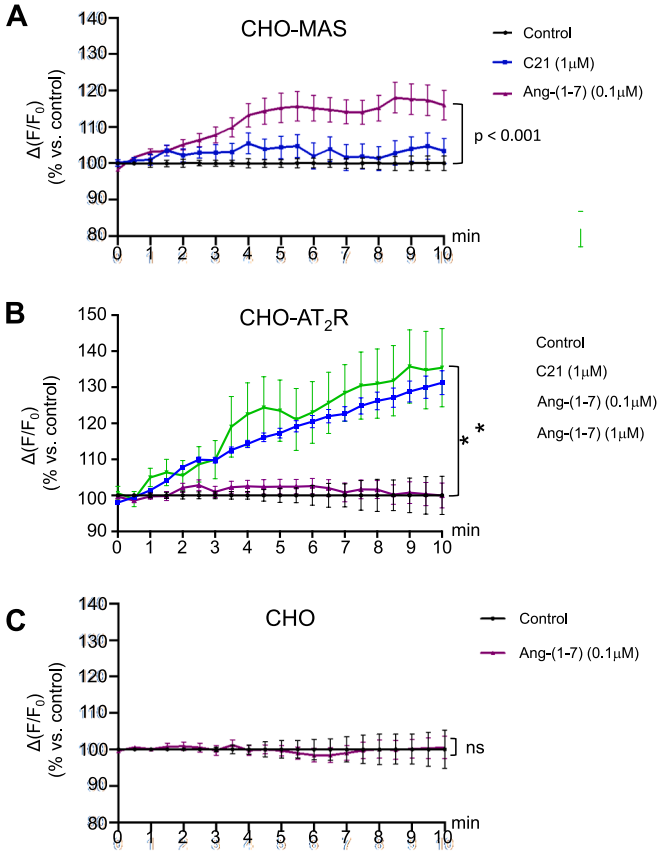

Fig. 4. Test of selectivity of C21 and angiotensin-(1–7) for the AT_2R or the receptor Mas. (A) Stimulation of Mas-transfected CHO cells with C21 (1 μM) had no significant effect on NO release. In contrast, stimulation with the positive control, Ang-(1–7) (0.1 μM) elicited a statistically significant increase in NO release compared to PBS controls. Two-way RM-ANOVA: * $p < 0.001$ Ang-(1–7) (0.1 μM) vs. control. (B) Stimulation of AT_2R-transfected CHO cells with the positive control, C21 (1 μM), elicited a statistically significant increase in NO release compared to PBS controls (Two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. control). Ang-(1–7) (0.1 μM) had no effect on NO release in these cells. However, treatment of AT_2R-transfected CHO cells with Ang-(1–7) at a concentration of 1 μM increased NO release to a similar extent as C21 (1 μM). Two-way RM-ANOVA: * $p < 0.001$ Ang-(1–7) (1 μM) vs. control, Ang-(1–7) (1 μM) vs C21 (1 μM): non-significant.**(C)** In non-transfected CHO cells, neither Ang-(1–7) nor C21 (see Fig. 3B) had any effect on NO release. Data shown in Fig. 3B and 4C originate from the same experiment with the same control. Results are expressed as the mean ± SEM of 2 to 4 independent experiments with 12–30 cells per experiment.

100 nM, 1 and 10 μM). All tested concentrations of C21 including the lowest (10 nM) led to a statistically significant (treatment, time, interaction) increase in NO release, and this effect was dose-dependent (Fig. 5).

3.5. Test of AT_2R agonists Ang II and CGP42112A

To further examine the accuracy of the assay in CHO-AT_2R, we first compared the stimulatory effect of C21 (1 μM) on NO release with that of the endogenous agonist Ang II (1 μM). As shown in Fig. 6A, both C21 and Ang II elicited statistically significant (treatment, time, interaction) increases in NO release to a very similar extent (there was no statistically significant difference between the effect of Ang II and C21), thus indicating that C21 is a full agonist for the AT_2R.

The AT_2R peptide agonist CGP42112A at 1 μM also elicited a statistically significant increase in NO release (treatment, time, interaction) (Fig. 6B). However, the effect of CGP42112A was significantly weaker than that produced by Ang II, suggesting that this peptide may be a partial agonist for the AT_2R (Fig. 6B).

Importantly, all tested agonists, C21, Ang II and CGP42112A, at a concentration of 1 μM did not increase NO release in non-transfected CHO-cells (Fig. 6C), indicating that the effect in CHO-AT_2R was AT_2R specific.

3.6. Test of AT_2R antagonists PD123319 and EMA401

In a further series of experiments, we tested the suitability of the assay for testing AT_2R antagonists. For this purpose, CHO-AT_2R cells were stimulated with C21 (1 μM) alone or in combination with the AT_2R antagonists PD123319 (10 μM) or EMA401 (10 μM), which were added to the cells 10 min prior to C21. Both antagonists elicited statistically significant (treatment, time, interaction) reductions in the C21-induced increase in NO release (Fig. 7A). However, this reduction of the C21-induced NO release was incomplete, at ~ 70% for PD123319 and ~ 50% for EMA401 at the 10 min time points (Fig. 7A). In fact, the stimulatory effect of C21 (1 μM) on NO release remained statistically significant versus the controls in the presence of PD123319 (10 μM) or EMA401 (10 μM) (Fig. 7A).

Since it is possible that the incomplete inhibition of AT_2R stimulation by PD123319 and EMA401 could be due to a potential agonistic activity of these ligands at the AT_2R, we incubated CHO-AT_2R cells with either PD123319 or EMA401 alone and compared their effects on NO release with that produced by C21 (1 μM). At 1 μM, PD123319 led to a slight, non-statistically significant increase in NO release compared to PBS controls (Fig. 7B). Therefore, we also tested PD123319 at a concentration of 10 μM and observed that it produced a strong, statistically significant (treatment, time, interaction) increase in NO release, which was comparable regarding efficacy to the response elicited by 1 μM C21 (Fig. 7B). EMA401 already elicited a statistically significant (treatment, time, interaction) increase in NO release at a concentration of 1 μM, however with lower efficacy than C21 at the same dose (Fig. 7C). Collectively, these data support that PD123319 and EMA401 have agonistic activity at the AT_2R.

To further strengthen evidence that EMA401 has agonistic and antagonistic properties at the AT_2R, we tested the compound in one, single experiment to rule out any differences in responses due to different batches or passages of cells. For this purpose, CHO-AT_2R cells were incubated either with EMA401 alone using the agonist concentration of 1 μM, or cells were treated with C21 (1 μM) as an agonist together with EMA401 at the antagonist concentration (10 μM; added to the cells 10 min prior to C21). As presented in Fig. 7D, C21 (1 μM) again elicited a statistically significant (treatment, time, interaction) increase in DAF-FM fluorescence intensity, indicating AT_2R-mediated release of NO. Similarly, EMA401 (1 μM) produced a statistically significant (treatment, time, interaction) increase in NO release but again to a lesser extent than C21. As in the previous experiment depicted in Fig. 7A, EMA401 (10 μM) acted as an antagonist and led to a statistically significant inhibition of the effect of C21 (1 μM), but the inhibition was again incomplete.

Importantly, neither PD123319 (10 μM) nor EMA401 (10 μM) had an agonistic effect on NO release in non-transfected CHO cells suggesting that the PD123319/EMA401-induced increase in NO release was AT_2R-mediated and not an off-target effect (Fig. 7E).

Preincubation with C21 (10 μM) had no antagonistic effect on Ang II (1 μM) induced NO release from CHO-AT_2R cells (data not shown), indicating that not all AT_2R ligands have agonistic and antagonistic properties, but that C21 is a full agonist.

Figs. 8 and 9 show a final set of control experiments. The first one served to provide evidence that the reduction in C21-induced NO release by preincubation with PD123319 or EMA401 as shown in Fig. 7A and D was really an antagonistic effect. Theoretically, it is also conceivable that the inhibitory effect shown in Fig. 7A/D was not due to receptor

Figure 5

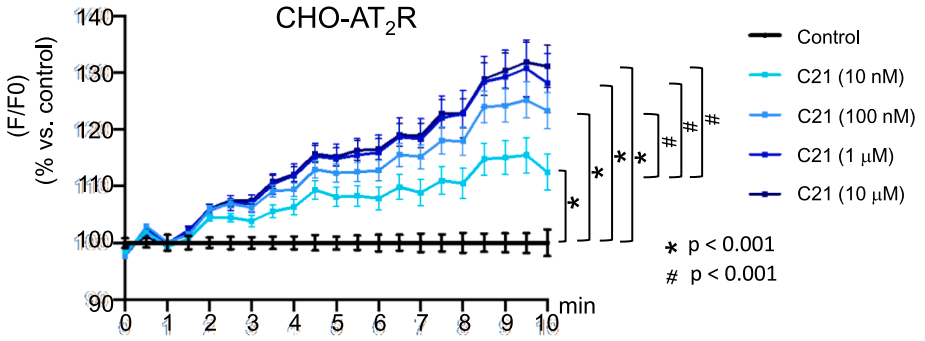

Fig. 5. Dose-response curve for C21 in CHO-AT_2R cells. Stimulation of CHO-AT_2R cells with C21 (10 nM to 10 μM) elicited a statistically significant increase in NO release compared to PBS-treated control cells in a dose-dependent manner. Two-way RM-ANOVA: * $p < 0.001$ C21 (10 nM) vs. control, * $p < 0.001$ C21 (100 nM) vs. control, * $p < 0.001$ C21 (1 μM) vs. control, * $p < 0.001$ C21 (10 μM) vs. control, # $p < 0.001$ C21 (10 nM) vs. C21 (100 nM), # $p < 0.001$ C21 (10 nM) vs. C21 (1 μM), # $p < 0.001$ C21 (10 nM) vs. C21 (10 μM). Results are expressed as the mean ± SEM of 2 to 4 independent experiments with 15–30 cells per experiment.

Figure 6

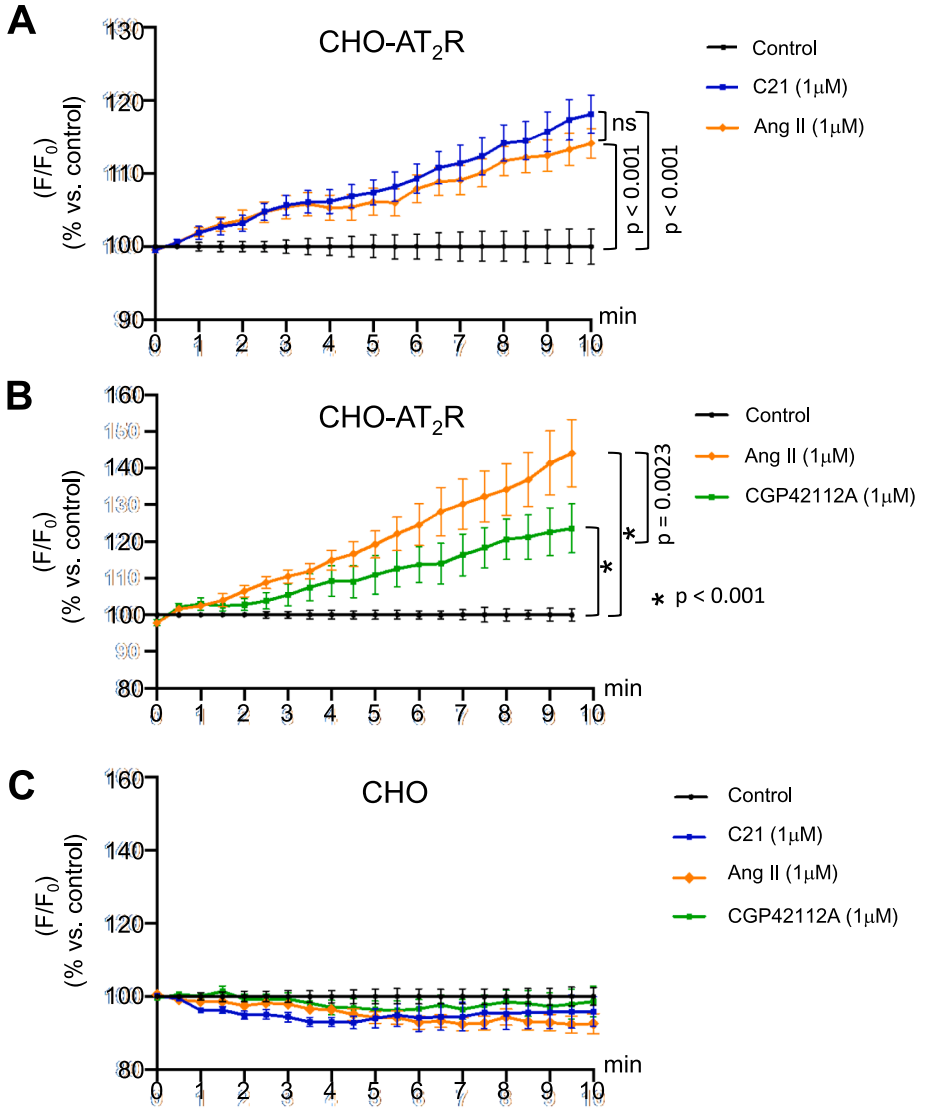

Fig. 6. Comparison of effects of various AT_2R agonists on NO release in CHO-AT_2R cells. (A) C21 (1 μM) and Ang II (1 μM) stimulated NO release from CHO-AT_2R cells in a statistically significant way and with the same efficacy. Two-way RM-ANOVA: * $p < 0.001$ Ang II (1 μM) vs. control, * $p < 0.001$ C21 (1 μM) vs. control, Ang II (1 μM) vs C21 (1 μM): non significant. (B) CGP42112A (1 μM) elicited a statistically significant increase in NO release from CHO-AT_2R cells, however with lower efficacy than Ang II (1 μM). Two-way RM-ANOVA: * $p < 0.001$ Ang II (1 μM) vs. control, * $p < 0.001$ CGP42112A (1 μM) vs. control, # $p = 0.0023$ CGP42112A (1 μM) vs. Ang II (1 μM). **(C)** In non-transfected CHO cells, all three AT_2R agonists, C21, Ang II and CGP42112A, had no effect on NO release.Results are expressed as the mean ± SEM of 15–40 cells analyzed per experiment; 2 to 4 independent experiments.

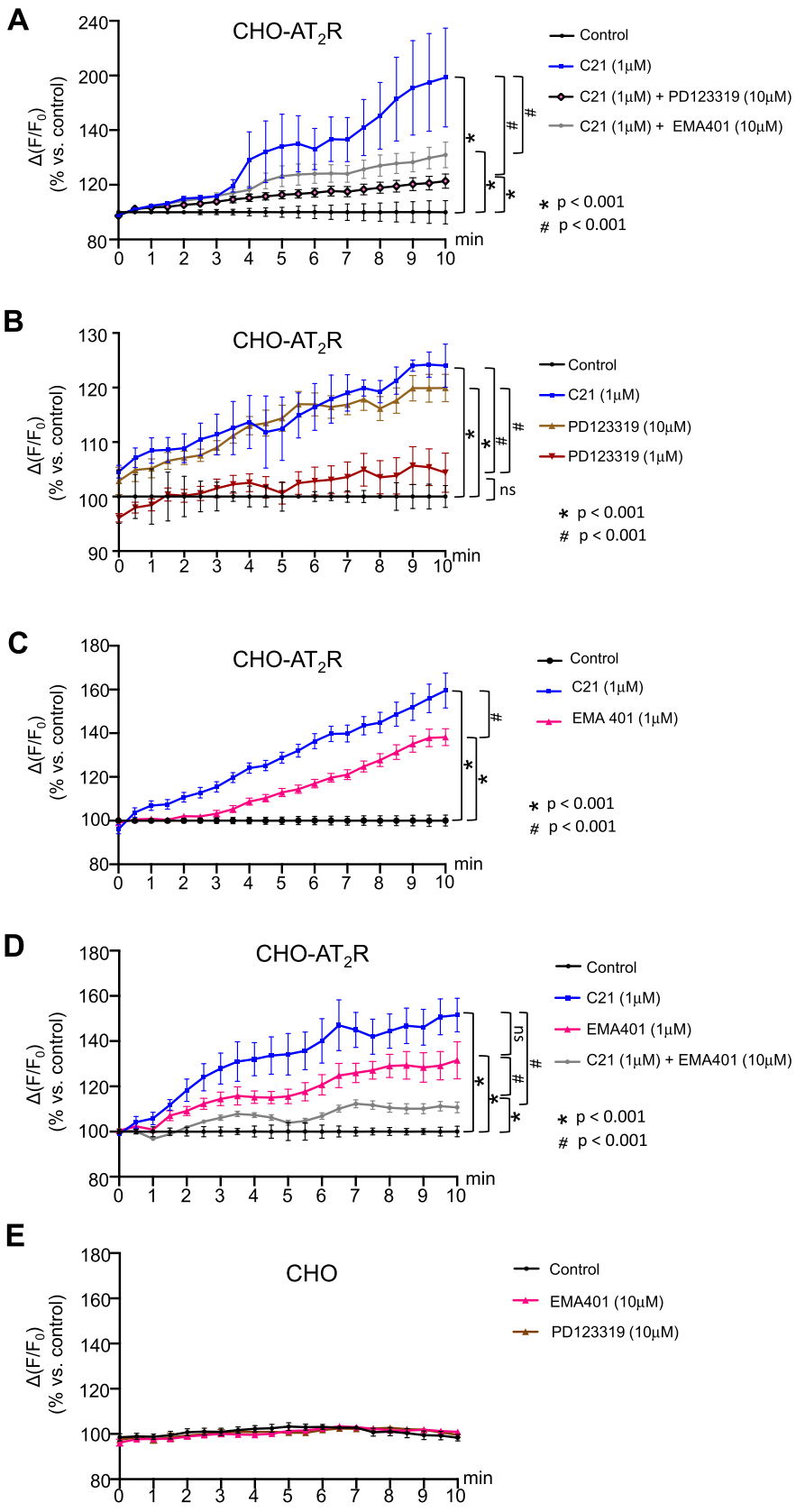

Fig. 7. Test of potential effects of the AT_2R antagonists PD123319 and EMA401 on NO release in CHO-AT_2R cells. (A) Stimulation of CHO-AT_2R cells with C21 (1 μM) significantly increased NO release compared to PBS-treated control cells. This effect was significantly, but not completely blocked by pre-incubation with the AT2R antagonists PD123319 (10 μM) or EMA401 (10 μM). Two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. control; *$p < 0.001$ C21 (1 μM) + EMA401 (10 μM) vs. control; # $p < 0.0001$ C21 (1 μM) + PD123319 (10 μM) vs. C21; # $p < 0.001$ C21 (1 μM) + EMA401 (10 μM) vs. C21 (1 μM). (B) Stimulation of CHO-AT_2R cells with PD123319 at a concentration of 10 μM, but not of 1 μM, led to a significant increase in NO release when compared to PBS controls. The efficacy of this effect of PD123319 was comparable to the effect of 1 μM C21. Two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. control; * $p < 0.001$ PD123319 (10 μM) vs. control; # $p < 0.001$ C21 (1 μM) vs. PD123319 (1 μM); * $p < 0.001$ PD123319 (10 μM) vs. PD123319 (1 μM), PD123319 (1 μM) vs control: non-significant. (C) Stimulation of CHO-AT_2R cells with EMA401 at a concentration of 1 μM significantly increased NO release compared to PBS control cells. The efficacy of this effect of EMA401 was significantly lower than the effect of C21 at the same concentration. Two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. control; * $p < 0.001$ EMA401 (1 μM) vs. control; # $p < 0.001$ C21 (1 μM) vs EMA401 (1 μM). (D) In CHO-AT_2R cells, both C21 (1 μM) and EMA 401 (1 μM) acted as agonists and significantly increased NO release. Efficacy of EMA401 was significantly lower than efficacy of C21. When cells were preincubated with EMA401 (10 μM), it acted as an antagonist and significantly, but not entirely inhibited the effect of C21 (1 μM). Two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. control; * $p < 0.001$ EMA401 (1 μM) vs. control;; # $p < 0.001$ EMA401 (1 μM) vs. C21 (1 μM) + EMA401 (10 μM), # $p < 0.001$ C21 (1 μM) vs. C21 (1 μM) + EMA401 (10 μM), C21 (1 μM) vs EMA401 (1 μM): non-significant. **(E)** In non-transfected CHO cells, PD123319 and EMA401 (both at 10 μM) did not have any effect on NO release. Results are expressed as the mean ± SEM of 2 to 4 independent experiments with 15–30 cells per experiment.

Figure 8

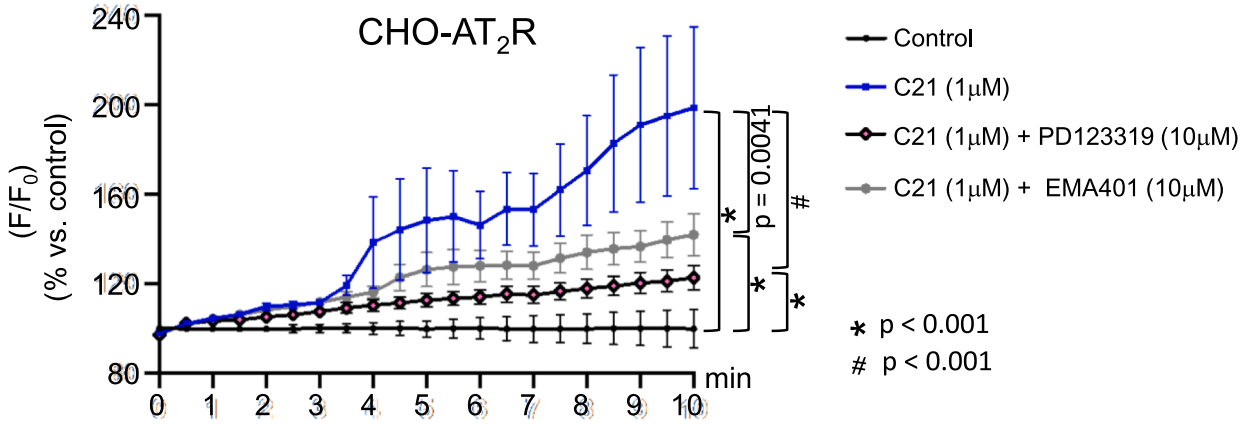

Fig. 8. Inhibition of the C21-induced effect on NO release by PD123319 and EMA401 added without preincubation. (A) Stimulation of CHO-AT_2R cells with C21 (1 μM) significantly increased NO release compared to PBS-treated control cells. This effect was significantly, but not completely blocked by the AT_2R antagonists PD123319 (10 μM) or EMA401 (10 μM), which were added to the cells simultaneously with C21. Inhibition by PD123319 was stronger than inhibition by EMA401. Two-way RM-ANOVA: * $p < 0.001$ C21 (1 μM) vs. control; * $p < 0.001$ C21 (1 μM) + EMA401 (10 μM) vs. control; # $p = 0.004$ C21 (1 μM) vs C21 (1 μM) + EMA401 (10 μM); # $p < 0.001$ C21 (1 μM) vs C21 (1 μM) + PD123319 (10 μM). Results are expressed as the mean ± SEM of 2 to 4 independent experiments with 15–30 cells per experiment.

Figure 9

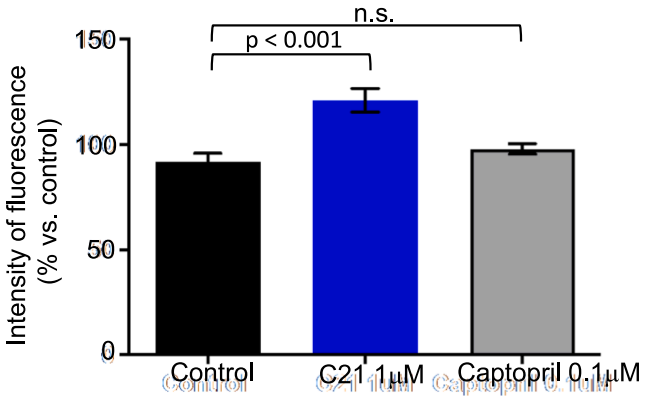

Fig. 9. Effect of the ACE-inhibitor captopril on NO release in CHO-AT_2R cells. A 10-minute treatment of CHO-AT_2R cells with the ACE-inhibitor captopril (0.1 μM) did not have any effect on NO release when compared to PBS controls, whereas there was a significant increase in NO release in response to the positive control, C21 (1 μM). (One-way ANOVA: * $p < 0.05$ C21 (1 μM) vs. control). Results are expressed as the mean ± SEM of 2 to 4 independent experiments with 15–30 cells per experiment.

blockade by PD123319 or EMA401, but that the two antagonists, which were added to the cells 10 min prior to the agonist (C21) may have increased NO release during these 10 min of preincubation (in which NO release was not yet recorded) by their agonistic activity. This may have caused that at time point 0, when C21 was added, NO levels in the medium were already increased and the maximal possible effect of C21 may have been reduced, since the assay has a ceiling effect. Therefore, we tested whether an antagonistic effect of PD123319 and EMA401 was also detectable when these antagonists and C21 were added to the cells at the same time. As depicted in Fig. 8, this was indeed the case; PD123319 and EMA401 both elicited statistically significant (treatment, time, interaction) decreases in the stimulatory effect of C21 on NO release; once again both agents failed to completely inhibit the effect of C21.

The second control experiment aimed at excluding a possible reaction of CHO-AT_2R cells to any RAS-interfering drug. Therefore, cells were treated with the ACE-inhibitor captopril (0.1 μM), which – unlike the positive control C21 (1 μM) – did not cause any increase in NO release (Fig. 9).

4. Discussion

In this study, we describe the development and verification of an assay, which we believe is suitable for evaluating AT_2R ligands for intrinsic activity (i.e., agonistic or antagonistic properties) and for selectivity for the AT_2R over the receptor Mas. The assay is based on a standard assay for measuring NO release in living cells by the compound DAF-FM, which upon binding to NO emits fluorescent light. The fluorescent signal is then detected by a fluorescence microscope and can be quantified by specific software.

This approach using a relatively simple assay may seem trivial at first glance. However, since the discovery of the AT_2R, finding an easy-to-perform assay for functional screening of AT_2R ligands has been a major and so far unresolved problem. This is because even though AT_2R couple to Gi, they do not elicit the traditional Gi-mediated decrease in cAMP, nor do they recruit β-arrestin, but their signaling includes activation of tyrosine - or serine/threonine phosphatase pathways, or of phospholipase A_2 [3,16–21]. Thus, standard screening assays that assess cAMP levels or which are based on the recruitment of β-arrestin [15], cannot be applied.

Another complicating factor in setting up an assay for AT_2R ligands is the fact that most AT_2R actions are of inhibitory nature (e.g. anti-inflammation, anti-fibrosis, anti-proliferation) [3,22], meaning that an assay based on one of these inhibitory actions would require that inflammation/fibrosis/proliferation, etc. will have to be induced first by additional compounds (cytokines, growth factors), which would make the assay complex and hardly suitable for high-throughput screening.

For AT_2R agonists, assays that have been used in the past to prove the AT_2R agonistic properties of a compound were, for example, the measurement of neurite outgrowth from neuronal cells [6,23], the determination of vasorelaxation in aortic rings, or blood pressure measurements under concomitant low-dose AT_1R blockade in

hypertensive rats [8,24]. All of these assays are complex, time-consuming and – in the case of neurite outgrowth – subjective.

The assay described in this study is easy to perform, can be run in a cell line and is highly reproducible (there was a positive response to C21 in each and every experiment we performed).

NO release is a direct response to AT_2R stimulation, meaning the assay is not measuring an inhibitory response and, therefore, no additional stimulating agents are needed.

The assay is sensitive (responding to a dose of the AT_2R agonist C21 as low as 10 nM) and seems suitable for the detection of concentration dependency.

Applying the NO-release assay in this study, we made four important, novel findings.

1) C21 is a full agonist for the AT_2R:

To our knowledge, whether C21 is a full agonist had not been tested before. Since in our assay, the NO release induced by C21 was equally strong as the NO release induced by the natural agonist, Ang II, we conclude that C21 is indeed a full agonist. This is supported by the fact that C21 showed no antagonistic activity when combined with Ang II.

2) C21 has no agonistic effect at the receptor Mas:

Since C21 produced a statistically significant induction in NO-release in AT_2R-transfected CHO cells, but not in Mas-transfected CHO cells, we conclude that C21 acts as an AT_2R agonist, but not as a Mas agonist. For unknown reasons, the affinity of compounds for Mas is difficult to determine by radioligand binding. Therefore, it is important to have a functional assay that can determine if a ligand exerts an agonistic effect on the receptor or not.

3) AT_2R-transfected HEK-293 cells are non-responsive to AT_2R stimulation:

Although HEK-293 cells transfected with the human AT_2R do indeed express the AT_2R and are suitable for binding studies [8,24], the observation that they do not respond to AT_2R stimulation with a physiological response is not really new. It has in fact been published by Victor Dzau's group more than 20 years ago [25], and it has been observed by other groups as well (Walter G. Thomas, Brisbane, and Robert E. Widdop, Monash; personal communications). Therefore, we conclude that AT_2R-transfected HEK-293 cells should be thoroughly tested for functionality before using them in any assay.

4) EMA401 and PD123319 have agonistic activity at the AT_2R:

In our experiments with the AT_2R antagonist EMA401 in CHO-AT_2R, we could confirm that EMA401 acts as an AT_2R antagonist, because it was able to inhibit the effect of C21. However, this inhibition was only partial. Moreover, when cells were treated with EMA401 alone, the compound stimulated NO release, i.e., it acted as an agonist, albeit not as efficiently as C21. The agonistic effect of EMA401 was absent in non-transfected CHO, which supports that the effect was truly AT_2R-mediated. These data strongly suggest that EMA401 is a partial agonist at the AT_2R. This means that EMA401 is able to stimulate the AT_2R, but with lower intrinsic activity as a full agonist such as C21. It also means that if EMA401 is combined with a full agonist in the same experiment, it will weaken the effect of the full agonist, because it occupies receptors, at which it only elicits the non-maximal effect and prevents the full agonist from eliciting a maximal effect. The net result of such an experiment then appears as a partial inhibition of the effect of the full agonist. However, competition for binding at the receptor may not be the only mechanism by which the net effect on NO release is determined when cells are incubated with two AT_2R (partial or full) agonists. It might also be an interference with the receptor activation mechanism of one ligand (i.e. the induced conformational change) by the receptor activation mechanism of the other ligand – and vice versa. This may explain, why incubation with C21 + PD123319 resulted in a smaller response than the effect of the "weaker" agonist, PD123319, alone.

In view of our finding that EMA401 has agonistic activity, the molecular mechanism underlying the analgesic effect of EMA401 in neuropathic pain may have to be re-evaluated. While it may indeed be the antagonistic component of the EMA401 effect, which is responsible for analgesia, it may as well be the agonistic component. Published, preclinical data with EMA401, which would be able to answer this question, do not exist, because to the best of our knowledge, EMA401 has never been shown in any *in vitro* or *in vivo* model related to pain to antagonise the effect of an AT_2R agonist. While the lack of conclusive data does not exclude that the analgesic effect of EMA401 is driven by its AT_2R antagonistic properties, there are also data available, which support that it is in fact an AT_2R agonistic effect, which relieves pain. This has for example been shown for analgesia in connection with Buruli ulcer, in resiniferatoxin-induced sensory small-fibre neuropathy and in vincristine-induced neuropathic pain [26–28].

Application of our assay further revealed that PD123319, which is commonly applied in preclinical research as an AT_2R antagonist, has agonistic activity as well, because – as EMA401 – it induced NO release in CHO-AT_2R when applied alone, while at the same time partially blocking C21-induced NO release. The agonistic effect was only clearly detectable at the highest tested dose, 10 μM, which, however, is the most commonly used dose of PD123319 in *in vitro* experiments.

While the partial agonistic effect of EMA401 has most relevance for the concept of AT_2R antagonists as treatment for neuropathic pain, the impact of PD123319 having agonistic activity mainly applies to basic, preclinical research addressing the AT_2R. Partial inhibition of AT_2R agonist effects by PD123319 is a commonly observed phenomenon and often leads to criticisms by reviewers, which can now be explained. It is probably of even greater relevance that according to our data, when applying PD123319 alone without concomitant application of an AT_2R agonist, it should be taken into consideration that the observed effects may be the result of AT_2R stimulation, not of AT_2R inhibition, and conclusions should be made with caution.

Since we did not observe any effects of EMA401 and PD123319 on NO release in non-transfected CHO cells, it can be assumed that the agonistic effects of the two compounds were truly AT_2R-mediated.

While the assay as presented here is – to our knowledge – the first easy-to-perform *in vitro* assay for the test of agonistic/antagonistic properties of AT_2R ligands and, therefore, a valuable tool for the test of future AT_2R-targeting drug candidates, the assay still has a number of limitations, which can be improved in future studies.

A major shortcoming of the assay is that it is very time-consuming and in its present form not suitable for high-throughput screening. Experiments are performed in live cells grown on coated coverslips, of which only a single one can be analyzed at a time. Approximately 12 coverslips per day can be measured. This means for example that running a concentration–response curve for one compound at 5 concentrations plus controls in 3 independent experiments will take about 3 full working days. Analysis of the results with manual selection of the region of interest (ROI) takes another 2–3 days.

A high-throughput assay should be suitable for analysis in multi-well plates (96 wells or more), which would accelerate the process significantly. Another way to accelerate the assay would be automated instead of manual selection of the ROI. We are currently working on an optimization of the assay taking these criteria into account to make the assay suitable for high-throughput screening of AT_2R-ligands.

Another limitation of this study, which originates in the slowness of the assay, is that – with the exception of C21 – we did not establish concentration–response curves (CRCs) for the tested compounds, but we tested the compounds only at one concentration. Important pharmacodynamic parameters such as efficacy and potency can only be determined with full CRCs with an up-titration of the dose until the effect

reaches a plateau. Although we tested our compounds at the highest reasonable dose of 1 μM (beyond 10 μM, compounds tend to produce off-target effects), we may have missed the maximal effect of some compounds and, therefore, underestimated their efficacy. Calculation of potency is not possible at all without a CRC.

Since we could not reliably determine the Emax of most compounds, we could not decide whether a compound is a full or partial agonist based on its efficacy. However, we still designated a compound to be a partial agonist, when in addition to its agonistic effects, it also had antagonistic effects meaning its intrinsic activity was not 100%. This applies to PD123319 and EMA401, whereas for CGP42112A, we only have data showing significantly lower efficacy at the tested dose compared to Ang II, which strongly suggests but does not provide final proof that CGP42112A is a partial agonist.

In conclusion, our data show that the measurement and quantification of NO release by a fluorescence-based method in primary endothelial cells or in AT_2R-transfected cells of the CHO cell line are suitable for determining the efficacy and intrinsic activity of AT_2R ligands. Therefore, this assay can be used in the future to better characterize the pharmacological properties of pre-existing or future AT_2R ligands for better accuracy and interpretability of AT_2R-related research and for the development of new drugs targeting the AT_2R.

CRediT authorship contribution statement

A. Augusto Peluso: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Igor M. Souza-Silva:** Formal analysis, Visualization. **Daniel C. Villela:** Resources. **Pernille B.L. Hansen:** Methodology. **Anders Hallberg:** Resources. **Michael Bader:** Writing – review & editing. **Robson Santos:** Resources, Writing – review & editing. **Colin Sumners:** Conceptualization, Formal analysis, Writing – review & editing, Visualization. **U. Muscha Steckelings:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: U. Muscha Steckelings reports financial support was provided by Independent Research Fund Denmark. U. Muscha Steckelings reports financial support was provided by Novo Nordisk Foundation.

Data availability

Data will be made available on request.

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