



Article

Development of a Non-Peptide Angiotensin II Type 1 Receptor Ligand by Structural Modification of Olmesartan as a Biased Agonist

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Abstract: As a biased agonist, peptide angiotensin II (Ang II) type 1 (AT₁) receptor ligand antagonizes Ang II-stimulated G protein signaling but stimulates several kinase pathways. Here, we developed a non-peptide AT₁ receptor compound as a biased ligand. We synthesized three non-peptide AT₁ receptor ligands (R239470, R781253, and R794847) as candidates of biased ligands. Extracellular signal-regulated kinase (ERK) 1/2 activation and inositol phosphate (IP) production were measured using a cell system that overexpressed AT₁ receptors (wild-type, L112A, Q257A, Y292A, and N295A receptors). We also examined the modes of receptor–ligand binding using a competition binding study. The *K_d* values of R239470, R781253, and R794847 for the AT₁ wild-type receptor were 0.8, 21, and 48 nM, respectively, as assessed in a competition binding study. Those of R239470, R781253, and R794847 for the L112A receptor were 37, 23, and 31 nM, respectively. R781253 and R794847 decreased and increased IP production, respectively, whereas R239470 did not change IP production. R781253 and R794847, but not R239470, activated ERK1/2. In conclusion, R239470, R781253, and R794847 act as a neutral antagonist, an inverse agonist, and an agonist with regard to IP production, respectively. On the other hand, R781253 and R794847, but not R239470, are agonists toward ERK1/2 activation. Thus, we developed a non-peptide AT₁ receptor compound as a biased ligand.

Keywords: AT₁ receptor; biased ligand; inositol phosphate production; extracellular signal-regulated kinase



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1. Introduction

Angiotensin II (Ang II) type 1 (AT₁) receptor, which is a member of the G-protein-coupled receptor (GPCR) family, mediates most known pathological cardiovascular functions in addition to physiological functions [1,2]. There are many interesting aspects of the functions of GPCRs [3], such as homo- and hetero-oligomerization, constitutive or spontaneous activity in the absence of agonists, nuclear localization, trafficking, intracellular protein-evoked receptor activation, and the GPCR receptor-associated proteins. Studies on these topics may lead to new and improved therapeutics for cardiovascular diseases.

AT₁ receptor blockers (ARBs) block the diverse effects of Ang II, such as vasoconstriction and cardiac hypertrophy. A biased ligand is a ligand that, when bound, activates the β -arrestin signal but not the G α_q signal, for example [4,5]. We previously reported that [Sar¹, Ile⁴, Ile⁸]Ang II, which is an Ang II analogue, did not increase G α_q -dependent inositol phosphate (IP) production. On the other hand, it still activated G α_q -independent extracellular signal-regulated kinase (ERK) 1/2 in human coronary artery smooth muscle cells, as well as in a stably expressed AT₁ receptor cell line [5]. Several ARBs show variable efficacies in addition to anti-hypertensive effects toward protection against organ damage in diabetic nephropathy, cardiac hypertrophy, arrhythmia, and renal failure [6]. Over the past several years, the efficacies of ARBs have been compared and differences have been observed, such as in reducing left ventricular mass, increasing nitric oxide production,

lowering monocyte chemoattractant protein-1 and plasma plasminogen activator inhibitor type-1 antigen, and inhibiting platelet activation, except with regard to the lowering of blood pressure (as a pleiotropic effect) [7]. In addition, many studies have suggested that additional tissue-protective benefits of ARB therapy may be mediated by AT₁ receptor β -arrestin signaling, although the structural basis for the differential activation of this mechanism by ARBs is not known [8,9].

Peptide-type AT₁ receptor-selective agonists, which are biased ligands, have been developed, and one of them, TRV120027 (Sar-Arg-Val-Tyr-Ile-His-Pro-DAla-OH), competitively antagonizes Ang-II-stimulated G protein signaling. It stimulates β -arrestin recruitment and activates several kinase pathways including ERK 1/2, Src, and so on [10]. In addition, the AT₁ receptor biased ligands TRV120023 and TRV120067 were reported to be useful in a mouse model of familial dilated cardiomyopathy [11] and in a mouse model of acute heart failure and dilated cardiomyopathy [12], respectively. On the other hand, a phase II trial with TRV027, which is similar to TRV120067, did not show an improved prognosis at day 30 compared with a placebo in patients with heart failure [13]. Peptide forms may be readily degradable, unstable, and inconsistent in their efficacy. In addition, although it activates the β -arrestin pathway, which is useful for treating heart failure, it is not clear whether it can activate other pathways that exacerbate heart failure because of the diversity of intracellular signals. If non-peptide AT₁ receptor ligands could be developed, it would be possible to verify these points in detail. Most ARBs have common molecular structures (biphenyl-tetrazol and imidazole groups), and it is clear that ARBs have ‘class effects’ [14]. On the other hand, recent studies have demonstrated that not all ARBs have the same effects. Some benefits conferred by ARBs may not be class effects, and instead may be ‘molecular effects’ [14]. In addition, small differences in the chemical structures of ligands can be responsible for agonism, neutral antagonism, or inverse agonism toward a GPCR. A small difference in the molecular structure of angiotensin II receptor blockers induces agonism, neutral antagonism, or inverse agonism toward AT₁ receptor [15]. Based on these reports, we thought that it may be possible to develop non-peptide AT₁ receptor biased ligands by slightly changing the structure of ARBs.

Therefore, by using olmesartan as the basic structure of ARBs, we created compounds with various slight structural changes and used them as candidates of biased ligands. For example, the carboxyl group of olmesartan was converted to a carbamoyl group, which eliminated its potent inverse agonism. Instead, we designed and produced an olmesartan-related compound by adding a 4-hydroxybenzyl group to the biphenyl group so that it may have agonistic action. Here, we developed a non-peptide AT₁ receptor compound as a biased ligand.

2. Materials and Methods

2.1. Materials

We purchased the following reagents: Ang II and [Sar¹, Ile⁸]Ang II (Peptide Institute Inc., Osaka, Japan); olmesartan (Toronto Research Inc., Toronto, ON, Canada); and ¹²⁵I-[Sar¹, Ile⁸]Ang II (PerkinElmer Japan Co. Ltd., Tokyo, Japan). Three non-peptide AT₁ receptor ligands (R239470, R781253, and R794847) as candidates of biased ligands were provided by Daiichi Sankyo Co., Ltd., Tokyo, Japan (Figure 1A).

2.4. Competition Binding Study

The K_d (nM) values of receptor binding were determined with ^{125}I -[Sar¹, Ile⁸]AngII-binding experiments under equilibrium conditions. Binding kinetics were determined following the methods described in the literature [9,15,18]. We carried out binding by using ^{125}I -[Sar¹, Ile⁸] AngII (2200 Ci/mmol) and an increasing concentration (10^{-11} – 10^{-5} M) of each unlabeled compound. Nonspecific binding of ^{125}I -[Sar¹, Ile⁸] Ang II was determined in the presence of a high concentration (10^{-6} M) of unlabeled [Sar¹, Ile⁸]. Next, the incubation mixture was separated by vacuum filtration through Whatman GF/C glass filters (Disposable Filter Funnels Grade GF/C, GE Healthcare), and presoaked with 0.1% polyethylenimine. The filter was washed with 3 mL of ice-cold stop buffer (25 mM Tris–HCl, pH 7.4, 1 mM MgCl₂). The radioactivity trapped on the filters was quantified using an automatic γ -counter. The percentage (%) of specific binding of ^{125}I -[Sar¹, Ile⁸]Ang II was calculated using the following formula: $100 \times (\% \text{ total binding of } ^{125}\text{I}\text{-[Sar}^1, \text{Ile}^8\text{]Ang II} - \text{nonspecific binding of } ^{125}\text{I}\text{-[Sar}^1, \text{Ile}^8\text{]Ang II}) / (\% \text{ total binding of } ^{125}\text{I}\text{-[Sar}^1, \text{Ile}^8\text{]Ang II})$.

2.5. IP Production Assay

Ligand (such as agonist or antagonist)-induced IP production through the AT₁ receptor in transfected COS7 cells transiently expressing the AT₁ receptor was measured to evaluate cell signaling as a second messenger for controlling blood pressure, since the increases in the intracellular Ca²⁺ concentration were the result of IP accumulation, which indicated vasoconstriction. As a surrogate model with higher levels of AT₁ receptor expression, COS7 cells are suitable for IP measurement because it is easy to detect differences in IP production. Cells were labeled for 24 h with [³H]-myoinositol at 37 °C in DMEM containing 10% FBS. Next, the cells were washed with buffer 3 times and incubated with a medium containing 10 mM LiCl for 20 min; 0.1 μM Ang II or 1 μM ligands were added and incubation was continued for another 30 min at 37 °C. After incubation, the medium was removed, and total soluble IP was extracted from the cells using the perchloric acid extraction method, as described previously [9,15,18]. Cells were plated at a field density of 5×10^3 cells/cm² in 60 mm dishes and cultured in a fresh growth medium at 37 °C in a DMEM 5% CO₂ incubator. After 24 h of plating, the cells were transfected with empty vector or expression vector for wild-type or mutant AT₁ receptors. The culture medium was changed 48 h after transfection and 1.5 mL of 1 $\mu\text{Ci}/\text{mL}$ of [³H]-myoinositol was added to each 60-mm dish at 37 °C in DMEM containing 10% FBS. After labeling, the cells were washed with buffer 3 times and incubated with a medium containing 10 mM LiCl for 20 min. The indicated concentration of ligands was added and incubation was continued for an additional 30 min at 37 °C. After incubation, the medium was removed, and total soluble IP was extracted from the cells using the perchloric acid extraction method. Total IP production was expressed as the sum of radioactivity, as described previously [9,15,18].

2.6. Immunoblotting of ERK 1/2 Activation

In *in vitro* studies, ERK 1/2 activation by 1 μM ligands or 0.1 μM Ang II incubated for 10 min was measured using COS7 cells that overexpressed AT₁ wild-type and mutant receptors. ERK activity was measured in cells maintained for 18 h in DMEM containing 0.1% serum. At the end of the stimulation, the culture medium was removed by aspiration, the cells were washed with ice-cold phosphate-buffered saline, and the dishes were frozen in liquid nitrogen. The cells were scraped using a scraper after melting, and the scraped cells were transferred to a tube. We homogenized the cells in lysis buffer plus protease inhibitors and determined the protein concentration using the bicinchoninic acid method. Immunoblotting was performed with primary antibodies as the specified total ERK (#9102, Cell Signaling, Danvers, MA, USA) or phospho-ERK (#9106, Cell Signaling, Danvers, MA, USA) antibody in each sample [5]. We fractionated total proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis; we then transferred the proteins to a Hybond nitrocellulose membrane and blocked the blotted membranes with 10% bovine serum albumin in tris-buffered saline buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl) for 1 h at

room temperature. Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (BIO-RAD170-6515 or 170-6516, Bio-Rad Laboratories Inc., Hercules, CA, USA) against total or phosphorylated ERK overnight at 4 °C. An enhanced chemiluminescent substrate system (Amersham, Buckinghamshire, UK) was used. The signal was independently quantified by digital image analysis (ImageJ, National Institutes of Health, Bethesda, MD, USA) [19].

2.7. Statistical Analysis

All data are expressed as the mean \pm standard error. Each experiment was performed by three or more independent determinations. The significance of differences among the measured values was evaluated with an analysis of variance using an unpaired or paired Student's *t*-test and Fisher's *t*-test, as appropriate. Statistical significance was considered to be present at <0.05 .

3. Results

3.1. Binding Affinities of [Sar¹,Ile⁸]Ang II, Olmesartan, R239470, R781253, and R794847 to AT₁ Wild-Type and Mutant Receptors

The K_d values of [Sar¹,Ile⁸]Ang II, olmesartan, R239470, R781253, and R794847 for the AT₁ wild-type receptor, as determined by a competition binding study, were 0.8, 2.3, 0.8, 21, and 48 nM, respectively (Table 1). Those of R239470, R781253, and R794847 for the AT₁ L112A receptor were 37, 23, and 31 nM, respectively. The K_d values of R239470, R781253, and R794847 were comparable to the K_d value of olmesartan for the receptor. The K_d values of R239470, R781253, and R794847 for the AT₁ Q257 receptor were better than that of olmesartan. Although R239470 could bind to the AT₁ N295A receptor, R781253 and R794847 could not ($K_d > 10,000$ nM).

Table 1. Binding affinities (K_d , nM) of [Sar¹,Ile⁸]Ang II, olmesartan, R239470, R781253, and R794847 to AT₁ wild-type and mutant receptors (n = 3).

Receptors	Wild-Type	L112A	Q257A	Y292A	N295A
Ligands					
[Sar ¹ ,Ile ⁸]Ang II	0.8 \pm 0.4	1.3 \pm 0.3	3.3 \pm 1.5	2.1 \pm 0.9	7.0 \pm 2.0
Olmesartan	2.3 \pm 0.8	27 \pm 7	228 \pm 38	47 \pm 15	334 \pm 105
R239470	0.8 \pm 0.3	37 \pm 12	20 \pm 2	6.3 \pm 3.8	69 \pm 8
R781253	21 \pm 10	23 \pm 7	14 \pm 6	95 \pm 7	>10,000
R794847	48 \pm 12	31 \pm 5	2.7 \pm 0.8	30 \pm 8	>10,000

3.2. Levels of IP Production Using Various Ligands in AT₁-WT, -L112A and Q257A Receptors

The levels of IP production using various ligands in AT₁ wild-type, -L112A, and Q257A receptors are shown in Figure 2. Ang II significantly increased IP production in AT₁ wild-type, L112A, and Q257A receptors in this cell system. Olmesartan induced a significant reduction in basal IP production levels in the AT₁ wild-type, L112A, and Q257A receptors and showed inverse agonism. In the AT₁ wild-type receptor, R781253, and R794847 significantly decreased and increased IP production, respectively, whereas R239470 did not change IP production (Figure 2). In the AT₁-L112A and -Q257A receptors, none of the compounds (R239470, R781253, and R794847) affected IP production, although olmesartan induced a significant reduction in basal IP production levels in the receptors.

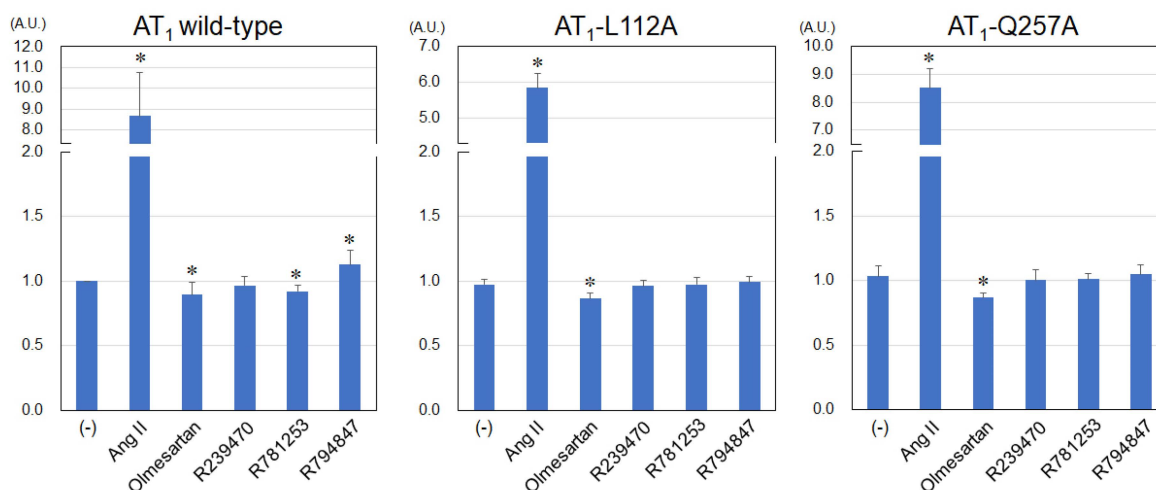


Figure 2. Levels of IP production using various ligands in AT₁ wild-type, -L112A, and Q257A receptors. IP, inositol phosphate; AT₁, angiotensin II type 1; A.U. arbitrary unit. n = 5. * $p < 0.05$ vs. (-).

3.3. Levels of ERK Activities Using Various Ligands in AT₁-WT, -L112A, and Q257A Receptors

Ang II significantly activated ERK1/2 in AT₁ wild-type, L112A, and Q257A receptors in this cell system (Figure 3). Olmesartan did not change ERK1/2 activation in AT₁ wild-type, L112A, or Q257A receptors. In the AT₁ wild-type receptor, R781253 and R794847, but not R239470, activated ERK1/2. R781253 and R794847, but not R239470, activated ERK1/2 in the AT₁-L112A receptor. None of the compounds activated ERK1/2 in the AT₁-Q257A receptor.

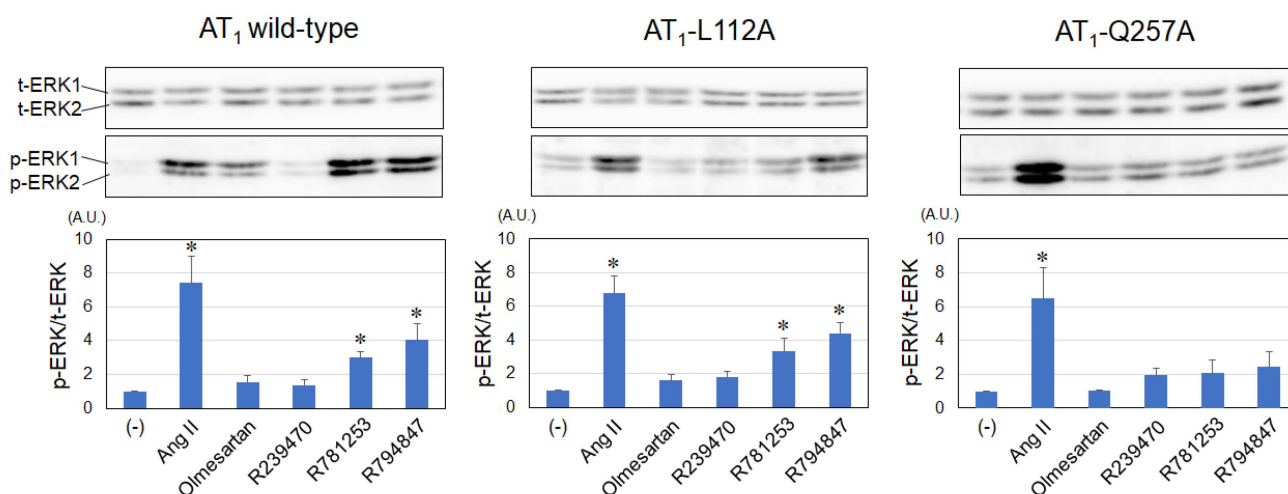


Figure 3. Levels of ERK activities using various ligands in AT₁ wild-type, -L112A, and Q257A receptors. t-ERK, total extracellular signal-regulated kinase; p-ERK, phospho-ERK; AT₁, angiotensin II type 1; A.U. arbitrary unit. n = 5. * $p < 0.05$ vs. (-).

4. Discussion

In this study, we developed a non-peptide AT₁ receptor compound, R781253, as a biased ligand (Figure 4). The positions of Lys¹¹² and Gln²⁵⁷ in the AT₁ receptor may play an important role in IP production with regard to R781253 as a biased ligand, whereas the position of Gln²⁵⁷, but not Lys¹¹², plays an important role in the ERK1/2 activation of R781253. Thus, a small difference in the molecular structure produced a biased ligand. In

addition, the differences in the binding position of the AT₁ receptor relative to R781253 influenced the function of the biased ligand.

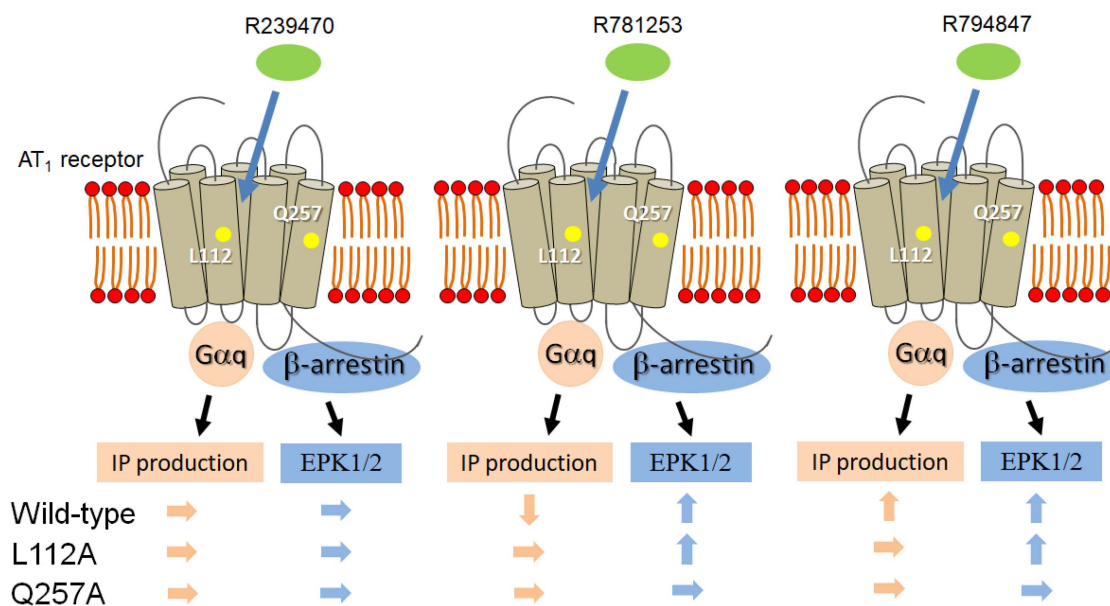


Figure 4. Summary of IP production and ERK activities by olmesartan and its related compounds in AT₁ wild-type, -L112A, and Q257A receptors. IP, inositol phosphate; ERK, extracellular signal-regulated kinase.

We confirmed that the non-peptide AT₁ receptor compound R781253 acted as a biased ligand (Figure 4). R239470, R781253, and R794847 were a neutral antagonist, an inverse agonist, and an agonist with regard to IP production, respectively. We previously reported that small differences in the chemical structures of ARBs can be responsible for agonism, neutral antagonism, or inverse agonism regarding IP production toward the AT₁ receptor [15]. We analyzed the ligand-specific changes in the receptor conformation with respect to stabilization around TM3. Although the agonist-, neutral antagonist and inverse-agonist-binding sites in the AT₁ receptor are similar, each ligand induced specific conformational changes in TM3. The hydroxyphenyl moiety, which represents the difference between the chemical structures of olmesartan and R794847, interacts with the Leu¹¹² of the AT₁ receptor. Coupled movements of Leu¹¹² and Asn¹¹¹ occur in molecular dynamics simulations of the AT₁ receptor [20]. The flipping of Asn¹¹¹ is essential for Gq-dependent signaling. In this study, we also analyzed the positions of Lys¹¹², which is adjacent to Asn¹¹¹, as well as Gln²⁵⁷, in the AT₁ receptor. Leu¹¹², in addition to Gln²⁵⁷, may play an important role in Gq-dependent IP production with regard to R781253 as a biased ligand. On the other hand, R781253 and R794847, but not R239470, were found to be agonists in terms of ERK1/2 activation. The additional 4-hydroxybenzyl groups of both R781253 and R794847 were predicted to extend to a sub-pocket on the bottom of the AT₁ receptor ligand-binding pocket, which includes Leu¹¹², Lys¹⁹⁹, Asn²⁰⁰, Trp²⁵³, His²⁵⁶, Gln²⁵⁷, and Thr²⁶⁰ in the AT₁ receptor [16]. Since the position of Gln²⁵⁷, but not of Lys¹¹², is important for the ERK1/2 activation by R781253, the hydroxy group in the 4-hydroxybenzyl group of R781253 may be crucial for the binding to Gln²⁵⁷ in the AT₁ receptor for ERK1/2 activation. In addition, the carbamoyl moieties of R239470 and R794847 cannot form a salt bridge to Arg¹⁶⁷ in the AT₁ receptor. Therefore, R781253 retains inverse agonism, while R239470 and R794847 do not. In addition, Arg¹⁶⁷ forms extensive networks of hydrogen bonds and salt bridges with the acidic tetrazole ring and the carboxyl group on the imidazole moiety of olmesartan in the AT₁ receptor–olmesartan complex. We might have been able to analyze IP production and ERK1/2 activation more effectively using the position of the Arg¹⁶⁷ mutated AT₁ receptor.

Lys¹⁹⁹ also played an important role in Ang II binding, as the K199A mutant completely abolishes Ang II binding, and we did not perform any further analysis.

The β -arrestin-biased ligand stabilizes an alternative TM7 conformation in the AT₁ receptor, whereas G-protein-biased ligands do not favor this conformation [21]. The binding of partial or β -arrestin-biased peptide agonists triggers a shift of TM6, which is a hallmark of GPCR activation, and a change in the conformation of Asn²⁹⁵ [20]. In addition, Tyr²⁹² and Leu¹¹² in the AT₁ receptor play important roles in coupling Ang II binding to Gq activation. In the active state of AT₁ receptor structures, the outward rotation of TM6 and its associated inward rotation of TM7 disrupts an inactive state for stabilizing interactions between Asn¹¹¹ and Asn²⁹⁵ underneath the ligand-binding pocket. Tyr²⁹² is also coupled to the ligand through two adjacent residues, Asn¹¹¹ and Leu¹¹², on TM3 [20]. To analyze the importance of the change in TM7 conformation by a non-peptide biased ligand R781253 in this study, we mutated the positions of Tyr²⁹² (Y292A) and Asn²⁹⁵ (N295A) in TM7. Since the K_d values of R781253 against Y292A and N295A were 95 nM and >10,000 nM, respectively, and since the binding affinities were very low, we did not confirm IP production or ERK activation using R781253 in the AT₁-Y292A and -N295A receptors. Further study is needed to resolve these issues.

Activation of the Gq/11 pathway is associated with a specific conformational transition that is stabilized by the agonist. On the other hand, activation of the β -arrestin pathway is linked to the stabilization of the ground state of the receptor [22]. Distinct conformations of the AT₁ receptor are associated with distinct cell signaling pathways [23]. The activities of GPCRs are finely regulated by GPCR kinases (GRKs), which phosphorylate agonist-occupied receptors and start the process of desensitization. GRK2 and GRK5 are predominantly expressed in the heart, where they have both canonical and non-canonical functions. Biased ligands induce the recruitment of distinct subtypes of GRK to the receptor as compared with balanced ligands. β -arrestin induces sustained G protein-induced cell signaling by a ternary GPCR–G-protein– β -arrestin complex. The activation of the G protein triggers the initiation of the β -arrestin system by releasing free G $\beta\gamma$ subunits and activating GRK2/3. Moreover, while β -arrestin recruitment depends on both GRK5/6 and GRK2/3 upon the binding of Ang II, it depends solely on GRK5/6 upon the binding of the β -arrestin-biased ligand TRV027 [24]. In this study, although we did not analyze the conformational transition or the importance of the kinds of GRK of R239470, R781253, and R794847, the biased ligand R781253 may induce a distinct conformational transition and the recruitment of different GRKs.

AT₁ receptor signaling has been implicated in Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infection [25]. SARS-CoV-2 enters cells by binding to angiotensin converting enzyme 2 (ACE2), which lead to the downregulation of ACE2 activity. Ang II, the ligand for the AT₁ receptor, activates a Gq signaling pathway, leading to vasoconstriction and adverse cellular responses in heart failure and acute lung injury. On the other hand, the action of Ang II is moderated by β -arrestin [25]. Cleavage of Ang II by ACE2 decreases Ang II levels and increases Ang(1-7) production, which is an endogenous β -arrestin-biased agonist of the AT₁ receptor with potential therapeutically beneficial effects. Compared with ARBs, the biased agonist of AT₁ receptor R781253, which can improve potency and pharmacodynamic properties compared with Ang(1-7), may be more advantageous in patients with SARS-CoV-2 infection because R781253 blocks Gq-dependent Ang II activity and induces β -arrestin signaling.

A limitation of this study is that it was conducted under limited conditions and in vitro, and no research has been conducted in vivo within the human body. However, it is considered to have sufficient reliability for verifying the binding mode between the ligand and receptor and subsequent specific intracellular signaling pathways. In addition, based on previous reports, mutated receptors were created and verified. Nonetheless, we performed experiments with only one type of cell. Each type of cell has different intracellular signal transduction systems, and the strength of the signals varies, so the results obtained in this study do not necessarily occur universally in all kinds of cells.

5. Conclusions

R239470, R781253, and R794847 were shown to be a neutral antagonist, an inverse agonist, and an agonist with regard to IP production, respectively. On the other hand, R781253 and R794847, but not R239470, were agonists with respect to ERK1/2 activation. Thus, we developed a non-peptide AT₁ receptor compound as a biased ligand.

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