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Engineered Mutation of Some Important Amino Acids in Angiotensin II Type 1 (AT_1) Receptor Increases the Binding Affinity of AT_1-Receptor Antagonists

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Abstract. The present study was designed to examine the binding affinity and functional potency of selective angiotensin II type 1 (AT_1)-receptor antagonists towards specific mutants of AT_1 receptor using site-directed mutagenesis. We also compared our results with the wild-type AT_1 receptor and investigated the possible reasons behind that. Both wild-type and mutant receptors were expressed in COS-7 cells and the binding affinities of the antagonists were determined by radioligand binding assay. Inhibition of agonist-stimulated inositol phosphate accumulation by the antagonists was also done. Substitution of asparagine^{235} of intracellular loop 3 of the AT_1 receptor by arginine increased the binding affinity of the antagonists 5 – 34-fold, whereas the increase in the binding affinity of the antagonists in the phenylalanine^{239} mutant by arginine and tryptophan (F239R and F239W) were 3 – 19-fold and 2 – 15-fold higher, respectively, compared to the wild-type AT_1 receptor. The results suggested that substitution by a positively charged or sterically hindered amino acid in the AT_1 receptor allows it to interact with the acidic tetrazole moiety and carboxylate groups of the antagonists more strongly compared to the wild-type receptor. These findings may play an important role to change the binding affinity of the antagonists to an effective level for the pharmacological function of the drugs.

Keywords: site-directed mutagenesis, angiotensin II type 1 (AT_1) receptor, radioligand binding, binding affinity, antagonist

Introduction

In our previous study we identified the binding sites of non-peptide angiotensin II (Ang II)–receptor antagonists with Ang II type 1 (AT_1) receptor by molecular modeling (1). We also showed the binding affinity and internalization behavior of the constitutively active mutant (CAM) N111G of AT_1 receptors in our earlier studies (2). The Ang II receptors are the members of the G-protein–coupled receptor (GPCR) superfamily, which form one of the largest protein families, with several hundred members in humans (3). Despite the wide variety of ligands and physiological roles, all these receptors are structurally characterized by seven transmembrane (TM) domains. Two subtypes of Ang II receptors have been identified (4, 5) and functionally characterized: Ang II type 1 (AT_1) and type 2 (AT_2) receptors (6). The AT_1 receptor mediates virtually all the known physiological actions of Ang II, including vascular contraction, aldosterone secretion, sodium and water retention, neuronal activation, and cardiovascular cell growth and proliferation (7, 8). The AT_1 receptor is an important target for drug development because abnormalities in its function are linked to hypertension, water-electrolyte imbalance, hyperaldosteronism, cardiac hypertrophy, and heart failure (9). Ang II–receptor blockers (ARBs) are highly selective for AT_1 receptors. In addition to their blood pressure–lowering effects, ARBs have been shown to

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promote regression of left ventricular hypertrophy and decrease cardiovascular morbidity and mortality in patients with heart failure or hypertensive diabetic nephropathy with proteinuria (7).

Although the receptor structure–function relationships vary markedly, changes in seven TM helical structures on GPCRs are essential for signal transduction. The activation of GPCRs has been proposed to involve a common pattern of movement of TM helices that is likely conserved in all GPCRs (10, 11). Ang II binds to the AT_1 receptor and induces cell signaling accompanied by changes in the TM3-TM6 conformation (12). The AT_1 receptor binds a peptide ligand and does not share significant overall homology with other receptors of the seven-TM domain family (13). Peptide antagonists have higher affinity toward the AT_1 receptor, suggesting that they bind in a unique conformation to the receptor (14). Recent mutational studies of the AT_1 receptor have shown that differential structural requirements exist for peptide and non-peptide ligand binding sites on the AT_1 receptor (15 – 18).

Binding of non-peptide AT_1-receptor antagonists may be dependent on non-conserved residues located deep in the hydrophobic TM segments of the AT_1 receptor, as demonstrated by mutational analysis of AT_1 receptors (13, 14, 17 – 22). Through site-directed mutagenesis, in our previous study, we showed the binding affinities of Ang II and several ARBs to the wild-type and CAM N111G of AT_1 receptors in which it was demonstrated that the binding affinities of the ARBs decreased in the N111G mutant compared to wild-type receptors (2). In this study, we mutated important amino acids (Fig. 1) of the AT_1 receptor using site-directed mutagenesis and analyzed these mutants by binding studies and inositol phosphate (IP) accumulation assay mutants. We confirmed that the binding affinities of the non-peptide antagonists of the AT_1 receptor increase in all the mutants compared to the wild-type receptor.

Materials and Methods

DNA constructs

The synthetic rat AT_1-receptor gene, cloned in the shuttle expression vector pMT-2, was used for expression and mutagenesis, as described in earlier studies (23). The mutants were prepared by the polymerase chain reaction (PCR) method with the QuichChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). DNA sequence analysis was done to confirm the mutation.

Cell culture, transfection, and membrane preparation

COS-7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. The wild-type and mutant AT_1 receptors were transfected transiently into COS-7 cells using Lipofectamine^{TM} 2000 according to the manufacturer's protocol (Invitrogen Life Technologies, Rockville, MD, USA). To express the AT_1-receptor protein, 12 μg of purified plasmid DNA/10^7 cells was used in the transfection. Transfected COS-7 cells that had been cultured for 48 h were harvested with ice-cold phosphate-buffered saline (PBS), pH 7.4; washed by Hank's buffered salt solution (HBSS) with 1.5% 0.5 M EDTA, 0.15% 50 mg/ml PMSF, and 0.15% 2 mg/ml aprotinin; and finally suspended in 0.25 M sucrose solution containing 1.5% 0.5 M EDTA, 0.15% 50 mg/ml PMSF, and 0.15% 2 mg/ml aprotinin. The cells were then disrupted by a Polytron Homogenizer for 10 s. The mass was centrifuged at 4°C for 5 min at 1260 × *g*, and the supernatant was ultra centrifuged at 4°C for 20 min at 30,000 × *g*. The resulting pellets were suspended in the binding assay buffer and used for binding experiments. The protein contents of the membranes were

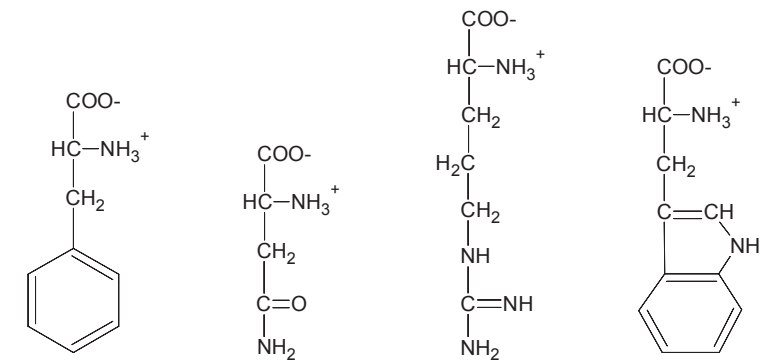

Fig. 1. Chemical structure of some important amino acids.

measured by the method of Lowry et al. (24) using bovine serum albumin as the standard.

Radioligand binding assay

Binding assays for the wild-type and mutants of AT_1 receptors were carried out in the incubation tube that contained 10 μg of membrane protein, [^{125}I]-Sar^1-Ilu^8-angiotensin II, unlabeled drug as required, and binding buffer containing 20 mM phosphate buffer, 100 mM sodium chloride, 20 mM magnesium chloride, 1 mM EGTA, and 0.2% BSA, pH 7.4 in a final volume of 125 μl. Both saturation and competition binding assays were carried out as described previously (25, 26). Briefly, for saturation binding studies, 6 – 7 concentrations (5 – 800 pM) of [^{125}I]-Sar^1-Ilu^8-angiotensin II were tested in duplicate. Nonspecific binding was defined as the amount of radioligand binding remaining in the presence of 10 μM Ang II. For competition binding studies, membranes were incubated with 250 pM of [^{125}I]-Sar^1-Ilu^8-angiotensin II and different concentrations of unlabelled drugs for 1 h at 25°C. The incubation was terminated by rapid filtration under vacuum through Whatman GF/C filters that had been presoaked in 0.5% polyethyleneimine followed by three times washing with ice-cold 50 mM tris HCl (pH 8.0). The bound ligand fraction was determined from the counts/min remaining on the membrane.

IP accumulation assay

COS-7 cells at about 90% confluence in 10-cm dishes were seeded into 24-well plates. The cells were then transiently transfected with plasmid DNA of the wild-type and mutants of AT_1 receptors. At 24 h after transfection, the cells were labeled with 1 μCi/ml [^{3}H]-myo-inositol in DMEM and incubated for 20 h at 37°C. The cells were washed with HBSS and exposed to HBSS containing 20 mM phosphate buffer and 20 mM LiCl, pH 7.4. The cells were incubated for 30 min at 37°C. Agonist and antagonists were added to each well and incubation continued for an additional 1 h at 37°C. At the end of the incubation, the medium was removed and the reaction was stopped by adding 1 ml of 10 mM formic acid (previously stored at 4°C) to each well. The plates were stored at 4°C for 2 h, and then the cells were neutralized by 1 ml 500 mM KOH and 9 mM sodium tetraborate per well. The contents of each well were extracted and centrifuged for 5 min at 1400 × *g* and the upper layer was transferred to a 1-ml AG1-X8 resin (100 – 200 mesh; Assist Co., Tokyo)–loaded column. The columns were washed 2 times with 5 ml 60 mM sodium formate and 5 mM borax. Total IPs were eluted with 5 ml 1 M ammonium formate and 0.1 M formic acid. Radioactivity was measured by a liquid scintillation spectrophotometry.

Data analyses

Nonlinear regression analyses of saturation and competition binding assay were performed by GraphPad Prism software (San Diego, CA, USA). The results of experiments were expressed as the mean ± S.E.M. In competition binding experiments, the values of inhibition constant (K_i) were calculated by the following equation (27):

$$K_i = IC_{50} / \{1 + ([L] / K_d)\}$$

, where the inhibition concentrations (IC_{50}) were determined as the concentrations of ligands that inhibited [^{125}I]-Sar^1-Ilu^8-angiotensin II binding by 50%, [L] = the concentration of [^{125}I]-Sar^1-Ilu^8-angiotensin II used, and K_d = the dissociation constant of [^{125}I]-Sar^1-Ilu^8-angiotensin II for the receptor. In the IP accumulation assay, IC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism software. Statistical analyses were performed by Student's unpaired *t*-test (two-tailed). A *P* value of less than 0.05 was taken as significant.

The pK_b values were estimated from the inhibitory effects of the antagonists on the concentration-dependent total IP accumulation curve for human Ang II according to the literature by Furchgott (28). pK_b = negative logarithm of K_b value, where K_b value was determined using the following equation:

$$(EC_{50} \text{ antagonist} / EC_{50} \text{ agonist}) - 1 = [B] / K_b$$

, where EC_{50} antagonist = concentration of agonist in presence of a particular concentration of antagonist ([B]) at which 50% of total IP is produced, EC_{50} agonist = concentration of agonist at which 50% of total IP is produced, K_b = dissociation constant of antagonist. The agonist and antagonist EC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism software. Statistical analyses were performed by Student's *t*-test.

Drugs

Ang II used in the study was purchased from Peptide Institute, Inc., Osaka. Candesartan was obtained as gift from Takeda Chemical Industries Ltd., Osaka. Losartan was obtained from Merck Research Laboratories, Rahway, NJ, USA. Valsartan was a gift from Novartis Institutes for BioMedical Research, Inc., Cambridge, MA, USA. Telmisartan was obtained from Nippon Boehringer Ingelheim, Tokyo. [^{125}I]-Sar^1-Ilu^8-angiotensin II (2200 Ci/mmol) was purchased from Perkin Elmer, Inc., Boston, MA, USA. [^{3}H]-Myo-inositol (83 Ci/mmol) was purchased from Amersham Biosciences, Piscataway, NJ, USA.

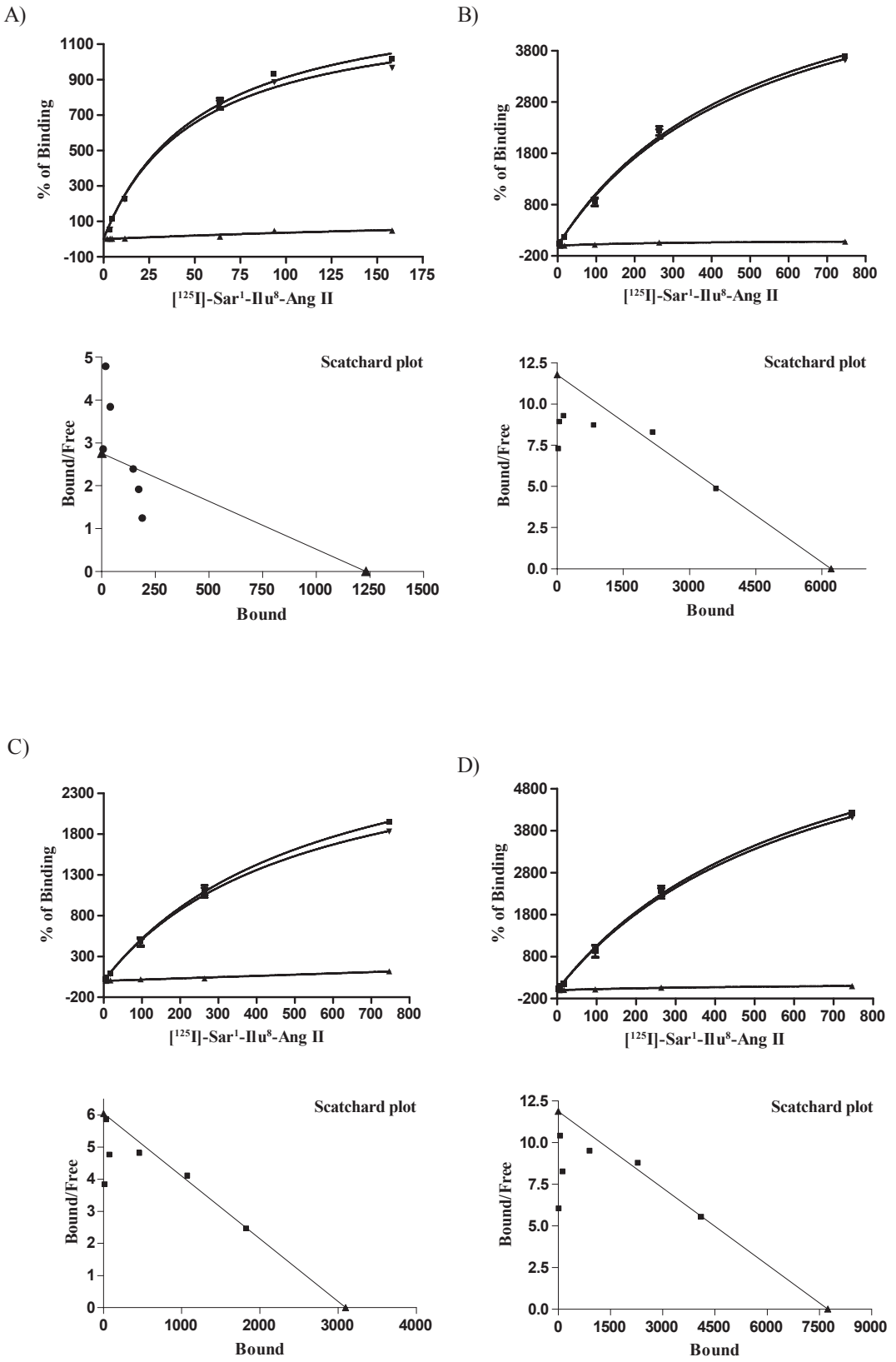

Fig. 2. Saturation analysis of wild-type (A), N235R (B), F239R (C), and F239W (D) mutants of AT_1 receptor labeled with [^{125}I]-Sar^1-Ilu^8-angiotensin II. Percentage of total binding (square), specific binding (inverted triangle), and nonspecific binding (triangle) are plotted against the concentration of the radioligand. Scatchard plots are also shown for each of the receptors. Each point represents the mean ± S.E.M. of four different experiments, each performed in duplicate.

Table 1. Dissociation constant (K_d) and maximum binding sites (B_{max}) of [^{125}I]-Sar^1-Ilu^8-angiotensin II for the wild-type and some mutants of AT_1 receptors

Receptor	K_d value (pM)	B_{max} (fmol/mg protein)
AT_1 WT	552.1 ± 20.0	1524.0 ± 70.1
N235R	515.6 ± 6.3	6173.0 ± 19.6**
F239R	467.3 ± 22.0*	2982.7 ± 55.9**
F239W	623.3 ± 14.9*	7538.3 ± 103.4**

[^{125}I]-Sar^1-Ilu^8-angiotensin II was used to label the wild-type and some mutants of AT_1 receptors transiently expressed in COS-7 cells. Data represent the mean ± S.E.M. of 4 independent experiments, each performed in duplicate. *$P < 0.05$, **$P < 0.001$ vs. wild-type.

Results

Radioligand binding of wild-type and mutant receptors

Saturation binding analysis: [^{125}I]-Sar^1-Ilu^8-angiotensin II was used to determine the dissociation constant (K_d) and maximum bound (B_{max}) values for the wild-type and each of the mutant receptors. Data are summarized in Table 1 and the representative saturation isotherms with Scatchard plots are shown in Fig. 2 (A – D). The wild-type and N235R mutant of AT_1 receptors bound the radioligand with high affinity (K_d = 552.1 ± 20.0 and 515.6 ± 6.3 pM respectively) (Table 1). The F239R mutant bound the radioligand with significantly higher affinity (K_d = 467.3 ± 22.0 pM) compared to the wild-type receptor, whereas lower affinity (K_d = 623.3 ± 14.9 pM) was found with the F239W mutant (Table 1). On the other hand, B_{max} values, calculated from the maximal specific binding of [^{125}I]-Sar^1-Ilu^8-angiotensin II, of the mutant receptors were 2 – 5-fold higher than those for the wild-type receptors (B_{max} = 1524.0 ± 70.1) (Table 1). The expression level for a given construct was found to be highly consistent in separate preparations.

Competition binding: Displacement of [^{125}I]-Sar^1-Ilu^8-angiotensin II by the AT_1-receptor agonist Ang II was analyzed and the wild-type receptor values were compared with the properties of the mutant receptors. The mutants showed 3 – 10-fold higher affinity towards Ang II compared to the wild-type receptor (Table 2). The binding affinities of several AT_1-receptor antagonists, candesartan, valsartan, losartan, and telmisartan, for the wild-type receptor were also determined in competition binding experiments for sites labeled with [^{125}I]-Sar^1-Ilu^8-angiotensin II. Figure 3 (A – D) shows representative competition curves and all data are summarized in Table 2. The results showed that candesartan had the highest binding affinity (K_i = 3.4 ± 0.3 nM) among all the antagonists used in this study towards the wild-type receptor. Telmisartan also had high binding affinity (K_i = 3.7 ± 1.1 nM), whereas valsartan showed moderate affinity (K_i = 8.2 ± 3.2 nM) to the wild-type AT_1 receptor. Losartan showed the lowest affinity (K_i = 59.4 ± 2.7 nM) among the antagonists. Competition binding experiments by the antagonists were also done for the mutant receptors to determine the change in binding affinity. All the antagonists of this study showed 2 – 34-fold higher binding affinities towards the mutants compared to the wild-type receptor (Table 2). However, the rank order of the binding affinity of the antagonists was same in the mutants as in the wild-type receptors, candesartan showing the highest and losartan having the lowest affinity to the receptors.

IP accumulation assay

The wild-type and mutant receptors were expressed in transiently transfected COS-7 cells and IP production was measured. The data of inhibition of Ang II–stimulated IP formation by several AT_1-receptor antagonists are summarized in Table 3, and Fig. 4 (A – D) shows representative Ang II–stimulated IP accumulation in the wild-type and mutant receptors. The results were qualitatively similar to the binding assay results. Candesartan showed the highest potency (pK_b = 8.43 ± 0.24) in inhibiting Ang II–stimulated IP formation. The lowest potency was shown by losartan (pK_b = 7.17 ± 0.08), whereas telmisartan and valsartan had pK_b values of 8.15 ± 0.12

Table 2. Binding affinities (K_i) of an agonist and antagonists (in nM) to the wild-type and some mutants of AT_1 receptors

Receptor	Ang II	Candesartan	Valsartan	Losartan	Telmisartan
AT_1 WT	30.6 ± 8.5	3.4 ± 0.3	8.2 ± 3.2	59.4 ± 2.7	3.7 ± 1.1
N235R	7.2 ± 0.2*	0.1 ± 0.0**	1.1 ± 0.1	8.6 ± 0.2**	0.7 ± 0.0*
F239R	3.1 ± 0.1*	0.5 ± 0.0**	2.5 ± 0.1	3.1 ± 0.1**	1.6 ± 0.1
F239W	8.9 ± 0.2*	1.0 ± 0.0**	3.0 ± 0.1	4.1 ± 0.0**	2.0 ± 0.0

[^{125}I]-Sar^1-Ilu^8-angiotensin II (250 pM) was used to label the wild-type and some mutants of AT_1 receptors transiently expressed in COS-7 cells. Data represent the mean ± S.E.M. of 4 independent experiments, each performed in duplicate. *$P < 0.05$, **$P < 0.001$ vs. wild-type.

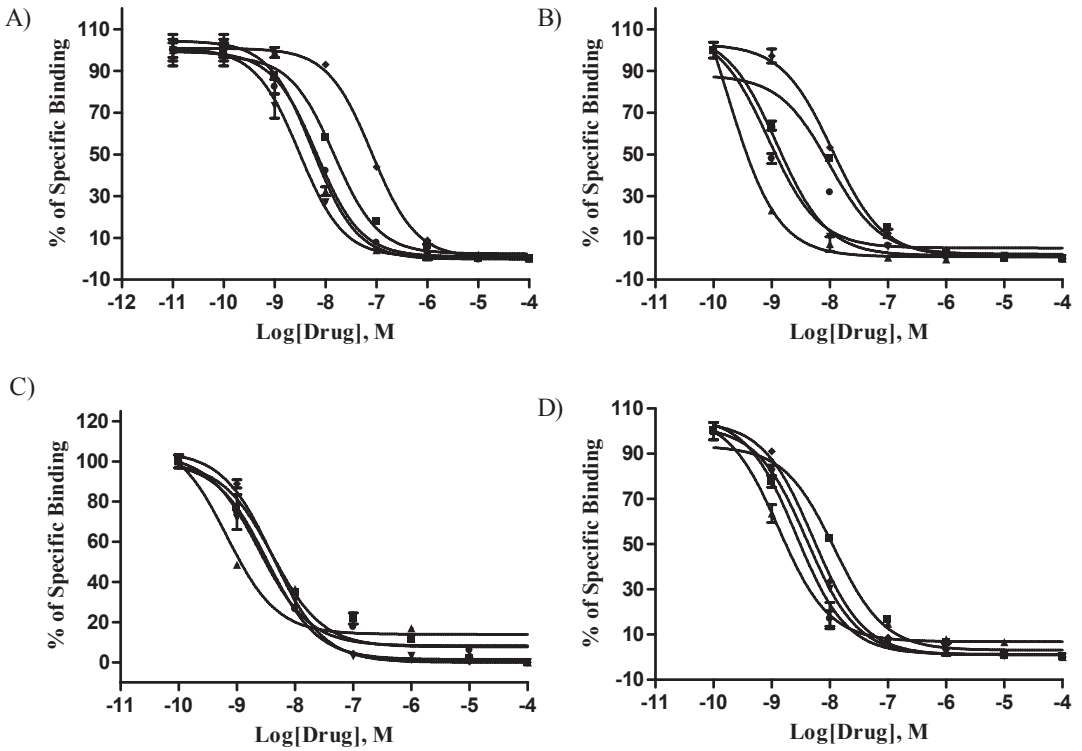

Fig. 3. Competition binding studies of Ang II (square) and the AT_1-receptor antagonists candesartan (triangle), valsartan (inverted triangle), losartan (diamond), and telmisartan (circle) for [^{125}I]-Sar^1-Ilu^8-angiotensin II binding to wild-type (A), N235R (B), F239R (C), and F239W (D) mutants of the AT_1 receptor. The data represent the mean ± S.E.M. of four different experiments, each performed in duplicate.

Table 3. Functional potencies (pK_b) of several AT_1 antagonists towards the wild-type and some mutants of AT_1 receptors

Receptor	AT_1 receptor antagonists			
	Candesartan	Valsartan	Losartan	Telmisartan
AT_1 WT	8.43 ± 0.24	7.89 ± 0.04	7.17 ± 0.08	8.15 ± 0.12
N235R	8.91 ± 0.03	7.98 ± 0.01	7.25 ± 0.02	8.51 ± 0.02*
F239R	7.66 ± 0.01*	7.03 ± 0.01**	6.58 ± 0.02**	7.60 ± 0.02*
F239W	7.52 ± 0.05**	7.19 ± 0.04**	6.74 ± 0.03*	7.11 ± 0.08**

[^{3}H]-Myo-inositol was used to label COS-7 cells transiently transfected with the wild-type and some mutants of AT_1 plasmid. Data represent the mean ± S.E.M. of 4 independent experiments, each performed in duplicate. *$P < 0.05$, **$P < 0.001$ vs. wild-type.

and 7.89 ± 0.04, respectively, in the wild-type receptor. The rank order of functional potency of the antagonists in the mutant receptors was also similar that in the wild-type receptor.

Discussion

The AT_1 receptor plays a major role in the regulation of cardiovascular homeostasis; thus the development of non-peptide AT_1-receptor antagonists represents a very important contribution in the effective treatment of hypertension and congestive heart failure (29 – 32). The mechanism of binding of the antagonists to the AT_1 receptor or its mutants is still not well understood. However, some reports have been published showing the important sites of interaction with different antagonists in the AT_1 receptor (25, 33 – 35). In our earlier study we evinced the binding affinities of different AT_1-receptor antagonists to the wild-type receptor and showed that the binding data were consistent with the number of interac-

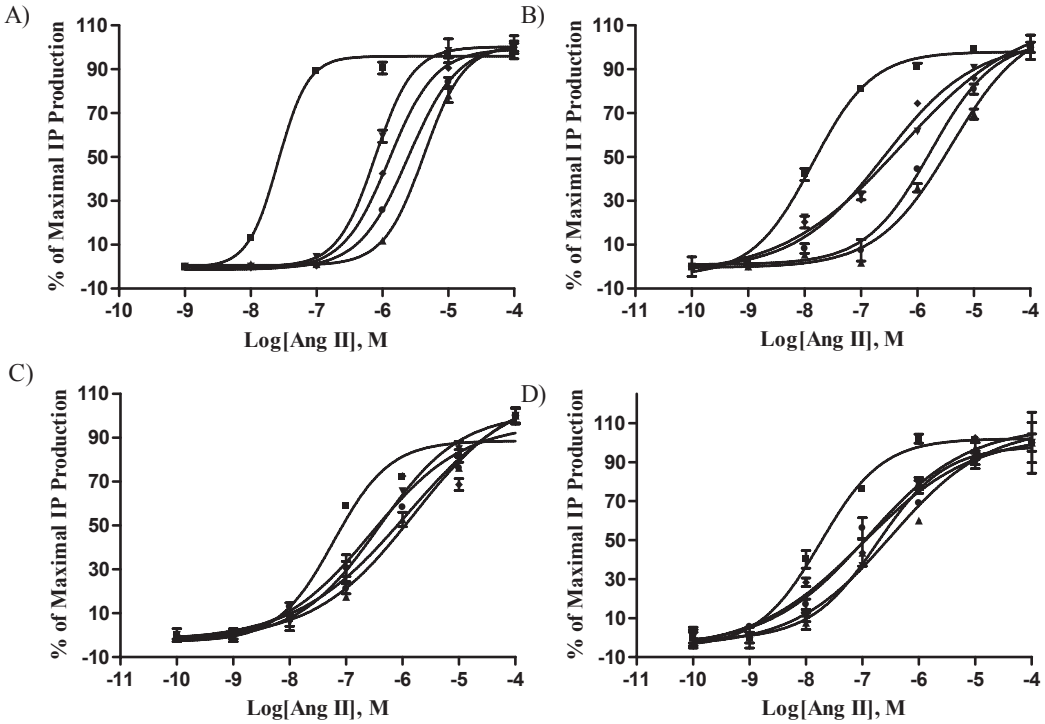

Fig. 4. Inhibition of Ang II (square)–stimulated IP production by the AT_1-receptor antagonists candesartan (triangle), valsartan (inverted triangle), losartan (diamond), and telmisartan (circle) in transiently transfected COS-7 cells expressing wild-type (A), N235R (B), F239R (C), and F239W (D) mutants of the AT_1 receptor. The data represent the mean ± S.E.M. of four different experiments, each performed in duplicate.

tion sites through molecular modeling of these drugs with the AT_1 receptor (1). We also demonstrated the binding affinities of non-peptide AT_1-receptor antagonists to the constitutively active AT_1-receptor mutant N111G (2) in which the affinity was decreased to a significant amount compared to the wild-type receptor. So the present study was performed to determine if there is increased affinity of the non-peptide antagonists towards some specific mutants of the AT_1 receptor and to investigate the importance of the amino acid residues responsible for such observations.

In saturation binding experiments, the data represent different levels of cell expression of the mutant receptors compared to the wild-type AT_1 receptor. The mutants showed 2 – 5-fold higher B_{max} value as assessed by the radioligand [^{125}I]-Sar^1-Ilu^8-angiotensin II binding compared to the wild-type receptor. There was no significant difference between the values of the dissociation constant (K_d) of [^{125}I]-Sar^1-Ilu^8-angiotensin II for the wild-type and N235R mutant of the AT_1 receptor, although F239R bound to the radioligand with significantly higher affinity whereas lower affinity with the ligand was found in the F239W mutant. It is evident from the competition binding study (Table 2) that all the antagonists of this study showed higher binding affinity towards the mutants compared to the wild-type AT_1 receptor (candesartan, 3 – 34-fold; valsartan, 3 – 7-fold; losartan, 7 – 19-fold; and telmisartan, 2 – 5-fold). It is obvious that the strength with which the investigated ARBs bind to the AT_1 receptor is associated with their chemical structure. Losartan possesses only one acidic tetrazole moiety, whereas candesartan and valsartan have an additional acidic carboxylate group. It can be reasoned that a positively charged amino acid at the receptor may serve as a counter ion for such a critical functional group. Site-directed mutagenesis studies involving the substitution of certain neutral amino acid residues with basic or positively charged amino acid residues in the AT_1 receptor offer a way to evaluate this hypothesis. Figure 1 illustrates the chemical structures of the amino acids involved in our mutagenesis study. Phenylalanine^{235} and asparagine^{239} of the third intracellular loop 3 (il3) of the AT_1 receptor are neutral amino acids. So substitution of these amino acids by positively charged arginine in the AT_1 receptor may result in a stronger interaction with the antagonists of this study. This may be the reason for the increased affinities of the AT_1-receptor antagonists towards N235R and F239R mutants. This finding is consistent with an earlier

study where the substitution of positively charged lysine[199] by non-charged glutamine decreased the binding affinity of non-peptide AT_1-receptor antagonists (36). Introduction of tryptophan in place of phenylalanine[239] of the AT_1 receptor may affect the conformation of the receptor due to steric hindrance of tryptophan itself, resulting in more room for ligand binding. Eventually the binding affinities of the antagonists were higher in F239W mutant compared to the wild-type AT_1 receptor, although the level of the increase in the binding affinity was less in comparison with the N235R and F239R mutants.

Figure 4 illustrates the concentration–response curves of Ang II–stimulated IP accumulation in wild-type and mutant receptors in the absence and presence of non-peptide AT_1-receptor antagonists. All the antagonists of this study shifted the concentration–response curve to the right without a significant change in the maximum response. The antagonists inhibited Ang II–stimulated IP production with the same pattern in the mutants, although the extent of inhibition was lower in the mutants compared to the wild-type receptor. The data of inhibition of Ang II–stimulated IP formation by the antagonists are summarized in Table 3. The data is consistent with the binding assay results with a point of view that higher binding affinity may lead to decrease in the functional potency of the antagonists in the mutant receptors, although it is hard to rationalize the functional potency data of the antagonists in the N235R mutant.

In summary, our results show in a novel fashion that the mutation of asparagine[235] and phenylalanine[239] by the positively charged amino acid arginine causes an increase in the binding affinity of the AT_1-receptor antagonists. Substitution of phenylalanine[239] by tryptophan also increases the binding affinity of the antagonists with a different mechanism. Thus the amino acids of these positions of il3 of AT_1 receptor are very important for the ligand binding affinity. If there is any natural mutation in such positions of the AT_1 receptor in a disease state, these findings may play an important role in determining the effective dose of the antagonists for pharmacological functions in the receptors.

References

1 Bhuiyan MA, Ishiguro M, Hossain M, Nakamura T, Ozaki M, Miura S, et al. Binding sites of valsartan, candesartan and losartan with angiotensin II receptor 1 subtype by molecular modeling. Life Sci. 2009;85:136–140.

2 Bhuiyan MA, Hossain M, Miura S, Nakamura T, Ozaki M, Nagatomo T. Constitutively active mutant N111G of angiotensin II type 1 (AT_1) receptor induces homologous internalization through mediation of AT_1-receptor antagonist. J Pharmacol Sci. 2009;111:227–234.

3 Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. Science. 2001;291: 1304–1351.

4 Whitebread S, Mele M, Kamber B, DeGasparo M. Preliminary biochemical characterization of two angiotensin II receptor subtypes. Biochem Biophys Res Commun. 1989;163:284–291.

5 Chiu AT, Herblin WF, McDall DE, Ardecky RJ, Carini DJ, Duncia JV, et al. Identification of angiotensin receptor subtypes. Biochem Biophys Res Commun. 1989;165:196–203.

6 Bumpus FM, Catt KJ, Chiu AT, DeGasparo M, Goodfriend T, Husain A, et al. Nomenclature for angiotensin receptors. A report of Nomenclature Committee of the Council for High Blood Pressure Research. Hypertension. 1999;17:720–721.

7 De Gasparo M, Catt KJ, Inagami T, Wright JW, Unger Th. The International Union of Pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev. 2000;52:415–472.

8 Burnier M. Angiotensin II type 1 receptor blockers. Circulation. 2001;103:904–912.

9 Peach MJ. Renin-angiotensin system. Biochemistry and mechanisms of action. Physiol Rev. 1977;57:313–370.

10 Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev. 2000;21:91–113.

11 Peleg G, Ghanouni P, Kobilka BK, Zare RN. Single-molecule spectroscopy of the β_2 adrenergic receptor: observation of conformational substates in a membrane protein. Proc Natl Acad Sci U S A. 2001;98:8469–8474.

12 Ghanouni P, Steenhuis JJ, Farrens DL, Kobilka BK. Agonist-induced conformational changes in the G-protein-coupling domain of the beta 2 adrenergic receptor. Proc Natl Acad Sci U S A. 2001;98:5997–6002.

13 Bihoreau C, Monnot C, Davies E, Teutsch B, Bernstein KE, Corvol P, et al. Mutation of Asp^{74} of the rat angiotensin II receptor confers changes in antagonist affinities and abolishes G-protein coupling. Proc Natl Acad Sci U S A. 1993;90:5133–5137.

14 Noda K, Saad Y, Kinoshita A, Boyle TP, Graham RM, Husain A, et al. Tetarzole and carboxylate groups of angiotensin receptor antagonists bind to the same subside by different mechanisms. J Biol Chem. 1995;270:2284–2289.

15 Hjorth SA, Schambye HT, Greenlee WJ, Schwartz TW. Identification of peptide binding residues in the extracellular domains of the AT_1 receptor. J Biol Chem. 1994;269:30953–30959.

16 Groblewski T, Maigret B, Nouet S, Larguier R, Lombard C, Bonnafous JC, et al. Amino acids of the third transmembrane domain of the AT_{1A} angiotensin II receptor are involved in the differential recognition of peptide and nonpeptide ligands. Biochem Biophys Res Commun. 1995;209:153–160.

17 Ji H, Leung M, Zhang Y, Catt KJ, Sandberg K. Differential structural requirement for specific binding of non-peptide and peptide antagonists to the AT_1 angiotensin receptor. Identification of amino acid residues that determine binding of the antihypertensive drug losartan. J Biol Chem. 1994;269:16533–16536.

18 Schambye HT, Hjorth SA, Bergsma DJ, Sathe G, Schwartz TW. Differentiation between binding sites of angiotensin II and nonpeptide antagonists on the angiotensin II type 1 receptors. Proc Natl Acad Sci U S A. 1994;91:7046–7050.

19 Ji H, Zheng W, Zhang Y, Catt KJ, Sandberg K. Genetic transfer of a non-peptide antagonist binding site to a previously unresponsive receptor. Proc Natl Acad Sci U S A. 1995;92:9240–9244.

20 Marie J, Maigret B, Joseph MP, Larguier R, Nouet S, Lombard C, et al. Tyr^{292} in the seventh transmembrane domain of the AT1A angiotensin II receptor is essential for its coupling to phospholipase C. J Biol Chem. 1994;269:20815–20818.
21 Monnot C, Bohireau C, Conchon S, Curnow KM, Corvol P, Clauser E. Polar residues in the transmembrane domains of the type 1 angiotensin II receptor are required for binding and coupling. Reconstitution of the binding site by co-expression of two deficient mutants. J Biol Chem. 1996;271:1507–1513.
22 Inoue Y, Nakamura N, Inagami T. A review of mutagenesis studies of angiotensin II type 1 receptor, the three-dimensional receptor model in search of the agonist and antagonist binding site and the hypothesis of a receptor activation mechanism. J Hypertens. 1997;15:703–714.
23 Jongejan A, Bruysters M, Ballesteros JA, Haaksma E, Bakker RA, Pardo L, et al. Linking agonist-binding to histamine H_1 receptor activation. Nat Chem Biol. 2005;1:98–103.
24 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–275.
25 Miura S, Fujino M, Hanzawa H, Kiya Y, Imaizumi S, Matsuo Y, et al. Molecular mechanism underlying inverse agonist of angiotensin II type I receptor. J Biol Chem. 2006;281:19288–19295.
26 John H, Jennifer NH, Steven JF, Daniel KY. Identification of angiotensin II type 2 receptor domains mediating high-affinity CGP 42112A binding and receptor activation. J Pharmacol Exp Ther. 2001;298:665–673.
27 Cheng Y-C, Prusoff WH. Relationship between the inhibition constant (Ki) and the concentration of inhibition, which causes 50% inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol. 1973;22:3099–3108.
28 Furchgott RF. The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In: Blaschko H, Muscholl E, editors. Handbook of experimental pharmacology. Vol. 33, Catecholamines. Berlin: Springer; 1972. p. 283–335.
29 Timmermans PB, Wong PC, Chiu AT, Herblin WF. Non-peptide angiotensin II receptor antagonists. Trends Pharmacol Sci. 1991;12:55–62.
30 Burnier M, Brunner HR. Angiotensin II receptor antagonist in hypertension. Kidney Intern. 1998;54 Suppl 68:S107–S111.
31 Awan NA, Mason DT. Direct selective blockade of the vascular angiotensin II receptor in therapy for hypertension and severe congestive heart failure. Am Heart J. 1996;131:177–185.
32 Ellis ML, Patterson JH. A new class of antihypertensive therapy: angiotensin II receptor antagonists. Pharmacotherapy. 1996;16:849–860.
33 Underwood DJ, Strader CD, Rivero R, Patchett AA, Greenlee W, Prendergast K. Structural model of antagonist and agonist binding to the angiotensin II, AT_1 subtype, G protein coupled receptor. Chem Biol. 1994;1:211–221.
34 Verheijen I, De Backer JP, Vanderheyden P, Vauquelin G. A two-state model of antagonist-AT_1 receptor interaction: further support by binding studies at low temperature. Biochem Pharmacol. 2004;67:397–399.
35 Verheijen I, Fierens FLP, De Backer JP, Vauquelin G, Vanderheyden P. Interaction between the partially insurmountable antagonist valsartan and human recombinant angiotensin II type 1 receptors. Fund Clin Pharmacol. 2000;14:577–585.
36 Fierens FLP, Vanderheyden PML, Gaborik Z, Minth TL, De Backer JP, Hunyady L, et al. Lys199 mutation of the human angiotensin type 1 receptor differentially affects the binding of surmountable and insurmountable non-peptide antagonists. J Renin-Angiotensin Syst. 2000;1:283–288.