ORIGINAL ARTICLE

Unique binding behavior of the recently approved angiotensin II receptor blocker azilsartan compared with that of candesartan

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The angiotensin II type 1 (AT₁) receptor blocker (ARB) candesartan strongly reduces blood pressure (BP) in patients with hypertension and has been shown to have cardioprotective effects. A new ARB, azilsartan, was recently approved and has been shown to provide a more potent 24-h sustained antihypertensive effect than candesartan. However, the molecular interactions of azilsartan with the AT₁ receptor that could explain its strong BP-lowering activity are not yet clear. To address this issue, we examined the binding affinities of ARBs for the AT₁ receptor and their inverse agonist activity toward the production of inositol phosphate (IP), and we constructed docking models for the interactions between ARBs and the receptor. Azilsartan, unlike candesartan, has a unique moiety, a 5-oxo-1,2,4-oxadiazole, in place of a tetrazole ring. Although the results regarding the binding affinities of azilsartan and candesartan demonstrated that these ARBs interact with the same sites in the AT₁ receptor (Tyr¹¹³, Lys¹⁹⁹ and Gln²⁵⁷), the hydrogen bonding between the oxadiazole of azilsartan-Gln²⁵⁷ is stronger than that between the tetrazole of candesartan-Gln²⁵⁷, according to molecular docking models. An examination of the inhibition of IP production by ARBs using constitutively active mutant receptors indicated that inverse agonist activity required azilsartan–Gln²⁵⁷ interaction and that azilsartan had a stronger interaction with Gln²⁵⁷ than candesartan. Thus, we speculate that azilsartan has a unique binding behavior to the AT₁ receptor due to its 5-oxo-1,2,4-oxadiazole moiety and induces stronger inverse agonism. This property of azilsartan may underlie its previously demonstrated superior BP-lowering efficacy compared with candesartan and other ARBs.

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INTRODUCTION

The angiotensin II (Ang II) type 1 (AT₁) receptor, which is a member of the G-protein-coupled receptor superfamily, has a widespread tissue distribution and mediates most known cardiovascular functions.¹ AT₁ receptor blockers (ARBs) are highly selective for the AT₁ receptor and block the diverse effects of Ang II.

Azilsartan, a new ARB, was recently approved for the treatment of hypertension and is the eight ARB in clinical use worldwide. Azilsartan medoxomil, a prodrug of azilsartan, was approved in the United States by the Food and Drug Administration in 2011 for the treatment of hypertension. Azilsartan was approved for use in Japan in 2012. Azilsartan medoxomil and azilsartan have been shown to have greater antihypertensive effects than other ARBs.^{2–5} Treatment with azilsartan medoxomil lowered 24-h blood pressure (BP) significantly more than treatment with olmesartan medoxomil or valsartan.^{2,3} In addition, a multicenter, randomized, double-blind study compared the efficacy and safety of azilsartan with that of

candesartan cilexetil in 622 Japanese patients with grade I–II essential hypertension. The results showed that azilsartan provided a more potent 24 h sustained antihypertensive effect than candesartan, but with equivalent safety.⁵ Azilsartan was discovered by modification of the tetrazole ring in candesartan, and has a unique moiety, a 5-oxo-1,2,4-oxadiazole, in place of a tetrazole ring.⁶ Most ARBs have class (or common) effects because they have common molecular structures (biphenyl-tetrazole and imidazole groups; Supplementary Figure 1). ARBs have been shown to have class- and molecule-specific differential effects in basic experimental studies.⁷ We also proposed that small differences in the molecular structures of ARBs could lead to differences in their abilities to influence the AT₁ receptor,^{7,8} as small differences in ligands for other G-protein-coupled receptors could lead to differences in pharmacological effects.^{9,10} In comparison with other ARBs, azilsartan bound tightly to and dissociated slowly from AT₁ receptors.¹¹ In addition, azilsartan induced the insurmountable antagonism of Ang II-induced vascular contractions against AT₁ receptor.

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We previously reported that the AT₁ receptor exhibits a low level of constitutive activity in the absence of any ligand.¹² Small differences in the chemical structures of ligands can be responsible for agonism, neutral antagonism or inverse agonism toward a G-protein-coupled receptor.^{13,14} Therefore, we hypothesized that the 5-oxo-1,2,4-oxadiazole moiety of azilsartan, which represents a small difference in the molecular structures of azilsartan and candesartan, may be responsible for the molecular effects of azilsartan, such as inverse agonism. We examined the binding affinities of azilsartan and candesartan to the AT₁ receptor, along with their inverse agonist activity, and we constructed molecular docking models for the comparison of the interactions between these ARBs and the receptor.

MATERIALS AND METHODS

Materials

The following antibodies and reagents were purchased or provided: candesartan, azilsartan and azilsartan-7H, which does not contain a carboxyl group in the benzimidazole ring compared with azilsartan (Takeda Pharm, Osaka, Japan); [Sar¹]Ang II and [Sar¹, Ile⁸]Ang II (Sigma-Aldrich, St Louis, MO, USA); and [¹²⁵I]-[Sar¹, Ile⁸]Ang II (Amersham Biosciences, Buckinghamshire, UK).

Mutagenesis and expression of the AT₁ receptor and membrane preparation

The synthetic wild-type (WT) AT₁ receptor gene, cloned in the shuttle expression vector pMT-3, was used for expression and mutagenesis studies, as described previously.^{13,15}

Cell cultures, transfections and membrane preparation

COS1 cells were cultured in 10% fetal bovine serum and penicillin- and streptomycin-supplemented Dulbecco's modified Eagle's essential medium (Invitrogen, Carlsbad, CA, USA) in 5% CO₂ at 37 °C. In the experiments, cells without cell-growth supplement were used. Cell viability in control experiments was >95% by trypan blue exclusion analysis. The WT and mutant AT₁ receptors were transiently transfected into COS1 cells using Lipofectamine 2000 liposomal reagent (Invitrogen) according to the manufacturer's instructions. Cell membranes were prepared by the nitrogen Parr bomb disruption method in the presence of protease inhibitors.

Competition binding study

The K_d values of receptor binding were determined by [¹²⁵I]-[Sar¹, Ile⁸] AngII-binding experiments under equilibrium conditions, as described previously.^{13,15} Cell membranes expressing the WT or mutant receptor were incubated at room temperature for 1 h with [¹²⁵I]-[Sar¹, Ile⁸] Ang II. All binding experiments were carried out at 22 °C in a volume of 125 µl. Nonspecific binding to the membranes was determined from [¹²⁵I]-[Sar¹, Ile⁸] Ang II binding in the presence of 10 µM [Sar¹, Ile⁸] Ang II. After equilibrium was reached, the binding experiments were stopped by filtering the binding mixture through Whatman GF/C glass fiber filters, which were extensively washed further with binding buffer to wash the free radioligand. The bound ligand fraction was determined from the c.p.m. remaining on the membrane. Equilibrium-binding kinetics were determined as described previously.^{13,15}

Inositol phosphate (IP) production assay

Agonist- or ARB-induced IP formation by WT and mutant AT₁ receptors in transfected cells was measured to evaluate cell signaling for vasoconstriction. Semi-confluent COS-1 cells transfected in 60 mm petri dishes were labeled for 24 h with [³H]-myoinositol at 37 °C in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. On the day of the functional assay, the labeled cells were washed with Hank's balanced salt solution three times and incubated with Hank's balanced salt solution containing 10 mM LiCl for 20 min; 1 µM ARBs or 0.1 µM [Sar¹]Ang II was added and incubation was continued for another 10 min at 37 °C. At the end of incubation, the medium was removed, and total soluble IP was extracted from the cells by the perchloric acid extraction method, as described previously.^{13,15}

Molecular modeling of AT₁ receptor-ARBs

The amino-acid sequences of human AT₁ receptor, bovine rhodopsin (PDBID:1U19), human β₂ adrenergic receptor (PDBID:2RH1) and human A_{2A} adenosine receptor (PDBID:3EML) were added to a multiple sequence alignment comprising 68 sequences in the purine receptor and to the peptide clusters reported by Costanzi *et al.*¹⁶ We carried out multiple sequence alignment including these sequences using the MOE software (version 2010.10, Chemical Computing Group, Montreal, Quebec, Canada). The BLOSUM62 matrix¹⁷ was applied, with a gap start penalty of 5 and a gap extend penalty of 0.2.

Homology models were built with the program MODELLER 9v7 (Accelrys, San Diego, CA, USA) using rhodopsin, β₂ adrenergic receptor or A_{2A} adenosine receptor as a template protein. Three thousand homology models were generated from each template protein. The extracellular 2 (EL2) loop was built completely *de novo*, without any alignment between the sequences of AT₁ receptor and the template protein, but the disulfide bridge of the conserved Cys residues on the transmembrane (TM)3 helix and EL2 loop was enforced.

Azilsartan was docked into the homology models using the SP algorithm within Schrödinger Glide 5.6 software (Schrödinger, New York, NY, USA). The protein preparation and grid-generation processes were performed automatically using the XGlide python script (Schrödinger). Amino-acid residues Gln²⁵⁷, Lys¹⁹⁹ and Asn²⁹⁵ were used to define the cavity of the grid. Gln²⁵⁷ and Lys¹⁹⁹ were identified as important residues for the interaction between azilsartan and AT₁ receptor by mutagenesis experiments as described in the Results section, and Asn²⁹⁵ was used to dock the ligand inside the membrane protein. No constraints were added to grid generation to ensure that subsequent dockings were not biased in any way. One pose per protein in the docking process was stored for analysis. Model selection was performed using the protein ligand interaction fingerprints module within MOE. We chose five models with ligands that showed both hydrogen bonding with Gln²⁵⁷ and ionic interaction with Lys¹⁹⁹. To refine these models, the induced fit docking protocol within Schrödinger software was applied, and generated 100 refined models from each docking model. Among these models, the model that had the best induced fit docking score¹⁸ was selected. For comparison with the binding model of azilsartan, candesartan was docked into the structure of the best model of the azilsartan/AT₁ receptor using the Glide SP algorithm (Schrödinger). Finally, the docking model of candesartan/AT₁ receptor was minimized to only amino-acid residues that are within 5 Å around the ligand using MacroModel 9.8 within Schrödinger software.

Statistical analysis

The results are expressed as the mean ± s.d. of four or more independent determinations. Significant differences in measured values were evaluated with an analysis of variance using Fisher's *t*-test and unpaired Student's *t*-test. Statistical significance was set at <0.05.

RESULTS

Binding affinities of azilsartan, azilsartan-7H and candesartan to WT and mutant AT₁ receptors

The K_d of azilsartan for the WT AT₁ receptor was comparable to that of candesartan. Next, we selected candidate amino acids in the AT₁ receptor (Ser¹⁰⁵, Ser¹⁰⁹, Tyr¹¹³, Val¹¹⁶, Phe¹⁸², Tyr¹⁸⁴, Lys¹⁹⁹, Phe²⁰⁸, Trp²⁵³, His²⁵⁶, Gln²⁵⁷, Thr²⁸⁷, Tyr²⁹² and Asn²⁹⁵, Supplementary Figure 2) for consideration as specific binding sites of azilsartan based on the molecular models of the AT₁ receptor complex described in previous reports.^{12,13,19,20} To determine the specific site that binds to azilsartan, we examined the binding affinities of azilsartan, azilsartan-7H and candesartan, the chemical structures of which are shown in Figure 1, to AT₁ receptors that were mutated at the candidate amino acids mentioned above. The results are shown in Table 1. The affinities of [Sar¹, Ile⁸]Ang II were almost the same for some mutants and were decreased for other mutants, but not to <1/10 the affinity for the WT AT₁ receptor. The affinity of azilsartan for the Y113A, K199A, Q257A and N295A mutants was reduced by >10-fold compared with the WT AT₁ receptor, suggesting that Tyr¹¹³, Lys¹⁹⁹, Gln²⁵⁷ and Asn²⁹⁵ in the AT₁ receptor are involved in binding

Figure 1 Chemical structures of ARBs.

Table 1 Binding affinities (K_d) of Ang II and ARBs to AT₁ WT and mutants receptors

Receptor	[Sar ^I , Ile ⁸]			
	Ang II	Candesartan	Azilsartan	Azilsartan-7H
WT	0.7 ± 0.1 (1.0)	2.6 ± 0.5 (1.0)	3.1 ± 0.2 (1.0)	49 ± 9 (1.0)
S105A	0.5 ± 0.3 (0.7)	5.4 ± 2.0 (2.1)	12 ± 3 (3.9)	33 ± 5 (0.7)
S109A	1.4 ± 0.1 (2.0)	5.6 ± 0.7 (2.2)	11 ± 3 (3.5)	29 ± 9 (0.6)
N111G	0.8 ± 0.5 (1.0)	15 ± 5 (5.8)	48 ± 6 (15)	2362 ± 756 (48)
N111G/K199Q	1.3 ± 0.9 (1.9)	241 ± 41 (93)	754 ± 54 (243)	13654 ± 667 (279)
N111G/Q257A	1.0 ± 0.2 (1.4)	64 ± 18 (25)	71 ± 27 (15)	4477 ± 437 (91)
Y113A	1.0 ± 0.4 (1.4)	173 ± 45 (67)	232 ± 96 (75)	14794 ± 482 (302)
Y113F	1.8 ± 1.0 (2.6)	7.9 ± 3.6 (3.0)	14 ± 0.3 (4.5)	523 ± 128 (11)
V116A	1.0 ± 0.4 (1.4)	7.4 ± 2.7 (2.8)	9.6 ± 0.8 (3.1)	76 ± 23 (1.6)
F182A	0.6 ± 0.2 (0.9)	3.2 ± 0.5 (1.2)	7.1 ± 0.9 (2.3)	23 ± 5 (0.5)
Y184A	1.0 ± 0.4 (1.4)	2.0 ± 0.7 (1.2)	4.8 ± 1.3 (2.3)	24 ± 9 (0.5)
K199A	3.3 ± 0.5 (4.7)	53 ± 5 (20)	103 ± 7 (33)	2059 ± 344 (42)
F208A	0.7 ± 0.3 (1.0)	3.6 ± 1.2 (1.4)	7.5 ± 1.9 (2.4)	30 ± 12 (0.6)
W253A	2.2 ± 0.8 (3.1)	13 ± 2 (5.0)	25 ± 4 (8.1)	40 ± 7 (0.8)
H256A	0.8 ± 0.3 (1.1)	4.3 ± 1.6 (1.7)	5.6 ± 2.4 (1.8)	54 ± 11 (1.1)
Q257A	2.5 ± 0.2 (3.6)	37 ± 8 (14)	309 ± 22 (100)	5028 ± 1216 (103)
T287A	1.3 ± 0.2 (1.9)	15 ± 5 (5.8)	30 ± 7 (9.7)	741 ± 35 (15)
Y292A	1.7 ± 0.8 (2.4)	4.6 ± 1.4 (1.8)	18 ± 5 (5.8)	22 ± 4 (0.4)
N295A	6.4 ± 2.1 (9.3)	99 ± 16 (38)	212 ± 97 (68)	2291 ± 861 (47)

Abbreviations: Ang II, angiotensin II; ARB, AT₁ receptor blocker; AT₁, angiotensin II type 1; WT, wild type.

Numbers in parentheses show ratio of K_d (mutant)/ K_d (WT).

to azilsartan. Interestingly, azilsartan (100-fold reduction in binding affinity to WT AT₁ receptor) exhibited about a sevenfold greater reduction in binding affinity to the Q257A receptor compared with candesartan (14-fold reduction), indicating that the oxadiazole of azilsartan probably binds to Gln²⁵⁷ of the AT₁ receptor. In addition, the affinity of azilsartan-7H (49 nM) for the AT₁-WT receptor was about 16-fold less than that of azilsartan (3.1 nM), and the affinity of azilsartan was comparable to that of candesartan, suggesting that the carboxyl moiety of azilsartan is also important for binding to the AT₁-WT receptor.

Binding and inverse agonism properties of azilsartan, azilsartan-7H and candesartan

Next, we examined whether azilsartan, azilsartan-7H and candesartan retained the ability to bind to N111G mutant receptor (Table 1). As we previously reported that the AT₁-N111G receptor had high basal activity in the absence of Ang II²¹ and could be used to determine the

inverse agonism of azilsartan, we also analyzed the binding affinities of these ARBs in the mutant receptor. The affinity of azilsartan-7H (5754 nM) for the AT₁-N111G receptor was 113-fold less than that of azilsartan (51 nM). These data suggest that the carboxyl moiety of azilsartan is important for binding to the N111G receptor.

We analyzed whether azilsartan induced inverse agonism toward IP production on WT, N111G, N111G/K199Q and N111G/Q257A receptors (Figure 2). Azilsartan significantly suppressed the basal activity of WT receptor. However, the basal activity of the WT AT₁ receptor is too low to evaluate the differences in inverse agonistic activity among ARBs. To confirm these differences, we used N111G receptor. Although both azilsartan and candesartan significantly suppressed the basal activities of the mutant receptor, azilsartan showed significantly stronger inverse agonism than candesartan. Modification of the carboxyl moiety of azilsartan gave azilsartan-7H, which did not show inverse agonism and instead showed neutral antagonism. In addition, azilsartan did not induce inverse agonism in the N111G/K199Q and N111G/Q257A receptors. Thus, the results suggested that the insertion of oxadiazole in the tetrazole ring in azilsartan induced stronger inverse agonism and the position of Gln²⁵⁷ in addition to Lys¹⁹⁹ in the AT₁ receptor may have a role in the inverse agonism of azilsartan.

Molecular model of the interaction between azilsartan and the AT₁ receptor

A molecular model was constructed based on the three main interactions between the AT₁ receptor and azilsartan that were suggested from the mutation experiments (Figure 3). Gln²⁵⁷ binds to the oxadiazole ring by hydrogen bonding, with a bond distance of 2.6 Å. This distance was shorter than that between Gln²⁵⁷ and the tetrazol ring of candesartan (3.3 Å), indicating that the interaction may form stronger hydrogen bonding. Tyr¹¹³ binds to the biphenyl group of azilsartan by Van der Waals interaction, as the mutagenesis data indicated that the phenyl group of Tyr¹¹³ would be more important for interaction with azilsartan than the hydroxyl group, and the atomic distance was 3.4 Å. This distance was shorter than that between Tyr¹¹³ and the biphenyl group of candesartan (4.0 Å). In addition, Lys¹⁹⁹ was a candidate for binding to the carboxyl group of azilsartan, and the bond distances were 2.6 and 3.2 Å.

DISCUSSION

This study demonstrated that azilsartan induces stronger inverse agonism than candesartan and this ability of azilsartan may be associated with its unique moiety, a 5-oxo-1,2,4-oxadiazole, in place of a tetrazole ring. The oxadiazole ring in azilsartan is not found in any other clinically approved ARBs, as most ARBs, including candesartan, have a biphenylmethyl moiety with an acidic group

Figure 2 Percent of maximum IP production with or without 1 μ M of the ARBs or 0.1 μ M [Sar¹]Ang II in COS1 cells transiently expressing the WT, N111G, N111G/K199Q or N111G/Q257A AT₁ receptor. [Sar¹]Ang II or ARB was added to the medium for 10 min. Percentage (%) maximum IP production indicates [Sar¹]Ang II-induced IP production (1920 c.p.m.) in WT AT₁ receptor-transfected cells (100%) after adjusting for basal IP production (130 c.p.m.) without treatment in mock-treated cells (0%). **P* < 0.05 vs. no treatment. #*P* < 0.05 vs. candesartan.

Figure 3 Molecular model of the interaction between azilsartan (magenta) or candesartan (blue) and the AT₁ receptor. (a) and (b) are side and top views, respectively. The AT₁ receptor is shown as a ribbon. Numbers (in parentheses) show the distances between azilsartan (candesartan) and the sites of the AT₁ receptor.

(either a tetrazole or carboxylic acid). A molecular model suggested that Gln²⁵⁷ binds to the oxadiazole ring by hydrogen bonding, and the bond distance was shorter than that between Gln²⁵⁷ and the tetrazole ring of candesartan.

Fabia *et al.*²² studied 36 reports in which BP was measured using ambulatory BP monitoring. The antihypertensive activities of ARBs differed, and the magnitude of the reduction in BP did not essentially depend on the initial BP values or on the dose used. In addition, azilsartan showed a more potent 24 h sustained antihypertensive effect than candesartan.⁵ Thus, not all ARBs may have the same antihypertensive effects. Although spontaneous mutations have not been reported for the AT₁ receptor, we reported that the WT AT₁ receptor shows slight but significant constitutive activity with regard to the accumulation of IP, which is a cell signaling molecule that has a role in vasoconstriction.¹² An inverse agonist can inhibit the constitutive activity of AT₁ receptor and may induce a stronger

BP-lowering effect than a neutral antagonist toward IP production. Azilsartan showed stronger inverse agonism than candesartan, and this could help explain why azilsartan showed a stronger antihypertensive effect than candesartan. Although WT AT₁ receptor shows only slight constitutive activity, Morisset *et al.*²³ clearly showed that inverse agonists are useful in a therapeutic strategy even if nonmutated receptors are expressed at normal levels in G-protein-coupled receptors, H₃ receptor. Although we understand that the inverse agonism of azilsartan based on an experimental study may not necessarily directly influence the clinical outcome, we believe that it is reasonable to consider this possibility because the concentration of azilsartan (1 μ M) in the experimental study was comparable to the azilsartan concentration in human plasma after the administration of azilsartan in a dose of 20–40 mg.

Moreover, there have been several reports that may explain why azilsartan lowers BP more than other ARBs.^{11,24,25} First, Ojima *et al.*¹¹

reported that azilsartan inhibited the accumulation of Ang II-induced IP in a cell-based assay, and this effect was resistant to washout. Although olmesartan and valsartan inhibited Ang II-induced IP accumulation, the activities of these compounds were markedly reduced after washout. Time-course studies of the abilities of different ARBs to persistently block Ang II binding to AT₁ receptors after drug washout for 240 min have also indicated that azilsartan dissociates from AT₁ receptors more slowly than other ARBs, including olmesartan, telmisartan and valsartan. Second, azilsartan medoxomil inhibited Ang II-induced pressor responses in rats, and its inhibitory effects lasted 24 h after oral administration, whereas the inhibitory effects of olmesartan medoxomil disappeared within 24 h.²⁴ Third, azilsartan blocked Ang II-induced activation of mitogen-activated protein kinase in vascular smooth muscle cells 4–8 h after washout of the drug from the incubation media.²⁵

Azilsartan has a unique moiety, a 5-oxo-1,2,4-oxadiazole, in place of a tetrazole ring. On the basis of molecular modeling, Gln²⁵⁷ binds to the oxadiazole ring by hydrogen bonding that is stronger than the hydrogen bonding of Gln²⁵⁷ to the tetrazole ring of candesartan, as the bond distance in azilsartan was shorter than that in candesartan. In addition, Lys¹⁹⁹ and Tyr¹¹³ may bind to the carboxyl group and biphenyl group of azilsartan, respectively. These three interactions are critical for the tight binding of azilsartan to AT₁ receptor. When the receptor is viewed from the top, azilsartan seems to form the letter 'S' when it binds (Supplementary Figure 3). The oxadiazole in azilsartan, which represents a small difference in the chemical structure compared with candesartan, induced stronger inverse agonism, and the position of Gln²⁵⁷ (TM6) in addition to Lys¹⁹⁹ (TM5) in the AT₁ receptor may have a role in the inverse agonistic activity of azilsartan. We previously reported that olmesartan and valsartan showed inverse agonism toward IP production.^{12,13} Cooperative interactions between the hydroxyl group and Tyr¹¹³ (TM3) and between the carboxyl group and His²⁵⁶ (TM6) are crucial for the inverse agonist activity of olmesartan,¹² whereas the most critical interaction for the inverse agonism of valsartan involves Lys¹⁹⁹ of AT₁ receptor. The molecular basis of the inverse agonism of azilsartan was found to be distinct from that of olmesartan and valsartan.

One of the critical features of ARBs is selectivity for the AT₁ receptor over the AT₂ receptor. The high selectivity is expected to lead to additional AT₂ stimulation because local free Ang II levels will rise after ARB treatment.²⁶ AT₂ receptor stimulation induces vasodilation through bradykinin and nitric oxide release, cell growth inhibition and natriuresis.²⁷ The greatest difference in affinities for AT₁ and AT₂ has been reported for valsartan, at 30 000 times greater affinity for the AT₁ receptor than for the AT₂ receptor.²⁸ In the case of azilsartan, the selectivity was about 39 000 times higher for the AT₁ receptor than for the AT₂ receptor according to our experiment (the K_d of azilsartan for the AT₁ and AT₂ receptors was 3.1 nM and 121 μM, respectively; Miura *S et al.* our unpublished data), indicating that azilsartan may have a beneficial effect in this respect.

Although ARBs are generally well tolerated, the development of new ARBs is important because the currently available ARBs cannot achieve optimal BP levels, and many patients continue to suffer from cardiovascular events and metabolic disturbances despite being treated with an ARB.^{29,30} In this respect, as azilsartan bound tightly to and dissociated slowly from AT₁ receptors compared with other ARBs, azilsartan is expected to be a desirable ARB because it not only shows superior BP control compared with other ARBs but also improves insulin resistance in animal models.^{24,25}

In conclusion, this study demonstrated that azilsartan induces stronger inverse agonism than candesartan, and this ability of

azilsartan may be associated with its unique moiety, a 5-oxo-1,2,4-oxadiazole, in place of a tetrazole ring. The oxadiazole ring in azilsartan is not found in any other clinically approved ARB. A mutagenesis study and molecular modeling revealed that Gln²⁵⁷ in the AT₁ receptor binds to the oxadiazole ring of azilsartan by stronger hydrogen bonding than with candesartan. This interaction may tightly bind azilsartan to the AT₁ receptor and could be important for inducing inverse agonism.

CONFLICT OF INTEREST

SM has received lecture honoraria from Takeda Pharm Co. Ltd. AO is a full-time employee of Takeda Pharm Co. Ltd.

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