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Ability of the new AT_1 receptor blocker azilsartan to block angiotensin II-induced AT_1 receptor activation after wash-out

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Abstract

Introduction: The recently approved angiotensin II (Ang II) type 1 (AT_1) receptor blocker (ARB) azilsartan strongly reduces blood pressure (BP) in patients with hypertension. We previously reported that azilsartan showed unique binding behavior to the AT_1 receptor because of its 5-oxo-1,2,4-oxadiazole moiety. However, the ability of azilsartan to block Ang II-dependent AT_1 receptor activation is not yet clear.

Materials and methods: Azilsartan and a derivative of azilsartan (azilsartan-7H) that lacks a carboxyl group at the benzimidazole ring were used. Ang II-induced inositol phosphate (IP) production and extracellular signal-regulated kinase (ERK) activation were analyzed in a cell-based wash-out assay.

Results: Azilsartan, but not azilsartan-7H, completely blocked Ang II-induced IP production and ERK activation. Our previous report demonstrated that azilsartan mainly interacts with Tyr^{113}, Lys^{199}, and Gln^{257} in the AT_1 receptor. The interactions between azilsartan and Tyr^{113} and Gln^{257}, but not Lys^{199}, were critical for blocking Ang II-induced IP production and ERK activation after wash-out.

Conclusions: Although our findings regarding the molecule-specific effects of azilsartan are based on basic research, they may lead to an exciting insight into the mechanism of azilsartan.

Keywords

Azilsartan, cell-based wash-out assay, extracellular signal-regulated kinase activation, inositol phosphate production, molecule-specific effects

Introduction

Angiotensin II (Ang II) type 1 (AT_1) receptor mediates most known patho-physiological cardiovascular functions.[1] AT_1 receptor blockers (ARBs) inhibit the diverse effects of Ang II, such as vasoconstriction and cell proliferation, which are evoked mainly by Gq protein-dependent inositol phosphate (IP) production and Gq protein-independent extracellular signal-regulated kinase (ERK) activation, respectively.

A new ARB, azilsartan, was recently approved for the treatment of hypertension (HT). Azilsartan medoxomil and azilsartan have been shown to have greater antihypertensive effects than other ARBs.[2–5] Treatment with azilsartan medoxomil lowered 24-hour blood pressure (BP) significantly more than treatment with olmesartan medoxomil or valsartan.[2,3] ARBs have been shown to have class- and molecule-specific effects in basic experimental studies.[6] We have also proposed that small differences in the molecular structures of ARBs could lead to differences in their abilities to influence the AT_1 receptor.[6,7] There have been some reports based on experimental studies on why the depressor effect of azilsartan is superior to those of other ARBs.[8,9] Azilsartan has been shown to bind tightly to and dissociate slowly from AT_1 receptors in comparison to other ARBs.[8] In addition, azilsartan induces the insurmountable antagonism of Ang II-induced vascular contractions against AT_1 receptor. In addition, we recently demonstrated that azilsartan induces stronger inverse agonism independent of Ang II stimulation than candesartan, and this ability of azilsartan may be associated with its unique moiety,

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a 5-oxo-1,2,4-oxadiazole, in place of a tetrazole ring.[9] A molecular model suggested that Gln^{257} binds to the oxadiazole ring by hydrogen bonding, and the bond distance was shorter than that between Gln^{257} and the tetrazole ring of candesartan. Azilsartan may also interact with Tyr^{113} and Lys^{199} in addition to Gln^{257} in the AT_1 receptor.

However, the ability of azilsartan to block Ang II-dependent AT_1 receptor activation is not clear. Therefore, we investigated the ability of azilsartan to block Ang II-induced Gq-dependent IP production and Gq-independent ERK activation through AT_1 receptor using a cell-based wash-out assay. We also explored whether the interactions between azilsartan and Tyr^{113}, Lys^{199} or Gln^{257} in the AT_1 receptor are important for inducing this ability.

Materials and methods

Materials

The following antibodies and reagents were purchased or provided: azilsartan and azilsartan-7H, which does not contain a carboxyl group in the benzimidazole ring compared to azilsartan (Figure 1) (Takeda Pharm Co. Ltd, Osaka, Japan); [Sar^1]Ang II and [Sar^1, Ile^8]Ang II (Sigma-Aldrich, St. Louis, MO, USA); ^{125}I-[Sar^1, Ile^8]Ang II (Amersham Biosciences, Buckinghamshire, UK); and *anti*-ERK 1/2 and phospho (p)-ERK antibodies (Cell Signaling Technology, MA, USA).

Mutagenesis and expression of the AT_1 receptor and membrane preparation

The synthetic AT_1 wild-type (WT) receptor gene, cloned in the shuttle expression vector pMT-3, was used for expression and mutagenesis, as described previously.[10]

Cell cultures, transfections, and membrane preparation

COS1 cells were cultured and maintained in fetal bovine serum and penicillin- and streptomycin-supplemented Dulbecco's modified Eagle's essential medium (Invitrogen, Carlsbad, CA, USA) in 5% CO^2 at 37°C. In the experiments, cells that were not treated with cell-growth supplement were used. The AT_1 WT and mutant 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.

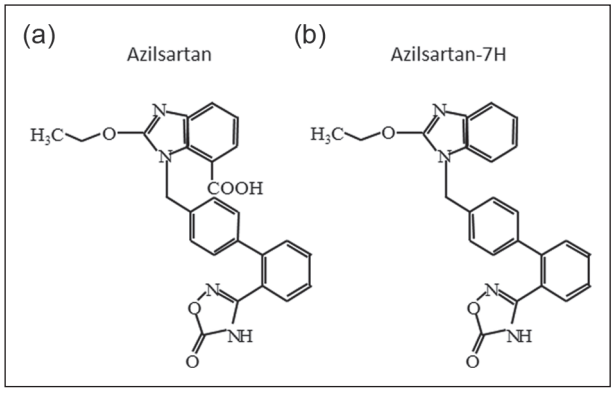

Figure 1. Chemical structures of angiotensin II type 1 receptor blockers ((a), azilsartan; (b), azilsartan-7H).

Percentage of AT_1 receptor occupied by ARB after wash-out

Cell membranes expressing the AT_1 WT or mutant receptors were prepared. The membranes were incubated for 30 minutes at 22°C with or without the indicated concentrations of ARBs. After the membranes were washed-out one to four times with the use of excess cold phosphate buffer, they were centrifuged for 10 minutes at 16,000 × g at 4°C. The membranes were used in the assay for the specific binding of ^{125}I-[Sar^1, Ile^8]Ang II for 30 minutes at 22°C. The percentage of AT_1 receptor occupied by ARB was calculated by the following formula: 100 – {1 – [(specific binding using cell membrane without ARB treatment with no wash-out) – (specific binding using cell membrane with ARB treatment at the indicated wash-out times)/(specific binding using cell membrane without ARB treatment with no wash-out) – (specific binding using cell membrane with ARB treatment with no wash-out)]} × 100(%).[10]

IP production and ERK activation after wash-out

Cells expressing the AT_1 WT and mutant receptors were grown and incubated with or without 10^{-6} M of ARBs for 30 minutes at 37°C in 5% CO_2. After the cells were washed-out once with the use of excess Hank's balanced salt solution, they were incubated with or without 10^{-7} M of [Sar^1] Ang II for 10 minutes at 37°C in 5% CO_2. The cells were used in the assay for IP production and ERK activation. Total soluble IP was measured by the perchloric acid extraction method, as described previously.[11] In addition, Western blotting was performed to detect ERK activation. Cytoplasmic fractions were prepared as described previously.[12] Equal amounts of samples on a protein basis were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was performed with primary antibodies as specified in each case. Horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescent substrate system were used for detection. The signal was independently quantified by a digital image-analysis system.

Statistical analysis

The results are expressed as the mean ± standard deviation of three or more independent determinations. Significant

differences in measured values were evaluated with an analysis of variance using Fisher's *t* test and paired or paired Student's *t* test, as appropriate. Statistical significance was set at < 0.05.

Results

Percentage AT_1 WT receptor occupied by azilsartan and azilsartan-7H after wash-out

First, we evaluated the percentage AT_1 WT receptor occupied by azilsartan and azilsartan-7H after wash-out. After the first washout (Figure 2(a)), azilsartan showed a significantly higher percentage AT_1 WT receptor occupied by ARBs than azilsartan-7H. In addition, the percentage values of azilsartan were also significantly higher than those of azilsartan-7H after the second, third, and fourth washouts, indicating that the carboxyl group of azilsartan is an important chemical structure that allows it to bind to AT_1 WT receptor after wash-out.

Next, we previously indicated that azilsartan may interact with Tyr^{113}, Lys^{199}, and Gln^{257} in the AT_1 receptor.[9] Therefore, we analyzed whether the positions of Tyr^{113}, Lys^{199}, and Gln^{257} were also critical for the ability of azilsartan to bind to AT_1 receptor after wash-out, and changed Tyr^{113}, Lys^{199}, and Gln^{257} to Ala (Y113A, K199A, and Q257A) (Figure 2(b)). The percentage AT_1 Y113A and Q257A receptors occupied by azilsartan after the first wash-out were significantly lower than that with the AT_1 WT receptor. The percentage AT_1 K199A receptor occupied by azilsartan was significantly lower than that with the AT_1 WT receptor, but relatively higher than those with AT_1 Y113A and Q257A receptors. Interactions between azilsartan and all three of the positions of Tyr^{113}, Lys^{199}, and Gln^{257} in the AT_1 WT receptor are critical for the ability of azilsartan to bind to the AT_1 receptor after wash-out.

IP production with azilsartan and azilsartan-7H in the AT_1 WT and mutant receptors

Since ARBs block the diverse effects of Ang II, such as vasoconstriction, we first measured IP production after the first wash-out using azilsartan and azilsartan-7H with the AT_1 WT receptor (Figure 3). As shown in Figure 3(a), azilsartan completely blocked Ang II-induced IP production before and after wash-out, while azilsartan-7H did not block Ang II-induced IP production before and after wash-out.

We next analyzed the binding sites of the AT_1 WT receptor to azilsartan that are important for blocking Ang II-induced IP production after the first wash-out using AT_1 Y113A, K199A, and Q257A receptors (Figure 3(b–d)). The levels of Ang II-induced IP production with azilsartan before and after wash-out were similar to those without azilsartan in the AT_1 Y113A receptor (Figure 3(b)). The level of Ang II-induced IP production with azilsartan after wash-out but not before wash-out was similar to the level without azilsartan in the AT_1 Q257A receptor (Figure 3(d)). On the other hand, in AT_1 K199A receptor, Ang II-induced IP production was significantly blocked by azilsartan before and after wash-out (Figure 3(c)). Thus, azilsartan antagonized IP production after wash-out in the AT_1 Y113A

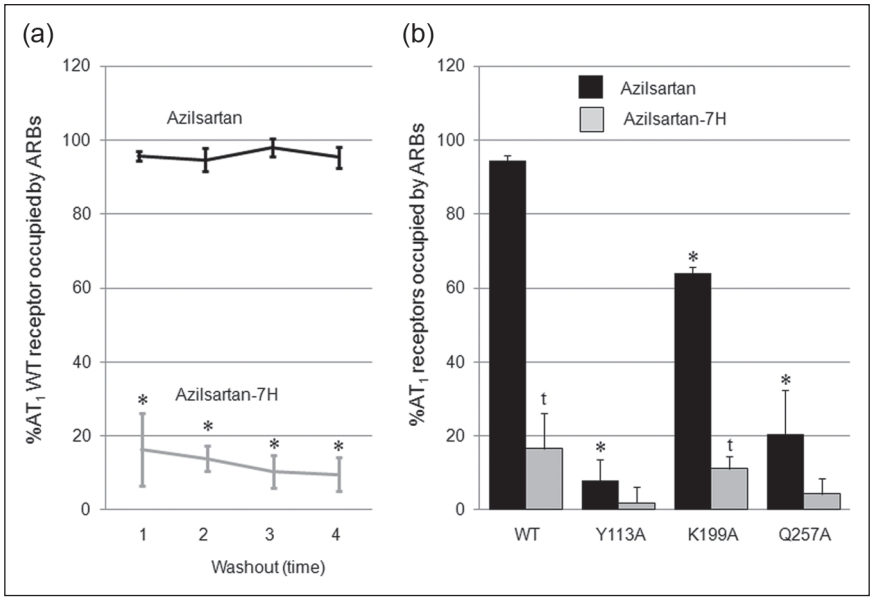

Figure 2. (a) Percentage angiotensin II (Ang II) type 1 (AT_1) wild-type (WT) receptor occupied by 1 μM azilsartan or azilsartan-7H after the first to fourth wash-outs. *$p < 0.05$ vs. azilsartan after each wash-out. ARBs: AT_1 receptor blockers Percentage angiotensin II (Ang II) type 1 (AT_1) wild-type (WT) and mutant receptors occupied by 1 μM azilsartan or azilsartan-7H after the first wash-out. *$p < 0.05$ vs. AT_1 WT receptor using azilsartan. t$p < 0.05$ vs. azilsartan in each receptor. ARBs: AT_1 receptor blockers.

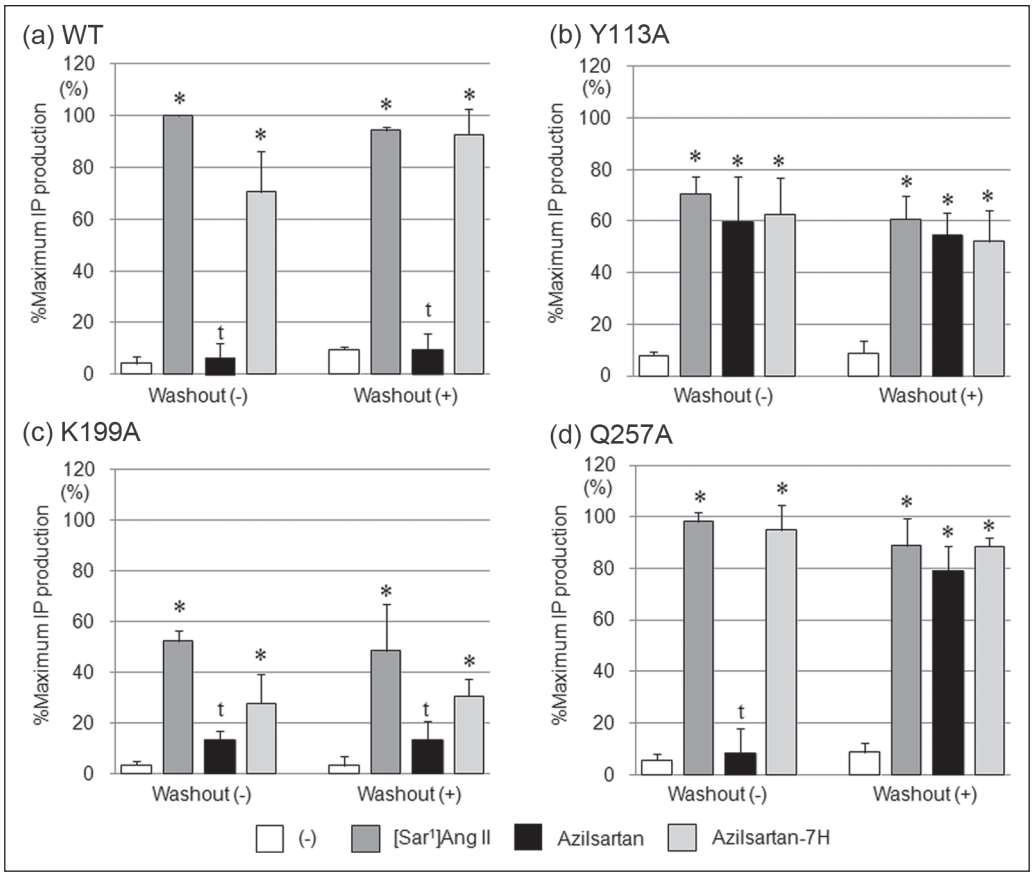

Figure 3. Percentage of (%) maximum IP production with or without 1 μM of azilsartan or azilsartan-7H and 0.1 μM [Sar^1]Ang II without or with a first wash-out in COS1 cells transiently expressing the AT_1 WT (a), Y113A (b), K199A (c), and Q257A (d) receptors. [Sar^1]Ang II or ARB was added to the medium for 10 minutes. Percentage maximum IP production indicates [Sar^1]Ang II-induced IP production (1410 counts/min) in WT AT_1 receptor-transfected cells (100%) after adjusting for basal IP production (160 counts/min) without treatment in mock-treated cells (0%). *$p < 0.05$ vs. no treatment. t$p < 0.05$ vs. [Sar^1]Ang II. Ang II: angiotensin II; AT_1 WT: Ang II type 1 (AT_1) wild-type; ARB: AT_1 receptor blockers; IP: inositol phosphate.

and Q257A receptor, which indicated that Tyr^{113} and Gln^{257} in the AT_1 WT receptor play important roles in the effect of azilsartan.

ERK activation with azilsartan and azilsartan-7H in the AT_1 WT and mutant receptors

We determined ERK activation after the first wash-out using azilsartan and azilsartan-7H with the AT_1 WT receptor because ARBs block cell proliferation by Ang II (Figure 4). As shown in Figure 4(a), azilsartan completely blocked Ang II-induced ERK activation before and after wash-out, while azilsartan-7H did not block Ang II-induced ERK activation. We next analyzed the effect of ARBs in Ang II-induced ERK activation before and after wash-out using AT_1 Y113A, K199A, and Q257A receptors (Figure 4(b–d)). Ang II-induced ERK activation was not blocked by azilsartan or azilsartan-7H in the AT_1 Y113A and Q257A receptors after wash-out. In the AT_1 K199A receptor, Ang II-induced ERK activation was significantly blocked by azilsartan before and after wash-out (Figure 4(c)). Thus, the interactions between azilsartan and Tyr^{113} and Gln^{257}, but not Lys^{199}, in the AT_1 WT receptor are important for the blocking of Ang II-induced ERK activation by azilsartan after wash-out.

Discussion

We have provided direct evidence that azilsartan has a strong blocking effect against Ang II-induced AT_1 receptor activation using a cell-based wash-out assay, and is resistant to wash-out. The interactions between azilsartan and Tyr^{113} or Gln^{257} in the AT_1 receptor may be associated with its ability to block Ang II-induced IP production and ERK activation after wash-out.

Azilsartan medoxomil and azilsartan have been shown to have greater antihypertensive effects than other ARBs.[2–5] Previous studies with basic experiments, including ours, have examined why the depressor effect of azilsartan is superior to those of other ARBs.[8,9,13,14] Azilsartan

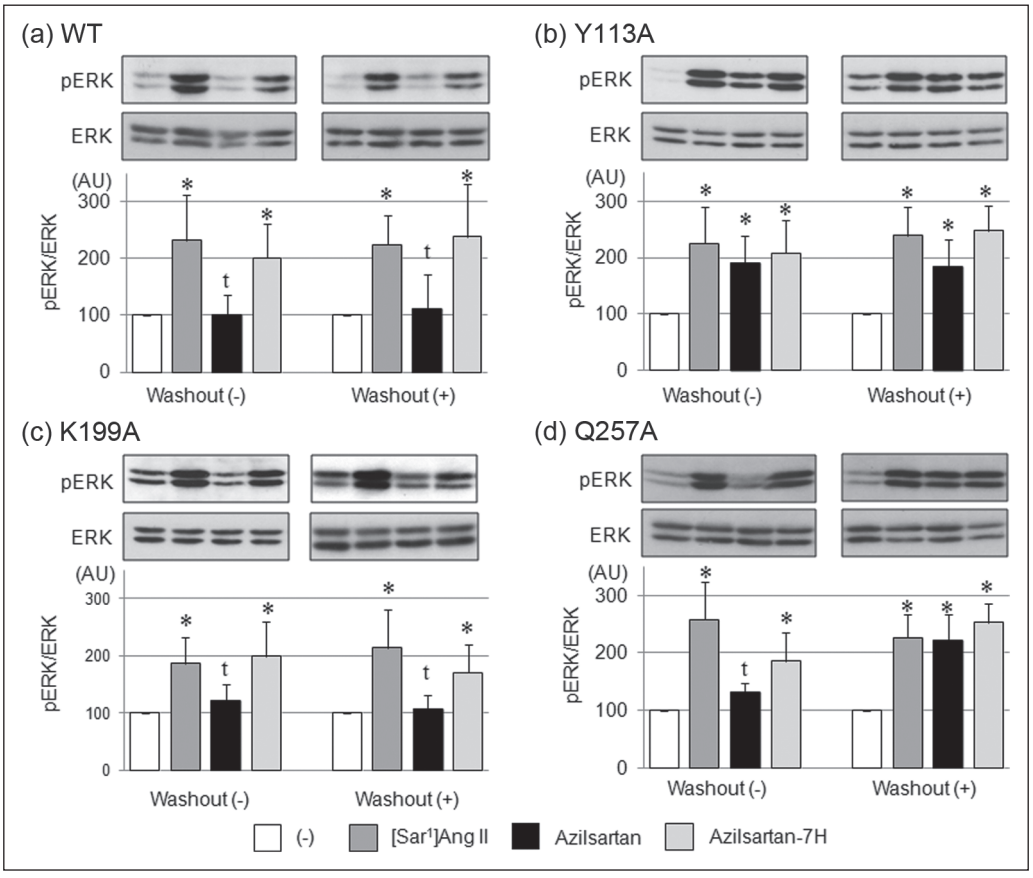

Figure 4. Extracellular signal-regulated kinase (ERK) activation after 0.1 μM [Sar^1]angiotensin II (Ang II) stimulation under pretreatment with 1 μM azilsartan or azilsartan-7H without or with a first wash-out in COS1 cells transiently expressing the Ang II type 1 (AT_1) wild-type (WT) (a), Y113A (b), K199A (c), and Q257A (d) receptors. ERK activation is shown as the ratio of ERK1/2 to *p*-ERK1/2, and ERK activation without treatment as a control was considered to be 100. AU: arbitrary unit. **p* < 0.05 vs. no treatment. t*p* < 0.05 vs. [Sar^1]Ang II.

binds tightly to and dissociates slowly from AT_1 receptors in comparison to other ARBs[8] and induces stronger inverse agonism independent of Ang II stimulation.[9] The main finding in this study was that azilsartan showed a strong blocking effect toward Ang II-induced AT_1 receptor activation after cell-based wash-out. Our observation may also help to explain the stronger depressor effect of azilsartan.

We previously reported that candesartan also blocked Ang II-induced AT_1 receptor activation after wash-out.[10] Candesartan induced more beneficial effects than other ARBs (e.g. olmesartan, telmisartan, valsartan, irbesartan, and losartan), and a carboxyl group of candesartan was a critical molecular structure for the blockade of Ang II-induced AT_1 receptor activation. Based on the data from the present study, the blocking effect of azilsartan is similar to that of candesartan. In addition, azilsartan was a highly potent and slowly dissociating ARB after wash-out compared with the other ARBs tested (olmesartan, telmisartan, and valsartan).[8] Thus, we confirmed that azilsartan shows the same ability to block as candesartan after wash-out, and is superior to other ARBs. The carboxyl group of azilsartan is also a critical structure for this blockade.

Our previous report demonstrated that candesartan mainly interacts with Tyr^{113}, Lys^{199}, and Gln^{257} in the AT_1 receptor.[10] The interactions between azilsartan and Tyr^{113} and Gln^{257}, but not Lys^{199}, were critical for blocking Ang II-induced activation. As shown in Figure 2(a), the percentage AT_1 K199A receptor occupied by azilsartan was higher than that in the AT_1 Y113A or Q257A receptor. The oxadiazole ring in azilsartan is a unique structure that is not found in other ARBs. Most ARBs, including candesartan, have a biphenylmethyl moiety with an acidic group (either a tetrazole or carboxylic acid).[6] Gln^{257} binds to the oxadiazole ring of azilsartan by hydrogen bonding, and Lys^{199} and Tyr^{113} may bind to the carboxyl group and biphenyl group of azilsartan, respectively.[10] In the case of the AT_1 K199A receptor, the unique structure of azilsartan might maintain the ability to block Ang II-induced IP production and ERK activation after wash-out. Interestingly, the position of Gln^{257} as well as Lys^{199} in the AT_1 receptor may play a role in the inverse agonistic activity of azilsartan.[10] Although

azilsartan bound tightly to the AT_1 receptor, the interactions between azilsartan and the binding sites of AT_1 receptor for inducing each ability of azilsartan, such as blockade of Ang II-dependent and Ang II-independent AT_1 receptor activation (antagonism and inverse agonism, respectively), may be slightly different.

In conclusion, this study demonstrated that the interaction between Tyr^{113} or Gln^{257} in the AT_1 receptor and azilsartan may be associated with the blockade of Ang II-induced AT_1 receptor activation by azilsartan after washout. Although our finding regarding this molecule-specific effect of azilsartan is based on basic research, it may lead to an exciting insight into the mechanism of azilsartan.

Conflict of interest

None declared.

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