In Vitro Antagonistic Properties of a New Angiotensin Type 1 Receptor Blocker, Azilsartan, in Receptor Binding and Function Studies

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Pharmacology Research Laboratories (M.O., H.I., M.T., H.F., Y.I., K.Kus., H.N.), Medicinal Chemistry Research Laboratories (H.S., T.K., Y.K.), and Strategic Research Planning Department (K.Kub.), Pharmaceutical Research Division, Takeda Pharmaceutical Company, Ltd., Osaka, Japan

ABSTRACT

The angiotensin II (AII) antagonistic action of azilsartan (AZL) [2-ethoxy-1-{[2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl}-1H-benzimidazole-7-carboxylic acid] was investigated in radioligand binding and function studies. AZL inhibited the specific binding of ^{125}I-Sar^1-Ile^8-AII to human angiotensin type 1 receptors with an IC$_{50}$ of 2.6 nM. The inhibitory effect of AZL persisted after washout of the free compound (IC$_{50}$ value of 7.4 nM). Olmesartan, telmisartan, valsartan, and irbesartan also inhibited the specific binding with IC$_{50}$ values of 6.7, 5.1, 44.9, and 15.8 nM, respectively. However, their inhibitory effects were markedly attenuated with washout (IC$_{50}$ values of 242.5, 191.6, >10,000, and >10,000 nM). AZL also inhibited the accumulation of AII-induced inositol 1-phosphate (IP1) in the cell-based assay with an IC$_{50}$ value of 9.2 nmol; this effect was resistant to washout (IC$_{50}$ value of 81.3

nM). Olmesartan and valsartan inhibited IP1 accumulation with IC$_{50}$ values of 12.2 and 59.8 nM, respectively. The activities of these compounds were markedly reduced after washout (IC$_{50}$ value of 908.5 and 22,664.4 nM). AZL was defined as an inverse agonist in an experiment by using a constitutively active mutant of human angiotensin type 1 receptors. In isolated rabbit aortic strips, AZL reduced the maximal contractile response to AII with a pD′$_2$ value of 9.9. The inhibitory effects of AZL on contractile responses induced by AII persisted after the strips were washed; these inhibitory effects were more potent than those of olmesartan. These results suggest that AZL is a highly potent and slowly dissociating AII receptor blocker. Its tight receptor binding might be expected to produce potent and long-lasting antihypertensive effects in preclinical and clinical settings.

Introduction

Hypertension is associated with an increased risk of cardiovascular morbidity and mortality, including coronary heart disease, heart failure, stroke, and renal disease (Chobanian et al., 2003; Mancia et al., 2007). Current guidelines in Europe, Japan, and the United States for the management of patients with hypertension emphasize the importance of managing hypertension to reduce the substantial morbidity and mortality associated with cardiovascular events (Chobanian et al., 2003; Mancia et al., 2007; Ogihara et al., 2009).

Therefore, the achievement of BP control is an important factor in the management of hypertension and its associated cardiovascular complications.

Angiotensin II (AII) is the major effector hormone of the renin-angiotensin-aldosterone system. AII plays a key role in the regulation of BP and fluid-electrolyte balance and in the pathophysiology of hypertension (de Gasparo et al., 2000). The numerous effects of AII, including its roles in vasoconstriction, secretion of aldosterone and vasopressin, cellular proliferation, and hypertrophy are dominantly mediated through the activation of the angiotensin type 1 (AT$_1$) receptor, a member of the superfamily of G protein-coupled receptors (de Gasparo et al., 2000). Therefore, an AII receptor blocker (ARB) that blocks AT$_1$ receptors is considered to be a

ABBREVIATIONS: BP, blood pressure; AII, angiotensin II; AT$_1$, angiotensin type 1; ARB, angiotensin receptor blocker; EXP3174, 2-n-butyl-4-chloro-1-((2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl)imidazole-5-carboxylic acid; AZL, azilsartan; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IP1, inositol 1-phosphate; DMSO, dimethyl sulfoxide; CHO, Chinese hamster ovary; TAK-491, 5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 2-ethoxy-1-{[2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl}-1H-benzimidazole-7-carboxylate monopotassium salt.

logical therapeutic strategy in the management of hypertension and cardiovascular and renal diseases.

The clinical efficacy of ARBs, such as losartan, candesartan cilexetil, valsartan, irbesartan, telmisartan, and olmesartan medoxomil, has been confirmed in hypertensive patients (Oparil 2000; Easthope and Jarvis, 2002; Smith 2008). In addition, the beneficial effects of ARBs on other pathophysiological conditions, such as congestive heart failure and diabetic nephropathy, have also been reported (Ram 2008; Steckelings et al., 2009). However, results of randomized comparative clinical trials have suggested differences in antihypertensive efficacy and 24-h BP control among ARBs (Oparil 2000; Smith 2008). Although ARBs possess common structural features that are required for effective antagonism of the AT_1 receptor, their side chains differ structurally. These differences may contribute to the characteristic binding kinetics of these agents to AT_1 receptors that result in their differential pharmacological potency and efficacy.

ARBs are classified as surmountable or insurmountable antagonists on the basis of functional data, such as AII-mediated contractions in isolated vascular tissues and AII-mediated inositol phosphate production in intact cells (Wienen et al., 1992; Fierens et al., 1999). Surmountable antagonists, such as losartan, produce parallel rightward shifts in the dose-response curves of AII without affecting the maximal response. In contrast, most insurmountable ARBs, including telmisartan, irbesartan, valsartan, and 2-n-butyl-4-chloro-1-((2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl)imidazole-5-carboxylic acid (EXP3174; active metabolite of losartan), partially reduce the maximal response to AII (Van Liefde and Vauquelin, 2009). Others, such as candesartan (active metabolite of candesartan cilexetil) and olmesartan (active metabolite of olmesartan medoxomil), almost completely suppress the response (Shibouta et al., 1993; Mizuno et al., 1995). The insurmountable inhibition by some ARBs might be due to slow dissociations from AT_1 receptors and has been proposed to contribute to long-lasting clinical actions (Ojima et al., 1997; Oparil 2000). Indeed, results of comparative clinical trials suggest that insurmountable ARBs, such as candesartan cilexetil (Bakris et al., 2001; Vidt et al., 2001; Smith 2008) and olmesartan medoxomil (Oparil et al., 2001; Scott and McCormack, 2008), may be more effective than the surmountable antagonist losartan or the partially insurmountable antagonists valsartan and irbesartan in reducing the BP of patients with mild to moderate hypertension.

Azilsartan (AZL) [2-ethoxy-1-{[2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl}-1H-benzimidazole-7-carboxylic acid; TAK-536] (Fig. 1) is a selective AT_1 receptor antagonist (Kohara et al., 1996). It is structurally similar to candesartan except that it bears a 5-oxo-1,2,4-oxadiazole moiety in place of the tetrazole ring. AZL has a carboxyl group at the 7-position of the benzimidazole ring, which is the group that is believed to have a role in producing the insurmountable receptor antagonistic activity of candesartan (Noda et al., 1993). This insurmountable, or tight, binding of candesartan to the AT_1 receptor may contribute to its potent and long-lasting antihypertensive activity.

In this study, we investigated the AII antagonistic properties of AZL in binding studies and in a cell-based assay using human AT_1 receptors and in contraction studies using rabbit aortic strips. In both binding studies and the cell-based as-
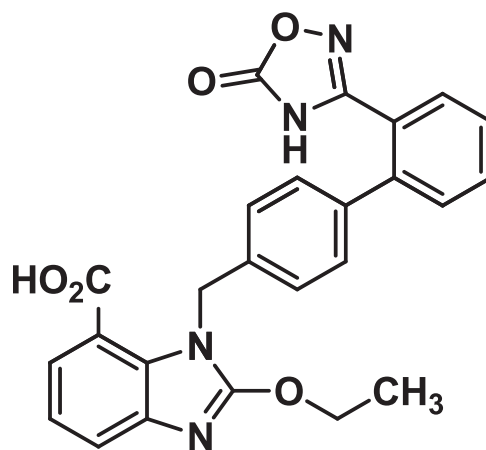
AZL

Fig. 1. Chemical structure of AZL.

say, we evaluated the dissociation of AZL and the ARBs (olmesartan, telmisartan, valsartan, and irbesartan) from AT_1 receptors using indirect kinetic methods after we removed the compounds by washout. Finally, we evaluated the inverse agonist activity of AZL using constitutively active mutants of the AT_1 receptor.

Materials and Methods

Radioligand Binding Studies on Human AT_1 Receptors. A radioligand binding assay was performed by using human AT_1 receptor-coated microplates (FlashPlate; PerkinElmer Life and Analytical Sciences, Waltham, MA) containing 4.4 to 6.2 fmol of receptors/well (10 μg of membrane protein/well). Membrane-coated wells were incubated with 45 μl of assay buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.005% CHAPS, pH 7.4) containing various concentrations of test compounds at room temperature. After 90 min, 5 μl of ^{125}I-Sar[1]-Ile[8]-AII (final concentration 0.6 nM) dissolved in assay buffer was added to the wells, and the plate was incubated for 5 h. In each step, the plate was briefly and gently shaken on a plate shaker.

In washout experiments, the membranes were incubated with the compounds for 90 min, then immediately washed twice with 200 μl/well of assay buffer to remove unbound compounds, and further incubated for 5 h with ^{125}I-Sar[1]-Ile[8]-AII. Membrane-bound radioactivity was counted using a TopCount Microplate Scintillation and Luminescence Counter (PerkinElmer Life and Analytical Sciences).

In the experiments to estimate the dissociation rate of the compounds from AT_1 receptors, membranes were incubated for 90 min with each compound at a concentration of 30 nM for AZL, olmesartan, and telmisartan and a concentration of 300 nM for valsartan. At these respective concentrations, they inhibited the specific binding of ^{125}I-Sar[1]-Ile[8]-AII to human AT_1 by approximately 90%. The membranes were then immediately washed twice with 200 μl/well of assay buffer and further incubated with ^{125}I-Sar[1]-Ile[8]-AII for 240 min. Membrane-bound radioactivity was counted using the TopCount Microplate Scintillation and Luminescence Counter (PerkinElmer Life and Analytical Sciences) at 30, 60, 90, 120, 150, 180, or 240 min.

Nonspecific binding of ^{125}I-Sar[1]-Ile[8]-AII was estimated in the presence of 10 μM unlabeled AII. Unlabeled AII was added again after washout for the washout experiment. Specific binding was defined as total binding minus nonspecific binding.

Cell-Based Assay with Intact COS-7 Cells Expressing Human AT$_1$ Receptors. The expression vector pCAGGS was kindly donated by Dr. Junichi Miyazaki, Osaka University, Osaka, Japan (Niwa et al., 1991). We constructed an expression vector pCAGGS-MCS (pCAGGS having multicloning sites) by introducing the SacI, SmaI, KpnI, EcoRI, EcoRV, NotI, XhoI, BalI, and BstI sites to the original pCAGGS vector.

An AT$_1$-pCAGGS expression vector was constructed as follows. A human AT$_1$ cDNA fragment was obtained by digesting AGTR1-pcDNA3.1 vector (purchased from Missouri S&T cDNA Resource Center, Rolla, MO) with EcoRI and XhoI. This fragment was cloned into the pCAGGS-MCS vector.

An AT$_1$ cDNA fragment having the I245T mutation was generated by means of a polymerase chain reaction by using a primer pair (5′-GATAATTATGGCAAC*T*GTGCTTTTCTTT-3′ and 5′-AAAGAAAAGCACA*G*TTGCCATAATTATC-3′) and the AT$_1$-pCAGGS vector as template. This fragment was digested with EcoRI and XhoI and cloned into the pCAGGS-MCS vector.

Cell Culture and Transfections. COS-7 cells were maintained in 10-cm-diameter dishes at 37°C in a 5% CO$_2$ atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin. Expression plasmids of 0.133 µg/well were transfected into the cells at a density of 2 × 10^4/well in collagen I-coated 96-well plates (IWAKI, Funabashi, Japan) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Measurement of Inositol 1-Phosphate Accumulation. Twenty-four hours after transfections, the cells were starved by changing the culture medium to starvation buffer (1 mM CaCl$_2$, 0.5 mM MgCl$_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, and 10 mM HEPES, pH 7.3). Then, 5 µl/well of the test compounds dissolved in starvation buffer was added to the cells at the indicated concentrations, and they were pretreated for the indicated times. Two hours after starvation, LiCl was added to a final concentration of 50 mM with or without AII 10 nM, and the cells were further incubated for the indicated times at 37°C. In washout experiments, the cells were washed once with 100 µl/well of starvation buffer to remove unbound compounds before stimulation with AII.

The accumulation of inositol 1-phosphate (IP1) was measured by using a IP-One Tb kit (CIS Bio International, Gif-sur-Yvette, France) according to the manufacturer's instructions. The fluorescence resonance energy transfer signal was measured on a plate reader (EnVision; PerkinElmer Life and Analytical Sciences).

Contraction Studies in Rabbit Aorta. All animal experiments were performed according to the guidelines of the Takeda Experimental Animal Care and Use Committee. Japanese white male rabbits (2–4 kg; Kitayama Laboratory Ltd., Kyoto, Japan) were sacrificed by bleeding from the carotid artery under anesthesia with sodium pentobarbital (30 mg/kg i.v.). The thoracic aorta was removed and cut into helical strips (2 mm in width; 12–17 mm in length). The aortic strips were mounted at a resting tension of 2 g in organ baths containing 20 ml of Krebs-Henseleit solution (120 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$ · 7H$_2$O, 1.2 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$ · 2H$_2$O, 25 mM NaHCO$_3$, and 10 mM *d*-glucose). The Krebs-Henseleit solution was kept at 37°C and oxygenated with 95% O$_2$ and 5% CO$_2$. Isometric contraction was measured with a force displacement transducer (UL-10GR or UL-20GR; Minebea Co., Ltd., Tokyo, Japan) connected to an amplifier (2238 or 6M 92; NEC San-ei Instruments, Tokyo, Japan; or AP-641G, Nihon Kohden Co., Tokyo, Japan) and recorded on a polygraph (polygraph system, Nihon Kohden Co.; Linearcorder, Graphtec Co., Yokohama, Japan; or Unicorder, Pantos Co., Kyoto, Japan).

Inhibitory Effects on the Concentration-Contractile Response Curve for AII. The aortic strips were stimulated twice by the addition of 10 nM AII to the bath after stabilization, followed by washing with Krebs-Henseleit solution, and allowed to relax to the baseline tension. Thereafter, a control cumulative concentration-

contractile response curve for AII was obtained. Repeated washout was performed after we obtained the control response curve, and the strip was incubated with AZL, olmesartan, or vehicle [0.1% dimethyl sulfoxide (DMSO)] for 90 min. The concentration-response curve for AII was then determined again in the presence of the test compound. The contractile response obtained in the second cumulative addition of AII was normalized to the maximal contraction generated by the first concentration-response curve in each aortic strip and expressed as a percentage of the maximal response to AII obtained in the presence of vehicle in each aorta. Because AZL and olmesartan were found to exert insurmountable AII antagonism, pD′$_2$ values, the negative logarithm values of the concentration of drug that inhibits the maximum response by 50%, were calculated according to the method described by van Rossum (1963).

Inhibitory Effects on AII-Induced Contraction after Washout. The aortic strips were stimulated twice by the addition of 10 nM AII to the bath after stabilization, washed with Krebs-Henseleit solution, and restabilized. The strips were again challenged with 10 nM AII. The maximal contractile tension obtained in this challenge was taken as the control response. The strips were then incubated with 0.1, 0.3, or 1 nM AZL, 0.1, 0.3, or 1 nM olmesartan, or vehicle [20 µl of DMSO (final DMSO concentration of 0.1%)] for 30 min. The maximal contractile response to additional AII within 20 min was obtained in the presence of the drugs. The strips were then washed four times, and this washing procedure was repeated twice over 2 h. Final addition of AII was performed, and the maximal response within 20 min was measured as washout data. Contractile responses to AII in drug-treated strips were corrected for changes occurring in vehicle-treated strips in each aorta. The inhibition rate from the control response was calculated at each measurement point.

Selectivity. The aortic strips were repeatedly stimulated by the addition of 60 mM KCl, 1 µM norepinephrine, 1 µM 5-hydroxytryptamine, or 2 µM prostaglandin F$_{2\alpha}$. After the response became stable, the strips were incubated with AZL for 30 min, and the vasoconstrictor-induced contraction was measured again. The inhibition rate was calculated from the values obtained before and after the AZL treatment.

Drugs and Materials. AZL was synthesized in Takeda Pharmaceutical Company Limited (Osaka, Japan). Olmesartan (Yanagisawa et al., 1996), telmisartan (Ries et al., 1993), valsartan (Bühlmayer et al., 1994), and irbesartan (Bernhart et al., 1993) were used in this experiment as competitors. Olmesartan was prepared from olmesartan medoxomil (Daiichi-Sankyo, Tokyo, Japan). Telmisartan, valsartan, and irbesartan were extracted from 80-mg telmisartan tablets (Micardis; Boehringer Ingelheim GmbH, Ingelheim, Germany), 160-mg valsartan tablets (Diovan; Novartis, Basel, Switzerland), and 300-mg irbesartan tablets (Aprovel; Sanofi-aventis, Winthrop, UK), respectively, in KNC Laboratories Co., Ltd. (Kobe, Japan).

^{125}I-Sar^1-Ile^8-AII and human AT$_1$ receptor expressed in CHO cells, which were already immobilized into 96-well FlashPlates (Screen-Ready hAT$_1$ AII receptor target), were purchased from PerkinElmer Life and Analytical Sciences. AII was purchased from Peptide Institutes, Inc. (Osaka, Japan). Norepinephrine, 5-hydroxytryptamine, and prostaglandin F$_{2\alpha}$ were purchased from Wako Chemical Industries, Ltd. (Osaka, Japan).

AZL, olmesartan, telmisartan, valsartan, and irbesartan were dissolved in DMSO at 10 mM and diluted to the desired concentration with DMSO.

Data Analysis. Data are shown as the mean ± S.E.M. Concentrations of compounds required for 50% inhibition of the ^{125}I-Sar^1-Ile^8-AII binding to human AT$_1$ receptors in the receptor assay or 50% inhibition of IP1 accumulation in the cell-based assay (IC$_{50}$) were calculated by nonlinear logistic regression analysis using SAS software (version 8.2; SAS Institute, Inc., Cary, NC). For dissociation kinetic data, differences between values with and without removal of compounds by washing the membranes were assessed for each time point by applying a Student's *t* test or an Aspin-Welch test, followed by the Holm correction for multiple time-point comparisons by use of

SAS software. All induced accumulation of IP1 was compared using a Student's t test. A difference was considered statistically significant at $p < 0.05$.

To compare the inhibitory effects of drugs on AII-induced contraction before and after washout procedures, data were analyzed by performing a Student's paired t test followed by Holm correction. Differences were considered statistically significant at $p < 0.05$.

Results

Radioligand Binding Studies on Human AT_1 Receptors. Pretreatment of AZL for 90 min inhibited the specific binding of ^{125}I-Sar^1-Ile^8-AII to human AT_1 receptors expressed in CHO cells in a concentration-related manner with an IC_{50} of 2.6 nM, indicating a high affinity for AT_1 receptors (Fig. 2A; Table 1). Olmesartan, telmisartan, valsartan and irbesartan also produced concentration-dependent inhibition of ^{125}I-Sar^1-Ile^8-AII binding with IC_{50} values of 6.7, 5.1, 44.9, and 15.8 nM, respectively.

A potent inhibitory effect of AZL at AT_1 receptors was maintained even 5 h after washout with an IC_{50} value of 7.4 nM (Fig. 2B; Table 1). In contrast, the inhibitory effects of olmesartan, telmisartan, irbesartan, and valsartan were markedly attenuated after washout. Their IC_{50} values were 242.5, 191.6, >10,000, and >10,000 nM, respectively (Fig. 2B; Table 1). On the basis of the IC_{50} values after washout, AZL was 32.7-, 25.8-, >1349-, and >1349-fold more potent as an AT_1 receptor antagonist than olmesartan, telmisartan,
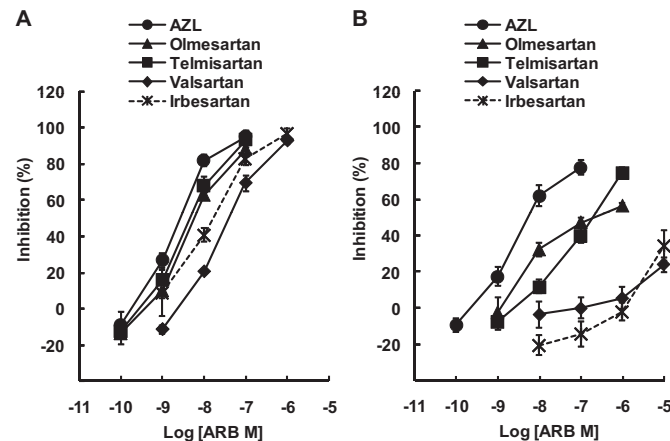
valsartan, and irbesartan, respectively. Thus, the inhibitory effect of AZL after washout was only 3 times less potent than that before washout, whereas the inhibitory effects of olmesartan, telmisartan, valsartan, and irbesartan were markedly attenuated after washout; IC_{50} values after washout were 36, 37, >223, and >635 times higher than those in the presence of compounds, respectively (Table 1).

Next, we examined the dissociation rate of these compounds except for irbesartan because of its weak potency after washout. Concentrations of 30 nM for AZL, olmesartan, and telmisartan and 300 nM for valsartan inhibited ^{125}I-Sar^1-Ile^8-AII binding to human AT_1 receptors by approximately 90% (Fig. 2A). The inhibitory effect of AZL on ^{125}I-Sar^1-Ile^8-AII binding after washout persisted even when the incubation period was extended to 240 min after the addition of the radioligand; the inhibitory effect of AZL was reduced by 25% (Fig. 3).

On the contrary, the inhibition of ^{125}I-Sar^1-Ile^8-AII binding by olmesartan, telmisartan, and valsartan after washout decreased gradually as incubation time increased, and their inhibitory effects were reduced by 44, 70, and 99% at 240 min, respectively. Furthermore, reductions in the inhibitory effects of olmesartan, telmisartan, and valsartan at concentrations up to 100, 100, and 1000 nM, which almost completely inhibited the binding of ^{125}I-Sar^1-Ile^8-AII (99, 102, and 100% inhibition, respectively), reached 52, 54, and 85%, respectively, at 240 min ($n = 2$, data not shown). These results suggest that AZL is a highly potent and slowly dissociating AT_1 receptor antagonist compared with the other ARBs tested.

Cell-Based Assay with Intact COS-7 Cells Expressing Human AT_1 Receptor. Pretreatment of cells with AZL, olmesartan, or valsartan for 2 h inhibited the accumulation of IP1 with IC_{50} values of 9.2, 12.2, and 59.8 nM, respectively

Fig. 2. Inhibition of the specific binding of ^{125}I-Sar^1-Ile^8-AII to human AT_1 receptors by AZL, olmesartan, telmisartan, valsartan, and irbesartan. Membranes of CHO cells expressing AT_1 receptor were preincubated for 90 min with each compound and further incubated with ^{125}I-Sar^1-Ile^8-AII for 5 h with or without washout. Results shown are in presence of compounds (A) and after washout of compounds (B). Data are the mean± S.E.M. of four experiments performed in duplicate.

TABLE 1
Inhibitory effects on the specific binding of ^{125}I-Sar^1-Ile^8-AII to human AT_1 receptors
Data in parentheses are 95% confidence intervals.

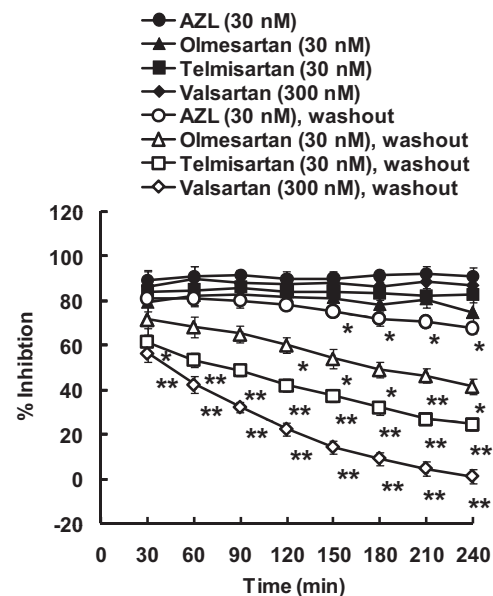| Compound | IC_{50} | | Ratio: Washout (+)/ Washout (−) |
	Washout (−)	Washout (+)	
	nM		
AZL	2.6 (1.7–4.1)	7.4 (3.9–14.2)	3
Olmesartan	6.7 (3.8–10.8)	242.5 (91.0–1056.8)	36
Telmisartan	5.1 (3.0–8.1)	191.6 (124.1–303.2)	37
Valsartan	44.9 (30.5–64.7)	>10,000	>223
Irbesartan	15.8 (8.5–29.7)	>10,000	>635

Fig. 3. Time course of dissociation of AZL, olmesartan, telmisartan, and valsartan from human AT_1 receptors. Membranes of CHO cells expressing AT_1 receptor were preincubated for 90 min with each compound and further incubated with ^{125}I-Sar^1-Ile^8-AII in the presence of compounds (solid symbols) or after washout of compounds (open symbols) for 240 min. Data are the mean ± S.E.M. of four experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$, versus the presence of compound (Student's t test followed by the Holm correction).
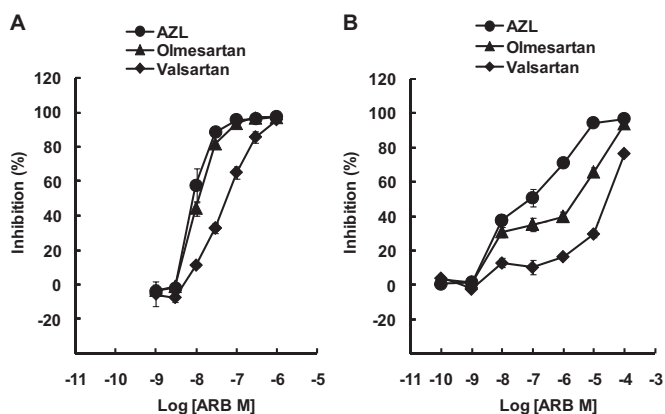
Fig. 4. Antagonistic activities of AZL, olmesartan, and valsartan. COS-7 cells expressing human AT_1 receptors were pretreated for 2 h with each compound and further incubated with AII (10 nM) for 1 h with or without washout. Results shown are in the presence of compounds (A) and after washout of compounds (B). Data are the mean $\pm$ S.E.M. of three experiments performed in triplicate.

(Fig. 4A; Table 2). Antagonism of AZL against AT_1 receptors was almost equal to that of olmesartan, and it was stronger than that of valsartan.

AZL inhibited the AII-stimulated accumulation of IP1 after washout with an IC_{50} value of 81.3 nM. The ratio of IC_{50} values with and without washout was 9 (Fig. 4B; Table 2). In comparison, the inhibitory effects of olmesartan and valsartan markedly decreased after washout in comparison with AZL; their IC_{50} values were 908.5 and 22,664.4 nM, respectively, and the ratios of IC_{50} values with and without washout were 74 and 379, respectively (Fig. 4B; Table 2).

Inhibitory Effects on the Concentration-Contractile Response Curve for AII. Pretreatment with 0.03, 0.1, 0.3, or 1 nM AZL for 90 min reduced AII-induced maximal contraction of rabbit aortic strips in a concentration-related manner (Fig. 5A). Olmesartan also inhibited the maximal response to AII in a concentration-related manner (Fig. 5B), which was consistent with data reported previously (Mizuno et al., 1995). The pD'_2 values for AZL and olmesartan were both 9.9.

Inhibitory Effects on AII-Induced Contraction after Washout. Pretreatment with 0.1, 0.3, or 1 nM AZL and olmesartan for 30 min inhibited AII-induced aortic contraction in a concentration-related manner (Fig. 6A). The inhibitory effects of AZL were still observed even after several washout procedures, although they were slightly reduced after washout, especially at the highest dose tested (Fig. 6A). Olmesartan also potently inhibited the AII-induced contraction before washout; however, its inhibitory effects were significantly reduced after washout at all doses tested (Fig. 6B). Thus, the antagonistic effect of AZL persisted longer after
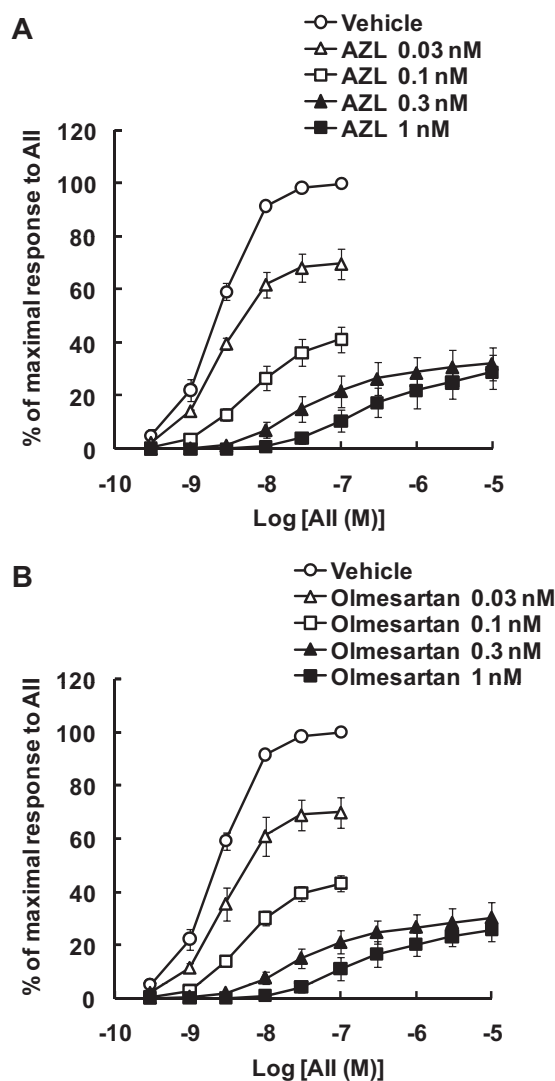

Fig. 5. Effects of AZL (A) and olmesartan (B) on the concentration-contractile response curves for AII in rabbit aortic strips. Data are the mean $\pm$ S.E.M. ($n = 4$).

washout than did that of olmesartan, which suggests that the dissociation of AZL from binding sites in rabbit aorta is much slower than that of olmesartan.

Selectivity. AZL 10 μM did not inhibit the vasoconstriction induced by KCl, norepinephrine, 5-hydroxytryptamine, or prostaglandin $F_{2\alpha}$ (results not shown).

Inverse Agonist Activity. Ligands capable of suppressing the agonist-independent activation of receptors are defined "inverse agonists." AII-independent activation of wild-type AT_1 receptors without stimuli such as stretch is very low, so constitutive active mutants of AT_1 receptors have

TABLE 2

Inhibitory effects of AZL, olmesartan, and valsartan on AII-stimulated production of IP1

Data in parentheses are 95% confidence intervals.

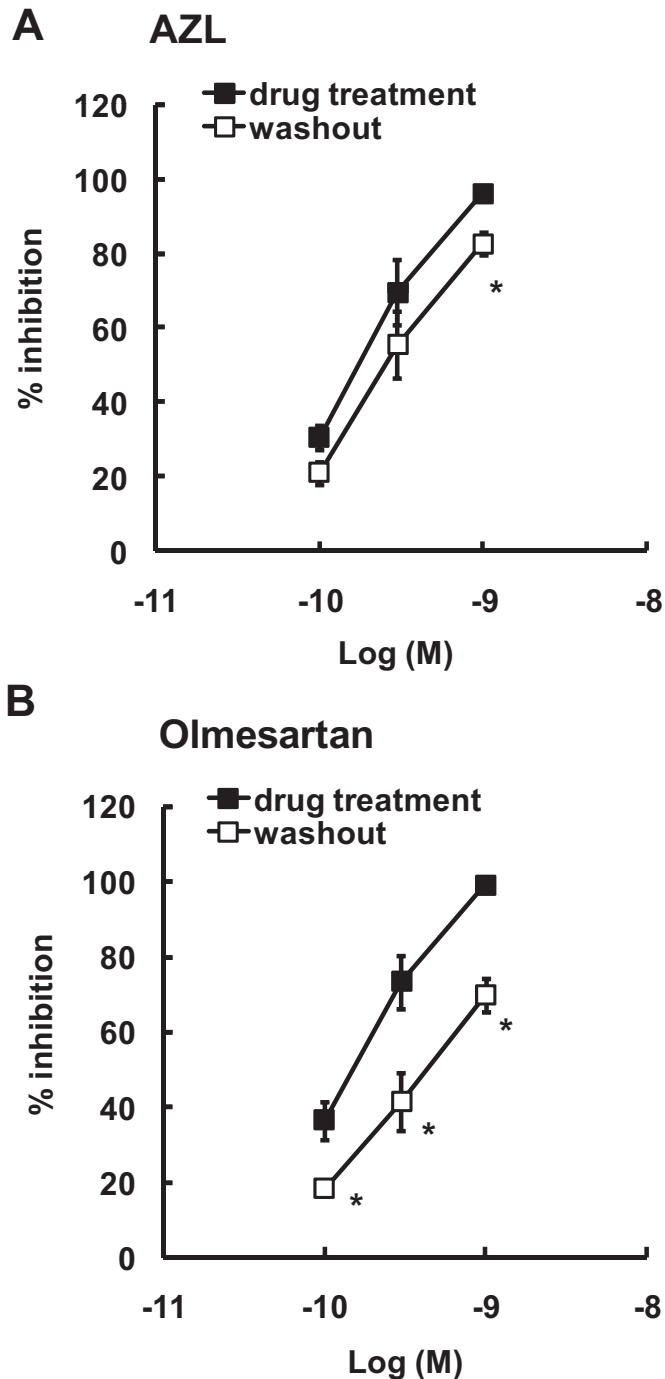| Compound	IC_{50}		Ratio: Washout (+)/Washout (−)
	Washout (−)	Washout (+)	
	nM		
AZL	9.2 (7.5–10.6)	81.3 (49.4–133.8)	9
Olmesartan	12.2 (10.7–14.0)	908.5 (424.8–2019.1)	74
Valsartan	59.8 (48.1–74.2)	22,664.4 (13673.6–39909.5)	379

A AZL

B Olmesartan

Fig. 6. Effects of washing on the inhibition of the contractile response to AII by AZL (A) and olmesartan (B) in rabbit aortic strips. After the contractile response to AII (10 nM) was determined in the presence of a compound (solid symbols), the strip was washed over 2 h. The strip was then rechallenged with AII (10 nM), and the contractile response was measured (open symbols). Data are the mean $\pm$ S.E.M. ($n = 4$). $*$, $p < 0.05$ drug treatment versus washout (Student's paired t test followed by the Holm correction).

been used to investigate the inverse agonism of ARBs (Miura et al., 2006; Yasuda et al., 2008). As reported previously (Parnot et al., 2000), the I245T mutation increased basal IP1 production without AII stimulation (Fig. 7A). Furthermore, pretreatment with AZL for 30 min suppressed basal IP1 accumulation in cells expressing I245T-AT$_1$ in a concentration-dependent manner (Fig. 7B). AZL is therefore defined as
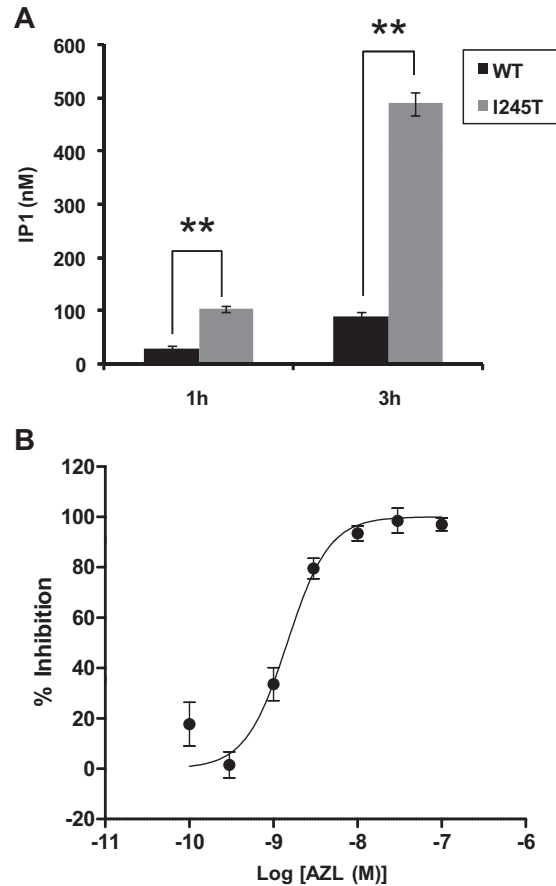
A

B

Fig. 7. Inverse agonist activity of AZL on constitutive activation of I245T-AT$_1$. A, COS-7 cells were transfected with vector expressing the constitutively active I245T-AT$_1$ receptor, and basal IP1 accumulation without AII for 1 or 3 h was measured. Data are presented as mean $\pm$ S.E.M. ($n = 4$). B, also shown is the concentration-response curve of inverse agonist activity of AZL against the basal IP1 accumulation for 3 h. Data are the mean $\pm$ S.E.M. ($n = 3$). $**$, $p < 0.01$ versus cells expressing wild-type (WT)-AT$_1$ (Student's t test).

an inverse agonist of AT$_1$ receptor because it decreases the basal activity level of the receptor on IP1 accumulation.

Discussion

This study demonstrated that AZL was a selective and insurmountable AT$_1$ receptor antagonist and that it had high affinity to AT$_1$ receptors. In particular, it remained substantially bound to the receptors after washout of the compound compared with other ARBs, such as olmesartan, telmisartan, valsartan, and irbesartan. Furthermore, the inhibitory effects of AZL on IP1 accumulation and vasoconstriction induced by AII persisted even after washout, whereas those of other ARBs were markedly attenuated.

We could not directly determine the dissociation rates of compounds from the AT$_1$ receptor because the relevant radiolabeled compounds were not available. However, the potency of compounds for AT$_1$ receptor antagonism for olmesartan, telmisartan, valsartan, and irbesartan observed in the washout experiment was almost comparable to the rank order of their dissociation half-life reported in recent studies; that is, olmesartan > telmisartan > valsartan > irbesartan (Van Liefde and Vauquelin, 2009). Therefore, the concordance of these findings suggests that the potency of these compounds at the AT$_1$ recep-

tor observed in the washout experiment may have been related to their dissociation rates from the AT_1 receptor. Therefore, AZL may tightly bind to and dissociate more slowly from AT_1 receptors compared with other ARBs tested.

Of note, AZL induced persistent inhibition of AII-induced IP1 accumulation even after washout of the compound compared with olmesartan and valsartan in the cell-based assay, and the rank order of IC_{50} values was consistent with that obtained in the binding assay (Tables 1 and 2). The slow dissociation of AZL from AT_1 receptors contributed to the functional antagonism against AII.

The pharmacological properties of ARBs generally have been defined by AII-induced contraction studies in isolated vascular tissues. Among clinically available ARBs, olmesartan and candesartan are potent and insurmountable antagonists of AII-induced contractility (Mizuno et al., 1995; Shibouta et al., 1993). Therefore, we investigated the inhibitory effects of AZL on contraction induced by AII in rabbit aortas to clarify its mode of action and potency, and we compared the results obtained with those of olmesartan. AZL and olmesartan caused a marked depression in the maximal response to AII and the pD'_2 value for both compounds was 9.9 (Fig. 5). AZL did not inhibit the contraction induced by KCl, norepinephrine, 5-hydroxytryptamine, or prostaglandin $F_{2\alpha}$ in the rabbit aorta. This observation supported the specificity of this compound for AT_1 receptors, which was also supported by the results of 128 broad-screening assays for activity of 10 μM AZL across various receptors (including human AT_2 receptor), ion channels, and enzymes (including rabbit angiotensin-converting enzyme and human renin) (data not shown).

Several molecular mechanisms, such as slow dissociation from the receptor (Ojima et al., 1997), slow removal from tissue compartments (Panek et al., 1995), allosteric modulation of receptor (Wienen et al., 1992), and stimulation of receptor internalization (Liu et al., 1992), may contribute to the insurmountable behavior of AII antagonists. However, increasing evidence suggests that slow dissociation from the receptor is one of the leading mechanisms of insurmountable behavior (Van Liefde and Vauquelin, 2009). The slow dissociation of AZL from the AT_1 receptor demonstrated in the current study probably contributed to the insurmountable action of the molecule.

The insurmountable behavior of ARBs such as candesartan, olmesartan, and EXP3174 (an active metabolite of losartan) is thought to be linked to a carboxyl group at their imidazole-derived moiety (Wienen et al., 1992; Noda et al., 1993; Van Liefde and Vauquelin, 2009) because ARBs that do not contain this group (e.g., candesartan derivatives and losartan) cannot display insurmountable antagonism (Wienen et al., 1992; Noda et al., 1993). Fierens et al. (2000) reported that Lys199 substitution of AT_1 receptors resulted in the loss of high affinity for candesartan, indicating that the carboxyl group was likely to interact with Lys199 to produce the tight binding to AT_1 receptors. Therefore, the tight or insurmountable binding of AZL to AT_1 receptors may be related to a carboxyl group at the 7-position of the benzimidazole ring, similar to candesartan (Noda et al., 1993). In addition, most ARBs, including candesartan, have a biphenylmethyl moiety with an acidic group (either a tetrazole or carboxylic acid) (Van Liefde and Vauquelin, 2009), which is thought to affect the initial interaction between antagonists and receptors (Vauquelin et al., 2001). Of interest, AZL has a

unique moiety, a 5-oxo-1,2,4-oxadiazole, in place of a tetrazole ring (Kohara et al., 1996). Because the tetrazole ring of existing ARBs may interact with the Gln257, Lys199, or Asn295 residues in AT_1 receptors (Miura et al., 2006; Bhuiyan et al., 2009; Qin et al., 2009), the oxadiazolone in AZL may interact with these residues more strongly than with the tetrazole ring.

The inverse agonist activity that some ARBs, such as candesartan and olmesartan, exert against the constitutive activity at AT_1 receptors could be an important pharmacological property related to their beneficial effects on organ protection (Miura et al., 2006; Yasuda et al., 2008; Qin et al., 2009). For example, pressure overload induces cardiac hypertrophy in angiotensinogen-deficient mice (lacking AII), as well as in wild-type mice, and the hypertrophy is suppressed by candesartan (Zou et al., 2004). Because AZL has inverse agonist activity, it may also be expected that it shows organ-protective effects (e.g., prevention of cardiac hypertrophy). The carboxyl groups at their imidazole-derived moiety of candesartan and olmesartan are suggested to be responsible for their inverse agonist activities and for their insurmountable antagonism (Yasuda et al., 2008; Qin et al., 2009). Therefore, this group of AZL is expected to contribute to its inverse agonism.

Compensatory elevation of renin and AII in vivo during ARB treatment is well known, and increased AII levels are expected to compete with the antagonist-receptor complex to attenuate the antagonistic actions of an ARB. However, AZL is thought to maintain its antagonistic activity when AII levels increase and even when the AZL concentration in the circulation becomes low 24 h after administration because of the unique antagonistic profile of AZL observed in vitro, such as the insurmountable antagonism and slow dissociation from the receptor. Therefore, the unique antagonistic profile of AZL allows potent inhibition of AII-induced responses throughout 24 h and induces potent and long-lasting antagonistic action in vivo. In fact, the inhibitory effect of AZL on AII-induced pressor responses lasted longer and was more potent than that of olmesartan medoxomil in conscious rats (K. Kusumoto, H. Igata, and M. Ojima, unpublished observations). Furthermore, clinical studies have demonstrated that azilsartan medoxomil or TAK-491 (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 2-ethoxy-1-{[2′-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]-methyl}-1H-benzimidazole-7-carboxylate monopotassium salt), a prodrug of AZL, is more effective than valsartan and olmesartan medoxomil in lowering BP in patients with mild to moderate hypertension (Bakris et al., 2010; Sica et al., 2010).

Currently available ARBs are very well tolerated. However, the next generation of ARBs is important because the currently available ARBs cannot completely control BP, and there is a risk of cardiovascular disease and diabetes in many patients (Kurtz and Klein 2009). In this respect, AZL is expected to be a desirable ARB, because it not only shows superior control of BP but also shows greater improvement of insulin resistance in animal models (Kurtz and Klein 2009).

In conclusion, AZL bound tightly to and dissociated slowly from AT_1 receptors compared with other ARBs. In addition, AZL induced insurmountable antagonism of AII-induced vascular contractions and inverse agonism against AT_1 receptors. The high-affinity and tight binding properties of AZL are expected to induce potent and long-lasting antihypertensive effects in preclinical and clinical settings.

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