Pharmacological Profiles of a Highly Potent and Long-Acting Angiotensin II Receptor Antagonist, Fimasartan, in Rats and Dogs after Oral Administration

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The pharmacological profile of fimasartan, [2-\(n\)-butyl-5-dimethylamino-thiocarbonyl-methyl-6-methyl-3-[[2-(1H-tetrazole-5-yl)biphenyl-4-yl]methyl]-pyrimidin-4(3H)-one, a new non-peptide angiotensin type 1 (AT\(_1\))-selective angiotensin receptor antagonist, has been investigated in a variety of in vitro and in vivo experimental models. In the present study, fimasartan showed slow dissociation and irreversible binding to AT\(_1\) subtype receptors in membrane fractions of HEK-293 cells with a \(K_d\) of 0.03 nM and a \(T_{1/2}\) of 63.7 min. The inhibitory effect of fimasartan on angiotensin II (Ang II)-induced contraction persisted longer after washout than that of losartan or candesartan. In conscious rats, a single dose of fimasartan (0.3, 1, or 3 mg/kg; \(p.o.\)) dose-dependently antagonized Ang II-induced pressor responses. Both orally administered fimasartan and losartan dose-dependently decreased mean arterial pressure in furosemide-treated rats and dogs, and fimasartan administered orally at 1, 3, or 10 mg/kg reduced blood pressure in conscious spontaneously hypertensive rats. Taken together, these findings indicate that fimasartan has potent and sustained binding affinity at the AT\(_1\) receptor subtype, and reveal the molecular basis responsible for the marked lowering of blood pressure in various conscious rats and dogs models after its oral administration.

Key words  angiotensin type II receptor blocker; blood pressure; fimasartan; hypertension

Hypertension increases the risk of cardiovascular and renal diseases, including myocardial infarction, renal failure, stroke, and diabetic nephropathy. Thus, it has been emphasized that the management and prevention of hypertension is crucial to reduce the risks of cardiovascular-associated diseases.\(^{1,2}\)

Angiotensin II (Ang II) receptor blockers (ARBs), such as, azilsartan, candesartan, eprosartan, irbesartan, losartan, olmesartan, telmisartan, valsartan, and fimasartan, are recommended as initial medications for hypertensive patients, and are strongly recommended in some special circumstances according to guidelines issued for the management of hypertension.\(^{3,4}\) Although ARBs possess common structural features for effective antagonism of angiotensin type 1 (AT\(_1\)) receptor, their side chains differ structurally, which might explain the characteristic binding kinetics of these agents to AT\(_1\) receptors, and thus, their different pharmacological potencies and pharmacokinetic and pharmodynamic profiles.\(^{5–7}\)

Fimasartan (BR-A-657, Boryung Pharm. Co., Ltd., Seoul, Republic of Korea) was approved by the Korea Food and Drug Administration (KFDA) in 2010 for the treatment of essential hypertension. Several nonclinical and clinical studies have been conducted on fimasartan, for example, (1) clinical studies have been undertaken on its efficacy and safety in large populations\(^{8,9}\) and interactions with other concomitant drugs\(^{10–12}\) and (2) nonclinical studies have investigated the mechanistics underlying its other pharmacological activities, such as, its inhibitory effect on catecholamine secretion,\(^{13}\) its cardioprotective effect, which was attributed to the prevention of mitochondrial damage,\(^{14}\) its anti-atherosclerotic effect,\(^{15}\) and its anti-inflammatory potential.\(^{16}\)

In a previous study, we found fimasartan selectively inhibits AT\(_1\) receptors and described the molecular basis responsible for the marked lowering of blood pressure in conscious rats observed after its intravenous administration.\(^{17}\) However, no report has been issued on its anti-hypertensive activities after oral administration, except in renal hypertensive rats (RHRs), or the molecular mechanisms involved. In the present study, we further compared the pharmacological properties of fimasartan with those of other ARBs. This paper adds to knowledge regarding the pharmacodynamic characteristics of fimasartan and supports its use for the treatment of hypertensive patients and as a component of combination therapies targeting cardiovascular diseases.

MATERIALS AND METHODS

Materials  Fimasartan (Fig. 1A) used for this study was synthesized at Boryung Pharm. Co., Ltd. (Seoul, Republic of Korea), and its purity (> 97%) was determined by HPLC-MS analyses.\(^{17}\) Candesartan, telmisartan, valsartan and losartan were purchased from Jesambiozam Co., Ltd. (Seoul, Republic of Korea). Ang II was purchased from Sigma Co. (St. Louis, MO, U.S.A.). Sodium pentobarbital was purchased from Hanlin (Seoul, Republic of Korea). \([^{125}\text{I}]\text{[Sar}^2\text{-Ile}^6\text{]}\text{Ang II (2200 Ci/}

mmol, Cat. No. NET-2480) was obtained from Perkin-Elmer (CA, U.S.A.). Other chemicals were the best grade commercially available. All materials were prepared immediately before use.

Binding Assay  A radioligand binding assay was performed by using human angiotensin II AT\(_1\) receptor in transfected HEK-293 cells. In washout experiments, the receptors (16\(\mu\)g proteins) were first incubated with 0.05 nM
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  \begin{align*}
    &\text{[^{125}I]}\text{[Sar}^1\text{-Ile}^8\text{]}\text{Ang II in test compounds (candesartan, telmisartan, valsartan, or fimasartan) in assay buffer} \\
    &\text{containing 50 mM Tris–HCl (pH 7.4), 5 mM MgCl}_2, \text{ 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1\% bovine serum albumin for 120 min at 37°C. Free compounds were} \\
    &\text{removed by dilution and centrifugation, then an excess of} \text{[^{125}I]}\text{[Sar}^1\text{-Ile}^8\text{]}\text{Ang II (0.1 nM) was added, and a second incubation} \\
    &\text{was performed. As a control, a similar treatment without washout was performed for all test compounds and each incubation time. Following second incubation, the samples} \\
    &\text{were filtered rapidly under vacuum through glass fiber filters pre-soaked with 0.3\% polyethyleneimine (PEI) and rinsed several times with ice-cold 50 mM Tris–HCl using a 96-sample cell} \\
    &\text{harvester. The filters were dried then counted for radioactivity in a scintillation counter. To determine and compare IC}_{50} \text{ ratios with and without washout, the second incubation time} \\
    &\text{was fixed to 120 min and each test compounds were tested at 6 concentrations (10}^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, \text{and } 10^{-6}\text{ M). The washout and second incubation procedures were conducted as described above. To compare the time–courses of receptor binding after washout, each test compounds at one concentration (approximately predetermined IC}_{50} \text{ concentration for each compounds) were incubated with receptor for 120 min at 37°C. The washout and second incubation procedures were also the same as in IC}_{50} \text{ experiments except that the bindings of} \text{[^{125}I]}\text{[Sar}^1\text{-Ile}^8\text{]}\text{Ang II were quantified after 0.5, 1, 1.5, 2, 4, 6, and 8 h of second incubation. In kinetic assay, association of} \\
    &\text{test compounds was measured by incubating for 12 different times (4, 8, 12, 16, 20, 25, 30, 45, 90, 120, and 140 min) with} \\
    &\text{13 concentrations of each test compound ranging from 0 to } 100\times \text{K}_i \text{ in triplicate, according to the } \text{K}_i \text{ value determined} \\
    &\text{in previous studies were used.}^{17–19} \text{ Following incubation, the} \\
    &\text{samples were filtered, rinsed, and then counted for radioac-}
  \end{align*}

Fig. 1. Effects of Fimasartan, Candesartan, Telmisartan, Valsartan, and Losartan on the Inhibition of the Specific Binding of \text{[^{125}I]}\text{[Sar}^1\text{-Ile}^8\text{]}\text{Ang II to the AT}_1 \text{ Receptors of HEK-293 Cells}

(A) Chemical structure of fimasartan. (B) Membranes of HEK-293 cells expressing AT_1 \text{ receptor were preincubated for 2 h with each compound (10}^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, \text{and } 10^{-6}\text{ M) and further incubated with} \text{[^{125}I]}\text{[Sar}^1\text{-Ile}^8\text{]}\text{Ang II for 2 h with or without washout. (C) Membranes of HEK-293 cells expressing AT}_1 \text{ receptor were preincubated for 2 h with each compound (IC}_{80} \text{ concentration) and further incubated with} \text{[^{125}I]}\text{[Sar}^1\text{-Ile}^8\text{]}\text{Ang II for 8 h with or after washout. Scatter plots of candesartan (○), telmisartan (△), valsartan (□), losartan (●) and fimasartan (▲) without washout and of candesartan (○), telmisartan (△), valsartan (□), losartan (●) and fimasartan (▲) with washout. The experiments were performed in triplicate. *p<0.05 vs. without washout group.}
tivity as in washout experiments. $K_{\text{inc}}$ and $K_{\text{off}}$ values were calculated in GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.) by fitting the association curves using the radioligand kinetic constants determined previously. $K_i$ at equilibrium was also calculated from predetermined radioligand $K_d$ (0.04 nM) and $IC_{50}$ obtained after enough time of incubation. For each condition, total binding in absence of compound and non-specific binding using 10 $\mu$M Ang II were determined and specific binding was defined as total binding minus nonspecific binding.

**Animals** All procedures were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (#KHP-2006-08-15). Male Sprague–Dawley (SD) rats and male beagle dogs were obtained from Orient Bio (Sungnam, Gyeonggi, Republic of Korea) and New Zealand white male rabbits were obtained from Samtako (Osan, Gyeonggi, Republic of Korea). The animals were housed in an environmentally controlled animal room (20±2°C; relative humidity 40–60%) under 12 h dark/light cycle for at least two weeks, and were allowed water only for 24 h before the experiment. To account for diurnal enzyme activity variations, animals were anesthetized at a fixed time (10:00–12:00 a.m.).

**In Vitro Potency in Rabbit Aorta** New Zealand white male rabbits (Samtako) weighing 2.0–2.5 kg were sacrificed by bleeding from the carotid artery under intravenous anesthesia with pentobarbital sodium (30 mg/kg). The thoracic aorta was removed, dissected free of surrounding tissue soaked in modified Kreb's bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM KH2PO4, 25 mM NaHCO3 and 11 mM glucose, pH 7.4) and cut into rings mounted in 20 mL organ baths. The solution was maintained at 37°C and oxygenated with a 95:5% O2:CO2 mixture (pH 7.4). Isometric contraction was measured with a force transducer (Grass FT03, Grass Instruments, Quincy, MA, U.S.A.) and linked with physiograph (Grass model-7 polygraph, Grass Instruments). After stabilization, the aortic strips were stimulated by the addition of 10 nM of Ang II to the bath, followed by washing 3–4 times with Kreb's bicarbonate solution to relax to the baseline tension and these steps were conducted one more time to obtain the reference. The aortic strips were incubated with fimasartan (1, 3, or 10 nM), losartan (30, 100, or 300 nM), candesartan (1, 3, or 10 nM) or vehicle in each organ bath for 30 min. The maximal contractile response to the addition of Ang II was obtained in the presence of the test compounds and then the strips were washed three times with an interval of 10 min. These steps were repeated three times (at 1, 2, and 3 h after administration of test compounds) to observe the time courses of contractile responses.

**Ang II-Induced Pressor Response in Conscious Normotensive Rats** Male SD rats (Crl:CD) weighing 350–400 g underwent the surgery described above. To activate the renin–angiotensin system, furosemide (10 mg/kg) was subcutaneously injected at 20 and 2 h before drugs administration. Fimasartan (1, 3, or 10 mg/kg), losartan (3 or 10 mg/kg) or vehicle (D.W.) were administered by oral and blood pressure was measured at 20, 40, 60 min and every 30 min from 1 h until 8 h.

**Hypotensive Effects in Furosemide-Treated Beagle Dogs** Male beagle dogs (Covance) weighing 8–11 kg were anesthetized with intramuscular injection of atropine sulfate (0.1 mg/kg) and intravenous injection of Zoletil 50® (1 mg/kg; Tiletamine/Zolazepam) and the cannula filled with heparinized saline (100 unit/mL) to prevent blood clot were passed through a subcutaneous tunnel, placed over occipital region and fixed to the skin. After completion of surgery, the dogs were given Radacl®, (20 mg/kg; ceforanide) twice a day for one week. In all experiments, the cannulated tube was connected to a pressure transducer linked with physiograph. The dogs were allowed water only for 18 h before the experiment started. After stabilization of the blood pressure and heart rate for at least 1 h, they were continuously infused with Ang II (20 ng/kg/min, intravenously (i.v.)) and then increased blood pressure by Ang II was measured for 90 min to obtain the reference value prior to the treatment of test compounds. Fimasartan (0.3, 1, or 3 mg/kg) in gelatin capsules were orally treated and an empty capsule was used as vehicle. Blood pressure was measured at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h after drug administration.

**Hypotensive Effects in Furosemide-Treated Beagle Dogs** Male beagle dogs (Covance) weighing 8–11 kg were anesthetized using intraperitoneal injection of pentobarbital sodium (50 mg/kg). Long incision of 1.5–2.0 cm was made in the left inguinal region to expose the femoral vein and/or artery and then catheters were inserted into them. The catheters were passed through a subcutaneous tunnel, placed over occipital region and fixed to the skin. The catheters were filled with heparinized saline (100 unit/mL) to prevent blood clot. In all experiments, the cannulated tube was connected to a pressure transducer (CDX III, Modular Ins., Malvern, PA, U.S.A.) linked with physiography. When the blood pressure and heart rate were stabilized, Ang II (0.1 µg/kg) was intravenously administered to the left femoral vein of rats three times with an interval of 20 min prior to treatment of test compounds to obtain the reference. Fimasartan (0.3, 1, or 3 mg/kg), losartan (1, 3, or 10 mg/kg) or vehicle distilled water (D.W.) were administered orally and then Ang II were intravenously treated as a bolus at various time points (20, 40, 60 min, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, and 24 h) to determine the Ang II-induced blood pressure response.

**Hypotensive Effects in Spontaneously Hypertensive Rats** Male spontaneously hypertensive rats (SHR/NCrjOri) weighing 350–370 g underwent the surgery described above. After stabilization period, fimasartan (1, 3, or 10 mg/kg), losartan (3 or 10 mg/kg) and vehicle (D.W.) were orally administered. Blood pressure was measured at 20, 40, 60 min, every 30 min from 1 h until 8 h, 23.5 and 24 h after administration of test compounds.

**Statistical Analysis** The hypotensive effect was assessed by measuring systolic arterial pressure (SAP) and diastolic arterial pressure (DAP), and then mean arterial pressure.
(MAP) was calculated as follows; MAP=\[DAP+(SAP-DAP)/3\]. MAP and heart rate (HR) were expressed as percentage changes from a condition before test compounds administration. Figures were obtained by using Sigmaplot 8.0 (Systat Software, Chicago, IL, U.S.A.). All results are expressed as means±standard error of the mean (S.E.M.) Changes in response to fimasartan or losartan during in vivo studies were compared to that in vehicle-treated controls over corresponding periods. One-way ANOVA was used for intergroup comparisons and Dunnett’s test was used for multiple comparisons. SPSS ver. 8.0 (SPSS Inc., IL, U.S.A.) was used throughout, and statistical significance was accepted for p values of <0.05.

RESULTS

Effect of Washout on Fimasartan-Induced \[^{125}\text{I}[^{\text{Sar}}-\text{Ile}]\text{Ang II}\] Binding to AT\(_1\) Receptors in Human Recombinant HEK-293 Cells Pretreatment of fimasartan for 2h concentration-dependently inhibited specific binding of \[^{125}\text{I}[^{\text{Sar}}-\text{Ile}]\text{Ang II}\] to human AT\(_1\) receptors expressed in HEK-293 cells with an IC\(_{50}\) of 0.5 nm, indicating a high affinity for AT\(_1\) receptors (Fig. 1B). Candesartan, telmisartan, valsartan, and losartan also concentration-dependently inhibited these bindings with IC\(_{50}\) values of 2.0, 2.2, 3.2, and 42.6 nm, respectively. In addition, fimasartan retained a potent inhibitory effect on AT\(_1\) receptors after washout with an IC\(_{50}\) value of 0.5 nm. In contrast, the inhibitory effects of candesartan, telmisartan, valsartan, and losartan were markedly attenuated by washout and their IC\(_{50}\) values were 3.2, 88.8, >1000, and >1000 nm, respectively. Based on these IC\(_{50}\) values obtained after washout, fimasartan was 6.4, 177.6, >2000, and >2000 times more potent as AT\(_1\) receptor antagonist than candesartan, telmisartan, valsartan, and losartan, respectively (Fig. 1B).

We also examined the reversibility of these compounds on AT\(_1\) receptor for up to 8h after washout. The inhibitory effects of fimasartan and candesartan on \[^{125}\text{I}[^{\text{Sar}}-\text{Ile}]\text{Ang II}\] binding to human AT\(_1\) receptors were slightly reduced by washout from 82 to 67% and from 77 to 63%, respectively, at 2h. Moreover, the inhibitory effect of fimasartan on \[^{125}\text{I}[^{\text{Sar}}-\text{Ile}]\text{Ang II}\] binding after washout persisted even when the incubation period was extended to 8h after the addition of radioligand. However, the inhibitory effects of telmisartan, valsartan, and losartan were significantly reduced at 2h after washout from 80 to 31%, from 63 to 9%, and from 53 to 7%, respectively, and then gradually decreased to 8h (Fig. 1C).

Next, we measured the kinetic parameters of these ligand interactions with AT\(_1\) receptor, that is, association rate constant (\(K_{\text{on}}\)), dissociation rate constant (\(K_{\text{off}}\)), and kinetically derived affinity (\(K_i\) or \(K_c\)). \(K_{\text{on}}\) ranged from 0.0280 nm\(^{-1}\)min\(^{-1}\) for valsartan to 0.3620 nm\(^{-1}\)min\(^{-1}\) for fimasartan, and \(K_{\text{off}}\) from 0.0109 min\(^{-1}\) for fimasartan to 0.1822 min\(^{-1}\) for losartan. \(K_i\) at AT\(_1\) receptors was 0.03 nm for fimasartan, and 0.19, 0.13, 1.98 and 1.5 nm for candesartan, telmisartan, valsartan, and losartan, respectively. \(K_i\) values at equilibrium were 0.08, 0.48, 0.27, 5.49, and 4.2 nm for fimasartan, candesartan, telmisartan, valsartan and losartan, respectively (Supplementary Table S1). For fimasartan, its dissociation half-life (\(T_{1/2}\)) calculated from its dissociation rate constant; \(K_{\text{off}}\) was 63.7 min. These results suggest that fimasartan is a highly potent and slowly dissociating AT\(_1\) receptor antagonist as compared with the other ARBs tested.

Inhibitory Effects of Fimasartan on Ang II-Induced Contraction in Isolated Rabbit Thoracic Aorta after Washout A functional in vitro study was performed to characterize the mode of the interaction between fimasartan and AT\(_1\) receptor in rabbit thoracic aorta. Pretreatment with fimasartan (1, 3, or 10 nm) or candesartan (1, 3, or 10 nm) reduced Ang II-induced maximal aortic contraction to 59.2±6.49, 32.8±6.06, and 5.8±1.74 or to 32.2±7.33, 9.2±3.29, and 0%, respectively, and these inhibitory effects persisted for 3h even after three washouts. At 3h after these washouts, Ang II-induced maximal aortic contraction was reduced to 25.2±4.17, 14.2±2.62, and 5.8±1.58 or 38.0±3.49, 29.0±7.22 and 8.3±1.45% by 1, 3, or 10 nm of fimasartan or candesartan, respectively (Figs. 2A, B). Losartan also potently inhibited Ang II-induced contraction before washout, to 46.8±8.51, 24.4±5.27 and 2.3±1.58% at 30, 100, and 300 nm, respectively, but these reductions were significantly recovered after washout to 102.0±2.16, 87.4±2.06 and 60.8±6.31%, respectively, at 3h (Fig. 2C). These results suggest that dissociation of fimasartan from binding sites in rabbit aorta is much slower than those of candesartan and losartan.

Effects of Single Orally Administered Fimasartan on Ang II-Induced Pressor Response in Rats Administration of Ang II (0.1 mg/kg, i.v.) induced a mean pressor response of 38.31±0.86 mmHg for 3 consecutive doses over baseline MAP. Fimasartan or losartan had significant dose-dependent inhibitory effects on Ang II induced pressor responses. Fimasartan maximally reduced MAP by 41.01±4.72% at 3h (0.3 mg/kg, per os (p.o.)) and by 100.0% at 40 min (1 mg/kg, p.o.) and 20 min (3 mg/kg, p.o.) post-administration. In the 3 mg/kg orally-treated group, complete inhibitory effects were maintained for approximately 6h (Fig. 3A). However, losartan maximally reduced MAP by 34.35±9.22% at 4h (1 mg/kg, p.o.), 73.85±9.46% at 6h (3 mg/kg, p.o.), and by 100% at 3, 4, 5, and 8h (10 mg/kg, p.o.) post-administration (Fig. 3B). These results suggest that oral administration of fimasartan has more potent, rapid, sustained hypotensive effects than losartan on Ang II-induced hypertension in rats.

Effects of Orally Administered Fimasartan on Blood Pressure in Furosemide-Treated Rats The antihypertensive effects of fmasartan (1, 3, or 10 mg/kg, p.o.) or losartan (3 or 10 mg/kg p.o) on MAP were examined in furosemide (10 mg/kg)-treated rats (Figs. 4A, B). Predose mean MAP values before test compound administration were 108.81±0.77 mmHg in all groups. Fimasartan (3 or 10 mg/kg, p.o) significantly reduced MAP from 20 min post-administration, whereas in the losartan-treated group, MAP was significantly reduced from 4.5 and 1.5h at doses of 3 and 10 mg/kg, respectively. The hypotensive effects of both compounds at 3 and 10 mg/kg were lasted significantly until 8h post-administration. According to these results, fimasartan acted more rapidly and had a stronger blood pressure-lowering effect than losartan.

Effects of Orally Administered Fimasartan on Blood Pressure in Furosemide-Treated Beagle Dogs The effects of a single administration of fimasartan (0.3, 1, or 3 mg/kg, p.o) or losartan (10 or 30 mg/kg, p.o) on blood pressure were evaluated in furosemide-treated dogs. In furosemide-treat-
ed dogs, predose baseline values for MAP and HR were 95.22±0.94 mmHg and 109.00±4.04 bpm, respectively, and were similar in all groups. The anti-hypertensive effect of fimasartan (1 or 3 mg/kg, p.o.) dose-dependently reduced MAP with gradual onset (40 min at 3 mg/kg) and significantly lasted for at least 8 h (p<0.05). Maximum hypotensive effects of oral administration of fimasartan at 0.3, 1, or 3 mg/kg were 14.25±1.59% (at 4.5 h), 20.14±1.56% (at 5 h), and 32.12±2.09% (at 4 h), respectively (Fig. 5A). Although losartan (10 or 30 mg/kg, p.o.) also significantly decreased MAP, the reduction was smaller and its effective duration was shorter than those of fimasartan (Fig. 5B). Neither fimasartan nor losartan caused a significant change in HR (data not shown).

Effects of Orally Administered Fimasartan on Blood
Pressure in Spontaneously Hypertensive Rats

The effects of a single administration of fimasartan (1, 3, or 10 mg/kg, p.o.) or losartan (3 or 10 mg/kg, p.o.) on MAP in SHRs are shown in Fig. 6. Predose MAP and HR were 159.08 ± 1.61 mmHg and 316.02 ± 11.56 bpm, respectively and similar in all groups. A significant MAP reduction was observed at 20 min post-administration and this rapid reduction lasted at least 8h at fimasartan doses of 3 and 10 mg/kg (Fig. 6A). Although fimasartan (1 mg/kg, p.o.) caused a gradual MAP reduction, it did not produce a significant effect versus vehicle-treated control groups for up to 24h. On the other hand, the blood pressure lowering effects of losartan were significant from 2 and 0.5 h after administering 3 or 10 mg/kg, respectively, and the effects of both doses lasted for up to 8h (Fig. 6B). A maximal MAP reduction at losartan dose of 10 mg/kg was about 30 mmHg (about 20% change of MAP), which is consistent with the results of other previous studies. Both fimasartan and losartan administered orally decreased MAP in a dose-dependent manner but did not cause a significant change in HR (data not shown).

DISCUSSION

This study demonstrated that fimasartan 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. Furthermore, these potent and sustained binding affinities at the AT_1 receptor subtype of fimasartan reveal the molecular basis responsible for the marked lowering of blood pressure in various conscious rats and dogs models after its oral administration.

Previously, we reported fimasartan is a potent and specific antagonist of Ang II at the AT_1 receptor, and described the mechanism responsible for marked lowering of blood pressure after the intravenous administration in high renin furosemide-treated and renal artery-ligated rats. Furthermore, these find-
ings suggested an association between the renin–angiotensin system (RAS) and the hypotensive effect of fimasartan.\textsuperscript{17} The present study shows that fimasartan binds rapidly with high affinity to AT\textsubscript{1} receptors, and that it remains substantially bound after washout as compared with other ARBs, candesartan, telmisartan, valsartan, and losartan. In addition, the inhibitory effect of fimasartan on Ang II-induced vasoconstriction persisted even after washout. Furthermore, the study demonstrates the anti-hypertensive effects of fimasartan after oral administration in several animal models of hypertension, that is, in Ang II-pressor hypertensive rats, furosemide-treated rats, and dogs, and SHRs.

In our Ang II at AT\textsubscript{1} subtype receptor binding assay, IC\textsubscript{50} values of the test compounds were in good agreement with previously reported values.\textsuperscript{17,26–28} Moreover, our reversibility experimental results suggest that fimasartan and candesartan are irreversible antagonists, whereas telmisartan, losartan, and valsartan are reversible antagonists. The order of dissociation half-life of the five antagonists was similar to that observed in the reversibility experiment involving binding after washout, and was: fimasartan > candesartan > telmisartan > valsartan > losartan. Furthermore, the concordance of these findings suggests that the potencies of these compounds at AT\textsubscript{1} receptor as observed by washout may be related to their dissociation rates from AT\textsubscript{1} receptor. Notably, fimasartan bound more rapidly to and dissociated more slowly from AT\textsubscript{1} receptor than the other ARBs tested. The slow dissociation of fimasartan from AT\textsubscript{1} receptors seems to contribute to the durability of its functional antagonism of Ang II. As it is known, more potent AT\textsubscript{1} selective antagonists such as telmisartan and valsartan were developed based on the replacement of the imidazole ring of losartan with five-membered, six-membered, fused or acyclic moieties. Through the replacement of the imidazole ring of losartan with pyrimidin-4(3\text{H})-one ring, we discovered the more potent and AT\textsubscript{1}-selective antagonist fimasartan.\textsuperscript{29} Therefore, we expect that the pyrimidinone ring of fimasartan seems to be one of the major factors of high affinity to AT\textsubscript{1} receptors, and that it remains substantially bound after washout.
We previously reported that fimasartan reduces maximal contractile response to Ang II due to insurmountable antagonism in rabbit aorta. In the present study, duration of blockade of vascular contractile response to Ang II was examined, and the results obtained supported insurmountable inhibitory effect of fimasartan on tissue contracture due to protracted receptor binding. The results obtained for candesartan and losartan correlated well with those of previous studies. Of the several mechanisms proposed to explain the insurmountable behavior of Ang II antagonists, which manifests a slow dissociation from the receptor, slow removal from tissue compartments, allosteric modulation of receptor, and the stimulation of receptor internalization, slow dissociation from the receptor is accepted as the leading mechanism by other researchers. To explain the extent of insurmountable inhibition in terms of binding kinetics, a two-step model of antagonist–receptor complex was proposed. According to this model, antagonist–receptor complexes are able to adopt rapidly reversible or tight-binding/slow reversible state, and that the equilibrium between these two states is reflected by the kinetic constant for conversion from one state to the other. Furthermore, this parameter matches with experimentally determined dissociation rates and the extent of insurmountable inhibition. At present, insurmountable antagonist is accepted as an in vitro feature of most ARBs, except losartan. Surmountable antagonists, such as, losartan, produce parallel rightward shifts in the dose–response curves of Ang II without affecting maximal response, whereas most insurmountable ARBs, such as, telmisartan, irbesartan, valsartan, and EXP3174 (an active metabolite of losartan), partially reduce maximal response to Ang II. Although the clinical roles and relevances of in vitro insurmountability, especially its impact on organ protection and long-term outcomes in disease backgrounds, such as, stroke and myocardial infarction are still being explored, their potential therapeutic benefits are generally acknowledged. In accordance with underlying slow dissociation from AT1 receptor, insurmountable antagonism may increase the half life of fimasartan at effect sites, and extend its activity regardless of its pharmacokinetic properties at the organ level. Receptor binding half-lives are therefore likely to be at least as important as plasma half-lives in determining the durations of ARBs. Insurmountable antagonism and slow dissociation of fimasartan at AT1 receptor are expected to cause potent anti-hypertensive effects in animal models. Fimasartan and losartan after oral administration inhibited Ang II-induced pressor responses; these inhibitory effects gradually subsided but remained significant at 24h post-administration for both compounds. The persistence of fimasartan was observed in Ang II-induced pressor dogs (Supplementary Figure S1) when administered at the same dosages as in rats, although maximal blood pressure reductions in dogs (5h for all doses) occurred slightly later than in rats (3h, 40, and 20 min for doses of 0.3, 1, or 3 mg/kg, respectively). However, this difference was probably due to the use of different formulations (a solution in rats and a capsule of power in dogs) or the method of Ang II administration (intravenous bolus in rats and continuous infusion in dogs). In our previous study, maximal inhibitory effects of fimasartan (1 or 3 mg/kg p.o.) on Ang II-induced pressor responses in rats occurred earlier (0.33 to 0.66h) than those in RHRs (6 to 8h). These discrepancies in time courses of responses in the two rat models may be explained by mediation of the transient vasoconstriction induced by exogenous Ang II by AT1 receptors located on vascular smooth muscle cells close to vascular lumen, whereas the vasocostriction mediated by sustained levels of endogenous Ang II is mediated within deeper vessel walls. Furosemide-treated conscious rats and dogs have high plasma renin levels, but because they have relatively normal blood pressures as compared with Ang II-pressor models and RHRs, predose baseline blood pressures was not much increased over those of normotensive rats or dogs. In both animals, fimasartan and losartan dose-dependently decreased MAP. ED20 values (doses required to reduce MAP by 20% of predose blood pressure) of fimasartan or losartan were 6.70 or 6.62 mg/kg in rats and 1.10 or 11.58 mg/kg in dogs, respectively, suggesting fimasartan has a more potent, rapid, and lasting effects in dogs. One possible explanation for this species difference of losartan is that its active metabolite, EXP3174, which plays an important role in its hypotensive activity, is not produced in dogs. Insurmountable antagonism and slow dissociation of fimasartan at AT1 receptor after oral administration means that its BP-reducing effects occur rapidly and persist at least for 24h in vivo, which suggests fimasartan is a suitable once daily treatment for hypertension in preclinical and clinical environments. In line with its in vitro data, fimasartan exhibited greater potency and longer lasting anti-hypertensive activity than losartan in vivo in Ang II-pressor rats and dogs, furosemide-treated rats and dogs, and SHRs. Thus, our preclinical findings suggest that fimasartan could demonstrate this kind of potency in the clinical setting.

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Conflict of Interest S.H.P., Y.H.C., and J.H.L. are employees of Boryung Pharmaceutical Co., Ltd. H.-S.H. and K.-T.L. have no conflict of interest to disclose.

Supplementary Materials The online version of this article contains supplementary materials.
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