In Vitro Pharmacology of a Novel Non-Peptide Angiotensin II-Receptor Antagonist, E4177

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ABSTRACT—E4177, 3-[(2'-carboxybiphenyl-4-yl)methyl]-2-cyclopropyl-7-methyl-3*H*-imidazo[4,5-*b*]pyridine, was characterized by in vitro autoradiography and by examining functional antagonism upon angiotensin II (Ang II)-induced contraction of isolated vessels. In rat adrenal cortex and liver, E4177 competitively inhibited the specific binding of ^{125}I-[Sar^1,Ile^8]Ang II, with IC_{50} being $(5.2\pm1.0)\times10^{-8}$ M for the adrenal cortex and $(1.2\pm0.3)\times10^{-7}$ M for the liver. These IC_{50} values were similar to those for losartan, which showed an IC_{50} of $(6.0\pm0.9)\times10^{-8}$ M for the adrenal cortex and $(1.3\pm0.5)\times10^{-7}$ M for the liver. In contrast, E4177 and losartan had little effect on the binding to rat adrenal medulla where AT_2-receptors predominate. These results indicate that E4177 is AT_1-specific as is losartan. E4177 and losartan competitively antagonized the Ang II-induced contraction of human and rabbit arterial strips without any agonistic action. The obtained IC_{50} values indicated that E4177 was twice as potent as losartan in human arteries and three times more so in rabbit aortic strips. Responses to norepinephrine, serotonin, histamine or KCl were not affected by E4177. In addition, E4177 (10^{-5} M) had no effect on angiotensin-converting enzyme activity. These data indicate that E4177 is a potent AT_1 Ang II-receptor antagonist that may be clinically useful for the treatment of cardiovascular diseases such as hypertension.

Keywords: Losartan, Autoradiography (in vitro), Angiotensin II-receptor antagonist (non-peptide), Receptor subtype

The renin-angiotensin system (RAS) plays a critical role in blood pressure regulation and fluid and electrolytes homeostasis through angiotensin II (Ang II), an active principle of the system (1, 2). In addition to such well-known actions of Ang II, it is assumed that Ang II may have diverse actions (3). To explore further the potential role of Ang II, an agent that interrupts the formation of Ang II or its action at the receptor sites would be an essential tool. Angiotensin-converting enzyme (ACE) inhibitors have been successfully used as such; they are excellent therapeutics for hypertension and heart failure, and they have served to introduce the concept of "tissue" RAS (4). Recent studies suggested that ACE inhibitors prevent the neointimal thickening after intimal injury (5) and in atherosclerotic vessels (6, 7), indicating the involvement of Ang II in such trophic disorders. However, since ACE acts on many substrates other than Ang I such as bradykinin, substance P and enkephalins, the actions of ACE inhibitors are not completely specific. In fact, antihypertrophic effects of ACE inhibitors on balloon-injured blood vessels have been claimed to involve factors other than Ang II (8, 9). Furthermore, there are Ang II-generating enzyme systems other than ACE in dog and primate blood vessels (10) and human heart (11), indicating persistent Ang II generation even after ACE inhibition. In view of these limitations of ACE inhibitors, specific Ang II-receptor antagonist should be advantageous for evaluating the pathophysiological meanings of the angiotensin system.

Losartan (also called DuP753) is a breakthrough novel Ang II-receptor antagonist which is non-peptide and devoid of any agonist activity (12). Following the development of new classes of Ang II-receptor antagonists, two distinct subtypes of Ang II receptors have been identified. A standard nomenclature for Ang II receptor subtypes has been proposed, in which the Ang II receptors inhibited by losartan, a prototypic AT_1-receptor antagonist, are designated AT_1-receptors; and those inhibited by

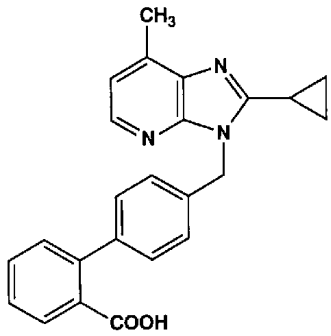

Fig. 1. Chemical structure of E4177.

PD123177, PD123319, or CGP42112A are designated as AT_2-receptors (13). AT_1-receptors are believed to be responsible for known biological actions of Ang II but the function of AT_2-receptors is not clear at present (3).

In the present study, we describe the in vitro pharmacological properties of a chemically novel, non-peptide Ang II-receptor antagonist, E4177 (Fig. 1). Our findings indicate that this compound is an AT_1-selective and competitive antagonist of Ang II.

MATERIALS AND METHODS

Binding assay for Ang II receptors in rat adrenal gland and liver

Ang II receptors were labelled by in vitro autoradiography by using ^{125}I-[Sar^1,Ile^8]Ang II ligand as described elsewhere (14). Male Wistar rats (300–350 g) were decapitated, and then their adrenal glands and liver were removed quickly, frozen in isopentane-dry ice (−40℃) and stored at −80℃. Serial 20-μm sections were cut on a cryostat, thaw-mounted onto gelatin-coated slides and dried in a desiccator under reduced pressure overnight at 4℃. Sections were then preincubated in 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 5 mM Na_2EDTA, 0.1% NaN_3, and 0.2% bovine serum albumin (buffer A) for 15 min at room temperature. Sections were then incubated in a fresh volume of buffer A containing 0.1 μCi (~50 pM) of ^{125}I-[Sar^1,Ile^8]-Ang II for 2 hr at room temperature. After incubation, sections were washed by four successive 1 min-washes in ice-cold buffer A without bovine serum albumin. Then the sections were dried under a stream of cold air and apposed to Fuji RX-X ray films in a cassette and exposed for 1.5 days for the adrenal glands or 4 days for the liver. After development of the films, the autoradiograms were quantitated with a Microcomputer Imaging Device run on an IBM AT computer (Imaging Research Co., Ontario, Canada). Non-specific binding was determined in the presence of 0.5 μM unlabeled [Sar^1,Ile^8]Ang II. To determine the IC_{50} values of the compounds, unlabeled [Sar^1, Ile^8]Ang II, E4177, or losartan, at concentrations ranging from 10^{-10} M to 10^{-5} M, was added in the incubation mixtures which contained 0.1 μCi of ^{125}I-[Sar^1,Ile^8]Ang II, like the routine incubations.

Effect of E4177 on ACE activity

To examine if E4177 affects ACE activity in vitro, beagle lung ACE extracts were incubated with or without 10^{-5} M E4177 using Hip-His-Leu as a substrate. Hippuric acid liberated during the incubations was measured by high-performance liquid chromatography to quantitate the ACE activity (15). Trandolaprilat (16), a highly potent ACE inhibitor, was used as a positive control.

Effect of E4177 on angiotensin-induced contraction in isolated blood vessels

The effect of E4177 on vascular Ang II receptors was examined in human gastroepiploic artery and rabbit aorta. Gastroepiploic artery specimens were isolated from omenta which were attached to the resected stomachs from 24 patients, aged 24–75 years, by operation for the treatment of gastric cancer or gastric ulcer. Descending thoracic aortae were removed from male Japanese White rabbits (3.1–3.3 kg; Keari Co., Osaka) under sodium pentobarbital anesthesia (60 mg/kg). Human gastroepiploic arteries and rabbit aortae were cut into helical strips 2- to 2.5-mm-wide and 20-mm-long and 3.0-mm-wide and 20-mm-long, respectively. These strips were mounted in a tissue bath containing Tyrode's solution composed of 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$ and 5.7 mM glucose. The Tyrode's solution was kept at 37℃ and gassed continuously with 5% CO_2 in oxygen. The initial resting tension was set at 1.5 g for human arterial strips and 2.0 g for rabbit aortic strips. Isometric tension signals were detected with a force-displacement transducer (Nihon Kohden, Tokyo), amplified and displayed on a chart recorder (Pantos, Kyoto). After an equilibration period of 90–120 min, the strips were stimulated by adding 30 mM KCl to which the response of each strip served as the reference for agonist-induced contraction of the corresponding strip. The control cumulative concentration-response curve for Ang II (10^{-10}–3×10^{-7} M) was first obtained. Following this, the tissues were washed 5 times over a 1-hr period, and then various concentrations of antagonists were added and allowed 20-min contact prior to the addition of agonists. To measure the potency of E4177 and losartan, the pA_2 values (negative logarithms of dissociation constant) were determined according to the methods described by Arunlakshana and Schild (17). To determine the IC_{50} values, another series of experiments were carried out where the inhibition of 20 nM Ang II-induced responses was assessed with various concentrations of

Ang II antagonists.

Verification of the specificity of E4177

To test the specificity of E4177, contractile responses to KCl (30 mM), norepinephrine ($10^{-7}-3\times10^{-5}$ M), and serotonin ($3\times10^{-7}-10^{-5}$ M) were examined before and after a 20-min incubation with 1 μM E4177. Whether Ang II antagonists affect the relaxations induced by histamine ($10^{-7}-10^{-5}$ M) was also examined in human arteries precontracted with 30 mM KCl.

Drugs

E4177 and losartan were synthesized at Tsukuba Research Laboratories, Eisai Pharmaceutical Co., Ltd. Trandolaprilat was a gift from Nippon Roussel (Tokyo).

Statistics

All numerical data were expressed as the mean± S.E.M. Differences were considered significant when the P values were less than 0.05 with Student's *t*-test or Duncan's multiple range test where appropriate.

RESULTS

Interaction of E4177 and losartan with Ang II receptors evaluated by in vitro autoradiography

The ability of E4177 to interact with Ang II receptors in rat adrenal glands and livers was evaluated. Because nonspecific binding determined in the presence of 0.5 μM unlabeled [Sar^1,Ile^8]Ang II was very weak and no visible image was observed on the X-ray films, the data presented in-

Fig. 2. Effect of E4177 and losartan on the ^{125}I-[Sar^1,Ile^8]Ang II binding in rat adrenal gland. (A) is the binding in the absence of Ang II antagonists. (B) is the binding in the presence of 1 μM E4177. For comparison, the binding in the presence of losartan (1 μM) is shown in (C). White in the tissue represents a high level of binding, while black indicates the background level.

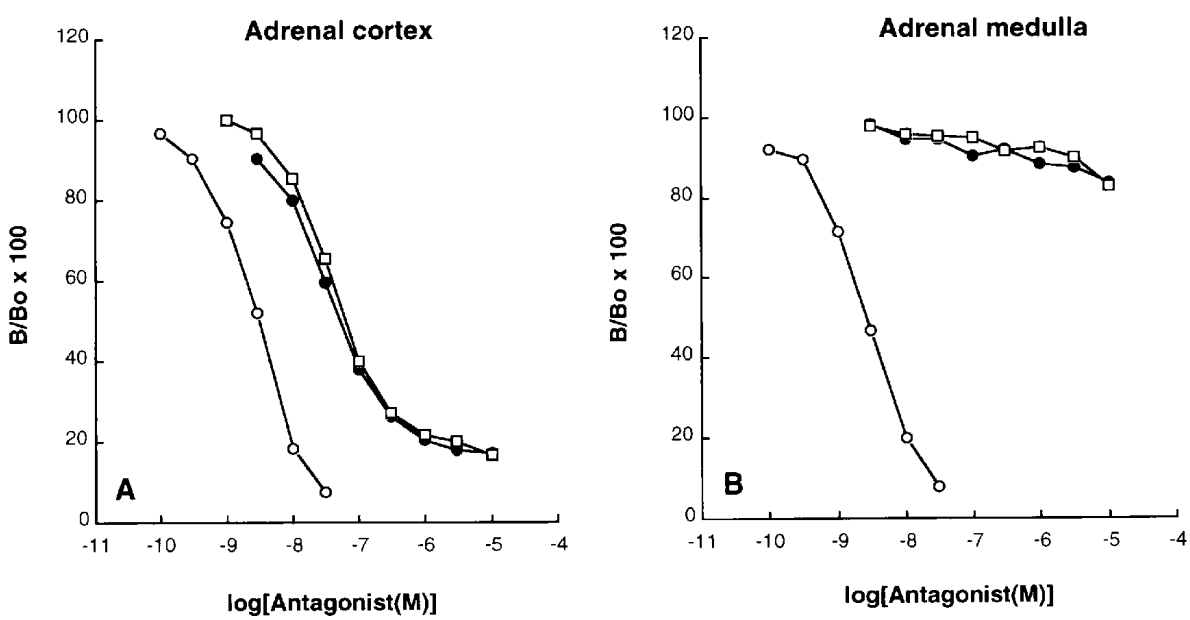

Fig. 3. Binding isotherms showing the inhibition of ^{125}I-[Sar^1,Ile^8]Ang II binding to the adrenal cortex (A) and medulla (B) by unlabeled [Sar^1,Ile^8]Ang II (_), E4177 (●), and losartan (□).

dicated the specific binding. In rat adrenal glands, E4177 potently inhibited the ligand binding to the cortex where AT_1-receptors are known to be predominant. In contrast, E4177 only slightly inhibited the binding to the medulla where AT_2-receptors are known to be predominant (Fig. 2). In the rat liver, which exclusively contains AT_1-receptors, E4177 inhibited the binding potently. Similar results were observed with losartan, an established selective AT_1-receptor antagonist, both in the adrenal glands and livers. All the above findings indicated that E4177 was selective towards AT_1-receptors.

Interaction with Ang II receptors were further evaluated by competitive binding experiments. As shown in Fig. 3A, [Sar^1,Ile^8]Ang II displayed high affinity for the Ang II receptors in the adrenal cortex with an IC_{50} of 1.6×10^{-9} M. E4177 inhibited the binding in a biphasic manner with an IC_{50} of $(5.2\pm 1.0)\times 10^{-8}$ M for the AT_1-sites and $>10^{-5}$ M for the AT_2-sites. IC_{50} values of losartan were $(6.0\pm 0.9)\times 10^{-8}$ M for the AT_1-sites and $>10^{-5}$ M for the AT_2-sites, being very similar to those of E4177. In rat adrenal medulla, unlabelled [Sar^1,Ile^8]Ang II inhibited the binding of radioligand in a monophasic manner as in the adrenal cortex, whereas neither E4177 nor losartan showed substantial inhibition of the binding, indicating again that E4177 was selective for AT_1-receptors (Fig. 3B). In the rat liver where AT_1-receptors are exclusively recognized, both E4177 and losartan were potent in competing with ^{125}I-[sar^1,Ile^8]Ang II with IC_{50}s of $(1.2\pm 0.3)\times 10^{-7}$ M and $(1.3\pm 0.5)\times 10^{-7}$ M, respectively (Fig. 4).

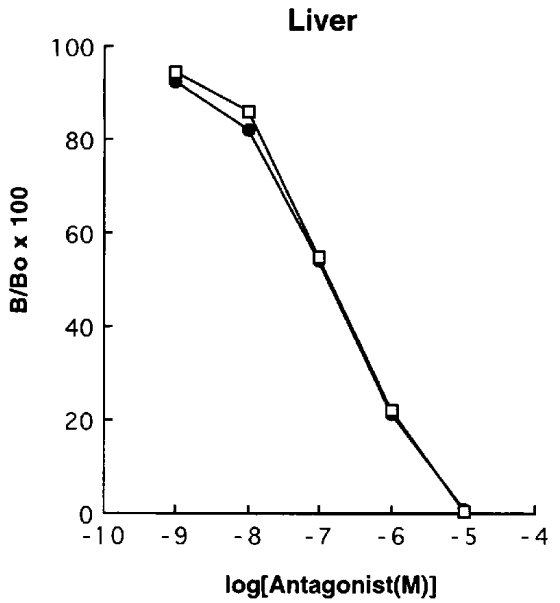

Fig. 4. Binding isotherms showing the inhibition of ^{125}I-[Sar^1,Ile^8]-Ang II binding to the liver by E4177 (●) and losartan (□).

Effect of E4177 on angiotensin-converting enzyme activity

The effect of E4177 and the ACE inhibitor trandolaprilat on ACE activity was investigated with beagle lung ACE extract. Trandolaprilat (1 μM) completely inhibited ACE activity, whereas E4177 (10 μM) did not exhibit substantial inhibition on ACE (data not shown).

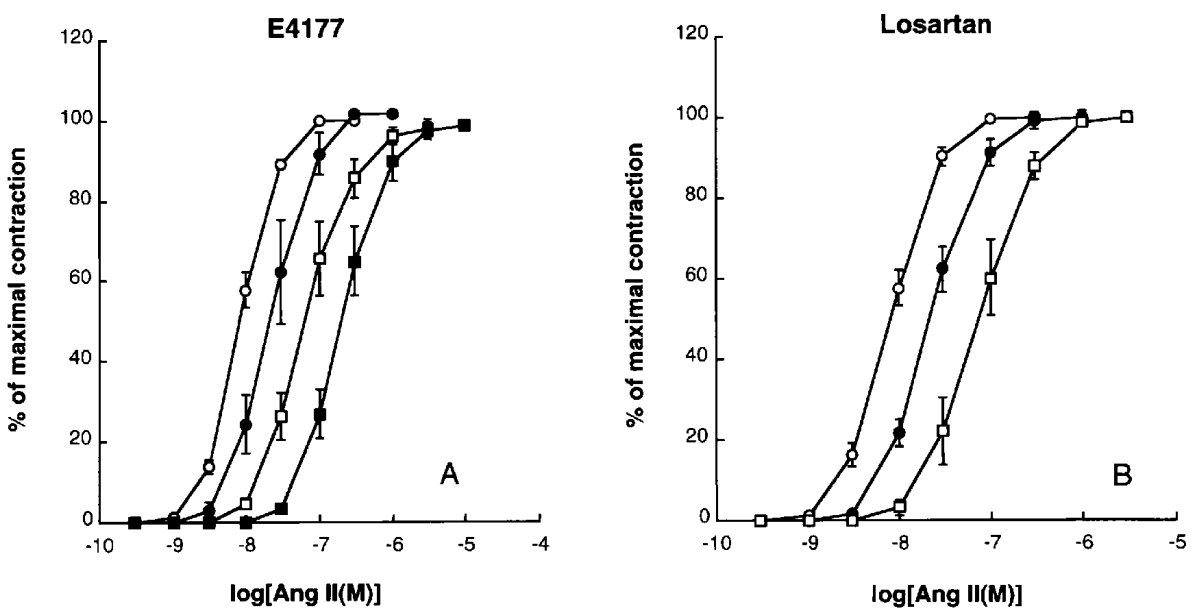

Fig. 5. Effects of E4177 (A) and losartan (B) on the concentration-contractile response curve to Ang II in isolated rabbit aortic strips. Each point represents the mean±S.E.M. (n=5). Control (○); E4177: 3×10^{-9} M (●), 10^{-8} M (□), 3×10^{-8} M (■); Losartan: 10^{-8} M (●), 3×10^{-8} M (□).

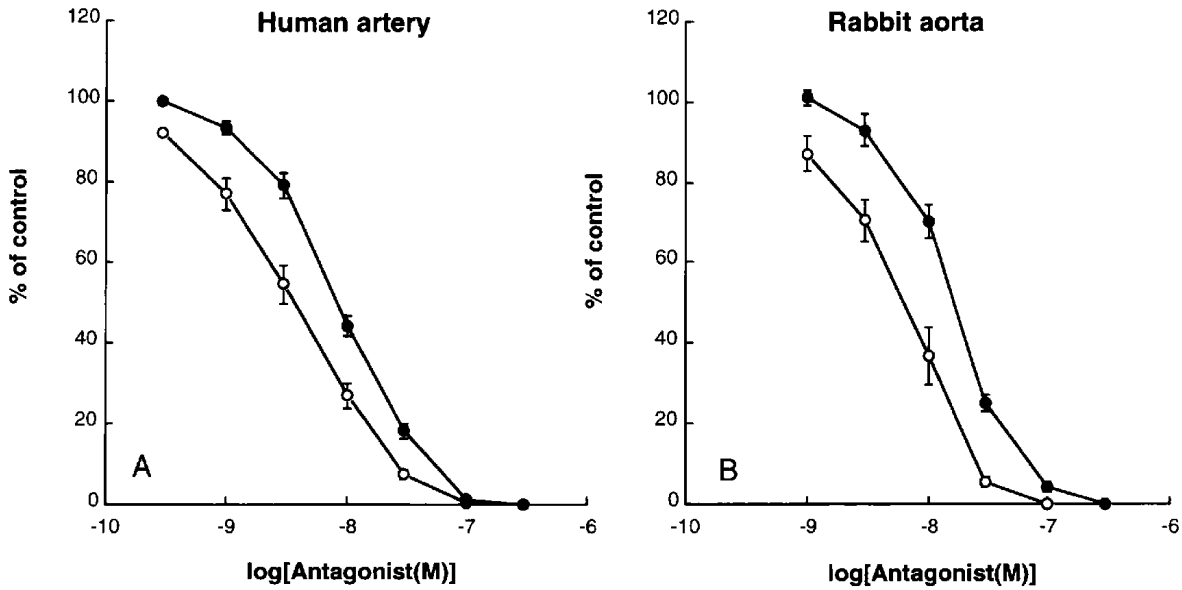

Fig. 6. Effects of E4177 and losartan on the contractile responses evoked by 2×10^{-8} M Ang II in human gastroepiploic arteries (A) and isolated rabbit aortic strips (B). Each point represents the mean±S.E.M. (n=11 for human artery, n=5 for rabbit aorta). E4177 (○), losartan (●).

Antagonism to Ang II-induced contractile responses

E4177 competitively antagonized the contractions induced by Ang II in isolated rabbit aortae. With increasing concentrations of E4177 ($3\times10^{-9}-3\times10^{-8}$ M), the concentration-contractile response curve to Ang II was characterized by a parallel rightward shift (Fig. 5). Schild analysis of the data gave a pA_2 value of 8.8±0.1 and a slope of 1.1±0.1, indicating that the antagonism shown by E4177 was competitive. The antagonism produced by losartan ($10^{-8}-3\times10^{-8}$ M) was also characterized by a rightward shift of the Ang II concentration-contractile response curve, and the pA_2 value and slope were estimated as 8.3±0.1 and 1.2±0.1, respectively. Maximal contractile responses were well maintained even at the highest concentrations of E4177 and losartan. This indicates the effects of these compounds were completely reversible.

In both human gastroepiploic artery and rabbit aorta, E4177 inhibited the contractile responses to 20 nM Ang II in a dose-dependent manner, with IC_{50} values of $(4.3\pm0.7)\times10^{-9}$ M (n=13) for human artery and $(5.3\pm1.8)\times10^{-9}$ M (n=5) for rabbit aorta. On the other hand, IC_{50} values obtained with losartan were $(7.8\pm0.9)\times10^{-9}$ M (n=11) in human artery and $(1.6\pm0.2)\times10^{-8}$ M (n=5) in rabbit aorta (Fig. 6, A and B).

Specificity of the antagonism

E4177 (10^{-6} M) did not significantly affect the concentration-response curve to norepinephrine ($10^{-7}-3\times10^{-5}$ M) or serotonin ($3\times10^{-8}-10^{-5}$ M) (data not shown). The concentration of E4177 employed was more than sufficient to antagonize Ang II-induced vascular contraction in human gastroepiploic artery. A range of concentrations of E4177 ($10^{-9}-10^{-4}$ M) did not alter the contractile response to 30 mM KCl (data not shown). Relaxing responses to histamine in human gastroepiploic artery were not affected by $10^{-7}-10^{-6}$ M of E4177 (data not shown). In the absence of Ang II, E4177 did not affect the basal resting tension of these tissues at any of the concentrations examined.

DISCUSSION

In the present study, we characterized the in vitro pharmacological properties of a chemically novel Ang II receptor antagonist, E4177.

AT_1-receptors predominate in rat adrenal cortex, whereas AT_2-receptors are predominant in the adrenal medulla (12, 18). E4177 inhibited ^{125}I-[Sar^1,Ile^8]Ang II binding to the cortex in a biphasic manner, and even at the highest concentration of E4177, about 20% of the binding still persisted. In the medulla, E4177 had little effect on the radioligand binding. These data indicate that E4177 binds selectively to AT_1-receptors. Consistent with this, E4177 blocked ^{125}I-[Sar^1,Ile^8]Ang II binding in a monophasic manner to rat liver which contains only AT_1-receptors (12, 18). Our data on the effects of losartan on ^{125}I-[Sar^1,Ile^8]Ang II binding in the adrenal glands and liver agreed well with previous observations (14, 19). In the competitive binding experiments, E4177 was as potent as losartan, as indicated by the similarity in their IC_{50}

values.

E4177, as well as losartan, exhibited a reversible and competitive antagonism, as shown by the parallel shift of the concentration-response curve produced in isolated rabbit aortae without affecting the maximal responses.

The resting tension was not affected by E4177 at any of the concentrations tested in either human gastroepiploic artery or rabbit aortae. These findings indicate that E4177 has no agonistic effect.

E4177 is specific to Ang II receptors; it did not affect the contractile responses evoked by norepinephrine, serotonin, or KCl. Furthermore, E4177 had no effect on the relaxing responses to histamine in human arteries precontracted with KCl. ACE activity was not affected by E4177 at a concentration as high as 10^{-5} M, which was sufficient to fully antagonize the contractions induced by Ang II.

In the experiment using isolated vessels, since E4177 was found to be more potent than losartan in antagonizing Ang II-induced contraction, it may be expected that the blood pressure-lowering effect of E4177 is also more potent than that of losartan. This possibility is supported by our in vivo study, which also indicates the oral bioavailability of E4177 (K. Ishii et al., to be published elsewhere).

In conclusion, the present data obtained from in vitro binding studies and isolated vascular contraction assay demonstrate that E4177 is a competitive and reversible AT_1-specific antagonist without agonistic activity. This newly developed agent has an in vitro potency comparable to or slightly higher than the known AT_1-antagonist losartan. These properties suggest that E4177 would be useful for exploring the as yet undefined role of Ang II in both normal and disease states as well as being useful for treating cardiovascular diseases in which Ang II is pathogenically involved.

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