In Vitro Pharmacological Properties of KRH-594, a Novel Angiotensin II Type 1 Receptor Antagonist

Koichi TAMURA,*a Masayasu OKUHIRA,* Imao MIKOSHIAb and Ken HASHIMOTO*  
Institute of Medical Research, Wakanaga Pharmaceutical Co., Ltd., 1624 Shinokochi, Koda-cho, Takata-gun, Hiroshima 739-11, Japan and R&D, Kissei Pharmaceutical Co., Ltd., 4365-1 Kasubara, Hotaka, Minamiaso, Nagano 399-83, Japan. Received February 5, 1997; accepted May 20, 1997

This report describes the in vitro pharmacological properties of dipotassium (Z)-2-[5-ethyl-3-[2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl-1,3,4-thiadiazolin-2-ylidine]aminocarbonyl]-1-cyclopentene carboxylate, called KRH-594, a novel angiotensin II (AII) type 1 (AT1) receptor antagonist. We exposed rabbit aortic rings to KRH-594 (0.1 nM) for increasing contact times and observed an increasing degree of insurmountable suppression of AII-induced contractions. KRH-594 (0.01, 0.1 and 1.0 nM) caused a concentration-related, insurmountable suppression of the AII concentration-response curve. Repeated washing of rabbit aortic rings preincubated with KRH-594 (0.1, 1.0 and 10 nM) slowly reversed the insurmountable suppression. The marked suppression of AII-induced contractions by KRH-594 (0.1 nM) was restored by co-incubation with losartan (100 nM). KRH-594 (10 μM) had no effect on bradykinin-, acetylcholine-, or histamine-induced contractions of guinea pig ileum, demonstrating its high specificity for AT1 receptors. These results demonstrate that KRH-594 is a potent, specific and insurmountable AT1 receptor antagonist. KRH-594 activity in rabbit aorta appears to be that of a slowly reversible (pseudo-irreversible) antagonist.

Key words: KRH-594; angiotensin II receptor; insurmountable antagonism

Recently, potent nonpeptide angiotensin II (AII) receptor antagonists have been synthesized and used in the treatment of hypertension and cardiovascular disease. Studies of this new class of compounds, exemplified by losartan and its analogues, led to the discrimination of two AII receptor subtypes designated AT1 and AT2. AT1 receptors have a high affinity for losartan and its active metabolite, EXP3174, whereas AT2 receptors have high affinity for compounds such as PD-123177 and the peptide CGP-42112A.1-3

Inhibition by AT1 receptor antagonists of the contractile response to the AII of blood vessels prepared from many organs has been reported. Some of them produced nonparallel shifts to the right of the AII concentration-contraction response curves and reduced the maximal response to AII, indicating noncompetitive AII antagonism, whereas other antagonists produced parallel shifts to the right without depression of the maximal response. These apparently noncompetitive and competitive antagonism effects have been described as insurmountable and surmountable antagonism,4,5 respectively.

In this paper, the pharmacological profile of dipotassium (Z)-2-[5-ethyl-3-[2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl-1,3,4-thiadiazolin-2-ylidine]aminocarbonyl]-1-cyclopentene carboxylate (Fig. 1), called KRH-594, was investigated and compared with that of the well-characterized surmountable AT1 receptor antagonist losartan, in vitro.

MATERIALS AND METHODS

Functional Studies in Rabbit Aorta The thoracic aortas were removed from male New Zealand white rabbits (Kitayama Rabes, Japan) weighing 2-3 kg. The vascular endothelium was removed by gently rubbing the intimal surface of the blood vessel with a piece of wire. The aorta was cut into 5 mm rings, and the rings were mounted in 20-ml organ baths containing oxygenated (95% O2, 5% CO2) Krebs bicarbonate solution at 37 °C. The composition of the Krebs solution was as follows (in mM): NaCl, 118; KCl, 4.7; MgSO4, 1.2; NaHCO3, 25; KH2PO4, 1.2; glucose, 10; CaCl2, 2.5, pH 7.4. Under 2.0 g resting tension, isometric tension changes were recorded with a force displacement transducer (TB-611T, Nihon Kohden, Tokyo, Japan) connected to an amplifier (AP-620G, Nihon Kohden, Tokyo, Japan) and analyzed with a computer (NEC, PC-9801, Tokyo, Japan). After 120 min, 50 mM KCl was added to stimulate the preparations, after which the preparations were rinsed with fresh bathing fluid at 15-min intervals for 60 min.

After the resting tension stabilized, an initial cumulative concentration-contraction response curve to AII (0.1-100 nM) was obtained. The tissues were then washed several times and allowed to relax to the baseline tension. Then, a second AII response curve was obtained and used as a 'pretest curve'.

The first series of experiments was performed to study the AII response of rings equilibrated with an antagonist. Each ring was exposed for 1, 2, 3, and 4 h to KRH-594 (0.1 nM) or for 15 min and 1 h to losartan (100 nM). Simultaneously, another ring was exposed to the vehicle (distilled water) alone, which served as a time-matched control. After exposure to the antagonist, a final

![Chemical Structure of KRH-594](image)

© 1997 Pharmaceutical Society of Japan
cumulative AII concentration–response curve was constructed, which was called the ‘test curve’. Results were expressed as a percentage of the maximum AII response obtained with the pretest curve.

The second series of experiments was performed to study the concentration–related response. Each ring was incubated for 3 h with KRH-594 (0.01, 0.1 and 1 μM) or for 1 h with losartan (10, 100 and 1000 μM) to allow it to reach equilibrium before a third concentration–response curve to AII was constructed. Time-matched control rings were not exposed to an antagonist.

The third series of experiments was performed to study the interaction between KRH-594 and losartan. Four separate rings were prepared, two of which were exposed to 0.1 μM KRH-594 for 3 h. The other two were not exposed to KRH-594 and served as a time-matched control. One of the rings being treated with KRH-594 and one of control rings were co-incubated with 100 μM losartan for the final 1 h of the 3 h incubation period. Test AII response curves were then obtained.

In the fourth series of experiments, each ring was exposed to KRH-594 (0.1, 1.0 or 10 μM) or the vehicle for 30 min, then test AII response curves were constructed. All the rings were washed with fresh bathing fluid for 3 h; the fluid was changed at intervals of 15 min. Then, test AII response curves were obtained again.

**Specificity Study in Guinea Pig Ileum** Guinea pig ileum has been used to determine the specificity of AII antagonists because it is established that this tissue is contractile to various agonists. Segments of ileum were removed from male Hartley strain guinea pigs (400—600 g, Japan SLN, Inc., Hamamatsu, Japan). Longitudinal muscle strips, 15 mm in length, were dissected and mounted in 20 ml tissue baths containing oxygenated (95% O2, 5% CO2) Tyrodes solution (in mm): NaCl, 137.9; KCl, 2.7; MgCl2·6H2O, 0.5; NaH2PO4· 2H2O, 1.1; CaCl2·2H2O, 1.8; NaHCO3, 11.9; and glucose, 5.6, kept at 37°C. The resting tension of each preparation was adjusted to 0.5 g. The strips were allowed to equilibrate and were washed every 15 min. After a 60-min stabilization period, 80 mM KCl was added to stimulate the preparations, after which the preparations were rinsed with fresh medium at 5-min intervals for 20 min. This procedure was repeated 2–3 times. After the resting tension had stabilized, the response to angiotensin I (AI, 11 nm), AII (4.7 nm), bradykinin (BK, 42 nm), histamine (His, 250 nm) or acetylcholine (Ach, 69 nm) was recorded before and after a 30-min incubation with KRH-594. The concentrations in parentheses are the 50% effective concentrations (EC50) for AI, AII, His and Ach, and the EC50 value for BK, all of which were estimated in pilot studies. Each strip was utilized for one agonist each.

**Drugs** KRH-594 and losartan were synthesized at the Institute for Medical Research, Wako Pharmaceuticals Co., Ltd. (Hirosima, Japan). Angiotensin I acetate salt, angiotensin II acetate salt and bradykinin acetate salt were purchased from Sigma (St. Louis, MO, U.S.A.). Acetylcholine was purchased from Daiichi Seiyaku Co., Ltd. (Tokyo, Japan). Histamine dihydrochloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**RESULTS**

**Effect of Equilibration of Antagonism of KRH-594 and Losartan on AII-Induced Contraction in Rabbit Aortic Rings** The cumulative addition of AII (0.1—300 μM) caused concentration-related contractions of rabbit isolated aortic rings. When the contact time with KRH-594 (0.1 μM) was increased, a greater degree of antagonism to AII was observed. The AII response curves of rabbit aorta rings incubated with KRH-594 (0.1 μM) for 3 h and 4 h were almost the same. Thus, KRH-594 seemed to have reached equilibrium after approximately 3 h of incubation (Fig. 2A). The concentration–response curve for AII-induced contractions for tissues incubated with losartan (100 μM) for 15 min and 1 h did not differ (Fig. 2B).

Losartan seemed to reach equilibrium with AT1 receptors more rapidly than KRH-594.

**Antagonism of KRH-594 and Losartan** KRH-594 (0.01, 0.1 and 1 μM) caused a concentration–related

---

**Fig. 2.** Effects of Equilibration of KRH-594 (0.1 μM) (A) and Losartan (100 μM) (B) on AII-Induced Concentration–Response Curves in Isolated Rabbit Thoracic Aorta

Preparations were incubated for 1, 2, 3 and 4 h with KRH-594 or for 15 min and 60 min with losartan. Contraction is expressed as percentage of the maximum contractile force obtained during the pretest curve for AII. Each point is the mean ± S.E. of 4—8 animals.
slightly rightward displacement of the concentration-response curve to AII and a decreased maximal response to AII (Fig. 3A). Using the method of Kenakin, the pKₐ of KRH-594 was derived from four separate experiments in which KRH-594 (0.1 nm) was incubated with aortic tissue for 3 h; a pKₐ of 10.4 ± 0.3 was calculated. For comparison, Fig. 3B shows the effect of the surmountable, competitive angiotensin receptor antagonist, losartan (10, 100 and 1000 nm) on AII-induced contractions in the rabbit aorta; losartan did not affect the maximum response to AII. The pA₂ of losartan was calculated to be 8.6 ± 0.2 by Schild analysis. At a concentration of 10 μM, neither compound displayed agonist activity in this assay.

The Effect of Losartan on KRH-594-Induced Antagonism to AII As described above, in aortic rings incubated with KRH-594 (0.1 nm) for 3 h, the AII concentration-response curve was noticeably suppressed compared with time-matched controls. In preparations which were co-incubated with losartan (100 nm) for the final 1 h of the KRH-594 incubation period (3 h), the subsequent AII concentration-response curves were displaced upward and to the right with losartan, as compared with that treated with KRH-594 alone (Fig. 4).

The Effect of Washing on Antagonism After a 30 min incubation period, KRH-594 (0.1, 1.0 or 10 nm) produced a concentration-related, insurmountable suppression of the contractile response to AII (Fig. 5A). KRH-594 (1 nm) caused approximately 80% suppression of the maximum response to AII. After this treatment, each tissue was washed with bathing fluid for another 3 h, during which time the fluid was changed at intervals of 15 min. After washing, the suppression of the maximum response to AII caused by KRH-594 (1 nm) was reduced to approximately 56% (Fig. 5B).

Specificity in Guinea Pig Ileum KRH-594 effectively inhibited the contractile responses to A1 and AII in a dose-dependent manner with IC₅₀ values of 0.31 ± 0.028 nm and 0.34 ± 0.11 nm, respectively, but KRH-594 did not alter the contractile responses to BK, Ach or His, even at a high concentration of 10 μM (Fig. 6).

DISCUSSION

In this paper we report the in vitro pharmacological properties of KRH-594, a novel compound recently shown to be a potent AT₁ antagonist in a previous study. In rabbit aorta, KRH-594 caused insurmountable antagonism of the concentration-response curve to AII. Several possible mechanisms could account for the insurmountable antagonism of AII by KRH-594: (1) non-specific antagonism, (2) subpopulation of receptors, (3) internalization, (4) interaction of tissue compartments, (5) an irreversible antagonism, (6) pseudo-irreversible interactions, (7) allosteric modulation of receptors.

![Figure 3. Effects of KRH-594 (A) and Losartan (B) on the AII Concentration-Contraction Curve in Isolated Rabbit Thoracic Aortic Rings](image1)

Preparations were incubated before the addition of AII, with losartan for 1 h, and with KRH-594 for 3 h, respectively. A contraction is expressed as a percentage of the maximum contractile force obtained during the pretest curve for AII. Each point is the mean ± S.E. of 4–5 separate experiments.

![Figure 4. The Contractile Effect of AII in Isolated Rabbit Aorta, in the Presence of KRH-594 (0.1 nm, 3 h Incubation) or Losartan (100 nm, 1 h Incubation) Alone or KRH-594 Co-incubated with Losartan for the Final 60 min of the 3 h Incubation Period](image2)

A contraction is expressed as a percentage of the maximum contractile force obtained during the pretest curve for AII. Each point is the mean ± S.E. of 5 animals.
We previously demonstrated that KRH-594 inhibited the binding of AII to AT₁ receptors in rat liver membranes, with a more than 25000 fold higher affinity than for AT₂ receptors in bovine cerebellar membranes. In this study, even at a relatively high concentration of 10 μM, KRH-594 did not affect the contractile response to Ach, His or BK in guinea pig ileum (Fig. 6). These findings indicate not only that KRH-594 is highly selective for AT₁ receptors, but also that KRH-594 does not interfere with the intracellular signal-transduction process that mediates the contractions caused by agents other than AII.

Recently, Weinert et al. proposed the existence of an AT₁ receptor subpopulation sensitive to insurmountable antagonists such as CI-996, EXP-3174, L-158809 and SKF-108834 because the reduction in the maximal AII contractile response was restricted to about 20% of the control AII contraction at concentrations of antagonist as high as 10 μM. However, the almost complete inhibition of the AII-induced contraction by pretreatment with KRH-594 (10 nM, 91.7% inhibition, Fig. 5) was observed here, and 97% inhibition by CV-11974 (1 nM) has been reported. Therefore, the existence of a receptor subpopulation may not account for the insurmountable antagonism exhibited by KRH-594 and CV-11974.

It has been proposed that the insurmountable antagonism exhibited by certain peptide antagonists of AII in rabbit aorta is related to the peptide-dependent internalization of AII receptors which results in a decreasing receptor concentration and causes tachyphylaxis. A recently published report showed that the functional response to AII in rabbit aorta after complete tachyphylaxis is restored by the addition of losartan or the peptide antagonist saralasin, so it argues against the assumption of receptor internalization. It is difficult to imagine how the internalization model could account for the finding that losartan can restore pre-established tachyphylaxis once receptor internalization has occurred. The problem here is that the insurmountable antagonism of KRH-594 was reversed by co-incubation with losartan (Fig. 4). It is unlikely that the insurmountable antagonism of KRH-594 is caused by receptor internalization, and losartan can reverse the internalization.

The insurmountable antagonism may be thought to be produced by an irreversible antagonism. In rabbit aorta, the suppression of the AII-induced contractile response by a 30-min exposure to KRH-594 was partially removed when the rabbit aortic rings were repeatedly washed with bathing fluid for 3 h (Fig. 5). This result indicates that KRH-594 can slowly dissociate from AII receptors and that the functional response to AII might be restored following more extensive washing. In addition, the degree of suppression of the maximal AII response induced by KRH-594 was reduced by co-incubation with losartan, which reached equilibrium with the AT₁ receptors more rapidly than KRH-594, as shown in Fig. 4. If KRH-594 had bound covalently to the AT₁ receptors, co-incubation
with a competitive antagonist would not have been expected to have any effect on the degree of suppression. These results show that KRH-594 dissociates slowly from the AT₁ receptors and that the vacated receptors are then occupied by losartan, which associates more quickly with AT₁ receptors than KRH-594 does. Subsequently administered AII could then access the receptors occupied by losartan. These results suggest that KRH-594 is a slowly reversible (pseudo-irreversible) antagonist.

Pseudo-irreversible antagonism is thought to be produced by a slow dissociation of the antagonist-receptor complex. Taylor et al. demonstrated slow dissociation accompanied by insurmountable antagonism for ligands of the serotonin receptors. Pseudo-irreversible interactions of the insurmountable antagonists, DuP532, GR117289 and SC-54629 with AT₁ binding sites have also been suggested. The effect of KRH-594 was reversed by washing or co-incubation with losartan, as described above. These results indicate that KRH-594 may slowly dissociate from the AT₁ receptors. KRH-594 may be a pseudo-irreversible antagonist.

Panek et al. suggested that insurmountable antagonism may in part be due to a slow removal of the antagonist from tissue compartments, cells or the matrix surrounding the AT₁ receptor which may be influenced by such factors as compound lipophilicity and the kinetics of distribution and metabolism. This, in effect, could provide the compartments for the sequestration of AT₁ antagonists, thereby preventing their rapid removal. Robertson et al. reported that GR117289 is a highly lipophilic agent (log P = 7.5) and that the profile of activity of GR117289 is attributable, at least in part, to its retention or perhaps even its concentration within the lipid membrane. In our preliminary experiment, the lipophilicity of KRH-594 was much lower than that of losartan (data not shown). Therefore, it is likely that the insurmountable antagonism of KRH-594 is directly due to its interaction with AT₁ receptors.

The pseudo-irreversible antagonism of KRH-594 could account for the reversibility of its insurmountable antagonism by losartan as described above. However the reversibility of AII-induced complete tachyphylaxis by losartan could not be explained by the pseudo-irreversible agonism of AII, because the pseudo-irreversible binding of AII to its receptor has been never been reported. There is another simple explanation for this phenomenon. Recently, Robertson et al. proposed that the properties of AT₁ receptors may be explained by the simple two-state receptor model of Gero. This model, which assumes that a receptor exists in two interchangeable states (the active (R) and the inactive (R') states), appears to be the simplest of the models proposed until now. Any ligand possesses two different affinities governing the occupancy of R and R'. The basal ratio of R/R' will depend on the characteristics of the ligand. It appears that losartan has an ability to reverse the AII receptors to R state, which allows subsequently administered AII to fully activate the contraction. Our results obtained in the functional experiments with KRH-594 may be explained by postulating that KRH-594 has a higher affinity for R' than for R. The addition of the antagonist would then result in a new equilibrium between R and R', in which fewer R are available to bind the agonist, thus explaining the decrease in the maximal response to AII. Since losartan could have a higher affinity for R than for R' and compete with KRH-594 in the binding site, losartan is capable of preventing or reversing the insurmountable antagonism. These two states of AT₁ receptors have been poorly characterized since selective ligands discriminating between them are not available. Further experimental support for the relevance of this model may come from the identification of the two receptor states.

In conclusion, the in vitro pharmacological properties of a novel non-peptide AT₁ receptor antagonist, KRH-594, have been characterized and are shown to differ from those of the well known AT₁ receptor antagonist, losartan. These data suggest that KRH-594 is a slowly reversible (pseudo-irreversible) antagonist and they clarify AT₁ receptors.

Acknowledgments The authors thank Dr. Susumu Ohno (Beckmann Research Institute, City of Hope, CA, U.S.A.) and Dr. Hidetada Komatsu (Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan) for critical review of the manuscript. We also thank Terukage Hirata (Institute for Medical Research, Wakunaga Pharmaceutical Co., Ltd.) for the synthesis of KRH-594 and losartan.

REFERENCES