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# Characterization of angiotensin II antagonism displayed by KR-31081, a novel nonpeptide AT1 receptor antagonist

**Sunghou Lee**[1*]

[1]**Department of Biomedical Technology, Sangmyung University**

# 안지오텐신 수용체 길항제 KR-31081의 특성에 관한 연구

이승호[1*]

[1]상명대학교 공과대학 의생명공학과

**Abstract** The pharmacological profile of KR-31081, a nonpeptide $AT_1$ selective angiotensin receptor antagonist, was investigated by receptor binding studies, functional *in vitro* assays with rabbit aorta. KR-31081 inhibited the specific binding of [$^{125}$I] [Sar$^1$, Ile$^8$]-angiotensin II to human recombinant $AT_1$ receptor with an 8.6-fold greater potency than losartan ($IC_{50}$: 1.43 and 12.3 nM, respectively), but it did not inhibit the binding of [$^{125}$I] CGP 42112A to human recombinant $AT_2$ receptor ($IC_{50}$: higher than 10 $\mu$ M for both). The Hill coefficient for the competition curve of KR-31081 against $AT_1$ receptor was not significantly different from unity (0.99). Scatchard analysis showed that KR-31081 interacted with human recombinant $AT_1$ receptor in a competitive manner, as with losartan. In functional studies with rabbit aorta, KR-31081 competitively inhibited the contractile response to angiotensin II ($pK_B$ values: 8.66) with 20-70% decrease in the maximum contractile responses, unlike losartan that showed competitive antagonism without any change in the maximum contractile responses to angiotensin II ($pA_2$ values: 7.59). These results suggest that KR-31081 is a highly potent $AT_1$ selective angiotensin II receptor antagonist with a mode of insurmountable antagonism to be developed as the exploratory potential of this compound.

**요 약** 비펩타이드성 안지오텐신 수용체 길항제로 새롭게 개발된 KR-31081은 재조합 수용체 결합실험에서 기존 의약인 로사탄에 비하여 8.6배 이상의 월등한 효과를 나타내었으며, 기능성 혈관실험에서도 대조물질인 로자탄보다 혈관수축 억제효과가 10배 이상 탁월하였다. 이러한 KR-31081의 특징들은 제 1형의 안지오텐신 수용체에 특이적으로 나타났으며 제 2형의 안지오텐신 수용체에 대한 수용체 결합친화력이 발견되지 않았다. 기능성 혈관실험에서는 KR-31081이 안지오텐신에 의한 혈관수축 효과를 경쟁적으로 저하시켰지만 표준물질인 로자탄과는 달리 농도가 증가함에 따라 혈관자체의 최고 수축효과의 감소가 관찰되었다. 안지오텐신 수용체에 선택적으로 작용하는 것으로 나타난 KR-31081은 고혈압 및 혈관질환에 대한 연구 및 진단에 활용될 수 있을 것이라고 판단된다.

**Key Words :** KR-31081, Antihypertension, Angiotensin, AT1 receptor antagonist, receptor ligands, diagnostics

## 1. Introduction

The pivotal role of the renin-angiotensin system in the regulation of blood pressure has been well established [1]. Many attempts have been made to reduce the contribution of angiotensin II (AII) to the development of high blood pressure by inhibiting the synthesis of A II by renin or angiotensin-converting enzyme (ACE). Most recently, Aliskiren, the direct renin inhibitor, showed that it provided effective blood pressure lowering with a good safety and tolerability profile in alone or combination with other antihypertensive therapies [2]. On the other hand, several ACE inhibitors have proven to be clinically effective in the treatment of hypertension and congestive

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*Corresponding Author : Lee, Sunghou (sunghou.lee@gmail.com)
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heart failure with good oral activity and long duration of action [3-4]. However, some evidence suggested that their unwanted side effects such as dry cough and angioedema result from the lack of specificity of ACE for angiotensin I [5-6]. These problems prompted many studies to be conducted for the development of more useful drugs that block the action of AII directly and more completely without undesirable side effects of ACE inhibitors.

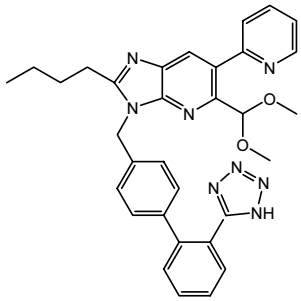
The discovery of losartan, a competitive $AT_1$ receptor antagonist [7-8] without partial agonistic activity [9], has opened a new era toward the development of new selective, orally active AII receptor antagonists as novel antihypertensive agents with the property of direct and more specific interruption of AII receptor itself. Currently losartan is on the market as the first AII receptor antagonist that was launched as a novel antihypertensive agent since proven to be orally active in animals [9-11] and humans [12-14]. Recently, the application of AII receptor antagonists has expanded to ongoing trials for noninvasive diagnostics as in positron emission tomography imaging [15-17], therefore upcoming $AT_1$ receptor antagonist candidates are meaningful developments as new diagnostic tools in future.

KR-31081 belongs to a novel class of nonpeptide AII receptor antagonists with a high affinity for $AT_1$ receptor (Fig. 1). In the present study, the pharmacological properties of KR-31081 as an AII receptor antagonist in comparison with losartan by examining its antagonistic effects on the binding of [$^{125}$I] [Sar$^1$, Ile$^8$]-AII and [$^{125}$I] CGP 42112A to human recombinant $AT_1$ and $AT_2$ receptor subtype, and on the AII-induced contraction of rabbit aortic segments were characterized.

## 2. Materials and Methods

### 2.1 Chemicals

KR-31081(2-butyl-5-dimethoxymethyl-6-(pyridyn-2-yl)-3-[[2'-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-3H-imidazo[4,5-b]pyridine, US patent #5691348), L-158809, PD-123177 and losartan [9] were synthesized at the Bio-Organic Science Division, KRICT.



[Fig. 1] Chemical structure of KR-31081

Sodium pentobarbital was purchased from Hanlim Pharm. Co. (Seoul, Korea) and ketamine hydrochloride from Yuhan Co. (Seoul, Korea). [Sar$^1$, Ile$^8$]-AII, AII acetate, arterenol bitartarate, vasopressin acetate, isoproterenol hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [$^{125}$I] [Sar$^1$, Ile$^8$]-AII and [$^{125}$I] CGP 42112A (2200 Ci/mmol) were obtained from PerkinElmer Life Sciences (Waltham, MA, USA.). KR-31081 and losartan were dissolved in dimethyl sulfoxide and further diluted in the buffer for AII binding assay and for isolated tissue experiments, and dissolved in 0.05 N KOH in saline for the intravenous administration in rats. All chemicals were prepared just before use.

### 2.2 Radioligand binding assay

For the competition and equilibrium binding studies on $AT_1$ and $AT_2$ receptors, human recombinant $AT_1$ and $AT_2$ receptor (PerkinElmer Life Sciences., Waltham, MA, USA) were used to exclude the possible interaction between drugs and other receptors that would exist already in various tissues. Binding assays were performed in 96-well plates by incubating aliquots of the human recombinant $AT_1$ and $AT_2$ receptor with 0.21 nM of [$^{125}$I] [Sar$^1$, Ile$^8$]-AII and 0.5 nM of [$^{125}$I] CGP 42112A, respectively. From the preliminary experiments with those concentrations of radioligands, the binding parameters were well fit to the control data provided by the receptor vendor (data not shown). Test compounds were dissolved at 2.5 mM in dimethylsulfoxide and serially diluted to 10 concentrations for the evaluation of activity in the total assay volume of 250 μl. The assay buffer contained 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA and 0.1% bovine serum albumin (pH 7.4). Specific [$^{125}$I] [Sar$^1$, Ile$^8$]-AII and [$^{125}$I] CGP 42112A binding were determined

experimentally from the difference between counts in the absence and the presence of unlabelled AII and [$Sar^1$, $Ile^8$]-AII at the concentration of 10 μM, respectively. After incubation at 37 °C for 60 minutes (or 180 minutes for $AT_2$ receptor), the incubation mixtures were filtered through GF/C glass-fiber filters (PerkinElmer Life Sciences., Waltham, MA, USA) which were presoaked in 0.3% polyethylenimine and rapidly washed nine times with 200 μl of ice cold 50 mM Tris buffer (pH 7.4) using the Inotech harvester (Inotech, Switzerland). The filters were covered with MeltiLex (melted on scintillator, PerkinElmer Life Sciences., Waltham, MA, USA), sealed in sample bag, followed by drying in the microwave oven, and counted by MicroBeta (PerkinElmer Life Sciences., Waltham, MA, USA). The assays were performed in three separate experiments run in quadruplicate.

The ability of antagonists to inhibit specific [$^{125}$I] [$Sar^1$, $Ile^8$]-AII and [$^{125}$I] CGP 42112A binding was estimated by $IC_{50}$ values, which are the molar concentrations of unlabeled drugs necessary to displace 50% of specific binding. The $K_i$ value was calculated from the equation $K_i = IC_{50}/(1+L/K_d)$, where L equals the concentration of [$^{125}$I] [$Sar^1$, $Ile^8$]-AII or [$^{125}$I] CGP 42112A [18]. The data from binding experiments were analyzed by the nonlinear regression method, using the PRISM computer program (GraphPad Software Inc, CA, USA).

### 2.3 *In vitro* antagonist potency in rabbit aorta

This study conformed to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institute of Health. The descending thoracic aorta was isolated from male New Zealand white rabbits (2-3 kg, Samyook Experimental Animal Co., Suwon, Korea). The endothelial layer of aorta was destroyed by gentle rubbing of the luminal surface with a cotton swab moistened with Krebs' solution. The aorta was cut into ring segments of 3-4 mm in width, and the vascular rings were mounted in 20 ml organ baths containing Krebs' bicarbonate buffer of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2 and glucose 11.0. The Krebs' buffer was kept at pH 7.4 by continuous bubbling with a gas mixture (95% $O_2$, 5% $CO_2$) at 37 °C. The isometric contraction was recorded with force displacement transducers (Grass FT03, Grass Ins., Quincy, MA, U.S.A.) and displayed on a chart recorder (Multicorder MC 6625, Hugo Sachs Electronic, March, Germany).

The rings were allowed to equilibrate for 90 min under a resting tension of 2 g. The first control cumulative concentration-contractile response curve for AII ($10^{-10}$ - $10^{-5}$ M) was determined to ensure stable reactivity to subsequently added AII. Then, the tissue was washed three times until baseline tension was recovered. After each ring was treated for 30 min with a single dose of KR-31081 ($10^{-9}$, 3 x $10^{-9}$, $10^{-8}$ M), losartan ($10^{-7}$, 3 x $10^{-7}$, $10^{-6}$ M) or vehicle (0.1% dimethyl sulfoxide), the second cumulative concentration-contractile response curve for AII was established. To exclude any influence of multiple dosing with KR-31081 and losartan on the concentration-contractile response curve, each tissue was incubated only with one concentration of the antagonist. Responses from rabbit aorta were expressed as percentage of the maximal AII response obtained from the first cumulative concentration-response curve. The $pA_2$ values were determined according to the Schild equation with $pK_B$ values being calculated from the equation of [antagonist]/(dose ratio-1).

To test the specificity of KR-31081 as an AII receptor antagonist, the concentration-contractile responses to norepinephrine, KCl, serotonin and histamine were also examined in the endothelium-removed rabbit aorta in the presence and the absence of KR-31081 at 1 μM. Responses in this study were expressed as percentage of the maximal response obtained from the first cumulative concentration-response curve.

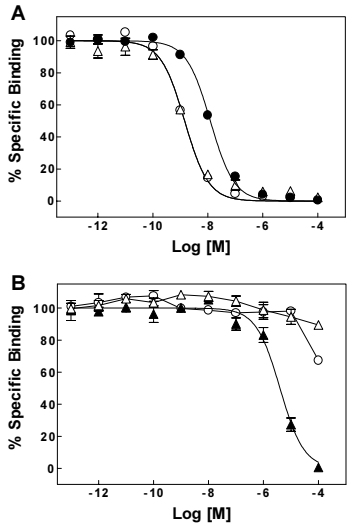
### 2.4 Statistical analysis

All values are expressed as mean ± S.E.M. Data were analyzed by Student's t-test or one-way analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparisons (Sigma Stat , Jandel Co., San Rafael, CA, U.S.A.). In all comparisons, the difference was considered to be statistically significant at $p < 0.05$.

## 3. Results

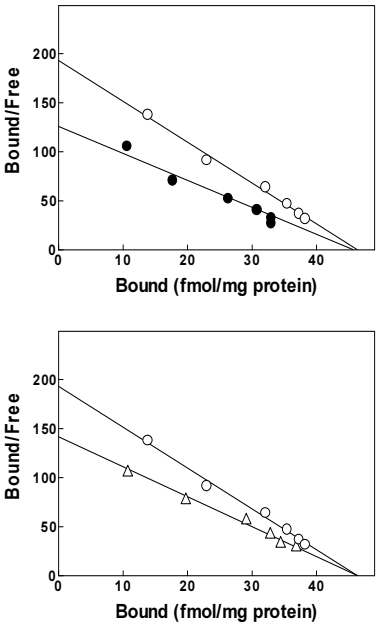
### 3.1 Radioligand binding assay

Against the human recombinant $AT_1$ receptor, [$^{125}$I] [Sar$^1$, Ile$^8$]-AII interacted with a single population of binding sites with the dissociation constant ($K_d$) of 0.24 ± 0.01 nM. The corresponding number of binding sites labeled by the radioligand was 46.3 ± 0.7 fmol/mg of protein. KR-31081, losartan, L-158809 and PD-123177 competed dose-dependently with 0.21 nM [$^{125}$I] [Sar$^1$, Ile$^8$]-AII against the binding sites of the human recombinant $AT_1$, where they appeared to exhibit monophasic inhibition curves (Fig. 2).




[Fig. 2] Inhibition of specific [$^{125}$I] [Sar$^1$, Ile$^8$]-AII binding to human recombinant $AT_1$ (A) and of specific [$^{125}$I] CGP 42112A binding to human recombinant $AT_2$ receptor (B) by KR-31081 (open circles), losartan (solid circles), L-158809 (open triangles) and PD-123177 (solid triangles), respectively. The dose-response curve for the inhibition of specific binding by these compounds was determined by incubating the radioligand with 10 concentrations of each compound in the medium of receptor source. The data points represent the mean ± S.E.M of three separate experiments run in quadruplicate.

KR-31081 displayed high specific affinity for the human recombinant $AT_1$ receptor ($IC_{50}$ = 1.43 ± 0.19 nM) without any binding affinity for the human recombinant $AT_2$ subtype against which PD-123177 showed moderate activity ($IC_{50}$ = 4.3 ± 1.4 μ M). KR-31081 was about 8.6 times more potent than losartan ($IC_{50}$ = 12.30 ± 1.42 nM) and equipotent to L-158809 ($IC_{50}$ = 1.44 ± 0.34 nM) in displacing labeled AII from the human recombinant $AT_1$ receptor. The Hill coefficients for the inhibition by KR-31081, losartan and L-158809 were 0.99, 0.86 and 0.99, respectively, which were not significantly different from unity. The results from saturation binding assay using [$^{125}$I] [Sar$^1$, Ile$^8$]-AII conducted in the presence of KR-31081 (1 nM) and losartan (10 nM) were depicted in Fig. 3.

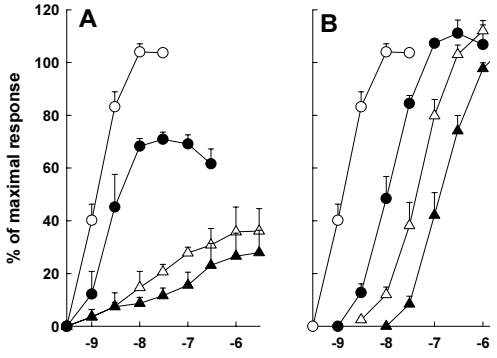



[Fig. 3] Scatchard transformations of saturation binding data for specific [$^{125}$I] [Sar$^1$, Ile$^8$]-AII binding to human recombinant $AT_1$ receptor in the absence (open circles) or presence of KR-31081 (1 nM, solid circles) and losartan (10 nM, open triangles). The data points represent the mean of three separate experiments run in quadruplicate.

The Scatchard transformations of [$^{125}$I] [Sar$^1$, Ile$^8$]-AII saturation curves revealed that these two antagonists did not affect the total number of binding sites labelled by [$^{125}$I] [Sar$^1$, Ile$^8$]-AII, but increased the dissociation constant of the radioligand by a factor of 1.38 ± 0.40 with KR-31081 and 1.37 ± 0.15 with losartan. Saturation binding assay also has shown their competitive interaction with the receptor.

### 3.2 *In vitro* antagonist potency in rabbit aorta

KR-31081 and losartan inhibited the AII-induced contractions of the rabbit aorta in a concentration-dependent manner (Fig. 4), but with dissimilar types of antagonism.

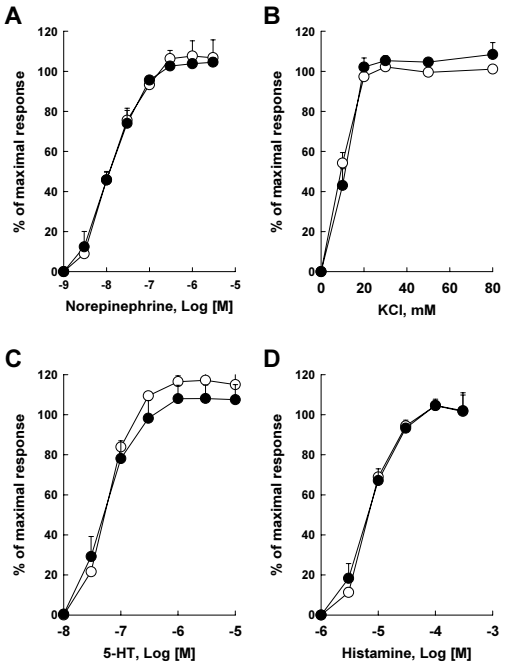



[Fig. 4] Effects of KR-31081 and losartan on the concentration-contractile response curve to AII in isolated rabbit aorta. A: KR-31081: Vehicle (open circles), $10^{-9}$ M (solid circles), 3 x $10^{-9}$ M (open triangles), $10^{-8}$ M (solid triangles). B: Losartan: Vehicle (open circles), $10^{-7}$ M (solid circles), 3 x $10^{-7}$ M (open triangles), $10^{-6}$ M (solid triangles). The data points represent the mean percentage of the maximal response ± S.E.M. (n=4-8).

KR-31081 ($10^{-9}$, 3 x $10^{-9}$ and $10^{-8}$ M) produced a rightward shift in the concentration-contractile response curve to AII with a significant reduction in the maximum contractile response by 29.3, 64.8 and 73.3% at each concentration, respectively (the calculated $pK_B$: 8.66) (Fig. 4A). In contrast, losartan ($10^{-7}$, 3 x $10^{-7}$ and $10^{-6}$ M) produced a parallel rightward shift in the concentration-response curve without any changes in the maximal contractile response ($pA_2$ value: 7.59; slope of the Schild plot: 1.34) (Fig. 4B). At the higher concentration (1 μM), KR-31081 did not change the concentration -response curve to norepinephrine, KCl, serotonin and histamine in rabbit aortic preparations (Fig. 5).

## 4. Discussion

The result from the present study with various experimental models have shown that KR-31081 containing pyridylimidazole moiety is a potent $AT_1$ selective antagonist *in vitro*.




[Fig. 5] Effects of vehicle (open circles) and KR-31081 ($10^{-6}$ M, solid circles) on the concentration-contractile response curve to norepinephrine (A), potassium chloride (B), serotonin (C) and histamine (D) in isolated rabbit aorta. The data points represent the mean percentage of the maximal response ± S.E.M. (n=4-5).

In radioligand binding studies, KR-31081 totally displaced specifically bound [$^{125}$I] [Sar$^1$, Ile$^8$]-AII from human recombinant angiotensin $AT_1$ receptor with 8.6 times greater potency than losartan, but without interaction with human recombinant angiotensin $AT_2$ receptor, from which PD 123177, a $AT_2$ selective antagonist, displaced specifically bound [$^{125}$I] CGP 42112A. The analysis of the competition curve showing characteristics of monophasic inhibition indicated binding of KR-31081 to a single class of $AT_1$ receptors with a Hill coefficient of 0.99. In the further studies with radioligand saturation experiments, KR-31081 caused an increase in dissociation constant of [$^{125}$I] [Sar$^1$, Ile$^8$]-AII without reduction in the maximum binding capacity ($B_{max}$)

of human recombinant $AT_1$ receptor. All these data provide strong evidence that KR-31081 competitively interacts with $AT_1$ receptors as for losartan.

Functional *in vitro* studies were performed to characterize the mode of interaction of KR-31081 with $AT_1$ and $AT_2$ receptors. Unlike losartan, that exerted a parallel rightward shift in the concentration-response curve without any changes in the maximal contractile response in rabbit aorta, KR-31081 caused a rightward shift in the concentration-response curve to AII with a reduction of maximal contractile response by 20 to 70%, suggesting an insurmountable antagonism of AII-induced contraction. This unusual pharmacological behavior exerted by KR-31081 has also been reported previously for other nonpeptide $AT_1$ antagonists such as BIBR 277, GR 138950 or EXP3174 [19, 20]. Several hypothetical mechanisms proposed to explain insurmountable antagonism exhibited by $AT_1$ receptor antagonists include the action on multiple receptors, a slow dissociation of the receptor-antagonist complex, and allosteric modification of receptors [7, 21]. At present, however, it remains unclear how an insurmountable antagonism can be displayed in isolated vessels by nonpeptide $AT_1$ receptor antagonists including KR-31081 that showed competitive antagonism in the binding study. The insurmountable antagonism may not be limited to a single mechanism and may be influenced by factors such as the agonist/antagonist used, tissues, species and experimental conditions as suggested by Bond et al. [22].

As in the binding assay, KR-31081 was shown to be significantly more potent than losartan in other experiments: over 50-fold in blocking the contractile effect of AII in rabbit aorta (on the basis of calculated $pK_B$). The selective and specific interaction of KR-31081 with AII receptors was further substantiated by the results from the functional experiments demonstrating no effects of KR-31081 on the contractile response to norepinephrine, KCl and 5-HT in the isolated rabbit aorta. Together with *in vivo* experimental results, KR-31081 can be an important ligand to study rennin-angiotensin-aldosterone system and for the development of diagnostic tools labeled by fluorescence or radioactive molecules.

In summary, the results from the present study with various binding and functional experiments demonstrate that KR-31081 is a highly potent selective nonpeptide $AT_1$ receptor antagonist with a mode of insurmountable antagonism. Thus, the further studies are needed to evaluate and develop the exploratory potential of this compound.

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**Sunghou Lee** [Regular member]

- Feb. 1990 : Fairleigh Dickinson Univ., Dept. of Biochem., MS
- May 2001 : Georgetown Univ., Dept. of Physiology, PhD
- July 1990 ~ Aug. 2004 : KRICT, Sr. Res. Scientist
- Sep. 2004 ~ current : Sangmyung Univ., Dept. of Biomed. Technology, Professor

<Research Interests>
High throughput screening, GPCR biochemistry