Shared determinants of receptor binding for subtype selective, and dual endothelin-angiotensin antagonists on the AT_1 angiotensin II receptor

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Abstract Site-directed interspecies amino acid exchange was used to compare the binding determinants of a novel dual endothelial-angiotensin receptor ligand, L-746,072, with type-1 angiotensin receptor (AT_1) selective antagonists on AT receptors expressed in COS cells. These studies suggest that residues on AT receptors which are non-conserved between amphibian and mammalian species play a greater role in subtype selective ligand recognition than for dual receptor ligands. These data also support the hypothesis that a common non-peptide binding site exists within transmembrane domains on peptidergic receptors.

Key words: Angiotensin; Endothelin; AT_1 receptor; Non-peptide; Biphenylimidazole; Dual receptor antagonist

1. Introduction

The renin-angiotensin system (RAS) plays a critical role in the control of blood pressure and water-electrolyte homeostasis. Elevated blood levels of the peptide hormone, angiotensin II (Ang II), is implicated in the pathophysiology of hypertension, congestive heart failure, coronary ischemia and renal insufficiency. Consequently, a major target site for drug intervention in treating many of these pathological conditions has focused on blockade of the RAS [1,2]. Endothelins are another class of potent endogenous vasoconstrictor and pressor agents which play a key role in regulation of vascular function and have also been implicated in the pathophysiology of cardiovascular disease [3,4]. Ang II and endothelin mediate their physiological effects by binding to receptors in the plasma membrane which belong to the peptide ligand subfamily of G protein-coupled receptors. Thus, the discovery of a dual Ang II and endothelin receptor antagonist, L-746,072 (Fig. 1), provides the intriguing possibility of developing a novel therapeutic agent for pathophysiological conditions involving Ang II and endothelin action [5].

The development of non-peptide ligands for peptide binding receptors has led to the interesting observation that structural determinants of peptide and non-peptide ligand recognition are distinct [6–9]. We have investigated the structural determinants of non-peptide ligand recognition on the Ang II receptor (AT receptor) by taking advantage of the pharmacological differences between mammalian and amphibian AT receptors. While receptors from both species recognize Ang II peptides with equivalent high affinities, amphibian AT receptors generally bind non-peptide ligands with significantly lower affinity than mammalian AT receptors. Thus, site-directed mutagenesis in which amino acids are exchanged between mammalian and amphibian receptors, permits analysis of the role non-conserved amino acids play in non-peptide ligand recognition in the AT receptor.

Interspecies amino acid exchange previously revealed 13 non-conserved residues in the transmembrane (TM) domains that were crucial to the formation of the biphenylimidazole, Losartan (Fig. 1), binding site on the rat type 1b AT (rAT_{1b}) receptor as evidenced by construction of a combinatorial amphibian mutant receptor, xCM46 (Fig. 2), which gained $>25\,000$-fold affinity for Losartan [10]. This variant frog receptor also gained significant affinity towards other biphenylimidazoles as well as members of the imidazoleacrylic acid class of AT_1 selective non-peptides [11]. These studies suggested that a common binding site for AT_1 selective non-peptides is conserved within the TM domains of the AT_1 receptor. In this study, we further explore this hypothesis by examining the binding determinants of a novel dual antagonist of the Ang II and endothelin receptor and compare these determinants with KR-31,016, a member of the biphenylimidazole class of AT_1 selective non-peptides (Fig. 1).

2. Materials and methods

2.1. Ligands

Ang II, [Sar[1],Ile[8]]Ang II and [Sar[1],Ala[8]]Ang II were purchased from Peninsula Laboratories (Belmont, CA). The non-peptide antagonists were kindly provided as follows: KR-31,016 (S.-E. Yoo, KRICT, Taejon, South Korea) and L-746,072 (T.F. Walsh, Merck, Sharp and Dohme, Rahway, NJ).

2.2. Transfections and cell culture [12]

The monkey kidney epithelial COS-7 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and 4 mM glutamine supplemented with 10% fetal calf serum, and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 and 95% air. Three days after plating in tissue culture flasks (2×10^6 cells/150 cm^2), the cells were transfected with 50 μg of plasmid DNA coding for rAT_{1b} [13], xAT_a [14], the combinatorial mutant, xCM46 [10] and single point mutants of the rAT_{1b} receptor [10,12] cloned into pCDNAI/AMP using the calcium phosphate method.

2.3. Membrane preparation [12]

Two days after transfection, the medium was replaced with 10 ml of

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Abbreviations: RAS, renin angiotensin system; Ang II, angiotensin II; AT receptor, angiotensin receptor; rAT_{1b}, rat (Sprague-Dawley) angiotensin receptor type 1b; xAT_a, frog (*Xenopus laevis*) angiotensin receptor type a; xCM46, xAT_a combinatorial mutant receptor defined in Table 1; COS-7, monkey kidney epithelial cells; TM, transmembrane

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ice-cold Versene. After 10–15 min incubation, the COS-7 cells were removed from the flask by trituration, pelleted by centrifugation at 4°C for 10 min at 1000×*g*, and washed twice in 30 ml of Hanks' balanced salt solution. The washed cells were resuspended in 3 ml of buffer A (10 mM Tris-HCl, pH 7.4; 5 mM EDTA) and disrupted by two cycles of freeze-thawing. The membranes were then washed twice with buffer B (50 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$) by pelleting at 4°C for 15 min at 10 000×*g*, resuspended in buffer B by homogenizing with a disposable pellet pestle, and analyzed for protein content using the Bradford protein reagent (Bio-Rad). The membranes were then frozen and stored at −70°C until they were used for receptor binding assays.

2.4. Binding assays [10]

Monoiodinated ^{125}I-[Sar^1,Ile^8]Ang II was obtained from Peptide Radioiodination Center (Pullman, WA). Cell membrane fractions were sonicated for 5 s with a Sonifer Cell Disrupter immediately before use in binding assays and incubated (20–40 μg/tube) for 1–2 h at room temperature with 100 000 cpm of radioligand and indicated concentrations of cold antagonists in 0.3 ml of buffer B containing 0.1% bovine serum albumin. Binding reactions were terminated by rapid filtration and bound radioligand was measured by gamma spectrometry. All determinations were performed in triplicate. Non-specific binding was defined as the binding of radioligand in the presence of 500 nM cold [Sar^1,Ile^8]Ang II and specific binding was defined as total radioligand bound minus non-specific binding. The IC_{50} values from the specific binding data were determined by computerized non-linear regression analysis using the 'Kaleidagraph' program.

3. Results and discussion

The AT_1 subtype selective non-peptide, KR-31,016 (Fig. 3), and the dual antagonist L-746,072 (Fig. 4), exhibited significantly greater affinity towards the rAT_{1b} than to the xAT_a receptor. These findings are consistent with previous reports demonstrating that AT_1 non-peptide ligands are in general markedly less potent at binding amphibian compared with mammalian AT receptors [11,15,16]. The dual Ang II-endothelin antagonist bound the rAT_{1b} receptor with one order of magnitude greater affinity compared to the xAT_a receptor. In comparison, KR-31,016 bound the rAT_{1b} with 95 000-fold greater affinity than the xAT_a receptor. This finding strongly suggests that residues on the AT receptor which are non-conserved between amphibian and mammalian species play a far greater role in subtype selective ligand recognition than for dual receptor ligands. This concept is further supported by the observation that a series of AT_1 selective non-peptides displayed greater differences in the affinities towards mammalian AT_1 receptors than to amphibian receptors [11,15–17] compared with non-peptides which bound AT_1 and AT_2 receptors with equivalent affinities [18,19].

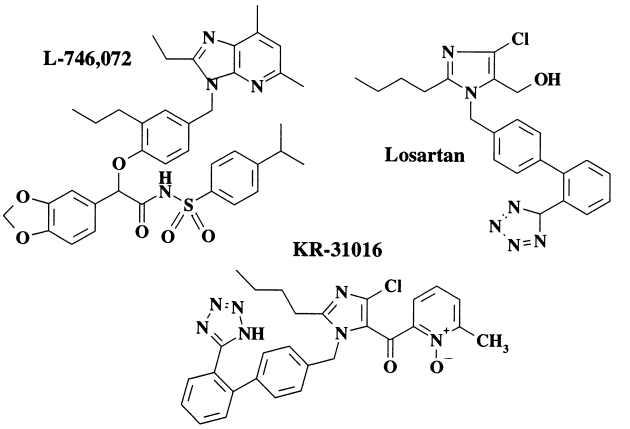

Fig. 1. Structure of angiotensin receptor non-peptide ligands.

The variant of the amphibian xAT receptor, xCM46, in which thirteen amino acids in TMII-VII were exchanged for the corresponding residues in the mammalian rAT_{1b} receptor, was previously shown to gain 25 000-fold affinity towards Losartan when compared to its affinity towards the xAT_a wild type receptor [10]. In this study, we found that the biphenylimidazole derivative, KR-31,016, gained nearly 100 000-fold affinity towards xCM46 compared to the xAT_a receptor (Fig. 3, Table 1). Thus, these results lend support to previous studies which suggested that the class of biphenylimidazoles share a common binding site on the AT_1 receptor [11]. In

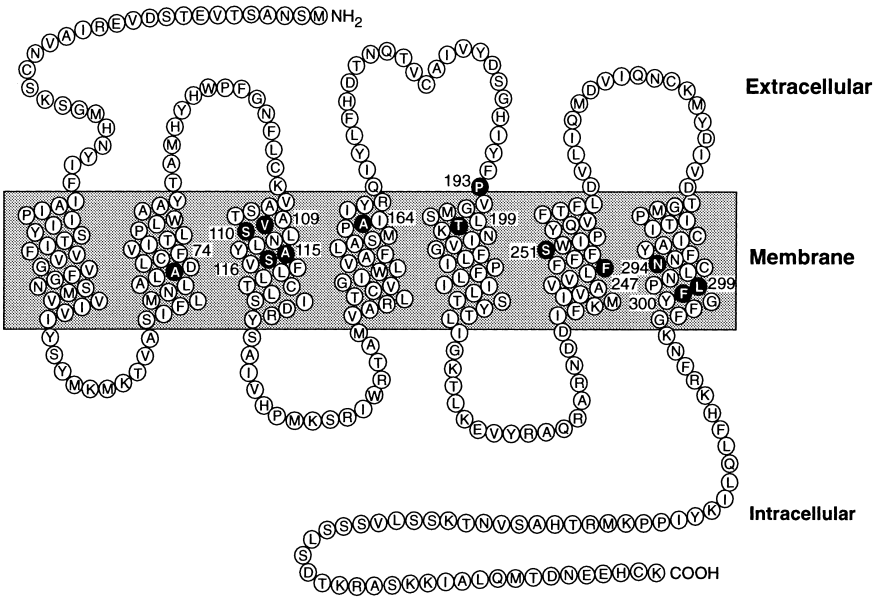

Fig. 2. Schematic of the xAT_a combinatorial mutant receptor which binds Losartan with affinities equivalent to the mammalian rAT_1 receptor. Residues exchanged for their mammalian counterparts are highlighted in black.

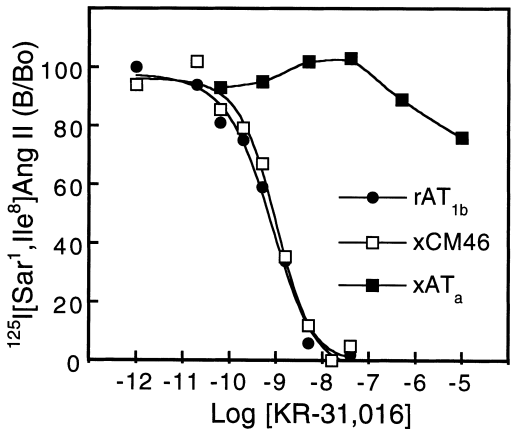

Fig. 3. Competition binding curves for the AT_1 selective antagonist, KR-31,016. The binding profiles of KR-31,016 are shown for the gain of function xAT_a mutant receptor (xCM46) and the wild type rAT_{1b} and xAT_a receptors. The standard error of each point was less than 10%. IC_{50} values for AT receptor ligands are listed in Table 1.

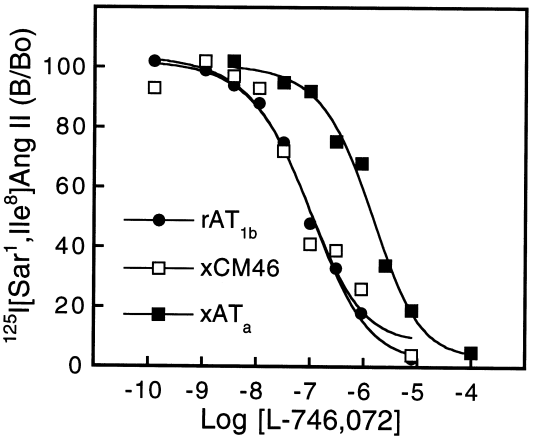

Fig. 4. Competition binding curves for the dual AT and ET receptor antagonist, L-746,072. The binding profiles for L-746,072 are shown, as described in Fig. 3.

order to determine whether residues which were influential in biphenylimidazole binding share common determinants with the dual Ang II-endothelin antagonist, L-746,072 was examined for its affinity towards the xCM46 variant receptor (Fig. 4, Table 1). The fact that L-746,072 exhibited affinities towards xCM46 which were nearly identical to the rAT_{1b} receptor suggests that the dual antagonist shares common determinants for ligand recognition with AT_1 selective non-peptides (Table 1).

Analysis of 13 single point mutants of the rAT_{1b} receptor in which mammalian residues were exchanged for amphibian amino acids revealed that V108I and S109T in transmembrane III (TMIII), S252C in TMVI, and N295S in TMVII, had significantly reduced affinities for L-726,072 and KR-31,016 as evidenced by F_{mut} values (= mutant IC_{50}/rAT_{1b} IC_{50}) greater than 5.0. These mutants were shown to exhibit reduced affinities for Losartan [10,12] and the balanced affinity AT_1/AT_2 receptor ligand, L-163,017 [19]. Previous studies also demonstrated that these single point mutants of the rAT_{1b} receptor exhibited Bmax values and binding affinities towards the peptide ligands, Ang II and [Sar^1,Ile^8]Ang II, which were not significantly different between species which suggests that these mutations did not cause major conformational changes in receptor structure [10,12]. These results suggest that the dual Ang II-endothelin antagonist, the AT_1 selective non-peptide antagonists, and the AT_1/AT_2 balanced affinity ligand share overlapping binding sites on the AT_1 receptor.

The most significant attenuation in KR-31,016 binding affinities among the 13 single point mutants was seen in the following rank order of F_{mut} values: S109T > N295S > V108I > A163S > S252C which is identical to the rank order of F_{mut} values for the other well studied biphenylimidazole, Losartan [10,12]. This was in contrast to the rank order of F_{mut} values for L-746,072 which was N295S > S252C > V108I = S109T > F248L = A114T. Thus, single point mutants revealed distinctions in the determinants of AT_1 specific and

Table 1
Binding affinities for angiotensin receptor non-peptide ligands

Receptor	KR-31,016		L-746,072	
Wild type	IC_{50} (nM)	F_{mut}	IC_{50} (nM)	F_{mut}
rAT_{1b}	0.52 ± 0.064	1.0	97 ± 10	1.0
xAT_a	> 50 μM		1400 ± 200	
Mutants				
xCM46	0.49 ± 0.05	0.94	103 ± 20	1.1
A73S	1.2 ± 0.3	2.3	85 ± 10	0.88
V108I	17 ± 2	33	892 ± 100	9.2
S109T	83 ± 14	160	860 ± 90	8.9
A114T	1.8 ± 0.4	3.5	580 ± 70	6.0
S115T	1.9 ± 0.3	3.6	86 ± 10	0.89
A163S	4.9 ± 0.7	9.0	190 ± 30	2.0
P192M	0.47 ± 0.05	0.91	100 ± 10	1.0
T198A	1.7 ± 0.2	3.0	82 ± 10	0.84
S252C	3.0 ± 0.6	5.8	1200 ± 2	12
F248L	0.50 ± 0.05	0.96	650 ± 100	6.7
N295S	22.5 ± 20	42	2000 ± 300	21
L300F	1.6 ± 0.3	3.1	360 ± 70	3.7
F301L	1.8 ± 0.3	3.5	420 ± 80	4.3

Data represent the mean of the IC_{50} values ± S.E. obtained from three independent experiments each performed in triplicate using ^{125}I-[Sar^1,Ile^8]Ang II as the radioligand; F_{mut} = mutant IC_{50}/rAT_{1b} IC_{50}.

dual receptor non-peptide ligand binding. Of all 13 single point mutants, S109T caused the greatest reduction in the binding affinity for KR-31,016 (Table 1) and Losartan [10], while having relatively smaller ranking effects on the binding affinities of L-746,072 (Table 1) and the balanced affinity AT_1/AT_2 non-peptide, L-163,017 [19]. Conversely, S252C caused the second largest reduction in affinities towards L-746,072 (Table 1) and L-163,017 [19] compared with the other 13 mutants while this mutant had a much lower F_{mut} ranking for KR-31,016 (Table 1) and Losartan [12]. Thus, our data suggest that distinct molecular differences exist between receptor subtype selective antagonist binding sites and dual receptor ligand binding sites on the AT_1 receptor.

In conclusion, these findings provide a focus for refining molecular models of non-peptide ligand docking in the Ang II and endothelin receptors. Elucidation of the structural determinants of dual receptor Ang II-endothelin ligand recognition may lead to improved therapeutics for the treatment of cardiovascular diseases involving both Ang II and endothelin action. Our results also lend further support to the hypothesis that a general non-peptide binding site exists within the transmembrane domain of peptide hormone receptors regardless of the nature of the ligand [6,20]. These findings in the AT_1 receptor may be applicable to other members of the peptide ligand receptor subfamily of G protein-coupled receptors. Investigations into the molecular mechanisms of ligand recognition ultimately may lead to improved therapeutics for the myriad of diseases involving this large class of receptors.

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