Identification of nonconserved amino acids in the AT_1 receptor which comprise a general binding site for biphenylimidazole antagonists

Vaneet Nirula[a], Wei Zheng[a], Kamakshi Krishnamurthi[b], Kathryn Sandberg[a,b,*]

[a]*Division of Nephrology and Hypertension, Department of Medicine, Georgetown University Medical Center, 4000 Reservoir Road, NW, Washington, DC 20007, USA*

[b]*Department of Physiology and Biophysics, Georgetown University Medical Center, 4000 Reservoir Road, NW, Washington, DC 20007, USA*

Received 9 August 1996

Abstract Mutational analysis based on pharmacological differences between mammalian and amphibian angiotensin II receptors (AT receptors) previously led to construction of a mutant receptor that gained >25000-fold affinity for the biphenylimidazole, Losartan. This variant frog receptor also bound with high affinity other nonpeptides in the biphenylimidazole chemical class according to the following rank order of potency (expressed in F_{mut} values = mutant IC_{50}/rAT_{1b} IC_{50}): Losartan, 0.91; L-162,389, 1.0; L-163,491, 1.9; L-158,809, 3.5; L-163,017, 3.9; SC-51,316, 3.9. In contrast, the imidazoleacrylic acids, SKF-108,566 (F_{mut} = 160) and SB-203,220 (F_{mut} = 170), bound with markedly less affinity. Thus, nonconserved residues determining the molecular requirements for biphenylimidazole recognition are conserved in general, but are not identical to nonconserved residues necessary for high affinity binding of imidazoleacrylic acids.

Key words: Angiotensin AT_1 receptor; Nonpeptide antagonist; Biphenylimidazole; Imidazoleacrylic acid; Site-directed mutagenesis

1. Introduction

The fact that the renin-angiotensin system (RAS) plays a critical role in the control of blood pressure and water and electrolyte homeostasis [1] has led to pharmaceutical intervention in the RAS as an effective treatment of hypertension and congestive heart failure [2]. The effector hormone in the RAS is the octapeptide angiotensin II (Ang II), which exerts its effects through type-1 (AT_1) and type-2 (AT_2) receptors located in the plasma membrane. These cloned peptide hormone receptors belong to the seven transmembrane (TM) domain superfamily of G-protein coupled receptors [3]. The AT_1 receptor mediates all of the classic effects of Ang II on cardiovascular and renal function whereas the function of the AT_2 receptor is not as yet well understood [2,4].

Discovery of the prototype CV-2947 compound, which is a specific nonpeptide antagonist of the AT_1 receptor and orally active [5], has led to the development of two series of nonpeptide receptor antagonists. The majority of these compounds are biphenylimidazole derivatives while a second series is derived from imidazoleacrylic acids (Fig. 1). Although different strategies were employed in the development of these compounds, both approaches were based on molecular modeling of Ang II. The biphenylimidazoles were developed from aligning the imidazole of CV-2947 with the imidazole of His^6 in Ang II [6] whereas alignment of the CV-2947 imidazole with the aromatic component of Pro^7 led to the imidazoleacrylic acid nonpeptides [7].

Compounds from both classes of nonpeptides are potent ligands of the mammalian AT_1 receptor but have little affinity for amphibian AT receptors [8–12]. We previously employed interspecies amino acid exchange to create single point and combinatorial mutations of the rat (rAT_{1b}) and frog (xAT_a) receptors [9,13]. These studies identified 13 nonconserved amino acids in TM domains that are crucial to the formation of a Losartan (biphenylimidazole) binding site on the rAT_{1b} receptor as evidenced by construction of a combinatorial amphibian mutant receptor, xCM46, which exhibited an affinity for Losartan that was identical to the wild type rAT_{1b} receptor [13]. In the present study, we examined whether the residues identified in the *gain-of-function* xCM46 mutant (Fig. 2), defined a general nonpeptide binding pocket for biphenylimidazoles and/or imidazoleacrylic acid nonpeptide ligands.

2. Materials and methods

2.1. Ligands

Angiotensin II, [Sar^1,Ile^8]angiotensin II and [Sar^1,Ala^8]angiotensin II were purchased from Peninsula Laboratories (Belmont, CA). The nonpeptide antagonists were kindly provided as follows: SK&F-108,566 and SB-203,220 (J. Weinstock, SmithKline Beecham Pharmaceuticals, King of Prussia, PA); L-163,017, L-162,313, L-163,491 and L-162,389 (W.J. Greenlee, Merck, Sharp&Dohme, Rahway, NJ), SC-51,316 (G.M. Olins, Searle&Co., St. Louis, MO) and Losartan (P.C. Wong, DuPont Pharmaceutical Co., Wilmington, DE).

2.2. Transfections and cell culture [9,13]

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} [14], xAT_a [15], and the combinatorial mutant, xCM46 [13] cloned into pCDNAI/AMP using the calcium phosphate method.

2.3. Membrane preparation [9,13]

Two days after transfection, the medium was replaced with 10 ml of 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 $1000\times g$, 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

*Corresponding author. Georgetown University Medical Center, Building D, Room 394, 4000 Reservoir Road, NW, Washington, DC, 20007, USA. Fax: (1) (202) 687-7278.
E-mail: sandberg@medlib.georgetown.edu

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

PII S0014-5793(96)00961-1

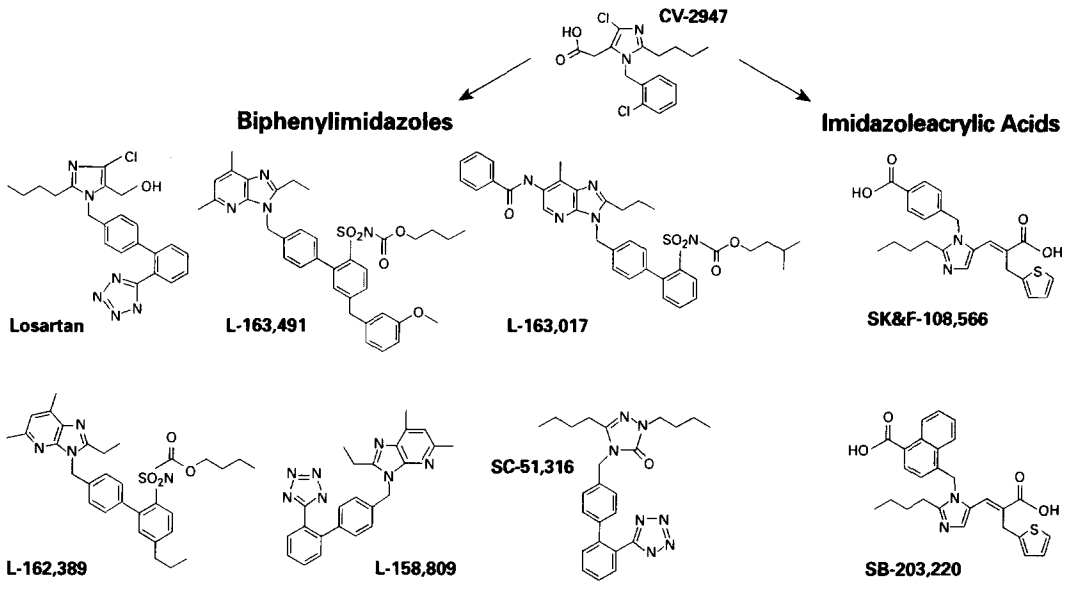

Fig. 1. Structure of angiotensin receptor nonpeptide ligands.

with buffer B (50 mM Tris HCl, pH 7.4; 5 mM $MgCl_2$) by pelleting at 4°C for 15 min at 10000 × *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 used for receptor binding assays.

2.4. Binding assays [9,13]

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 100000 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. Nonspecific binding was defined as the binding of radioligand in the presence of 500 nM cold Saralasin and specific binding was defined as total radioligand bound minus nonspecific binding. The IC_{50} and B_{max} values from the specific binding data were determined by computerized nonlinear regression analysis using the 'Kaleidagraph' program.

3. Results and discussion

The rAT_{1b}, xAT_a and xCM46 receptors expressed in COS-7 cell membranes exhibited B_{max} values and affinities towards the peptide ligands, Ang II and [Sar^1,Ile^8]Ang II, which were not significantly different between species (Table 1). In contrast, the AT_1 nonpeptide ligands had very low affinity for xAT_a receptors (IC_{50} values > 50 μM) (Table 1) whereas all of the nonpeptide ligands examined had nanomolar affinities towards the rAT_{1b} receptor with the following rank order of potency: L-158,809 > SC-51,316 > Losartan > SK&F-108,566 > L-163,017 > SB-203,220 > L-163,491 > L-162,389 (Fig. 3, Table 1). These findings are consistent with previous reports demonstrating that AT_1 nonpeptide ligands are several orders of magnitude less potent at binding amphibian xAT compared with mammalian AT_1 receptors [8–12].

The pharmacological and sequence differences in cloned AT receptors between species previously facilitated the identifica-

Table 1
Binding affinities for AT receptor ligands: wild type and mutant angiotensin II receptors

Ang II ligands	rAT_{1b} IC_{50} (nM)	xAT_a		xCM46	
		IC_{50} (nM)	$F_{(xATa/rAT1b)}$	IC_{50}(nM)	F_{mut}
Peptides					
Angiotensin II	1.0 ± 0.1	1.1 ± 0.1	1	1.4 ± 0.1	1
[Sar^1,Ile^8]Ang II	2.2 ± 0.1	2.1 ± 0.2	1	2.5 ± 0.3	1
Biphenylimidazoles					
Losartan	2.2 ± 0.2	> 50000	> 25000	2.0 ± 0.1	0.91
L-162,389	130 ± 30	> 50000	> 380	125 ± 30	1.0
L-163,491	57 ± 30	> 50000	> 880	110 ± 70	1.9
L-158,809	0.2 ± 0.08	> 50000	> 250000	0.7 ± 0.06	3.5
L-163,017	13 ± 2	> 50000	> 3800	51 ± 8	3.9
SC-51,316	0.51 ± 0.1	> 50000	> 100000	2 ± 0.3	3.9
Imidazoleacrylic acids					
SK&F-108,566	7.4 ± 1.6	> 50000	> 6800	1200 ± 200	160
SB-203,220	32 ± 5	> 50000	> 1500	5400 ± 700	170

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. $F_{(xATa/rAT1b)} = xAT_a$ IC_{50}/rAT_{1b} IC_{50}; F_{mut} = xCM46 IC_{50}/rAT_{1b} IC_{50}. B_{max} values (mean ± S.E.M., n = 3–12) in fmol/10^5 cells: rAT_{1b}, 9.3 ± 1.8; xAT_a, 10 ± 1.1; xCM46, 7.9 ± 1.5.

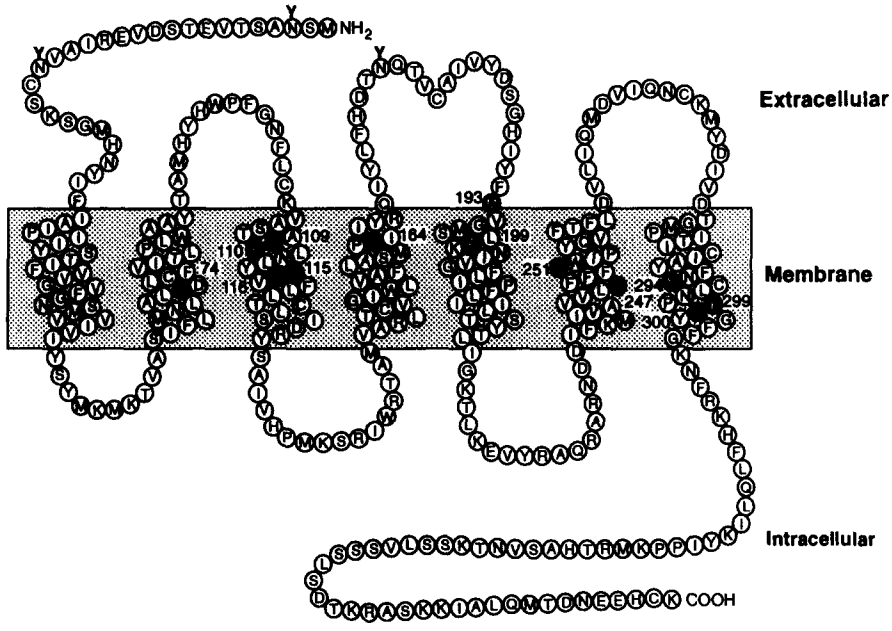

Fig. 2. Schematic of the amphibian xAT_a receptor. Solid circles indicate the residues replaced by mammalian amino acids by site-directed mutagenesis to create the combinatorial mutant, xCM46.

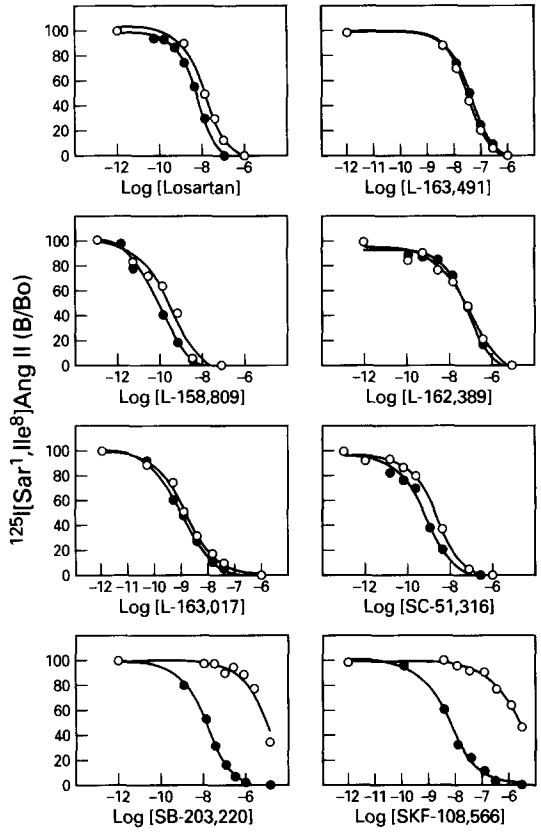

Fig. 3. Competition binding curves for angiotensin II receptor nonpeptides. Data are expressed as percent of control (specific bound radioligand in the absence of unlabeled ligand). The binding profiles of rAT_{1b} (■) and the combinatorial xAT_a mutant, xCM46 (□) for nonpeptide antagonists are shown. Representative examples of 3–4 similar experiments (each performed in triplicate) are shown. The standard error of each point was less than 10%. IC_{50} values for AT receptor ligands are listed in Table 1.

tion of nonconserved amino acids in the rAT_{1b} receptor which were critical structural determinants of Losartan binding [9]. These studies led to the construction of a combinatorial mutant of the amphibian AT receptor, xCM46, in which 13 amino acids in TMII-VII were exchanged for the corresponding amino acids in the mammalian rAT_{1b} receptor. In contrast to the amphibian wild type xAT receptor which did not bind Losartan, even at micromolar concentrations, xCM46 exhibited nanomolar affinity towards Losartan that was indistinguishable from the rAT_{1b} wild type receptor [13]. In order to determine whether these residues which were influential in Losartan binding are of general importance, a series of biphenylimidazoles were examined for their affinities towards the xCM46 mutant (Fig. 3, Table 1). All of the biphenylimidazoles examined exhibited affinities towards xCM46 which were similar to the rAT_{1b} receptor as evidenced by *small* (1–4) F_{mut} values (= xCM46 IC_{50}/rAT_{1b} IC_{50}). In contrast, the imidazoleacrylic acid antagonists had *large* (160–170) F_{mut} values for xCM46 (Fig. 3, Table 1).

These results suggest that the nonconserved residues which determine the molecular requirements for Losartan binding are shared within the class of biphenylimidazoles in general but are not identical to the nonconserved residues necessary for high affinity binding of imidazoleacrylic acids. Thus, these findings are consistent with a previous mutagenesis study of the human AT_1 receptor in which His^{256} in TMVI, highly conserved among mammalian and nonmammalian AT receptors, was shown to interact with imidazoleacrylic acids but not biphenylimidazoles [11]. Although distinct structural determinants exist, previous reports demonstrate that there are also common points of interaction between these two classes of nonpeptides. Interspecies amino acid exchange and alanine mutagenesis suggests that Val^{108} in TMIII [16] and Asn^{295} in TMVII [11] are common structural requirements of biphenylimidazole and imidazoleacrylic acid binding. Taken together, these data indicate that distinct but overlapping molecular determinants in the TM region exist for

biphenylimidazole and imidazoleacrylic acid recognition on the AT_1 receptor.

These findings also support the concept that a common binding site for small-molecule ligands is conserved within the TM domain of all G-protein coupled receptors regardless of the native ligand [17,18]. This is particularly intriguing since recent reports suggest that peptide hormone ligands such as Ang II are too large too fit within the TM domains of their respective receptors and interact with residues in the extracellular domains of the receptor [19,20]. Further investigations into the molecular basis of nonpeptide recognition could lead to fundamental insights into the molecular basis of ligand recognition for peptide hormone receptors in general and provide a rational framework for ligand design for this large class of seven TM domain G-protein coupled receptors.

Acknowledgements: This study was supported by grants from the American Heart Association, Nation's Capitol Affiliate and the National Kidney Foundation of the National Capitol Area.

References

[1] Raizada, M.K., Phillips, I.A. and Sumners, C. (1993) Cellular and Molecular Biology of the Renin Angiotensin System, CRC Press, Boca Raton, FL.
[2] Timmermans, P.B., Wong, P.C., Chiu, A.T., Herblin, W.F. and Smith, R.D. (1993) J. Hum. Hypertens. 7 Suppl. 2, S19–31.
[3] Sandberg, K. (1994) Trends Endo. Metab. 5, 28–35.
[4] Timmermans, P.B.M.W.M., Wong, P.C., Chiu, A.T., Herblin, W.F., Benfield, P., Carini, D.J., Lee, R.J., Wexler, R.R., Saye, J.A.M. and Smith, R.D. (1993) Pharmacol. Rev. 45, 205–251.
[5] Furukawa, Y., Kishimoto, S. and Nishikawa, K. (1982) United States Patent (1982) 4, 340,598.
[6] Smith, R.D., Chiu, A.T., Wong, P.C., Herblin, W.F., and Timmermans, P.B.M.W.M. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 135–165.
[7] Keenan, R.M., Weinstock, J., Finkelstein, J.A., Franz, R.G., Gaitanopoulos, D.E., Girard, G.R., Hill, D.T., Morgan, T.M., Samanen, J.M., Peishoff, C.E., Tucker, L.M., Aiyar, N., Griffin, E., Ohlstein, E.H., Stack, E.J., Weidley, E.F. and Edwards, R.M. (1993) J. Med. Chem. 36, 1880–1892.
[8] Sandberg, K., Ji, H., Millan, M.A. and Catt, K.J. (1991) FEBS Lett. 284, 281–284.
[9] Ji, H., Leung, M., Zhang, Y., Catt, K.J. and Sandberg, K. (1994) J. Biol. Chem. 269, 16533–16536.
[10] Schambye, H., Hjorth, S.A., Bergsma, D.J., Sathe, G. and Schwartz, T.W. (1994) Proc. Natl. Acad. Sci. USA 91, 7046–7050.
[11] Schambye, H., Hjorth, S.A., Weinstock, J. and Schwartz, T.W. (1995) Mol. Pharmacol. 47, 425–431.
[12] Groblewski, T., Maigret, B., Nonet, S., Larguier, R., Lombard, C., Bonnafous, J.C. and Marie, J. (1995) Biochem. Biophys. Res. Commun. 209, 153–160.
[13] Ji, H., Zheng, W., Zhang, Y., Catt, K.J. and Sandberg, K. (1995) Proc. Natl. Acad. Sci. USA 92, 9240–9244.
[14] Sandberg, K., Ji, H., Clark, A.J., Shapira, H. and Catt, K.J. (1992) J. Biol. Chem. 267, 9455–9458.
[15] Ji, H., Sandberg, K., Zhang, Y. and Catt, K.J. (1993) Biochem. Biophys. Res. Commun. 194, 756–762.
[16] Nirula, V., Zheng, W., Sothinathan, R. and Sandberg, K. (1996) Br. J. Pharmacol., in press.
[17] Schwartz, T.W. (1994) Curr. Opin. Biotech. 5, 434–444.
[18] Cascieri, M.A., Gong, T.M. and Strader, C.D. (1995) J. Pharmacol. Toxicol. Methods 33, 179–185.
[19] Hjorth, S.A., Schambye, H.T., Greenlee, W.J. and Schwartz, T.W. (1994) J. Biol. Chem. 269, 30953–30959.
[20] Feng, Y.-H., Noda, K., Saad, Y., Liu, X-p., Husain, A. and Karnik, S.S. (1995) J. Biol. Chem. 270, 12846–12850.