Br. J. Pharmacol. (1994), **113**, 331–333

SPECIAL REPORT

Mutations in transmembrane segment VII of the AT_1 receptor differentiate between closely related insurmountable and competitive angiotensin antagonists

[1]Hans T. Schambye, Bjørn v. Wijk, Siv A. Hjorth, *Wolfgang Wienen, *Michael Entzeroth, †Derk J. Bergsma & Thue W. Schwartz

Laboratory for Molecular Endocrinology, University Dept. of Clinical Biochemistry, Rigshospitalet 6321, DK-2100 Copenhagen, Denmark; *Department of Biochemical Research, Cardiovascular Pharmacology, Dr. Karl Thomae GmbH, D-88397 Biberach an der Riss, Germany and †Dept. of Molecular Genetics, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406, U.S.A.

Chimeric constructs between the human and the *Xenopus laevis* AT_1 receptor have demonstrated, that the binding of non-peptide angiotensin antagonists is dependent on non-conserved residues located deep in transmembrane segment VII of the AT_1 receptor. Here we have studied four pairs of closely related antagonists each consisting of a competitive and an insurmountable compound differentiated by one out of three different types of minor chemical modifications. None of the antagonists bound to the *Xenopus* receptor and the binding of all of the compounds to the human receptor was severely impaired by the introduction of non-conserved residues from transmembrane segment VII of the *Xenopus* receptor. In all four pairs of antagonists the competitive compound was affected more by these substitutions than the corresponding insurmountable one (209 vs. 22, 281 vs. 29, 290 vs. 29 and 992 vs. 325-fold increase in K_i values). A similar pattern was observed in response to substitution of a single non-conserved residue in transmembrane segment VII, Asn^{295} to Ser. These results indicate that a common molecular mechanism distinguishes the interaction of insurmountable and competitive antagonists with the AT_1 receptor.

Keywords: Angiotensin AT_1 receptor; non-peptide antagonists; insurmountable antagonism; competitive antagonism; chimeric receptors; mutagenesis

Introduction The newly developed non-peptide antagonists for the angiotensin AT_1 receptor behave as competitive compounds in binding assays with radiolabelled angiotensin II (AII) (Wexler *et al.*, 1992; Wienen *et al.*, 1992; 1993). However, in bioassays a subset of these compounds exhibit insurmountable antagonism; i.e. the dose-response curve for the agonist is not only shifted to the right, but the maximal response is also reduced (Kenakin, 1987; Wexler *et al.*, 1992; Wienen *et al.*, 1992; 1993).

By construction of chimeric receptors between the human and the *Xenopus* AT_1 receptor, which does not bind the non-peptide compounds (Bergsma *et al.*, 1993), we recently identified residues in transmembrane segment (TM) VII, especially Asn^{295}, to be important for the binding of non-peptide ligands but not for angiotensin II (Schambye *et al.*, 1994). Surprisingly, insurmountable and competitive antagonists were differently affected by these exchanges in TM-VII as a larger decrease in binding affinity was observed for competitive than for insurmountable compounds (Schambye *et al.*, 1994).

In the present study we have selected four pairs of closely related angiotensin antagonists in which only a minor chemical modification distinguishes the insurmountable compound from its competitive counterpart. The chemical differences were located in three different parts of the compounds' common molecular scaffold (Figure 1).

Methods Rabbit aorta contraction after angiotensin II stimulation was measured as previously described (Wienen *et al.*, 1992). The apparent dissociation constants, pK_B, for competitive and insurmountable antagonism were calculated as described (Kenakin, 1987).

Chimeric receptors were constructed using an overlap extension technique based on PCR, as described by Schambye *et al.* (1994). Constructs were verified by dideoxynucleotide sequencing (Sequenase kit, U.S. Biochemicals Co.).

The binding to wildtype human and *Xenopus* AT_1 and the mutant receptors were studied in transfected COS 7 and CHO cells (Gether *et al.*, 1993). Binding experiments were performed on whole cells for 24 h at 4°C, as described by Schambye *et al.* (1994).

Peptide and non-peptide ligands Angiotensin II (AII) and [Sar^1,Leu^8]-AII were purchased from Peninsula (St. Helens, Merseyside, UK). BIBR247, BIBR179, BIBR277, BIBR255, BIBS39 and BIBR237 were synthesized by Drs N. Hauel and B. Narr at Dr Karl Thomae GmbH, Germany. Losartan and EXP3174 were kindly provided by P.B.M.W.M. Timmermans [The DuPont Merck Pharmaceutical Company (Wexler *et al.*, 1992)].

Results All of the tested compounds were potent inhibitors of AII-induced aortic contraction with a pK_B of between 7.0 and 9.78 (Table 1). Four compounds (losartan, BIBR255, BIBS39 and BIBR237) acted as classical competitive antagonists and induced a parallel shift of the concentration-contractile curve without depression of the maximal response (Table 1), whereas four other compounds (EXP3174, BIBR247, BIBR179 and BIBR277) acted as insurmountable antagonists, thus reducing the maximal response to agonist stimulation by 24% to 60% (Table 1).

All of the non-peptide antagonists were bound with high affinities by the wildtype human AT_1 receptor with K_I values ranging from 0.87 nM to 25.4 nM (Table 1). In contrast, none of the non-peptide antagonists were able to displace the ^{125}I-[Sar^1,Leu^8]-AII from the *Xenopus* AT_1 receptor (results not shown).

The chimeric AT_1 receptor constructs HX7-hAT_1, where TM-VII and the C-terminal tail of the human receptor were exchanged with the corresponding segments from the

[1] Author for correspondence.

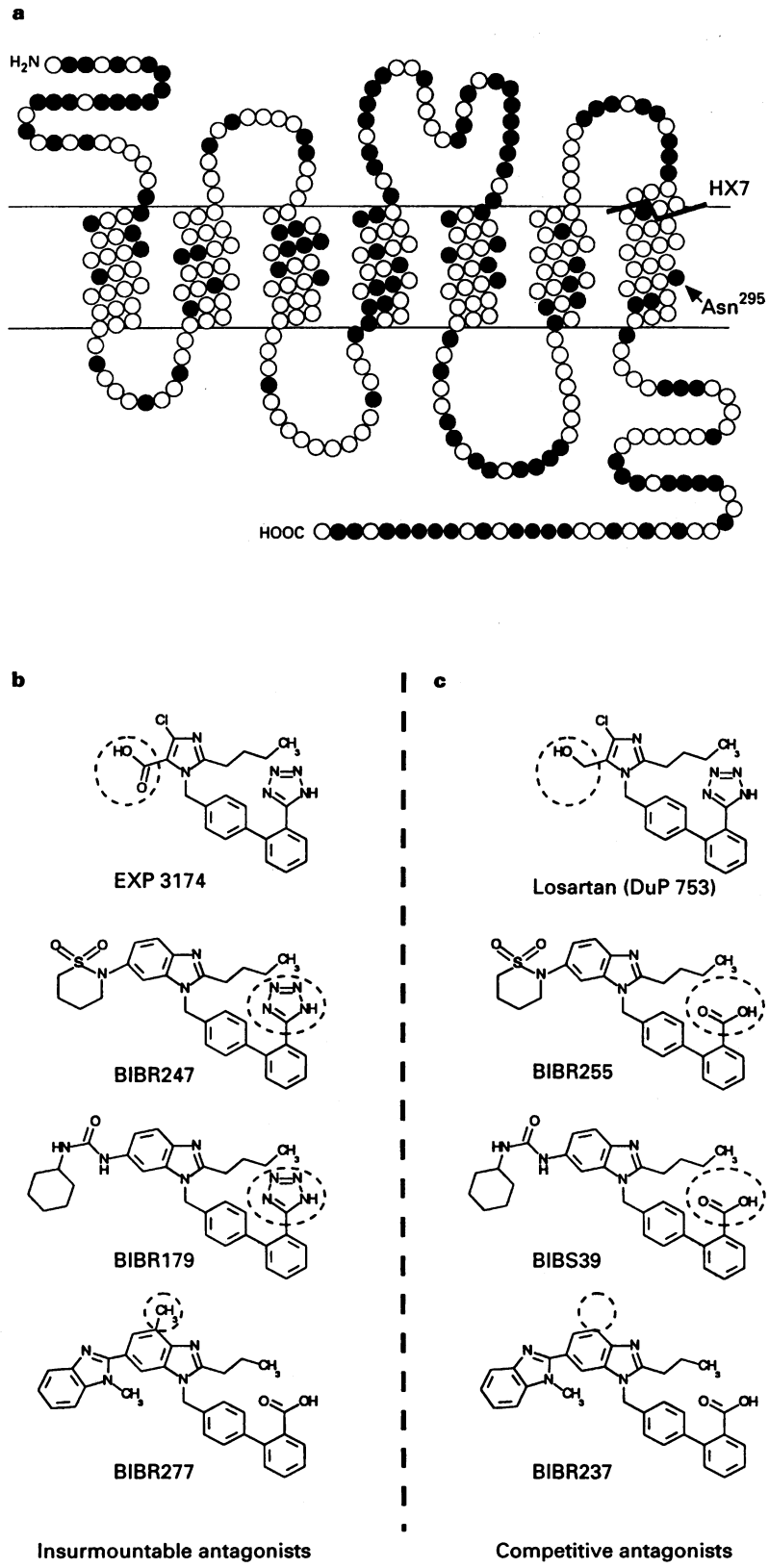

Figure 1 Structure of the human angiotensin II AT_1 receptor and of the employed non-peptide angiotensin antagonists. (a) Diagram of the human angiotensin AT_1 receptor (Bergsma *et al.*, 1992) with the splice-junction for the chimeric receptor HX7-hAT_1 indicated by a crossing line and the Asn^{295} residue located in TM-VII indicated by an arrow: (○) amino acid conserved between the human and the *Xenopus* AT_1 receptors (Bergsma *et al.*, 1993); (●) amino acid residues specific for the human AT_1 receptor. (b) and (c) The non-peptide antagonists are grouped according to their antagonist properties. The chemical differences between the two compounds in each pair are indicated by circles.

Table 1 Inhibition of angiotensin II (AII)-induced aortic contractions and binding affinity of peptide and non-peptide ligands for the human wildtype receptor and chimeric human/*Xenopus* receptor constructs

	Aorta contraction			*Receptor binding*							
Ligand	pK_B	(*n*)	*Reduction of Max. response*	*Human AT_1* K_I (nM)	(*n*)	*HX7-hAT_1* K_I (nM)	(*n*)	F_{mut}	*(Asn^{295}→Ser) hAT_1* K_I (nM)	(*n*)	F_{mut}
Angiotensin II	–		–	0.19 ± 0.03	(7)	0.34 ± 0.05	(3)	1.8	0.51 ± 0.05	(7)	2.7
BIBS39	8.14 ± 0.08	(4)	NR	8.90 ± 2.02	(6)	1865 ± 91	(4)	209	244 ± 26	(5)	27
BIBR179	7.00	(4)	32%	25.4 ± 3.9	(9)	550 ± 9	(5)	22	150 ± 10	(5)	6
BIBR237	7.85 ± 0.05	(3)	NR	5.68 ± 0.62	(9)	1594 ± 140	(4)	281	168 ± 15	(5)	30
BIBR277	9.48	(6)	60%	4.86 ± 0.98	(8)	143 ± 18	(5)	29	17.6 ± 2.7	(5)	4
BIBR255	7.85 ± 0.05	(4)	NR	6.02 ± 0.77	(9)	1748 ± 168	(4)	290	318 ± 34	(5)	53
BIBR247	7.90	(3)	39%	1.77 ± 0.49	(9)	52 ± 7	(5)	29	9.9 ± 1.3	(5)	6
Losartan (DuP753)	8.01 ± 0.02	(6)	NR	1.42 ± 0.35	(7)	1406 ± 207	(5)	992	107 ± 11	(5)	75
EXP3174	9.78	(4)	24%	0.87 ± 0.16	(7)	282 ± 33	(5)	325	14.8 ± 1.3	(5)	17

The pK_B value for the compounds are given (mean ± s.e.mean, $n = 3-6$, mean only for insurmountable compounds). The reduction of the maximum contractile response is measured in the presence of 1 μM of antagonist. NR - no reduction. The antagonists are listed as the chemically related pairs (see Figure 1). K_I values are presented in nM (mean ± s.e.). The effect of the mutations on the binding affinity is expressed as the mutation factor, $F_{mut} = K_I$ (mutant receptor)/K_I (human AT_1 receptor). B_{max} values (mean ± s.e.mean, $n = 5-8$): wildtype human AT_1, 3.0 ± 0.8 fmol/10^5 cells; HX7-hAT_1, 13.7 ± 3.8 fmol/10^5 cells; (Asn^{295}→Ser)hAT_1 4.9 ± 1.3 fmol/10^5 cells.

Xenopus receptor (Figure 1) and the (Asn^{295}-Ser)hAT_1 mutant both bound AII and [Sar^1,Leu^8]-AII with affinities comparable to those of the wildtype receptors suggesting that the mutant receptors have a normal overall structure (Table 1) (Schambye *et al.*, 1994). In contrast, the affinity of all of the non-peptide compounds was severely reduced by the HX7-hAT_1 exchange, the K_I value increased 22 to 992 fold (the mutational factor, F_{mut}, in Table 1). Substitution of Asn^{295} with Ser in TM-VII led to a decrease in binding affinity of 4 to 75 fold (Table 1). For each of the pairs of antagonists the binding affinity of the competitive compound was more reduced by the chimeric exchanges in TM-VII, than the insurmountable counterpart was (Table 1). Thus, in HX7-hAT_1 the mutational factor for the four competitive antagonists BIBS39, BIBR237, BIBR255 and losartan was 209, 281, 290 and 992, respectively, as compared to 22, 29, 29 and 325 for their insurmountable counterparts, BIBR179, BIBR277, BIBR247 and EXP3174 (Table 1). In the (Asn^{295}→Ser)hAT_1 mutant the same pattern was observed, albeit the effect of this minor modification of the receptor was smaller, with a mutational factor between 27 and 75 for the competitive antagonists and between 4 and 17 for the corresponding insurmountable compounds (Table 1).

Discussion The binding of all of the non-peptide antagonists is critically dependent on non-conserved residues in TM-VII, especially Asn^{295}, – residues which do not seem to participate in peptide binding (Schambye *et al.*, 1994). DuP753 and EXP3174 were more affected by the exchange of TM-VII than the other six compounds. Possibly these relatively smaller compounds (Figure 1) have fewer points of interaction with the receptor, making binding more dependent upon interaction with the non-conserved residues in TM-VII.

Despite the fact, that the chemical modifications, which differentiate the four competitive compounds from their insurmountable counterparts, are located in three different parts of the compounds common molecular scaffold (Figure 1), the competitive analogue was in all four cases affected approximately 10 fold more than the insurmountable one by the substitutions in TM-VII (Table 1).

It is difficult to conceive of a direct common interaction between, for example, Asn^{295} and the decisive chemical moiety in all four pairs of antagonists, as the chemical differences are located so differently (Figure 1 and Table 1). More likely Asn^{295} either interacts directly with a common chemical moiety of the antagonists, or the Asn^{295} to Ser substitution changes the overall structure of the receptor and thereby indirectly affects the binding of the antagonists in a way which is more detrimental to competitive than the insurmountable compounds. In any case, as there is a strong correlation between the compounds pharmacological properties and the degree to which they were affected by the mutations these results indicate, that a common molecular mechanism distinguishes insurmountable and competitive antagonists in their interaction with AT_1 receptors.

We thank Dorte Frederiksen, Lisa Zeh and Tina Jakobsen for expert technical assistance. Supported by grants from the Danish Heart Association, the Danish Medical Research Foundation, the Novo Nordisk Foundation.

References

BERGSMA, D.J., ELLIS, C., KUMAR, C., NUTHULAGANTI, P., KERSTEN, H., ELSHOURBAGY, N., GRIFFIN, E., STADEL, J.M. & AIYAR, N. (1992). Cloning and characterization of a human angiotensin-II type-1 receptor. *Biochem. Biophys. Res. Commun.*, **183**, 989–995.

BERGSMA, D.J., ELLIS, C., NUTHULAGANTI, P.R., NAMBI, P., SCAIFE, K., KUMAR, C. & AIYER, N. (1993). Isolation and expression of a novel angiotensin-II receptor from Xenopus-laevis heart. *Mol. Pharmacol.*, **44**, 277–284.

GETHER, U., JOHANSEN, T.E. & SCHWARTZ, T.W. (1993). Chimeric NK1 (substance P)/NK3 (neurokinin B) receptors. *J. Biol. Chem.*, **268**, 7893–7898.

KENAKIN, T.P. (1987). Drug antagonism. In *Pharmacological Analysis of Drug Receptor Interaction*. ed. Kenakin, T.P. pp. 205–244. New York: Raven Press.

SCHAMBYE, H.T., HJORTH, S.A., BERGSMA, D.J., SATHE, G. & SCHWARTZ, T.W. (1994). Differentiation between binding sites for angiotensin II and non-peptide antagonists on the angiotensin II type I receptors. *Proc. Natl. Acad. Sci. U.S.A.*, (in press).

WEXLER, R.R., CARINI, D.J, DUNCIA, J.V., JOHNSON, A.L., WELLS, G.J., CHIU, A.T., WONG, P.C. & TIMMERMANS, P.B.M.W.M. (1992). Rationale for the chemical development of angiotensin II receptor antagonists. *Am. J. Hypertens.*, **5**, 209–220.

WIENEN, W., HAUEL, N., VAN MEEL, J.C.A., NARR, B., RIES, U. & ENTZEROTH, M. (1993). Pharmacological characterization of the novel nonpeptide angiotensin-II receptor antagonist, BIBR-277. *Br. J. Pharmacol.*, **110**, 245–252.

WIENEN, W., MAUZ, A.B.M., VAN MEEL, J.C.A. & ENTZEROTH, M. (1992). Different types of receptor interaction of peptide and nonpeptide angiotensin-II antagonists revealed by receptor binding and functional studies. *Mol. Pharmacol.*, **41**, 1081–1088.

(Received May 10, 1994
Accepted June 27, 1994)