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Interaction between the Nonpeptide Angiotensin Antagonist SKF-108,566 and Histidine 256 (HisVI:16) of the Angiotensin Type 1 Receptor

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SUMMARY

His^{256} (HisVI:16) of transmembrane segment (TM)-VI of the rat angiotensin type 1 (AT_1) receptor was targeted for mutagenesis to investigate its potential involvement in ligand binding. Substitution of His^{256} with alanine, phenylalanine, glutamine, or isoleucine did not affect the binding of either angiotensin II or nine different biphenylimidazole AT_1 antagonists. In contrast, the binding affinity of the prototype imidazoleacrylic acid antagonist SKF-108,566 was reduced 15-fold by the exchange of His^{256} with alanine. Substitution of His^{256} with either isoleucine or phenylalanine yielded similar results, whereas a glutamine residue was able to substitute for His^{256}, suggesting that the ϵ-nitrogen of His^{256} could be involved in the interaction with the imidazoleacrylic acid. To identify the chemical groups on SKF-108,566 that interact with His^{256} and with Asn^{295}, a previously identified interaction point for nonpeptide antagonists located in TM-VII, we tested the binding of 15 analogs of SKF-108,566 in which different chemical moieties were systematically exchanged. The results indicated that the carboxyphenyl group of SKF-108,566 interacts with the imidazole side chain of His^{256}. The data did not point to any particular contact group on the antagonist for Asn^{295}. It is concluded that the imidazoleacrylic acid antagonists share some interactions in TM-VII of the AT_1 receptor with the biphenylimidazole antagonists, but the binding of the imidazoleacrylic acid compounds is uniquely dependent on His^{256} in TM-VI, possibly through the carboxyphenyl moiety.

The renin/angiotensin system is of critical importance in the control of blood pressure and in water and electrolyte homeostasis. Angiotensin II, the main effector hormone in the system, exerts its effects through at least two different receptors, designated AT_1 and AT_2 (1). All of the cardiovascular and renal effects of angiotensin II are mediated by the AT_1 receptor, whereas the function of the AT_2 receptor remains to be determined (2). The cDNAs encoding both the AT_1 and AT_2 receptors have been cloned, demonstrating that these receptors belong to the large superfamily of G protein-coupled receptors characterized by seven TMs (3–6).

Pharmacological intervention in the renin/angiotensin system using angiotensin-converting enzyme inhibitors is an effective treatment for hypertension and congestive heart failure. Blockade of the AT_1 receptor, which represents a more direct approach, has recently become feasible with the discovery of orally active, specific, nonpeptide antagonists (7). From a common lead compound, the Takeda compound CV-2947 (8), two series of antagonists have been developed (7, 9) (Fig. 1). The majority of the compounds, including losartan (DuP753) (7), are biphenylimidazole derivatives, whereas one, SKF-108,566, is an imidazoleacrylic acid derivative (9) (Fig. 1). Molecular modeling of angiotensin II was used in the development of both types of compounds, but models and strategies differ substantially (7, 9). The major difference is that the imidazole of CV-2947 was aligned with the imidazole of His^6 of angiotensin II in the development of losartan (7), whereas the imidazole of SKF-108,566 was superimposed on the aromatic part of Pro^7 (9). The basic as-

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ABBREVIATIONS: AT_1 and AT_2 receptors, angiotensin type 1 and type 2 receptors; TM, transmembrane segment; PCR, polymerase chain reaction.

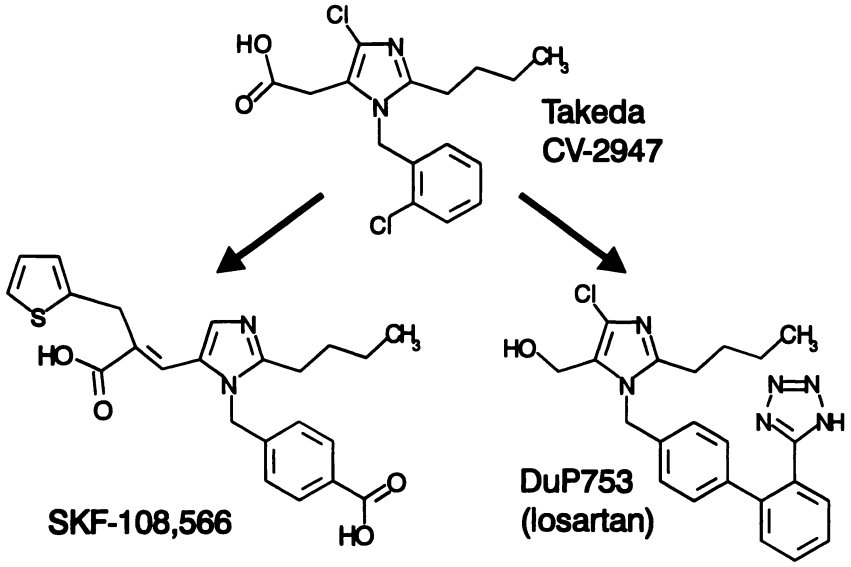


Fig. 1. Chemical structures of DuP753 (losartan) and SKF-108,566 and their common lead compound, the so-called Takeda (CV-2947) compound.

sumption in both cases was, however, that the compounds, in spite of their very limited chemical resemblance to angiotensin II, structurally mimic the peptide and act by binding to the same epitope(s) on the receptor as does the native ligand (7, 9, 10).

In a study of chimeric human and *Xenopus laevis* angiotensin AT_1 receptors, we recently found that the binding of both types of nonpeptide antagonists is critically dependent on nonconserved amino acid residues in TM-VI and -VII of the human AT_1 receptor, residues that apparently have no interaction with the peptide ligands (11). An asparagine residue, Asn^{295} (AsnVII:13) (12), in the middle of TM-VII (Fig. 2) was found to be important for binding of all of the nonpeptide antagonists, including losartan and SKF-108,566 (11). In contrast, the binding of angiotensin II was found to be dependent on residues in the amino-terminal extension and in extracellular loops 1 and 3 (13). Exchange of these residues did not affect the binding of the nonpeptide ligands.

Both angiotensin II and all of the nonpeptide ligands contain acidic moieties, which are important for their binding (7). A possible counterpart for such acidic moieties could be His^{256} (HisVI:16), which is conserved in all mammalian AT_1 receptors (Fig. 2) (3, 4, 14). This residue presumably points inward in the receptor and corresponds to a tyrosine residue (Tyr^{506} in the rat M3 receptor) in muscarinic receptors that has been shown previously to be involved in ligand binding (12, 15). In the present study we examine the role of His^{256} in the binding of AT_1 receptor ligands by exchanging it with a series of other amino acid residues.

Materials and Methods

Peptide and nonpeptide ligands. Angiotensin II and [Sar^1,Leu^8]-angiotensin II were purchased from Peninsula (St. Helens, Merseyside, UK). SKF-108,566 and its analogs was synthesized as described (9, 16–18). The following nonpeptide biphenylimidazole antagonists were kindly provided: DuP753 (losartan, MK-954) (19) (P. B. M. W. M. Timmermans, The DuPont Merck

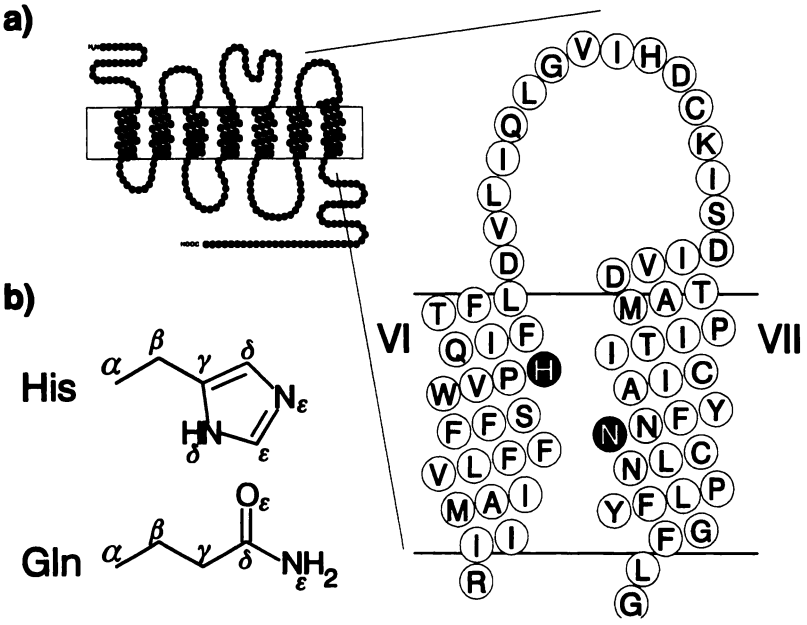


Fig. 2. a, Structure of TM-VI and -VII of the rat AT_1 receptor. *Black circles*, His^{256} (HisVI:16) and Asn^{295} (AsnVII:13). b, Side chains of histidine and glutamine residues, with indication of the positions of the ϵ-nitrogens (42).

Pharmaceutical Co., Wilmington, DE), L-158,809 (20) (W. J. Greenlee, Merck, Sharp & Dohme, Rahway, NJ), SC 51,316 (21) (G. M. Olins, Searle & Company, St. Louis, MO), SR 47,436 (22) (D. Nisato, Sanofi SA, Paris, France), ICI D8731 (23) (A. A. Oldham, ICI Pharmaceuticals, Cheshire, UK), GR117,289 (24) (D. Middlemiss, Glaxo, Ware, UK), BMS 180,560 (25) (M. A. Poss, Bristol-Myers Squibb Pharmaceuticals, Princeton, NJ), and BIBS 39 (26) and BIBR 277 (27) (M. Entzeroth, Dr. Karl Thomae GmbH, Biberach, Germany).

Receptor mutagenesis. The rat AT_1 receptor cDNA was generously provided by Dr. T. J. Murphy (Emory University, Atlanta, GA) (3) and the human AT_1 receptor cDNA by Dr. D. J. Bergsma (SmithKline Beecham, King of Prussia, PA) (4). A 'cassette' gene was initially generated from the rat receptor cDNA, as described previously (13). Mutant receptor cDNAs were constructed using the PCR overlap extension technique (13, 28). The PCR fragments were digested with *Nde*I and subsequently inserted, as approximately 200-base pair fragments, into the similarly digested cassette gene cloned in the expression vector pTEJ8 (29). PCRs employed *Pyrococcus furiosus* polymerase (Stratagene), using conditions recommended by the manufacturer. Temperature cycling consisted of 30 cycles of 94° for 1 min, 50° for 1 min, and 72° for 1 min. All mutants were initially identified by the presence of a diagnostic restriction site and subsequently verified by dideoxynucleotide sequencing (Sequenase kit; United States Biochemicals).

Transfections and cell culture. The wild-type AT_1 receptors and the mutated receptors were transiently transfected into COS-7 cells by the calcium phosphate precipitation method, according to previously reported procedures (30). COS-7 cells were grown in Dulbecco's modified Eagle's medium 1885, supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.1 mg/ml gentamicin, at 37° in a humidified atmosphere of 10% CO_2/90% air. All materials and media used for tissue culture were purchased from GIBCO (Paisley, Scotland).

Binding experiments. Monoiodinated ^{125}I-[Sar^1,Leu^8]-angiotensin II was prepared by the Iodogen method and purified by reverse phase high performance liquid chromatography, as described (11). The transfected COS-7 cells were transferred to six-, 12-, or 24-well culture plates (1–7 × 10^4 cells/well) 1 day after transfection and 24 hr before the binding experiments were performed. The number of cells per well was based on the expression efficiency of the individual plasmids, aiming at 5–10% binding of the added radioligand in the competition binding experiments. The cells were washed once in 25 mM Tris buffer, pH 7.4, containing 140 mM NaCl, 5 mM $MgCl_2$, and 0.1% (w/v) bovine serum albumin (Sigma), and binding experiments were performed for 24 hr at 4° with 50 pM ^{125}I-[Sar^1,Leu^8]-angiotensin II and a variable amount of unlabeled peptide or nonpeptide ligand, in 0.5–1 ml of 25 mM Tris buffer, pH 7.4, containing 5 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin (Sigma), and 100 μg/ml bacitracin (Sigma). All determinations were performed in triplicate, and the nonspecific binding was determined as the binding in the presence of 1 μM unlabeled [Sar^1,Leu^8]-angiotensin II. The specific binding constituted >95% of the total binding. The binding data were analyzed and IC_{50} values were determined by computerized nonlinear regression analysis using Inplot 4.0 (GraphPad Software, San Diego, CA). K_d values for binding of radiolabeled [Sar^1,Leu^8]-angiotensin II to the different receptors were estimated from competition binding experiments with variable amounts of the corresponding unlabeled peptide, using the equation $K_d = IC_{50} - L$ (where L is the concentration of free radioligand) (31). K_i and B_{max} values were calculated using the equations $K_i = IC_{50}/[1 + (L/K_d)]$ and $B_{max} = B_0(IC_{50}/L)$.

Results

Exchanges of His^{256}. Substitution of His^{256} (HisVI:16) in the rat AT_1 receptor with alanine, isoleucine, phenylalanine, or glutamine had minimal effect on the binding of the peptide ligands angiotensin II and [Sar^1,Leu^8]-angiotensin II (Fig. 3; Table 1). The binding of losartan (Figs. 1 and 3) and eight other antagonists of the biphenylimidazole class (L-158,809, BIBR 277, BIBS 39, GR117,289, BMS 180,560, SR 47,436, ICI D8731, and SC 51,316) (data not shown) was likewise unaffected by these mutations. In contrast, exchange of His^{256} with alanine reduced the binding affinity of the imidazoleacrylic acid SKF-108,566 by a factor of 15.3 (Table 2) [the effect of the mutations on the binding affinity is expressed as the mutation factor, F(mut) = K_i(mutant receptor)/K_i(wild-type AT_1 receptor)]. Exchange with isoleucine or phenylalanine led to the same degree of reduction of affinity, whereas substitution with glutamine did not change the affinity for SKF-108,566 (Table 1).

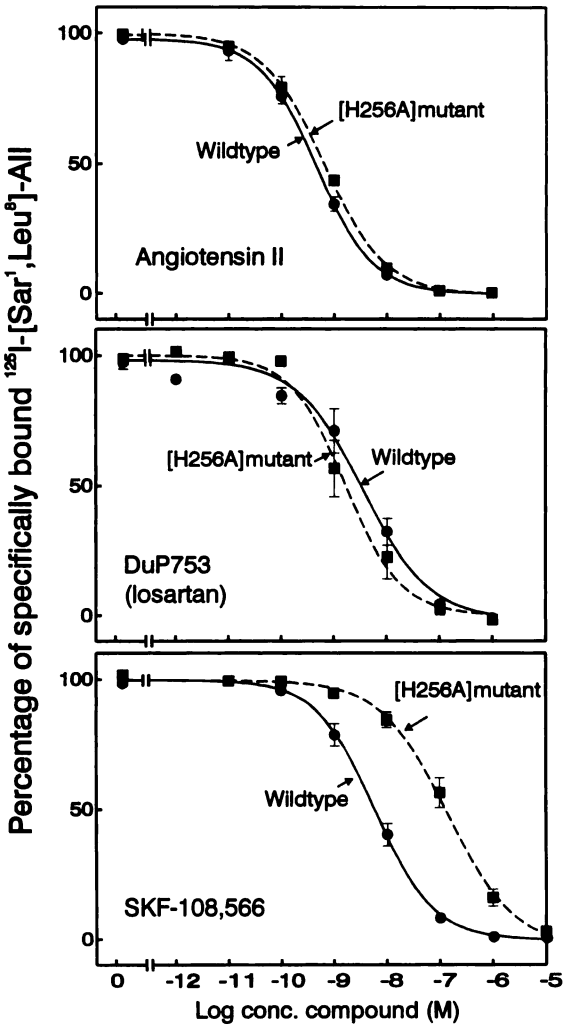


Fig. 3. Competition binding profiles for angiotensin II (*upper*), DuP753 (losartan) (*middle*), and SKF-108,566 (*lower*) with the wild-type rat AT_1 receptor and the His^{256} to alanine rat AT_1 mutant receptor transiently expressed in COS-7 cells. ●, Wild-type; ■, His^{256} to alanine mutant. Data are expressed as percentage of maximum specifically bound ^{125}I-[Sar^1,Leu^8]-angiotensin II (mean ± standard error, three to five experiments).

Binding of SKF-108,566 analogs to the wild-type rat AT_1 receptor. Analogs in which the carboxyl group in the *para*-position of the phenyl ring (SKF-107,011, SKF-108,728, SKF-108,867, and SKF-108,815) (Table 2) or the corresponding carboxyl group on the naphthalene ring (SB-205,390) (Table 3) was removed or changed were bound with reduced

TABLE 1

K_i values of SKF-108,566, angiotensin II, and [Sar^1,Leu^8]-angiotensin II for the wild-type rat receptor and the His^{256} to alanine, glutamine, asparagine, and phenylalanine mutants

The effect of the mutations on the binding affinity is expressed as the mutation factor, F(mut) = K_i(mutant receptor)/K_i(rat AT$_1$ receptor). B_{max} values (mean ± standard error, four to 21 experiments) were as follows: wild-type rat AT$_1$ receptor, 91 ± 16 fmol/10^5 cells; His^{256} to alanine rat AT$_1$ receptor mutant ([H256A]rAT$_1$), 49 ± 10 fmol/10^5 cells; His^{256} to glutamine mutant ([H256Q]rAT$_1$), 59 ± 21 fmol/10^5 cells; His^{256} to isoleucine mutant ([H256I]rAT$_1$), 59 ± 15 fmol/10^5 cells; His^{256} to phenylalanine mutant ([H256F]rAT$_1$), 132 ± 28 fmol/10^5 cells.

Ligand	Wild-type rat AT$_1$		[H256A]rAT$_1$			[H256Q]rAT$_1$			[H256I]rAT$_1$			[H256F]rAT$_1$		
	K_i	n[a]	K_i	n	F(mut)	K_i	n	F(mut)	K_i	n	F(mut)	K_i	n	F(mut)
	nM		nM			nM			nM			nM		
SKF-108,566	5.4 ± 0.9	20	82.8 ± 13.2	8	15.3	5.5 ± 1.0	3	1.0	62.9 ± 8.2	4	11.0	67.7 ± 9.9	5	12.5
Angiotensin II	0.44 ± 0.05	21	0.61 ± 0.10	4	1.4	ND[b]			0.31 ± 0.03	3	0.7	0.71 ± 0.11	4	1.60
[Sar^1,Leu^8]-Angiotensin II	0.31 ± 0.02	44	0.22 ± 0.04	9	0.71	0.22 ± 0.02	4	0.7	0.41 ± 0.04	5	1.3	0.49 ± 0.05	6	1.58

[a] n, number of experiments.
[b] ND, not determined.

affinity by the wild-type AT$_1$ receptor, compared with SKF-108,566 and the remaining analogs [F(ana) = 35 ± 7.5, compared with 7.3 ± 3.2] [the effect of the chemical modifications is expressed here as the analog factor, F(ana) = K_i(analog)/K_i(SKF-108,566), where K_i is the affinity for the wild-type receptor].

In contrast, SB-206,754, SB-204,889, and SKF-109,059, in which the other carboxyl group was changed, were bound with higher affinity by the AT$_1$ receptor than was the prototype compound, SKF-108,566 (Tables 2 and 3). Removal or change of the thiophene did not affect the binding affinity to a large degree (compounds SB-200,491, SB-201,529, and SB-201,972) (Table 2). However, the affinity decreased when both the thienyl and the propylenic acid groups were removed (SB-204,403) (Table 3), demonstrating the importance of the entire side chain.

The compounds in which the phenyl ring was replaced by a naphthalene group were all bound with a lower affinity than was SKF-108,566 [F(ana) = 2.7–33.8] (Table 3), indicating that the conformational restriction induced by the double-ring structure impairs the interaction between the antagonist and the receptor. Another explanation may be that the presence of 0.1% bovine serum albumin in the binding buffer reduced the apparent afffinity of the naphthalene analogs because these compounds are bound by plasma proteins to a higher degree than is SKF-108,566 (18).

Binding of SKF-108,566 analogs to mutated AT$_1$ receptors. Exchange of His^{256} with alanine reduced the affinity of all of the analogs, except for the naphthalene derivatives (Table 3) and the compound that has a biphenyl group, similarly to losartan (SKF-108,815) (Table 2), indicating that the latter compounds do not interact with His^{256}. Compounds in which the carboxyl moiety of the benzyl group was removed (SKF-107,011, SKF-108,728, SKF-108,867, and SKF-108,815) were less susceptible to the His^{256} to alanine mutation than were the other monophenyl derivatives (Table 2) [F(mut) = 5.3 ± 1.2, compared with 15.1 ± 2.5].

As previously observed with the human AT$_1$ receptor (11, 32), exchange of Asn^{295} (AsnVII:13) with serine in the rat AT$_1$ receptor affected the binding of both biphenylimidazole derivatives and the imidazoleacrylic acid, whereas the binding of peptide analogs was only slightly affected. The affinities of all of the analogs were reduced by the mutation [F(mut) = 6 to ⩾100] (Tables 2 and 3).

Examination of the binding to the wild-type human AT$_1$ receptor and the human Asn^{295} to serine mutant receptor showed that most of the analogs had approximately 10 times higher affinity for the human receptor than for the rat receptor, but the affinities were affected to the same degree by the mutation (data not shown).

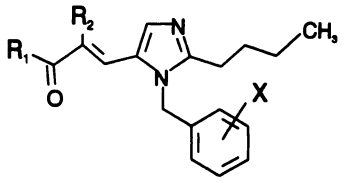
Discussion

In the present study we have characterized the binding of both peptide and nonpeptide angiotensin ligands to wild-type and mutated AT$_1$ receptors. The data demonstrate that the binding of nonpeptide ligands is affected by mutations in the TMs of the receptor that do not affect the peptide ligands. Furthermore, the data indicate that the nonpeptide antagonist SKF-108,566 shares some interaction points on the receptor with the biphenyl nonpeptide antagonists, e.g., losartan. However, the binding of SKF-108,566 is also specifically

TABLE 2

K_i values of SKF-108,566 and analogs for the wild-type rat receptor and the His^{256} to alanine ([H256A]rAT$_1$) and Asn^{295} to serine ([N295S]rAT$_1$) mutants

The effect of the chemical difference between SKF-108,566 and the analogs on the affinity for the wild-type rat AT$_1$ receptor is expressed as the analog factor, *F*(ana) = K_i(analog)/K_i(SKF-108,566). The effect of the mutations on the binding affinity is expressed as the mutation factor, *F*(mut) = K_i(mutant receptor)/K_i(rat AT$_1$ receptor).



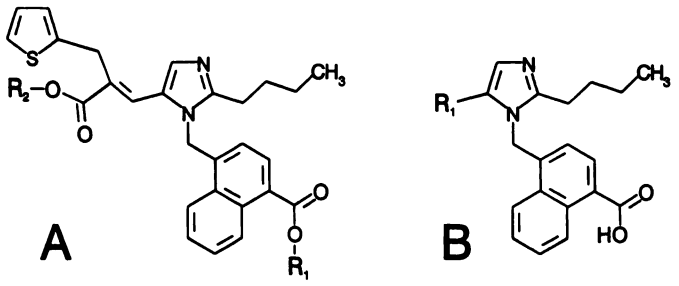
Compound	X	R_1	R_2	Wild-type rat AT$_1$			[H256A]rAT$_1$			[N295S]rAT$_1$		
				K_i	*n*[a]	*F*(ana)	K_i	*n*	*F*(mut)	K_i	*n*	*F*(mut)
				nM			nM			nM		
SKF-108,566	4-CO_2H	OH	CH_2-2-thienyl	5.4 ± 0.9	20	1.0	82.8 ± 13.2	8	15.3	134 ± 9.8	4	25
SKF-107,011	2-Cl	OH	CH_2-2-thienyl	101.6 ± 19.0	5	18.7	463.9 ± 39.2	3	4.6	>10,000	3	>100
SKF-108,728	3-CH_3, 4-OH	OH	CH_2-2-thienyl	195.5 ± 24.8	6	36.1	1,450 ± 180.1	3	7.4	7,658 ± 2,206	3	39
SKF-108,867	4-CN	OH	CH_2-2-thienyl	280.5 ± 9.5	3	51.8	1,924 ± 10.8	3	6.9	~10,000	3	~36
SKF-108,815	4-2′-Carboxyphenyl	OH	CH_2-2-thienyl	278.9 ± 39.4	6	51.5	614.9 ± 112.0	3	2.2	3,896 ± 1,377	3	14
SB-200,424	4-CH_2CO_2H	OH	CH_2-2-thienyl	5.0 ± 0.9	3	0.9	126.2 ± 25.4	3	25.3	810 ± 41	3	163
SKF-109,059	4-CO_2H	NH_2	CH_2-2-thienyl	1.5 ± 0.1	6	0.3	26.7 ± 5.7	3	17.6	86.7 ± 6.4	3	57
SB-206,754	4-CO_2H	OC_2H_5	CH_2-2-thienyl	1.1 ± 0.2	7	0.2	9.9 ± 2.0	3	9.3	36.4 ± 4.4	3	37
SB-200,491	4-CO_2H	OH	*n*-Butyl	17.8 ± 2.3	6	3.3	337.1 ± 31.8	3	18.9	747 ± 165	3	42
SB-201,529	4-CO_2H	OH	CH_2-phenyl	16.2 ± 2.5	6	3.0	234.7 ± 11.9	3	14.5	705 ± 133	3	44
SB-201,972	4-CO_2H	OH	H	49.5 ± 2.8	6	9.1	225.1 ± 32.8	3	4.6	3,540 ± 211	3	72
Angiotensin II[b]				0.19 ± 0.03	10		ND[c]			0.51 ± 0.05	7	2.7

[a] *n*, number of experiments.
[b] The affinity of angiotensin II was measured in the homologous wild-type and Asn^{295} to serine human AT$_1$ receptors. The B_{max} value for the Asn^{295} to serine mutant was 202 ± 34 fmol/10^5 cells (mean ± standard error, seven experiments).
[c] ND, not determined.

TABLE 3

K_i values of naphthalene derivatives for the wild-type rat receptor and the His^{256} to alanine ([H256A]rAT$_1$) and Asn^{295} to serine ([N295S]rAT$_1$) mutants

Compounds SB-203,220, SB-204,889, and SB-205,390 refer to structure A, whereas SB-204,403 and SB-205,706 refer to structure B. The effect of the chemical difference between SKF-108,566 and the analogs on the affinity for the wild-type rat AT$_1$ receptor is expressed as the analog factor, *F*(ana) = K_i(analog)/K_i(SKF-108,566). The effect of the mutations on the binding affinity is expressed as the mutation factor, *F*(mut) = K_i(mutant receptor)/K_i(rat AT$_1$ receptor).



Compound	Structure	R_1	R_2	Wild-type rat AT$_1$			[H256A]rAT$_1$			[N295S]rAT$_1$		
				K_i	*n*[a]	*F*(ana)	K_i	*n*	*F*(mut)	K_i	*n*	*F*(mut)
				nM			nM			nM		
SB-203,220	A	H	H	47.0 ± 5.2	5	8.7	37.1 ± 2.6	3	0.8	268 ± 65	3	6
SB-204,889	A	H	C_2H_5	14.6 ± 2.7	6	2.7	20.1 ± 0.5	3	1.4	243 ± 67	3	17
SB-205,390	A	C_2H_5	H	95.2 ± 11.9	6	17.6	106.9 ± 10.4	3	1.1	≫10,000	3	≫100
SB-204,403	B	H		183.2 ± 51.1	3	33.8	317.2 ± 41.2	3	1.7	7,526 ± 315	3	41
SB-205,706	B	*trans*-CH=CHCO$_2$H		60.8 ± 7.5	3	11.2	94.0 ± 1.3	3	1.4	4,205 ± 64	3	69

[a] *n*, number of experiments.

and uniquely dependent on His^{256} in TM-VI, conceivably through a carboxyphenyl/imidazole interaction.

His^{256} (HisVI:16) corresponds to a known ligand-binding residue in muscarinic and adrenergic receptors (12, 15, 33). It has been speculated that all agonists for G protein-coupled receptors, including peptides, bind in a pocket that corresponds to the ligand binding site in the small-messenger receptors (34, 35). Thus, it would be expected that the binding of angiotensin II would be affected by substitution of His^{256}. However, exchange of His^{256} with alanine, isoleucine,

phenylalanine, or glutamine had no effect on the binding of the peptide ligands angiotensin II and [Sar^1,Leu^8]-angiotensin II (Fig. 3; Table 1). This demonstrates that His^{256} is not the counterpart for the terminal carboxyl group in angiotensin II, which is known to be very important for binding and activity (36). More likely, the carboxyl-terminal carboxyl group interacts with Lys^{199} (LysV:08), as suggested previously (37). Interestingly, it was recently shown that mutation of Val^{108} in the AT_1 receptor, which corresponds to the important Asp^{113} in TM-III of the hamster β_2-adrenoceptor (AspIII:08), a residue that is totally conserved in monoamine receptors and is known to interact with both agonists and antagonists (12, 33), also did not affect the binding of angiotensin II but reduced the affinity of losartan 38-fold (38). These observations are analogous to previous findings in the tachykinin system, where a substitution in TM-VI did not affect peptide binding, whereas the binding affinity of some nonpeptide tachykinin antagonists was decreased (39). Consequently, it is likely that peptide agonists, at least in the angiotensin and tachykinin systems, do not bind in a pocket corresponding to the agonist binding site in the small-messenger receptors.

Substitution of His^{256} with alanine, isoleucine, or phenylalanine reduced the binding affinity of the imidazoleacrylic acid nonpeptide antagonist SKF-108,566. The effect of the mutation of His^{256} on the binding affinity of SKF-108,566 may be indirect, but because only the monophenylimidazole analogs and neither the biphenyl compounds nor the naphthalene analogs were affected by the mutations it seems likely that the decreased binding affinity was due to a direct effect.

We previously found that the binding of SKF-108,566 was affected quantitatively similarly to that of the other antagonists by mutations in TM-VI and -VII, e.g., Asn^{295} (11). It is concluded that, although SKF-108,566 appears to share many points of receptor interaction with the biphenylimidazole antagonists, it is uniquely dependent on His^{256}, which is located in the middle of TM-VI of the AT_1 receptor.

Analyses of the binding of analogs may provide information about the receptor binding domains of ligands (33, 40). Analogs lacking a moiety that in the prototype compound interacts with a particular residue would be expected to be resistant to mutational exchanges of this residue. In contrast, the prototype compound and other analogs would bind with reduced affinity to the mutated receptor (40). In other words, the binding energy that has already been diminished by removal of the moiety from the compound would not be further reduced by removal of the corresponding interaction point on the receptor.

The present data suggest that the carboxyl group in the *para*-position of the phenyl ring of SKF-108,566 interacts with His^{256}, because analogs lacking this group were bound with lower affinities by the wild-type receptor and were less susceptible to exchanges of His^{256} (Table 2). Furthermore, it appears that in the naphthalene compounds conformational restriction prevents interaction between the carboxyl group and His^{256}, because the affinities of these analogs for the wild-type receptor were lower than that of SKF-108,566 and the binding of these compounds was unaffected by the His^{256} to alanine mutation.

It is interesting to note that SB-200,424, in which the carboxyl group is in the *para*-position but there is a CH_2 group between the phenyl ring and the carboxyl group, interacted as well as SKF-108,566 with the wild-type AT_1 receptor and was affected by the His^{256} to alanine mutation just as was SKF-108,566. It is thus suggested that the carboxyl group in this analog retains the interaction with His^{256}. The present findings thus do not support the proposal that the carboxyl group of SKF-108,566 and the tetrazole of losartan (Fig. 1) should mimic each other and bind to the same area of the receptor as Tyr^4 of angiotensin II (7, 9, 10).

Exchange of His^{256} with glutamine led to affinities to SKF-108,566 comparable with those of the wild-type receptor (Table 1). This is in agreement with findings with tachykinin receptors, where it has been shown that glutamine can substitute for histidine residues in TM-V and -VI (39, 40). The ϵ-nitrogen of a histidine residue is in approximately the same position as the nitrogen atom in glutamine, indicating that the ϵ-nitrogen of His^{256} interacts with the carboxyl group of SKF-108,566, presumably through a charged or hydrogen bond interaction (39, 40).

The binding of all analogs was affected by substitution of Asn^{295} (Tables 2 and 3). This indicates that Asn^{295} is an important interaction point for these compounds, suggesting that a chemical group common to all of the analogs interacts with Asn^{295}. Because only the phenyl ring, the imidazole, and the butane group are common to all analogs (Fig. 1; Tables 2 and 3), it is likely that Asn^{295} interacts with the phenyl or imidazole group in an amino/aromatic interaction (41). This is supported by previous findings that chemically divergent biphenylimidazole derivatives sharing only these two ring structures were all affected by exchange of Asn^{295} (11, 32). We cannot, however, exclude an indirect effect of the exchange of Asn^{295}, because this residue may be important for stabilizing the receptor in a conformation that binds antagonists with high affinity.

Interestingly, the binding of SB-205,390, in which the carboxyl group in the *para*-position of the naphthalene ring is replaced by an ester, was reduced much more than the binding of the homologous SB-203,220 and SB-204,889, which both retain the carboxyl group. This further corroborates the idea that this carboxyl group does not interact with Asn^{295}, because exchange of the carboxyl group and the mutation reduce the binding affinity synergistically. In conclusion, the present data suggest that the imidazoleacrylic acid SKF-108,566 shares some interaction points, in particular Asn^{295}, on the AT_1 receptor with the biphenylimidazole derivatives. However, the dependence on His^{256} is unique, and presumably the ϵ-nitrogen of His^{256} could be involved in the interaction with the carboxyl group in position 4 of the phenyl ring (Fig. 1).

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References

1. Bumpus, F. M., K. J. Catt, A. T. Chiu, M. DeGasparo, T. Goodfriend, A. Husain, M. J. Peach, D. G. Taylor, Jr., and P. B. M. W. M. Timmermans. Nomenclature for angiotensin receptors: a report of the Nomenclature Committee of the Council for High Blood Pressure Research. *Hypertension (Dallas)* **17**:720–721 (1991).
2. Dudley, D. T., R. L. Panek, T. C. Major, G. H. Lu, R. F. Bruns, B. A. Klinkefus, J. C. Hodges, and R. E. Weishaar. Subclasses of angiotensin II binding sites and their functional significance. *Mol. Pharmacol.* **38**:370–377 (1990).
3. Murphy, T. J., R. W. Alexander, K. K. Griendling, M. S. Runge, and K. E.

Bernstein. Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature (Lond.)* **351**:233–236 (1991).
4. Bergsma, D. J., C. Ellis, C. Kumar, P. Nuthulaganti, H. Kersten, N. Elshourbagy, E. Griffin, J. M. Stadel, and N. Aiyar. Cloning and characterization of a human angiotensin-II type-1 receptor. *Biochem. Biophys. Res. Commun.* **183**:989–995 (1992).
5. Sandberg, K., H. Ji, A. J. L. Clark, H. Shapira, and K. J. Catt. Cloning and expression of a novel angiotensin II receptor subtype. *J. Biol. Chem.* **267**:9455–9458 (1992).
6. Mukoyama, M., M. Nakajima, M. Horiuchi, H. Sasamura, R. E. Pratt, and V. J. Dzau. Expression cloning of type-2 angiotensin-II receptor reveals a unique class of 7-transmembrane receptors. *J. Biol. Chem.* **268**:24539–24542 (1993).
7. Wexler, R. R., D. J. Carini, J. V. Duncia, A. L. Johnson, G. J. Wells, A. T. Chiu, P. C. Wong, and P. B. M. W. M. Timmermans. Rationale for the chemical development of angiotensin II receptor antagonists. *Am. J. Hypertens.* **5**:209–220 (1992).
8. Furukawa, Y., S. Kishimoto, and K. Nishikawa. Hypotensive imidazole derivatives. United States Patent 4,340,598 (1982).
9. Keenan, R. M., J. Weinstock, J. A. Finkelstein, R. G. Franz, D. E. Gaitanopoulos, G. R. Girard, D. T. Hill, T. M. Morgan, J. M. Samanen, C. E. Peishoff, L. M. Tucker, N. Aiyar, E. Griffin, E. H. Ohlstein, E. J. Stack, E. F. Weidley, and R. M. Edwards. Potent nonpeptide angiotensin-II receptor antagonists. 2. 1-(Carboxybenzyl)imidazole-5-acrylic acids. *J. Med. Chem.* **36**:1880–1892 (1993).
10. Moore, G. J. Designing peptide mimetics. *Trends Pharmacol. Sci.* **15**:124–129 (1994).
11. Schambye, H. T., S. A. Hjorth, D. J. Bergsma, G. Sathe, and T. W. Schwartz. Differentiation between binding sites for angiotensin II and nonpeptide antagonists on the angiotensin II type 1 receptors. *Proc. Natl. Acad. Sci. USA* **91**:7046–7050 (1994).
12. Schwartz, T. W. Locating ligand binding sites in 7TM receptors by protein engineering. *Curr. Opin. Biotechnol.* **5**:434–444 (1994).
13. Hjorth, S. A., H. T. Schambye, W. J. Greenlee, and T. W. Schwartz. Identification of peptide binding residues in the extracellular domains of the AT_1 receptor. *J. Biol. Chem.* **269**:30953–30959 (1994).
14. Sandberg, K. Structural analysis and regulation of angiotensin II receptors. *Trends Endocrinol. Metab.* **5**:28–35 (1994).
15. Wess, J. Molecular basis of muscarinic acetylcholine receptor function. *Trends Pharmacol. Sci.* **14**:308–313 (1993).
16. Weinstock, J., R. M. Keenan, J. Samanen, J. Hempel, J. A. Finkelstein, R. G. Franz, D. E. Gaitanopoulos, G. R. Girard, J. G. Gleason, D. T. Hill, T. M. Morgan, C. E. Peishoff, N. Aiyar, D. P. Brooks, T. A. Fredrickson, E. H. Ohlstein, R. R. Ruffolo, E. J. Stack, A. C. Sulpizio, E. F. Weidley, and R. M. Edwards. 1-(Carboxybenzyl)imidazole-5-acrylic acids: potent and selective angiotensin II receptor antagonists. *J. Med. Chem.* **34**:1514–1517 (1991).
17. Keenan, R. M., J. Weinstock, J. A. Finkelstein, R. G. Franz, D. E. Gaitanopoulos, G. R. Girard, D. T. Hill, T. M. Morgan, J. M. Samanen, J. Hempel, D. S. Eggleston, N. Aiyar, E. Griffin, E. H. Ohlstein, E. J. Stack, E. F. Weidley, and R. Edwards. Imidazole-5-acrylic acids: potent nonpeptide angiotensin-II receptor antagonists designed using a novel peptide pharmacophore model. *J. Med. Chem.* **35**:3858–3872 (1992).
18. Weinstock, J., D. T. Hill, R. M. Keenan, R. G. Franz, D. E. Gaitanopoulos, G. R. Girard, D. S. Eggleston, N. Aiyar, E. Griffin, E. Ohlstein, D. P. Brooks, M. Gellai, T. A. Frederickson, E. F. Weidley, and R. M. Edwards. A potent long-acting imidazole-5-acrylic acid angiotensin II AT-1 receptor antagonist. *Bioorg. Med. Chem. Lett.* **4**:23–28 (1994).
19. Carini, D. J., J. V. Duncia, P. E. Aldrich, A. T. Chiu, A. L. Johnson, M. E. Pierce, W. A. Price, J. B. Santella, G. J. Wells, R. R. Wexler, P. C. Wong, S. E. Yoo, and P. B. M. W. M. Timmermans. Nonpeptide angiotensin II receptor antagonists: the discovery of a series of *N*-(biphenylylmethyl)imidazoles as potent, orally active antihypertensives. *J. Med. Chem.* **34**:2525–2547 (1991).
20. Chang, R. S. L., P. K. S. Siegl, B. V. Clineschmidt, N. B. Mantlo, P. K. Chakravarty, W. J. Greenlee, A. A. Patchett, and V. J. Lotti. *In vitro* pharmacology of L-158,809, a new highly potent and selective angiotensin II receptor antagonist. *J. Pharmacol. Exp. Ther.* **262**:133–138 (1992).
21. Olins, G. M., V. M. Corpus, E. G. McMahon, M. A. Palomo, J. R. Schuh, D. J. Blehm, H. C. Huang, D. B. Reitz, R. E. Manning, and E. H. Blaine. *In vitro* pharmacology of a nonpeptidic angiotensin-II receptor antagonist, SC-51316. *J. Pharmacol. Exp. Ther.* **261**:1037–1043 (1992).
22. Cazaubon, C., J. Gougat, F. Bousquet, P. Guiraudou, R. Gayraud, C. Lacour, A. Roccon, G. Galindo, G. Barthelemy, B. Gautret, C. Bernhart, P. Perreaut, J. C. Breliere, G. Lefur, and D. Nisato. Pharmacological characterization of SR-47436, a new nonpeptide AT(1) subtype angiotensin-II receptor antagonist. *J. Pharmacol. Exp. Ther.* **265**:826–834 (1993).
23. Bradbury, R. H., C. P. Allott, M. Dennis, E. Fisher, J. S. Major, B. B. Masek, A. A. Oldham, R. J. Pearce, N. Rankine, J. M. Revill, D. A. Roberts, and S. T. Russell. New nonpeptide angiotensin-II receptor antagonists. 2. Synthesis, biological properties, and structure-activity relationships of 2-alkyl-4-(biphenylylmethoxy)quinoline derivatives. *J. Med. Chem.* **35**:4027–4038 (1992).
24. Middlemiss, D., G. M. Drew, B. C. Ross, M. J. Robertson, D. I. C. Scopes, M. D. Dowle, J. S. Akers, K. Cardwell, K. L. Clark, S. Coote, C. D. Eldred, J. Hamblett, A. Hilditch, G. C. Hirst, T. Jack, J. Montana, T. A. Panchal, J. M. S. Paton, P. Shah, G. Stuart, and A. Travers. Bromobenzofurans: a new class of potent, non-peptide antagonists of angiotensin II. *Bioorg. Med. Chem.* **1**:711–716 (1991).
25. Dickinson, K. E. J., R. B. Cohen, S. Skwish, C. L. Delaney, R. P. Serafino, M. A. Poss, Z. Gu, D. E. Ryono, S. Moreland, and J. R. Powell. BMS-180560, an insurmountable inhibitor of angiotensin II-stimulated responses: comparison with losartan and EXP3174. *Br. J. Pharmacol.* **113**:179–189 (1994).
26. Zhang, J. S., M. Entzeroth, W. Wienen, and J. C. A. Van Meel. Characterization of BIBS-39 and BIBS-222: two new nonpeptide angiotensin-II receptor antagonists. *Eur. J. Pharmacol.* **218**:35–41 (1992).
27. Wienen, W., N. Hauel, J. C. A. Vanmeel, B. Narr, U. Ries, and M. Entzeroth. Pharmacological characterization of the novel nonpeptide angiotensin-II receptor antagonist, BIBR-277. *Br. J. Pharmacol.* **110**:245–252 (1993).
28. Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**:61–68 (1989).
29. Johansen, T. E., M. S. Schøller, S. Tolstoy, and T. W. Schwartz. Biosynthesis of peptide precursors and protease inhibitors using new constitutive and inducible eukaryotic expression vectors. *FEBS Lett.* **267**:289–294 (1990).
30. Gether, U., T. E. Johansen, and T. W. Schwartz. Chimeric NK1 (substance P)/NK3 (neurokinin B) receptors. *J. Biol. Chem.* **268**:7893–7898 (1993).
31. DeBlasi, S., K. O'Reilly, and H. J. Motulsky. Calculating receptor number from binding experiments using the same compound as radioligand and competitor. *Trends Pharmacol. Sci.* **10**:227–229 (1989).
32. Schambye, H. T., B. C. v. Wijk, S. A. Hjorth, W. Wienen, M. Entzeroth, D. J. Bergsma, and T. W. Schwartz. Mutations in transmembrane segment VII of the AT(1) receptor differentiate between closely related insurmountable and competitive angiotensin antagonists. *Br. J. Pharmacol.* **113**:331–333 (1994).
33. Strader, C. D., I. S. Sigal, and R. A. F. Dixon. Structural basis of *beta*-adrenergic receptor function. *FASEB J.* **3**:1825–1832 (1989).
34. Hoflack, J., M. F. Hibert, and S. Trumpp-Kallmeyer. Three-dimensional models of gonadothyrotropin hormone receptor transmembrane domain. *Drug Design Discovery* **10**:157–171 (1993).
35. Hoflack, J., S. Trumpp-Kallmeyer, and M. Hibert. Molecular modelling of G protein-coupled receptors, in *3D QSAR in Drug Design. Theory, Methods and Applications* (H. Kubinyi, ed.). Escom, Leiden, The Netherlands, 355–372 (1993).
36. Khosla, M. C., R. R. Smeby, and F. M. Bumpus. Structure-activity relationship in angiotensin II analogs, in *Angiotensin* (I. H. Page and F. M. Bumpus, eds.). Springer-Verlag, Berlin, 126–161 (1974).
37. Yamano, Y., K. Ohyama, S. Chaki, D. F. Guo, and T. Inagami. Identification of amino acid residues of rat angiotensin-II receptor for ligand binding by site directed mutagenesis. *Biochem. Biophys. Res. Commun.* **187**:1426–1431 (1992).
38. Ji, H., M. Leung, Y. Zhang, K. J. Catt, and K. Sandberg. Differential structural requirements for specific binding of nonpeptide and peptide antagonists to the AT_1 angiotensin receptor: identification of amino acid residues that determine binding of the antihypertensive drug, losartan. *J. Biol. Chem.* **269**:16533–16536 (1994).
39. Zoffmann, S., U. Gether, and T. W. Schwartz. Conserved His-VI-17 of the NK-1 receptor is involved in binding of non-peptide antagonists but not substance P. *FEBS Lett.* **336**:506–510 (1993).
40. Fong, T. M., M. A. Cascieri, H. Yu, A. Bansal, C. Swain, and C. D. Strader. Amino-aromatic interaction between histidine 197 of neurokinin-1 receptor and CP 96345. *Nature (Lond.)* **362**:350–353 (1993).
41. Burley, S. K., and G. A. Petsko. Weakly polar interactions in proteins. *Adv. Protein Chem.* **39**:125–189 (1988).
42. Creighton, T. E. *Proteins. Structures and Molecular Properties*. W. H. Freeman, New York (1993).

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