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Molecular Determinants of the Species Selectivity of Neurokinin Type 1 Receptor Antagonists

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SUMMARY

Most nonpeptide neurokinin (NK)1 antagonists display a marked difference in affinity for rat versus human NK1 receptors. The molecular basis for the species selectivity of RP67580 and CP96,345 has been previously addressed [*J. Biol. Chem.* **267**:25668–25671 (1992); *J. Biol. Chem.* **268**:2319–2323 (1993)]. We are extending these previous results to additional NK1 antagonists, which are members of different chemical families. Included is a new perhydroisoindolol, RPR100893, which unlike its parent compound (RP67580) is human receptor selective. Chimeric rat/human NK1 receptors, as well as rat and human mutant NK1 receptors, were constructed and expressed in COS-1 cells, and affinities for substance P and the various antagonists were determined in binding studies. With human receptor-selective antagonists, the rat R290(S→I) mutation was the most effective in increasing antagonist affinity (from 7- to 23-fold). Combination with the R116(L→V) mutation led to an additional increase in affinity for *trans*-4-hydroxy-1-(1*H*-indol-3-ylcarbonyl)-L-prolyl-*N*-methyl-*N*-(phenylmethyl)-L-tyrosineamide (a derivative of FK888) and to nearly full human receptor affinity for RPR100893 and (±)-CP99,994. Based on the gains in affinities, these results confirm and extend the role of residues 116 and 290 of the NK1 receptor in the species selectivity of these three new human receptor-selective NK1 antagonists. In comparison, the affinity of RP67580, the least selective molecule, was most affected by changes at position 116, and combination with mutations at either position 97 (V→E) or position 290 led to the human receptor phenotype. For the heterosteroid KAN610857, modifications of the rat receptor at positions 97 and 290, and to a lesser degree position 116, were the most effective in reducing affinity. Two double-mutants [R(97,290) and R(116,290)], although different from those identified for RP67580, also displayed human receptor-like affinity. Therefore, the molecular determinants of the species selectivity appear to be different, in part, between rat and human receptor-selective compounds, even between closely related chemical families.

The tachykinins (SP, NKA, and NKB) form a family of neuropeptides sharing a carboxyl-terminal consensus sequence and mediating their biological effects by interaction with three related receptors, i.e., NK1, NK2, and NK3. The recent molecular cloning of the three receptors has revealed that they belong to the large family of seven-TMR receptors (1) and are highly homologous (41–51% identity in pairwise comparison). In parallel, specific nonpeptide antagonists of NK1 receptors, for instance CP96,345 (2) and RP67580 (3), and NK2 receptors (4) have been recently developed and have allowed a better characterization of the biological and pharmacological properties of these receptors (5).

Due to the structural similarity of the peptide agonists and of their receptors, as well as to the availability of selective nonpeptide ligands, the tachykinin family has thus represented an interesting system to address the issue of identification of both agonist and antagonist binding sites. By using a combination of deletion mutants (6) and chimeric receptors (7, 8), the binding site of peptide agonists was shown to include the amino terminus as well as part of the three extracellular loops, which is a quite different situation than in the monoamine receptors (9). Several key residues for agonist binding have been identified on the NK1 receptor, in the amino terminus, the first and second extracellular loops (10), and TMR II and TMR VII (11). Interestingly, the peptide binding sites, although likely to be largely overlapping, do not appear to involve the same individual residues for the three tachykinin receptors (6–8). In sharp contrast to agonists, the binding site for the NK1 antagonists appears to extend from TMR V to TMR VII (10–12). His-197 in TMR V (13) and Tyr-272 in the third extracellular loop (14) could possibly interact with CP96,345, and His-265 in TMR VI (15, 16) and Tyr-287 in TMR VII (11) could interact with RP67580.

A different approach to the identification of amino acids on the receptor involved in antagonist binding has been to take advantage of the pronounced species selectivity of NK1 antag-

ABBREVIATIONS: SP, substance P; HIPT, *trans*-4-hydroxy-1-(1*H*-indol-3-ylcarbonyl)-L-prolyl-*N*-methyl-*N*-(phenylmethyl)-L-tyrosineamide; TMR, transmembrane regions; NK, neurokinin; RT, reverse transcription; PCR, polymerase chain reaction.

onists, in contrast to the endogenous ligand SP. CP96,345 and FK888 display 90- and 600-fold higher affinity, respectively, for the human versus the rat NK1 receptor (17, 18), whereas RP67580 and WIN 51708 have opposite selectivity, being 5- and >1000-fold more potent at the rat receptor (19, 20). Rat and human receptors differ by only 22 of 407 amino acids, and the molecular basis of the species selectivity of CP96,345 and RP67580 has been mapped to residues 116 (TMR III) and 290 (TMR VII) by Fong *et al.* (21). Those authors favored a conformational role for these two residues in the antagonist binding pocket. Similar results have been recently obtained for FK888 (22). In comparison, Sachais *et al.* (23) identified residue 290 and residues in the second extracellular loop as contributing to the human receptor selectivity of CP96,345. In parallel, using a similar approach (rat/human NK1 receptor chimeras as well as point mutants), we have extended these previous studies to new antagonists, of different chemical families, that exhibit even greater species selectivity. In particular, we have studied a new derivative of the perhydroisoindolone RP67580, RPR100893 (24), that, unlike its parent compound, displays strong selectivity for the human receptor. Binding studies were conducted on membrane preparations using tritiated ligand, compared with binding to intact cells using an iodinated label as in the previous reports. Preliminary results of the present study have been presented previously (25).

Materials and Methods

Peptide and nonpeptide ligands. [Prolyl^{2,4}-3,4(N)-^{3}H]SP was obtained from Amersham (Buckinghamshire, UK). SP was purchased from Bachem (Butendorf, Switzerland). RP67580, RPR100893 [(3a*S*,4*S*,7a*S*)-7,7-diphenyl-4-(2-methoxyphenyl)-2-[(*S*)-2-(2-methoxyphenyl)propionyl]perhydroisoindol-4-ol], (±)-CP99,994, and HIPT were synthesized in the Rhône-Poulenc Rorer Chemistry Department. KAN610857 was kindly provided by Dr. Bruce Tomczuk, Eastman Kodak Company (Rochester, NY).

Construction. The rat NK1 receptor cDNA was obtained by RT-PCR from rat brain mRNA, using the published sequence (26). A partial human NK1 receptor cDNA was obtained by RT-PCR from the human U-373 MG cell line mRNA, using degenerate oligonucleotides. A full length cDNA was later obtained by hybridization screening of a human brain cDNA library and RT-PCR of the 3′ end. Full length cDNAs were subcloned in the pSV2 eukaryotic expression vector. For rat/human chimeric constructions, three restriction sites conserved in both species were used, defining four segments in the receptor that were shuffled between species for the desired constructs. Chimeras were named with a four-letter code according to the species origin (R for rat or H for human) of each segment, e.g., HHHR represents the chimera made of the first three segments of the human receptor and the fourth of its rat counterpart. The restriction sites were *Stu*I (nucleotide 235), *Ppu*MI (nucleotide 465), and *Bgl*II (nucleotide 825), with nucleotide 1 being the first nucleotide of the coding sequence. The corresponding amino acid positions were Ala-79, Val-156, and Leu-277, respectively. For digestion with *Ppu*MI, plasmids were prepared from a *dam*$^-$ *Escherichia coli* strain.

Site-directed mutagenesis was performed on single-stranded DNA (M13 mp19) using the thionucleotide method (Amersham). Mutations were verified by automated sequencing using fluorescent dideoxy nucleotides, and the absence of additional mutations was verified for the entire sequence before subcloning into the expression vector.

Transfection. COS-1 cells were transfected by the Transfectam method (Sepracor). Briefly, cells (2×10^6/10-cm dish) were rinsed in serum-free Dulbecco's modified Eagle's medium for about 15 min before transfection. The Transfectam stock solution (in ethanol) was diluted in water (75 μg in 300 μl/dish), as was the expression DNA (8 μg in 300 μl/dish), and each was mixed thoroughly. After a 5-min incubation, Transfectam and the DNA solutions were pooled and vortex-mixed thoroughly. After an additional 5 min, the solution was applied to the cells in serum-free Dulbecco's modified Eagle's medium for 3–5 hr. The cells were then rinsed and serum-containing medium (10% fetal calf serum) was added. After 72 hr, the cells were harvested by scraping in hypo-osmotic homogenization buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$) and were lysed in a Dounce homogenizer, followed by two centrifugation steps. Crude membrane preparations were then resuspended in homogenization buffer at approximately 1 mg/ml, divided into aliquots, and stored at −80° for further use.

Binding. [^{3}H]Prolyl-SP binding experiments were performed as described by Fardin *et al.* (19), except that the final reaction volume was 300 μl. The reaction solution contained 50 mM Tricine, pH 7.4, 10 mM $MgCl_2$, 1 mg/ml glucose, 0.2 mg/ml bovine serum albumin, 40 μg/ml bacitracin, 5 μg/ml leupeptin, and 5 μg/ml bestatin. The membrane protein concentration varied from 5 to 30 μg/ml, and for displacement studies the concentration of labeled SP was approximately 1 nM (ranging from 0.93 to 1.09 nM). The incubation was carried out at room temperature (22°) for 30 min (except for displacement experiments with RPR100893, which used an incubation time of 90 min) before filtration, using a cell harvester (Skatron, Sterling, VA), onto a Whatman GF/C filter that had been presoaked in polyethylenimine (0.3%). Bound radioactivity on the filters was determined by scintillation counting (Ready Gel; Beckman). Equilibrium dissociation constants (K_d) and binding densities (B_{max}) were determined on membranes by saturation binding experiments (ligand concentrations ranging from 0.1 to 8 nM) carried out in triplicate. In displacement studies, experiments were carried out in duplicate.

Data were analyzed using EBDA/LIGAND software, and K_i values for antagonists were derived from the half-maximal inhibitory concentrations using the Cheng-Prusoff equation, $K_i = IC_{50}/(1 + [[^3H]SP]/K_d)$, where K_d represents the dissociation constant of SP for the chimera.

Protein concentrations were determined using the Bio-Rad protein assay system, with bovine serum albumin as a standard.

Results

SP Binding Characteristics

To understand the molecular basis of the strong species selectivity (rat versus human) of most NK1 antagonists, we have constructed rat/human NK1 receptor chimeras as well as point mutants (resulting in the alteration of rat to human receptor sequence at the mutated positions), either individually or in combination. Briefly, using relevant restriction sites, the receptor sequence was divided into four segments whose limits were in the lower TMR II, the upper TMR IV, and the third extracellular loop (Fig. 1). When transiently expressed in COS-1 cells, all chimeras and mutants resulted in high densities of high affinity SP binding sites, with maximal binding densities ranging from 1 to 8 pmol/mg of protein (crude membrane preparations). The rat receptor displayed a slightly higher affinity for [^{3}H]SP (with a K_d value of 0.56 ± 0.18 nM) than did the human receptor (K_d value of 0.91 ± 0.06 nM; difference not statistically significant at this power of analysis) (Table 1). In functional studies measuring SP-induced inositol phosphate hydrolysis, the same rank order of potency for SP was found in rat and human receptor-transfected cells, with half-maximal activation values of 0.05 ± 0.02 and 0.09 ± 0.03 nM, respectively (data not shown). Most chimeric and mutant receptors displayed SP dissociation constants similar to rat and human receptor values. Substitution at position 97 appeared to be the most effective at altering SP affinity (Table 2), with the difference in affinity between the mutant (position 97) and wild-type receptors being statistically significant ($p < 0.05$, two-tailed

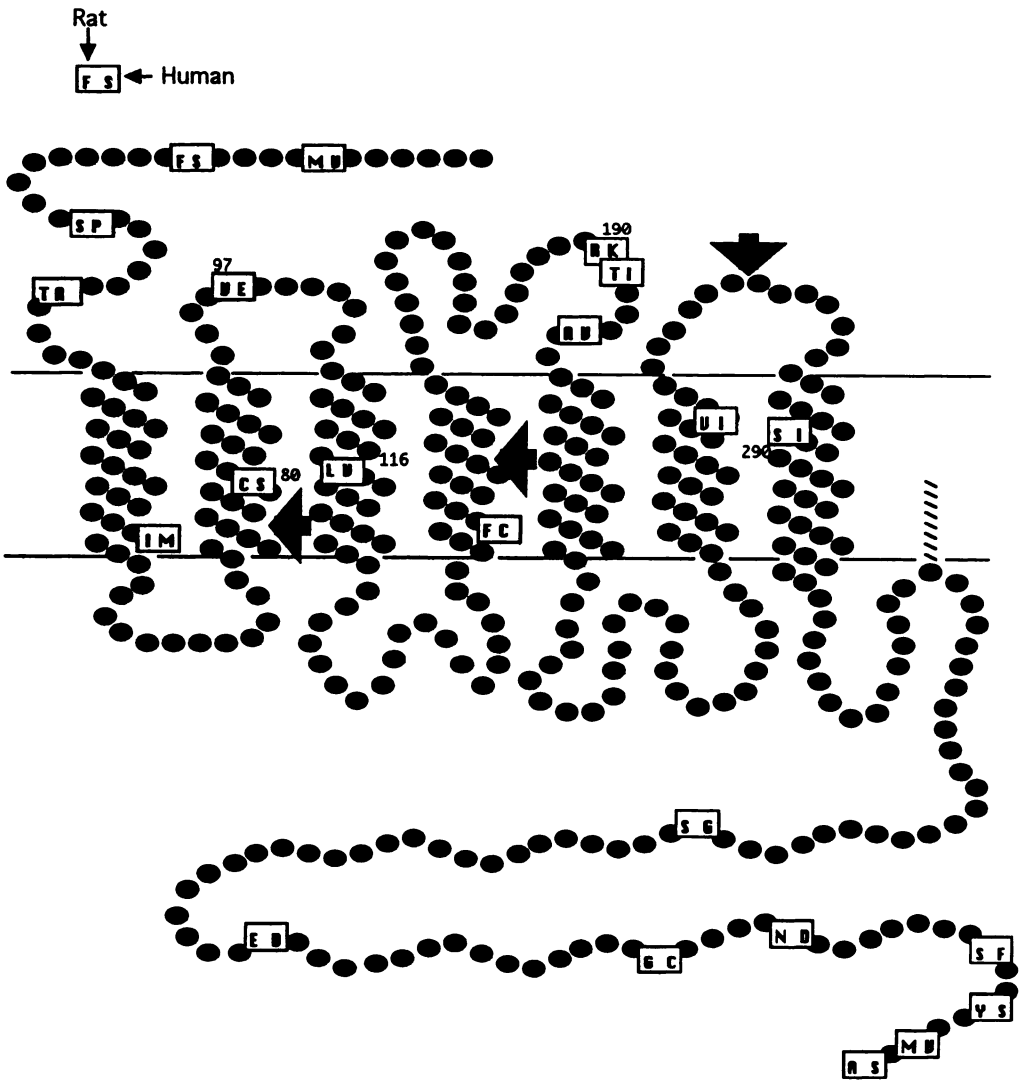


Fig. 1. Comparison of rat and human NK1 receptor sequences. The structural model of the NK1 receptor indicates the rat/human differences in primary structure (*boxes*). The one-letter code is used, and *residues on the left* correspond to those in the rat receptor. Positions of rat/human differences referred to in the text are also indicated by numbers. *Arrows*, positions of the restriction sites used to construct chimeras.

TABLE 1

Equilibrium dissociation constants of tachykininergic ligands for chimeric NK1 receptors

Chimeric receptors were constructed as described in Materials and Methods, and [^{3}H]SP binding studies were performed on membranes of transfected COS-1 cells. For SP, K_d values were obtained from saturation experiments. For competition experiments, K_i values were derived from IC_{50} values according to the Cheng-Prusoff equation, $K_i = IC_{50}/(1 + [[^3H]SP]/K_d)$, where K_d represents the SP dissociation constant for this chimera. Data represent means ± standard errors of three to five experiments.

Receptors	K_d or K_i							
	Rat NK1	Human NK1	HHHR	RRRH	RHHR	HRRH	RHRR	RRHR
	nM							
SP[a]	0.56 ± 0.18	0.91 ± 0.06	1.09 ± 0.09	0.88 ± 0.28	1.13 ± 0.14	0.95 ± 0.04	1.97 ± 0.22	0.49 ± 0.05
RPR100893	336 ± 42	4.5 ± 0.7	76 ± 5	46 ± 10	75 ± 19	47 ± 4	88 ± 8	127 ± 9
(±)-CP99,994	106 ± 22	0.4 ± 0.1	16 ± 3	3.4 ± 0.7	8 ± 1	4.5 ± 0.5	18 ± 3	83 ± 5
HIPT	2,290 ± 72	8 ± 1	349 ± 42	237 ± 35	280 ± 34	189 ± 24	1,290 ± 245	1,300 ± 300
RP67580	10 ± 2	54 ± 5	42 ± 11	20 ± 3	49 ± 1	29 ± 1	73 ± 9	16 ± 5
KAN610857 (1E)	301 ± 45	24,939 ± 1,821	>10,000	>10,000	>10,000	>10,000	>10,000	261 ± 91

[a] K_d.

Student's *t* test) in both the rat and human backgrounds. The SP affinity of the R97(L→V) mutant was not statistically different from that of the human receptor.

NK1 Receptor Antagonist Species Selectivity

Several NK1 antagonists with marked species selectivity were selected for this study. (±)-CP99,994 and RP67580 are well characterized compounds, whereas HIPT is a derivative of FK888 (18) and KAN610857 is a derivative of WIN51708, described as compound **1e** by Appell *et al.* (20). RPR100893 has been recently described (24) and is a derivative of RP67580, although the stereoselectivity of the heterocycle is opposite (3a*S*,7a*S*). For chimeric/mutant receptors, the affinity for the various antagonists generally fell between the values for rat

TABLE 2

Equilibrium dissociation constants of tachykininergic ligands for species-related mutant NK1 receptors

Mutants were constructed either from the rat receptor [i.e., R97(V→E)] or, in some instances, from the human NK1 receptor [i.e., H97(E→V)]. Binding experiments were performed as described in the legend to Table 1. Data represent means ± standard errors of at least three experiments.

Receptor	K_d SP	K_i				
		Human NK1-selective antagonists			Rat NK1-preferring antagonists	
		RPR100893	(±)-CP99,994	HIPT	RP67580	KAN610857
	nM	nM	nM	nM	nM	nM
Rat	0.56 ± 0.18	336 ± 42	106 ± 22	2290 ± 72	10 ± 2	0.3 ± 0.04
Human	0.91 ± 0.06	4.5 ± 0.7	0.6 ± 0.1	7.9 ± 1	54 ± 5	24.9 ± 1.8
R80(C→S)	0.71 ± 0.11	247 ± 27	ND[a]	>1000	8 ± 2	0.3 ± 0.04
R97(V→E)	1.43 ± 0.25	421 ± 50	250 ± 37	3078 ± 370	15 ± 3	5.6 ± 0.8
H97(E→V)	0.60 ± 0.05	22 ± 9	ND	17 ± 1	46 ± 7	22.6 ± 2.8
R116(L→V)	0.87 ± 0.20	106 ± 9	13 ± 2	977 ± 112	33 ± 4	0.9 ± 0.04
R290(S→I)	0.93 ± 0.13	39 ± 3	4.5 ± 1.1	332 ± 31	19 ± 5	8.7 ± 0.85
H290(I→S)	1.39 ± 0.26	109 ± 1	16 ± 3	327 ± 25	41 ± 5	24.1 ± 0.9
R(97,116)	1.23 ± 0.15	79 ± 10	40 ± 3	505 ± 79	51 ± 7	10.2 ± 1.0
R(97,290)	1.72 ± 0.27	27 ± 4	8.9 ± 2.4	ND	18 ± 5	19.7 ± 1.5
R(116,290)	0.67 ± 0.09	7.8 ± 1.5	1 ± 0.1	34 ± 6	60 ± 11	19.5 ± 1.8
R(97,116,290)	0.92 ± 0.07	7.8 ± 1.6	1.4 ± 0.2	19 ± 6	89 ± 11	24.4 ± 2.9

[a] ND, not determined.

and human receptors (Tables 1 and 2). Exchange of rat for human receptor sequences in the third segment (from extracellular loop 2 to extracellular loop 3) (chimera RRHR) only marginally altered antagonist affinities, neither favoring human receptor-preferring compounds (RPR100893 or CP99,994) nor decreasing affinities for rat receptor-preferring antagonists (RP67580 or KAN610857) (Table 1). Accordingly, the double mutation R190–191(RT→KI) in this third segment did not affect antagonist binding (data not shown) and, although species differences in this region might contribute somewhat to antagonist binding, they were not further resolved.

Human Receptor-Selective Antagonists

RPR100893 and CP99,994. RPR100893 is a perhydroisoindolol derivative (Fig. 2) that, unlike RP67580, exhibits a strong human receptor preference (K_i value of 4.5 ± 0.7 nM with the human NK1 receptor), having weak activity with the rat receptor (K_i value of 336 ± 42 nM). With the rat receptor, exchange of the fourth segment (chimera RRRH) increased affinity for RPR100893 (Table 1), which was confirmed with the single-point mutant R290(S→I) (K_i value of 39 ± 3 nM) (Table 2). Reciprocally, the symmetrical human receptor mutant H290(I→S) exhibited an even more pronounced 24-fold loss of affinity (Table 2). To a lesser extent, segment 2 (chimeras RHRR and RHHR) also contributed to species selectivity (Table 1), with the mutation from leucine to valine at position 116 being the most significant [R116(L→V)] (see Table 2), especially when combined with mutations at residue 80 or 97 [K_i values of 45 ± 2 and 79 ± 4 nM for R(80,116) (data not shown) and R(97,116), respectively]. Combination of mutations at positions 290 and 116 (but not any other) resulted in a receptor with an affinity for RPR100893 similar to that of the human receptor (Table 2). The gain in binding energies was almost additive when the two human receptor residues were combined (Fig. 3), suggesting independent mechanisms. For CP99,994, the binding profile with the different mutant receptors was qualitatively identical to that of RPR100893 (Fig. 3; Table 2) and similar to previous results obtained with CP96,345.

HIPT. From studies with the chimeric constructs, it is clear that no segment of the receptor individually plays a major role in the species selectivity of the pseudo-dipeptide HIPT (Table 1). This was confirmed with point mutants, for which the largest gain in affinity was a modest 7-fold increase for mutant R290(S→I) (Fig. 3; Table 2). There was a marked synergistic

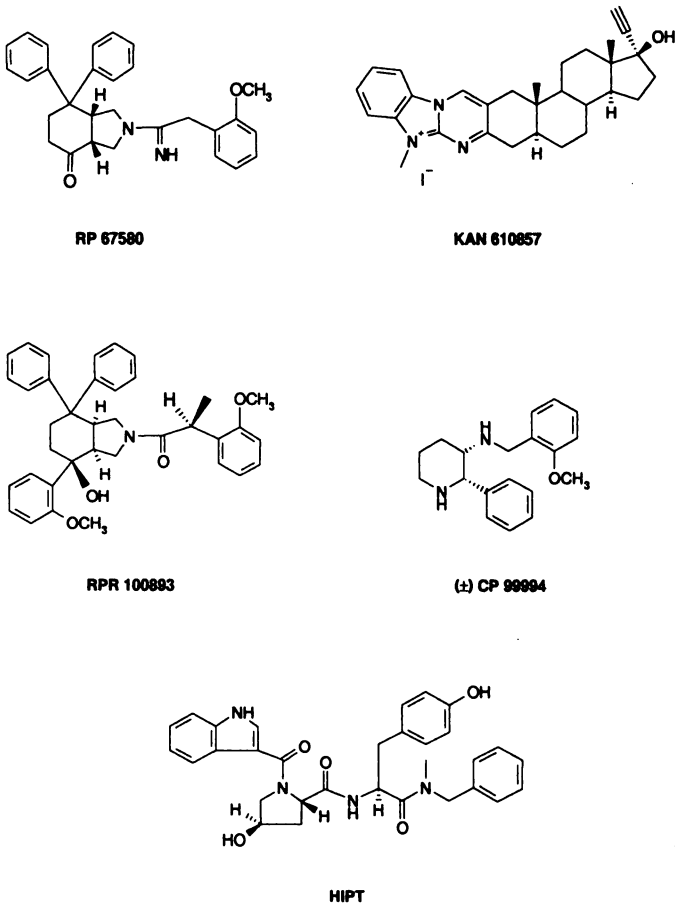


Fig. 2. NK1 receptor antagonists. Chemical structures of five distinct NK1 receptor antagonists displaying marked species selectivity are shown. HIPT is a derivative of FK888, and KAN610857 is compound **1e** in the work of Appell *et al.* (20).

HUMAN-NK1 SELECTIVE ANTAGONISTS

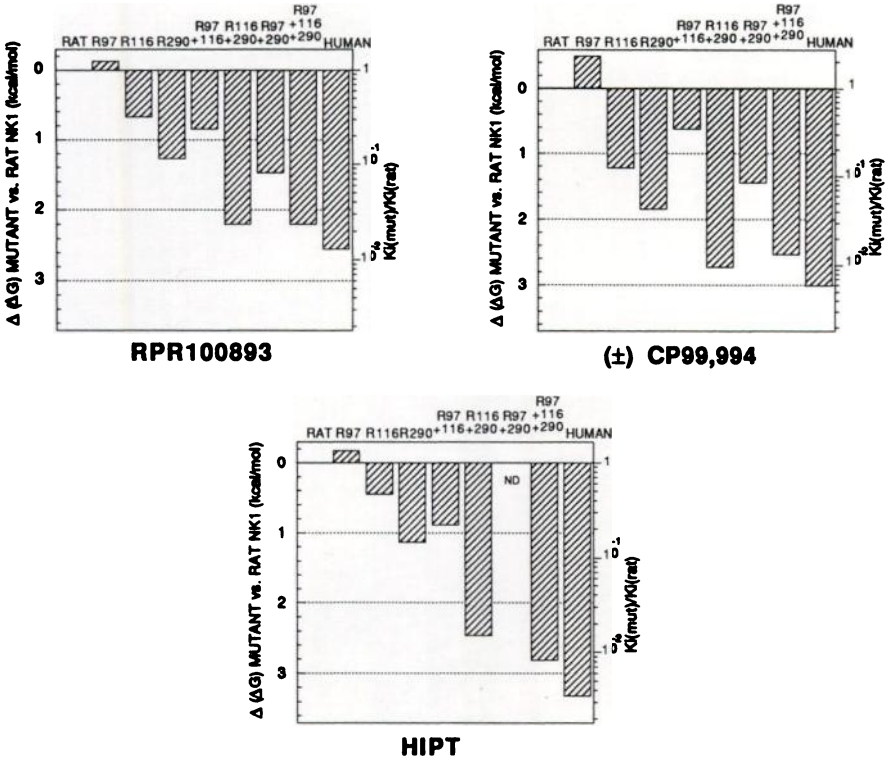
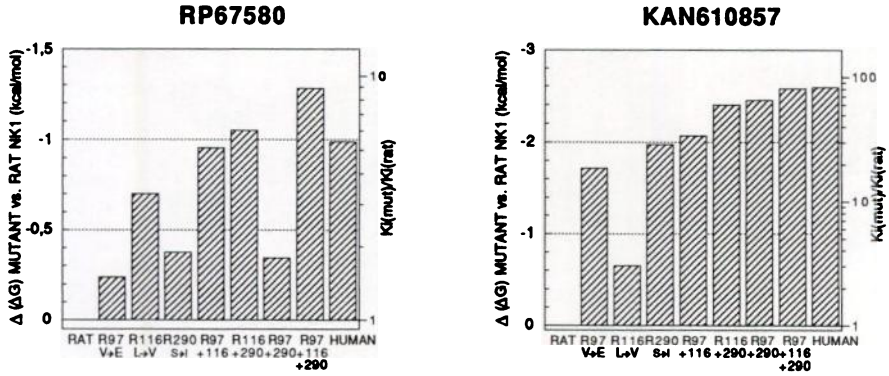


Fig. 3. Energy contribution of the different mutant NK1 receptors to the species selectivity of NK1 antagonists. Changes in energy of the receptor/antagonist interaction were obtained according to the equation $\Delta(\Delta G) = -RT\ln[K_{i(mut)}/K_{i(ratNK1)}]$, where $K_{i(mut)}$ and $K_{i(ratNK1)}$ represent the antagonist affinities for the mutant and rat NK1 receptors, respectively, and are derived from Table 2. An upward shift corresponds to a loss of binding energy. *ND*, not determined.

RAT-NK1 SELECTIVE ANTAGONISTS



effect between mutations at positions 116 and 290, with the gain in binding energy of the double-mutant being larger (2.4 kcal/mol) than the sum of the values for the individual mutants (1.6 kcal/mol) (Fig. 3). The gain in binding energy for R(116,290) is comparable to values obtained with RPR100893 and CP99,994, further emphasizing the importance of these two residues in the species selectivity of human receptor-preferring antagonists. However, the double mutation does not confer the full human receptor phenotype (with the difference from the human receptor affinity being highly significant, $p < 0.01$). Additional residues are likely to be involved in conferring species selectivity, such as Glu-97 (Table 2) and possibly residues in the third segment of the protein, as deduced from the significant gain in affinity of chimera RHHR, compared with RHRR (K_i values of 280 ± 34 and 1290 ± 245 nM, respectively) (Table 1). In this regard, HIPT displays a profile of species selectivity more complex than those of RPR100893 and CP99,994.

Rat Receptor-Preferring Antagonists

RP67580. RP67580 displayed a 5-fold difference in affinities for rat and human receptors, as published previously (19). All mutants displayed a fairly high affinity for RP67580, which, together with the similar results on SP affinity, confirmed that no gross conformational modification of the receptors (either agonist or antagonist sites) had occurred. From chimeric studies (Table 1), it appeared that segment 2 contributed most to the

species preference of RP67580 for the rat receptor, with chimera RHRR possessing an even lower affinity for RP67580 (K_i value of 73 ± 9 nM) than the human receptor (54 ± 5 nM). The mutation R116(L→V) had the largest effect on binding (Table 2) and, interestingly, two different combinations, R(116,97) and R(116,290), could confer the human receptor phenotype. Combination of these three mutations, in R(97,116,290), resulted in a lower affinity than that of the human receptor ($p < 0.05$), suggesting that in the latter the additional species differences might have a compensatory effect for RP67580 affinity. Contributions in the loss of binding energy were almost additive for all three mutations (Fig. 3).

KAN610857. Finally, KAN610857 displayed a larger species selectivity (80-fold) for the rat receptor. In this receptor, exchange of any segment (except for the third, as mentioned previously) led to a sharp loss of affinity (Table 1). The individual mutations R97(V→E) and R290(S→I) were the most effective in reducing KAN610857 affinity (19- and 29-fold, respectively), in contrast to the results observed with RP67580. Accordingly, the double-mutant R(97,290) displayed the same affinity as the human receptor. Mutation at position 116 by itself had a small effect on KAN610857, but its contribution was additive with that of mutation at position 290, resulting again in a double-mutant with a human receptor phenotype. Therefore, the human receptor configuration could be obtained in two different ways, as for RP67580. However, these two combinations are different from those obtained with RP67580, although they involve the same three residues. The triple-mutant R(97,116,290) and the human receptor had similar affinities. Individual reciprocal mutations in the human receptor did not improve affinity for KAN610857 (Table 2), suggesting that loss of affinity is achieved more readily than gain of affinity. All three mutations could have a synergistic effect, as suggested by the nonadditivity of the loss of binding energies of the rat receptor. Alternatively, additional residues could be needed to confer high affinity in a human background.

Discussion

There is a major interest in mapping the binding sites of both agonists and antagonists on G protein-coupled receptors. In this respect, the present study was undertaken to identify the amino acids on the NK1 receptor involved in the strong species selectivity (human versus rat) of several NK1 antagonists belonging to different chemical families. Identification of such residues could possibly provide clues to the binding site of antagonists. The binding properties of several rat/human NK1 receptor chimeras, as well as point mutants corresponding to species differences, were analyzed.

Three human receptor-selective antagonists were tested with our constructs and displayed a large gain in affinity for point mutants constructed on the rat receptor background. Two residues, Ile-290 and Val-116, primarily contributed to the human receptor selectivity of all three compounds, as described previously for CP96,345 (21–23) and for FK888 (22) with human reciprocal mutants. These results are thus extended to new, structurally different antagonists. For RPR100893 and CP99,994, the contributions of the two residues were additive in terms of binding energy, suggesting independent effects, and resulted in a human receptor-like receptor. CP99,994, a simpler derivative of CP96,345 bearing only one benzyl group at the C2-position, behaved similarly to CP96,345, demonstrating that the second benzyl moiety does not contribute to the species selectivity of this compound. Concerning HIPT, the two mutations displayed a pronounced cooperative effect. Unlike for RPR100893 and CP99,994, additional residues, including position 97 and residues in the third segment of the receptor, contribute to the full human receptor phenotype, as indicated by some of the present chimera results.

Altogether, the species selectivity of the human receptor-selective antagonists is determined in a largely similar way by residues 116 and 290. Without knowledge regarding direct interactions with the receptor, this similarity indicates that the overall steric binding sites of the three antagonists are overlapping.

RP67580 (the parent compound of RPR100093) has been extensively characterized (3) and has a small affinity preference for the rat receptor (19). The individual mutation at position 116 had the largest effect on affinity, unlike the small effect of mutation at position 290. Interestingly, two different double mutations, R(97,116) and R(116,290), could confer the human receptor phenotype (5-fold loss of affinity), indicating that several possible arrangements could lead to the human binding site conformation, as observed at the chimeric level by others (21). Combination of all three mutations yielded an even lower affinity, suggesting that, with the human receptor, additional mutations compensate for the loss of affinity. Finally, the molecular determinants of the species selectivity of the heterosteroid KAN610857 were shown to be different from those for all other compounds, with the mutation R97(V→E) along with R290(S→I) resulting in the greatest loss of binding affinity. However, the reciprocal mutations of the human receptor did not produce significant gain in affinity. Again, two double mutations, R(97,290) and R(116,290), could lead to an affinity similar to that of the human receptor. Although the same three residues were involved, these two combinations were different from those obtained for RP67580, which, along with the more pronounced effects of the individual mutations at positions 97 and 290, emphasized the differences between the two compounds. In comparison with human receptor-selective antagonists, the contribution of residue 97 to the species selectivity of rat receptor-preferring antagonists indicates a significant difference in the molecular determinants of this selectivity between the two types of NK1 antagonists.

A key question regarding mutagenesis experiments is whether the affinity modifications observed with mutants are due to a direct interaction of the amino acid residue with the antagonist, thereby providing information on the binding site determinants, or are due to a conformational modification of the receptor. At position 116, the leucine to valine mutation corresponds to a very small change in the physicochemical nature of the side chain and results in a 0.4–1.1 kcal/mol modification in binding energy. Therefore, this position is likely involved in a slight conformational modification of the binding site. Regarding position 97, the presence of an acidic group on the human receptor corresponds to a drastic difference, compared with the rat valine residue. The negative charge is detrimental to the binding of rat receptor-selective antagonists, but the symmetrical mutation in the human receptor does not lead to any gain in affinity. Therefore, a direct electrostatic interaction with the antagonists appears less likely. At position 290, the serine (rat) and isoleucine (human) residues also have significantly different physicochemical properties, which could

contribute directly to the binding of antagonists. For most antagonists, reciprocal modifications of position 290 in the rat and human receptors have symmetrical effects, and involvement of the nearby residue Tyr-287 in the binding of agonists and of RP67580 has also been reported recently (11). Interestingly, position 290 on the NK1 receptor is adjacent to the residue equivalent to Asn/Thr-355 in 5-hydroxytryptamine type 1B/1Dβ receptors (27, 28), which is also involved in a marked species selectivity of ligand binding, or to Asn/Phe-312 in α- and β-adrenergic receptors (29) or to Asn-355 in the 5-hydroxytryptamine type 1A receptor (30), which have all been suggested to interact directly with antagonists. In these examples, the asparagine (or glutamine) side chain has been shown to interact directly with the ether oxygen of pindolol (31). Structural modeling of G protein-coupled receptors based on structural data from rhodopsin also indicates that the amino acid corresponding to Ser-290 in TMR VII (i.e., VII-6) could face the interior of the cleft delimited by the seven transmembrane helices (32) and could potentially provide interactions with antagonists. However, our present results are also consistent with a conformational effect of the mutation at position 290, and further experiments with the introduction of chemically different residues at position 290, as well as testing of additional perhydroisoindoles, will be needed to decide between these two hypothesis. The residues identified in the present study are different from those shown to interact with CP96,345 or RP67580, i.e., His-197 in TMR V (13), His-265 in TMR VI (15, 16), Tyr-272 in the third extracellular loop (14), and Tyr-287 (11). However, the involvement of multiple residues of the receptor in the binding of antagonists is well documented (9).

In conclusion, the molecular basis of the marked species selectivity of NK1 antagonists differs with the type of antagonist, especially between human and rat receptor-selective compounds. Although most antagonists certainly share the same general steric binding site, the antagonist-receptor binding interactions are likely to be different for each compound, even for two members of closely related chemical families. While the present paper was in the review process, results similar to those obtained with KAN610857 were published by Sachais and Krause (33), using another derivative, WIN51708.

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