0026-895X/95/040660-06$3.00/0

MOLECULAR PHARMACOLOGY, 47:660–665 (1995).

Characterization of the Interaction of Diacylpiperazine Antagonists with the Human Neurokinin-1 Receptor: Identification of a Common Binding Site for Structurally Dissimilar Antagonists

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Received December 27, 1994; Accepted January 25, 1995

SUMMARY

We recently described a novel series of diacylpiperazine antagonists of the human neurokinin (NK)-1 receptor. The diacylpiperazine compounds are structurally dissimilar from previously described NK-1 antagonists. L-161,664 [1-(*N*,*N*-diphenylaminocarbonyl)-4-(*N'*,*N'*-di-*n*-pentylaminocarbonyl) piperazine-2-diethylaminopropylcarboxamide] inhibits ^{125}I-substance P binding to the human NK-1 receptor with an IC_{50} of 43 ± 21 nM but has 50-fold and 200-fold lower affinity for the human NK-2 and NK-3 receptors, respectively. L-161,664 inhibits substance P-stimulated inositol monophosphate accumulation in Chinese hamster ovary cells expressing the human NK-1 receptor by increasing the EC_{50} for substance P but not its maximal effect. The compound decreases the apparent affinity of the NK-1 receptor for ^{125}I-substance P and does not alter the rate of dissociation of ^{125}I-substance P from the receptor. These data indicate that L-161,664 is a potent and selective competitive antagonist of the human NK-1 receptor. L-161,664 has reduced affinity for mutants of the NK-1 receptor in which alanine has replaced Gln-165 in transmembrane helix 4, His-197 in helix 5, His-265 in helix 6, or Tyr-287 in helix 7. Similarly, a novel series of acyclic 2-benzhydryl-2-aminoethyl ethers that we have recently shown to be competitive NK-1 receptor antagonists have reduced affinity for the Q165A, H197A, and H265A mutant receptors. These residues have been shown to be important for binding of quinuclidine, tryptophan benzyl ester, and perhydroisoindole antagonists to the receptor. Analysis of the interaction of structural analogs of L-161,664 with the Q165A mutant receptor suggests that this residue interacts with the 2-diethylaminopropylcarboxamide side chain of L-161,664. Thus, even though the diacylpiperazine antagonists are structurally dissimilar from other classes of antagonists described to date, these data suggest that a common antagonist binding site that accomodates much structural diversity is present in the human NK-1 receptor. Furthermore, these data, combined with those obtained from medicinal chemistry approaches, suggest a minimum pharmacophore map for the interaction of these diverse ligands with the NK-1 binding site.

The neuropeptide SP is a member of a family of peptides, termed tachykinins, that share the carboxyl-terminal sequence Phe-X-Gly-Leu-Met-NH_2. These peptides interact with three distinct NK receptors, the NK-1, NK-2, and NK-3 receptors. The cloning of the genes encoding these proteins revealed that they are members of the family of G protein-coupled receptors characterized by seven transmembrane helical domains (1–6). These receptors and peptides are widely distributed within the central and peripheral nervous systems, and the NK-1 receptor has been shown to mediate the effects of the tachykinins on peripheral neurogenic inflammation (7, 8), pain transmission (9), and emesis (10, 11). Therefore, nonpeptide NK-1 receptor antagonists may be useful as novel anti-inflammatory, antinociceptive, and antiemetic agents.

Since the discovery of the quinuclidine amine CP 96,345 as the first nonpeptide NK-1 antagonist by Lowe and co-workers (12), several different structural classes of antagonists have been discovered. These include the perhydroisoindole RP 67,580 (13), quinuclidine ethers such as L-709,210 (14, 15), the phenylpiperidine CP 99,994 (16), tryptophan benzyl esters such as L-732,138 (17), and acyclic 2-benzhydryl-2-aminoethyl ethers such as L-732,106 (18) (Fig. 1). These antagonists all contain a substituted benzyl moiety linked via a heteroatom to a core scaffold that is further appended by aromatic substitutions. More recently, a novel class of

ABBREVIATIONS: SP, substance P; NK, neurokinin; CHO, Chinese hamster ovary; Gpp(NH)p, guanosine-5'-(β,γ-imido)triphosphate.

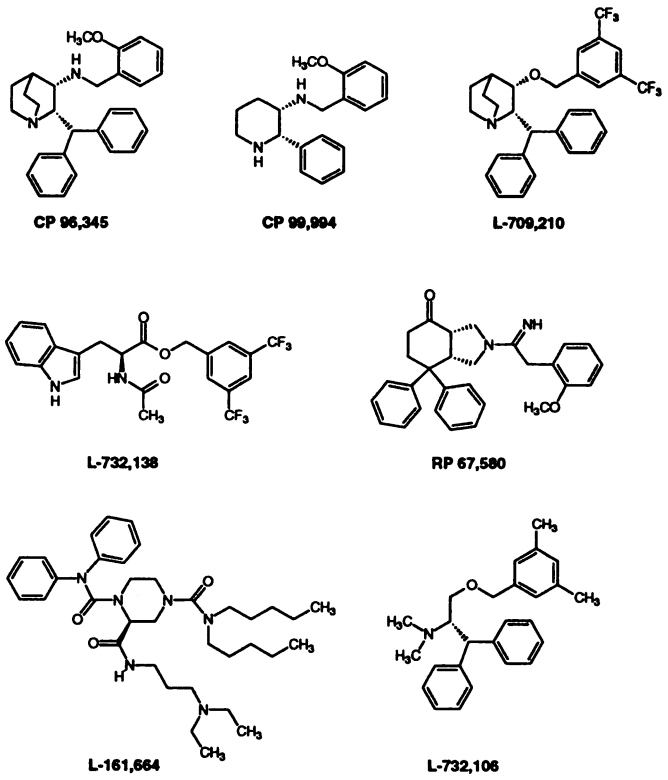


Fig. 1. Structures of the quinuclidine amine (CP 96,345), quinuclidine ether (L-709,210), perhydroisoindole (RP 67,580), phenylpiperidine (CP 99,994), *N*-acyl-tryptophan benzyl ester (L-732,138), acyclic 2-benzhydryl-2-aminoethyl ether (L-732,106), and diacylpiperazine (L-161,664) NK-1 antagonists.

potent diacylpiperazine NK-1 antagonists has been described that has little structural homology with the previously described compounds (19). Analysis of the activity of various diacylpiperazine analogs with the human NK-1 receptor has shown that a carboxamide side chain at position 2 of the piperazine ring enhances potency in this series (19).

Site-directed mutagenesis of the human NK-1 receptor has led to the identification of five residues within the interhelical domain of the receptor that appear to be directly involved in binding of various nonpeptide antagonists (20–24). These experiments suggest that His-197 in the fifth transmembrane helix interacts with the benzhydryl moiety of the quinuclidine antagonists and with the indole moiety of the tryptophan benzyl ester antagonists (20, 21). The substituted benzyl ether group of the quinuclidine ether antagonists and the benzyl ester group of tryptophan benzyl ester antagonists appear to interact with His-265 in transmembrane helix 6 (21, 22). More recently, Gln-165 in transmembrane helix 4 has been identified as the residue that participates in a hydrogen bonding interaction with the linking nitrogen or oxygen heteroatom in the quinuclidine amine and quinuclidine ether antagonists (23). In addition, Ser-169 and Tyr-287 in transmembrane helices 4 and 7 have been shown to be important for binding of the perhydroisoindole RP 67,580 but not for binding of the quinuclidine amine or quinuclidine ether antagonists (23, 24).

We have now characterized the pharmacological and molecular interactions of the novel diacylpiperazine NK-1 antagonists with the human NK-1 receptor. These compounds are potent and competitive NK-1 antagonists and demonstrate selectivity for this receptor over the related NK-2 and NK-3 receptors. Although the structures of the diacylpiperazine antagonists suggest that their molecular interactions with the receptor might be quite different from those of the previously discovered antagonists described above, the characterization of the interaction of the diacylpiperazine antagonists with NK-1 receptor mutants indicates that these compounds interact with the receptor at a common antagonist binding site.

Materials and Methods

Cells and reagents. L-161,664 [1-(*N*,*N*-diphenylaminocarbonyl)-4-(*N*′,*N*′-di-*n*-pentylaminocarbonyl)piperazine-2-diethylaminopropylcarboxamide] and the other diacylpiperazine antagonists used in these experiments were synthesized as described previously (19). Receptor assays and functional assays were performed using stable CHO cell lines, expressing 1×10^5 human NK-1 receptors/cell, 1×10^6 human NK-2 receptors/cell, or 1.8×10^5 human NK-3 receptors/cell, that were selected and maintained as described previously (25). Mutants of the NK-1 receptor were prepared as described previously and were assayed after transient expression in COS cells (19–23).

Receptor binding assays. Human NK-1/CHO cells (5×10^4) or membranes (1–2 μg) were incubated with ^{125}I-Tyr^8-SP (0.1 nM, 2200 Ci/mmol; New England Nuclear) at room temperature for 45 min, as described previously (25), and then filtered over GF/C filters that had been presoaked in 0.1% polyethylenimine, using a Tomtec 96-well harvester. Experiments with mutant receptors were carried out under the same conditions after transient expression of the receptors in COS cells and were performed in parallel with experiments with the wild-type receptor expressed in COS cells. Assays using [^{125}I]L-703,606 were performed in a similar fashion, as described in detail previously (25). Binding affinity for the human NK-2 and NK-3 receptors was measured using ^{125}I-NKA or ^{125}I-Bolton Hunter-labeled eledoisin (0.1 nM, 2200 Ci/mmol; New England Nuclear), respectively, under the same conditions as described above for the NK-1 receptor. The free energy of binding and the change in the free energy of binding were calculated using the formulas $\Delta G = -RT\ln K_d$ and $\Delta(\Delta G) = -RT\ln (K_{d_{wt}}/K_{d_{mut}})$.

SP-induced inositol phosphate production. The assay was performed as described by Berridge *et al.* (26), using cells grown to confluence in 12-well tissue culture dishes. Cells were prelabeled with *myo*-[2-^{3}H]inositol for 24 hr, washed, and incubated with SP and LiCl, in the presence or absence of L-161,664, as described previously (25). Inositol monophosphate was isolated after extraction and ion exchange chromatography (25).

Results

The substituted diacylpiperazine L-161,664 (Fig. 1) inhibits its binding of ^{125}I-SP to the human NK-1 receptor expressed in CHO cells with an IC_{50} of 43 ± 21 nM (19 experiments) (Fig. 2). This compound inhibits binding of the quinuclidine antagonist [^{125}I]L-703,606 to the human NK-1 receptor with the same affinity (see below). In contrast, L-161,664 is 46-fold, 50-fold, and 200-fold less potent as an inhibitor of binding to the rat NK-1 (data not shown), human NK-2, and human NK-3 receptors, respectively (Fig. 2). In functional assays, increasing concentrations of L-161,664 increase the EC_{50} for SP-stimulated phosphatidylinositol turnover in human NK-1/CHO cells, without altering the maximal response to SP (Fig. 3). Schild analysis of these data gives a slope of 0.9, suggesting that the compound is a competitive antagonist of SP with a K_b of 27 nM.

Scatchard analysis of the binding of ^{125}I-SP to the human

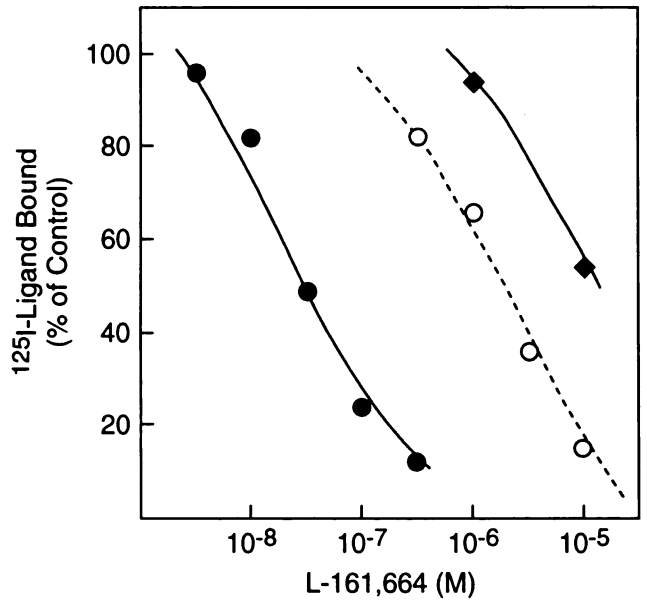


Fig. 2. Inhibition by L-161,664 of ligand binding to the human NK-1 (●), NK-2 (○), and NK-3 (♦) receptors. Assays were performed using whole cells expressing the human receptors, with ^{125}I-SP, ^{125}I-NKA, and ^{125}I-Bolton Hunter-labeled eledoisin as ligands for the NK-1, NK-2, and NK-3 receptors, respectively, as described in Materials and Methods.

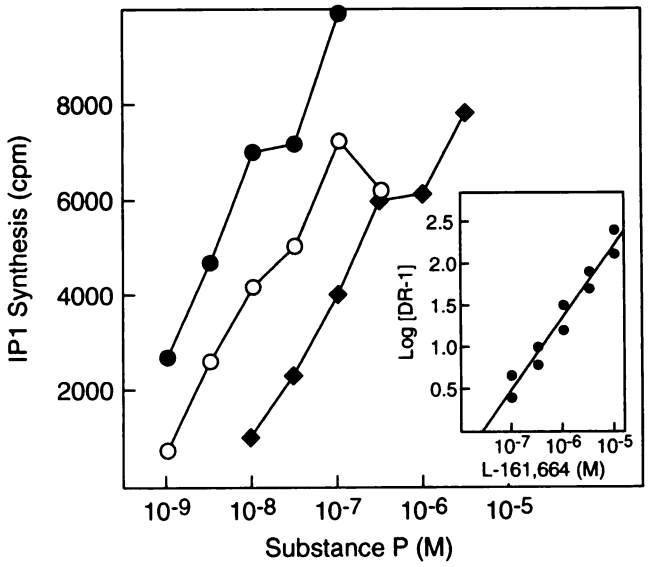


Fig. 3. Inhibition by L-161,664 of SP-induced inositol phosphate (*IP1*) synthesis in human NK-1/CHO cells. ●, Control; ○, 300 nM L-161,664; ♦, 3 μM L-161,664. *Inset*, Schild analysis of these data.

NK-1 receptor expressed in CHO cell membranes indicates that the K_d for ^{125}I-SP is 41 ± 7 pM. Inclusion of 30 nM L-161,664 in this experiment results in an increase in the apparent K_d for ^{125}I-SP to 212 ± 59 pM (data not shown). The dissociation of ^{125}I-SP from the receptor is biphasic, and the data can be fit to a model with 87% of the ^{125}I-SP binding being to receptors in the high affinity, G protein-coupled state and 13% of binding being to receptors in the low affinity, uncoupled state (Fig. 4). This model indicates that the rate of dissociation from the slowly dissociating, high affinity site is 0.0078 ± 0.0008 min^{-1} and the rate of dissociation

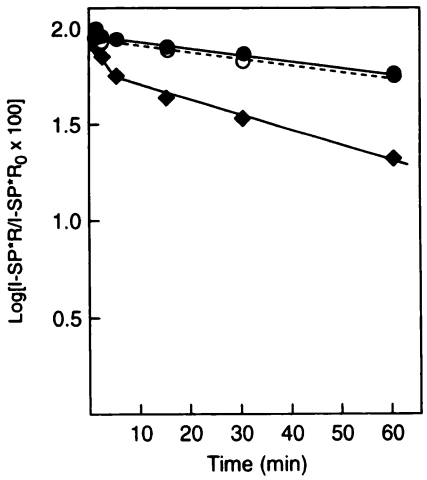


Fig. 4. Effects of L-161,664 on the dissociation of ^{125}I-SP from the human receptor. Ligand and NK-1/CHO cell membranes were incubated until equilibrium, and dissociation was initiated by the addition of 100 nM SP in the absence (●) or presence of 1 μM L-161,664 (○) or 10 μM Gpp(NH)p (♦). Data were analyzed as described by Duggleby (30), and theoretical curves were generated using the formula $Y = R_1e^{-(Xk_{-1})} + R_2e^{-(Xk_{-2})}$, where Y is the ratio of ^{125}I-SP·R/^{125}I-SP·R$_0$, X is time, R_1 and R_2 are the percentages of receptors in the low and high affinity states, and k_{-1} and k_{-2} are the rates of dissociation from the low and high affinity sites, respectively.

from the rapidly dissociating, low affinity site is 2.6 ± 1.9 min^{-1}. Inclusion of 10 μM Gpp(NH)p results in an increase in the percentage of receptors in the low affinity state from 13 ± 6% to 36 ± 3%. In contrast, inclusion of excess L-161,664 (1 μM) does not alter the rate of dissociation of ^{125}I-SP from the receptor. Both the equilibrium binding data and the kinetic data indicate that L-161,664 is a competitive antagonist of SP at the NK-1 receptor.

To characterize the molecular interactions of the diacylpiperazine antagonists with the human NK-1 receptor, the affinity of L-161,664 for receptor mutants in which alanine was substituted for Gln-165 in helix 4, His-197 in helix 5, His-265 in helix 6, or Tyr-287 in helix 7 was measured. These experiments were performed with wild-type or mutant receptors transiently expressed in COS cells. L-161,664 has 10-fold, 6-fold, 30-fold, and 78-fold reduced affinity for the Q165A, H197A, H265A, and Y287A mutant NK-1 receptors, respectively (Fig. 5). These data indicate that these amino acid residues are important for the binding of this antagonist to the receptor. As described previously, the H197A and H265A mutant receptors have normal affinity for ^{125}I-SP (20, 22), the Q165A mutant has 7-fold reduced affinity for ^{125}I-SP but normal affinity for the tryptophan benzyl ester antagonist L-732,138 (23, 27), and the Y287A mutant has low affinity for ^{125}I-SP but normal affinity for the quinuclidine amine antagonist [^{125}I]L-703,606 (24). These data suggest that these substitutions do not affect the overall folding of the receptor protein.

The interactions of L-161,664 with His-197, His-265, and Tyr-287 were further characterized using mutant receptors with various amino acid substitutions at these positions. Glutamine and phenylalanine, but not serine, can substitute for His-197 in maintaining the high affinity of the receptor for L-161,664 (Table 1). In contrast, substitution of His-265 with glutamine, tyrosine, or phenylalanine results in a 5–6-fold reduced affinity of L-161,664, compared with its affinity

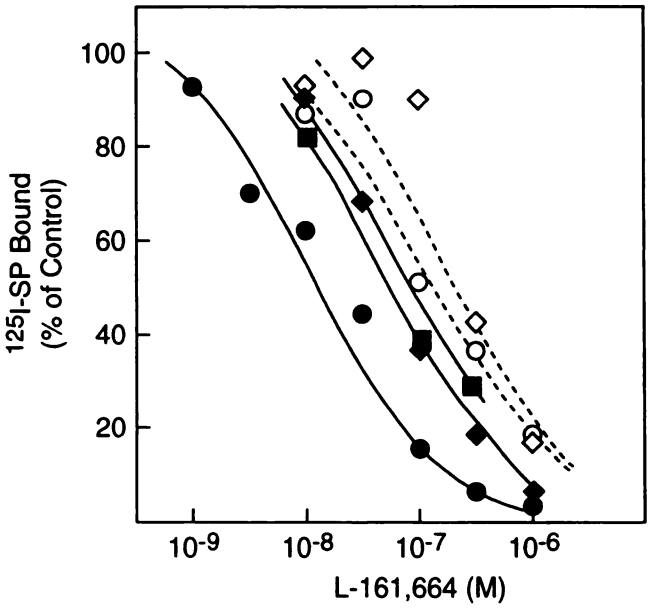


Fig. 5. Inhibition by L-161,664 of ligand binding to the human NK-1 receptor (●), the Q165A mutant (○), the H197A mutant (♦), the H265A mutant (◇), and the Y287A mutant (■). Assays were performed using receptors transiently expressed in COS cells. ^{125}I-SP was the ligand for experiments using the Q165A, H197A, and H265A mutants, and [^{125}I]L-703,606 was the ligand for experiments using the Y287A mutant.

for the wild-type receptor, suggesting that these residues can only partially substitute for His-265 in maintaining the high affinity of the receptor for L-161,664 (Table 1).

Because the mutant in which Tyr-287 is substituted with alanine has poor affinity for ^{125}I-SP, experiments using this mutant were conducted using the iodinated quinuclidine amine antagonist [^{125}I]L-703,606 (24). This ligand has the same affinity for the Y287A mutant receptor as for the wild-type receptor (24). Histidine can substitute for Tyr-287 in the interaction of L-161,664 with the receptor, but phenylalanine, glutamine, serine, and tryptophan are poor substitutes (Table 1).

L-159,588, an analog of L-161,664 in which the diethylaminopropyl carboxamide side chain at position 2 of the piperazine ring has been replaced with a carboxyl group, has 20-fold reduced affinity for the human NK-1 receptor (Table 1 and Ref. 19). This reduction in affinity is not due to the introduction of the negatively charged carboxylate anion, because a compound with a primary alcohol in this position has the same affinity as L-159,588 (19). These data suggest that the aminoalkyl amide side chain of L-161,664 is important for maintaining affinity. L-159,588 has similar affinity for the Q165A mutant and for the wild-type receptor (Table 1). In contrast, L-159,588 has 5-fold, 15-fold, and 29-fold lower affinity for the H197A, H265A, and Y287A mutants, respectively, than for the wild-type receptor, suggesting that interactions with these residues are maintained in the absence of the carboxamide side chain (Table 1). As is the case with L-161,664, glutamine and phenylalanine can substitute for His-197 in the interaction with L-159,588. In contrast to the interaction of L-161,664 with His-265, tyrosine can also substitute for His-265 in maintaining affinity for L-159,588 (Table 1).

The acyclic aminoethyl ether L-732,106 inhibits binding to the human NK-1 receptor with an IC_{50} of 5.5 ± 3.7 nM (five experiments). This compound has 20-fold, 60-fold, and 80-fold reduced affinity for the Q165A, H197A, and H265A mutants, respectively, and has normal affinity for the Y287A mutant.

Discussion

We have previously shown that the quinuclidine and perhydroisoindole antagonist binding site includes residues in transmembrane helices 4, 5, 6, and 7 of the human NK-1 receptor. A combination of receptor mutagenesis and medicinal chemistry approaches suggests that the benzydryl moiety of the quinuclidine antagonists interacts with His-197 in helix 5 (20), the disubstituted benzyl ether interacts with His-265 in helix 6 (22), and the linking nitrogen or oxygen heteroatom interacts with Gln-165 in helix 4 of the receptor (23). The acyclic 2-aminoethyl ether L-732,106 also interacts with these three residues, and the loss in binding affinity is similar for this compound and for L-709,210. These data suggest that L-732,106 and L-709,210 interact with the NK-1 receptor in similar fashions. The tryptophan benzyl ester antagonists also interact with His-197 and His-265, suggesting that the indole and benzyl ester pharmacophores in this series correspond to the benzhydryl and benzyl ether moieties in the quinuclidine antagonists (21). The related perhydroisoindole antagonists also interact with His-265 (22) and, in addition, interact with Ser-169 and Tyr-287 in helices 4 and 7 (23, 24). Therefore, these structurally similar antagonists bind to overlapping sites within the transmembrane domain of the NK-1 receptor.

The diacylpiperazines represent a novel, structurally diverse series of NK-1 antagonists (19). L-161,664 is a potent, selective, and competitive antagonist of the interaction of SP with the human NK-1 receptor. As with the compounds other than RP 67,580 shown in Fig. 1, L-161,664 has reduced affinity for the rat NK-1 receptor. Previous data suggest that this loss in affinity is due to the change of Val-116 to leucine in the third transmembrane helix and Ile-290 to serine in the seventh transmembrane helix (28, 29). Because the nature of these changes does not support a direct interaction of these two residues with antagonist, it has been proposed that changes in the local helical packing occur as a result of these amino acid differences in the rat and human receptors and that this change in local structure of the receptor results in the loss of affinity (28, 29).

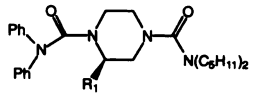
Although L-161,664 is structurally dissimilar from the other classes of NK-1 antagonists that have been described, L-161,664 binding requires the same residues in the NK-1 receptor that were shown previously to be important for binding of the quinuclidine, tryptophan benzyl ester, and perhydroisoindole classes of antagonists. These data suggest that a common antagonist binding site is present in the human NK-1 receptor and accomodates much structural diversity.

Calculation of the difference in the free energy of binding [$\Delta(\Delta G)$] of L-161,664 to the wild-type and mutant receptors suggests that Gln-165, His-197, His-265, and Tyr-287 contribute 1.3, 1.0, 1.9, and 1.1 kcal/mol, respectively, of binding energy to the interaction of this compound with the receptor. Because the calculated binding energy (ΔG) for binding of this compound to the wild-type receptor is 10.7 kcal/mol,

TABLE 1

Interaction of piperazine NK-1 antagonists with Gln-165, His-197, His-265, and Tyr-287 of the human NK-1 receptor

Data are expressed as mean ± standard deviation of the number of determinations in parentheses. Analysis of binding to the Y287 mutant was performed using [^{125}I]L-703,606 as ligand instead of ^{125}I-SP. L-161,664 and L-159,588 inhibit binding of [^{125}I]L-703,606 to the wild-type receptor with IC_{50} values equal to 14 ± 10 (four experiments) and 250 ± 30 (two experiments), respectively. Ph, phenyl.



	IC_{50}	
	L-161,664 [R_1 = CO-NH-$(CH_2)_3$-N$(C_2H_5)_2$]	L-159,588 (R_1 = CO_2)
	nM	
Human NK-1 receptor (wild-type)	11 ± 0.9 (4)	265 ± 41 (4)
Q165A	115 ± 5 (2)	650 ± 50 (2)
H197A	65 ± 5 (2)	1,200 ± 10 (2)
H197S	65 ± 5 (2)	1,350 ± 150 (2)
H197F	19 ± 4 (2)	350 ± 50 (2)
H197Q	10 ± 1 (2)	275 ± 25 (2)
H265A	300 ± 50 (2)	4,000 ± 500 (2)
H265Q	50 ± 5 (2)	1,350 ± 150 (2)
H265Y	50 ± 5 (2)	275 ± 25 (2)
H265F	62 ± 18 (2)	775 ± 125 (2)
Y287A	78 ± 25 (4)	7,250 ± 250 (2)
Y287Q	98 ± 33 (2)	5,300 ± 500 (2)
Y287S	120 ± 30 (2)	5,500 ± 500 (2)
Y287W	65 ± 15 (2)	13,000 ± 7,000 (2)
Y287F	43 ± 8 (2)	4,200 ± 800 (2)
Y287H	13 ± 8 (2)	400 ± 50 (2)

these four interactions combined potentially account for approximately half of the total energy of binding.

Replacement of the aminoalkyl amide side chain of L-161,664 with a carboxylic acid to form L-159,588 results in a 1.8 kcal/mol loss in binding energy for the wild-type receptor. Replacement of Gln-165 with alanine, thereby removing the amide side chain from this position in the receptor, results in a 1.3 kcal/mol loss in binding energy for L-161,664. Thus, replacement of the substituted carboxamide side chain in the ligand and removal of the amide side chain from Gln-165 result in similar losses in binding energy between the ligand and the receptor. The observation that replacement of the carboxamide moiety of the ligand and removal of the amide side chain from position 165 in the receptor are not additive (i.e., that L-159,588 is not sensitive to the nature of the residue at position 165) suggests that Gln-165 interacts with the carboxamide side chain of L-161,664.

In contrast, substitution of His-197, His-265, or Tyr-287 with alanine results in a further loss of 0.9, 1.6 or 2.0 kcal/mol, respectively, of binding energy for L-159,588. Comparison of these data with those for L-161,664 suggests that removal of the carboxamide side chain does not decrease the sensitivity of the compound to the nature of the residue at these positions of the receptor, suggesting that these residues do not interact with the amide side chain of L-161,664.

An overlay of the structures of L-709,210, L-732,138, L-732,106, and L-161,664 is shown in Fig. 6. L-161,664 was positioned to overlay the aminoalkyl amide side chain with the heteroatom in the benzyl ether side chain of L-709,210 and L-732,106, because all of these moieties appear to interact with Gln-165. Interestingly, we have shown that the benzyl ester of L-732,138 does not interact with this residue,

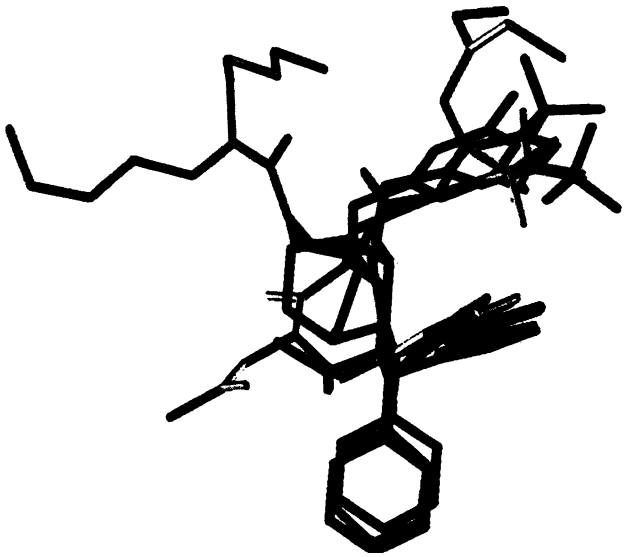


Fig. 6. Overlay of the structures of L-709,210, L-732,106, L-732,138, and L-161,664. Overlay of L-709,210 and L-732,138 was performed as described by Cascieri *et al.* (21). L-161,664 is positioned so that the aminoalkyl amide side chain overlays the linking heteroatom in L-709,210.

and modeling studies support the hypothesis that the oxygen atoms of L-732,138 do not interact with the receptor in the same manner as does the oxygen in the benzyl ether moieties (27).

The modeled overlay shown in Fig. 6 suggests that a general pharmacophore map for small ligands interacting with the NK-1 receptor comprises aromatic moieties capable of

interacting with His-197 and His-265 and a heteroatom moiety capable of hydrogen bonding with Gln-165. Although the presence of a nitrogen in the quinuclidine or piperazine ring, acyclic amino ether, and *N*-acyl-tryptophan antagonists is required to maintain affinity, an interaction of this heteratom with the receptor has yet to be identified (17, 18, 20). Although it is hypothetical in the absence of physical data to support the model of antagonist interaction with the receptor that the mutagenesis data suggest, this pharmacophore map is a useful tool to predict the design of novel antagonists.

These experiments suggest that the structurally dissimilar diacylpiperazines interact within the same binding site as do the quinuclidine, tryptophan benzyl ester, and perhydroisoindole NK-1 antagonists. In addition, our data on the NK-1 receptor and the data generated using other G protein-coupled receptors suggest that this binding site may be common to many receptors, although the precise molecular interactions involved differ among receptors and antagonists (21, 22). Additional experiments will be required to more fully define the nature of this site and its relationship to the agonist binding site.

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