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# Two related neurokinin-1 receptor antagonists have overlapping but different binding sites

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**Abstract**

The neuropeptide substance P binds to the G protein-coupled neurokinin-1 (NK-1) receptor and elicits cellular responses thought to be involved in pain, neurogenic inflammation, vasodilatation, and plasma exudation. Several small molecule nonpeptide antagonists of the substance P/NK-1 receptor interaction have been developed. Mutational analysis of the receptor protein sequence has led to the conclusion that the binding site for these nonpeptide antagonists lies within the bundle created by transmembrane domains IV-VII of the receptor. This current investigation employs site directed mutagenesis of the NK-1 receptor to compare the binding site of CP-96,345 with that of a related compound CP-99,994. The data demonstrate that while both compounds appear to bind within the transmembrane domain bundle, the contribution of individual amino acid residues to the binding of each compound differs. 

*Keywords*: NK-1 antagonists, site-directed mutagenesis, ligand-receptor interactions, CP-96,345, CP-99,994.

## 1. Introduction

The tachykinins (or neurokinins), substance P, neurokinin A, and neurokinin B act as neurotransmitters in mammals. Each mediates responses through a distinct G protein-coupled receptor [1]. These receptors have been cloned and characterized. Substance P binds with highest affinity to the neurokinin 1 (NK-1) receptor. Neurokinin A binds to the neurokinin 2 (NK-2) receptor and neurokinin B binds to the neurokinin 3 (NK-3) receptor. The neurokinin peptides are postulated to have roles in a number of biological actions including pain transmission, smooth muscle contraction, vasodilatation and secretion, and neurogenic inflammation [2]. These observations have led to the development of a number of peptide and nonpeptide neurokinin receptor antagonists.

Mutational studies of the neurokinin receptors (particularly of the NK-1 receptor) have shown that the binding sites for peptide agonists and antagonists overlap but differ from those of nonpeptide antagonists [3]. Much of this comes from work done to characterize the binding site of the nonpeptide NK-1 receptor antagonist CP-96,345 [4]. A combined approach of mutagenesis and use of antagonist analogs has led to an understanding of the amino acids important for binding of CP-96,345 to the NK-1 receptor [5–11]. We have used site-directed mutagenesis of the NK-1 receptor and compared the residues important for the binding of CP-96,345 with those of another nonpeptide antagonist CP-99,994 to determine if antagonists of similar chemical structure indeed contact the receptor in similar manners [12]. We have uncovered a previously uncharacterized interaction of CP-96,345. Additionally, we have found that, while the binding sites of these two compounds overlap, the particular residues important for binding differ.

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## 2. Results

### 2.1 Effects of NK-1 receptor mutations on agonist binding affinity

The binding sites of two related nonpeptide NK-1 receptor antagonists have been examined by site-directed mutagenesis of specific amino acid residues in the human NK-1 receptor. Each mutation was assessed for its effect on the binding of agonist ([$^3$H]-Sar-substance P) and on the antagonist activity of CP-96,345 and CP-99,994. Mutagenesis was focused in the transmembrane domains IV-VII of the receptor where nonpeptide antagonists have been shown previously to bind [11]. Of the fifteen amino acid substitutions made, four resulted in undetectable binding of the agonist (Table 1). Substitution of Trp-155 in transmembrane domain (TM) IV with either Ala or Phe resulted in no detectable [$^3$H]-Sar-substance P binding. The same results were obtained when Glu-193 in TM-V was substituted with Lys and Phe-267 in TM-VI was substituted with Ala. The effects of the remaining mutations on agonist binding are not great and do not likely indicate amino acids that are directly involved in substance P binding. It is more likely that these amino acid substitutions generate conformational changes in the receptor that subtly affect agonist binding.

### 2.2 Effects of receptor mutations on NK-1 receptor antagonist affinities

The activities of two chemically related NK-1 receptor antagonists, CP-96,345 and CP-99,994 (Fig. 1) were determined on each of the NK-1 receptor constructs that maintained binding to [$^3$H]-Sar-substance P. The most drastic effects on the activity of CP-96,345 were found when substitutions were made of Gln-165 (TM-IV) and His-197 (TM-V) (Table 1). Changing either of these residues to Ala resulted in a ~20- and 70-fold reduction in antagonist activity, respectively. We also observed that His-197 could be substituted by Phe or Gln with little effect (< tenfold) on activity. These data are consistent with those of previous investigators, although the absolute effects of individual substitutions do vary slightly [9,10]. These subtle differences between investigations may be explained by slightly different assay conditions and the use of different radio-labeled ligands.

Not reported by previous investigators is a role for either Leu-203 or Ile-204 (TM-V) in the activity of

Table 1
The effects of NK-1 receptor mutants on agonist and antagonist binding affinity

| Mutation | $K_d$ (nM) ± SE[a] | Sites/cell[c] ± SE×10$^6$ | CP-96,345 $K_i$ (nM) ±SE | Fold effect[c] | CP-99,994 $K_i$ (nM) ± SE | Fold effect |
|---|---|---|---|---|---|---|
| WT | 2.6±0.3 | 2.4±0.4 | 3.0±0.7 | — | 1.0±0.2 | — |
| W155A | NSB[b] | — | — | — | — | — |
| W155F | NSB | — | — | — | — | — |
| Q165A | 8.3±1.2 | 1.6±0.5 | 53.6±34.7 | 17.8 | 99.7±39.1 | 99.7 |
| E193A | 9.5±3.5 | 0.4±0.1 | 7.5±5.0 | 2.5 | 1.4±1.1 | 1.4 |
| E193K | NSB | — | — | — | — | — |
| H197A | 2.4±0.4 | 2.0±0.4 | 209±31.4 | 69.6 | 9.9±6.0 | 9.9 |
| H197F | 0.8±0.2 | 1.3±0.1 | 1.4±0.7 | 0.5 | 1.1±0.5 | 1.1 |
| H197Q | 1.7±0.2 | 1.3±0.2 | 19.8±6.5 | 6.6 | 1.5±0.6 | 1.5 |
| L203A/I204A | 0.7±0.1 | 1.7±0.2 | 101±3.9 | 33.7 | 1.7±0.3 | 1.7 |
| F264A | 10.7±0.2 | 1.6±1.0 | 8.6±4.5 | 2.9 | 4.4±0.1 | 4.4 |
| H265A | 0.7±0.1 | 1.6±0.1 | 3.7±2.9 | 1.2 | 1.6±1.1 | 1.6 |
| F267A | NSB | — | — | — | — | — |
| Y272A | 1.1±0.1 | 1.5±0.3 | 12.6±2.4 | 4.2 | 0.1±0.01 | 0.1 |
| Q165A/Y272A | 8.5±2.8 | 0.5±0.1 | 99.8±4.4 | 33.2 | 114±11.1 | 114.0 |
| I283A | 5.2±1.2 | 1.2±0.2 | 12.2±4.1 | 4.1 | 0.3±0.05 | 0.3 |
| Y287F | 1.9±0.6 | 1.3±0.5 | 0.4±0.3 | 0.1 | 0.4±0.12 | 0.4 |

[a]Standard error of the mean.
[b]No specific binding detected.
[c]Fold effect = $K_i$ mutant/$K_i$ wild-type.
Each of the mutant NK-1 receptors shown in the table were transiently expressed in COS-7 cells. The equilibrium binding affinity of [$^3$H]-(9-Sar,11-Met($O_2$)-substance P) was determined by saturation binding on whole-cells with six concentrations of radioligand. The reported data are the result of experiments from at least three independent transient transfections. The activities of CP-96,345 and CP-99,994 were determined by inhibition of 1 nM [$^3$H]-(9-Sar,11-Met($O_2$)-substance P) in competitive binding assays on whole cells. The $K_i$ value was calculated from the graphically determined $IC_{50}$ using the equation of Cheng and Prusoff [25].

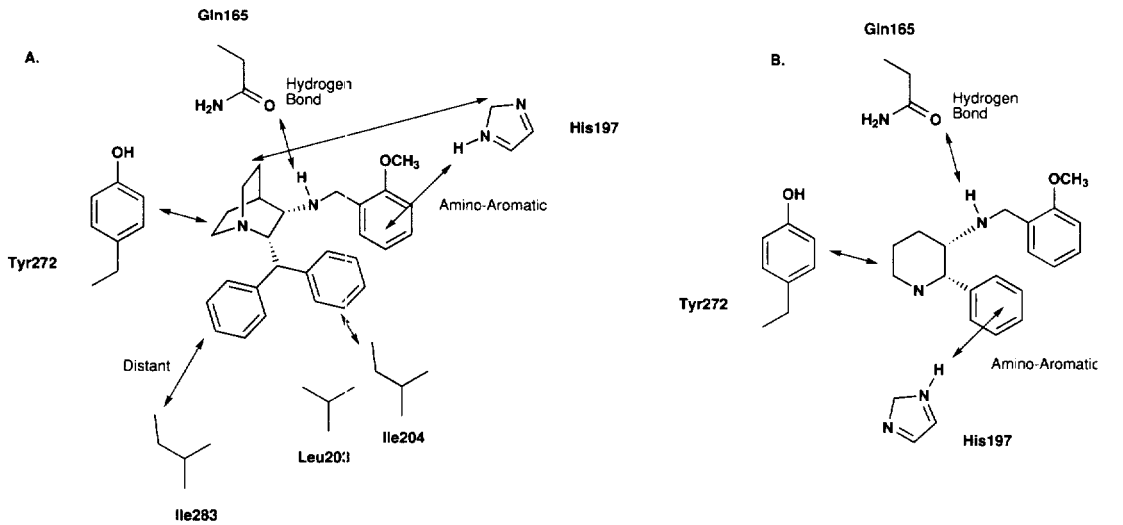



Fig. 1. Schematic diagrams of proposed interactions of CP-96,345 (**A**) and CP-99,994 (**B**) with the human NK-1 receptor. The benzylic amino group is proposed to hydrogen bond with Gln-165 for both CP-96,345 and CP-99,994. His-197 forms an amino aromatic interaction with the benzyl ring in the C-3 position and possibly with the ethylene of the quinuclidine bridge of CP-96,345. His-197 forms an amino aromatic interaction with the phenyl moiety in the C-2 position of CP-99,994. Ile-204 forms an interaction with the phenyl moiety in the C-2 position of CP-96,345 but forms no interaction with CP-99,994.

CP-96,345. Substitution of both of these residues with Ala resulted in a ~30-fold decrease in antagonist activity (Table 1). This suggests that either one or both of these amino acids may have an important previously unrecognized interaction with the antagonist.

The effects of mutations on the activity of CP-99,994 were largely similar to those on CP-96,345. However, there are some clear differences. Both the Q165A and H197A substitutions affected the activity of CP-99,994 but each with different magnitudes than for CP-96,345 (Table 1). While His-197 appears to be more important than Gln-165 for the activity of CP-96,345, the opposite is true for CP-99,994. In addition, the double substitution L203A/I204A had little effect on the activity of CP-99,994 (2-fold, Table 1) in stark contrast to CP-96,345.

The results obtained for the Y272A substitution in TM-VI also underscore differences in the manner in which these two compounds contact the receptor. This mutation results in a ~fourfold decrease in the activity of CP-96,345 (Table 1) while it results in a tenfold increase in the activity of CP-99,994. This result suggests that an Ala residue in position 272 may be able to form a new interaction with CP-99,994 that Tyr could not. An additional explanation is that Tyr-272, due to its bulky nature, impedes CP-99,994 and that its substitution with a smaller residue allows tighter binding to other residues. These possibilities were addressed by making the double mutant Q165A/Y272A.

Structural predictions of the NK-1 transmembrane domains suggest that Gln-165 and Tyr-272 are in close proximity near the top of the opening formed by the transmembrane bundle. If the replacement of Tyr-272 with Ala indeed created a new site of interaction with CP-99,994 then this substitution may be able to partially offset the effect of the Q165A mutant. The effect of the double mutation on the activity of CP-99,994 is not significantly different from that of the Q165A substitution alone (Table 1) suggesting that an Ala in position 272 does not interact directly with CP-99,994 but rather that the increase in activity in the Y272A substitution is due to the removal of the Tyr residue. Interestingly, the Y272A substitution in the rat NK-1 receptor results in a ~20-fold decrease in CP-96,345 affinity highlighting species differences in the C96,345 binding site [13].

## 3. Discussion

Site-directed mutagenesis has been employed to analyze the binding sites of the two similar NK-1 receptor antagonists CP-96,345 and CP-99,994. Most strikingly, we have uncovered a previously unidentified interaction for CP-96,345. Substitution of Leu-203 and Ile-204 in TM-V with Ala results in a ~30-fold decrease in activity (Table 1). It is also possible that the L203A/I204A mutations cause a subtle effect on the conformation of residues which lie higher in the binding pocket, thereby affecting CP-96,345 binding. Future studies with modified derivatives of CP-96,345 and combination mutants will be required to fully address the role(s) of Leu-203/Ile-204 in the binding of CP-96,345. Our studies also indicate that the binding sites of these two compounds

overlap but that the specific receptor interactions for each compound differ.

CP-99,994 was identified in an attempt to develop a constrained analog of CP-96,345 [14]. X-ray analysis originally suggested that CP-99,994 occupied the same NK-1 receptor space as CP-96,345. More recent analysis of the crystal structures of these two compounds has also suggested identical binding sites for these two compounds [15]. Our current study demonstrates that while the binding sites of CP-96,345 and CP-99,994 overlap, the specific NK-1 receptor interactions are not identical. The most dramatic difference between the two compounds is the fact that CP-99,994 is unaffected by substitution at Leu-203 and Ile-204 (Table 1). In addition, the magnitude of the affects of the Q165A and H197A mutations are different for CP-99,994 than they are for CP-96,345. Our data for the single Gln-165 substitutions have large experimental errors making it difficult to interpret the differences that were identified. However, the data for the double Gln-165/Tyr-272 mutants have much smaller errors and still have the same effects on the compounds as the single Gln-165 mutation supporting the interpretation that substitution of Gln-165 affects the two compounds differently.

Different binding orientations for these compounds is further supported by a variety of recent SAR studies on CP-96,345 and CP-99,994. These studies demonstrate differential tolerances for variation of the steric bulk of the quinuclidine and piperidyl moieties. Variation of the quinuclidine moiety of CP-96,345 leads to a dramatic loss of receptor affinity: aza-tricyclic analogs and even slight ring expansion from a [2.2.2] to a [3.2.2] bicyclo ring system reduces binding substantially [16,17]. In contrast, substantial variation is tolerated in the steric bulk of the piperidyl moiety of CP-99,994: bridges across the nitrogens and morphan bridges across the piperidyl ring retain high binding affinity [18,19]. Only formation of a spiro derivative at the C-2 position loses affinity [20].

When CP-96,345 was first discovered, a three-point interaction model with the NK-1 receptor was proposed in which the quinuclidine nitrogen ion paired with Glu 78, the methoxyphenyl moiety interacted with a specificity site, and the diphenyl moiety interacted with an accessory site [21]. Later studies by the same group, however, indicated that the quinuclidine nitrogen could not ion pair with the NK-1 receptor. N-alkyl salts were shown to retain high receptor affinity and X-ray analysis confirmed their preservation of the geometry of CP-96,345. Since the lone nitrogen pair would have to interact through the space of the alkyl substituent, the quinuclidine nitrogen could not form a hydrogen bond or ion pair and the role of the nitrogen was suggested as structural [16]. Mapping of CP-96,345 analogs has suggested that only one of the two benzhydryl rings is required for activity and that this ring may interact with His-197 [9,22]. Previous mutagenesis studies have also suggested that Gln-165 interacts with the benzylic amino group via a hydrogen bond interaction [10].

Our results suggest a slightly varied model of CP-96,345 binding to the NK-1 receptor (Fig. 1(a)). Gln-165 in TM-IV hydrogen bonds to the benzylic amino group as previously proposed [10]. In order to accommodate an interaction with either Leu-203 or Ile-204 we propose that His-197 in TM-V interacts with the benzyl ring in the C-3 position and possibly with the ethylene of the quiniclidine bridge. Taking into account the geometry of CP-96,345, our model would then suggest that Ile-204 interacts with the benzhydral moiety in the C-2 position previously thought to interact with His-197.

We propose a model of CP-99,994 binding to the NK-1 receptor that is reminiscent of the original model proposed for CP-96,345. (Fig. 1(b)) [3,9] In our model Gln-165 hydrogen bonds to the benzylic amino group. This is consistent with the observation that removal of this amino group leads to a dramatic loss of activity that is only recovered by its replacement with oxygen or an epoxide [23]. We propose that His-197 forms an amino-aromatic interaction with the phenyl moiety in the C-2 position. As stated above, previous studies have shown that formation of a spiro derivative at the C-2 position of CP-99,994 has a dramatic effect on affinity [20]. This is consistent with the orientation of the C-2 phenyl toward His-197 in our current model. In this same study an interaction of the C-3 aryl was proposed with His-265 in TM-VI. Our data indicate that substitution of His-265 with Ala has no effect on the binding of CP-99,994 ruling out such an interaction.

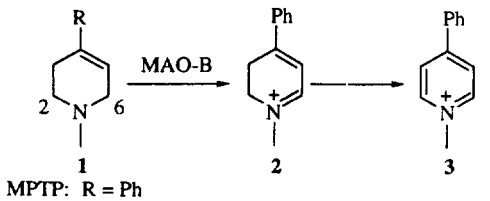
## 4. Conclusion

The data presented indicate that two compounds, with similar structures and similar affinities, each bind to the NK-1 receptor in different manners. These results have important implications for the development of NK-1 receptor antagonists. Since very similar compounds can bind differently, it may not be straightforward to apply pharmacophore hypotheses from one functional series to a different series of compounds. These results also indicate that a previously unidentified interaction, either at Leu-203 or at Ile-203, could be exploited to increase the affinity of NK-1 antagonists.

## 5. Experimental

### *5.1 Bacterial strains and plasmids*

Human neurokinin-1 receptor cDNA (J. Krause) was subcloned from pBluescript II SK(-) into pSR$\alpha$-SPORT for expression in COS-7 cells. The plasmid pSR$\alpha$-




Scheme 1.

SPORT is a derivative of pSV-SPORT (Life-Technologies) in which the SV-40 early promoter has been replaced with the SR$\alpha$ promoter. Plasmids were propagated in DH5$\alpha$ *E. coli* (Life-Technologies). Site-directed mutagenesis of human NK-1 receptor DNA was carried out by polymerase chain reaction (PCR) overlap-extension [24]. The DNA sequence of the amplified region of each mutant was determined prior to evaluation.

### *5.2 NK-1 receptor antagonists*

CP-96,345 and CP-99,994 were synthesized at Schering–Plough Research Institute. CP-96,345 was synthesized as a racemic mixture containing the 2*S*, 3*S*, 2*R*, and 3*R* configurations. The presence of the inactive diastereomers in this mixture is expected only to lower the apparent affinity of the compound since the actual concentration of active compound is reduced by half. This is not expected to have any effect on the relative effects of mutations on the binding affinity. CP-99,994 was synthesized as the (+)-enantiomer. Stock solutions for both compounds were 10 mM in dimethyl-sulfoxide.

### *5.3 Mammalian cell culture and transfections*

Each NK-1 receptor construct was transiently expressed in COS-7 cells (American Type Culture Collection) for evaluation. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Life Technologies, Inc.). DNA constructs were transfected into COS-7 cells by electroporation using a GENE-PULSER (Bio-Rad, Inc.) following the manufacturer's procedure.

### *5.4 Binding assays*

Transfected COS-7 cells were plated onto 24-well tissue culture plates (Falcon, Inc.) and incubated 48–72 h in culture medium. Duplicate binding assays were incubated 45 min at room temperature in binding buffer 50 mM Tris-HCl pH 7.4/1 mM $MgCl_2$/1 mM $MnCl_2$. Saturation binding was performed by incubating transfected cells in the presence of increasing concentrations of [$^3$H]-(9-Sar,11-Met($O_2$)-substance P ([$^3$H]-Sar-substance P) (DuPont-NEN, Inc.) with and without excess cold substance P (10 $\mu$M) (Penninsula Labs, Inc.) as competitor to define non-specific and total binding. Competition binding was performed by incubating transfected cells in the presence of a given concentration of [$^3$H]-Sar-substance P and increasing concentrations of either specific NK-1 receptor antagonist CP-96,345 or CP-99,994. Each competition experiment also included total and non-specific binding controls. After incubation, cells were washed in the wells twice with phosphate buffered saline (PBS) and harvested by addition of 0.5% sodium dodecyl sulfate (SDS). The harvested cell lysate was combined with scintillation fluid (Ready Safe, Beckman, Inc.). The samples were then counted in a LKB RackBeta at 40–50% efficiency.

### *5.5 Analysis of binding data*

Agonist affinities ($K_d$) and receptor numbers ($B_{max}$) were determined from saturation binding by nonlinear regression curve fitting using the program PRISM (Graphpad Software). $IC_{50}$ values were determined graphically by plotting antagonist concentration versus percent of maximum specific binding. $K_i$ values were determined using the Cheng and Prusoff equation [25].

#### *5.5.1 Molecular modeling*

The homology model of the human NK-1 receptor was constructed from the electron diffraction structure of bacteriorhodopsin with the Homology program (v. 95.0, Molecular Simulations Inc., San Diego, CA) using a method previously described for muscarinic receptor modeling [26,27]. The selected alignment was consistent with the three-dimensional arrangement of residues in helices TM V and TM VI that permit formation of an artificial zinc binding site [28]. The crystal structures of CP96,345 and CP99,994 were docked to these models on the basis of interaction sites mapped by mutagenesis and the resulting complex models were refined with the Discover program (v. 95.0, Molecular Simulations Inc., San Diego, CA) [14,21].

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