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The Neurokinin-1 and Neurokinin-2 Receptor Binding Sites of MDL103,392 Differ

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Abstract—Several small molecule non-peptide antagonists of the NK-1 and NK-2 receptors have been developed. Mutational analysis of the receptor protein sequence has led to the conclusion that the binding site for these non-peptide antagonists lies within the bundle created by transmembrane domains IV–VII of the receptor and differs from the binding sites of peptide agonists and antagonists. The current investigation uses site-directed mutagenesis of the NK-1 and NK-2 receptors to elucidate the amino acids that are important for binding and functional activity of the first potent dual NK-1/NK-2 antagonist MDL103,392. The amino acids found to be important for MDL103,392 binding to the NK-1 receptor are Gln-165, His-197, Leu-203, Ile-204, Phe-264, His-265 and Tyr-272. The amino acids found to be important for MDL103,392 binding to the NK-2 receptor are Gln-166, His-198, Tyr-266 and Tyr-289. While residues in transmembrane (TM) domains IV and V are important in both receptors (Gln-165/166 and His-197/198), residues in TM V and VI are more important for the NK-1 receptor and residues in TM VII play a more important role in the NK-2 receptor. These data are the first report of the analysis of the binding site of a dual tachykinin receptor antagonist and indicate that a single compound (MDL103,392) binds to each receptor in a different manner despite there being a high degree of homology in the transmembrane bundles. In addition, this is the first report in which a model for the binding of a non-peptide antagonist to the NK-2 receptor is proposed.

Introduction

The tachykinins (or neurokinins) substance P, neurokinin A and neurokinin B act as neurotransmitters in mammals. Each mediates responses through distinct G protein-coupled receptors (reviewed by ref. 1) which 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 non-peptide neurokinin receptor antagonists for the treatment of various conditions.

The binding sites for peptide agonists and antagonists overlap but differ from those of non-peptide antagonists (for review see ref. 3). Site-directed mutational analysis of the agonist and non-peptide antagonist human NK-1 and NK-2 receptor binding sites provides substantial proof of these distinct binding sites.[3–7] Additional support for non-overlapping binding sites for agonists and non-peptide antagonists comes from studies employing photoreactive and photolabile analogues of both peptides and small molecules.[8–11]

There have been many studies characterizing the binding site of the non-peptide NK-1 receptor antagonist CP96,345.[12] A combined approach of mutagenesis and use of antagonist analogues has led to an understanding of the amino acids important for binding of CP96,345 to the NK-1 receptor.[4,5,13–18] Several studies have also uncovered the binding site of the non-peptide NK-2 receptor antagonist SR48,968.[4,6,7,19] We have used site-directed mutagenesis of the NK-1 and NK-2 receptors to compare the residues important for the binding of MDL103,392 (Fig. 1), an NK-1/NK-2 receptor dual antagonist with the same potency at both receptors[20]

Key words: Antagonists; mutagenesis; tachykinins; receptors.
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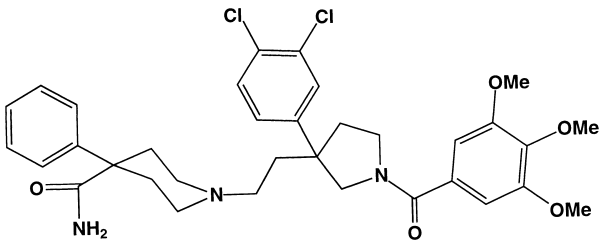

Figure 1. Chemical structure of MDL103,392.

and which is capable of blocking both SP and NKA induced respiratory effects in vivo.[21] We have found that, while the binding sites for the two receptors overlap, the particular residues important for binding differ substantially. These data were used to generate molecular models of the NK-1 and NK-2 binding sites of MDL103,392.

Results

Effects of receptor mutations on agonist binding affinity

The binding sites of the non-peptide NK-1/NK-2 receptor antagonist MDL103,392 have been examined by site-directed mutagenesis of specific amino acid residues in the human NK-1 and NK-2 receptors. Each mutation was assessed for its effect on the binding of agonist ([^{3}H]Sar-sub-P and [^{3}H]NKA, respectively) and on the antagonist activity of MDL103,392. Mutagenesis was focused in transmembrane domains IV–VII of the receptors where non-peptide antagonists have been shown previously to bind.[5] Of the amino acid substitutions made in the NK-1 receptor, four resulted in undetectable binding of the agonist. Substitution of Trp-155 with either Ala or Phe resulted in no detectable [^{3}H]Sar-sub-P binding. The same results were obtained when Glu-193 was substituted with Lys and Phe-267 was substituted with Ala (Table 1). Mutation of Trp-156 in the NK-2 receptor to either Ala or Phe resulted in undetectable agonist binding, as did substitution of His-198, Tyr-266, His-267, Tyr 269, Ile-285 and Tyr-289 with Ala (Table 2). Similar results have also been reported previously for the Y266A and Y289A mutations in the NK-2 receptor.[6,7]

The remaining amino acid substitutions had varying effects on agonist affinity, ranging from ~3-fold increased to ~4-fold reduced affinity (Tables 1 and 2). The effects of these mutations on agonist binding are modest and do not likely indicate amino acids that are directly involved in Sar-sub-P or NKA binding. It is more likely that these amino acid substitutions generate conformational changes in the receptor that subtly affect agonist binding.

Effects of receptor mutations on antagonist binding affinity

The activity of MDL103,392 (Fig. 1) was determined on each of the NK-1 and NK-2 receptor constructs that maintained agonist binding. The greatest effects on the NK-1 activity of MDL103,392 were found when

Table 1. Effects of NK-1 receptor mutants on agonist and antagonist binding affinities[a]

Mutation	Residue position		K_d (nM) ± SE[b] agonist	K_i (nM) ± SE MDL103,392	Fold effect on antagonist activity[d]
WT NK-1			2.6 ± 0.3	6.2 ± 2.0	
W155A	TM IV	(Mid)	NSB[c]		
W155F	TM IV	(Mid)	NSB		
Q165A	TM IV	(Top)	8.3 ± 1.2	42.0 ± 10.4	6.8
E193A	TM V	(Top)	9.5 ± 3.5	0.9 ± 0.4	0.1
E193K	TM V	(Top)	NSB		
H197A	TM V	(Top)	2.4 ± 0.4	48.9 ± 12.6	7.9
Q165A/H197A			8.6 ± 1.0	1023.0 ± 15.0	165.0
H197F	TM V	(Top)	0.8 ± 0.2	10.4 ± 1.7	1.7
H197Q	TM V	(Top)	1.7 ± 0.2	5.6 ± 2.5	0.9
L203A/I204A	TM V	(Mid)	0.7 ± 0.1	89.4 ± 3.4	14.4
L203A	TM V	(Mid)	2.7 ± 0.1	4.1 ± 0.5	0.7
I204A	TM V	(Mid)	1.8 ± 0.2	55.5 ± 6.4	9.0
F264A	TM VI	(Top)	10.7 ± 0.2	159.0 ± 24.6	25.6
F264Y	TM VI	(Top)	2.2 ± 0.5	> 3000	> 480.0
H265A	TM VI	(Top)	0.7 ± 0.1	102.2 ± 30.5	16.5
F267A	TM VI	(Top)	NSB		
F267Y	TM VI	(Top)	5.2 ± 0.6	9.4 ± 1.4	1.5
Y272A	TM VI	(Top)	1.1 ± 0.1	8.2 ± 1.2	1.3
Q165A/Y272A			8.5 ± 2.8	976.8 ± 157.0	157.5
I283A	TM VII	(Top)	5.2 ± 1.2	2.5 ± 1.2	0.4
Y287F	TM VII	(Top)	1.9 ± 0.6	4.1 ± 3.2	0.7

[a] 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]Sar-sub-P was determined by saturation binding on whole-cells with six concentrations of radioligand. The inhibition constants for MDL103,392 were determined by competition binding experiments of MDL103,392 versus radiolabeled agonist as tracer. The reported data are the result of experiments from at least three independent transient transfections.

[b] Standard error of the mean.

[c] No specific binding detected.

[d] Fold effect = K_i (mutant receptor)/K_i (wild-type receptor).

Table 2. Effects of NK-2 receptor mutants on agonist and antagonist binding affinities[a]

Mutation	Residue position		K_d (nM) ± SE[b] agonist	K_i (nM) ± SE MDL103,392	Fold effect on antagonist activity[d]
WT NK-2			0.6 ± 0.4	5.3 ± 1.6	
W156A	TM IV	(Mid)	NSB[c]		
W156F	TM IV	(Mid)	NSB		
Q166A	TM IV	(Top)	0.5 ± 0.1	36.9 ± 9.5	7.0
H198A	TM V	(Top)	NSB		
H198F	TM V	(Top)	1.1 ± 0.1	4.3 ± 0.5	0.8
H198Q	TM V	(Top)	0.3 ± 0.1	8.8 ± 3.4	1.7
Q166A/H198A			12.6 ± 1.2	131.0 ± 16.4	24.7
L204A/I205A	TM V	(Mid)	1.2 ± 0.1	3.7 ± 0.9	0.7
L204A	TM V	(Mid)	2.3 ± 0.2	8.0 ± 0.7	1.5
I205A	TM V	(Mid)	5.8 ± 0.2	6.8 ± 0.5	1.3
Y266A	TM VII	(Top)	NSB		
H267A	TM VII	(Top)	NSB		
Y269A	TM VII	(Top)	NSB		
S274A	TM VI	(Top)	0.2 ± 0.1	2.2 ± 0.5	0.4
Q166A/S274A			0.9 ± 0.2	41.1 ± 6.2	7.8
I285A	TM VII	(Top)	NSB		
Y289A	TM VII	(Top)	NSB		
Y289F	TM VII	(Top)	0.3 ± 0.1	1597.0 ± 249.5	301.3

[a] Each of the mutant NK-2 receptors shown in the table were transiently expressed in COS-7 cells. The equilibrium binding affinity of [^{3}H]NKA was determined by saturation binding on whole-cells with six concentrations of radioligand. The inhibition constants for MDL103,392 were determined by competition binding experiments of MDL103,392 versus radiolabeled agonist as tracer. The reported data are the result of experiments from at least three independent transient transfections.
[b] Standard error of the mean.
[c] No specific binding detected.
[d] Fold effect = K_i (mutant receptor)/K_i (wild-type receptor).

substitutions were made of Leu-203/Ile-204, Phe-264 and His-265 (Table 1). Changing these residues to Ala resulted in ~14-, ~26- and ~16-fold reductions in antagonist activity respectively. Several of the other NK-1 receptor mutations resulted in modest decreases in the activity of MDL103,392 (i.e. ~7-fold for Q165A and H197A).

There is a high degree of homology between the NK-1 and NK-2 receptors within the transmembrane domain regions and particularly among the amino acids that appear important from the above NK-1 mutagenesis. For this reason, homologous mutations were created in the NK-2 receptor. Interestingly mutation of the Leu/Ile pair in NK-2 (L204A/I205A) had no effect on antagonist activity, yielding the first suggestion that the binding sites for the two receptors may be different (Table 2). As stated above, substitution of Tyr-266, His-267 and Tyr-269 (homologous to Phe-264, His-265 and Phe-267 in NK-1) resulted in undetectable binding of NKA to the NK-2 receptor and so could not be assessed by binding assays. Substitution of Tyr-289 with Phe resulted in a ~300-fold reduction in the activity of MDL103,392. The homologous mutation in NK-1 (Y287F) did not alter the activity of MDL103,392 (Tables 1 and 2). Only minor effects were seen with other NK-2 receptor mutations, suggesting that Tyr-289 is a major contact residue for this molecule in the NK-2 receptor.

Structural predictions of the NK-1 and NK-2 transmembrane domains suggest that Gln-165/166, His-197/198 and Tyr-272/Ser-274 are in close proximity near the top of the opening formed by the transmembrane bundle. It is, therefore, possible that the small effects of the Q165A substitution in NK-1 and the Q166A substitution in NK-2 (Tables 1 and 2, 7-fold) are due to masking by interactions at either His-197/198 or Tyr-272/Ser-274. Mutation of His-197 to Ala in NK-1 resulted in a ~8-fold decrease in MDL103,392 activity while the H198A mutation in NK-2 had no effect on the antagonist. Substitution of Tyr-272 in NK-1 and Ser-274 in NK-2 with Ala had no effect on antagonist activity.

The double mutants NK-1 Q165A/H197A, NK-1 Q165A/Y272A, NK-2 Q166A/H198F and NK-2 Q166A/S274A were made to test whether any of these amino acids had a greater role in MDL103,392 binding than the single mutations indicated. The mutants Q165A/H197A and Q165A/Y272A in the NK-1 receptor resulted in much greater effects than the individual mutations on MDL103,392 binding (170- and 160-fold respectively) with little effect on agonist binding (Table 1). The Q166A/H198F mutation in the NK-2 receptor had a 26-fold effect on MDL103,392 activity while the Q166A/S274A mutation had no greater affect on MDL103,392 binding than the Q166A mutation alone (Table 2). The results from analysis of these double mutants reveal interactions in both receptors that the single mutations did not.

Effects of NK receptor mutations on agonist and antagonist functional activity

Due primarily to the lack of binding of agonist to the NK-2 receptor mutants Y266A, H267A and Y269A, studies were initiated to examine the consequences of various mutations on the receptor functional activity.

Both the NK-1 and NK-2 receptors have been previously shown to signal through the release of intracellular calcium (as well as through cAMP production).[1] Intracellular calcium release was assayed using a fluorometric imaging plate reader (FLIPR) which allows for the detection of fluorescent calcium-binding dyes in a 96-well format.

Functional analysis was carried out by determining the effects of various concentrations of MDL103,392 on the dose–response curve of the agonists. Cells were preincubated for 10 min either in buffer alone or in the presence of MDL103,392. Preincubation time courses were run for each mutant and 10 min was found to be adequate for full antagonist function (data not shown). Agonist was then added to the cells and relative intracellular calcium release was determined by fluorescent imaging. Each point was run in quadruplicate and all reported values are the result of at least two independent experiments.

The functional system was first validated by examining the effects of mutants for which binding data had already been generated. Wild-type NK-1 and NK-1 H265A (Fig. 2a and b) as well as wild-type NK-2 and NK-2 Y289F (Fig. 3a and b) were examined to test the functional system. Agonist EC_{50} values were determined from the buffer alone control samples and the shift of the agonist dose–response curve was used to determine an average apparent K_b value for MDL103,392 over the dose range tested. For those cases in which the functional inhibition by MDL103,392 appeared to be non-competitive, separate experiments were also run to determine an IC_{50} value for the antagonist.

The EC_{50} values for Sar-sub-P and NKA at the wild-type NK-1 and NK-2 receptors were 1.1 nM and 3.2 nM respectively (Table 3). These values are in good agreement with the K_d values that were determined by saturation binding (Tables 1 and 2). The K_b values for MDL103,392 against the NK-1 and NK-2 receptors were 9 nM and 8 nM respectively (Table 3), again in good agreement with the K_i values determined from binding analysis (Tables 1 and 2).

The K_b value for MDL103,392 against the NK-1 H265A mutant was 282 nM (Table 3) which represents a 31-fold decrease in antagonist potency at this mutant receptor. This is consistent with the 17-fold effect seen by binding analysis. Closer examination of the curves for the H265A mutant (Fig. 2b) indicates that MDL103,392 depresses the maximal response as well as shifting the dose–response curve at all of the concentrations tested. This differs from the wild-type NK-1 receptor (Fig. 2a) in which MDL103,392 shows significant depression of maximal response only at concentrations >100-fold the K_i. The depression of maximal response is characteristic of non-competitive antagonism. It is not strictly valid to report K_b values for non-competitive antagonists so the IC_{50} values for MDL103,392 against the wild-type and H265A NK-1 receptors were determined in separate experiments and used to estimate the K_d value using a derivative of the

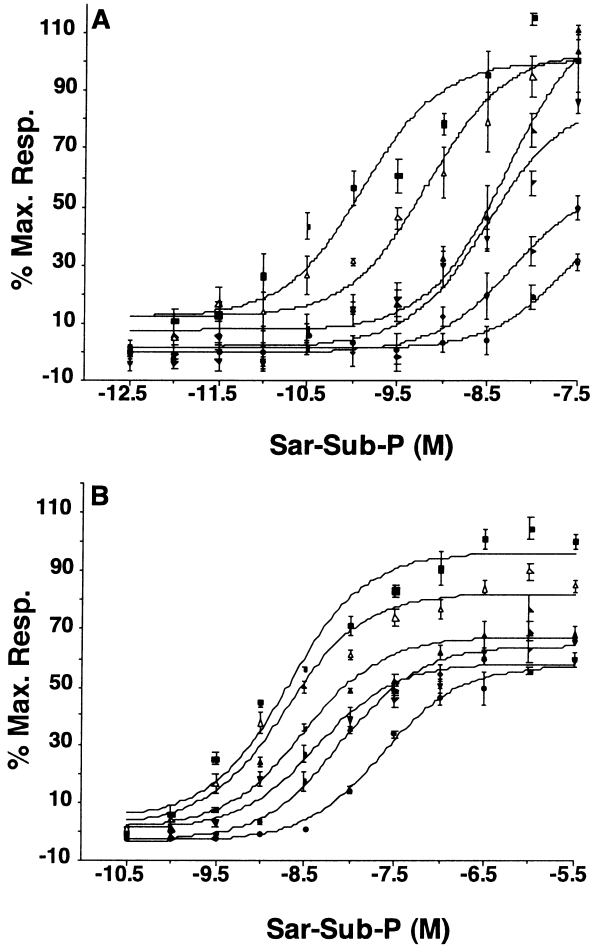

Figure 2. Measurement of the effect of MDL103,392 on the dose–response curve of agonists at the NK-1 and NK-2 receptors. Shown are the data from representative experiments carried out as outlined in Experimental. The X-axis is the log concentration of agonist and the Y-axis is percent maximum response. The receptors and concentrations of MDL103,392 tested in each panel are as follows: (a) Wild-type NK-1 receptor: ■-none, △-30 nM, ▲-100 nM, ▼-300 nM, ◆-1000 nM, ●-3000 nM; (b) NK1 H265A receptor: ■-none, △-30 nM, ▲-100 nM, ▼-300 nM, ◆-1000 nM, ●-3000 nM.

Gaddum equation (data not shown).[28] The IC_{50}-derived K_d values for the wild-type and H265A receptors were 7 and 194 nM respectively (Table 3). These values indicate a 28-fold shift in the potency of MDL103,392 by the H265A mutant, again consistent with both the apparent K_b and K_i values. As is also shown in Table 3, the fold effect of the Y289F mutation in the NK-2 receptor is 112-fold which compares favorably with the 301-fold effect seen in the binding assay. The consistency between the functional assay and the binding assay in determining the effects of mutations on MDL103,392 activity indicated that the functional assay could be used to analyze those mutants for which there was no longer detectable binding of the agonist.

The Y266A, H267A and Y269A mutants of the NK-2 receptor were run in functional assays as described above. Each of these mutants retained functional activity (Fig. 3c, Table 3) and resulted in 90–160-fold increases in the agonist EC_{50} value (Y266A 300 nM,

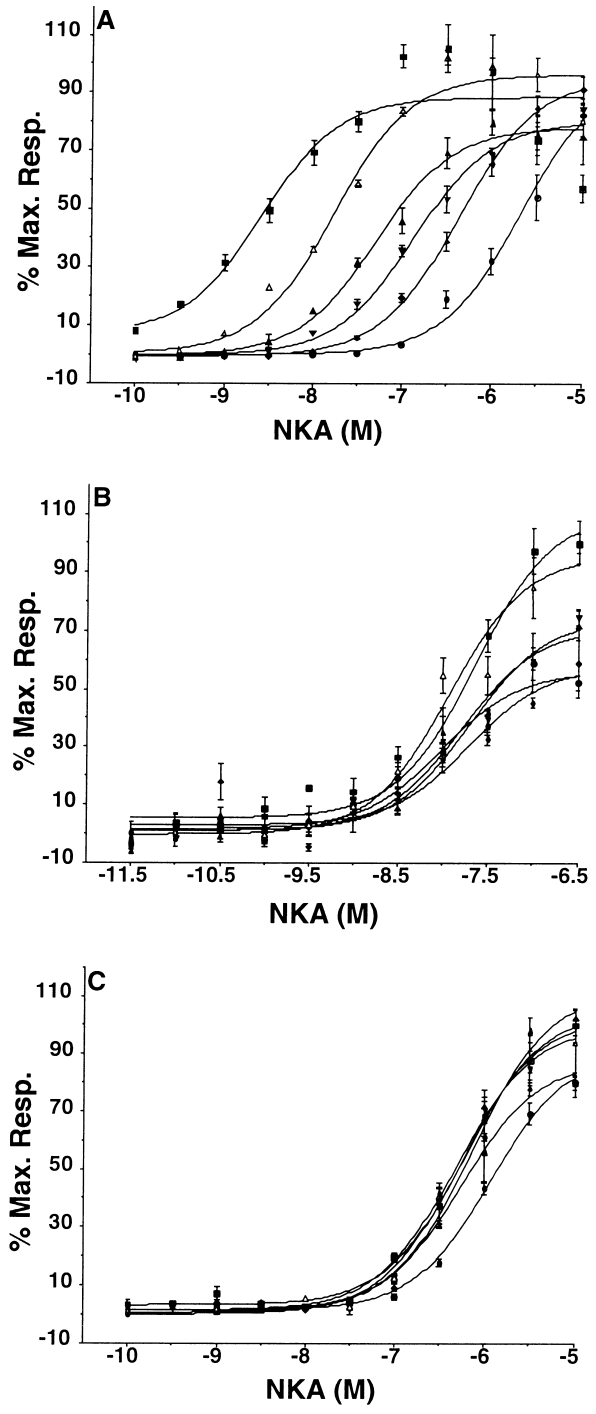

Figure 3. Measurement of the effect of MDL103,392 on the dose–response curve of agonists at NK-2 receptor mutants. Shown are the data from representative experiments carried out as outlined in Experimental. The X-axis is the log concentration of agonist and the Y-axis is percentage of maximum response. The receptors and concentrations of MDL103,392 tested in each panel are as follows: (a) Wild-type NK-2 receptor: ■-none, △-100 nM, ▲-300 nM, ▼-1000 nM, ◆-3000 nM, ●-10,000 nM; (b) Y289F NK-2 receptor: ■-none, △-100 nM, ▲-300 nM, ▼-1000 nM, ◆-3000 nM, ●-10,000 nM; (c) Y266A NK-2 receptor: ■-buffer, △-30 nM, ▲-100 nM, ▼-300 nM, ◆-1000 nM, ●-3000 nM.

H267A 526 nM and Y269A 437 nM) (Table 3). MDL103,392 had a K_b value of 6300 nM against the Y266A mutant (representing a 778-fold increase over wild-type). The H267A and Y269A substitutions had no effect on the MDL103,392 activity (Table 3). The IC_{50}-derived K_d was also determined for each of these mutant receptors since there is evidence of non-competitive antagonism at some concentrations of MDL103,392 (Fig. 3c, data not shown). As with the NK-1 H265A mutant the effects of these NK-2 substitutions were the same when either method of analysis was employed. These data reveal an additional site of interaction for MDL103,392 in the NK-2 receptor at Tyr-266 and indicate that His-267 and Tyr-269 are not involved in the binding of MDL103,392.

Discussion

Site-directed mutagenesis has been employed to analyze the NK-1 and NK-2 receptor binding sites of the non-peptide dual antagonist MDL103,392. Our results indicate that the binding sites in each receptor lie within the bundle formed by transmembrane domains IV–VII as for other nonpeptide antagonists; however, the specific residues important for binding to either receptor are quite different.

In the NK-1 receptor Gln-165, His-197, Leu-203, Ile-204, Phe-264, His-265 and Tyr-272 all contribute to the binding site of MDL103,392 (Tables 1 and 2). Gln-165, His-197 and Tyr-272 have previously been shown to be important interaction sites, particularly for the quinuclidine NK-1 receptor antagonists such as CP96,345.[12,13,18,17,30,29] His-265 has also been shown to be an important residue for antagonist binding, however, not for the quinuclidine class of antagonists but rather for the piperazine antagonists.[31,32] We have previously reported that mutation of Leu-203 and Ile-204 affects the binding of NK-1 receptor antagonists.[13] Unique to MDL103,392 is the effect of substitution of Phe-264. This residue has not been previously implicated in the binding site of non-peptide NK-1 receptor antagonists. The binding interactions of MDL103,392 in the NK-1 receptor comprise those amino acids that have been shown to be important for a number of diverse structural classes of antagonists as well as a novel interaction at Phe-264.

In the NK-2 receptor Gln-166, His-198, Tyr-266 and Tyr-289 are important residues for the binding of MDL103,392 (Tables 1 and 2). In earlier reports single amino acid substitutions at Gln-166 and His-198 had little effect on the binding of the NK-2 receptor selective antagonist SR48,968.[7,19] Our data regarding MDL103,392 and these single substitutions are consistent with these previous results; however, when both residues were mutated simultaneously there was a significant effect on the activity of MDL103,392 revealing interactions at both residues (Table 2). Tyr-266 and Tyr-289 have also been shown to be important residues for the binding of SR48,968.[7] Huang et al. also indicated that His-267 is involved in the SR48,968 binding site. Our data indicate that this residue is not involved in the binding site of MDL103,392 (Table 3). The binding interactions of MDL103,392 in the NK-2 receptor are quite different from those in the NK-1 receptor and

Table 3. Effects of NK receptor mutants on functional responses[a]

Mutation	Residue position		EC_{50} (nM) ± SE[b]	K_b (nM) ± SE MDL103,392	K_d[c] ± SE MDL103,392
WT NK-1			1.1 ± 0.9	9.0 ± 0.8	7.0 ± 1.0
W155A	TM IV	(Mid)	na[d]	nd[e]	nd
E193K	TM V	(Top)	na	nd	nd
H265A	TM VI	(Top)	2.0 ± 0.5	282 ± 10.0	193.5 ± 26.5
WT NK-2			3.2 ± 1.3	8.1 ± 0.7	6.3 ± 2.8
W156A	TM IV	(Mid)	na	nd	nd
I285A	TM VII	(Top)	na	nd	nd
H198A	TM V	(Top)	na	nd	nd
Y266A	TM VI	(Top)	300.0 ± 200.0	6300 ± 1.8	3550 ± 750
H267A	TM VII	(Top)	526.0 ± 134.0	6.3 ± 0.6	13.1 ± 6.9
Y269A	TM VII	(Top)	437.0 ± 49.0	4.0 ± 0.9	5.1 ± 3.8
Y289F	TM VII	(Top)	9.0 ± 6.0	912.0 ± 0.6	nd

[a] Each of the mutant NK receptors shown in the table were transiently expressed in COS-7 cells. The EC_{50} of the agonist (Sar-sub-P for NK-1 and NKA for NK-2) was determined by Ca^{++} flux assay. Receptor mutants were assessed for the effects on inhibition of agonist induced responses by MDL103,392. The reported data are the results of experiments from at least two independent transient transfections.
[b] Standard error of the mean.
[c] K_d calculated from the Gaddum equation, $K_d = IC_{50}'/(([\text{agonist}]/EC_{50}')-1)$.[28]
[d] Not active.
[e] Not determined.

suggest that different conformations of the same molecule bind to either receptor. Constrained analogues mimicking either bound conformation lose activity for the other receptor (data not shown), indicating that conformational flexibility may be required to achieve high dual potency.

A trivial explanation for the differences obtained through mutational analysis of these two receptors is that the compound tested was actually a mixture of both the (*R*)- and (*S*)-enantiomers. If one enantiomer binds to the NK-1 receptor while the other binds to the NK-2 receptor one could explain the observed differences as effects on two different molecules. This explanation is unlikely, however, since it has previously been shown that all of the binding activity of MDL103,392 can be accounted for in the (*R*)-enantiomer with the (*S*)-enantiomer being ~100-fold weaker at either receptor.[20] If anything the present study may be underestimating the effects of mutations on the active enantiomer.

The above data, in concert with results from previous studies, were used to generate models of the binding of MDL103,392 to the NK-1 and NK-2 receptors. Schematic representations of these binding sites are shown in Figure 4 and three-dimensional depictions of these models are shown in Figure 5. Due to the fact that extensive structure–activity relationship studies were not conducted around MDL103,392 these models must be considered highly speculative. Further studies employing compound analogues or photoaffinity labeling will be required to accurately determine the binding sites for MDL103,392. Nonetheless, the following models are presented in order to more clearly represent the interpretation of the current mutational data.

In the NK-1 receptor model, Gln-165 is proposed to hydrogen bond with the benzamide carbonyl of MDL103,392, similar to the interaction with the benzylic nitrogen of CP96,345, as has been suggested previously (Fig. 4a).[13,18] This interaction is likely stabilized by further interactions of Gln-165 and either His-197 or Tyr-272 as suggested by the data from the Q165A/H197A and Q165A/Y272A double mutants (Table 1). Leu-203 and Ile-204 are proposed to form an interaction with the 3,4-dichloro-phenyl moiety. Phe-264 forms an aromatic–aromatic interaction with the phenyl moiety in the 4 position of the piperidine. The role of His-265 is less clear. His-265 could form a pocket along with Leu-203 and Ile-204 serving to stabilize the position of the dichloro-phenyl moiety or could have a role in stabilizing the position of the 3,4,5-tri-methoxy phenyl moiety analogous to the proposed role of this residue in the binding of quinuclidine antagonists.[5,32]

The proposed binding site for MDL103,392 in the NK-2 receptor shares some similarities with that of NK-1 but also has distinct interactions (Fig. 4b). Gln-166 is proposed to hydrogen bond with the benzamide carbonyl just as the homologous residue does in NK-1. Within the predicted model of the NK-2 binding site, His-198 is in close enough proximity to the 3,4,5-tri-methoxy phenyl moiety so that a hydrogen bond interaction with the methoxy group in the 3 position is a possibility; however, structure–activity relationship studies of MDL103,392 have shown that a derivative with an unsubstituted phenyl ring is more potent at the NK-2 receptor than the tri-methoxy substituted group.[20] His-198 could also serve to stabilize Gln-166, as His-197 does in NK-1. The data from the Q166A/S274A double mutant (Table 2) do not suggest a role for Ser-274 in the NK-2 binding site (differing from the corresponding residue in NK-1, Tyr-272). The effects of substitutions at Tyr-266 and Tyr-289 are very substantial (Tables 2 and 3) and indicate that these are important residues in the NK-2 binding site of MDL103,392. Tyr-266 hydrogen bonds to the nitrogen of the piperidine ring and Tyr-289 hydrogen bonds with the piperidyl amide. The assertion that the Tyr-266 interaction is a hydrogen bond with the piperidyl nitrogen is supported

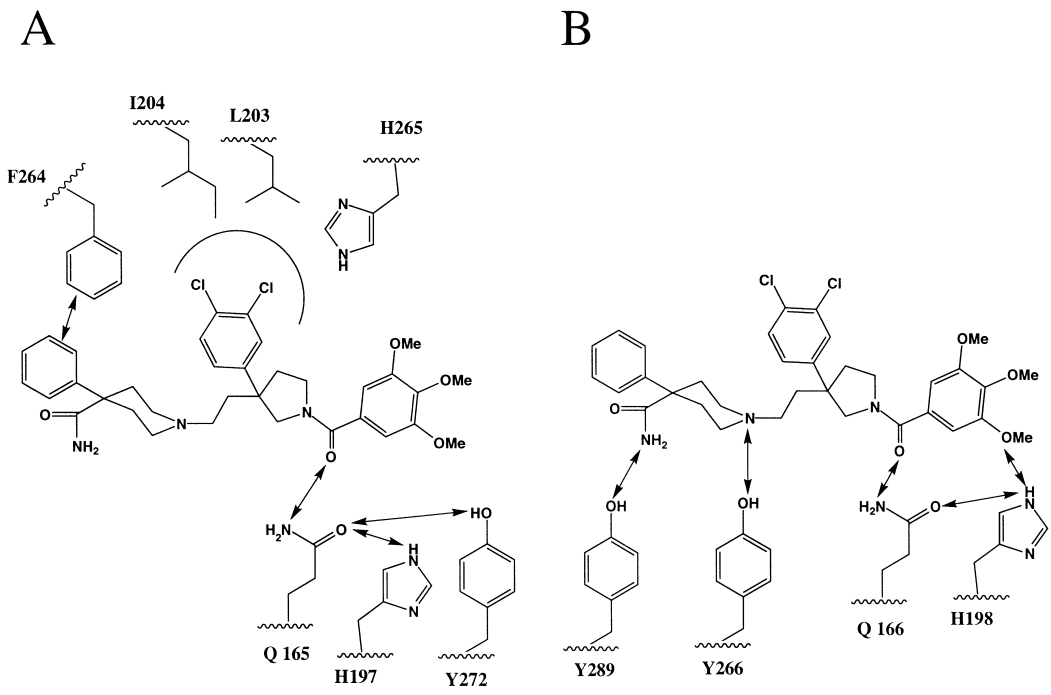

Figure 4. Schematic representations of the NK-1 (A) and NK-2 (B) receptor binding sites of MDL103,392 determined from the current mutagenesis data. Specific interactions are discussed in the text.

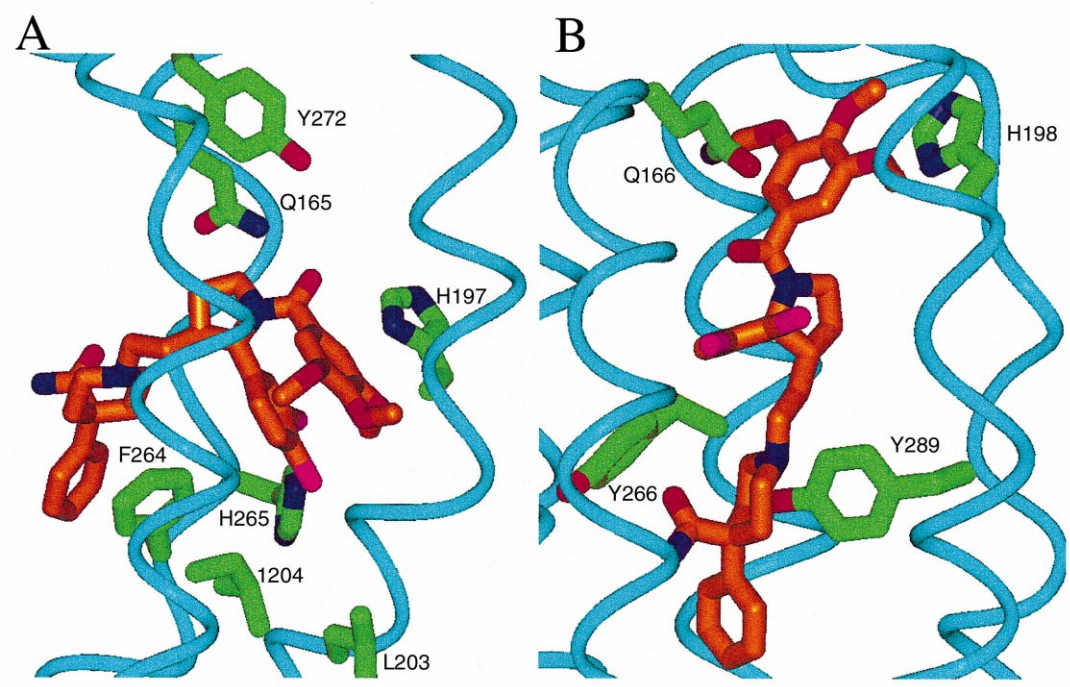

Figure 5. Three-dimensional models of the NK-1 (A) and NK-2 (B) receptor binding sites of MDL103,392. Transmembrane helices are shown in turquoise and are oriented with the extracellular space at the top of the page. Amino acid side-chains are shown in green. MDL103,392 is shown in orange.

by dramatic loss of NK-2 binding affinity in analogues in which this moiety is substituted with a carbon isosteric or quaternized nitrogen (data not shown).

The 3,4-dichloro-phenyl substituent of MDL103,392 is clearly an important group for the activity at both the NK-1 and NK-2 receptors.[33] The fact that our model does not propose an interaction within the NK-2 receptor at this moiety either reflects the need to continue mutagenesis studies of this receptor or that the 3,4-dichloro-phenyl moiety plays a central role in the conformation of MDL103,392 and does not serve as a

specific interaction point. The latter explanation is unlikely, since it is clear that the nature of the substitutions on the phenyl ring are important with a single chloro- or a dimethoxy-substitution being less potent, especially at the NK-2 receptor.[33] These substitutions would not be expected to alter the conformation of this ring dramatically and so suggest that they form a direct interaction with an amino acid side-chain within the receptor.

The three-dimensional models of binding of MDL103,392 to the NK-1 and NK-2 receptors are shown in Figure 5 (a and b respectively). The orientations of the transmembrane helices are such that the extracellular surface of the receptor is at the top of the image. Comparison of Figure 5a and b shows the contrast in the predicted binding orientation of MDL103,392 at each of the receptors. In both receptors the compound is oriented with the 3,4,5-trimethoxyphenyl ring toward the top of the helical bundle near Gln-165. In the NK-1 receptor MDL103,392 is predicted to lie transverse to the helices and shows no apparent interactions with transmembrane helix VII. In contrast in the NK-2 receptor model the compound is predicted to lie parallel to the transmembrane helices and contact residues on helices IV, V, VI and VII. The alternative binding conformations of the dual antagonist MDL103,392 and the differences in relative importance of conserved residues such as NK-2 Tyr-289 and NK-1 Leu-203 and Ile-204 might not have been expected a priori given the high sequence identity within the helical bundle between the two receptors. Of the four sequence differences in this cavity, our study suggests the orientation difference is likely due, at least in part, to direct interactions with NK-1 Phe-264 and its counterpart, NK-2 Tyr-266.

Conclusion

The data presented in this manuscript clearly indicate that the amino acids important for the binding of MDL103,392 at the NK-1 and NK-2 receptors are different. This further suggests that different conformations of the compound bind to either receptor resulting in an equipotent dual antagonist. These data will aid in the design of future generations of neurokinin antagonists and may prove applicable to other G protein-coupled peptide receptors.

Experimental

Bacterial strains and plasmids

Human neurokinin-1 and -2 receptor cDNAs (J. Krause) were subcloned from pBluescript II SK(−) into pSRα-SPORT for expression in COS-7 cells. The plasmid pSRα-SPORT is a derivative of pSV-SPORT (Life-Technologies, Gaithersburg, MD) in which the SV-40 early promoter has been replaced with the SRα promoter. Plasmids were propagated in DH5α *E. coli* (Life-Technologies, Gaithersburg, MD). Site-directed mutagenesis of human NK receptor DNAs was carried out by polymerase chain reaction (PCR) overlap-extension.[22] Oligonucleotide primers were supplied by Life-Technologies (Gaithersburg, MD). The DNA sequence of the amplified region of each mutant was determined prior to evaluation.

NK receptor antagonists

MDL103,392 was synthesized at Schering-Plough Research Institute as an equimolar mixture of both the (*R*)- and (*S*)-enantiomers. Stock solution for the compound was 10 mM in dimethyl-sulfoxide.

Mammalian cell culture and transfections

Each NK receptor construct was transiently expressed in COS-7 cells (American Type Culture Collection, Rockville, MD) 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, Gaithersburg, MD). DNA constructs were transfected into COS-7 cells by electroporation using a GENE-PULSER (Bio-Rad Inc., Hercules, CA) following the manufacturer's procedure.

Binding assays

Transfected COS-7 cells were plated onto 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) 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 NK receptor transfected cells in the presence of increasing concentrations of [^{3}H](9-Sar,11-Met(O_2)-substance P ([^{3}H]Sar-sub-P) (NK-1) (DuPont-NEN, Boston, MA) or [4,5-^{3}H-Leu^9]neurokinin A ([^{3}H]NKA) (NK-2) (Zeneca, Bilingham, Cleveland, UK) with and without excess cold Sar-sub-P (10 μM) (Penninsula Labs, Belmont, CA) or cold NKA (Penninsula Labs, Belmont, CA) (10 μM) respectively 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-sub-P or [^{3}H]NKA and increasing concentrations of MDL103,392 in duplicate. Each competition experiment also included total and non-specific binding controls. After incubation, cells were washed in the wells twice with phosphate buffered saline and harvested by addition of 0.5% sodium dodecyl sulfate. The harvested cell lysate was combined with scintillation fluid (Ready Safe, Beckman, Inc.). The samples were then counted in an LKB RackBeta (LKB-Wallac, Gaithersburg, MD) at 40–50% efficiency.

Functional assays

Transiently transfected COS-7 cells were plated into 96-well black Viewplates (Packard Instruments, Meriden, CT) and grown for 72 h post transfection. Medium was removed and cells were incubated for 1 h at 37°C in 0.1 mL loading buffer (Hanks balanced salt solution

[Life-Technologies, Gaithersburg, MD]/0.2% bovine serum albumin [Sigma, St. Louis, MO]/20 mM HEPES [Life-Technologies, Gaithersburg, MD]/2.5 mM probenecid [Sigma, St. Louis, MO]) containing 4 μM calcium green-1AM dye (Molecular Probes, Eugene, OR). Stock solution of dye was mixed 1:1 with 20% pluronic acid (Molecular Probes, Eugene, OR) prior to addition to loading buffer. After incubation cells were washed with loading buffer on a Labsystems plate washer to remove excess dye. Intracellular calcium release assays were carried out using a Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA) according to the manufacturer's instructions. For all assays either buffer of buffer containing various concentrations of antagonist were added and the plate incubated at room temperature for at least 10 min. Agonist (Sar-sub-P or NKA) was added and calcium release was monitored continuously for 2 min post agonist addition. The data shown are percent maximum response based on the maximal response obtained for a given receptor construct in the absence of antagonist. For the determination of the effects of MDL103,392 on the agonist dose–response curve (apparent K_b values) a fixed concentration of antagonist was preincubated with cells followed by activation by multiple concentrations of agonist. For the determination of MDL103,392 IC_{50} values multiple concentrations of antagonist were preincubated with cells followed by activation with a fixed concentration of agonist. Each data point was run in quadruplicate.

Data analysis

Agonist affinities (K_d) were determined from saturation binding by non-linear regression curve fitting using the program PRISM (Graphpad Software, San Diego, CA). IC_{50} values were determined graphically by plotting antagonist concentration versus percent of maximum specific binding. K_i values were determined from IC_{50} values using the Cheng and Prusoff equation.[23] Agonist EC_{50} values were determined by non-linear regression curve fitting using the program PRISM (Graphpad Software, San Diego, CA). Apparent K_b values were determined using the agonist dose ratio and the values reported are the average determined over the range of antagonist concentrations tested.[24] IC_{50} values were determined by non-linear regression curve fitting using the program PRISM.

Molecular modeling

The homology models of the human NK-1 and NK-2 receptors were constructed from the electron diffraction structure of bacteriorhodopsin[25] with the Homology program (v. 95.0, Molecular Simulations Inc., San Diego, CA) using a method previously described for muscarinic receptor modeling.[26] 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.[27]

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References

1. Maggi, C. A. *General Pharmacology* **1995**, *26*, 911.
2. Longmore, J.; Swain, C. J.; Hill, R. J. *DN & P* **1995**, *8*, 5.
3. Fong, T. M.; Strader, C. D. *Medicinal Research Reviews* **1994**, *14*, 387.
4. Huang, R.-R. C.; Yu, H.; Strader, C. D.; Fong, T. M. *Biochemistry* **1994**, *33*, 3007.
5. Fong, T. M.; Huang, R.-R. C.; Yu, H.; Swain, D. J.; Underwood, D; Cascieri, M. A.; Strader, C. D. *Canadian Journal of Physiology and Pharmacology* **1995**, *73*, 860.
6. Bhogal, N.; Donnely, D.; Findlay, J. B. C. *Journal of Biological Chemistry* **1994**, *269*, 27269.
7. Huang, R.-R. C.; Vicario, P. P.; Strader, C. D.; Fong, T. M. *Biochemistry* **1995**, *34*, 10048.
8. Boyd, N. D.; Kage, R.; Dumas, J. J.; Krause, J. E.; Leeman, S. E. *Proceedings of the National Academy of Science USA* **1996**, *93*, 433.
9. Kersey, I. D.; Fishwick, C. W. G.; Findlay, J. B. C.; Ward, P. *Bioorganic and Medical Chemistry Letters* **1995**, *5*, 1271.
10. Li, Y. M.; Marnerakis, M.; Stimson, E. R.; Maggio, J. E. *Journal of Biological Chemistry* **1995**, *270*, 1213.
11. Turcatti, G.; Vogel, H.; Chollet, H. *Biochemistry* **1995**, *34*, 3972.
12. Snider, R. M.; Constantine, J. W.; Lowe, J. A. I.; Longo, K. P.; Lebel, W. S.; Woody, H. A.; Drozda, S. E.; Desai, M. C.; Vinick, F. J.; Spencer, R. W.; Hess, H. J. *Science* **1991**, *251*, 435.
13. Greenfeder, S.; Cheewatrakoolpong, B.; Anthes, J.; Billah, M.; Egan, R. W.; Brown, J. E.; Murgolo, N. J. *Bioorganic and Medical Chemistry* **1998**, *6*, 189.
14. Gether, U.; Yokota, Y.; Emonds-Alt, X.; Breliere, J.-C.; Lowe, J. A. I.; Snider, R. M.; Nakanishi, S.; Schwartz, T. W. *Proceedings of the National Academy of Science* **1993**, *90*, 6194.
15. Gether, U.; Johansen, T. E.; Snider, R. M.; Lowe, J. A. I.; Nakanishi, S.; Schwartz, T. W. *Nature* **1993**, *362*, 345.
16. Gether, U.; Emonds-Alt, X.; Breliere, J. C.; Fujii, T.; Hagiwara, D.; Pradier, L.; Garret, C.; Johansen, T. E.; Schwartz, T. W. *Molecular Pharmacology* **1993**, *45*, 500.
17. Fong, T. M.; Cascieri, M. A.; Yu, H.; Bansal, C.; Swain, D. J.; Strader, C. D. *Nature* **1993**, *362*, 350.
18. Fong, T. M.; Yu, H.; Cascieri, M. A.; Underwood, D.; Swain, D. J.; Strader, C. D. *Journal of Biological Chemistry* **1994**, *269*, 14957.
19. Edmonds-Alt, X.; Vilain, P.; Goulaouic, P.; Proietto, V.; Van Broeck, D.; Advenier, C.; Naline, E.; Neliat, G.; Le Fur, G.; Breliere, J. C. *Life Science and Pharmacological Letters* **1992**, *50*, 101.
20. Burkholder, T. P.; Kudlacz, E. M.; Le, T.-B.; Knippenberg, R. W.; Shatzer, S. A.; Maynard, G. D.; Webster, M. E.; Horgan, W. *Bioorganic and Medical Chemistry* **1996**, *6*, 951.
21. Kudlacz, E. M.; Shatzer, S. A.; Knippenberg, R. W.; Logan, D. E.; Poirot, M.; Van Giersbergen, P. L. M.; Burkholder, T. P. *Journal of Pharmacology and Experimental Therapeutics* **1996**, *277*, 840.
22. Higuchi, R. Recombinant PCR. In *PCR Protocols:A Guide to Methods and Applications*; Innis, M. A.; Gelfand, D. H.; Sninsky, J. J.; White, T. J., Eds.; Academic Press: San Diego, 1990; p 177.

23. Cheng, Y.; Prusoff, W. H. *Biochemistry and Pharmacology* **1973**, *22*, 3099.
24. Tallarida, R. J. *Life Sciences* **1988**, *43*, 2169.
25. Henderson, R.; Baldwin, J. M.; Ceska, T. A.; Zemlin, F.; Beckmann, E.; Downing, K. H. *Journal of Molecular Biology* **1990**, *213*, 899.
26. Murgolo, N. J.; Kozlowski, J.; Tice, M. A. B.; Hollinger, F. P.; Brown, J. E.; Zhou, G.; Taylor, L. A.; McQuade, R. D. *Bioorganic Medicinal and Chemical Letters* **1996**, *6*, 785.
27. Elling, C. W.; Nielsen, S. M.; Schwartz, T. W. *Nature* **1995**, *374*, 74.
28. Lazareno, S.; Birdsall, N. J. M. *Trends in Pharmacological Science* **1993**, *14*, 237.
29. Lundstrom, K.; Hawcock, A. B.; Vargas, A.; Ward, P.; Thomas, P.; Naylor, A. *European Journal of Pharmacology* **1997**, *337*, 73.
30. Huang, R.-R. C.; Yu, H.; Strader, C. D.; Fong, T. M. *Molecular Pharmacology* **1994**, *45*, 690.
31. Cascieri, M. A.; Shiao, L.-L.; Mills, S. G.; MacCoss, M.; Swain, C. J.; Yu, H.; Ber, E.; Sadowski, S.; Wu, M. T.; Strader, C. D.; Fong, T. M. *Molecular Pharmacology* **1995**, *47*, 660.
32. Fong, T. M.; Yu, H.; Cascieri, M. A.; Underwood, D.; Swain, D. J.; Strader, C. D. *Journal of Biological Chemistry* **1994**, *269*, 2728.
33. Burkholder, T. P.; Kudlacz, E. M.; Maynard, G. D.; Liu, X.-G.; Le, T.-B.; Webster, M. E.; Horgan, S. W.; Wenstrup, D. L.; Freund, D. W.; Boyer, F.; Bratton, L.; Gross, R. S.; Knippenberg, R. W.; Logan, D. E.; Jones, B. K.; Chen, T.-M. *Bioorganic and Medicinal Chemistry Letters* **1996**, *7*, 2531.