Tyrosine 220 in the 5th Transmembrane Domain of the Neuromedin B Receptor Is Critical for the High Selectivity of the Peptoid Antagonist PD168368*

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Peptoid antagonists are increasingly being described for G protein-coupled receptors; however, little is known about the molecular basis of their binding. Recently, the peptoid PD168368 was found to be a potent selective neuromedin B receptor (NMBR) antagonist. To investigate the molecular basis for its selectivity for the NMBR over the closely related receptor for gastrin-releasing peptide (GRPR), we used a chimeric receptor approach and a site-directed mutagenesis approach. Mutated receptors were transiently expressed in Balb 3T3. The extracellular domains of the NMBR were not important for the selectivity of PD168368. However, substitution of the 5th upper transmembrane domain (uTM5) of the NMBR by the comparable GRPR domains decreased the affinity 16-fold. When the reverse study was performed by substituting the uTM5 of NMBR into the GRPR, a 9-fold increase in affinity occurred. Each of the 4 amino acids that differed between NMBR and GRPR in the uTM5 region were exchanged, but only the substitution of Phe220 for Tyr in the NMBR caused a decrease in affinity. When the reverse study was performed to attempt to demonstrate a gain of affinity in the GRPR, the substitution of Tyr220 for Phe caused an increase in affinity. These results suggest that the hydroxyl group of Tyr220 in uTM5 of NMBR plays a critical role for high selectivity of PD168368 for NMBR over GRPR. Receptor and ligand modeling suggests that the hydroxyl of the Tyr220 interacts with nitrophenyl group of PD168368 likely primarily by hydrogen bonding. This result shows the selectivity of the peptoid PD168368, similar to that reported for numerous non-peptide analogues with other G protein-coupled receptors, is primarily dependent on interaction with transmembrane amino acids.

Recently the “peptoid” approach was described for the design of low molecular weight, nonpeptide ligands (peptoid), using the chemical structure of mammalian neuropeptides as a starting point (1, 2). These peptoids may act as either agonists or antagonists at neuropeptide receptors. To date, several classes of peptoid antagonists for receptors of gastrointestinal (GI) hormones/neurotransmitters have been described including for cholecystokinin (3–5), somatostatin (6), tachykinins (7–9), or bombesin (10) receptors. They have been proven useful in helping to examine the role of these receptors in mediating various physiological and pathophysiological processes and they may be useful as therapeutic agents in such conditions as panic attacks (1, 2, 5). Whereas there have been a number of studies of the molecular basis of action of nonpeptide antagonists for various GI hormone/neurotransmitter receptors (11–15) within the heptahelical G protein-coupled receptors (GPCRs), almost nothing is known about the molecular basis of action for peptoid antagonists. For most small molecule ligands for GPCRs such as nonpeptide antagonists, transmembrane regions play an important role in determining the high affinity binding (16, 17). However, the essential receptor domains for the high selectivity of peptoid binding are still unclear.

Neuromedin B (NMB) and gastrin-releasing peptide (GRP), mammalian homologues of the amphibian tetradecapeptide bombesin, are small amidated peptides with structurally related carboxyl termini (18). These peptides mediate a spectrum of biological activities by binding to two structurally and pharmacologically distinct receptors, the NMB receptor (NMBR) and GRP receptor (GRPR) (19, 20). These peptides have important effects in the central nervous system including thermoregulation (21), satiety (22), control of circadian rhythm (23), and peripheral tissues causing stimulation of gastrointestinal hormone release (18, 24, 25), activation of macrophages (26), and effects on development (27, 28). These peptides also have potent growth effects causing proliferation of normal cells (18, 29) and various tumor cell lines (18, 30). The NMBR and GRPR are members of the bombesin receptor family within the GPCR superfamily and share ~50% overall amino acid sequence identity (19, 20). Both the NMBR and GRPR are widely distributed in the central nervous system and alimentary tract (18). Although the potential physiological role of GRP and its receptor has been a major focus of research (18, 20), the role of NMB in physiological or pathophysiological processes has received much less attention. Some studies suggest that NMB may play an important role in a number of biological processes, including causing growth of some tumor cells (31), a modulatory role in suppression of feeding behavior or gastric emptying (32), con-
control of the hypothalamic-pituitary-adrenocortical axis and thyrotropin release (33), sensory transmission in the spinal cord (34), excitation of serotonin neurons in the dorsal raphe nucleus (35), control of potassium secretion by the blood-brain barrier (36), and smooth muscle contractility (37). However, which of these are physiological or the principal roles of NMBR activation in pathological processes remains unclear. The development of selective, high affinity NMBR agonists and antagonists would enable a more precise definition of the role of NMBR in these processes.

In contrast to GRPR antagonists for which numerous classes of high affinity antagonists have been described (38), the discovery process for NMBR antagonists has been slower. None of the strategies successfully used previously yielded potent antagonists when applied to the NMBR (38, 39). However, a peptoid based on a 3-amino acid template, PD165929, was recently characterized as a high affinity NMBR antagonist (10). In a previous study, we evaluated the pharmacology of a second generation peptoid from this series, PD168368 (40, 41). The results confirmed that PD168368 was a potent and selective antagonist for NMBR over GRPR regardless of species origin, which could prove generally useful in understanding the role of NMBR in physiological and pathological processes.

To attempt to provide insight into the molecular basis of the specificity of the peptoid PD168368 for the NMBR receptor, in the present study we have investigated in detail the high affinity of PD168368 for the NMBR, and its selectivity for the NMBR over the GRPR using a chimeric and mutagenesis approach. To identify which domains of the NMBR are important for high affinity binding of PD168368, we used a chimeric receptor approach, which has proven useful in elucidating the structural basis of GPCR interaction with ligands (42). A site-directed mutagenesis approach was then used to identify critical amino acid(s) within these domains. Here we report the peptoid antagonist PD168368’s selectivity for the NMBR over GRPR depends primarily on an interaction with amino acids in the 5th upper transmembrane region of the NMBR. Detailed site-directed mutagenesis studies demonstrate that Tyr$^{220}$ in this region is a key amino acid for high affinity binding PD168368. Computer modeling of the receptor and analysis of the ligand support the conclusion that PD168368 interacts with the hydroxyl group of Tyr$^{220}$ principally through hydrogen bonding.

**Experimental Procedures**

**Materials**—pDNA3 was from Invitrogen (Carlsbad, CA). Oligonucleotides were from Midland Certified Reagent Company (Midland, TX) and Life Technologies, Inc. Sequence cloning kit and QuikChange® site-directed mutagenesis kit were from Stratagene (La Jolla, CA). Restriction endonucleases (HindIII, XbaI, and EcoRI), fetal bovine serum (FBS), penicillin-streptomycin, LipofectAMINE® reagent, LipofectAMINE® Plus reagent, and trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na) were from Life Technologies, Inc. Dulbecco’s modified Eagle’s medium (DMEM) and Dulbecco’s phosphate-buffered saline were from Biofluids, Inc. (Rockville, MD). Balb 3T3 cells were from American Type Culture Collection (Rockville, MD). A 100 × 20-mm tissue culture dish (Falcon® 3003) was from Becton Dickinson (Plymouth, United Kingdom). Bombesin (Bn) was from Peninsula Laboratories, Inc. (Belmont, CA). Na$^{131}$I (2,200 Ci/mmol) was from Amersham Pharmacia Biotech. 1,3,4,6-Tetracloro-3a,6a-diphenylglycouril (IODO-GEN®) and dithiothreitol were from Pierce. Bovine serum albumin fraction V and HEPES were from ICN Pharmaceutical Inc. (Aurora, OH). Soybean trypsin inhibitor type I-S and bacitracin were from Sigma. Nyoil M20 oil (specific gravity 1.0337) was from Nye Lubricants Inc. (New Bedford, MA). PD168368 was a gift from Robert Pinnock (Medicinal Chemistry Department, Pfizer Global Research, Cambridge, United Kingdom). All other chemicals were of the highest purity commercially available.

**Construction of Chimeric and Mutant Receptors**—The cDNAs of the mouse GRPR and rat NMBR were identical to those described previously (42). The receptor extracellular domains or upper transmembrane domains used to make chimeric receptors were those identified using hydroxyphyt plots for the GRPR and for the NMBR. The amino acids in these regions of the two receptors were aligned using the Wisconsin Package (Version 9.1; Genetics Computer Group, Madison, WI) for comparisons. The cDNA of the wild-type mouse GRPR was inserted between the HindIII site and XbaI site of pcDNA3, and the wild-type rat NMBR was inserted into the EcoRI site of pcDNA3. Both the GRPR/NMBR extracellular domain chimeras and upper transmembrane chimeras were constructed using the Seamless® cloning kit. Mutant receptors were made by using the QuikChange® site-directed mutagenesis kit, following the manufacturer’s instructions except that the annealing temperature was 60 °C and the DpnI digestion was for 2 h. Nucleotide sequence analysis of the entire coding region was performed using an automated DNA sequence analyzer (ABI Prism® 377 DNA sequence; Applied Biosystems Inc., Foster City, CA).

**Cell Transfection**—Balb 3T3 cells were seeded in a 10-cm diameter tissue culture dish at a density of 10$^6$ cells/dish and grown overnight at 37 °C in DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin. The following morning cells were transfected with 5 μg of plasmid DNA by cationic lipid-mediated method using 30 μl of LipofectAMINE® reagent and 20 μl of LipofectAMINE® Plus reagent in serum-free DMEM for 3 h at 37 °C. At the end of the incubation period, the medium was replaced with DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37 °C, with a 5% CO$_2$ atmosphere and were used 48 h later for binding assays.

**Preparation of $^{125}$I-Tyr$^4$Bn and $^{125}$I-[Tyr$^6$]$\beta$-Ala$^{11}$,Phe$^{13}$,Nle$^{14}$Bn$^{6-14}$]** $^{125}$I-Tyr$^4$Bn and $^{125}$I-[Tyr$^6$]$\beta$-Ala$^{11}$,Phe$^{13}$,Nle$^{14}$Bn$^{6-14}$ at a specific activity of 2,200 Ci/mmol were prepared by a modification of the methods described previously (39, 40, 43). Briefly, 0.5 μg of IODO-GEN in chloroform was transferred to a vial, dried under a stream of nitrogen and washed with 100 μl of KH$_2$PO$_4$ (pH 7.4). To this vial, 20 μl of 0.5 M KH$_2$PO$_4$ (pH 7.4), 8 μg of peptide in 4 μl of water, and 2 μl of 20% (v/v) Na$^{131}$I were added, mixed gently and incubated at room temperature for 6 min. The incubation was stopped by the addition of 100 μl of distilled water and in the case of $^{125}$I-Tyr$^4$Bn, 300 μl of 1.5 M dithiothreitol was also added to reduce the oxidized methionines. The elution mixture was re-incubated at 80 °C for 60 min. The reaction mixtures were applied to a Sep-Pak (Waters Associates, Milford, MA), and free $^{131}$I was eluted with 5 ml of water followed by 5 ml of 0.1% (v/v) trifluoroacetic acid. The radiolabeled peptides were eluted with 200 μl of sequential elutions (×10) with 60% acetonitrile in 0.1% trifluoroacetic acid. The two or three fractions with the highest radioactivity were...
combined and purified on a reverse-phase, high performance liquid chromatography with a μBondapak column (0.46 × 25 cm). The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (v/v) from 16–60% acetonitrile in 60 min, and 1-ml fractions were collected and checked for radioactivity and receptor binding. The pH of the pooled fractions were adjusted to 7 using 0.2 M Tris (pH 9.5), and radioligands were stored in aliquots with 0.5% bovine serum albumin at −20 °C.

Whole Cell Radioligand Binding Assays—Competitive binding assays were performed 48 h after transfection as described previously (43, 44). Briefly, disaggregated transiently transfected cells were incubated for 1 h at room temperature in 250 μl of binding buffer (pH 7.4) with the ligands 125I-[Tyr8]-Bn (2,200 Ci/mmol) or 125I-[Glu6,-Ala8]-Phe16,Nle14-Bn (6–14) (2,200 Ci/mmol) in the presence of the indicated concentration of unlabeled peptides. The binding buffer contained 98 mM NaCl, 6 mM KCl, 11.5 mM glucose, 5 mM fumarate, 5 mM glutamate, 5 mM pyruvate, 24.5 mM HEPES, 0.2% (v/v) essential amino acid solution, 2.5 mM KH2PO4, 1 mM MgCl2, 0.5 mM CaCl2, 0.2% (w/v) bovine serum albumin, 0.05% (w/v) bacitracin, and 0.01% (w/v) soybean trypsin inhibitor. The cell concentration was adjusted to 0.15–9 × 106 cells/ml to assure that no more than 20% of the total added radioactive ligand bound. Bound tracer was then separated from unbound tracer by laying 100 μl of the binding reaction on top of an oil phase (100 μl of Nyoisil M20, Nye Lubricants Inc., New Bedford, MA) in a 0.4-ml microcentrifuge tube (PGC Scientific, Frederick, MD), and pelleting the cells through the oil by centrifugation at 10,000 g in a Microfuge E67 (Beckman, Palo Alto, CA) for 3 min. The supernatant was aspirated, and the pellet cells were rinsed twice with distilled water. The amount of radioactivity bound to the cells was measured in a Cobra II γ counter (Packard, Downers, IL). Aliquots (100 μl) of the incubation mixture were taken in duplicate to determine the total radioactivity. Binding was expressed as the percentage of total radioactivity that was associated with the cell pellet. All binding values represented saturable binding (i.e. total binding minus nonsaturable binding). Nonsaturable binding was <15% of the total binding in all experiments. Each point was measured in duplicate, and each experiment was replicated at least three times. Calculation of IC50 values was performed with a curve-fitting program, KaleidaGraph graphing software (Synergy Software, Reading, PA).

Visualization of PD168368—In an attempt to visualize the hydrogen bonding potential and likely hydrogen bonding donor and acceptor sites of PD168368, we generated a model for the ligand in the molecular modeling suite Sybyl 6.6 (Tripos Inc., St. Louis, MO). The model was initially generated in a reasonable low energy conformation using Con- cord (45, 46), a program widely used to generate three-dimensional structures. This model was then optimized by energy minimization using the Sybyl 6.6 Tripos force field with atomic charges calculated by the method of Gasteiger-Hückel (47–49). The molecular surface was visualized as a Connolly dot surface. The molecular volume was 558 Å3.

Binding Site Model—The coordinates of the NMB transmembrane receptor model (NMBR_Vriend) were retrieved from the GPCR data base server on the World Wide Web (50). In an attempt to better define the NMBR binding pocket of PD168368, the receptor was modeled using the α-carbon template from bacteriorhodopsin, a seven-transmembrane receptor characterized by electron cryomicroscopy (51), the WHAT IF program (52), and Sybyl 6.6 (Tripos, Inc.). Following optimization of the model receptor transmembrane bundle by extensive energy minimization using the Kollman all force field (51) in Sybyl 6.6, putative solvent accessible binding sites were explored using the SiteID module in Sybyl 6.6. The surface of the solvent cluster filling the binding site was visualized using the Molcad module of Sybyl.

Table I

| Peptide/peptoid | IC50 (nM) GRPR | IC50 (nM) NMBR |
|-----------------|--------------|--------------|
| Bn              | 0.49 ± 0.01  | 1.5 ± 0.1    |
| GRP             | 1.9 ± 0.1    | 21 ± 1       |
| NMB             | 100 ± 2      | 1.0 ± 0.1    |
| PD168368        | 3,500 ± 110  | 96 ± 8       |

Wild-type NMBR and GRPR—The Bn-related natural occurring agonists Bn and GRP (Fig. 1, Table I) had high affinities (IC50 values of 0.5 and 2 nM, respectively) for the GRPR, and NMB (Fig. 1, Table I) for the NMBR (IC50 = 1.0 nM). Bn had a 3-fold and GRP an 11-fold selectivity for the GRPR, whereas NMB had 100-fold selectivity for the NMBR (Table I). PD168368 (Fig. 1) had 96-fold lower affinity for the NMBR (IC50 = 96 ± 8 nM) than NMB (Table I). However, PD168368 also had a very low affinity for the GRPR (IC50 = 3,500 ± 110 nM) with the result that PD168368 had a 36-fold selectivity for the NMBR over the GRPR (Table I).

Extracellular Chimeric Receptors—To begin to explore the molecular basis for this difference in affinity of PD168368 for NMBR and GRPR, four chimeric receptors with the extracellular domains of GRPR substituted for the comparable domains of the NMBR.
in NMBR (loss of affinity chimeras, see Fig. 2) and four chimeras with the extracellular domains of NMBR substituted into GRPR (gain of affinity chimeras, see Fig. 3) were made. The affinities of PD168368 for the NMBR chimeras in which the 1st, 2nd, and 4th extracellular domain in the NMBR was substituted by the comparable domain of GRPR were similar to wild-type NMBR (Fig. 2, Table II). Substitution of the 3rd extracellular domain in the NMBR in the comparable domain of the GRPR decreased the affinity almost 2-fold from 96 ± 7.8 nM to 180 ± 17 nM (Fig. 2, Table II). However, this represented only a small change compared with the 36-fold lower affinity PD168368 had for the wild type GRPR compared with the wild type NMBR (Fig. 2, Tables I and II). To confirm these results, gain of affinity chimeras were made by substituting in the GRPR, the comparable extracellular domains of the NMBR (Fig. 3). The 3rd extracellular domain substitution into the GRPR increased potency, causing a 3-fold gain in affinity from 3,500 ± 110 nM to 1,100 ± 49 nM (Fig. 3, Table II). However, chimeras formed by the insertions of the 1st, 2nd, or 4th extracellular loop of NMBR into GRPR caused no change in affinity (Fig. 3, Table II).

Upper Transmembrane Chimeric Receptors—Because substitutions of the extracellular domains caused only a small change in affinity of PD168368, suggesting they were playing only a minor role in the selectivity of PD168368 for the NMBR, we applied a similar chimeric approach to the receptor’s upper transmembrane regions, which are the transmembrane areas most likely to interact with the ligand. The affinities of potential loss of affinity NMBR chimeras in which 1st, 2nd, 3rd, 4th, and 7th upper transmembrane domain in the NMBR in the comparable domain from GRPR showed no change in affinity for PD168368 compared with the wild type NMBR (Fig. 4, Table III). However, substitution of the 5th or the 6th upper transmembrane domain of the NMBR by the comparable GRPR domains decreased the affinity 16-fold (i.e. 96 ± 7.8 nM to 1,540 ± 150 nM) and 2-fold (96 ± 7.8 nM to 190 ± 9.4 nM), respectively (Fig. 4, Table III). When the reverse study was performed to make potential gain of affinity chimeras by substituting into the GRPR the 5th upper transmembrane domain of NMBR, a 9-fold increase in affinity (3,500 ± 110 nM to 390 ± 36 nM) occurred (Fig. 5, Table III). In contrast, substitutions of the other upper transmembrane domains of NMBR into the GRPR, including the upper 6th transmembrane region of NMBR, caused no gain of affinity (Fig. 5, Table III). A simultaneous substitution into the GRPR of the 3rd extracellular domain and the 5th upper transmembrane domain of NMBR resulted in a 36-fold increase in affinity for PD168368 and the resultant receptor had similar affinity for PD168368 as the wild type NMBR (Fig. 5, Table III).

Point Mutants in the 5th Upper Transmembrane Domain—To identify which amino acid(s) in the 5th upper transmembrane domain of the NMBR and GRPR were responsible for the selectivity of PD168368 for the NMBR, the amino acid sequence of this region of the GRPR and NMBR was compared (Fig. 6). The two receptors differed in 4 amino acids in the 5th transmembrane region (Fig. 6). Specifically, the isoleucine in position 216 in the NMBR was replaced by a serine in the GRPR, tyrosine 220 by phenylalanine, phenylalanine 221 by tyrosine, and leucine 222 in NMBR by valine in the comparable position of GRPR (Fig. 6). To determine the importance of these four amino acid differences, potential loss of affinity NMBR point mutations were made by substituting into the NMBR the comparable amino acid from the GRPR (Figs. 6 and 7) and four potential gain of affinity GRPR point mutations were made by substituting into the GRPR the comparable amino acid from the NMBR (Figs. 6 and 8). For the potential loss of affinity NMBR point mutations, three of the substitutions (Ser<sup>216</sup> for

![Diagram](Image 254x496 to 555x730)

**TABLE II**

Affinities of PD168368 for wild-type NMBR, loss of affinity NMBR, and gain of affinity GRPR extracellular loop chimeric receptors and wild-type GRPR

| Receptor | IC<sub>50</sub> for PD168368 |
|----------|-----------------|
| Wild-type NMBR | 96 ± 8 |
| [e1-NMBR]GRPR | 96 ± 9 |
| [e2-NMBR]GRPR | 180 ± 17 |
| [e3-NMBR]GRPR | 98 ± 4 |
| [e4-NMBR]GRPR | 1,100 ± 49 |
| Wild-type GRPR | 1,100 ± 49 |
| [e1-GRPR]NMBR | 4,600 ± 260 |
| [e2-GRPR]NMBR | 2,100 ± 50 |
| [e3-GRPR]NMBR | 1,100 ± 49 |
| [e4-GRPR]NMBR | 3,000 ± 92 |

![Diagram](Image 254x496 to 555x730)
Values represent the mean ± S.E. from at least three independent experiments.

Hydrogen bond acceptor regions associated with the pyridyl group and amide bonds (Fig. 9).

**Basis of NMBR Selectivity of PD168368**

There are now more than 30 GPCRs mediating the action of GI hormones or neurotransmitters such as bradykinin, GRP, or cholecystokinin (CCK) (53). In most cases their roles in physiological processes or in pathological conditions are still unclear because only recently have high affinity antagonists been developed for some of these receptors. The antagonists for these receptors generally fall into one of three types: peptide antagonists, nonpeptide antagonists, or peptoid antagonists, which have features of both peptides and nonpeptides (1, 2). In general the molecular basis of action of these antagonists is poorly understood, and it is unclear whether it is similar to the well studied adrenergic or muscarinic cholinergic receptor antagonists (54–56). Whereas there have been a number of studies of the molecular basis of action of nonpeptide antagonists for various GI hormone receptors (11–15), there are only a few studies of peptide antagonists (12, 57, 58) and almost none for peptoid antagonists (59). In this study we examined the molecular basis of action of the novel peptoid receptor antagonist PD168368, which is reported to be selective for the NMBR in the bombesin family of receptors (10, 40, 41).
extracellular domains of the GRPR were substituted into the NMBR to assess loss of affinity for PD168368, only the substitution of the 3rd extracellular domain in the NMBR by the comparable domain of the GRPR altered affinity and the decrease in affinity was less than 2-fold. Second, when the reverse study was performed by substituting into the GRPR the extracellular domain of the NMBR to produce gain of affinity chimeras, only the substitution of the 3rd extracellular domain in the GRPR by the comparable domain of the NMBR altered affinity causing an almost 3-fold increase for PD168368. The contribution of the 3rd extracellular domain for high selectivity of PD168368 was small compared with the almost 40-fold higher affinity PD168368 had for the native NMBR over GRPR. These results therefore suggest that differences in the 3rd extracellular domain of these two receptors play only a small role in the selectivity of PD168368. This result has both similarities and differences from studies of the interaction of peptide and nonpeptide agonists and antagonists with other GPCRs. Mutagenesis and biophysical analyses of several GPCRs indicate that the receptor extracellular domain can be an important receptor domain for determining high affinity ligand binding (16). Only a few studies have explored whether peptide antagonists interacting with receptor extracellular domains. Such an interaction is important for determining high affinity of the peptide antagonist JMV179 for the human CCK-A receptor (12); however, for other peptide antagonists such as the interaction of losartan with the AT1b angiotensin II receptor (13), the receptor extracellular domain can be important for determining high affinity interaction. Numerous studies show that with most nonpeptide antagonists numbering neurotransmitter/hormones that function as agonists 

### TABLE IV

| Receptor                  | IC\textsubscript{50} for PD168368 |
|---------------------------|----------------------------------|
| Wild-type NMBR            | 96 ± 8                           |
| [uTM5-GRPR]NMBR           | 1,500 ± 150\textsuperscript{a}    |
| [I216S]NMBR               | 110 ± 6\textsuperscript{a}        |
| [V220F]NMBR               | 830 ± 63                         |
| [F221Y]NMBR               | 150 ± 3                          |
| [L222V]NMBR               | 81 ± 3                           |
| Wild-type GRPR            | 3,500 ± 110                       |
| [uTM5-NMBR]GRPR           | 390 ± 96                         |
| [S215I]GRPR               | 3,600 ± 290                      |
| [F219Y]GRPR               | 550 ± 14                         |
| [V220F]GRPR               | 3,600 ± 170                      |
| [V221L]GRPR               | 4,900 ± 320                      |

**FIG. 5.** Importance of the uTM regions in determining selectivity of PD168368 for NMBR over GRPR; gain of affinity GRPR transmembrane chimeras. The chimeric uTM GRPRs were formed by replacing each of the transmembrane domains of GRPR by the comparable domain of the NMBR one at a time as shown in the diagrams at the top of the figure. Affinities were measured by competitive radioligand displacement of 50 pm \textsuperscript{125}I-Tyr\textsuperscript{4}Bn by PD168368 at the concentrations shown. Each point on the dose-inhibition curve is the mean from three separate experiments, and in each experiment each point was measured in duplicate. uTM5-, uTM2-, uTM3-, uTM4-, uTM5-, uTM6-, and uTM7-NMBR-GRPR refer to replacement by this transmembrane domain of the GRPR by that from the NMBR. Arrows indicate large changes in affinity of the GRPR.

**FIG. 6.** Alignment of amino acid sequences in the 5th upper transmembrane domain of NMBR and GRPR. Boxes indicate divergent amino acids between these two receptors in the 5th upper transmembrane region. Shown are the four NMBR and four GRPR point mutants made to explore the importance of each of the four amino acid differences for determining PD168368 selectivity.

**TABLE IV**

Affinities of PD168368 for the wild-type, receptor uTM5 loss and gain of affinity point mutants of NMBR and GRPR

Affinities were calculated by competitive displacement of 50 pm \textsuperscript{125}I-[Tyr\textsuperscript{4}]Bn or \textsuperscript{125}I-[D-Tyr\textsuperscript{6},Phe\textsuperscript{11},Nle\textsuperscript{14}]Bn-(6–14) (*) by PD168368. Values represent the mean ± S.E. from at least three independent experiments. The numbers refer to the position in either the NMBR or GRPR of the amino acid substitutions as shown in Fig. 6. [I216S]NMBR refers to replacement of the isoleucine in position 216 of the NMBR by serine, which exists in the comparable position in GRPR (see Fig. 6).

A. **Importance of the 3rd Extraglandular Domain**

- Wild-type NMBR: 96 ± 8
- [uTM5-GRPR]NMBR: 1,500 ± 150\textsuperscript{a}
- [I216S]NMBR: 110 ± 6\textsuperscript{a}
- [V220F]NMBR: 830 ± 63
- [F221Y]NMBR: 150 ± 3
- [L222V]NMBR: 81 ± 3
- Wild-type GRPR: 3,500 ± 110
- [uTM5-NMBR]GRPR: 390 ± 96
- [S215I]GRPR: 3,600 ± 290
- [F219Y]GRPR: 550 ± 14
- [V220F]GRPR: 3,600 ± 170
- [V221L]GRPR: 4,900 ± 320

B. **Comparability of Amino Acid Differences**

- Wild-type NMBR: 96 ± 8
- [uTM5-GRPR]NMBR: 1,500 ± 150\textsuperscript{a}
- [I216S]NMBR: 110 ± 6\textsuperscript{a}
- [V220F]NMBR: 830 ± 63
- [F221Y]NMBR: 150 ± 3
- [L222V]NMBR: 81 ± 3
- Wild-type GRPR: 3,500 ± 110
- [uTM5-NMBR]GRPR: 390 ± 96
- [S215I]GRPR: 3,600 ± 290
- [F219Y]GRPR: 550 ± 14
- [V220F]GRPR: 3,600 ± 170
- [V221L]GRPR: 4,900 ± 320
agonists such as α- and β-adrenergic agents, nucleosides, eicosanoids, muscarinic cholinergic agents, and lipid moieties, the extracellular receptor domains are not important generally in determining receptor selectivity and high affinity interaction (17). However, with small molecular nonpeptide agonists for the G protein-coupled Ca2+-sensing receptor; Ca2+- and Gd3+ interaction with the large NH2-terminal extracellular segment of the receptor is important for high affinity interaction (65).

Because the extracellular domains play only a minor role in the selectivity of PD168368 for the NMBR over the GRPR, we applied a similar chimeric approach to the receptor’s upper transmembrane region, which is also likely to interact with small ligands (16, 17). Our results support the conclusion that amino acids in the 5th upper transmembrane region play a major role, and in the 6th upper transmembrane region a minor role in determining the high selectivity of PD168368 for the NMBR over the GRPR. In contrast, the amino acids in the other five upper transmembrane regions did not play a significant role in this ligand’s selectivity. The importance of the 5th upper transmembrane region for PD168368 selectivity is consistent with the interaction of some small ligands (α- and β-adrenergic agents, nucleosides, eicosanoids, or muscarinic cholinergic agents) (16, 17) but not others (Ca2+, glutamate, or γ-aminobutyric acid) with their GPCRs, which provide evidence that upper transmembrane domains can be important regions for determining the affinity of some small molecular ligands to interact with receptors (17). High affinity receptor binding of some peptide agonists have been shown to primarily depend on interactions with the receptor upper transmembrane domains. This includes interaction of various peptide agonists with angiotensin II receptor (66), the endothelin-A receptor (ET_A-R) (15), neuropeptide Y1 receptor (67), and the thyrotropin-releasing hormone receptor (68). However, with peptide agonists for the B2 bradykinin receptor (63), the corticotropin-releasing factor receptor (69), or µ- and δ-opioid receptors (70), the transmembrane regions are not important. In similar fashion, several nonpeptide agonists have been shown to primarily interact with GPCR upper transmembrane regions including nonpeptide agonists for adrenergic receptors (54, 55), muscarinic cholinergic receptors (56, 71, 72), or eicosanoid (73, 74). High affinity receptor binding of some peptide antagonists also have been shown to depend primarily on interaction with the upper transmembrane domains of receptors such as the interaction of NPC567 and Hoe140 with the B2 bradykinin receptor (57, 58) or BQ-123 with the ET_A-R (13), whereas with other GPCRs such as interaction of CCK-JMV179 with the CCK-A receptor (12), the transmembrane regions are not important. A number of studies show that for most nonpeptide antagonists the interaction with the GPCRs’ transmembrane domains are the primary determinants of high affinity receptor interaction (13, 14, 60, 75–77). For example, with the AT_1b angiotensin II receptor, the nonpeptide antagonist losartan
requires specific amino acid residues within the 3rd to 7th transmembrane regions for high affinity binding (60). Similarly, the high affinity binding of the nonpeptide antagonist L365,260 with CCK-B/gastrin receptor (75), BMS-182874 with ET₁-R (13), SB209670 and Ro 46-2005 with ET₄-R (14), or CP96345 and l-161,664 with NK1 receptor (76, 77) are all dependent primarily on interaction with receptor upper transmembrane regions. Our results show that the peptoid antagonist PD168368 primarily resembles nonpeptide receptor antagonist’s interaction with GPCRs in that its high affinity and selectivity for the NMBR depends primarily on interactions with the receptor upper transmembrane areas.

For a few other ligands, the 5th transmembrane region is also a critical region to interact with a GPCR as we found in the present study for PD168368. For example, the critical binding site for the 8-amino acid peptide agonist angiotensin II is in the 5th transmembrane region of AT₁a angiotensin II receptor (66). The high affinity interaction of the nonpeptide agonists acetylcholine and carbachol depends on interaction with the 5th transmembrane region of the m3 muscarinic receptor (72). Furthermore, high affinity receptor binding and selectivity for several nonpeptide antagonists depend on interact with specific amino acids in the 5th transmembrane region, including the binding of the nonpeptide corticotropin releasing factor receptor 1 antagonist NBI27914 (69) to the human corticotropin releasing factor receptor 1 and the binding of the NK1 receptor antagonist L161,664 to the NK1 receptor (77).

To determine which amino acids in the 5th upper transmembrane region of the NMBR account for the PD168368’s selectivity, we performed a comparative alignment of the amino acids in this region between the NMBR and the GRPR. Within the 5th upper transmembrane domain, there were four amino acid residues that differ between NMBR and GRPR (Fig. 6). These differences included a Ser for Ile change at position 216 of the NMBR and Phe, Tyr, and Val at positions 219–221 of the GRPR for Tyr, Phe, and Leu at positions 220–222 of the NMBR. Our results support the conclusion that the tyrosine residue in position 220 of NMBR instead of a phenylalanine in this position in the GRPR is the key amino acid residue in determining the selectivity of the antagonist PD168368 caused by the differences in the receptors’ 5th transmembrane region.

![Fig. 9. An energy-minimized structure for PD168368. PD168368 is shown as a ball and stick model (hydrogen, cyan; carbon, white; nitrogen, blue; oxygen, red). It is surrounded by a Connolly molecular surface that has been colored to indicate hydrogen bond acceptor (blue) and donor (red) regions. Hydrophobic regions are colored gray.](image)

![Fig. 10. The NMB transmembrane receptor putative binding site surrounding the critical residue Tyr²²⁰. Helices 1–7 are colored light cyan, orange, red, purple, blue, green, and yellow, respectively. The putative binding site, established by computer modeling using SiteID, is depicted as a gray opaque space-filling model. Residues surrounding the binding site, including Tyr²²⁰ (green), are shown as stick models. Amino acid side chains not facing the center of the transmembrane region are omitted for clarity.](image)
In a previous study (42), we showed that the 5th transmembrane domain of NMBR was critical also for high affinity NMB binding. However, the isoleucine in position 216 was the key amino acid for high affinity interaction of NMB with the NMBR over the GRPR. These results demonstrate that the key receptor determinants for the selectivity of the agonist NMB and peptoid antagonist PD168368 for the NMBR over the GRPR are in very close proximity in the NMBR. This result is consistent with the strategy used to design PD168368, which depended strongly on incorporating critical structural features for determining high affinity NMB binding to NMBR into the PD168368 (10). However, despite these considerations the peptoid antagonist PD168368 and the peptide agonist NMB show a significant difference in their determinants of high affinity interaction with the NMBR. Specifically, the key amino acid in the 5th transmembrane domain (Tyr220) for high affinity NMBR interaction of PD168368 is different from the key amino acid for the high affinity interaction of NMB (Ile216) with the NMBR, supporting the conclusion that significantly different molecular interactions are likely responsible for their high affinities for the NMBR.

In several studies, a tyrosine residue in the GPCRs plays a critical role in ligand-receptor interaction (13, 66, 68, 73, 77). The tyrosine of the 3rd transmembrane domain of the thyrotropin-releasing hormone receptor binds the pyrogallvlum moiety of thyrotropin-releasing hormone through hydrogen bonding (68). The tyrosine of the 6th transmembrane domain of the m2 muscarinic receptor is a critical amino acid for the interaction with nonpeptide agonists (aceetylcholine, carbachol, oxotremorexine M, and pilocarpine) (71), likely by a hydrogen bonding mechanism. The tyrosine hydroxyl moiety characteristically interacts with ligands by functioning as a hydrogen bond donor or as a strong locus of cation-π binding (73). The cation-π binding occurs through the side-chains of aromatic amino acids such as phenylalanine, tyrosine, or tryptophan (78). In our study the substitution of phenylalanine for tyrosine in the NMBR resulted in a marked decrease in affinity for PD168368. Therefore, it might be argued that the interaction of the tyrosine hydroxyl with PD168368 through hydrogen bonding is likely more important than cation-π binding. The calculated ∆G° value of 0.5 kcal/mol for the peptoid antagonist PD168368 obtained from the difference in affinities by replacing Tyr220 of the NMBR by the comparable Phe219 of the GRPR is consistent with the elimination of a hydrogen bond (79). However, tyrosine is predicted to have a higher cation-π binding potential than phenylalanine secondary to the negative electrostatic potential of the oxygen (80). Therefore, whether the tyrosine hydroxyl is involved in increasing negative electrostatic potential due to cation-π bonding site interaction or to hydrogen bonding cannot be resolved by our studies.

To further explore the putative binding site for PD168368, three-dimensional modeling of the NMBR based on the structure of bacteriorhodopsin (51) was employed. In this model, the critical Tyr220 residue on the 5th transmembrane domain of the NMBR was found to face the interior of a large binding pocket formed primarily by transmembrane domains 3–7 (Fig. 10). Examination of a minimum energy conformation of the ligand showed that it is dominated by a large hydrogen bond accepting region around the nitrophenyl group, and smaller acceptor regions associated with the pyridyl moiety and amide bonds (Fig. 9). The ligand also has smaller hydrogen bond donor regions associated with the Trp moiety and the amide linkages. The rest of the molecule consists of uncharged hydrocarbon structures that prefer a lipid or hydrophobic environment (Fig. 9). Thus, it is feasible for the ligand PD168368 to be able to enter the intramembrane binding pocket to interact with Tyr220 while simultaneously being capable of interacting with extracellular loops. If this interaction is primarily dependent on hydrogen bonding as our data suggest, it is most likely that the hydroxyl of the Tyr220 interacts with nitrophenyl group of PD168368 or perhaps one of the other hydrogen acceptor groups on PD168368. This proposal is supported by the fact that, in PD168368, the largest hydrogen bond accepting group is the nitro group on the phenyl ring (Fig. 9). This is also the most accessible of the hydrogen bond acceptors in the ligand. The other hydrogen bond acceptors, the pyridyl group, and the backbone carbonyls are much less accessible. Our receptor model indicates that Pro130, Gln132, Leu134, and Val127 of TM helix 3; Trp168, Ser171, and Glu178 of TM helix 4; Ile216 and Tyr220 of TM helix 5; Trp173, Asn176, His179, and Tyr256 of TM helix 6; and Ser213 of TM helix 7 are all facing the putative binding pocket and could interact with a small ligand such as PD168368 (Figs. 9 and 10). Pro130, Gln132, Leu134, and Val127 of TM helix 3; Trp168, Ser171, and Glu178 of TM helix 4; Trp279, Asn282, His285, and Tyr296 of TM helix 6; and Ser313 of TM helix 7 in the NMBR are the same as the comparable amino acids in the GRPR and therefore are unlikely to be important in the selectivity of PD168368 for the NMBR over the GRPR. Of the other two amino acids, Ile216 and Tyr220, our mutagenesis studies show only Tyr220 is important in determining the selectivity of PD168368 for the NMBR over the GRPR. Therefore, we conclude that Tyr220 is the important amino acid in determining the selectivity of PD168368 of the transmembrane amino acids facing the binding pocket. Our studies suggest that amino acids within the 3rd extracellular domain have a small effect on determining the affinity of PD168368. Although the 3rd extracellular domain of the NMBR is in close proximity to the putative binding pocket of PD168368 (Fig. 10) in the present study because of its relative small effect on PD168368 affinity, we did not investigate in detail which amino acids within this domain are responsible for the slight differences in affinity. We therefore cannot speculate on the type of ligand-receptor interaction responsible for this small effect of the 3rd extracellular domain.

In conclusion, our receptor chimeric studies showed that the 5th transmembrane domain of the NMBR was a responsible region for the high affinity and selectivity of the peptoid antagonist PD168368 for the NMBR over the GRPR. Our mutagenesis studies show Tyr220 in the 5th upper transmembrane domain of the NMBR was the key amino acid interacting with this antagonist. To our knowledge, this is the first study that reveals the molecular basis of action between a peptoid antagonist and receptor. The described data from these studies allowed us to obtain a more detailed picture of the receptor-ligand interaction and propose a model that could account for important receptor-ligand interactions. The availability of this model could be of use not only for studying the molecular basis of the interaction of natural agonists, but also for peptoid antagonists or peptide antagonists for this important family of receptors.

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