0026-895X/96/010198-09$3.00/0

MOLECULAR PHARMACOLOGY, 49:198–206 (1996).

The Binding of Propranolol at 5-Hydroxytryptamine$_{1D\beta}$ T355N Mutant Receptors May Involve Formation of Two Hydrogen Bonds to Asparagine

RICHARD A. GLENNON, MALGORZATA DUKAT, RICHARD B. WESTKAEMPER, ABD M. ISMAIEL, DARCY G. IZZARELLI, and ERIC M. PARKER

Department of Medicinal Chemistry, School of Pharmacy, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0540 (R.A.G., M.D., R.B.W., A.M.I.), and Department of Central Nervous System Biology, Bristol-Myers Squibb, Pharmaceutical Research Institute, Wallingford, Connecticut 06492-7660 (D.G.I., E.M.P.)

Received August 3, 1995; Accepted October 3, 1995

SUMMARY

Although the β-adrenergic receptor antagonist (−)-propranolol binds with relatively low affinity at human 5-hydroxytryptamine$_{1D\beta}$ receptors (K_i = 10,200 nM), it displays significantly higher affinity (K_i = 17 nM) at its species homolog, 5-HT$_{1B}$ receptors, and at a mutant 5-HT$_{1D\beta}$ receptor (K_i = 16 nM), where the threonine residue at position 355 (T355) is replaced with an asparagine residue (i.e., a T355N mutant). Propranolol contains two oxygen atoms, an ether oxygen atom and a hydroxyl oxygen atom, and it has been speculated that the enhanced affinity of propranolol for the T355N mutant receptor is related to the ability of the asparagine residue to hydrogen bond with the ether oxygen atom. However, the specific involvement of the propranolol oxygen atoms in binding to the wild-type and T355N mutant 5-HT$_{1D\beta}$ receptors has never been addressed experimentally. A modification of a previously described 5-HT$_{1D\beta}$ receptor graphic model was mutated by replacement of T355 with asparagine. Propranolol was docked with the wild-type and T355N mutant 5-HT$_{1D\beta}$ receptor models in an attempt to understand the difference in affinity of the ligand for the receptors. The binding models suggest that the asparagine residue of the mutant receptor can form hydrogen bonds with both oxygen atoms of propranolol, whereas the threonine moiety of the wild-type receptor can hydrogen-bond only to one oxygen atom. To test this hypothesis, we prepared and examined several analogues of propranolol that lacked either one or both oxygen atoms. The results of radioligand binding experiments are consistent with the hypothesis that both oxygen atoms of propranolol could participate in binding to the mutant receptor, whereas only the ether oxygen atom participates in binding to the wild-type receptor. As such, this is the first investigation of serotonin receptors that combines the use of molecular modeling, mutant receptors generated by site-directed mutagenesis, and synthesis to investigate structure/affinity relationships.

The cloning and expression of more than a dozen 5-HT receptor subtypes (members of the 5-HT$_1$–5-HT$_7$ families) have resulted in a greater appreciation of their structural similarities and differences and aided in their classification (for reviews, see Refs. 1–6). 5-HT receptors may be conveniently divided into two broad families: G protein-coupled receptors (5-HT$_1$, 5-HT$_2$, 5-HT$_4$, 5-HT$_6$, and 5-HT$_7$), and ion-channel receptors (5-HT$_3$); the former type are associated with either an adenylate cyclase (5-HT$_1$, 5-HT$_4$, 5-HT$_6$, and 5-HT$_7$) or a phospholipase (5-HT$_2$) second messenger system. 5-HT$_1$ receptors are further subclassified as 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, or 5-HT$_{1F}$ receptors. Second messenger systems associated with the 5-HT$_5$ receptor are unknown.

With an adrenergic receptor gene used as a probe, the 5-HT$_{1A}$ receptor was one of the first populations of 5-HT receptors to be cloned (7). Interestingly, 5-HT$_{1A}$ receptors display greater transmembrane homology with adrenergic receptors than with most other 5-HT receptors (1). As if to underscore this observation, aryloxyalkylamine β-adrenergic antagonists such as propranolol and pindolol bind with significant affinity at 5-HT$_{1A}$ receptors (2). Because it had been previously demonstrated that binding of these aryloxyalkylamines at α_2-adrenergic receptors is significantly enhanced on conversion of a phenylalanine residue in TM7 to an asparagine (8) and because 5-HT$_{1A}$ receptors possess an asparagine at a homologous position, it has been suggested that this asparagine moiety is an important feature for the binding of aryloxyalkylamines (9). To establish a role for this asparagine residue for 5-HT$_{1A}$ binding, Guan *et al.* (9) mutated N385 of human 5-HT$_{1A}$ receptors to valine and found that this single point mutation markedly decreased the affinity of aryloxyalkylamines without altering the affinity of other classes of 5-HT$_{1A}$ ligands.

Aryloxyalkylamines also bind with significant affinity at

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); TM, transmembrane helix; NIONE, 1-isopropylamino-2-(1-naphthyloxy)ethane hydrogen oxalate or *N*-isopropyl-O-naphthylethanolamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium.

5-HT$_{1B}$ receptors; for example, (−)-propranolol binds at rat 5-HT$_{1B}$ receptors with a K_i of 15–60 nM (2, 10, 11). Although 5-HT$_{1B}$ receptors represent 5-HT autoreceptors in rodents, most other species, including human, possess a species homolog of 5-HT$_{1B}$ receptors: 5-HT$_{1D}$ receptors. Two distinct populations (intraspecies subtypes) (12) of human 5-HT$_{1D}$ receptors have been identified: 5-HT$_{1D\alpha}$ (13) and 5-HT$_{1D\beta}$ (12–16). Although there is >90% TM homology between rat 5-HT$_{1B}$ and human 5-HT$_{1D\beta}$ receptors (17) and although most 5-HT$_{1B}$ ligands bind at 5-HT$_{1D\beta}$ receptors (2), the latter receptors are characterized by an unexpectedly low affinity for (−)-propranolol and (−)-pindolol (K_i = 5,000–12,000 nM) (10, 12, 18). Interestingly, 5-HT$_{1B}$ receptors contain an asparagine residue in TM7, whereas the corresponding amino acid in human 5-HT$_{1D\beta}$ receptors is a threonine (T355). On the basis of the above studies implicating a role for the TM7 asparagine in the binding of aryloxyalkylamines, T355 of 5-HT$_{1D\beta}$ receptors was mutated to an asparagine by several groups of investigators (10, 17–19). Due to the enhanced affinity of (−)-propranolol (K_i = 13–22 nM) (10, 18, 19) for the T355N mutant, each group concluded that this single point mutation accounts for the binding differences of aryloxyalkylamines at wild-type versus the mutant 5-HT$_{1D\beta}$ receptors and suggested that this asparagine is a primary binding feature that differentiates 5-HT$_{1B}$ from 5-HT$_{1D\beta}$ receptors.

Suryanarayana *et al.* (8) speculated that the ether oxygen atom of aryloxyalkylamines forms a hydrogen bond with the critical asparagine residue in TM7 of adrenergic receptors and that this interaction is responsible for the high affinity binding of these ligands. However, because threonine residues are also capable of participating in hydrogen bond formation, it is puzzling why aryloxyalkylamines display low affinity at 5-HT$_{1D\beta}$ receptors. Furthermore, propranolol contains two oxygen atoms: an ether oxygen atom and a hydroxyl oxygen atom. Although it is generally thought by analogy to the above discussion of the binding of aryloxyalkylamines at other receptors (e.g., Refs. 8 and 17) that the ether oxygen atom is a major contributor to 5-HT$_{1D}$ binding (e.g., Ref. 19), this hypothesis has not been directly addressed experimentally. In addition, a potential binding role for the hydroxyl oxygen atom of aryloxyalkylamines has never been considered.

Over the past several years, we constructed and refined three-dimensional graphic models of various 5-HT receptors with the intention of eventually applying these models to receptor-based drug design (e.g., Refs. 20–23). However, before such a goal can be realized, proposed receptor models require validation. To this extent, we previously published a provisional graphic model of 5-HT$_{1D\beta}$ receptors (23); the anomalous behavior of propranolol (i.e., the differential binding of propranolol at 5-HT$_{1D\beta}$ versus the 5-HT$_{1D\beta}$ T355N mutant receptors) offers us an opportunity to evaluate differences in potential binding modes and to explore the issue of hydrogen bond formation. Accordingly, we report here the construction of a graphic model of the 5-HT$_{1D\beta}$ mutant receptor based on a modified graphic model of the 5-HT$_{1D\beta}$ receptor and a comparison of binding modes of propranolol for the wild-type versus mutant receptors. Binding hypotheses were developed, and several compounds were synthesized and evaluated in radioligand binding assays to test these hypotheses.

Materials and Methods

Molecular Modeling

Modeling procedures were performed using version 6.1a of the SYBYL molecular modeling package from Tripos Associates (St. Louis, MO). Molecular mechanics minimizations were performed using the Tripos force field with explicit consideration of all atoms without constraints. Conjugate gradient minimizations were carried out to a root-mean-squares gradient of <0.1 kcal/mol-Å2 with a dielectric constant of 4. The 5-HT$_{1D\beta}$ receptor model was constructed as described previously (23) from the experimental structure of bacteriorhodopsin (24) with two modifications. The current model reflects slightly different alignments among 5-HT$_{1D\beta}$ TM helices 1, 5, and 6 and is consistent with the more recent alignment reported for the 5-HT$_2$ receptor family (21). The starting structure of bacteriorhodopsin was modified by translation of TM helix 4 ~3 Å in the positive direction on the *z*-axis, as suggested by the authors of the original article.[1] Models of the 5-HT receptors were constructed by graphic mutation of bacteriorhodopsin side chains with retention of the experimental backbone structure. Obviously unfavorable side-chain interactions were removed manually, and the resulting structures were energy minimized. A model of the T355N mutant 5-HT$_{1D\beta}$ receptor was constructed from the native 5-HT$_{1D\beta}$ receptor model and subjected to energy minimization as described above.

Radioligand Binding

Materials. The wild-type human 5-HT$_{1D\beta}$ receptor gene and the T355N human 5-HT$_{1D\beta}$ receptor gene were prepared as previously described (18) and inserted into the expression vector pCMV5 (25) (obtained from Dr. David Russell, University of Texas Southwestern Medical Center, Dallas, TX). [1,2-^{3}H]-5-Carboxamidotryptamine (15–20 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Cell culture media and reagents were obtained from BioWhittaker (Walkerville, MD), and fetal calf serum was purchased from Flow/ICN (Costa Mesa, CA). DEAE-dextran was obtained from Pharmacia (Piscataway, NJ), and chloroquine was purchased from Sigma Chemical Co. (St. Louis, MO). All chemical reagents were of the highest grade commercially available.

Transfection of COS-1 cells and preparation of crude plasma membranes. COS-1 cells (American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum. The cells were maintained in a humidified incubator at 37° with 5% CO_2. COS-1 cells were transfected by the DEAE-dextran procedure. Briefly, 3 × 10^6 COS-1 cells were seeded in 150-mm tissue dishes (Nunc, Naperville, IL) in DMEM with 10% heat-inactivated fetal calf serum. Twenty-four hours after seeding, the medium was replaced with DMEM, 10% heat-inactivated fetal calf serum, 20 mM HEPES, pH 7.3, 100 units/mL penicillin, and 100 μg/mL streptomycin. Plasmid DNA (final concentration, 100 μg/mL), DEAE-dextran (final concentration, 50 μg/mL), and chloroquine (final concentration, 100 μM) were then added, and the cells were incubated in this medium for 3.5 hr. The cells were subsequently incubated for 2–3 min in 10% dimethylsulfoxide in Dulbecco's phosphate-buffered saline; washed once with phosphate-buffered saline; and incubated in DMEM, 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. At 60–72 hr after transfection, crude plasma membranes were prepared from the transfected COS-1 cells as previously described (18).

Binding assays. Competition binding assays were carried out as described previously (18, 26). Membranes were incubated in a buffer consisting of 50 mM Tris-Cl, pH 7.4 at 22°, 12.5 mM $MgCl_2$, 1 mM EDTA, 100 μM ascorbic acid, 1 nM [1,2-^{3}H]-5-carboxamidotryptamine, and various concentrations of competing agents. Nonspecific binding was defined as binding in the presence of 10 μM 5-HT.

[1] R. Henderson, personal communication.

After 1-hr incubation at 22°, the binding assay was stopped by the addition of 5 ml ice-cold wash buffer (20 mM Tris, pH 7.4 at 4°, 2 mM $MgCl_2$) followed by filtration over Whatman GF/C filters. Filters were washed twice with 5 ml of ice-cold wash buffer and counted by liquid scintillation counting. The binding data were analyzed by nonlinear least-squares regression (27). All comparisons between the wild-type and the T355N human 5-$HT_{1D\beta}$ receptors were carried out on the same day with the same drug solutions.

Synthesis

Synthesis of the novel compounds was performed as described below. The final target compounds were submitted for microanalysis (Atlantic Microlab, Norcross, GA), and results are within 0.4% of theory. All products were homogeneous by thin layer chromatography, and assigned structures are consistent with the spectral data.

Deshydroxypropranolol or 1-isopropylamino-3-(1-naphthyloxy)propane hydrochloride. Dibromopropane (0.7 g, 3.5 mmol) was added to a solution of 1-naphthol (0.5 g, 3.5 mmol) and NaOH (0.14 g, 3.5 mmol) in H_2O (10 ml). The reaction mixture was heated at reflux for 2 hr and then stirred at room temperature overnight. The mixture was diluted with H_2O (5 ml), extracted with CH_2Cl_2 (2 × 10 ml) and Et_2O (1 × 10 ml), and washed well with 10% NaOH (2 × 10 ml) and then H_2O (2 × 30 ml). The extract was dried ($MgSO_4$); solvent was removed under reduced pressure to give 0.42 g (46%) of the intermediate naphthopropoxy halide as an oil, which was used without further characterization in the next step. *N*-Isopropylamine (0.14 g, 2.4 mmol) was added dropwise to a solution of the naphthopropoxy halide (0.42 g, 1.6 mmol) in MeCN (10 ml) in the presence of K_2CO_3 (0.22 g, 1.6 mmol). The reaction mixture was heated at reflux for 4 hr. The solvent was removed under reduced pressure, and the residue was dissolved in H_2O (15 ml), extracted with Et_2O (3 × 10 ml), and dried ($MgSO_4$); the Et_2O was evaporated *in vacuo* to produce a crude oil (0.2 g). The free base of the title compound was isolated by column chromatography using silica gel (60–200 mesh) and CH_3OH as an eluent. The ethereal solution of the free base was treated with ethereal solution of HCl gas to produce 0.04 g (11%) of the target compound as an ivory-colored solid (m.p. 186–188°). It was analyzed for $C_{16}H_{21}NO{\cdot}HCl$.

Desoxypropranolol or 1-(3-hydroxy-4-isopropylaminobutyl)naphthalene hydrochloride. The title compound was prepared according to published procedure (28) as a white solid in 22% yield: m.p. 178–181° after recrystalization from chloroform; literature (28) m.p. 181–183°.

Didesoxopropranolol or 1-(4-isopropylaminobutyl)naphthalene hydrochloride. A mixture of 4(1-naphthyl)butanal (29) (0.25 g, 1.3 mmol) and *N*-isopropylamine (0.7 g, 12 mmol) in absolute EtOH/MeOH (1:1) (50 ml) was hydrogenated (60 p.s.i.) over 10% Pd/C (0.1 g) at room temperature for 30 hr. The catalyst was removed by filtration, and filtrate was evaporated under reduced pressure. The free base of the title compound (0.08 g) was isolated by column chromatography using silica gel (60–200 mesh) and MeOH as an eluent. An ethereal solution of the free base was treated with an ethereal solution of HCl gas to produce 0.04 g (11%) of the title compound as an ivory-colored solid (m.p. 168–170°). It was analyzed for $C_{17}H_{23}N{\cdot}HCl{\cdot}0.2H_2O$.

NIONE or 1-isopropylamino-2-(1-naphthyloxy)ethane hydrogen oxalate. A mixture of 2-(1-naphthyloxy)ethanol-*p*-toluene sulfonate (0.33 g, 1.0 mmol), *N*-isopropylamine (0.12 g, 2.0 mmol), and K_2CO_3 (0.14 g, 1.0 mmol) in dioxane (20 ml) was heated overnight at reflux. The solvent was removed under reduced pressure, and the residue was treated with 10% NaOH (5 ml) and extracted three times with Et_2O (10 ml). The combined extracts were washed with H_2O (3 × 10 ml), dried ($MgSO_4$), and treated with a saturated ethereal solution of oxalic acid to produce 0.04 g (20%) of the desired salt after recrystallization from an absolute EtOH/anhydrous Et_2O mixture (m.p. 228–230°). It was analyzed for $C_{15}H_{19}NO{\cdot}C2H_2O_4{\cdot}0.25H_2O$.

Compound F or 1-(3-isopropylaminopropyl)naphthalene hydrogen oxalate. Triethylamine (0.29 g, 2.8 mmol) was gradually added to a stirred mixture of 1-naphthyl-β-acrylic acid (0.50 g, 2.8 mmol) in CH_2Cl_2 (10 ml) at 0°. The resulting clear solution was maintained at this temperature during the addition of ethyl chloroformate (0.34 g, 3.2 mmol). After 30 min, *N*-isopropylamine (0.17 g, 2.8 mmol) in CH_2Cl_2 (10 ml) was added, the temperature was allowed to rise to room temperature, and stirring was allowed to continue for an additional 2 hr. The solvent was removed under reduced pressure, and the solid residue was recrystallized from aqueous EtOH to produce 0.62 g (90%) of the amide intermediate: m.p. 214–216°. The amide (1.0 mmol) in anhydrous tetrahydrofuran (10 ml) was added in a dropwise manner to a stirred suspension of $LiAlH_4$ (0.42 g, 10.6 mmol) in tetrahydrofuran (20 ml) at ice-bath temperature. The reaction mixture was heated overnight at reflux, and excess hydride was decomposed by the dropwise addition of H_2O (2 ml), 10% NaOH (2 ml), and H_2O (2 ml) at 0°. The inorganic material was removed by filtration, and the filtrate was dried ($MgSO_4$) and evaporated to dryness to produce an oily product. An ethereal solution of the oil was treated with a saturated solution of oxalic acid in anhydrous Et_2O to give 0.16 g (48%) of the target compound (m.p. 175–177°) after recrystallization from an absolute EtOH/anhydrous Et_2O mixture. It was analyzed for $C_{16}H_{21}N{\cdot}C_2H_2O_4$.

Results and Discussion

Construction of the 5-$HT_{1D\beta}$ Receptor Graphic Models

A human 5-$HT_{1D\beta}$ receptor graphic model was constructed from the experimental structure of bacteriorhodopsin; the alignments of the 5-$HT_{1D\beta}$ receptor and bacteriorhodopsin sequences are shown in Table 1. Models of the 5-HT receptors were constructed by graphical mutation of bacteriorhodopsin side chains with retention of the experimental backbone structure. A graphic model of the T355N mutant 5-$HT_{1D\beta}$ receptor was constructed from the native 5-$HT_{1D\beta}$ receptor model and subjected to energy minimization. There are inherent uncertainties in the construction of graphical models of G protein receptors. This is true of models derived from bacteriorhodopsin (which is not a G protein-coupled receptor) as well as those derived from other starting points. For example, a low resolution structure of rhodopsin (which is a G protein-coupled receptor) (30, 31) has been used by some as a starting point for neurotransmitter receptor modeling (32–34). Although both approaches are used, controversy continues as to which is the most appropriate approach (21, 35, 36). In the present investigation, we elected to evaluate bacteriorhodopsin as a template for the 5-$HT_{1D\beta}$ receptor models; nevertheless, all models should be considered provisional, the usefulness of which will be determined by consistency with experimental data and validation of predictions based thereon.

Refinement and Analysis of the Receptor Models

The goal of the modeling investigations was simply to determine whether the 5-$HT_{1D\beta}$ models are consistent with the results of site-directed mutagenesis studies implicating a role for N355 in the binding of aryloxyalkylamine ligands, presumably via hydrogen bond formation. The following questions were posed and investigated in a stepwise fashion: (i) In the absence of other receptor model features, are the translational and rotational orientations of TM3 and TM7 such that the TM3 aspartate and the TM7 threonine/asparagine can simultaneously interact with potential interaction sites of propranolol to produce complexes of reasonable geometries? If so, can any of the possible combinations of re-

TABLE 1

Alignment of the human 5-HT$_{1D\beta}$ and bacteriorhodopsin sequence as used in this study

52	
76	
LVMLLALITLATTLSNAFVIATVYR	TM1
PEWIWLALGTALMGLGTLYFLVKGM	
8	
32	
87	
111	
LIASLAVTDLLVSILVMPISTMYTV	TM2
DAKKFYAITTLVPAIAFTMYLSMLL	
38	
62	
117	
143	
LGQVVCDFWLSSDITCCTASILHLCVI	TM3
EQNPIYWARYADWLFTTPLLLLDLALL	
74	
100	
169	
190	
MIALVWVFSISISLPPFFWRQA	TM4
GTILALVGADGIMIGTGLVGAL	
106	
127	
209	
229	
TVYSTVGAFYFPTLLLIALYG	TM5
WWAISTAAMLYILYVLFFGFT	
137	
157	
312	
337	
ATKTLGIILGAFIVCWLPFFIISLVM	TM6
EVASTFKVLRNVTVVLWSAYPVVWLI	
166	
191	
345	
368	
WFHLAIFDFFTWLGYLNSLINPII	TM7
NIETLLFMVLDVSAKVGFGLILLR	
202	
225	

ceptor/ligand/donor/acceptor pairs be ruled out or strongly implicated on the basis of these simple considerations? (ii) Can any of the complexes implicated above be accommodated in the complete receptor models, and, if so, are there any unique conditions or restrictions in the manner in which propranolol and its analogues can form geometrically and energetically reasonable complexes?

The first question was addressed by evaluating constrained molecular dynamics simulations of propranolol complexed with extremely abbreviated representations of the receptors (i.e., a model consisting only of the TM3 aspartate and the TM7 threonine/asparagine residues constructed from the coordinates of the fully elaborated models according to alignments presented in Table 1). Dynamics simulations (100 psec) were performed at 300°, and with harmonic distance constraints of 3 Å, with the abbreviated wild-type and mutant receptor models as indicated schematically in Fig. 1. The non-side-chain atoms of the receptor residues were constrained to their original coordinates. All four sets of constraints produced trajectories of comparable mean energies. The side-chain torsion angle χ_1 for D129 and N355 appeared in two discrete states with values of $\sim -70°$ and $\sim -175°$. T355 χ_1 values similarly appeared in two states corresponding to $-70°$ and $-175°$. These values are consistent with those observed for asparagine and threonine residues in α-helices (37). The χ^2 values for D129 and N355 were evenly distributed throughout the entire range. Thus, on the basis of the simplest geometric constraints imposed by the disposition of D129 and T/N355 of the 5-HT$_{1D\beta}$ receptor model, each pattern of noncovalent bond formation seems equally plausible.

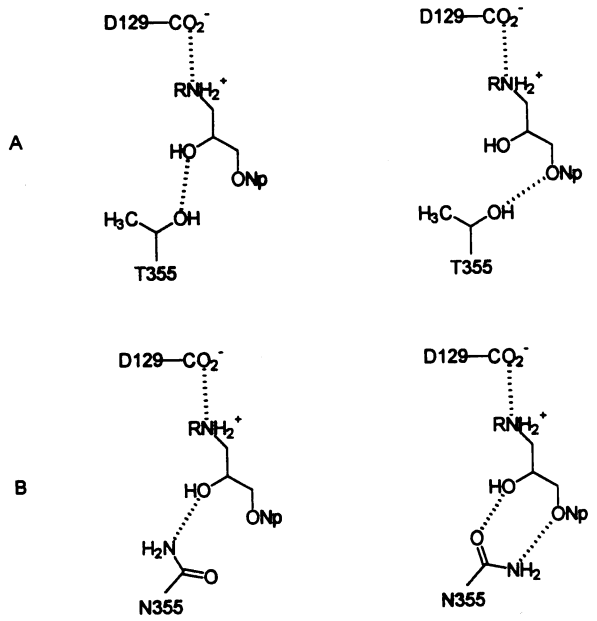

Fig. 1. Schematic of distance constraints used for molecular dynamics simulations with models of the 5-HT$_{1D\beta}$ (A) and T355N mutant 5-HT$_{1D\beta}$ (B) receptors.

Although the conformation of propranolol would be best characterized as extended in most of the dynamics simulations, there were significant differences in the isopropyl and naphthyl ring dispositions. In addition, location in space of the propranolol molecule varied markedly. The results from the dynamics runs performed with the minimal receptor models were replayed while being superimposed on the complete receptor models and evaluated visually for steric compatibility. Selected representative frames from each dynamics simulation were first energy minimized and then merged with the receptor model so that the conformations of D129 and T/N355 from the dynamics simulations were copied to the receptor model. The propranolol/receptor complexes were energy minimized, and the results were evaluated.

Evaluation in this manner produced several useful generalizations: (i) propranolol is always oriented such that the naphthyl ring is always pointed toward TM1, TM2, and TM7 and never toward TM4, TM5, or TM6. This is quite different from proposed orientations of other ligands (e.g., 5-HT) bound to 5-HT receptor models and is simply a consequence of the initial assumption that the TM7 asparagine/threonine residue interacts with a propranolol oxygen atom. Site-directed mutagenesis data and evaluation of receptor chimeras

strongly implicate TM5 and TM6 as being involved in interaction of the aromatic portion (including substituents) of typical serotonergic agents with various populations of 5-HT receptors (for reviews, see Refs. 38 and 39). (ii) The aromatic substituent of propranolol is best sterically accommodated in a pocket formed by residues of TM1 (A57, T60, L61, T64) and TM2 (V102, S106, Y109) oriented with the long axis of the naphthyl ring parallel to the helix axis with the unsubstituted ring pointed toward the extracellular side.

A representative propranolol/T355N/5-$HT_{1D\beta}$ receptor complex was selected for further analysis. The energy-minimized propranolol/N355 complex (Figs. 2A and 3A) shows distances compatible with formation of an ionic bond between the receptor carboxylate (i.e., aspartate moiety) and the ligand ammonium ion as well as a bifunctional hydrogen bond between the receptor asparagine moiety and both the ether and hydroxyl oxygen atoms of propranolol. It should be noted that the distance between the hydroxyl oxygen atom of propranolol and the N355 amide oxygen is near the maximum distance compatible with specific hydrogen-bonded interactions experimentally observed in protein structures. A cutoff distance of 3.5 Å between heteroatoms is typically used in studies of experimental crystal structures of proteins to determine the presence or absence of interacting atoms (40, 41). However, it has been argued that longer hydrogen bond lengths are probably also energetically significant (42).

A model of propranolol bound to the wild-type 5-$HT_{1D\beta}$ receptor was constructed from the T355N mutant complex by graphic mutation of asparagine to threonine followed by molecular mechanics energy minimization without constraints. A hydrogen bond between T355 and the ether oxygen atom of (−)-propranolol is formed in the minimized structure with an

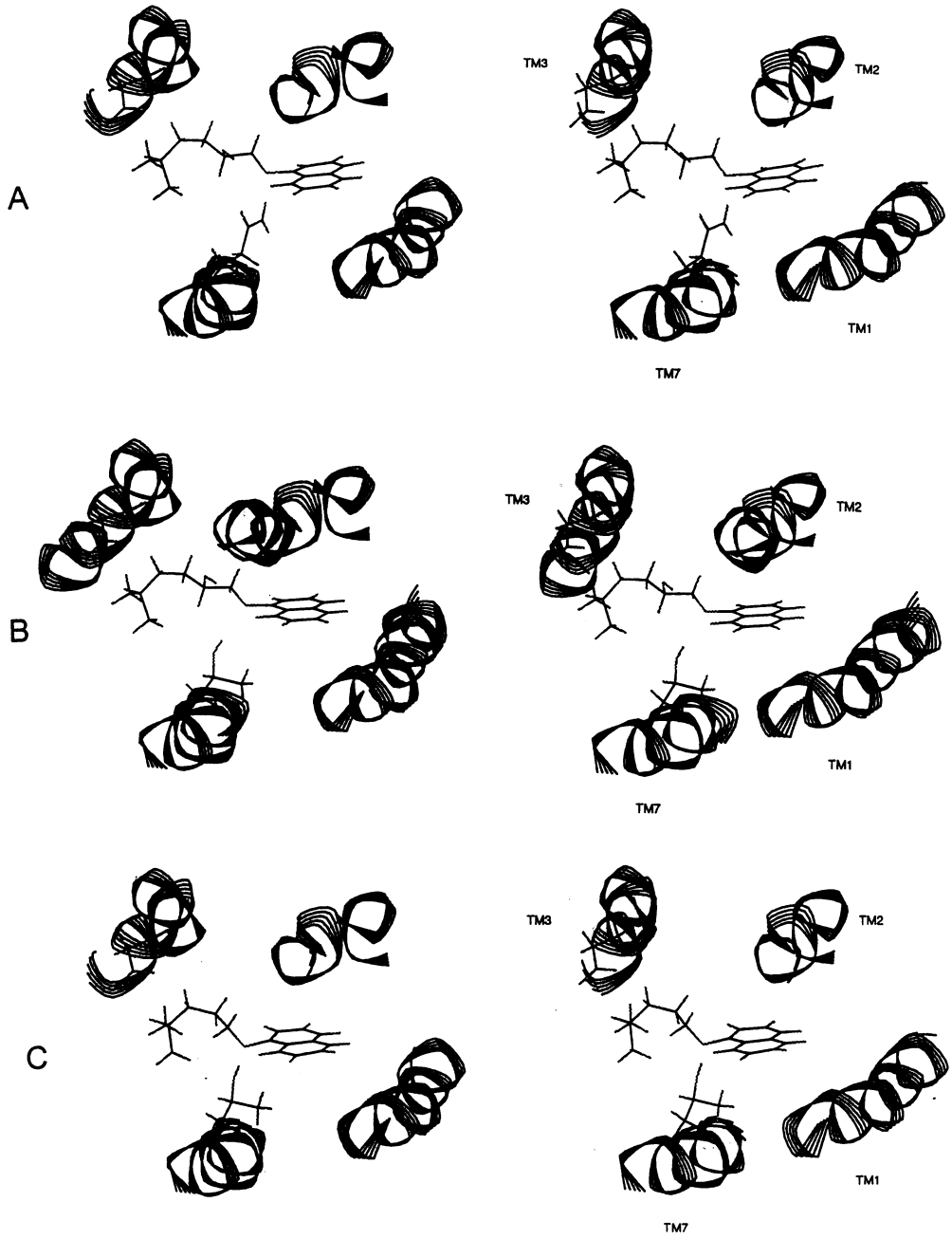

Fig. 2. Stereodiagrams of complexes between propranolol and the 5-$HT_{1D\beta}$ T355N mutant receptor model (A), propranolol and the wild-type 5-$HT_{1D\beta}$ receptor model (B), and compound E and the wild-type 5-$HT_{1D\beta}$ receptor model (C). Helices TM1, TM2, TM3, and TM7 are represented only by backbone traces for purpose of clarity. The side-chain structures for D129 of TM3 and T/N355 for TM7 are shown explicitly.

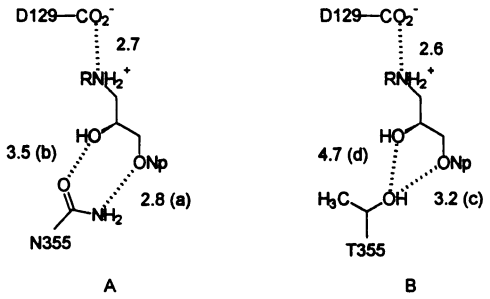

Fig. 3. Heteroatom distances (Å) between putative interacting functionalities for the propranolol T355N mutant (A) and wild-type (B) receptors. *a–d*, Hydrogen bonds discussed in the text.

O—O distance of 3.2 Å (Figs. 2B and 3B). Systematic conformational analysis by rotation of the threonine side chain (χ^1) revealed that the closest distance that can be achieved between the threonine oxygen atom and the hydroxyl oxygen atom of propranolol is ~4.6 Å (range, 4.6–6.4 Å); this distance is most likely too great to accommodate an energetically favorable hydrogen bond. Given the limitations of both the experimental determination of hydrogen bond geometries for proteins and the very large geometric errors associated with any molecular mechanics-based model construction, it is unjustified to attach a great deal of significance to the value of the distances reported for models of the ligand/receptor complex. It is clear, however, that the pattern of distances observed in the model structures are most consistent with the following order of hydrogen bond strengths: a > c > b > d (Fig. 3). Interestingly, this order is consistent with the ligand affinity data for the wild-type and mutant 5-HT$_{1D\beta}$ receptors, as is discussed below. Thus, this model is consistent with the concept that T355 functions as a hydrogen bond donor to the ether oxygen atom of (−)-propranolol and suggests that the hydroxyl oxygen atom contributes little to affinity with regard to an interaction with T355.

On the basis of these relative hydrogen bond strengths, it can be predicted that propranolol and compounds that contain an ether functionality analogous to that of propranolol should have measurable, but probably not optimal, affinities for 5-HT receptors bearing a threonine residue at position 355 by virtue of formation of a hydrogen bond of type c (Fig. 3). Removal of the hydroxyl functionality of propranolol should have little effect because of the inability of the T355 of 5-HT$_{1D\beta}$ receptors to form hydrogen bond d. Because of the potential importance of a hydrogen bond of type a for N355, it can be predicted that compounds with an ether functionality should bind with affinities somewhat higher than with T355 (i.e., the wild-type receptor) and that those that lack the ether oxygen should have greatly reduced affinities. In contrast to the wild-type receptor, affinities at the T355N mutant receptor are predicted to be significantly affected by removal of the propranolol hydroxy group because of formation of an energetically significant hydrogen bond of type b.

Empirical Evaluation of Binding Hypotheses

Binding of propranolol to the 5-HT$_{1D\beta}$ receptor. We and others have made the tacit assumption that the aspartate in TM3 is the amine binding site for serotonergic ligands at G protein-coupled 5-HT receptors. Although this has yet to be conclusively demonstrated for 5-HT$_{1D\beta}$ receptors, the cognate aspartate has been shown on the basis of site-directed mutagenesis to be important for binding at, for example, 5-HT$_{2A}$ receptors (43). Thus, with the assumption that D129 of TM3 is the amine binding site, T355 of TM7 offers a second anchoring point with which to begin our investigation of 5-HT$_{1D}$ receptor binding interactions. As shown in Figs. 2B and 3B, the ether oxygen atom of propranolol is within hydrogen bonding distance of the TM7 threonine residue. Thus, this model is consistent with the concept that the ether oxygen atom of propranolol can hydrogen bond with the TM7 T355 residue. In addition to threonine being unable to form as strong a hydrogen bond as the amide of asparagine (40), two interesting features were observed that might account for the reduced affinity of propranolol at wild-type 5-HT$_{1D\beta}$ receptors. First, the methyl group of T355 may somewhat sterically interfere with hydrogen bond formation by influencing the conformation of the threonine hydroxyl group; second, the length of the aryloxyalkylamine alkyl chain is such that the aryl (i.e., naphthyl ring) portion is thrust toward other residues (particularly those of TM1 and TM2), which could also contribute to unfavorable steric interactions.

Binding of propranolol to the T355N 5-HT$_{1D\beta}$ mutant. Figs. 2A and 3A show a mode of binding for propranolol to 5-HT$_{1D\beta}$ receptors where T355 has been replaced by asparagine. To some extent, the aryloxyalkylamine is shifted so as to relieve some of the unfavorable TM1 interactions. More importantly, however, is that the model suggests the possible existence of two hydrogen-bonded interactions. That is, not only can the ether oxygen atom of the aryloxyalkylamines participate in hydrogen bond formation, but also the alkyl chain hydroxyl group can form a second hydrogen bond with this same asparagine moiety. Fig. 4 is a close-up schematic of the binding of propranolol to the two types of 5-HT$_{1D\beta}$ receptors. Relief of unfavorable interactions, coupled with formation of two hydrogen bonds, may account for the higher affinity of propranolol for the T355N 5-HT$_{1D\beta}$ mutant (as well as for 5-HT$_{1B}$) receptors compared with the wild-type 5-HT$_{1D\beta}$ receptors.

Binding of desoxy analogues. By analogy to the 5-HT$_{1A}$ and adrenergic situations (8, 9), it has been proposed that the

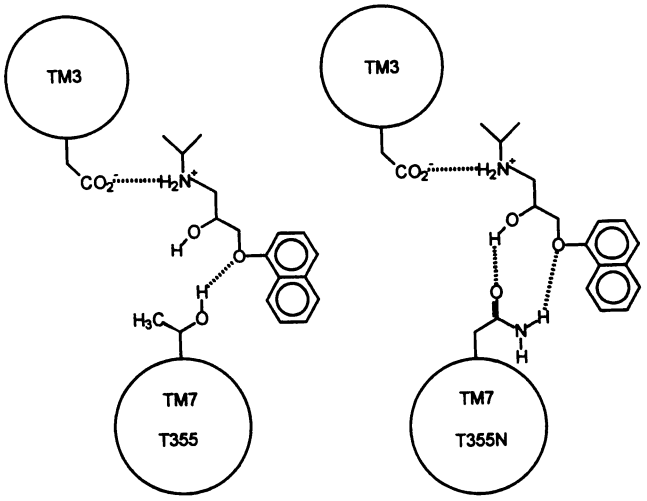

Fig. 4. Schematic of the most probable ionic and hydrogen bonding interactions between propranolol and the 5-HT$_{1D\beta}$ wild-type and T355N 5-HT$_{1D\beta}$ mutant receptors based on structure/activity relationships, mutagenesis, and model-building studies.

TM7 asparagine of 5-HT_{1B} and T355N 5-$HT_{1D\beta}$ mutant receptors participates in a hydrogen bond interaction with the ether oxygen of the aryloxyalkylamines. Although the model shown in Fig. 3 is consistent with this suggestion, it also indicates the possible formation of an unexpected second hydrogen bond to the alkyl hydroxyl group. To determine the relative importance of the ether oxygen atom and the hydroxyl group of propranolol for binding at the wild-type and T355N mutant 5-$HT_{1D\beta}$ receptors, we synthesized and evaluated the binding affinities of (−)-propranolol, deshydroxypropranolol, and desoxypropranolol (Fig. 5). Deshydroxypropranolol lacks the hydroxyl group of propranolol, whereas desoxypropranolol lacks the ether oxygen atom (i.e., the ether oxygen has been replaced by a methylene group, which cannot participate in hydrogen bond formation) but retains the hydroxyl group. For comparison, we also examined didesoxopropranolol, which lacks the hydroxyl group of propranolol and where the ether oxygen atom has been replaced by a methylene group. Consistent with other reports (10, 12, 18), (−)-propranolol (K_i = 10,200 nM; Table 2) binds with low affinity at 5-$HT_{1D\beta}$ receptors. Deshydroxypropranolol, the analogue of propranolol devoid of the hydroxyl group, binds with nearly identical affinity (K_i = 7660 nM). Neither desoxypropranolol nor didesoxopropranolol binds at 5-HT_{1D} receptors. The relatively low affinities of (−)-propranolol and deshydroxypropranolol make it difficult to reach any definitive conclusions; nevertheless, it appears that the hydroxyl group is not a major contributor to binding because its removal has essentially no effect on affinity. In contrast, deshydroxypropranolol and didesoxopropranolol, both of which lack the ether oxygen atom, are essentially inactive. Thus, it appears that the ether oxygen atom participates in binding.

The same four compounds were examined at T355N mutant 5-$HT_{1D\beta}$ receptors (Table 2). As previously reported (10, 18, 19), (−)-propranolol binds with high affinity (K_i = 16 nM)

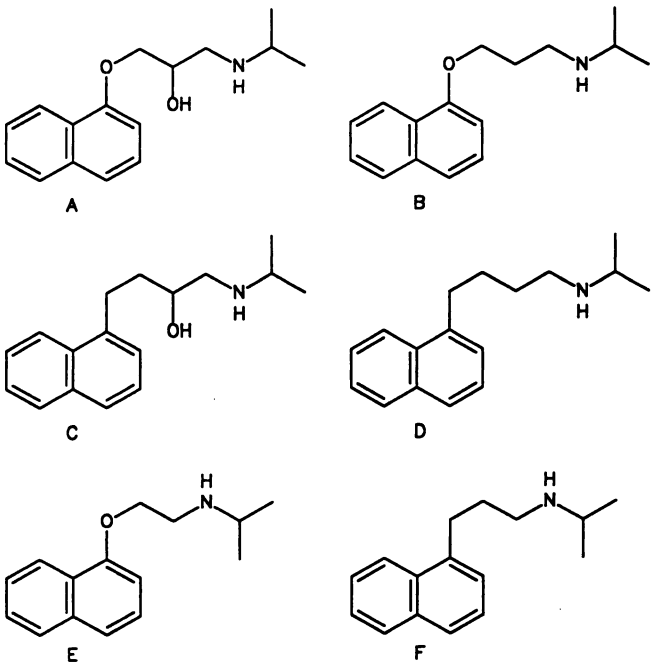

Fig. 5. Structures of agents used in the present investigation: A, propranolol; B, deshydroxypropranolol; C, desoxypropranolol; D, didesoxopropranolol; E, NIONE; F, the desoxy analogue of NIONE.

TABLE 2

Binding data for propranolol analogues at wild-type and T355N mutant 5-$HT_{1D\beta}$ receptors

Agent[a]	5-$HT_{1D\beta}$	5-$HT_{1D\beta}$ Mutant
	K_i nM ± standard error	
(−)-Propranolol	10,200 ± 910	16 ± 0.5
Deshydroxypropranolol	7,660 ± 1350	2,890 ± 550
Desoxypropranolol	≈100,00	1,530 ± 425
Didesoxopropranolol	≈100,00	>100,000
NIONE	260 ± 30	1,025 ± 340
Compound F	≈100,00	>100,000

[a] See Fig. 5 for structures of agents used in this study.

and with affinity comparable to that reported at 5-HT_{1B} receptors (K_i = 17 nM) (11). Deshydroxypropranolol binds at the mutant receptors with significantly reduced affinity (K_i = 2890 nM); desoxypropranolol similarly binds with reduced affinity (K_i = 1530 nM). Didesoxopropranolol is essentially inactive (Table 2). If the ether oxygen atom of propranolol was solely responsible for its enhanced affinity (via hydrogen bond formation with asparagine) at the mutant receptor, deshydroxypropranolol and propranolol might have been expected to bind with comparably high affinity. The reduced affinity of deshydroxypropranolol suggests that the hydroxy group of propranolol plays an important role. The observation that desoxypropranolol binds with higher affinity to the T355N mutant receptor than to the wild-type receptor also argues that the ether oxygen atom of propranolol cannot be solely responsible for the increased affinity of propranolol for the T355N receptors. If the high affinity of propranolol was solely attributable to the hydroxyl group, then desoxypropranolol might have been expected to bind with an affinity comparable to that of propranolol. It does not. It appears, then, that both oxygen atoms are important for optimal binding at the mutant receptor.

These results support the idea that propranolol binds at T355N mutant 5-$HT_{1D\beta}$ receptors by formation of two hydrogen bonds, as schematically displayed in Fig. 4.

Binding of chain-shortened analogues. As described above, there are some unfavorable interactions between (−)-propranolol and TM1 residues when (−)-propranolol is docked with 5-$HT_{1D\beta}$ wild-type receptors (Fig. 2B). It might be possible to reduce these unfavorable interactions by shortening the length of the alkyl chain of propranolol by one methylene group. Due to the inherent hydrolytic instability of compounds possessing a hydroxyl group α to an ether oxygen or amine function, we prepared and evaluated NIONE (Fig. 5) or the chain-shortened homolog of deshydroxypropranolol. It was expected that this agent would bind at wild-type 5-$HT_{1D\beta}$ receptors with higher affinity than (−)-propranolol or deshydroxypropranolol due to a reduction in unfavorable steric interactions (Fig. 2C). In contrast, lacking the side-chain hydroxyl group of (−)-propranolol and the resulting inability to participate in the formation of two hydrogen bonds, it was expected that NIONE would bind at mutant 5-$HT_{1D\beta}$ receptors with lower affinity than (−)-propranolol but with an affinity comparable to that of the other analogues that can participate only in single hydrogen bond formation (i.e., deshydroxypropranolol and desoxypropranolol). It was further expected that this compound, because of the possibility of more favorable interactions at the wild-type versus the T355N mutant receptors, might even display reversed selectivity relative to propranolol. If binding of NIONE occurs in a manner similar to those shown in Figs. 2A and 2B,

the carbon counterpart of NIONE (i.e., Compound F; Fig. 5) should be inactive. As shown in Table 2, NIONE (K_i = 260 nM) binds at wild-type 5-HT$_{1D\beta}$ receptors with 30–40 times the affinity of (−)-propranolol and deshydroxypropranolol. Its affinity at the T355N mutant 5-HT$_{1D\beta}$ receptors (K_i = 1025 nM) is significantly lower than that of (−)-propranolol (K_i = 16 nM) and is consistent with formation of a single hydrogen bond. Compound F (Fig. 5), the desoxy analogue of NIONE, is essentially inactive (K_i = >100,000 nM) at 5-HT$_{1D}$ receptors.

Conclusion

In the absence of three-dimensional structural information on 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors, we attempted to gain information on the receptors by construction of graphic models (23). The differential binding of certain aryloxyalkylamines at 5-HT$_{1D\beta}$ and T355N mutant 5-HT$_{1D\beta}$ receptors provides a unique opportunity to explore these differences because the differences are attributable to a change of only a single amino acid residue. A slightly modified graphic model of the 5-HT$_{1D\beta}$ receptor was constructed; the model was mutated by replacing T355 with an asparagine moiety. Docking studies suggested that (−)-propranolol should bind with higher affinity at the mutant 5-HT$_{1D\beta}$ receptors than at the wild-type receptors, primarily because the presence of the asparagine moiety allows the formation of two hydrogen bonds (see Fig. 4 for a schematic comparison). One hydrogen bond involves the side-chain ether oxygen, as previously suggested by other investigators (19), and a second hydrogen bond uses the side-chain hydroxyl group. The mode of interaction shown in Fig. 2B (and shown schematically in Fig. 4) for 5-HT$_{1D\beta}$ binding of (−)-propranolol is supported by a lack of effect on removal of the hydroxyl group and by reduction of affinity when the ether oxygen atom is replaced by a methylene group. The mode of interaction of (−)-propranolol with mutant 5-HT$_{1D\beta}$ receptors, as shown in Fig. 2A (and schematically in Fig. 4), is supported by the reduced affinity of propranolol analogues lacking either the ether oxygen or the hydroxyl oxygen atom. Removal of both oxygen atoms abolishes ligand affinity for both wild-type 5-HT$_{1D\beta}$ and mutant 5-HT$_{1D\beta}$ receptors. The effect of shortened chain length was also investigated, and the results were consistent with the models. Thus, we propose that (−)-propranolol displays higher affinity for the mutant 5-HT$_{1D\beta}$ receptors (and, by analogy, at 5-HT$_{1B}$ receptors) than for wild-type 5-HT$_{1D\beta}$ receptors, primarily because of its ability to participate in the formation of dual hydrogen bond interactions with N355, which is present in the mutant 5-HT$_{1D\beta}$ (and 5-HT$_{1B}$) receptors but not in wild-type 5-HT$_{1D\beta}$ receptors; unfavorable steric interactions may also contribute to the lower affinity of propranolol for wild-type 5-HT$_{1D\beta}$ receptors.

References

1. Sandou, F., and R. Hen. 5-HT receptor subtypes: molecular and functional diversity. *Med. Chem. Res.* **4**:16–84 (1994).
2. Zifa, E., and G. Fillion. 5-Hydroxytryptamine receptors. *Pharmacol. Rev.* **44**:401–458 (1992).
3. Martin, G. R., and P. P. A. Humphrey. Receptors for 5-hydroxytryptamine: current perspectives on classification and nomenclature. *Neuropharmacology* **33**:261–273 (1994).
4. Boess, F. G., and I. L. Martin. Molecular biology of 5-HT receptors. *Neuropharmacology* **33**:275–317 (1994).
5. Hoyer, D., D. E. Clarke, J. R. Fozard, P. R. Hartig, G. R. Martin, E. J. Mylecharane, P. R. Saxena, and P. P. A. Humphrey. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol. Rev.* **46**:157–203 (1994).
6. Glennon, R. A., and M. Dukat. Serotonin receptor subtypes, in *Psychopharmacology: The Fourth Generation of Progress* (R. E. Bloom and D. J. Kupfer, eds.). Raven Press, New York, 415–429 (1994).
7. Kobilka, B. K., T. Frielle, S. Collins, T. Yang-Feng, T. S. Kobilka, V. Francke, R. J. Lefkowitz, and M. G. Caron. An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulation proteins. *Nature (Lond.)* **329**:75–79 (1987).
8. Suryanarayana, S., D. A. Daunt, M. von Zastrow, and B. A. Kobilka. A point mutation in the seventh hydrophobic domain of the α_2 adrenergic receptor increases its affinity for a family of β receptor antagonists. *J. Biol. Chem.* **266**:15488–15492 (1991).
9. Guan, X.-M., S. J. Peroutka, and B. K. Kobilka. Identification of a single amino acid residue responsible for the binding of a class of β-adrenergic receptor antagonists to 5-hydroxytryptamine-1A receptors. *Mol. Pharmacol.* **41**:695–698 (1992).
10. Oksenberg, D., S. A. Marsters, B. F. O'Dowd, H. Jin, S. Havlik, S. J. Peroutka, and A. Ashkenazi. A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT1B receptors. *Nature (Lond.)* **360**:161–163 (1992).
11. Pierson, M. E., R. A. Lyon, M. Titeler, P. Kowalski, and R. A. Glennon. Design and synthesis of propranolol analogues as serotonergic agents. *J. Med. Chem.* **32**:859–863 (1989).
12. Weinshank, R. L., J. M. Zgombick, M. J. Macchi, T. Bronchek, and P. R. Hartig. Human serotonin 1D receptor is encoded by a subfamily to two distinct genes: 5-HT1Dα and 5-HT1Dβ. *Proc. Natl. Acad. Sci. USA* **89**:3630–3634 (1992).
13. Hamblin, M. W., and M. A. Metcalf. Primary structure and functional characterization of a human 5-HT1D-type serotonin receptor. *Mol. Pharmacol.* **40**:143–148 (1991).
14. Demchyshyn, L., R. K. Sunahara, K. Miller, M. Teitler, B. J. Hoffman, J. L. Kennedy, P. Seeman, H. H. M. van Tol, and H. B. Niznik. A human serotonin 1D receptor variant (5-HT1Dβ) encoded by an intronless gene on chromosome 6. *Proc. Natl. Acad. Sci. USA* **89**:5522–5526 (1992).
15. Jin, H., D. Oksenberg, A. Ashkenazi, S. J. Peroutka, A. M. V. Duncan, R. Rozmahel, Y. Yang, G. Mengod, J. M. Palacios, and B. F. O'Dowd. Characterization of the human 5-hydroxytryptamine-1B receptor. *J. Biol. Chem.* **267**:5735–5738 (1992).
16. Levy, F. O., T. Gudermann, E. Perez-Reyes, M. Birnbaumer, A. J. Kaumann, and L. Birnbaumer. Molecular cloning of a human serotonin receptor (S12) with a pharmacological profile resembling that of the 5-HT$_{1D}$ subtype. *J. Biol. Chem.* **267**:7553–7562 (1992).
17. Metcalf, M. A., R. W. McGuffin, and M. W. Hamblin. Conversion of the human 5-HT1Dβ serotonin receptor to the rat 5-HT1B ligand-binding phenotype by Thr^{355}Asn site directed mutagenesis. *Biochem. Pharmacol.* **44**:1917–1920 (1992).
18. Parker, E. M., D. A. Grisel, L. G. Iben, and R. A. Shapiro. A single amino acid difference accounts for the pharmacological distinctions between rat and human 5-hydroxytryptamine-1B receptors. *J. Neurochem.* **60**:380–383 (1993).
19. Adham, N., J. A. Tamm, J. A. Salon, J.-J. Vaysse, R. L. Weinshank, and T. Branchek. A single point mutation increases the affinity of serotonin 5-HT1Dα, 5-HT1Dβ, 5-HT1E and 5-HT1F receptors for β-adrenergic antagonists. *Neuropharmacology* **33**:387–391 (1994).
20. Glennon, R. A., and R. B. Westkaemper. Serotonin receptors, 5-HT ligands and receptor modeling, in *Trends in Receptor Research* (P. Ageli, U. Gulin, and W. Quaglia, eds.). Elsevier Science Publishers, Amsterdam, 185–207 (1992).
21. Westkaemper, R. B., and R. A. Glennon. Molecular graphics models of members of the 5-HT2 subfamily: 5-HT2A, 5-HT2B and 5-HT2C receptors. *Med. Chem. Res.* **3**:317–334 (1993).
22. Westkaemper, R. B., and R. A. Glennon. Molecular modeling of the interaction of LSD and other hallucinogens with 5-HT2 receptors, in *Hallucinogens: An Update* (G. Lin and R. A. Glennon, eds.). United States Government Printing Office, Washington, D. C., 263–283 (1994).
23. Glennon, R. A., and R. B. Westkaemper. 5-HT1D receptors: a serotonin receptor population for the 1990s. *Drug News Perspect.* **6**:390–405 (1993).
24. Henderson, R., J. M. Baldwin, and T. A. Ceska. Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.* **148**:179–191 (1990).
25. Anderon, S., D. L. Davis, H. Dahlback, H. Jornvall, and D. W. Russell. Cloning, structure and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264**:8222–8229 (1989).
26. Parker, E. M., D. A. Grisel, L. G. Iben, H. P. Nowak, C. D. Mahle, F. D. Yocca, and G. T. Gaughan. Characterization of human 5-HT1 receptors expressed in Sf9 insect cells. *Eur. J. Pharmacol.* **268**:43–53 (1994).
27. Munson, P. J., and D. Robard. LIGAND: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
28. Have, R. β-Adrenergic blocking agents. VIII. Reactions of β-haloalkylamines related to pronethalol and propranolol. *J. Med. Chem.* **13**:398–403 (1970).
29. Stokker, G. E., W. F. Hoffman, A. W. Alberts, E. J. Cragoe, Jr., A. A. Deana, J. L. Gilfillan, J. W. Huff, F. C. Novello, J. D. Prugh, R. J. Smith,

and A. K. Willard. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. I. Structural modifications of 5-substituted 3,5-dihydroxypentanoic acid and their lactone derivatives. *J. Med. Chem.* **28**:347–358 (1985).
30. Schertler, G. F. X., C. Villa, and R. Henderson. Projection structure of rhodopsin. *Nature (Lond.)* **362**:770–772 (1993).
31. Unger, V. M., and G. F. X. Schertler. Low resolution structure of bovine rhodopsin determined by electron cryo-microscopy. *Biophys. J.* **68**:1776–1786 (1995).
32. Baldwin, J. M. The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.* **12**:1693–1703 (1993).
33. Cronet, P., C. Sander, and G. Vriend. Modeling of transmembrane seven helix bundles. *Protein Eng.* **6**:59–64 (1993).
34. Donnelly, D., J. B. Findlay, and T. L. Blundell. The evolution and structure of aminergic G protein-coupled receptors. *Recept. Channels* **2**:61–78 (1994).
35. Hoflack, J., S. Trumpp-Kallmeyer, and M. Hibert. Re-evaluation of bacteriorhodopsin as a model for G protein-coupled receptors. *Trends Pharmacol. Sci.* **15**:7–9 (1994).
36. Teeter, M. M., M. Froimowitz, B. Stec, and C. J. DuRand. Homology modeling of the dopamine D2 receptor and its testing by docking of agonists and tricyclic antagonists. *J. Med. Chem.* **37**:2874–2888 (1994).
37. McGregor, M. J., S. A. Islam, and M. J. E. Sternberg. Analysis of the relationship between sidechain conformation and secondary structure in globular proteins. *J. Mol. Biol.* **198**:295–310 (1987).
38. Branchek, T. Site directed mutagenesis of serotonin receptors. *Med. Chem. Res.* **3**:287–296 (1993).
39. Roth, B. L., M. S. Choudhary, and S. Craigo. Mutagenesis of 5-HT2 serotonin receptors: what does an analysis of many mutant receptors tell us? *Med. Chem. Res.* **3**:297–305 (1993).
40. Baker, E. N., and R. E. Hubbard. Hydrogen bonding in globular proteins. *Prog. Biophys. Mol. Biol.* **44**:97–179 (1984).
41. Thanki, N., J. M. Thornton, and J. M. Goodfellow. Distributions of water around amino acid residues in proteins. *J. Mol. Biol.* **202**:637–657 (1988).
42. Jeffrey, G. A., and W. Saenger. *Hydrogen Bonding in Biological Structures*. Springer-Verlag, Berlin, 475 (1991).
43. Wang, C.-D., T. K. Gallaher, and J. C. Shih. Site-directed mutagenesis of the serotonin 5-hydroxytryptamine-2 receptor: identification of amino acids necessary for ligand binding and receptor interaction. *Mol. Pharmacol.* **43**:931–940 (1993).

Send reprint requests to: Dr. Richard A. Glennon, Department of Medicinal Chemistry, School of Pharmacy, Box 980540 MCV/VCU, Richmond, VA 23298-0540.