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Benzimidazole derivatives. 4. The recognition of the voluminous substituent attached to the basic amino group of 5-HT_4 receptor antagonists

María L. López-Rodríguez[a,*], Bellinda Benhamú[a], Marta Murcia[a], Elsa Álvaro[a], Mercedes Campillo[b] & Leonardo Pardo[b,*]

[a]*Departamento de Química Orgánica I, Facultad de Ciencias Químicas, Universidad Complutense, E-28040 Madrid, Spain;* [b]*Laboratori de Medicina Computacional, Unitat de Bioestadística and Institut de Neurociències, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain*

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Summary

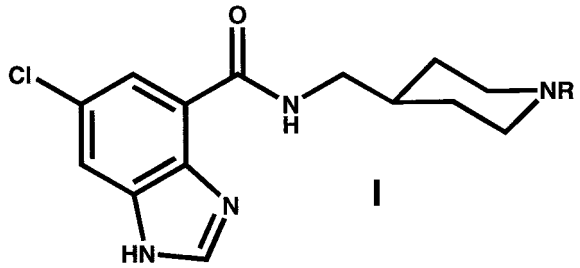
We report a structure–affinity analysis of an important element in the pharmacophore model for the recognition of 5-HT_4 receptor antagonists: the voluminous substituent attached to the basic nitrogen of the ligand. We have designed, synthesized and pharmacologically evaluated a series of benzimidazole derivatives **I** containing a common molecular skeleton formed by *N*-[(4-piperidyl)methyl]-6-chlorobenzimidazole-4-carboxamide and four different substituents (R = butyl, 2-[(methylsulfonyl)amino]ethyl, 5-[(phenylacetyl)amino]pentyl, and 5-[(benzylsulfonyl)amino]pentyl). These compounds possess binding affinities in the nM range (K_i = 0.11–1.50 nM). Moreover, a ligand that contains a hydrogen atom attached to the basic nitrogen (R = H; K_i = 150 nM) is used as a control for structure–affinity relationships.

Introduction

Rational drug design for lead finding benefits from the knowledge of the three-dimensional (3D) structure of the target protein. In recent years, the number of detailed 3D structural information of soluble proteins and their complexes with various ligands has increased in a significant manner and has revolutionized the drug design process. However, design of ligands targeting transmembrane proteins, such as transporters, ligand-gated ion channels, or G protein-coupled receptors (GPCRs), remains a more difficult challenge due to the limited or unavailable structures of this type deposited in the Brookhaven protein data bank. GPCRs belong to a central family of proteins because they are targeted by 60% of the drugs clinically used [1], form one of the largest protein families identified in the human genome [2, 3], and are related to a vast number of pathologies [4]. Unfortunately, atomic-level details of a 3D structure of a GPCR is only known for Rhodopsin (RHO) [5], the light photoreceptor protein of rod cells. RHO and probably the entire family of GPCRs are formed by a highly organized heptahelical transmembrane bundle. Thus, in the absence of a structural description of any other member of the GPCR family, homology modeling using the transmembrane domain of RHO as template is a common procedure in structure–affinity studies of ligands interacting with GPCRs. This procedure seems appropriate because of the large number of conserved sequence patterns in the transmembrane segments among the members of the family [6]. This structural homology does probably not extend to the extracellular domain, for which there is very little homology, and is highly structured in RHO, blocking the access of the extracellular ligand to the core of the receptor [7].

*To whom correspondence should be addressed. E-mail: mluzlr@quim.ucm.es; Leonardo.Pardo@uab.es

Table 1. Experimental 5-HT_4 receptor binding affinities (K_i) and solvation free energies (ΔG_{solv}) of benzimidazole derivatives **1–5**.



I

Compound	R	$K_i \pm$ SEM[a] (nM)	ΔG_{solv} (kcal/mol)
1[b]	-$(CH_2)_3$-CH_3	0.32 ± 0.07	−54.1
2[b]	-$(CH_2)_2$-$NHSO_2$-CH_3	0.11 ± 0.03	−69.2
3	-$(CH_2)_5$-NHCO-CH_2-Ph	0.31 ± 0.03	−68.1
4	-$(CH_2)_5$-$NHSO_2$-CH_2-Ph	1.50 ± 0.20	−74.2
5	H	150 ± 3.7	−65.3

[a] K_i values are mean ± SEM of two to four assays performed in triplicate.
[b] Binding affinities previously reported [10].

In the course of a program aimed at the discovery of new serotonin 5-HT_4 receptor (5-HT_4R) ligands we developed a computational model of the transmembrane domain of the 5-HT_4R, constructed from the crystal structure of RHO, complexed with various ligands of general structure **I** (Table 1) [8–10]. This model, together with the putative residues of the ligand-binding site experimentally determined by site-directed mutagenesis [11], facilitated the identification of the structural elements of the ligands that are key to high 5-HT_4R affinity. The most salient features are the ionic interaction between the NH group of the protonated piperidine of the ligand and the carboxylate group of $Asp^{3.32}$ (nomenclature of Ballesteros and Weinstein [12]); the interaction of the electron-rich clouds of the aromatic ring of $Phe^{6.51}$, which is a key residue for producing an inverse agonists effect [13], and the electron-poor hydrogens of the carbon atoms adjacent to the protonated piperidine nitrogen of the ligand; the hydrogen bond between the carbonylic oxygen of the ligand and the hydroxyl group of $Ser^{5.43}$; the occupancy of a small cavity between transmembrane helices (TMH) 5 and 6 by a chloro atom of the ligand [10]; and a voluminous substituent (R) in the basic amino framework of the molecule that expands towards TMH 7 [14]. In this work we have designed, synthesized, and pharmacologically evaluated new benzimidazole derivatives **I** in which the substituent (R) was modified to optimally interact with the residues of the receptor. This approach has led to ligands that expand all the way through TMHs 2, 3, 5, 6, and 7 and possess affinities in the nM or sub-nM range. These compounds are of considerable interest, from a medicinal chemistry point of view, because 5-HT_4R ligands are involved in (patho)physiological processes both in peripheral and central nervous systems [15].

Materials and methods

Model of the 5-HT_4 receptor

The previously reported 3D model of the transmembrane domain of the 5-HT_4R [9, 10], constructed from the crystal structure of RHO, has been used during the study. This computer model maintains the position of the TMHs as in RHO with the exception of TMH 3. TMH 3 is slightly bent towards TMH 5, at position 3.37, to facilitate the experimentally derived interactions between the ligand and $Asp^{3.32}$ and $Ser^{5.43}$. This structural effect is due to the *gauche*-conformation of the $Thr^{3.37}$ side chain [16]. We have recently provided experimental evidence for this structural difference of TMH 3 in RHO and the serotonin family by designing and testing ligands that contain comparable functional groups but at different interatomic distances [17].

The mode of recognition of compound **3** was first determined by ab initio geometry optimization with the 3-21G* basis set. The model system consisted of Asp$^{3.32}$ and Asn$^{7.45}$ (only the C_α atom of the backbone is included) of the 5-HT$_4$R and the designed compound **3** (Table 1). All free valences were capped with hydrogen atoms. The C_α atoms of the residues and the benzimidazole-4-carboxamide moiety of the ligand were kept fixed at the positions previously obtained in the model of the complex between compound **1** (see Table 1) and the 5-HT$_4$R [10]. Compounds **2** and **3** were docked into the entire transmembrane domain of the 5-HT$_4$R by computer-aided tools. Subsequently, the structures of these complexes were placed in a rectangular box (~72 Å × 67 Å × 53 Å in size) containing methane molecules (~4600 molecules in addition to the transmembrane domain) to mimic the hydrophobic environment of the transmembrane helices. The density of 0.4–0.5 g cm^{-3} of the methane box is approximately half of the density observed in the hydrophobic core of the membrane. This is due to the different equilibrium distance between carbons in the methane box and in the polycarbon chain of the lipid. However, it has been shown that this procedure reproduces several structural characteristics of membrane embedded protein [18]. Finally, the complete systems were energy minimized using the particle mesh Ewald method to evaluate electrostatic interactions.

Ab initio geometry optimizations were performed with the GAUSSIAN-98 system of programs [19]. Solvation free energies (ΔG_{solv}) of the isolated ligands were calculated with a polarized continuum model us-

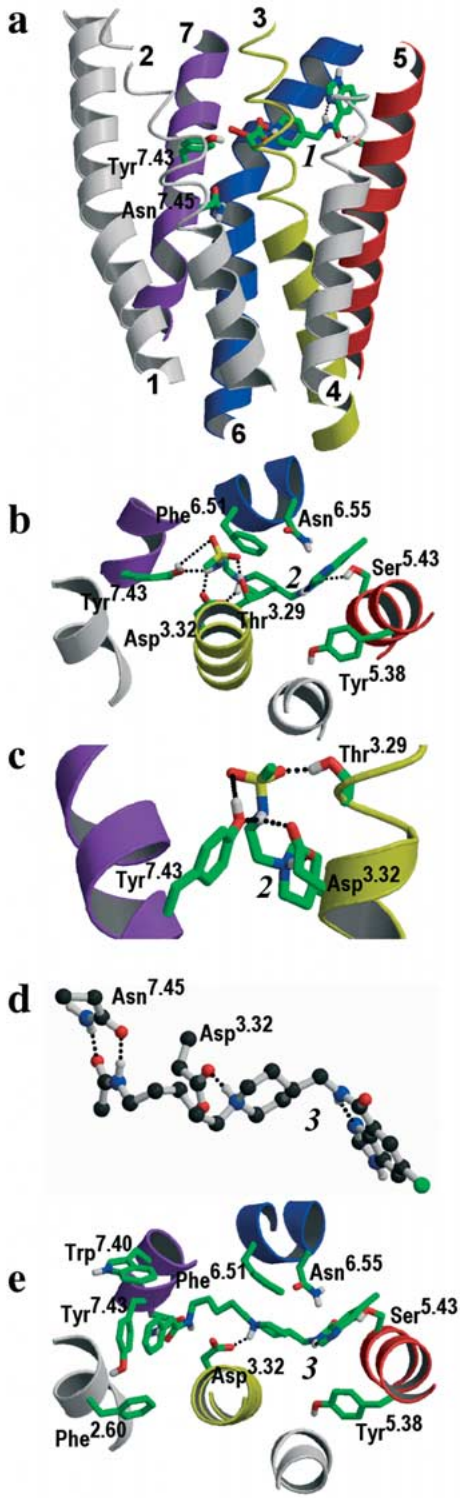


Figure 1. (a) Molecular model of the previously reported complex between compound **1** and the transmembrane domain of the 5-HT$_4$R [10], in a view parallel to the membrane. The butyl side chain of compound **1** is shown in red. The C_α traces of TMHs 1 (white), 2 (white), 3 (yellow), 4 (white), 5 (red), 6 (blue), and 7 (purple) are shown. (b,c) Detailed view of the transmembrane helix bundle of the 5-HT$_4$R complexed with compound **2**. The sulfonamide moiety and the piperidine nitrogen of the ligand form a complex hydrogen bond network with Thr$^{3.29}$, Asp$^{3.32}$, and Tyr$^{7.43}$. (d) Ab initio geometry optimization, at the HF/3-21G* level of theory, of compound **3** inside the side chains of Asp$^{3.32}$ and Asn$^{7.45}$. Only polar hydrogens are depicted to offer a better view. (e) Detailed view of the transmembrane helix bundle of the 5-HT$_4$R complexed with compound **3**. The phenyl ring of the ligand is positioned in the face-to-edge orientation (T-shaped) to both Phe$^{2.60}$ and Trp$^{7.40}$ and in the parallel orientation to Tyr$^{7.43}$. Figures were created using MolScript v2.1.1 [36] and Raster3D v2.5 [37].

ing the 6-31G* basis set. Energy minimization was run with the Sander module of AMBER 7 [20] and the all-atom force field [21]. Parameters for **2** and **3** were obtained with the antechamber program of AMBER 7 using the 'general Amber force field' and RESP point charges [22].

Chemistry

Melting points (uncorrected) were determined on a Gallenkamp electrothermal apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 250-AM spectrometer at 250 and 62.5 MHz, respectively. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (*J*) are in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad). Elemental analyses (C, H, N) were determined at UCM's analysis services and were within ±0.4% of the theoretical values. Analytical thin-layer chromatography (tlc) was run on Merck silica gel plates (Kieselgel 60 F-254) with detection by UV light, iodine, or acidic vanillin solution. For flash chromatography, Merck silica gel type 60 (size 230–400 mesh) was used. Unless stated otherwise, all starting materials and reagents were high-grade commercial products purchased from Aldrich, Fluka or Merck. All solvents were distilled prior to use. Dry DMF was obtained by stirring with CaH_2 followed by distillation under argon.

General procedure for the synthesis of (1-substituted -4-piperidyl) methylamines 7 and 8.

To an ice-cold solution of 4-piperidylmethylamine (1.1 g, 10 mmol) in dry acetonitrile (10 ml) was added dropwise a solution of *N*-(5-chloropentyl)phenylacetacetamide or *N*-(5-chloropentyl)phenylmethanesulfonamide (8 mmol) in acetonitrile (8 ml). The reaction was allowed to warm to room temperature and stirred overnight. Then a 4% aqueous solution of NaOH was added dropwise (50 ml), the mixture was extracted with chloroform (3 × 50 ml), and the combined organic layers were washed with brine and dried over Na_2SO_4. After evaporation of the solvent, the crude oil was purified by column chromatography to afford the corresponding alkylated amine as a pure compound. In both cases, small amounts of the dialkylated compound (17% and 20%, respectively) were observed in the ^{1}H NMR spectra of the reaction crudes.

[1-[5-[(Phenylacetyl)amino]pentyl]-4-piperidyl]methylamine (7).

Yield 30%; chromatography chloroform/methanol/ammonia, from 9:4:0.1 to 9:4:1; mp 136–138 °C (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.14–1.71 (m, 13H, $C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2NH$, $2H_3$, $2H_5$, H_4, NH_2), 1.85 (t, J = 10.5, 2H, H_{2ax}, H_{6ax}), 2.23 (dd, J = 8.1, 7.3, 2H, NCH_2), 2.54 (d, J = 4.9, 2H, $C\underline{H}_2NH_2$), 2.88 (d, J = 11.5, 2H, H_{2eq}, H_{6eq}), 3.17 (q, J = 6.6, 2H, $C\underline{H}_2NHCO$), 3.54 (s, 2H, CH_2Ph), 5.43 (br s, 1H, CONH), 7.21–7.37 (m, 5H, Ph); ^{13}C NMR (DMSO-d_6) δ 24.7 ($\underline{C}H_2CH_2CH_2NH$), 26.5 ($\underline{C}H_2CH_2NH$), 29.2 ($NCH_2\underline{C}H_2$), 29.8 ($C_3$, C_5), 39.2, 39.4 (C_4, CH_2Ph), 43.7 ($\underline{C}H_2NHCO$), 48.0 (CH_2NH_2), 53.6 (C_2, C_6), 58.7 (NCH_2), 127.2, 128.8, 129.3, 134.9 (Ph), 170.7 (NHCO).

[1-[5-[(Benzylsulfonyl)amino]pentyl]-4-piperidyl] methylamine (8).

Yield 30%; chromatography chloroform/methanol/ammonia, from 9:4:0.1 to 9:4:1; mp 128–129 °C (toluene); ^{1}H NMR ($CDCl_3$) δ 1.18–1.55 (m, 11H, $C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2NH$, H_{3ax}, H_{5ax}, H_4, NH_2), 1.67 (d, J = 9.0, 2H, H_{3eq}, H_{5eq}), 1.88 (t, J = 11.2, 2H, H_{2ax}, H_{6ax}), 2.29 (dd, J = 7.8, 7.1, 2H, NCH_2), 2.53 (d, J = 5.1, 2H, $C\underline{H}_2NH_2$), 2.87-2.89 (m, 4H, H_{2eq}, H_{6eq}, $C\underline{H}_2NHSO_2$), 4.22 (s, 2H, CH_2Ph), 7.36 (s, 5H, Ph); ^{13}C NMR (DMSO-d_6) δ 24.2 ($\underline{C}H_2CH_2CH_2NH$), 26.2 ($\underline{C}H_2CH_2NH$), 29.6, 29.9 ($C_3$, C_5, $NCH_2\underline{C}H_2$), 39.2 (C_4), 43.5 (CH_2NHSO_2), 47.9 (CH_2NH_2), 53.7 (C_2, C_6), 58.4, 58.6 (NCH_2, CH_2Ph), 128.6, 128.8, 129.6, 130.6 (Ph).

Synthesis of (1-tert-butoxycarbonyl-4-piperidyl)methylamine (9).

To an ice-cold solution of 4-piperidylmethylamine (1.0 g, 8.7 mmol) in dioxane (9 ml), water (9 ml) and 1 M NaOH (9 ml) was added dropwise a solution of di-*tert*-butyl dicarbonate (2.1 g, 9.5 mmol) in dioxane (50 ml). The reaction was allowed to warm to room temperature and stirred for 4 h. The solution was concentrated under reduced pressure (40 ml), and extracted with chloroform (3 × 40 ml), and the combined organic layers were dried over Na_2SO_4. After evaporation of the solvent, the crude oil was purified by column chromatography (from chloroform to chloroform/methanol 9:1) to afford pure amine **9** (oil), in 47% yield. Dicarbamate was also isolated as a side product from the reaction crude in 31% yield. ^{1}H NMR ($CDCl_3$) δ 1.02 (qd, J = 12.4, 4.4, 2H, H_{3ax}, H_{5ax}), 1.39 (s, 10H, H_4, $3CH_3$), 1.60–1.68 (m, 4H,

H_{3eq}, H_{5eq}, NH_2), 2.53 (d, J = 6.6, 2H, $C\underline{H}_2NH_2$), 2.62 (t, J = 12.2, 2H, H_{2ax}, H_{6ax}), 4.05 (d, J = 12.7, 2H, H_{2eq}, H_{6eq}); ^{13}C NMR (DMSO-d_6) δ 28.3 ($3CH_3$), 29.6 (C_3, C_5), 39.3 (C_4), 43.6 (C_2, C_6), 47.7 (CH_2NH_2), 79.1 ($\underline{C}(CH_3)_3$), 154.7 (COO).

General procedure for the synthesis of 6-chlorobenzimidazole-4-carboxamides 3, 4 and 6.

To a solution of 6-chlorobenzimidazole-4-carboxylic acid [23] (1 g, 5 mmol) in dry *N,N*-dimethylformamide (DMF, 5 ml) under an argon atmosphere was added 1,1′-carbonyldiimidazole (CDI, 811 mg, 5 mmol). The mixture was stirred at 40 °C for 1 h, and then a solution of 6 mmol of the corresponding (1-substituted-4-piperidyl)methylamine **7**–**9** and 1,8-diazabicyclo[5.4.0]undec-7ene (DBU, 761 mg, 5 mmol) in DMF (10 ml) was added dropwise, and the reaction mixture was stirred at 50 °C for 20–24 h. The solvent was removed under reduced pressure, and the crude was taken up in chloroform (50 ml) and washed with water (20 ml) and 20% aqueous potassium carbonate (20 ml). The organic layer was dried over sodium sulphate and the solvent was evaporated to afford the crude product, which was purified by column chromatography and recrystallization from the appropriate solvents.

N-[[1-[5-[(Phenylacetyl)amino]pentyl]-4-piperidyl]methyl]-6-chlorobenzimidazole-4-carboxamide (3).

Yield 54%; chromatography from chloroform to chloroform/methanol, 4:1; mp 151–153 °C (toluene); ^{1}H NMR ($CDCl_3$) δ 1.18–1.57 (m, 8H, $C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2NH$, $H_{3'ax}$, $H_{5'ax}$), 1.74 (m, 1H, $H_{4'}$), 1.82 (d, J = 12.0, 2H, $H_{3'eq}$, $H_{5'eq}$), 2.06 (t, J = 11.7, 2H, $H_{2'ax}$, $H_{6'ax}$), 2.34–2.39 (m, 2H, NCH_2), 3.02 (d, J = 11.4, 2H, $H_{2'eq}$, $H_{6'eq}$), 3.17 (q, J = 7.2, 2H, $C\underline{H}_2NHCOBn$), 3.46 (t, J = 4.8, 2H, $CONHC\underline{H}_2$), 3.54 (s, 2H, CH_2Ph), 5.74 (br t, 1H, NHCOBn), 7.21–7.34 (m, 5H, Ph), 7.54 (s, 1H, H_7), 7.97 (s, 2H, H_5, H_2); ^{13}C NMR ($CDCl_3$) δ 24.6 ($\underline{C}H_2CH_2CH_2NH$), 25.9 ($\underline{C}H_2CH_2NH$), 29.2, 29.4 ($NCH_2\underline{C}H_2$, $C_{3'}$, $C_{5'}$), 35.9 ($C_{4'}$), 39.5 (CH_2Ph), 43.6 ($\underline{C}H_2NHCO$), 44.8 ($CONH\underline{C}H_2$), 53.3 ($C_{2'}$, $C_{6'}$), 58.4 (NCH_2), 115.3 (C_7), 123.2 (C_4), 123.5 (C_5), 127.3 (Ph), 128.5 (C_6), 128.9, 129.3 (Ph), 134.6 (C_{7a}), 134.9 (Ph), 138.8 (C_{3a}), 142.0 (C_2), 165.0 (CONH), 171.4 (NHCO). Anal. ($C_{27}H_{34}ClN_5O_2$) C, H, N.

N-[[1-[5-[(Benzylsulfonyl)amino]pentyl]-4-piperidyl]methyl]-6-chlorobenzimidazole-4-carboxamide (4).

Yield 73%; chromatography chloroform/methanol, from 9:1 to 7:3; mp 161–163 °C (chloroform/hexane); ^{1}H NMR ($CDCl_3$) δ 1.21–1.50 (m, 8H, $C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2NH$, $H_{3'ax}$, $H_{5'ax}$), 1.65–1.77 (m, 3H, $H_{4'}$, $H_{3'eq}$, $H_{5'eq}$), 1.97 (t, J = 11.1, 2H, $H_{2'ax}$, $H_{6'ax}$), 2.32 (dd, J = 7.8, 7.2, 2H, NCH_2), 2.91–2.96 (m, 4H, $H_{2'eq}$, $H_{6'eq}$, $C\underline{H}_2NHSO_2$), 3.46 (br t, 2H, $CONHC\underline{H}_2$), 4.20 (s, 2H, CH_2Ph), 4.85 (br s, 1H, $NHSO_2$), 7.30–7.35 (m, 5H, Ph), 7.57 (s, 1H, H_7), 7.95 (s, 1H, H_5), 7.99 (s, 1H, H_2), 9.71 (br s, 1H, CONH); ^{13}C NMR ($CDCl_3$) δ 24.1 ($\underline{C}H_2CH_2CH_2NH$), 25.7 ($\underline{C}H_2CH_2NH$), 29.3, 29.7 ($C_{3'}$, $C_{5'}$, $NCH_2\underline{C}H_2$), 35.9 ($C_{4'}$), 43.5 (CH_2NHSO_2), 44.8 ($CONH\underline{C}H_2$), 53.4 ($C_{2'}$, $C_{6'}$), 58.1, 58.6 (NCH_2, CH_2Ph), 114.8 (C_7), 122.9, 123.6 (C_4, C_5), 128.7, 128.8, 129.4, 130.6 (C_6, Ph), 134.9 (C_{7a}), 138.9 (C_{3a}), 141.9 (C_2), 164.9 (CONH). Anal. ($C_{26}H_{34}ClN_5O_3S$) C, H, N.

N-[(1-tert-butoxycarbonyl-4-piperidyl)methyl]-6-chlorobenzimidazole-4-carboxamide (6).

Yield 60%; chromatography from chloroform to chloroform/methanol 7:3; mp 178–179 °C (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.12–1.34 (m, 2H, $H_{3'ax}$, $H_{5'ax}$), 1.44 (s, 9H, $3CH_3$), 1.73–1.84 (m, 3H, $H_{3'eq}$, $H_{5'eq}$, $H_{4'}$), 2.71 (t, J = 11.5, 2H, $H_{2'ax}$, $H_{6'ax}$), 3.49 (m, 2H, $CONHC\underline{H}_2$), 4.12 (d, J = 12.5, 2H, $H_{2'eq}$, $H_{6'eq}$), 7.61 (s, 1H, H_7), 8.10 (m, 2H, H_2, H_5), 9.98 (t, J = 5.1, 1H, CONH), 11.25 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 28.4 ($3CH_3$), 29.9 ($C_{3'}$, $C_{5'}$), 36.5 ($C_{4'}$), 43.7 ($C_{2'}$, $C_{6'}$), 45.2 ($CONH\underline{C}H_2$), 79.7 ($\underline{C}(CH_3)_3$), 115.6 (C_7), 123.2 (C_4), 123.7 (C_5), 128.9 (C_6), 134.8 (C_{7a}), 138.7 (C_{3a}), 141.9 (C_2), 155.0 (COO), 165.2 (CONH). Anal. ($C_{19}H_{25}ClN_4O_3$) C, H, N.

Synthesis of N-[(4-piperidyl)methyl]-6-chlorobenzimidazole-4-carboxamide (5).

5 ml (126 mmol) of 98% formic acid was added to 500 mg (1.27 mmol) of **6**, and the solution was stirred at room temperature for 4 h. After evaporation of the solvent under reduced pressure the crude was taken up in 1 ml of ice-cold water. The resulting solution was basified with an ice-cold solution of aqueous 1 N KOH, and amide **5** was obtained after filtration as a solid which was purified by recrystallization from methanol/chloroform: yield 73%; p.f. 238–240 °C. ^{1}H NMR ($CDCl_3$) δ 1.19 (br q, J = 11.5, 2H, $H_{3'ax}$, $H_{5'ax}$), 1.64–1.70 (m, 3H, $H_{3'eq}$, $H_{5'eq}$, $H_{4'}$), 2.49 (br

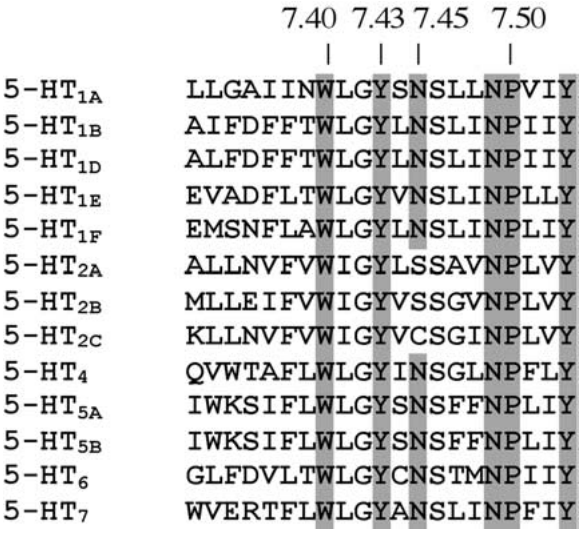


Figure 2. Multiple sequence alignment of TMH 7 in the serotonin family of GPCRs. Numbers at the top define the general numbering scheme to identify residues in the transmembrane segments of different receptors [12]. The highly conserved NPxxY motif and the residues referenced in the paper are highlighted.

t, J = 11.0, 2H, $H_{2'ax}$, $H_{6'ax}$), 2.98 (br d, J = 10.7, 2H, $H_{2'ec}$, $H_{6'ec}$), 3.30 (br t, 2H, CONHC$\underline{H}_2$), 7.72 (s, 1H, H_5), 7.77 (s, 1H, H_7), 8.36 (s, 1H, H_2), 9.88 (br s, 1H, CONH); ^{13}C NMR ($CDCl_3$) δ 29.9 ($C_{3'}$, $C_{5'}$), 36.2 ($C_{4'}$), 44.9, 45.2 ($C_{2'}$, $C_{6'}$, CONH$\underline{C}H_2$), 117.0 (C_7), 120.9 (C_5), 122.1; 125,4 (C_4. C_6), 139.1; 139.5 (C_{3a}, C_{7a}), 147.0 (C_2), 164.4 (CONH). Anal. ($C_{14}H_{17}ClN_4O$) C, H, N.

Radioligand binding assays at the 5-HT$_4$ receptor

Male Sprague-Dawley rats (*Rattus norvegicus albinus*), weighing 180–200 g, were killed by decapitation and the brains rapidly removed and dissected. Tissues were stored at −80 °C for subsequent use and homogenized on a Polytron PT-10 homogenizer. Membrane suspensions were centrifuged on a Beckman J2-HS instrument. Binding assays were performed according to the procedure previously described by Grossman et al. [24]. The data were analyzed by an iterative curve-fitting procedure (program Prism, Graph Pad), which provided IC_{50}, K_i, and r^2 values for test compounds, K_i values being calculated from the Cheng–Prusoff equation [25]. Values are means of 2–4 experiments performed in triplicate. The protein concentrations of the rat striatum were determined by the method of Lowry [26], using bovine serum albumin as the standard.

Results

Figure 1a shows the previously obtained computational model of the complex between compound **1** (see Table 1) and the transmembrane domain of the 5-HT$_4$R [10]. The butyl group of the side chain (R) attached to the piperidine nitrogen (shown in red in Figure 1a) expands between TMHs 3 and 7. This voluminous substituent is an important element in the pharmacophore model for the recognition of 5-HT$_4$R antagonists [14]. It is plausible to hypothesize that this bulky substituent impedes the counterclockwise rotation (viewed from the extracellular side) of TMHs 3 and 6 necessary for receptor activation [27–29]. Moreover, we have recently shown that the interaction between the sulfonamide moiety of EF-7412, an arylpiperazine derivative acting at the 5-HT$_{1A}$R sites, and Asn$^{7.39}$ in TMH 7 is a key element for converting the ligand to an antagonist in pre- and post-synaptic sites [30, 31]. Thus, it seems important to modify this voluminous substituent to enhance the interaction with the residues of the receptor, principally the residues located in TMH 7.

Molecular modeling of ligands containing a [(methylsulfonyl)amino]ethyl side chain attached to the piperidine nitrogen

A common voluminous substituent attached to the piperidine nitrogen (R) of several 5-HT$_4$R antagonists (i.e., GR 113808, RS 39604) is [(methylsulfonyl)amino]ethyl (see [32] for a review). We have recently synthesized compound **2** that replaces the butyl side chain of **1** by [(methylsulfonyl)amino]ethyl (see Table 1) [23]. It has been shown by site-directed mutagenesis that replacement of Tyr$^{7.43}$ (the position of this residue in the 5-HT$_4$R model is shown in Figure 1a) by Ala avoids the binding of GR 113808 to the receptor [11]. Thus, it is reasonable to assume that Tyr$^{7.43}$ anchors the common sulfonamide moiety of GR 113808 and compound **2**. Figures 1b and 1c show detailed views of the energy-optimized structure of compound **2** inside the transmembrane domain of the 5-HT$_4$R (see Materials and methods for computational details). The sulfonamide moiety and the piperidine nitrogen of the ligand form a complex hydrogen bond network with the receptor: the N-H group of the protonated piperidine interacts with the O_δ atom of Asp$^{3.32}$, the N-H group of the sulfonamide moiety interacts with both the O_δ atom of Asp$^{3.32}$ and the O_η atom of Tyr$^{7.43}$, and the S=O groups of the sulfonam-

ide moiety interact with the H_η atom of $Tyr^{7.43}$ and the H_γ atom of $Thr^{3.29}$, respectively. The interactions between the piperidine and benzimidazole moieties of the ligand and the residues of the receptor located in TMHs 5 and 6 have been described in detail elsewhere [10].

Molecular design of 5-HT$_4$ receptor ligands

Figure 2 shows the multiple sequence alignment of TMH 7 of the serotonin family of GPCRs. Inspection of this alignment reveals a key polar residue that might be susceptible to forming hydrogen bond interactions with the ligand: $Asn^{7.45}$. The position of this residue in the 5-HT$_4$R model is shown in Figure 1a. Based on this hypothesis we have designed derivatives of general chemical structure I (R=$(CH_2)_n$NHCOR′), which contain a –NHCO– group that would optimally interact with the side chain of $Asn^{7.45}$. To identify the optimal length (n) of R for achieving this proposed interaction we performed ab initio geometry optimization of structures with $n = 3$–5 and $R' = CH_3$ inside the side chains of $Asp^{3.32}$ and $Asn^{7.45}$. The positions of the C_α atoms of the receptor side chains and the benzimidazole-4-carboxamide moiety of the ligand were kept fixed at the values obtained in the model of the complex between compound **1** and the 5-HT$_4$R [10] (see Materials and methods for computational details). Figure 1d shows the obtained structure for the optimal –CH_2– chain, connecting the piperidine ring and the –NHCO– group, of $n = 5$. The N–H moiety of the –NHCO– group acts as a hydrogen bond donor in the hydrogen bond interaction with the O_δ atom of Asn, and the C=O moiety of the –NHCO– group acts as a hydrogen bond acceptor in the hydrogen bond interaction with the N_δ–H moiety of Asn. The presence of several aromatic residues within TMHs 2 ($Phe^{2.60}$) and 7 ($Trp^{7.40}$, $Tyr^{7.43}$) made us introduce a phenyl ring into the structure. A methylene –CH_2– group was inserted between the phenyl ring and the –NHCO–group (see Table 1) to let the aromatic ring expand within the TMHs (see Figure 1e). The phenyl ring of the ligand is positioned in the face-to-edge orientation (T-shaped) to both $Phe^{2.60}$ and $Trp^{7.40}$ and in the parallel orientation to $Tyr^{7.43}$. Finally, compound **4** replaces the –NHCO– group of compound **3** for the sulfonamide –$NHSO_2$– group (see Table 1).

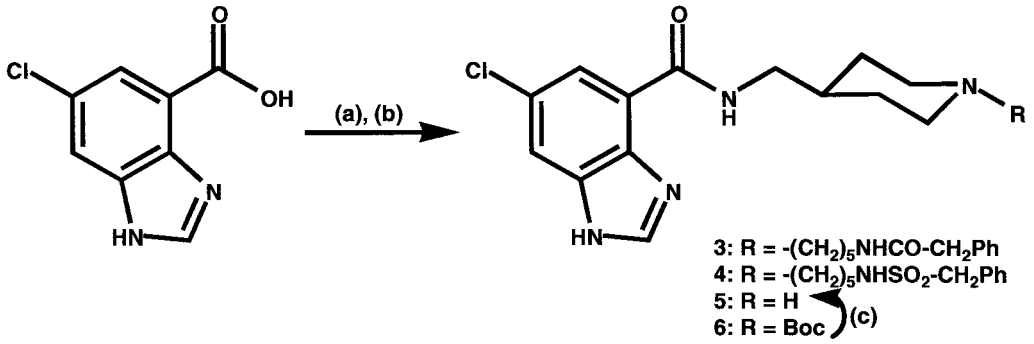
Synthesis and pharmacological evaluation of 5-HT$_4$ receptor ligands

Compounds **3, 4** and **6** were synthesized from 6-chlorobenzimidazole-4-carboxylic acid [23], as described in Scheme 1, by activation with 1,1′-carbonyldiimidazole (CDI), and subsequent coupling with the (1-substituted-4-piperidyl)methylamine **7-9** in the presence of 1,8-diazabicycloundec-7-ene (DBU) in *N,N*-dimethylformamide (DMF) as solvent. Deprotection of N-Boc derivative **6** with formic acid afforded the desired compound **5**. The (1-substituted-4-piperidyl)methylamines **7–9** were prepared from (4-piperidyl)methylamine, by reaction with the appropriate halide (*N*-(5-chloropentyl)phenylacetamide or *N*-(5-chloropentyl)phenylmethanesulfonamide) in dry acetonitrile or with di-*tert*-butyl dicarbonate in a solution of dioxane, water and 1 M sodium hydroxide (Scheme 2). New compounds were characterized by IR and ^{1}H and ^{13}C NMR spectroscopy, and gave satisfactory combustion analyses (C, H, N) as reported in Materials and methods. Table 1 reports the in vitro affinity at 5-HT$_4$R sites by radioligand binding assays, using [^{3}H]GR 113808 in rat striatum membranes (see Materials and methods) [24].

Discussion

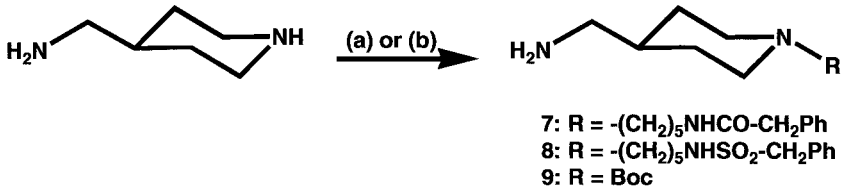
We present in this study a structural analysis of the voluminous substituent (R), attached to the piperidine nitrogen of the molecule, that expands towards TMH 7. It has been proposed that this voluminous substituent is an important element in the pharmacophore model for the recognition of 5-HT$_4$R antagonists [14]. Four different substituents have been modeled, synthesized, and pharmacologically evaluated (see Table 1): a butyl group (compound **1**), a 2-[(methylsulfonyl)amino]ethyl group (compound **2**), a 5-[(phenylacetyl)amino]pentyl group (compound **3**), and a 5-[(benzylsulfonyl)amino]pentyl group (compound **4**). Moreover, in order to test the role of the investigated side chain in receptor binding, compound **5**, that contains a hydrogen atom attached to the basic nitrogen, has been synthesized as a control. All these compounds share a common molecular skeleton formed by *N*-[(4-piperidyl)methyl]-6-chlorobenzimidazole-4-carboxamide and only differ in the substituent which makes them appropriate for structure–affinity relationships.

Compounds **1**–**3** all possess 5-HT$_4$R binding affinities in the sub-nM range (0.11–0.32 nM, see Table 1).



Reagents: (a) CDI, DMF; (b) (1-substituted-4-piperidyl)methylamines (7-9), DBU, DMF; (c) HCOOH

Scheme 1. Synthesis of 6-chlorobenzimidazole-4-carboxamides **3**–**6**.



Reagents: (a) Cl-$(CH_2)_5$-NHR, acetonitrile (7, 8); (b) $(Boc)_2O$, dioxane / H_2O / 1 M NaOH (9)

Scheme 2. Synthesis of (1-substituted-4-piperidyl)methylamines **7**–**9**.

However, the mechanism by which these ligands elicit their binding affinities differs. K_i is a function of both the stabilization of the complex formed between the ligand molecule and the receptor and the solvation energy of the ligand. Replacement of the hydrogen atom of compound **5** by the non polar butyl side chain of compound **1** enhances the affinity of the ligand for the receptor (K_i (**1**) = 0.32 nM *vs.* K_i (**5**) = 150 nM, Table 1) due to a significant decrease of the solvation energy facilitating the entrance of the ligand to the binding site (ΔG_{solv} (**1**) = −54.1 kcal/mol *vs.* ΔG_{solv} (**5**) = −65.3 kcal/mol) and hydrophobic interactions with the receptor. In contrast, the polar sulfonamide side chain of compound **2** possesses a higher energy penalty to displace the ligand from the extracellular aqueous environment to the binding pocket (ΔG_{solv} (**2**) = −69.2 kcal/mol), but it is able to form a complex hydrogen bond network with $Thr^{3.29}$, $Asp^{3.32}$, and $Tyr^{7.43}$, compensating the larger solvation energy. Compound **3** forms hydrogen bond interactions with $Asn^{7.45}$, throughout the –NHCO– group, and aromatic–aromatic interactions with $Phe^{2.60}$, $Trp^{7.40}$, and $Tyr^{7.43}$, throughout the phenyl ring. The solvation energy of compound **3** is ΔG_{solv} (**3**) = −68.1 kcal/mol, comparable in magnitude with compound **2** (ΔG_{solv} (**2**) = −69.2 kcal/mol). The difficulty in predicting, by computational methods, the magnitude of the interaction energy between a given compound and the 5-HT_4R model impedes a further quantitative comparison between compounds **2** and **3**. Compound **4** possesses lower 5-HT_4R binding affinity (K_i = 1.5 nM, see Table 1) than compounds **1**–**3**. Substitution of the –NHCO– group of compound **3** for the sulfonamide –$NHSO_2$– group of compound **4** introduces a second hydrogen bond acceptor into the structure. This increases the energy penalty for ligand desolvation to ΔG_{solv} (**4**) = −74.2 kcal/mol (see Table 1). The absence of a polar side chain (results not shown), in addition to $Asn^{7.45}$, in this part of the receptor, discards the interaction of this second hydrogen bond acceptor with the receptor. Thus, the additional energy penalty of solvation of compound **4**, relative to compound **3**, is not compensated by additional interactions between the ligand and the receptor, resulting in a ligand with lower binding affinity.

It is important to note that the role of the highly conserved $Asp^{3.32}$ in the binding of 5-HT_4R ligands to the 5-HT_4R is unclear. It has recently been shown, in a very interesting study, that substitution of

$Asp^{3.32}$ by Ala converts the 5-HT_4R in a G_s-coupled receptor activated exclusively by synthetic ligands (RASSL) and not by the natural agonist serotonin [33]. The binding of GR 113808, that contains the basic piperidine nitrogen of compounds **1**–**4** and the [(methylsulfonyl)amino]ethyl group of compound **2**, is only moderately affected by the mutation of $Asp^{3.32}$ [33]. Thus, the following factors should be taken into account. First, the basic nitrogen of some 5-HT_4R ligands might interact with a negative charge of the receptor but not with $Asp^{3.32}$ as it has been suggested [33]. Second, the absence of the interaction between the ligand and $Asp^{3.32}$ in the mutant receptor might be compensated by the larger energy penalty of Asp to disrupt its side chain environment in the ligand-free form, and the larger interaction of other residues of the receptor with the ligand [9]. And third, the ligand might bind in different manners to wild type and mutant receptors. This would be in agreement with the fact that GR 113808, which behaves as an antagonist in wild type receptor, exhibits agonist properties in the $Asp^{3.32}$ Ala mutant receptor [33].

The previously reported 3D model of the complex between compound **1** and the transmembrane domain of the 5-HT_4R [10], based on the rhodopsin crystal structure, has provided a coherent framework for guiding the design of new ligands acting at the 5-HT_4R sites. Designed compound **3** deserves special attention. It possesses a binding affinity of 0.31 nM for the 5-HT_4R and in contrast to the other compounds expands all the way through TMHs 2 (interacting with $Phe^{2.60}$), 3 ($Asp^{3.32}$), 5 ($Tyr^{5.38}$, $Ser^{5.43}$), 6 ($Phe^{6.51}$, $Asn^{6.55}$), and 7 ($Trp^{7.40}$, $Tyr^{7.43}$, $Asn^{7.45}$) (see Figure 1). The flexibility of compound **3**, which might be an impediment for better binding affinity, allows the ligand to trail the helices forming the tertiary structure of GPCRs. Only TMHs 1 and 4 (TMH 4 is involved in forming the interface for receptor dimerization [34, 35]) are not participating in the binding of compound **3**. Thus, the high binding affinity of this designed compound, for the receptor binding site, provides experimental support to the proposed model for the interaction of this class of ligands with the 5-HT_4R.

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