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Tania de La Fuente, Mar Martín-Fontecha, Jessica Sallander, Bellinda Benhamú, Mercedes Campillo, et al.. Benzimidazole Derivatives as New Serotonin 5-HT₆ Receptor Antagonists. Molecular Mechanisms of Receptor Inactivation. *Journal of Medicinal Chemistry*, 2010, 53 (3), pp.1357-1369. 10.1021/jm901672k . hal-02483302

HAL Id: hal-02483302

<https://hal.science/hal-02483302>

Submitted on 18 Feb 2020

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Benzimidazole Derivatives as New Serotonin 5-HT₆ Receptor Antagonists. Molecular Mechanisms of Receptor Inactivation

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New serotonin 5-HT₆ receptor antagonists

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^a Abbreviations: GPCR, G protein-coupled receptor; CNS, central nervous system; h5-HT₆R, human 5-HT₆ receptor; PI, positive ionizable atom; HBA, H-bonding acceptor group; HYD, hydrophobic; TM, transmembrane helix; EL, extracellular loop; SEM, 2-(trimethylsilyl)ethoxymethyl; BOC, *tert*-butoxycarbonyl; WT, wild-type

Abstract

Based on our previously described pharmacophore model for serotonin 5-HT₆ receptor (5-HT₆R) antagonists, we have designed, synthesized, and pharmacologically characterized a series of benzimidazole derivatives **1-20**. The new compounds contain a benzimidazole scaffold in the central core, a piperazine ring, either a carbonyl (-CO-) or a methylene (-CH₂-) group, and an aromatic ring (benzene or naphthalene). Most of the benzimidazole-4-substituted derivatives show high 5-HT₆R affinity, whereas 5-substituted analogues are inactive. The functional activity of the ligands has been assessed in the human receptor, and the compounds were found to represent a new family of potent 5-HT₆R antagonists. Site-directed mutagenesis and a β_2 -adrenoceptor-based homology model of the 5-HT₆R were used to predict the mode of binding of antagonist SB-258585 and these benzimidazole derivatives. Substitution of W6.48, F6.52, or N6.55 by Ala fully impedes compound **4**, the most potent antagonist, to block 5-HT-induced activation. Thus, we propose that D3.32 in TM 3 anchors the protonated piperazine ring, the benzimidazole ring expands parallel to EL 2 to hydrogen bond N6.55 in TM 6, and the final aromatic ring is placed between TMs 3 and 5 in CH₂-containing compounds and between TMs 3 and 6 in CO-containing compounds. These binding modes of benzimidazole derivatives are compared with the binding mode of SB-258585. This combined experimental and computational study has permitted to propose the molecular mechanisms by which the new benzimidazole derivatives act as 5-HT₆R antagonists.

G protein-coupled receptors (GPCRs), serotonin 5-HT₆ receptor antagonists, structure-affinity relationships, computational models, site-directed mutagenesis

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) research is now more than 50 years old,¹⁻³ but still represents one of the most attractive areas in medicinal chemistry.⁴ Fourteen serotonin receptor subtypes have been identified so far, that are classified into seven families (5-HT₁₋₇)^{5,6} based on amino acid sequences, pharmacology, and intracellular mechanisms. With the exception of the ionotropic 5-HT₃ receptor, all of the 5-HT subtypes are G protein-coupled receptors (GPCRs^a).⁷⁻¹⁰ To date many serotonergic agents acting at 5-HT₁₋₄ receptors are clinically used¹¹ for the treatment of certain central nervous system (CNS) illnesses. One of the most recent additions to the family of serotonin receptors is the human 5-HT₆ receptor (h5-HT₆R), that was cloned in 1996^{12,13} as a gene codifying a polypeptide chain of 440 amino acids^{13,14} that is positively coupled to the adenylate cyclase¹⁵⁻¹⁷ cascade via the G_s protein. The high affinity of several antipsychotic and antidepressant agents^{13,18,19} boosted the first studies exploring the 5-HT₆R potential for the treatment of schizophrenia and bipolar affective disorder. However, contradictory results¹⁹ were obtained about the role of the receptor in these therapeutic indications. Later on, 5-HT₆R function was associated with the control of cholinergic neurotransmission,^{20,21} which prompted much interest into the possible implication of the receptor in cognitive impairment^{19,22,23} (memory and learning) related to neurological diseases such as Alzheimer's. In the last five years it has been shown that selective 5-HT₆R ligands are able to reduce food intake,^{24,25} though this effect had been unnoticed in early studies. Thus, the 5-HT₆R has emerged as a promising target for the treatment of obesity and related metabolic syndrome,^{19,22,26} a disease with an increasing global prevalence and high unmet clinical need. Clearly, there is much evidence that the h5-HT₆R is involved in the pathogenesis of CNS diseases^{4,19,22,23,26,27} related to cognitive or eating disorders, so it appears to be an attractive therapeutic target that should be exploited for drug development. Since it is known to be expressed almost exclusively in the CNS^{18,28-31} and mainly in the olfactory tubercle, striatum, nucleus accumbens, and hippocampus, it is

possible that new therapeutic agents targeting this receptor might have relatively few peripheral side effects. The first 5-HT₆R antagonists Ro 04-6790,³² Ro 63-0563³² and SB-258585³³ (Chart 1) were identified in the late 1990s by high-throughput screening at Roche and GlaxoSmithKline. Soon afterward, Glennon *et al.*,³⁴⁻³⁷ pioneers in the synthesis of 5-HT₆R ligands, reported the first selective agonist EMDT³⁵ and antagonist MS-245³⁶ (Chart 1), concurrently discovered by Merck Sharp & Dohme.³⁸ Since then, the number of patent applications and scientific publications concerning the 5-HT₆R experienced a dizzying growth, mostly in the last few years. At present, several research groups from both academia and the pharmaceutical industry have contributed to the development of structurally different 5-HT₆R agonists and antagonists^{4,19,22,26,27} (such as WAY-181187, E-6801 and SB-357134, Chart 1), that have been used as pharmacological tools to study the receptor. To our knowledge²⁶ eight of these compounds have entered clinical trials, and five of them are still under active development in Phase I or II for the treatment of cognitive dysfunction associated with Alzheimer's disease or schizophrenia, as well as for the treatment of obesity. However, no 5-HT₆R ligand has been marketed so far, and the search for new compounds acting at this receptor remains an area of high interest for medicinal chemists, aiming at either highly selective agents or compounds combining 5-HT₆R activity with other serotonin activities thought to be beneficial for the treatment of CNS disorders.

In this context, we are involved in a project aimed at the development of new serotonin 5-HT₆R ligands using a rational approach. As a starting point, using Catalyst software,³⁹ we generated a ligand-based pharmacophore model for 5-HT₆R antagonism⁴⁰ from a training set of 45 structurally diverse compounds previously described. Based on this model, here we have designed new benzimidazole derivatives containing a piperazine ring, either a carbonyl (-CO-) or a methylene (-CH₂-) group, and an aromatic ring (benzene or naphthalene) (Figure 1). We report the synthesis of new compounds **1-20** and their binding affinity for the 5-HT₆R (Table 1). The identified ligands have been characterized as antagonists in COS-7 cells

transfected with the h5-HT₆R, representing a new family of 5-HT₆R antagonists. Site-directed mutagenesis and a β_2 -adrenoceptor-based homology model of the 5-HT₆R were used to predict the mode of binding of these ligands. This combined experimental and computational study has permitted us to propose the molecular mechanisms by which the new benzimidazole derivatives act as 5-HT₆R antagonists.

Results and Discussion

Pharmacophore-based Design of New 5-HT₆R Ligands. Our previously reported 3-D pharmacophore model for 5-HT₆R antagonists showed four key pharmacophore elements: a positive ionizable atom (PI), an aromatic ring-hydrophobic site (AR), a hydrogen bond acceptor group (HBA), and a hydrophobic site (HYD).⁴⁰ Based on these structural requirements, we have designed a series of new compounds (Figure 1A) that contain a piperazine ring as PI; the AR feature is a benzimidazole system; the HBA feature is a carbonyl group ($X = CO$); and a benzene or naphthalene ring as HYD. Hence, the designed derivatives could represent a new structural class of 5-HT₆R ligands, containing a benzimidazole scaffold in the central core. In the new compounds the piperazine ring is attached to the benzimidazole system at either position 4 or position 5, providing two different orientations of the AR, HBA, and HYD moieties relative to the receptor-anchoring PI group. Figure 1B shows designed compound **3**: $Y = 4\text{-(piperazin-1-yl)}$, $X = CO$, $Ar = 1\text{-naphthyl}$ mapped onto the 3-D model. Additionally, compounds lacking the HBA feature or the HYD region were studied in order to assess the influence of these pharmacophore elements on the binding to the receptor. Therefore, we also synthesized analogues (Figure 1A) where the carbonyl group ($X = CO$) was replaced by a methylene ($X = CH_2$) with no hydrogen bond accepting capability, or where the Ar moiety was missing ($X = H$).

Synthesis. The synthesis of new benzimidazole derivatives **1-20** (Figure 1A, Table 1) was performed by using two different strategies, depending on the nature of X moiety ($X = CH_2$ or

X = CO, see Schemes 1-3). Compounds **10-20** (X = CH₂) were obtained by nucleophilic substitution of 3-chloro- or 5-chloro-2-nitroaniline with piperazine or 1-methylpiperazine, followed by catalytic hydrogenation of the resulting piperazinyl-2-nitroanilines **21-24**, and subsequent oxidative cyclization of the obtained *o*-phenylenediamines **25-28** with the appropriate readily available arylacetaldehydes (Scheme 1). On the other hand, those benzimidazole derivatives having a carbonyl group (X = CO, **1-9**) were synthesized by butyllithium-mediated acylation at the 2-position of *N*-protected derivatives followed by removal of 2-(trimethylsilyl)ethoxymethyl (SEM) or *tert*-butoxycarbonyl (BOC) groups (Schemes 2 and 3). Thus, the reduction of commercially available 3-chloro-2-nitroaniline, Phillips cyclization, and SEM protection of the obtained 4-chlorobenzimidazole (**29**)^{41,42} afforded a non-separable mixture of *N*-protected regioisomers **30**, in a 1:1 ratio (Scheme 2). Subsequent Pd(0)-catalyzed coupling⁴³ of **30** with 1-BOC- or 1-methylpiperazine yielded only 4-substituted intermediates **31,32**. Finally, derivatives **31,32** were acylated via butyllithium deprotonation, and SEM or BOC groups were subsequently removed to obtain desired compounds **1-5** (X = CO). Similarly, the cyclization of *o*-phenylenediamines **27,28**⁴⁴ followed by the protection of 5-substituted benzimidazoles **38,43** and the 2-acylation of *N*-protected derivatives **39,44** afforded final compounds **6-9** (X = CO), after deprotection of intermediates **40-42,45** (Scheme 3).

Binding Affinities and Structure-Affinity Relationships. Target compounds **1-20** were assessed for *in vitro* affinity at the h5-HT₆R by radioligand binding assays, using [³H]LSD in transfected HEK-293 cells (see Experimental Section for details). The competitive inhibition assays were first performed at a fixed dose of 10⁻⁶ M, and the complete dose-response curve, at six different concentrations of the ligand, was determined for those compounds that presented a displacement of the radioligand over 50%. The inhibition constant *K_i* was calculated from the IC₅₀ value using the Cheng-Prusoff equation,⁴⁵ and the values in Table 1 are the mean of two to four experiments. Compounds binding the 5-HT₆R were tested for

selectivity over the 5-HT₇ receptor, using [³H]LSD in transfected HEK-293 cells (see Experimental Section for details), and found to be inactive ($K_i > 500$ nM) at the 5-HT₇R. The following structure-affinity relationships can be drawn from the 5-HT₆R binding affinity data presented in Table 1. Most of the 4-substituted benzimidazole derivatives show high affinity ($K_i = 13$ -66.8 nM) whereas 5-substituted analogues are inactive ($K_i > 1000$ nM). These experimental data show that 4-substituted benzimidazoles display a favorable orientation of the AR, HBA, and HYD moieties relative to the receptor-anchoring PI group. In 4-substituted derivatives containing a carbonyl group as HBA (**1-5**: X = CO) only compounds **3** and **4**, both containing 1-naphthyl ring as HYD moiety, show 5-HT₆R affinity [$K_i(\mathbf{3}) = 13$ nM; $K_i(\mathbf{4}) = 34$ nM]. In contrast, phenyl [$K_i(\mathbf{1}) > 1000$ nM vs $K_i(\mathbf{3}) = 13$ nM, $K_i(\mathbf{2}) > 1000$ nM vs $K_i(\mathbf{4}) = 34$ nM] is insufficient for high-affinity binding, and 2-naphthyl [$K_i(\mathbf{5}) > 1000$ nM vs $K_i(\mathbf{4}) = 34$ nM] aromatic system is not tolerated at the HYD region. However, when the carbonyl group is replaced by a methylene moiety all 4-substituted compounds (**10-14**: X = CH₂) display affinity for the 5-HT₆R ($K_i = 16$ -66.8 nM). These results show that the hydrogen bonding capability proposed for HBA pharmacophore element is not essential for the binding of this new family of ligands to the 5-HT₆R. Also, comparison of the affinity data obtained for compounds **4** (X = CO, $K_i = 34$ nM) vs **13** (X = CH₂, $K_i = 36.22$ nM) and **3** (X = CO, $K_i = 13$ nM) vs **12** (X = CH₂, $K_i = 16$ nM) suggest that the aromatic ring (Ar) is accommodated at different parts of the receptor for compounds with X = CO and X = CH₂ (see *Computer-based Analysis of the Structure-affinity Relationships*). The inactivity of 4-(4-methylpiperazin-1-yl)-1*H*-benzimidazole ($K_i > 1000$ nM, data not shown), lacking the aromatic ring Ar, supports the hypothesis that the presence of HYD region is important for the receptor binding.

Functional Activity of 5-HT₆R Ligands. Compounds displaying affinity for the 5-HT₆R were evaluated for their functional profile, by determining the adenylate cyclase activity in COS-7 cells expressing the human receptor. Cells were treated with the ligand for 10 min at 37 °C, in the absence (agonist effect) or the presence (antagonist effect) of serotonin (100

μM), and the cAMP content was then quantified by homogeneous time-resolved fluorescence (HTRF®) (see Experimental Section for details). Adenylate cyclase activity is expressed as the percentage of the maximal effect obtained with serotonin alone. All assayed compounds show a dose-dependent effect in the presence of 5-HT, and no effect in the absence of 5-HT, indicating their antagonist profile. The K_i values in Table 2 are the mean of three experiments, using eight different concentrations of the tested compound. These data ($K_i = 5\text{--}31\text{ nM}$) show that 4-substituted benzimidazoles represent a new family of potent 5-HT₆R antagonists.

Defining the Mode of Binding of Ligand 4 by Site-directed Mutagenesis and Computational Modeling. Figure 2 shows the sequence alignment of transmembrane helices (TMs) 3, 5, 6, and 7 and extracellular loop (EL) 2 for β_1 - and β_2 -adrenergic receptors, selected serotonin receptors, and the 5-HT₆R. These domains form the ligand binding site in the crystal structures of β_1 -⁴⁶ and β_2 -^{47,48} adrenergic receptors, as they were previously identified by site-directed mutagenesis.⁴⁹ In order to characterize the amino acid residues of the 5-HT₆R involved in the binding of ligand **4**, mutagenesis assays were performed with Ala substitution at C3.36, S5.43, W6.48, F6.52, and N6.55 sites. Receptor expression levels at the plasma membrane relative to the expression of wild-type (WT) receptor were determined by an ELISA assay. These mutant receptors are expressed at the 80-110% range with the exception of W6.48A (30%). Figure 3A shows the antagonist activity of **4** relative to 5-HT-induced activation in the five mutant receptors and in the WT 5-HT₆R. Notably, the substitution of W6.48, F6.52, or N6.55 by Ala fully impedes compound **4** to block 5-HT-induced activation. The C3.36A mutation decreases the antagonist activity of the ligand by 7-fold [$K_i(\text{WT}) = 5\text{ nM}$ vs $K_i(\text{C3.36A}) = 34\text{ nM}$]. Surprisingly, the S5.43A mutation substantially increases the antagonist activity of **4** by 98-fold [$K_i(\text{WT}) = 5\text{ nM}$ vs $K_i(\text{S5.43A}) = 0.051\text{ nM}$].

In order to understand these observed effects at the molecular level, we constructed a computer 3-D model of the complex between compound **4** and a β_2 -adrenoceptor-based model of the 5-HT₆R (see Experimental Section). In this model (Figure 3B) D3.32 anchors

the protonated piperazine ring; the benzimidazole ring expands toward TM 6, parallel to EL 2, to hydrogen bond O₆ of the key N6.55; the coplanar carbonyl group also hydrogen bonds the N₆-H moiety of N6.55; and the aromatic naphthalene ring enters deeply into a small cavity between TMs 3 and 6, to form edge-to-face interactions with the key W6.48 and F6.52 side chains in TM 6. This computational model explains the experimentally determined importance of W6.48, F6.52 and N6.55 of the 5-HT₆R in the binding of ligand **4** (Figure 3A). The finding that the S5.43A mutation enhances the activity of **4** (Figure 3A) is attributed to the fact that S5.43 hydrogen bonds N6.55 (Figure 3B); thus, the mutation at this site increases the flexibility of N6.55 favoring its interaction with **4**. Other significant interactions between compound **4** and the 5-HT₆R, as revealed by the computational model, are: the interaction between the highly polar methyl group attached to the protonated piperazine ring and W3.28; the interaction of the aromatic naphthalene ring with V3.33 in TM 3; and the interaction between the aromatic benzimidazole ring and F6.51 in TM 6, F7.35 in TM 7, and L182 in EL 2 (Figures 3B and 3C).

Computer-based Analysis of the Structure-affinity Relationships. In an attempt to get a better understanding of the above described structure-affinity relationships, we have performed additional computational simulations of the complexes between 4-substituted benzimidazole derivatives and the 5-HT₆R. Regarding HBA, replacement of the sp² carbonyl group (X = CO) by the sp³ methylene unit (X = CH₂) allows the benzimidazole ring and the aromatic systems (Ar = phenyl, 1-naphthyl, 2-naphthyl) in the HYD region to form an angle of approximately 90°. Thus, in compounds with X = CH₂ the HYD moiety is located between TMs 3 and 5, to interact with the hydrophobic side chains of V3.33 and A5.42 (Figure 4A). In contrast, the Ar aromatic moiety in compounds containing X = CO is located between TMs 3 and 6, interacting with V3.33, W6.48 and F6.52 side chains (Figure 4B). The different location of the HYD feature in the receptor explains the observed different structure-affinity relationships at this pharmacophore element in both types of compounds (X = CH₂ or X =

CO). Clearly, all tested aromatic rings phenyl, 1-naphthyl, and 2-naphthyl are permitted in the cavity between TMs 3 and 5 ($X = \text{CH}_2$, Figure 4A), whereas phenyl and 1-naphthyl are permitted in the cavity between TMs 3 and 6 ($X = \text{CO}$, Figure 4B). In the cavity between TMs 3 and 6 the smaller phenyl ring, relative to 1-naphthyl, does not optimally interact with F6.52 (Figure 4B), resulting in the corresponding loss of affinity [$K_i(\mathbf{1}) > 1000 \text{ nM}$, $K_i(\mathbf{2}) > 1000 \text{ nM}$]. The presence of the 2-naphthyl system is not permitted because it clashes with TM 3 (see orange ring in Figure 4B), leading to an inactive analogue [$K_i(\mathbf{5}) > 1000 \text{ nM}$].

Comparison of the binding of SB-258585 with the binding of benzimidazole derivatives 4 and 13 to the 5-HT₆R. SB-258585³³ (Chart 1) is a commercially available potent 5-HT₆R antagonist that contains the common piperazine ring as the PI pharmacophore element, a methoxyphenyl system as AR, a sulfonamide group as HBA, and a iodophenyl ring as HYD.⁴⁰ In order to compare the binding mode of this 5-HT₆R antagonist with the binding mode of benzimidazole derivatives we evaluated the antagonist activity of SB-258585 in the C3.36A, S5.43A, W6.48A, F6.52A, and N6.55A mutant receptors (Figure 5A). Clearly, with the only exception of W6.48A, all these mutations decrease the antagonist activity of SB-258585: 72-fold, relative to WT, in the C3.36A mutant receptor; 8-fold in S5.43A; 152-fold in F6.52A; and 36-fold in N6.55A (Figure 5A). These mutational data are compatible with a mode of binding of SB-258585 to the 5-HT₆R as depicted in Figure 5B: D3.32 anchors the protonated piperazine ring, the methoxyphenyl system interacts with F6.52, the methoxy group hydrogen bonds C3.36, the sulfonamide group hydrogen bonds both S5.43 and N6.55, and the substituted benzene interacts with the hydrophobic side chains of V3.33 and A5.42. Previously reported rhodopsin-based models have suggested that arylsulfonyltryptamines bind between TMs 1, 6 and 7;⁵⁰ the sulfonyl oxygens of MS-245 (Chart 1) bind S3.37 and T5.46;⁵¹ or N6.55 in SB-357134 (Chart 1) and related sulfonamide derivatives.^{31,40}

Figure 5C shows SB-258585 (white) and benzimidazole derivatives **4** (green) and **13** (orange) bound to the 5-HT₆R for comparison purposes. With respect to the HBA feature, the

large sulfonamide moiety of SB-258585 is capable to hydrogen bond both S5.43 and N6.55 while the carbonyl group of compound **4** hydrogen bonds exclusively N6.55, which also binds the N-H of the benzimidazole ring (AR feature) of compounds **4** and **13**. Clearly, the piperazine-benzimidazole rings of **4** and **13** are placed near the extracellular side, parallel to EL 2, while the piperazine-methoxyphenyl rings of SB-258585 are located deeper in the TM bundle. Notably, the methoxyphenyl system of SB-258585 (AR feature) is placed at similar position than the naphthalene ring (HYD feature) of compound **4**. On the other hand, the position of the HYD feature of SB-258585 (iodophenyl ring) is similar to the HYD feature of compound **13** (naphthalene ring). Thus, the different location of the AR features in the binding site suggests that compounds **4** and **13** elicit their antagonist function by a different mechanism than ligand SB-258585 (see below).

Molecular Mechanisms of Receptor Inactivation. The understanding of the molecular mechanisms by which these benzimidazole derivatives act as 5-HT₆R antagonists, and GPCR function in general, is a key subject in medicinal chemistry. The recent knowledge of the molecular steps of agonist-induced GPCR activation (see Smit *et al.*⁵² and Nygaard *et al.*⁵³ for recent reviews) facilitates this task. It has been proposed, from engineered GPCRs with metal ion binding sites, that TM 6 performs an inward movement of the extracellular part toward TM 3.⁵⁴ Thus, in the new family of 5-HT₆R antagonists the role of the benzimidazole ring may be to maintain, by forming a hydrogen bond interaction with N6.55, TM 6 away from the center of the bundle (Figure 6A). In addition, L182 in EL 2 forms favorable C-H $\cdots\pi$ interactions⁵⁵ with the benzimidazole ring, which may serve to immobilize both EL 2 and TM 6 in the inactive conformation (Figure 6A). Similar interactions are observed between the antagonist cyanopindolol and the partial inverse agonist carazolol with the homologous F201⁴⁶ and F193⁴⁸ of the β_1 - and β_2 -adrenergic receptors, respectively. Fluorescence spectroscopy in the β_2 -adrenergic receptor provides evidence that agonist binding induces or stabilizes relocation of the extracellular side of TM 5, to facilitate binding of the catechol

hydroxyls of the adrenergic agonists.⁵⁶ The Ar system in CH₂-containing compounds is located between TMs 3 and 5, interacting with V3.33 and A5.42, and probably blocking these helices in the inactive conformation (Figure 6B). In addition to these activation pathways, agonist binding might also trigger the rotamer toggle switch of W6.48 from pointing toward TM 7 to pointing toward the binding site crevice,^{49,57,58} through the formation of specific hydrogen bonds.⁵⁹ The 1-naphthyl ring in CO-containing compounds occupies a small cavity between TMs 3 and 6 (Figure 6C), and might act by blocking the proposed conformational transition of W6.48 and the subsequent steps in the activation process.⁵² We have also shown that the conformational transition of W6.48 is accompanied, in a concerted manner, by the conformational transition of the side chain at position 3.36 from pointing toward TM 6 to pointing toward TM 7.⁶⁰ Thus, the methoxy group of SB-258585 might block receptor activation by hydrogen bonding C3.36 in the inactive conformation (Figure 6D). Accordingly, the C3.36A mutation has a significant effect in the antagonist activity of SB-258585 (the effect for compound **4** is less significant), while the W6.48A mutation impairs the antagonist activity of compound **4** (the effect for SB-258585 is minor).

Conclusions

Based on our previously described pharmacophore model for 5-HT₆R antagonists,⁴⁰ we have identified benzimidazole derivatives as a new structural class of serotonin 5-HT₆R ligands. Computational modeling and site-directed mutagenesis have allowed us to characterize the amino acid residues of 5-HT₆R involved in ligand binding (Figure 3). The distinctive positively charged piperazine ring anchors the ligand to D3.32 in TM 3, whereas the benzimidazole ring is situated parallel to EL 2, to hydrogen bond N6.55 in TM 6 (Figures 3 and 4). An important feature of these molecules is the linker between the benzimidazole ring and the terminal Ar system (phenyl, 1-naphthyl, 2-naphthyl). The sp² carbonyl group (-CO-) enforces both aromatic domains to be coplanar, whereas the sp³ methylene unit (-CH₂-)

allows the rings to form an angle of approximately 90°. Thus, in CH₂-containing compounds the Ar system is located between TMs 3 and 5, to interact with V3.33 and A5.42 (Figure 4A), whereas in CO-containing compounds the Ar aromatic moiety is located between TMs 3 and 6, interacting with V3.33, W6.48 and F6.52 (Figure 4B). Both types of compounds display high binding affinity for the 5-HT₆R as shown for ligands **12** [$K_i(\mathbf{12}) = 16$ nM] and **13** [$K_i(\mathbf{13}) = 36$ nM], having CH₂ as a linker; and ligands **3** [$K_i(\mathbf{3}) = 13$ nM] and **4** [$K_i(\mathbf{4}) = 34$ nM] containing CO (Table 1). The new compounds were pharmacologically characterized as potent 5-HT₆R antagonists ($K_i = 5$ -31 nM).

This combined experimental and computational study has permitted to understand how ligand binding blocks receptor activation (Figure 6), which may contribute to the rational design of new compounds acting at the serotonin 5-HT₆R. As some of the residues involved in ligand binding and receptor inactivation are strongly conserved, these findings can likely be extended to other members of the rhodopsin-like family of GPCRs.

Experimental Section

Chemistry. Melting points (uncorrected) were determined on a Stuart Scientific electrothermal apparatus. Infrared (IR) spectra were measured on a Shimadzu-8300 or Bruker Tensor 27 instrument equipped with a Specac ATR accessory of 5200-650 cm⁻¹ transmission range; frequencies (ν) are expressed in cm⁻¹. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Avance 500 (¹H, 500 MHz; ¹³C, 125 MHz), Bruker Avance 300-AM (¹H, 300 MHz; ¹³C, 75 MHz) or Bruker 200-AC spectrometer (¹H, 200 MHz; ¹³C, 50 MHz) at the UCM's NMR facilities. 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), app (apparent). Mass spectrometry (MS) was carried out on a Bruker LC-Esquire in electrospray mode (ESI) or a HP 5989 A in electron

impact mode (EI, 70 eV). Elemental analyses (C, H, N) were obtained on a LECO CHNS-932 apparatus at the UCM's analysis services and were within 0.5% of the theoretical values, confirming a purity of at least 95% for all tested compounds. Analytical thin-layer chromatography (TLC) was run on Merck silica gel plates (Kieselgel 60 F-254) with detection by UV light (254 nm), ninhydrin solution, or 10% phosphomolybdic acid solution in ethanol. Flash chromatography was performed on glass column using silica gel type 60 (Merck, particle 230-400 mesh) or on a Supelco VersaFlash station using silica gel cartridges (Supelco, particle size 20-45 μ m). Unless stated otherwise, starting materials, reagents and solvents were purchased as high-grade commercial products from Sigma-Aldrich, Lancaster, Scharlab or Panreac, and were used without further purification. THF was distilled from sodium benzophenone ketyl and used immediately. Dichloromethane was distilled from calcium hydride.

The following compounds were synthesized according to described procedures: **23**,⁶¹ **24**,⁴⁴ **28**,⁶² and **29**,⁴² and their spectroscopic data are in agreement with those previously described.

Spectroscopic data of all described compounds were consistent with the proposed structures. For series **1-8**, **10-20** we include the data of compounds **5**, **6**, **8**, **11**, **12**, **15**, and **19**. For intermediates **25-28**, **33-37**, **40-42** the data of compounds **26**, **35** and **42** are described.

General Procedure for the Synthesis of 2-Nitro- ω -piperazin-1-ylanilines (21-24**).** To a solution of 3- or 5-chloro-2-nitroaniline (5.12 g, 29.5 mmol) in anhydrous DMF (70 mL), piperazine or 1-methylpiperazine (78 mmol), Cs₂CO₃ or K₂CO₃ (78 mmol) were added, respectively. The reaction was heated at 120 °C for 24 h. Upon cooling, the mixture was poured into ice-cold water and the solid was filtered, washed with cold water and dried under vacuum. The solid was purified by column chromatography using the appropriate eluent, to afford pure **21-24**.

2-Nitro-3-piperazin-1-ylaniline (21). Obtained from 3-chloro-2-nitroaniline and piperazine, in 40% yield. Chromatography: CH₂Cl₂/EtOH, 8:2; mp 212-213 °C. IR (KBr):

3308, 1514, 1443. ^1H NMR (CDCl_3 , δ): 1.90 (br s, 1H), 3.01 (m, 8H), 4.95 (br s, 2H), 6.57 (d, $J = 8.1$, 2H), 7.14 (t, $J = 8.1$, 1H). ^{13}C NMR (CDCl_3 , δ): 46.2, 53.0, 109.3, 111.2, 122.0, 132.9, 142.9, 148.0. MS (ESI): 223.0 (M+H).

3-(4-Methylpiperazin-1-yl)-2-nitroaniline (22). Obtained from 3-chloro-2-nitroaniline and 1-methylpiperazine, in quantitative yield. Chromatography: $\text{CH}_2\text{Cl}_2/\text{EtOH}$, 9:1. IR (CHCl_3): 3383, 1605, 1573, 1511, 1459. ^1H NMR (CDCl_3) δ 2.32 (s, 3H), 2.53 (br t, $J = 4.7$, 4H), 3.01 (br t, $J = 4.7$, 4H), 4.80 (br s, 2H), 6.38 (d, $J = 8.1$, 2H), 7.11 (t, $J = 8.1$, 1H). ^{13}C NMR (CDCl_3) δ 46.1, 51.7, 55.1, 109.1, 111.2, 121.3, 132.9, 142.6, 147.6. MS (ESI): 258.9 (M+Na).

2-Nitro-5-piperazin-1-ylaniline (23). Obtained from 5-chloro-2-nitroaniline and piperazine, in quantitative yield. Chromatography: $\text{CH}_2\text{Cl}_2/\text{EtOH}$, 9:1; mp 164-165 °C (lit.⁶¹ 170 °C).

5-(4-Methylpiperazin-1-yl)-2-nitroaniline (24). Obtained from 5-chloro-2-nitroaniline and 1-methylpiperazine, in 89% yield. Chromatography: $\text{CH}_2\text{Cl}_2/\text{EtOH}$, 98:2; mp 153-154 °C (lit.⁴⁴ 156-157 °C).

General Procedure for the Synthesis of ω -Piperazin-1-ylbenzene-1,2-diamines (25-28). To a suspension of **21-24** (1.3 mmol) in absolute MeOH (10 mL), 10% Pd(C) (25 mg) was added and the reaction was hydrogenated at room temperature for 24 h, with an initial hydrogen pressure of 50 p.s.i. The mixture was filtered over celite and the solvent was evaporated to dryness to give **25-28**, which were used in the next step without further purification.

3-Piperazin-1-ylbenzene-1,2-diamine (25). Obtained from **21** in quantitative yield.

3-(4-Methylpiperazin-1-yl)benzene-1,2-diamine (26). Obtained from **22** in quantitative yield. IR (CHCl_3): 3325, 1600, 1478, 1455. ^1H NMR (CDCl_3) δ 2.31 (s, 3H), 2.60 (m, 4H), 2.94 (br t, $J = 4.7$, 4H), 6.54 (dd, $J = 6.9$, 1.4, 1H), 6.63-6.72 (m, 2H). ^{13}C NMR (CDCl_3) δ

46.1, 51.3, 55.9, 111.6, 112.7, 118.9, 130.1, 134.4, 140.2. MS (ESI) 207.0 (M+H), 228.9 (M+Na).

4-Piperazin-1-ylbenzene-1,2-diamine (27). Obtained from **23** in quantitative yield.

4-(4-Methylpiperazin-1-yl)benzene-1,2-diamine (28). Obtained from **24** in 89% yield; mp 101-103 °C (lit.⁶² 101-102 °C).

General Procedure for the Synthesis of Compounds 29, 38, 43. To a suspension of 3-chlorobenzene-1,2-diamine,⁴¹ **27** or **28** (9.8 mmol) in water (14 mL), formic acid (1.2 mL, 29 mmol) was added and the mixture was heated at 100 °C for 3 h. After the complete disappearance of the starting material (tlc), the reaction was poured on ice and 1 M KOH was added until pH 9-10. The resulting solid was filtrated, dried and recrystallized; otherwise, the aqueous solution was extracted with CH₂Cl₂ (3 x 50 mL), the organic layers were dried (Na₂SO₄), and evaporated to dryness. The residue was purified by column chromatography using the appropriate eluent, to afford pure benzimidazoles **29, 38, 43**.

4-Chloro-1H-benzimidazole (29). Obtained from 3-chlorobenzene-1,2-diamine in quantitative yield; mp 167-169 °C (lit.⁴² 170-171 °C) (EtOAc).

5-(4-Methylpiperazin-1-yl)-1H-benzimidazole (38). Obtained from **28** in 60% yield. Chromatography: CHCl₃/MeOH, 95:5; mp 81-83 °C (lit.⁴³ 82-84 °C). Spectroscopic data are in agreement with those previously described.

5-Piperazin-1-yl-1H-benzimidazole (43). Obtained from **27** in 87% yield. Chromatography CHCl₃/MeOH/NH₃ 6:4:0.1; mp 123-125 °C (lit.⁴³ 126-128 °C). Spectroscopic data are in agreement with those previously described.

General Procedure for the Synthesis of Compounds 30 and 39. To a suspension of K₂CO₃ (2.86 g, 20.7 mmol) or NaH (444 mg, 11.1 mmol, 60% mineral oil) in anhydrous DMF (10 mL), **29** or **38** (6.9 mmol) was added portionwise, respectively, under an argon atmosphere. The mixture was stirred at room temperature for 1 h, then 2-(trimethylsilyl)ethoxymethyl chloride (SEMCl) (2.1 mL, 11.1 mmol) was added dropwise,

and the reaction was stirred for 2 h. The reaction was quenched with water and extracted with CH₂Cl₂ (**30**) or EtOAc (**39**). The organic layers were dried (Na₂SO₄) and evaporated under vacuum, and the residue was purified by column chromatography, using the appropriate eluent, to provide **30** or **39**.

4- and 7-Chloro-1-[(2-(trimethylsilyl)ethoxy)methyl]-1H-benzimidazoles (30). Obtained from **29** in quantitative yield, as a non-separable mixture of regioisomers in a 1:1 ratio. Chromatography: hexane/EtOAc, 7:3. IR (CHCl₃) 1613, 1576, 1499, 1428. ¹H NMR (CDCl₃) δ 0.03 (s, 9H), 0.93, 0.99 (br t, *J* = 4.5, 2H), 3.55, 3.62 (app t, *J* = 8.0, 2H), 5.59, 5.88 (s, 2H), 7.20-7.36 (m, 2H), 7.46, 7.72 (dd, *J* = 5.4, 1.0, 1H), 7.99, 8.03 (s, 1H). ¹³C NMR (CDCl₃) δ -1.4, -1.3, 17.8, 17.9, 66.3, 66.8, 74.6, 75.1, 109.2, 116.8, 119.4, 122.8, 123.4, 124.2, 125.0, 125.2, 130.1, 133.4, 143.4, 145.1, 146.3. MS (ESI) 283.0 (M+H).

6-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-benzimidazole (39). Obtained from **38** in 44% yield, after the separation from its 5-substituted regioisomer* (38% yield) by column chromatography (CH₂Cl₂/EtOH, 9:1). IR (CHCl₃) 1625, 1581, 1502, 1490, 1454. ¹H NMR (CDCl₃) δ -0.03 (s, 9H), 0.91 (app t, *J* = 8.2, 2H), 2.39 (s, 3H), 2.64 (br t, *J* = 4.8, 4H), 3.26 (br t, *J* = 4.8, 4H), 3.51 (app t, *J* = 8.2, 2H), 5.49 (s, 2H), 7.00 (d, *J* = 2.1, 1H), 7.05 (dd, *J* = 9.0, 2.3, 1H), 7.69 (d, *J* = 8.8, 1H), 7.84 (s, 1H). ¹³C NMR (CDCl₃) δ -1.5, 17.6, 46.0, 50.5, 55.1, 66.2, 74.0, 96.9, 114.5, 120.4, 134.9, 137.9, 141.9, 149.5. MS (EI) *m/z* (%) 346 (M, 100), 331 (21), 229 (21), 199 (9), 73 (46), 43 (40).

*Spectroscopic data of 5-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-benzimidazole: IR (CHCl₃) 1624, 1586, 1491, 1454. ¹H NMR (CDCl₃) δ -0.05 (s, 9H), 0.90 (app t, *J* = 8.2, 2H), 2.39 (s, 3H), 2.65 (br t, *J* = 4.9, 4H), 3.24 (br t, *J* = 4.9, 4H), 3.50 (t, *J* = 8.2, 2H), 5.49 (s, 2H), 7.10 (dd, *J* = 8.8, 2.4, 1H), 7.34 (d, *J* = 2.1, 1H), 7.43 (d, *J* = 9.0, 1H), 7.90 (s, 1H). ¹³C NMR (CDCl₃) δ -1.6, 17.6, 46.0, 50.9, 55.2, 66.2, 74.2, 107.2, 110.2,

116.0, 128.3, 142.9, 144.9, 148.3. MS (EI) m/z (%) 346 (M, 100), 331 (21), 229 (21), 199 (9), 73 (46), 43 (40).

General Procedure for the Synthesis of Compounds 31 and 32. To a solution of **30** (537 mg, 1.9 mmol) in *o*-xylene (17 mL) under an argon atmosphere, 1-BOC- or 1-methylpiperazine (2.7 mmol), NaOt-Bu (250 mg, 2.7 mmol), tris(dibenzylideneacetone)dipalladium(0) (190 mg, 0.19 mmol) and tri-*t*-butylphosphine (150 mg, 0.8 mmol) were added. The mixture was heated at 110 °C for 24 h. Upon cooling to room temperature, the reaction was filtered over celite, the solvent was removed under reduced pressure, and the residue was purified by column chromatography using the appropriate eluent, to afford pure **31** or **32**.

***Tert*-butyl 4-(1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-4-yl)piperazine-1-carboxylate (31).** Obtained from **30** and 1-BOC-piperazine in 49% yield. Chromatography: hexane/EtOAc, 8:2. IR (CHCl₃) 1697, 1603, 1543, 1477, 1450. ¹H NMR (CDCl₃) δ -0.01 (s, 9H), 1.08 (app t, *J* = 8.6, 2H), 1.46 (s, 9H), 2.72 (br t, *J* = 4.8, 4H), 3.40-3.50 (m, 4H), 3.73 (app t, *J* = 8.3, 2H), 5.70 (s, 2H), 6.77 (dd, *J* = 8.0, 1.2, 1H), 7.26 (t, *J* = 8.0, 1H), 7.99 (dd, *J* = 8.4, 1.1, 1H), 8.04 (s, 1H). ¹³C NMR (CDCl₃, δ): -1.7, 18.1, 28.2, 51.8, 67.5, 75.3, 79.7, 114.7, 116.0, 125.0, 129.5, 138.0, 144.3, 149.9, 154.5.

4-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazole (32). Obtained from **30** and 1-methylpiperazine in 43% yield. Chromatography: CH₂Cl₂/EtOH, 98:2. IR (CHCl₃) 1499, 1453. ¹H NMR (CDCl₃) δ -0.04 (s, 9H), 0.90 (app t, *J* = 8.4, 2H), 2.44 (s, 3H), 2.73 (br t, *J* = 4.8, 4H), 3.50 (app t, *J* = 8.1, 2H), 3.61 (m, 4H), 5.52 (s, 2H), 6.69 (d, *J* = 8.3, 1H), 7.12 (d, *J* = 8.3, 1H), 7.24 (t, *J* = 8.1, 1H), 7.90 (s, 1H). ¹³C NMR (CDCl₃, δ): -1.5, 17.8, 46.0, 52.8, 55.5, 65.9, 74.8, 115.3, 116.7, 122.8, 128.1, 139.9, 144.3, 145.4. MS (ESI) 347.0 (M+H).

Synthesis of *Tert*-butyl 5- and 6-[4-*tert*-butoxycarbonyl]piperazin-1-yl]-1*H*-benzimidazole-1-carboxylates (44). To a solution of **43** (200 mg, 1 mmol) in anhydrous

CH₂Cl₂ (6 mL), di-*tert*-butyl dicarbonate (648 mg, 3 mmol), triethylamine (0.4 mL, 3 mmol) and 4-(dimethylamino)pyridine (37 mg, 0.3 mmol) were added, and the mixture was stirred at room temperature overnight. The reaction solution was washed with 1 M NaOH and the organic layer was dried (Na₂SO₄) and evaporated at reduced pressure. The resulting crude was purified by column chromatography to give **44** in 69% yield, as a non-separable mixture of regioisomers in a 1:1 ratio. IR (KBr) 1755, 1740, 1694, 1624, 1487, 1451. ¹H NMR (CDCl₃) δ 1.50, 1.70 (s, 18H), 3.14-3.21, 3.62-3.66 (m, 8H), 7.05, 7.12 (dd, *J* = 8.9, 2.3, 1H), 7.31, 7.59 (br s, 1H), 7.66, 7.87 (d, *J* = 10.3, 1H), 8.30, 8.39 (s, 1H). ¹³C NMR (CDCl₃) δ 28.3, 28.6, 50.7, 51.1, 80.0, 85.4, 102.2, 108.2, 114.7, 115.9, 117.3, 120.9, 132.8, 133.0, 138.4, 140.2, 142.4, 147.6, 149.1, 149.7, 154.7. MS (EI) *m/z* (%) 402 (M, 18), 346 (44), 302 (24), 290 (100), 246 (50), 216 (23), 57 (45), 41 (37).

General Procedure for the Synthesis of Compounds 33-37, 40-42, and 45. To a solution of SEM-protected or BOC-protected benzimidazole **31**, **32**, **39** or **44** (1.3 mmol) in anhydrous THF (5.2 mL), BuLi (1.8 mmol, 1.6 M in hexane) was added under an argon atmosphere at -78 °C. The mixture was stirred at this temperature for 1 h, then corresponding alkyl benzoate or naphthoate (1.4 mmol) was added slowly and the reaction mixture was allowed to reach room temperature and stirred overnight. The reaction was quenched with a saturated aqueous solution of NH₄Cl, then extracted with CH₂Cl₂, and the organic layers were washed with water and brine, dried (Na₂SO₄), and evaporated to dryness. The residue was purified by column chromatography using the appropriate eluent, to afford pure **33-37**, **40-42**, **45**.

***tert*-Butyl 4-(2-benzoyl-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-4-yl)piperazine-1-carboxylate (33).** Obtained from **31** and ethyl benzoate, in 21% yield. Chromatography: hexane/EtOAc, 8:2.

[4-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-2-yl](phenyl)methanone (34). Obtained from **32** and ethyl benzoate, in 47% yield. Chromatography: CH₂Cl₂/EtOH, 95:5.

tert-Butyl 4-[2-(1-naphthoyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-4-yl]piperazine-1-carboxylate (35). Obtained from **31** and ethyl 1-naphthoate, in 27% yield. Chromatography: hexane/EtOAc, 98:2. ¹H NMR (CDCl₃) δ -0.05 (s, 9H), 0.95 (app t, *J* = 8.3, 2H), 1.50 (s, 9H), 3.50 (m, 8H), 3.69 (app t, *J* = 8.3, 2H), 6.17 (s, 1H), 6.65 (d, *J* = 8.0, 1H), 7.21 (d, *J* = 8.4, 1H), 7.36 (t, *J* = 8.0, 1H), 7.51-7.58 (m, 3H), 7.93-7.96 (m, 1H), 8.10 (t, *J* = 8.9, 2H), 8.42-8.45 (m, 1H). ¹³C NMR (CDCl₃) δ -1.3, 18.0, 28.5, 49.7, 66.5, 74.0, 79.8, 103.9, 108.3, 124.1, 125.7, 126.3, 127.6, 128.6, 131.5, 131.8, 132.9, 133.8, 134.3, 135.0, 138.1, 144.8, 144.9, 154.9, 188.7.

[4-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-2-yl](1-naphthyl)methanone (36). Obtained from **32** and ethyl 1-naphthoate, in 55% yield. Chromatography: CH₂Cl₂/EtOH, 98:2.

[4-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-2-yl](2-naphthyl)methanone (37). Obtained from **32** and methyl 2-naphthoate, in 59% yield. Chromatography CH₂Cl₂/EtOH, 98:2.

[6-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-2-yl](phenyl)methanone (40). Obtained from **39b** and ethyl benzoate, in 63% yield. Chromatography: CH₂Cl₂/EtOH, 95:5.

[6-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-2-yl](1-naphthyl)methanone (41). Obtained from **39b** and ethyl 1-naphthoate, in 57% yield. Chromatography: CH₂Cl₂/EtOH, 95:5.

[6-(4-Methylpiperazin-1-yl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1*H*-benzimidazol-2-yl](2-naphthyl)methanone (42). Obtained from **39b** and methyl 2-naphthoate, in 56% yield. Chromatography: CH₂Cl₂/EtOH, 97:3. ¹H NMR (CDCl₃) δ -0.11 (s, 9H), 1.02 (app t, *J* = 8.2, 2H), 2.52 (s, 3H), 2.80 (br t, *J* = 4.9, 4H), 3.48 (br t, *J* = 4.9, 4H), 3.76 (app t, *J* = 8.2, 2H), 6.16 (s, 2H), 7.13 (d, *J* = 2.0, 1H), 7.26 (dd, *J* = 9.1, 2.2, 1H), 7.60-7.75 (m, 2H), 7.92 (d, *J* = 9.0, 1H), 7.98-8.13 (m, 3H), 8.38 (dd, *J* = 8.6, 1.7, 1H), 9.03 (s, 1H). ¹³C NMR (CDCl₃) δ -

1.6, 17.7, 45.8, 49.5, 54.8, 66.2, 73.7, 96.3, 116.3, 122.2, 125.7, 126.4, 127.6, 127.9, 128.5, 129.9, 132.3, 133.7, 134.5, 135.6, 136.2, 137.3, 145.9, 150.7, 185.6.

***tert*-Butyl 4-[2-(2-naphthoyl)-1*H*-benzimidazol-5-yl]piperazine-1-carboxylate (45).**

Obtained from **44** and ethyl 2-naphthoate, in 33% yield. Chromatography: hexane/EtOAc, 9:1. IR (CHCl₃) 3453, 3288, 1693, 1632, 1516, 1428. ¹H NMR (CDCl₃) δ 1.52 (s, 9H), 3.24 (br t, *J* = 5.1, 4H), 3.64-3.67 (m, 4H), 6.95 (d, *J* = 2.1, 1H), 7.14 (dd, *J* = 9.1, 2.2, 1H), 7.56-7.68 (m, 2H), 7.87 (d, *J* = 9.3, 1H), 7.88 (d, *J* = 9.3, 1H), 7.98 (d, *J* = 8.7, 1H), 8.12 (d, *J* = 7.9, 1H), 8.53 (dd, *J* = 8.7, 1.7, 1H), 9.51 (s, 1H), 10.36 (br s, 1H). ¹³C NMR (CDCl₃) δ 28.3, 49.9, 50.4, 50.6, 79.9, 96.8, 116.6, 122.5, 125.6, 126.4, 127.6, 128.0, 128.6, 130.1, 132.4, 132.8, 134.0, 134.4, 135.7, 138.7, 147.3, 150.9, 154.6, 182.9. MS (EI) *m/z* (%) 456 (M, 36), 400 (77), 356 (33), 314 (100), 155 (29), 127 (36), 56 (32), 41 (25).

General Procedure for the Synthesis of Benzimidazole Derivatives 1-8 (X = CO). To a solution of **33-37**, **40-42** (0.34 mmol) in freshly distilled CH₂Cl₂ (3 mL), BCl₃ (1.8 mmol, 1 M in CH₂Cl₂) was added dropwise and under an argon atmosphere. The reaction was stirred at room temperature for 16 h, then quenched with saturated NaHCO₃. The reaction mixture was extracted with EtOAc, and the organic layers were dried (Na₂SO₄) and evaporated at reduced pressure. The residue was purified by column chromatography using the appropriate eluent, to afford pure **1-8**.

Phenyl(4-piperazin-1-yl-1*H*-benzimidazol-2-yl)methanone (1). Obtained from **33** in 58% yield. Chromatography: CH₂Cl₂/MeOH, 7:3; mp 123-125 °C.

[4-(4-Methylpiperazin-1-yl)-1*H*-benzimidazol-2-yl](phenyl)methanone (2). Obtained from **34** in 53% yield. Chromatography: CH₂Cl₂/EtOH, 98:2; mp 151-154 °C.

1-Naphthyl(4-piperazin-1-yl-1*H*-benzimidazol-2-yl)methanone (3). Obtained from **35** in 64% yield. Chromatography: CH₂Cl₂/MeOH, 7:3; mp 210-213 °C.

[4-(4-Methylpiperazin-1-yl)-1*H*-benzimidazol-2-yl](1-naphthyl)methanone (4). Obtained from **36** in 58% yield. Chromatography: CH₂Cl₂/EtOH, 9:1; mp 213-214 °C.

[4-(4-Methylpiperazin-1-yl)-1*H*-benzimidazol-2-yl](2-naphthyl)methanone (5).

Obtained from **37** in 50% yield. Chromatography CH₂Cl₂/EtOH, 98:2; mp 210-211 °C. IR (KBr) 3442, 3301, 1635, 1525, 1486, 1440. ¹H NMR (CDCl₃) δ 2.47 (s, 3H), 2.83 (br t, *J* = 4.8, 4H), 3.81 (br t, *J* = 5.0, 4H), 6.67 (d, *J* = 7.7, 1H), 7.11 (d, *J* = 7.7, 1H), 7.32 (t, *J* = 8.0, 1H), 7.60-7.68 (m, 2H), 7.89-8.03 (m, 3H), 8.54 (dd, *J* = 8.7, 1.6, 1H), 9.78 (s, 1H), 10.50 (br s, 1H). ¹³C NMR (CDCl₃) δ 46.4, 49.9, 55.4, 104.4, 108.2, 126.1, 127.2, 127.9, 128.2, 128.4, 129.3, 130.5, 132.9, 133.1, 135.2, 135.5, 136.3, 136.6, 145.4, 145.9, 186.8. MS (ESI) 371.1 (M+H). Anal. (C₂₃H₂₂N₄O) C, H, N.

[5-(4-Methylpiperazin-1-yl)-1*H*-benzimidazol-2-yl](phenyl)methanone (6). Obtained from **40** in 75% yield. Chromatography: CHCl₃/MeOH/NH₃, 9:1:0.1; mp 210-212 °C. IR (KBr) 3420, 3304, 1624, 1597, 1574, 1516, 1485, 1452. ¹H NMR (CDCl₃) δ 2.40 (s, 3H), 2.65 (br t, *J* = 4.9, 4H), 3.31 (br t, *J* = 4.9, 4H), 6.91 (d, *J* = 2.1, 1H), 7.12 (dd, *J* = 9.1, 2.2, 1H), 7.53-7.58 (m, 2H), 7.62-7.67 (m, 1H), 7.81 (d, *J* = 9.1, 1H), 8.66 (dd, *J* = 7.0, 1.6, 2H), 10.60 (br s, 1H). ¹³C NMR (CDCl₃) δ 46.5, 50.1, 55.4, 96.8, 116.8, 122.9, 128.8, 131.5, 133.8, 135.2, 136.2, 138.9, 147.5, 150.5, 183.9. MS (ESI) 321.0 (M+H). Anal. (C₁₉H₂₀N₄O) C, H, N.

[5-(4-Methylpiperazin-1-yl)-1*H*-benzimidazol-2-yl](1-naphthyl)methanone (7).

Obtained from **41** in 77% yield. Chromatography: CH₂Cl₂/EtOH, 9:1; mp 142-144 °C.

[5-(4-Methylpiperazin-1-yl)-1*H*-benzimidazol-2-yl](2-naphthyl)methanone (8).

Obtained from **42** in 65% yield. Chromatography: CH₂Cl₂/EtOH, 97:3; mp 231-233 °C. IR (KBr) 3444, 3299, 1633, 1616, 1570, 1517, 1493, 1446. ¹H NMR (CDCl₃) δ 2.39 (s, 3H), 2.63 (br t, *J* = 4.8, 4H), 3.31 (br t, *J* = 4.8, 4H), 6.93 (d, *J* = 2.0, 1H), 7.13 (dd, *J* = 9.3, 2.0, 1H), 7.50-7.68 (m, 2H), 7.84 (d, *J* = 9.3, 1H), 7.90-7.98 (m, 2H), 8.10 (d, *J* = 7.8, 1H), 8.52 (dd, *J* = 8.6, 1.7, 1H), 9.48 (s, 1H), 10.23 (br s, 1H). ¹³C NMR (CDCl₃) δ 45.6, 48.8, 54.6, 96.0,

115.9, 121.6, 125.8, 126.9, 127.7, 127.9, 128.8, 129.9, 131.9, 132.9, 133.3, 135.0, 135.6, 137.6, 147.2, 150.3, 182.5. MS (ESI) 371.0 (M+H). Anal. (C₂₃H₂₂N₄O) C, H, N.

Synthesis of 2-naphthyl-(5-piperazin-1-yl-1*H*-benzimidazol-2-yl)methanone (9). To a solution of **45** (69 mg, 0.15 mmol) in anhydrous CH₂Cl₂ (1 mL), trifluoroacetic acid (0.25 mL, 3 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 5 h, then evaporated under reduced pressure. The resulting crude was resuspended in CH₂Cl₂ and washed with 1 M NaOH. The organic layer was dried (Na₂SO₄) and evaporated, and the obtained solid was purified by recrystallization from Et₂O, to afford pure **9** in quantitative yield; mp 155-157 °C. IR (KBr) 3442, 1626, 1600, 1516, 1461. ¹H NMR (CD₃OD) δ 3.22-3.39 (m, 8H), 7.17 (br s, 1H), 7.23 (dd, *J* = 9.0, 2.2, 1H), 7.54-7.69 (m, 2H), 7.70 (d, *J* = 9.0, 1H), 7.95-8.09 (m, 3H), 8.29 (dd, *J* = 8.7, 1.7, 1H), 9.20 (s, 1H). ¹³C NMR (CD₃OD) δ 44.5, 49.2, 93.4, 111.7, 118.2, 125.6, 127.1, 127.9, 128.3, 129.0, 130.0, 132.0, 132.1, 132.8, 133.6, 133.8, 136.2, 148.2, 150.3, 183.9. MS (ESI) 357.0. Anal. (C₂₂H₂₀N₄O) C, H, N.

General Procedure for the Synthesis of Benzimidazole Derivatives 10-20 (X = CH₂). To a solution of previously synthesized **25-28** (1.5 mmol) and the appropriate aldehyde (2.3 mmol) in freshly distilled CH₂Cl₂ (8 mL), ytterbium triflate (93 mg, 0.2 mmol) was added and the reaction was stirred at room temperature overnight. The mixture was filtered over celite, the solvent was evaporated under reduced pressure, and the residue was purified by column chromatography using the appropriate eluent, to afford pure **10-20**.

2-Benzyl-4-piperazin-1-yl-1*H*-benzimidazole (10). Obtained from **25** and phenylacetaldehyde, in 45% yield. Chromatography: CH₂Cl₂/EtOH, 9:1; mp 126-128 °C.

2-Benzyl-4-(4-methylpiperazin-1-yl)-1*H*-benzimidazole (11). Obtained from **26** and phenylacetaldehyde, in 42% yield. Chromatography: CH₂Cl₂/EtOH, 8:2; mp 120-123 °C. IR (KBr) 3356, 1593, 1533, 1494, 1456. ¹H NMR (CDCl₃) δ 2.44 (s, 3H), 2.79 (m, 4H), 3.62 (m, 4H), 4.30 (s, 2H), 6.66 (d, *J* = 8.0, 1H), 7.09 (m, 1H), 7.12 (t, *J* = 8.0, 1H), 7.30-7.38 (m,

5H). ^{13}C NMR (CDCl_3) δ 36.3, 45.7, 49.6, 55.2, 105.6, 110.0, 123.6, 127.7, 129.4, 129.5, 135.1, 136.5, 137.0, 141.5, 151.3. MS (ESI) 307.0 (M+H). Anal. ($\text{C}_{19}\text{H}_{22}\text{N}_4$) C, H, N.

2-(1-Naphthylmethyl)-4-piperazin-1-yl-1H-benzimidazole (12). Obtained from **25** and 1-naphthylacetaldehyde, in 45% yield. Chromatography: $\text{CH}_2\text{Cl}_2/\text{EtOH}$, 9:1; mp 163-165 °C. IR (KBr) 3454, 1617, 1597, 1531, 1455. ^1H NMR (CD_3OD) δ 3.47-3.52 (m, 8H), 4.74 (s, 2H), 6.73-6.76 (m, 1H), 7.11-7.14 (m, 2H), 7.35 (d, J = 6.8, 1H), 7.42-7.50 (m, 3H), 7.83 (d, J = 8.2, 1H), 7.88-7.91 (m, 1H), 8.10-8.14 (m, 1H). ^{13}C NMR (CD_3OD) δ 34.0, 45.3, 106.2, 108.7, 124.3, 124.7, 126.8, 127.1, 127.5, 128.3, 129.2, 129.9, 132.2, 134.2, 135.6, 136.2, 136.9, 142.2, 151.9. MS (ESI) 343.2 (M+H). Anal. ($\text{C}_{22}\text{H}_{22}\text{N}_4$) C, H, N.

4-(4-Methylpiperazin-1-yl)-2-(1-naphthylmethyl)-1H-benzimidazole (13). Obtained from **26** and 1-naphthylacetaldehyde, in 48% yield. Chromatography: $\text{CH}_2\text{Cl}_2/\text{EtOH}$, 9:1; mp 95-96 °C.

4-(4-Methylpiperazin-1-yl)-2-(2-naphthylmethyl)-1H-benzimidazole (14). Obtained from **26** and 2-naphthylacetaldehyde, in 51% yield. Chromatography: $\text{CH}_2\text{Cl}_2/\text{EtOH}$, 8:2.

2-Benzyl-5-piperazin-1-yl-1H-benzimidazole (15). Obtained from **27** and phenylacetaldehyde, in 67% yield. Chromatography: $\text{CHCl}_3/\text{MeOH}/\text{NH}_3$, 9:1:0.1; mp 173-175 °C. IR (KBr) 3450, 1633, 1493, 1455. ^1H NMR (Acetone- d_6) δ 2.78-2.92 (m, 8H), 2.97 (br s, 1H), 4.05 (s, 2H), 6.76 (dd, J = 8.7, 2.3, 1H), 6.84 (d, J = 2.1, 1H), 7.07-7.25 (m, 6H), 7.90 (br s, 1H). ^{13}C NMR (Acetone- d_6) δ 35.7, 46.6, 52.6, 102.0, 114.3, 114.4, 126.9, 128.8, 129.2, 133.9, 136.1, 138.4, 149.3, 152.8. MS (ESI) 293.0 (M+H). Anal. ($\text{C}_{18}\text{H}_{20}\text{N}_4$) C, H, N.

2-Benzyl-5-(4-methylpiperazin-1-yl)-1H-benzimidazole (16). Obtained from **28** and phenylacetaldehyde, in 53% yield. Chromatography: $\text{CH}_2\text{Cl}_2/\text{EtOH}$, 97:3; mp 108-109 °C.

2-(1-Naphthylmethyl)-5-piperazin-1-yl-1H-benzimidazole (17). Obtained from **27** and 1-naphthylacetaldehyde, in 44% yield. Chromatography: $\text{CH}_2\text{Cl}_2/\text{EtOH}$, 9:1; mp 112-114 °C.

5-(4-Methylpiperazin-1-yl)-2-(1-naphthylmethyl)-1*H*-benzimidazole (18). Obtained from **28** and 1-naphthylacetaldehyde, in 75% yield. Chromatography: CH₂Cl₂/EtOH, 9:1; mp 121-123 °C.

2-(2-Naphthylmethyl)-5-piperazin-1-yl-1*H*-benzimidazole (19). Obtained from **27** and 2-naphthylacetaldehyde, in 42% yield. Chromatography: CH₂Cl₂/EtOH 9:1; mp 157-159 °C. IR (KBr) 3443, 1632, 1490, 1456. ¹H NMR (CD₃OD) δ 3.25-3.32 (m, 8H), 4.27 (s, 2H), 6.92 (dd, *J* = 8.8, 2.3, 1H), 7.02 (d, *J* = 2.0, 1H), 7.29-7.39 (m, 4H), 7.67-7.73 (m, 4H). ¹³C NMR (CD₃OD) δ 36.3, 45.3, 50.1, 103.9, 116.5, 116.7, 127.0, 127.5, 128.0, 128.5, 128.8, 129.7, 134.1, 135.3, 135.5, 135.8, 140.0, 148.6, 155.3. MS (ESI) 343.0 (M+H). Anal. (C₂₂H₂₂N₄) C, H, N.

5-(4-Methylpiperazin-1-yl)-2-(2-naphthylmethyl)-1*H*-benzimidazole (20). Obtained from **28** and 2-naphthylacetaldehyde, in 59% yield. Chromatography: CH₂Cl₂/EtOH, 95:5; mp 149-152 °C.

Pharmacology. Radioligand Binding Assays. Membranes from HEK-293 cells expressing human 5-HT₆ or 5-HT₇ receptors, were purchased from Perkin-Elmer and conserved at -80 °C in packaging buffer for subsequent use. Specific radioligand [³H]LSD (79.2 Ci/mmol) was from NEN (Perkin-Elmer). Competitive inhibition assays were performed according to standard procedures, briefly detailed below.

5-HT₆ Receptor. Cell paste (6.0 mg/mL) was homogenized in 200 volumes of assay buffer (50 mM Tris-HCl, 10 mM MgCl₂, 0.5 mM EDTA, pH 7.4 at 25 °C). Fractions of 20 µL of the membranes suspension were incubated at 37 °C for 60 min with 2.6 nM [³H]LSD, in the presence or absence of the competing drug, in a final volume of 200 µL of assay buffer. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 500 µM serotonin, and represented less than 10% of total binding.

5-HT₇ Receptor. Cell paste (6.8 mg/mL) was homogenized in 200 volumes of assay buffer (50 mM Tris-HCl, 10 mM MgSO₄, 0.5 mM EDTA, pH 7.4 at 25 °C). Fractions of 500 µL of

the membranes suspension were incubated at 27 °C for 120 min with 3 nM [³H]LSD, in the presence or absence of the competing drug, in a final volume of 540 µL of assay buffer. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 25 µM clozapine, and represented less than 15% of total binding.

For all binding assays, competing drug, nonspecific, total, and radioligand bindings were defined in triplicate. Incubation was terminated by rapid vacuum filtration through Wallac Filtermat A filters, presoaked in poly(ethylenimine) (0.5% for the 5-HT₆R and 0.3% for the 5-HT₇R), using a FilterMate Unifilter 96-Harvester. The filters were then washed 5-9 times with 500 µL of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 25 °C), and heated at 80 °C. The radioactivity bound to the filters was measured by scintillation spectrometry, using a Microbeta TopCount instrument. The data were analyzed by an iterative curve-fitting procedure using GraphPad Prism, which provided IC₅₀, K_i, and *r*² values for test compounds, K_i values being calculated from the Cheng-Prusoff equation.⁴⁵

Plasmid Construct. HA-tagged-5-HT₆ receptor cDNAs in pRK5 were generated by fusing the sequence of the HA epitope (YPYDVPDYA) to a cleavable signal peptide (MVLLILSVLLLKEDVRGSAQS) derived from the metabotropic glutamate receptor 5 (mGlu5R). This sequence was inserted into the pRK5 vector using Xba I and BsrG I restriction sites. Then, full-length mouse 5-HT₆R cDNA was subcloned in frame using BsrG I and Hind III. HA-5-HT₆-C3.36A, HA-5-HT₆-S5.43A, HA-5-HT₆-W6.48A, HA-5-HT₆-F6.52A, HA-5-HT₆-N6.55A receptor mutants were generated from HA-5-HT₆R, cloned in pRK5, with the Quick Change Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, Netherlands).

Cell Surface ELISA. COS-7 cells were transfected with HA-tagged-5-HT₆R, WT or mutants cDNAs. Cells were grown on 96-well plates in DMEM medium with 10% dFCS and fixed as described previously.⁶³ Cells were then incubated with anti-HA antibody at 0.6 µg/mL for 60 min in the same buffer and incubated with anti-rabbit/HRP conjugate

(Amersham Pharmacia Biotech) at 1 µg/mL for 60 min. Chromogenic substrate was added (Supersignal® ELISA femto-maximum sensitivity, Pierce, Perbio-Brebières, France). Chemiluminescence was detected and quantified by a Wallac Victor2 luminescence counter.

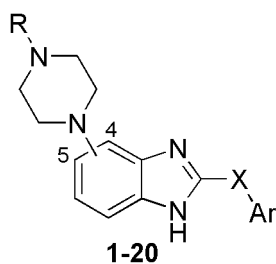
Determination of cAMP Production in Transfected Cells. COS-7 cells were transfected with the appropriate cDNA and seeded into 24-well plates (100 000 cells/well). 24 hours post-transfection, a 10 min-stimulation with the appropriate concentrations of each tested compound in absence or presence of 100 µM 5-HT was performed, as previously described.⁶¹ Quantification of cAMP production was performed by homogenous time resolved fluorescence (HTRF®) using the cAMP Dynamic Kit (Cisbio International, Bagnols-sur-Cèze, France), according to the manufacturer's instructions.

Data Analysis. The dose-response curves were fitted using GraphPad Prism and the following equation for monophasic dose-response curves: $y = (y_{\max} - y_{\min}) / 1 + [(x / IC_{50})^{n_H}] + y_{\min}$, where IC_{50} is the concentration of the antagonist necessary to reduce 50% of the maximal effect induced by 5-HT and n_H is the Hill coefficient. All data represented correspond to the mean ± SEM of three independent experiments performed in triplicate.

Computational Methods. Models of the Ligand-Receptor Complexes. Modeller v9.5⁶⁴ was used to build a homology model of the human 5-HT₆R using the crystal structure of the β₂-adrenergic receptor (PDB code 2RH1)^{47,65} as template. Internal water molecules 506, 519, 528, 529, 532, 534, 537, 543, 546, and 548 that mediate a number of interhelical interactions,⁴⁸ and seem conserved in the rhodopsin-like family of GPCRs,⁶⁶ were also included in the model. The Duan *et al.* (2003) force field⁶⁷ was used for receptors and the general Amber force field (GAFF)⁶⁸ and HF/6-31G*-derived RESP atomic charges were used for the ligands. Molecular dynamics simulations of the ligand-receptor complexes were performed with the Sander module of AMBER 9⁶⁹ using the protocol previously described.⁷⁰

Acknowledgment. This work has been supported by grants from the Spanish Ministerio de Educación (MEC, SAF-2007/67008), Comunidad Autónoma de Madrid (CAM, S-SAL-249-2006), and Instituto de Salud Carlos III (RD07/0067/0008). The authors thank Fundación Ramón Areces and MEC for predoctoral grants to T.F., J.S, and R.A.M.

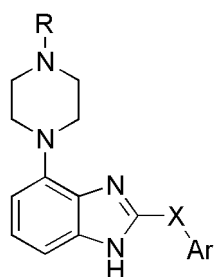
Supporting Information Available. Spectral characterization data of compounds **1-4**, **7**, **10**, **13**, **14**, **16-18**, **20**, **25**, **27**, **29**, **33**, **34**, **36**, **37**, **40**, and **41**, and combustion analysis data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Table 1. Binding Affinity of Synthesized Compounds **1-20** at the h5-HT₆R.

compd	position	R	X	Ar	$K_i \pm \text{SEM}$ (nM) ^a
1	4	H	CO	phenyl	> 1000
2	4	CH ₃	CO	phenyl	>1000
3	4	H	CO	1-naphthyl	13 ± 1
4	4	CH ₃	CO	1-naphthyl	34 ± 2
5	4	CH ₃	CO	2-naphthyl	> 1000
6	5	CH ₃	CO	phenyl	> 1000
7	5	CH ₃	CO	1-naphthyl	> 1000
8	5	CH ₃	CO	2-naphthyl	> 1000
9	5	H	CO	2-naphthyl	> 1000
10	4	H	CH ₂	phenyl	58 ± 3
11	4	CH ₃	CH ₂	phenyl	66.8 ± 0.7
12	4	H	CH ₂	1-naphthyl	16 ± 2
13	4	CH ₃	CH ₂	1-naphthyl	36.22 ± 0.08
14	4	CH ₃	CH ₂	2-naphthyl	49 ± 3
15	5	H	CH ₂	phenyl	> 1000
16	5	CH ₃	CH ₂	phenyl	> 1000
17	5	H	CH ₂	1-naphthyl	> 1000
18	5	CH ₃	CH ₂	1-naphthyl	> 1000
19	5	H	CH ₂	2-naphthyl	> 1000
20	5	CH ₃	CH ₂	2-naphthyl	> 1000

^a Values are the mean of two to four experiments performed in triplicate.

Table 2. Potency of New 5-HT₆R Antagonists.



compd	R	X	Ar	$K_i \pm \text{SEM}$ (nM) ^a
3	H	CO	1-naphthyl	15 ± 2
4	CH ₃	CO	1-naphthyl	5 ± 2
10	H	CH ₂	phenyl	31 ± 4
11	CH ₃	CH ₂	phenyl	16 ± 5
12	H	CH ₂	1-naphthyl	16 ± 3
13	CH ₃	CH ₂	1-naphthyl	27 ± 3
14	CH ₃	CH ₂	2-naphthyl	17 ± 4

^a Values are the mean of three experiments performed in triplicate.

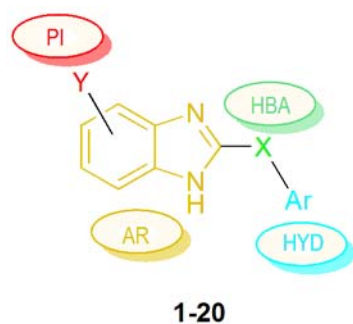
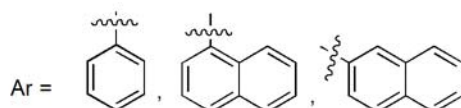
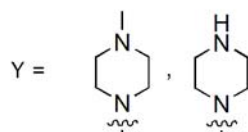
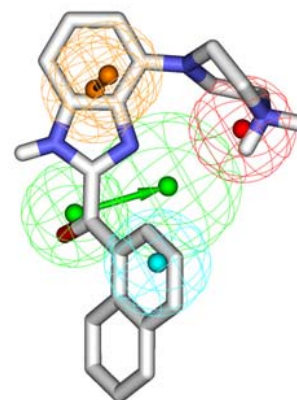
A $X = \text{CO}, \text{CH}_2$ **B**

Figure 1. A previously reported 3-D pharmacophore model for 5-HT₆R antagonists showed four key structural elements: a positive ionizable atom (PI, red), an aromatic ring-hydrophobic site (AR, yellow), a hydrogen bond acceptor group (HBA, green), and a hydrophobic site (HYD, blue). (A) Pharmacophore-based designed benzimidazole derivatives **1-20**. (B) Designed compound **3**: Y = 4-(piperazin-1-yl), X = CO, Ar = 1-naphthyl mapped into the pharmacophore model.

	TM3 3.28 – 3.37	EL2	TM5 5.42 – 5.50	TM6 6.47 – 6.55	TM7 7.35 – 7.40
β_1	WTS L DVLCVT	CD F V T NR	SS I I S FYIP	CW L P P FFLVN	FVAFNW
β_2	WTS I DVLCVT	CD F FTNQ	SS I V S FYVP	CW L P F FIVN	YILLNW
5-HT _{1A}	F I ALDVLCCT	CT I SKDH	ST F GAFYIP	CW L P P FFIVA	GAIINW
5-HT _{2A}	W I YLDVLFST	CL L A-DD	G S FV S FFIP	MW C P F FFITN	LN V FVW
5-HT ₄	R T SLDVLLTT	CV F MVNK	C S VVAFYIP	CW A P F FFVTN	WT A FLW
5-HT ₇	F I AMDVMCCT	CL I SQDF	ST A VAFYIP	CW L P F FLLS	ERTFLW
5-HT ₆	WT A FDVMCCS	CR L LASL	AS G L T FFLP	TW L P F FFVAN	FDVLTW

Figure 2. Sequence alignment of TMs 3, 5-7 and EL2 of β_1 - and β_2 -adrenergic receptors, and serotonin 5-HT_{1A}, 5-HT_{2A}, 5-HT₄, 5-HT₇, and 5-HT₆ receptors. Residues referenced in the manuscript are boxed. The highly conserved WxPFF motif in TM 6 is shown in dark grey.

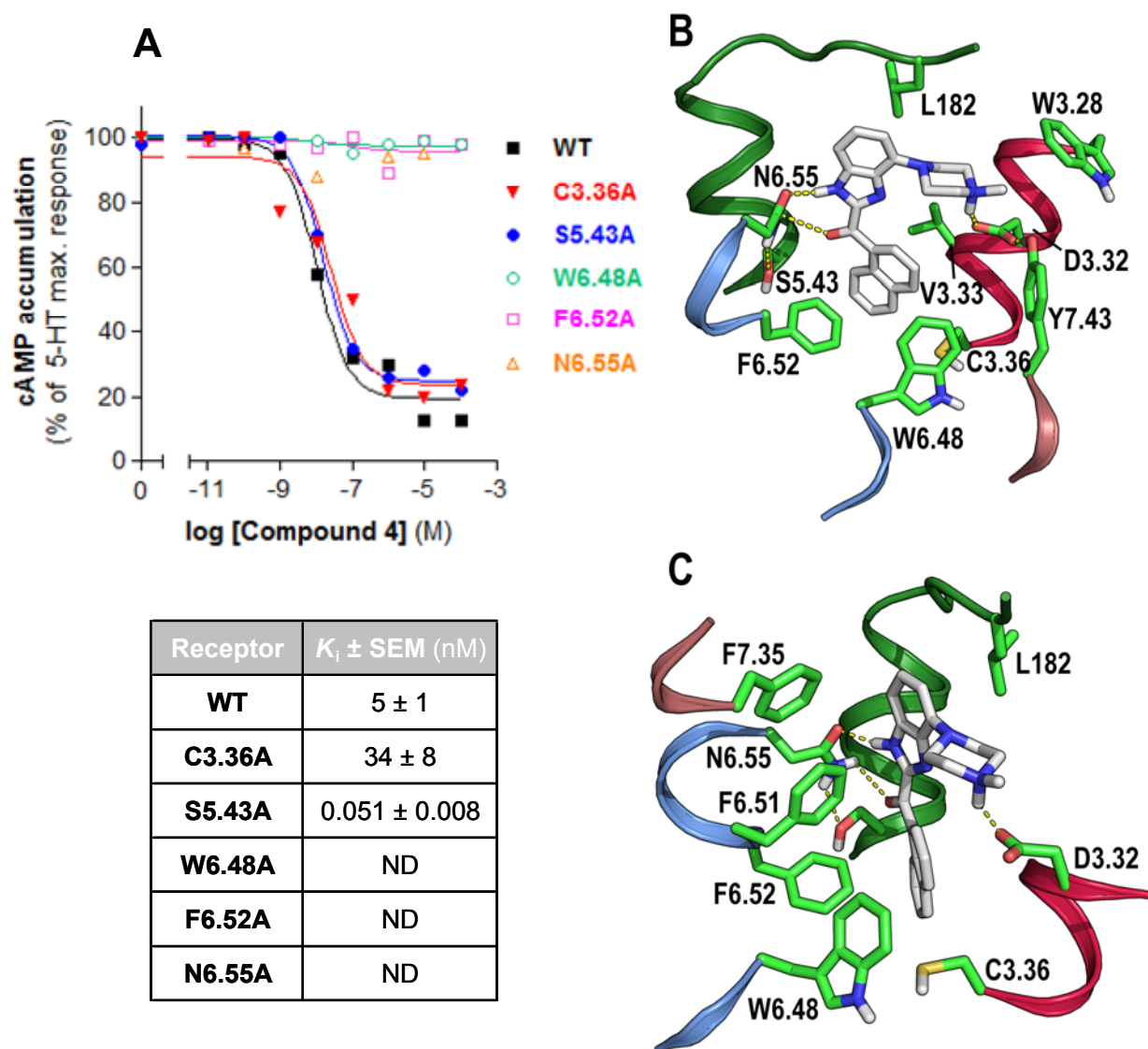


Figure 3. (A) The antagonist activity of compound **4** was tested relative to 5-HT-induced activation in WT 5-HT₆R and C3.36A, S5.43A, W6.48A, F6.52A, and N6.55A mutant receptors. The antagonist effect is represented as the percentage of inhibition of 5-HT (100 μ M)-induced stimulation of cAMP taken as 100%. Each value represents the mean \pm SEM determined from three independent experiments performed in triplicate. The inhibition constant K_i was calculated from the IC_{50} value using the Cheng-Prusoff equation. IC_{50} was assessed as the concentration required to inhibit 50% of 5-HT (100 μ M)-induced stimulation of cAMP. EC_{50} values of 5-HT on WT, C3.36A and S5.43A receptors were 5 ± 1 nM, 0.8 ± 0.1 μ M, and 3 ± 1 nM, respectively. (B, C) Computational model of the complex between ligand **4** (in white) and the 5-HT₆R. In this model D3.32 anchors the protonated piperazine

ring, the benzimidazole ring hydrogen bonds N6.55 and interacts with F6.51, F7.35, and L182 in EL 2, and the aromatic naphthalene ring enters into a small cavity between TMs 3 and 6 to interact with V3.33, W6.48 and F6.52. Panels B and C are rotated 90°. The color code of the helices is: TM 3 (red), 5 (green), 6 (blue), and 7 (brown).

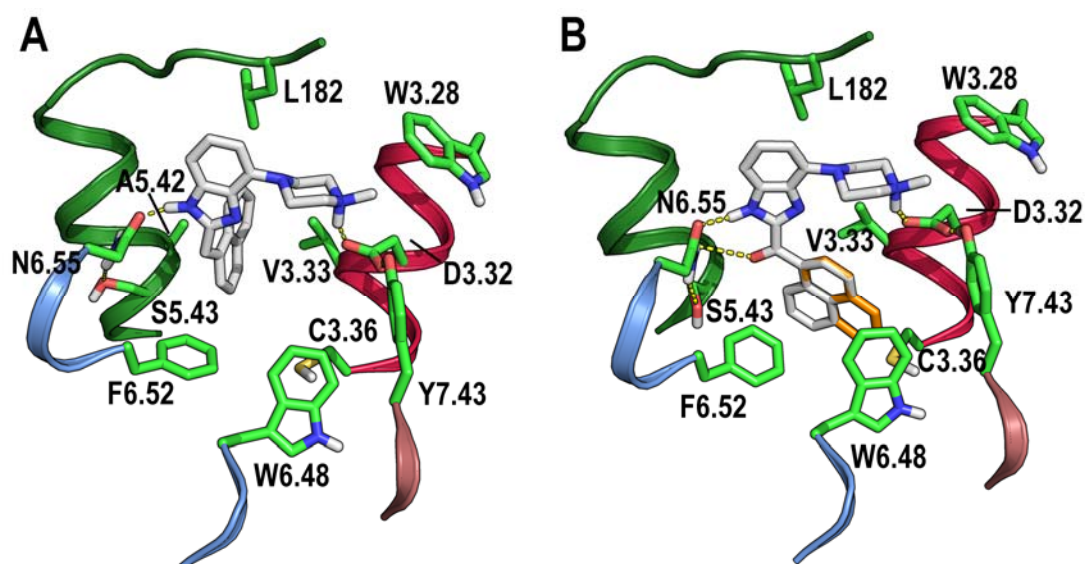


Figure 4. Computational models of the complexes between ligand **13** (A) and **4** (B) and the 5-HT₆R. An important feature of these molecules is the linker between the benzimidazole ring and the terminal aromatic ring. The methylene unit (-CH₂-) of ligand **13** allows both rings to form an angle of approximately 90° (A), whereas the carbonyl group (-CO-) of ligand **4** enforces both aromatic domains to be coplanar (B). In CH₂-containing compounds the aromatic ring is located between TMs 3 and 5, to interact with V3.33 and A5.42 (A). In CO-containing compounds the aromatic moiety is located between TMs 3 and 6, interacting with V3.33, W6.48 and F6.52 (B). 2-Naphthyl (in orange) is not tolerated within the cavity between TMs 3 and 6 due to the clash with TM 3 (B). The color code of the helices is: TM 3 (red), 5 (green), 6 (blue), and 7 (brown).

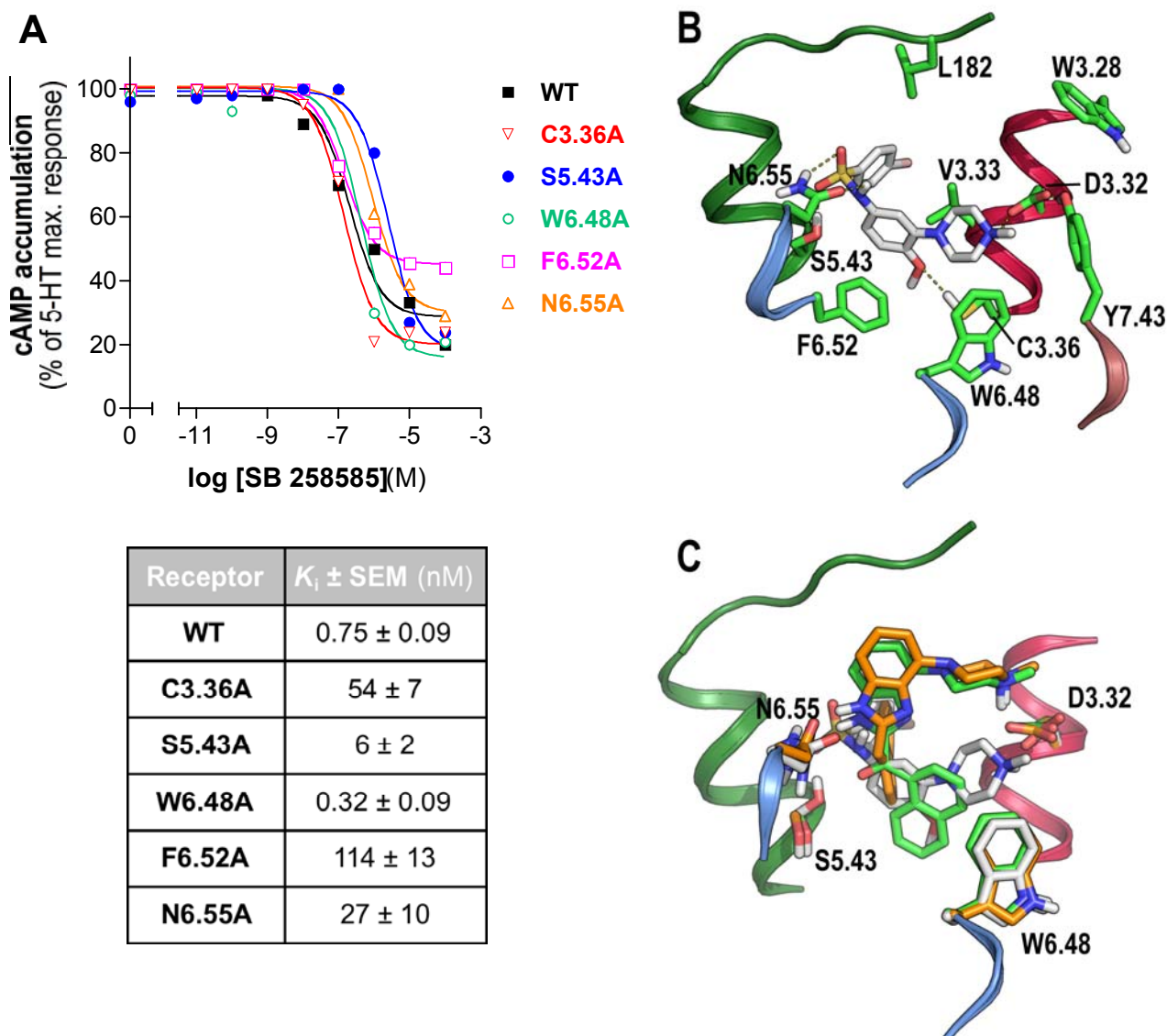


Figure 5. (A) The antagonist activity of SB-258585 was tested relative to 5-HT-induced activation in WT 5-HT₆R and C3.36A, S5.43A, W6.48A, F6.52A, and N6.55A mutant receptors. The antagonist effect is represented as the percentage of inhibition of 5-HT (100 μ M)-induced stimulation of cAMP taken as 100%. Each value represents the mean \pm SEM determined from three independent experiments performed in triplicate. The inhibition constant K_i was calculated from the IC_{50} value using the Cheng-Prusoff equation. IC_{50} was assessed as the concentration required to inhibit 50% of 5-HT (100 μ M)-induced stimulation of cAMP. EC_{50} values of 5-HT were 5 ± 1 nM on WT, 0.8 ± 0.1 μ M on C3.36A, 3 ± 1 nM on S5.43A, 1.1 ± 0.1 nM on W6.48A, 0.18 ± 0.06 μ M on F6.52A, and 45 ± 2 nM on N6.55A receptor. (B) Computational model of the complex between SB-258585 (in white) and the 5-

HT₆R. In this model D3.32 anchors the protonated piperazine ring, the methoxyphenyl system interacts with F6.52, the methoxy group hydrogen bonds C3.36, the sulfonamide moiety hydrogen bonds both S5.43 and N6.55, and the iodophenyl ring interacts with the hydrophobic side chains of V3.33 and A5.42. (C) Comparison of the binding modes of SB-258585 (white) and benzimidazole derivatives **4** (green) and **13** (orange) to the 5-HT₆R. The color code of the helices is: TM 3 (red), 5 (green), 6 (blue), and 7 (brown).

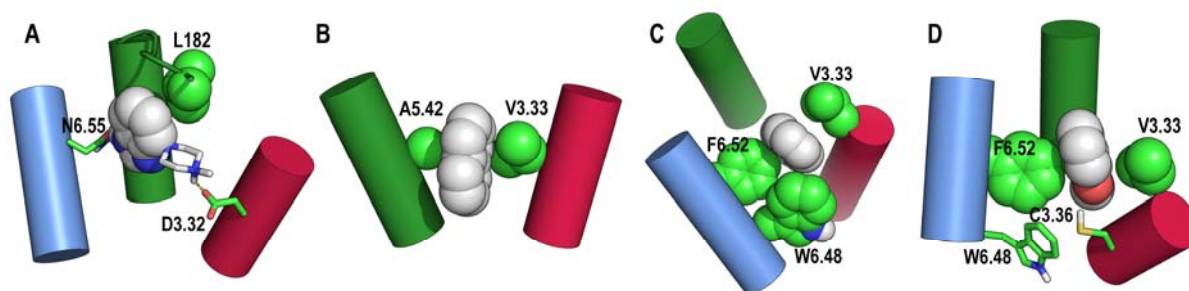
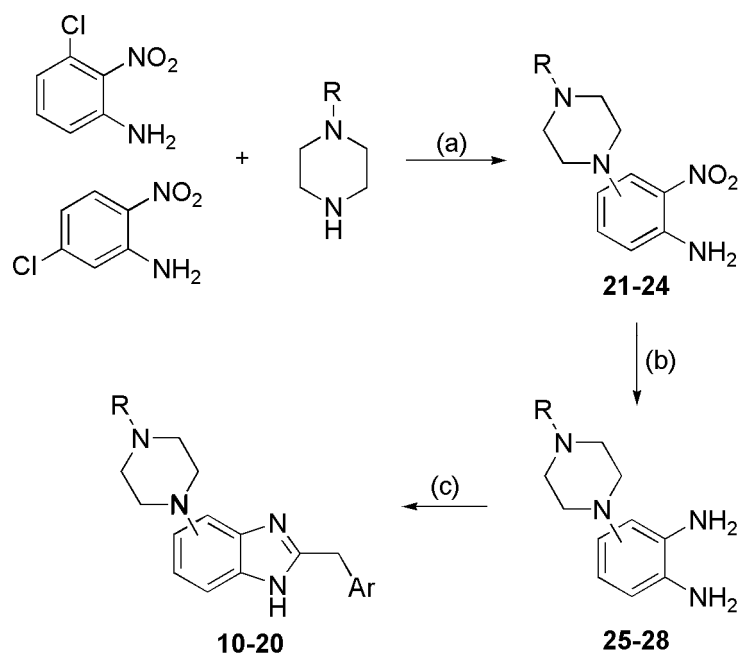


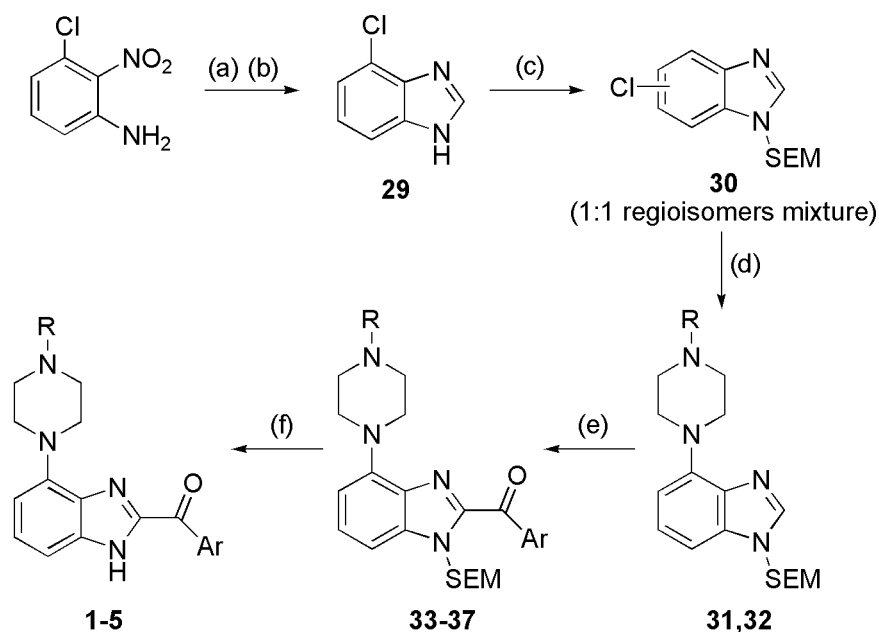
Figure 6. Molecular mechanisms of receptor inactivation. (A) The benzimidazole ring (in white spheres) impedes the proposed inward movement of the extracellular part of TM 6 toward TM 3 required for receptor activation.⁵⁴ The interaction of L182 in EL 2 with the benzimidazole ring may serve to immobilize both EL 2 and TM 6 in the inactive conformation. (B) The aromatic naphthalene ring (in white spheres) in CH₂-containing compounds is located between TMs 3 and 5, interacting with V3.33 and A5.42, and probably blocking the movement of these helices necessary for receptor activation.⁵⁶ (C) The aromatic naphthalene ring (in white spheres) in CO-containing compounds is located between TMs 3 and 6, interacting with V3.33, W6.48, and F6.52. This mode of binding blocks the proposed rotamer toggle switch of W6.48 and the subsequent steps in the activation process.⁵⁰ (D) The methoxyphenyl system of SB-258585 (in white spheres) is located between TMs 3 and 6, interacting with V3.33, C3.36 and F6.52. The hydrogen bond between the methoxy group and C3.36 blocks the conformational transition of C3.36 toward TM 7 during the process of receptor activation.⁶⁰ The color code of the helices is: TM 3 (red), 5 (green), and 6 (blue).

Scheme 1^a



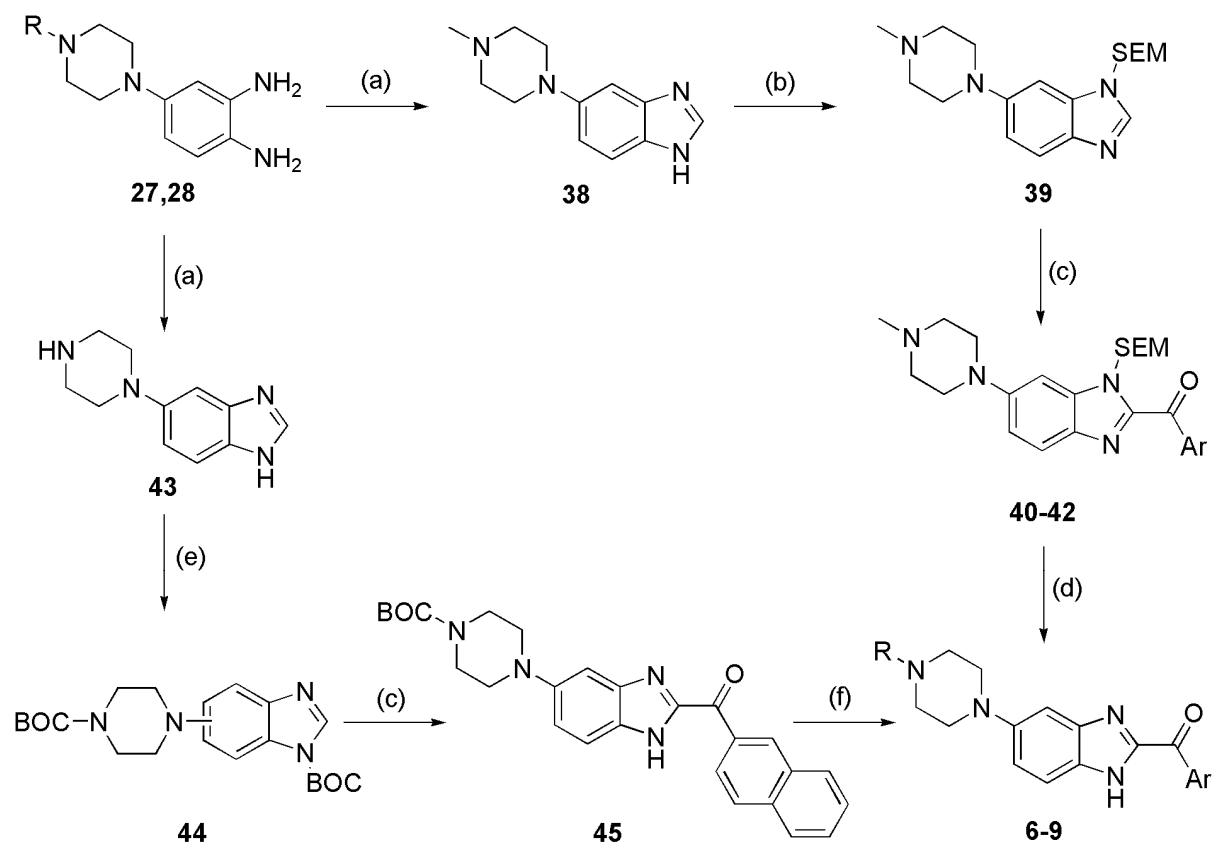
^a Reagents and conditions: (a) Cs₂CO₃ or K₂CO₃, DMF, 120 °C, 24 h, 40% to quantitative yield; (b) H₂, Pd(C), MeOH, rt, 24 h, 89% to quantitative yield; (c) ArCH₂CHO, Yb(OTf)₃·2H₂O, CH₂Cl₂, rt, overnight, 42-75%.

Scheme 2^a



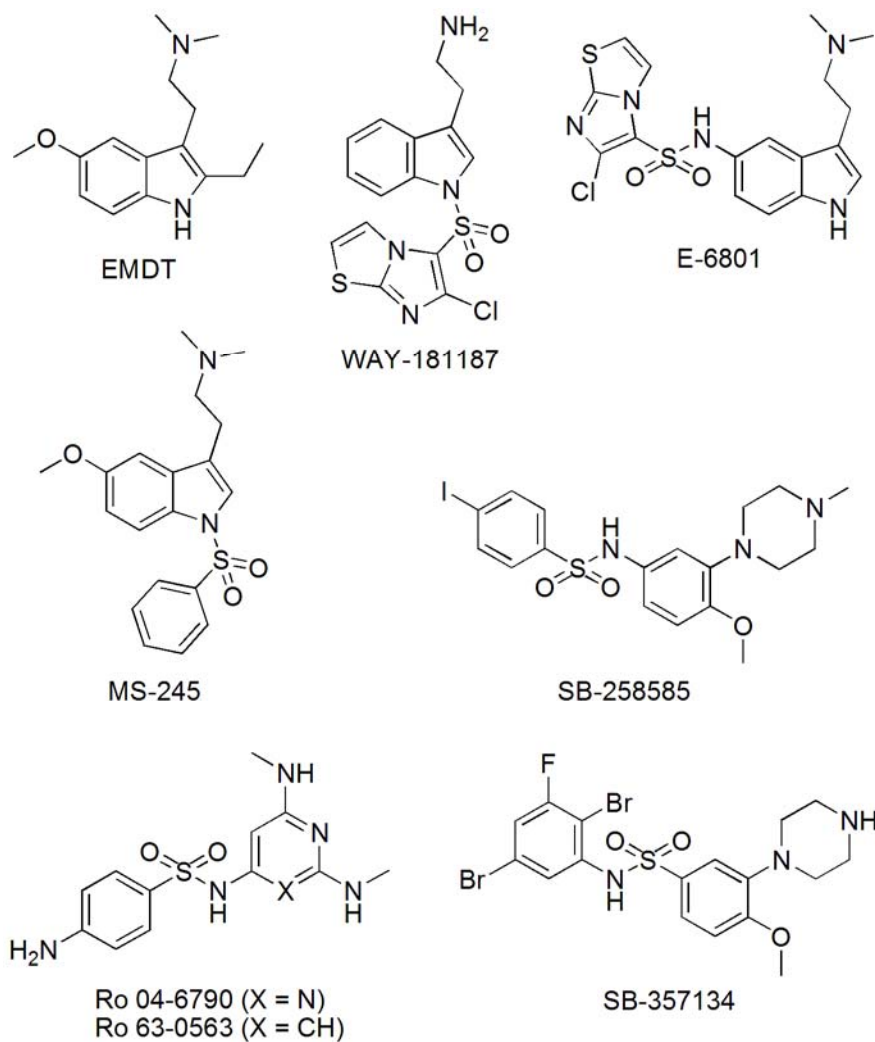
^a Reagents and conditions: (a) SnCl₂, HCl, rt, 3 h, quantitative yield; (b) HCOOH, H₂O, 100 °C, 3 h, quantitative yield; (c) SEMCl, K₂CO₃, DMF, 0 °C to rt, 3 h, quantitative yield; (d) 1-BOC-piperazine or 1-methylpiperazine, NaO^tBu, Pd₂dba₃, P(^tBu)₃, *o*-xylene, 110 °C, 24 h, 43-49%; (e) ArCOOR, BuLi, THF, -78 °C to rt, overnight, 21-59%; (f) BCl₃, CH₂Cl₂, rt, 5 h, 50-64%.

Scheme 3^a



^a Reagents and conditions: (a) HCOOH, H₂O, 100 °C, 3 h, 60-87%; (b) i. SEMCl, NaH, DMF, 0 °C to rt, 3 h, ii. SiO₂ column chromatography (CH₂Cl₂/EtOH, 9:1), 44%; (c) ArCOOR, BuLi, THF, -78 °C to rt, overnight, 33-63%; (d) BCl₃, CH₂Cl₂, rt, 5 h, 65-77%; (e) BOC₂O, DMAP, Et₃N, rt, overnight, 69%; (f) TFA, CH₂Cl₂, rt, 5 h, quantitative yield.

Chart 1. Structures of 5-HT₆R agonists EMDT, WAY-181187 and E-6801, and antagonists MS-245, SB-258585, Ro 04-6790 and SB-357134.



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