5-HT_4 Receptor Antagonists: Structure-Affinity Relationships and Ligand-Receptor Interactions

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Abstract: Among serotonin receptors (5-HTRs), the 5-HT_4 subtype is of considerable interest because it is involved in (patho)physiological processes both in peripheral and central nervous systems. In addition to the clinical use of 5-HT_4R agonists in the treatment of gastrointestinal motility disorders, the potential use of antagonists in the treatment of irritable bowel syndrome, arrhythmias and micturition disturbances are currently under investigation. This article will review the development of the most important classes of 5-HT_4R antagonists with an emphasis on benzimidazole derivatives, their structure-affinity relationships, ligand-receptor interactions and pharmacological applications.

INTRODUCTION

Over the last two decades the family of serotonin (5-hydroxytryptamine, 5-HT) receptors (5-HTRs) has been the subject of intense research in Medicinal Chemistry, due to their involvement in (patho)physiological processes both in peripheral and central nervous systems [1-9]. From the initial subdivision by Gaddum and Picarelli into D and M sites [10], fourteen 5-HTR subtypes have been identified and classified in seven classes (5-HT_{1-7}) [11-14]. 5-HTRs belong to the superfamily of G protein-coupled receptors (GPCRs), except the 5-HT_3 subtype which is a ligand-gated cation channel receptor [15-17]. Studies of receptor binding have shown that the majority of compounds with affinity for 5-HTRs display a high to moderate affinity for more than one 5-HTR subtype and are often non-selective over other GPCRs (e.g. dopaminergic receptors, adrenoceptors). For this reason, considerable attention has centred around the identification of agents which act selectively at each of these receptor subtypes.

The 5-HT_4R was discovered in 1988 as a serotoninergic receptor positively linked to adenylyl cyclase in the central nervous system (CNS) [18], which was shown to be similar to the receptor responsible for stimulating motility in the gastrointestinal (GI) tract [19-21]. Since then, considerable progress has been made in the study of the 5-HT_4R and their ligands [22-24], with the development of the specific radioligands [^{3}H]GR 113808 [25, 26] and [^{125}I]SB 207710 [27]. In addition to the CNS [28-31] and the GI system [32-43] the 5-HT_4R has been localized in several animal and human peripheral tissues, such as the heart [44-46], the urinary bladder [47, 48] and the adrenal gland [49, 50], where they mediate a variety of responses. Thus, certain 5-HT_4R agonists are clinically used as prokinetic agents in the treatment of GI motility disorders [51-55], and their possible utility in memory deficits is currently under investigation [29, 56-66]. Additionally, a number of therapeutic indications have been proposed for antagonists of this receptor, including irritable bowel syndrome (IBS) [36, 67-71], cardiac atrial arrhythmias [46, 72, 73] and urinary incontinence [74, 75].

The 5-HT_4R was first cloned from rat brain in 1995 and two isoforms (5-HT_{4S} and 5-HT_{4L}) were found that differ in the length and sequence of their carboxy termini [76, 77]. More recently, several groups have reported the cloning of five splice variants of the human 5-HT_4R (5-HT_{4a}, 5-HT_{4b}, 5-HT_{4c}, 5-HT_{4d}, and 5-HT_{4e}) [78-81]. The expression of these isoforms depends on the tissue: three or four variants were expressed in heart atrium, brain and intestine, while only one was found in bladder, and the 5-HT_{4d} isoform was only present in the intestine [79]. The availability of the cloned 5-HT_4Rs has stimulated more interest in the study of this serotoninergic receptor subtype.

Within this field, the search for new ligands with high affinity and selectivity at the 5-HT_4R continues to generate interest because of the potential to find new therapeutic drugs. This review covers the development of 5-HT_4R antagonists with an emphasis on the benzimidazole class, their structure-affinity relationships and ligand-receptor interactions.

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5-HT$_4$ ANTAGONIST PHARMACOPHORE

A comparative receptor mapping, following the active analog approach (AAA), of serotoninergic 5-HT$_3$ and 5-HT$_4$R recognition sites, using compounds belonging to different chemical classes (active and inactive), has been reported [82]. This study lent support to the three-component pharmacophore model for antagonists of the 5-HT$_3$R proposed by Hibert [83] and Evans [84], as well as to the proposal of a model for antagonists of the 5-HT$_4$ binding site [82]. This model consists of an aromatic moiety, a coplanar carbonyl group with the oxygen situated at *ca.* 3.6 Å from the centroid of the aromatic ring, a nitrogen atom situated at *ca.* 8.0 Å from this centroid and *ca.* 5.4 Å from the oxygen of the carbonyl group, and a voluminous substituent in the basic amino framework of the molecule. The basic nitrogen is 3.6 Å-4.0 Å above the plane of the aromatic moiety (Figure **1**).

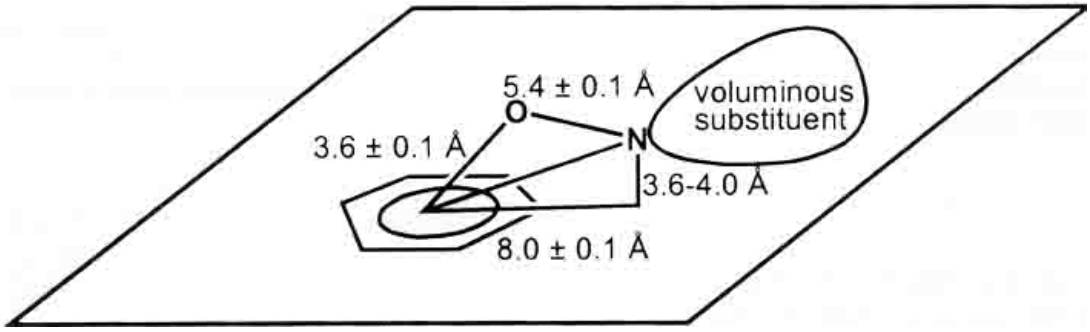


Fig. (1). Pharmacophore model for 5-HT$_4$R antagonists.

The major difference between the 5-HT$_3$ and 5-HT$_4$R cavities is in the region occupied by the substituent of the basic nitrogen. The 5-HT$_3$R can only accommodate relatively small substituents at the nitrogen atom, whereas the 5-HT$_4$ requires more voluminous groups [82, 85]. Thus, the interaction or the lack of interaction with the hydrophobic pocket around the basic nitrogen would be responsible for 5-HT$_4$/5-HT$_3$ or 5-HT$_3$/5-HT$_4$ selectivity, respectively [82]. Also, the basic nitrogen is located at *ca.* 8.0 Å from the aromatic moiety in the 5-HT$_4$R antagonist pharmacophore, whereas this distance is *ca.* 7.5 Å in the 5-HT$_3$ model.

Regions of steric tolerance and intolerance, based on the van der Waals surfaces of active and inactive 5-HT$_3$ and 5-HT$_4$ ligands for both receptors have been proposed [82], and these models have received some support from the design and synthesis [82, 86-88] of two active and selective antagonist ligands (Figure **2**): UCM-30593 [K_i (5-HT$_3$) = 3.7 nM; K_i (5-HT$_4$) > 1000 nM] and UCM-21195 [K_i (5-HT$_4$) = 13.7 nM; K_i (5-HT$_3$) > 10000 nM]. The novel ligand UCM-21195 was used as a lead compound for the

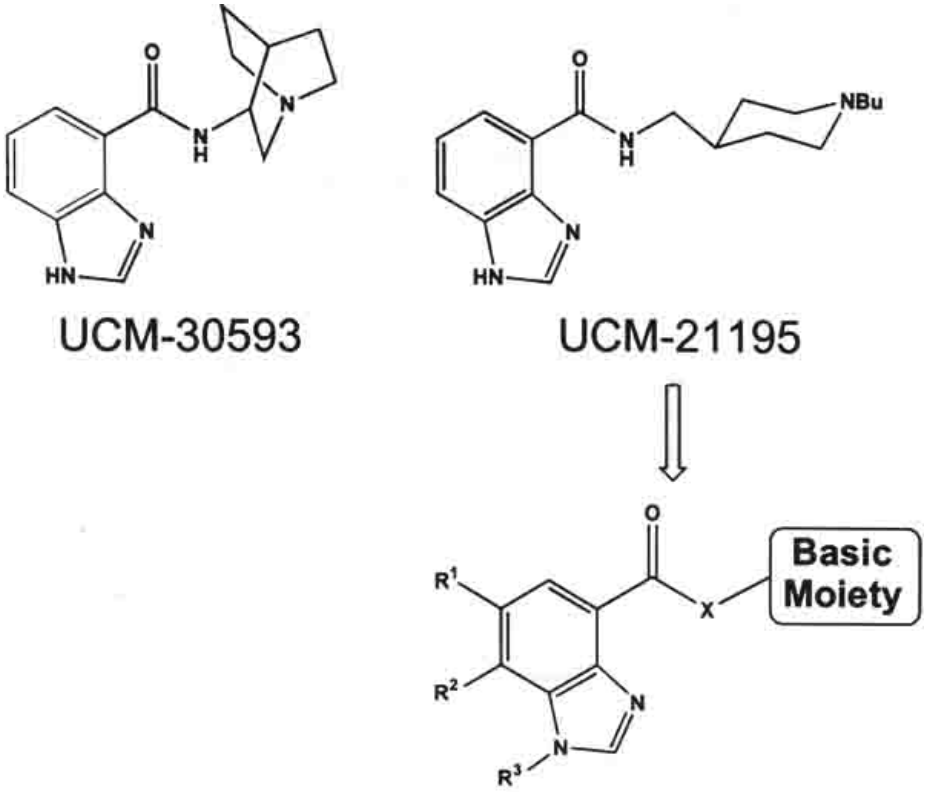


Fig. (2). Structures of UCM-30593, UCM-21195, and benzimidazole derivatives **1**.

design of a new series of benzimidazole-4-carboxylic acid derivatives **1** (Figure **2**), in order to analyze the influence of some structural variations of the different pharmacophore elements on the affinity for the 5-HT$_4$R, and to develop a novel class of potent and selective 5-HT$_4$R antagonists [86, 88, 89].

5-HT$_4$R ANTAGONISTS

From a chemical structure standpoint, 5-HT$_4$R antagonists can be classified in three classes: antagonists derived from non-selective agonist benzamides, antagonists derived from the non-selective antagonist tropisetron (ICS 205-930), and benzimidazole derivatives.

Antagonists Derived from Agonist Benzamides

The study of certain gastroprokinetic benzamides, such as metoclopramide [90], renzapride (BRL 24924) [91] and cisapride [92], revealed that they exert their GI motility stimulation via activation of 5-HT$_4$Rs [93]. These benzamides (Figure **3**) were the first 5-HT$_4$R ligands identified, but they showed a very poor selectivity due to their high activity as 5-HT$_3$R antagonists.

In the search for selective 5-HT$_4$R antagonists, the first approach was to convert the agonist or partial agonist activity of these benzamides into antagonist properties. In this way, benzoate SDZ 205-557 [94-96] (the ester equivalent of metoclopramide) and its analogs LY 297524 [97] and RS 23597-190 [98] were the first moderately potent and selective 5-HT$_4$R antagonists identified (Table **1**). Alternatively, the replacement of the benzamide ring of the agonist SC 53116 (Figure **3**) with an imidazopyridine system led to the antagonist SC 53606 [99], with moderate selectivity for the 5-HT$_4$R (Table **1**). SC 53606 was under preclinical investigation as a potentially useful drug for the treatment of IBS, but was discontinued in 1997. More recently, the introduction of two *cis*-methyl groups at positions 3 and 5 of the piperidine ring in the benzoate agonist ML 10302 (Figure **3**) has afforded the antagonist ML 10375 [100], improving 5-HT$_4$R affinity but without activating the receptor (Table **1**). Again, a slight structural modification induces a dramatic change in the pharmacological profile. Langlois *et al.* [101] have suggested the existence of two different binding sites for the 5-HT$_4$R: the agonists would initially bind the inactive site inducing a conformational rearrangement of the ligand and the receptor site into the active state, mediating the coupling of G proteins and thus the agonist activity. The antagonists would only bind the inactive site, and no biological activity is induced because of their inability to bind the receptor in its active state. In this way, antagonism of ML 10375 and agonism of ML 10302 could be explained because the presence of the two methyl groups in the antagonist would avoid binding to the active site probably due to a steric hindrance. The same authors have recently reported a new series of arylpiperazine derivatives **2** (Figure **4**), designed from ML 10302 (Figure **3**) by replacing the piperidine ring with an aryl or heteroarylpiperazine system [102]. These analogs have been characterized as 5-HT$_4$R antagonists, supporting their theory of two binding sites for agonists and antagonists of this receptor [101]. However, further evidence is required to test this hypothesis.

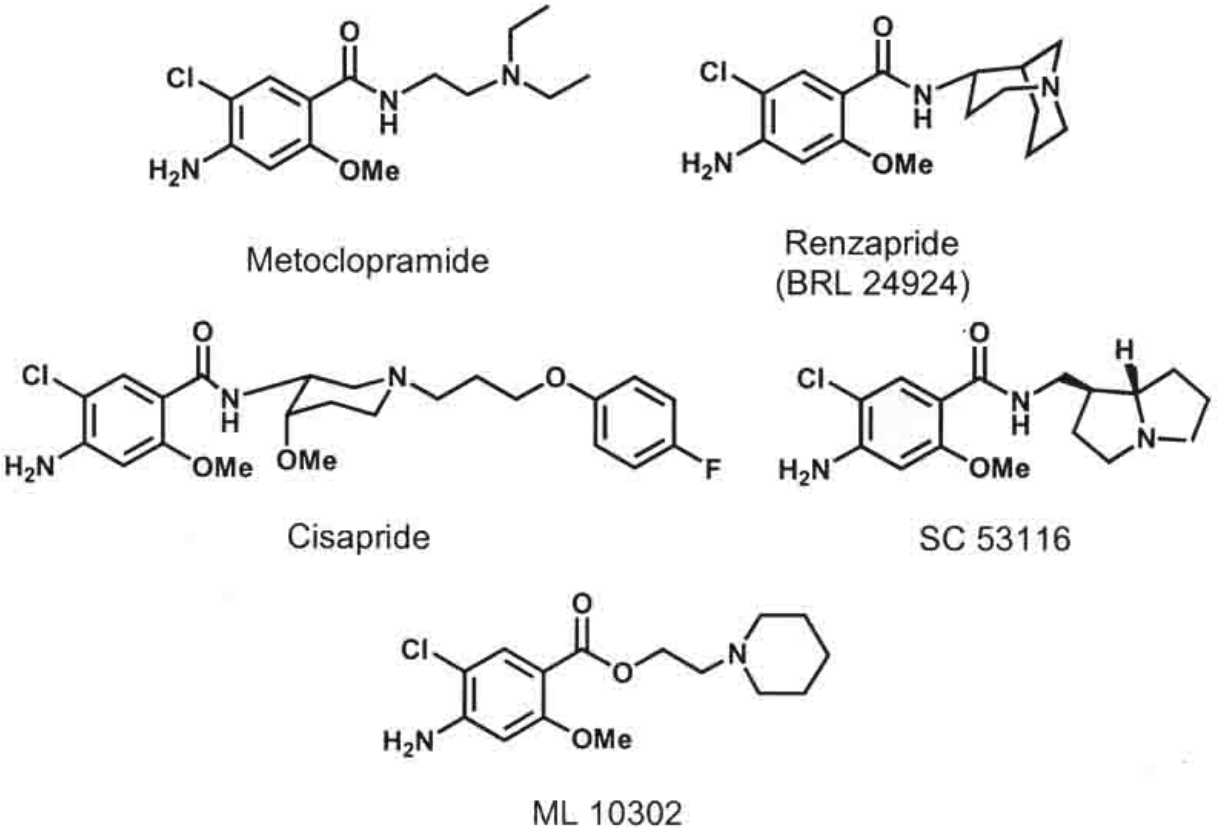


Fig. (3). Structures of 5-HT$_4$R agonists.

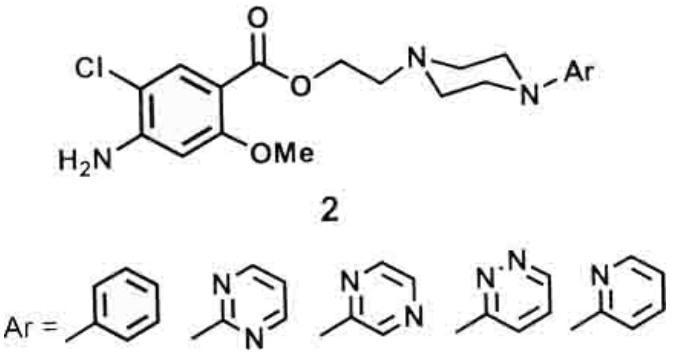


Fig. (4). Structure of arylpiperazine derivatives **2**.

A significant advance in the development of highly potent and selective 5-HT_4R antagonists is the family of benzodioxanes (see Table **1**). Indeed, SB 204070 [103, 104] is one of the most potent 5-HT_4R antagonists reported to date, though the lability of its ester linkage represents a major drawback for its use as a potential drug. The equivalent amide SB 205800 [85] (Figure **5**) retains the affinity and selectivity for the 5-HT_4R but in addition it shows oral activity *in vivo*. To date, SB 205800 is at the preclinical stage of development with GlaxoSmithKline for the treatment of atrial fibrillation, IBS, stroke and CNS disorders. The most important contribution among benzodioxane derivatives is doubtless the 7-iodo analog of SB 204070, SB 207710 (Table **1**), which has been radiolabelled [27]. [^{125}I]SB 207710 is widely used for studies of affinity assessment by radioligand binding assays and of 5-HT_4R localization and distribution by radiographic analysis [105].

In an attempt to improve bioavailability, the ester group of several 5-HT_4R antagonists has been replaced with a carbonyl group. Thus, ketones RS 39604 [106], RS 67532 [106], RS 100235 [107] and RS 100302 [107] (Table **1**) are potent and selective 5-HT_4R antagonists with *in vivo* activity. In particular, RS 39604 is orally active and long-acting *in vivo* [108]. This antagonist and RS 100235 underwent preclinical trials for their potential use in the treatment of IBS. However, no further development has been reported for any of them.

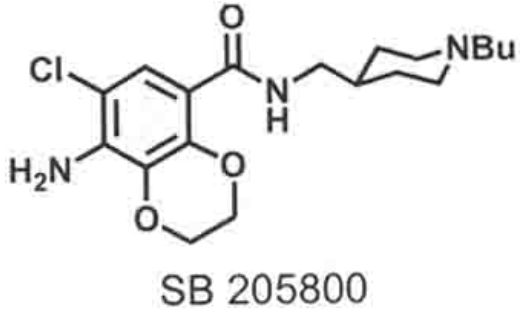


Fig. (5). Structure of SB 205800.

Antagonists Derived from Tropisetron

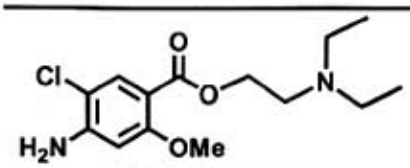
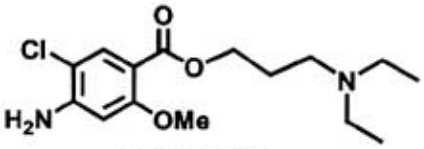
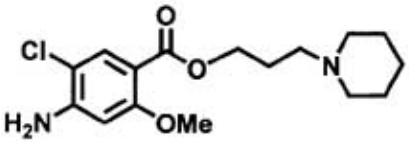
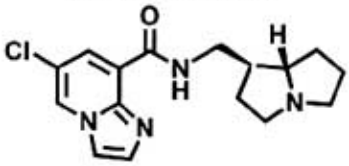
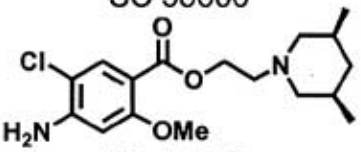
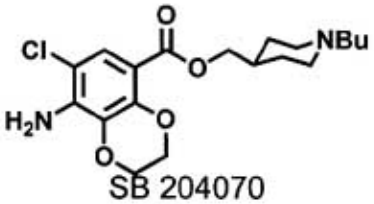
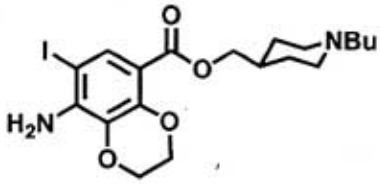
Tropisetron [18, 20] was the earliest 5-HT_4R antagonist identified, but as with the agonist benzamides it showed poor affinity and selectivity mainly due to its high affinity for 5-HT_3 receptors (Table **2**). Subsequently, the indole ring of tropisetron has been used in the search for new antagonists with high 5-HT_4R affinity and selectivity (see Table **2**). With SB 203186 [109] high affinity and moderate selectivity were achieved, and this antagonist was under preclinical evaluation for the treatment of cardiac arrhythmias, but has been discontinued. A major advance came with the identification of GR 113808 [25]. Due to its high affinity (in the subnanomolar range) and selectivity (>1000 fold) for the 5-HT_4R, this antagonist was tritiated and to date [^{3}H]GR 113808 [26] is the most widely used radioligand in the study of 5-HT_4R localization and in the assessment of affinity of new ligands of this receptor. Considering the poor *in vivo* activity of GR 113808 [110], analog GR 125487 [111] was synthesized by the same authors, with improved potency and activity in *in vivo* models of 5-HT_4R function. GR 125487 underwent preclinical evaluation for the treatment of IBS, but no recent development has been reported. GR 113808 is still used as a lead compound in the search for novel 5-HT_4R antagonists.

In a series of fused indole esters and amides, SB 207266 (Piboserod) [112, 113] (Table **2**) exhibited the best pharmacological profile with high 5-HT_4R affinity and selectivity, and long-acting oral activity [69-71, 114, 115]. This antagonist was evaluated in Phase II trials as a potential new drug for the treatment of IBS, but it appears to no longer be in development for this indication. An indazole amide, LY 353433 [116] (Figure **6**), has also shown to be an orally active 5-HT_4R antagonist with long-lasting effects and an excellent safety profile in animal studies [117, 118]. LY 353433 was under preclinical investigation for the treatment of IBS and other GI disorders, but there has been no recent development reported for this antagonist in this indication.

Benzimidazole Derivatives

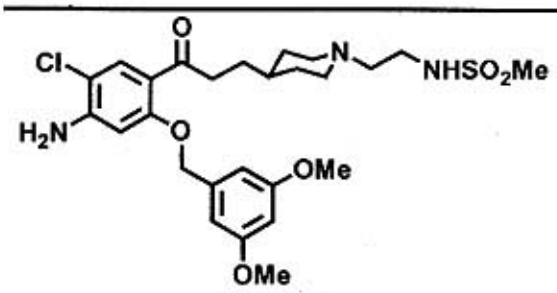
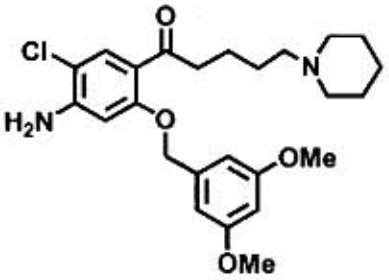
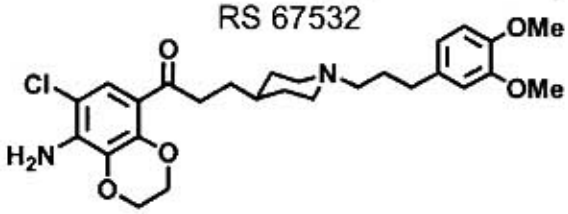
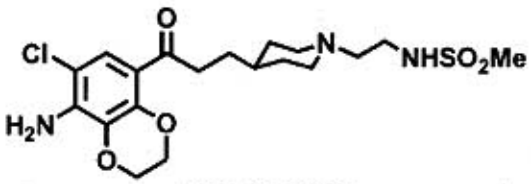
Another structural class of 5-HT_4R antagonists has been investigated where the indole ring of tropisetron is replaced with a benzimidazole system. On the basis of the pharmacophore model proposed by us [82] for 5-HT_4R antagonists, we have designed and synthesized [88] a series of benzimidazole-4-carboxylic acid derivatives of general structure **1** (see Figure (**2**) and Figure (**7**)), and analyzed the influence of some structural variations of the different pharmacophore elements on the affinity for the 5-HT_4R and selectivity over 5-HT_3Rs. Structure-affinity relationship (SAFIR) observations show that the presence of at least one methylene unit between the acyl group and the 1-alkyl-4-piperidyl ring is necessary for selective binding at the 5-HT_4R. In the case of piperidinoalkyl derivatives it has been observed that only a piperidinopropyl chain leads to compounds with affinity and selectivity for 5-HT_4Rs. In order to understand the influence of the methylene chain length for 5-HT_4R affinity and selectivity, a structural analysis of compounds **1a**, UCM-21195, **1b** and **1c** was carried out, and the structural parameters of lowest energy conformers are given in Table **3**. We can observe that the distance between the aromatic ring and the basic nitrogen atom (Ar-N) is the key structural parameter. In non-selective analogs **1a** (K_i (5-HT_4) = 290 nM, K_i (5-HT_3) = 198 nM) and **1b** (K_i (5-HT_4) = 157 nM, K_i (5-HT_3) = 290 nM) the

Table 1. Affinities and Activities of 5-HT$_4$R Antagonists Derived from Agonist Benzamides

Compound	pK_i		pA_2
	5-HT$_4$	5-HT$_3$	
 SDZ 205-557	8.2[a]	6.9[b]	7.4[c,d]
 LY 297524	8.3[e]	---	7.7[b,f]
 RS 23597-190	8.4[g]	5.7[h]	7.8[f,h]
 SC 53606	7.9[i]	6.6[i]	7.9[f,i]
 ML 10375	9.6[j]	---	8.6[f,j]
 SB 204070	9.9[k]	6.6[l]	10.8[l,m]
 SB 207710	9.2[n]	<6.3[n]	10.0[m,n]

[a]Ref. [25]. [b]Ref. [85]. [c]Ref. [94]. [d]Guinea pig ileum. [e]Ref. [31]. [f]Rat oesophagus. [g]Ref. [29]. [h]Ref. [98]. [i]Ref. [99]. [j]Ref. [101]. [k]Ref. [112]. [l]Ref. [103]. [m]Guinea pig distal colon. [n]Ref. [27].

(Table 1) contd....

Compound	pK_i 5-HT$_4$	pK_i 5-HT$_3$	pA_2
 RS 39604	9.1[o]	6.0[p]	9.3[f,p]
 RS 67532	8.7[g]	---	8.5[f,q,r]
 RS 100235	10.0[s]	6.3[s]	11.2[f,r,s]
 RS 100302	---	---	9.9[f,r,s]

[o]Ref. [105]. [p]Ref. [108]. [q]Ref. [106]. [r]pK_B value. [s]Ref. [107].

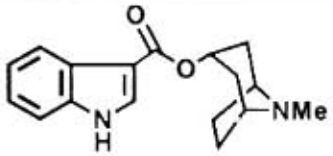
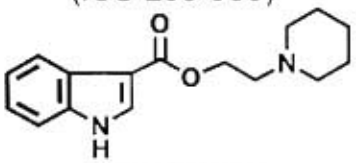
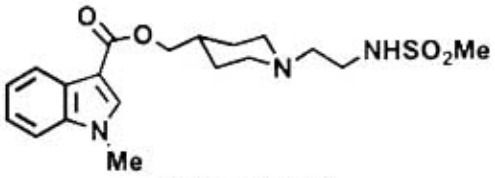
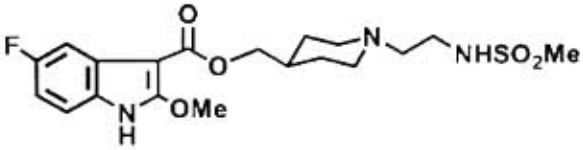
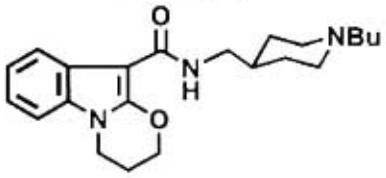
distance is 7.82 Å and 7.49 Å, respectively, whereas in selective compounds UCM-21195 (K_i (5-HT$_4$) = 13.7 nM, K_i (5-HT$_3$) > 10000 nM) and **1c** (K_i (5-HT$_4$) = 499 nM, K_i (5-HT$_3$) > 10000 nM) the nitrogen atom is situated at a further distance from the aromatic ring (8.48 Å and 8.34 Å, respectively). These structural data are in agreement with the proposed hypothesis for 5-HT$_4$R affinity and 5-HT$_4$/5-HT$_3$ selectivity [82], in which the distance between the aromatic ring and the basic nitrogen atom is *ca.* 8.0 Å for the 5-HT$_4$ antagonist pharmacophore and *ca.* 7.5 Å for the 5-HT$_3$ antagonist model.

On the other hand, analogs with a substituent on the nitrogen atom less voluminous than a butyl group are devoid of affinity at the 5-HT$_4$R. These results confirm that both the presence of a voluminous substituent in the basic nitrogen of the amino moiety and the distance from this nitrogen to the aromatic ring are of great importance for high affinity and selectivity for 5-HT$_4$Rs.

Four compounds of this series **1d-g** [88] displayed subnanomolar affinity at central 5-HT$_4$Rs, high selectivity over other serotonin receptors (5-HT$_3$, 5-HT$_{2A}$, and 5-HT$_{1A}$), and potent 5-HT$_4$R antagonist activity in the guinea pig ileum (Table **4**).

Benzimidazolone derivatives have also been identified as classical 5-HT$_4$R ligands with moderate selectivity. Among them, the most interesting antagonist is DAU 6285 [121-123] (Figure **8**), which underwent preclinical investigation as an antiarrhythmic agent [124, 125] and in GI disorders [126]. However, no recent development has been reported for

Table 2. Affinities and Activities of 5-HT$_4$R Antagonists Derived from Tropisetron

Compound	pK_i		pA_2
	5-HT$_4$	5-HT$_3$	
 Tropisetron (ICS 205-930)	7.2[a] 6.0[b]	8.5[b]	6.5[c,d]
 SB 203186	7.2[e]	---	8.9[f,g,h]
 GR 113808	9.5[a]	6.0[i]	9.5[a,j]
 GR 125487	10.4[k]	---	10.0[j,k]
 SB 207266	9.3[l]	<6.0[l]	10.6[l,m]

[a]Ref. [25]. [b]Ref. [122]. [c]Ref. [20]. [d]Guinea pig ileum. [e]Ref. [24]. [f]Ref. [119]. [g]pK_B value. [h]Human heart atrium. [i]Ref. [110]. [j]Rat oesophagus. [k]Ref. [120]. [l]Ref. [113]. [m]Guinea pig distal colon.

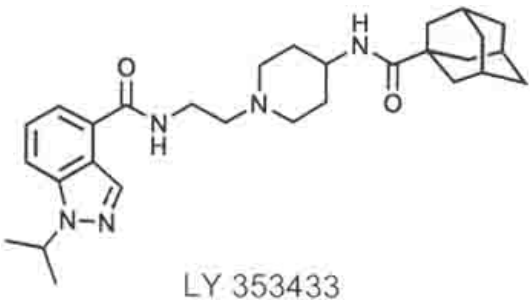

LY 353433

Fig. (6). Structure of LY 353433.

this compound. More recently, Orjales *et al.* [127] have described a series of benzimidazolones **3** as 5-HT$_4$R ligands (Figure **8**) with selectivity over 5-HT$_3$ and D$_2$ receptors. In these amides, 5-HT$_4$R functional activity is modulated by the substituent at position 3 of the aromatic ring (R) and the substituent on the piperazine ring (R'), and this trend seems to support the hypothesis of two different binding sites for 5-HT$_4$R agonists and antagonists, suggested by Langlois *et al.* [101].

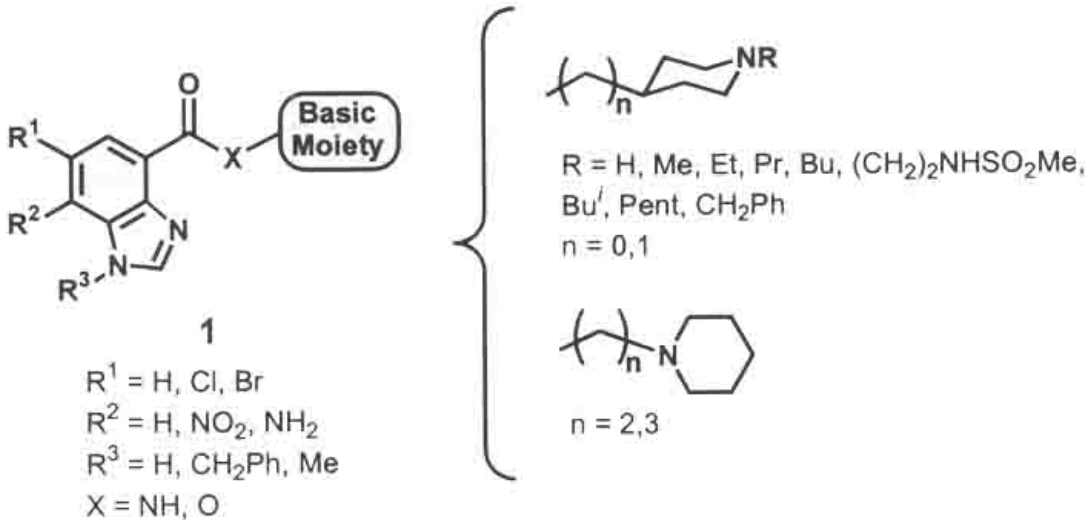


Fig. (7). Structure of benzimidazole derivatives **1**.

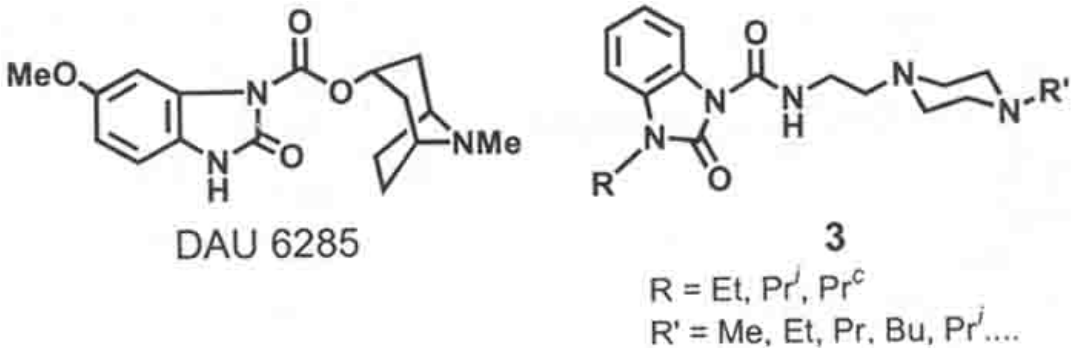
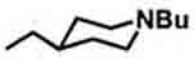
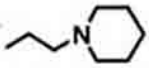
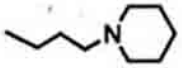


Fig. (8). Structures of benzimidazolones DAU 6285 and **3**.

QUANTITATIVE STRUCTURE-AFFINITY RELATIONSHIPS (QSAR)

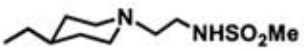
A three dimensional quantitative structure-affinity relationship (3D-QSAR) study using comparative molecular field analysis (CoMFA) has been successfully applied to explain the binding affinities for the 5-HT_4R of a series of benzimidazole-4-carboxamides and carboxylates **1** [89], in which the basic moiety is 1-alkyl-4-piperidyl or (1-alkyl-4-piperidyl)methyl subunits. The prototropic equilibrium

Table 3. Energy and Structural Parameters of Lowest Energy Conformers of Benzimidazole-4-Carboxamides 1 (X=NH, R^1=R^2=R^3=H)[a]

Comp.	Basic Moiety	Energy (Kcal.mol^{-1})	Ar-O[b] (Å)	Ar-N[c] (Å)	O-N[d] (Å)	h[e] (Å)
1a		97.88	3.74	7.82	4.38	0.07
UCM-21195		135.34	3.74	8.48	5.62	3.55
1b		150.11	3.75	7.49	4.48	1.42
1c		128.04	3.74	8.34	5.20	2.56

[a]Ref. [88]. [b]Distance between the centroid of the aromatic ring and the oxygen of the carbonyl group. [c]Distance between the centroid of the aromatic ring and the basic nitrogen of the amine. [d]Distance between the oxygen of the carbonyl group and the basic nitrogen of the amine. [e]Distance of the basic nitrogen with respect to the aromatic ring.

Table 4. Affinities and Activities of 5-HT$_4$R Antagonists 1 (X = NH, R^1 = Cl, R^2 = R^3 = H)

Comp.	Basic Moiety	K_i[a] (nM) 5-HT$_4$	K_i[a] (nM) 5-HT$_3$	pA$_2$[a,b]
1d		0.32	>1000	7.6
1e		0.11	>1000	7.9
1f		0.29	>1000	8.2
1g		0.54	>1000	7.9

[a]Ref. [88]. [b]Guinea pig ileum.

present in the benzimidazole ring was studied, by NMR and IR techniques and theoretical methods, in order to estimate the predominating tautomer of these molecules in solution [128]. Carboxamides incorporate tautomer I, in which an intramolecular hydrogen bond between the amide NH and the nitrogen of the benzimidazole ring is formed; and carboxylates incorporate tautomer II, in which the hydrogen bond is formed between the NH of the benzimidazole ring and the carboxylate group (Figure **9**). Biological data [128] evidence the importance of the tautomeric bias of carboxamides (I) and carboxylates (II) in their interaction with the 5-HT$_4$R (Table **5**). Thus, affinity for the 5-HT$_4$R decreased after *N*3-methylation of **1j** [K_i (**1j**) = 2.9 nM *vs* K_i (**1k**) = 241 nM], suggesting that the intramolecular hydrogen bond present in **1j** contributes to the coplanarity of the carbonyl group and the benzimidazole system, important elements for high affinity [82]. Consequently, *N*1-methylated analog **1i** still exhibits high 5-HT$_4$R affinity [K_i (**1i**) = 2.2 nM *vs* K_i (**1h**) = 0.29 nM]. Additional support to the proposed bioactive conformation was found upon examination of the 5-HT$_4$R affinity of benzimidazole-5-carboxamide **1l** (Figure **10**). Biological assays reveal this regioisomer is completely inactive (K_i > 1000 nM). The position of the amide moiety (C-5) in this derivative prevents the formation of an intramolecular hydrogen bond, which permits the tautomeric equilibrium at the benzimidazole ring, and allows the carbonyl group to be out of coplanarity with the aromatic benzimidazole system.

This study has provided insight into the *bioactive conformation* of this family of 5-HT$_4$R antagonists, which was used in the alignment of these compounds in the 3D-QSAR/CoMFA analysis [89] (Figure **11a**) illustrates the CoMFA electrostatic and steric maps using compound UCM 21195 as the reference structure. With respect to the aromatic moiety, the electrostatic map shows a high electron density favoring red shaded region at the 6-position of the benzimidazole ring, which is indicative of the favorable

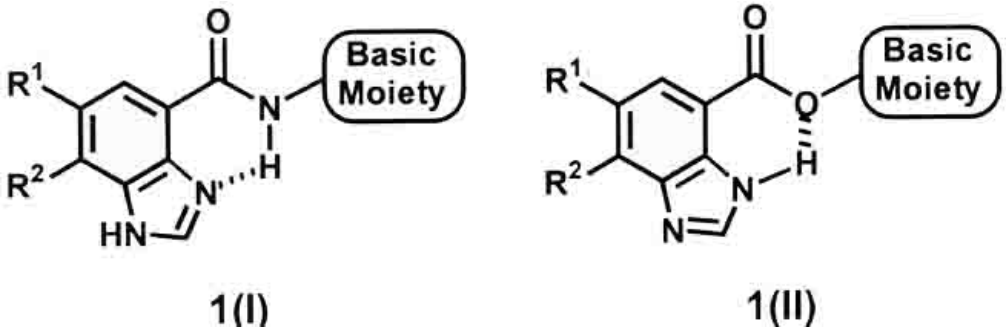


Fig. (9). Bioactive conformation of benzimidazole-4-carboxamides and carboxylates **1**.

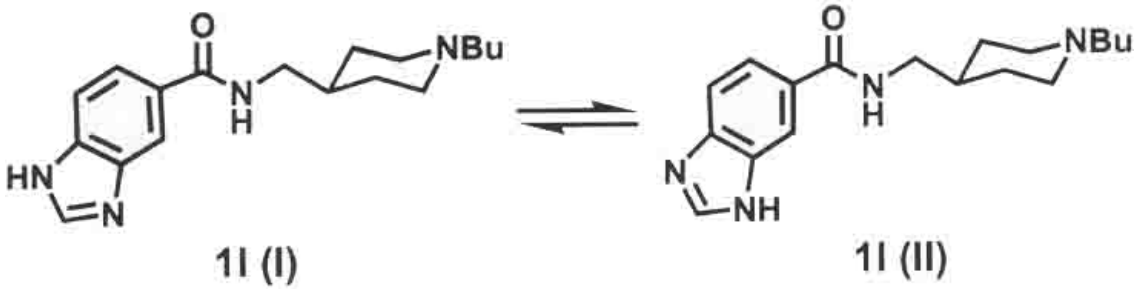
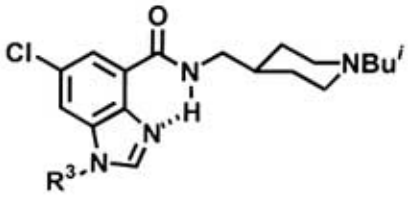
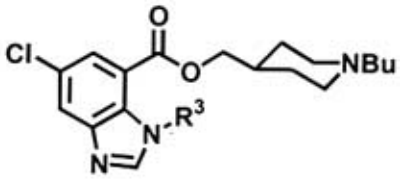


Fig. (10). Tautomeric equilibrium of benzimidazole-5-carboxamide **11**.

Table 5. 5-HT$_4$R Affinity Data of Benzimidazole Derivatives 1





Comp	R^3	K_i[a] (nM)
1h	H	0.29
1i	Me	2.2

Comp	R^3	K_i[a] (nM)
1j	H	2.9
1k	Me	241

[a]Ref. [128].

effect of electronegative substituents in this position. Steric contours depict also a small favorable (green) area in the aromatic 6-position and an unfavorable (yellow) region near the 7-position of the benzimidazole ring. In regard to the basic amino moiety, the electrostatic map shows a region in which positive charge enhances affinity (blue) near to the nitrogen atom. The steric map shows a large favorable (green) region of the terminus side chain. The occupation of this area by a bulky group will have positive effect on the biological affinity.

LIGAND-RECEPTOR INTERACTIONS: MOLECULAR MODELS OF G PROTEIN-COUPLED RECEPTORS (GPCRS)

The high resolution crystal structure of bovine rhodopsin [129] provides for the first time a detailed atomic description of a GPCR molecule in an inactive conformation. Rhodopsin and most likely the other rhodopsin-like GPCRs are formed by a highly organized heptahelical transmembrane (TM) bundle. This structure provides a solid basis for modelling the structures of other GPCRs, due to the conservation of functionally important sequence motifs within the transmembrane domain [130]. This structural homology does probably not extend to the extracellular domain, for which there is very little homology, and is highly structured in rhodopsin, blocking the access of the extracellular ligand to the core of the receptor [131]. Such models can then be used to help rationalize the many observations made on the relations between the structure of the ligand and the biological response.

The modification of the amino acid sequence of members of the GPCR family of receptors, using methods of molecular biology, is a common procedure to define the amino acid side chains of the receptor that form the ligand binding pocket (see GPCRDB in http://www.gpcr.org/7tm/ [132]). Recently, the ligand-binding site of the human 5-HT$_4$ has been explored by site-directed mutagenesis [133]. Serotonin anchors the conserved Asp$^{3.32}$ (see Visiers *et al.* [130] for receptor-numbering scheme), in TM 3, throughout its protonated amine, as revealed by the lack of binding affinity of serotonin to the Asp$^{3.32}$Asn point mutation (Table **6**). Surprisingly, the antagonist GR 113808 seems uninfluenced by this mutation (Table **6**), despite the finding that Asp$^{3.32}$ binds both agonists and antagonists, in the other members of the neurotransmitter family of receptors (see [134] for a discussion). Besides, substitution of Ser$^{5.43}$, in TM 5, by Ala avoids the binding of GR 113808 (Table **6**), suggesting a direct hydrogen bond with the carbonyl oxygen of the ester group of the ligand [133]. It has also been shown that the Asn$^{6.55}$Leu point mutation, in TM 6, abolishes the binding of serotonin (Table **6**). However, the influence of this mutation in the binding of the GR 113808 antagonist is not clear. Despite the observation that the single Asn$^{6.55}$Leu or Phe$^{6.52}$Val mutation moderately reduces the affinity for GR 113808, the double Phe$^{6.52}$Ala/Asn$^{6.55}$Leu mutation totally avoids the binding of GR 113808 to the 5-HT$_4$R (Table **6**). TM 6 possesses the Pro$^{6.50}$PhePhe motif in both the adrenergic and serotoninergic subfamilies of receptors. Replacement of Phe$^{6.51}$ by Ala abolishes the binding of the GR 113808 antagonist, suggesting a direct interaction (Table **6**).

Computational models (see Figure **11b-f**) of the transmembrane domain of the 5-HT$_4$R complexed with GR 113808 [134], and the benzimidazole-4-carboxamide UCM 21195 and the equivalent carboxylate UCM 26995 [89, 135] have recently been constructed from the crystal structure of rhodopsin and these putative residues of the ligand-binding site. The protonated amine of the ligands is located between Asp$^{3.32}$ and Phe$^{6.51}$ (Figure **11c** shows these interactions for GR 113808). The interaction is formed through the ionic pair between the N-H group of the protonated amine and the Oδ atom of Asp$^{3.32}$, and the electrostatic interactions between the electron-rich clouds of the aromatic ring of Phe$^{6.51}$ and the electron-poor hydrogens of the carbon atoms adjacent to the protonated nitrogen of the ligands. Besides, the carbonylic oxygen of the ligands is involved in the hydrogen bond to Ser$^{5.43}$ (see Figs. **11d** for GR 113808, **11e** for UCM 21195, and **11f** for UCM 26995). Additionally, Asn$^{6.55}$ hydrogen bonds either the ether oxygen of GR 113808 and UCM 26995, or the π electron clouds of the benzimidazole ring of UCM 21195. The aromatic ring of the ligands is positioned in the face-to-edge

orientation (T-shaped) to Phe$^{6.52}$ (GR 113808) or Tyr$^{5.38}$ (UCM 21195 and UCM 26995). Finally, the voluminous side chain of the ligands attached to the protonated nitrogen is pointing towards TM 7 (results not shown). It is important to note that the mode of binding of these ligands follows the proposed 5-HT$_4$ pharmacophore model [82] consisting on an aromatic moiety, a coplanar carbonyl group, a protonated nitrogen atom, and a voluminous substituent (see above).

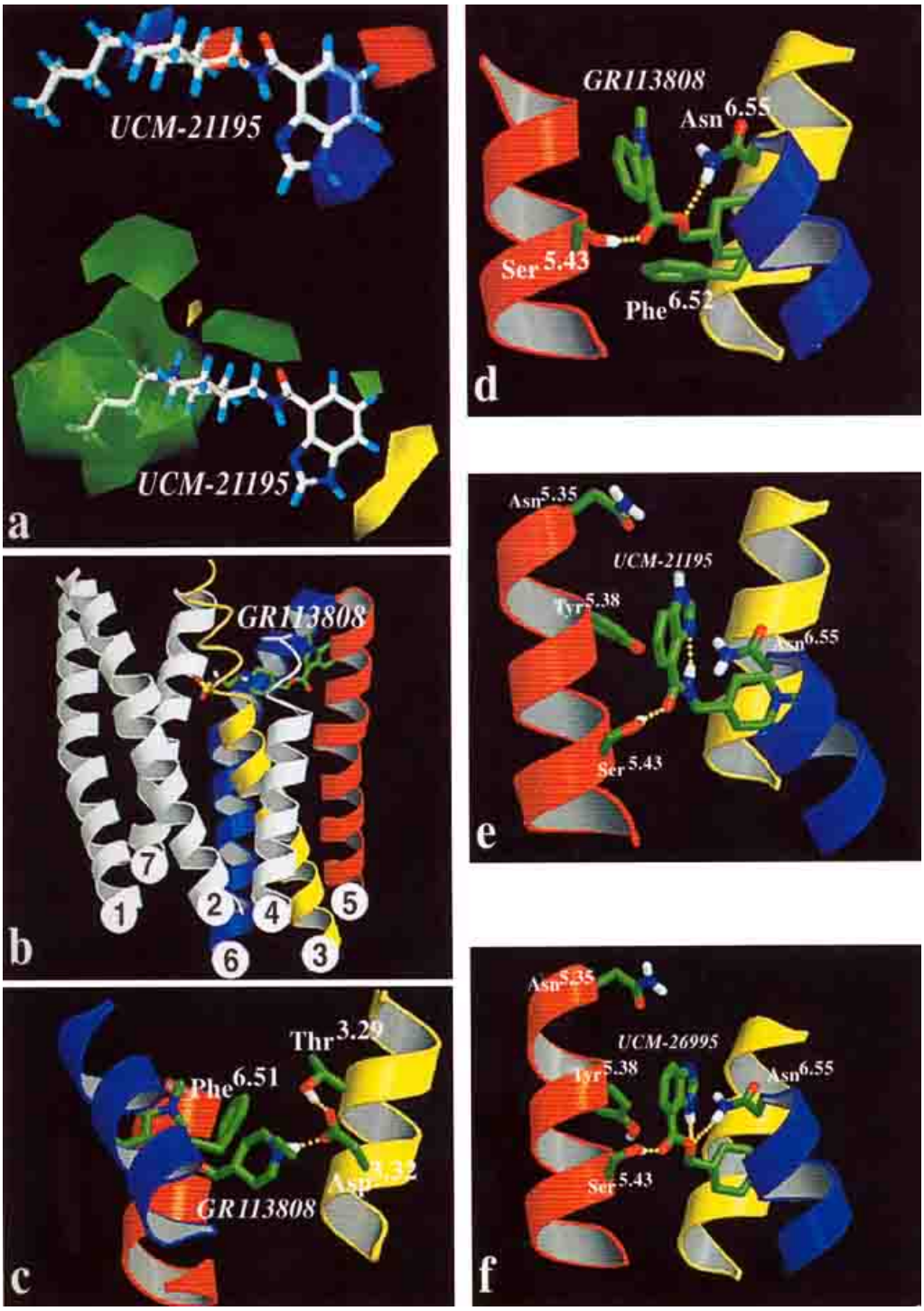


Fig. (11). a. Electrostatic and steric CoMFA maps showing contributions to 5-HT$_4$ affinity. The colour code is as follows: red denotes regions where positive charge is detrimental to affinity and blue denotes regions where positive charge enhances the affinity; yellow denotes regions where steric bulk is detrimental to affinity and green denotes regions where steric bulk enhances the affinity. **b.** Molecular model of the complex between GR 113808 and the transmembrane helix bundle of the human 5-HT$_4$R constructed from the crystal structure of rhodopsin [129], in a view parallel to the membrane. **c, d, e, f.** Detailed view of the transmembrane helix bundle of the 5-HT$_4$R complexed with GR 113808 (c, d), UCM 21195 (e), and UCM 26995 (f). The C_α traces of the extracellular part (top) of TM 3 (yellow), 5 (red), and 6 (blue) are shown.

Table 6. Results of Binding Experiments on Wild-Type and Mutant 5-HT$_4$Rs[a]

	K_D (nM) [^{3}H]GR 113808	Bmax fmoles mg^{-1} prot	K_i (nM) 5-HT
Wild type	0.23	850	772
Asp$^{3.32}$Asn	0.32	345	>100000
Ser$^{5.43}$Ala	no binding		
Cys$^{5.42}$Ala	0.07	436	378
Phe$^{6.51}$Ala	no binding		
Phe$^{6.52}$Val	0.45	828	695
Asn$^{6.55}$Leu	1.20	2270	>100000
Phe$^{6.52}$Val/Asn$^{6.55}$Leu	no binding		

[a]Ref. [133].

These results provide the tools for predicting the affinity of related compounds and for guiding the design and synthesis of new ligands with predetermined affinities and selectivity. These studies are now in progress and the results will be reported in due course.

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