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Comparative receptor mapping of serotoninergic 5-HT_3 and 5-HT_4 binding sites*

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Summary

The clinical use of currently available drugs acting at the 5-HT_4 receptor has been hampered by their lack of selectivity over 5-HT_3 binding sites. For this reason, there is considerable interest in the medicinal chemistry of these serotonin receptor subtypes, and significant effort has been made towards the discovery of potent and selective ligands. Computer-aided conformational analysis was used to characterize serotoninergic 5-HT_3 and 5-HT_4 receptor recognition. On the basis of the generally accepted model of the 5-HT_3 antagonist pharmacophore, we have performed a receptor mapping of this receptor binding site, following the active analog approach (AAA) defined by Marshall. The receptor excluded volume was calculated as the union of the van der Waals density maps of nine active ligands ($pK_i \geq 8.9$), superimposed in pharmacophoric conformations. Six inactive analogs ($pK_i < 7.0$) were subsequently used to define the essential volume, which in its turn can be used to define the regions of steric intolerance of the 5-HT_3 receptor. Five active ligands ($pK_i \geq 9.3$) at 5-HT_4 receptors were used to construct an antagonist pharmacophore for this receptor, and to determine its excluded volume by superimposition of pharmacophoric conformations. The volume defined by the superimposition of five inactive 5-HT_4 receptor analogs that possess the pharmacophoric elements ($pK_i \leq 6.6$) did not exceed the excluded volume calculated for this receptor. In this case, the inactivity may be due to the lack of positive interaction of the amino moiety with a hypothetical hydrophobic pocket, which would interact with the voluminous substituents of the basic nitrogen of active ligands. The difference between the excluded volumes of both receptors has confirmed that the main difference is indeed in the basic moiety. Thus, the 5-HT_3 receptor can only accommodate small substituents in the position of the nitrogen atom, whereas the 5-HT_4 receptor requires more voluminous groups. Also, the basic nitrogen is located at ca. 8.0 Å from the aromatic moiety in the 5-HT_4 antagonist pharmacophore, whereas this distance is ca. 7.5 Å in the 5-HT_3 antagonist model. The comparative mapping of both serotoninergic receptors has allowed us to confirm the three-component pharmacophore accepted for the 5-HT_3 receptor, as well as to propose a steric model for the 5-HT_4 receptor binding site. This study offers structural insights to aid the design of new selective ligands, and the resulting models have received some support from the synthesis of two new active and selective ligands: **24** (K_i(5-HT_3) = 3.7 nM; K_i(5-HT_4) > 1000 nM) and **25** (K_i(5-HT_4) = 13.7 nM; K_i(5-HT_3) > 10 000 nM).

Introduction

Since the discovery of the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) in 1948, its role has been associated with many central nervous system-related activities [1–3]. 5-HT receptors [4–6] can be divided into two major superfamilies: G-protein-coupled and ion channels. The former can be further characterized according to their second messenger system: adenyl cyclase-coupled (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_4, 5-HT_{5A},

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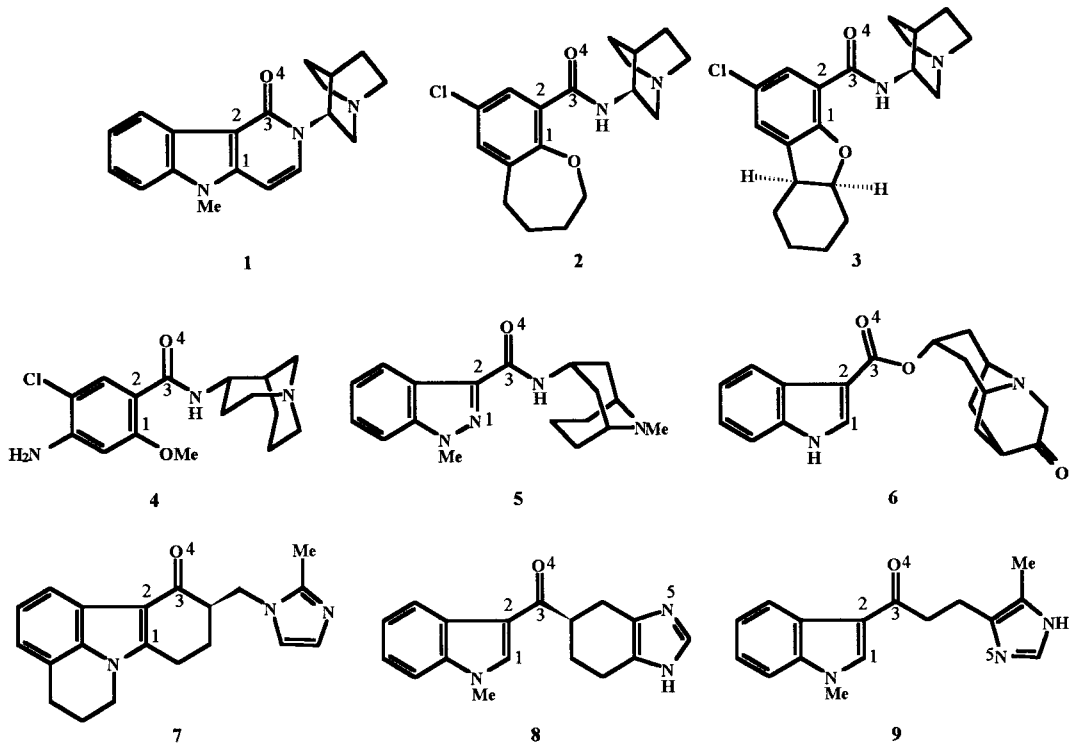


Fig. 1. Chemical structures of the antagonists used to define the 5-HT_3 receptor excluded volume ($pK_i \geq 8.9$).

5-HT_{5B}, 5-HT_6, 5-HT_7) or phosphoinositol-coupled (5-HT_{2A} – previously 5-HT_2 –, 5-HT_{2B} and 5-HT_{2C} – previously 5-HT_{1C} –). The 5-HT_3 receptor is unique in that it belongs to the superfamily of ion-channel receptors [7,8].

In recent years, most interest has been paid to the two sub-populations of the serotoninergic receptor: 5-HT_3 [9,10] and 5-HT_4 [11], due to their implication in various (patho)physiological processes, both periferally and cen-

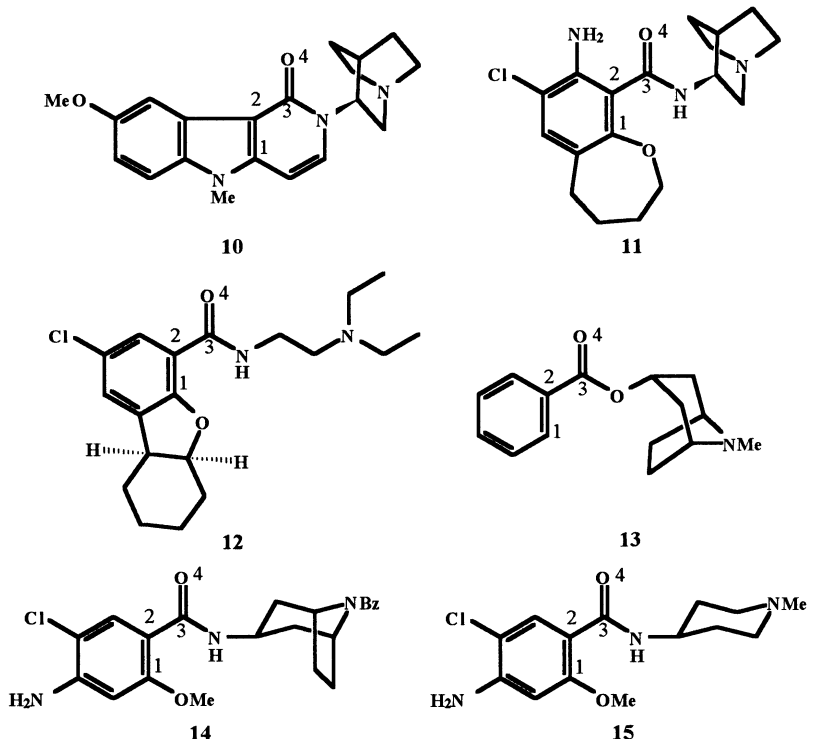


Fig. 2. Chemical structures of the inactive analogs used to define the 5-HT_3 receptor included volume ($pK_i < 7.0$).

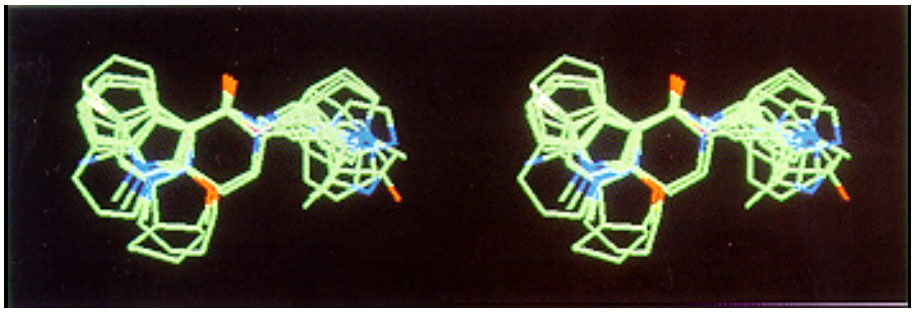


Fig. 3. Stereo representation of 5-HT_3 receptor antagonists superimposed in pharmacophoric conformations.

trally. Indeed, 5-HT_3 receptor antagonists (e.g. ondansetron and granisetron) are a novel class of therapeutic agents that are highly effective in the control of cancer chemotherapy emesis [12]. They are also promising in the control of central nervous system conditions, such as anxiety, schizophrenia, drug withdrawal and cognitive disorders [13]. On the other hand, the clinical use of currently available drugs acting at the 5-HT_4 receptor has been hampered by their lack of selectivity, and specific 5-HT_4 receptor antagonists could open the door to new therapies for the treatment of various pathophysiological disorders, such as IBS (irritable bowel syndrome) [14], arrythmias [15] and micturition disturbances [16].

As most of the known 5-HT_3 receptor antagonists also exhibit affinity for the 5-HT_4 subtype, there is considerable interest in the medicinal chemistry of both receptors and significant efforts have been made towards the discovery of potent and selective ligands. Several structure–affinity relationship and molecular modeling studies [17–20] have led to the definition of an accepted three-dimensional model of the 5-HT_3 antagonist pharmacophore. Although little is known about the 5-HT_4 receptor recognition site, it has been shown that 5-HT_3 and 5-HT_4 receptor ligands are structurally similar, although the selectivity is believed to depend upon the structure of the basic moiety [21–23].

In this paper, we have used computer-aided conformational methods to characterize serotoninergic 5-HT_3 and 5-HT_4 receptor recognition. We have applied the active analog approach (AAA) [24,25] as the modeling strategy to obtain the main steric differences between both receptor binding sites. The models have received some support from the synthesis of two new active and selective ligands.

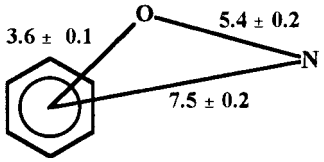


Fig. 4. Proposed pharmacophore model for 5-HT_3 receptor antagonists.

Methods

Computer-assisted conformational analysis and molecular modeling

The general methodology that has been applied for each studied receptor can be outlined as follows. (i) A set of compounds was selected from the literature, and the compounds were divided into two groups (active and inactive) based on their binding affinity at the target receptor. (ii) The pharmacophoric elements were defined. (iii) A conformational search of the active and inactive compounds was performed to determine their lowest energy conformations. (iv) Flexible superimposition of the low-energy conformations of active compounds was done to determine common pharmacophoric conformations, and to construct the receptor pharmacophore. (v) The *essential receptor volume* was evaluated after calculation of the *excluded* and *included receptor volumes*, in order to determine steric limitations in the binding site. All molecular modeling studies were carried out with a Silicon Graphics INDIGO2 computer and the multifaceted software package INSIGHT II [26], and implementation of each of these routines is described briefly below.

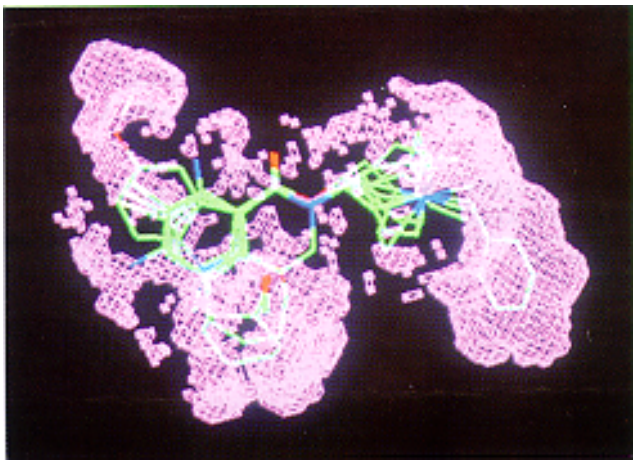


Fig. 5. Essential volume required by the 5-HT_3 receptor. Superimposition of inactive analogs. The volumes defined by the benzyl group of **14** and the methoxy group of **10** represent the main regions unavailable for drug interactions.

TABLE 1
BINDING CONSTANT DATA OF IN VITRO AFFINITY FOR THE 5-HT_3 RECEPTOR

Compound	pK_i	Ref.
1	10	36
2	8.9	37
3	9.8	38
4	9.1	39
5	9.1	39
6	9.8	9
7	9.7	39
8	10.5	40
9	9.5	39
10	6.5	36
11	< 7.0	37
12	< 7.0	38
13	6.5	9
14	< 6.0	41
15	6.6	41

(i) Evaluation of binding affinity. Since the identification of 5-HT_3 receptor binding sites in brain [27], a number of radioligands ([^{3}H]GR 65630 [27], [^{3}H]LY 278584 [28], ...) have been used to assess 5-HT_3 receptor affinity. There is relatively good consistency between the results obtained in the different in vitro binding assays. Thus, the compounds given in Table 1 were selected from the literature and divided into two groups, based on their in vitro affinity at 5-HT_3 receptors: active ($pK_i \geq 8.9$) and inactive ($pK_i < 7.0$). On the other hand, since the more recent discovery of the 5-HT_4 receptor, [^{3}H]GR 113808 is the most widely used radioligand for in vitro binding assays. The affinities of active ($pK_i \geq 9.3$) and inactive ($pK_i \leq 6.6$) analogs chosen for the 5-HT_4 receptor mapping are summarized in Table 2.

(ii) Pharmacophoric elements of 5-HT_3 and 5-HT_4 receptor antagonists. A number of pharmacophore models and alignments of antagonists binding to the 5-HT_3 receptor have been presented [17–20]. All of the previous models are in general agreement as to which pharmacophoric elements are important for significant binding affinity. Based on these models, the following key pharmacophoric elements were considered in the conformational analyses: an aromatic moiety, a carbonyl function, and a basic nitrogen atom. Although little is known about the 5-HT_4 receptor recognition site, it has been shown that 5-HT_3 and 5-HT_4 receptor ligands are structurally similar. Therefore, we have considered the same three structural features as the pharmacophoric elements considered for the molecular modeling of 5-HT_4 receptor antagonists. Thus, the task of the molecular modeling analysis was to identify a conformation for each compound where these key pharmacophoric elements were spatially arranged in a similar way for all active compounds.

TABLE 2
BINDING CONSTANT DATA OF IN VITRO AFFINITY FOR THE 5-HT_4 RECEPTOR

Compound	pK_i	Ref.
16	10	42
17	10.9	43
18	10	44
19	9.3	45
20	10.2	43
14	< 6.0	41
15	6.6	41
21	< 6.0	46
22	6	41
23	< 6.0	47

(iii) Lowest energy conformations. The molecules were built de novo in their protonated forms, which is believed to be the bioactive form, using the Builder module of INSIGHT II. Their geometry was optimized by using the Optimize option of this module, which combines three different minimization algorithms through the DISCOVER program (steepest descents, conjugate gradients, and the BFGS algorithm). A conformational search was performed on each compound to identify its lowest energy conformation(s). For this step, we applied a systematic conformational search routine as implemented in the Search and Compare module of INSIGHT II, assigning rotatable bonds and allowing them to rotate with a 15° stepwise increment of the dihedral angles (unless stated otherwise in the Results and Discussion section). We then found common low-energy conformations for all of the compounds. The commonality was assessed by comparing (i) the distances between the centroid of the aromatic ring, the oxygen of the carbonyl function and the basic nitrogen atom; (ii) the coplanarity of the carbonyl group with the aromatic ring; and (iii) the situation of the basic nitrogen with respect to this plane.

(iv) Construction of the pharmacophore. The most common low-energy conformations of the active molecules were subject to flexible superimposition in order to determine the pharmacophoric conformations. The structural features of the molecules that have been considered in the overlapping process are: the centroid of the aromatic ring supporting the carbonyl group, the vector

TABLE 3
CONFORMERS OF COMPOUND **1**

Conf.	Energy (kcal mol^{-1})	τ^a (°)	Ar–N^b (Å)	O–N^c (Å)	h^d (Å)
A	195.01	179.88	6.95	3.69	0.19
B	200.79	–179.3	7.35	5.33	0.27

[a] Dihedral angle defined by [C1-C2-C3-O4] (see Fig. 1).
[b] Distance between the centroid of the aromatic ring and the basic nitrogen of the amine.
[c] Distance between the oxygen of the carbonyl group and the basic nitrogen of the amine.
[d] Deviation of the basic nitrogen with respect to the aromatic ring.

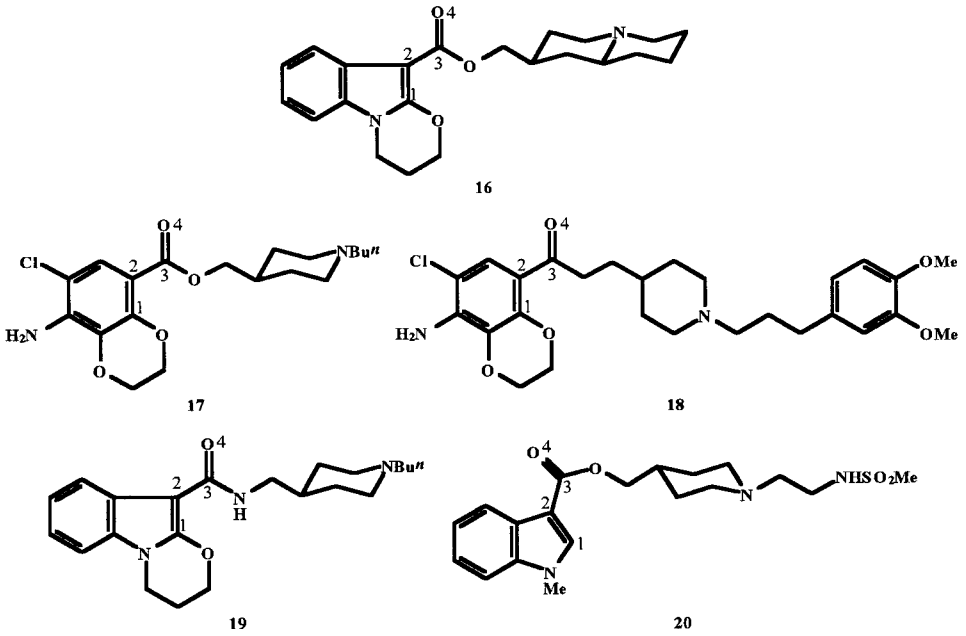


Fig. 6. Chemical structures of the antagonists used to define the 5-HT_4 receptor excluded volume ($pK_i \geq 9.3$).

corresponding to the carbonyl bond, and the nitrogen atom of the amino moiety. Flexible superimposition was performed using the Overlap routine as implemented in the INSIGHT II Search and Compare module. When the superimposition forced the molecule into high-energy conformations in order to obtain maximum similarity, a minimization was applied using the cvff force field as implemented in the DISCOVER program of INSIGHT II.

(v) Receptor mapping. The steric receptor mapping of each binding site involved the construction of pseudo-electron density maps for the compounds superimposed in pharmacophoric conformations, using the Volume routine as implemented in the Search and Compare module of INSIGHT II. The active ligands were superimposed in pharmacophoric conformations, and the van der Waals density map of this active supermolecule defined the receptor excluded volume, which represents the volume of the receptor site that is available for ligand binding. The essential feature of the AAA is a comparison of active and inactive molecules. A commonly accepted hypothesis to explain the lack of activity of inactive molecules pres-

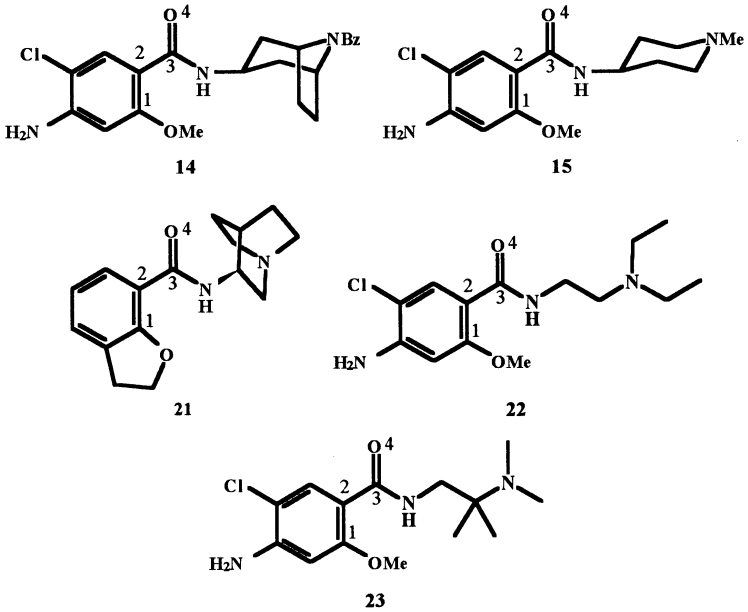


Fig. 7. Chemical structures of the compounds used to define the combined volume of 5-HT_4 receptor inactive ligands ($pK_i \leq 6.6$).

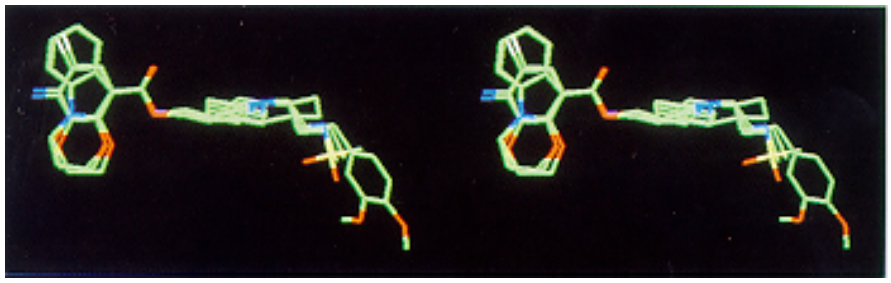


Fig. 8. Stereo representation of 5-HT_4 receptor antagonists superimposed in pharmacophoric conformations.

enting the pharmacophoric requirements is that their molecular volume (termed included volume) exceeds the receptor excluded volume. This additional volume, termed the receptor essential volume, is apparently filled by the receptor and unavailable for ligand binding. Following this approach, the receptor included volume was evaluated by superimposition of the inactive analogs, and the construction of the pseudoelectron density map of the resulting inactive supermolecule. The active and inactive supermolecules were then superimposed along the same four points mentioned in (iv), and the receptor essential volume was calculated by subtracting from the included volume the common regions with the excluded volume.

These receptor mapping techniques supplied topographical data that allowed us to confirm the steric model of the 5-HT_3 receptor site and to propose a steric model for the 5-HT_4 receptor site.

Results and Discussion

Conformational analysis of 5-HT_3 receptor antagonists

The compounds selected for the 5-HT_3 receptor mapping were classified as active or inactive based on their in vitro receptor binding constant data from the literature (see Table 1). The structures of the active ($pK_i \geq 8.9$) and inactive ($pK_i < 7.0$) analogs that were used in the molecular modeling analysis are shown in Figs. 1 and 2, respectively. In order to identify the lowest energy conformations of the compounds, we carried out a computerized conformational analysis. Analog **1** was chosen as the template compound for the determination of pharmacophoric conformations because of its high affinity at the 5-HT_3 receptor ($pK_i = 10.0$) and its conformationally restricted structure, which considerably reduces the number of low-energy conformations. Thus, we initially performed a systematic conformational search on the most rigid active ligand, **1**, allowing its rotatable bond to rotate with an increment of 1°. Two low-energy conformations were found within a window of 10 kcal mol^{-1} above the minimum energy (Table 3). Conformer B was chosen as the pharmacophoric conformation, since the distance (O-N) between the oxygen of the carbonyl group and the basic nitrogen is approximately 5 Å in previously reported models of the 5-HT_3 receptor pharmacophore [18–20]. The geometry of all other compounds was optimized and the resulting low-energy conformations were compared to the template (conformer B of ligand **1**) for maximum similarity, according to the following structural parameters: τ, dihedral angle defined by [C1(N1)-C2-C3-O4] (see Figs. 1 and 2); Ar-O, distance between the centroid of the aromatic ring and the oxygen of the carbonyl group; Ar-N, distance between the centroid of the aromatic ring and the nitrogen atom of the amino moiety; O-N, distance between the oxygen of the carbonyl group and the nitrogen atom of the amino moiety; and h, deviation of the nitrogen atom with respect to the aromatic plane. In most of the ligands, the coplanarity of the carbonyl group with the aromatic or heteroaromatic ring involved two low-energy conformations: $\tau \simeq 0°$ or $\tau \simeq 180°$. We have consid-

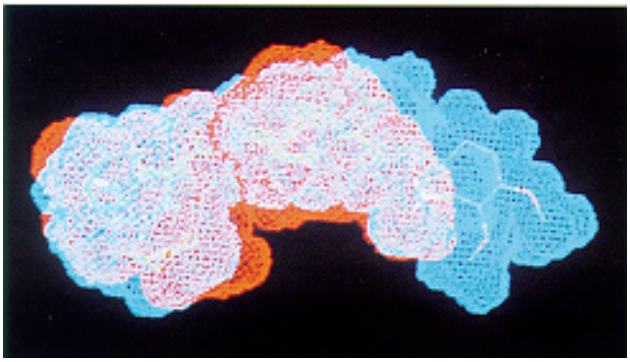


Fig. 9. 5-HT_4 receptor excluded volume (blue) and combined volume of 5-HT_4 receptor inactive analogs (red). Superimposition of active ligands.

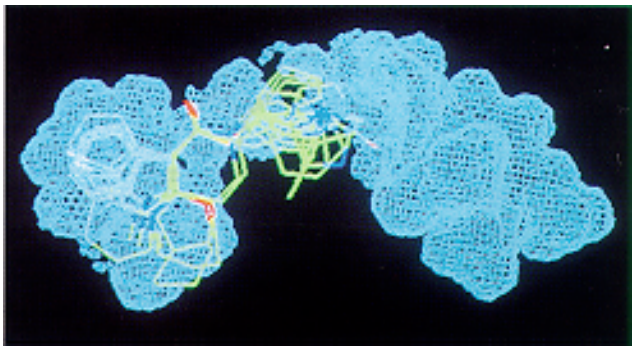


Fig. 10. 'Difference volume': subtraction of 5-HT_3 and 5-HT_4 receptor excluded volumes. Superimposition of 5-HT_3 receptor active ligands.

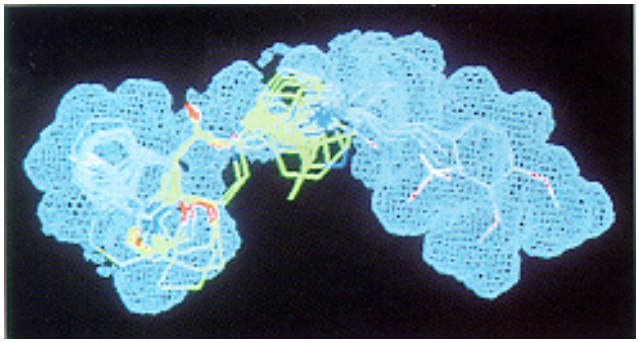


Fig. 11. 'Difference volume': subtraction of 5-HT_3 and 5-HT_4 receptor excluded volumes. Superimposition of 5-HT_4 receptor active ligands.

ered the conformation with $\tau \simeq 180°$, supported by the high affinity of the conformationally restricted ligand **1** (Fig. 1). This orientation also allows the existence, in some ligands, of an intramolecular hydrogen bond between the NH of the amide group and the oxygen situated at the *ortho* position of the benzene ring. On the other hand, in those compounds with the carbonyl group linked to an indole or indazole ring, the conformation with $\tau \simeq 180°$, i.e. with a transoid orientation of the C=O bond relative to the indole C1-C2 or indazole N1-C2 bond, was chosen since an additional ring constraint forced the carbonyl group of potent compounds, such as ondansetron [29] and ligand **7** (cilansetron, Fig. 1) to maintain a transoid conformation. Concerning the compounds with a quinuclidine moiety [**1**–**3** (Fig. 1), **10** and **11** (Fig. 2)], the asymmetric atom was oriented in the *S* configuration, since this enantiomer has been shown to be more potent than the corresponding *R* configuration [30,31]. The N-substituents of compounds containing a [3.2.1] or [3.3.1] azabicyclo system (**5**, **13** and **14**) were oriented in an equatorial position, as it is the preponderant orientation in DMSO-d_6 solution and in the solid state conformation of similar 5-HT_3 antagonists [32], shown by NMR studies and X-ray analysis, respectively.

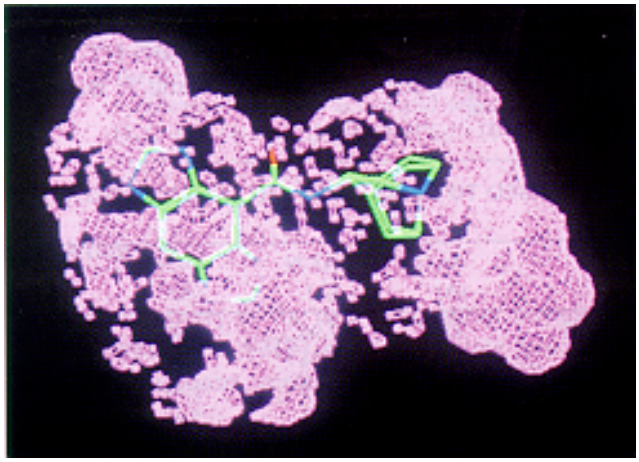


Fig. 13. Fitting of compound **24** (conformations I and II) in the 5-HT_3 receptor cavity.

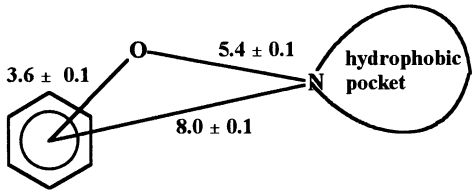


Fig. 12. Proposed pharmacophore model for 5-HT_4 receptor antagonists.

Having determined the lowest energy conformation(s) of each compound, the 5-HT_3 pharmacophoric conformation of each molecule was determined by flexible superimposition with the template compound (conformer B of ligand **1**). The structural determinants considered in the fitting process are: the centroid of the aromatic ring supporting the carbonyl group, the extremities of the carbonyl bond, and the nitrogen of the amino moiety. The superimposition of analogs **8** and **9** was only achieved with N5 (Fig. 1), whereas the best fitting for ligand **7** was found with the centroid defined by both nitrogens of the imidazole ring. As demonstrated by previous work [33], the lowest energy conformations are not necessarily the active forms of a given ligand, since the binding process of a ligand to its receptor may involve compensation for the required increase in conformational energy. Therefore, it seems reasonable to consider that for all the molecules studied, it was possible to find stable conformations ($\Delta E \leq 8.7$ kcal mol^{-1}) where these points were superimposed with an rms (root mean square) deviation ≤ 0.4 (Table 4, Fig. 3). The parameters used to define the 5-HT_3 antagonist pharmacophore, summarized in Table 4, included distances between pharmacophoric atoms (Ar-O, Ar-N, O-N), the coplanarity of the carbonyl group and the aromatic moiety (defined by τ [C1(N1)-C2-C3-O4]), and the situation of the basic nitrogen with respect to this plane (defined by h). From these geometric calculations, it appears that 5-HT_3 receptor antagonists can share a pharmacophore (Fig. 4) made up of the following structural features: an aromatic moiety, a coplanar carbonyl function (with the oxygen atom situated at an average distance of 3.6 ± 0.1 Å from the centroid of the aromatic

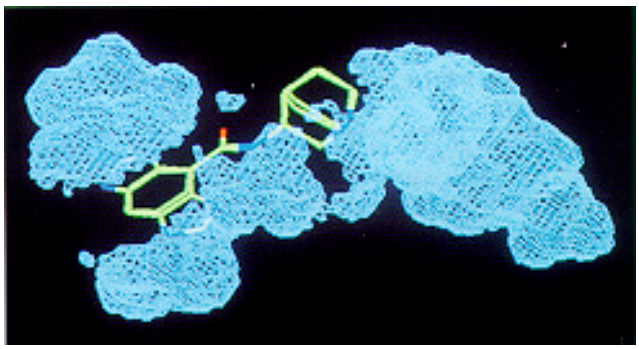


Fig. 14. Superimposition of compound **24** (conformations I and II) on the difference volume of 5-HT_3 and 5-HT_4 receptors.

TABLE 4
ENERGY AND STRUCTURAL PARAMETERS OF THE CONFORMATIONS USED TO DEFINE THE 5-HT_3 ANTAGONIST PHARMACOPHORE

Compound	Energy (kcal mol^{-1})	ΔE[a] (kcal mol^{-1})	Rms[b]	τ[c] (°)	Ar–O[d] (Å)	Ar–N[e] (Å)	O–N[f] (Å)	h[g] (Å)
1	200.79	5.78	0	−179.30	3.58	7.35	5.33	0.27
2	188.39	4.69	0.06	−179	3.71	7.51	5.3	0.59
3	181.12	3.04	0.06	178.45	3.73	7.51	5.33	0.34
4	135.12	4.3	0.21	−160.2	3.71	8.02	5.68	0.74
5	99.77	0.02	0.09	176.55	3.56	7.35	5.57	1.44
6	153.9	2.94	0.08	−169.8	3.58	7.36	5.36	0.35
7	92.88	2.96	0.42	179.1	3.57	7.12	5.37	1.91
8	161.15	0.05	0.19	−176.2	3.56	7.54	5.45	1.72
9	69.47	8.7	0.22	177.08	3.56	7.51	5.09	0.49

[a] Increment of energy with respect to the lowest energy conformation.
[b] Rms deviation resulting from superimposition with template compound **1**.
[c] Dihedral angle defined by [C1(N1)-C2-C3-O4] (see Fig. 1).
[d] Distance between the centroid of the aromatic ring and the oxygen of the carbonyl group.
[e] Distance between the centroid of the aromatic ring and the basic nitrogen of the amine.
[f] Distance between the oxygen of the carbonyl group and the basic nitrogen of the amine.
[g] Deviation of the basic nitrogen with respect to the aromatic ring.

ring), and a basic nitrogen atom (situated at an average distance of 5.4 ± 0.2 Å from the oxygen of the carbonyl group, and 7.5 ± 0.2 Å from the centroid of the aromatic ring). This nitrogen atom is almost aligned in the same plane as the aromatic ring (h = 0.3–1.9 Å). Our pharmacophore model for 5-HT_3 receptor antagonists confirms those previously reported by Hibert [18] and Evans [20].

5-HT_3 receptor mapping

Once the pharmacophoric conformations had been determined, the volume available for binding at the receptor site could be mapped. The receptor excluded volume, which represents the volume that is available to drugs interacting with the receptor, was calculated as the van der Waals density map of the supermolecule represented in Fig. 3, resulting from the superimposition of the nine active ligands in Fig. 1.

By defining the shape of the receptor binding pocket as the complement to the common volume of the active ligands, the receptor regions responsible for recognition and molecular stabilization are naturally positioned in three-dimensional space. This is critical in defining the receptor topography and modeling the active site. The 5-HT_3 receptor site topography was envisioned further by the spatial information described by the inactive molecules. The inactive analogs were superimposed in order to calculate the receptor included volume. Even though these molecules possess the pharmacophoric elements, their inability to be active is likely due to some part of the molecule interacting with the receptor intolerable regions. The essential volume, which represents the regions that are required by the receptor and may not be available for drug interactions, was determined by subtracting from the included volume the common regions with the excluded volume. This receptor essential volume (Fig. 5) can be used to define the 5-HT_3 receptor regions of steric intolerance, here represented by the volumes defined by the methoxy group of compound **10** and the benzyl group of compound **14**.

Conformational analysis of 5-HT_4 receptor antagonists

The chemical structures of the active ($pK_i \geq 9.3$) and inactive ($pK_i \leq 6.6$) analogs selected for the 5-HT_4 receptor mapping are represented in Figs. 6 and 7. Based on

TABLE 5
ENERGY AND STRUCTURAL PARAMETERS OF THE CONFORMATIONS USED TO DEFINE THE 5-HT_4 ANTAGONIST PHARMACOPHORE[a]

Compound	Energy (kcal mol^{-1})	ΔE (kcal mol^{-1})	Rms[b]	τ (°)	Ar–O (Å)	Ar–N (Å)	O–N (Å)	h (Å)
16	122.92	2.56	0.00	−176.20	3.58	7.96	5.46	3.72
17	156.65	2.61	0.03	−178.99	3.68	8.14	5.48	3.63
18	209.39	8.06	0.07	174.99	3.69	8.13	5.34	3.93
19	133.27	4.47	0.01	−176.95	3.57	7.95	5.46	4.02
20	116.29	2.71	0.01	−175.35	3.58	7.94	5.46	3.88

[a] See definitions in the footnotes of Table 4.
[b] Rms deviation resulting from superimposition with compound **16**.

TABLE 6
ENERGY AND STRUCTURAL PARAMETERS OF THE NEW DESIGNED COMPOUNDS **24** AND **25**[a]

Compound	Energy (kcal mol^{-1})	ΔE (kcal mol^{-1})	Rms	τ (°)	Ar–O (Å)	Ar–N (Å)	O–N (Å)	h (Å)
24-I	181.14	4.47	0.08[b]	174.63	3.75	7.45	5.34	0.01
24-II	190.25	6.05	0.03[b]	1.18	3.77	7.55	5.3	0.38
25-I	135.39	0.25	0.13[c]	–178.61	3.74	8.5	5.52	3.21
25-II	141.62	0.33	0.12[c]	–2.91	3.77	8.51	5.53	3.31

[a] See definitions in the footnotes of Table 4.
[b] Rms deviation resulting from superimposition with template compound **1**.
[c] Rms deviation resulting from superimposition with compound **16**.

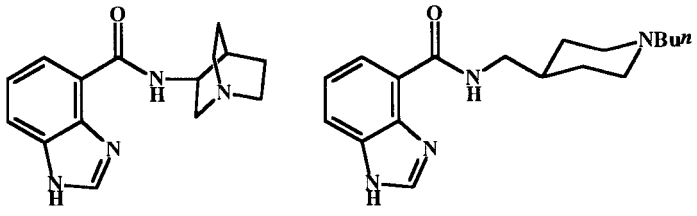
the structural similarity of 5-HT_3 and 5-HT_4 receptor ligands, we have considered the same three structural features as the pharmacophoric elements considered for the molecular modeling of 5-HT_3 receptor ligands: an aromatic moiety, a carbonyl group, and a basic nitrogen atom. Due to the flexibility of the molecules, it was a difficult task to choose the template compound. Therefore, the geometry of the five active compounds (**16**–**20**) was optimized considering only conformations with $\tau \simeq 180°$, and the resulting low-energy conformations were superimposed allowing rotation of the two bonds between the carboxamide or carboxylate group and the amino moiety (see Fig. 6). The flexible superimposition was performed considering the following points: the centroid of the aromatic ring supporting the carbonyl group, the vector defined by the carbonyl group and the basic nitrogen atom. Then, a systematic conformational search was performed on the resulting propharmacophoric conformations of molecules **17**–**20** about the flexible bonds of the N-substituent with a 30° step of the dihedral angles. Of the multiple conformers resulting from this search, for each compound we chose as the pharmacophoric conformation the one that best matched the alkyl chain with the quinolizidine ring of **16**. Thus, the N-substituent of the amino moiety was oriented to occupy the same cavity in the receptor. From this analysis, for all the active ligands studied it was possible to find stable pharmacophoric conformations ($\Delta E \leq 8.06$ kcal mol^{-1}) where the four points mentioned above were superimposed with an rms deviation ≤ 0.07 (Table 5, Fig. 8). The structural features that define the 5-HT_4 antagonist pharmacophore, summarized in Table 5, are an aromatic moiety, a coplanar carbonyl function (with the oxygen situated at an average distance of 3.6 ± 0.1 Å from the centroid of the aromatic ring), and a basic nitrogen atom (situated at an average distance of 5.4 ± 0.1 Å from the oxygen of the carbonyl group and 8.0 ± 0.1 Å from the centroid of the aromatic ring). In this case, the nitrogen atom seems to deviate from the plane of the aromatic moiety (h = 3.6–4.0 Å). To our knowledge, this is the first model reported for the 5-HT_4 antagonist pharmacophore.

5-HT_4 receptor mapping

In order to evaluate the steric limitations of the 5-HT_4 binding site, we mapped its topography. The pharmacophoric conformations of the five ligands represented in Fig. 6 were superimposed, and the van der Waals volume of this supermolecule (Fig. 8) was calculated to determine the receptor excluded volume. The van der Waals volume defined by the supermolecule resulting from superimposing the five inactive analogs in Fig. 7 did not exceed the excluded volume calculated for the receptor, as shown in Fig. 9. In this case, the inactivity may be due to the lack of positive interaction of the amino moiety with a hypothetical hydrophobic pocket, which would interact with the voluminous substituents of the basic nitrogen of active ligands.

Comparative analysis of 5-HT_3 and 5-HT_4 receptor cavities

From the comparison of both serotoninergic receptor excluded volumes, it is observed that the steric requirements of the aromatic moiety are very similar. However, the subtraction of the common elements, termed the 'difference volume' (Figs. 10 and 11), shows that the main steric difference is indeed in the basic moiety of the molecule, in particular, in the region occupied by the substi-



24 **25**

Scheme 1. Structures of compounds **24** and **25**.

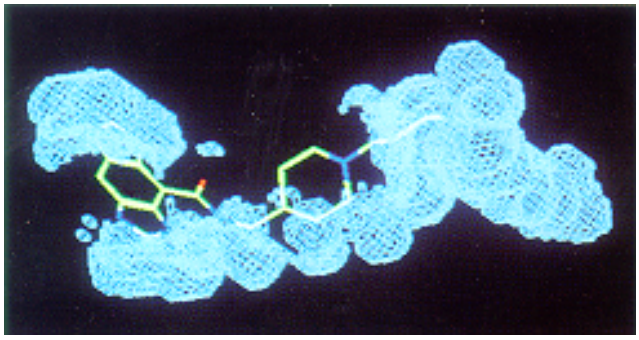


Fig. 15. Superimposition of compound **25** (conformations I and II) on the difference volume of 5-HT_3 and 5-HT_4 receptors.

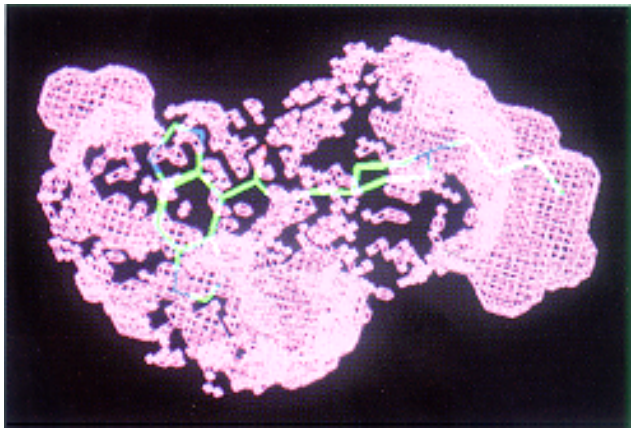


Fig. 16. Intolerable steric interaction of compound **25** (conformations I and II) with the 5-HT_3 receptor essential volume.

tuent of the basic nitrogen. Thus, the 5-HT_3 receptor can only accommodate small substituents in the position of the nitrogen atom, whereas the 5-HT_4 receptor requires more voluminous groups. Also, the basic nitrogen is located at ca. 8.0 Å from the aromatic moiety in the 5-HT_4 pharmacophore, whereas this distance is ca. 7.5 Å in the 5-HT_3 model (see Tables 4 and 5).

The comparative mapping of both serotoninergic receptors has allowed us to confirm the three-component pharmacophore for antagonists at the 5-HT_3 receptor, which is consistent with that proposed earlier by Hibert [18] and Evans [20], as well as to propose a model for the 5-HT_4 receptor binding site. This model would consist 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 hydrophobic accessory region in the basic amino framework of the molecule (Fig. 12). The interaction or lack of interaction with this hydrophobic pocket would be responsible for 5-HT_4/5-HT_3 or 5-HT_3/5-HT_4 selectivity, respectively. *N*-(3-Quinuclidinyl)benzimidazole-4-carboxamide (**24**) is a potent and selective 5-HT_3 receptor ligand recently reported by us [K_i(5-HT_3) = 3.7 nM; K_i(5-HT_4) > 1000] [34]. The perfect fitting of this new ligand in the 5-HT_3 receptor cavity (Fig. 13) and the lack of interaction with the hydrophobic region of the difference volume (Fig. 14) give some support to this model. In order to test the 5-HT_4 receptor model, compound **25** was designed, which retains the benzimidazole ring and the carboxamide group of **24**, but replaces the quinuclidine cycle for a (1-butyl-4-piperidyl)methyl moiety. A check prior to synthesis indicated a positive steric interaction of the *n*-butyl group with the hydrophobic pocket of the difference volume, and an intolerable steric interaction with the essential volume required by the 5-HT_3 receptor, as depicted in Figs. 15 and 16, respectively. The determination of pharmacophoric conformations for analogs **24** and **25** was achieved according to the method described for the rest of the ligands in the Methods section. For each compound, two pharmacophoric conformations were considered (I: $\tau \simeq 0°$ and II: $\tau \simeq 180°$), since both conformers may present an intramolecular hydrogen bond between the benzimidazole and the amide moieties. The structural parameters of these conformers are given in Table 6. As is shown, compounds **24** and **25** verify our proposed pharmacophoric models for 5-HT_3 and 5-HT_4 antagonists, respectively.

Compound **25** was synthesized and evaluated for binding affinity at 5-HT_3 [28] and 5-HT_4 [35] receptors: K_i(5-HT_4) = 13.7 nM; K_i(5-HT_3) > 10 000 nM. Thus, [*N*-(1-butyl-4-piperidyl)methyl]benzimidazole-4-carboxamide (**25**) is a new active and selective 5-HT_4 receptor lead compound, and its design and synthesis have given additional support to our proposed 5-HT_4 receptor model.

Conclusions

The structural and biological properties of a variety of known compounds were used in the present study to map the topography of 5-HT_3 and 5-HT_4 receptors. Together, the results were employed to generate a three-dimensional steric model for the 5-HT_4 receptor binding site, as well as to confirm the models previously reported for the 5-HT_3 receptor. The proposed models offer structural insights to aid the design of new 5-HT_3 and 5-HT_4 receptor ligands and provide a frame of reference to assess their selectivity prior to their synthesis. Thus, the comparative receptor mapping has allowed us to design the novel and selective 5-HT_4 receptor ligand **25**, which represents the lead compound of a structurally new class of 5-HT_4 receptor ligands.

Further studies based on compounds with totally rigid amino moieties are in progress in order to determine the size of the hydrophobic pocket, and to test our hypothesis. Also, determination of pharmacological properties of **24**, and synthesis of new analogs of **24** as well as related derivatives of **25** are currently in progress in our laboratory. The results will be reported in due course.

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