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Structure–affinity relationship studies on benzotriazole derivatives binding to 5-HT receptor subtypes

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Summary — A number of benzotriazole derivatives have been assayed in radioligand binding experiments involving the following recombinant human serotonin receptors: 5-HT_{2A}, 5-HT_{1A}, 5-$HT_{1D\beta}$ and 5-HT_{2C}. Several of the compounds tested show interesting selectivity profiles. In particular, the affinities of **3d**, **4a** and **4d** for the 5-HT_{2A} subtype (with pK_i values of 7.4, 7.4 and 8.0, respectively) are between 100 and 1000 times higher than for the other investigated receptors. Compound **5l**, characterized by a pK_i value of 7.4 on the 5-HT_{1A} receptor, binds with 100- to 1000-fold lower potencies on the other receptors. Our benzotriazole derivatives are generally weak ligands of the 5-$HT_{1D\beta}$ and 5-HT_{2C} receptors. Structure–affinity relationship data suggest that not all the compounds exhibit the same binding mode at the 5-HT_{2A} and 5-HT_{1A} receptors.

benzotriazole / 5HT-receptor binding / SAR

Introduction

An increasing amount of evidence indicates that serotonin (5-HT) is involved in the regulation of several physiological functions including affective behavior, sleep, food intake, sexual behavior, memory and thermoregulation [1]. Moreover, a dysregulation of the serotonergic system is thought to underly the pathogenesis of psychiatric illnesses such as depression, anxiety and obsessive compulsive disorders [2–4]. 5-HT probably attains such a variety of functions by acting on distinct receptor subtypes, most of which have been sequenced and cloned [5–7]. The availability of compounds that act on various 5-HT receptor subtypes has allowed progress in the understanding of the relative contribution of these receptors in the control of various aspects of behavior. As an example, 5-HT_{1A} receptor agonists stimulate locomotor activity and increase food intake, whereas 5-HT_{2C} agonists cause hypolocomotion and reduce eating [8–11]. Recently, 5-HT_{2C} receptor antagonists have been proposed as potential anxiolytic agents [12], whereas 5-HT_{2A} blockers might be helpful in the treatment of psychotic disorders [13].

However, most of the serotonergic agents used in pharmacological tests are not selective enough to permit an accurate investigation on the role played by the various 5-HT receptors. The lack of selectivity is probably due to structural similarities between 5-HT receptor subtypes. This fact is particularly evident in the case of 5-HT_{2A} and 5-HT_{2C} receptors which have about 80% sequence homology within the seven transmembrane domains [14]. Thus, it is important to develop new compounds with high affinity and selectivity for the various 5-HT receptors. This research strategy could increase the number of compounds with potential clinical usefulness for the treatment of various psychiatric disorders. Moreover, acquisition of data regarding the structure–affinity relationships for 5-HT receptor ligands will allow a more rational approach to the design of new compounds targeted selectively at 5-HT receptor subtypes.

We have recently reported [15, 16] the synthesis and the preliminary pharmacological screening of a series of benzotriazole derivatives (**1a–h**, **2a–h**, **3a–h**, **4a–h**, **5a–e** and **5g,h** in table I) designed as structural analogues of trazodone [17] (fig 1). The promising

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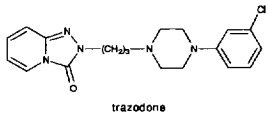


Fig 1. Structural formula of trazodone.

results of these investigations aroused our interest in an accurate evaluation of the same set of molecules in radioligand binding experiments involving 5-HT receptors. In the present study, recombinant 5HT receptor subtypes (5-HT_{2A}, 5-HT_{1A}, 5-$HT_{1D\beta}$ and 5-HT_{2C}) are used to gain information about structure–affinity relationships for the previously reported benzotriazoles [15, 16] and a number of newly prepared analogues.

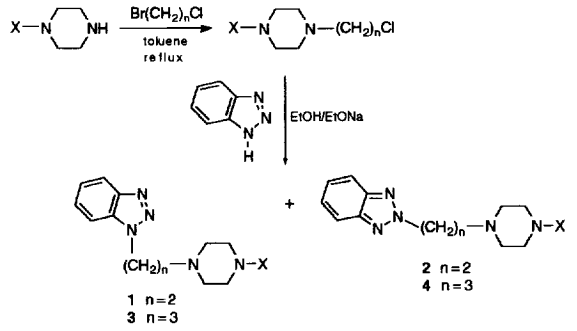
The use of recombinant receptor subtypes presents two major advantages over conventional studies in which animal tissues are used as sources of receptors: (i) a single subtype of receptor is selectively expressed in membranes from transfected cells; and (ii) affinity–selectivity data relative to human receptors are obviously more relevant from a clinical point of view. The latter is an important issue because there are examples of dramatic species-specific differences in the binding properties of the same receptor subtype, such as 5-$HT_{1D\beta}$/5-HT_{1B} or β_3-adrenergic receptors.

Chemistry

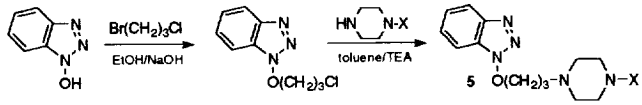
The synthetic route used to prepare the benzotriazole derivatives listed in table I involves two steps of nucleophilic substitution with yields ranging between 20 and 70% (schemes 1 and 2).

Scheme 1 outlines the steps for the preparation of 1- and 2-{2-[4-(*X*)-1-piperazinyl]ethyl}benzotriazoles (**1a–l** and **2a–l**) and 1- and 2-{3-[4-(*X*)-1-piperazinyl]-propyl}benzotriazoles (**3a–l** and **4a–l**). Compounds **1a–h**, **2a–h**, **3a–h** and **4a–h** have been reported in our previous articles [15, 16], whereas derivatives **1i,l**, **2i,l**, **3i,l** and **4i,l** were newly synthesized. Compounds **1i**, **2i**, **3i** and **4i**, which were described independently by Mokrosz et al [18] during the preparation of this paper, were synthesized according to the same general procedure for preparation as compounds **1l**, **2l**, **3l** and **4l** reported in the *Experimental protocols* (the physicochemical data of **1i**, **2i**, **3i** and **4i** are omitted in this section).

The 1-(2- and 4-methoxyphenyl)piperazines were usually commercially available, whereas the *N*-phenylethylpiperazine was prepared by reaction of piperazine with 2-bromoethylbenzene according to a procedure described in our previous paper [15].



Scheme 1.



Scheme 2.

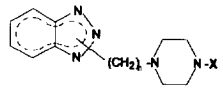
The 1-(2-chloroethyl)- and 1-(3-chloropropyl)-4-substituted piperazines were obtained by reaction of 1-bromo-2-chloroethane or 1-bromo-3-chloropropane with the corresponding *N*-substituted piperazine. Condensation of 1-(2-chloroethyl)- or 1-(3-chloropropyl)-4-substituted piperazines with benzotriazole in anhydrous ethanol and sodium ethoxide [15] gave a mixture of 1- and 2-substituted isomers **1** and **2** or **3** and **4**, respectively. The 1- and 2-substituted isomers were separated as reported in the *Experimental protocols*. Generally, compounds bearing the substituent in the 2-position of the benzotriazole system were obtained in higher yields.

Scheme 2 outlines the steps for preparation of 1-{3-[4-(*X*)-1-piperazinyl]propoxy}benzotriazoles (**5a–l**). This procedure was found to be more advantageous than that described in scheme 1 for higher yields. Compounds **5a–e** and **5g,h** were reported in our previous paper [16], while compounds **5f** and **5i,l** were synthesized as described in the *Experimental protocols*.

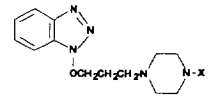
The 1-(3-chloropropoxy)benzotriazole was prepared by reaction of 1-bromo-3-chloropropane with 1-hydroxybenzotriazole in ethanol and sodium hydroxide. Condensation of 1-(3-chloropropoxy)benzotriazole with various *N*-substituted piperazines in toluene and triethylamine solution gave the expected compounds **5** in yields between 51 and 63%.

Analytical purification of each product was obtained by crystallization from the appropriate solvent. The ^1H NMR spectra clearly differentiated

Table I. 5-HT_{1A}, 5-HT_{2A} and 5-$HT_{1D\beta}$ binding affinities of benzotriazole derivatives.



1–4



5

Compound	*X*	*n*	*pK_i*		
			5-HT_{1A}	*5-HT_{2A}*	*5-$HT_{1D\beta}$*
1-Substituted benzotriazoles					
1a	C_6H_5	2	5.58 ± 0.06	5.89 ± 0.03	4.64 ± 0.01
1b	C_6H_4-2-Cl	2	5.78 ± 0.01	5.92 ± 0.29	5.04 ± 0.10
1c	C_6H_4-3-Cl	2	6.14 ± 0.12	6.67 ± 0.03	5.27 ± 0.70
1d	C_6H_4-4-Cl	2	5.97 ± 0.21	6.73 ± 0.14	4.74 ± 0.05
1e	$CH_2C_6H_5$	2	4.76 ± 0.01	5.29 ± 0.10	4.57 ± 0.08
1f	$(CH_2)_2C_6H_5$	2	≤ 4.00	6.75 ± 0.13	5.35 ± 0.11
1g	CH_3	2	≤ 4.00	4.90 ± 0.14	4.94 ± 0.06
1h	$(CH_2)_2OH$	2	≤ 4.00	4.87 ± 0.02	5.12 ± 0.04
1i	C_6H_4-2-OCH_3	2	7.51 ± 0.11	6.04 ± 0.05	5.51 ± 0.07
1l	C_6H_4-4-OCH_3	2	5.02 ± 0.20	5.32 ± 0.14	≤ 4.00
3a	C_6H_5	3	5.95 ± 0.09	7.20 ± 0.04	4.87 ± 0.03
3b	C_6H_4-2-Cl	3	6.19 ± 0.07	6.60 ± 0.12	4.75 ± 0.15
3c	C_6H_4-3-Cl	3	6.64 ± 0.08	7.30 ± 0.05	5.69 ± 0.01
3d	C_6H_4-4-Cl	3	5.74 ± 0.43	7.42 ± 0.05	5.37 ± 0.08
3e	$CH_2C_6H_5$	3	5.18 ± 0.15	5.14 ± 0.10	4.97 ± 0.10
3f	$(CH_2)_2C_6H_5$	3	4.77 ± 0.26	5.83 ± 0.12	5.72 ± 0.26
3g	CH_3	3	≤ 4.00	5.51 ± 0.03	≤ 4.00
3h	$(CH_2)_2OH$	3	≤ 4.00	5.29 ± 0.09	5.16 ± 0.01
3i	C_6H_4-2-OCH_3	3	7.89 ± 0.26	6.38 ± 0.09	5.44 ± 0.13
3l	C_6H_4-4-OCH_3	3	6.12 ± 0.11	6.37 ± 0.11	≤ 4.00
2-Substituted benzotriazoles					
2a	C_6H_5	2	5.69 ± 0.02	5.70 ± 0.18	4.64 ± 0.01
2b	C_6H_4-2-Cl	2	6.08 ± 0.02	5.56 ± 0.09	5.14 ± 0.02
2c	C_6H_4-3-Cl	2	6.62 ± 0.13	6.96 ± 0.01	5.24 ± 0.01
2d	C_6H_4-4-Cl	2	5.15 ± 0.02	6.21 ± 0.12	4.98 ± 0.03
2e	$CH_2C_6H_5$	2	4.37 ± 0.01	4.44 ± 0.04	4.64 ± 0.31
2f	$(CH_2)_2C_6H_5$	2	≤ 4.00	6.92 ± 0.10	5.33 ± 0.22
2g	CH_3	2	≤ 4.00	5.09 ± 0.24	4.77 ± 0.04
2h	$(CH_2)_2OH$	2	≤ 4.00	≤ 4.00	≤ 5.00
2i	C_6H_4-2-OCH_3	2	7.58 ± 0.23	5.52 ± 0.04	4.97 ± 0.10
2l	C_6H_4-4-OCH_3	2	4.87 ± 0.16	4.70 ± 0.01	≤ 4.00
4a	C_6H_5	3	5.42 ± 0.43	7.39 ± 0.11	4.26 ± 0.02
4b	C_6H_4-2-Cl	3	6.17 ± 0.10	6.43 ± 0.04	4.85 ± 0.07
4c	C_6H_4-3-Cl	3	6.48 ± 0.04	7.54 ± 0.05	4.67 ± 0.01
4d	C_6H_4-4-Cl	3	6.05 ± 0.01	7.96 ± 0.05	4.90 ± 0.10
4e	$CH_2C_6H_5$	3	5.92 ± 0.27	5.76 ± 0.15	5.00 ± 0.04
4f	$(CH_2)_2C_6H_5$	3	4.88 ± 0.10	6.36 ± 0.12	4.74 ± 0.19
4g	CH_3	3	≤ 4.00	4.89 ± 0.33	≤ 5.00
4h	$(CH_2)_2OH$	3	≤ 4.00	5.20 ± 0.05	≤ 4.00
4i	C_6H_4-2-OCH_3	3	6.99 ± 0.17	6.19 ± 0.17	4.70 ± 0.31
4l	C_6H_4-4-OCH_3	3	6.05 ± 0.15	6.41 ± 0.13	≤ 4.00
5a	C_6H_5		6.76 ± 0.10	6.55 ± 0.07	4.96 ± 0.16
5b	C_6H_4-2-Cl		6.78 ± 0.26	6.34 ± 0.06	5.63 ± 0.16
5c	C_6H_4-3-Cl		6.75 ± 0.16	6.84 ± 0.11	5.71 ± 0.01
5d	C_6H_4-4-Cl		5.78 ± 0.23	7.01 ± 0.13	4.67 ± 0.02
5e	$CH_2C_6H_5$		5.68 ± 0.20	6.26 ± 0.15	4.73 ± 0.03
5f	$(CH_2)_2C_6H_5$		≤ 4.00	5.30 ± 0.01	≤ 4.00
5g	CH_3		≤ 4.00	6.48 ± 0.09	≤ 4.00
5h	$(CH_2)_2OH$		≤ 4.00	5.26 ± 0.06	≤ 4.00
5i	C_6H_4-2-OCH_3		7.79 ± 0.14	6.35 ± 0.11	5.44 ± 0.01
5l	C_6H_4-4-OCH_3		7.44 ± 0.03	5.77 ± 0.05	≤ 4.00
Trazodone			6.43 ± 0.20	7.48 ± 0.01	4.60 ± 0.03

between 1- and 2-benzotriazole isomers, in the characteristic pattern of their spectra [19] (details are given in the *Experimental protocols*). Compounds of general formula **5** showed proton patterns similar to those of the other 1-substituted analogues **1** and **3**.

In order to increase the stability of the synthesized products, some of them were converted into the corresponding hydrochlorides.

Results and discussion

The affinities of the benzotriazole derivatives for the human receptor subtypes 5-HT_{1A}, 5-$HT_{1D\beta}$, 5-HT_{2A} and 5-HT_{2C} were determined by radioligand binding competition experiments. We transiently expressed human 5-HT receptors in COS-7 cells and used their membranes as a source of receptors. Affinity constants, expressed as pK_i values, are summarized in table I.

Three main substructures are present in the compounds under investigation: a 4-substituted piperazine ring, a flat cyclic system constituted of a 1- or 2-substituted benzotriazole, and an alkyl or alkyloxy chain connecting the piperazine and benzotriazole moieties. From the binding data listed in table I, some general trends can be easily identified. The majority of the compounds exhibit their highest potency on the 5-HT_{2A} receptor; only a few bind to the 5-HT_{1A} receptor, with less than micromolar affinity; none of the compounds show appreciable affinity for the 5-$HT_{1D\beta}$ receptor.

On the 5-HT_{2A} receptor, pK_i values greater than 7 are displayed only by some derivatives of sets **3** and **4** featuring, like trazodone (pK_i = 7.5), a propylene bridge. In these compounds (**3a,c,d**, and **4a,c,d**) a Ph, a 3-Cl-Ph or a 4-Cl-Ph group is attached to the N4-position of the piperazine ring. Replacement of these substituents generally lowers the potency to a considerable extent. Compound **5d** is the only one of the oxypropylene analogues that retains a pK_i value (7.0) for the 5-HT_{2A} receptor comparable with that of the reference drug.

As regards the 5-HT_{1A} receptor, the potency is significantly increased by the presence of a 2-MeO-Ph substituent at the N4-position (**1i**, **2i**, **3i** and **4i**). Only in the oxypropylene series are the 2-MeO-Ph and 4-MeO-Ph groups both capable of promoting 5-HT_{1A} binding (**5i** and **5l**).

The most favorable affinity–selectivity profiles are shown by compounds **3d**, **4a** and **4d** (5-HT_{2A} receptor) and **2i**, **3i**, **5i** and **5l** (5-HT_{1A} receptor).

Meaningful structure–affinity relationships for the 5-$HT_{1D\beta}$ receptor cannot be outlined due to the very low affinities of the compounds and the extremely poor variance of the pK_i data.

An important issue which deserves further discussion, especially considering the high flexibility of our compounds, is whether the effect of the N4-substituent (X) on the 5-HT_{2A} and 5-HT_{1A} affinity is constant in all five sets of benzotriazoles. By addressing this question we might indirectly gain information about the relative alignment of the ligands in their receptor-recognized conformations. As an example, if all the tested ligands anchored the piperazine-X moiety at a common position on the receptor, the change of potency upon a given X replacement should ideally be the same within each set. Insights into ligand–receptor interaction mechanisms, through comparative analyses of structure–affinity relationships, could also be useful for establishing a sensible 'alignment rule' in 3D-QSAR studies [20].

In figure 2 we have plotted, for each set, the 5-HT_{2A} pK_i values against the type of X substituent. Three distinct patterns are detectable according to the degree of parallelism existing among different curves: one for sets **1** and **2** (black line), one for sets **3** and **4** (red line), and another for set **5** (blue line). This finding supports the following hypotheses: (i) there are roughly three different binding modes at the 5-HT_{2A} receptor depending on the type of intermediate chain (propylene, ethylene or oxypropylene); and (ii) for each of these binding modes, the X substituent is located in a relatively fixed position at the receptor.

Analogous diagrams are shown in figure 3, where the effects of the X substituent on the binding to the 5-HT_{1A} receptor are compared. The five plots can again be grouped into the above-mentioned three clusters: sets **1** and **2** (black line), sets **3** and **4** (red line) and set **5** (blue line). It is therefore likely that also at the 5-HT_{1A} receptor three different positions for the X substituent are possible.

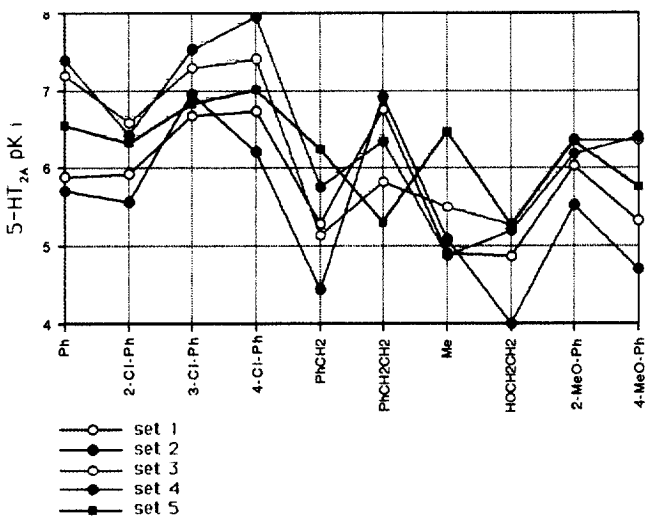


Fig 2. Plot of 5-HT_{2A} pK_i values against the type of substituent attached to the N4-position of the piperazine ring.

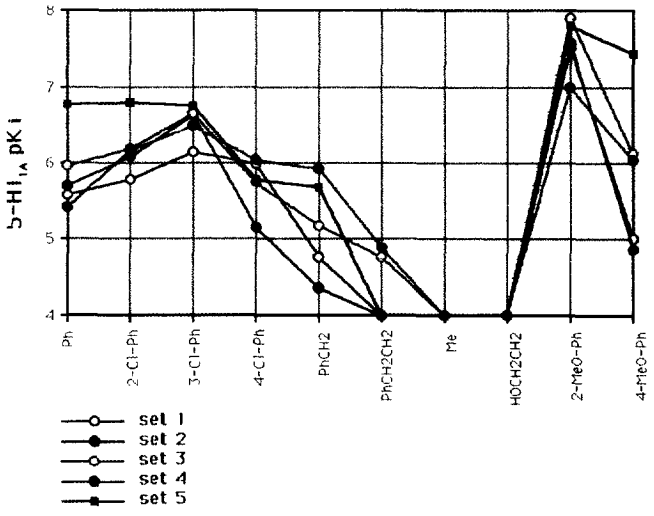


Fig 3. Plot of 5-HT_{1A} pK_i values against the type of substituent attached to the N4-position of the piperazine ring.

A closer look at figures 2 and 3 reveals, however, that our mechanistic model is far from ideal. In fact, differences in binding affinities between isomers of the same cluster are not perfectly constant (see pK_i variations in sets **1** and **2** from X = Me to $HOCH_2CH_2$, and in sets **3** and **4** from X = $PhCH_2CH_2$ to Me for 5-HT_{2A} receptor subtypes). These 'irregularities', which may arise partly from a different substitution mode of the benzotriazole system, indicate that a crude 'lock-and-key' scheme of receptor–ligand interaction is an oversimplification.

In conclusion, our graphical approach to structure–affinity relationships (similar to the well-known quantitative methods of Free–Wilson [21] or Fujita–Ban [22]) suggests that the effect of the X substituent on affinity toward 5-HT_{2A} and 5-HT_1A receptors depends, within a certain approximation, on the nature of the intermediate chain. This means that not all the assayed ligands bind to the receptor by fitting the piperazine-X moiety into the same complementary site. Well-behaved structure–affinity relationships show up only within each of the above three classes of analogues. What we have hypothesized appears to be consistent with the recently reported findings of Ismaiel et al [23], who postulated that certain ketanserin analogues bind to the 5-HT_{2A} receptor in different modes.

Table II lists the affinities for the 5-HT_{2C} receptor of a subset of benzotriazole derivatives chosen as the most potent on 5-HT_{2A} and 5-HT_{1A} receptors.

It was actually hoped that the selected compounds would appreciably discriminate among the three receptors by binding weakly to the 5-HT_{2C} subtype. Indeed, several of the tested compounds exhibit

Table II. 5-HT_{2C} binding affinities of some benzotriazole derivatives.

Compound	*pK_i*
1i	5.04
2i	6.52
3a	5.63
3b	6.10
3c	6.24
3d	5.82
3i	7.00
4a	5.64
4b	5.89
4c	6.32
4d	5.78
5i	5.32
5l	< 4.30
Trazodone	7.13

5-HT_{2C} pK_i values below 6.0 under conditions yielding a pK_i value of 7.1 for trazodone. It should be noted that the best 5-HT_{2A}/5-HT_{2C} selectivity is achieved when X is a Ph or a 4-Cl-Ph group (**3a,d**, **4a,d**). Notably, compound **4d** is 100 times more potent on the 5-HT_{2A} than on the 5-HT_{2C} receptor. The methoxyphenyl analogues **1i**, **5i** and **5l** were the least potent on the 5-HT_{2C} receptor with pK_i values below 5.4. Among these derivatives, compound **5l** should be highlighted because its potency for the 5-HT_{2C} receptor is about 3000 times lower than for the 5-HT_{1A} receptor. From a comparison of the IC_{50} values of the pairs **1i/2i** and **1i/3i** it appears that 5-HT_{2C} potency is highly sensitive to the nature of the intermediate chain and to the position of attachment on the benzotriazole nucleus.

During the preparation of this manuscript, a paper was published by Mokrosz and coworkers [18], in which the 5-HT-receptor binding of some of the compounds in our series (**1i**, **2i**, **3i** and **4i**) were investigated using different experimental models. From the pharmacological data reported by Mokrosz [18] it is clear that **3i** acts as a full antagonist on the 5-HT_{1A} receptor. Such a finding, together with our results on selectivity, raises the potential interest in this series of compounds.

Experimental protocols

Chemistry

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. The structures described were supported by ^{1}H NMR spectra, GLC-MS analyses and microanalytical data. ^{1}H NMR spectra were recorded on a Bruker WM 250

spectrometer using CD_3OD as the solvent; chemical shifts (δ) are expressed in units (ppm) and the splitting patterns are designated as follows: s (singlet), dd (doublet of doublets), t (triplet) and m (multiplet). It is worth pointing out that the observed differences in the chemical shift values among the protons of 1- and 2-substituted derivatives confirm the different π-electronic delocalization of the two benzotriazole systems. Indeed, the benzotriazol-2-yl derivatives show a greater molecular symmetry. In fact, in compound **2l**, taken as an example, the aromatic protons of the benzotriazole ring appear as two doublets of doublets at δ 7.93, J = 9.5 and 3.2 Hz (H-4 and H-7) and 7.50, J = 9.5 and 3.2 Hz (H-5 and H-6). Larger differences occur in benzotriazol-1-yl derivatives, such as **1l**, in which the aromatic protons of the benzotriazole moiety appear as two doublets of doublets at δ 8.09 and 7.98, J = 8.5 and 1.1 Hz (H-4 and H-7) and two doublets of triplets at δ 7.69 and 7.52, J = 8.5 and 1.1 Hz (H-6 and H-5). Similar ^{1}H NMR data of the aromatic protons of the benzotriazole moiety occur in derivatives **4l** and **3l**. ^{1}H NMR data of the side chain of the compounds **1l**, **2l**, **3l** and **4l** are described in the general procedure for their preparation.

Compounds **5f**, **5i** and **5l** showed protonic patterns similar to those of the other 1-substituted analogues **1l** and **3l**. In compound **5l**, as an example, the aromatic protons of the benzotriazole moiety appear as two doublets of doublets at δ 8.01 and 7.85, J = 8.5 and 1.1 Hz (H-4 and H-7) and two doublets of triplets at δ 7.68 and 7.52, J = 8.5 and 1.1 Hz (H-6 and H-5). Similar ^{1}H NMR data for the aromatic protons of the benzotriazole ring were found for derivatives **5f** and **5i**. ^{1}H NMR data for the side chain of the compounds **5f**, **5i** and **5l** are described in the general procedure for their preparation.

Combined GLC-MS analyses were performed on a Hewlett-Packard 5890 gas chromatographer with a mass selective detector MSD HP 5970. A column 25 m x 0.20 mm HP-5 (cross-linked PhMe silicone 5%) with a 0.33 μm film thickness was employed.

Analytical TLC were performed on precoated silica-gel (0.2 mm GF 254, E Merck) or aluminum oxide glass-backed plates; the spots were located by UV (254 nm) light or by exposure to iodine vapor. Crude products were routinely passed through columns of silica gel (0.05 ÷ 0.20 mm, Carlo Erba) or basic aluminum oxide (Macherey Nagel) with an appropriate mixture of diethyl ether/*n*-hexane or ethanol as the eluent, respectively. The hydrochloride salts were potentiometrically titrated in glacial acetic acid by adding an excess of mercuric acetate and using a standard solution of acetous perchloric acid for titration. The equivalent weights of the compounds **1l**, **2l**, **3l**, **4l**, **5f**, **5i** and **5l** were consistent with monohydrochloride salts (experimental error 1%).

Final compounds gave satisfactory analyses (C, H, N) within ±0.4% of the theoretical values. Analytical and spectroscopic data were consistent with the structure of the corresponding compound.

Reagent grade materials were purchased from Aldrich Chemical Co and were used without further purification.

*General procedure for preparation of compounds **1l**, **2l**, **3l** and **4l***

1-Bromo-2-chloroethane or 1-bromo-3-chloropropane (0.15 mol) in toluene (80 mL) was refluxed under stirring and the appropiate *N*-substituted piperazine (0.1 mol) in toluene (30 mL) was added dropwise over 1 h. The reaction was monitored using TLC and diethyl ether as eluent. The mixture was then cooled and poured into water. The aqueous layer was alkalized with 2 N NaOH and extracted several times with chloroform. The organic layer was washed with water and dried over anhydrous $Na_2SO_4$4. It was then filtered and the solvent evaporated to dryness in vacuo. The resulting crude product was readily purified by passing it through a chromatographic column packed with silica gel using diethyl ether as eluent, to obtain pure 1-(2-chloroethyl)- or 1-(3-chloropropyl)piperazine-4-substituted derivatives. These compounds were reacted with benzotriazole in sodium ethoxide and anhydrous ethanol according to the reported procedure [15] and led to the expected products **1l**, **2l**, **3l** and **4l**.

TLC examination (diethyl ether/*n*-hexane 7:3 v/v) of the reaction mixture showed the formation of two UV-absorbing products, one of which was preponderant. Fractionation was performed on a silica-gel column using diethyl ether/*n*-hexane 7:3 v/v as eluent.

Characterization of pure products by ^{1}H NMR spectra showed that the first eluted compound was the 2-substituted benzotriazole derivative. Further purification of each product was obtained by crystallization.

*1-{2-[4-(4-Methoxyphenyl)-1-piperazinyl]ethyl}benzotriazole·HCl **1l**.* Yield 28%; mp 210–211 °C (EtOH/diethyl ether, 8:2); ^{1}H NMR δ 3.55–3.75 (m, 8H, CH_2-piperazine), 3.80 (s, 3H, OCH_3), 4.00 (t, 2H, NCH_2, J = 7.5 Hz), 5.30 (t, 2H, CH_2-Btz, J = 7.5 Hz), 6.95–7.28 (m, 4H, Ar-H); MS *m/z* 337 (M^+); $C_{19}H_{23}N_5O$·HCl (337): calc C 67.63%, H 6.87%, N 20.75%; found C 67.70%, H 6.85%, N 20.81%.

*2-{2-[4-(4-Methoxyphenyl)-1-piperazinyl]ethyl}benzotriazole·HCl **2l**.* Yield 50%; mp 230–231 °C (EtOH/diethyl ether, 8:2); ^{1}H NMR δ 3.60–3.78 (m, 8H, CH_2-piperazine), 3.80 (s, 3H, OCH_3), 4.13 (t, 2H, NCH_2, J = 7.5 Hz), 5.38 (t, 2H, CH_2-Btz, J = 7.5 Hz), 6.90–7.35 (m, 4H, Ar-H); MS *m/z* 337 (M^+); $C_{19}H_{23}N_5O$·HCl (337): calc C 67.63%, H 6.87%, N 20.75%; found C 67.76%, H 6.88%, N 20.71%.

*1-{3-[4-(4-Methoxyphenyl)-1-piperazinyl]propyl}benzotriazole·HCl **3l**.* Yield 30%; mp 213–214 °C (EtOH/diethyl ether, 8:2); ^{1}H NMR δ 2.63 (m, 2H, CH_2), 3.45 (t, 2H, NCH_2, J = 7.5 Hz), 3.57–3.80 (m, 8H, CH_2-piperazine), 3.84 (s, 3H, OCH_3), 4.95 (t, 2H, CH_2-Btz, J = 7.5 Hz), 7.00–7.38 (m, 4H, Ar-H); MS *m/z* 351 (M^+); $C_{20}H_{25}N_5O$·HCl (351): calc C 68.35%, H 7.16%, N 19.92%; found C 68.55%, H 7.15%, N 19.93%.

*2-{3-[4-(4-Methoxyphenyl)-1-piperazinyl]propyl}benzotriazole·HCl **4l**.* Yield 58%; mp 208–209 °C (EtOH/diethyl ether, 8:2); ^{1}H NMR δ 2.70 (m, 2H, CH_2), 3.44 (t, 2H, NCH_2, J = 7.5 Hz), 3.58–3.76 (m, 8H, CH_2-piperazine), 3.80 (s, 3H, OCH_3), 4.98 (t, 2H, CH_2-Btz, J = 7.5 Hz), 7.00–7.33 (m, 4H, Ar-H); MS *m/z* 351 (M^+); $C_{20}H_{25}N_5O$·HCl (351): calc C 68.35%, H 7.16%, N 19.92%; found C 68.42%, H 7.17%, N 19.94%.

*General procedure for preparation of compounds **5f**, **5i** and **5l***

To a solution of 1-hydroxybenzotriazole (0.05 mol) and NaOH (0.05 mol) in ethanol (50 mL) was added 1-bromo-3-chloropropane (0.05 mol). The reaction was heated under reflux for 3 h, and successively cooled, filtered and evaporated to dryness. The residue was dissolved in chloroform (150 mL) and washed with 2 N NaOH. The organic phase was washed with saturated aqueous NaCl, dried over Na_2SO_4 and evaporated to dryness. The resulting crude product was purified by passing it through a chromatographic column packed with silica gel using diethyl ether/*n*-hexane 9:1 v/v as eluent. Pure 1-(3-chloropropoxy)benzotriazole was obtained as pale yellow oil (55% yield). To a toluene solution (50 mL) of this compound (0.02 mol), triethylamine (0.02 mol) and the appropriate *N*-

substituted piperazine (0.02 mol) were gently added. The reaction mixture was vigorously stirred under reflux for 24–72 h and monitored by TLC. After cooling, the reaction mixture was extracted several times with 2N HCl. The aqueous layer was alkalinized with 2 N NaOH and extracted with chloroform. The combined organic extracts, washed with water and dried over Na_2SO_4, were evaporated in vacuo. The resulting crude product was purified by silica-gel column chromatography using diethyl ether as eluent. Further purification of each product was achieved by crystallization.

1-{3-[4-(Phenethyl)-1-piperazinyl]propoxy}benzotriazole•2HCl ***5f***. Yield 37%; mp 208–210 °C (EtOH/diethyl ether, 8:2); ^{1}H NMR δ 2.50 (m, 2H, CH_2), 2.65 (t, 2H, CH_2C, J = 7.5 Hz), 2.83 (t, 2H, CH_2N, J = 7.5 Hz), 3.70 (t, 2H, NCH_2, J = 7.5 Hz), 3.75–3.85 (m, 8H, CH_2-piperazine), 4.75 (t, 2H, CH_2-OBtz J = 7.5 Hz); 7.10–7.40 (m, 5H, Ar-H); MS *m/z* 385 (M^+); $C_{21}H_{27}N_5O$•2HCl (385): calc C 69.01%, H 7.44%, N 19.16%; found C 68.77%, H 7.45%, N 19.11%.

1-{3-[4-(2-Methoxyphenyl)-1-piperazinyl]propoxy}benzotriazole•HCl ***5i***. Yield 35%; mp 183–184 °C (EtOH/diethyl ether, 8:2); ^{1}H NMR δ 2.50 (m, 2H, CH_2), 3.72 (t, 2H, NCH_2, J = 7.5 Hz), 3.78–3.90 (m, 8H, CH_2-piperazine), 3.98 (s, 3H, OCH_3), 4.77 (t, 2H, CH_2-OBtz, J = 7.5 Hz); 6.98–7.53 (m, 4H, Ar-H); MS *m/z* 367 (M^+); $C_{20}H_{25}N_5O_2$•HCl (367): calc C 65.37%, H 6.85%, N 19.05%; found C 65.63%, H 6.84%, N 19.07%.

1-{3-[4-(4-Methoxyphenyl)-1-piperazinyl]propoxy}benzotriazole•HCl ***5l***. Yield 40%; mp 197–198 °C (EtOH/diethyl ether, 8:2); ^{1}H NMR δ 2.48 (m, 2H, CH_2), 3.60 (t, 2H, NCH_2, J = 7.5 Hz), 3.64–3.70 (m, 8H, CH_2-piperazine), 3.98 (s, 3H, OCH_3), 4.75 (t, 2H, CH_2-Btz, J = 7.5 Hz); 6.95–7.40 (m, 4H, Ar-H); MS *m/z* 367 (M^+); $C_{20}H_{25}N_5O_2$•HCl (367): calc C 65.37%, H 6.85%, N 19.05%; found C 65.50%, H 6.86%, N 19.02%.

Radioligand binding experiments

Competition ligand binding experiments were performed as described by Russo et al [24]. Membranes were prepared from COS-7 cells transiently transfected with cDNA encoding for 5-HT receptor subtypes. cDNA encoding for human 5-HT receptor subtypes were kindly donated by other laboratories: BK Kobilka (Department of Medicine and Biochemistry, Duke University Medicinal Center, Durham, NC, USA) for 5-HT_{1A}, L Demchyshyn (Department of Pharmacology, University of Toronto, Canada) for 5-$HT_{1D\beta}$, and A Saltzman (Rhone-Poulenc Rorer Central Research, Collegeville, PA, USA) for 5-HT_{2A} and 5-HT_{2C} receptors. Full length cDNAs were subcloned into eukaryotic expression vectors, as pcDNA/Amp for 5-HT_{2C} receptor and pPJIneo for the others and transfected transiently by the DEAE-dextran method.

Seventy-two hours after transfection, the cells were scraped into ice-cold PBS_{1X}, homogenized in Polytron and centrifuged at 2200 *g* for 10 min. The resulting pellets were resuspended in the appropriate binding buffers. The proteins concentration was determined using the method of Bradford with bovine serum albumin as standard. The membranes were stored at –20 °C until use. In a typical transfection experiment the level of expression was 300 fmol/mg of proteins for 5-HT_{1A}, 600 fmol/mg of proteins for 5-$HT_{1D\beta}$, 1600 fmol/mg of proteins for 5-HT_{2A} and 100 fmol/mg of proteins for 5-HT_{2C} receptors. None of these receptors were expressed in untransfected COS-7 cells.

The competition binding experiments were determined by displacement of [^{3}H]8-OH-DPAT (1 nM), [^{3}H]5-HT (2 nM), [^{3}H]ketanserin (2 nM) and [^{3}H]mesulergine (3 nM). Non-specific binding was defined by 1 μM 5-HT for 5-HT_{1A}, 10 μM 5-HT for 5-$HT_{1D\beta}$, and 10 μM methysergide for 5-HT_{2C} and 5-HT_{2A} receptors.

Radioligand binding studies were performed in duplicate in a total volume of 250 μL, containing 25 μL of radioligand, 25 μL of buffer or competing drug, and 200 μL of membranes (15–75 μg of proteins for tube). Incubation (60 min, 37 °C) was stopped by the addition of 5 mL of ice-cold PBS_{1x}, followed by rapid vacuum filtration through a Brandel harvester with Whatman GF/C filters, and three subsequent 5 mL washes.

IC_{50} values were calculated from competition curves analyzed by the Graphad Inplot 4.0 nonlinear fitting computer program. The pK_i values reported for 5-HT_{1A}, 5-HT_{2A} and 5-$HT_{1D\beta}$ receptors are means of three independent experiments, whereas the pK_i values for the 5-HT_{2C} receptor are means of two determinations. K_i values of competing drugs were calculated using the Cheng–Prusoff equation [25] with the K_D values for the following radioligands: [^{3}H]-8-OH-DPAT, 0.8 nM; [^{3}H]-5-HT, 4.5 nM; [^{3}H]ketanserin, 1.0 nM; and [^{3}H]mesulergine, 1.8 nM.

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