Full Paper

Synthesis and *In-vitro* Pharmacological Evaluation of New 5-HT$_{1A}$ Receptor Ligands Containing a Benzotriazinone Nucleus

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This paper reports the microwave-assisted synthesis and the binding assays on the 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors of new benzotriazinone derivatives, in order to identify selective ligands for the 5-HT$_{1A}$ subtype receptor. Conventional and microwave heating of the reactions were compared. Good yields and short reaction times are the main advantages of our synthetic route. More active compounds were selected and further evaluated for their binding affinities on D_1, D_2 dopaminergic and α_1, α_2 adrenergic receptors. The 3-(2-(4-(naphthalen-1-yl)piperazin-1-yl)ethyl)benzo[*d*][1,2,3]triazin-4(3*H*)-one **5** with K_i = 0.000178 nM was the most active and selective derivative for the 5-HT$_{1A}$ receptor with respect to other serotonin receptors and the most selective derivative compared to dopaminergic and adrenergic receptors.

Keywords: Benzotriazinone / Microwave-assisted synthesis / 5-HT Receptors / Serotonin

Received: July 17, 2007; accepted: September 5, 2007

DOI 10.1002/ardp.200700151

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is one of the most important targets for medicinal chemistry as it is implicated in numerous physiological and pathophysiological processes [1–4]. Serotonin receptors may be involved in impulsivity and alcoholism [5–6] and in the different phases of sleep [7], sexual behavior, appetite control, thermoregulation, and cardiovascular function [8, 9]. In particular, the 5-HT$_{1A}$ receptor, found in high concentration in the limbic system where it is thought to play a role in emotional processes, is a major target for research and drug development due to its implication in many physiological processes.

5-HT$_{1A}$R belongs to the G protein-coupled receptors (GPCRs) [10] and the members of this family possess a similar amino acid composition. In particular, the 5-HT$_{1A}$R transmembrane amino acid sequence presents 45% homology with the respective part of the α_1-adrenergic receptor [11].

Several agents are already known for their high affinity toward these receptors and, from a chemical point of view, they can be subdivided into different classes. The most studied group is that of long-chain arylpiperazine (LCAPs) [12, 13] that, for a long time, has drawn great interest as a source of both neuropsychiatric drugs (buspirone, ziprasidone, aripiprazol) and compounds with a high therapeutical potential (adatanserin, mazapertine, flesinoxan, lecozotan, bifeprunox) which among others exert their action via 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. Their diversified receptor-binding profile and intrinsic activity, depending on either the kind of substituent attached to the N-4 atom of the piperazine moiety or the nature of an

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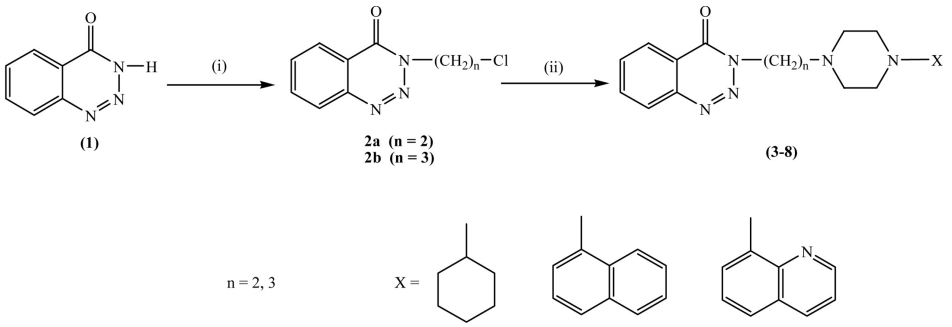
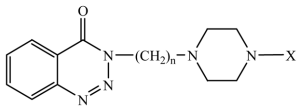
Abbreviations: 5-hydroxytryptamine (5-HT); G protein-coupled receptors (GPCRs); of long-chain arylpiperazine (LCAP); structure-activity relationship (SAR) studies

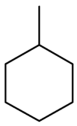
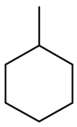
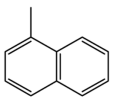
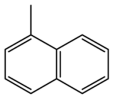
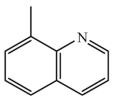
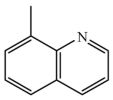


Reagents and conditions: (i) $Br(CH_2)_nCl$, K_2CO_3, DMF, μν; (ii) 4-X-piperazine, K_2CO_3, NaI, DMF, μν.

Scheme 1. Synthesis route of compounds **1–8**.

Table 1. Physiochemical properties of final compounds **3–8**.



Compound	n	X	Formula[a)]	Mp. (°C)[b)]	Yield (%)[c)]
3	2		$C_{19}H_{27}N_5O \times 2HCl$	248–250	65
4	3		$C_{20}H_{29}N_5O \times 2HCl$	243–245	87
5	2		$C_{23}H_{23}N_5O \times HCl$	246–248	71
6	3		$C_{24}H_{25}N_5O \times HCl$	251–253	82
7	2		$C_{22}H_{22}N_6O \times HCl$	208–210	62
8	3		$C_{23}H_{24}N_6O \times HCl$	200–202	79

[a)] Satisfactory microanalyses obtained: C, H, N values are within ± 0.4% of theoretical values.
[b)] All compounds were crystallized by ethyl alcohol and diethyl ether.
[c)] Yields of the last step are referred to microwave irradiation.

amide or imide terminal fragment, opens possibilities for the discovery of new potent therapeutic agents [14]. The influence of each part of the LCAP structures on the 5-HT$_{1A}$ receptor affinity, intrinsic activity, and selectivity has been the subject of many structure-activity relationship (SAR) studies. In particular, much effort has been devoted to understand the role of the terminal part in the ligand-receptor interaction and, in consequence, a

great number of many different fragments were used [15]. However, a limitation of many 5-HT_{1A} receptor ligands in the potential use as drugs or pharmacological tools is their undesired high affinity for other receptor subtypes. The dopaminergic D_2 receptor and α_1-adrenoceptor are two examples of receptor sites to which several 5-HT_{1A} ligands bind with high affinity.

Our group has undertaken a research program aimed at developing new 5-HT_{1A} agents [16–23] with high affinity and selectivity over other serotoninergic, dopaminergic and adrenergic receptors. In continuation of our research program, we have analyzed a new set of derivatives where the piperazine-*N*-alkyl moiety has been linked to a 1,2,3-benzotriazinone fragment (Scheme 1); this heterocyclic nucleus was already investigated by our group, in order to develop aryl piperazine derivatives with high affinity and selectivity for the 5-HT_{1A} receptor [21]. Aiming to further explore its influence on the serotoninergic activity, this bicyclic nucleus was linked to not yet studied 4-substituted piperazines (cyclohexyl, naphthyl and quinolyl) via two or three methylene spacing units. All the new compounds were tested for their affinity for 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors. Moreover, the multireceptor profiles of promising derivatives were also evaluated in terms of binding affinities for dopaminergic D_1, D_2 and α_1, α_2 adrenoreceptors.

Chemistry

The synthesis of final compounds **3–8**, reported in Scheme 1, was performed using a microwave oven (ETHOS 1600, Milestone Inc.) especially designed for organic synthesis. The experimental conditions used in our work were similar to those used by conventional heating, with the same amount of starting reagent and volume of solvent. Synthesis by microwave irradiation gave the desired compounds in better yields than those obtained by conventional heating. The overall times for the synthesis were considerably reduced. All the 4-X-piperazines (Scheme 1) employed for the preparation of compounds **3–8** are commercially available.

Alkylation of the starting heterocycle 1,2,3-benzotriazin-4(3*H*)-one with 1-bromo-2-chloroethane or 1-bromo-3-chloropropane, in presence of K_2CO_3 in DMF, gave the corresponding chloroalkyl benzotriazinone derivatives **2a** and **2b**. The obtained intermediates were condensed with the desired 4-X-piperazine in DMF in the presence of K_2CO_3, and NaI, under reflux to give the final compounds **3–8**.

All the final compounds were transformed into the corresponding hydrochloride salts using dry gaseous HCl in anhydrous diethyl ether.

Physicochemical data of the final compounds are reported in Table 1. The parameters of time, power, temperature used for the microwave irradiation, and the conditions used for the conventional heating are reported in Table 2. Analytical purification of each final product was obtained by chromatography on silica-gel column and further by crystallization from the appropriate solvent. All new compounds gave satisfactory elemental analyses (C, H, N) and were characterized by mass spectrometry (Finnigan LCQ–MS ThermoQuest-Ion trap) and ^{1}H-NMR, performed on the free base of each compound. ^{1}H-NMR and MS data for all final compounds were consistent with the proposed structures.

Results and discussion

The syntheses of these novel compounds, characterized by benzotriazinonic nucleus, were successfully improved with the application of microwave heating to our reactions; in particular, under controlled microwave irradiation, the reaction times were extensively reduced, from 2–3 h to 30 min in the syntheses of intermediates (**2a** and **2b**) and from 24 h to 70 min in the obtaining of final compounds (Table 2, **3–8**).

The obtained six new arylpiperazine derivatives **3–8** were evaluated for activity and selectivity. Introduction of a 1,2,3-benzotriazinonic nucleus as terminal part, slight modifications concerning the alkyl spacer chain length (two or three units), and the introduction of new rings on the N-4 piperazine moiety are depicted in Scheme 1 and were performed in an effort to obtain compounds with high affinity and selectivity for 5-HT_{1A}R over other serotoninergic receptors, as well as dopaminergic and adrenergic receptors. As anticipated, two of these compounds are potent 5-HT_{1A} receptor ligands. In fact, they showed nanomolar or even subnanomolar 5-HT_{1A} receptor affinities (Table 3). Besides the outstanding 5-HT_{1A} receptor affinity of compound **5** (K_i = 0.000178 nM), K_i values were clustered in a relatively narrow range from 7.79 nM for **6** to 105 nM (compound **4**). Compounds **7**, **8**, and **3** where less active showing K_i values of 370, 317, and 5090 nM, respectively.

Concerning the most active compounds **5** and **6**, the derivative with piperazinylethyl chain linked to the benzotriazinonic nucleus **5** showed high and preferential affinity for the 5-HT_{1A}R, relative to the compound in which the spacer is one atom longer **6**. The influence of the alkyl chain length, observed for our compounds, is in accordance with recently reported structure affinity relationships (SARs) of new models of arylpiperazines [23].

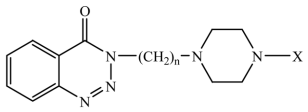
Table 2. Conventional heating versus microwave irradiation for intermediates **2a, 2b**, and the final compounds **3–8**.

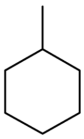
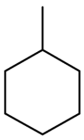
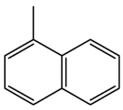
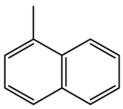
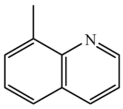
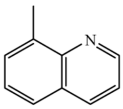
Compd.	Conventional heating[a]			Microwave irradiation[b]			
	Yield (%)	Time (min)	Temp. (°C)	Yield (%)	Time (min)	Power (Watt)	Temp. (°C)
2a–2b	72–85	120–180	80	90–96	5	200	90
					5	80	90
					20	400	80
3–8	22–48	24h	reflux	62–87	5	80	80
					60	400	120
					5	200	110

[a] Oil bath.

[b] The experimental conditions used on microwave irradiation were similar to those used by conventional heating, with the same amounts of starting reagent and volume of solvent.

Table 3. Affinities of compounds **3–8** for 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors.



Compound	Substituent		Receptor affinity K_i ± SD (nM)		
	X	n	5-HT$_{1A}$ [^{3}H]8OH-DPAT	5-HT$_{2A}$ [^{3}H]ketanserin	5-HT$_{2C}$ [^{3}H]mesulergine
3		2	5090 ± 203	>10^4	>10^4
4		3	105 ± 6	>10^4	>10^4
5		2	0.000178 ± 0.00003	5.29 ± 0.3	>10^4
6		3	7.79 ± 0.06	127 ± 17	>10^4
7		2	370 ± 4.7	315 ± 137	>10^4
8		3	317 ± 70	1630 ± 270	5670 ± 336

For purpose of comparison, 8-OH-DPAT, ketanserin, and mesulergine binds 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors with K_i values of 0.80, 0.85, and 1.90 nM, respectively, under these assay conditions.

Table 4. Affinities of compounds **5** and **6** for D_1, D_2, α_1, and α_2 receptors.

Compound	Receptor affinity $K_i \pm SD$ (nM)			
	D_1 [^{3}H]SCH-23390	D_2 [^{3}H]spiperone	α_1 [^{3}H]prazosin	α_2 [^{3}H]yohimbine
5	$>10^4$	$>10^4$	112 ± 47	1880 ± 107
6	$>10^4$	$>10^4$	562 ± 170	3600 ± 458

Moreover, regarding the influence of the substituent on the N-4 of the piperazine moiety, the naphthyl group for compounds **5** and **6** conferred the highest affinity for the 5-HT_{1A} receptor. Instead, the cyclohexyl or quinolyl moiety seems to show an unfavorable affinity profile compared to **5** and **6**. In fact, only compound **4** showed a quite moderate affinity for the 5-HT_{1A} receptor with K_i values of 105 nM.

The 5-HT_{2A} and 5-HT_{2C} receptor affinities of the tested compounds were always lower than those observed for 5-HT_{1A} receptors and ranged from 5.29 (**5**) to $>10^4$ nM (for **3**, **4**, **6**, **7**, and **8**) for 5-HT_{2A} and from 5670 (**8**) to $>10^4$ nM (for **3**–**7**) for 5-HT_{2C} receptors. The most 5-HT_{1A} active compound, 3-(2-(4-(naphthalen-1-yl)piperazin-1-yl)ethyl)benzo[*d*][1,2,3]triazin-4(3*H*)-one **5** (K_i = 0.000178 nM), was also the most selective derivative with respect to the serotonin receptors 5-HT_{2A} and 5-HT_{2C}.

Additionally, the affinity of the most active compounds **5** and **6** on several other receptors (α_1 and α_2 adrenergic and D_1 and D_2 dopaminergic receptors) was examined in order to verify their selectivity. Results are summarized in Table 4. The two derivatives proved highly selective against dopaminergic receptors with K_i values of above 10^4 nM. Regarding α_1 and α_2 adrenergic receptors, only compound **5** showed quite moderate affinity (112 nM). The high selectivity towards α_1 receptors, exhibited by compound **5**, is very interesting considering that the amino acid sequence of the transmembrane part of 5-HT_{1A}R is highly homologous to that of the α_1 adrenergic receptor.

These results support further on the choice of the 1,2,3-benzotriazinonic scaffold for the design and preparation of serotoninergic ligands endowed with high 5-HT_{1A} affinity. The high difference in affinity observed between **5** and **6** demonstrates again that the alkyl chain length represents a critical structural feature in determining 5-HT_{1A} receptor affinity and selectivity. In fact, in contrast to all other substituents generally reported in literature, the naphthyl derivative exhibits a higher affinity for the shorter chain compared to the longer chain. This aspect was demonstrated by a molecular modeling study, which could be due, as recently reported in literature, to the very hydrophobic nature of the substituent on the N-4 of the piperazine moiety [23]. Moreover, although the exceptionally high affinity of compound **5** is probably not due to its ability to form a hydrogen bond between a substituent and Asn-386 of the receptor (like the OCH_3 substituent), but rather solvent accessibility and hydrophobic interaction with the receptor are decisive. In fact, Zlatovic *et al.* [24] have recently reported that some arylpiperazine, such as naphthylpiperazine, can interact directly with the hydrophobic part of the 5-HT_{1A} receptor binding site. In particular the hydrophobic part of the binding site in the 5-HT_{1A} receptor, formed by Trp 358, Phe 361, and Tyr 390, is significant for the stabilization of the ligand-receptor complex. Finally, these aspects, correlated to the hydrophobic interaction with the receptor, can be useful to clarify also the smaller affinity correlated to the cyclohexyl or quinolyl derivatives, where a non-aromatic ring or the more hydrophilic quinolyl moiety are less favorable to the hydrophobic interaction with the receptor.

The NMR spectral data were provided by Centro di Ricerca Interdipartimentale di Analisi Strumentale, Università degli Studi di Napoli "Federico II". The assistance of the staff has been gratefully appreciated.

The authors have declared no conflict of interest.

Experimental

Synthesis – general procedures

Synthesis was performed using a microwave oven ETHOS 1600 (Milestone Inc.). All reactions were performed in standard pyrex glassware with a reflux condenser fitted through the roof of the microwave cavity and were performed by microwave program which was composed by appropriate temperature ramping and holding steps. The temperature of the stirred reaction mixture was monitored directly by a microwave-transparent fluoroptic probe inserted into the solution.

All reactions were followed by thin layer chromatography carried out on Merck silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) with fluorescent indicator and the plates were visualized with UV light (254 nm). Preparative chromatographic purifications were performed using silica gel column (Kieselgel 60). Melting points were determined using a Kofler hot-stage apparatus (C. Reichert, Vienna, Austria) and are uncorrected. The struc-

tures were verified spectroscopically by ^{1}H-NMR performed on the free base of each compound. Spectra were recorded on Bruker AMX-500 instruments (Bruker Bioscience, Billerica, MA, USA). Chemical shifts are given as δ with references to Me_4Si. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), m (multiplet). Mass spectra of the final products were performed on LCQ Thermo-Quest Ion trap mass spectrometry (Finnigan, Germany). Where analyses are indicated only by the symbols of the elements, results obtained are within ± 0.4% of the theoretical values.

*General procedure for preparation of 3-(n-chloroalkyl)-1,2,3-benzotriazin-4(3H)-one **2a, 2b***

A mixture of 1-bromo-2-chloroethane or 1-bromo-3-chloropropane (0.09 mol), 1,2,3-benzotriazin-4H-one **1** (0.03 mol), K_2CO_3 (0.05 mol) in DMF (70 mL) was introduced into the reaction vessel and the desired parameters (microwave power, temperature, and time) were set as reported in Table 2. After cooling, the mixture was concentrated to dryness and the residue was dissolved in water (50 mL); the solution was extracted several times with CH_2Cl_2. The organic phase was dried, concentrated, and chromatographed on silica gel column (diethyl ether/methanol, 9 : 1 v/v) to give compounds **2a** and **2b** as solids (obtained yields: **2a** 90%; **2b** 96%). ^{1}H-NMR spectra for all intermediates were consistent with the proposed structures.

*General procedure for preparation of 3-[2 or 3-(4-X-piperazin-1-yl)-alkyl]-1,2,3-benzotriazin-4(3H)-one **3–8***

A mixture of appropriate 3-(*n*-chloroalkyl)-1,2,3-benzotriazin-4(3*H*)-one **2a** or **2b** (0.03 mol), 4-X-piperazine (0.03 mol) and NaI (0.05 mol), K_2CO_3 (0.05 mol) in DMF (50 mL) was introduced into the reaction vessel and the desired parameters (microwave power, temperature, and time) were set as reported in Table 2. After cooling, the mixture was concentrated to dryness and the residue was dissolved in water (50 mL). The solution was extracted several times with CH_2Cl_2. The organic phase was dried on anhydrous Na_2SO_4, concentrated, and chromatographed on silica gel column (diethyl ether/ethanol 9 : 1 v/v) to give the final compounds **3–8** as white crystalline solids, (yield ranging between 65–84%).

*3-(2-(4-Cyclohexylpiperazin-1-yl)ethyl)benzo[d][1,2,3]-triazin-4(3H)-one **3***

^{1}H-NMR ($CDCl_3$) δ 1.05–1.86 (m, 11H, cyclohexyl), 2.56 (m, 4H), 2.61 (m, 4H), 2.89 (t, 2H, *J* = 6.8 Hz, CH_2-N-pip), 4.62 (t, 2H, *J* = 6.9 Hz, CH_2-N-benz), 7.80 (t, 1H, *J* = 7.6 Hz, ArH), 7.95 (t, 1H, *J* = 7.6 Hz, ArH), 8.15 (d, 1H, *J* = 8.1 Hz, ArH), 8.35 (d, 1H, *J* = 8.1 Hz, ArH). ESI-MS: 342.1 $[M + H]^+$.

*3-(3-(4-Cyclohexylpiperazin-1-yl)propyl)benzo[d][1,2,3]triazin-4(3H)-one **4***

^{1}H-NMR ($CDCl_3$) δ 1.04–1.78 (m, 11H, cyclohexyl), 2.10 (q, 2H, *J* = 6.9 Hz, -CH_2-), 2.12 (m, 4H), 2.44 (m, 4H), 2.49 (t, 2H, *J* = 6.8 Hz, CH_2-N-pip), 4.55 (t, 2H, *J* = 6.9 Hz, CH_2-N-benz), 7.80 (t, 1H, *J* = 7.6 Hz, ArH), 7.94 (t, 1H, *J* = 7.6 Hz, ArH), 8.14 (d, 1H, *J* = 8.1 Hz, ArH), 8.35 (d, 1H, *J* = 8.1 Hz, ArH). ESI-MS: 356.1 $[M + H]^+$.

*3-(2-(4-(Naphthalen-1-yl)piperazin-1-yl)ethyl)benzo[d][1,2,3]triazin-4(3H)-one **5***

^{1}H-NMR ($CDCl_3$) δ 2.88 (m, 4H), 3.05 (t, 2H, *J* = 6.8 Hz, CH_2-N-pip), 3.10 (m, 4H), 4.71 (t, 2H, *J* = 6.9 Hz, CH_2-N-benz), 7.06 (d, 1H, *J* = 8.1 Hz, ArH), 7.39 (t, 1H, *J* = 7.6 Hz, ArH), 7.46 (m, 2H, ArH), 7.53 (d, 1H, *J* = 8.1 Hz, ArH), 7.82 (m, 2H, ArH), 7.97 (t, 1H, *J* = 7.6 Hz, ArH), 8.19 (m, 2H, ArH), 8.38 (d, 1H, *J* = 8.1 Hz, ArH). ESI-MS: 386.2 $[M + H]^+$.

*3-(3-(4-(Naphthalen-1-yl)piperazin-1-yl)propyl)benzo[d][1,2,3]triazin-4(3H)-one **6***

^{1}H-NMR ($CDCl_3$) δ 2.21 (q, 2H, *J* = 6.9 Hz, -CH_2-), 2.65 (t, 2H, *J* = 6.8 Hz, CH_2-N-pip), 2.70 (m, 4H), 2.99 (m, 4H), 4.62 (t, 2H, *J* = 6.9 Hz, CH_2-N-benz), 6.96 (d, 1H, *J* = 8.1 Hz, ArH), 7.37 (t, 1H, *J* = 7.6 Hz, ArH), 7.45 (m, 2H, ArH), 7.52 (d, 1H, *J* = 8.1 Hz, ArH), 7.81 (m, 2H, ArH), 7.96 (t, 1H, *J* = 7.6 Hz, ArH), 8.16 (m, 2H, ArH), 8.37 (d, 1H, *J* = 8.1 Hz, ArH). ESI-MS: 400.2 $[M + H]^+$.

*3-(2-(4-(Quinolyn-8-yl)piperazin-1-yl)ethyl)benzo[d][1,2,3]triazin-4(3H)-one **7***

^{1}H-NMR ($CDCl_3$) δ 2.93 (m, 4H), 3.02 (t, 2H, *J* = 6.8 Hz, CH_2-N-pip), 3.37 (m, 4H), 4.69 (t, 2H, *J* = 6.9 Hz, CH_2-N-benz), 7.10 (t, 1H, *J* = 7.6 Hz, ArH), 7.35 (m, 2H,), 7.42 (d, 1H, *J* = 8.1 Hz, ArH), 7.80 (t, 1H, *J* = 7.6 Hz, ArH), 7.94 (t, 1H, *J* = 7.6 Hz, ArH), 8.08 (d, 1H, *J* = 7.6 Hz, ArH), 8.15 (d, 1H, *J* = 8.1 Hz, ArH), 8.36 (d, 1H, *J* = 7.6 Hz, ArH), 8.85 (d, 1H, *J* = 7.6 Hz, ArH). ESI-MS: 387.0 $[M + H]^+$.

*3-(3-(4-(Quinolyn-8-yl)piperazin-1-yl)propyl)benzo[d][1,2,3]triazin-4(3H)-one **8***

^{1}H-NMR ($CDCl_3$) δ 2.20 (q, 2H, *J* = 6.9 Hz, -CH_2-), 2.64 (t, 2H, *J* = 6.8 Hz, CH_2-N-pip), 2.77 (m, 4H), 3.30 (m, 4H), 4.60 (t, 2H, *J* = 6.9 Hz, CH_2-N-benz), 7.05 (t, 1H, *J* = 7.6 Hz, ArH), 7.34 (m, 2H), 7.40 (d, 1H, *J* = 8.1 Hz, ArH), 7.79 (t, 1H, *J* = 7.6 Hz, ArH), 7.93 (t, 1H, *J* = 7.6 Hz, ArH), 8.08 (d, 1H, *J* = 7.6 Hz, ArH), 8.14 (d, 1H, *J* = 8.1 Hz, ArH), 8.35 (d, 1H, *J* = 7.6 Hz, ArH), 8.84 (d, 1H, *J* = 7.6 Hz, ArH). ESI-MS: 401.1 $[M + H]^+$.

Hydrochloride salts: general procedure

The hydrochloride salts were prepared by adding HCl ethereal solution to an ethanolic solution of the free bases. All derivatives were recrystallized by diethyl ether and ethyl alcohol, formulae and melting points are reported in Table 1. The final compounds were obtained as white crystals.

Pharmacology – general procedures

The newly synthesized compounds were tested for *in-vitro* affinity for serotonin 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors by radioligand binding assays. The more active compounds on serotonin receptors have been selected and evaluated for their affinity for dopaminergic (D_1 and D_2) and adrenergic (α_1 and α_2) receptors. All the compounds were dissolved in ethanol or in 5% DMSO. The following specific radioligands and tissue sources were used: (a) serotonin 5-HT$_{1A}$ receptor, [^{3}H]-8-OH-DPAT, rat brain cortex, (b) serotonin 5-HT$_{2A}$ receptor, [^{3}H]ketanserin, rat brain cortex, (c) serotonin 5-HT$_{2C}$ receptor, [^{3}H]mesulergine, rat brain cortex, (d) dopamine D_1 receptor [^{3}H]SCH-23390, rat striatum, (e) dopamine D_2 receptor [^{3}H]spiperone, rat striatum, (f) α_1 adrenergic receptor [^{3}H]prazosin, rat brain cortex, and (g) α_2 adrenergic receptor [^{3}H]yohimbine, rat brain cortex. Non-specific binding

was determined as described in the Experimental (Section 4), and specific binding as the difference between total and non-specific binding. Blank experiments were carried out to determine the effect of 5% DMSO on the binding and no effects were observed. Competition experiments were analyzed by the "Easy Fit" program [25] to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding (IC_{50}), with six concentrations of test compounds, each performed in triplicate. The IC_{50} values obtained were used to calculate apparent inhibition constants (K_i) by the method of Cheng and Prussoff [26] from the following equation: $K_i = IC_{50}/(1 + S/K_D)$, where S represents the concentration of the hot ligand used and K_D its receptor-dissociation constant (K_D values, obtained by Scatchard analysis [27], were calculated for each labeled ligand).

5-HT$_{1A}$ binding assay

Radioligand-binding assays were performed following a published procedure [28]. Cerebral cortex from male Sprague-Dawley rats (180–220 g) was homogenized in 20 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.7 at 22°C) with a Polytron PT10, Brinkmann Instruments (setting 5 for 15 sec), and the homogenate was centrifuged at 50 000 *g* for 10 min at 0°C. The resulting pellet was then resuspended in the same buffer, incubated for 10 min at 37°C, and centrifuged at 50 000 *g* for 10 min. The final pellet was resuspended in 80 volumes of the Tris-HCl buffer containing 10 μM pargyline, 4 mM $CaCl_2$, and 0.1% ascorbate. To each assay tube was added the following: 0.1 mL of the drug dilution (0.1 mL of distilled water if no competing drug was added), 0.1 mL of [^{3}H]-8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]-8-OH-DPAT) (170.0 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) in the same buffer as above to achieve a final assay concentration of 0.1 nM, and 0.8 mL of resuspended membranes. The tubes were incubated for 30 min at 37°C, and the incubations were terminated by vacuum filtration through Whatman GF/B filters (Brandel Biomedical Research and Laboratories Inc., Gaithersburg, MD, USA). The filters were washed twice with 5 mL of ice-cold Tris-HCl buffer, and the radioactivity bound to the filters was measured by liquid scintillation spectrometer (Packard TRI-CARB® 2000CA – Packard BioScience s. r. l., Pero, Milan, Italy). Specific [^{3}H]-8-OH-DPAT binding was defined as the difference between binding in the absence and presence of 5-HT (10 μM).

Binding assays of 5-HT$_{2A}$ and 5-HT$_{2C}$

Radioligand-binding assays were performed as previously reported by Herndon *et al.* [29]. Briefly, frontal cortical regions of male Sprague-Dawley rats (180–220 g) were dissected on ice and homogenized (1 : 10 w/v) in ice-cold buffer solution (50 mM Tris HCl, 0.5 mM EDTA, and 10 mM $MgCl_2$ at pH 7.4) with a Polytron PT10 (setting 5 for 15 sec) and centrifuged at 3000 *g* for 15 min. The pellet was resuspended in buffer (1 : 30 w/v), incubated at 37°C for 15 min and then centrifuged twice more at 3000 *g* for 10 min (with resuspension between centrifugations). The final pellet was resuspended in buffer that also contained 0.1% ascorbate and 10^{-5} M pargyline.

Assays were performed in triplicate in a 2.0 mL volume containing 5 mg wet weight of tissue and 0.4 nM [^{3}H]ketanserin hydrochloride (88.0 Ci/mmol; Perkin Elmer Life Sciences) for 5-HT$_{2A}$ receptor assays, and 10 mg wet weight of tissue and 1 nM [^{3}H]mesulergine (87.0 Ci/mmol; Amersham Biosciences Europe GmbH, Freiburg, Germany) for 5-HT$_{2C}$ receptor assays. Cinanserin (1.0 μM) was used to define non-specific binding in the 5-HT$_{2A}$ assay. In the 5-HT$_{2C}$ assays, mianserin (1.0 μM) was used to define non-specific binding, and 100 nM spiperone was added to all tubes to block binding to 5-HT$_{2A}$ receptors. Tubes were incubated for 15 min at 37°C, filtered on Schleicher and Schuell (Keene, NH, USA) glass fibre filters presoaked in polyethylene imine, and washed with 10 mL of ice-cold buffer. Filters were counted at an efficiency of 50%.

D$_1$ Dopaminergic binding assay

The binding assay for D_1 dopaminergic receptors was that described by Billard *et al.* [30]. Corpora striata were homogenized in 30 vol. (w/v) ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25°C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged twice for 10 min at 50 000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris-HCl containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid and 10 μM pargyline (pH 7.1 at 37°C). Each assay tube contained 50 μL [^{3}H]SCH-23390 (85.0 Ci/mmol, Perkin Elmer Life Sciences) to achieve a final concentration of 0.4 nM, and 900 μL resuspended membranes (3 mg fresh tissue). The tubes were incubated for 15 min at 37°C and the incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25°C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]SCH-23390 binding was defined as the difference between binding in the absence or in the presence of 0.1 μM piflutixol.

D$_2$ Dopaminergic binding assay

The procedure used in the radioligand binding assay was reported in detail by Creese *et al.* [31]. Corpora striata were homogenized in 30 vol. (w/v) ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25°C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged twice for 10 min at 50 000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris-HCl containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid, and 10 μM pargyline (pH 7.1 at 37°C). Each assay tube contained 50 μL [^{3}H]spiperone (15.7 Ci/mmol, Perkin Elmer Life Sciences) to achieve a final concentration of 0.4 nM and 900 μL resuspended membranes (3 mg fresh tissue). The tubes were incubated for 15 min at 37°C and the incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25°C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]spiperone binding was defined as the difference between binding in the absence or in the presence of 1 μM (+)-butaclamol.

α$_1$ Adrenergic binding assay

The procedure used in the radioligand binding assay has been reported in detail by Greengrass and Brenner [32]. Brain cortex was homogenized in 30 vol. (w/v) ice-cold 50 mM Tris-HCl buffer, (pH 7.2 at 25°C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged twice for 10 min at 50 000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris-HCl, (pH 7.4 at 25°C). Each assay tube contained 50 μL drug solution, 50 μL [^{3}H]prazosine (80.5 Ci/mmol, Perkin Elmer Life Sciences) to achieve a final concentration of 0.4 nM, and 900 μL resuspended membranes (10 mg fresh tissue). The tubes were incubated for 30 min at 25°C and the

incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris-HCl, buffer (pH 7.2 at 25°C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]prazosin binding was defined as the difference between binding in the absence or in the presence of 10 μM phentolamine.

α_2 Adrenergic binding assay

The procedure used in the radioligand binding assay was reported in detail by Perry and U'Prichard [33]. Brain cortex was homogenized in 30 vol. (w/v) ice-cold 5 mM Tris-HCl, 5 mM EDTA buffer (pH 7.3 at 25°C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged three times for 10 min at 50 000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris-HCl, 0.5 mM EDTA (pH 7.5 at 25°C). Each assay tube contained 50 μL drug solution, 50 μL [^{3}H]yohimbine (80.5 Ci/mmol, Perkin Elmer Life Sciences) to achieve a final concentration of 1 nM, and 900 μL resuspended membranes (10 mg fresh tissue). The tubes were incubated for 30 min at 25°C and the incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris-HCl, 0.5 mM EDTA buffer (pH 7.5 at 25°C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]yohimbine binding was defined as the difference between binding in the absence or in the presence of 10 μM phentolamine.

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