European Journal of Medicinal Chemistry 47 (2012) 520–529

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

New potent 5-HT$_{2A}$ receptor ligands containing an N′-cyanopicolinamidine nucleus: Synthesis and in vitro pharmacological evaluation

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ARTICLE INFO

Article history:
Received 7 October 2011
Received in revised form
9 November 2011
Accepted 10 November 2011
Available online 20 November 2011

Keywords:
N′-cyanopicolinamidine derivatives
5-HT$_{1A}$ ligands
5-HT$_{2A}$ ligands
Binding assays

ABSTRACT

N′-cyanopicolinamidine derivatives, linked to an arylpiperazine moiety, were prepared and their affinity to serotonin 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors were evaluated. The combination of structural elements (heterocyclic nucleus, alkyl chain and 4-substituted piperazine) known to be critical for affinity to 5-HT$_{1A}$ receptors and the proper selection of substituents led to compounds with high specificity and affinity towards serotoninergic receptors. In binding studies, several molecules showed affinity in nanomolar and subnanomolar range at 5-HT$_{2A}$ and moderate to no affinity for other relevant receptors (5-HT$_{1A}$, 5-HT$_{2C}$, D$_1$, D$_2$, α_1 and α_2). N′-cyano-N-(3-(4-(3-chlorophenyl)piperazin-1-yl)propyl)-picolinamidine (**4l**) with $K_i = 0.000185$ nM, was the most active and selective derivative for the 5-HT$_{2A}$ receptor compared to other serotoninergic, dopaminergic and adrenergic receptors.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) [1–4] is involved in wide-ranging physiological and pathophysiological processes [5]. Pharmacological manipulation of the 5-HT system is believed to have therapeutic potential, and therefore the subject of intense research [4].

Among the 14 different serotonin receptors, belonging to the G protein coupled receptors superfamily [4], 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors are most frequently considered as important targets for neuro-biological research and drug development. Activation of these receptors leads to a number of physiological changes that can be easily quantified [6–8].

5-HT$_{1A}$ receptor, as a member of the group of G protein coupled receptors (GPCRs) [9], shows high similarity with other members of the family. A particular case is represented by the similarity between 5-HT$_{1A}$R and α_1-adrenoreceptor [10] that show a high degree of homology (45%) in their amino acid sequence. In addition to its effects on a wide range of psychiatric disorders, 5-HT$_{1A}$R is also involved in the proliferation of human tumour cells (PC3) and in human hormone refractory prostate cancer tissue [11]. The 5-HT$_2$ receptor family has three known subtypes, 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$, with 46–50% sequence homology [4]. Moreover, the 5-HT$_{2A}$R and 5-HT$_{2C}$R transmembrane domains share 80% sequence homology, suggesting similar pharmacological profiles [12]. Activation of 5-HT$_{2A}$ receptors stimulates the secretion of various hormones and influences neuronal plasticity; peripheral 5-HT$_{2A}$ receptors mediate several processes such as vasoconstriction and platelet aggregation [4]. The 5-HT$_{2C}$ receptor is involved in physiological functions such as locomotory activity, anxiogenesis and neuro-endocrine functions, and is implicated in sexual dysfunction in males [13,14].

On these basis drugs that target 5-HT$_2$ receptors could be useful for the treatment of several psychiatric disorders, including depression, anxiety, obsessive-compulsive disorders and schizophrenia [15].

Several chemical classes are already known for their high affinity towards these receptors and, from a chemical point of view, they can be subdivided into different classes. Among them one of the most studied group is that of long-chain arylpiperazines (LCAPs) [8]. In these derivatives the arylpiperazine moiety, tail of the molecule, and the heterocyclic head are separated with a spacer or linker. In the aryl position substituted phenyl is typically located while the heterocyclic nucleus is an amide or imide. The

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doi:10.1016/j.ejmech.2011.11.023

significance of the respective parts 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 associated with variation of the aromatic systems at the "head" and "tail" of the structure, with the length of the methylene linker, and with the replacement of the amide bond with other functions [16]. A huge number of SAR studies revealed the role of individual fragments, essential for high affinity for 5-HT_{1A} sites and selectivity for other receptors. However, a limitation of many 5-HT_{1A} receptor ligands is their undesired high affinity for other receptor subtypes such as the dopaminergic D_2 receptor and α_1-adrenoceptor.

In our laboratories, there has been an ongoing effort to develop more selective serotoninergic ligands [17–26] in order to have novel pharmacological tools that could improve our knowledge of the signal transduction mechanism leading to compounds with high affinity and selectivity.

A previously described study focused on the synthesis and pharmacological evaluation of a set of arylpiperazine derivatives containing an N′-cyanoisonicotinamidine nucleus; the binding data reported in this study identified this original scaffold as an optimal structural element to enhance 5-HT_{1A} receptor affinity [26].

In continuation of our research program, we designed a new set of derivatives where the piperazine-N-alkyl moiety has been linked to a novel original N′-cyanopicolinamidine fragment as terminal part of LCAPs (Scheme 1); this choice was made considering that it could be interesting to investigate the correlation between the nitrogen position in the pyridine ring and binding affinity/selectivity. The N′-cyanopicolinamidine scaffold was linked via three methylene spacing units to piperazines substituted in position 4 with aliphatic and/or aromatic substitutes. The alkyl chain length (three units) and the substitutes on the N-4 of the piperazine moiety were the same employed in our previous study [26]. This choice was done in order to obtain a complete and comparative structure-affinity and structure-selectivity relationship study between the N′-cyanoisonicotinamidine derivatives and the N′-cyanopicolinamidine derivatives. All the new compounds were tested for their affinity for 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors and the multireceptor profiles of promising derivatives were also evaluated in terms of binding affinities for dopaminergic (D_1, D_2) and adrenergic (α_1, α_2) receptors.

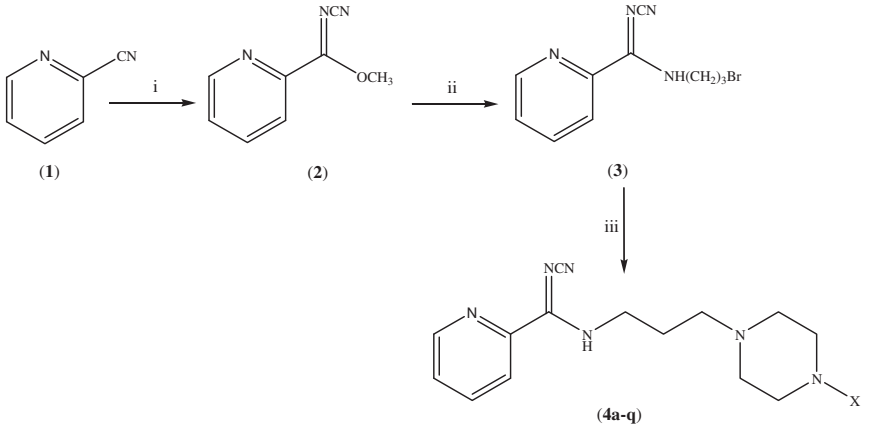
2. Chemistry

The synthetic strategy employed for the preparation of the target compounds (Table 1) is summarized in Scheme 1. The general procedure is as follows: methyl-N-cyano-2-pyridinecarboximidate (**2**) was prepared starting from 2-cyanopyridine (**1**) by base-catalyzed conversion in anhydrous methanol followed by treatment with cyanamide in aqueous phosphate buffer ($NaH_2PO_4 \cdot 2H_2O/Na_2HPO_4 = 4:1$). The conversion of methyl-N-cyano-2-pyridinecarboximidate (**2**) to N-(3-bromopropyl)-N′-cyanopicolinamidine (**3**) was obtained by reaction of (**2**) with 3-bromopropylamine·HBr and triethylamine in anhydrous methanol. Subsequent condensation of compound (**3**) with the desired 4-X-substituted piperazine, performed in CH_3CN in the presence of K_2CO_3 and NaI, under reflux, provided the final compounds **4a**–**q**. 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 and were characterized by ^{1}H NMR and mass spectrometry (API 2000 Applied Biosystem). ^{1}H NMR and MS data for all final compounds were consistent with the proposed structures. As already reported in literature, due to tautomeric equilibrium about sp^2 carbon, cyanoamidine derivatives may exist as a mixture of two tautomers (I and II in Fig. 1). The NMR spectra of **4a**–**q** confirmed that all final compounds exist predominantly as the cyanoimino form (I). Moreover, regarding the configurational determination of geometrical isomers (*E*/*Z*) on the amidine bond C=N, the N-cyanoamidine derivatives **4a**–**q** exhibited only one set of relevant ^{1}H NMR signals, implying the existence of either a single geometric form or, more probably, a fast equilibrium of two isomeric forms as already described in literature [27].

3. Results and discussion

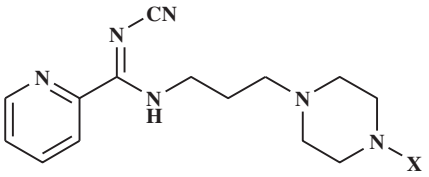
Several of the reported derivatives were potent 5-HT_{2A} receptor ligands, in fact, they showed nanomolar or even subnanomolar 5-HT_{2A} receptor affinities (Table 1). Besides the outstanding 5-HT_{2A} receptor affinity of compound **4l** ($K_i = 0.000185$ nM, Fig. 3), other interesting K_i values were those of compounds **4m** (0.00078 nM, Fig. 4), **4d** (0.00157 nM, Fig. 2), **4n** (0.0161 nM, Fig. 5) and **4h** (0.867 nM) while compounds **4o**, **4i**, **4p**, **4q**, **4g** and **4f** were less active with K_i values of 1.12, 27, 51.8, 21.5, 904 and 2730 nM, respectively. Other derivatives showed K_i values of above 10^4 nM or no affinity.

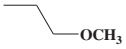
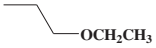
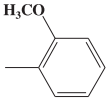
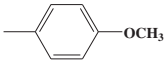
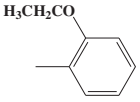
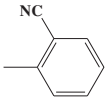
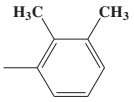
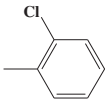
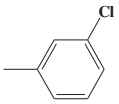
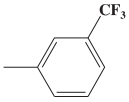
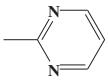
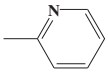
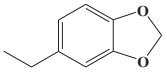
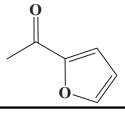
Derivatives **4a**–**q** are generally characterized by a binding affinity/selectivity profile that is surprisingly shifted towards 5-HT_{2A} receptors subtypes. Moreover the introduction of non



Scheme 1. Reagents and conditions: (i) CH_3ONa, H_2NCN, anhydrous MeOH; (ii) $Br(CH_2)_3NH_2 \cdot HBr$, TEA, anhydrous MeOH; (iii) 4-X-substituted piperazine, K_2CO_3, NaI, CH_3CN, 70 °C, 4 h.

Table 1
Affinities of compounds **4a**–**q** for 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors.



	Receptor affinity $K_i \pm$ SD (nM)			
Compd	X	5-HT_{1A} [^{3}H]8OH-DPAT	5-HT_{2A} [^{3}H]ketanserin	5-HT_{2C} [^{3}H]mesulergine
4a		No affinity	No affinity	$>10^4$
4b		No affinity	No affinity	389 ± 17.7
4c		No affinity	No affinity	597 ± 8.8
4d		4.68 ± 0.389	0.00157 ± 0.000106	1.61 ± 0.458
4e		0.0842 ± 0.00088	$>10^4$	No affinity
4f		17.2 ± 1.36	2730 ± 143	No affinity
4g		$>10^4$	904 ± 10.2	28.8 ± 2.67
4h		30 ± 1.79	0.867 ± 0.0308	29.7 ± 2.14
4i		170 ± 11.7	27 ± 0.97	No affinity
4l		6.67 ± 0.070	0.000185 ± 0.0000168	3.28 ± 0.223
4m		1.31 ± 0.263	0.000778 ± 0.0000331	1.81 ± 0.178
4n		3080 ± 154	0.0161 ± 0.00120	22.5 ± 1.74
4o		30.6 ± 2.20	1.12 ± 0.178	$>10^4$
4p		$>10^4$	51.8 ± 0.27	No affinity
4q		$>10^4$	21.5 ± 0.21	No affinity

For purpose of comparison, 8-OH-DPAT, ketanserine and mesulergine bind 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors with values of 0.80, 0.85 and 1.90 nM, respectively, under these assay conditions.

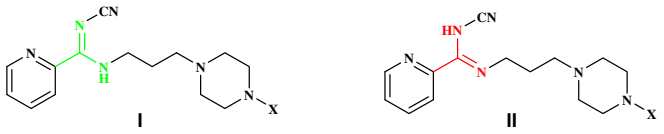


Fig. 1. Tautomeric equilibrium about sp^2carbon of the cyanoamidine derivatives.

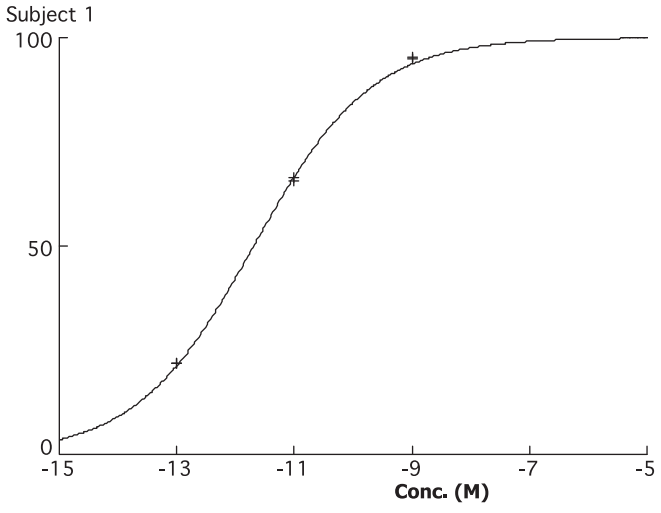
aromatic or larger substituents on the piperazine nitrogen, also in this series of derivatives, caused a dramatic decrease of the receptor affinity.

Concerning the influence of the N-4 substituent of the piperazine moiety, the 3-chlorophenyl group (**4l**), and the 3-trifluoromethylphenyl group (**4m**), conferred the highest affinity and selectivity for 5-HT_{2A} receptor. The presence of a 2-methoxyphenyl, pyrimidinyl or 2,3-dimethylphenyl group on the N-4 of the piperazine moiety, led also to compounds which exhibited high affinity for 5-HT_{2A} receptor (**4d** $K_i = 0.00157$ nM; **4n** $K_i = 0.0161$ nM and **4h** $K_i = 0.867$ nM). Non aromatic moiety as well as 2-ethoxyphenyl and 2-cyanophenyl groups furnished compounds characterized by a high decrease in binding affinity compared to the other N-4 substitutes at the piperazine ring.

The 5-HT_{1A} and 5-HT_{2C} receptor affinities of the tested compounds were always lower than those observed for 5-HT_{2A} receptors except for compounds **4e** and **4g** that showed higher affinities towards 5-HT_{1A} ($K_i = 0.0842$ nM) and 5-HT_{2C} receptors ($K_i = 28.8$ nM), respectively. N-(3-(4-(3-chlorophenyl)piperazin-1-yl)propyl)-N′-cyanopicolinamidine (**4l**) ($K_i = 0.000185$ nM) showed not only the highest affinity but also one of the best selectivity profiles with respect to the other serotoninergic receptors studied.

Additionally, the affinity of the most active compounds (**4d**, **4e**, **4f**, **4h**, **4l**, **4m**, **4n**, **4o**, **4p** and **4q**) on several other receptors (α_1 and α_2 adrenergic and D_1 and D_2 dopaminergic receptors) was

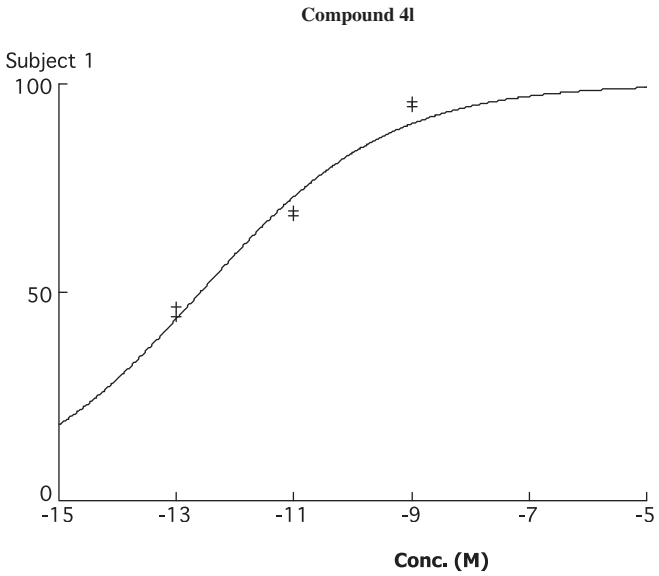
Compound 4d



[ ] final (M)	I% ($\overline{M}$ ± d.s.)	IC_{50} (M)
10^{-9}	95.11 ± 0.007	
10^{-11}	65.865 ± 0.516	2.12 x 10^{-12} ± 0.143
10^{-13}	21.85 ± 0.170	

Fig. 2. Binding curve of compound **4d**.

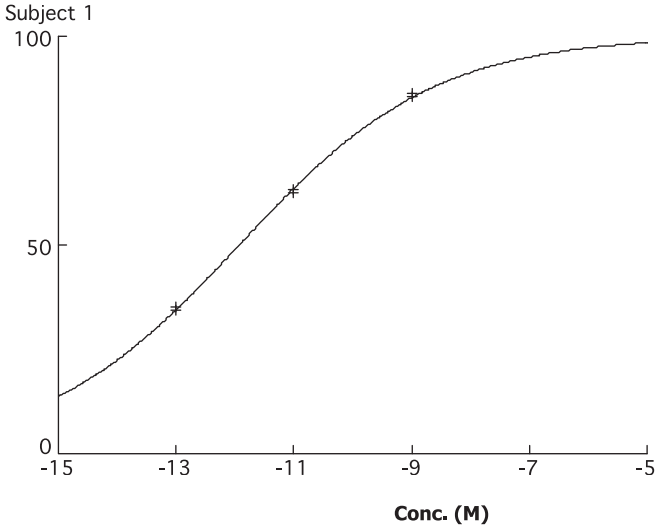
Compound 4l



[ ] final (M)	I% ($\overline{M}$ ± d.s.)	IC_{50} (M)
10^{-9}	95.095 ± 0.771	
10^{-11}	68.75 ± 0.940	2.50 x 10^{-13} ± 0.227
10^{-13}	45.32 ± 1.704	

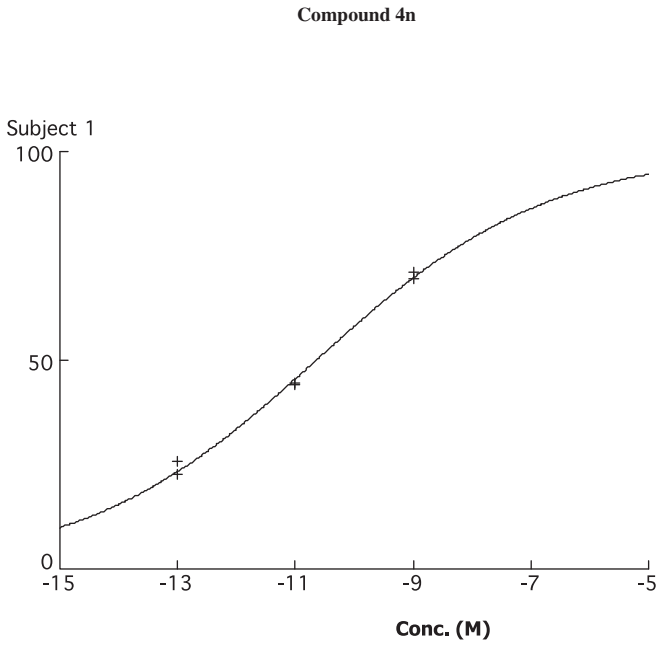
Fig. 3. Binding curve of compound **4l**.

Compound 4m



[ ] final (M)	I% ($\overline{M}$ ± d.s.)	IC_{50} (M)
10^{-9}	85.935 ± 0.389	
10^{-11}	62.825 ± 0.601	1.05 x 10^{-12} ± 1.447
10^{-13}	34.87 ± 0.481	

Fig. 4. Binding curve of compound **4m**.



[ ] final (M)	I% ($\overline{M}$ ± d.s.)	IC_{50} (M)
10^{-9}	70.42 ± 1.117	
10^{-11}	44.335 ± 0.488	2.17 x 10^{-11} ± 0.162
10^{-13}	24.16 ± 2.121	

Fig. 5. Binding curve of compound **4n**.

examined in order to verify the selectivity of these compounds. Results are summarized in Table 2. All the compounds proved highly selective against dopaminergic receptors with K_i values of above 10^4 nM except for compound **4f**, which exhibited K_i value of 52.4 nM on D_1 receptor and compound **4m**, which exhibited K_i value of 83.7 nM on D_2 receptor. Regarding α_1 and α_2 adrenergic receptors, only compound **4d** showed quite moderate affinity (105 nM and 245 nM respectively), while compound **4o** showed quite moderate affinity only towards α_1 receptors (130 nM); these data are very interesting considering the high degree of homology existing between these two receptors and demonstrate that these compounds possess a very good binding profile, preferring 5-$HT_{2A}R_s$ over all other evaluated receptors.

Table 2
Affinities of compounds **4d**, **4e**, **4f**, **4h**, **4l**, **4m**, **4n**, **4o**, **4p** and **4q** for D_1, D_2, α_1 and α_2 receptors.

Compd	Receptor affinity $K_i \pm$ SD (nM)			
	D_1	D_2	α_1	α_2
	[^{3}H]SCH-23390	[^{3}H]spiperone	[^{3}H]prazosin	[^{3}H]yohimbine
4d	$>10^4$	$>10^4$	105 ± 28.1	245 ± 37.7
4e	$>10^4$	$>10^4$	$>10^4$	No affinity
4f	52.4 ± 3.57	$>10^4$	152 ± 7.4	571 ± 38.4
4h	$>10^4$	$>10^4$	729 ± 9.3	318 ± 7.8
4l	381 ± 33	$>10^4$	174 ± 19.2	1360 ± 77
4m	$>10^4$	83.7 ± 3.23	770 ± 15.9	$>10^4$
4n	$>10^4$	$>10^4$	$>10^4$	No affinity
4o	$>10^4$	$>10^4$	130 ± 11.9	$>10^4$
4p	No affinity	No affinity	No affinity	964 ± 67.0
4q	No affinity	No affinity	No affinity	$>10^4$

A comparative structure-affinity and structure-selectivity relationship study between the N′-cyanoisonicotinamidine derivatives and the N′-cyanopicolinamidine derivatives emphasizes the high selectivity afforded by the discussed N′-cyanopicolinamidine scaffold to shift the binding affinity towards 5-HT_{2A} receptors subtypes. Moreover this study confirmed that the propyl chain associated to the appropriate aromatic ring substitution plays a critical role not only in determining 5-HT_{1A} receptor affinity and selectivity but it is also useful to determinate a favourable 5-HT_{2A} receptor affinity/selectivity profile.

The outstanding affinity of **4l,** supporting the 3-chlorophenyl group, and **4m**, supporting a very hydrophobic 3-trifluoromethylphenyl group, could be explained, as already reported by Parker et al. [28], in terms of correlation between lipophilicity and binding affinity towards 5-HT_{2A} receptors that appear decisive compared to all other compounds. Moreover, the interesting affinity/selectivity profile of **4d**, **4n**, and **4o**, supporting a 2-methoxyphenyl, a pyrimidinyl and a pyridin-2-yl group, respectively, are in accordance with data already reported in literature where, as general trend, the presence of an ether oxygen (**4d**) or a nitrogen (**4n** and **4o**) acts as hydrogen bond acceptors from key residues such as threonine and serine in the 5-HT_{2A} receptor binding pocket [29].

The main feature of these new derivatives was represented by the interest in the N′-cyanopicolinamidine fragment as a molecular scaffold that plays a critical role in determining 5-HT_{2A} receptor affinity and selectivity, evolved from our previous work [26] with the N′-cyanoisonicotinamidine scaffold. This result could be due to the different position of nitrogen into the aromatic system of the N′-cyanopicolinamidine nucleus compared to the N′-cyanoisonicotinamidine scaffold and could be explained considering also the topography of the 5-HT_{2A} receptor binding site. Molecular modelling of the 5-HT_{2A} receptor, together with detailed site-directed mutagenesis studies, suggests a hydrophobic binding pocket surrounding the ligands, with aromatic residues such as Trp-151, Phe-243, Phe-244, Trp336, Phe-339, Phe-340, Trp-367 and Tyr-370. Polar moieties may interact with Ser-159, Thr-160, Ser-239, Ser-242 and Asn-343 from the 5-HT_{2A} binding site [5] and protonated amine, like piperazine moiety, undergoes an electrostatic binding interaction with the conserved aspartate (Asp-159) residue in transmembrane helix 3 (TM3) [30]. Moreover human 5-HT_{2A} receptor and rat receptor (object of our study) are highly homologous in their amino acid sequences. Both contain Ser-239, but one of the three differences in the TMD sequence is seen at position 242 in fifth TMD, where the human receptor expresses a serine and the rat receptor expresses an alanine residue. The Ser-239 and Ser-242 in the human receptor are predicted to act as a hydrogen-bonding site for example for serotonin 5-hydroxyl group and indole NH respectively [31]. However, as already reported [32], H-bond of the indole NH to the side chains of Ser-159 or Thr-160 may also be possible since Ser-242 is mutated into Ala in the rat 5-HT_{2A}R. On the basis of these considerations we support the idea that, similarly to the indolic NH [31], the nitrogen lone pair into the aromatic system of the N′-cyanopicolinamidine nucleus can form H-bond with the side chains of Ser-159 or Thr-160. Therefore, the different position of nitrogen into the aromatic systems of the N′-cyanopicolinamidine and N′-cyanoisonicotinamidine nuclei can explain the different bent to form favourable H-bond with Ser-159 or Thr-160 and, consequently, the different affinity/selectivity binding profile of N′-cyanopicolinamidine derivatives towards 5-HT_{2A} receptors and N′-cyanoisonicotinamidine derivatives towards 5-HT_{1A} receptors.

In fact, compound **4e** (K_i value of 0.0842 nM) supporting *p*-methoxy-phenylpiperazine moiety, was the only derivative that showed a favourable affinity/selectivity binding profile towards 5-

HT_{1A} receptors. This result, apparently clashing with the low affinity and selectivity of the previously reported 4-methoxy-phenylpiperazine derivatives [25,26] could probably be attributed to favourable steric interaction of the methoxy group (**4e**) with the surrounding receptor residues that leads to the formation of a hydrogen bond with Asn-386 associated to the presence of the N′-cyanopicolinamidine scaffold.

Finally the interesting 5-HT_{2C} receptor affinities of some derivatives (**4d** $K_i = 1.61$ nM; **4h** $K_i = 29.7$ nM; **4l** $K_i = 3.28$ nM; **4m** $K_i = 1.81$ nM; **4n** $K_i = 22.5$ nM) are lower than those observed for 5-HT_{2A}R and comparable to those observed for 5-HT_{1A}R, determining for these derivatives a bad affinity/selectivity profile towards the 5-HT_{2C} receptor. Moreover, also in this series, the derivatives characterized by the presence of an aliphatic group (**4a**, **4b** and **4c**) on the N-4 position led to an unfavourable affinity/selectivity profile, confirming, as already reported [24–26], that a non aromatic moiety is less favourable to the hydrophobic interaction with the receptor, determining the formation of weaker complexes with the receptor.

4. Conclusion

We have described the synthesis of a new series of arylpiperazines as 5-HT_{2A} ligands (**4a**–**q**), containing a novel heterocyclic fragment. Some of the described compounds showed high in vitro affinity and selectivity towards 5-HT_{2A} receptors. Compound **4l** was the most potent ($K_i = 0.000185$ nM) and selective derivative for 5-HT_{2A} receptor with respect to the other serotonin, dopaminergic and adrenergic receptors, and the affinity/selectivity profile of compound **4m** ($K_i = 0.000778$ nM) supporting on the N-4 of the piperazine moiety the 3-trifluoromethylphenyl group appears interesting besides. The binding data presented in this study identified the N′-cyanopicolinamidine nucleus as an optimal structural element to shift the affinity/selectivity profile towards 5-HT_{2A} receptor binding, compared to the N′-cyanoisonicotinamidine scaffold that furnished a series of derivatives with good 5-HT_{1A} affinity and selectivity.

5. Experimental

5.1. Synthesis

5.1.1. General procedures

All reagents and substituted piperazines were commercial products purchased from Aldrich. Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian Mercury Plus 400 MHz instrument. Unless otherwise stated, all spectra were recorded in $CDCl_3$. Chemical shifts are reported in ppm using Me_4Si as internal standard. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), m (multiplet), q (quartet), qt (quintet), dd (double doublet), ddd (double dd), br s (broad singlet). Mass spectra of the final products were performed on API 2000 Applied Biosystem mass spectrometer. Where analyses are indicated only by the symbols of the elements, results obtained are within ±0.4% of the theoretical values. All reactions were followed by TLC, carried out on Merck silica gel 60 F_{254} plates with fluorescent indicator and the plates were visualized with UV light (254 nm). Preparative chromatographic purifications were performed using silica gel column (Kieselgel 60). Solutions were dried over Na_2SO_4 and concentrated with Buchi rotary evaporator at low pressure.

5.1.2. Methyl N-cyano-2-pyridinecarboximidate (*2*)

A mixture of 2-cyanopyridine (**1**) (10 g, 0.096 mol) and NaOMe (0.26 g, 0.0048 mol) in anhydrous methanol (180 ml) was stirred overnight at room temperature. AcOH (0.32 g, 0.0053 mol) was then added with stirring to neutralize the reaction solution and the solution was evaporated *in vacuo*. Diethyl ether (150 ml) was added to the residue and the resultant precipitate was filtered off. The filtrate was evaporated to give crude methyl-2-pyridinecarboximidate (7.0 g) as an oil. Crude compound was then added to a mixture of NH_2CN (4.33 g, 0.103 mol), $NaH_2PO_4 \cdot 2H_2O$ (32.14 g) and Na_2HPO_4 (7.30 g) in water (55 ml). After vigorous stirring for 4 h at room temperature, the reaction mixture was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and concentrated *in vacuo* to yield crude methyl-N-cyano-2-pyridinecarboximidate (**2**) (8.4 g), which was used directly in the following reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.16 (s, 3H); 7.63 (dd, 1H, $J = 9.4, 7.3$); 7.94 (d, 1H, $J = 3.4$); 7.98 (dd, 1H, $J = 7.3, 2.4$); 8.83 (ddd, 1H, $J = 9.4, 3.4, 2.4$). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 46.35; 116.90; 123.64; 126.80; 136.25; 148.13; 149.15, 153.10.

5.1.3. N-(3-Bromopropyl)-N′-cyanopicolinamidine (*3*)

To a solution of methyl N-cyano-2-pyridinecarboximidate (**2**) (8 g, 0.050 mol) in anhydrous MeOH (120 mL), 3-bromopropylamine·HBr (11.93 g, 0.055 mol) and triethylamine (5.56 g 0.055 mol) were successively added and the reaction mixture was stirred at room temperature for 18 h. After evaporation, the residue was dissolved in $CHCl_3$. The solution was washed with water, dried on anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (diethyl ether/ethanol 9:1 (v/v)). The combined and evaporated product fractions were crystallized from diethyl ether /hexane, yielding 8.4 g (63 %) of the desired product as a brown solid: mp: 73–74 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.15 (qt, 2H, $J = 6.2$); 2.75 (t, 2H, $J = 6.2$); 3.83 (q, 2H, $J = 6.2$); 7.47 (t, 1H, $J = 7.3$); 7.86 (t, 1H, $J = 7.3$); 8.5 (d, 1H, $J = 7.3$); 8.64 (d, 1H, $J = 7.3$); 9.62 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 27.33; 38.70; 53.91; 117.20; 122.54; 125.66; 135.10; 148.23; 150.70, 154.20.

5.1.4. General procedure for the condensation of 4-substituted arylpiperazines with derivative *3*

A mixture of N-(3-bromopropyl)-N′-cyanopicolinamidine (**3**) (0.006 mol) and NaI (0.009 mol) in acetonitrile was stirred under reflux for 30 min. Then the appropriate 4-substituted arylpiperazine (0.06 mol) and anhydrous K_2CO_3 (0.009 mol) were added. The reaction mixture was stirred under reflux for 4 h. After cooling, the mixture was filtered, concentrated to dryness and the residue was dissolved in water (50 mL). The solution was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and the solvent removed under vacuum. The crude mixture was purified by silica gel column chromatography using diethyl ether/methanol 8:2 (v/v) as eluent. The crude products were recrystallized from diethyl ether.

5.1.5. N-(3-(4-allylpiperazin-1-yl)propyl)-N′-cyanopicolinamidine (*4a*)

From **3** and 1-allylpiperazine.

Yield: 32%; mp 93–94 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.83 (m, 2H); 2.32 (br s, 4H, $2CH_2$ pip.); 2.51 (br s, 4H, $2CH_2$ pip.); 2.55 (t, 2H, N^1–CH_2, $J = 5.5$); 3.00 (d, 2H, $J = 6.6$); 3.81 (br s, 2H, N-CH_2); 5.14 (dd, 2H); 5.81 (m, 1H,); 7.48 (t, 1H, $J = 7.3$); 7.87 (t, 1H, $J = 7.7$); 8.50 (d, 1H, $J = 8.0$); 8.64 (d, 1H, $J = 4.4$); 9.75 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 24.36; 43.29; 52.96; 53.60; 57.91; 62.11; 117.55; 118.30; 123.54; 126.86; 135.14; 137.89; 148.24; 148.90, 151.80.

ESI-MS: 313.3 $[M+H]^+$; 335.2 $[M+Na]^+$.
Anal. ($C_{17}H_{24}N_6$), C, H, N.

*5.1.6. N′-cyano-N-(3-(4-(2-methoxyethyl)piperazin-1-yl)propyl) picolinamidine (**4b**)*

From **3** and 1-(2-methoxyethyl)piperazine.

Yield: 16%; mp 60–61 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.83 (m, 2H); 2.28 (br s, 4H, $2CH_2$ pip.); 2.38 (t, 2H, N^1–CH_2, $J = 5.4$); 2.55 (br s, 4H, $2CH_2$ pip.); 2.57 (t, 2H N^4–CH_2, $J = 5.5$); 3.35 (s, 3H, OCH_3); 3.50 (t, 2H, CH_2–O, $J = 5.5$); 3.81 (br s, 2H, N–CH_2,); 7.47 (t, 1H, $J = 7.3$); 7.86 (t, 1H, $J = 7.7$); 8.50 (d, 1H, $J = 8.0$); 8.64 (d, 1H, $J = 4.4$); 9.80 (s, 1H).

ESI-MS: 331.1 $[M+H]^+$.
Anal. ($C_{17}H_{26}N_6O$), C, H, N.

*5.1.7. N′-cyano-N-(3-(4-(2-ethoxyethyl)piperazin-1-yl)propyl) picolinamidine (**4c**)*

From **3** and 1-(2-ethoxyethyl)piperazine.

Yield: 56%; mp 195–197 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.17 (t, 3H, $J = 6.9$); 1.76 (br s, 4H, $2CH_2$ pip.); 1.81 (qt, 2H, $J = 5.8$); 2.55 (t, 2H, N^1–CH_2, $J = 6.2$); 2.56 (br s, 4H, $2CH_2$ pip.); 2.58 (t, 2H, N^4–CH_2, $J = 6.2$); 3.47 (q, 2H, O–CH_2, $J = 6.9$); 3.52 (t, 2H, CH_2–O, $J = 6.2$); 3.81 (br s, 2H, N–CH_2); 7.46 (t, 1H, $J = 7.3$); 7.86 (t, 1H, $J = 7.7$); 8.49 (d, 1H, $J = 8.0$); 8.64 (d, 1H, $J = 4.4$); 9.80 (s, 1H).

ESI-MS: 345.4 $[M+H]^+$; 367.1 $[M+Na]^+$; 383.0 $[M+K]^+$.
Anal. ($C_{18}H_{28}N_6O$), C, H, N.

*5.1.8. N′-cyano-N-(3-(4-(2-methoxyphenyl)piperazin-1-yl)propyl) picolinamidine (**4d**)*

From **3** and 1-(2-methoxyphenyl)piperazine.

Yield: 20%; mp 113–116 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.67 (m, 2H); 1.87 (qt, 2H, $-CH_2-$, $J = 5.5$); 2.65 (t, 2H, N^1–CH_2, $J = 5.1$); 2.72 (br s, 4H, $2CH_2$ pip.); 3.15 (br s, 4H, $2CH_2$ pip.); 3.87 (s, 3H, OCH_3); 6.87 (d, 1H, $J = 7.4$); 6.89 (d, 1H, $J = 7.4$); 6.93 (t, 1H, $J = 7.4$); 7.01 (t, 1H, $J = 7.4$); 7.41 (t, 1H, $J = 7.3$); 7.84 (t, 1H, $J = 7.7$); 8.51 (d, 1H, $J = 8.0$); 8.55 (d, 1H, $J = 4.4$); 10.06 (s, 1H).

ESI-MS: 379.4 $[M+H]^+$; 401.6 $[M+Na]^+$; 417.5 $[M+K]^+$.
Anal. ($C_{21}H_{26}N_6O$), C, H, N.

*5.1.9. N′-cyano-N-(3-(4-(4-methoxyphenyl)piperazin-1-yl)propyl) picolinamidine (**4e**)*

From **3** and 1-(4-methoxyphenyl)piperazine.

Yield: 15%; mp 106–108 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.90 (m, 2H); 2.63 (br s, 4H, $2CH_2$ pip.); 2.66 (t, 2H, N^1–CH_2, $J = 5.1$); 3.16 (br s, 4H, $2CH_2$ pip.); 3.78 (s, 3H, OCH_3); 3.88 (br s, 2H, N–CH_2); 6.84 (d, 2H, $J = 8.9$); 6.88 (d, 2H, $J = 8.9$); 7.39 (t, 1H, $J = 7.3$); 7.83 (t, 1H, $J = 7.7$); 8.44 (d, 1H, $J = 4.4$); 8.51 (d, 1H, $J = 8.0$); 9.97 (s, 1H).

ESI-MS: 379.9 $[M+H]^+$; 401.3 $[M+Na]^+$; 417.3 $[M+K]^+$.
Anal. ($C_{21}H_{26}N_6O$), C, H, N.

*5.1.10. N′-cyano-N-(3-(4-(2-ethoxyphenyl)piperazin-1-yl)propyl) picolinamidine (**4f**)*

From **3** and 1-(2-ethoxyphenyl)piperazine.

Yield: 14%; mp 122–125 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.43 (t, 3H, $J = 6.9$); 1.88 (m, 2H); 2.64 (t, 2H, N^1–CH_2, $J = 5.1$); 2.71 (br s, 4H, $2CH_2$ pip.); 3.17 (br s, 4H, $2CH_2$ pip.); 3.86 (br s, 2H, N–CH_2); 4.04 (q, 2H, OCH_2, $J = 6.9$); 6.85 (d, 1H, $J = 7.7$); 6.89 (d, 1H, $J = 7.7$); 6.91 (t, 1H, $J = 7.7$); 6.96 (t, 1H, $J = 7.7$); 7.41 (t, 1H, $J = 7.3$); 7.85 (t, 1H, $J = 7.7$); 8.50 (d, 1H, $J = 8.0$); 8.55 (d, 1H, $J = 4.4$); 10.10 (s, 1H).

ESI-MS: 393.5 $[M+H]^+$; 415.2 $[M+Na]^+$.
Anal. ($C_{22}H_{28}N_6O$), C, H, N.

*5.1.11. N′-cyano-N-(3-(4-(2-cyanophenyl)piperazin-1-yl)propyl) picolinamidine (**4g**)*

From **3** and 2-(piperazin-1-yl)benzonitrile.

Yield: 20%; mp 118–120 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.88 (m, 2H); 2.65 (t, 2H, N^1–CH_2, $J = 5.1$); 2.73 (br s, 4H, $2CH_2$ pip.); 3.30 (br s, 4H, $2CH_2$ pip.); 3.87 (br s, 2H, N–CH_2); 6.98 (d, 1H, $J = 7.1$); 7.02 (t, 1H, $J = 7.1$); 7.44–7.56 (m, 3H); 7.58 (d, 1H, $J = 7.3$); 7.86 (t, 1H, $J = 7.7$); 8.53 (br s, 1H); 9.78 (s, 1H).

ESI-MS: 374.4 $[M+H]^+$; 396.0 $[M+Na]^+$; 412.0 $[M+K]^+$.
Anal. ($C_{21}H_{23}N_7$), C, H, N.

*5.1.12. N′-cyano-N-(3-(4-(2,3-dimethylphenyl)piperazin-1-yl) propyl)picolinamidine (**4h**)*

From **3** and 1-(2,3-dimethylphenyl)piperazine.

Yield: 15%; mp 119–120 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.89 (m, 2H); 2.21 (s, 3H, CH_3); 2.27 (s, 3H, CH_3); 2.38 (br s, 4H, $2CH_2$ pip.); 2.65 (br s, 4H, $2CH_2$ pip.); 2.95 (t, 2H, N^1–CH_2, $J = 5.1$); 3.87 (br s, 2H, N–CH_2); 6.87 (d, 1H, $J = 7.7$); 6.91 (d, 1H, $J = 7.7$); 7.09 (t, 1H, $J = 7.7$); 7.44 (t, 1H, $J = 7.3$); 7.86 (t, 1H, $J = 7.7$); 8.51 (d, 1H, $J = 8.0$); 8.62 (d, 1H, $J = 4.4$); 10.01 (s, 1H).

ESI-MS: 377.9 $[M+H]^+$; 399.0 $[M+Na]^+$; 415.1 $[M+K]^+$.
Anal. ($C_{22}H_{28}N_6$), C, H, N.

*5.1.13. N-(3-(4-(2-chlorophenyl)piperazin-1-yl)propyl)-N′-cyanopicolinamidine (**4i**)*

From **3** and 1-(2-chlorophenyl)piperazine.

Yield: 26%; mp 86–88 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.88 (m, 2H); 2.65 (t, 2H, N^1–CH_2, $J = 5.1$); 2.71 (br s, 4H, $2CH_2$ pip.); 3.14 (br s, 4H, $2CH_2$ pip.); 3.87 (br s, 2H, N–CH_2); 6.97 (d, 1H, $J = 8.0$); 7.01(t, 1H, $J = 8.0$); 7.26 (t, 1H, $J = 8.0$); 7.36 (d, 1H, $J = 8.0$); 7.44 (t, 1H, $J = 7.3$); 7.86 (t, 1H, $J = 7.7$); 8.53 (d, 1H, $J = 8.0$); 8.57 (d, 1H, $J = 4.4$); 9.94 (s, 1H).

ESI-MS: 383.2 $[M+H]^+$.
Anal. ($C_{20}H_{23}ClN_6$), C, H, N.

*5.1.14. N-(3-(4-(3-chlorophenyl)piperazin-1-yl)propyl)-N′-cyanopicolinamidine (**4l**)*

From **3** and 1-(3-chlorophenyl)piperazine.

Yield: 30%; mp 148–149 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.89 (m, 2H); 2.63 (br s, 4H, $2CH_2$ pip.); 2.66 (t, 2H, N^1–CH_2, $J = 5.1$); 3.25 (br s, 4H, $2CH_2$ pip.); 3.87 (br s, 2H, N–CH_2); 6.77 (d, 1H, $J = 8.0$); 6.82 (d, 1H, $J = 8.0$); 6.87 (s, 1H); 7.16 (t, 1H, $J = 8.0$); 7.39 (t, 1H, $J = 7.3$); 7.83 (t, 1H, $J = 7.7$); 8.39 (d, 1H, $J = 4.4$); 8.51 (d, 1H, $J = 8.0$); 9.96 (s, 1H).

ESI-MS: 383.5 $[M+H]^+$.
Anal. ($C_{20}H_{23}ClN_6$), C, H, N.

*5.1.15. N′-cyano-N-(3-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)propyl)picolinamidine (**4m**)*

From **3** and 1-(3-(trifluoromethyl)phenyl)piperazine.

Yield: 24%; mp 115–116 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.90 (m, 2H); 2.64 (br s, 4H, $2CH_2$ pip.); 2.67 (t, 2H, N^1–CH_2, $J = 5.1$); 3.29 (br s, 4H, $2CH_2$ pip.); 3.89 (br s, 2H, N–CH_2); 7.06 (d, 1H, $J = 8.0$); 7.1 (s, 1H); 7.11 (d, 1H, $J = 8.0$); 7.35 (t, 1H, $J = 8.0$); 7.39 (t, 1H, $J = 7.3$); 7.84 (t, 1H, $J = 7.7$); 8.38 (d, 1H, $J = 4.4$); 8.51 (d, 1H, $J = 8.0$); 9.91 (s, 1H).

ESI-MS: 417.6 $[M+H]^+$; 439.4 $[M+Na]^+$; 455.2 $[M+K]^+$.
Anal. ($C_{21}H_{23}F_3N_6$), C, H, N.

*5.1.16. N′-cyano-N-(3-(4-(pyrimidin-2-yl)piperazin-1-yl)propyl) picolinamidine (**4n**)*

From **3** and 1-(pyrimidin-2-yl)piperazine.

Yield: 27%; mp 110–111 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.89 (m, 2H); 2.55 (br s, 4H, $2CH_2$ pip.); 2.61 (t, 2H, N^1–CH_2, $J = 5.1$); 3.58 (br s, 4H, $2CH_2$ pip.); 3.90 (br s, 2H, N–CH_2); 6.49 (t, 1H, $J = 4.4$); 7.49 (d, 2H, $J = 4.4$); 7.43 (t, 1H, $J = 7.3$); 7.85 (t, 1H, $J = 7.7$); 8.30 (d, 1H, $J = 4.4$); 8.52 (br s, 1H); 9.99 (s, 1H).

ESI-MS: 351.0 $[M+H]^+$; 373.2 $[M+Na]^+$; 389.0 $[M+K]^+$.
Anal. ($C_{18}H_{22}N_8$), C, H, N.

*5.1.17. N′-cyano-N-(3-(4-(pyridin-2-yl)piperazin-1-yl)propyl) picolinamidine (**4o**)*

From **3** and 1-(pyridin-2-yl)piperazine.

Yield: 16%; mp 90–92 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.91 (m, 2H); 2.62 (br s, 4H, $2CH_2$ pip.); 2.72 (t, 2H, N^1-CH_2, $J = 5.1$); 3.61 (br s, 4H, $2CH_2$ pip.); 3.88 (br s, 2H, N-CH_2); 6.64 (d, 1H, $J = 8.7$); 7.39 (m, 2H); 7.48 (t, 1H, $J = 8.7$); 7.83 (t, 1H, $J = 7.3$); 8.21 (br s, 1H); 8.42 (d, 1H, $J = 4.4$); 8.51 (d, 1H, $J = 8.0$); 10.01 (s, 1H).

ESI-MS: 350.0 $[M + H]^+$; 372.2 $[M + Na]^+$; 388.2 $[M + K]^+$.

Anal. ($C_{19}H_{23}N_7$), C, H, N.

*5.1.18. N-(3-(4-((benzo[d][1,3]dioxol-5-yl)methyl)piperazin-1-yl) propyl)-N′-cyanopicolinamidine (**4p**)*

From **3** and 1-((benzo[*d*][1,3]dioxol-6-yl)methyl)piperazine.

Yield: 63%; mp 104–105 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.82 (m, 2H); 2.48 (br s, 4H, $2CH_2$ pip.); 2.49 (br s, 4H, $2CH_2$ pip.); 2.54 (t, 2H, N^1-CH_2, $J = 5.1$); 3.44 (s, 2H, CH_2); 3.80 (br s, 2H, N–CH_2); 5.93 (s, 2H, OCH_2O); 6.73 (m, 1H, $J = 7.6$); 6.83 (m, 2H); 7.51 (t, 1H, $J = 7.3$); 7.87 (t, 1H, $J = 7.7$); 8.51 (m, 2H); 9.92 (s, 1H).

ESI-MS: 407.3 $[M + H]^+$; 429.1 $[M + Na]^+$; 445.2 $[M + K]^+$.

Anal. ($C_{22}H_{26}N_6O_2$), C, H, N.

*5.1.19. N-(3-(4-(2-furoyl)piperazin-1-yl)propyl)-N′-cyanopicolinamidine (**4q**)*

From **3** and 1-(2-furoyl)piperazine.

Yield: 29%; mp 102–104 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.88 (m, 2H); 2.57 (br s, 4H, $2CH_2$ pip.); 2.61 (t, 2H, N^1-CH_2, $J = 5.1$); 3.89 (br s, 4H, $2CH_2$ pip.); 3.92 (br s, 2H, N–CH_2); 6.48 (d, 1H, $J = 3.2$); 7.01 (d, 1H, $J = 3.2$); 7.48 (m, 2H); 7.87 (t, 1H, $J = 7.7$); 8.54 (m, 2H); 9.81 (s, 1H).

ESI-MS: 367.2 $[M + H]^+$; 389.3 $[M + Na]^+$; 405.1 $[M + K]^+$.

Anal. ($C_{19}H_{22}N_6O_2$), C, H, N.

5.2. In vitro receptor binding

5.2.1. General procedures

The compounds dissolved in ethanol or in 5% DMSO were tested for in vitro affinity for 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors by radioligand binding assays. The compounds showing the highest affinity towards serotonin receptors were selected and evaluated for their affinity for dopaminergic (D_1 and D_2) and adrenergic (α_1 and α_2) receptors. All 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; (g) α_2 adrenergic receptor, [^{3}H]yohimbine, rat brain cortex.

Non-specific binding was determined as described in Supplementary material, 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 [33] to obtain the concentration of unlabelled drug that caused 50% inhibition of ligand binding (IC_{50}), with six concentrations of tested 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 Prusoff [34], 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 [35], were calculated for each labelled ligand).

5.2.2. 5-HT_{1A} binding assay

Radioligand binding assays were performed following a published procedure [36]. 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 s), and the homogenate was centrifuged at 50,000 *g* for 10 min. 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).

5.2.3. 5-HT_{2A} and 5-HT_{2C} binding assays

Radioligand binding assays were performed as previously reported by Herndon et al. [37]. 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 s) 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, Boston, MA, USA) 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) 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 nonspecific-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 Schliecher 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%.

5.2.4. D_1 dopaminergic binding assay

The binding assay for D_1 dopaminergic receptors was that described by Billard et al. [38]. Corpora striata were homogenized in 30 volumes (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, Boston, MA, USA) 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.

5.2.5. D_2 dopaminergic binding assay

The procedure used in the radioligand binding assay was reported in detail by Creese et al. [39] 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, Boston, MA, USA) 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.

5.2.6. α_1 Adrenergic binding assay

The procedure used in the radioligand binding assay has been reported in detail by Greengrass and Brenner [40]. 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]prazosin (80.5 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) 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.

5.2.7. α_2 Adrenergic binding assay

The procedure used in the radioligand binding assay was reported in detail by Perry and U'Prichard [41]. 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, Boston, MA, USA) 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.

Acknowledgements

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 is gratefully appreciated.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.11.023.

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