Design, Synthesis, Lipophilic Properties, and Binding Affinities of Potential Ligands in Positron Emission Tomography (PET) for Visualization of Brain Dopamine D_4 Receptors

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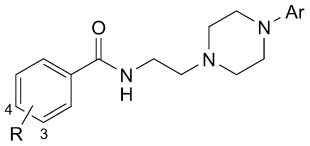
We report the synthesis of compounds structurally related to the high-affinity dopamine D_4 receptor ligand *N*-{2-[4-(3-cyanopyridin-2-yl)piperazin-1-yl]ethyl}-3-methoxybenzamide (**1e**). All compounds were specifically designed as potential PET radioligands for brain D_4 receptor visualization, having lipophilicity within a range for brain uptake and weak non-specific binding ($0.75 < \text{cLog}P < 3.15$) and bearing a substituent for easy access to labeling with the positron emitter isotope ^{11}C or ^{18}F. The best compound of the series, *N*-{2-[4-(4-chlorophenyl)piperazin-1-yl]ethyl}-6-fluoropyridine-3-carboxamide (**7a**), displayed excellent selectivity over D_2 and D_3 receptors (>100-fold), but its D_4 receptor affinity was suboptimal for imaging of brain D_4 receptors ($K_i = 30$ nM).

Introduction. – The dopamine D_2-like receptor family is composed of three receptor subtypes, D_2, D_3, and D_4, and it is characterized by the ability to interact with $G_{i/o}$ proteins, resulting in inhibition of adenylyl cyclase. Among the D_2-like receptors the D_4 receptor exhibits a specific pharmacological profile [1][2], and an extensive polymorphism of the human dopamine receptor D_4 gene is known [3][4]. Although the D_4 receptor was first cloned in 1991, there is still ambiguity about its pathophysiological functions, as well as about its exact localization and distribution density in the brain [5][6]. In particular, to this receptor an extremely low density in the brain is attributed [7][8]. Immunohistochemistry and hybridization studies revealed interspecies differences and contradictory findings, but suggested higher expression of D_4 receptors in the prefrontal cortex and the limbic system, and also in the temporal cortex, parts of tectum, and cerebellum [5][6][9–11]. Early interest in the D_4 receptor originated from the finding that clozapine, an atypical antipsychotic drug with high antipsychotic efficacy and reduced extrapyramidal and neuroendocrine side-effects, showed a tenfold higher affinity for D_4 than for D_2 receptor [1][2]. On such basis, it was hypothesized that the higher efficacy of clozapine to patients refractory to typical neuroleptics as well as to therapy of negative symptomatology was a consequence of D_4 binding. However, further studies did not confirm a direct link between schizophrenia and the D_4 receptor [12][13], indicating that the role in psychiatric diseases is uncertain. Great interest in the human dopamine D_4 receptor was generated by studies that indicated an association between D_4 receptor gene polymorphism and various complex behaviors including Attention Deficit Hyperactivity Disorder (ADHD), *Tourette*'s syndrome,

and the personality trait of novelty seeking. However, other studies did not confirm these associations. Only a meta-analysis provided strong evidence that the seven-repeat allele confers an increased risk for the development of ADHD, whereas the four-allele is protective [14]. Recent studies have shown that a variety of D_4 receptor agonists have a proerectile effect in animal experiments [15][16], indicating a role of D_4 receptors on the physiology of sexual functions.

Table 1. *Lipophilicity Values and Binding Affinities at Dopamine D_4 Receptors of Reference Benzamides*



	R	Ar	Clog*P*[a])	log *k*	K_i D_4 [nM][b])
1a	3-MeO	4-Cl–C_6H_4	3.72	0.88	4.97
1b	3-MeO	4-Me–C_6H_4	3.33	0.70	9.21
1c	3-MeO	1,2-Benzoxazol-3-yl	2.71	0.56	1.93
1d	3-MeO	4-CN–C_6H_4	2.66	0.29	63.95
1e	3-MeO	3-Cyanopyridin-2-yl	1.50	0.27	1.52
1f	3-MeO	5-Chloropyridin-2-yl	2.67	0.56	11.29
2a	4-F	4-Cl–C_6H_4	3.86	0.89	1.76
2b	4-F	4-Me–C_6H_4	3.33	0.67	2.64
2c	4-F	1,2-Benzoxazol-3-yl	2.86	0.57	0.34
2d	4-F	4-CN–C_6H_4	2.81	0.22	32.71
2e	4-F	3-Cyanopyridin-2-yl	1.64	0.22	0.93
2f	4-F	5-Chloropyridin-2-yl	2.82	0.73	2.92

[a]) Calculated with ClogP Biobyte software [24]. [b]) Data taken from [29].

Noninvasive imaging using selective radioligands for the D_4 receptor by positron emission tomography (PET) provides the opportunity to investigate the D_4-receptor expression in the living brain with high sensitivity. Various attempts have been made to identify a D_4-selective PET radioligand [17–23], but a major problem was the inadequate ratio of specific to nonspecific binding which is most obtrusive because of the very low D_4 receptor density. In particular, *Langer et al.* reported an attempt to visualize the dopamine D_4 receptor in primate brain with [^{11}C]**1a** (*Fig.*) [19]. The radioligand exhibited a very high background due to nonspecific binding. Similar results were obtained by *Zhang et al.* who synthesized and tested [^{11}C]**1a** and [^{11}C]YM-50001 (*Fig.*), with both radioligands being unsuitable for D_4-receptor imaging with PET [20]. In each case, it was suggested that the high nonspecific binding of [^{11}C]**1a** could be due to its relatively high lipophilicity (Clog*P* = 3.72) [24]. The nonspecific binding of PET radioligand candidates, such as binding to brain lipids, is hardly reversible and undisplaceable by non-radioactive ligands. Moreover, it is difficult to predict, and it has been correlated with lipophilicity of the substance [25–28].

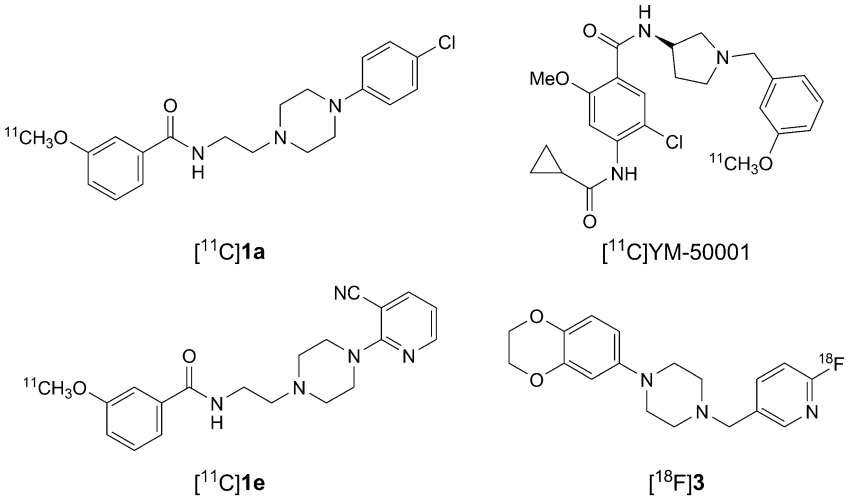


Figure. *Potential PET radiotracers for dopamine D_4 receptor imaging*

Recently we have reported the rational design of a set of benzamide derivatives structurally related to the high-affinity D_4 ligand **1a** as potential PET radiotracers (compounds **1a**–**1f** and **2a**–**2f**, *Table 1*) [29], which showed moderate lipophilicity in the Clog*P* range of 2.5–3.5 that is considered optimal for adequate brain penetration without an excessive level of nonspecific binding [30–32]. Among the target compounds, derivative **1e** (*Table 1*) was selected for PET studies *in vivo*, because it showed a good compromise between high D_4 receptor affinity and lipophilicity. However, [^{11}C]**1e** (*Fig.*) was not able to visualize D_4 receptor in monkey brain, but it showed fast kinetics, and it did not show nonspecific binding, suggesting that the lipophilicity of **1e** can be targeted to minimize nonspecific binding. It should be noted that, for **1e**, we observed a significant difference between experimental log *P* value measured by the pH metric technique (log $P = 2.55$) [29] and the calculated one (Clog$P = 1.5$). A recent study by *Kügler et al.* [33] has reported a significant correlation between the experimentally determined log *P* values of four potential D_4 receptor PET radiotracers and the extent of nonspecific binding as determined by competition experiments *in vitro*. The most promising radiotracer candidate therein reported was [^{18}F]**3** (*Fig.*) that showed D_4 receptor affinity in the low nanomolar range, good brain penetrance, and low nonspecific binding. The reported experimental log *P* value of [^{18}F]**3** determined with the shake flask method was 1.81, whereas the calculated log *P* value was 2.26 (ALOGPS 2.1 software). Collectively these data suggest that a new D_4 receptor PET radiotracer should possess lipophilicity comparable to that of compounds [^{11}C]**1e** and [^{18}F]**3** and D_4-receptor affinity lower than that of **1e** ($K_i = 1.52$ nM).

Thus, in keeping with the search of an effective D_4-receptor PET radiotracer, we modified further the structure of the compounds listed in *Table 1* with the aim to obtain new compounds that would have: *a*) high affinity for D_4 receptor ($K_i \leq 1.5$ nM) *b*) low affinity for dopamine D_2 and D_3 receptors ($K_i > 500$ nM) *c*) a good brain penetration as predicted by log *P* values in an optimal range as compound **1e**.

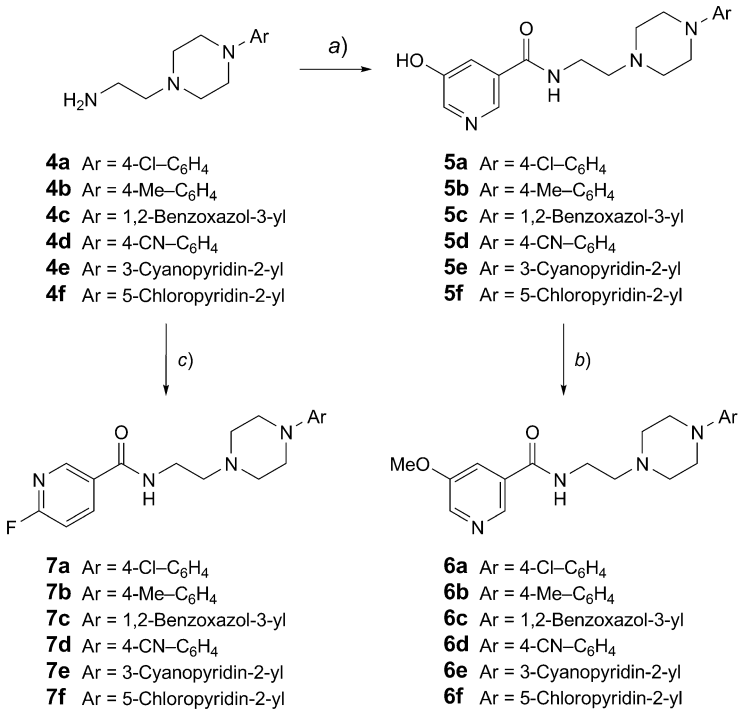
Previous structure–activity relationship studies on *N*-substituted-1-arylpiperazine derivatives suggested that the arylpiperazine moiety was responsible for the high D_4-receptor affinity, while the other aromatic end tethered by a chain with variable length to the protonatable N-atom of the *N*-substituted-1-arylpiperazine pharmacophore promotes D_4 selectivity over D_2 receptors [34][35]. Therefore, in order to achieve the aims *a* and *b* listed above, we left the arylpiperazine moieties of compounds **1a**–**1f** and **2a**–**2f** unchanged (also considering the restriction imposed by lipophilicity requirements), and, in order to achieve the aim *c* we modified the arenecarboxamide moiety by replacing a ring CH with an aza group, preferring the most accessible isomers. Thus, the 3-methoxybenzamide moieties of compounds **1a**–**1f** were replaced with 5-methoxypyridine-3-carboxamide moieties to give compounds **6a**–**6f**, and the 4-fluorobenzamide moieties of compounds **2a**–**2f** with 6-fluoropyridine-3-carboxamide moieties to give compounds **7a**–**7f**, respectively. The selection of the carboxylic acids was based on the stringent requirements for radiolabeling: [^{11}C]**6a**–[^{11}C]**6f** can be prepared from the corresponding phenolic derivatives **5a**–**5f** (see below), respectively, whereas [^{18}F]**7a**–[^{18}F]**7f** can be synthesized starting from the corresponding 6-bromopyridyl or 6-nitropyridyl derivatives *via* aromatic nucleophilic substitution with [^{18}F]F^-. Other possible pyridinecarboxylic acids, yet commercially available, cannot be radiolabelled.

Results and Discussion. – *Chemistry.* The target compounds were prepared as outlined in the *Scheme*. The 5-methoxypyridine-3-carboxamide derivatives **6a**–**6f** were prepared by condensing 5-hydroxy-3-pyridinecarboxylic acid [36] with amines **4a**–**4f**, prepared according to literature methods as detailed in the *Exper. Part*, in the presence of 1,1′-carbonyldiimidazole (CDI) to give the hydroxy derivatives **5a**–**5f**, respectively. The latters were methylated with CH_2N_2 to afford the target compounds **6a**–**6f**, respectively. The 6-fluoropyridine-3-carboxamide derivatives **7a**–**7f** were prepared by condensing the commercially available 6-fluoro-3-pyridinecarboxylic acid with amines **4a**–**4f**, respectively, using CDI as condensing agent.

Pharmacology. The target compounds **6a**–**6f** and **7a**–**7f** were assessed for their binding affinities at human cloned D_4, D_2, and D_3 receptors using [^{3}H]spiroperidol as radioligand. The K_i values (*Table 2*) were calculated from the IC_{50} values according to the equation of *Cheng* and *Prusoff* [37] by means of the Prism, v. 3.0, GraphPad software.

Structure–Activity Relationships. Previous studies on *N*-substituted 1-arylpiperazine derivatives have revealed that polar features of the *N*-substituent were well tolerated with respect to D_4-receptor affinity [38][39]. Instead, comparing affinities of the target compounds **6a**–**6f** and **7a**–**7f**, characterized by a pyridinecarboxamide moiety (*Table 2*), with those of the parent non-heteroaromatic compounds **1a**–**1f** and **2a**–**2f** (*Table 1*), a clear decrease in D_4-receptor affinity was evident. These data indicate that the aim *a* illustrated in the *Introduction* was not achieved. While the 4-fluorobenzamides **2a**–**2f** possessed higher D_4 affinities than their 3-methoxy counterparts **1a**–**1f**, the same trend was not displayed by the corresponding pyridinecarboxamides **6a**–**6f** and **7a**–**7f**. Having observed that the presence of the pyridinecarboxamide fragment was detrimental for affinity at D_4 receptor, we examined if there exists a relationship between lipophilicity values of the ligands and D_4-receptor affinities. A

Scheme

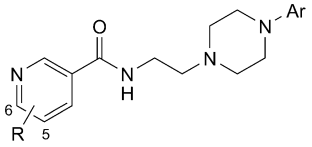


a) 5-Hydroxypyridine-3-carboxylic acid, 1,1′-carbonyldiimidazole (CDI), THF, 10–12 h, r.t., 40–50%. *b*) CH_2N_2, *t*-ButOH, 30 min, r.t. 40–50%. *c*) 6-Fluoropyridine-3-carboxylic acid, CDI, THF, 10–12 h, r.t., 40–50%.

linear relationship was not found when plotting pK_i *vs.* log *k* values (data not shown), suggesting that the interaction between the ligands and the receptor binding site does not rely solely on hydrophobic interactions. The amount of the decrease in affinities varied significantly from case to case, ranging from elevenfold (**1a** *vs.* **6a**) to over 2000-fold (**1e** *vs.* **6e**, **2c** *vs.* **7c**, and **2d** *vs.* **7d**). This suggests two observations. First, the presence of the pyridinecarboxamide moiety almost completely disrupted the interaction between compounds **6d**, **6e**, **7c**, **7d** and the D_4-receptor binding site: it is likely that the pyridinecarboxamide moiety interacts with the binding site in a way that hinders the decisive interaction between the protonated basic N-atom of **6d**, **6e**, **7c**, and **7d** with the highly conserved aspartic acid in the third transmembrane domain (TM3) of D_4-receptor binding site [35]. Second, the presence of the pyridinecarboxamide moiety exerted variable effects on the affinity of the ligands, because the local orientation of the arenecarboxamide moiety in the D_4-receptor binding site orients the arylpiperazine fragment toward transmembrane microdomains in different ways, resulting in acceptable or in poor binding affinity [35].

As far as the affinities for D_2 and D_3 receptors are concerned, the present data confirmed previous findings about the *N*-[2-(4-arylpiperazin-1-yl)ethyl]arenecarbox-

Table 2. *Lipophilicity Values and Binding Affinities at Dopamine D_4, D_2, and D_3 Receptors of Target Arenecarboxamides*



	R	Ar	Clog*P*[a])	log *k*	K_i [nM] ± S.E.M.[b])		
					D_4	D_2	D_3
6a	5-MeO	4-Cl–C_6H_4	3.15	0.64	56.0 ± 5	> 1000	> 1000
6b	5-MeO	4-Me–C_6H_4	2.77	0.49	528 ± 13	> 1000	> 1000
6c	5-MeO	1,2-Benzoxazol-3-yl	2.15	0.3	61.0 ± 8	> 1000	> 1000
6d	5-MeO	4-CN–C_6H_4	2.10	0.10	2236 ± 210	> 1000	> 1000
6e	5-MeO	3-Cyanopyridin-2-yl	0.93	0.06	> 3000	> 1000	> 1000
6f	5-MeO	5-Chloroyridin-2-yl	2.11	0.47	1384 ± 32	> 1000	> 1000
7a	6-F	4-Cl–C_6H_4	2.97	0.61	30.0 ± 0.6	> 1000	> 1000
7b	6-F	4-Me–C_6H_4	2.44	0.47	301 ± 19	> 1000	44.0 ± 3
7c	6-F	1,2-Benzoxazol-3-yl	1.97	0.28	1537 ± 135	> 1000	89.0 ± 7
7d	6-F	4-CN–C_6H_4	1.92	− 0.30	> 3000	> 1000	> 1000
7e	6-F	3-Cyanopyridin-2-yl	0.75	− 0.02	80.6 ± 13	357 ± 20	27.0 ± 2
7f	6-F	5-Chloroyridin-2-yl	1.93	0.41	372 ± 65	> 1000	> 1000
Haloperidol			–	–	4.2 ± 0.2	–	2.9 ± 0.7
Quinpirole			–	–	–	12.1 ± 1.9	–

[a]) Calculated with ClogP Biobyte software [24]. [b]) Binding data are the means of three independent experiments using standard displacement assays with [^{3}H]spiroperidol as the competitive ligand and human cloned receptor subtypes.

amide framework [40]: all the compounds were devoid of dopamine D_2 receptor affinity (except **7e**), while a few more compounds showed remarkable affinity for D_3 receptor (*i.e.*, **7b**, **7c**, and **7e**). These data indicate that the aim *b* mentioned in the *Introduction* was substantially achieved.

Collectively, these data confirmed that the *N*-[2-(4-arylpiperazin-1-yl)ethyl]arenecarboxamide structure can deliver selective D_4-receptor ligands if properly decorated, as in the case of compounds **6a**, **6b**, and **7a**.

Lipophilicity Evaluation. Lipophilicity can be evaluated in various theoretical and experimental ways. Theoretical partition coefficients are routinely calculated with various softwares that provide values generally well correlated with the experimental determinations. However, differences between the calculated log *P* values and the experimental ones have been reported as in the case described above compounds **1e** and [^{18}F]**3**. Therefore, we compared experimentally the lipophilicity values of the newly synthesized compounds **6a**–**6f** and **7a**–**7f** with those of the benzamides **1a**–**1f** and **2a**–**2f** studied previously. For this purpose, we adopted a reversed-phase HPLC method that we had previously used for arylpiperazine derivatives [41]. Plotting Clog*P* and log *k* values of compounds **1a**–**1f**, **2a**–**2f**, **6a**–**6f**, and **7a**–**7f** (*Tables 1* and *2*) led to a modest correlation ($r = 0.67$). 4-Cyano derivatives **1d**, **2d**, **6d**, and **7d** appeared more

hydrophilic than predicted: it can be hypothesized that solvation of the 4-cyano substituent results in higher solubility in the mobile phase. Instead, by excluding the 4-CN derivatives **1d**, **2d**, **6d**, and **7d**, Clog*P* and log *k* values were well correlated ($r = 0.941$). These observations confirm that calculated log *P* values reliably predict physicochemical properties of the compounds, but when lipophilicity must fall within a narrow range, and Clog*P* values are borderline, an experimental determination is prudent. The experimental determinations confirmed that compounds **6a**–**6f** and **7a**–**7f** were less lipophilic than the corresponding derivatives **1a**–**1f** and **2a**–**2f**, indicating that the aim *c* mentioned in the *Introduction* was accomplished. Among these, compound **7c** showed the same lipophilicity as **1e**.

Conclusions. – With the aim to obtain potential PET radiotracers for CNS dopamine D_4 receptors, we have structurally manipulated a set of previously reported high-affinity dopamine D_4 ligands characterized by the *N*-[2-(4-arylpiperazin-1-yl)ethyl]arenecarboxamide framework, in order to obtain molecules endowed with lipophilicity values that should account for low nonspecific binding. Although the modification was targeted at the arenecarboxamide moiety that previous structure–activity realtionship studies had indicated as less sensitive with respect to the interaction with the target receptor, we have observed a loss in D_4-receptor affinity that renders these compounds unsuitable candidates as PET radiotracers. On the other hand, compounds **6a**, **6c**, and **7a** display binding affinities and physicochemical properties still attractive for future investigations of the role of dopamine D_4 receptors.

Experimental Part

Chemistry. The purities of the tested compounds **1a**–**1f** and **2a**–**2f** have been assessed by RP-HPLC and combustion analyses. All compounds showed $\geq$ 95% purity. The following compounds were synthesized according to published procedures: *2-[4-(4-chlorophenyl)piperazin-1-yl]ethanamine* (**4a**) [42], *2-[4-(4-methylphenyl)piperazin-1-yl]ethanamine* (**4b**) [42], *2-[4-(1,2-benzoxazol-3-yl)piperazin-1-yl]ethanamine* (**4c**)[29], *4-[4-(2-aminoethyl)piperazin-1-yl]benzonitrile* (**4d**) [29], *2-[4-(2-aminoethyl)piperazin-1-yl]pyridine-3-carbonitrile* (**4e**) [29], *2-[4-(5-chloropyridin-2-yl)piperazin-1-yl]ethanamine* (**4f**) [29], 5-hydroxynicotinic acid [36]. Column chromatography (CC): *Merck* silica gel *60A* (SiO_2; 63–200 μm) as the stationary phase. M.p.: in open capillaries on a *Gallenkamp* electrothermal apparatus. ^{1}H-NMR Spectra: at 300 MHz on a *Varian Mercury-VX* spectrometer; all spectra were recorded on free bases; all chemical shift values in ppm (δ). MS: *HP6890–5973 MSD* gas chromatograph/mass spectrometer; only significant *m*/*z* peaks, with their percentage of relative intensity in parentheses, are reported. ESI$^+$/MS/MS: *Agilent 1100* Series *LC-MSD* trap System *VL* workstation. All spectra were in accordance with the assigned structures. When necessary, a standard procedure was used to transform final compounds into their HCl salts. Elemental analyses (C, H, N): *Eurovector Euro EA 3000* analyzer; results within $\pm$ 0.4% of the theoretical values.

General Procedure for Preparation of Compounds **5a**–**5f**. A mixture of 5-hydroxypyridine-3-carboxylic acid (0.07 g, 0.5 mmol) and 1,1′-carbonyldiimidazole (CDI; 0.10 g, 0.6 mmol) in 10 ml of anh. THF was stirred for 8 h. A soln. of amine **4a**–**4f** (0.5 mmol) in anh. THF (10 ml) was added, and then the mixture was stirred until the acid disappeared (TLC). The mixture was partitioned between AcOEt (20 ml) and H_2O (20 ml). The separated org. layer was washed with a sat. aq. soln. of Na_2CO_3 (20 ml), dried (Na_2SO_4), and concentrated *in vacuo*. The crude residue was chromatographed as detailed below to afford the pure arenecarboxamide in 40–50% yield.

N-*{2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl}-5-hydroxypyridine-3-carboxamide* (**5a**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 60%. ^{1}H-NMR ((D$_6$)DMSO): 2.47–2.49 (*m*, 2 H); 2.52–2.56 (*m*, 2 H); 3.10

(app. *t*, 2 H); 3.33 (br. *s*, 4 H); 3.37–3.42 (*m*, 2 H); 6.89–6.94 (*m*, 2 H); 6.99 (br. *s*, 1 H, D_2O exchanged); 7.17–7.22 (*m*, 2 H); 7.62 (br. *s*, 1 H); 8.21 (*d*, *J* = 2.8, 1 H); 8.43 (*d*, *J* = 1.9, 1 H); 8.53 (br. *s*, 1 H, D_2O exchanged). ESI-MS (pos.): 361 ($[M + H]^+$). ESI-MS/MS (pos.): 197 (19), 165 (100).

5-Hydroxy-N-*{2-[4-(4-methylphenyl)piperazin-1-yl]ethyl}pyridine-3-carboxamide* (**5b**). Eluted with $CHCl_3$/MeOH 19 : 1. Yield: 36%. ^{1}H-NMR ($CDCl_3$): 2.26 (*s*, 3 H); 2.68 (app. *t*, 6 H); 3.14 (app. *t*, 4 H); 3.58 (*q*, *J* = 5.8, 2 H); 6.83 (*d*, *J* = 8.5, 2 H); 7.07 (*d*, *J* = 8.5, 2 H); 7.18 (br. *t*, 1 H, D_2O-exchanged); 7.60–7.62 (*m*, 1 H); 8.37 (*d*, *J* = 2.8, 1 H); 8.43 (*d*, *J* = 1.9, 1 H); 8.44 (br. *s*, 1 H, D_2O-exchanged). ESI-MS (pos.): 341 ($[M + H]^+$). ESI-MS/MS (pos.): 177 (69), 165 (100).

N-*{2-[4-(1,2-Benzoxazol-3-yl)piperazin-1-yl]ethyl}-5-hydroxypyridine-3-carboxamide* (**5c**). Eluted with $CHCl_3$/MeOH 19 : 1. Yield: 52%. ^{1}H-NMR ($CDCl_3$): 2.67–2.72 (*m*, 6 H); 3.54–3.62 (*m*, 6 H); 7.20–7.24 (*m*, 3 H, D_2O-exchanged); 7.46–7.49 (*m*, 2 H); 7.60–7.64 (*m*, 1 H); 8.37 (*d*, *J* = 2.8, 1 H); 8.46 (*d*, *J* = 1.7, 1 H); 8.56 (br. *s*, 1 H, D_2O-exchanged). ESI-MS (pos.): 368 ($[M + H]^+$). ESI-MS/MS (pos.): 204 (35), 165 (100).

N-*{2-[4-(4-Cyanophenyl)piperazin-1-yl]ethyl}-5-hydroxypyridine-3-carboxamide* (**5d**). Eluted with $CHCl_3$/MeOH 9 : 1. Yield: 29%. ^{1}H-NMR ($CDCl_3$): 2.62–2.68 (*m*, 6 H); 3.32 (app. *t*, 4 H); 3.58 (*q*, *J* = 5.5, 2 H); 6.83–6.86 (*m*, 2 H); 7.02 (br. *s*, 1 H, D_2O-exchanged); 7.46–7.50 (*m*, 2 H); 7.61–7.62 (*m*, 1 H); 8.40 (*d*, *J* = 2.8, 1 H,); 8.43 (*d*, *J* = 1.9, 1 H); 8.64 (br. *s*, 1 H, D_2O-exchanged). ESI-MS (pos.): 352 ($[M + H]^+$). ESI-MS/MS (pos.): 165 (100).

N-*{2-[4-(3-Cyanopyridin-2-yl)piperazin-1-yl]ethyl}-5-hydroxypyridine-3-carboxamide* (**5e**). Eluted with $CHCl_3$/MeOH 19 : 1. Yield: 60% ^{1}H-NMR ($CDCl_3$): 2.63–2.69 (*m*, 6 H); 3.58 (*q*, *J* = 5.5, 2 H); 3.71 (app. *t*, 4 H); 6.76 (*dd*, *J* = 7.7, 1 H); 7.45 (br. *s*, 2 H, D_2O-exchanged); 7.61–7.62 (*m*, 1 H); 8.33 (*dd*, *J* = 4.7, 1.9, 1 H); 8.38 (*d*, *J* = 2.8, 1 H); 8.44 (*d*, *J* = 1.7, 1 H); 8.64 (br. *s*, 1 H, D_2O-exchanged). ESI-MS (pos.): 353 ($[M + H]^+$). ESI-MS/MS (pos.): 189 (17), 165 (100).

N-*{2-[4-(5-Chloropyridin-2-yl)piperazin-1-yl]ethyl}-5-hydroxypyridine-3-carboxamide* (**5f**). Eluted with $CHCl_3$/MeOH 19 : 1. Yield: 38%. ^{1}H-NMR ($CDCl_3$): 2.59 (app. *t*, 4 H); 2.65 (*t*, *J* = 6.1, 2 H); 3.50 (app. *t*, 4 H); 3.57 (*q*, *J* = 5.5, 2 H); 6.57 (*d*, *J* = 9.1, 1 H); 7.15 (br. *t*, 1 H, D_2O-exchanged); 7.41 (*dd*, *J* = 6.3, 2.8, 1 H); 7.61 (app. *t*, 1 H); 8.10 (*d*, *J* = 2.5, 1 H); 8.37 (*d*, *J* = 2.5, 1 H); 8.43 (*d*, *J* = 1.7, 1 H); 8.47 (br. *s*, 1 H, D_2O-exchanged). ESI-MS (pos.): 363 ($[M + H]^+$). ESI-MS/MS (pos.): 198 (41), 165 (100).

General Procedure for Preparation of Compounds **6a**–**6f**. A soln. of derivatives **5a**–**5f** (0.3 mmol) in *t*-BuOH (5 ml) was treated with an excess of CH_2N_2. The mixture was stirred at r.t. for 30 min, until the precursor disappeared (TLC). The solvents were removed *in vacuo*, and the residue was partitioned between AcOEt (20 ml) and H_2O (20 ml). The separated org. layer was washed with brine, dried (Na_2SO_4), and concentrated *in vacuo*. The crude residue was chromatographed as detailed below to give the pure arenecarboxamide in 40–50% yield.

N-*{2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl}-5-methoxypyridine-3-carboxamide* (**6a**). Eluted with $CHCl_3$/MeOH 98 : 2. Yield: 70%. M.p. 145–147°. ^{1}H-NMR ($CDCl_3$): 2.65–2.70 (*m*, 6 H); 3.18 (app. *t*, 4 H); 3.59 (*q*, *J* = 5.7, 2 H); 3.90 (*s*, 3 H); 6.81–6.87 (*m*, 2 H); 6.89 (br. *s*, 1 H, D_2O-exchanged); 7.18–7.23 (*m*, 2 H); 7.67–7.68 (*m*, 1 H); 8.41 (*d*, *J* = 3.0, 1 H); 8.50 (*d*, *J* = 1.9, 1 H). ESI-MS (pos.): 375 ($[M + H]^+$). ESI-MS/MS (pos.) 179 (100). Anal. calc. for $C_{19}H_{23}ClN_4O_2$ (374.87): C 60.88, H 6.18, N 14.95; found: C 60.94, H 6.26, N 14.65.

5-Methoxy-N-*{2-[4-(4-methylphenyl)piperazin-1-yl]ethyl}pyridine-3-carboxamide* (**6b**). Eluted with $CHCl_3$/MeOH, 19 : 1. Yield: 45%. M.p. 132–135°. ^{1}H-NMR ($CDCl_3$): 2.27 (*s*, 3 H); 2.75–2.79 (*m*, 6 H); 3.23 (app. *t*, 4 H); 3.65 (*q*, *J* = 5.2, 2 H); 3.90 (*s*, 3 H); 6.84 (*d*, *J* = 8.5, 2 H); 7.07 (*d*, *J* = 8.3, 2 H); 7.29 (br. *s*, 1 H, D_2O exchanged); 7.72–7.74 (*m*, 1 H); 8.40 (*d*, *J* = 3.0, 1 H); 8.56 (*d*, *J* = 1.6, 1 H). GC-MS: 355 (3, $[M + 1]^+$), 354 (14, M^+), 189 (100), 146 (20), 108 (14). Anal. calc. for $C_{20}H_{26}N_4O_2$ (354.45): C 67.77, H 7.39, N 15.81; found: C 67.50, H 7.34, N 15.44.

N-*{2-[4-(1,2-Benzoxazol-3-yl)piperazin-1-yl]ethyl}-5-methoxypyridine-3-carboxamide* (**6c**). Eluted with $CHCl_3$/MeOH, 19 : 1. 52% Yield. M.p. 88–90°. ^{1}H-NMR ($CDCl_3$): 2.76–2.83 (*m*, 6 H); 3.63–3.69 (*m*, 6 H); 3.90 (*s*, 3 H); 7.20–7.24 (*m*, 2 H, D_2O exchanged); 7.46–7.49 (*m*, 2 H); 7.66 (*d*, 1 H); 7.70–7.72 (*m*, 1 H); 8.40 (*d*, *J* = 2.7, 1 H); 8.57 (br. *s*, 1 H). ESI-MS/MS (pos.) 382 ($[M + H]^+$). ESI-MS/MS (pos.): 204 (15), 179 (100). Anal. calc. for $C_{20}H_{23}N_5O_3$ (381.43): C 62.98, H 6.08, N 18.36; found: C 63.10, H 6.22, N 18.11.

N-*{2-[4-(4-Cyanophenyl)piperazin-1-yl]ethyl}-5-methoxypyridine-3-carboxamide* (**6d**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 48%. M.p. 147–149°. ^{1}H-NMR ($CDCl_3$): 2.66–2.72 (*m*, 6 H); 3.36 (app. *t*, 4 H); 3.62 (*q*, *J* = 5.5, 2 H); 3.90 (*s*, 3 H); 6.86 (*d*, *J* = 9.1, 2 H); 6.94 (br. *s*, 1 H, D_2O-exchanged); 7.48–7.51 (*m*, 2 H); 7.68–7.70 (*m*, 1 H); 8.40 (*d*, *J* = 3.0, 1 H); 8.51 (*d*, *J* = 1.6, 1 H). ESI-MS/MS (pos.): 366 ($[M + H]^+$). ESI-MS/MS (pos.) 179 (100). Anal. calc. for $C_{20}H_{23}N_5O_2$ (365.43): C 65.73, H 6.34, N 19.16; found: C 66.05, H 6.21, N 19.46.

N-*{2-[4-(3-Cyanopyridin-2-yl)piperazin-1-yl]ethyl}-5-methoxypyridine-3-carboxamide* (**6e**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 38%. M.p. 112–115° (HCl salt). ^{1}H-NMR ($CDCl_3$): 2.86 (br. *s*, 6 H); 3.70 (*q*, *J* = 5.2, 2 H); 3.85 (app. *t*, 4 H); 3.92 (*s*, 3 H); 6.83 (*dd*, *J* = 7.7, 1 H); 7.45 (br. *s*, 1 H, D_2O-exchanged); 7.78–7.81 (*m*, 2 H); 8.36 (*dd*, *J* = 4.7, 1.9, 1 H); 8.41 (*d*, *J* = 2.7, 1 H); 8.60 (*d*, *J* = 1.1, 1 H). ESI-MS/MS (pos.): 367 ($[M + H]^+$). ESI-MS/MS (pos.): 179 (100). Anal. calc. for $C_{19}H_{22}N_6O_2 \cdot 4\ HCl \cdot 0.5\ H_2O$ (521.27): C 43.78, H 5.22, N 16.12; found: C 43.61, H 5.55, N 15.80.

N-*{2-[4-(5-Chloropyridin-2-yl)piperazin-1-yl]ethyl}-5-methoxypyridine-3-carboxamide* (**6f**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 34%. M.p. 143–145°. ^{1}H-NMR ($CDCl_3$): 2.70–2.77 (*m*, 6 H); 3.59–3.68 (*m*, 6 H); 3.90 (*s*, 3 H); 6.59 (*d*, *J* = 9.1, 1 H); 7.19 (br. *s*, 1 H, D_2O-exchanged); 7.43 (*dd*, *J* = 9.1, 2.8, 1 H); 7.72 (*dd*, *J* = 2.8, 1.9, 1 H); 8.11 (*d*, *J* = 2.2, 1 H); 8.40 (*d*, *J* = 3.0, 1 H); 8.56 (*d*, *J* = 1.6, 1 H). GC/MS: 377 (3, $[M + 2]^+$), 375 (9, M^+), 210 (100), 181 (35), 155 (38), 108 (24). Anal. calc. for $C_{18}H_{22}ClN_5O_2$ (375.85): C 57.52, H 5.90, N 18.63; found: C 57.15, H 5.82, N 18.33.

General Procedure for Preparation of Compounds **7a**–**7f**. A mixture of 6-fluoro-pyridine-3-carboxylic acid (0.07 g, 0.5 mmol) and CDI (0.10 g, 0.6 mmol) in 10 ml of anh. THF was stirred for 8 h. A soln. of amine **4a**–**4f** (0.5 mmol) in anh. THF (10 ml) was added, and then the mixture was stirred until the acid disappeared (TLC). The mixture was partitioned between AcOEt (20 ml) and H_2O (20 ml). The separated org. layer was washed with a sat. aq. soln. of Na_2CO_3 (20 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The crude residue was chromatographed as detailed below to afford the pure arenecarboxamide in 40–50% yield.

N-*{2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl}-6-fluoropyridine-3-carboxamide* (**7a**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 56%. M.p. 158–160°. ^{1}H-NMR ($CDCl_3$): 2.65–2.68 (*m*, 6 H); 3.17 (app. *t*, 4 H); 3.58 (*q*, *J* = 5.7, 2 H); 6.82–6.85 (*m*, 2 H); 6.87 (br. *s*, 1 H, D_2O-exchanged); 7.01 (*dd*, *J* = 8.4, 2.8, 1 H); 7.19–7.22 (*m*, 2 H); 8.22–8.27 (*m*, 1 H); 8.60 (*d*, *J* = 2.8, 1 H). GC/MS: 364 (4, $[M + 2]^+$), 362 (9, M^+), 211 (34), 209 (100), 166 (19), 124 (22). Anal. calc. for $C_{18}H_{20}ClFN_4O$ (362.83): C 59.59, H 5.56, N 15.44; found: C 59.29, H 5.55, N 15.16.

6-Fluoro-N-*{2-[4-(4-methylphenyl)piperazin-1-yl]ethyl}pyridine-3-carboxamide* (**7b**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 35%. M.p. 149–151°. ^{1}H-NMR ($CDCl_3$): 2.27 (*s*, 3 H); 2.73–2.76 (*m*, 6 H); 3.21 (app. *t*, 4 H); 3.63 (*q*, *J* = 5.2, 2 H); 6.83–6.86 (*m*, 2 H); 7.01 (*dd*, *J* = 8.5, 2.8, 1 H); 7.06–7.10 (*m*, 2 H); 7.16 (br. *s*, 1 H, D_2O-exchanged); 8.25–8.32 (*m*, 1 H); 8.65 (*d*, *J* = 2.7, 1 H). GC/MS: 343 (3, $[M + 1]^+$), 342 (14, M^+), 189 (100), 146 (22), 124 (20). Anal. calc. for $C_{19}H_{23}FN_4O$ (342.41): C 66.65, H 6.77, N 16.36; found: C 66.52, H 6.74, N 16.17.

N-*{2-[4-(1,2-Benzoxazol-3-yl)piperazin-1-yl]ethyl}-6-fluoropyridine-3-carboxamide* (**7c**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 50%. M.p. 152–154°. ^{1}H-NMR ($CDCl_3$): 2.78–2.85 (*m*, 6 H); 3.66–3.70 (*m*, 6 H); 7.01 (*dd*, *J* = 8.5, 2.7, 1 H); 7.15 (br. *s*, 1 H, D_2O-exchanged); 7.20–7.24 (*m*, 1 H); 7.45–7.53 (*m*, 2 H); 7.66 (*d*, *J* = 8.0, 1 H); 8.30 (*dt*, *J* = 8.5, 2.5, 1 H); 8.67 (*d*, *J* = 2.5, 1 H). ESI-MS/MS (pos.): 370 ($[M + H]^+$). ESI-MS/MS (pos.) 167 (100). Anal. calc. for $C_{19}H_{20}FN_5O_2$ (369.39): C 61.78, H 5.46, N 18.96; found: C 61.45, H 5.39, N 18.69.

N-*{2-[4-(4-Cyanophenyl)piperazin-1-yl]ethyl}-6-fluoropyridine-3-carboxamide* (**7d**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 42%. M.p. 155–157° (HCl salt). ^{1}H-NMR ($CDCl_3$): 2.73–2.76 (*m*, 6 H); 3.39 (app. *t*, 4 H); 3.63 (*q*, *J* = 5.5, 2 H); 6.84–6.89 (*m*, 2 H); 7.02 (*dd*, *J* = 8.5, 3.0, 1 H); 7.05 (br. *s*, 1 H, D_2O-exchanged); 7.48–7.53 (*m*, 2 H); 8.28 (*dt*, *J* = 8.5, 2.5, 1 H); 8.63 (*d*, *J* = 2.5, 1 H). GC/MS: 353 (0.4, M^+), 200 (100), 157 (15), 124 (17). Anal. calc. for $C_{19}H_{20}FN_5O \cdot 2\ HCl$ (426.32): C 53.53, H 5.20, N 16.43; found: C 53.85, H 5.51, N 16.25.

N-*{2-[4-(3-Cyanopyridin-2-yl)piperazin-1-yl]ethyl}-6-fluoropyridine-3-carboxamide* (**7e**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 78%. M.p. 165–166°. ^{1}H-NMR ($CDCl_3$): 2.73–2.75 (*m*, 6 H); 3.63 (*q*, *J* = 5.5, 2 H); 3.78 (app. *t*, 4 H); 6.81 (*dd*, *J* = 7.5, 1 H); 7.01 (*dd*, *J* = 8.5, 2.7, 1 H); 7.07 (br. *s*, 1 H, D_2O-exchanged); 7.78 (*dd*, *J* = 7.7, 1.9, 1 H); 8.29 (*dt*, *J* = 8.3, 2.5, 1 H); 8.35 (*dd*, *J* = 4.7, 1.9, 1 H); 8.65 (*d*, *J* =

2.2, 1 H). GC/MS: 354 (1, M^+), 201 (100), 124 (20). Anal. calc. for $C_{18}H_{19}FN_6O$ (354.38): C 61.01, H 5.40, N 23.71; found: C 60.85, H 5.40, N 23.54.

N-*{2-[4-(5-Chloropyridin-2-yl)piperazin-1-yl]ethyl}-6-fluoropyridine-3-carboxamide* (**7f**). Eluted with $CHCl_3$/MeOH 19:1. Yield: 35%. M.p. 180–182°. ^{1}H-NMR ($CDCl_3$): 2.67–2.75 (*m*, 6 H); 3.57–3.66 (*m*, 6 H); 6.59 (*d*, *J* = 9.1, 1 H); 7.01 (*dd*, *J* = 8.5, 2.2, 1 H); 7.09 (br. *s*, 1 H, D_2O-exchanged); 7.43 (*dd*, *J* = 9.1, 2.8, 1 H); 8.11 (*d*, *J* = 2.5, 1 H); 8.25–8.31 (*m*, 1 H); 8.64 (*d*, *J* = 2.5, 1 H). GC/MS: 365 (1, [*M* + 2]$^+$), 363 (5, M^+), 210 (100), 181 (34), 155 (37), 124 (39). Anal. calc. for $C_{17}H_{19}ClFN_5O$ (363.82): C 56.12, H 5.26, N 19.25; found: C 55.89, H 5.22, N 18.95.

Lipophilicity. Lipophilicity indices were determined by a reversed-phase (RP) HPLC method consisting in a *PerkinElmer* series *200 LC* apparatus equipped with a *PerkinElmer 785A* UV/VIS detector set at 254 nm. UV signals were monitored and the peaks obtained were integrated with a personal computer running *PerkinElmer* Turbochrom Software. The capacity factors (k) were measured with a *Phenomenex Kinetex C18-XB* (250 × 4.6 mm, 5 μm particle size) as non-polar stationary phase and with MeOH/0.01M phosphate buffer (pH 5.5) 60:40 (*v*/*v*) as mobile phase. This mobile phase composition was chosen for the analysis due to reasonable retention times for all compounds analyzed. All compounds were dissolved in MeOH, and the measurements were conducted at a flow rate of 1 ml/min. Capacity factors were calculated as $k = (t_R - t_0)/t_0$, where t_R is the retention time of the solute, and t_0 is the column dead time, measured as the solvent front.

Biological Methods. General. Human cloned dopamine D_{2L} receptors stably expressed in rat C6 glioma cells was kindly donated by Prof. *Roberto Maggio* (Università di L'Aquila, Italy). Cell culture reagents were purchased from *EuroClone* (IT-Milan). Human recombinant $D_{4.4}$ dopamine receptor expressed in CHO-K1 cells, human recombinant D_3 dopamine receptor expressed in CHO-K1 cells, and [^{3}H]spiroperidol were obtained from *PerkinElmer Life* and *Analytical Sciences* (Boston, MA, USA). Haloperidol, was purchased from *Sigma–Aldrich* (IT-Milan); quinpirole was obtained from *Tocris Bioscience* (UK-Bristol). For receptor binding studies, the compounds were dissolved in DMSO.

Cells Culture. Rat C6 glioma cells expressing human dopamine D_{2L} receptors were grown in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, in a humidified incubator at 37° in a 5% CO_2 atmosphere.

Radioligand-Binding Assay at Human Cloned D4.4 Dopaminergic Receptors. Binding of [^{3}H]spiroperidol at human cloned $D_{4.4}$ receptor was performed as described in [29] with minor modifications. The reaction buffer consisted of 50 mM *Tris*·HCl, pH 7.4, including 100 μl of dopamine $D_{4.4}$ receptor membranes, 0.5 nM of [^{3}H]spiroperidol (K_d = 0.17 nM), and 100 μl of the drug soln. (six to nine concentrations) for a total volume of 1 ml. Samples were incubated at 25° for 90 min, then the incubation was stopped by rapid filtration through *Whatman GF/C* glass fiber filters (presoaked in 0.3% polyethylenimine for 20 min). The filters were washed twice with 1 ml of ice-cold buffer (50 mM *Tris*·HCl; pH 7.4). Nonspecific binding was defined in the presence of 10 μM haloperidol. The radioactivity bound to the filters was measured by liquid scintillation using *LS6500 Multi-Purpose* scintillation counter, *Beckman*.

Radioligand-Binding Assay at Human Cloned D_{2L} Dopaminergic Receptors. Membranes of human dopamine D_{2L} receptors stably expressed in rat C6 glioma cells were prepared as described by *Colabufo et al.* [43]. Dopamine D_{2L} receptors were radiolabelled with [^{3}H]spiroperidol according to *Scarselli et al.* [44] with minor modifications. The incubation buffer (50 mM *Tris*·HCl pH 7.4, 120 mM NaCl, 5.0 mM KCl, 5.0 mM $MgCl_2$, 1 mM EDTA) contained 100 μg of dopamine D_{2L} receptor membranes, 0.50 nM [^{3}H]spiroperidol (K_d = 0.093 nM), and six to nine concentrations of drug soln. in a final volume of 500 μl. The samples were incubated for 120 min at 25°, then the incubation was stopped by rapid filtration through *Whatman GF/C* glass fiber filters (presoaked in 0.5 % polyethylenimine for 30 min). The filters were washed 3 × 1 ml of ice-cold 50 mM *Tris*, pH 7.4, 0.9% NaCl. Nonspecific binding was determined in the presence of 10 μM haloperidol. The radioactivity bound to the filters was measured by liquid scintillation using *LS6500 Multi-Purpose* scintillation counter, *Beckman*.

Radioligand-Binding Assay at Human Cloned D_3 Dopaminergic Receptors. Binding of [^{3}H]spiroperidol at human cloned D_3 receptor was performed as described to *Swarzenski et al.* [45] with minor modifications. The reaction buffer consisted of 50 mM *Tris*, 5 mM $MgCl_2$, 10 μg/ml saponine (pH 7.4), including 100 μl of dopamine D_3 receptor membranes, 0.4 nM of [^{3}H]spiroperidol (K_d = 0.60 nM), and

100 µl of the drug soln. (six to nine concentrations) for a total volume of 1 ml. Samples were incubated at 25° for 30 min, then the incubation was stopped by rapid filtration through *Whatman GF/C* glass fiber filters (pre-soaked in 0.5% polyethylenimine for 2 h). The filters were washed twice with 1 ml of ice-cold buffer (50 mM *Tris*, pH 7.4, 5 mM $MgCl_2$). Nonspecific binding was defined in the presence of 10 µM quinpirole. The radioactivity bound to the filters was measured by liquid scintillation using *LS6500 Multi-Purpose* scintillation Counter, *Beckman*.

Statistical Analysis. The inhibition curves on the different binding sites of the compounds were analyzed by nonlinear curve fitting utilizing the GraphPad Prism program. The value for the inhibition constant, K_i, was calculated by using the *Cheng–Prusoff* equation [38].

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