Full Paper

Novel Mannich Bases, 5-Arylimidazolidine-2,4-dione Derivatives with Dual 5-HT_{1A} Receptor and Serotonin Transporter Affinity

Anna Czopek[1], Marcin Kołaczkowski[1], Adam Bucki[1], Hanna Byrtus[1], Maciej Pawłowski[1], Agata Siwek[2], Andrzej J. Bojarski[3], Marek Bednarski[4], Dagmara Wróbel[5], and Anna Wesołowska[5]

[1] Department of Pharmaceutical Chemistry, Jagiellonian University Medical College, Kraków, Poland
[2] Department of Pharmacobiology, Jagiellonian University Medical College, Kraków, Poland
[3] Department of Medicinal Chemistry, Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland
[4] Department of Pharmacodynamics, Jagiellonian University Medical College, Kraków, Poland
[5] Department of Clinical Pharmacy, Jagiellonian University Medical College, Kraków, Poland

A computer aided ligand design study of imidazolidine-2,4-dione derivatives was conducted in order to obtain compounds with dual 5-HT_{1A} receptor and serotonin transporter (SERT) affinity. According to molecular modeling results, series of Mannich bases were chosen and synthesized. Investigated compounds were tested for 5-HT_{1A}, 5-HT_{2A}, α_1 and SERT affinity. Two selected compounds (**5**, **9**) were characterized in functional experiments and possessed a pharmacological profile which may enhance SERT blocking efficacy – 5-HT_{1A} partial agonism and 5-HT_{2A} antagonism in one molecule. Furthermore these compounds displayed satisfactory selectivity over adrenergic α_1 receptors. The most promising compounds, 5-arylimidazolidine-2,4-dione derivatives with 4-(3-chlorophenyl)piperazinylmethyl moiety were tested for antidepressant and anxiolytic activity. In particular, compound **5** (5-(2-methoxyphenyl)-3-{1-[4-(3-chlorophenyl)piperazin-1-yl]methyl}-imidazolidine-2,4-dione), tested in the forced swim test in mice, exhibited a favorable antidepressant-like profile without affecting spontaneous locomotor activity.

Keywords: 5-HT_{1A} receptor ligands / Antidepressants / Imidazolidine-2,4-dione / Mannich bases / Serotonin transporter inhibitors

Received: October 3, 2012; Revised: November 3, 2012; Accepted: November 9, 2012

DOI 10.1002/ardp.201200378

Introduction

Depression has been described by the World Health Organization as a mental disorder with symptoms of depressed mood, loss of interest or pleasure, feelings of guilt, low self-esteem, disturbed sleep, loss of appetite, low energy, and poor concentration. These symptoms, in the chronic or recurrent form, lead to impaired ability to cope with everyday duties, or, in the worst case, death. The effects of long-term depression not only lead to a significant reduction in mental activity, but they also have social and economic dimension [1, 2].

In past decades, monoamine reuptake inhibitors were the most significant drugs applied as a standard in the treatment of depression – in particular, the serotonin and norepinephrine reuptake inhibitors [3, 4]. Besides their side effects, the major drawback of these is the delayed onset of their antidepressant activity [5]. The therapeutic lag is believed to be caused by activation of 5-HT_{1A} autoreceptors, and the delayed onset is due to the time required for desensitization of these autoreceptors [6]. Therefore, one of the ways to accelerate the antidepressant activity may be introduction of additional interactions with serotonin 5-HT_{1A} receptors (5-HT_{1A}R). The mechanism of action, combining serotonin reuptake inhibition and 5-HT_{1A}R antagonism or partial agonism, should prevent the feedback that inhibits release of

Correspondence: Anna Czopek, Department of Pharmaceutical Chemistry, Jagiellonian University Medical College, 9 Medyczna Str, 30-688 Kraków, Poland.
E-mail: aczopek@cm-uj.krakow.pl
Fax: +48 12 657 02 62

serotonin from neurons, thus leading to increased efficiency of the antidepressant drug. This assumption has been based on clinical observations – it was found that patients treated with serotonin reuptake inhibitors (SSRIs) and pindolol (β-blocker/5-HT_{1A} antagonist/partial agonist) felt the positive effects of therapy more quickly than the group who received SSRIs only [7]. The most recently introduced antidepressant drug vilazodone acts as SSRI and partial agonist of 5-HT_{1A} receptor. An important advantage of vilazodone is that its antidepressant effect is observed after only 1 week of treatment [8, 9].

In previous studies [10], we reported the synthesis of 5,5-disubstituted- and 5-spiro-imidazolidine-2,4-diones with arylpiperazinylpropyl moiety which exhibited diversified affinity and intrinsic activity for serotonin 5-HT_{1A} and 5-HT_{2A} receptors (5-HT_{2A}R). The most interesting compounds behaved like agonists or partial agonists of 5-HT_{1A}Rs and they showed antidepressant activity. Bearing in mind the clinical significance of compounds with dual mechanism of action, being serotonin transporter (SERT) blockers and 5-HT_{1A} ligands, we decided to incorporate the serotonin reuptake inhibition into the profile of our compounds, interacting with 5-HT_{1A} receptors. Therefore we performed a computer-aided design of the dual ligands, resulting in development of Mannich bases of the arylpiperazinemethylimidazolidine-2,4-dione structure. The new compounds were tested for their affinity for SERT, 5-HT_{1A}, and 5-HT_{2A} receptors as well as for their selectivity over α_1-adrenergic receptors (α_1R). The most promising ones were characterized in functional experiments and tested for antidepressant and anxiolytic activity.

Results

Computer aided ligand design

To understand the most significant features of ligand binding to SERT we examined the binding mode of reference high affinity ligands. Therefore, we obtained a homology model of SERT, based on LeuT crystal structure (see Experimental part for details), and we examined the sertraline binding mode within the binding site. Sertraline was chosen as a model ligand due to its high affinity and selectivity for SERT as well as its fairly rigid structure, which made it suitable for ligand-steered binding site modeling. We also compared the docked pose of sertraline in the SERT model with its crystal structure in LeuT, which proved to be highly similar, being also an argument supporting the accuracy of the obtained homology model. Moreover, we projected structures of some other SERT blockers, crystalized in complex with LeuT in the binding site of modeled SERT. All of the structures presented quite similar binding mode, with the most notable feature of an aromatic ring penetrating the hydrophobic cavity, formed by Leu99, Trp103, Tyr176, Ile179, Phe335, and aliphatic chain of Arg104. Despite some differences in binding mode, resulting from diversified structures of the examined ligands, this interaction was present in all cases (Fig. 2A). As a starting point for dual ligands design, we used a compound from previously described series, an arylpiperazinepropyl derivative of 2′,3′-dihydrospiro[imidazolidine-4,1′-indene]-2,4-dione [10] (Fig. 1). This compound proved to be a partial agonist of 5-HT_{1A}R, but displayed no affinity for SERT. In order to explore this fact at a molecular level, we docked this compound to the homology model of SERT and examined its binding mode, paying particular attention to the possibility of emergence of the above-mentioned aromatic ring interaction. As a result, we found that the compound was docked to the SERT binding site with arylpiperazine moiety and therefore was unable to penetrate the hydrophobic binding cavity with the aromatic ring in the manner observed for the reference SSRIs (Fig. 2B). Based on this observation, we decided to shorten the alkyl linker from 3 to 1 unit, to enable the SERT binding site penetration with the aryl ring of the

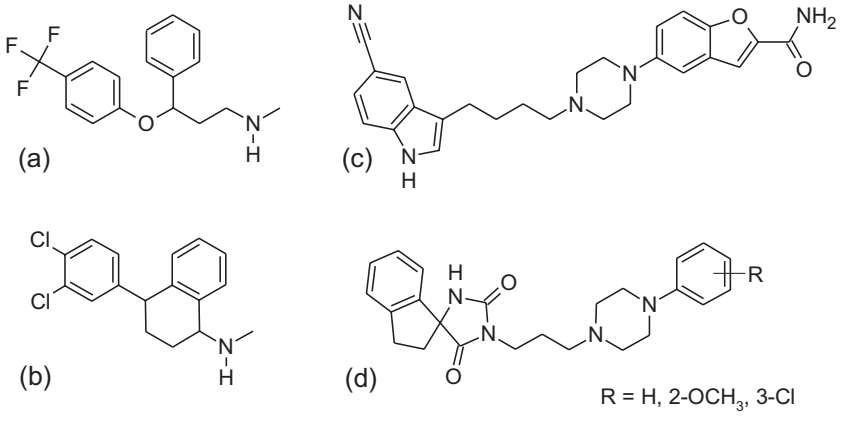


Figure 1. Structures of known antidepressants: fluoxetine (a), sertraline (b), vilazodone (c), and an arylpiperazinepropyl derivative of 2′,3′-dihydrospiro[imidazolidine-4,1′-indene]-2,4-dione (d) [10].

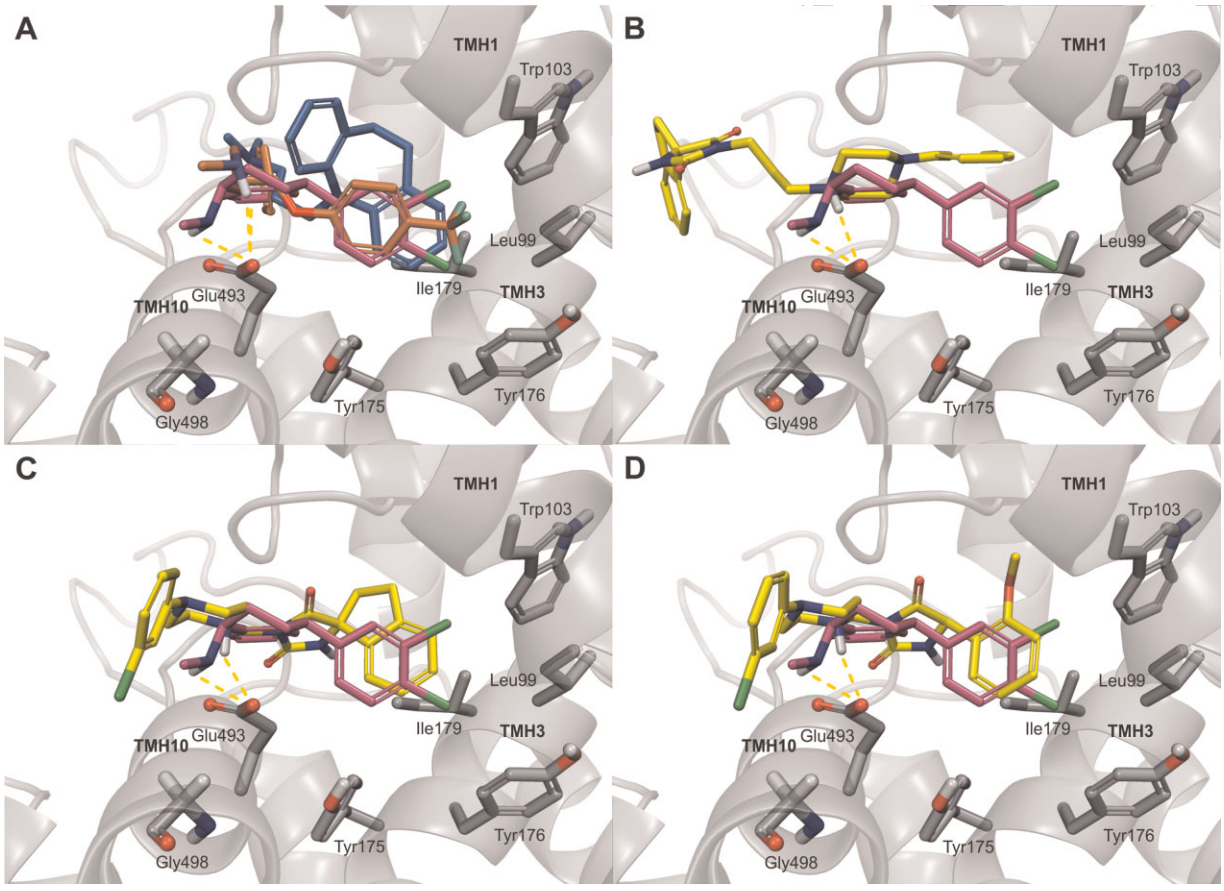


Figure 2. (A) Projection of SSRIs crystals with LeuT in the binding site of the SERT homology model showing the well superimposed aromatic rings interacting with the hydrophobic cavity between TMHs 1, 3, and 10. (B) Top-scored pose of a compound used as a starting point for modifications, showing its poor fit with reference sertraline. (C) Top-scored pose of a mid-stage modification compound with shortened alkyl chain, presenting its improved fit with the reference ligand. (D) Top-scored pose of a final structure in the SERT binding site. Residues forming important interactions with the ligands are presented as thick sticks. Dotted lines represent H-bonds with polar residues.

spirohydantoin moiety. The resulting Mannich base was found to interact with the SERT model in the desired mode, having the aromatic moiety well superimposable on those of the reference ligands (Fig. 2C). Bearing in mind the putative importance of good fit of the aryl portion to the binding cavity, we made it more flexible by replacing the spiro ring system with one substituted phenyl ring at position 5 of the imidazolidine-2,4-dione. The newly obtained structure presented good fit with the binding site of the SERT model, especially within the hydrophobic pocket, with better score of ligand–protein complex quality (Fig. 2D). In order to verify whether the modified structure is likely to maintain the originally occurring affinity for 5-HT_{1A}R it was docked to its homology model. The binding site of the 5-HT_{1A} serotonin receptor is placed on the extracellular side of the protein, along the third transmembrane helix (TMH 3), where ligands are anchored by a charge-reinforced hydrogen bonding to Asp3.32 (Ballesteros–Weinstein nomenclature [11]). It is divided into two pockets: (i) situated between TMHs 3, 5, and 6, where arylpiperazine moiety interacts with aromatic amino acids of the latter helix, particularly Phe6.51 and Phe6.52, and (ii) constituted by amino acids localized on TMHs 2, 3, and 7. 5-Arylhydantoin moiety was found to interact with this binding cavity, establishing van der Waals and polar contacts with Tyr2.64, Phe3.28, Trp7.40, and Tyr7.43. Moreover, the *o*-methoxyphenyl group is likely to form an H-bond with Asn7.39. The obtained ligand binding mode was in line with the one previously established for 5-HT_{1A}R ligands, thus promising its good *in vitro* activity (Fig. 3). Based on those findings we proposed a group of 5-phenylimidazolidine-2,4-dione derivatives with an arylpiperazinemethyl fragment, which were synthesized and subjected to pharmacological studies.

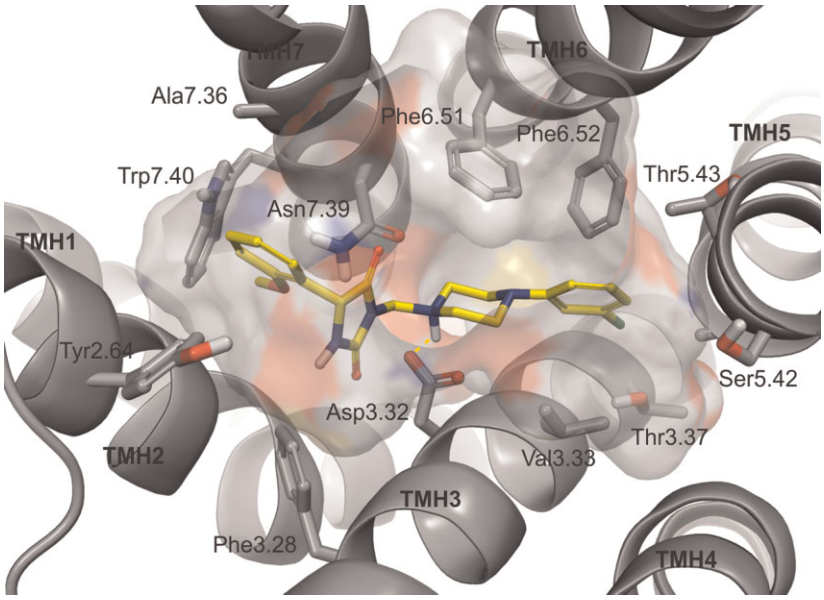
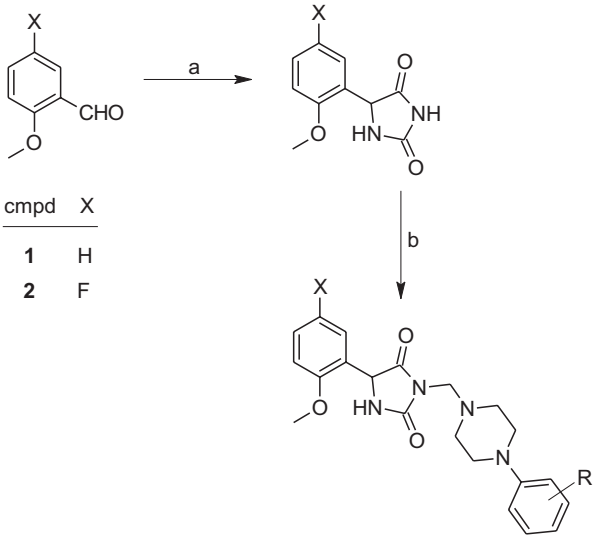


Figure 3. Top-scored ligand–receptor complex of compound **5** showing its binding mode in 5-HT$_{1A}$R (view from the extracellular side). Binding site of the receptor is shown as a transparent surface. Residues forming important interactions with the ligand are presented as thick sticks. Dotted lines represent H-bonds with polar residues.

Chemistry

The designed series of 5-arylhydantoin derivatives was prepared according to the synthetic routes shown in Scheme 1. The appropriate imidazolidine-2,4-diones were obtained from the 2-methoxybenzaldehyde and 5-fluoro-2-methoxybenzaldehyde by means of the Bucherer–Berg reaction with modifications described by Goodson et al. [12]. In the next step, aminoalkylation of the acidic proton of 5-arylhydantoins carried out in the presence of formaldehyde and substituted 1-phenylpiperazine enabled to obtain final compounds **3**–**10**. The structure of the final compounds **3**–**10** was established on the basis of the results of elemental (C, H, N) and spectral



cmpd	X
1	H
2	F

cmpd	X	R
3	H	H
4	H	2-OCH$_3$
5	H	3-Cl
6	H	3-CF$_3$
7	F	H
8	F	2-OCH$_3$
9	F	3-Cl
10	F	3-CF$_3$

Reagents, reaction conditions:

(a) KCN, $(NH_4)_2CO_3$, 50% ethanol.

(b) 4-Substituted piperazine derivatives, formaldehyde, 96% ethanol, reflux.

Scheme 1. Synthesis pathways of investigated compounds.

Table 1. Binding data for final compounds on 5-HT_{1A}, 5-HT_{2A}, and α_1 receptors and SERT.

Cmpd	K_i (nM)			K_i (μM)
	5-HT_{1A}	5-HT_{2A}	α_1	SERT
3	80 ± 7	693 ± 54	nd	4.1 ± 0.5
4	25 ± 3	782 ± 38	1100 ± 500	6.7 ± 0.2
5	38 ± 5	89 ± 5	555 ± 21	0.1665 ± 0.0086
6	86 ± 11	95 ± 12	403 ± 21	0.9644 ± 0.0286
7	269 ± 35	1084 ± 120	nd	5.0 ± 0.7
8	53 ± 4	788 ± 54	750 ± 46	5.2 ± 0.2
9	76 ± 8	58 ± 8	807 ± 27	0.2779 ± 0.0164
10	73 ± 5	68 ± 9	685 ± 20	0.9072 ± 0.0728

nd, not determined.

(^{1}H NMR, ^{19}F NMR) analysis. The detailed spectral data of the synthesized compounds are presented in the Experimental section.

Pharmacology

Affinity determination

The pharmacological *in vitro* studies included determination of affinity for SERT, 5-HT_{1A}, 5-HT_{2A} receptors as well as for adrenergic α_1 receptors. The compounds (**3**–**10**) were tested in radioligand binding experiments where their ability to displace [^{3}H]-citalopram, [^{3}H]-8-OH-DPAT, [^{3}H]-ketanserin, and [^{3}H]-prazosin, respectively, were evaluated [13–15]. The affinity data are shown in Table 1.

Intrinsic activity studies

The intrinsic activity of the selected compounds (**5**, **9**) at 5-HT_{1A}R was assessed by fluorescence detection of phosphorylated extracellular signal-regulated kinase (pERK) accumulation in Chinese hamster ovary (CHO)-K1 cells stably transfected with the human 5-HT_{1A}R (Fig. 4). The assay is based on the quantification of phosphorylated ERK (pERK) obtained due to agonist stimulation of 5-HT_{1A}R which induces phosphorylation of mitogen-activated protein (MAP) kinases such as extracellular signal-regulated kinase (ERK 1/2) [16]. In order to determine the central 5-HT_{2A}R properties of the tested compounds, their ability to evoke or inhibit the head twitches induced by (±)-DOI, a 5-HT_{2A}R agonist, was studied in mice [17, 18]. The results are presented in Table 2.

Antidepressant and anxiolytic activity

The potential antidepressant activity of compounds **5** and **9** was evaluated in the forced swim test in mice [19], whereas the potential anxiolytic properties were determined in the four-plate test in mice [20]. To control for the possibility of occurrence of drug-induced changes in locomotor activity of mice, which could contribute to behavior in both the forced swim and the four-plate tests, the influence of the investigated compounds on the spontaneous locomotor activity was also carried out. The obtained results are shown in Tables 3–5.

Discussion

Affinity of the tested compounds for SERT, 5-HT_{1A}R, 5-HT_{2A}R, and α_1R was diversified depending on the biological target, as well as substitution mode at the main scaffold, and ranged from nanomolar to low micromolar K_i values. As expected, based on similarity to the previously studied compounds, as well as on molecular modeling studies, affinity for the 5-HT_{1A}R was high in the case of all compounds ranging from 25 to 86 nM, excluding compound **7**, displaying moderate affinity (269 nM) for these receptors. Affinity of the newly synthesized compounds for 5-HT_{2A}R ranged from high to moderate

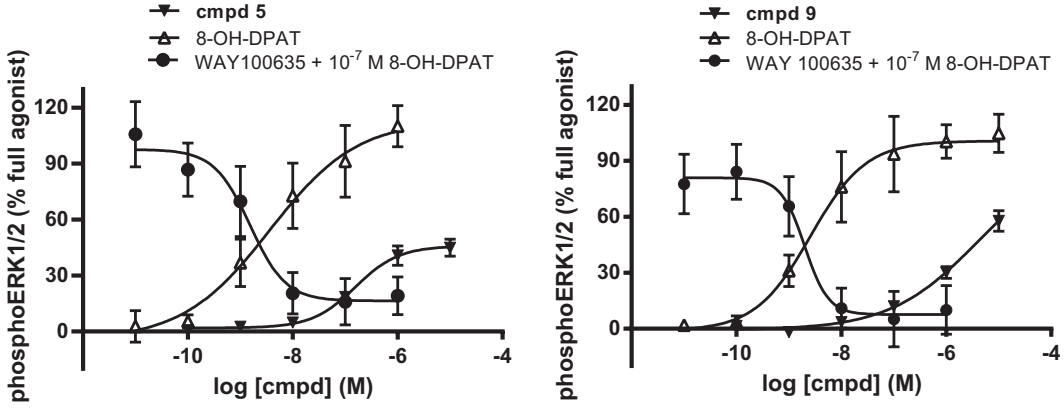


Figure 4. Concentration-dependence curves of compounds **5** and **9** to active extracellular signal-regulated kinase (ERK) phosphorylation in CHO-h5-HT_{1A} cells. Obtained values (%) are expressed as percent of the action of 0.1 μM of full agonist, 8-OH-DPAT (100%).

Table 2. The effect of **5** and **9** on the (±)-DOI (2.5 mg/kg)-induced head twitch response in mice.

Treatment	Dose (mg/kg)	Number of head twitches/20 min Mean ± SEM
Vehicle + (±)-DOI	–	20.2 ± 2.8
5 + (±)-DOI	10	11.0 ± 1.3[a)]
	20	9.2 ± 1.3[a)]
	40	6.8 ± 1.4[a)]
		$F(3,20) = 10.212$ $p < 0.001$
		ID_{50} = 20.10 (18.60–21.61) mg/kg
Vehicle + (±)-DOI	–	19.5 ± 4.1
9 + (±)-DOI	10	11.7 ± 2.3
	20	7.0 ± 1.0[a)]
	40	5.3 ± 1.0[a)]
		$F(3,20) = 6.744$ $p < 0.01$
		ID_{50} = 19.55 (18.05–21.05) mg/kg

Compounds **5** and **9** were administered 30 min before the test.
[a)] $p < 0.01$ *versus* vehicle + (±)-DOI group (Dunnett's test).
ID_{50} values were calculated by the method of Litchfield and Wilcoxon.

(58–1084 nM) indicating the superiority of 4-(3-chlorophenyl)piperazine or 4-(3-trifluoromethylphenyl)-piperazine moiety containing derivatives, which was in line with previously described SAR for arylpiperazine derivatives [13]. All the tested compounds possessed low affinity for α_1R (> 400 nM), which was also in compliance with the previously described impact of spacer shortening on α_1R affinity [21]. This property should be considered as an advantage of the studied compounds, given the putative implication of α_1R antagonism in adverse effects like sedation or hypotension. Unlike in the case of 5-HT$_{1A}$R, the predicted good fit between the designed ligands and SERT model was not reflected in high affinity of the whole group. On the other hand, the affinity for SERT was very diversified (166–6700 nM), depending strongly on the substitution pattern in the aromatic ring of phenylpiperazine moiety, which was not considered by us as crucial in the initial design approach. *Post hoc* analysis of the binding mode suggested that the arylpiperazine moiety might be involved in the interaction with the second hydrophobic pocket between TMHs 10 and 11, which seems to be as important in terms of affinity as the previously discussed interaction with the pocket between TMHs 1, 3 and 10 (Fig. 5). 3-Chloro substitution proved to be the most advantageous, and, in fact, the only one providing significant affinity for SERT. Despite a substantial body of work, both experimental and modeling, devoted to exploration of 3D structure of SERT, either the precise binding site (vestibular, buried) or the ligand binding mode remain, in fact, uncertain. The level of ambiguity is even higher than in the case of monoamine GPCRs, where considerable progress has been made recently. This may be a source of discrepancies between the structure–activity relationships, perceived based on modeling studies, and the actual experimental results. Nevertheless, the applied ligand design approach resulted in compounds possessing significant affinity for SERT, higher than that ever observed in our studies with the previously described compounds.

Based on the results of affinity studies, compounds **5** and **9**, possessing the desired profile, i.e., displaying significant affinity for both SERT and 5-HT$_{1A}$R, as well as 5-HT$_{2A}$R and

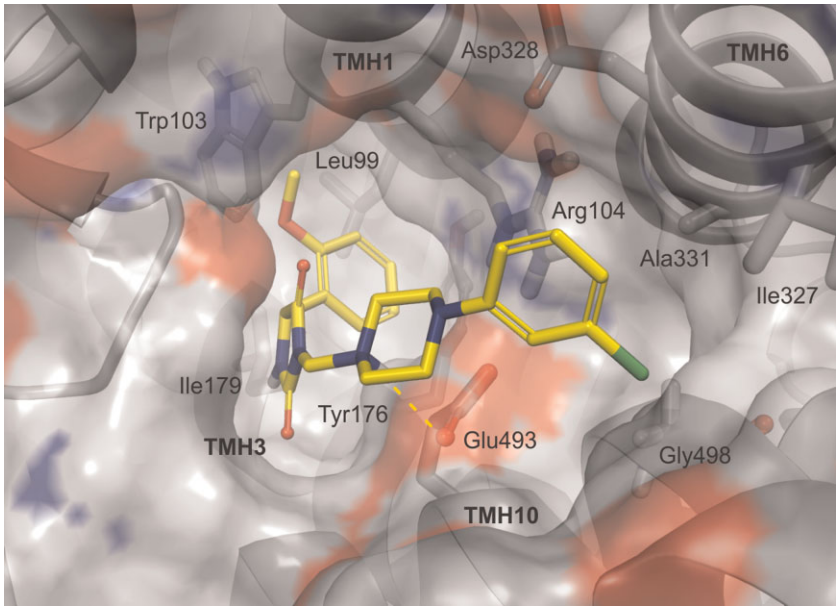


Figure 5. Top-scored pose of compound **5** in SERT model, showing important interaction between 3-chlorophenylpiperazine moiety and the hydrophobic cavity between TMHs 10 and 11 (view from the extracellular side). Binding site of the transporter is shown as a transparent surface. Residues forming important interactions with the ligand are presented as thick sticks. Dotted lines represent H-bonds with polar residues.

low affinity for α_1R, were selected for determination of intrinsic activity. Studies of intrinsic activity at human 5-HT_{1A}R showed that E_{max} values for stimulation of ERK phosphorylation by compounds **5** and **9** were 45 and 57.8%, respectively, indicating their partial agonist activity (0.1 μM 8-OH-DPAT = 100%). The EC_{50} values for the tested compounds in the agonist assay were 157 nM and 4.1 μM, respectively. In the 5-HT_{2A}R intrinsic activity test, both compounds administered alone had no ability to produce head twitches in mice as did (±)-DOI. However, both **5** and **9** dose-dependently and significantly abolished the *in vivo* effect produced by (±)-DOI with similar values of ID_{50} ~20 mg/kg (Table 2).

The functional profile of the investigated compounds determined *in vitro* and *in vivo*, i.e., partial agonists of 5-HT_{1A} receptors and 5-HT_{2A} antagonists with affinity for SERT, suggests that they may show antidepressant- and/or anxiolytic-like activity. To date, there is a lot of evidence indicating that such compounds produce behavioral activity relevant to depression and anxiety. These effects may be obtained by administration of agonists/antagonists of serotonin receptors in combination with other therapeutic compounds, for instance SSRIs, to augment their clinical effects [22]. Considering the premises described above, we selected compounds **5** and **9**, which combine all desirable functional activity in one molecule, for further *in vivo* preclinical studies employing the forced swim and the four-plate tests in mice.

Compounds **5** and **9** (20 and 30 mg/kg) dose-dependently and significantly reduced the immobility time of mice in the forced swim test (Table 3). Analog **9** was slightly less active, which can be connected with its sedative effect observed in the locomotor activity test (Table 5). However, it is noteworthy that the effective antidepressant doses of both **5** and **9** did not stimulate the spontaneous locomotor activity in mice during the 4–6-min, as well as the 30-min experimental sessions (Table 5), indicating that both compounds have specific antidepressant-like activity, and this effect cannot be explained by competing behaviors, such as locomotor activity. The results of our successive experiments show that both analogs produced no anti-anxiety-like effects in the four-plate test in mice (Table 4).

Conclusions

A computer-aided design of new imidazolidine-2,4-dione derivatives as dual 5-HT_{1A}/SERT ligands was performed. The structure optimization of the starting 5-HT_{1A}R ligands resulted in Mannich bases with an aryl substituent at position 5 of imidazolidine-2,4-dione, which showed the best fit with the SERT binding site. However, the affinity determination studies of the newly synthesized series revealed that,

Table 3. The effect of **5**, **9**, and imipramine in the forced swim test in mice.

Treatment	Dose (mg/kg)	Immobility time (s) Mean ± SEM
Vehicle	–	168.8 ± 4.9
5	10	149.7 ± 6.3
	20	125.7 ± 8.1[b]
	30	88.4 ± 9.9[b]
		$F(3,36) = 21.088$
		$p < 0.0001$
Vehicle	–	168.8 ± 4.9
9	10	151.2 ± 10.3
	20	139.1 ± 5.8[a]
	30	101.2 ± 8.3[b]
		$F(3,36) = 14.162$
		$p < 0.0001$
Vehicle	–	167.1 ± 6.7
Imipramine	10	149.1 ± 10.7
	20	107.8 ± 12.4[b]
		$F(2,27) = 8.760$
		$p < 0.01$

Compounds **5**, **9**, and imipramine were administered 30 min before the test. $n = 9$–10 mice per group.
[a] $p < 0.05$ *versus* vehicle.
[b] $p < 0.01$ *versus* vehicle (Dunnett's test).

Table 4. The effect of **5**, **9**, and diazepam in the four-plate test in mice.

Treatment	Dose (mg/kg)	Number of punished crossings Mean ± SEM
Vehicle	–	3.9 ± 0.3
5	10	3.3 ± 0.5
	20	4.6 ± 0.5
	30	4.9 ± 0.6
		$F(3,36) = 2.152$
		ns
Vehicle	–	3.9 ± 0.3
9	10	3.8 ± 0.4
	20	3.9 ± 0.4
	30	3.2 ± 0.4
		$F(3,36) = 0.770$
		ns
Vehicle*	–	3.5 ± 0.4
Diazepam	1.25	5.5 ± 0.5[a]
	2.5	6.8 ± 0.6[b]
	5	6.7 ± 0.6[b]
		$F(3,36) = 9.514$
		$p < 0.001$

Compounds **5** and **9** were administered 30 min before the test. $n = 10$ mice per group.
[a] $p < 0.05$.
[b] $p < 0.01$ *versus* vehicle (Dunnett's test).
ns, non-significant.
* Data taken from ref. [32].

Table 5. The effect of **5** and **9** on the spontaneous locomotor activity of mice.

Treatment	Dose (mg/kg)	Locomotor activity: number of crossings during:	
		4–6 min	30 min
Vehicle	–	197.1 ± 20.4	1344.9 ± 125.0
5	20	187.4 ± 21.7	560.1 ± 94.3[a)]
	30	201.8 ± 17.1	630.1 ± 85.5[a)]
		F(2,27) = 0.135	F(2,27) = 7.782
		ns	$p < 0.01$
Vehicle	–	197.1 ± 20.4	1344.9 ± 126.0
9	20	118.9 ± 8.8[a)]	411.4 ± 59.7[a)]
	30	41.8 ± 5.1[a)]	154.1 ± 16.3[a)]
		F(2,27) = 3.732	F(2,27) = 6.580
		$p < 0.05$	$p < 0.01$

Compound **5** and **9** were administered 30 min before the test.
$n = 10$ mice per group.
ns, non-significant.
[a)] $p < 0.01$ *versus* vehicle (Dunnett's test).

unlike it had been initially expected, only 3-chlorophenylpiperazine derivatives **5** and **9** were proven to possess significant affinity for both 5-HT$_{1A}$R and SERT, indicating high significance of the arylpiperazine moiety for proper interactions with the transporter binding site. The selected compounds **5** and **9** displayed partial agonist and antagonist properties at 5-HT$_{1A}$R and 5-HT$_{2A}$R sites, respectively, and a low affinity for α_1R. Such profile, combining SERT affinity with 5-HT$_{1A}$R partial agonism and 5-HT$_{2A}$R antagonism, prompted us to undertake the studies to determine the antidepressant and anxiolytic properties of these compounds. As a result, compounds **5** and **9** showed the potential and specific antidepressant activity in the forced swim test in mice. It is noteworthy that the antidepressant effect of compound **5** was comparable to that of the reference drug imipramine in doses not affecting the locomotor activity.

Experimental protocol

Molecular modeling

Homology model of the 5-HT$_{1A}$ serotonin receptor

Homology model of human 5-HT$_{1A}$ serotonin receptor was prepared in accordance with the method published previously [23]. The crystal structure of the β2-α_1R (PDB code 2rh1) was used as a template [24]. Our experience in homology modeling of monoaminergic GPCRs as well as the latest studies show the usefulness of this structure for modeling 5-HT$_{1A}$ receptor [25]. Amino acid sequence of the receptor was taken from the Uniprot database (accession number P08908), while sequence alignment was determined using the GeneSilico Metaserver's hhsearch method (https://genesilico.pl/meta2). The preliminary homology model was generated on SwissModel server (accessible from the program DeepView/SwissPdb-Viewer). Further model modifications were performed utilizing components of Schrödinger Suite 2009. The models were initially optimized using Protein Preparation Wizard and afterwards the extracellular loops were refined using Prime Refinement. In order to optimize the model taking into account crucial structural features of active ligands, induced fit docking (IFD) was involved for ligand-based optimization [26]. For this purpose a group of 12 possibly rigid compounds with high affinity for a given receptor was chosen. The top-scored complexes were inspected visually to check the compliance with common binding mode for monoaminergic receptor ligands. The above procedure resulted in 12 structures that served as molecular targets in further docking studies.

Homology model of serotonin transporter

The method of homology modeling developed by our group and introduced for 5-HT$_{1A}$ serotonin receptor was applied to model the structure of SERT. The X-ray structure of leucin transporter (LeuT) was used as the template for homology modeling (PDB id 2A65) since this protein is homologous to SERT. Amino acid sequence of human SERT, used in this study, figures in UniProt database under P31645 accession number. Building of the crude model and the following modifications were analogous to those applied for 5-HT$_{1A}$ modeling. At the IFD stage, spatial structure of sertraline as a possibly most conformationally rigid ligand of those which were crystalized together with LeuT [27] was used for binding site optimization. Using the ligand of known binding mode in LeuT was beneficial since no unambiguous binding mode information for SERT binding site is available. Best scored complex was verified in the context of proper binding mode, which resulted in final SERT model qualified to perform docking studies.

It is assumed that inhibitors of the SERT may act at two different active sites: (i) the substrate binding site, so called high affinity binding site, and (ii) low affinity vestibular binding site. On the basis of docking studies, we examined both of them to identify the one that binds our group of compounds. Since the first LeuT X-ray structures were published (e.g., PDB id 2A65 [28]), binding mode deliberations focused on the latter site. On the other hand, there were studies showing that SERT inhibitors act in a competitive manner in the area of the substrate binding site [29]. The previously mentioned structure was crystalized in occluded conformation, therefore there was no possibility to fit ligands in the tight pocket of the substrate binding site. Molecular docking to that site was possible since the outward-facing crystal structure was published [30]. Building homology model on the template of 3F3A enabled comparison of the binding modes of our compounds within both binding sites. It turned out that they were unable to dock correctly to the substrate binding site. What is noteworthy, in the low affinity binding site, they take analogous pose as SERT inhibitors (e.g., sertraline) co-crystallized with LeuT. For that reasons, molecular modeling studies were performed in the vestibular binding site, using homology model based on 2A65, which is identical in its spatial structure to crystals obtained together with SERT inhibitors (e.g., PDB id 3GWU).

Compound docking to the homology models

Docking of all compounds was carried out using Schrödinger's Glide application with XP (Extra Precision) mode set. In case of 5-HT$_{1A}$ model docking, an interaction constraint to form an H-bond between Asp3.32 and the protonated nitrogen of the ligand was applied, since such interaction was proved to be crucial for aminergic GPCR ligands binding [31]. Docking to

the SERT model was performed using the same conditions, constraining an H-bond interaction between the ligand and Glu493, since this residue was postulated to be engaged in the ligand binding in SERT [27]. The ligands were prepared with Schrödinger's LigPrep application employing OPLS_2001 force field. Beside of spatial optimization, it produced stereoisomers in possible protonation states.

Chemistry

Melting points (m.p.) were determined in open capillaries on an Electrothermal 9300 apparatus and were uncorrected. The purity of the compounds was confirmed by the thin-layer chromatography (TLC) performed on Merck silica gel 60 F_{254} aluminum sheets (Merck; Darmstadt, Germany), using the developing systems: benzene/ethyl acetate/acetone in the ratio of 10:5:1 by volume and acetone/isopropanol/chloroform in the ratio of 20:10:1 by volume. Spots were detected by their absorption under UV light ($\lambda = 254$ nm). The structures were confirmed by spectral (^{1}H NMR, ^{19}F NMR) and elemental (C, H, N) analysis. Nuclear magnetic resonance spectra were taken with Varian Mercury 300 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) in $CDCl_3$ (**3**–**10**) or d_6-DMSO (**1**, **2**) solution with TMS as internal standard. Chemical shifts were expressed in δ (ppm) and the coupling constants J in Hertz. Signal multiplicities are represented by the following abbreviations: s (singlet), br s (broad singlet), d (doublet), t (triplet), m (multiplet). For selected compounds (**1**, **3**–**5**) elemental analyses for C, H, N were carried out with an Elementar Vario EL III apparatus (Hanau, Germany). Additionally, the liquid chromatography/mass spectrometry (LC/MS) spectra for chosen compounds (**3**, **6**–**10**) were obtained on the Agilent 1100 HPLC coupled to a API 2000 (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer. Electrospray ionization (ESI) was used for ion production. All the analyses were performed on a C18 XBridge™ analytical column (2.1 mm × 30 mm, 3.5 μm, Waters, Ireland) with gradient elution using a mobile phase containing acetonitrile and water with an addition of 0.1% of formic acid.

Appropriate substituted 1-phenylpiperazine derivatives, 2-methoxybenzaldehyde, 5-fluoro-2-methoxybenzaldehyde, and other chemicals were commercially available (Aldrich or Fluka) and were used without purification.

*General procedure for preparation of imidazolidine-2,4-diones (**1**, **2**)*

The imidazolidine-2,4-diones (**1**, **2**) were prepared from the aldehyde by Bucherer–Bergs reaction with modifications described by Goodson et al. [12]. A solution of aldehyde (330 mmol) and ammonium carbonate (1 mol) in ethanol (330 mL) and water (220 mL) was warmed to 50°C, at which time potassium cyanide (350 mmol) dissolved in 50 mL of water was dropped in over a period of 15 min. The mixture was then heated at temperatures between 56 and 60°C for more than 20 h. The reflux condenser was then replaced by an air condenser and the temperature raised to 80°C for 1 h to remove the excess ammonium carbonate. Then the reaction solution was cooled and acidified. The precipitated solid was filtered off, washed with water, and recrystallized from a mixture of ethanol and acetone.

*5-(2-Methoxyphenyl)imidazolidine-2,4-dione (**1**)*

Following the general procedure **1** was obtained from 2-methoxybenzaldehyde (60° EtOH). Yield: 59%; m.p. 194–195°C; ^{1}H NMR (300 MHz, DMSO): δ 3.74 (s, 3H), 5.17 (s, 1H), 6.91–7.35 (m, 4H), 8.01 (s, 1H), 10.59 (s, 1H). Anal. calcd. for $C_{10}H_{10}N_2O_3$: C, 58.25; H, 4.89; N, 13.59. Found: C, 58.22; H, 5.08; N, 13.14.

*5-(5-Fluoro-2-methoxyphenyl)imidazolidine-2,4-dione (**2**)*

Following the general procedure **2** was obtained from 5-fluoro-2-methoxybenzaldehyde (60° EtOH). Yield: 64%; m.p. 227–229°C; ^{1}H NMR (300 MHz, DMSO): δ 3.72 (s, 3H), 5.19 (s, 1H), 7.02–7.20 (m, 3H), 8.07 (s, 1H), 10.73 (s, 1H); ^{19}F NMR (300 MHz, $CDCl_3$): δ −123.70–123.78 (m, 1F).

*General procedure for synthesis of compounds **3**–**10***

A mixture of 5-arylimidazolidine-2,4-dione (5 mmol), the substituted 1-phenylpiperazine (5 mmol) and 36% formalin solution (15 mmol) in 96% ethanol (30 mL) was refluxed for 25–30 h. After cooling, the precipitate was filtered off, then purified by column chromatography (ethyl acetate/methanol, 8:2) and recrystallized from anhydrous ethanol.

*[R,S]5-(2-Methoxyphenyl)-3-[1-(4-phenylpiperazin-1-yl)methyl]imidazolidine-2,4-dione (**3**)*

Following the general procedure **3** was obtained from **1**. Yield: 67%; m.p. 155–157°C; ESI–MS ($M+H^+$) 381.50; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.88–2.91 (t, $J = 4.80$ Hz, 4H), 3.20–3.23 (t, $J = 4.80$ Hz, 4H), 3.77 (s, 3H), 4.64–4.66 (d, $J = 5.40$ Hz, 2H), 5.18 (s, 1H), 6.83–6.99 (m, 6H), 7.02–7.29 (m, 4H). Anal. calcd. for $C_{21}H_{24}N_4O_3$: C, 66.30; H, 6.36; N, 14.73. Found: C, 66.60; H, 6.69; N, 14.62.

*[R,S]5-(2-Methoxyphenyl)-3-{1-[4-(2-methoxyphenyl)-piperazin-1-yl]methyl}imidazolidine-2,4-dione (**4**)*

Following the general procedure **4** was obtained from **1**. Yield: 55%; m.p. 147–150°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.92 (br s, 4H), 3.08 (br s, 4H), 3.82 (s, 3H), 3.85 (s, 3H), 4.63–4.65 (d, $J = 5.60$ Hz, 2H), 5.20 (s, 1H), 6.83–7.29 (m, 9H). Anal. calcd. for $C_{22}H_{26}N_4O_4$: C, 64.37; H, 6.38; N, 13.65. Found: C, 63.98; H, 6.23; N, 13.45.

*[R,S]5-(2-Methoxyphenyl)-3-{1-[4-(3-chlorophenyl)-piperazin-1-yl]methyl}imidazolidine-2,4-dione (**5**)*

Following the general procedure **5** was obtained from **1**. Yield: 71%; m.p. 163–165°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.84–2.88 (t, $J = 5.00$ Hz, 4H), 3.19–3.22 (t, $J = 5.00$ Hz, 4H), 3.75 (s, 3H), 4.62–4.64 (d, $J = 5.60$ Hz, 2H), 5.18 (s, 1H), 6.74–7.02 (m, 6H), 7.13–7.26 (m, 3H). Anal. calcd. for $C_{21}H_{23}N_4O_3Cl$: C, 60.79; H, 5.59; N, 13.50. Found: C, 60.45; H, 5.58; N, 13.24.

*[R,S]5-(2-Methoxyphenyl)-3-{1-[4-(3-(trifluoromethyl)-phenyl)piperazin-1-yl]methyl}-imidazolidine-2,4-dione (**6**)*

Following the general procedure **6** was obtained from **1**. Yield: 59%; m.p. 173–176°C; ESI–MS ($M+H^+$) 449.40; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.87–2.90 (t, $J = 5.00$ Hz, 4H), 3.23–3.25 (t, $J = 5.00$ Hz, 4H), 3.76 (s, 3H), 4.63–4.65 (d, $J = 5.10$ Hz, 2H), 5.18 (s, 1H), 6.90–7.08 (m, 6H), 7.21–7.26 (m, 3H); ^{19}F NMR (300 MHz, $CDCl_3$): δ −62.76 (m, F). Anal. calcd. for $C_{23}H_{25}F_3N_4O_3$: C, 59.73; H, 5.45; N, 12.11. Found: C, 59.79; H, 5.74; N, 12.18.

*[R,S]5-(5-Fluoro-2-methoxyphenyl)-3-[1-(4-phenylpiperazin-1-yl)methyl]imidazolidine-2,4-dione (**7**)*

Following the general procedure **7** was obtained from **2**. Yield: 61%; m.p. 194–196°C; ESI–MS ($M+H^+$) 399.40; ^{1}H NMR (300 MHz,

$CDCl_3$): δ 2.84–2.92 (t, J = 4.80 Hz, 4H), 3.12–3.15 (t, J = 4.80 Hz, 4H), 3.90 (s, 3H), 4.60 (s, 2H), 6.53 (s, 1H), 6.76–6.90 (m, 5H), 6.98–7.18 (m, 4H); ^{19}F NMR (300 MHz, $CDCl_3$): δ −123.71–123.98 (m, 1F). Anal. calcd. for $C_{21}H_{23}FN_4O_3$: C, 63.30; H, 5.82; N, 14.06. Found: C, 63.44; H, 6.05; N, 14.16.

*[R,S]5-(5-Fluoro-2-methoxyphenyl)-3-{1-[4-(2-methoxyphenyl)piperazin-1-yl]methyl}-imidazolidine-2,4-dione (**8**)*

Following the general procedure **8** was obtained from **2**. Yield: 50%; m.p. 172–174°C; ESI–MS (M+H$^+$) 429.30; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.44 (br s, 4H), 2.80 (br s, 4H), 3.67 (s, 3H), 3.73 (s, 3H), 4.81–4.85 (d, J = 5.00 Hz, 2H), 5.27 (s, 1H), 6.35 (s, 1H), 6.74–6.92 (m, 5H), 7.19–7.24 (m, 2H); ^{19}F NMR (300 MHz, $CDCl_3$): δ −123.31 (s, 1F). Anal. calcd. for $C_{22}H_{25}FN_4O_4$: C, 61.67; H, 5.88; N, 13.08. Found: C, 61.97; H, 6.22; N, 13.18.

*[R,S]5-(5-Fluoro-2-methoxyphenyl)-3-{1-[4-(3-chlorophenyl)piperazin-1-yl]methyl}-imidazolidine-2,4-dione (**9**)*

Following the general procedure **9** was obtained from **2**. Yield: 65%; m.p. 190–192°C; ESI–MS (M+H$^+$) 433.30; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.83–2.86 (t, J = 4.80 Hz, 4H), 3.19–3.22 (t, J = 4.80 Hz, 4H), 3.81 (s, 3H), 4.61–4.63 (d, J = 5.00 Hz, 2H), 5.14 (br s, 1H), 6.74–6.91 (m, 5H), 6.93–7.25 (m, 3H); ^{19}F NMR (300 MHz, $CDCl_3$): δ −123.65–123.83 (s, 1F). Anal. calcd. for $C_{21}H_{22}ClFN_4O_3$: C, 58.27; H, 5.12; N, 12.94. Found: C, 58.53; H, 5.43; N, 12.75.

*[R,S]5-(5-Fluoro-2-methoxyphenyl)-3-{1-[4-(3-(trifluoromethyl)phenyl)piperazin-1-yl]methyl}imidazolidine-2,4-dione (**10**)*

Following the general procedure **10** was obtained from **2**. Yield: 51%; m.p. 170–173°C; ESI–MS (M+H$^+$) 467.70; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.86–2.89 (t, J = 4.80 Hz, 4H), 3.23–3.26 (t, J = 4.80 Hz, 4H), 3.74 (s, 3H), 4.63–4.64 (d, J = 5.00 Hz, 2H), 5.14 (s, 1H), 6.83–6.88 (m, 1H), 6.96–7.09 (m, 7H); ^{19}F NMR (300 MHz, $CDCl_3$): δ −62.77 (s, 3F), −122.85 (s, 1F). Anal. calcd. for $C_{22}H_{22}F_4N_4O_3$: C, 56.65; H, 4.75; N, 12.01. Found: C, 56.82; H, 5.02; N, 11.80.

In vitro experiments

Serotonin 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor binding assays

Radioligand studies with native 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors were conducted according to the methods previously described [12]. Briefly, the following were used: for 5-HT$_{1A}$ assays, rat hippocampal membranes, [^{3}H]-8-OH-DPAT (170 Ci/mmol, NEN Chemicals), and 5-HT (10 μM) for non-specific binding; for 5-HT$_{2A}$ assays, [^{3}H]-ketanserin (88.0 Ci/mmol, NEN Chemicals) and methysergide (1 μM) for non-specific binding.

Adrenergic α$_1$ receptor binding assay

The assay was performed according to the method described by Cheng and Prusoff [14]. Rats' cerebral cortices were homogenized in 20 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.6), and centrifuged at 20.000 × g for 20 min (0–4°C). The cell pellet was resuspended in Tris–HCl buffer and centrifuged again. [^{3}H]Prazosin (19.5 Ci/mM, NEN Chemicals) was used for labeling α$_1$ receptors. The final incubation mixture (final volume 300 μL) consisted of 240 μL membrane suspension, 30 μL of a [^{3}H]Prazosin (0.2 nM) solution and 30 μL of buffer containing from seven to eight concentrations (10^{-11}–10^{-4} M) of investigated compounds. For measuring unspecific binding, phentolamine – 10 μM was applied. Radioactivity was measured in a WALLAC 1409 DSA – liquid scintillation counter. All assays were done in duplicates. Radioligand binding data were analyzed using iterative curve fitting routines (GraphPAD/Prism, Version 3.0 – San Diego, CA, USA).

Serotonin transporter binding assay

The experiment was performed according to the method described by Owens et al. [15]. Rats' cerebral cortices were homogenized in 30 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25°C) containing 150 mM NaCl and 5 mM KCl. Then the homogenate was centrifuged at 20 000 × g for 20 min. The resulting supernatant was decanted and the pellet was resuspended in the same quantity of the buffer, and centrifuged as above. The pellet was resuspended and centrifuged for further two times. [^{3}H]-Citalopram (50 Ci/mM, NEN Chemicals) was used for labeling SERT. The final examined mixture consisted of 240 μL tissue suspension, 30 μL of a 1 μM imipramine (displacer), 30 μL of 1 nM [^{3}H]-citalopram and 100 μL buffer containing seven concentrations (10^{-10}–10^{-4} M) of tested compounds. The incubation was performed in plates (MAFCNOB 10, Millipore) at 23°C. After 60 min the incubation was terminated by rapid vacuum filtration over glass filters (Watman GF/B). Next the filters were washed two times with 100 μL of ice-cold buffer (0–4°C) and placed in scintillation vials with scintillation cocktail. Radioactivity was measured in a WALLAC 1409 DSA – liquid scintillation counter. All assays were done in duplicates. Radioligand binding data were analyzed using iterative curve fitting routines (GraphPAD/Prism, version 3.0; San Diego, CA, USA).

Serotonin 5-HT$_{1A}$ receptor intrinsic activity assay

Chinese hamster ovary (CHO)-K1 cells stably transfected with the human serotonin 5-HT$_{1A}$ receptor were obtained commercially from PerkinElmer SA (Brussels, Belgium) and grown in RPMI supplemented with 10% fetal calf serum, 1.25 mg/mL geneticin and antibiotics. Cultures were maintained at 37°C in an air/CO_2 (95:5) water-saturated atmosphere and subcultured weekly. Cells were plated in 96-well culture plates at about 40 000 cells/well, grown until 90% confluent. For agonist studies, after removal of the medium, cells were stimulated for 15 min with compound diluted in 200 μL serum-free medium, based on preliminary studies showing the detection of maximum levels of phosphorylated extracellular signal-regulated kinase (pERK) after 15 min of stimulation (data not shown). In antagonist studies, cells were incubated for 15 min with compound diluted in 200 μL of the medium and then after removal of the medium, cells were stimulated with 0.1 μM (+)-8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) in the presence of tested compound dissolved in 200 μL of the medium. At the end of incubation the medium and compound were removed, and cells were lysed for 15 min at room temperature with cell lysis buffer (SureFire assay kit, Perkin Elmer). Lysates were allowed to equilibrate for 15 min at room temperature. Cell lysates were analyzed for content of pERK1/2 using an immunometric assay, therefore to 4 μL lysate was added 7 μL of reaction buffer plus activation buffer mix containing AlphaScreen® beads (PerkinElmer). Fluorescence detection was performed on a plate reader (EnVision, Perkin Elmer) set, using the method of time-resolved fluorescence energy transfer (TR-FRET). Efficacies of compounds tested in

CHO-h5-HT_{1A} cells are expressed relative to 0.1 μM 8-OH-DPAT (100%). Isotherms were analyzed by nonlinear regression, Rusing GraphPad Prism (GraphPad Software Inc., San Diego, CA) to yield EC_{50} and E_{max} values [16].

In vivo experiments

The experiments were performed on male Albino Swiss mice (24–28 g), purchased from a licensed breeder Staniszewska (Ilkowice, Poland) in an environmentally controlled, experimental room (ambient temperature 21 ± 2°C; relative humidity 50–60%; 12:12 light/dark cycle, lights on at 8:00). Standard laboratory food and filtered water were freely available. Animals were assigned randomly to treatment groups. All the experiments were performed by two observers unaware of the treatment applied between 9 a.m. and 2 p.m. on separate groups of animals. All the experimental procedures were approved by the I Local Ethics Commission for Animal Experiments at Jagiellonian University in Kraków. (±)-2,5-Dimethoxy-4-iodoamphetamine (hydrochloride, (±)-DOI, Sigma–Aldrich, Inc., USA) was dissolved in saline. The investigated compounds were suspended in a 1% aqueous solution of Tween 80. (±)-DOI and the tested compounds were given intraperitoneally (ip), in a volume of 10 mL/kg. The tested compounds (**5**, **9**) were administered 30 min before the experiments. Each experimental group consisted of six to ten animals per dose, and all the animals were used only once.

Head twitch response in mice

To habituate mice to the experimental environment, each animal was randomly transferred to a 12 cm (diameter) × 20 cm 20 cm (height) glass cage lined with sawdust, 30 min before treatment. Head twitches in mice were induced by (±)-DOI (2.5 mg/kg). Immediately after the treatment, the number of head twitches was counted throughout 20 min [17]. The tested compounds (**5**, **9**) were administered 60 min before (±)-DOI.

Four-plate test in mice

The box was made of an opaque plastic and was rectangular (25 cm × 18 cm × 16 cm) in shape. The floor was covered with four rectangular metal plates (11 cm × 8 cm), separated by a 4 mm gap. The plates were connected to a source of continuous current which enabled a 120 V difference of potential between two adjacent plates for 0.5 s when the experimenter pressed the switch. Individual mice were placed gently onto the plate, and were allowed to explore for 15 s. Afterwards, each time a mouse passed from one plate to another, the experimenter electrified the whole floor which evoked a visible flight reaction of the animal. If the animal continued running, it received no new shocks for the following 3 s. The episodes of punished crossing were counted for 60 s [20].

Forced swim test in mice

The experiment was carried out according to the method of Porsolt et al. [19]. Briefly, mice were individually placed in a glass cylinder (25 cm high; 10 cm in diameter) containing 6 cm of water maintained at 23–25°C, and were left there for 6 min. A mouse was regarded as immobile when it remained floating on the water, making only small movements to keep its head above it. The total duration of immobility was recorded during the last 4 min of a 6-min test session.

Locomotor activity in mice

The locomotor activity of mice was recorded in photoresistor actometers (24 cm in diameter) illuminated by two light beams, which were connected to a counter for the recording of light-beam interruptions. The mice were placed individually in the actometers, and the number of crossings of the light beams was counted twice: between 2 and 6 min (i.e., the time equal to the observation period in the forced swim test), and during 30-min experimental sessions.

Statistics

The obtained data were analyzed by one-way analysis of variance followed by Dunnett's test. ID_{50} values were calculated by the method of Litchfield and Wilcoxon.

The authors wish to thank the Polish Ministry of Science and Higher Education for financial support (grant no. 2P05F04329). Part of this work was presented at XII BDSHC in Tihany, Hungary. Schrödinger Suite 2009 was licensed for Jagiellonian University Collegium Medicum.

The authors have declared no conflict of interest.

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