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Synthesis and activity of di- or trisubstituted *N*-(phenoxyalkyl)- or *N*-{2-[2-(phenoxy)ethoxy]ethyl}piperazine derivatives on the central nervous system

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ABSTRACT

Aim of the study was evaluation of anxiolytic, antidepressant, anticonvulsant and analgesic activity in a series of a consistent group of compounds. A series of eleven new *N*-(phenoxyalkyl)- or *N*-{2-[2-(phenoxy)ethoxy]ethyl}piperazine derivatives has been obtained. Their affinity towards 5-HT_{1A}, 5-HT_{2A}, 5-HT_6, 5-HT_7, D_2 and α_1 receptors has been assessed, and then functional assays were performed. The compounds were evaluated in mice, *i.p.* for their antidepressant-like (forced swim test), locomotor, anxiolytic-like (four-plate test) activities as well as – at higher doses – for anticonvulsant potential (MES) and neurotoxicity (rotarod). Two compounds (**3**, **6**) were also evaluated for their analgesic activity in neuropathic pain models (streptozocin test, oxaliplatin test) and they were found active against allodynia in diabetic neuropathic pain at 30 mg/kg. Among the compounds, anxiolytic-like, anticonvulsant or analgesic activity was observed but antidepressant-like activity was not. One of the two most interesting compounds is 1-{2-[2-(2,4,6-trimethylphenoxy)ethoxy]ethyl}-4-(2-methoxyphenyl)piperazine dihydrochloride (**9**), exhibiting anxiolytic and anticonvulsant activity in mice, *i.p.* 30 min after administration (at 2.5 mg/kg and ED_{50} = 26.33 mg/kg, respectively), which can be justified by the receptor profile: 5-HT_{1A} K_i = 5 nM (antagonist), 5-HT_7 K_i = 70 nM, α_1 K_i = 15 nM, D_2 K_i = 189 nM (antagonist). Another interesting compound is 1-[3-(2,4,6-trimethylphenoxy)propyl]-4-(4-methoxyphenyl)piperazine dihydrochloride (**3**), exhibiting anxiolytic, anticonvulsant and antiallodynic activity in mice, *i.p.*, 30 min after administration (at 10 mg/kg, ED_{50} = 23.50 mg/kg, at 30 mg/kg, respectively), which can be related with 5-HT_{1A} weak antagonism (K_i = 146 nM), or other possible mechanism of action, not evaluated within presented study. Additionally, for the most active compound in the four-plate test (**7**), molecular modeling was performed (docking to receptors 5-HT_{1A}, 5-HT_{2A}, 5-HT_7, D_2 and α_{1A}).

Central nervous system disorders, such as depression, anxiety, epilepsy and neuropathic pain are sometimes comorbid.[1] The reason of this phenomenon might be their common etiology, including trauma, intoxication, neurodegeneration or genetic factors.[2–4] The mentioned disorders also share common treatments, *e.g.* some anticonvulsants such as carbamazepine, valproates, or gabapentin improve mood. On the other hand, tianeptine may cause depression and seizures can be caused by maprotiline and clomipramine. Among antidepressants, drugs such as venlafaxine and selective serotonin reuptake inhibitors are considered relatively safe.[5,6] It has been reported that serotonergic system impairment may be another common factor for these diseases, which may justify the above mentioned phenomena.[7,8]

Taking into account possible multidirectional activity of newly designed compounds within CNS seems particularly important in

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terms of drug design. Providing drugs targeting more than one comorbid disorder would allow to reduce polypragmasy and a risk of side effects. On the other hand, including research on various activities within CNS on early stages of drug development would prevent unexpected off-target activities and adverse effects when aiming in treatment of one particular disorder.[9] Due to involvement of serotonergic system dysregulation in several central nervous system disorders, including broad pharmacological research is particularly important in case of serotonergic receptors ligands.

Phenyl piperazine (including particularly 2-methoxyphenylpiperazine) derivatives constitutes a group of compounds widely investigated in terms of serotonergic activity.[10–13] Thus, this group of compounds is of interest regarding search for multidirectional agents.

During our ongoing studies in terms of design and synthesis of new centrally active compounds, derivatives exhibiting anticonvulsant, analgesic and/or antidepressant-like activity have been synthesized. Therefore, inclusion of analysis of all these activities constitute natural consequence in our research. In order to design molecules which may possibly be active in treatment of the above disorders, we based on our former findings in groups of derivatives of *N*-(2-methoxyphenyl)piperazine, as well as phenoxyalkyl- or phenoxyethoxyethyl derivatives of piperazine,[14–19] where we found reference compounds **I**–**IV** for this study (Fig. 1), or of aminoalkanols.[20,21]

The majority of active compounds which have been obtained in our laboratory so far, possessed affinities towards various serotonergic receptors and additionally towards receptors D_2 and α_1. Based on our former research including structure-activity relationship as well as on literature review, the serotonergic mechanism of action seems to be the most probable one in case of compounds of interest. Thus, it was decided to include the multiplicity of serotonergic system influence on major depressive disorder (MDD),[22] seizures, neuropathic pain and anticonvulsant activity. Within current study, *in vitro* serotonergic ($5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$, $5\text{-}HT_6$, $5\text{-}HT_7$), D_2 and α_1 receptors binding assays and *in vivo* pharmacological tests were planned. We expected presented phenylpiperazine derivatives to be multitarget centrally active compounds with mechanism of action basing mainly on serotonergic modulation. Receptors α_1 were incorporated due to known fact that compounds possessing affinity towards $5\text{-}HT_{1A}$ often exhibit an off-target activity towards receptors α_1 and there are many potent α_1 ligands among phenylpiperazine derivatives.[23,24] More than one type of central activity *in vivo* was expected, basing on our previous experience with this group of compounds as well as potential multitarget activity.

Preliminary experiments with the use of serotonergic antagonist WAY100635 indicated that mechanism other than serotonergic might be involved in case of some phenylpiperazine derivatives synthesized before in our laboratory, however particular targets have been not defined yet.[7] For that reason, we decided to test all compounds *in vivo*, regardless their receptor profile, in order to correlate serotonergic receptor profile with *in vivo* activity. Potent central activity of close structural analogs served as a

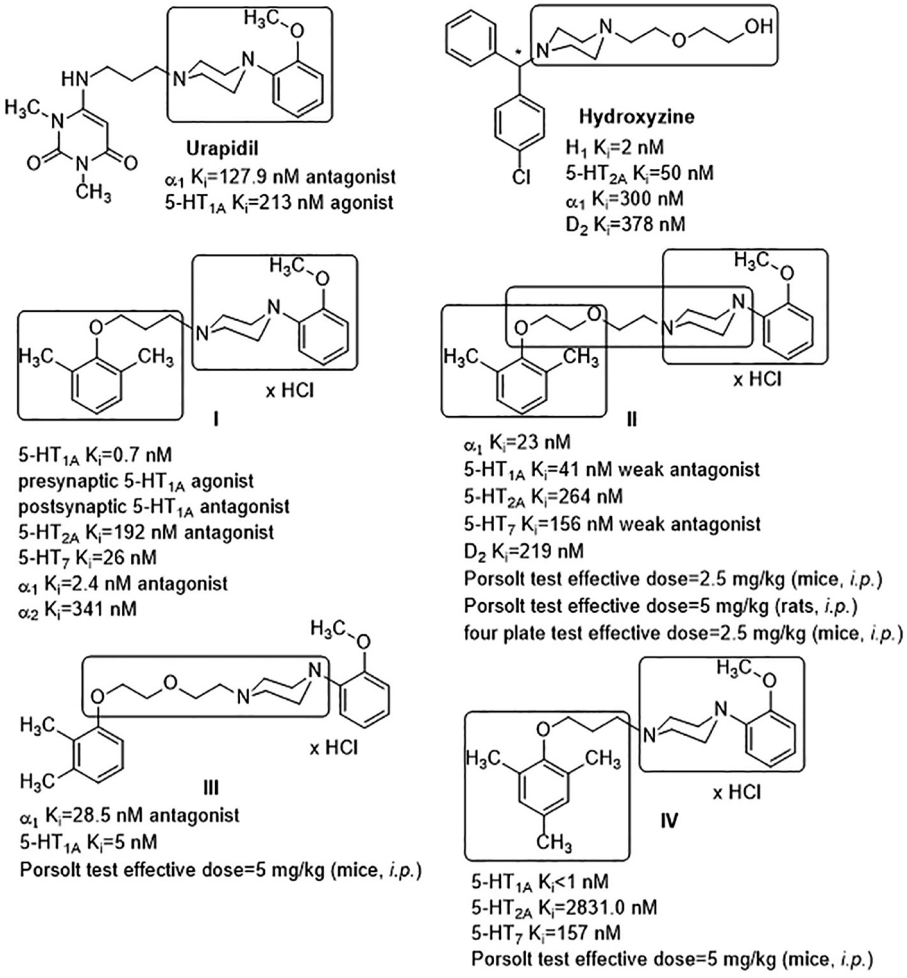


Fig. 1. Chemical structures of reference drugs (urapidil[14,25] and hydroxyzine) as well as compounds **I**,[14,15] **II**,[16,17] **III**,[18] **IV**.[15,16]

premise for such investigations. The results will be indication for our future studies in terms of rationality of including receptors other than serotonergic in searching for compounds' mechanism of action.

The aim of our study was design, synthesis and examination of receptor binding, functional assays (for agonism or antagonism) and evaluation of pharmacological activity in a group of new phenoxyalkyl or phenoxyethoxyethyl derivatives of piperazine, where the other substituent in the piperazine ring may significantly improve the demanded activity. Another aim was to find possible correlation between the receptor profiles of the compounds and their activity on the central nervous system.

This study presents eleven di- or trisubstituted compounds such as: 2,4-$(CH_3)_2$-, 4-Cl-3-CH_3-, 2,4,6-$(CH_3)_3$-phenoxyalkyl as well as 2,3-$(CH_3)_2$-, 2,3,5-$(CH_3)_3$- or 2,4,6-$(CH_3)_3$-phenoxyethoxyethyl derivatives of piperazine (Table 1).

It was decided to perform all pharmacological testing on the same species (mice), using the same route of administration (intraperitoneal *i.p.*), and at the same time after administration (30 min), in order to receive comparable results and enable proper conclusions. However, we needed to take into account different doses for *in vivo* testing of activity on anxiety or mood disorders and seizures, where typically higher doses are used for screening.

Chemistry

Eleven derivatives of *N*-phenoxyalkyl- (**1**–**4**) and *N*-{2-[2-(phenoxy)ethoxy]ethyl}piperazine (**5**–**11**) in the forms of hydrochlorides have been obtained. Compounds disubstituted in the phenyl ring with two methyl groups have been extensively studied within our previous research (*e.g.* reference compounds **I**–**III**). We also obtained several trisubstituted compounds, among others reference compound **IV**, which exhibited favorable receptor profile and interesting *in vivo* activity. The design of compounds within current study based on structural modification of reference compounds **I**–**IV**, including change of phenyl ring's substitution positions (in case of compound **4** – incorporating chlorine as one of substituents), manipulation with linker longevity and modification of arylpiperazine substituent. In case of compounds **2**, **6** and **8** an aryl moiety has been removed from amine component in order to obtain structural derivatives of hydroxyzine.

Calculation of several physicochemical parameters by means of Molinspiration online toolkit was an important part in our research.[26] Such calculations are useful at early stage of drug discovery process in terms of predicting essential parameters and facilitating rational drug design. The parameters include logP (for lipophilicity), topological polar surface area TPSA, molecular volume, and violations from the Lipinski rule of five. The structures and the results are presented in Table 1. The first calculated parameter miLogP was found in the range 2.38–5.14, while in the literature the optimal value for CNS active compounds is about 2–3.[27] Our experience with phenylpiperazine derivatives indicates that in case of this group of compounds deviations from this rule are possible. We previously obtained centrally active compounds which were relatively safe in preliminary examinations and exhibited logP values >4, *e.g.* reference compounds **I**–**IV** (Fig. 1). For that reason, the design of current study did not involve logP value as a decisive parameter in design of compounds. However, logP values were calculated for providing information and possible discussion of relationship between lipophilicity and central activity of presented compounds. The most advantageous value of TPSA should be <120 Å^2 for orally administered drugs and <60–70 Å^2 for compounds designed to penetrate blood-brain barrier. The proposed structures are consistent with both the rules for oral drugs and for CNS drugs (calculated values are in the range 15.71–45.17 Å^2). The volumes range 326.87–405.85 Å^3. None of the compounds exhibited any violations from the Lipinski rule of five,[28] which presumes that they are potential drug-like agents.

All title compounds (Table 1) were obtained *via* multistep chemical synthesis (Scheme 1). The detailed description of reaction conditions as well as physicochemical properties of obtained products are available in Supplementary materials.

Receptor studies

The synthesized compounds were subject to receptor binding studies for 5-HT_{1A}, 5-HT_{2A}, 5-HT_6, 5-HT_7, as well as D_2, and α_1 receptors. The assay was executed according to previously described protocol.[29] The results are presented in Table 2. The most advantageous binding properties were observed for compounds **1**, **4**, **7**, and **9**, where nanomolar affinities have been achieved. Among them, compounds **1**, **7** and **9** are substituted with 2-methoxy group in phenylpiperazine moiety. The advantageous impact of such modification is consistent with literature and our former findings.

Comparing the results with the reference compounds, compound **1** is 2,4-dimethyl analog of the reference compound **I** and exhibits comparable receptor binding (*e.g.* for compound **1** K_i = 2.0 nM towards 5-HT_{1A} and K_i = 15 nM towards 5-HT_7, while for compound **I** K_i = 0.7 nM towards 5-HT_{1A} and K_i = 26 nM towards 5-HT_7). Compound **7** – the 2,3,5-trimethyl analog of **III** – exhibits slightly higher binding affinity towards α_1 receptors compared to the reference **III** (K_i = 14 nM vs K_i = 28.5 nM), but lower toward 5-HT_{1A} (K_i = 32 nM vs K_i = 5 nM). Compound **9** as the 2,4,6-trimethyl analog of **III** exhibits the same affinity toward 5-HT_{1A} receptors (K_i = 5.0 nM towards 5-HT_{1A}) and slightly better affinity toward α_1 receptors (K_i = 15.0 nM for compound **9** vs K_i = 28.5 for compound **III**). It also should be noticed that compounds **6** and **8** as hydroxyethyl analogs of **7** and **9**, respectively, do not exhibit such advantageous receptor profiles.

Upon completion of affinity assays, functional studies were performed for relevant compounds (compounds **1**, **4**, **7**, and **9** – 5-HT_{1A}, 5-HT_{2A}, 5-HT_7 and D_2 receptors, compounds **3** and **11** – 5-HT_{1A} receptor and compound **5** – 5-HT_{2A} receptor). Antagonistic properties were proved for the majority of performed evaluations. The exception was weak agonistic properties of compound **4** towards receptor D_2. The data are presented in Supplementary materials.

Pharmacology

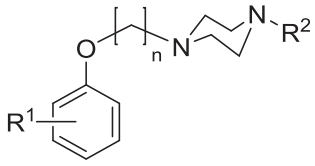
All compounds were screened for antidepressant-like and anxiolytic-like properties *in vivo*. For this purpose we used the forced swim test in mice[30,31] and the four-plate test in mice,[17,32] respectively. The results are presented in Table 3. Influence on locomotor activity in mice was evaluated in 1-min session[22] (Table 4).

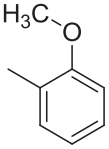
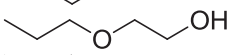
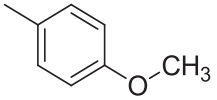
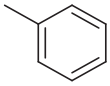
Compounds **2**–**3** and **7**–**10** exhibited anxiolytic-like properties, while compounds **1**, **4**–**6**, and **11** were inactive in the four-plate test. Compound **2** (2-(2-{4-[2-(Mesityloxy)ethyl]piperazin-1-yl}ethoxy)ethanol dihydrochloride) and **9** (1-{2-[2-(mesityloxy)ethoxy]ethyl}-4-(2-methoxyphenyl)piperazine dihydrochloride) were active at the doses 2.5 and 5 mg/kg ($p < 0.05$) and compound **7** (2,3,5-trimethyl substituted analog of **9**) at the doses 2.5 ($p < 0.0001$) and 5 mg/kg ($p < 0.01$), which was the strongest effect demonstrated in this group of compounds in this test. Compound **3**, **8**, and **10** exhibited anxiolytic-like activity at the dose 10 mg/kg, while the reference clorazepate – at the dose 1.25 mg/kg.

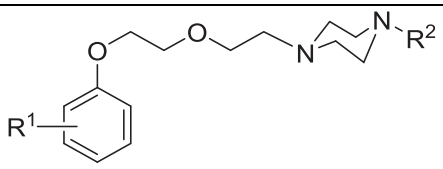
As neither of the compounds affected locomotor activity in mice at anxiolytic-like doses, the observed effect was specific.

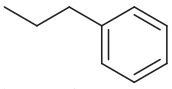
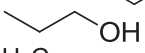
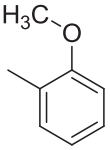
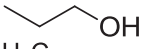
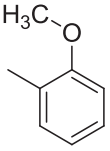
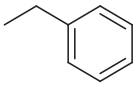
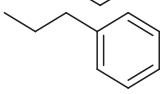
Anticonvulsant evaluation was performed independently from results of receptor and antidepressant-like or anxiolytic-like activ-

Table 1
Structures of the title compounds **1**–**11**.



Compd.	R^1	n	R^2	m	miLogP[a]	TPSA (Å^2)	Volume (Å^3)
1	2,4-$(CH_3)_2$	3		1	4.72	24.94	355.44
2	2,4,6-$(CH_3)_3$	2		2	2.38	45.17	342.46
3		3		2	5.14	24.94	372.00
4	4-Cl-3-CH_3	3		2	4.94	15.71	326.87

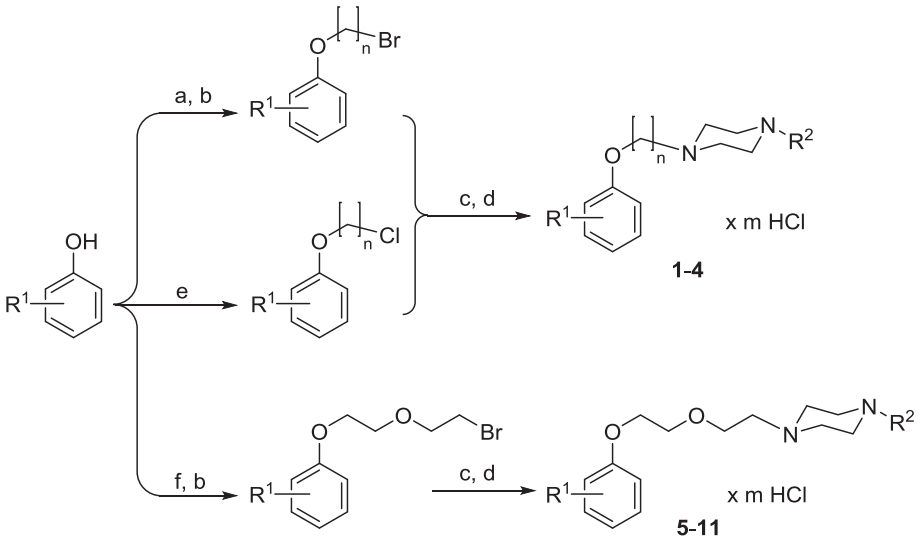


Compd	R^1	R^2	m	miLogP[a]	TPSA (Å^2)	Volume (Å^3)
5	2,3-$(CH_3)_2$		2	4.41	24.94	389.29
6	2,3,5-$(CH_3)_3$		2	2.38	45.17	342.46
7			2	4.71	34.18	397.79
8	2,4,6-$(CH_3)_3$		2	2.38	45.17	342.46
9			2	4.71	34.18	397.79
10			2	4.41	24.94	389.05
11			2	4.81	24.94	405.85

[a] Calculations performed with use of Molinspiration online toolkit for base forms.[26]

ity. Typical mode of search for anticonvulsants is performed with use of *in vivo* screening. Previously, we performed screening research related with this group of compounds, on the basis of some active compounds in depression or anxiety, and in the PTZ test we did not find them active although we saw some activity in MES.[33] Therefore, this time anticonvulsant activity was screened only in MES at 30 mg/kg b.w. (mice, *i.p.*) and in case of positive results ED_{50} was derived. Concomitantly, rotarod test was performed for neurotoxicity assessment.

The assays were performed for compounds **1**–**7**, **9**, and **11** (Table 5), according to procedure described by Löscher et al.[34] together with neurotoxicity evaluation by standard rotarod procedure.[35] The most promising compounds **2**–**3**, **5**–**6**, **9**, and **11** were subject to quantitative assays, resulting in ED_{50} for MES



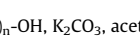
Scheme 1. Synthesis of the title compounds. a: Cl-$(CH_2)_n$-OH, K_2CO_3, acetone/EtOH; b: PBr_3; c:  , K_2CO_3, toluene, or *N*-methylmorfoline, DMF; d: gaseous HCl, EtOH; e: Br-$(CH_2)_n$-Cl, K_2CO_3, acetone; f: Cl-$(CH_2)_2$-O-$(CH_2)_2$-OH, K_2CO_3, acetone/EtOH; R^1, R^2, n, m as in Table 1.

Table 2
Binding results for the title compounds **1–11** and reference drugs.

Compound	K_i (nM)					
	5-HT_{1A} [^{3}H] – 8-OH-DPAT	5-HT_{2A} [^{3}H] – ketanserin	5-HT_6 [^{3}H] – LSD	5-HT_7 [^{3}H] – LSD	D_2 [^{3}H] – methylspiperone	α_1 [^{3}H] – prazosin
1	**2.0** ± 0.2	527.0 ± 4.0	>5000	**15.0** ± 3.0	**97.0** ± 3.6	**11.0** ± 1.0
2	3168.0 **±** 112.0	|	–	–	|	–
3	**146.0** ± 28.4	772.0 ± 45.2	>5000	3027.0 ± 208.0	4963.0 ± 479.0	2117.0 ± 180.0
4	**20.0** ± 1.2	**76.0** ± 2.7	>5000	**90.0** ± 8.0	299.0 ± 9.5	**93.0** ± 3.0
5	–	220.0 ± 7.3	200.0 ± 3.5	388.0 ± 7.0	1206.0 ± 13.5	**36.0** ± 1.1
6	–	>5000	>5000	3142.0 ± 218.0	–	–
7	**32.0** ± 3.6	**159.0** ± 12.4	1540.0 ± 103.0	**89.0** ± 10.0	**162.0** ± 10.3	**14.0** ± 0.6
8	–	>5000	>5000	–	–	–
9	**5.0** ± 0.6	245.0 ± 5.5	2427.0 ± 270.0	**70.0** ± 0.5	189.0 ± 19.3	**15.0** ± 1.5
10	–	498.0 ± 135	>5000	3160.0 ± 34.5	–	–
11	552 ± 14.0	2805.0 ± 249.0	2155.0 ± 129.0	1050.0 ± 54.0	–	772.0 ± 29.5
Methiothepin	4.0 ± 0.4	|	0.9 ± 0.07	1.7 ± 0.2	|	|
Mianserin	|	3.2 ± 0.2	|	|	|	|
Haloperidol	|	|	|	|	5.9 ± 0.2	|
Phentolamine	|	|	|	|	|	12.0 ± 0.6

– No binding at 10^{-5} M; | no data.

and TD_{50} in rotarod. Both ED_{50} and TD_{50} values with 95% confidence limits were calculated by probit analysis.[36] Further determination of protection index (PI = TD_{50}/ED_{50}) indicated that the most favorable neuronal stabilizing properties and safety were observed for compounds **2**, **3**, **6**, and **11** (PIs 1.88, 2.89, 3.07 and 2.96, respectively).

It was decided that within the tested group of piperazine derivatives, two compounds with the most favorable PIs and MES ED_{50} about 20 mg/kg, were chosen for further evaluation of activity in neuropathic pain models: **3** and **6** (*i.e.* 1-[1-(2,4,6-trimethylphenoxy)propyl]-4-(4-methoxyphenyl)piperazine dihydrochloride and 1-{2-[2-(2,3,5-trimethylphenoxy)ethoxy]ethyl}-4-(2-hydroxyethyl)piperazine dihydrochloride, respectively).

Evaluation of antiallodynic or antihyperalgesic activity of compounds **3** and **6** in mouse models of neuropathic pain was realized with use of streptozocin test (STZ) and oxaliplatin test (OXA). The mechanism of deterioration of nerves in the streptozocin model resembles the process of human diabetic neuropathy.[37] Evaluation of antiallodynic activity was performed with use of von Frey test.

Moreover, activity against hyperalgesia was also tested in both STZ and OXA models – with use of hot plate or cold plate test, respectively.[37–39] The results are presented in Figs. 2–5 and they indicate that both compounds are active in mechanical allodynia in STZ test at the higher dose 30 mg/kg, but not in thermal hyperalgesia (hot plate or cold plate tests). Both compounds differ structurally in terms of substitution in the phenyl ring (although they are both trisubstituted), they have different linker between phenoxy group and piperazine (propyl or ethoxyethyl) and the piperazine moiety contains different substituent at the distal nitrogen (4-methoxyphenyl or 2-hydroxyethyl).

Molecular modeling

The final step of our research was the docking study of compound **7**. Despite it was not the one possessing the strongest affinities for the presented receptors, it was the most active anxiolytic agent among compounds **1–11** tested *in vivo*. Putative binding mode of compound **7** was determined by docking to homology models of the 5-HT_{1A}, 5-HT_{2A} and 5-HT_7 serotonin receptors, as well as for the dopamine D_2 and adrenergic α_{1A} receptors, performed according to the published procedures.[40–45] The GPCR

Table 3
Antidepressant-like and anxiolytic-like activity of **1**–**11** in mice, *i.p.*

Compound	Forced swim test (antidepressant-like activity)			Four-plate test (anxiolytic-like activity)		
	Dose (mg/kg)	Immobility time ± SEM (s)	F statistic (degrees of freedom)	Dose (mg/kg)	Number of punished crossings ± SEM (s)	F statistic (degrees of freedom)
Vehicle	–	164.0 ± 11.7			2.6 ± 0.3	
1	5	182.6 ± 17.3	F(3,28) = 1.674, ns	5	2.1 ± 0.4	F(2,21) = 1.736, ns
	10	198.1 ± 8.3		10	1.5 ± 0.5	
	20	197.3 ± 9.9				
Vehicle	–	159.6 ± 19.6	–	–	3.3 ± 0.4	
2	5	152.5 ± 17.6	F(3,28) = 1.114, ns	1.25	3.3 ± 0.4	F(3,30) = 5.807, p < 0.01
	10	135.3 ± 7.0		2.5	5.5 ± 0.5*	
	20	128.0 ± 15.9		5	5.0 ± 0.5*	
Vehicle	–	194.5 ± 18.5	–	–	3.9 ± 0.2	
3	5	179.9 ± 18.5	F (3,28) = 0.388, ns	5	3.8 ± 0.4	F(2,21) = 14.02, p < 0.001
	10	202.0 ± 13.2		10	7.5 ± 0.9***	
	20	183.9 ± 13.8				
Vehicle	–	201.9 ± 16.0		–	3.0 ± 0.3	
4	5	189.1 ± 14.2	F(3,28) = 0.6499, ns	5	2.8 ± 0.4	F(2,21) = 3.190, ns
	10	188.9 ± 18.0		10	1.9 ± 0.3	
	20	172.1 ± 11.6				
Vehicle	–	153.9 ± 11.7		–	3.5 ± 0.5	
5	5	157.6 ± 16.3	F(3,28) = 0.088, ns	5	3.8 ± 0.5	F(2,21) = 0.938, ns
	10	161.1 ± 11.7		10	2.9 ± 0.4	
	20	162.1 ± 10.0				
Vehicle	–	190.9 ± 16.1		–	2.8 ± 0.4	
6	5	171.8 ± 15.7	F(3,28) = 0.141, ns	5	2.8 ± 0.3	F(2,21) = 0.769, ns
	10	184.9 ± 15.6		10	3.0 ± 0.5	
	20	176.4 ± 12.7				
Vehicle	–	163.9 ± 6.7		–	3.1 ± 0.5	
7	5	165.6 ± 19.1	F(3,28) = 0.3591, ns	1.25	3.0 ± 0.4	F(3,32) = 11.89, p < 0.0001
	10	141.9 ± 21.9		2.5	6.1 ± 0.4****	
	20	156.1 ± 20.4		5	5.1 ± 0.4**	
Vehicle	–	164.0 ± 11.7		–	4.4 ± 0.4	
8	5	182.6 ± 17.3	F(3,28) = 1.575, ns	5	3.3 ± 0.5	F(2,29) = 15.31, p < 0.0001
	10	198.1 ± 8.3		10	7.4 ± 0.7***	
	20	197.3 ± 9.9				
Vehicle	–	166.9 ± 20.2		–	3.1 ± 0.4	
9	5	138.5 ± 17.9	F(3,36) = 0.7080, ns	1.25	3.1 ± 0.3	F(3,36) = 6.254, p < 0.01
	10	131.3 ± 14.5		2.5	4.6 ± 0.5*	
	20	150.6 ± 20.8		5	4.9 ± 0.3*	
Vehicle	–	194.5 ± 18.5		–	3.6 ± 0.2	
10	5	176.5 ± 9.6	F(3,28) = 0.828, ns	5	3,6 ± 0.9	F(2,21) = 12.24, p < 0.001
	10	182.4 ± 14.1		10	8.1 ± 0.9***	
	20	157.5 ± 22.8				
Vehicle	–	156.5 ± 21.1		–	3.6 ± 0.4	
11	5	170.5 ± 13.4	F(3,28) = 0.324, ns	5	3.9 ± 0.6	
	10	173.8 ± 19.5		10	4.0 ± 0.3	F(2,21) = 0.176, ns
	20	178.8 ± 10.9				
Vehicle[18]	–	178.4 ± 9.1				
Fluoxetine[18]	10	152.1 ± 12.9	F(2,27) = 11.65, p < 0.001			
	15	106.6 ± 9.8***				
Vehicle				–	3.0 ± 0.3	
Clorazepate				0.625	3.3 ± 0.3	F(2,21) = 5.574, p < 0.0001
				1.25	4.8 ± 0.5*	

All studied compounds and fluoxetine or clorazepate were administered 30 min before the test. Vehicle-treated groups received 0.9% NaCl. The values are expressed as mean ± SEM, n = 8–10 mice per group. Statistical analysis: one-way ANOVA (Newman-Keuls *post hoc*) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. respective vehicle-treated group, ns – not significant.

structures were represented by several homology models,[46,29,47] which simulated conformational flexibility of the proteins. Visual inspection of the resulting complexes allowed us to capture crucial ligand-receptor interactions responsible for binding to the biological targets.

The predicted binding mode of **7** was considered representative for other derivatives characterized by high affinity for the main biological targets *in vitro*. The molecule posed extended conformation across the two cavities forming the binding sites in monoaminergic G protein-coupled receptors. The main anchoring interactions, important for affinity for the selected receptors, involved the phenylpiperazine moiety: (i) a charge-reinforced hydrogen bond between the protonated nitrogen atom and the carboxyl group of Asp3.32, as well as (ii) the CH-π stacking with Phe6.52 (Fig. 6). The compounds lacking the above-mentioned moiety were unable to bind effectively to the receptors (Table 2). The substituted *N*-[(2,3,5-trimethylphenoxy)ethoxyethyl] fragment of the molecule occupied the opposite cavity and found favorable aromatic/hydrophobic interactions there, which were distinctive for each receptor type. In the case of serotonin receptors, the 5-HT_{1A} receptor interacted with the phenyl ring of Tyr2.64 (π–π stacking, Fig. 6A), whereas in the 5-HT_7 receptor – with Phe3.28 (CH-π interactions, Fig. 6B). In the 5-HT_{2A} receptor site, the latter fragment formed VdW interactions with hydrophobic interface of Ile2.65, Trp3.28, Val7.39 and Tyr7.43 (Fig. 6C). The latter binding mode was similar to the one in the dopamine D_2 receptor (Leu2.65, Phe3.28, Val3.29 and Trp71 from extracellular loop 1, Fig. 6D). The compound bounds to the adrenergic α_{1A} recep-

Table 4
Influence of the studied compounds on locomotor activity of mice in 1-min session.

Compound	Dose (mg/kg)	Number of crossings ± SEM (s)	t statistic (degrees of freedom)
Vehicle	–	68.4 ± 12.1	t(14) = 1.821, ns
2	5	41.4 ± 8.6	
Vehicle	–	69.0 ± 13.7	t(14) = 1.640, ns
3	10	41.1 ± 10.0	
Vehicle	–	68.4 ± 12.1	t(14) = 1.122, ns
7	5	51.0 ± 9.7	
Vehicle	–	69.0 ± 13.7	t(14) = 0.937, ns
8	10	54.4 ± 7.4	
Vehicle	–	72.2 ± 9.7	t(18) = 0.4732, ns
9	5	66.2 ± 8.2	
Vehicle	–	69.0 ± 13.7	t(14) = 0.025, ns
10	10	68.5 ± 14.2	

All studied compounds were administered *i.p.* 30 min before the test. Vehicle-treated groups received 0.9% NaCl. The values are expressed as mean ± SEM, n = 8–10 mice per group. Statistical analysis: Student *t*-test; ns – not significant.

tors *via* π–π stacking with Tyr2.64 and cation-π interaction with Lys7.39 (Fig. 6E). The presented simulations provided retrospective explanation of SAR data and support for future rational design of multifunctional ligands.

The most important interactions of the tested ligand were in line with the common binding mode for monoaminergic receptor ligands determined experimentally,[48] and with the results of our previous studies[49] which demonstrated the accuracy of the predictions based on the developed homology models.

Structure-activity relationship

Nine out of eleven synthesized compounds exhibited pharmacological activity *in vivo*, including anxiolytic, anticonvulsant and/or antiallodynic activities. Interestingly, *in vivo* activity was not always convergent with preferred receptor profile. For that reason it seems reasonable to discuss SAR in terms of receptor profile and *in vivo* activity independently.

Within presented series, all compounds possessing phenyl ring connected directly to piperazine moiety were characterized with interesting receptor profiles, while other compounds lacked affinity towards tested receptors (apart from compound **5**, which binds to receptors α_1 and possesses a linker between piperazine and phenyl ring). The results of molecular modeling performed for compound **7** serve for an explanation, showing that phenyl ring takes part in ligand binding in case of all receptors of interest. Its presence and proper distance from nitrogen atom of piperazine seem to be crucial for binding of compound **7** and it might be also important when discussing other active compounds.

All compounds binding to receptors 5-HT_{1A} possess also affinity towards receptors α_1, apart from compound **3** – 4-methoxy analog of reference compound **IV**. Such a selectivity of compound **3** is

Table 5
Anticonvulsant activity of compounds **1**–**7**, **9** and **11** (mice, *i.p.*).

Compd.	Dose (mg/kg)	MES[a]	Deaths	NT[b] (10 rpm)	ED_{50} (confidence interval) (mg/kg)	TD_{50} (confidence interval) (mg/kg)	PI
Control	–	0/6	4/6				
1	30	1/6	0/6	1/6			
2	100	6/6	0/6	4/6	45.42	85.26 (75.16–96.71)	1.88
	60	3/6	1/6		(37.17–55.51)		
	30	1/6	2/6	1/6			
3	100	6/6	0/6	3/6	23.50	67.88 (38.47–119.77)	2.89
	45	5/6	0/6	2/6	(13.65–40.45)		
	30	3/6	0/6	1/6			
	15	2/6	0/6	0/6			
4	30	1/6	2/6	3/6			
5	100	3/3	0/3	3/3	22.19	35.50 (20.77–60.67)	1.60
	45	6/6	0/6	4/6	(18.36–26.82)		
	30	3/6	0/6	2/6			
	15	1/6	2/6	1/6			
6	100	6/6	0/6	4/6	24.39	74.92 (42.22–132.94)	3.07
	30	4/6	0/6	1/6	(17.66–33.67)		
	20	2/6	1/6				
	15	1/6	2/6				
7	30	1/6	0/6	3/6			
9	40	5/6	0/6	4/6	26.33	31.48 (22.86–43.35)	1.19
	30	3/6	0/6	3/6	(18.85–36.78)		
	20	2/6	0/6	1/6			
11	100		1/1		18.00	53.33 (49.47–57.48)	2.96
	30	6/6	0/6	0/6	(16.12–20.11)		
	20	4/6	0/6				
	15	1/6	0/6				

[a] The data indicate: number of mice protected against MES seizures/number of mice tested.
[b] The data indicate: number of mice in which motor impairment was observed/number of mice tested.

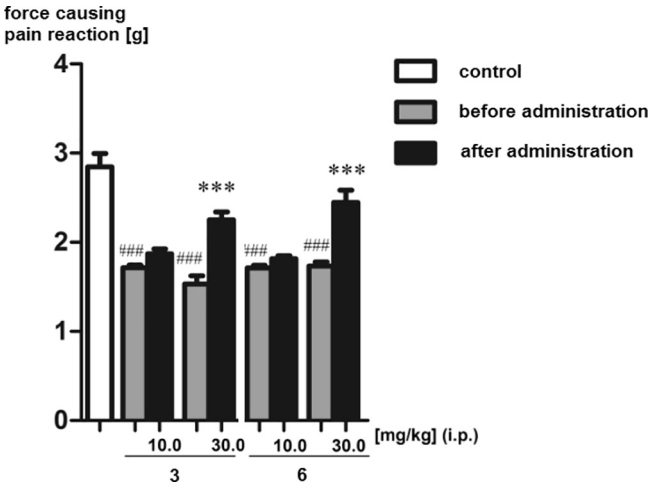


Fig. 2. Evaluation of mechanical nociceptive threshold with use of von Frey test for compounds **3** and **6** in STZ test. Results are presented as average force against the plantar surface of the hind paw of the mouse [g] (±SEM), causing pain reaction. Statistical analysis: one-way ANOVA and Tukey's *post hoc* test. Statistical significance compared to control group: $^{###}p < 0.001$ and compared to measurement before administration of compound: $^{***}p < 0.001$.

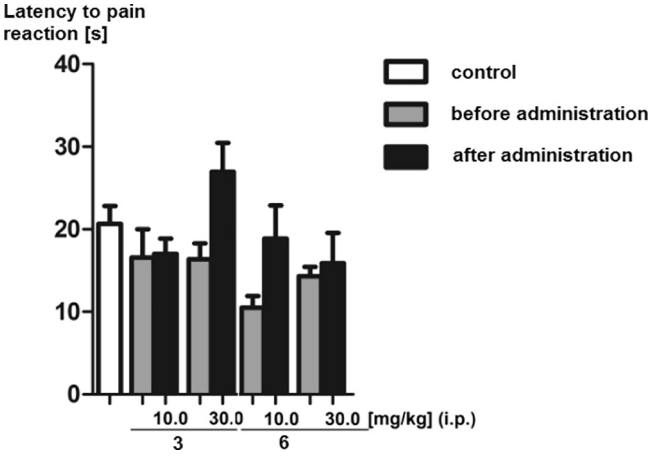


Fig. 3. Evaluation of thermal nociceptive threshold in diabetic mice – hot plate test for compounds **3** and **6** in STZ model. Results are presented as average latency of pain reaction [s] (±SEM). Statistical analysis: one-way ANOVA and Tukey's *post hoc* test.

noteworthy, as search for 5-HT_{1A} ligands devoid off-target α_1 affinity is an important scientific goal in terms of drug design.[50]

Compounds **1**, **4**, **7** and **9** possessed notable affinity towards receptors 5-HT_7, **4** and **7** – towards 5-HT_{2A}, while **1** and **7** – D_2. However, it is difficult to draw extensive conclusions regarding SAR.

Functional studies showed antagonistic properties of tested compounds in all performed evaluations, with the exception of compound **4** being weak agonist. As the affinity of this compound towards receptors D_2 is not very high (K_i = 299 nM), this activity might not be the crucial one for *in vivo* activity. Nevertheless, it worth noted that this is the only compound possessing chlorine as a substituent in the phenyl ring.

In terms of correlation of receptor profile and *in vivo* activity as well as SAR regarding pharmacological studies, the following observations can be made.

Compound **2** (2-(2-{4-[2-(mesityloxy)ethyl]piperazin-1-yl} ethoxy)ethanol dihydrochloride) exhibits no particular affinity towards the tested receptors, and still shows anxiolytic-like activity at 2.5 mg/kg (mice, *i.p.*) as well as anticonvulsant properties in MES test with ED_{50} = 45.42 mg/kg (mice, *i.p.*) at neurotoxicity in rotarod test with TD_{50} = 85.26 mg/kg (mice, *i.p.*). This compound

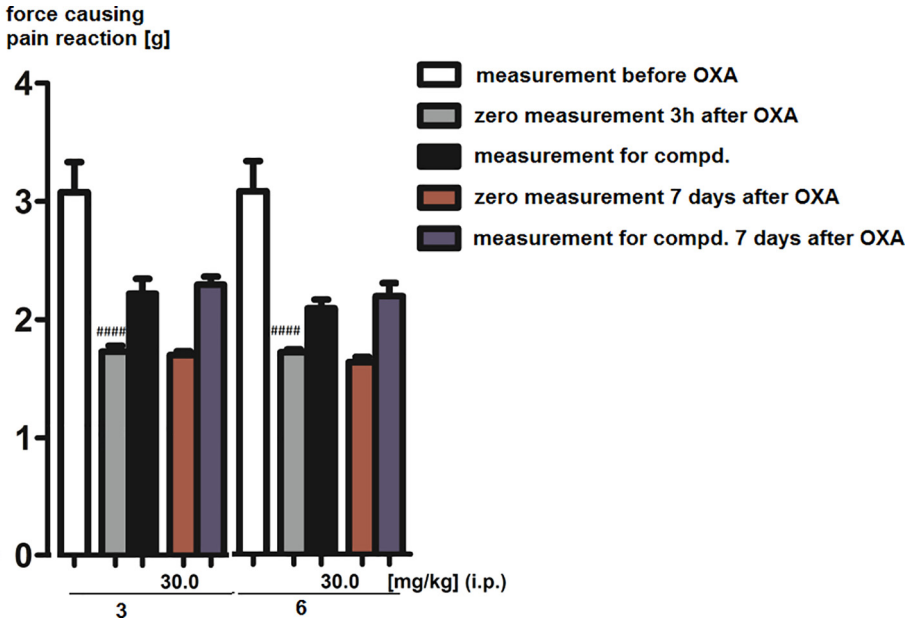


Fig. 4. Evaluation of mechanical nociceptive threshold with use of von Frey test for **3** and **6** in OXA test. Results are presented as average force against the plantar surface of the hind paw of the mouse [g] (±SEM), causing pain reaction. Statistical analysis: ANOVA with repeated measurements and Bonferroni's *post hoc* test. Statistical significance compared to control group: $^{###}p < 0.0001$.

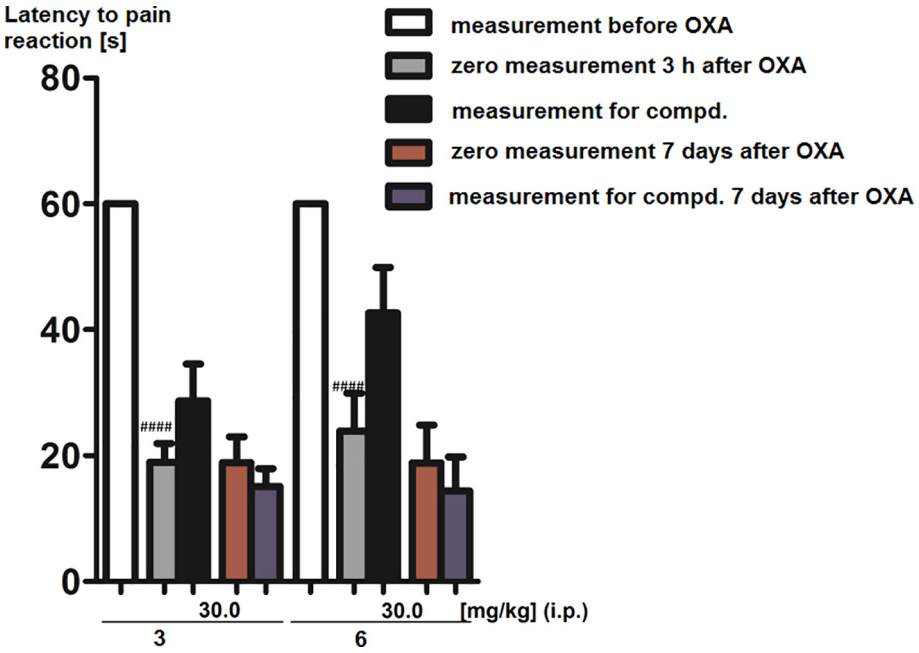


Fig. 5. Evaluation of thermal nociceptive threshold in oxaliplatin-treated mice – cold plate test for **3** and **6**. Results are presented as average latency of pain reaction [s] (±SEM). Statistical analysis: one-way ANOVA with repeated measurements and Bonferroni's *post hoc* test. Statistical significance compared to control group: $^{####}p < 0.0001$.

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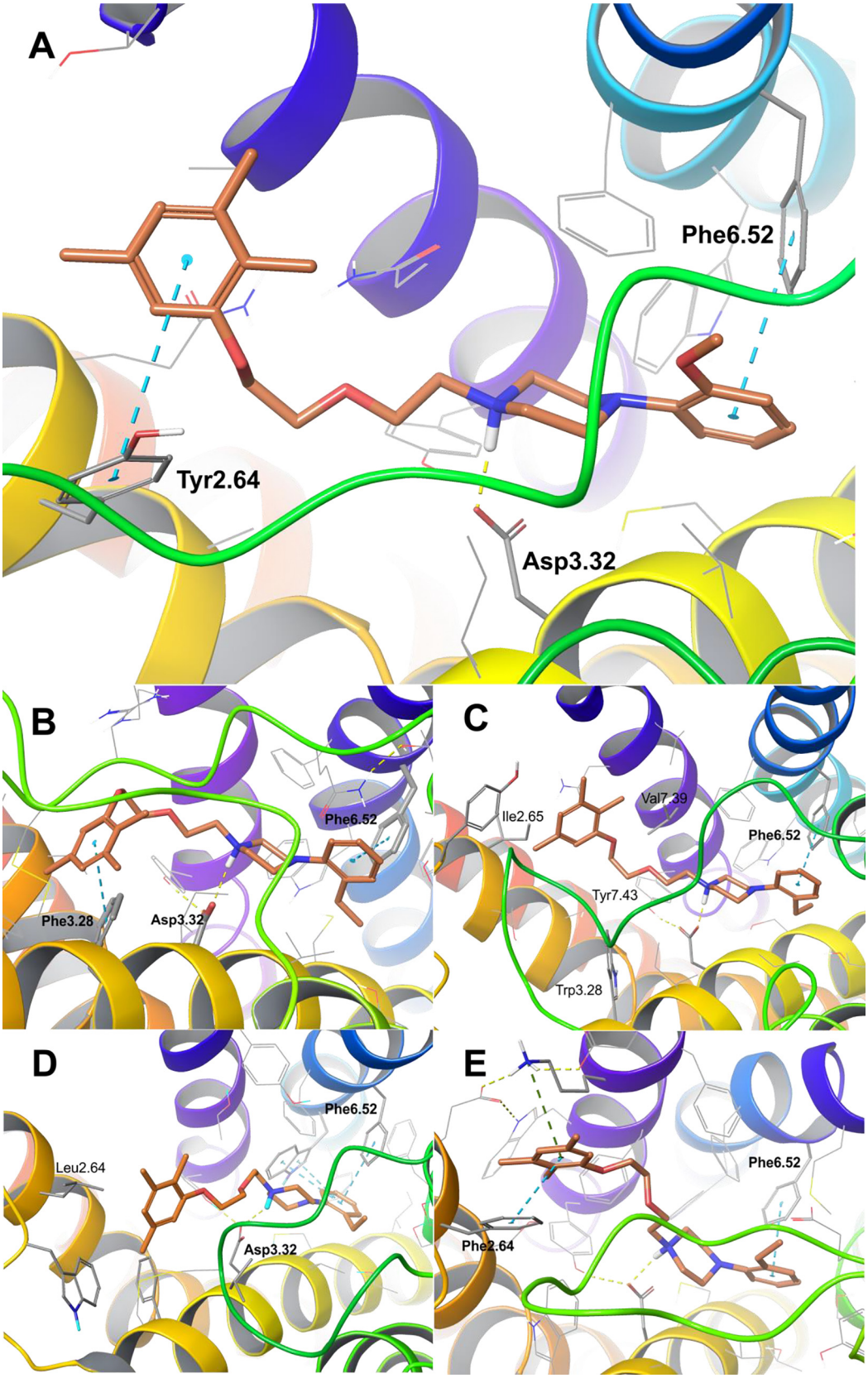

Fig. 6. Binding modes of compound **7** in the sites of serotonin 5-HT_{1A} (A), 5-HT_7 (B) and 5-HT_{2A} (C) receptors, as well as the dopamine D_2 and the adrenergic α_{1A} receptors. Amino acid residues engaged in ligand binding (within 4 Å from the ligand atoms) are displayed as sticks, whereas those forming H-bonds (dotted yellow lines), π–π stacking (dotted cyan lines) and cation-π stacking interactions (dotted green lines) are represented as thick sticks. For the sake of clarity, a part of TMH5 and extracellular loop 2 residues were undisplayed. TMH – transmembrane helix.

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is also a derivative which contains the same elements as compound **8**, but in different order. The structure of **2** with unsubstituted hydroxyl group of *N*-hydroxyethoxyethylpiperazine as in hydroxyzine is more advantageous in terms of pharmacological profile.

Compound **3** (1-[3-(2,4,6-trimethylphenoxy)propyl]-4-(4-methoxyphenyl)piperazine dihydrochloride) exhibits binding to 5-HT_{1A} receptors (K_i = 146 nM) and anticonvulsant properties (MES ED_{50} = 23.50 mg/kg at TD_{50} = 67.88 mg/kg). Due to the broad PI it was subject to evaluation of analgesic activity, where it proved active in mechanical allodynia at 30 mg/kg (STZ model). The analogy with reference compound **I** shows that incorporation of methyl substituent in position 4 of the phenyl ring and using the methoxy substituent not in position 2 but 4 in phenyl at piperazine, changes completely the biological properties of the obtained compound.

Incorporation of phenethylpiperazine as in compound **5** ((2,3-dimethylphenoxy)ethoxyethyl derivative) produced affinity to α_1 receptors, anticonvulsant activity, but not satisfying protection index (PI = 1.60) in the obtained compound. On the contrary, **11** as also a phenethylpiperazine derivative of (2,4,6-trimethylphenoxy)ethoxyethyl exhibits much broader protection index (PI = 2.96) at its anticonvulsant evaluation, disregard of not satisfying affinity towards the tested receptors.

Compound **6** (1-(2-hydroxyethyl)-4-{2-[2-(2,3,5-trimethylphenoxy)ethoxy]ethyl}piperazine dihydrochloride), similarly to compound **3**, exhibits anticonvulsant and analgesic activity, disregard of no particular affinity to any of the tested receptors or no similarity to **3**.

Compound **7** (1-(2-methoxyphenyl)-4-{2-[2-(2,3,5-trimethylphenoxy)ethoxy]ethyl}piperazine dihydrochloride) exhibits affinity towards 5-HT_{1A} (K_i = 32 nM), 5-HT_{2A} (K_i = 159 nM), 5-HT_7 (K_i = 89 nM), and D_2 (K_i = 162 nM). It also exhibits anxiolytic-like properties at 2.5 mg/kg (mice, *i.p.*).

Compound **9** (1-{2-[2-(mesityloxy)ethoxy]ethyl}-4-(2-methoxyphenyl)piperazine dihydrochloride) exhibits affinity towards 5-HT_{1A} (K_i = 5 nM), 5-HT_7 (K_i = 70 nM), D_2 (K_i=189 nM), α_1 (K_i = 15 nM). Its anticonvulsant activity is observed in MES test with ED_{50} = 26.33 mg/kg (mice, *i.p.*) at neurotoxicity observed by rotarod test with TD_{50} = 31.48 mg/kg (mice, *i.p.*).

It is worth noticing that compound **9** is a 2,4,6-trimethylphenoxy analog of compound **7** – a derivative of 2,3,5-trimethylphenol, and change of positions of methyl substituents modified the pharmacological profiles from anxiolytic-like (both derivatives) to anticonvulsant at elevated dose (2,4,6-trimethyl derivative **9** only).

Compound **9** is also the 4-methyl derivative of reference compound **III**. Its receptor profile is comparable, but **9** proved more neurotoxic *in vivo* since its TD_{50} in rotarod is much lower than TD_{50} for the reference **III**.

Discussion

Summarizing the presented results, it must be concluded that each of the obtained compounds present particular receptor profile and unique pharmacological output, although they share structural characteristics.

Tested compounds showed central activity regardless of their lipophilicity being optimal for CNS drugs.[16] This observation is consistent with our former findings and indicates that in case of this group of compounds logP values in the range 2–5 is enough to allow exhibiting activity within central nervous system in tests performed on mice, *i.p.*

Like the reference drugs described in the introductory part, some of the synthesized compounds (**2**–**3**, **6**, **9**) present more than one central activity. Compound **3** (1-[3-(2,4,6-trimethylphenoxy)propyl]-4-(4-methoxyphenyl)piperazine dihydrochloride) exhibited anxiolytic, anticonvulsant and antiallodynic activity in mice, *i.p.*, 30 min after administration (at 10 mg/kg, ED_{50} = 23.50 mg/kg, at 30 mg/kg, respectively). The observed coexistence of the three activities is consistent with assumptions described in the introduction, however, shows some new perspective on search for new drugs for the central nervous system diseases. Verification of other possible activities but the searched for in the process of drug discovery seems to be advantageous, due to possibility of finding other desirable properties and increase in knowledge of pharmacological output of a future drug candidate.

It can be concluded, that among presented compounds possessing affinity towards tested receptors, the possible main mechanism of action is serotonergic antagonism. However, other molecular targets, including those not evaluated within current study, might also be involved.

Five compounds (**2**, **6**, **8**, **10** and **11**) possessed poor pharmacological profile, but promising *in vivo* results, including anxiolytic, anticonvulsant and antiallodynic activities. For that reason, it seems reasonable to include other than serotonergic, dopaminergic and adrenergic molecular targets in our future research in this group of compounds, especially in case of derivatives which do not possess phenyl ring connected directly to piperazine moiety.

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The experimental protocol was approved by the First Local Ethics Committee on Animal Testing at the Jagiellonian University in Krakow (No 108/2014, 232/2015 and 233/2015) and was in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.04.059. These data include MOL files and InChiKeys of the most important compounds described in this article.

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