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# Synthesis of *N*-(phenoxyalkyl)-, *N*-{2-[2-(phenoxy)ethoxy] ethyl}- or *N*-(phenoxyacetyl)piperazine Derivatives and Their Activity Within the Central Nervous System

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Depression, anxiety and epilepsy share some etiology factors, causing frequently observed multimodal activity of centrally active compounds. This might raise the risk of central adverse effects of potential drugs, but on the other hand - in a light of common comorbidity of these diseases - also make an opportunity for avoiding polypragmasy. The presented study combines rational drug design methods, chemical synthesis, receptor studies and *in vivo* pharmacological screening (mice, *i.p.*) in order to obtain new centrally active piperazine derivatives in a context of their potential multimodality, investigate the mechanism of their activity and establish relationship between their structure, molecular mechanism and *in vivo* central activity(-ies). The most promising pharmacological profile showed 1-(2-(2,5-dimethylphenoxy)ethyl)-4-phenylpiperazine dihydrochloride (**1**), which was active in the four-plate test (anxiolytic-like activity) at 1.25 mg/kg b.w. and possessed high affinities towards several tested molecular targets (5-$HT_{1A}$ $K_i = 35$ nM - weak antagonist, 5-$HT_{2A}$ $K_i = 121$ nM, 5-$HT_7$ $K_i = 130$ nM - weak antagonist, $\alpha_1$ $K_i = 82$ nM, $\mu$ $K_i = 240$ nM).

## Introduction

Central nervous system disorders such as depression, anxiety and epilepsy are important health and social challenges. Despite the availability of many centrally active drugs on the market, some patients do not receive satisfactory health improvement. Drug-resistant epilepsy and therapy-resistant depression, which concern about 20%-40% of epileptic patients and 12%-20% of depressed patients, respectively, may serve as an example.[1,2] Genome variability (polymorphisms leading to modification of drug metabolism, drug targets or drug transporters), disease-related mechanisms (alterations in drug target (s) or drug uptake into the brain, structural brain alterations) and drug-related mechanisms (functional tolerance, induction of drug-metabolizing enzymes/drug transporters, ineffective mechanism of drug activity) are considered crucial reasons of observed drug-resistance in brain diseases.[3] Poor response of large amount of patients to treatment serves as a premise for

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search for new centrally active compounds targeting various molecular targets.

The disorders mentioned above are sometimes comorbid. The rate of depressive symptoms in drug-resistant epilepsy patients is about 23%[4] and the prevalence of anxiety within population suffering from epilepsy is estimated as high as 11–25% in Canada, US and UK. Depression and anxiety share negative affective symptoms and it is often difficult to evaluate them separately in clinical practice[5] – anxiety may be considered a risk marker for major depressive disorder onset and risk factor for its persistence.[6]

The coexistence and common etiology of epilepsy, depression and anxiety include factors such as trauma, intoxication or neurodegeneration.[7,8] On the molecular level, the classical hypothesis on pathophysiology of depression and anxiety correlates these disorders with impaired monoamine signaling, particularly serotonergic/adrenergic/dopaminergic and GABAergic/serotonergic transmission in case of depression and anxiety, respectively.[5] However, recently in case of depression other mediators are mentioned in this context, e.g. glutamate, GABA, BDNF, allopregnanolone, neuropeptides, thyroid hormone, corticosteroids, sexual hormones and cannabinoids.[9] The involvement of many of these pathways has been also proved for pathophysiology of epilepsy.[10,11] These findings are supported by the observed diverse therapeutic or adverse effects of drugs targeting monoamine pathways within central nervous system depending on the dose. It is known that anticonvulsants such as carbamazepine or valproates improve mood, but on the other hand, epilepsy can be caused by some antidepressants, e.g. maprotyline and clomipramine.[7,12,13] Such multidirectional activity of centrally active compounds should be taken into account in the process of drug design in order to prevent unexpected off-target activities and adverse effects, as well as reduce polypragmasy while targeting comorbid diseases.

Our research team achieved satisfying results in terms of design and synthesis of new compounds for antidepressant-like, anticonvulsant and anxiolytic-like activity in a group of phenoxyalkyl and phenoxyethoxyethyl derivatives of piperazine (particularly *N*-(2-methoxyphenyl)piperazine),[14–18] as well as derivatives of aminoalkanols.[19,20] In case of piperazine derivatives our preliminary research has focused on the potential antidepressant-like activity of new compounds, their potential anxiolytic-like or anticonvulsant activities were usually tested only for selected compounds. These studies resulted in piperazine derivatives possessing antidepressant-like activity (e.g. compound **IV** in Figure 1), but also multidirectional activity within central nervous system (e.g. antidepressant-like and anxiolytic-like activities of compounds **I**, **III** in Figure 1). The results encouraged us to include evaluation of more than one central activity *in vivo* within preliminary activity screening of synthesized compounds, including antidepressant-like, anxiolytic-like, as well as anticonvulsant and analgesic (for selected compounds). Recent studies on di- and trisubstituted phenoxyalkyl- and phenoxyethoxyethyl derivatives of piperazine[21] resulted in several active compounds, among others reference compound **VI**, possessing anxiolytic-like, anticonvulsant and

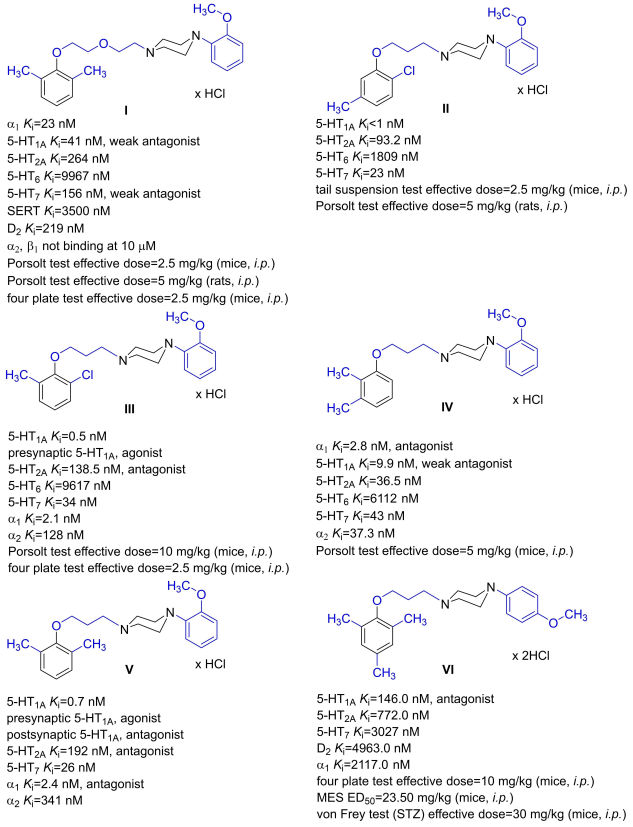


**Figure 1.** Chemical structures of reference compounds I,[14,15] II,[14,23] III–V,[16,17,23] VI.[21] Sites of possible modifications resulting in the title compounds are marked in blue.

antiallodynic activities (Figure 1). Interestingly, the anxiolytic-like activity occurred to be the most frequently observed within tested compounds, not the expected antidepressant-like one.

The molecular mechanism lying behind the observed *in vivo* results remains another challenge. The majority of compounds obtained in our laboratory so far, possessed affinity towards serotonergic (mainly 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_7$), dopaminergic $D_2$ and adrenergic $\alpha_1$ receptors; some of them were classified as antagonists of these receptors in functional studies (see reference compounds **I–VI**, Figure 1). Previously, serotonergic mechanism of central activity observed *in vivo* seemed to be the most probable and we have drawn preliminary conclusions on structure-*in vitro* profile relationship, including docking studies of the most active compounds.[21,22] However, with increasing number of tested compounds the relationship between favorable receptor profile and *in vivo* activity became less clear. Active compounds binding to 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_7$, $D_2$ and $\alpha_1$ receptors (e.g. reference compounds **I–IV** and **VI**, Figure 1) have been obtained in parallel to compounds with similarly advantageous receptor profile lacking expected pharmacological activity (e.g. compound **V** in Figure 1, tested for antidepressant-like activity). The research resulted in a conclusion that mechanisms other than investigated ones may be involved in case of this group of compounds. For that reason, the design of new derivatives basing mainly on


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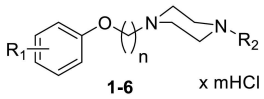
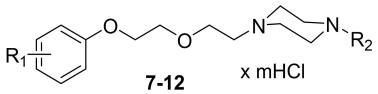
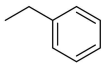
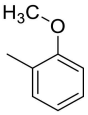
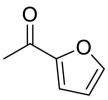
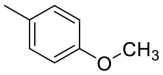
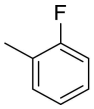
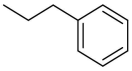
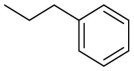
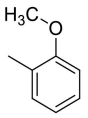
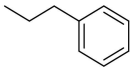
**Table 1.** Structures of the title compounds **1–16.**

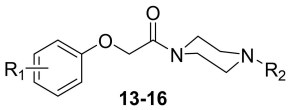


**1-6** x mHCl

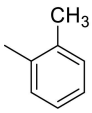
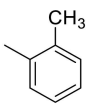


**7-12** x mHCl

| Compd. | $R_1$ | n | $R_2$ | m | Compd. | $R_1$ | $R_2$ | m |
|---|---|---|---|---|---|---|---|---|
| 1 | 2,5-$(CH_3)_2$ | 2 |  | 2 | 7 | 2,5-$(CH_3)_2$ |  | 2 |
| 2 | 2,5-$(CH_3)_2$ | 2 |  | 1 | 8 | 2,5-$(CH_3)_2$ |  | 1 |
| 3 | 2,5-$(CH_3)_2$ | 3 |  | 2 | 9 | 2,6-$(CH_3)_2$ |  | 2 |
| 4 | 2,5-$(CH_3)_2$ | 4 |  | 2 | 10 | 2,6-$(CH_3)_2$ |  | 1 |
| 5 | 2,6-$(CH_3)_2$ | 4 |  | 2 | 11 | 2,6-$(CH_3)_2$ |  | 1 |
| 6 | 2-Cl-6-$CH_3$ | 4 |  | 1 | 12 | 2-Cl-6-$CH_3$ |  | 2 |



**13-16**

| Compd. | $R_1$ | $R_2$ | Compd. | $R_1$ | $R_2$ |
|---|---|---|---|---|---|
| 13 | 2,5-$(CH_3)_2$ |  | 15 | 2-Cl-6-$CH_3$ |  |
| 14 | 2,5-$(CH_3)_2$ |  | 16 | 2-Cl-6-$CH_3$ |  |

Calculations performed with the use of Molinspiration online toolkit for base forms.[29]

structure-*in vivo* activity analysis of previously tested compounds may be advantageous, as well as performing *in vitro* studies together with *in vivo* evaluations for all synthesized derivatives. Such an approach would allow analysis of structure-*in vitro* profile, structure-*in vivo* activity and *in vitro* profile-*in vivo* activity relationships.

Within current study, we aimed to: a) rationally design new compounds potentially possessing anxiolytic-like, antidepressant-like and/or anticonvulsant activity; b) explore the mechanism of their activity, going beyond previous hypotheses; c) analyze and determine correlation between potentially coexisting central activities; d) investigate safety profile of tested compounds, with a view of evaluating this group of compounds as potential drug candidates.

## Results and Discussion

### Chemistry

Sixteen derivatives of *N*-(phenoxyalkyl)- (**1-6**), *N*-[(phenoxy) ethoxyethyl]- (**7-12**) and *N*-(phenoxyacetyl)piperazine (**13-16**) have been designed and synthesized. The structure of compound **2** was subject to patent protection,[23] the structure of **5** (monohydrochloride) has been published elsewhere,[24] without the pharmacological activity described in this paper, and compounds **13**, **14** possess CAS numbers (however their chemical and pharmacological characterization have never been published).

The designed compounds (Table 1) are analogs or homologs of reference compounds (Figure 1) disubstituted in the phenyl ring with two methyl groups or methyl group and chlorine in positions 2,5 or 2,6. A prerequisite for such a choice of substituents ware favorable properties of 2,5- and 2,6-disubstituted derivatives extensively studied by our group (e.g. reference compounds **I–III**, **V** in Figure 1).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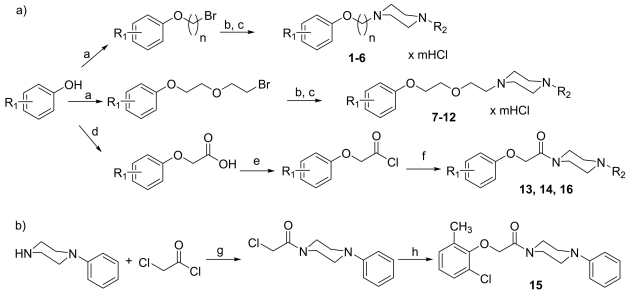
The linker between phenoxyl group and piperazine varies in length and structure (alkyl chain for compounds **1–6**, ethoxyethyl for **7–12** and acetyl for **13–16**). Design of amide derivatives **13–16** resulted from analysis of literature, where centrally active amide derivatives of *N*-phenylpiperazine are widely described,[25,26] as well as our former experience with some phenoxyalkyl derivatives of aminoalkanols showing anticonvulsant activity, where incorporation of amide moiety resulted in reduced *in vitro* cytotoxicity and *in vivo* neurotoxicity (rotarod).[27,28]

Amine components differ in aryl substituent of piperazine. Our previous research covered mostly derivatives of *N*-(2-methoxyphenyl)piperazine,[14–18] however, during our ongoing studies we aim to further explore the importance of piperazine substitution on *in vivo* activity.[21] In current paper, we introduce various aryl substituents, including phenyl, 2-(methoxy)phenyl, 2-(fluoro)phenyl, *o*-tolyl, 4-(methoxy)phenyl, benzyl, phenethyl, pyridin-2-yl or 2-furoyl.

The design process has been supported by calculation of physicochemical parameters by means of Molinspiration online toolkit[29] (all calculated values available in Supporting Information, Table S1). None of the compounds exhibited more than one violation from the Lipinski rule of five,[30] making them potentially promising drug-like agents. The most beneficial value of topological polar surface area (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 the rules for both oral and CNS drugs (calculated values are in the range 15.71-37.84 Å$^2$). The molecular volumes of presented compounds range 313.10-389.29 Å$^3$.

Synthesis of the title compounds is presented in Scheme 1. Compounds **1–12** were obtained by *N*-alkylation of *N*-substituted piperazine using respectively substituted phenoxyethyl (**1**, **2**), phenoxypropyl (**3**), phenoxybutyl (**4-6**), or phenoxyethoxyethyl (**7-12**) bromides. The reaction was performed in presence of $K_2CO_3$ in toluene. The yield of *N*-alkylation was in the range of 50–75%. Appropriate phenoxyalkyl and phenoxyethoxyethyl bromides were achieved according to formerly published methods (described in Supporting Information, Experimental Section), and crude products were used for further alkylation. All amines received as oily products were converted into hydrochlorides upon treatment with excess of EtOH solution of gaseous HCl. The raw hydrochlorides were recrystallized from the mixture of acetone/EtOH 1:1 (v/v).




**Scheme 1.** Synthesis of the title compounds. a: 1. Cl-$(CH_2)_n$-OH for **1–6** or Cl-$(CH_2)_2$-O-$(CH_2)_2$-OH for **7–12**, $K_2CO_3$, acetone/EtOH, 2. $PBr_3$; b: piperazine-$R_2$, $K_2CO_3$, toluene; c: gaseous HCl, EtOH; d: $C_2H_5ONa$, Cl–$CH_2$-COOH; e: $SOCl_2$; f: piperazine-$R_2$, toluene, $K_2CO_3$; g: Et-O–Et, TEA; h: 2-chloro-6-methylphenol, acetone, $K_2CO_3$; $R_1$, $R_2$, n, m as in Table 1.

For synthesis of amide derivatives **13**, **14** and **16**, appropriately substituted phenoxyacetic chlorides were obtained according to previously published procedure.[31,32] In the next step chlorides were used as acylating agents in the reaction with appropriate amines in order to achieve final compounds (reaction carried out in toluene, in the presence of $K_2CO_3$ as a proton acceptor). Compound **15** has been synthesized *via* alternative two-step (instead of a three-step) synthetic pathway, involving acylation of phenylpiperazine with chloroacetic acid chloride and subsequent reaction of the product with 2-chloro-6-methylphenol. The yield of the final *N*-alkylation was 70%. However, it was difficult to scale-up the first step of synthesis (acylation of phenyl piperazine) and in consequence, the standard protocol used in our laboratory seems to remain the most efficient. Final compounds were purified by crystallization from organic solvents (hexane, heptane, toluene).

## Pharmacology

### *Receptor binding studies*

Compounds were subject to receptor binding preliminary screening (Table 2) successively, in the order of their synthesis. Basing on literature review[10] and our former research,[22] evaluations covered receptors 5-$HT_{1A}$, 5-$HT_{2A}$, 5-$HT_6$, 5-$HT_7$, $D_2$ and $\alpha_1$. Compounds **1**, **2**, **5**, **6**, **9** and **11** showed promising receptor profiles, where $K_i$ are in nanomolar range. Most compounds exhibited affinity towards receptors 5-$HT_{1A}$ and compounds which lacked this type of activity possessed no affinity towards other types of tested receptors. For that reason, compounds **3**, **8** and **13–16** were tested for potential affinity towards receptors 5-$HT_{1A}$ only.

In order to check the impact of other molecular targets, which we have not taken into account so far, we chose compound **1** (the one characterized by advantageous receptor profile basing on preliminary screening and the one most active *in vivo)* for extensive binding studies involving a whole panel of receptors. The assay has been performed at Eurofins CEREP SA. Percent inhibition of control specific binding for compound **1** was $>80$% for receptors: $\alpha_1$, $\alpha_2$, $D_1$, $D_{2S}$, μ-opioid (MOP) ($K_i=240$ nM, Table 2), 5-$HT_{1A}$ ($K_i=34$ nM), 5-$HT_{2A}$ ($K_i=150$ nM), 5-$HT_{2B}$, 5-$HT_7$ ($K_i=390$ nM). Results obtained for receptors $\alpha_1$, $D_2$, 5-$HT_{1A}$, 5-$HT_{2A}$ and 5-$HT_7$ were consistent with those obtained before within preliminary assays. However, the study proved that compound **1** is not selective for receptors $\alpha_1$ and $D_2$, but binds also to receptors $\alpha_2$ and $D_1$. It also enabled identification of additional important targets, such as 5-$HT_{2B}$ and μ-opioid receptor. The results of binding panel for compound **1** including all tested molecular targets are presented in Supporting Information (Figure S3).


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**Table 2.** Binding results for the title compounds **1–16** and reference compounds.

| Compd. | $K_i$ (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 | $\alpha_1$ [$^3$H] - prazosin | $\mu^{[a]}$ [$^3$H] - DAMGO |
|---|---|---|---|---|---|---|---|
| 1 | 35.0 ± 2.5 | 121.0 ± 6.2 | 2722.0 ± 82.5 | 130.0 ± 3.5 | 933.0 ± 61.7 | 82.0 ± 3.8 | 240 |
| 2 | 5.0 ± 0.5 | 355.0 ± 19.5 | 2035.0 ± 122.0 | 40.0 ± 3.5 | 162.0 ± 7.9 | 7.0 ± 0.7 | \| |
| 3 | 28.0 ± 2.0 | \| | \| | \| | \| | \| | \| |
| 4 | - | 812.0 ± 10.0 | 336.0 ± 19.5 | 659.0 ± 13.0 | 1697.0 ± 17.5 | 344.0 ± 11.5 | \| |
| 5 | 18.0 ± 1.3 | 217.0 ± 6.5 | 4805.0 ± 46.0 | 20.0 ± 0.5 | 425.0 ± 3.0 | 45.0 ± 1.0 | \| |
| 6 | 2.0 ± 0.2 | 314.0 ± 25.5 | 3190.0 ± 143.0 | 18.0 ± 2.0 | 40.0 ± 2.2 | 5.0 ± 0.3 | \| |
| 7 | 596.0 ± 7.0 | 2006.0 ± 288.0 | 4734.0 ± 229.0 | 905.0 ± 12.0 | - | 2663.0 ± 275.0 | \| |
| 8 | 380.0 ± 35.0 | \| | \| | \| | \| | \| | \| |
| 9 | 36.0 ± 3.2 | 836.0 ± 47.5 | 3424.0 ± 57.0 | 244.0 ± 4.9 | 306.0 ± 33.0 | 47.0 ± 4.0 | \| |
| 10 | - | 969.0 ± 73.0 | 1645.0 ± 79.0 | 1055.0 ± 74.0 | - | 670.0 ± 15.5 | \| |
| 11 | 88.0 ± 3.0 | 1385.0 ± 46.5 | > 5000 | 450.0 ± 6.4 | 1953.0 ± 53.5 | 391.0 ± 38.8 | \| |
| 12 | - | 1716.0 ± 133.0 | 745.0 ± 55.0 | 448.0 ± 30.0 | - | 538.0 ± 12.5 | \| |
| 13 | - | \| | \| | \| | \| | \| | \| |
| 14 | - | \| | \| | \| | \| | \| | \| |
| 15 | - | \| | \| | \| | \| | \| | \| |
| 16 | - | \| | \| | \| | \| | \| | \| |
| 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 | \| |
| DAMGO | \| | \| | \| | \| | \| | \| | 0.36 |

- no binding at $10^{-5}$ M; | no data; [a] results obtained within receptor binding panel performed by Eurofins CEREP SA for compound **1**.

Upon completion of receptor profile, functional assays were performed for receptors 5-$HT_{1A}$ (compounds **1**, **2**, **5**, **6**, **7**, **9** and **11**), 5-$HT_{2A}$ (compounds **1**, **2**, **5**, **6**, and **9**), as well as 5-$HT_7$ and $D_2$ (compounds **1**, **2**, **6** and **9**). The results showed antagonistic properties for most compounds possessing high affinity towards particular receptors, e.g. receptors 5-$HT_{1A}$ – compounds **1**, **2**, **5**, **6**, **9**, 5-$HT_7$ - compounds **1**, **2**, **6**, $D_2$ – compounds **2** and **6** ($E_{max}$ values are presented in Supporting Information, Tables S4-S7).

Analysis of the results obtained for new and reference compounds (Figure 1) allowed us to draw conclusions on the influence of amine moiety on the receptor profile. 2-methoxy substituent in the phenyl ring seems to positively influence the receptor profile, as compound **1** (desmethoxy analog of **2**) exhibited weaker affinities toward all the tested receptors. However, in most cases they were still in nanomolar range. All compounds possessing aryl ring separated from piperazine moiety by 1 or 2 carbon atoms (**4**, **7**, **10**, **12**) exhibited poor receptor profile. It indicates that such a modification may be unfavorable, similarly to the presence of pyridinylpiperazine scaffold, as compound **11** showed weaker affinities than its 2-fluorophenyl (**9**) and 2-methoxyphenyl (reference compound **I**) analogs.

The influence of phenyl ring substitution and the structure of linker is less clear. We observed that compounds **5** and **6** possessed better receptor profiles than compounds **1** and **2**, respectively. It might be caused by the substitution of the phenyl ring in positions 2 and 6 (instead of 2 and 5) or elongation of the linker (from 2 to 4 carbon atoms). Amide derivatives **13–16** (compounds with acetyl linker) did not bind to 5-$HT_{1A}$ receptors at $10^{-5}$ M.

### *In vivo* pharmacological studies

All compounds were screened for potential anxiolytic-like activity (the four-plate test in mice, *i.p.*) and compounds **1**, **2**, **4–7** and **9–12** also for antidepressant-like properties (the forced swim test in mice, *i.p.*). The results are presented in Table 3 and Table 4, respectively. For active compounds, we analyzed their influence on locomotor activity (mice, *i.p.*) in order to exclude the amphetamine-like effect. The results confirmed that the observed pharmacological activity was specific (experimental data are presented in Supporting Information, Table S8).

Compounds **1**, **2** and **9** exhibited anxiolytic-like properties. The strongest effect was demonstrated by compound **1** (anxiolytic-like activity comparable to that of clorazepate). Compounds **5** and **6** possessed potentially beneficial receptor profile and contained the same amine moieties as active compounds **1** and **2** and compound **6** is additionally the butyl homolog of **III**, whose four-plate test effective dose is equal to 2.5 mg/kg. However, both compounds **5** and **6** lacked the expected anxiolytic-like activity. Also amide derivatives showed no preferential pharmacological properties (compound **13** escalated anxiety in mice at the dose 2.5 mg/kg).

Only compound **4** (1-[4-(2,5-dimethylphenoxy)butyl]-4-phenethylpiperazine dihydrochloride) produced specific antidepressant-like effect in a dose 20 mg/kg, however its activity was not as strong as the effect of fluoxetine. It is noteworthy, that this compound showed weak affinity towards receptors tested in the receptor binding assay, so the mechanism of its antidepressant-like activity is likely to be sought elsewhere.

Compounds active in the four-plate test or forced swim test (**1**, **2**, **4** and **9**) and compound **6** were subject to anticonvulsant screening in maximum electroshock test (mice, *i.p.*) (Table 5).


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**Table 3.** Anxiolytic-like activity of **1–16** in mice, *i.p.*

| Compd. | Four-plate test (anxiolytic-like activity) Dose (mg/kg) | Number of punished crossings ± SEM (s) | F statistic (degrees of freedom) |
|---|---|---|---|
| Vehicle | - | 2.8 ± 0.4 | |
| **1** | 0.625 | 3.1 ± 0.2 | F(4,35) = 15.61, |
| | 1.25 | 4.8 ± 0.5** | p < 0.0001 |
| | 2.5 | 5.5 ± 0.3**** | |
| | 5 | 6.1 ± 0.4**** | |
| Vehicle | - | 3.1 ± 0.4 | |
| **2** | 1.25 | 4.1 ± 0.2 | F(3,36) = 3.331, |
| | 2.5 | 4.6 ± 0.4* | p < 0.05 |
| | 5 | 4.5 ± 0.4* | |
| Vehicle | - | 4.8 ± 0.5 | |
| **3** | 2.5 | 4.8 ± 0.6 | F(2,21) = 0.020, |
| | 5 | 4.6 ± 0.4 | p = 0.9798 |
| Vehicle | - | 3.0 ± 0.5 | |
| **4** | 5 | 2.9 ± 0.1 | F(2,21) = 1.239, ns |
| | 10 | 3.8 ± 0.5 | |
| Vehicle | - | 3.6 ± 0.3 | |
| **5** | 5 | 3.6 ± 0.5 | F(2,21) = 0.556, ns |
| | 10 | 2.9 ± 0.8 | |
| Vehicle | - | 3.1 ± 0.5 | |
| **6** | 5 | 2.4 ± 0.5 | F(2,21) = 1.020, ns |
| | 10 | 3.4 ± 0.5 | |
| Vehicle | - | 3.4 ± 0.4 | |
| **7** | 5 | 3.4 ± 0.5 | F(2,21) = 2.807, ns |
| | 10 | 2.3 ± 0.3 | |
| Vehicle | - | 4.8 ± 0.5 | |
| **8** | 2.5 | 5.3 ± 0.9 | t(14) = 0.505, |
| | | | p = 0.6217 |
| Vehicle | - | 2.6 ± 0.3 | |
| **9** | 2.5 | 4.1 ± 0.6 | F(2,21) = 4.078, |
| | 5 | 4.6 ± 0.5* | p < 0.05 |
| Vehicle | - | 3.5 ± 0.5 | |
| **10** | 5 | 2.8 ± 0.3 | F(2,21) = 2.358, ns |
| | 10 | 2.4 ± 0.3 | |
| Vehicle | - | 3.8 ± 0.3 | |
| **11** | 5 | 4.6 ± 0.4 | F(2,21) = 1.241, ns |
| | 10 | 4.0 ± 0.5 | |
| Vehicle | - | 3.9 ± 0.4 | |
| **12** | 5 | 4.4 ± 0.4 | F(2,21) = 0.769, ns |
| | 10 | 3.4 ± 0.8 | |
| Vehicle | - | 4.8 ± 0.5 | |
| **13** | 2.5 | 2.8 ± 0.4** | t(14) = 3.266, |
| | | | p = 0.0056 |
| Vehicle | - | 4.8 ± 0.5 | |
| **14** | 2.5 | 4.3 ± 0.5 | t(14) = 0.695, |
| | | | p = 0.4985 |
| Vehicle | - | 4.8 ± 0.5 | |
| **15** | 2.5 | 5.1 ± 0.6 | F(2,21) = 1.288, |
| | 5 | 3.8 ± 0.7 | p = 0.2966 |
| Vehicle | - | 4.8 ± 0.5 | |
| **16** | 2.5 | 3.1 ± 0.7 | t(14) = 1.963, |
| | | | p = 0.0698 |
| Vehicle | - | 3.0 ± 0.3 | |
| Clorazepate | 0.625 | 3.3 ± 0.3 | F(2,21) = 5.574, |
| | 1.25 | 4.8 ± 0.5* | p < 0.0001 |
| Vehicle[33] | - | 3.6 ± 0.4 | |
| Buspirone[33] | 1.25 | 5.0 ± 0.6 | F(3,36) = 5.660, |
| | 2.5 | 5.7 ± 0.4 | p < 0.01 |
| | 5 | 6.4 ± 0.6 | |

All studied compounds and clorazepate 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: unpaired t test if two groups were analysed or one-way ANOVA (Newman-Keuls *post hoc*) for two or more groups; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 *vs.* respective vehicle-treated group, ns – not significant

**Table 4.** Antidepressant-like activity of **1**, **2**, **4–7** and **9–12** in mice, *i.p.*

| Compd. | Forced swim test (antidepressant-like activity) Dose (mg/kg) | Immobility time ± SEM (s) | F statistic (degrees of freedom) |
|---|---|---|---|
| Vehicle | - | 196.1 ± 6.3 | |
| **1** | 5 | 181.6 ± 12.8 | F(3,28) = 0.8911, ns |
| | 10 | 168.3 ± 17.0 | |
| | 20 | 186.3 ± 10.5 | |
| Vehicle | - | 166.9 ± 20.2 | |
| **2** | 5 | 140.3 ± 13.3 | F(3,36) = 0.4906, ns |
| | 10 | 156.9 ± 14.2 | |
| | 20 | 158.4 ± 14.9 | |
| Vehicle | - | 152.5 ± 6.4 | |
| **4** | 5 | 164.4 ± 11.8 | F(3,28) = 5.405, p < 0.01 |
| | 10 | 145.9 ± 13.1 | |
| | 20 | 103.8 ± 12.8* | |
| Vehicle | - | 178.4 ± 14.8 | |
| **5** | 5 | 212.6 ± 12.8 | F(3,28) = 1.777, ns |
| | 10 | 173.1 ± 17.4 | |
| | 20 | 152.9 ± 26.5 | |
| Vehicle | - | 152.4 ± 15.7 | |
| **6** | 5 | 128.9 ± 13.9 | F(3,28) = 3.270, ns |
| | 10 | 161.1 ± 12.4 | |
| | 20 | 193.4 ± 16.7 | |
| Vehicle | - | 147.9 ± 10.7 | |
| **7** | 5 | 163.3 ± 13.0 | F(3,28) = 0.167, ns |
| | 10 | 163.4 ± 25.2 | |
| | 20 | 157.4 ± 18.8 | |
| Vehicle | - | 179.0 ± 16.3 | |
| **9** | 5 | 178.1 ± 12.9 | F(3,28) = 0.4560, ns |
| | 10 | 170.5 ± 14.3 | |
| | 20 | 191.8 ± 6.6 | |
| Vehicle | - | 184.4 ± 7.8 | |
| **10** | 5 | 176.6 ± 11.7 | F(3,28) = 1.802, ns |
| | 10 | 170.5 ± 10.1 | |
| | 20 | 150.8 ± 12.6 | |
| Vehicle | - | 165.5 ± 16.3 | |
| **11** | 5 | 153.0 ± 27.2 | F(3,28) = 0.0662, ns |
| | 10 | 154.3 ± 23.4 | |
| | 20 | 160.4 ± 21.8 | |
| Vehicle | - | 182.6 ± 9.5 | |
| **12** | 5 | 192.6 ± 11.6 | F(3,28) = 0.492, ns |
| | 10 | 178.0 ± 8.5 | |
| | 20 | 174.5 ± 14.3 | |
| Vehicle[34] | - | 178.4 ± 9.1 | |
| Fluoxetine[34] | 10 | 152.1 ± 12.9 | F(2,27) = 11.65, |
| | 15 | 106.6 ± 9.8*** | p < 0.001 |

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: unpaired t test if two groups were analysed or one-way ANOVA (Newman-Keuls *post hoc*) for two or more groups; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 *vs.* respective vehicle-treated group, ns – not significant

Only compound **4** exhibited activity in this test, however it also showed high toxicity in rotarod.

Basing on pharmacological profile of tested compounds, it can be concluded, that anxiolytic-like activity is probably a result of serotonergic and adrenergic modulation, which is consistent with our previous findings. However, affinity of compound **1** towards μ receptors suggest that this receptor might be also important for activity and it should be included in preliminary screening in future studies. There is evidence for possible involvement of μ receptors in anxiety modulation.[35]


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**Table 5.** Anticonvulsant activity of compounds **1**, **2**, **4**, **6** and **9** (maximum electroshock test, mice, *i.p.*).

| Compd. | Dose (mg/kg) | MES [a] | Deaths | NT (10 rpm) [b] |
|---|---|---|---|---|
| Control | - | 0/6 | 4/6 | - |
| 1 | 100 | 0/6 | 3/6 | 3/6 |
| 2 | 30 | 0/6 | 5/6 | 1/6 |
| 4 | 50 | 4/4 | 0/4 | 4/4 |
| | 10 | 1/6 | - | 0/6 |
| 6 | 30 | 0/6 | 1/6 | 1/6 |
| 9 | 30 | 0/6 | 4/6 | 2/6 |

All studied compounds were administered *i.p.* 30 min. before the test. [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.

Immuno-cytochemical studies showed that GABA neurons in the CNS express μ-opioid receptor, especially the hippocampal neurons of rats, and studies *in vivo* on mice suggest that μ receptor modulation influences anxiolytic behavior regulated by GABA-mediated synaptic transmission.[36] Their blockade in the rats ventrolateral periaqueductal grey matter retards fear extinction.[37] Additionally, it has been proven that deletion of μ-receptor in knock-out mice blocks the anxiolytic-like and locomotor stimulant effects of low to moderate doses of ethanol.[38]

Antidepressant-like and anticonvulsant activities seem to be non-related to serotonergic/adrenergic or opioid transmission, although we previously obtained serotonergic and adrenergic receptors ligands among piperazine derivatives active in forced-swim test and/or MES test (Figure 1). Probably, they activity was a result of mechanisms which were not tested.

## Metabolic stability

### *In vitro studies*

Metabolism of compounds **1**, **2**, and **6** was studied *in vitro* in mouse liver microsomes (MLMs). Compounds **1** and **2** were chosen due to their activity observed *in vivo,* for determining the possible involvement of metabolites in pharmacological activity and/or toxicity. Compound **6** possessed interesting receptor profile, however no *in vivo* activity. Metabolic stability evaluation would uncover, if such results might be due to low stability of compound **6**.

Six metabolites (**M1**-**M6**) of **2** and three metabolites (**M1**-**M3**) of **1** and **6** were identified in compounds treated MLMs (Table 6). On the basis of the LC–MS/MS analysis, it can be assumed that major metabolites of all tested compounds resulted from hydroxylation of methyl substituent of the phenyl ring. The exemplary ions fragmentation (MS/MS) spectra for major metabolites (**M1**) of compounds **1**, **2** and **6** are presented in Supporting Information (Figure S9).

Compound **6** was the most stable in MLMs as evidenced by its longest $t_{1/2}$ and slowest $Cl_{int}$, whereas compound **2** was the least stable (Table 7). Additionally, **1** and **6** $Cl_{int}$ values were lower than those reported previously for a registered tricyclic antidepressant - imipramine.[39] According to accepted norms of $Cl_{int}$,[40] compounds **1** and **2** can be categorized as moderately stable, which is considered a desirable feature of potential drug candidates. Both compounds possessed advantageous receptor profiles, however compound **2** was less potent anxiolytic *in vivo*. The reason might be its lower metabolic stability. As compound **6** occurred to be the most stable within tested compounds, its low metabolic stability does not seem to be a reason of the poor *in vivo* pharmacological profile.

Chromatograms of tested compounds as well as plots demonstrating their depletion in time are presented in Supporting Information (Figures S10 and S11, respectively).

**Table 6.** Summary of **1**, **2** and **6** metabolites generated inmurine liver microsomal system.

| Compd. | Metabolite | Mass shift (Da) | *m/z* | Retention time (min) | Content among metabolites (%) |
|---|---|---|---|---|---|
| 1 | Parent | 0 | 311 | 5.26 | - |
| | M1 | +16 | 327 | 4.43 | 36.9 |
| | M2 | +16 | 327 | 4.11 | 36.0 |
| | M3 | +16 | 327 | 4.20 | 27.1 |
| 2 | Parent | 0 | 341 | 5.24 | - |
| | M1 | +16 | 357 | 4.52 | 21.3 |
| | M2 | +16 | 357 | 4.11 | 20.4 |
| | M3 | +2 | 343 | 3.66 | 20.1 |
| | M4 | +2 | 343 | 3.79 | 14.1 |
| | M5 | -14 | 327 | 4.86 | 12.7 |
| | M6 | +16 | 357 | 4.19 | 11.4 |
| 6 | Parent | 0 | 389 | 5.40 | - |
| | M1 | +16 | 405 | 4.79 | 48.7 |
| | M2 | +16 | 405 | 4.49 | 26.5 |
| | M3 | +16 | 405 | 4.63 | 24.8 |

**Table 7.** Stability ($t_{1/2}$ and $Cl_{int}$) of **1**, **2** and **6** in murine liver microsomal system.

| Compd. | Protein concentration (mg/mL) | $t_{1/2}$ (min) | $Cl_{int}$ (μL/min/mg) |
|---|---|---|---|
| 1 | 0.4 | 17.7 | 98.0 |
| 2 | 0.4 | 10.8 | 160.4 |
| 6 | 0.4 | 27.5 | 63.0 |
| Imipramine[39] | 0.5 | 11.0 | 125.5 |

### *In silico studies*

*In silico* methods facilitate research by providing fast prediction of properties of interest, as well as enable choosing the most promising compounds at early stages of drug design. The use of MetaSite software[41] would allow to detect potential metabolically unstable compounds at early stages of our future studies. In order to evaluate its utility regarding a group of piperazine derivatives of interest, we predicted the metabolism of compound **1** and compared the results with the outcomes of *in vitro* evaluations.


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The results indicate that the main site of metabolism should be methyl substituent in position 5 of phenyl ring (100% relative probability). The next two with relative probability 40–50% were C4 atom of phenyl ring connected with piperazine ring and methyl substituent in position 2 of phenyl ring (Figure 2A).

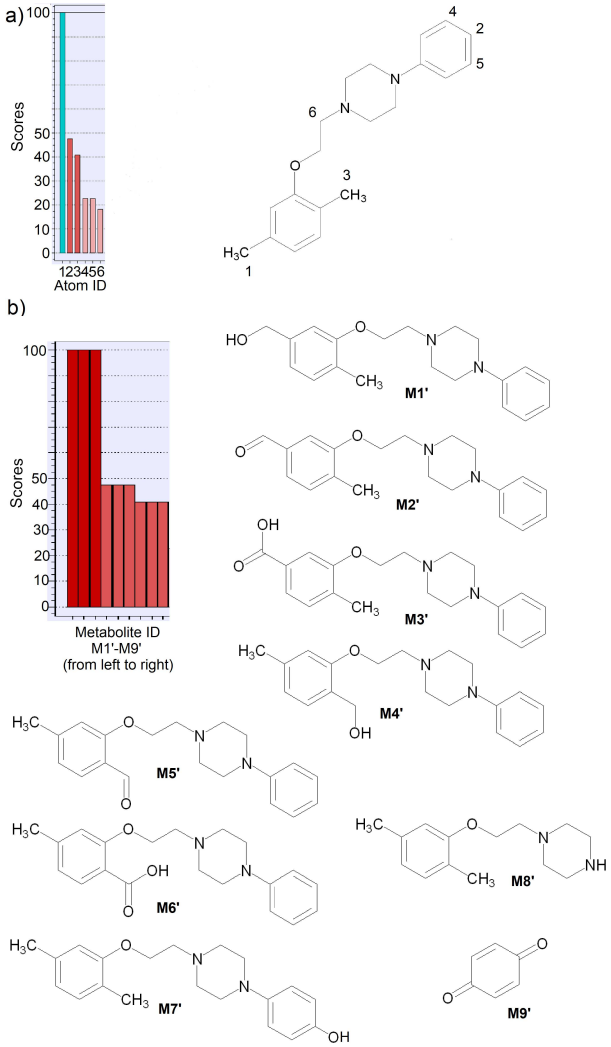


**Figure 2.** *In silico* metabolism prediction for compound **1**: a) – sites of metabolism prediction; b) - major metabolites prediction.

Structures of major metabolites were also predicted, those with the highest probability scores are presented in Figure 2B. The three most probable predicted metabolites **M1'-M3'** are the result of reactions of methyl group in position 5 of the phenyl ring: aliphatic hydroxylation for metabolite **M1'** and aliphatic carbonylation (oxidation) for metabolites **M2'** and **M3'**. It is consistent with previously performed site of metabolism identification. The less probable metabolites resulted from analogous reactions of methyl group in position 2 of the phenyl ring (**M4'-M6'**), aromatic hydroxylation in position 4 of the phenyl ring (**M7'**) and *N*-dearylation (**M8'** and **M9'**).

The results are generally consistent with results obtained *in vitro,* as three products of hydroxylation are present among metabolites predicted by MetaSite (**M1'**, **M4'** and **M7'**), which most probably reflect metabolites **M1-M3** detected for compound **1** in *in vitro* assay. It might be concluded, that MetaSite program can properly detect possible sites of metabolism and types of possible metabolic reactions. However, our study shows that it is not useful for estimation of the exact probability scores and content of particular metabolites in case of this group of compounds, as m/z characteristic for many important predicted metabolites (e.g. **M2'**, **M3'** – probability score 100%) are absent among *in vitro* study results.

## Cytotoxicity

Potential cytotoxic effect of compound **1** (LDH release assay and MTT test, including basal and $H_2O_2$-induced) was examined against human neuroblastoma SH-SY5Y cells as well as human astrocytes (MTT test). SH-SY5Y cell line of human origin, with dopaminergic phenotype, is a commonly used cell culture model in studies related to neurodegenerative disorders, neurotoxicity and oxidative stress.[42] Similarly, addition of hydrogen peroxide to cell medium, as the source of detrimental reactive oxygen species, is a well-validated and frequently used model for oxidative stress. Astrocytes derived from iPSC cells constitute a valuable model for gliotoxicity testing of central nervous system - active compounds.[43]

Two viability tests were used to evaluate the cellular cytotoxicity. LDH assay measure the ability of compound to cause the cell membrane damage by colorimetric reaction mediated by enzyme – lactate dehydrogenase which is transferred from cells to culture medium. MTT assay measure the ability of compound to change cellular metabolism. The assay is based on the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan product.

Exposure of SH-SY5Y cells to compound **1** increased LDH amount in culture medium, which reflects the cell membrane damage. The strongest effect was observed in high concentrations 75 and 100 μM. In MTT reduction assay, the test compound at low (0.1 μM) concentration caused a positive effect on cells by potentiating their viability. However, at concentrations from 10 μM it decreased MTT reduction in concentration-dependent manner. Addition of hydrogen peroxide alone significantly caused damage to cells by increasing approximately three-fold the release of LDH and reducing by approx. 20% MTT reduction. At the highest examined concentration, 10 μM, compound **1** statistically significantly potentiated the damaging effects of hydrogen peroxide in both LDH release and MTT reduction assays. In case of human astrocytes, compound **1** did not affect cellular metabolism in concentration range 0,1-1 μM (data not shown) while in higher concentration decrease MTT reduction in


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concentration-dependent manner in all evaluated concentrations (10 to 200 μM).

Graphical representation of the results are available in Supporting Information (Figures S12-S16).

### Crystallography and docking studies

In the presented series of *N*-phenoxyalkylpiperazine derivatives, selected representatives were characterised by a substantial affinity for the targeted serotonin 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_7$ receptors. However, analysis of the structure-activity relationship (SAR) showed that modifications in arylpiperazine moiety might have resulted in serious affinity loss. To find out molecular interactions underlying these observations, crystallographic studies were carried out for compound **1**, followed by putative binding modes in receptors 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_7$.

#### *Crystallography*

The results of crystal structure analysis for compound **1** are shown in Figure 3. The bond lengths have typical values. The

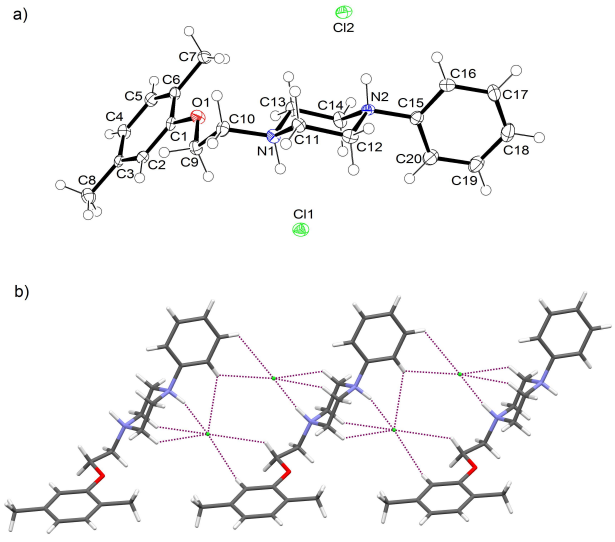


**Figure 3.** a) - The molecular structure of **1** showing the atom numbering scheme. Displacement ellipsoids are drawn at the 50% probability level. b) - The intermolecular interactions of three molecules of **1** in the crystal structure. Dashed lines indicate hydrogen bonds.

methyl substituents at phenoxy moiety are coplanar with benzene ring. The deviation of C7 and C8 atoms from the benzene plane is −0.038(8) Å and −0.035(8) Å, respectively. Two aromatic rings in the molecules are close to perpendicularity with the interplanar angle of 78.3(1)°. The oxygen atom (O1) is involved in weak intramolecular hydrogen bond C13-H13 A···O1.

The piperazine moiety adopts chair conformation with equatorial substituents at N1 and N2 atoms. The phenyl ring at the N2 atom is not coplanar with piperazine moiety, the torsion angle C16-C15-N2-C12 and C20-C15-N2-C12 are −100.2(5)° and 77.1(5)°, respectively. This conformation is less frequent in comparison to other crystal structures with phenyl ring at the N2 atom in piperazine moiety deposited in the Cambridge Structural Database (CSD, Version 5.40).[44] In the most of the crystal structures with phenylpiperazine moiety, the phenyl ring is more or less coplanar with piperazine ring (the torsion angles in the vicinity of 0° or +/-180°).

Both nitrogen atoms in piperazine moiety of **1** are protonated and are involved in hydrogen bonds with chlorine anions (Cl1, Cl2). The chlorine anions are also engaged in weak C–H···Cl interactions. The parameters of the chlorine anions interactions are listed in Supporting Information (Table S17).

#### *Docking studies*

Flexible docking to the previously developed homology models of the receptors of interest was performed. The GPCR structures were represented by several homology models, which mimicked conformational flexibility of the proteins. Visual inspection of the resulting complexes allowed us to capture basic interactions which clarified the input of the arylpiperazine moiety in the ligand-receptor recognition. The most important interactions of the tested ligand in the 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_7$ receptors occurred to be consistent with the common binding mode for serotonin receptor ligands determined experimentally,[45] and the results of our previous studies.[46] The compound was used in the modelling procedure in the form of monohydrochloride (compound protonated at the nitrogen connected to the alkyl linker). At the pH of the receptor environment phenoxyalkyl derivatives of arylpiperazine are present only as monocation and non-protonated forms, of which only the first bind to the receptor. The molecule took extended conformation and was situated across the two cavities of the binding site: the deeper one formed between the transmembrane helices (TMHs) 3–6 (orthosteric binding site) and the second one, located between TMHs 2 and 7 (allosteric binding site). The main anchoring interactions, common for the binding sites of the examined subtypes of serotonin receptors, were (i) a charge-reinforced hydrogen bond between phenylpiperazine (the protonated nitrogen atom) and the carboxyl group of Asp3.32, as well as (ii) the CH-π stacking with Phe6.52 (Figure 4). The above interactions highlight the importance of aromatic ring coupled directly to the piperazine (e.g. in compound **1**; $K_i < 130$ nM), and explains considerably lower affinity of phenethylpiperazine derivative, compound **4** ($K_i > 659$ nM) for the 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_7$ receptors. Noteworthy, the predicted chair conformation of piperazine and its equatorial substitution in compound **1** were in line with the crystallographic data presented above. The substituted phenoxyalkyl fragment of the molecule occupied the second cavity and found favourable aromatic/hydrophobic interactions there, which were distinctive for each receptor type. In the case of the 5-HT$_{1A}$ receptor, it interacted with the phenyl ring of Tyr2.64 (π-π stacking, Figure 4A). 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


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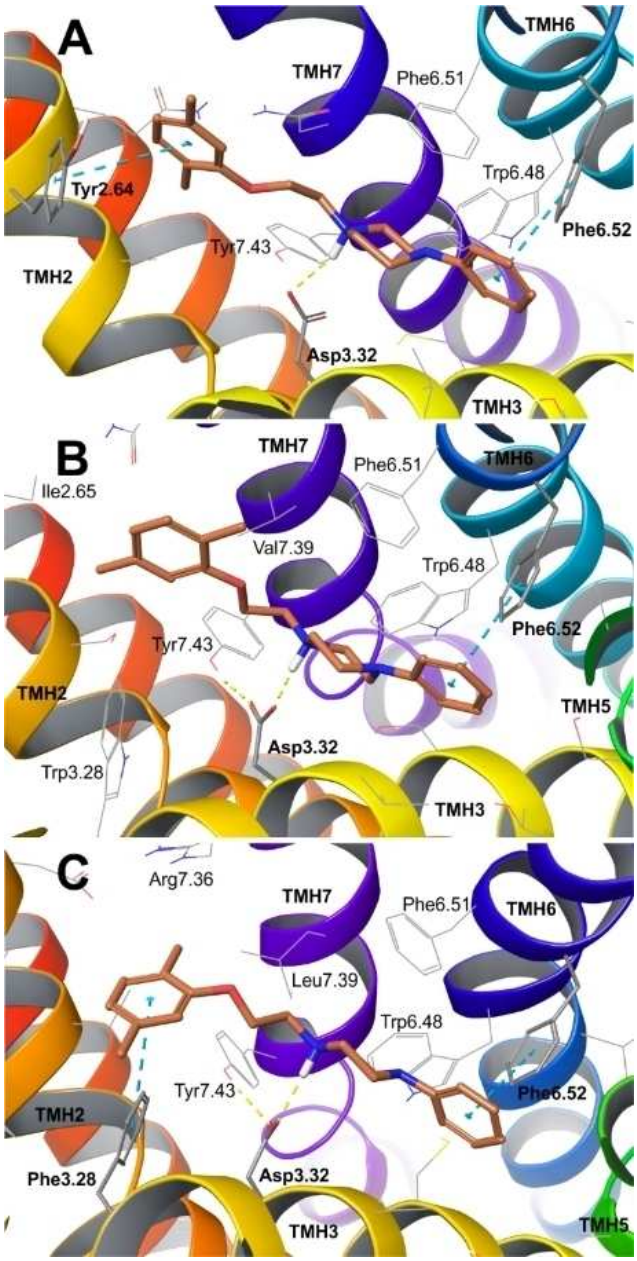


**Figure 4.** Binding modes of compound **1** in the sites of 5-$HT_{1A}$ (A), 5-$HT_{2A}$ (B) and 5-$HT_7$ (C) receptors. Amino acid residues engaged in ligand binding (within 4 Å from the ligand atoms) are displayed as sticks, whereas those forming H-bonds (dotted yellow lines), CH-π and π-π stacking interactions (dotted cyan lines) are represented as thick sticks. For the sake of clarity, a part of TMH5 and extracellular loop 2 residues were undisplayed.

(Figure 4B), whereas in the 5-$HT_7$ receptor - specific CH-π interactions with Phe3.28 (Figure 4C). The above-described predictions provided retrospective explanation of SAR data and support for future rational design of serotonin receptor ligands.

## Conclusions

Within the presented study we obtained a series of arylpiperazine derivatives, including several compounds exhibited notable receptor profile (affinity towards serotonergic, adrenergic and dopaminergic receptors). Broad panel of receptor studies performed for compound 1 proved the binding of this compound also to μ receptors – the mechanism which we have not observed before in the studies regarding this group of compounds. Functional studies allowed to classify some of tested compounds as 5-$HT_{1A}$, 5-$HT_7$ and/or $D_2$ receptors antagonists. Three compounds (1, 2, 9) showed statistically significant anxiolytic-like activity *in vivo* and one compound (4) both antidepressant-like and anticonvulsant activities (in appropriate doses). High neurotoxicity of compound 4 makes it poor candidate for further development as anticonvulsant agent, however there are premises for search for active compounds within the group of its derivatives.

These findings might be a prerequisite for future search for anxiolytics targeting, beyond preyiously proposed monoamine signaling, also opioid system among phenoxyalkyl derivatives of piperazine. Compound 4 showed poor affinity to the tested receptors, while exhibited multidirectional central activity *in vivo*. The mechanism is probably not related with the tested molecular targets.

Selected active compounds, including the most promising compound 1, were subjected to further investigation in order to predict their usefulness and important properties in the treatment process. Metabolic stability assay performed simultaneously for compounds 1, 2 and 6, verified that compound 1 (not the most metabolically stable in the group) was more stable than imipramine. The cytotoxicity of compound 1 was tested against human neuroblastoma SH-SY5Y cells (effect was found from 10 μM, however at 0.1 μM the compound increased cell viability) and human astrocytes (cytotoxic in concentrations 10–200 μM).

In order to support further studies on rational design of serotonergic receptor ligands in a group of arylpiperazine derivatives, we performed crystallographic analysis and docking of compound 1 to receptors 5-$HT_{1A}$, 5-$HT_{2A}$ and 5-$HT_7$. The results proved, among other observations, the importance of aromatic ring coupled directly to the piperazine moiety, which is consistent with the observed *in vitro* receptor profiles.

### Supporting Information Summary

The detailed description of all experimental procedures, as well as Tables/Figures S1-S17 are available in Supporting Information.

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## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** 5-HT • antidepressant-like • anticonvulsant • anxiolytic-like • piperazine derivatives

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