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Anti-Alzheimer's multitarget-directed ligands with serotonin 5-HT$_6$ antagonist, butyrylcholinesterase inhibitory, and antioxidant activity

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Abstract

Serotonin 5-HT$_6$ receptors, butyrylcholinesterase (BuChE) and oxidative stress are related to the pathophysiology of Alzheimer's disease. Inhibition of BuChE provides symptomatic treatment of the disease and the same effect was demonstrated for 5-HT$_6$ antagonists in clinical trials. Oxidative stress is regarded as a major and primary factor contributing to the development of Alzheimer's disease; therefore, antioxidant agents may provide a disease-modifying effect. Combining BuChE inhibition, 5-HT$_6$ antagonism, and antioxidant properties may result in multitarget-directed ligands providing cognition-enhancing properties with neuroprotective activity. On the basis of the screening of the library of 5-HT$_6$ antagonists against BuChE, we selected two compounds and designed their structural modifications that could lead to improved BuChE inhibitory activity. We synthesized two series of compounds and tested their affinity and functional activity at 5-HT$_6$ receptors, BuChE inhibitory activity and antioxidant properties. Compound **12** with K_i and K_b values against 5-HT$_6$ receptors of 41.8 and 74 nM, respectively, an IC_{50} value of 5 μM against BuChE and antioxidant properties exceeding the activity of ascorbic acid is a promising lead structure for further development of anti-Alzheimer's agents.

KEYWORDS

5-HT$_6$ antagonists, Alzheimer's disease, antioxidant properties, butyrylcholinesterase inhibitors, multimodal agents, multitarget-directed ligands, serotonin receptors

1 | INTRODUCTION

Alzheimer's disease (AD) is a predominant neurodegenerative disorder, which affects approximately 35 million people worldwide.[1] This number is constantly increasing and it has been projected that by 2050, the number of individuals suffering from AD will reach 115 million.[2] The pathogenesis of AD is multifactorial in origin, and it results from complex processes that affect each other at various levels, leading to neuronal loss.[3,4]

The brain areas primarily affected by progressive neurodegeneration involve cholinergic neurons in the cerebral cortex and hippocampus.[5] As the cholinergic system is associated with memory and cognition, impaired cholinergic neurotransmission induces symptoms of cognitive decline and memory impairment in AD patients. Therefore, alleviating the level of acetylcholine (ACh) in

cholinergic synapses has been proposed to improve the symptoms of AD.[6] It is possible to elevate ACh levels in the synaptic cleft by inhibiting enzymes involved in ACh hydrolysis: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE).[7] The activity of both enzymes changes along with the AD progression. In an advanced stadium of AD, activity of BuChE prevails, and thus may account for the persistence of symptoms and disease progression.[8] In fact, first therapeutic agents for AD that appeared on the market act through inhibition of cholinesterases (donepezil, rivastigmine, and galantamine).[9] However, their clinical efficacy is rather disappointing considering the fact that improvement in cognitive symptoms is modest and temporary.[10] Therefore, development of novel therapeutic agents for Alzheimer's disease is urgently needed.

Recently, many novel molecular targets, which might open new therapeutic opportunities for the treatment of AD, have been suggested. A growing body of evidence highlights a promising therapeutic potential of serotonin 5-HT_6 receptor antagonists.[11] Serotonin 5-HT_6 receptors are expressed in the brain areas (hippocampus and cerebral cortex) responsible for cognitive functions, and it has been proved in animal studies that 5-HT_6 antagonists enhance memory and learning processes. Molecular studies demonstrated that this effect is related to an indirect enhanced release of ACh in the cortex.[12] Results of phase II clinical trials showed that combined therapy using 5-HT_6 receptor antagonist (idalopirdine) and AChE inhibitor (donepezil) results in a superior therapeutic effect in AD patients compared with monotherapy.[13] Further studies revealed that 5-HT_6 antagonists enhance neuroplasticity and provide neuroprotective effect against amyloid β-induced neurotoxicity, by lowering reactive oxygen species (ROS) levels and maintaining the proper neurite outgrowth.[14] Moreover, among many interesting pharmacological activities of 5-HT_6 antagonists, antidepressant, and anxiolytic activities might be particularly beneficial for AD patients, because approximately 80% of AD patients exhibit comorbid behavioral disturbances.[12] Consequently, the 5-HT_6 receptor has been regarded as a potential therapeutic target for the treatment of AD, as its inhibition may provide both symptomatic and the disease-modifying effects in the treatment of AD.

ROS have been considered as one of the contributing factors related to the onset and advancement of AD.[15] The oxidative stress hypothesis of AD states that mitochondrial dysfunction mediates increased release of ROS, which contributes to the enhanced expression of transmembrane amyloid-β protein precursor (APP) and initiates accumulation of amyloid β.[16] In fact, numerous clinical studies found elevated levels of oxidative markers as well as antioxidant enzymes in AD patients.[17] In addition, enhanced release of ROS and disrupted antioxidant protection may directly impair synaptic function and neurotransmission resulting in cognitive decline. These data have suggested that AD patients may benefit from the treatment with antioxidant agents.[18]

Given the complex etiology of AD, targeting exclusively one selected biological target may not result in finding a sufficient treatment for AD.[19] As a consequence, scientists follow a recent trend focused on the design of multitarget-directed ligands (MTDLs), which can simultaneously interact with several crucial biological targets and offer potentially more effective treatment of AD.[20,21] Herein, we describe the design, synthesis and in vitro evaluation of a series of MTDLs combining inhibitory activity against BuChE, 5-HT_6 receptor antagonism and antioxidant activity. The biological studies comprise evaluation of inhibitory activity toward BuChE as well as AChE, binding affinity and intrinsic activity on 5-HT_6 receptors and antioxidant evaluation using ferric reducing antioxidant power assay (FRAP).

2 | RESULTS AND DISCUSSION

2.1 | Design

In the course of our work on MTDLs, we have screened a series of 5-HT_6 receptor antagonists synthesized in our laboratory against AChE

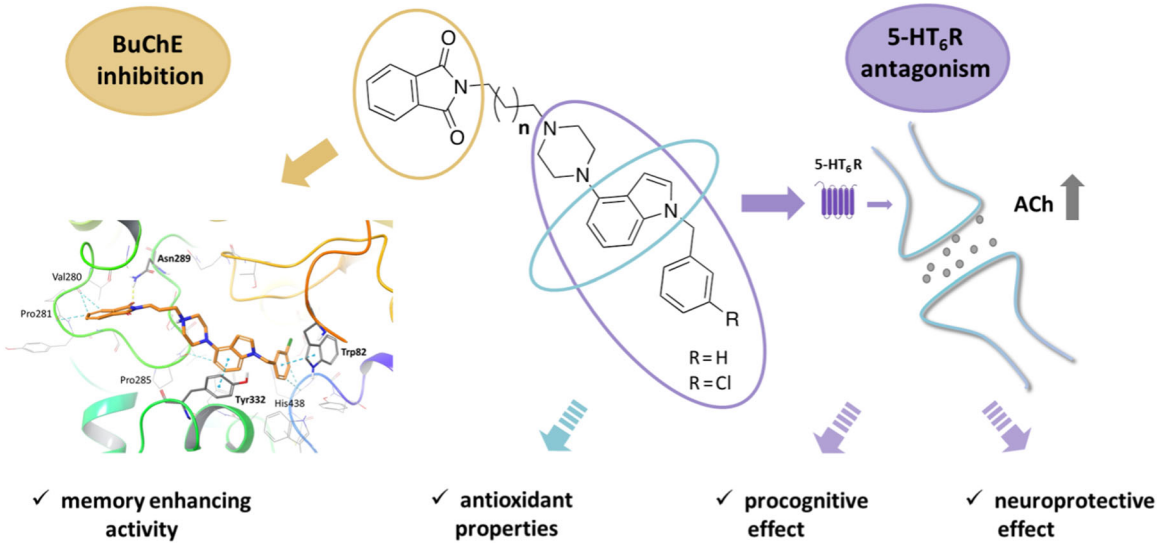


FIGURE 1 Design strategy of multitarget-directed ligands combining butyrylcholinesterase inhibitory activity, 5-HT_6 antagonism and antioxidant properties

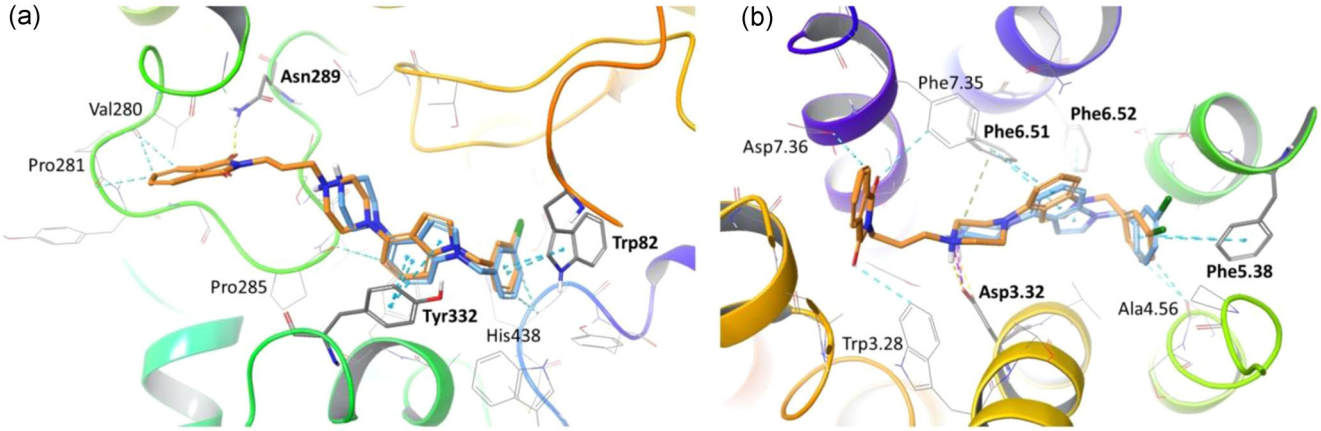


FIGURE 2 The predicted binding mode of compound **5** (orange) and **12** (blue) in the active site of butyrylcholinesterase (a) and the binding site of the 5-HT$_6$ receptor (b). Amino acid residues engaged in ligand binding (within 4 Å from the ligand atoms) are shown as sticks, whereas residues identified as crucial for ligand binding, for example, forming H-bonds (dotted yellow lines), π–π stacking interactions (dotted cyan lines) and aromatic H-bonds (dotted pale blue lines) are represented as thick sticks

and BuChE. Among the tested compounds we have selected two: compounds **4** and **5** (Scheme 1) that displayed high nanomolar affinity toward 5-HT$_6$ receptor (K_i = 17 and 3 nM, respectively) and micromolar activity against BuChE (IC_{50} = 6.82 and 12.37 μM, respectively). Following the "designing in" strategy[21] and our recent success on the development of highly active multifunctional ligands based on these pharmacophores[22,23] we have designed a novel series of MTDLs with *N*-substituted 4-(piperazin-1-yl)-1*H*-indole core (Figure 1).

To improve the activity of the compounds against BuChE we have designed a series of derivatives with piperazine moiety modified by a phthalimide fragment attached with alkyl linkers of a different length (Figure 1). We have previously used this fragment in the design of selective BuChE inhibitors.[24]

A number of studies confirmed that indole-based compounds capture free radicals and thus protect lipids, proteins, and other biological systems from peroxidation.[25,26] We have postulated that free radical scavenger activity of indole moiety combined with neuroprotective activity resulting from interaction with the 5-HT$_6$ receptor may protect neurons from further degeneration.

The design of new molecules was supported by docking studies, using optimized crystal structure of BuChE and homology model of the 5-HT$_6$ receptor. In the active site of BuChE, as presented for representative compound **5**, the molecule forms π–π interactions with Trp82 (benzyl group) and Tyr332 (indole moiety) (Figure 2a). Among the newly designed structures, the best-performing compound **12**, containing the phthalimide fragment with propylene linker, favorably fills the volume and interacts with amino acid residues of a hydrophobic pocket composed of i.a. Val280, Pro281, Pro285 and forms an additional hydrogen bond with Asn289 (Figure 2a). The proposed structural modifications were expected to increase inhibitory potency against BuChE, as the predicted ligand binding energy (MM-GBSA methodology) dropped from −58,476 to −80,280 kcal/mol (compound **5** vs. **12**, respectively). Furthermore, the planned introduction of spatial phthalimide substituent to the indole moiety does not appear to have a negative impact on affinity for the 5-HT$_6$ receptor. The binding mode of compound **5** presents interactions in the orthosteric binding site (helices 3, 5, and 6), namely the charged-reinforced hydrogen bond between the basic amine and Asp3.32, the π–π stacking between indole moiety and Phe6.51/6.52, as well as the aromatic interaction of benzyl ring with Phe5.38 (Figure 2b). Its derivative, compound **12**, displays the same binding pattern, expanding its interactions to the accessory pocket between helices 2 and 7. Moreover, values of the MM-GBSA scoring function used to compare the free energies of the receptor's complexes with compound **5** and its phthalimide counterpart compound **12** (−65,411 vs. −93,328 kcal/mol, respectively) indicate a beneficial effect of the modifications planned.

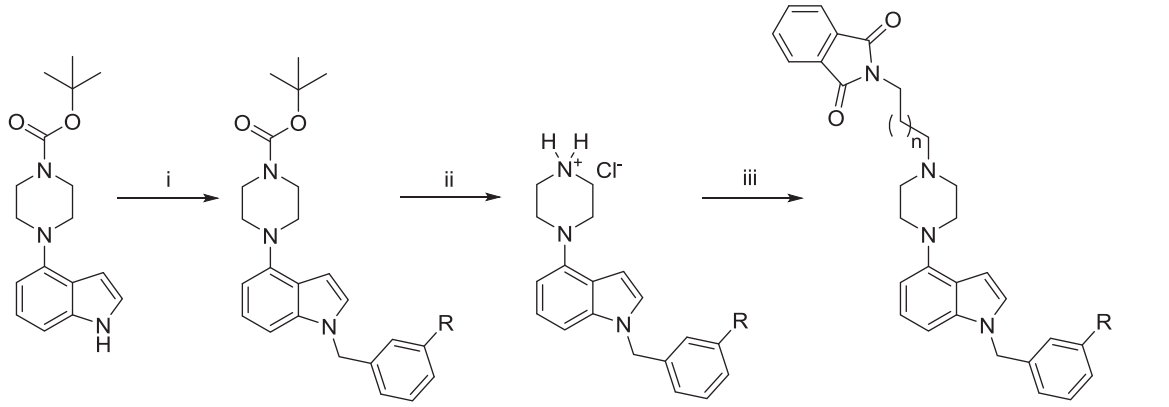
2.2 | Chemistry

We have synthesized a series of 12 compounds (**6**–**17**) according to the synthetic pathway presented in Scheme 1. The key intermediates for the synthesis (**4** and **5**) were prepared according to the procedure that we have used and described before.[27] Briefly, commercially available *tert*-butyl 4-(1*H*-indol-4-yl)piperazine-1-carboxylate was alkylated with benzyl bromide or 1-(bromomethyl)-3-chlorobenzene in the presence of potassium *tert*-butoxide and 18-crown-6. The obtained tertiary amines **2** and **3** were subsequently deprotected in acidic conditions to give compounds **4** and **5** as hydrochloride salts. In the last step, both compounds were alkylated in acetonitrile reflux with ω-bromoalkylphthalimides in the presence of K_2CO_3 as a base, to yield final compounds **6**–**17**.

2.3 | In vitro studies

2.3.1 | Affinity and functional activity on 5-HT$_6$ receptor

We assessed the affinity of the final compounds **6**–**17** for the 5-HT$_6$ receptor, in a radioligand binding assay with methiothepin as a



SCHEME 1 Synthesis of compounds **4–17**. Reagents and conditions: (i) Benzyl bromide or 1-(bromomethyl)-3-chlorobenzene, THF, potassium *tert*-butoxide, 18-crown-6, RT, 24 hr, (ii) 1 M HCl in ethyl acetate, RT, 24 hr, (iii) ω-bromoalkylphthalimide derivative, MeCN, K_2CO_3, KI, reflux, 24 hr

reference compound.[28] The results of this study are summarized in Table 1. All of the compounds displayed an affinity for 5-HT_6 receptor ranging from 21 to 252 nM. We could see that the length of an alkyl linker clearly affects the affinity of the compounds for the 5-HT_6 receptor. Among 3-chlorobenzyl derivatives, the highest affinity was detected for compound **13** with butylene linker (K_i = 27 nM). Elongation of the linker resulted in significant decrease of affinity for compound **14** with pentylene linker (K_i = 238 nM). Further extension of the linker resulted in K_i values ranging from 127 to 207 nM. Among nonsubstituted benzyl derivatives, compound **9** with hexylene linker showed the highest affinity (K_i = 91 nM) while the lowest activity was detected for compound **10** with heptylene linker (K_i = 250 nM).

To determine the antagonistic mode of action, we have selected three compounds: **6**, **12**, and **13** for cell-based functional studies.[28] The K_b values measured for these compounds: 1670 (**6**), 74 (**12**), and 193 nM (**13**), confirm that the compounds are 5-HT_6 antagonists.

2.3.2 | Cholinesterase (AChE/BuChE) inhibitory activity

We evaluated the ability of the synthesized compounds to inhibit *ee*AChE and *eq*BuChE using Ellman's method.[29] These enzymes are routinely used as a cheaper alternative and give a good prediction for potency against expensive human enzymes. After an initial screening at 10 μM, for the compounds with inhibitory potency higher than 50%, we determined IC_{50} values. We used tacrine and donepezil as reference compounds in this assay. Additionally, to confirm that the results of the assay on *eq*BuChE correspond well with human BuChE we have tested compound **6** on the human enzyme. The results are collected in Table 1.

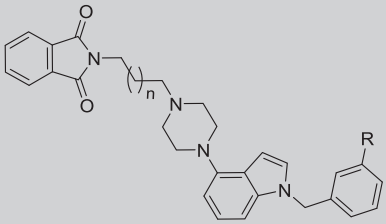
Comparing with the potency of their precursors, **4** and **5**, compounds **6** and **12** displayed approximately twofold higher inhibitory potency against *eq*BuChE with IC_{50} values of 3.44 and 5.07 μM, respectively. Both compounds are derivatives with 3-carbon atom linker connecting phthalimide moiety with 4-piperazineindole core. Compounds with longer linkers (**7**–**9**, **11**, **14**, and **15**) displayed rather modest inhibitory potency against *eq*BuChE ranging from 18–40%. Compounds **10**, **13**, and **16** were not active at the screening concentration. Regarding the influence of *meta*-chlorine substituent at benzyl moiety on *eq*BuChE inhibition, we noticed higher activities for unsubstituted compounds (**4** vs. **5**, **6** vs. **12**, **7** vs. **13**, and **11** vs. **17**) or no differences in activities between them (**8** vs. **14**, **9** vs. **15**, and **10** vs. **16**).

2.3.3 | Determination of the antioxidant activity by FRAP assay

The FRAP aims to determine potential reducing/antioxidant activity[30] of the tested compounds. The antioxidant activity is measured by the ability of the compound to reduce 2,4,6-tripyridyl-*s*-triazine (Fe^{3+}-TPTZ salt) to its blue colored product (Fe^{2+}-TPTZ salt).[31] The assay enables to detect molecules with redox potential, which is crucial for maintaining the redox status in cells. For FRAP assay we have selected the most promising compounds acting as BuChE inhibitors/5-HT_6 antagonists, namely: **6**, **12**, and **13**.

The compounds and a reference drug—ascorbic acid (vitamin C)—were tested at three concentrations: 10, 100, and 1000 μM. The results are presented in Figure 3 as a concentration of Fe^{2+} ions formed by the reduction of Fe^{3+} by the tested compounds. All the compounds displayed antioxidant activity similar or exceeding the activity of ascorbic acid at the corresponding concentration. Compound **6** showed significantly higher antioxidant potential in all three concentrations. These data confirm the validity of the applied structural design and are in agreement with previous studies, which

TABLE 1 Cholinesterase inhibitory potency and 5-HT_6 receptor affinity of compounds **4** and **5** and new multifunctional ligands **6–17**



Compounds	R	*n*	*Ee*AChE[a] % inh.[d]	*Eq*BuChE [b] IC_{50} (μM)[e] /% inh[d]	*h*5-HT_6R[c] K_i (nM)[f]
4	H	–	<10%	6.82 ± 0.23	17.0 ± 1.5
5	Cl	–	<10%	12.37 ± 0.35	3.0 ± 0.2
6	H	1	<10%	3.44 ± 0.16 8.27 ± 0.20[i]	149.8 ± 8.4 1670.0 ± 1.5[g]
7	H	2	10.9%	34.8%	n. a.
8	H	3	<10%	40.0%	189.3 ± 18.1
9	H	4	<10%	14.5%	90.6 ± 9.8
10	H	5	<10%	<10%	252.2 ± 23.1
11	H	6	<10%	28.5%	180.6 ± 12.7
12	Cl	1	<10%	5.07 ± 0.20	41.8 ± 4.8 74.0 ± 0.3[g]
13	Cl	2	<10%	<10%	27.2 ± 2.5 193.0 ± 3.5[g]
14	Cl	3	<10%	37.7%	238.1 ± 6.1
15	Cl	4	<10%	18.2%	127.0 ± 12.1
16	Cl	5	<10%	<10%	207.6 ± 58.1
17	Cl	6	<10%	< 10%	174.0 ± 11.3
Donepezil			0.011 ± 0.0002[e]	1.83 ± 0.04[e]	n.d.[h]
Tacrine			0.023 ± 0.0004[e]	0.015 ± 0.0001[e]	n.d.[h]
Methiothepin					0.5 ± 0.007

Abbrevations: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; n.a., not available.
[a] AChE from the electric eel.
[b] BuChE from equine serum.
[c] Human 5-HT_6 receptor.
[d] Inhibition % at 10 μM.
[e] IC_{50} values are expressed as mean ± standard error of the mean (SEM) of at least three experiments.
[f] K_i values expressed as mean ± standard error of the mean (SEM) of two experiments.
[g] K_b value.
[h] Not determined.
[i] Human BuChE.

showed that indole-based compounds exhibit high antioxidant potential.[25,26]

3 | CONCLUSIONS

Currently, available anti-Alzheimer's drugs offer, at most, symptomatic treatment for some patients. Although relief of symptoms is important, it is crucial to find effective disease-modifying drugs. Mitochondrial cascade hypothesis points to oxidative stress as a primary factor that triggers a neurodegenerative cascade leading to Aβ accumulation, tau phosphorylation, synaptic loss and, finally, development of AD. Thus, compounds with antioxidant properties are thoroughly studied in terms of their applicability in the treatment of AD. Based on mitochondrial cascade hypothesis and cholinergic hypothesis we have designed, synthesized, and evaluated a new series of indole-based MTDLs combining 5-HT_6 antagonistic activity, BuChE inhibition, and antioxidant properties. Our study confirms our previous findings that *N*-substituted 4-(piperazin-1-yl)-1*H*-indole core is a suitable pharmacophore for the development of MTDLs. The applied structural modifications that resulted in enhanced interactions with BuChE did not affect the affinity at the 5-HT_6 receptor and the obtained compounds retained high potency. Compound **12** displayed favorable dual activity against selected targets: 5-HT_6

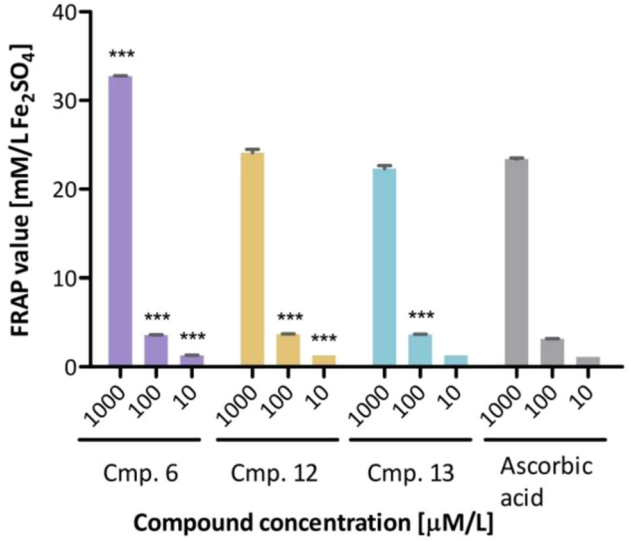


FIGURE 3 Antioxidant effect of compounds **6**, **12**, **13** and ascorbic acid in ferric reducing antioxidant power assay (FRAP). The reducing power (FRAP value) is represented as mM/l of Fe_2SO_4. The values are represented as mean ± standard deviation ($n = 3$). ***$p < 0.001$, the significant differences in the antioxidant activity of the tested compound in comparison to ascorbic acid at the same concentration (the Student *t* test)

(K_i = 41.8 nM) and BuChE (IC_{50} = 5.07 μM). When tested in FRAP assay, compound **12** displayed favorable antioxidant properties, exceeding the activity of the reference ascorbic acid. The antioxidant activity is likely to be derived from an indole moiety, which previously showed powerful antioxidant properties. Compound **12** might be considered as a promising starting point for further optimization toward the development of multifunctional ligands against Alzheimer's disease.

4 | EXPERIMENTAL

4.1 | Molecular modeling

The structures of ligands were built in Maestro 2D Sketcher and optimized using LigPrep tool, generating protonation states according to Epik. Glide SP flexible docking procedure was carried out using default parameters and OPLS3 force field. Post-docking minimization was performed to retrieve five low energy complexes for each ligand. The obtained poses were then evaluated based on Glide gscore values and visual inspection. The selected complexes were optimized using Prime MM-GBSA tool. The free energy of ligand binding (MMGBSA dG Bind) was calculated using VSGB solvation model and OPLS3 force field. A homology model of serotonin 5-HT_6 receptor and a crystal structure-based BuChE model served as molecular targets.

Homology modeling procedure was reported previously[32] and was applied for the preparation of a model based on the serotonin 5-HT_{1B} receptor crystal structure (PDB ID: 4IAR).[22] H-bond constraints, as well as the centroid of a grid box (25 Å) for docking studies were located on Asp3.32.

The BuChE model was developed on the basis of the experimental structure of the enzyme (PDB ID: 1P0P) was used.[33] The initial structure was refined using the Protein Preparation Wizard. Water molecules and hetero groups other than the ligand were deleted, the missing protein side chain atoms were predicted using Prime and the energy of the whole system was minimized (OPLS3). Grid boxes for docking in BuChE models were placed in a centroid of cocrystallized ligand. The model was tested throughout docking studies involving BuChE inhibitors of experimentally proven affinity. The obtained consistent binding modes of the reference compounds verified the accuracy of the crystal-based model.

4.2 | Chemistry

4.2.1 | General

^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on Varian Mercury 300 MHz (Varian, Inc., Palo Alto, CA) or Jeol 500 MHz (Jeol Inc., Peabody, MA). The chemical shifts for ^{1}H NMR are referenced to tetramethylsilane (TMS) via residual solvent signals (1 H, $CDCl_3$ at 7.26 ppm, DMSO-d_6 at 2.50 ppm). Mass spectra (MS) were recorded on UPLC-MS/MS system consisting of a Waters ACQUITY UPLC (Waters Corporation, Milford, MA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Column chromatography was performed on Merck (Darmstadt, Germany) silica gel 60 (63–200 μm). For the TLC and column chromatography following solvents were used: dichloromethane (DCM), methanol (MeOH), petroleum ether, hexane (Hex), diethyl ether (Et_2O), chloroform ($CHCl_3$), ethyl acetate (EtOAc), petroleum ether (PET), 25% ammonia–water solution. The purity of the final compounds was determined using an analytical RPLC-MS on Waters Acquity TQD using an Aquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm) at 214 and 254 nm. CH_3CN/H_2O gradient with 0.1% HCOOH was used as the mobile phase at a flow rate of 0.3 ml/min. All the compounds showed purity above 95%. All of the reagents were purchased from commercial suppliers and were used without further purification.

The following compounds: *tert*-butyl 4-(1-benzyl-1*H*-indol-4-yl)-piperazine-1-carboxylate (**2**), *tert*-butyl 4-(1-(3-chlorobenzyl)-1*H*-indol-4-yl)piperazine-1-carboxylate (**3**), 4-(1-benzyl-1*H*-indol-4-yl)piperazin-1-hydrogen chloride (**4**), and 4-(1-(3-chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-hydrogen chloride (**5**) were reported previously.[27]

The InChI codes of the investigated compounds together with some biological activity data are provided as Supporting Information.

4.2.2 | General procedure for the synthesis of compounds 6–17 (procedure A)

The appropriate 4-(1-benzyl-1*H*-indol-4-yl)piperazin-1-hydrogen chloride derivative (1 equiv.) with the 2-(ω-bromoalkyl)isoindoline-1,3-dione (1 equiv.) in the presence of K_2CO_3 (1 equiv.) and KI (1 equiv.) in MeCN was stirred at 70°C for 24 hr. After that time, the reaction mixture was filtered and concentrated under reduced pressure. The solvent was evaporated and the crude mixture was purified over column chromatography.

2-(3-(4-(1-Benzyl-1*H*-indol-4-yl)piperazin-1-yl)propyl)-isoindoline-1,3-dione (6)

Following procedure A, reaction of 4-(1-benzyl-1*H*-indol-4-yl)-piperazin-1-hydrogen chloride (135 mg, 0.414 mmol) with 2-(3-bromopropyl)isoindoline-1,3-dione (111 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (DCM/MeOH, 9.8:0.2, v/v); yield: 149 mg (75%); MW = 478.60; formula $C_{30}H_{30}N_4O_2$; MS *m/z* 479.46 ($M+H^+$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.96–7.79 (m, 2 H), 7.74–7.62 (m, 2 H), 7.33–7.17 (m, 3 H), 7.15–7.01 (m, 4 H), 6.94 (d, *J* = 8.2 Hz, 1 H), 6.58–6.45 (m, 2 H), 5.28 (s, 2 H), 3.81 (t, *J* = 6.8 Hz, 2 H), 3.17 (m, 4 H), 2.71 (t, *J* = 4.6 Hz, 4 H), 2.58 (t, *J* = 7.1 Hz, 2 H), 2.09, 1.86 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.5, 145.6, 137.6, 137.5, 133.9, 132.3, 128.7, 127.5, 126.7, 123.2, 122.4, 121.9, 106.6, 104.5, 100.2, 56.0, 53.3, 50.9, 50.2, 36.6, 25.1.

2-(4-(4-(1-Benzyl-1*H*-indol-4-yl)piperazin-1-yl)butyl)-isoindoline-1,3-dione (7)

Following procedure A, reaction of 4-(1-benzyl-1*H*-indol-4-yl) piperazin-1-hydrogen chloride (135 mg, 0.414 mmol) with 2-(4-bromobutyl)isoindoline-1,3-dione (117 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (DCM/MeOH, 9.8:0.2, v/v); yield: 167 mg (82%); MW = 492.62; formula $C_{31}H_{32}N_4O_2$; MS *m/z* 493.36 ($M+H^+$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.85–7.81 (m, 2 H), 7.72, 7.67 (m, 2 H), 7.29–7.22 (m, 3 H), 7.10–7.04 (m, 4 H), 6.99–6.93 (m, 1 H), 6.63–6.55 (m, 1 H), 6.51 (dd, *J* = 0.8, 3.3 Hz, 1 H), 5.28 (s, 2 H), 3.73 (t, *J* = 6.5 Hz, 2 H), 3.44–3.34 (m, 4 H), 2.91–2.82 (m, 4 H), 2.68, 2.59 (m, 2 H), 1.82, 1.66 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 145.2, 137.5, 137.5, 133.9, 132.1, 128.7, 127.6, 126.9, 126.7, 123.7, 122.4, 121.9, 106.8, 104.9, 100.1, 57.7, 53.3, 50.5, 50.3, 37.5, 26.5, 23.3.

2-(5-(4-(1-Benzyl-1*H*-indol-4-yl)piperazin-1-yl)pentyl)-isoindoline-1,3-dione (8)

Following procedure A, reaction of 4-(1-benzyl-1*H*-indol-4-yl)-piperazin-1-hydrogen chloride (135 mg, 0.414 mmol) with 2-(5-bromopentyl)isoindoline-1,3-dione (123 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (Hex/Et_2O/DCM/MeOH, 40:30:29:1, v/v/v/v); yield: 208 mg (99%); MW = 506.65; formula $C_{32}H_{34}N_4O_2$; MS *m/z* 507.39 ($M+H^+$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.79–7.73 (m, 2 H), 7.66–7.59 (m, 2 H), 7.24–7.13 (m, 3 H), 7.05–6.96 (m, 4 H), 6.87 (d, *J* = 8.2 Hz, 1 H), 6.51 (d, *J* = 7.4 Hz, 1 H), 6.46 (d, *J* = 3.1 Hz, 1 H), 5.20 (s, 2 H), 3.63 (t, *J* = 7.3 Hz, 2 H), 3.26–3.17 (m, 4 H), 2.65 (d, *J* = 3.8 Hz, 4 H), 2.44–2.34 (m, 2 H), 1.73–1.62 (m, 2 H), 1.60–1.49 (m, 2 H), 1.41–1.29 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 145.7, 137.7, 137.5, 133.9, 132.0, 128.6, 127.5, 126.7, 123.1, 122.4, 121.8, 106.5, 104.5, 100.2, 58.4, 53.5, 50.8, 50.1, 37.8, 28.4, 26.2, 24.8.

2-(6-(4-(1-Benzyl-1*H*-indol-4-yl)piperazin-1-yl)hexyl)-isoindoline-1,3-dione (9)

Following procedure A, reaction of 4-(1-benzyl-1*H*-indol-4-yl)-piperazin-1-hydrogen chloride (135 mg, 0.414 mmol) with 2-(6-bromohexyl)isoindoline-1,3-dione (128 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (DCM/MeOH, 95:5, v/v); yield: 170 mg (79%); MW = 520.68; formula $C_{33}H_{36}N_4O_2$; MS *m/z* 521.48 ($M+H^+$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.88–7.83 (m, 2 H), 7.81–7.76 (m, 3 H), 7.27–7.21 (m, 3 H), 7.12 (d, *J* = 1.8 Hz, 2 H), 7.07–7.02 (m, 2 H), 6.63–6.57 (m, 1 H), 6.52 (d, *J* = 3.3 Hz, 1 H), 5.35 (s, 2 H), 3.73–3.66 (m, 2 H), 3.34 (m, 4 H), 3.23 (m, 4 H), 3.00–2.82 (m, 2 H), 1.72 (m 2 H), 1.49–1.39 (m, 2 H), 1.31–1.21 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 145.8, 137.7, 137.5, 133.7, 132.2, 129.3, 127.5, 126.6, 123.5, 122.4, 121.9, 106.5, 104.4, 100.3, 58.9, 53.7, 51.3, 50.2, 38.1, 29.6, 29.5, 28.5, 27.5.

2-(7-(4-(1-Benzyl-1*H*-indol-4-yl)piperazin-1-yl)heptyl)-isoindoline-1,3-dione (10)

Following procedure A, reaction of 4-(1-benzyl-1H-indol-4-yl)piperazin-1-hydrogen chloride (135 mg, 0.414 mmol) with 2-(7-bromoheptyl) isoindoline-1,3-dione (134 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (Hex/Et_2O/DCM/MeOH, 40:30:29:1, v/v/v/v); yield: 117 mg (53%); MW = 534.70; formula $C_{34}H_{38}N_4O_2$; MS *m/z* 535.51 ($M+H^+$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.88–7.81 (m, 2 H), 7.73–7.66 (m, 2 H), 7.31–7.22 (m, 3 H), 7.13–7.05 (m, 4 H), 6.95 (d, *J* = 8.2 Hz, 1 H), 6.60 (d, *J* = 7.4 Hz, 1 H), 6.55 (d, *J* = 3.1 Hz, 1 H), 5.29 (s, 2 H), 3.69 (t, *J* = 7.3 Hz, 2 H), 3.36–3.26 (m, 4 H), 2.76–2.66 (m, 4 H), 2.48–2.39 (m, 2 H), 1.77–1.63 (m, 2 H), 1.63–1.51 (m, 2 H), 1.43–1.23 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 145.8, 137.7, 137.6, 133.8, 132.2, 129.3, 127.5, 126.7, 123.5, 122.4, 121.9, 106.5, 104.4, 100.3, 58.9, 53.7, 51.3, 50.2, 38.0, 29.7, 29.5, 28.5, 27.5, 26.8.

2-(8-(4-(1-Benzyl-1*H*-indol-4-yl)piperazin-1-yl)octyl)isoindoline-1,3-dione (11)

Following procedure A, reaction of 4-(1-benzyl-1*H*-indol-4-yl)piperazin-1-hydrogen chloride (135 mg, 0.414 mmol) with 2-(8-bromooctyl)isoindoline-1,3-dione (140 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (Hex/Et_2O/DCM/MeOH, 40:30:29:1, v/v/v/v); yield: 48 mg (21%); MW = 548.73; formula $C_{35}H_{40}N_4O_2$; MS *m/z* 549.47 ($M+H^+$); ^1H NMR (300 MHz, $CDCl_3$) δ 7.87–7.79 (m, 2 H), 7.74–7.65 (m, 2 H), 7.31–7.21 (m, 4 H), 7.12–7.02 (m, 3 H), 6.95 (d, *J* = 8.5 Hz, 1 H), 6.62–6.57 (m, 1 H), 6.53 (dd, *J* = 0.6, 3.2 Hz, 1 H), 5.29 (s, 2 H), 3.74–3.63 (m, 2 H), 3.32 (d, *J* = 4.4 Hz, 4 H), 2.75 (m, 4 H), 2.53–2.41 (m, 2 H), 1.75–1.62 (m, 2 H), 1.57 (m, 2 H), 1.39–1.27 (m, 8 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.5, 145.7, 137.6, 137.5, 133.9, 132.2, 128.7, 127.5, 126.7, 123.1, 122.4, 121.9, 106.6, 104.6, 100.2, 58.8, 53.5, 51.0, 50.2, 38.0, 29.7, 29.4, 29.1, 28.6, 27.5, 26.8.

2-(3-(4-(1-(3-Chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-yl)propyl)isoindoline-1,3-dione (12)

Following procedure A, reaction of 4-(1-(3-chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-hydrogen chloride (150 mg, 0.414 mmol) with 2-(3-bromopropyl)isoindoline-1,3-dione (111 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (DCM/MeOH, 9.8:0.2, v/v); yield: 155 mg (73%); MW = 513.04; formula $C_{30}H_{29}ClN_4O_2$; MS *m/z* 513.50 ($M+H^+$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90–7.79 (m, 2 H), 7.75–7.66 (m, 2 H), 7.27–7.16 (m, 2 H), 7.12–7.02 (m, 3 H), 6.96–6.85 (m, 2 H), 6.65–6.51 (m, 2 H), 5.26 (s, 2 H), 3.74 (t, *J* = 7.1 Hz, 2 H), 3.29 (m, 4 H), 2.72 (m, 4 H), 2.55–2.43 (m, 2 H), 1.76 (quin, *J* = 7.1 Hz, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.5, 145.8, 139.7, 137.3, 134.7, 133.9, 132.1, 130.0, 127.8, 126.7, 126.6, 124.8, 123.2, 122.7, 121.9, 106.7, 104.3, 100.7, 58.1, 53.6, 51.2, 49.6, 37.9, 24.2.

2-(4-(4-(1-(3-Chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-yl)butyl)isoindoline-1,3-dione (13)

Following procedure A, reaction of 4-(1-(3-chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-hydrogen chloride (150 mg, 0.414 mmol) with 2-(4-bromobutyl)isoindoline-1,3-dione (117 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (DCM/MeOH, 9.8:0.2, v/v); yield: 175 mg (80%); MW = 527.07; formula $C_{31}H_{31}ClN_4O_2$; MS *m/z* 527.50 ($M+H^+$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.94–7.78 (m, 2 H), 7.75–7.62 (m, 2 H), 7.31–7.14 (m, 2 H), 7.06 (dd, *J* = 3.7, 9.1 Hz, 3 H), 6.96–6.82 (m, 2 H), 6.57–6.42 (m, 2 H), 5.25 (s, 2 H), 3.81 (t, *J* = 6.9 Hz, 2 H), 3.24–3.02 (m, 4 H), 2.73–2.58 (m, 4 H), 2.54 (t, *J* = 6.9 Hz, 2 H), 1.94 (quin, *J* = 6.7 Hz, 2 H), 160–1.52 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.5, 145.8, 139.7, 137.3, 134.6, 133.9, 132.3, 130.0, 127.8, 126.7, 126.6, 124.8, 123.2, 122.6, 121.9, 106.6, 104.2, 100.2, 56.2, 53.5, 51.2, 49.3, 36.7, 25.3, 24.1.

2-(5-(4-(1-(3-Chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-yl)pentyl)isoindoline-1,3-dione (14)

Following procedure A, reaction of 4-(1-(3-chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-hydrogen chloride (150 mg, 0.414 mmol) with 2-(5-bromopentyl)isoindoline-1,3-dione (123 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography ($CHCl_3$/PET/MeOH, 77:20:3, v/v/v); yield: 177 mg (79%); MW = 541.09; formula $C_{32}H_{33}ClN_4O_2$; MS *m/z* 541.36 ($M+H^+$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87–7.79 (m, 2 H), 7.72–7.64 (m, 2 H), 7.23–7.15 (m, 2 H), 7.09–7.03 (m, 3 H), 6.96–6.88 (m, 2 H), 6.62–6.57 (m, 1 H), 6.52 (dd, *J* = 0.6, 3.2 Hz, 1 H), 5.24 (s, 2 H), 3.70 (t, *J* = 7.2 Hz, 2 H), 3.38 (d, *J* = 4.4 Hz, 4 H), 2.86 (m, 4 H), 2.64–2.51 (m, 2 H), 1.72 (qd, *J* = 7.1, 13.9 Hz, 4 H), 1.48–1.35 (m, 2 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 145.3, 139.7, 137.3, 134.6, 133.9, 132.1, 130.0, 127.8, 126.8, 126.7, 124.8, 123.2, 122.7, 121.6, 107.0, 104.7, 100.5, 58.2, 53.2, 50.4, 49.6, 37.7, 28.3, 25.5, 24.6.

2-(6-(4-(1-(3-Chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-yl)hexyl)isoindoline-1,3-dione (15)

Following procedure A, reaction of 4-(1-(3-chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-hydrogen chloride (150 mg, 0.414 mmol) with 2-(6-bromohexyl)isoindoline-1,3-dione (128 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography ($CHCl_3$/Hex/MeOH, 78:20:2, v/v/v); yield: 207 mg (90%); MW = 555.12; formula $C_{33}H_{35}ClN_4O_2$; MS *m/z* 555.38 ($M+H^+$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82 (dd, *J* = 3.1, 5.4 Hz, 2 H), 7.68 (dd, *J* = 3.1, 5.4 Hz, 2 H), 7.23–7.15 (m, 2 H), 7.12–7.03 (m, 3 H), 6.96–6.85 (m, 2 H), 6.68–6.52 (m, 2 H), 5.23 (s, 2 H), 3.69 (t, *J* = 7.3 Hz, 2 H), 3.30 (m, 4 H), 2.71 (m, 4 H), 2.52–2.38 (m, 2 H), 1.70 (m, 2 H), 1.57 (m, 2 H), 1.47–1.33 (m, 4 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 145.9, 139.7, 137.4, 134.6, 133.8, 132.1, 130.0, 127.7, 126.7, 126.6, 124.8, 123.1, 122.7, 121.9, 106.8, 104.3, 100.7, 58.7, 53.6, 51.2, 49.6, 38.0, 28.5, 27.2, 26.8, 26.7.

2-(7-(4-(1-(3-Chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-yl)heptyl)isoindoline-1,3-dione (16)

Following procedure A, reaction of 4-(1-(3-chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-hydrogen chloride (150 mg, 0.414 mmol) with 2-(7-bromoheptyl)isoindoline-1,3-dione (134 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (Hex/Et_2O/DCM/MeOH, 40:30:29:1, v/v/v/v); yield: 183 mg (78%); MW = 569.15; formula $C_{34}H_{37}ClN_4O_2$; MS *m/z* 569.41 ($M+H^+$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87–7.81 (m, 2 H), 7.70 (dd, *J* = 3.2, 5.3 Hz, 2 H), 7.22–7.17 (m, 2 H), 7.12–7.03 (m, 3 H), 6.97–6.87 (m, 2 H), 6.60 (d, *J* = 7.4 Hz, 1 H), 6.56 (d, *J* = 3.1 Hz, 1 H), 5.26 (s, 2 H), 3.69 (t, *J* = 7.2 Hz, 2 H), 3.36–3.26 (m, 4 H), 2.77–2.67 (m, 4 H), 2.50–2.38 (m, 2 H), 1.69 (td, *J* = 6.7, 13.5 Hz, 2 H), 1.63–1.50 (m, 2 H), 1.44–1.31 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 145.9, 139.7, 137.4, 134.7, 133.8, 132.1, 130.0, 127.8, 126.7, 126.5, 124.8, 123.1, 122.7, 121.7, 106.8, 104.3, 100.7, 58.8, 53.6, 51.2, 49.6, 38.0, 29.8, 29.1, 28.5, 27.5, 26.7.

2-(8-(4-(1-(3-Chlorobenzyl)-1*H*-indol-4-yl)piperazin-1-yl)octyl)isoindoline-1,3-dione (17)

Following procedure A, reaction of 4-(1-(3-chlorobenzyl)-1*H*-indol-4-yl)-piperazin-1-hydrogen chloride (150 mg, 0.414 mmol) with 2-(8-bromooctyl)isoindoline-1,3-dione (140 mg, 0.414 mmol) in the presence of K_2CO_3 (57 mg, 0.414 mmol) and KI (69 mg, 0.414 mmol) in 20 ml MeCN was performed. Purification: column chromatography (DCM/Hex/acetone/MeOH, 25:19:5:1, v/v/v/v); yield: 167 mg (69%); MW = 583.17; formula $C_{35}H_{39}ClN_4O_2$; MS *m/z* 583.44 ($M+H^+$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.88–7.77 (m, 2 H), 7.72–7.59 (m, 2 H), 7.22–7.01 (m, 5 H), 6.97–6.84 (m, 2 H), 6.66–6.51 (m, 2 H), 5.23 (s, 2 H), 3.76–3.59 (m, 2 H), 3.39–3.23 (m, 4 H), 2.80–2.63 (m, 4 H), 2.51–2.37 (m, 2 H), 1.74–1.62 (m, 2 H), 1.55 (m, 2 H), 1.43–1.28 (m, 8 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.4, 145.9, 139.8, 137.4, 134.6, 133.8, 132.1, 130.0, 127.7, 126.8, 126.5, 124.8, 123.3, 122.7, 122.0, 106.7, 104.2, 100.8, 58.9, 53.7, 51.3, 49.6, 38.0, 29.4, 29.1, 28.6, 27.6, 26.8, 26.7.

4.3 | Biological assays

4.3.1 | Serotonin 5-HT$_6$ receptor binding assay[22]

The assay was performed on membranes from CHO-K1 cells stably transfected with the human 5-HT$_6$ receptor (PerkinElmer, Waltham, MA). All assays were carried out in duplicates. Fifty microliters dimethyl sulfoxide (DMSO) solution (10^{-5} to 10^{-12} M) of the tested compounds, 50 µl [^{3}H]-LSD (final concentration 1.3 nM) and 150 µl diluted membranes (8 µg protein per well) prepared in assay buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA) were transferred to polypropylene 96-well microplate using 96-wells pipetting station Rainin Liquidator (Mettler-Toledo, Columbus, OH). Methiothepin (10 µM) was used to define nonspecific binding. Microplate was covered with a sealing tape, mixed and incubated for 60 min at 37°C. The reaction was terminated by rapid filtration through GF/B filter mate presoaked with 0.5% polyethyleneimine for 30 min. Ten rapid washes with 200 µl 50 mM Tris buffer (4°C, pH 7.4) were performed using automated harvester system Harvester-96 MACH III FM (Tomtec, Chicago, IL). The filter mates were dried at 37°C in forced air fan incubator and then solid scintillator MeltiLex was melted on filter mates at 90°C for 5 min. Radioactivity was counted in MicroBeta2 scintillation counter (PerkinElmer). Data were fitted to a one-site curve-fitting equation with Prism 6 (GraphPad Software, San Diego, CA) and K_i values were estimated from the Cheng −Prusoff equation.

4.3.2 | Functional assays for the 5-HT$_6$ receptor[28]

A cellular aequorin-based functional assay was performed on recombinant CHO-K1 cells expressing mitochondrially targeted aequorin, human GPCR and the promiscuous G protein α16 for 5-HT$_6$. After thawing, cells were transferred to assay buffer (DMEM/HAM's F12 with 0.1% protease free bovine serum albumin [BSA]) and centrifuged. The cell pellet was resuspended in assay buffer and coelenterazine h was added at final concentrations of 5 µM. The cells suspension was incubated at 16°C, protected from light with constant agitation for 16 hr and then diluted with assay buffer to the concentration of 100,000 cells/ml. After 1 hr of incubation, 50 µl of the cells suspension was dispensed using automatic injectors built into the radiometric and luminescence plate counter MicroBeta2 LumiJET (PerkinElmer) into white opaque 96-well microplates preloaded with test compounds (eight concentrations in DMSO). Immediate light emission generated following calcium mobilization was recorded for 60 s. In antagonist mode, after 30 min of incubation the reference agonist was added to the above assay mix and light emission was recorded again. Final concentration of the reference agonist was equal to EC80 (40 nM serotonin).

4.3.3 | In vitro AChE and BuChE inhibition assay

The inhibitory activity was measured according to a method described by Ellman et al.,[34] as modified for 96-well microplates. AChE from *Electrophorus electricus*, BuChE from equine serum, 5,5′-dithiobis-(2-nitrobenzoic acid; DTNB), acetylthiocholine iodide (ATC), and butyrylthiocholine iodide (BTC) were purchased from Sigma-Aldrich, human plasma BuChE was a gift from Vivonics. The enzymes were prepared at a final concentration of 0.384 U/ml, aqueous solutions DTNB at 0.0025 M and ATC/BTC at 0.00375 M. At first, 25 µl of the test compound (or water; i.e., blank samples) was incubated (5 min) in 200 µl of 0.1 M phosphate buffer (pH 8.0) with DTNB (20 µl) and the enzyme (20 µl) at 25°C. After incubation 20 µl of acetylthiocholine iodide or butyrylthiocholine iodide solutions were added to start the reaction. Finally, after 5 min of the reaction, changes in absorbance were measured at 412 nm, using the microplate reader (EnSpire Multimode; PerkinElmer). The IC_{50} values were calculated from seven concentrations of compounds using nonlinear regression (GraphPad Prism 5; GraphPad Software).

4.3.4 | Determination of the antioxidant activity by FRAP assay

The FRAP assay was done according to Benzie and Strain[35] with some modifications. The FRAP reagent was prepared by mixing 10 parts of 300 mmol/l acetate buffer pH 3.6 (3.1 g $CH_3COONa \times 3 H_2O$ and 16 ml CH_3COOH), 1 part of 10 mmol/l 2,4,6-tripyridyl-*s*-triazine (TPTZ) solution in 40 mmol/l HCl, and 1 part of 20 mmol/l $FeCl_3 \times 6H_2O$ solution. Next, 300 µl of the FRAP reagent was mixed with 10 µl of tested compound (solution in ethanol) and incubated at room temperature for 10 min in the dark. The absorbance of the resulting colored product, the ferrous tripyridyltriazine complex, was recorded at 593 nm. Results are presented as the concentration of Fe^{2+} generated in the test. Ascorbic acid was used as reference compound.

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