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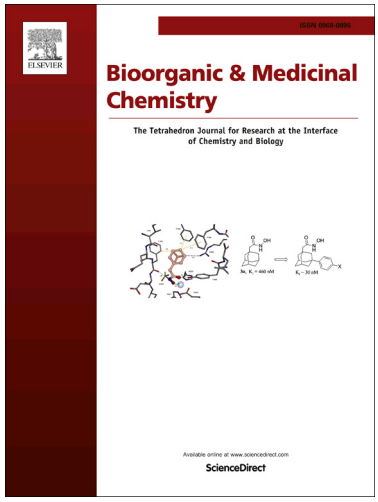

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PII: S0968-0896(15)00069-3
DOI: http://dx.doi.org/10.1016/j.bmc.2015.01.045
Reference: BMC 12048

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 25 November 2014
Revised Date: 22 January 2015
Accepted Date: 25 January 2015

Please cite this article as: Guzior, N., Bajda, M., Rakoczy, J., Brus, B., Gobec, S., Malawska, B., Isoindoline-1,3-dione derivatives targeting cholinesterases: Design, synthesis and biological evaluation of potential anti-Alzheimer's agents, *Bioorganic & Medicinal Chemistry* (2015), doi: http://dx.doi.org/10.1016/j.bmc.2015.01.045

Isoindoline-1,3-dione derivatives targeting cholinesterases: Design, synthesis and biological evaluation of potential anti-Alzheimer's agents

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Abstract

Alzheimer's disease is a fatal neurodegenerative disorder with a complex etiology. Because the available therapy brings limited benefits, the effective treatment for Alzheimer's disease remains the unmet challenge. Our aim was to develop a new series of donepezil-based compounds endowed with inhibitory properties against cholinesterases and β-amyloid aggregation. We designed the target compounds as dual binding site acetylcholinesterase inhibitors with *N*-benzylamine moiety interacting with the catalytic site of the enzyme and an isoindoline-1,3-dione fragment interacting with the peripheral anionic site of the enzyme. The results of pharmacological evaluation lead us to identify a compound **3b** as the most potent and selective human acetylcholinesterase inhibitor (*h*AChE IC_{50} = 0.361 μM). Kinetic studies revealed that **3b** inhibited acetylcholinesterase in non-competitive mode. The result of the parallel artificial membrane permeability assay for the blood-brain barrier indicated that the compound **3b** would be able to cross the blood-brain barrier and reach its biological targets in the central nervous system. The selected compound **3b** represents a potential lead structure for further development of anti-Alzheimer's agents.

Key words:

cholinesterase inhibitors; β-amyloid aggregation; Alzheimer's disease, multi-target-directed ligands, PAMPA-BBB assay

1. Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative disorder with complex etiology that remains the largest unmet challenge in neurology.[1] Worldwide, it is estimated that 36 million people suffer from dementia and this number is still growing.[2]

AD is the most common form of dementia that is characterized by the loss of memory and cognitive functions.[3] The major pathological changes associated with the disease include the extensive loss of neurons (particularly cholinergic) and the presence of altered protein deposits: senile plaques and neurofibrillary tangles.[4] These observations led to two major hypotheses of the disease. Cholinergic hypothesis was proposed in 1976 and it relates memory impairments in AD with the decreased number of cholinergic neurons that is accompanied by a progressive decline in the acetylcholine (ACh) level.[5,6] The amyloid hypothesis relates the cause of the disease with β-amyloid (Aβ) cascade. Aβ is cleaved from the amyloid precursor protein (APP) with two enzymes: β- and γ-secretase[7,8] and Aβ species (especially $A\beta_{1-42}$) are highly fibrillogenic, able to form oligomers and senile plaques.

The available AD drug therapy is based mainly on cholinergic hypothesis and includes cholinesterase inhibitors: donepezil, galantamine and rivastigmine. These drugs mostly inhibit acetylcholinesterase (AChE), the key enzyme in the cholinergic system involved in the hydrolysis of ACh, and less so butyrylcholinesterase (BuChE), the auxiliary enzyme in the cholinergic system.[9] In addition to its hydrolytic function, AChE performs also other functions, such as regulation of neurite growth[10,11], maintenance of synapses[12] and mediation of cell adhesion.[13] Some studies revealed that AChE co-localizes with senile plaques[14], AChE/Aβ complexes display higher neurotoxicity compared with Aβ fibrils[15] and AChE accelerates the assembly of Aβ into amyloid fibrils.[16] Crystallographic studies revealed that AChE has two binding sites: the active site at the bottom of a deep narrow 20 Å gorge – with the AChE catalytic triad and the anionic subsite called the catalytic anionic site (CAS) – and the peripheral anionic site (PAS) near the entrance of the gorge.[17] PAS is believed to promote

the Aβ aggregation process.[18] Finding an additional role of PAS resulted in the development of a novel class of compounds – bivalent AChE inhibitors – that interact with CAS and PAS at the same time.[19] Recently, many cholinesterase inhibitors targeting Aβ aggregation have been developed.[20-24] According to the multi-target-directed ligands (MTDLs) strategy,[25] modulation of multiple biological targets with one compound can be beneficial for treatment of complex diseases.[26] Numerous reviews indicate that searching for ligands with a specific multi-target profile (also called multiple ligands) seems to be the current trend in the development of anti-AD therapy.[27-31]

AD requires a novel therapy because the available treatment is insufficient and no new drugs have been approved since 2004.[32] Currently, 94 drug-candidates are in clinical trials and only 14 in phase III. This relatively small number, followed by high failure rate of AD clinical trials suggest there is an urgent need to increase the number of compounds that enter the AD drug-development process.[33]

Herein, we present the design, synthesis and biological evaluation of a novel series of isoindoline-1,3-dione derivatives as potential cholinesterase and Aβ inhibitors. All the compounds were tested against AChE, BuChE and self-induced $A\beta_{1-42}$ aggregation. We also tested their blood-brain barrier (BBB) permeability using the parallel artificial membrane permeability assay (PAMPA).

2. Results and discussion

2.1. Design

Recently, we have developed a new series of AChE inhibitors that were designed as dual binding site cholinesterase inhibitors able to bind to both the CAS and PAS.[34,35] Those donepezil-based compounds consisted of the isoindoline-1,3-dione moiety, which was reported to interact with the PAS[36] and the benzylamine fragment, which was reported to interact with the CAS (compound 1, Figure 1).[37] In this series, we identified potent and selective human AChE (*h*AChE) inhibitors endowed with additional biological properties such as Aβ aggregation inhibition and neuroprotective effect against Aβ toxicity.

To further explore the biological properties of isoindoline-1,3-dione derivatives, we designed a new series of donepezil-based compounds with *N*-methylbenzylamine fragment (series A, Figure 1). We assumed that the introduction of a methyl group in benzylamine will

provide hydrophobic interactions of this group with an aromatic side chain of Phe330 in AChE. We also introduced a benzene ring into the alkyl chain spacer of compound **1** (series B, Figure 1). We expected that the presence of the aromatic ring in an alkyl chain would provide additional π- π stacking and CH- π interaction with Phe331 and Tyr334 in AChE. Finally, we introduced different substituents in the benzylamine fragment. They were selected based on our previous knowledge[35] that a fluorine atom at position 2 or a chlorine atom at position 3 in the phenyl ring can improve the inhibitory potency towards AChE. We assumed that these modifications would allow us to identify new AChE inhibitors with a multifunctional profile.

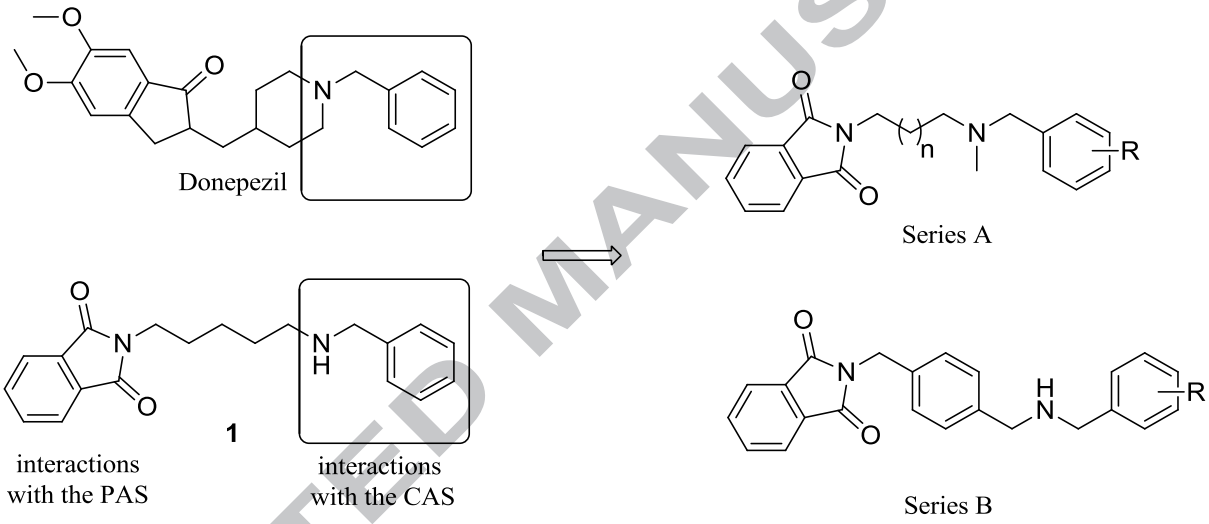
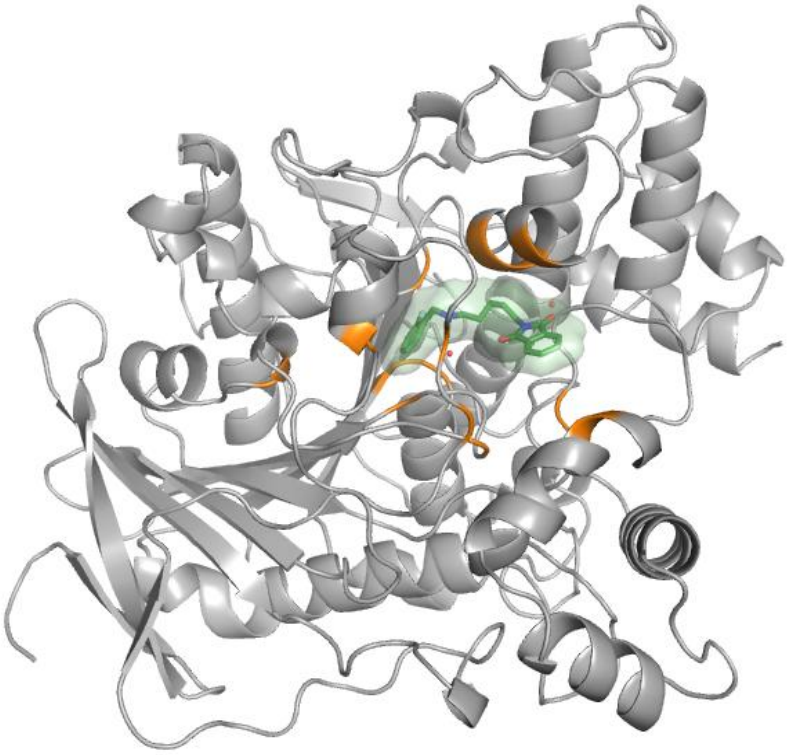


Figure 1. Structure of the isoindolino-1,3-dione derivative **1** and general structures of the designed compounds.

Molecular modeling

In order to examine possible interactions of the designed compounds with the enzyme we performed molecular modeling studies (Figure 2). According to the docking studies, all the designed compounds were arranged along the gorge of AChE to create optimal interactions with both the CAS and PAS at the same time. The phthalimide fragment was engaged in π-π stacking with Trp279 and CH- π interactions with Tyr70 in the PAS. Both carbonyl groups of phthalimide formed H-bonds – with water molecule and Tyr121, respectively. The benzylamine fragment was responsible for π-π stacking with Trp84 in the CAS. The protonated amine group formed cation- π interactions with Phe330 and a hydrogen bond network with Tyr121 *via* water molecule. It was noted that changing secondary amine to tertiary (with *N*-methyl substituent) decreased the basicity of the compounds and weakened

cation-π interaction, but on the other hand provided hydrophobic interactions of *N*-methyl group with the aromatic side chain of Phe330. The alkyl linker formed hydrophobic interactions with aromatic residues such as Phe290, Phe331 and Tyr334 in the middle of the active gorge. When we introduced a phenyl group into the linker (series B, Figure 1), we expected that it would provide extra interactions in the middle of the active site gorge. Further modifications included the introduction of different halogen atoms in the benzylamine moiety. A fluorine atom at *ortho* position created a hydrogen bond with a hydroxyl group of Ser200 while a chlorine atom at *meta* position made interactions with a carboxyl group of Glu199 and backbone of Gly441.



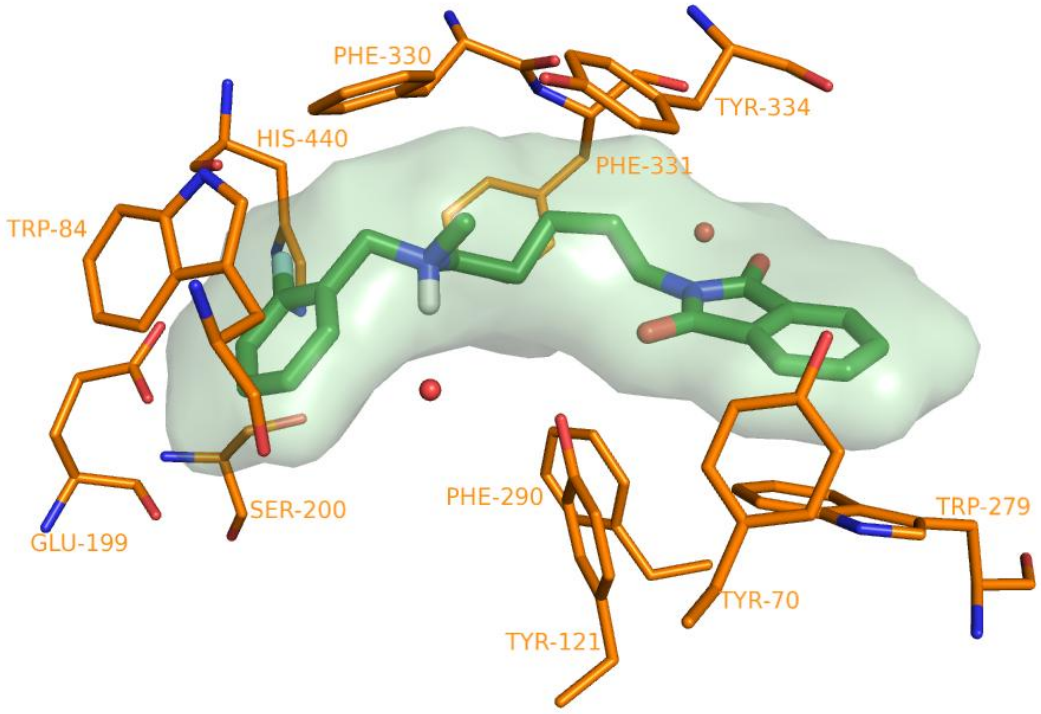
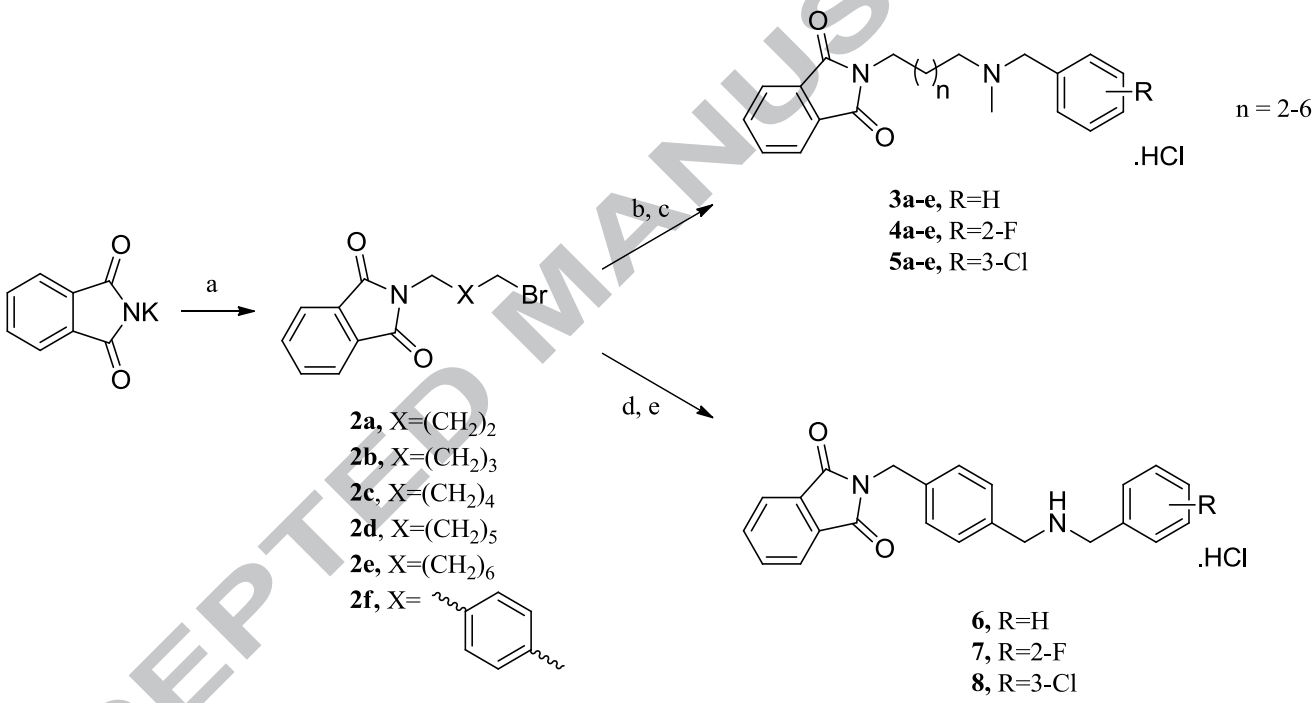


Figure 2. The binding mode of compound **4b** in the active site of acetylcholinesterase obtained by docking. General view (top; orange represents amino acids involved in binding) and detailed representation (bottom).

2.2. Chemistry

The synthesis of the designed isoindoline-1,3-dione derivatives was accomplished as shown in Scheme 1. Compounds **2a-e** were the key intermediates, prepared as previously described.[38] Phthalimide potassium was alkylated with the appropriate α,ω-dibromoalkane or α,α′-dibromo-*p*-xylene in the presence of a phase transfer catalyst, tetra-*n*-butylammonium bromide (TBAB). The reactions were carried out in acetonitrile for 15 h under reflux. In the next step, compounds **2a-e** were used as alkylating agents in reactions with *N*-methylbenzylamine or its *2*-fluoro or *3*-chloro derivatives. Reactions were carried out in acetonitrile in the presence of potassium carbonate for 16 h, under reflux. Following purification by silica gel column chromatography, the final 2-(ω-(*N*-methylbenzylamino)alkyl)isoindoline-1,3-dione derivatives **3a-e** – **5a-e** were isolated with satisfactory yields (72 – 99%) and converted into hydrochloride salts. Compound **2f** was used as an alkylating agent in reactions with an appropriate *N*-benzylamine according to the method reported previously.[34] Reactions were carried out in acetonitrile in the presence of potassium carbonate for 48 h at room temperature. After purification by silica gel column

chromatography, the final 2-(4-((benzylamino)methyl)benzyl)isoindoline-1,3-dione derivatives **6** – **8** were isolated with satisfactory yields (39 – 48%) and converted into hydrochloride salts.



Scheme 1. Synthesis of the target isoindoline-1,3-dione derivatives. Reagents and conditions: (a) α,ω-dibromoalkane or α,α′-dibromo-*p*-xylene, TBAB, MeCN, reflux, 15 h; (b) *N*-methylbenzylamine or substituted *N*-methylbenzylamine , K_2CO_3, MeCN, reflux, 16 h; (c) HCl in 2-propanol (d) *N*-benzylamine or substituted *N*-benzylamine, K_2CO_3, MeCN, rt, 48 h; (e) HCl in 2-propanol.

2.3. Biological activity evaluation

We determined the inhibitory potency of the compounds against cholinesterases using the method of Ellman.[39] We tested the compounds against AChE from *Electrophorus electricus*

(*Ee*AChE) and BuChE from equine serum (*Eq*BuChE). In the next step, we tested the active compounds against human recombinant AChE (*h*AChE). Compounds with inhibitory potency lower than 50% at a screening concentration (10 μM) were considered as inactive. The IC_{50} values are given in Table 1. Compound **1**, donepezil and tacrine were included as a positive control.

In the series A (compounds **3a-e**, **4a-e** and **5a-e** with different lengths of linkers) we identified potent *Ee*AChE inhibitors with IC_{50} values in the low micromolar to nanomolar range. In each subseries we observed that the most active were the compounds with five or six carbon atom linkers. Compound **4b** (a five carbon linker, 2-fluoro derivative) was found to be the most potent and selective *Ee*AChE inhibitor with an IC_{50} value of 67 nM. The activity significantly decreased with shortening of the tether (to a four carbon linker) and slightly decreased with the elongation of the tether (to seven and eight carbon linkers). Regarding the influence of substituents in benzylamine on *Ee*AChE inhibitory potency, we confirmed that the introduction of a fluorine atom at position 2 was beneficial for inhibitory potency (compound **4b**) due to an extra hydrogen bond in the CAS. In the case of *m*-chloro derivatives the halogen atom could be engaged in a halogen bond interaction, however it did not increase the potency.

For *h*AChE we observed similar structure-activity relationship (SAR) trends with slightly higher IC_{50} values. The most active were compounds with five carbon atom linkers and among them compound **3b** with an unsubstituted benzylamine fragment was the most potent (IC_{50} = 361 nM). In the case of a six carbon atom alkyl chain, we observed a significant decrease in potency compared with *Ee*AChE activity. This is due to the difference in the structure of both enzymes. In the case of *h*AChE, the six carbon linker prevents phthalimide and benzylamine fragments from optimal interactions with Trp286 and Trp86, at the same time. We observed that the introduction of fluorine or chlorine atoms in the phenyl ring decreased the potency against *h*AChE.

In the case of *Eq*BuChE, we observed that the potency increased according to the length of the linker and the most potent *Eq*BuChE inhibitors were compounds **3e** and **5e** with octamethylene linkers (IC_{50} = 0.543 μM and IC_{50} = 0.909 μM, respectively). The longer linker provided higher flexibility of a molecule and enabled the compounds occurr in bent conformation. Benzylamine and phthalimide fragments better fit to Trp82 from CAS and Leu286, Val288 and Trp231, forming lipophilic pocket, respectively. Moreover, the octamethylene tether created more hydrophobic interactions.

Regarding the introduction of a phenyl group in the alkyl linker in the series B, we observed that this modification deteriorated the potency against *Ee*AChE but improved the potency against BuChE. In the case of AChE, the length of the alkyl linker between a phenyl group and a phthalimide moiety was probably too short to provide a good fit of phthalimide to PAS. On the contrary in the case of BuChE π- π stacking and CH- π interactions of the phenyl group in the middle of the active gorge were strong enough to inhibit the enzyme regardless the length of the linker. Through the replacement of methylene groups by aromatic fragments we can reach a more balanced profile of inhibition towards both cholinesterases. The introduction of a fluorine atom was beneficial for inhibition of both AChE and BuChE, while a chlorine atom was beneficial only for BuChE.

Targeting the self-induced Aβ aggregation represents an emerging approach for discovering drug candidates for AD. $A\beta_{1-42}$ has a high tendency to form fibrils and aggregates. Also, its oligomers are neurotoxic and cause membrane disruption in neuronal cells.[40] Within the previous series of compounds[35] we identified potent inhibitors of self-induced Aβ aggregation (62 to 66.0% at 10 μM), however we did not observed the relevant structure-activity relationships among the tested compounds. This may suggest that the mechanisms of Aβ aggregation inhibition are nonspecific. In the presented series of compounds we expected to identify more Aβ aggregation inhibitors. We tested all the compounds in the thioflavin T based assay to investigate their ability to inhibit self-induced Aβ aggregation.[41] We screened them at relatively low concentration (10 μM), to obtain the results that are comparable with the Ellman's assay. The tested compounds turned out to be very poor inhibitors of self-induced Aβ aggregation (percentages of inhibition lower than 5% at 10 μM).

Summarizing the results of biological evaluation of the presented series of compounds, we can conclude that we identified potent and selective AChE inhibitors among *N*-methylbenzylamine derivatives. We noticed that changing a secondary amine (benzylamine) to tertiary amine (*N*-methylbenzylamine) allowed us to develop potent AChE inhibitors. They displayed similar potency to a compound **1** and donepezil. We also observed that incorporation of a benzene ring in an alkyl linker improved the potency against BuChE in comparison with the compound **1**.

Table 1. Inhibitory potency of isoindoline-1,3-dione derivatives against *Ee*AChE, *h*AChE and *Eq*BuChE.

Compd	*Ee*AChE IC_{50} [μM][a]	*Eq*BuChE IC_{50} [μM][a]	SI[b]	*h*AChE IC_{50} [μM][a]
3a	2.326±0.090	nd[c]	-	nd[c]
3b	0.080±0.001	nd[c]	-	0.361±0.010
3c	0.089±0.002	nd[c]	-	1.831±0.048
3d	0.172±0.009	1.011±0.025	5.9	0.997±0.018
3e	0.139±0.006	0.543±0.011	3.9	0.860±0.022
4a	1.522±0.018	nd[c]	-	nd[c]
4b	0.067±0.001	nd[c]	-	0.525±0.010
4c	0.075±0.001	10.460±0.097	139.5	1.992±0.037
4d	0.525±0.048	7.157±0.062	13.6	0.625±0.012
4e	0.661±0.012	4.678±0.047	7.1	0.644±0.010
5a	1.430±0.051	nd[c]	-	nd[c]
5b	0.110±0.003	5.826±0.057	53.0	0.537±0.012
5c	0.095±0.001	2.435±0.080	25.6	1.189±0.075
5d	0.500±0.016	1.499±0.052	3.0	1.303±0.082
5e	1.363±0.050	0.909±0.017	0.7	1.044±0.028
6	nd[c]	2.080±0.028	-	nd[c]
7	2.582±0.078	2.899±0.041	1.1	nd[c]
8	6.311±0.320	1.319±0.079	0.3	nd[c]
1[d]	0.078±0.001	nd[c]	-	0.202±0.004
Donepezil	0.010±0.001	1.830±0.176	183	0.006±0.001
Tacrine	0.024±0.001	0.002±0.001	<0.1	0.131±0.002

[a] IC_{50} values are expressed as mean ± standard error of the mean (SEM) of at least three experiments.
[b] SI – selectivity index; IC_{50} *Eq*BuChE/IC_{50} *Ee*AChE
[c] IC_{50} value not determined; % inhibition at 10 μM lower than 50%.
[d] Data from Ref.[35]

2.3. Kinetic studies

We performed kinetic studies to investigate the mechanism of *Ee*AChE inhibition for compound **3b**. Substrate-velocity curves in the absence and presence of the tested compound were recorded. Analysis of Lineweaver-Burk double reciprocal plots showed that with increasing concentrations of the tested inhibitor, x-intercepts were the same (unaffected K_m) but slopes were different (decreased V_{max}) (Figure. 3). This means that compound **3b** is a non-competitive AChE inhibitor, the same as donepezil[42]. The non-competitive type of inhibition may indicate preferential binding to the PAS rather than the CAS.

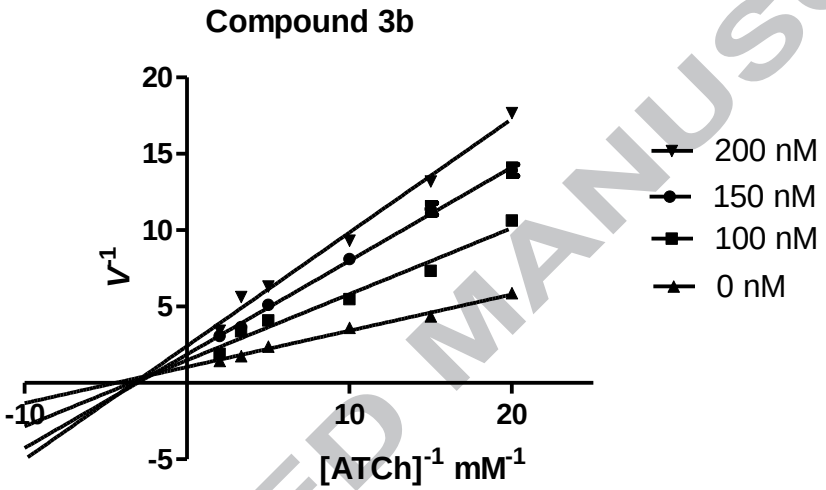


Figure 3. Lineweaver–Burk plots illustrating non-competitive type of *Ee*AChE inhibition by compound **3b**. ATCh = acetylthiocholine; *V* = initial velocity rate. Lines were derived from a weighted least-square analysis of data points.

2.4. Blood brain barrier permeability assay

The permeability through BBB of the anti-AD drug candidates should be assessed as early as possible in the drug discovery process. We used a parallel artificial membrane permeability assay for BBB (PAMPA-BBB) to determine the brain permeability of the synthesized compounds.[43] We used 7 commercial drugs as the reference compounds and we established the following ranges of permeability: $\log P_e > -5.4$ for compounds with high BBB permeability; $\log P_e < -7.3$ for compounds with low permeability and $-7.3 > \log P_e > -5.4$ for compounds with uncertain BBB permeability. We chose compounds **3b**, **4b** and **5b** for this

assay. The results of the PAMPA-BBB assay (Table 2) indicates that all the tested compounds would be able to cross the BBB and reach their biological targets in the CNS.

Table 2. Permeability ($logP_e$) in the PAMPA-BBB assay for commercial drugs and the selected compounds with prediction of their penetration to the CNS.

Compd	$LogP_e$ [a]	Prediction	Compd	$LogP_e$ [b]	Prediction
Theophylline	-7.3	CNS-	**3b**	-3.7	CNS+
Verapamil.HCl	-3.5	CNS+	**4b**	-3.6	CNS+
Lidocaine	-4.3	CNS+	**5b**	-3.4	CNS+
Quinidine.HCl	-3.5	CNS+			
Progesterone	-5.1	CNS+			
Corticosterone	-5.4	CNS±			
Propranolol.HCl	-3.5	CNS+			

[a] Results are the mean of three replicates (n = 3).
[b] Results are the mean of two replicates (n = 2).

3. Conclusions

In this paper we have presented a new series of isoindoline-1,3-dione cholinesterase inhibitors. We designed the target compounds based on the results of our previous studies and with the assistance of molecular modeling. The donepezil-based compounds consist of an isoindoline-1,3-dione fragment connected to *N*-methylbenzylamine by alkyl linkers of different lengths or to benzylamine by the alkyl linker containing a phenyl group. Halogen atoms were introduced in a phenyl group in benzylamine at positions validated in our previous studies.[35] We tested all the compounds against cholinesterases and self-induced $A\beta_{1-42}$ aggregation. The results allowed us to identify selective and potent *h*AChE inhibitors among *N*-methylbenzylamine derivatives. The most active was compound **3b**, which comprises of *N*-methylbenzylamine and a five carbon atom linker. We also identified compounds with balanced inhibitory activity towards AChE and BuChE: compounds **3e** and **5e**. Kinetic studies revealed that the developed compounds are non-competitive AChE inhibitors. According to the results of self-induced Aβ aggregation assay, these compounds are poor aggregation inhibitors at 10 μM. Finally, the preliminary blood brain barrier permeability assay results showed that the compounds would be able to reach the CNS.

Taking into account all the results presented in this paper, we developed the novel cholinesterase inhibitors with the ability to reach their targets in the CNS. They serve as the lead compounds for further development as potential therapeutics for AD.

4. Experimental part

4.1.Molecular modeling

Three-dimensional structures of ligands were built with the Corina online tool.[44] Subsequently, by using Sybyl 8.0[45], Gasteiger-Marsili charges were assigned following checks of atom types and protonation of compounds. Finally, ligand structures were saved in the mol2 format. Docking was performed with AChE from 1EVE and BuChE from the 1P0I crystal structure[46] using the Gold 4.1 program.[47] The targets were prepared as follows: all histidine residues were protonated at Ne, hydrogen atoms added, ligand molecules removed, and binding sites defined as all amino acid residues within 10 Å from donepezil (for AChE) and within 20 Å from the glycerol molecule present in the active center of BuChE. The presence of some conserved water molecules was also taken into account. A standard set of genetic algorithms with population size 100, number of operations 100 000 and clustering tolerance of 1 Å were applied. As a result, 10 ligand conformations were obtained and sorted according to ChemScore function values. Results were visualized by PyMOL.

4.2. Chemistry

4.2.1. General methods

^{1}H NMR spectra were recorded on Varian Mercury 300 at 300 MHz. The chemical shifts for ^{1}H NMR are referenced to TMS via residual solvent signals (^{1}H, $CDCl_3$ at 7.26 ppm, DMSO-d_6 at 2.50 ppm). Mass spectra (MS) were obtained on an UPLC-MS/MS system consisting of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Analytical thin layer chromatography was done using aluminum sheets precoated with silica gel 60 F254. Column chromatography was performed on Merck silica gel 60 (63 – 200 μm). Flash chromatography was performed on Isolera™ Spektra (Biotage). 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 x 100 mm) at 214 nm 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%.

The following compounds: 2-(4-bromobutyl)isoindoline-1,3-dione **(2a)**[38], 2-(5-bromopentyl)isoindoline-1,3-dione **(2b)**[38], 2-(6-bromohexyl)isoindoline-1,3-dione **(2c)**[38], 2-(7-bromoheptyl)isoindoline-1,3-dione **(2d)**[38], 2-(8-bromooctyl)isoindoline-1,3-dione **(2e)**[38] and 2-(4-(bromomethyl)benzyl)isoindoline-1,3-dione **(2f)**[48] had been previously reported.

4.2.2. General procedure for the preparation of hydrochloride salts

The hydrochloride salts were prepared by dissolving the compounds in a minimum quantity of dichloromethane. Then the solution was treated with 5 M solution of HCl in 2-propanol, evaporated under reduced pressure, washed with pentane and dried.

*4.2.3. General procedure for the synthesis of compounds **(3a-e – 5a-e)***

2-(ω-Bromoalkyl)-isoindoline-1,3-dione (1 eq) with *N*-methylbenzylamine (1 eq) or its substituted analog in the presence of K_2CO_3 (3 eq) was stirred in acetonitrile under reflux for 16 h. Once the reaction was finished, the solvent was evaporated under vacuum, producing a residue that was further dissolved in 50 mL of 5 M NaOH and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (dichloromethane to dichloromethane/methanol 98:2), yielding a yellow oil. The final product was obtained in the form of hydrochloride salt. The following compounds were obtained.

*4.2.4. 2-(4-(Benzyl(methyl)amino)butyl)isoindoline-1,3-dione **(3a)***

Following the General procedure, reaction of 2-(4-bromobutyl)isoindoline-1,3-dione **(2a)** (564 mg, 2 mmol) with *N*-methylbenzylamine (260 μL, 2 mmol) and K_2CO_3 (829 mg, 6 mmol) in acetonitrile (30 mL), after 16 h, column chromatography and preparation the salt gave product **3a** (708 mg, 98%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.29; mp 107 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.52 – 1.66 (m, 2H), 1.72 – 1.80 (m, 2H), 2.58 (d, 2H, J = 4.85 Hz), 2.90 – 3.16 (m, 2H), 3.56 (t, 2H, *J* = 6.86 Hz), 4.19 (dd, 1H, *J*1 = 6.04, *J*2=13], 4.33 (dd,1H, *J*1 = 4 .57, *J*2 = 12.96),7.40 – 7.42 (m, 3H), 7.60 – 7.63 (m, 2H), 7.81 – 7.89 (m, 4H), 11.06 (s br, 1H); MS: m/z 323.18 [M+H$^+$].

*4.2.5. 2-(5-(Benzyl(methyl)amino)pentyl)isoindoline-1,3-dione **(3b)***

Following the General procedure, reaction of 2-(5-bromopentyl)isoindoline-1,3-dione **(2b)** (590 mg, 2 mmol) with *N*-methylbenzylamine (260 μL, 2 mmol) and K_2CO_3 (829 mg, 6 mmol) in acetonitrile (30 mL), after 16 h, column chromatography and preparation the salt gave product **3b** (719 mg, 97%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.26; mp 102 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.23 – 1.27 (m, 2H), 1.55 – 1.60 (m, 2H), 1.72 – 1.80 (m, 2H), 2.57 (d, 3H, *J* = 4.87), 2.84 – 3.02 (m, 2H), 3.54 (t, 2H, *J* = 6.91), 4.13 – 4.19 (dd, 1H, *J*1 = 5.99, *J*2 = 12.99), 4.28 – 4.34 (dd, 1H, *J*1 = 4.62, *J*2 = 12.99), 7.40 – 7.42 (m, 3H), 7.58 – 7.61 (m, 2H), 7.79 – 7.86 (m, 4H), 10.95 (s br 1H); MS: m/z 337.20 [M+H$^+$].

*4.2.6. 2-(6-(Benzyl(methyl)amino)hexyl)isoindoline-1,3-dione **(3c)***

Following the General procedure, reaction of 2-(6-bromohexyl)isoindoline-1,3-dione **(2c)** (620 mg, 2 mmol) with *N*-methylbenzylamine (260 μL, 2 mmol) and K_2CO_3 (829 mg, 6 mmol) in acetonitrile (30 mL), after 16 h, column chromatography and preparation the salt gave product **3c** (771 mg, 99%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.20; mp 130 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.20 – 1.33 (m, 4H), 1.55 – 1.59 (m, 2H),1.72 – 1.75 (m, 2H), 2.58 (d, 3H, *J* = 4.85), 2.81 – 3.07 (m, 2H), 3.54 (t, 2H *J* = 6.97), 4.16 – 4.23 (dd, 1H, *J*1= 5.87, *J*2 = 12.98), 4.29 – 4.35 (dd, 1H, *J*1 = 4.74, *J*2=12.96), 7.41 – 7.43 (m, 3H), 7.61 – 7.64 (m, 2H), 7.80 – 7.87 (m, 4H), 11.24 (s br, 1H); MS: m/z 351.23 [M+H$^+$].

*4.2.7. 2-(7-(Benzyl(methyl)amino)heptyl)isoindoline-1,3-dione **(3d)***

Following the General procedure, reaction of 2-(7-bromoheptyl)isoindoline-1,3-dione **(2d)** (648 mg, 2 mmol) with *N*-methylbenzylamine (260 μL, 2 mmol) and K_2CO_3 (829 mg, 6 mmol) in acetonitrile (30 mL), after 16 h, column chromatography and preparation the salt gave product **3d** (723 mg, 90%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.21; mp 125 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.20 – 1.27 (m, 6H), 1.52 – 1.56 (m, 2H), 1.67 – 1.72 (m, 2H), 2.57 (d, 3H, *J* = 4.87), 2.81 – 4.07 (m, 2H), 3.52 (t, 2H, *J* = 7.09), 4.14 – 4.21 (dd, 1H, *J*1 = 5.95, *J*2 = 12.99), 4.28 – 4.34 (dd, 1H, *J*1 = 4.74, *J*2 =12.98), 7.39 – 7.42 (m, 3H), 7.59 – 7.62 (m, 2H), 7.79 – 7.85 (m, 4H), 11.01 (s br, 1H); MS: m/z 365.26 [M+H$^+$].

*4.2.8. 2-(8-(Benzyl(methyl)amino)octyl)isoindoline-1,3-dione **(3e)***

Following the General procedure, reaction of 2-(8-bromooctyl)isoindoline-1,3-dione **(2e)** (676 mg, 2 mmol) with *N*-methylbenzylamine (260 μL, 2 mmol) and K_2CO_3 (829 mg, 6

mmol) in acetonitrile (30 mL), after 16 h, column chromatography and preparation the salt gave product **3e** (657 mg, 75%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.19; mp 137 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.19 – 1.22 (m, 8H), 1.52 – 1.57 (m, 2H), 1.66 – 1.69 (m, 2H), 2.57 (d, 3H, J = 4.86), 2.81 – 3.00 (m, 2H), 3.53 (t, 2H, *J* =7.07), 4.14 – 4.20 (dd, 1H, *J*1 = 5.97, *J*2 = 12.97), 4.28 – 4.34 (dd, 1H, *J*1 = 4.72, *J*2 = 12.99), 7.40 – 7.43 (m, 3H), 7.57 – 7.61 (m, 2H), 7.81 – 7.86 (m, 4H), 10.85 (s br, 1H); MS: m/z 379.28 [M+H$^+$].

4.2.9. 2-(4-((2-Fluorobenzyl)(methyl)amino)butyl)isoindoline-1,3-dione ***(4a)***

Following the General procedure, reaction of 2-(4-bromobutyl)isoindoline-1,3-dione **(2a)** (282 mg, 1 mmol) with 2-fluoro-*N*-methylbenzylamine (133 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **4a** (278 mg, 74%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.43; mp 137 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.59 – 1.67 (m, 2H), 1.72 – 1.82 (m, 2H), 2.64 (d, 3H, *J* = 4.31), 2.97 – 3.18 (m, 2H), 3.58 (t, 2H, *J* = 6.78), 4.22 – 4.28 (dd, 1H, *J*1 = 5.60, *J2* = 13.32), 4.35 – 4.41 (dd, 1H, *J*1 = 3.43, *J*2 = 13.38), 7.27 – 7.35 (m, 2H), 7.48 – 7.51 (m, 1H), 7.53 – 7.56 (m, 1H), 7.73 – 7.90 (m, 4H), 10.82 (s br, 1H); MS: m/z 341.26 [M+H$^+$].

4.2.10. 2-(5-((2-Fluorobenzyl)(methyl)amino)pentyl)isoindoline-1,3-dione ***(4b)***

Following the General procedure, reaction of 2-(5-bromopentyl)isoindoline-1,3-dione **(2b)** (295 mg, 1 mmol) with 2-fluoro-*N*-methylbenzylamine (133 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **4b** (331 mg, 85%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.31; mp 138 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.21 – 1.34 (m, 2H), 1.56 – 1.65 (m, 2H), 1.75 – 1.80 (m, 2H), 2.63(s 3H), 2.98 – 3.05 (m, 2H), 3.57 (t, 2H, *J* = 6.89), 4.22 – 4.27 (dd, 1H, *J*1 = 3.89, *J*2 = 13.04), 4.37 (d, 1H, *J* = 13.11), 7.27 – 7.34 (m, 2H), 7.48 – 7.58 (m, 1H), 7.76 – 7.83 (m, 1H), 7.84 – 7.88 (m, 4H), 11.00 (s br, 1H); MS: m/z 355.29 [M+H$^+$].

4.2.11. 2-(6-((2-Fluorobenzyl)(methyl)amino)hexyl)isoindoline-1,3-dione ***(4c)***

Following the General procedure, reaction of 2-(6-bromohexyl)isoindoline-1,3-dione **(2c)** (310 mg, 1 mmol) with 2-fluoro-*N*-methylbenzylamine (133 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **4c** (336 mg, 83%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.33; mp 135 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.23 – 1.33 (m, 4H), 1.55 – 1.59 (m, 2H),

1.71 – 1.75 (m, 2H), 2.62 (d, 3H, *J*=2.61), 2.96 – 3.04 (m, 2H), 3.54 (t,2H, *J* = 6.98), 4.20 – 4.26 (dd, 1H, *J*1 = 4.59, *J*2 = 12.95), 4.36 (d, 1H, *J* = 12.83), 7.25 – 7.32 (m, 2H), 7.47 – 7.50 (m, 1H), 7.52 – 7.54 (m, 1H), 7.69 – 7.85 (m, 4H), 10.80 (s br, 1H); MS: m/z 369.31 [$M+H^+$].

4.2.12. 2-(7-((2-Fluorobenzyl)(methyl)amino)heptyl)isoindoline-1,3-dione ***(4d)***

Following the General procedure, reaction of 2-(7-bromoheptyl)isoindoline-1,3-dione **(2d)** (324 mg, 1 mmol) with 2-fluoro-*N*-methylbenzylamine (133 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **4d** (302 mg, 72%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.32; mp 142 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.21 – 1.26 (m, 6H), 1.53 – 1.58 (m, 2H), 1.70 – 1.75 (m, 2H), 2.59 (d, 3H, J = 2.74), 2.89 – 2.99 (m, 2H), 3.51 (t, 2H, *J* = 7.09), 4.21-4.24 (dd, 1H, *J*1 = 4.95, *J*2 = 12.98), 4.36 (d, 1H, *J* = 12.77), 7.25 – 7.33 (m, 2H), 7.51 – 7.58 (m, 1H), 7.73 – 7.79 (m, 1H), 7.82 – 7.87 (m, 4H), 10.95 (s br, 1H); MS: m/z 385.33 [$M+H^+$].

4.2.13. 2-(8-((2-Fluorobenzyl)(methyl)amino)octyl)isoindoline-1,3-dione ***(4e)***

Following the General procedure, reaction of 2-(8-bromooctyl)isoindoline-1,3-dione **(2e)** (338 mg, 1 mmol) with 2-fluoro-*N*-methylbenzylamine (133 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **4e** (402 mg, 93%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.34; mp 136 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.20 – 1.27 (m, 8H), 1.53 – 1.58 (m, 2H), 1.70 – 1.75 (m, 2H), 2.62 (d, 3H, *J* = 2.90), 2.94 – 3.01 (m, 2H), 3.53 (t, 2H, *J* = 7.06), 4.21 – 4.27 (dd, 1H, *J*1 = 4.97, *J*2 = 13.08), 4.36 (d, 1H, *J* = 12.40), 7.25 – 7.33 (m, 2H), 7.47 – 7.54 (m, 1H), 7.74 – 7.79 (m, 1H), 7.81 – 7.86 (m, 4H), 10.89 (s br, 1H); MS: m/z 397.36 [$M+H^+$].

4.2.14. 2-(4-((3-Chlorobenzyl)(methyl)amino)butyl)isoindoline-1,3-dione ***(5a)***

Following the General procedure, reaction of 2-(4-bromobutyl)isoindoline-1,3-dione **(2a)** (282 mg, 1 mmol) with 3-chloro-*N*-methylbenzylamine (146 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **5a** (357 mg, 91%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.48; mp 172 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.56 – 1.64 (m, 2H), 1.69 – 1.80 (m, 2H), 2.57 (d, 3H, *J* = 3.89), 2.91 – 3.09 (m, 2H), 3.56 (t, 2H, *J* = 6.75), 4.15 – 4.21 (dd, 1H, *J*1 =

5.87, *J*2=12.84), 4.32 – 4.37 (dd, 1H, *J*1 = 3.65, *J*2 = 13.03), 7.41 – 7.49 (m, 2H), 7.55 – 7.58 (m, 1H), 7.74 (s , 1H), 7.81 – 7.85 (m, 4H), 10.93 (s br, 1H); MS: m/z 357.28 [M+H$^+$].

4.2.15. 2-(5-((3-Chlorobenzyl)(methyl)amino)pentyl)isoindoline-1,3-dione ***(5b)***

Following the General procedure, reaction of 2-(5-bromopentyl)isoindoline-1,3-dione **(2b)** (295 mg, 1 mmol) with 3-chloro-*N*-methylbenzylamine (146 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **5b** (377 mg, 93%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.62; mp 177 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.24 – 1.31 (m, 2H), 1.56 – 1.63 (m, 2H), 1.65 – 1.81 (m, 2H), 2.60 (d, 3H, *J* = 3.71), 2.90 – 3.05 (m, 2H), 3.57 (t, 2H, *J* = 6.89), 4.16 – 4.26 (dd, 1H, *J*1 = 5.70, *J*2 = 12.84), 4.33 – 4.39 (dd, 1H, *J*1 = 3.31, *J*2 = 13.05), 7.46 – 7.49 (m, 2H), 7.59 (d, 1H, *J* = 6.84), 7.76 (s, 1H), 7.81 – 7.88 (m, 4H), 10.92 (s br, 1H); MS: m/z 371.24 [M+H$^+$].

4.2.16. 2-(6-((3-Chlorobenzyl)(methyl)amino)hexyl)isoindoline-1,3-dione ***(5c)***

Following the General procedure, reaction of 2-(6-bromohexyl)isoindoline-1,3-dione **(2c)** (310 mg, 1 mmol) with 3-chloro-*N*-methylbenzylamine (146 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **5c** (399 mg, 95%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.50; mp 150 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.25 – 1.29 (m, 4H), 1.56 – 1.61 (m, 2H), 1.69 – 1.75 (m, 2H), 2.58 (d, 3H, *J* = 4.19), 2.88 – 3.04 (m, 2H), 3.54 (t, 2H, *J* = 6.99), 4.17 – 4.26 (dd, 1H, *J*1 = 5.88, *J*2 = 12.97), 4.33 – 4.39 (dd, 1H, *J*1=3.97, *J*2 = 13.00), 7.46 – 7.52 (m, 2H), 7.58 – 7.60 (m, 1H), 7.76 (s, 1H), 7.81 – 7.88 (m, 4H), 10.93 (s br, 1H); MS: m/z 385.33 [M+H$^+$].

4.2.17. 2-(7-((3-Chlorobenzyl)(methyl)amino)heptyl)isoindoline-1,3-dione ***(5d)***

Following the General procedure, reaction of 2-(7-bromoheptyl)isoindoline-1,3-dione **(2d)** (324 mg, 1 mmol) with 3-chloro-*N*-methylbenzylamine (146 μL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **5d** (412 mg, 95%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.58; mp 135 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.20 – 1.28 (m, 8 H), 1.55 – 1.62 (m, 2H),

1.67 – 1.72 (m, 2H), 2.61 (d, 3H, *J* = 2.59), 2.90 – 3.06 (m, 2H), 3.56 (t, 2H, *J* = 7.07), 4.17 – 4.23 (dd, 1H, *J*1 = 5.37, *J*2 = 12.74), 4.33 – 4.39 (dd, 1H, *J*1 = 2.35, *J*2 = 13.25), 7.45 – 7.59 (m, 3H), 7.75 (s, 1H), 7.83 – 7.88 (m, 4H), 10.74 (s br, 1H); MS: m/z 399.29 [M+H^+].

*4.2.18. 2-(8-((3-Chlorobenzyl)(methyl)amino)octyl)isoindoline-1,3-dione **(5e)***

Following the General procedure, reaction of 2-(8-bromooctyl)isoindoline-1,3-dione **(2e)** (338 mg, 1 mmol) with 3-chloro-*N*-methylbenzylamine (146 µL, 1 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (15 mL), after 16 h, column chromatography and preparation the salt gave product **5e** (403 mg, 90%) as a white solid: R_f (DCM/MeOH, 95:5) = 0.51; mp 164 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.20 – 1.33 (m, 6H), 1.54 – 1.60 (m, 2H), 1.66 – 1.73 (m, 2H), 2.58 (d, 3H, *J* = 4.68), 2.87 – 3.07 (m, 2H), 3.54 (t, 2H, *J* = 7.06), 4.15 – 4.21 (dd, 1H, *J*1 = 6.12, *J*2 = 12.98), 4.31 – 4.37 (dd, 1H, *J*1 = 4.39, *J*2 = 13.01), 7.43 – 7.51 (m, 2H), 7.55 – 7.57 (m, 1H), 7.73 (s, 1H), 7.79 – 7.86 (m, 4H), 10.75 (s br, 1H) MS: m/z 413.32 [M+H^+].

*4.2.19. General procedure for the synthesis of compounds **(6 – 8)***

2-(4-(Bromomethyl)benzyl)isoindoline-1,3-dione (1 eq) with threefold excess of benzylamine (3 eq) in the presence of K_2CO_3 (3 eq) was stirred in acetonitrile at room temperature for 48 h. Once the reaction was finished, the solvent was evaporated under vacuum, producing a residue that was further dissolved in 20 mL of $NaHCO_3$ and extracted with dichloromethane (4 x 25 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (dichloromethane to dichloromethane/acetone 95:5), yielding a yellow oil. The final product was obtained in the form of hydrochloride salt. The following compounds were obtained.

*4.2.20. 2-(4-((Benzylamino)methyl)benzyl)isoindoline-1,3-dione **(6)***

Following the General procedure, reaction of 2-(4-(bromomethyl)benzyl)isoindoline-1,3-dione **(2f)** (330 mg, 1 mmol) with benzylamine (327 µL, 3 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (20 mL), after 48 h, column chromatography and preparation the salt gave product **6** (157 mg, 39%) as a white solid: R_f (DCM/acetone, 95:5) = 0.26; mp 270 °C;

^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 3.94 – 4.23 (m, 4H), 4.79 (s, 2H), 7.32 – 7.44 (m, 5H), 7.49 – 7.58 (m, 4H), 7.79 – 8.04 (m, 4H), 9.72 (s br, 2H); MS: m/z 357.28 [M+H$^+$].

4.2.21. 2-(4-(((2-Fluorobenzyl)amino)methyl)benzyl)isoindoline-1,3-dione ***(7)***

Following the General procedure, reaction of 2-(4-(bromomethyl)benzyl)isoindoline-1,3-dione **(2f)** (330 mg, 1 mmol) with 2-fluorobenzylamine (343 μL, 3 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (20 mL), after 48 h, column chromatography and preparation the salt gave product **7** (197 mg, 48%) as a white solid: R_f (DCM/acetone, 95:5) = 0.22; mp 269 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 4.10 – 4.24 (m, 4H), 4.79 (s, 2H), 7.17 – 7.74 (m, 8H), 7.98 – 7.81 (m, 4H), 9.70(s br, 2H); MS: m/z 375.36 [M+H$^+$].

4.2.22. 2-(4-(((3-Chlorobenzyl)amino)methyl)benzyl)isoindoline-1,3-dione ***(8)***

Following the General procedure, reaction of 2-(4-(bromomethyl)benzyl)isoindoline-1,3-dione **(2f)** (330 mg, 1 mmol) with 2-fluorobenzylamine (365 μL, 3 mmol) and K_2CO_3 (415 mg, 3 mmol) in acetonitrile (20 mL), after 48 h, column chromatography and preparation the salt gave product **7** (192 mg, 45%) as a white solid: R_f (DCM/acetone, 95:5) = 0.23; mp 283 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 3.95 – 4.33 (m, 4H), 4.79 (s, 2H), 7.31 – 7.55 (m, 7H), 7.67 (s, 1H), 7.81 – 7.96 (m, 4H), 9.74 (s br, 2H); MS: m/z 391.25 [M+H$^+$]

4.3. Biological Activity

4.3.1. In vitro inhibition of AChE and BuChE

To assess the inhibitory activity of the target compounds towards cholinesterases, we performed Ellman's assay using AChE from *Electrophorus electricus* (Sigma–Aldrich), human recombinant AChE (Sigma – Aldrich) and BuChE from horse serum (Sigma–Aldrich). 500 U of AChE or BuChE were dissolved in 1 ml of a gelatine solution (1% in water) and diluted with demineralized water to give a stock solution of 5 U/ml. The AChE solution was further diluted before use to give a final concentration of 3.125 U/ml. The 0.0125 M 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) solution containing 0.15% (w/v) sodium carbonate and the 0.01875 M acetylthiocholine (ATC)/butryltiocholine (BTC) iodide solution were prepared in demineralized water. All assays were performed in 0.1 M phosphate

buffer pH 8.0. The tested compounds or water in a blank sample (25 µl) were incubated with the enzyme (20 µl) for 5 min at 25 °C (*h*AChE – 37 °C) in 765 µl buffer prior to starting the reaction. Then, 20 µL of DTNB and 20 µl of ATC/BTC were added. After 5 minutes of the reaction, changes in absorbance were measured at 412 nM in EnSpire Multimode Microplate Reader (PerkinElmer). The percentage of inhibition of enzymes was calculated by comparison with a blank sample (100% activity). Calculation of IC_{50} values was performed with GraphPad Prism 5. Each concentration was measured in triplicate. Data is expressed as mean ± SEM.

4.3.2. Inhibition of $A\beta_{1-42}$ aggregation

To investigate the inhibition of β amyloid peptide aggregation by the tested compounds,we performed thioflavin T-based fluorometric assay. Recombinant human HFIP-pretreated $A\beta_{1-42}$ peptide (Merck Millipore, Darmstadt, Germany) was solubilized in DMSO to give a 100 µM stock solution. Prior to incubation, peptide stock solution was diluted in 150 mM HEPES buffer (pH 7.4) containing 150 mM NaCl to give a final concentration of 10 µM. A volume of 10 µL Aβ was mixed with 10 µL of the tested compound at the final concentration 10 µM. The mixture was immediately added to the corresponding wells in black-walled 96-well plate and filled with the HEPES buffer to the final volume of 100 µL (2 µM final Aβ concentration). Each sample was prepared in quadruplicate and the content of the DMSO was always 2%. The plate was sealed and placed on a Synergy™ H4 plate reader at room temperature. After 24 h of incubation and continuous shaking, 50 µL of the thioflavin-T solution (15 µM stock solution in HEPES, 5 µM final concentration) was added to each well using the built-in injector of Synergy™ H4 to quantify the amyloid fibril formation. After incubating for 5 min, fluorescence was measured at an excitation wavelength of 446 nm and emission wavelength of 490 nm on a Synergy™ H4 plate reader. Fluorescence intensities at the plateau were averaged, and the average fluorescence of the buffer and DMSO only, or of the buffer and the tested compound, was subtracted. The equation, F_i/F_0 x 100%, where F_i is the fluorescence of Aβ treated with the tested compound, and F_0 is the fluorescence of Aβ alone, was used to quantify the inhibition of $A\beta_{1-42}$ self-induced aggregation.

4.3.3. Kinetic characterization of EeAChE inhibition

To estimate the type of inhibition of *Ee*AChE we performed the same experimental protocol as reported at section *4.3.1.* The different concentrations of the substrate ATC (0.05

– 0.5 mM) were used to create Lineweaver-Burk plots by plotting the inverse initial velocity (1/*V*) as a function of the inverse of the substrate concentration (1/[S]). The stock solution of ATC (0.5 mM in a well) was prepared in water and diluted before use to obtained 0.3, 0.2, 0.1 and 0.05 mM concentrations of substrate. Compound **3b** was tested at three different concentrations. The double reciprocal plots were assessed by a weighted least square analysis that assumed the variance of *V* to be a constant percentage of *V* for the entire data set. Each experiment was performed in triplicate.

4.3.4. PAMPA-BBB assay

The brain penetration of compounds **3b**, **4b**, **5b** was assessed using the parallel artificial membrane permeability assay for blood brain barrier. The BBB PAMPA Explorer Test System was purchased from pION Inc. The *in vitro* permeability through BBB-1 lipid membrane was determined for 7 commercial drugs and the tested compounds. The compounds were dissolved in DMSO (10 mM stock solution) and diluted with Prisma HT buffer (5 µl/1 ml). Then, the acceptor 96-well microplate was filled with solution of the tested compounds in buffer (200 µl/well). The filter membrane in acceptor 96-well microplate was impregnated with BBB-1 lipid solution (5 µl/well) and the acceptor plate was filled with Brain Sink Buffer (200 µl/well). The acceptor plate was carefully placed on the donor plate to form a sandwich that was left undisturbed for 2 h at 37 °C. After incubation, the donor plate was carefully removed. The concentration of compounds in the acceptor, the donor, and the reference wells were measured using EnSpire Multimode Microplate Reader (PerkinElmer). Logarithm of the effective permeability ($logP_e$) of the compounds was calculated using the pION software. Assay validation was made by comparing experimental permeability of the commercial drugs with the reference value. Taking into account the limits established by Di *et al.* for BBB permeation, we established the following ranges of permeability: compounds with high permeability (CNS+), $\log P_e > -5.4$; compounds with low permeability (CNS-), $logP_e < -7.3$; compounds with uncertain BBB permeability (CNS±), $-7.3 > logP_e > -5.4$.

Acknowledgments

Financial support from the Polish Ministry for Science and High Education (grant No N N405 163339), the European Regional Development Fund (POIG.02.01.00-12-023/08) and

the Slovene Research Agency is gratefully acknowledged. Ellman's assays were performed at the Department of Technology and Biotechnology of Drugs, Jagiellonian University Medical College, Faculty of Pharmacy, Kraków, Poland. We also thank Dr Bálint Sinkó for his help with establishing and managing PAMPA-BBB assay.

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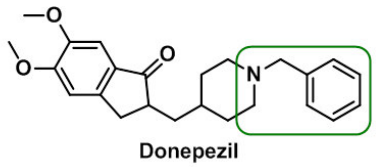

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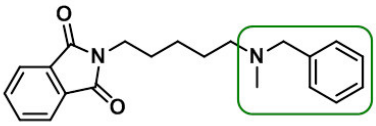

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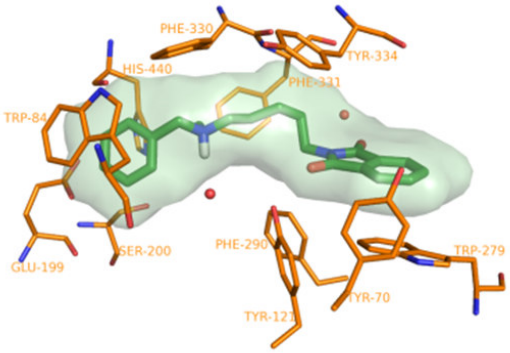

***h*AChE IC_{50} = 361nM**
***Eq*BuChE IC_{50} > 10000 nM**
PAMPA-BBB: CNS+