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Multiple Ligands Targeting Cholinesterases and β-Amyloid: Synthesis, Biological Evaluation of Heterodimeric Compounds with Benzylamine Pharmacophore

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Alzheimer's disease (AD) is a fatal and complex neurodegenerative disorder for which effective treatment remains the unmet challenge. Using donepezil as a starting point, we aimed to develop novel potential anti-AD agents with a multidirectional biological profile. We designed the target compounds as dual binding site acetylcholinesterase inhibitors, where the *N*-benzylamine pharmacophore is responsible for interactions with the catalytic anionic site of the enzyme. The heteroaromatic fragment responsible for interactions with the peripheral anionic site was modified and three different heterocycles were introduced: isoindoline, isoindolin-1-one, and saccharine. Based on the results of the pharmacological evaluation, we identified compound **8b** with a saccharine moiety as the most potent and selective human acetylcholinesterase inhibitor ($IC_{50} = 33$ nM) and beta amyloid aggregation inhibitor. It acts as a non-competitive acetylcholinesterase inhibitor and is able to cross the blood–brain barrier *in vitro*. We believe that compound **8b** represents an important lead compound for further development as potential anti-AD agent.

Keywords: Alzheimer's disease / Beta amyloid aggregation inhibitors / Cholinesterase inhibitors / Multitarget-directed ligand

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Introduction

Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disorder which requires an effective therapy. The World Health Organization estimates that 36 million people suffer from dementia worldwide and projects that the number of patients will double by 2030 and more than triple by 2050 [1]. AD is the fifth-leading cause of death for those aged 65 years and over. In addition to being a leading cause of death, AD is a leading cause of disability and morbidity [2].

AD is characterized by the loss of central cholinergic neurons, and disruption of protein folding and aggregation [3]. According to the cholinergic hypothesis proposed in 1976, memory impairments in AD are associated with a decreased number of cholinergic neurons [4, 5]. The reduced number of these neurons results in a lower level of acetylcholine. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are enzymes involved in the hydrolysis of acetylcholine. Thus, the accessible treatment for mild to moderate AD includes cholinesterase inhibitors—donepezil, rivastigmine, and galantamine—that enhance cholinergic neurotransmission [6]. For a long time, AChE was considered as a symptomatic target; however, numerous studies have shown that AChE is involved also in non-hydrolytic processes such as neurite growth [7, 8],

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synaptic maintenance [9], and beta amyloid (Aβ) aggregation [10]. 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 [11]. The PAS is believed to accelerate the assembly of Aβ into amyloid fibrils [12, 13].

According to hypotheses related to the cause of the disease, Aβ and τ protein are two proteins altered in AD [14]. The amyloid cascade hypothesis has become the conceptual framework for investigating disease-modifying therapy since its proposal in 1991 [15–18]. It states that Aβ deposition results from an imbalance between the production and clearance of Aβ. The deposition of Aβ in the brain triggers τ protein phosphorylation, neurofibrillary tangles formation, and neurons death. Thus, disruption of the amyloid cascade can positively affect the course of the disease and has become one of the most researched strategies in the development of disease modifying therapy for AD [19].

Unfortunately, all attempts to discover an effective therapy have failed; only one drug has been approved since 2003 (memantine—NMDA receptor antagonist) [20]. An appraisal of the 30 years of drug development for AD revealed that there is still a need to better understand the complex nature of the disease [21]. The wide range of biological targets make it unlikely that affecting one alone will lead to satisfactory therapeutic effects [22]. Therefore, the development of multitarget-directed ligands (MTDLs, multiple ligands), which simultaneously hit crucial targets responsible for the causes of the disease, seems to be an emerging approach in searching for the treatment of AD. In recent years, many multifunctional compounds, which affect different targets involved in pathology of AD, have been developed [23–28]. Among them, there are compounds that influence cholinesterases as a symptomatic target and Aβ as a disease-modifying target. In many cases, these compounds also possess neuroprotective, antioxidant, or metal chelating properties [29–35].

We aimed to develop novel potential anti-AD agents with a multifunctional profile among heterodimeric compounds with a benzylamine pharmacophore. The purpose of this study was to explore the structure–activity relationship (SAR) in a new series of benzylamine derivatives and to select the lead compound for further development. In this article, we present the design, synthesis, and biological evaluation of a novel series of compounds. We assessed their inhibitory potency against AChE, BuChE, and $A\beta_{1-42}$ aggregation. We also tested their blood–brain barrier (BBB) permeability using the parallel artificial membrane permeation assay (PAMPA-BBB).

Results and discussion

Design

Recently, we developed a new series of potent AChE inhibitors composed of an isoindoline-1,3-dione moiety connected by alkyl linkers to benzylamine [36, 37]. We designed the target compounds as dual binding site AChE inhibitors capable of binding to both CAS and PAS (compound **1**, Fig. 1). Benzylamine part of compound **1** was selected as an analog of benzylpiperidine in donepezil, where it is responsible for interactions with the CAS [38]. Isoindoline-1,3-dione was selected as the second moiety, as it was reported that it is able to interact with the PAS [39]. Among those hybrid molecules, we identified potent and selective human AChE (*h*AChE) inhibitors with additional biological properties such as Aβ aggregation inhibition and neuroprotective effect against Aβ toxicity. Our further studies confirmed the importance of the benzylamine pharmacophore

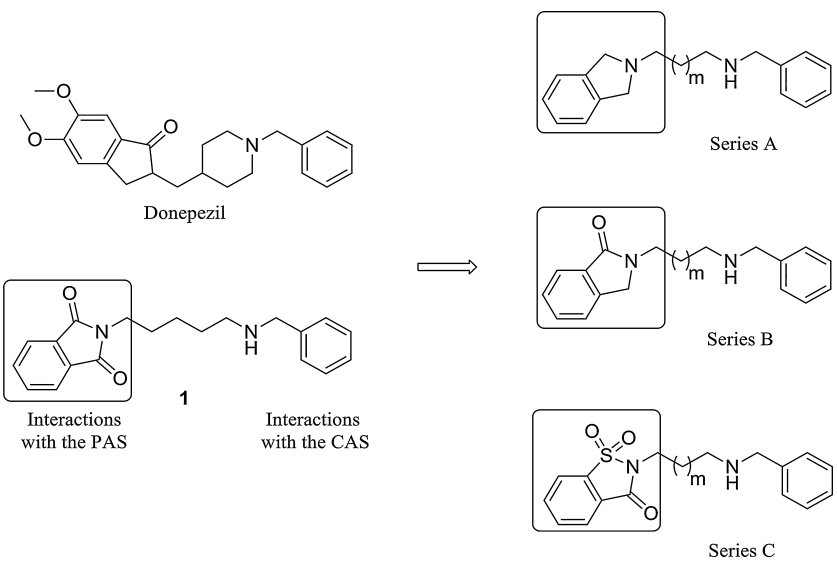


Figure 1. Structure of isoindoline-1,3-dione derivative **1** and general structures of the designed compounds.

for the inhibitory potency against AChE [40]. But those studies did not confirm that substituents in a phenyl ring of benzylamine (chlorine and fluorine atoms) may bring beneficial effects for their inhibitory potency. Thus, we used compound **1** with the unsubstituted benzylamine pharmacophore as a model inhibitor in the design of new series of donepezil-based compounds.

In the presented study, we decided to systematically modify the heteroaromatic fragment, which is presumably responsible for interactions with the PAS, in order to investigate the SAR in a novel series of compounds. We designed a series of compounds where the benzylamine moiety is connected by alkyl linkers of different lengths to isoindolin-1-one, isoindoline, or 2,3-dihydro-1,2-benzisothiazol-3-one-1,1-dioxide. We assumed that the reduction of both carbonyl groups in phthalimide (series **A**, Fig. 1) would increase the basicity and provide additional cation–π interactions in the PAS. We also reduced a single carbonyl group in phthalimide (series **B**, Fig. 1) for the purpose of SAR analysis, although isoindolin-1-one was reported to reduce the potency against AChE in comparison with phthalimide [41]. We also postulated that the replacement of a phthalimide fragment in compound **1** with saccharin (series **C**, Fig. 1) would provide additional hydrogen bond interactions in the PAS.

Molecular modeling

In order to examine the possible interactions of the compounds presented in this article with AChE, we performed molecular modeling studies (a docking position for the typical inhibitor **8b** is presented in Fig. 2). Compound **8b** was oriented in the gorge of AChE to create interactions with the CAS and PAS. The saccharine fragment was engaged in π–π stacking with Trp279 and CH–π interactions with Tyr70 in the PAS. Three oxygen atoms from carbonyl and sulfone groups created four hydrogen bonds. The carbonyl group formed a H-bond with a water molecule while oxygen atoms from sulfone with Tyr121 and two other water molecules. The benzylamine fragment was responsible for π–π stacking with Trp84 in the CAS. The protonated amino group formed cation–π interactions with Phe330 and a hydrogen bond network with Tyr121 via a water molecule. The alkyl linker formed hydrophobic interactions with aromatic residues such as Phe290, Phe331, and Tyr334 in the middle of the active gorge.

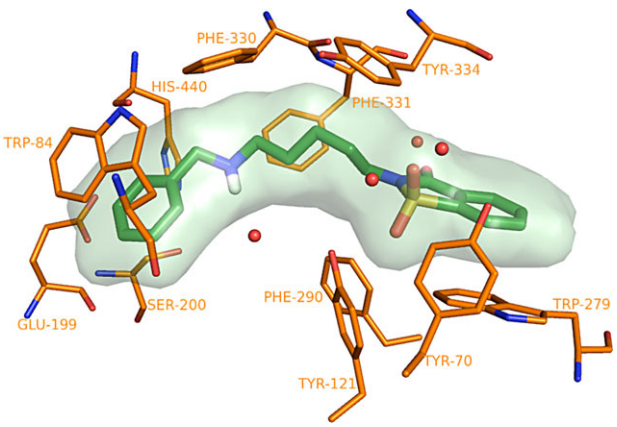


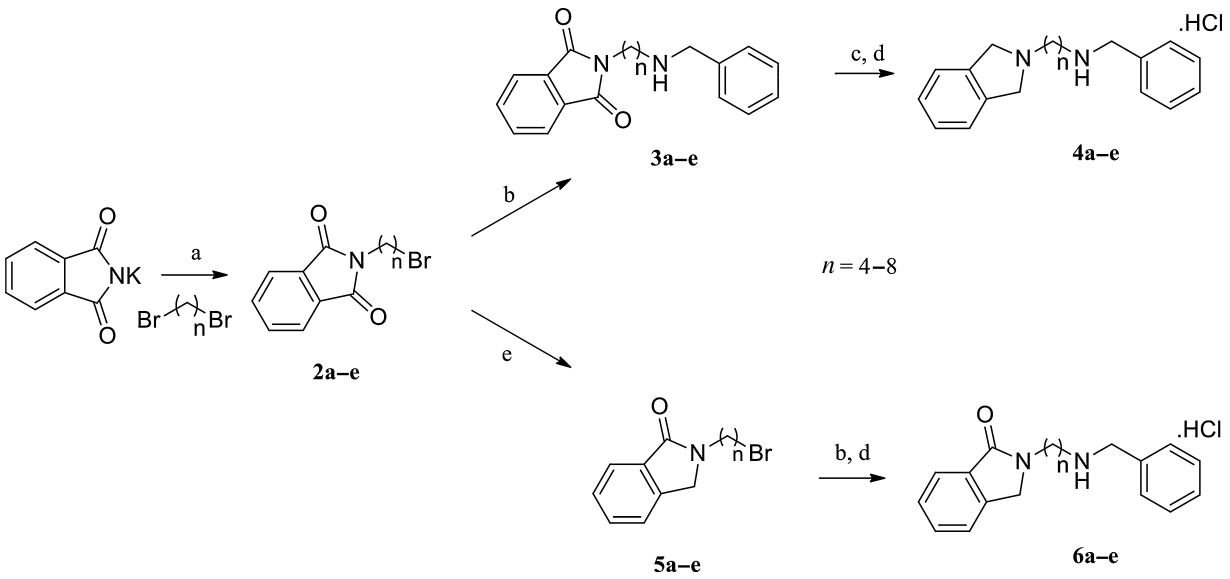
Figure 2. The proposed binding mode of compound **8b** in the active site of acetylcholinesterase obtained by docking.

According to the docking studies, all the designed compounds presented similar binding modes with their benzylamine fragments and alkyl linkers as compound **8b**. The designed compounds only revealed differences in the binding mode of their heteroaromatic fragments. The reduction of carbonyl groups in phthalimide provided a new basic center with potential to form cation–π interactions with Trp279 in the PAS, but also removed two hydrogen bonds, previously formed by CO groups with Tyr121 and a water molecule. It was also noted that an elongation of a linker in this series of compounds provided favorable hydrophobic and cation–π interactions of isoindoline with Trp279. Binding modes of the target compounds showed that these inhibitors can bind to both CAS and PAS in AChE and therefore could be described as dual binding site inhibitors. The dual binding mode is characteristic for donepezil [38] as well as for previously described isoindoline-1,3-dione derivatives [36, 37, 39].

Chemistry

The synthesis of the designed isoindoline and isoindolin-1-one derivatives was accomplished as shown in Scheme 1. Compounds **2a–e** were the key intermediates and were prepared as previously described [42]. Phthalimide potassium was alkylated with the appropriate α,ω-dibromoalkane 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 benzylamine according to the previously reported method [36]. The reactions were carried out in acetonitrile in the presence of potassium carbonate for 48 h at room temperature. Following purification by silica gel column chromatography, the final 2-(ω-(benzylamino)alkyl)-isoindoline-1,3-dione derivatives **3a–e** were isolated with satisfactory yields. Then, compounds **3a–e** were reduced with lithium aluminum hydride ($LiAlH_4$) [43]. The reactions were carried out in THF for 3 h at reflux, followed by silica gel column chromatography purification, which afforded the expected compounds **4a–e** that were finally converted into hydrochloride salts.

Subsequently, imides **2a–e** were selectively reduced to corresponding isoindolinones using silane derivatives as reducing agents in the presence of catalytic amounts of fluoride ions [44, 45]. The reactions of compounds **2a–e** with poly(methylhydrosiloxane) in THF in the presence of tetra-*n*-butylammonium fluoride (TBAF) were carried out at room temperature. After 24 h DCM, trifluoroacetic acid and triisopropylsilane were added and stirred for 15 min. The expected lactams **5a–e** were isolated after silica gel column



Scheme 1. Synthesis of the target isoindoline and isoindolin-1-one derivatives. Reagents and conditions: (a) TBAB, MeCN, reflux, 15 h; (b) benzylamine, K_2CO_3, MeCN, rt, 48 h; (c) $LiAlH_4$, THF, reflux, 3 h; (d) HCl in 2-propanol; (e) i: PMHS, TBAF, THF, rt, 24 h; ii: *i*-$PrSiH_3$, TFA, DCM, rt, 0.25 h.

chromatography purification. In the next step, compounds **5a–e** were used as alkylating agents in reactions with benzylamine. Reactions were carried out in acetonitrile in the presence of potassium carbonate for 48 h at room temperature. Following purification by silica gel column chromatography, the final 2-(ω-(benzylamino)alkyl)isoindolin-1-one derivatives **6a–e** were isolated with satisfactory yields and then converted into hydrochloride salts.

2,3-Dihydro-1,2-benzisothiazol-3-one 1,1-dioxide derivatives were prepared according to the pathway described in Scheme 2. In the first step, alkylation of saccharin sodium salt with the appropriate α,ω-dibromoalkane gave intermediate compounds **7a–e** [46]. The reactions were carried out in DMF for 24 h under reflux, followed by purification by flash chromatography. Subsequently, compounds **7a–e** were used as alkylating agents in reactions with benzylamine in DMSO. *N*-Alkylation of benzylamine with compounds **7a–e** provided after purification by flash chromatography the target compounds **8a–e** with satisfactory yield [47]. The compounds **8a–e** were converted into hydrochloride salts.

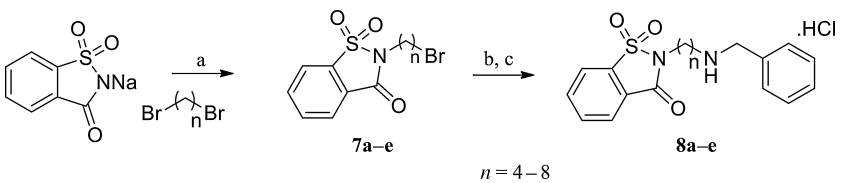
All the final compounds were characterized in the form of hydrochloride salts and the analytical and spectroscopic data are detailed in the experimental section. The biological evaluation was also performed with the hydrochloride salts.

Biological activity

In vitro evaluation of the inhibition of cholinesterases

To test the potency against cholinesterases, we used Ellman's protocol [48]. We assessed the potency against AChE from *Electrophorus electricus* (*Ee*AChE), human recombinant AChE (*h*AChE), and BuChE from equine serum (*Eq*BuChE). Compound **1**, donepezil, and tacrine were included as the references. The potency measured is given as IC_{50} values for the active compounds (Table 1).

We found that the majority of compounds were potent or moderate inhibitors of *Ee*AChE, with IC_{50} values ranging from 0.036 to 2.847 μM (Table 1). Analyzing the impact of the modifications in a heteroaromatic fragment, we found that the introduction of saccharin with an additional carbonyl group in a place of phthalimide (series **C**) increased the potency against *Ee*AChE. Compound **8b** (*Ee*AChE $IC_{50} = 36$ nM) was two times more potent than its analog **1** (*Ee*AChE $IC_{50} = 78$ nM) and also displayed selectivity toward AChE. The results confirmed our previous findings that a five carbon atom



Scheme 2. Synthesis of the target 2,3-dihydro-1,2-benzisothiazol-3-one-1,1-dioxide derivatives. (a) DMF, reflux, 24 h; (b) benzylamine, DMSO, 60°C, 3.5 h; (c) HCl in 2-propanol.

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Table 1. Inhibitory potency against *Ee*AChE, *h*AChE, and *Eq*BuChE.

Compounds	*n*	*Ee*AChE IC_{50} (μM)[a]	*Eq*BuChE IC_{50} (μM)[a]	*h*AChE IC_{50} (μM)[a]	$A\beta_{1-42}$ aggreg. inh. (%)[b]
4a	4	>10[c]	6.582 ± 0.262	>10[c]	26.36 ± 19.78
4b	5	>10[c]	7.691 ± 0.222	>10[c]	<10[d]
4c	6	>10[c]	3.401 ± 0.075	>10[c]	27.43 ± 14.60
4d	7	2.847 ± 0.079	2.134 ± 0.030	3.452 ± 0.134	<10[d]
4e	8	0.461 ± 0.008	1.807 ± 0.029	0.920 ± 0.037	<10[d]
6a	4	>10[c]	>10[c]	>10[c]	<10[d]
6b	5	0.460 ± 0.007	>10[c]	0.577 ± 0.017	<10[d]
6c	6	0.407 ± 0.005	2.306 ± 0.067	3.261 ± 0.111	<10[d]
6d	7	0.306 ± 0.003	1.914 ± 0.051	1.261 ± 0.030	25.00 ± 13.34
6e	8	1.119 ± 0.028	0.363 ± 0.010	2.264 ± 0.069	<10[d]
8a	4	0.727 ± 0.025	>10[c]	1.475 ± 0.104	23.15 ± 6.87
8b	5	0.036 ± 0.001	>10[c]	0.033 ± 0.001	22.19 ± 16.68
8c	6	0.288 ± 0.013	2.735 ± 0.070	0.906 ± 0.039	<10[d]
8d	7	0.405 ± 0.024	1.222 ± 0.076	0.519 ± 0.022	18.12 ± 13.42
8e	8	0.991 ± 0.040	1.776 ± 0.072	0.890 ± 0.055	<10[d]
1[e]	5	0.078 ± 0.001	>10[c]	0.202 ± 0.004	<10[d]
Donepezil	–	0.010 ± 0.001	1.830 ± 0.176	0.006 ± 0.001	13.80 ± 6.8
Tacrine	–	0.024 ± 0.001	0.002 ± 0.001	0.131 ± 0.002	–

[a] IC_{50} values are expressed as mean ± standard error of the mean (SEM) of at least three experiments.
[b] % Inhibition of self-induced $A\beta_{1-42}$ aggregation at 10 μM; values are expressed as mean ± standard error (SD) of at least four independent experiments.
[c] % Inhibition at 10 μM lower than 50%.
[d] % Inhibition at 10 μM lower than 10%.
[e] Data from Ref. [36].

linker was the most suitable and elongation or shortening of a linker caused a decrease in potency against *Ee*AChE (**8a**: *Ee*AChE IC_{50} = 727 nM vs. **8b**: *Ee*AChE IC_{50} = 36 nM). In series **A** and **B**, we noticed that the reduction of carbonyl groups in a phthalimide fragment adversely affected potency against *Ee*AChE. The compounds with an isoinodolin-1-one fragment were 5–14 times less potent *Ee*AChE inhibitors than compound **1**. Among them, the most potent were compounds **6c** and **6d** with six and seven carbon atom linkers. The reduction of a second carbonyl group resulted in a lack of potency against *Ee*AChE among compounds with shorter linkers (**4a–c**). Only compounds with longer linkers (**4d–e**) inhibited *Ee*AChE in the submicromolar range. We expected that reduction of two carbonyl groups would improve the potency against AChE, but we observed an opposite effect. Presumably, the interactions between a protonated amine and Tyr279 in the PAS did not compensate for the lack of two hydrogen bonds between carbonyl groups and Tyr121 and water in the PAS.

Since most of the compounds were potent *Ee*AChE inhibitors, we also tested them against *h*AChE. We found that the overall results were related to those obtained for *Ee*AChE and we observed similar SAR trends. The most potent and selective *h*AChE inhibitor was compound **8b** with an IC_{50} value of 33 nM. Only compounds **6c**, **6d**, and **8c** displayed much lower potency in comparison to potency against *Ee*AChE.

Regarding the inhibition of *Eq*BuChE, most of the compounds displayed moderate inhibitory potency with IC_{50} values in the low micromolar range. The potency against *Eq*BuChE increased with the length of the linker. The most potent BuChE inhibitor was compound **6e** comprising an eight carbon atom spacer and isoinodolin-1-one fragment with an IC_{50} value of 0.363 μM. It also inhibited AChE, but was preferential toward BuChE. We identified also selective inhibitors of *Eq*BuChE among the compounds with an isoindoline fragment (compounds **4a–c**).

Kinetic studies

To investigate the mechanism of *Ee*AChE inhibition, kinetic studies with the representative compounds from each series were performed. Substrate–velocity curves in the absence and the presence of the compounds **4e**,**6d**, and **8b** were recorded. Analysis of Lineweaver–Burk double reciprocal plots showed that with increasing concentrations of the tested inhibitors, *x*-intercepts were the same (K_m is unaffected) but slopes were different (V_{max} is decreased) (Fig. 3). This means that the tested compounds display non-competitive type of inhibition which is also characteristic for donepezil [49]. Non-competitive type of inhibition may indicate prevailing interactions with the PAS rather than with the CAS.

Inhibition of self-induced $A\beta_{1-42}$ aggregation

Although senile plaques are one of the hallmarks of AD in patients' brains, they are not specific to AD [50]. Senile plaques are composed of the neurotoxic Aβ that is cleaved from amyloid

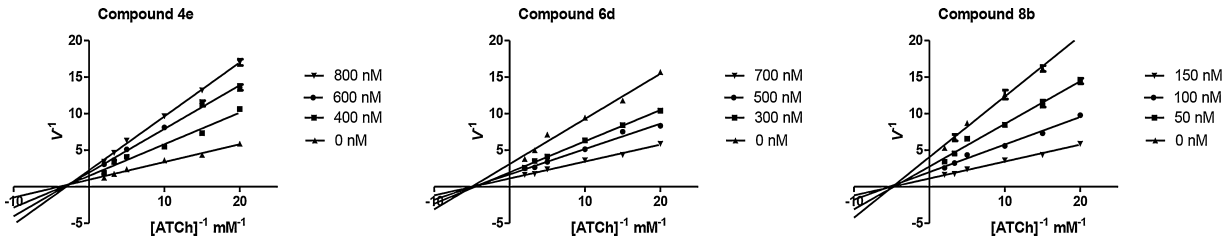

Figure 3. Lineweaver–Burk plots illustrating non-competitive type of *Ee*AChE inhibition by compounds **4c**, **6d**, and **8b**. ATCh, acetylthiocholine; *V*, initial velocity rate. Lines were derived from a weighted least-square analysis of data points.

precursor protein (APP). The accumulation and aggregation of Aβ species (mainly $A\beta_{1-42}$) is considered to be responsible for neurotoxicity in AD. $A\beta_{1-42}$ is highly fibrillogenic and its oligomers cause membrane disruption in neuronal cells [51]. Therefore, targeting the Aβ self-induced aggregation represents an emerging approach in discovering drug candidates with neuroprotective properties. Among the previous benzylamine derivatives [36], we identified potent inhibitors of self-induced Aβ aggregation (62–66.0% at 10 μM). In the presented series of compounds, we expected to identify more Aβ aggregation inhibitors. We tested all the compounds in thioflavin T-based assay [52] to investigate their ability to inhibit self-induced Aβ aggregation. Among the tested compounds, six of them displayed inhibition of $A\beta_{1-42}$ aggregation at 10 μM with the percentages of inhibition ranging from 18.12 to 27.43% (Table 1). They were found to be slightly more potent inhibitors of $A\beta_{1-42}$ aggregation than donepezil but not as potent as our previous inhibitors. We did not observe the relevant SARs among the tested compounds. This may suggest that the mechanisms of Aβ aggregation inhibition are non-specific.

Summarizing the results of biological assays against cholinesterases and self-induced Aβ aggregation, we found that compounds **4a**, **4c**, **6d**, **8a**, **8b**, and **8d** displayed moderate potency toward two or three targets. Each of them inhibited self-induced Aβ-aggregation at 10 μM. They also inhibited cholinesterases in the low micromolar range. Compounds **4a** and **4c** selectively inhibited BuChE, compound **6d** inhibited both enzymes, and compounds **8a** and **8d** inhibited AChE. We found compound **8b** the most promising, as it is selective AChE inhibitor with potency similar to donepezil and improved anti-aggregation properties. This compound is an interesting multifunctional agent for further development as a potential therapeutic for AD.

Blood–brain barrier permeation assay

Permeation through the BBB of the CNS drug candidates should be assessed as early as possible in the drug discovery process. Therefore, we performed a parallel artificial membrane permeation assay for the BBB (PAMPA-BBB) to determine the brain permeability of the synthesized benzylamine derivatives (Table 2) [53]. Seven commercial drugs were used as the reference compounds and the following ranges of permeability were established: $\log P_e > -5.4$ 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 tested the compounds **4e**, **6d**, **8b**, and **1**. The results of the PAMPA-BBB assay indicate that all the tested compounds would be able to cross the BBB and reach their biological targets in the CNS.

Conclusions

We have presented here a continuation of our studies in the group of heterodimeric compounds with a benzylamine

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

Compounds	$\log P_e$ [a)]	Prediction	Compounds	$\log P_e$ [b)]	Prediction
Theophylline	−7.3	CNS−	**4e**	−4.2	CNS+
Verapamil HCl	−3.5	CNS+	**6d**	−3.9	CNS+
Lidocaine	−4.3	CNS+	**8b**	−3.2	CNS+
Quinidine HCl	−3.5	CNS+	**1**	−3.9	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 ($n = 2$).

moiety. We designed novel series of compounds as dual binding site cholinesterase inhibitors based on the results of our previous studies and with the assistance of molecular modeling. We found that most of the synthesized compounds are potent AChE inhibitors or dual AChE/BuChE inhibitors with IC_{50} values in the low micromolar to nanomolar range. The introduction of a saccharine fragment increased the potency against AChE. Compound **8b** (*h*AChE $IC_{50} = 33$ nM) is the most potent and selective *h*AChE inhibitor with an IC_{50} value of 33 nM, which is comparable to donepezil and seven times more potent than the prototype compound **1** (*h*AChE $IC_{50} = 202$ nM). Kinetic studies revealed that the developed compounds act as non-competitive AChE inhibitors. We also identified selective BuChE inhibitors among the compounds with an isoindoline fragment. These compounds **(4a–c)** inhibit *Eq*BuChE in the low micromolar range. All the compounds were also assessed for their Aβ anti-aggregation potency in a self-induced aggregation assay. Six of them (**4a**, **4c**, **6d**, **8a**, **8b**, and **8d**) inhibit Aβ aggregation at 10 μM concentration. The results of the PAMPA-BBB assay indicate that the synthesized compounds will be able to cross the BBB.

This study proves that our novel compounds, especially compound **8b**, are viable candidates for further development as potential anti-AD agents. Moreover, the presented modifications of the heteroaromatic fragment provided us with some interesting conclusions about the interactions of molecules with AChE, which allowed us to expend our AChE computational model. We found that the introduced saccharine fragment can be a novel lead pharmacophore due to its positive effect on potency against *h*AChE.

Experimental

The Experimental part as well as the InChI codes of the new compounds are provided in the online Supporting Information at http://onlinelibrary.wiley.com/doi/10.1002/ardp.201500117/suppinfo.

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The authors have declared no conflict of interest.

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