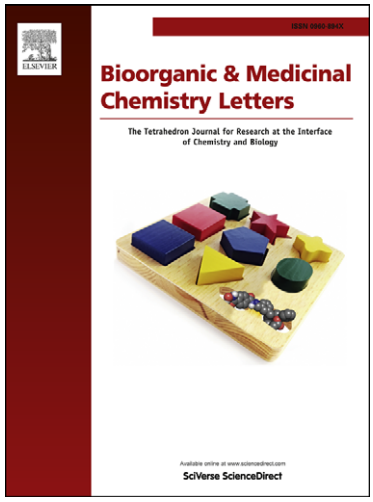

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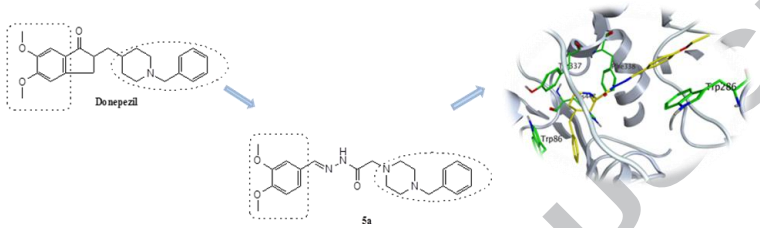

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Synthesis, Molecular Modeling and Evaluation of Novel N'-2-(4-Benzylpiperidin-/piperazin-1-yl)acylhydrazone Derivatives as Dual Inhibitors for Cholinesterases and Aβ Aggregation

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ABSTRACT

To develop new drugs for treatment of Alzheimer's disease, a group of N'-2-(4-Benzylpiperidin-/piperazin-1-yl)acylhydrazones was designed, synthesized and tested for their ability to inhibit acetylcholinesterase, butyrylcholinesterase and aggregation of amyloid beta peptides (1-40, 1-42 and 1-40_1-42). The enzyme inhibition assay results indicated that compounds moderately inhibit both acetylcholinesterase and butyrylcholinesterase. β-Amyloid aggregation results showed that all compounds exhibited remarkable Aβ fibril aggregation inhibition activity with a nearly similar potential as the reference compound rifampicin, which makes them promising anti-Alzheimer drug candidates. Docking experiments were carried out with the aim to understand the interactions of the most active compounds with the active site of the cholinesterase enzymes.

Alzheimer's disease (AD), the most common cause of dementia, is a neurodegenerative disorder characterized by progressive deterioration of memory and cognition[1]. One of the major therapeutic strategies adopted for primarily symptomatic AD is based on the cholinergic hypothesis targeting cholinesterase enzymes (acetylcholinesterase and butyrylcholinesterase)[2]. Inhibition of the hydrolysis of acetylcholine by blocking its metabolic enzyme acetylcholinesterase (AChE) increases the ACh concentration and provides a possible symptomatic treatment option for AD. On the other hand, butyrylcholinesterase (BuChE) has recently been considered as a potential target because it also plays an important role in regulating ACh level[3]. AChE inhibitors currently approved as drugs for the treatment of Alzheimer's disease are donepezil, rivastigmine, galanthamine and tacrine. ~~Although~~, donepezil is most commonly used AChE inhibitor, its Aβ formation inhibitor activity is weak (%22)[4]. Recently, the accumulation of the β-amyloid peptide (Aβ) in the brain has been thought to be a key factor in the pathogenesis of the disease[5]. It has been shown that AChE interacts with Aβ and promotes amyloid fibril formation by a hydrophobic environment close to the peripheral anionic binding site (PAS) of the enzyme[6, 7]. It is well known that two distinct binding sites exist in the active pocket of AChE peripheral binding site (PAS) and catalytic active site (CAS), located at the entrance and the bottom of the active-site gorge, respectively. These sites are characterized by two tryptophan residues, Trp84 at the active site and Trp279 at the mouth of the gorge (PAS)[8-11].

The dual-binding inhibitors, which target simultaneously both the catalytic and the peripheral-binding sites, not only inhibit the

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hydrolysis of ACh by AChE, but also prevent self aggregation of Aβ[12, 13].

Lately, it was found that the presence of benzyl piperidine moiety in the AChE inhibitors contribute to inhibitor activity by interacting with the catalytic site of the AChE[14-16]. On the other hand, it is known that potent dual-binding inhibitors of AChE were established to have aromatic stacking interactions with aromatic residues lining the wall of the AChE gorge.

In this study we designed N'-2-(4-benzylpiperidin-/piperazin-1-yl)acylhydrazone derivatives, which were expected to inhibit both AChE, BuChE, and Aβ aggregation by combining 4-benzylpiperidine-/piperazine and 3,4-dimethoxybenzyl scaffolds linked with N-acylhydrazone moiety **(Figure 1)**.

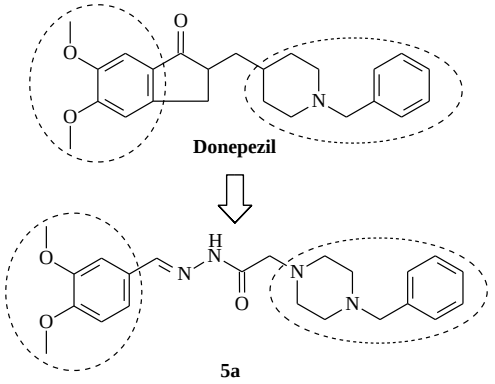


Figure 1. Design strategy for the target compounds

To predict the drug-likeness of the designed compounds, Lipinski's "rule of five" was calculated. All the compounds (**5a-6c**) were in agreement with "rule of five" (cLogP ≤ 5, MW ≤ 500, number of hydrogen bond donors ≤ 5 and number of hydrogen acceptors ≤ 10) (**Table 1**). It has been shown that the compounds penetrating the blood-brain barrier typically have polar surface area (PSA) values less than 70Å[17, 18]. For this reason, PSA values of the target compounds were also calculated. The calculated theoretical PSA values (**Table 1**) for the compounds (**5a-6c**) may support their ability to penetrate the blood-brain barrier according to this hypothesis.

The target compounds (**5a-6c**), were synthesized via the route outlined in **Scheme 1**. The commercially available secondary amines were transformed into ethyl 2-(4-benzylpiperidin-/piperazin-1-yl)acetate **1** or **2** by the reaction with ethyl bromoacetate in the presence of potassium carbonate. The reaction of **1** or **2** with excess hydrazine hydrate gave 2-(4-benzylpiperidin-/piperazin-1-yl)acetohydrazide **3** or **4**. Finally, these hydrazides were treated with appropriate aldehydes in the presence of hydrochloric acid to obtain **5a-6c** with yields between 37-62%.

The compounds (**5a-6c**) were tested for their *in vitro* inhibitory activities against human recombinant AChE and equine BuChE according to the Ellman method. The ChE results were summarized in **Table 1**. As shown in the Table 1, IC_{50} values were failed to meet the expectations since all compounds inhibited human recombinant AChE at higher concentrations than donepezil (donepezil IC_{50}: 23.1 nM for human AChE[19], IC_{50}: 7.4 μM for equine BuChE[20]). On the other hand equine BuChE inhibitory activities of the compounds were more promising. The inhibitory potential of the compounds in this series showed that none of them was selective for both enzymes.

6c and **6b** were selected for further kinetic analysis due to slightly better activities against AChE and BuChE, respectively. The Lineweaver-Burk plot of **6b** for BuChE indicated the mixed type inhibition and Dixon plot of **6b** for AChE indicated a uncompetitive inhibition (**Figure 2**). Ki values were determined for both enzymes with both inhibitors using inhibition data and results were summarized in **Table 2**. **6b** was found to act as an uncompetitive and linear mixed type inhibitor against AChE and BuChE with Ki values of 17.9 and 4.3 μM respectively (**Figure 2**). On the other hand, **6c** showed uncompetitive and competitive inhibition against AChE and BuChE with Ki values of 35.8 and 9.49 μM respectively. Uncompetitive and linear mixed type inhibitory patterns of the compounds indicated their interactions with the residues at the gorge other than the catalytic active site. These findings were compatible with their destabilising effects on amyloid fibrils since amino acids at the peripheral anionic sites (PAS) of the cholinesterases could promote Aβ fibrillation.

Analysis of AChE with **6c** showed a concentration dependent inhibition pattern. At concentrations below IC_{50} (53 μM) it was shown that the interaction of the inhibitor with the enzyme was possible only after the formation of enzyme-substrate complex (uncompetitive inhibition).

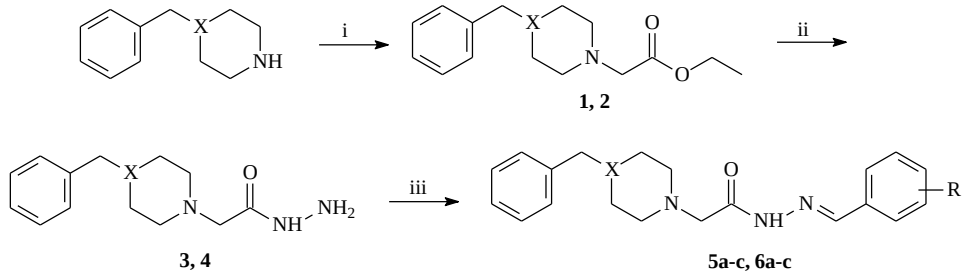
It was concluded that the inhibitor interacted with a site other than the active site due to a new conformation depending on the formation of enzyme-substrate complex. A noncompetitive inhibition was observed at high inhibitor concentrations above IC_{50}. It can be assumed that this concentration dependent inhibition type might be due to the multi-interaction sites of the enzyme. An average of inhibitor binding sites calculated as 1.35 by using Hill plot. The obtained n values for 20-40 and 60-100 μM inhibitor concentrations were 0.73 and 2.5 respectively.

Table 1. The physicochemical parameters and *in vitro* AChE / BuChE enzyme inhibition data for the target compounds

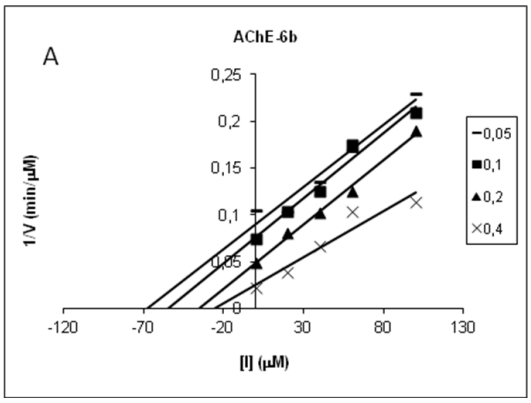
Comp.	X	R	PSA[a]	LogP[a]	MW	H-Bond Donor/Acceptor	IC_{50} (μM)[b] ± SEM	
							AChE	BuChE
5a	N	3,4-diOCH_3	66,4	2,24	396	1/6	81,1 ± 4,82	71,7 ± 3,69
5b	N	4-OCH_3	57,1	2,23	366	1/5	88,5 ± 3,61	88,3 ± 4,41
5c	N	4-OC_2H_5	57,2	2,62	380	1/5	81,4 ± 3,73	98,8 ± 4,09
6a	C	3,4-diOCH_3	63,2	3,11	395	1/5	62,8 ± 6,07	55,1 ± 6,11
6b	C	4-OCH_3	53,9	3,10	365	1/4	73,5 ± 4,62	48,8 ± 4,99
6c	C	4-OC_2H_5	53,9	3,49	379	1/4	53,1 ± 4,56	67,3 ± 5,24

[a] PSA and LogP calculated using MOE 2011.10 (Molecular Operating Environment, Chemical Computing Group)

[b] IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss



Scheme 1. Synthesis of the compounds. Reagents and conditions: (i) $BrCH_2COOC_2H_5$, K_2CO_3, reflux (ii) $NH_2NH_2.H_2O$, reflux (iii) appropriate carbonyl compounds, rt.



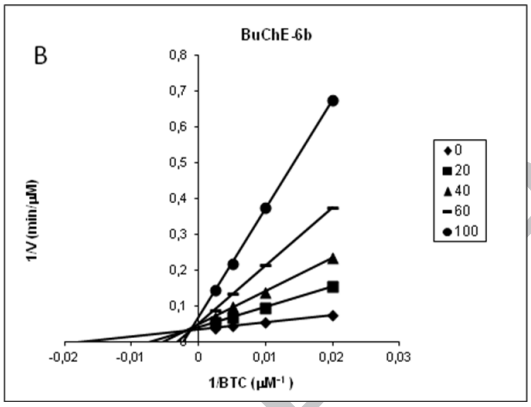


Figure 2. A) Dixon plot for the inhibition of AChE by **6b**, B) Lineaweaver-Burk plot for the inhibition of BuChE by **6b**

All newly synthesized compounds were tested for their ability to inhibit aggregation of amyloid beta peptides (1-40, 1-42 and 1-40_1-42) by using a Thioflavin T fluorescence method. Compared with the reference compound rifampicin, all target compounds showed significant destabilization effects on the aggregation of fibriles (**Figure 3**). The percentage of inhibition was calculated by taking the non-inhibitor fibril fluorescence intensity comparing with the reference and the results are given in **Figure 4**. Compared with rifampicin for the inhibition of Aβ aggregation, all compounds showed similar inhibitory activity at 100 µM, with inhibition ratio from 69% to 90%.

The docking study was performed by using MOE software to clarify the binding mode of the active ligands (**6c** and **6b**) in human AChE and BuChE enzymes respectively. The major difference in crystal structures of torpedo californica AChE (1EVE) and human AChE (1B41) is the conformations of Phe330 and Tyr337 respectively[21]. Phe330 is open-gate conformation in 1EVE while the analogous, Tyr337, is closed in 1B41. It is known that the side-chain orientation of Phe330 is responsible for substrate trafficking down the gorge. The similar behavior can be expected by Tyr 337. That is why, the conformation of Tyr337 was changed before docking studies for human AChE. Previous studies on X-ray crystal structure of the E2020-TcAChE complex showed that bivalent inhibitor donepezil span along the active center to peripheral binding site. All three major functional groups of donepezil, the benzyl moiety, the piperidine nitrogen, and the dimethoxyindanone moiety, interact with residues Trp84 at the bottom of the gorge, Phe330, and Trp279 in the peripheral binding site respectively[9].

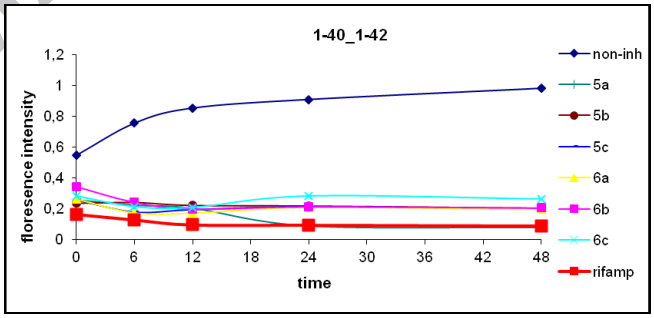


Figure 3. Inhibitory effects of the compounds on the aggregation of 1-40_1-42 amyloid peptides.

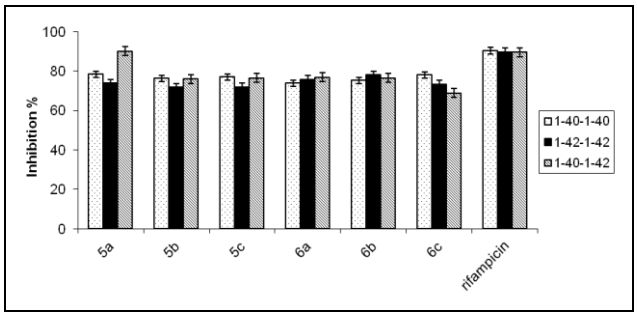


Figure 4. Amyloid fibril inhibition of the compounds compared to rifampicin

Table 2. Cholinesterase inhibitory effects of **6b** and **6c**

Inhibitor	Enzyme	Inhibition Type	Ki, µM	α
6b	BuChE	Linear mixed type	30,5 ± 0,08	12,5 ± 0,18
6b	AChE	Uncompetitive	19,5 ± 0,06	-
6c	BuChE	Competitive	31,5 ± 0,05	-
6c	AChE	Uncompetitive (20-40 µM of I) Noncompetitive (60-100 µM of I)	50,8 ± 0,04 83,8 ± 0,04	-

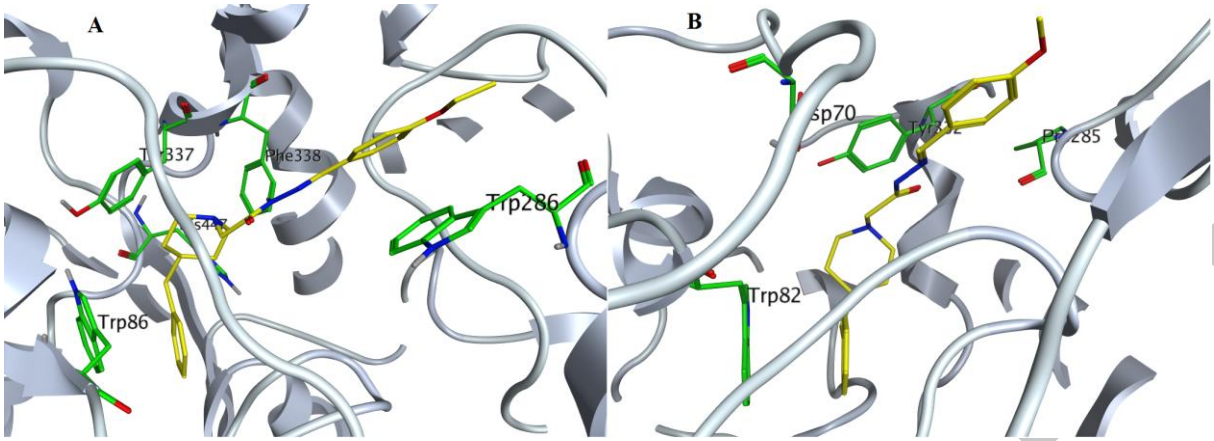


Figure 5. Best docking pose of A) **6c** in AChE B) **6b** in BuChE

It was demonstrated that **6c** interacts with PAS and CAS of human AChE. At the entrance of the enzyme, π-π interaction was formed between the 4-ethoxybenzylidene group of the compound and the indole ring of PAS residue Trp286 while the benzylpiperidine moiety of the compound interacted with Trp86, by means of a π-π stacking (**Figure 5A**). When we looked into orientations of **6b** which had remarkable inhibitory activity against BuChE, only the terminal benzyl group of **6b** established π-π interaction with Trp82 due to lack of Trp279 in BuChE (**Figure 5B**). This may explain the similar inhibition potential of our newly synthesized compounds against both AChE and BuChE.

In summary, six new N'-2-(4-benzylpiperidin-/piperazin-1-yl)acylhydrazone derivatives (**5a-6c**) were synthesized and tested for their ability to inhibit acetylcholinesterase, butyrylcholinesterase and Aβ aggregation. The results showed that these compounds possessed a moderate and non-selective inhibitory activity against both enzymes. Analysis of kinetics data exhibited that, **6b** was a mixed type and uncompetitive inhibitor of BuChE and AChE, respectively whereas **6c** was competitive and uncompetitive inhibitor in the order mentioned. Also the docking studies indicated that **6c** interacted with PAS and CAS of human AChE. The results of kinetic analysis and docking studies support the multi-site binding of the compounds to AChE. On the other hand, β-amyloid aggregation results showed that all compounds exhibited a good activity against Aβ aggregation. Although our novel compounds showed weak cholinesterase inhibitory activity, their remarkable Aβ fibril aggregation inhibitory activities make us hopeful to study on these compounds for further optimization.

Supplementary Data

Supplementary data associated with this article can be found in the online version, at doi:

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