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Biological evaluation and molecular docking studies of 4-aminobenzohydrazide derivatives as cholinesterase inhibitors

Zuleyha Almaz[a,*], Aykut Oztekin[b], Ayse Tan[c], Hasan Ozdemir[d]

[a] Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Mus Alparslan University, Mus, 49250, Turkey
[b] Agri Ibrahim Cecen University, Vocational School of Health Services, Department of Medical Services and Techniques, Agri, 04100, Turkey
[c] Vocational School of Technical Sciences, Mus Alparslan University, Mus, 49250, Turkey
[d] Atatürk University, Science Faculty, Chemistry Department, Erzurum, 25240, Turkey

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ABSTRACT

Nowadays, inhibition of the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes have emerged as an encouraging approach in the treatment of dementia and remission of symptoms of Alzheimer's disease. Therefore, inhibition of cholinesterases is one of the main targets by researchers. Benzohydrazides are biologically active compounds and have various pharmacological effects, bearing these in mind, we investigated the inhibitory effects of some mono or di-substituted 4-aminobenzohydrazide derivatives **(1a-11a)** against AChE and BChE. For this purpose, we studied the inhibition effects (IC_{50}, K_i values, and inhibition types) of these molecules on AChE and BChE enzymes. Based on the results, compound **3a** showed potent AChE and BChE inhibition (IC_{50} = 0.59 and 0.15 μM). The K_i values of the compounds (**3a, 4a,** and **8a**) showing the best inhibition effect against AChE and BChE were calculated and these values ranged from 0.10 ± 0.04 to 5.10 ± 2.14 μM. To determine the possible binding mechanisms with the active sites of both enzymes of compounds **3a, 4a**, and **8a** having strong inhibitory effects, docking analyses were performed. According to the docking results, compound **3a** showed the best binding affinity (-7.3 kcal/mol for AChE and -6.8 kcal/mol for BChE) against both enzymes.

1. Introduction

Alzheimer's disease (AD) is considered a neurodegenerative disease that progresses over time and is very difficult to diagnose at an early stage [1]. Dementia is the most common type of mental pathology seen in middle-aged and elderly individuals. The most important changes detected in the brains of individuals with AD are synapse loss with neuron death, amyloid plaque formation, and decreased neurotransmitter content. Since the most dramatic abnormalities occur in the cholinergic system, this situation is referred to as the cholinergic hypothesis of AD. Therefore, AD is mainly associated with change in cholinesterase (ChE) metabolism and degeneration [2]. ChE's are hydrolyzed acetylcholine and two types, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). About 80% of acetylcholine is hydrolyzed by AChE, while BChE plays a complementary role[3]. ChE inhibitors have been supported by the cholinergic hypothesis with the finding that they have positive effects on cognition in AD [4]. These inhibitory compounds have been suggested for the symptomatic treatment of moderate AD [5]. AChE is a membrane-bound enzyme and is found in brain, muscles and cholinergic neurons [6]. AChE lowers neurotransmitter levels by hydrolyzing ACh to acetate and choline. It terminates the communication between nerve cells, thus playing an important role in cholinergic transition [5]. BChE is found in the liver, pancreas, blood serum and central nervous system (CNS) [7]. In AD, AChE decreases by up to 45% while BChE increases by up to 90%. Thus BChE compensates for the role of AChE. The development of ChE inhibitors that act as dual-target inhibitors of AChE and BChE is therefore very important [3]. There are a number of drugs for treating the most common symptoms of dementia [8]. These drugs, which partially inhibit AChE, can cross the blood–brain barrier. They can increase ACh levels and potentiate their physiological effects. In this way, they can also provide symptomatic relief [9]. Commonly prescribed anti-AD drugs, which are cholinesterase inhibitors, act by increasing ACh levels through inhibition of this enzyme [10]. Unfortunately, the medications developed have only been able to relieve AD symptoms. But, if brain cell damage is severe, the ability of cholinesterase inhibitors for curing the symptoms becomes less and less. It was determined that the effects of cholinesterase inhibitors reported in studies differ in for each individual [11]. The first drug approved for the treatment of AD (in 1993) is tacrine, an AChE and BChE

* Corresponding author.
E-mail address: z.turkoglu@alparslan.edu.tr (Z. Almaz).

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inhibitor. However, the use of tacrine has been limited because it causes side effects such as nausea, diarrhea, dizziness, and vomiting. More tolerated and less toxic drugs were approved instead of tacrine. To date, four cholinesterase inhibitors with different pharmacological and pharmacokinetic profiles have been licensed for the symptomatic treatment of AD, such as rivastigmine, donepezil and galantamine, along with tacrine [12]. These drugs will continue to be developed as they are a proven symptomatic therapy with a known goal. In the light of all this information, the synthesis of new molecules has increased as cholinesterase inhibitors, which are an important treatment option for AD. In order to contribute to these pharmacological studies, we focused on the effect of benzohydrazide derivatives against AChE and BChE. Benzohydrazides, which are aromatic derivatives of hydrazine and benzoic acid derivatives, have become important due to their various pharmacological effects. It has also been found that benzohydrazide derivatives have inhibitory activities against AChE and BChE. In the recent studies, amide benzohydrazides have emerged as potential candidates as anticholinesterases and have remarkable inhibition potential against AChE and BChE [13]. It has also been found that the 6-substituted-3 (2H) –pyridazinone 2-yl-propionhydrazide derivatives (expecially 4 -Fluorophenyl) exhibits significant AChE inhibitory activities [14]. Furthermore, they have been found to act as analgesic and anti-inflammatory agents [15,16]. In addition, benzohydrazide derivatives have significant antitubercular and anti-HIV activities [17,18]. In the light of above, in this study, mono or di-substituted 4-aminobenzohydrazide derivatives were synthesized and their inhibition potential on AChE and BChE enzymes was examined in vitro by comparing with a reference drug tacrine.

2. Results and discussion

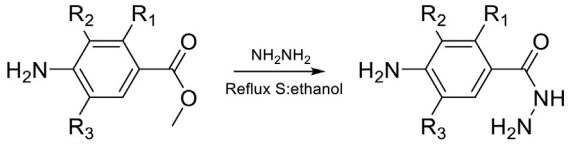
2.1. Chemistry

Methyl aminobenzoate derivatives (**1**–**11**) were purchased from fluorochem ltd U.K. In our previous studies, 4-aminobenzohydrazide derivatives (**1a**-**11a**) were synthesized from methyl 4-aminobenzoate derivatives according to previous described procedure [19-21] (Fig. 1). For this purpose, compounds **1**–**11** were reacted with hydrazine to obtain the target compounds (**1a**-**11a**) in good yields.

2.2. Anti-cholinesterase activity and enzyme kinetics studies

ChE inhibitors are commonly used in the treatment of AD, which is known for decreased cholinergic transmission, knot formation, amyloid plaques and neuron loss [22]. We evaluated the inhibitory effects of 11 different 4-aminobenzohydrazide derivatives and tacrine on AChE and BChE under in vitro conditions in the present study. The concentration of the 4-aminobenzohydrazide derivatives (**1a**-**11a**) required to inhibit 50% of the enzyme activities were calculated from different inhibitor concentrations and are summarized in Table 1. Compound 3a showed a better AChE inhibitory effect than tacrine, an AChE inhibitor used therapeutic agent in AD treatment. Three compounds (4a, 8a, and 10a) showed notable inhibitory effects against AChE and BChE with IC_{50} values. The IC_{50} values for compound 3a, which showed the best inhibition activity from these derivative inhibitors, were 0.59 μM for AChE and 0.15 μM for BChE. A selectivity index (SI) was calculated to understand whether or not a given compound was selective for enzymes. Thereafter, Lineweaver-Burk plots were drawn to determine the K_i values and inhibition types of the compounds (3a, 4a, 8a) showing the best inhibitory effect against both AChE and BChE enzymes. As seen in Table 2, K_i values range from 0.28 ± 0.02 to 5.10 ± 2.14 for AChE and 0.10 ± 0.04 to 3.84 ± 1.76 for BChE. Compound 3a had the best K_i value, which also had the best docking score with AChE and BChE. These three compounds for AChE, compound 4a for BChE showed a noncompetitive inhibition type. Compounds 3a and 8a showed competitive inhibition type for BChE. Activity (%)-[3a] and Lineweaver–Burk graphs of compound 3a showing the best activity against AChE and BChE are given in Figs. 2 and 3 respectively.

When the structure-activity relationships of the compounds are examined, compound **3a** bearing the Br and F substituents at the C3 and C5 positions of the benzene ring showed the best inhibitory activity against AChE and BChE. In contrast to **3a**, it was seen to have weaker the inhibition potential of compound 1a, which contains the Cl substituent at the C5 position and Br substituent at the C3 position of the benzene ring. Compounds **4a, 8a**, and **10a**, which contain the methoxy group, Br or Cl were attached to the C2 position of the benzene ring respectively, showed significant inhibitory activities against both AChE and BChE. The Br substituent at the C2 position of the benzene ring of compound **8a**, compared to the C3 position, decreased significantly the inhibition potential for both enzymes. The methoxy, Br, and Cl groups at the C2 and C3 positions of the benzene ring (for compounds **5a, 9a**, and **11a**, respectively), were significantly decreased the inhibitory activities against both enzymes. While the methyl group at the C2 of the benzene ring of compound **6a** showed an approximately two-fold reduction in inhibition of AChE compared with the methyl group at the C3 of compound **7a**. On the other hand in inhibition with BChE of **6a**, a slightly increase was observed.

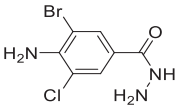
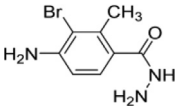
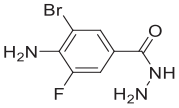
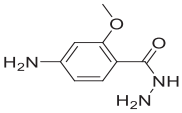
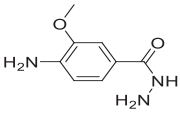
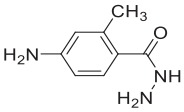
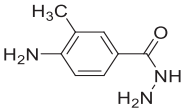
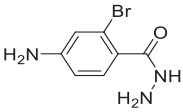
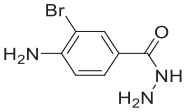
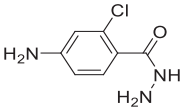
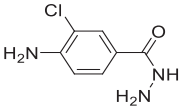
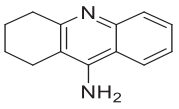


(1-11) **(1a-11a)**

	R_1	R_2	R_3		R_1	R_2	R_3		R_1	R_2	R_3		R_1	R_2	R_3
1:	-H	-Br	-Cl	**7**:	-H	$-CH_3$	-H	**1a**:	-H	-Br	-Cl	**7a**:	-H	$-CH_3$	-H
2:	$-CH_3$	-Br	-H	**8**:	-Br	-H	-H	**2a**:	$-CH_3$	-Br	-H	**8a**:	-Br	-H	-H
3:	-H	-Br	-F	**9**:	-H	-Br	-H	**3a**:	-H	-Br	-F	**9a**:	-H	-Br	-H
4:	-OMe	-H	-H	**10**:	-Cl	-H	-H	**4a**:	-OMe	-H	-H	**10a**:	-Cl	-H	-H
5:	-H	-OMe	-H	**11**:	-H	-Cl	-H	**5a**:	-H	-OMe	-H	**11a**:	-H	-Cl	-H
6:	$-CH_3$	-H	-H					**6a**:	$-CH_3$	-H	-H				

Fig. 1. The general synthesis pathway of mono or disubstituted 4-aminobenzohydrazides (**1a**-**11a**).

Table 1
IC_{50} values (μM) and SI indexes of the 4-aminobenzohydrazide derivatives (**1a**-**11a**).

Inhibitors		AChE	R^2	BChE	R^2	SI index[a]
1a		346.50	0.957	441.43	0.985	1.27
2a		173.28	0.986	86.63	0.981	0.50
3a		0.59	0.989	0.15	0.961	0.25
4a		2.82	0.996	3.63	0.993	1.29
5a		693.14	0.977	>1000	-	–
6a		346.57	0.935	573.92	0.979	1.65
7a		693.14	0.953	428.12	0.967	0.62
8a		4.62	0.925	3.81	0.961	0.82
9a		693.14	0.956	475.98	0.958	0.69
10a		8.45	0.925	63	0.974	7.45
11a		693.14	0.964	>1000	-	–
Tacrine *		0.71	0.982	0.11	0.987	0.15

[a]Calculated by the formula IC_{50} BChE/IC_{50} AChE for each compound to show selectivity toward either enzyme.
*Used as a positive control for AChE and BChE enzymes.

Table 2
K_i values (μM) and inhibition types of compounds **3a, 4a** and **8a** having strong inhibitory effects against AChE and BChE.

Inhibitors	AChE (μM)	Inhibition type	BChE (μM)	Inhibition type
3a	0.28±0.02	Noncompetitive	0.10±0.04	Competitive
4a	2.95±0.55	Noncompetitive	1.37±0.60	Noncompetitive
8a	5.10± 2.14	Noncompetitive	3.84±1.76	Competitive

Comparing our IC_{50} values with a study examining the biological activities of sulfonyl hydrazones, benzoyl hydrazones, and chalcone derivatives, the authors found that the IC_{50} values showing the best AChE inhibitory activity were 10.16±0.80 μM for [N'-(1-(4-morpholinophenyl)ethylidene)−2-(trifluoromethoxy) benzenesulfonohydrazide], and 17.20±1.86 μM for [4-Chloro-N'-(1-(4-morpholinophenyl)ethylidene)benzohydrazide] while our 5-Fluoro-substituted compound 3a was in the 0.59 μM. In the

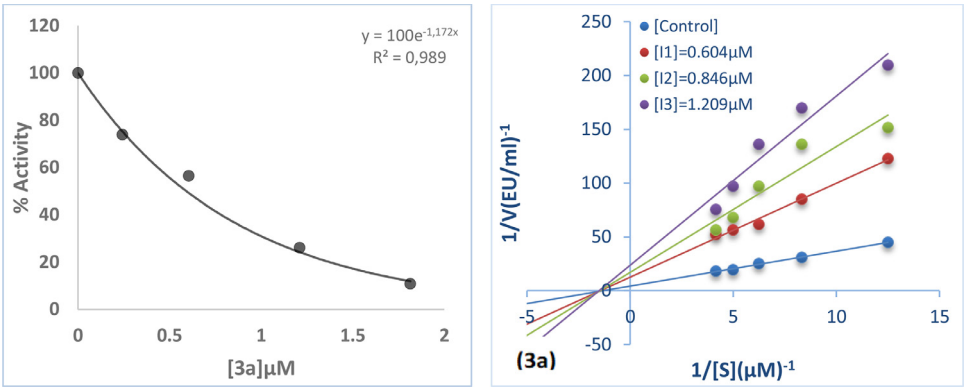


Fig. 2. IC_{50} graph and Lineweaver-Burk graph of compound **3a** for AChE.

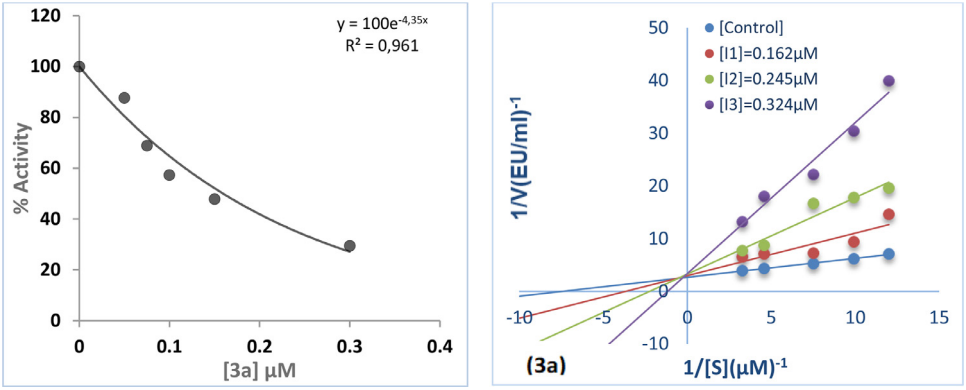


Fig. 3. IC_{50} graph and Lineweaver-Burk graph of compound **3a** for BChE.

same study BChE assay, the authors found that the IC_{50} values indicated the best BChE inhibitory activity were 16.52±0.80 µM for [[N'-(1-(4-morpholinophenyl) ethylidene)−2-(trifluoromethoxy) benzenesulfonohydrazide] and 19.21±0.75 for µM [4-(methylthio)-N'-(1-(4-morpholinophenyl) ethylidene)benzohydrazide] [23]. Concordantly, we observed that the IC_{50} value for compound 3a showing best BChE inhibition was in the 0.15 µM.

2.3. Molecular docking

Compounds **3a, 4a**, and **8a** showed significant inhibition effects against AChE and BChE. Therefore, their molecular docking studies were carried out on the compounds. The compounds were similarly docked into the active sites of both receptors. According to the docking results, the compounds could easily bound with the active sites of AChE and BChE. 2D ligand-receptor interaction diagrams of the compounds with AChE and BChE are presented in Fig. 5. While the binding energy values of compounds **3a, 4a**, and **8a** in the interaction with AChE, were calculated as −7.3 kcal/mol, −7.0 kcal/mol, and −6.9 kcal/mol, those of **3a, 4a**, and **8a** in the interaction with BChE were calculated as −6.8 kcal/mol, −6.1 kcal/mol, and −6.4 kcal/mol, respectively. The best binding poses and 3D ligand-receptor interaction diagrams of compound **3a**, which is the best inhibitor against both receptors, are presented in Fig. 4 for AChE and Fig. 6 for BChE. In the interaction with AChE, compound **3a** formed van der Waals interactions with Tyr115, Trp113, Ile447, Gly444, Tyr129, Ser 199, Gly117, Gly118, and Tyr120 residues. The hydrazide moiety of compound **3a** formed a conventional hydrogen bond with Gly116, a π−cation interaction with Trp82, a carbon-hydrogen bond with Gly444, salt bridge, and attractive charge interactions with Glu198 residue. The Br substituent on the benzene ring of **3a** formed π−alkyl interactions with Phe293, His443, and Phe334 residues. The benzene ring of the compound formed $\pi-\pi$ T-shaped interactions with Trp82, Tyr333 residues. In the interaction with BChE, compound **3a** formed van der Waals interactions with Gly437, Ser196, Tyr126, Gly114, Asn81, Thr118, and Asp68 residues. The amine group on the benzene ring and the hydrazide moiety of **3a** formed conventional hydrogen bonds with Glu195 and Trp80 residues, respectively. While the Br substituent on the benzene ring of **3a** formed π−alkyl interactions Trp80 and His436 residues, the F substituent formed halogen (fluorine) interactions Glu195 and Gly113 residues. Finally, the carbonyl group formed a carbon-hydrogen bond with Gly119 residue.

3. Experimental

3.1. Chemicals

Purified AChE from electric eel (*Electrophorus electricus*) and butyrylcholinesterase from equine serum, acetylthiocholine iodide (AChI), butyrylcholine iodide (BChI), 5,5′dithiobis-2-nitrobenzoic acid (DTNB,), dimethylsulfoxide (DMSO) and other chemicals were obtained from (Sigma-Aldrich, St. Louis, MO).

3.2. Synthesis of 4-aminobenzohydrazide derivatives (1a-11a)

4-aminobenzohydrazide derivative compounds (**1a**-**11a**) were synthesized from commercially available 4-aminobenzoate forms according to the protocol previously described [21]. Experimental details had been presented in our previous studies [19,20].

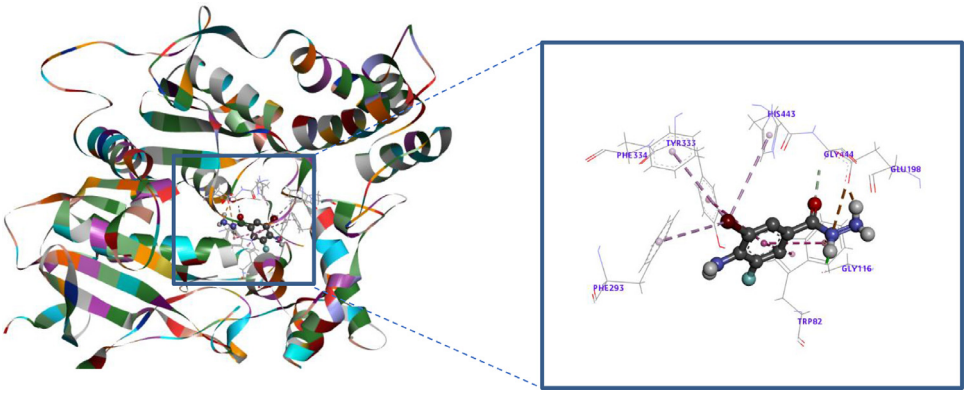


Fig. 4. The best binding pose and 3D interaction diagram of compound **3a** with AChE,.

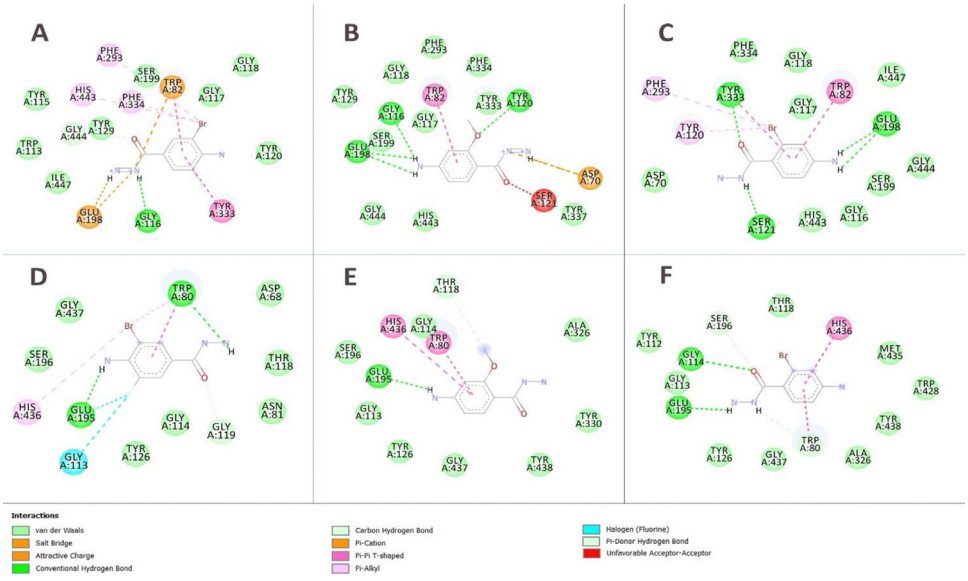


Fig. 5. 2D ligand-receptor interaction diagrams of compounds: A) **3a-**AChE, B) **4a-**AChE, C) **8a**- AChE, D) **3a-**BChE, E) **4a-**BChE, F) **8a-**BChE.

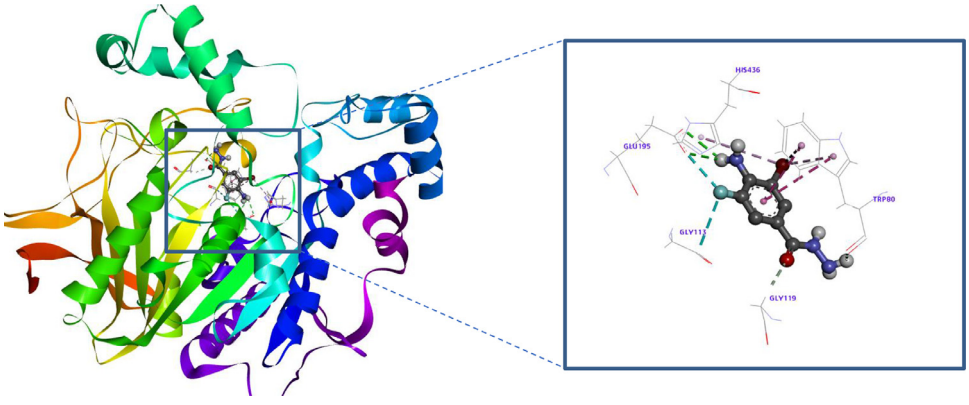


Fig. 6. The best binding pose and 3D interaction diagram of compound **3a** with BChE.

3.3. In vitro inhibition assay of cholinesterases

The inhibitory effects of synthesized (1a-11a) inhibitory molecules on AChE and BChE activity were measured using a modification of Ellman's spectrophotometric method [24] as described previously [25,26]. The essence of this method is to form 5-mercapto-2-nitrobenzoic acid by reacting thiocholine with 5,5′-Dithio-bis (2-nitro-benzoic acid)(DTNB). AChI and BChI were used as a substrate. DTNB was used for determination of AChE and BChE enzyme activitiy. İnitially, 2 mg of each inhibitor synthesized was taken, dissolved in 1 mL of DMSO and diluted in various concentrations with deionized water. The reaction system was composed of 2-200 μL inhibitor sample, 50 μL (AChI)/(BChI), 100 μL of buffer (1 M, pH 8.0; Tris-HCl buffer for the AChE assay and phosphate buffer for the BChE assay), 50 μL DTNB, and 20μL enzyme (0.2 units/mL for the AChE assay and 0.3 units/mL for the BChE assay).

The absorbance of the reaction mixture starting with the enzyme addition was measured at 412 nm for 5 min on a Shimadzu UV-1800 Spectrophotometer. Activity (%) - [1a-11a] (inhibitory concentration) was plotted to determine the inhibition effect of derivative molecules on AChE and BChE. IC_{50} values were obtained by activity (%) versus compound plots. Three different inhibitory concentrations were used to calculate K_i values. Lineweaver - Burk curves were plotted and calculations were performed [27].

3.4. Molecular docking studies

3D crystallographic structures of AChE and BChE receptors were downloaded from the Protein Data Bank (www.rcsb.org) [28] (PDB ID: 1c2b [29] for AChE, PDB ID:6esy [30] for BChE). 2D structures of compounds **3a, 4a**, and **8a** were drawn on ChemDraw Ultra 12.0. 2D structures of the compounds were optimized with Avogadro software [31]. Docking studies were performed with the program UCSF Chimera (1.13.1) [32]. Polar hydrogens and Gasteiger partial charges were added and a grid box was created with tools-Autodock Vina [33] in UCSF Chimera. The results of docking were visualized with Discovery Studio Visualizer [34].

4. Conclusions

In recent years, many studies have been carried out to examine the inhibition of AChE and BChE in the symptomatic treatment of AD. In view of these studies, we synthesized some 4-aminobenzohydrazide derivatives (**1a**-**11a**) and investigated their inhibitory properties against AChE and BChE. The results showed that some of these compounds have moderate to high inhibitory activity. Compound **3a** is a good inhibitor for both AChE and BChE. Moreover, it showed a stronger inhibitory effect against AChE than the reference drug, tacrine. Compounds 5a, 6a, 9a and 11a showed very weak inhibitory activity against AChE and BChE compared to tacrine and other compounds. Molecular docking studies were carried out for three compounds (**3a, 4a**, and **8a**) and their best binding modes were determined. According to the ligand-receptor interactions of the compounds, compound **3a** showed the best binding affinity against both enzymes. The binding energy values of **3a** were calculated as −7.3 kcal/mol for AChE and −6.8 kcal/mol for BChE. The hydrazide moiety, the benzene ring, the Br, F, and amine groups of compound **3a** were detected to be effective in interactions with AChE and BChE. All in all, compounds **3a, 4a**, and **8a** (especially compound **3a**) can be considered promising inhibitors for AChE and BChE enzymes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Zuleyha Almaz: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Aykut Oztekin:** Visualization, Writing – review & editing. **Ayse Tan:** Visualization, Writing – review & editing. **Hasan Ozdemir:** Methodology.

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