

Synthesis and Biological Evaluation of Tacrine Derivatives as Potential Acetylcholinesterase and Butyrylcholinesterase Inhibitors for Alzheimer's Disease

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SUMMARY

Some tacrine derivatives bearing unsubstituted and para-substituted benzylidene moieties linked to the hydrazine function were synthesized and screened for potential acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities. They were purified and structurally characterized by FT-IR, ¹H NMR, and ESI-MS spectroscopy, confirming the proposed molecular structures. Enzyme inhibition assays for AChE and BChE were conducted using a modified Ellman colorimetric method. All compounds exhibited significant inhibitory potency, with IC₅₀ values ranging from 0.088 to 0.359 μ M on AChE and from 0.025 to 0.093 μ M on BChE. Among the derivatives, compound 1b bearing a methyl substituent on the benzylidene ring showed the highest AChE inhibition (IC₅₀= 0.088 μ M), while the unsubstituted derivative 1a exhibited the highest BChE inhibition (IC₅₀= 0.025 μ M). Molecular docking studies were also conducted to elucidate the possible binding mode of the most active compounds (1b for AChE; 1a for BChE), and the ADMET profiles of all synthesized compounds were evaluated using the ADME and toxicity prediction tools available on the PreADMET web server.

Keywords: Acetylcholinesterase inhibitors, Butyrylcholinesterase inhibitors, Alzheimer's disease, tacrine derivatives

Alzheimer Hastalığı İçin Potansiyel Asetilkolinesteraz ve Butirikolinesteraz İnhibitörleri Olarak Takrin Türevlerinin Sentezi ve Biyolojik Değerlendirilmesi

ÖZ

Hidrazin fonksiyonuna bağlı nonsüstitüe ve para-süstitüe benziliden grubu taşıyan bazı takrin türevleri sentezlendi ve potansiyel asetilkolinesteraz (AChE) ve butirilkolinesteraz (BChE) inhibitör aktiviteleri açısından değerlendirildi. Bileşikler saflaştırıldıktan sonra molekül yapıları FT-IR, ¹H NMR ve ESI-MS spektroskopisi ile teyit edildi. AChE ve BChE enzimlerine karşı inhibitör aktiviteler, modifiye Ellman kolorimetrik yöntemi ile değerlendirildi. Tüm bileşikler, AChE'ye karşı 0,088-0,359 μ M ve BChE'ye karşı 0,025-0,093 μ M aralığında değişen IC₅₀ değerleriyle önemli inhibitör potansiyeli gösterdi. Türevler arasında, benziliden halkasında metil süstitüenti taşıyan 1b bileşiği, AChE'ye karşı en iyi inhibisyonu (IC₅₀= 0,088 μ M) gösterirken, nonsüstitüe 1a bileşiği BChE'ye karşı en iyi inhibisyonu (IC₅₀= 0,025 μ M) sergiledi. En aktif bileşiklerin (AChE için 1b; BChE için 1a) olası bağlanma biçimini açıklığa kavuşturmak amacıyla moleküler kenetlenme çalışmaları da gerçekleştirildi. Ayrıca, sentezlenen tüm bileşiklerin ADMET profilleri, PreADMET web sunucusunda bulunan ADME ve toksisite tahmin araçları kullanılarak değerlendirildi.

Anahtar Kelimeler: Asetilkolinesteraz inhibitörleri, butirilkolinesteraz inhibitörleri, Alzheimer hastalığı, takrin türevleri

Received: 11.03.2026

Revised: 09.04.2026

Accepted: 28.04.2026

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INTRODUCTION

Alzheimer's disease (AD), a neurodegenerative disorder, predominantly affects the geriatric population and is characterized by its progressive nature. Augusta Deter was diagnosed with AD for the first time in literature by a German scientist, Alois Alzheimer, in 1906. Despite more than a century of its clinical recognition and substantial research efforts, a detailed understanding of the disease's underlying pathophysiology, especially at a molecular level, remains elusive. Clinically, the most common symptom of AD is a gradual decline in mental abilities, mainly memory deficits. As neuronal damage progresses, neuronal transmission impairments and synaptic depletion lead to advanced cognitive dysfunction (Alzheimer's Association Report, 2020).

According to the World Alzheimer Report 2018, a new case of dementia occurs every 3 seconds. While there were approximately 50 million individuals with dementia in 2018, projections indicate a threefold increase in this number by 2050. In parallel with this epidemiological growth, the global economic burden is projected to reach the 2 trillion USD threshold as early as 2030 (Scheltens et al., 2021; World Alzheimer Report 2018, 2018).

Given the immense socio-economic burden of the disease on global healthcare systems, the high failure rate in Alzheimer's drug development could be controversial, but it is largely attributed to the multifaceted pathology of AD, the clinical challenges for early diagnosis, and difficulties in differentiating AD from other forms of age-related cognitive decline (Cumings, 2021; Cummings et al., 2018, 2021, 2022).

The multifactorial etiology of AD causes difficulties for both precise diagnosis and effective therapeutic design. The pleiotropic pathophysiological profile of the disease includes impaired cholinergic transmission, amyloid-beta deposition, tau-mediated neurofibrillary tangles, and biometal dyshomeostasis,

alongside oxidative stress and mitochondrial impairment. Despite their clear involvement in neuronal demise, the chronology and the link between these pathophysiological changes remain elusive (Cavalli et al., 2008).

The cholinergic hypothesis remains the most long-standing paradigm in AD research, serving as the pharmacological foundation for the most currently approved FDA treatments. Acetylcholine (ACh), a neurotransmitter, is essential for mediating memory and cognitive processes. Following ACh's interaction with nicotinic and muscarinic receptors, the transmission is terminated via enzymatic degradation. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes are responsible for the hydrolysis of ACh into choline and acetate at the synaptic cleft (Scarpini et al., 2003).

In the later stages of AD, AChE levels decrease significantly, and BChE levels increase to compensate for the decline in AChE levels. Moreover, BChE is associated with neurofibrillary tangles. As a result, in addition to AChE, BChE becomes an important therapeutic target as the disease progresses (Greig et al., 2001; Guillozet et al., 1997). Inhibition of AChE and BChE is still the main strategy in the fight against AD, even nowadays.

As AD progresses, the cholinesterase enzyme profile undergoes remodeling in the brain. BChE adapts to declining AChE activity, helping to preserve cholinergic homeostasis. Beyond its hydrolytic function, BChE is associated with neurofibrillary tangles. Given its presence in neurofibrillary tangles and its compensatory capacity, BChE represents a high-priority target for drug development in late-stage AD (Greig et al., 2001; Guillozet et al., 1997). Thus, targeting both AChE and BChE persists as an important strategy for AD drug development.

The AChE's active site is situated within a deep and continuously narrowing channel on the surface of the protein. The enzyme possesses two essential

binding sites within its internal topography: the catalytic anionic site (CAS), nestled at the bottom of the active-site gorge, and the peripheral anionic site (PAS), which is strategically located at the peripheral entry to facilitate initial substrate recognition (Bajda et al., 2013; Inestrosa et al., 1996). ACh initially interacts with the PAS through electrostatic interactions and subsequently migrates along the gorge toward the CAS, where catalytic hydrolysis occurs. Given that AChE and BChE exhibit approximately 65% sequence homology, BChE similarly contains both CAS and PAS regions (Nicolet et al., 2003).

Tacrine, approved by the FDA as the first medication for AD, was withdrawn from the market four years later due to serious adverse effects, primarily hepatotoxicity (Gracon et al., 1998). Although tacrine is associated with certain clinical limitations, its core structure continues to serve as a valuable template for the design of novel AChE inhibitors with enhanced pharmacological properties (Lin et al., 2017). Numerous tacrine-derived compounds have demonstrated strong inhibitory potency toward AChE while exhibiting reduced toxicity compared to the parent molecule. Accordingly, a wide range of tacrine-based analogues have been synthesized and biologically evaluated as potential AChE inhibitors (Przybyłowska et al., 2019; Sameem et al., 2017).

On the other hand, the hydrazone-type compounds are associated with a wide range of bioactivity profiles, demonstrating antimicrobial, anticonvulsant, and anti-inflammatory properties, which makes them versatile candidates in drug discovery. Besides, there are a few examples of hydrazone-containing compounds with antioxidant and neuroprotective biological profiles (Narang et al., 2012; Padmini et al., 2013; Rollas & Güniz Küçükgülzel, 2007; Verma et al., 2014). Previously, we have reported hydrazone-containing derivatives as potential anti-Alzheimer compounds with low μM range ChE inhibitory (Parlar et al., 2016a; Prinz et al., 2020). Additionally, in our pursuit to develop novel multifunctional compounds

against AD, hydrazone-bearing tacrine-donepezil hybrids were introduced possessing good ChE inhibitory properties (Bayraktar et al., 2024).

In light of these findings and as part of our continued research efforts, we synthesized tacrine-based hydrazone derivatives bearing different substituents on the benzylidene ring. They have been reported to exhibit various pharmacological activities (Dontsova et al., 2001; Fathhalla et al., 2013; Fattorusso et al., 2008; Gemma et al., 2012; Pellerano & Savini, 1983; Sahu et al., 2012). In the present study, we focused on evaluating their inhibitory activity against AChE and BChE to evaluate their potential against Alzheimer's disease.

The inhibitory activities of all synthesized derivatives against AChE and BChE were evaluated using *in vitro* assays. Subsequently, molecular docking studies were performed to characterize their interaction patterns within the enzymes' active sites. Finally, the pharmacokinetic and toxicity parameters (ADMET profiles) were calculated *in silico*.

MATERIALS AND METHODS

Chemistry

All chemicals, reagents, and solvents used in this study were obtained as high-purity commercial products from Sigma-Aldrich, Merck, and Fluka. Reaction progress was monitored by thin-layer chromatography (TLC) on silica gel-coated aluminum plates (Merck Silica gel 60 F₂₅₄). The developed TLC plates were visualized under ultraviolet light at 254 nm. The structures of the synthesized compounds were confirmed through melting point determination as well as FT-IR, ¹H NMR, and ESI-MS spectroscopic analyses. FT-IR spectra were recorded using a PerkinElmer Spectrum 100 FT-IR spectrometer equipped with an attenuated total reflectance (ATR) accessory. ¹H NMR spectra were obtained on a Varian AS 400 Mercury Plus spectrometer operating at 400 MHz using deuterated dimethyl sulfoxide (DMSO-d₆) as the solvent. Chemical shifts (δ) are expressed in parts per million (ppm), while coupling constants (*J*) are reported in hertz (Hz). Mass spectrometric analyses were carried

out on a Thermo MSQ Plus LC-MS instrument using analytical-grade solvents and employing the electrospray ionization (ESI) technique. The melting points of the synthesized compounds were measured with a Barnstead Electrothermal IA9100 series melting point apparatus and are reported without correction.

Synthesis of compound 9-chloro-1,2,3,4-tetrahydroacridine hydrochloride

A mixture of anthranilic acid (5.4 mmol) and cyclohexanone (5.2 mmol) was slowly combined with 3 ml of POCl₃ while maintaining the reaction in an ice bath. The resulting mixture was then heated under reflux at 100 °C for 2 hours. After cooling to room temperature, the mixture was neutralized with a saturated K₂CO₃ solution and extracted three times with ethyl acetate. The pooled organic layers were washed with brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The crude product was purified by recrystallization from acetone, affording 9-chloro-1,2,3,4-tetrahydroacridine hydrochloride (Teponnou et al., 2017).

Synthesis of compound 9-hydrazinyl-1,2,3,4-tetrahydroacridine hydrochloride (1)

The intermediate (3.7 mmol) obtained in the first step was refluxed in n-propanol with hydrazine hydrate (3.7 mmol) for 18 h. Once the reaction was complete, the mixture was cooled to ambient temperature, and the precipitate formed was collected by filtration and subsequently washed with n-propanol (Douglas et al., 1977).

Synthesis of the final compounds (1a-1e)

Compound **1** (1.4 mmol) obtained in the second step was stirred with various aldehydes (1.4 mmol; benzaldehyde, 4-methylbenzaldehyde, 4-methoxybenzaldehyde, 4-chlorobenzaldehyde, 4-nitrobenzaldehyde) in ethanol for 6-12 hours. The resulting crude product was treated with cold ethanol and filtered. Then, the final compounds (**1a-1e**) were obtained by crystallization with ethanol (Scheme 1.) (Douglas et al., 1977).

9-(2-Benzylidenehydrazinyl)-1,2,3,4-tetrahydroacridine hydrochloride (1a)

Yield: 68%; mp 157 °C; IR ν_{maks} : 3328, 2934, 2690, 1634, 1577 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆) δ : 1.84-1.87 (4H, m), 2.91 (2H, bs), 3.09 (2H, bs), 7.47-7.55 (3H, m, Ar-H), 7.64 (1H, ddd, J = 1.2/7.2/7.8 Hz, Ar-H), 7.76 (2H, dd, J = 1.6/8.0 Hz, Ar-H), 7.88 (1H, td, J = 0.8/8.4 Hz, Ar-H), 8.07 (1H, d, J = 8.4 Hz, Ar-H), 8.89 (1H, s, Ar-CH=N-), 9.28 (1H, d, J = 8.4 Hz, Ar-H), 11.39 (1H, bs, NH) ppm. ESI-MS m/z : 302.28 [M+H]⁺

9-(2-(4-Methylbenzylidene)hydrazinyl)-1,2,3,4-tetrahydroacridine hydrochloride (1b)

Yield: 82%; mp 217 °C; IR ν_{maks} : 3243, 2768, 1636, 1586 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆) δ : 1.85 (4H, bs), 2.37 (3H, s), 2.88 (2H, bs), 3.08 (2H, bs), 7.30 (2H, d, J = 8.4 Hz, Ar-H), 7.60-7.65 (3H, m, Ar-H), 7.87 (1H, ddd, J = 1.2/7.2/7.8 Hz, Ar-H), 8.06 (1H, d, J = 8.4 Hz, Ar-H), 8.84 (1H, s, Ar-CH=N-), 9.28 (1H, d, J = 8.8 Hz, Ar-H), 11.30 (1H, bs, NH) ppm. ESI-MS m/z : 316.27 [M+H]⁺

9-(2-(4-Methoxybenzylidene)hydrazinyl)-1,2,3,4-tetrahydroacridine hydrochloride (1c)

Yield: 75%; mp 279 °C; IR ν_{maks} : 3238, 2710, 1635, 1585 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆) δ : 1.85 (4H, bs), 2.87 (2H, bs), 3.07 (2H, bs), 3.84 (3H, s), 7.07 (2H, d, J = 8.8 Hz, Ar-H), 7.62 (1H, td, J = 1.2/7.6 Hz, Ar-H), 7.70 (2H, d, J = 8.8 Hz, Ar-H), 7.87 (1H, t, J = 7.2 Hz, Ar-H), 8.04 (1H, d, J = 8.4 Hz, Ar-H), 8.81 (1H, s, Ar-CH=N-), 9.31 (1H, d, J = 8.8 Hz, Ar-H), 11.25 (1H, bs, NH) ppm. ESI-MS m/z : 332.25 [M+H]⁺

9-(2-(4-Chlorobenzylidene)hydrazinyl)-1,2,3,4-tetrahydroacridine hydrochloride (1d)

Yield: 72%; mp 120 °C; IR ν_{maks} : 3220, 2929, 2780, 1633, 1580 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆) δ : 1.87 (4H, bs), 2.91 (2H, bs), 3.10 (2H, bs), 7.57 (2H, dt, J = 2/8.4 Hz, Ar-H), 7.67 (1H, ddd, J = 1.2/7.2/7.8 Hz, Ar-H), 7.79 (2H, dt, J = 2/8.8 Hz, Ar-H), 7.91 (1H, ddd, J = 1.2/7.2/7.8 Hz, Ar-H), 8.04 (1H, d, J = 8.8 Hz, Ar-H), 8.83 (1H, s, Ar-CH=N-), 9.22 (1H, d, J = 8.8

Hz, Ar-H), 11.39 (1H, bs, NH) ppm. ESI-MS m/z : 336.16 (100%) $[M+H]^+$, 338.16 (40%) $[M+H+2]^+$

9-(2-(4-Nitrobenzylidene)hydrazinyl)-1,2,3,4-tetrahydroacridine hydrochloride (1e)

Yield: 93%; mp 285°C (decomposition); IR ν_{max} : 2950, 2575, 1634, 1574 cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ : 1.85 (4H, bs), 2.93 (2H, bs), 3.10 (2H, bs), 7.65 (1H, td, $J = 1.2/7.6$ Hz, Ar-H), 7.86-7.93 (3H, m, Ar-H), 8.06 (2H, d, $J = 8.4$ Hz, Ar-H), 8.26 (2H, d, $J = 8.8$ Hz, Ar-H), 8.95 (1H, s, Ar-CH=N-), 9.13 (1H, d, $J = 8.4$ Hz, Ar-H) ppm. ESI-MS m/z : 347.21 $[M+H]^+$

Biological activity assays

In vitro inhibition of AChE and BChE

AChE (E.C. 3.1.1.7, type VI-S, from *electric eel*), BChE (E.C. 3.1.1.8, from *equine serum*), DTNB [Ellman's reagent; (5,5'-Dithiobis(2-nitrobenzoic acid)]], acetylthiocholine iodide (ATC), S-butyrylthiocholine iodide (BTC) and DMSO were purchased from Sigma-Aldrich. Potassium dihydrogen phosphate, potassium hydroxide, and sodium bicarbonate were purchased from Fluka. Deionized water was produced using a Millipore® purification system (Eschborn, Germany). Spectral measurements were carried out on a Shimadzu 160A instrument.

The inhibitory effects of the synthesized compounds on AChE and BChE were assessed using a modified Ellman colorimetric assay (Ellman et al., 1961; Parlar et al., 2016b; Prinz et al., 2013), with tacrine hydrochloride and galantamine serving as reference standards. The IC_{50} values were obtained from the logarithmic concentration-inhibition plots using GraphPad Prism 4.03 (GraphPad Software Inc.) and are presented in Table 1.

Docking studies

The chemical structures of studied compounds were generated with MarvinSketch chemical drawing tool (ChemAxon, 2012). Hydrogens were added with USCF Chimera 1.16, charges were calculated with antechamber using AM1-BCC charged method and compounds were parametrized using GAFF force

field (Jakalian et al., 2002; Pettersen et al., 2004; Wang et al., 2004, 2006). The prepared compounds were exposed to an energy minimization with USCF Chimera 1.16 (Pettersen et al., 2004).

The crystal structure of tacrine in complex with *ee*AChE (PDB id: 1ACJ resolved at 2.80 Å) was taken from the RCSB Protein Data Bank (Harel et al., 1993). Waters and heteroatoms were removed from the PDB file and *ee*AChE were subjected to an energy minimization using USCF Chimera 1.16 (Pettersen et al., 2004). Homology model of *eq*BChE was prepared from the crystal structure of human butyrylcholinesterase (PDB id: 4TPK resolved at 2.70 Å). The preparations of homology models were described for *eq*BChE in the Sarıkaya et al. (2018).

ADMET

ADMET properties of the synthesized compounds were estimated through the ADME and toxicity prediction tools provided by the PreADMET server (Lee et al., 2004).

RESULTS AND DISCUSSION

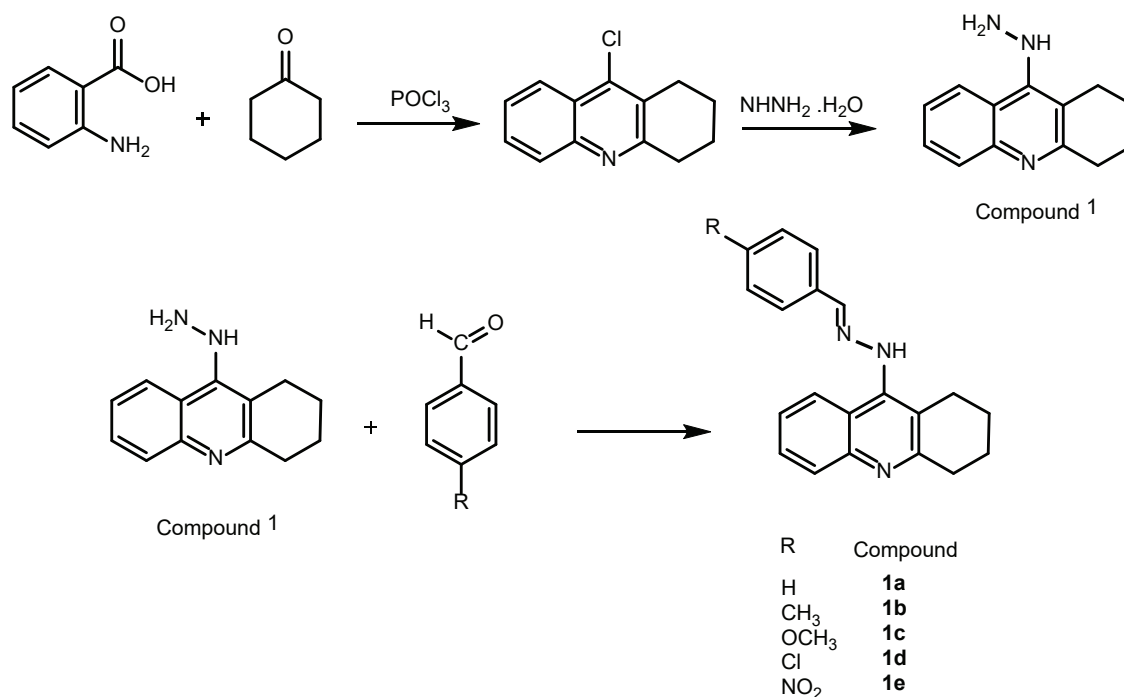
In this study, five tacrine derivatives were synthesized, and their structures were elucidated by melting point values and FT-IR, ^1H NMR, and ESI-MS spectroscopic methods. The ability of the final compounds to inhibit AChE and BChE was assessed using a modified version of the Ellman colorimetric assay (Ellman et al., 1961; Parlar et al., 2016b; Prinz et al., 2013). The inhibitory potencies were reported as IC_{50} values. Tacrine hydrochloride and galantamine were used as the reference standards. The IC_{50} values for AChE and BChE inhibition are presented in Table 1.

Chemistry and structural characterization

The final compounds were synthesized in three steps. In the first step, anthranilic acid and cyclohexanone were reacted in the presence of phosphoroxychloride to obtain the intermediate 9-chloro-1,2,3,4-tetrahydroacridine. In the second step, aromatic nucleophilic substitution of 9-chloro-1,2,3,4-tetrahydroacridine with hydrazine hydrate yielded 9-hydraz-

inyl-1,2,3,4-tetrahydroacridine. In the final step, the intermediate 9-hydrazinyl-1,2,3,4-tetrahydroacridine was condensed with unsubstituted benzaldehyde or

benzaldehydes bearing methyl, methoxy, chloro and, nitro substituents at *para* position to obtain the target compounds (Scheme 1.).



Scheme 1. General synthetic route for the title compounds (**1a-1e**)

Characteristic absorption peaks observed in the IR spectra of the final compounds were consistent with their suggested molecular structures. The bands observed in the range of 3328-3220 cm⁻¹ were assigned to N-H stretching vibrations, and absorptions seen between 2950-2710 cm⁻¹ were assigned to aromatic and aliphatic C-H stretching vibrations (Silverstein et al., 2014). The intense absorption bands in the IR spectra were seen between 1634-1574 cm⁻¹ region. These bands, as expected, were attributed to the N=CH stretching vibration of the hydrazone moiety and the aromatic C=C stretching vibrations (Silverstein et al., 2014). In addition, the symmetrical stretching vibration of the nitro substituent of compound **1e** was detected as a sharp band at 1330 cm⁻¹, confirming the presence of the nitro atom (Silverstein et al., 2014).

The ¹H NMR spectra, recorded in DMSO-d₆, confirmed the presence of aromatic and aliphatic protons with chemical shifts and splitting patterns consistent

with the proposed structures. The proton signals due to the NH group were recorded as a broad singlet between δ 11.25-11.39 ppm. The singlet signal observed in the aromatic region between δ 8.81-8.95 ppm was assigned to the hydrazone N=CH proton, confirming that 9-hydrazinyl-1,2,3,4-tetrahydroacridine ring condensed with benzaldehydes to give 9-benzylidenehydrazinyl-1,2,3,4-tetrahydroacridine derivatives. In all spectra, the doublet signal detected at δ 9.13-9.31 ppm (*J* = 8.4-8.8 Hz) was assigned to the H-8 proton of the 1,2,3,4-tetrahydroacridine ring, consistent with its expected downfield chemical shift (Silverstein et al., 2014).

Regarding the proton signals of the benzylidene ring, in the spectra of **1b**, **1c**, and **1d**, the signals observed between δ 7.07-7.57 ppm, integrating for two protons, were assigned to the H-2' and H-6' protons. In the spectra of **1e**, these protons are thought to resonate within the multiplet detected at δ 7.86-7.93 ppm,

owing to the electron-withdrawing nature of the nitro substituent (Silverstein et al., 2014). On the other hand, the H-3' and H-5' proton signals appeared between δ 7.60-7.79 ppm in the spectra of **1b**, **1c**, and **1d**, and the doublet signal observed at δ 8.26 ppm in the spectrum of **1e** was attributed to these protons. The downfield shift of these signals in **1e** can be attributed to the electron-withdrawing influence of the nitro group, as expected (Silverstein et al., 2014). In the spectra of compound **1a**, which contains an unsubstituted benzylidene ring, three aromatic protons of the benzylidene ring appeared as a multiplet between δ 7.47-7.55 ppm, whereas the remaining two aromatic protons were observed as a doublet of doublets at δ 7.76 ppm, resulting from *ortho* and *meta* couplings.

The aliphatic proton signals of the tetrahydroacridine were detected as a broad singlet integrating for four protons at δ 1.84-1.87 ppm, and as broad singlets, each integrating for two protons, at δ 2.87-2.93 ppm and δ 3.07-3.10 ppm. In derivatives **1b** and **1c**, the

methyl and methoxy substituents were detected as sharp singlets at δ 2.37 and δ 3.84 ppm, respectively, each integrating for three protons, as expected (Silverstein et al., 2014).

The mass spectra of the synthesized compounds were confirmed by ESI spectra showing molecular ion peaks (M^+) consistent with their calculated molecular weights. The isotopic peak is also clearly observed in the mass spectrum of the compound **1d** carrying the chloro substituent.

Biological activity evaluation

Following synthesis and structural characterization, the compounds were tested for their potential anti-Alzheimer's activity. The inhibitory potential of the compounds toward AChE and BChE was investigated *in vitro* via Ellman's colorimetric technique (Ellman et al., 1961; Parlar et al., 2016b; Prinz et al., 2013). As the reference standards, tacrine and galantamine were used. The biological evaluation results of the synthesized compounds are summarized in Table 1.

Table 1. Cholinesterase (AChE and BChE) inhibitory activities of the title compounds

Compound	$IC_{50} \pm S.E.M. (\mu M)^a$		Selectivity $eeAChE/eqBChE$
	<i>eeAChE</i>	<i>eqBChE</i>	
1a	0.359 ± 0.029	0.025 ± 0.001	14.36
1b	0.088 ± 0.005	0.077 ± 0.006	1.14
1c	0.103 ± 0.012	0.066 ± 0.003	1.56
1d	0.165 ± 0.010	0.093 ± 0.008	1.77
1e	0.134 ± 0.011	0.070 ± 0.008	1.91
Tacrine HCl	0.0758 ± 0.02	0.0098 ± 0.0002	7.73
Galantamine	0.43 ± 0.03	14.92 ± 0.57	0.02

^a Results are expressed as mean $\pm$ standard error of the mean (SEM) from triplicate independent experiments.

IC_{50} values were obtained using GraphPad 5 software.

Different substituents, including methyl, methoxy, chloro and, nitro, were introduced at *para* position of the benzylidene ring to evaluate their effects on inhibitory potency. According to the AChE inhibitory activity results, all compounds exhibited significant inhibition in the range of 0.088-0.359 μM . The *para*-substituted compounds (**1b**, **1c**, **1d**, **1e**) showed

higher affinity than the unsubstituted compound (**1a**) towards AChE. Among the *para*-substituted derivatives, the AChE inhibitory activity increased in the order of *p*-Cl < *p*-NO₂ < *p*-OCH₃ < *p*-CH₃ substitutions on the benzylidene ring. The most active compound was found to be **1b**, bearing a methyl substituent on the benzylidene ring, with an IC_{50} value of 0.088

μM . Notably, derivatives **1b** ($\text{IC}_{50} = 0.088 \mu\text{M}$) and **1c** ($\text{IC}_{50} = 0.103 \mu\text{M}$) showed comparable AChE inhibition to that of the reference drug tacrine ($\text{IC}_{50} = 0.0758 \mu\text{M}$).

In terms of BChE activity, all compounds displayed inhibitory activity in the nanomolar range ($\text{IC}_{50} = 0.025\text{--}0.093 \mu\text{M}$), showing higher potency against BChE than AChE, thus indicating BChE selectivity. The unsubstituted derivative **1a** exhibited the highest BChE inhibition ($\text{IC}_{50} = 0.025 \mu\text{M}$), but the lowest activity against AChE, demonstrating approximately 14-fold selectivity for BChE over AChE. And, the *para*-substituted compounds (**1b–1e**) exhibited relatively modest and comparable selectivity towards BChE, with selectivity ratios ranging from 1.14 to 1.91, suggesting only a slight preference for BChE inhibition. Within the series, the inhibitory activity in-

creased in the following order: *p*-Cl < *p*-CH₃ < *p*-NO₂ < *p*-OCH₃, with the chloro-substituted compound exhibiting the lowest activity against both enzymes. Moreover, all compounds showed stronger inhibition of both enzymes compared to the reference compound galantamine ($\text{IC}_{50} = 0.43 \mu\text{M}$ on AChE; $14.92 \mu\text{M}$ on BChE).

Docking studies

In this study, the non-covalent interactions of tacrine analogs, determined as the most potent inhibitors of *ee*AChE and *eq*BChE, were analyzed through molecular docking within the active sites of *ee*AChE (PDB ID: 1ACJ) and *eq*BChE (homology model). The DOCK6 program was employed to predict the binding geometries of these compounds within the active site gorge of AChE and BChE. The docking results are given in Table 2.

Table 2. The calculated docking scores of the title compounds inside the active sites of *ee*AChE (PDB ID:1ACJ) and *eq*BChE (homology model) with the absolute ranked positions

Compound	<i>ee</i> AChE (Gridscore)	<i>eq</i> BChE (Gridscore)
1a	-34.56 (16)	-40.62 (2)
1b	-40.04 (3)	-40.38 (2)
1c	-39.95 (10)	-40.15 (11)
1d	-35.30 (10)	-39.69 (4)
1e	-43.54 (3)	-39.84 (13)
Tacrine	-27.26 (23)	-26.55 (10)

The proposed docking pose of compound **1b** revealed that its 1,2,3,4-tetrahydroacridine ring and hydrazine group were positioned within CAS of AChE, while the 4-methylbenzylidene group was located between the CAS and the PAS of AChE (Figure 1A.). Notably, the 1,2,3,4-tetrahydroacridine ring of compound **1b** adopted a similar orientation to tacrine within the CAS of AChE (Figure 1B.). This ring extended towards His440, nestled between Trp84 and Phe330. Furthermore, the 4-methylbenzylidene group was positioned between the aromatic rings of Tyr121

and Phe330. Within the CAS, compound **1b** established critical interactions, including sandwich-type π - π , T-shape π - π , and hydrophobic interactions, which significantly influenced its binding orientation. The sandwich-type π - π interactions occurred as the 1,2,3,4-tetrahydroacridine ring of compound **1b** positioned between the indole ring of Trp84 and the benzene ring of Phe330. Meanwhile, T-shaped π - π interactions were observed as the 4-methylbenzylidene group of compound **1b** was situated between the benzene rings of Tyr121 and Phe330 (Figure 1A.).

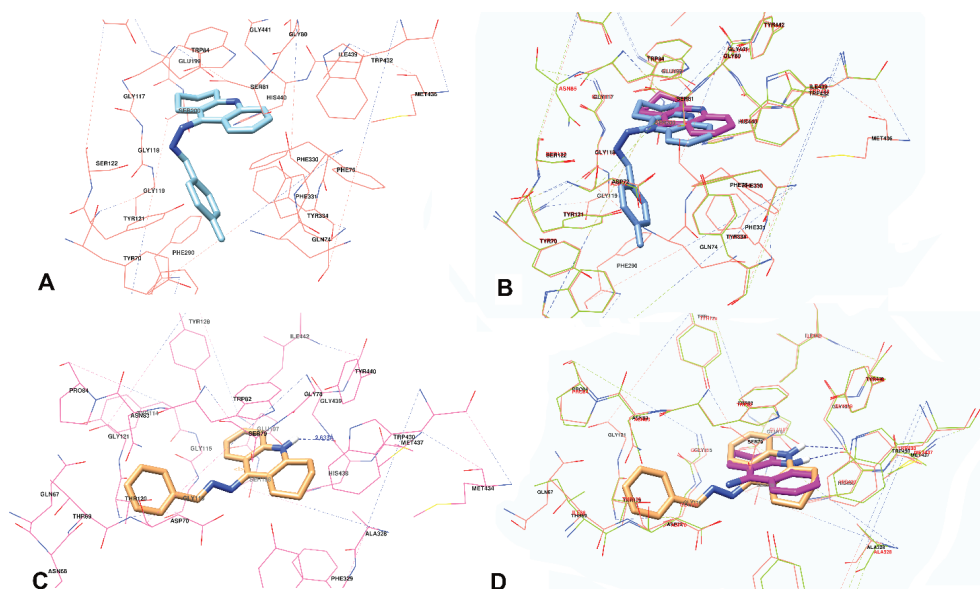


Figure 1. (A) The estimated binding geometry of compound **1b** in *eeAChE*. (B) Superposition of compound **1b-eeAChE** complex and cocrystal structure of tacrine-*eeAChE* complex (PDB ID:1ACJ). (C) The estimated binding geometry of compound **1a** in *eqBChE*. (D) Superposition of compound **1a-eqBChE** complex and cocrystal structure of tacrine-*huBChE* complex (PDB ID:4BDS). Sandy brown and light blue sticks represent compound **1a** and **1b**, respectively, and salmon-colored sticks represent the active site residues of *eeAChE* and *eqBChE*. Magenta sticks represent tacrine, and the chartreuse green sticks represent the active site residues of crystal *eeAChE* and crystal *huBChE*. Hydrogen bonds were given as blue lines. The active site residues are named using three letters code. For a clear image, the hydrogen atoms, excluding the formed hydrogen bonds, were ghosted

The suggested docking pose of compound **1a** demonstrated that its 1,2,3,4-tetrahydroacridine ring and hydrazine group were positioned within the CAS of BChE. Remarkably, the 1,2,3,4-tetrahydroacridine ring of compound **1a** exhibited an orientation similar to tacrine within the CAS of *huBChE* (Nachon et al., 2013) (Figure 1C and 1D.). This ring is positioned between Trp82, Glu197 and His438. Additionally, the benzylidene group occupied a pocket formed by Gln67, Asn68, Thr69, Asp70, Asn83, Pro84, Tyr120, and Gly121 (Figure 1C.).

Inside the *eqBChE*, compound **1a** establishes crucial interactions, including hydrogen bonding, face-to-face π - π and hydrophobic interactions, which play a vital role in stabilizing its binding orientation. The protonated nitrogen atom of the 1,2,3,4-tetrahydroacridine ring and the carbonyl oxygen of His438 formed a hydrogen bond. The face-to-face π - π interaction involved the 1,2,3,4-tetrahydroacridine ring of

compound **1a** directly aligning with the indole ring of Trp82. Additionally, the benzylidene group is also engaged in hydrophobic interactions with residues within the pocket residues, further enhancing stability.

ADMET

The ADMET properties of compounds can provide an opportunity to evaluate their potential as drugs to treat the target disease. For this purpose, the PreAdmet web server was used to estimate the ADMET properties of compounds **1a** and **1b** in our study (Lee et al., 2004). The calculation results for the compounds are reported in Table 3. Compounds **1a** and **1b** were determined to cross the blood-brain barrier at high levels (Lee et al., 2004). The compounds bound strongly to plasma proteins. They were determined to have low skin permeability, good human intestinal absorption, and moderate CaCO_2 permeability. The estimated effects of compounds **1a** and **1b** on liver enzymes were found to be variable. They were found

to have an inhibitory effect against the CYP 2D6 enzyme, while they had no inhibitory effects against the CYP 2C19, CYP 2C9, and CYP 3A4 enzymes.

Estimated toxicity data of the compounds were determined by acute algal toxicity (Algae at), Ames test, cancerous mouse, cancerous rat, acute water flea toxicity (Daphnia at), hERG inhibition, acute fish toxicity (medaka at and minnow at), *in vitro* Ames tests in TA100 strains (TA100-10RLI, TA100-NA), *in vitro* Ames tests in TA1535 strains (TA1535-10RLI,

TA1535-NA). Positive and negative results were predicted for the compounds **1a** and **1b** for the cancerous mouse test and the cancerous rat test, respectively. A positive result in the experimental models indicates the absence of carcinogenic activity. The Ames test indicated that compounds **1a** and **1b** have possible mutagenic properties. *In vitro* Ames tests in TA100 and TA1535 strains showed negative results because the compounds had no metabolic activity. The potential effect of compounds **1a** and **1b** on hERG inhibition has been determined to carry a medium risk.

Table 3. Predicted ADME/Tox properties of the title compounds

Properties	Compound 1a	Compound 1b
BBB	10.6872	12.0365
Buffer solubility (mg/L)	938.726	671.6
Caco2	23.4448	23.8804
CYP 2C19 inhibition	Non	Non
CYP 2C9 inhibition	Non	Non
CYP 2D6 inhibition	Inhibitor	Inhibitor
CYP 2D6 substrate	Substrate	Substrate
CYP 3A4 inhibition	Non	Non
CYP 3A4 substrate	Weakly	Weakly
HIA	93.18373	93.314987
MDCK	1.4742	0.0626938
Pgp inhibition	Inhibitor	Inhibitor
Plasma Protein Binding	85.928114	84.205497
Pure water solubility (mg/L)	0.402596	0.119116
Skin Permeability	-3.20097	-3.0572
SKlogD value	3.8802	4.36657
SKlogP value	3.8802	4.36657
SKlogS buffer	-2.50804	-2.67316
SKlogS pure	-5.87571	-6.4243
Algae at	0.0183023	0.00809627
Ames test	mutagen	mutagen
Carcino Mouse	positive	positive
Carcino Rat	negative	negative
Daphnia at	0.0249753	0.00889742
hERG inhibition	medium_risk	medium_risk
Medaka at	0.00123452	0.00017401
Minnow at	0.00145865	0.000320213
TA100 10RLI	negative	positive
TA100 NA	negative	negative
TA1535 10RLI	negative	negative
TA1535 NA	negative	negative

CONCLUSION

In conclusion, a set of tacrine-derived compounds was prepared and tested for their ability to inhibit AChE and BChE. All compounds displayed significant inhibitory activity, with selectivity towards BChE. Notably, all derivatives demonstrated better inhibitory potency on both enzymes than the reference drug galantamine. Among them, the unsubstituted derivative **1a** showed the strongest BChE inhibitory activity, whereas compound **1b**, having a methyl substituent, displayed the highest AChE inhibitory activity. Introduction of a substituent at the *para* position of the benzyldiene ring enhanced the AChE activity, but reduced BChE activity, and no clear correlation was observed between the electron-withdrawing or electron-donating nature of the substituents and the enzyme inhibition. Molecular docking analyses offered insights into the probable binding interactions of the most potent compounds, and *in silico* ADMET predictions suggested favorable pharmacokinetic and toxicity properties. Overall, these results highlight the potential of these derivatives as promising lead candidates for the development of novel therapeutics for Alzheimer's disease.

ACKNOWLEDGEMENTS

This work was funded by the Ege University Research Foundation (Project No. 16-ECZ-028). The authors are grateful to the Pharmaceutical Sciences Research Centre (FABAL) at the Faculty of Pharmacy, Ege University, for access to spectral analysis instruments and molecular docking software.

AUTHOR CONTRIBUTION STATEMENT

GB: Synthesis of the compounds, bioactivity studies, and writing; SP: conceptualism, bioactivity studies, and writing; GC: molecular modelling studies and writing; VA: conceptualism and writing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUPPORTING INFORMATION

Supplementary data associated with this study, including FT-IR, ¹H NMR, and ESI-MS spectra of the final compounds, are accessible online.

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