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Design, Synthesis, Molecular Docking, and Pharmacological Evaluation of 2-Aminobenzimidazole Derivatives as Potential Anti-Alzheimer's Agents

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ABSTRACT

Some new aminobenzimidazole derivatives were synthesized and investigated their pharmacological potential including anti-oxidant and acetylcholinesterase inhibitory activity to treat AD. A series of eight benzimidazole derivatives was synthesized by two-step procedure. All the synthesized derivatives were characterized by spectroscopic techniques such as NMR (^{1}H-NMR) and FTIR. All the compounds were further evaluated for their anti-oxidant and AchE inhibitory potential. Four compounds 2g, 2a and 2b have shown potent inhibitory activity as 91.86%, 88.69%, and 81.91% respectively. Among eight synthesized compounds, the highest anti-oxidant activity 86.64% was exhibited by compound 2d, and compound 2d showed higher potency with IC50 of 4.96 µg/mL. Next docking studies were performed by using Auto Dock Vina program for which acetylcholinesterase has been used as target. Compounds 2e, 2a and 2d have exhibited the highest binding affinity with acetylcholinesterase among the synthesized derivatives with a value of -10.5 kcal/mol, -10.4 kcal/mol and -10.3 kcal/mol respectively. The results indicated that benzimidazole derivatives could be used as lead molecules for acetylcholinesterase inhibitors and can be further evaluated for their therapeutic potential for the treatment of AD.

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent dementia and is defined by a progressive loss of cognitive functions [1]. Initial symptoms usually include short-term memory impairment, mood changes, difficulty in learning, and difficulty in executing daily tasks [2]. AD is a multifactorial neurodegenerative disorder that emerges from multiple interrelated pathological processes ultimately resulting in neuronal dysfunction and death [3]. The major neuropathological features of AD are extracellular amyloid plaques, intracellular neurofibrillary tangles, and dysfunction of the cholinergic neurotransmission system [4]. In addition, oxidative stress and synaptic loss additionally contribute to the worsening of cognitive impairment. The deposition of amyloid-β (Aβ) peptides—produced by the amyloidogenic cleavage of amyloid precursor protein (APP)—lies at the core of senile plaque formation and is involved in disease causation [5]. High levels of pro-inflammatory mediators like interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), and cyclooxygenase-2 (COX-2) have been found in AD brains and have been used as markers of neuroinflammation and progression of the disease. Of these, COX-2 is the premier enzyme mediating neuroinflammatory cascades and is significantly upregulated in the brains of AD patients, and hence is a

potential therapeutic target for drug development. This enzyme is over-expressed in AD patient brain and has also been utilized as a drug target to design drugs [6]. Loss of cholinergic neurons, especially in the basal forebrain, is coupled with loss of neurotransmitter acetylcholine. Reduced levels of acetyl choline in the brain of AD patients seem to be an essential component in causing dementia [7].

Acetylcholine plays very significant role in attention, learning, memory and motivation. A widely distributed hydrolase enzymes are classified into Cholinesterases such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The imbalance between AChE and BChE leads to the development of AD in patients. Since AChE enzymes are more hydrolytic than BChE, so to save this AChE inhibitors are the best choice to treat AD [8]. Davies and Maloney in 1976 proposed this theory regarding AD pathogenesis named as cholinergic hypothesis [9]. As a result of this hypothesis the first drug to be approved for the treatment of AD is Tacrine. According to this hypothesis the cholinergic neurons in the brain undergo degeneration which leads to the decrease in the level of neurotransmitter acetylcholine as well as its associated enzyme choline acetyltransferase (ChAT). The specific regions of the brain are hippocampus and cortex where low level of AChE occurs resulting in learning and memory dysfunction [10].

In order to prevent and treat AD, the researchers need to develop new therapies. As the AD pathogenesis mechanisms need more clarification, up till now, there is difficulty in development of effective drugs. The researchers need to develop multiple drug target therapies as multiple factors are involved in progression of AD [11]. So far, the USA Food and Drug Administration (FDA) approved only five drugs for the treatment of AD, but all of these five drugs are used for symptomatic treatment with temporary relief. Among the currently available therapeutic options for Alzheimer's disease, four drugs function as acetylcholinesterase (AChE) inhibitors tacrine (now withdrawn from clinical use), donepezil (DNP), rivastigmine, and galantamine while memantine acts as an N-methyl-D-aspartate (NMDA) receptor antagonist [12]. However, these agents are often associated with adverse effects, including gastrointestinal discomfort, muscle pain, nausea, heartburn, headaches, loss of appetite, diarrhea, imbalance, hepatotoxicity, and short biological half-lives. Consequently, ongoing research efforts are focused on discovering and developing novel therapeutic candidates with improved efficacy and fewer side effects for the management of Alzheimer's disease [13].

The heterocyclic benzimidazole scaffold is an important structural motif in the synthesis of various pharmacologically active compounds [14]. Benzimidazole compounds such as ricobendazole, thiabendazole, albendazole, and oxfendazole have been shown to inhibit cholinesterases in several studies. New compounds with cholinesterase inhibitory action have been discovered involving benzimidazole moiety. The anticholinesterase and antioxidant effects of a variety of Mannich bases of benzimidazole derivatives with a phenolic group were investigated. Ellman's method was used to assess the inhibitory activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in vitro. All of the compounds had moderate to good AChE inhibitory action, according to the activity data [15-16]. The benzimidazole ring system is a privileged scaffold in medicinal chemistry, known for its wide range of pharmacological applications. Benzimidazole-based molecules including albendazole, thiabendazole, and ricobendazole have been reported to inhibit cholinesterase enzymes, highlighting their potential in neuroprotective drug development. Recent studies have also demonstrated that Mannich base derivatives of benzimidazole possessing phenolic groups exhibit both antioxidant and AChE inhibitory properties. Given their structural versatility, benzimidazole derivatives offer a promising framework for designing multifunctional agents that can simultaneously target oxidative stress and cholinergic dysfunction two critical factors implicated in AD progression. Synthesis of amides have been the focus of organic synthesis since decades and a number of methods have been developed. Using succinic anhydride with amines is a convenient and inexpensive method of bond formation, therefore it was selected in the current research to synthesize new amide containing benzimidazole derivatives.

In this context, the present study was designed to synthesize and characterize a novel series of 2-aminobenzimidazole derivatives. The compounds were evaluated for their antioxidant and acetylcholinesterase inhibitory potential, complemented by molecular docking studies to explore ligand–target interactions and binding affinities. This integrated approach aims to identify promising lead structures for the development of safer and more effective therapeutic agents against Alzheimer's disease.

MATERIALS AND METHODS

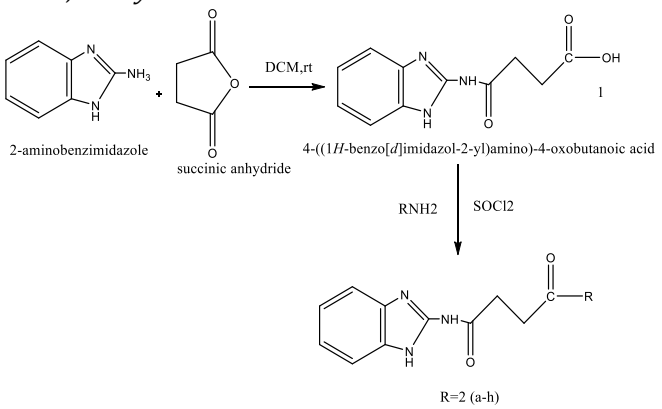
Drugs and Chemicals: Materials required for initiation were obtained from Sigma Aldrich (St. Louis, MO, USA). Melting points of all newly synthesized benzimidazole derivatives were recorded via the A Digital Gallen Kamp apparatus (Sanyo, Osaka, Japan) was utilized for melting point determination. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker AM-300 spectrometer (Billerica, Massachusetts, UK) operating at 300 MHz, using DMSO-d_6 as the solvent and tetramethylsilane (TMS) as the internal reference. Fourier-transform infrared (FTIR) spectra were obtained with an ATR eco ZnSe spectrophotometer, and the absorption maxima (V_max) were expressed in cm^{-1}. The progress of all chemical reactions was monitored using thin-layer chromatography (TLC). Analytical-grade reagents, including 2-aminobenzimidazole, dichloromethane, thionyl chloride, triethylamine, and succinic anhydride, were employed throughout the study. All solvents, chemicals, and reagents used were of 99% HPLC purity.

General Procedure for One-Pot Synthesis of 2-aminobenzimidazole Derivatives (2a-2h): Equimolar amounts of succinic anhydride and 2-aminobenzimidazole were dissolved in dry dichloromethane and stirred at ambient temperature for approximately 20 minutes. The reaction progress was monitored using thin-layer

chromatography (TLC). Solvent systems comprising chloroform:methanol in ratios of 4:1 and 7:1 were utilized to assess the purity of the synthesized compounds. After completion, the resulting solid product was filtered and recrystallized from methanol to yield the desired compound (1) [17]. Subsequently, 1 mmol of cyclohexylamine-4-oxobutanoic acid was reacted with 1 mmol of the corresponding amine and 3 mmol of triethylamine (Et_3N). To this mixture, 1 mmol of thionyl chloride ($SOCl_2$) was added at room temperature. The reaction mixture was stirred for 30–60 minutes, and its progress was monitored by TLC. Following completion, the solvent was evaporated under reduced pressure to obtain the crude product. The residue was dissolved in dichloromethane and sequentially washed with 1 N HCl and 1 N NaOH. The organic phase was then dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and evaporated to dryness, affording the corresponding amide derivatives 2(a–h). (Figure 1: General synthetic pathway; Figure 2: Structures of the newly synthesized 2-aminobenzimidazole derivatives).

Figure 1

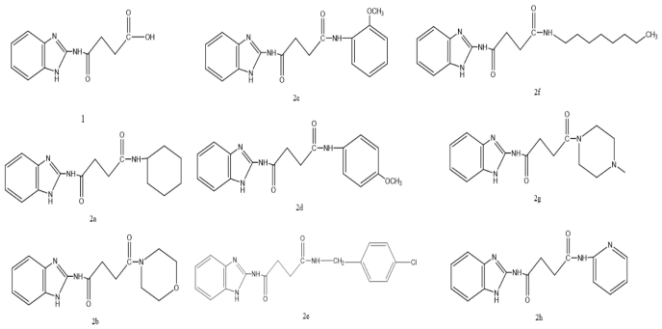
Scheme for the synthesis of new 2-aminobenzimidazole derivatives. DCM, dichloromethane; rt, room temperature; SOCl2, thionyl chloride.



Compound = R
2a = Cyclohexylamine
2b = Morpholine
2c = o-anisidine
2d = p-anisidine
2e = 4-chlorobenzylamine
2f = N-octylamine
2g = N-methyl piperazine
2h = 2-amino pyridine

Figure 2

Structures of all newly synthesized 2-aminobenzimidazole derivatives.



DPPH Free Radical Scavenging Assay: The synthesized derivatives were evaluated for anti-oxidant activity by diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. As positive and negative control, Ascorbic acid (10 µL) was used as the standard, while a blank solution containing 10 µL of DMSO and 190 µL of DPPH solution served as the control. Various concentrations (10, 20, and 40 µL) of each test compound were mixed with 0.1 mM DPPH solution in methanol and vigorously shaken. The resulting mixtures were incubated at 37°C for 30 minutes. After incubation, the absorbance was recorded at 517 nm using a UV–visible spectrophotometer. The IC_{50} values, representing the concentration required to achieve 50% radical scavenging activity, were determined by plotting the percentage inhibition against concentration in Microsoft Excel. The corresponding x-values were obtained from the linear regression equation. The plotted graphs indicated that the tested compounds demonstrated maximum percentage inhibition with the lowest IC_{50} values. The percentage inhibition or radical scavenging activity was calculated using the following formula [18].

$$\% \text{ scavenging activity} = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Acetylcholinesterase Assay: The acetylcholinesterase (AChE) inhibitory activity was assessed using the Hestrin hydroxamic acid method with minor modifications to Ellman's procedure. A total reaction volume of 100 µL was prepared, consisting of 60 µL of 50 mM Na_2HPO_4 buffer (pH 7.7), 10 µL of the test compound (0.5 mM/well), and 10 µL of AChE enzyme (0.005 unit/well). The mixture was gently mixed, and an initial absorbance reading was taken at 405 nm. Subsequently, the samples were preincubated at 37°C for 10 minutes. The enzymatic reaction was initiated by adding 10 µL of acetylthiocholine iodide (0.5 mM/well) as the substrate, followed by 10 µL of DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid)] at a concentration of 0.5 mM/well. After incubation for 37 minutes at 37°C, the absorbance was recorded at 405 nm using a microplate reader (Synergy HT, BioTek, USA). Physostigmine (0.5 mM/well) served as the reference inhibitor. The percentage inhibition of AChE activity was calculated using the following formula

$$\% \text{ Inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

where control is the total enzyme activity without inhibitor and test is the enzyme activity in the presence of test compound [19].

***In-silico* studies:** Docking were performed using free license software packages. Machines used for molecular docking was laptop built of Intel core i5-6300U CPU with 6GB DDR3 RAM running Windows 10 operating system. Chemoinformatics was used to determine chemo-informatics of synthesized compounds. Chemdbiodraw, ChemSketch v2.5, Discovery Studio Visualizer v17.2.0.16349 were used to find ligand and protein interaction and their binding affinities. PyRX was used for molecular docking. All the synthesized ligand were docked against the specific protein (AchE PDB ID: 1GQR) using PyRX. Docking analysis of 2-aminobenzimidazole derivatives (2a-2h) against acetylcholinesterase was performed to compare the relative binding affinity of

ligand and protein. Protein structure (PDB ID: 1GQR) was downloaded from RCSB from protein data bank site. Discovery studio was used to remove water molecule and ligand to clean the protein and saved in the form of PDBQT file format for further use. Discovery Studio Visualizer was utilized to analyze the optimal conformational poses and visualize ligand–target molecular interactions. The docking protocol was validated by superimposing and comparing the conformations of the co-crystallized ligands with those obtained from the re-docking simulations [20].

RESULTS

Table 1

Spectral Analysis and Properties of Synthesized Compounds (2a-2h)

Compound	Physical State / Color	Melting Point (°C)	Rf Value	FTIR (cm^{-1}) *(Key Bands)*	^{1}H-NMR (δ, ppm, DMSO-d_6, 300 MHz) *(Key signals)*
2a N_1-(1H-benzo[d]imidazol-2-yl)-N_4-cyclohexylsuccinamide	White solid	160–180	0.71	3304 (N–H), 1649 (C=O), 1542 (C=C)	8.64 (s, 1H, amide-NH), 7.14 (s, 1H, amide-NH), 8.31–6.35 (m, 4H, Ar-H), 3.63–3.56 (m, 2H, CH_2), 3.45 (d, 2H, CH_2), 3.15 (m, 10H, CH_2)
2b N-(1H-benzo[d]imidazol-2-yl)-4-morpholino-4-oxobutanamide	Light brown solid	60–90	0.85	3395 (N–H), 1720 (C=O), 1569 (C=C)	8.04 (s, 1H, amide-NH), 7.14 (s, 1H, amide-NH), 8.22–6.15 (m, 4H, Ar-H), 3.43–3.21 (m, 2H, CH_2), 3.75 (d, 2H, CH_2), 3.15 (m, 8H, CH_2)
2c N_1-(1H-benzo[d]imidazol-2-yl)-N_4-(2-methoxyphenyl)succinamide	Off-white solid	150–170	0.83	3384 (N–H), 1669 (C=O), 1524 (C=C)	9.64 (s, 1H, amide-NH), 9.14 (s, 1H, amide-NH), 8.01–6.15 (m, 4H, Ar-H), 3.83–3.76 (m, 2H, CH_2), 3.35 (d, 2H, CH_2), 2.76–2.69 (d, 3H, OCH_3, J = 21 Hz)
2d N_1-(1H-benzo[d]imidazol-2-yl)-N_4-(4-methoxyphenyl)succinamide	Black solid	160–180	0.90	3302 (N–H), 1648 (C=O), 1505 (C=C)	8.24 (s, 1H, amide-NH), 7.47 (s, 1H, amide-NH), 8.72–6.35 (m, 8H, Ar-H), 3.33–3.11 (m, 2H, CH_2), 3.25 (d, 2H, CH_2), 2.86–2.59 (d, 3H, OCH_3, J = 21 Hz)
2e N_1-(1H-benzo[d]imidazol-2-yl)-N_4-(4-chlorophenyl)succinamide	Light brown solid	60–100	0.92	2929 (N–H), 1721 (C=O), 1569 (C=C)	8.29 (s, 1H, amide-NH), 7.71 (s, 1H, amide-NH), 8.52–6.15 (m, 8H, Ar-H), 3.33–3.11 (m, 6H, CH_2)
2f N_1-(1H-benzo[d]imidazol-2-yl)-N_4-octylsuccinamide	Light brown solid	120–170	0.83	3295 (N–H), 1628 (C=O), 1543 (C=C)	7.97 (s, 1H, amide-NH), 7.74 (s, 1H, amide-NH), 7.10–6.83 (m, 4H, Ar-H), 2.98 (d, 2H, CH_2), 2.26 (d, 2H, CH_2), 1.35–1.33 (d, 14H, alkyl-H), 0.879–0.840 (t, 3H, CH_3)
2g N-(1H-benzo[d]imidazol-2-yl)-4-(4-methylpiperazin-1-yl)-4-oxobutanamide	Yellow solid	180–185	0.85	3369 (N–H), 1625 (C=O), 1569 (C=C)	8.54 (s, 1H, amide-NH), 7.74 (s, 1H, amide-NH), 8.32–6.45 (m, 4H, Ar-H), 3.43–3.21 (m, 2H, CH_2), 3.75 (d, 2H, CH_2), 3.15 (m, 8H, CH_2), 2.56–2.29 (d, 3H, OCH_3, J = 21 Hz)
2h N_1-(1H-benzo[d]imidazol-2-yl)-N_4-(pyridin-2-yl)succinamide	Dark brown solid	155–160	0.90	3216 (N–H), 1675 (C=O), 1548 (C=C)	8.34 (s, 1H, amide-NH), 7.23 (s, 1H, amide-NH), 8.52–6.25 (m, 8H, Ar-H), 3.53–3.31 (d, 2H, CH_2), 3.35 (d, 2H, CH_2), 2.56–2.29 (d, 3H, OCH_3, J = 21 Hz)

DPPH Free Radical Scavenging Assay

The anti-oxidant activity of the synthesized compounds was evaluated by monitoring the discoloration of stable purple color of 2,2-diphenyl-1-picrylhydrazyl to yellow color [21]. The highest activity was 86.4% shown by 2d with IC_{50} value 4.96 µg/mL. The compounds 2b and 2g also showed good activity with % inhibition 56.7 and 55.6 and IC_{50} values were 9.68 and 8.56 µg/mL respectively. As the % inhibitions of compounds 2f, 2e, 2c and 2a were below 50% so their IC_{50} values were not calculated. Ascorbic acid was used as standard or positive control.

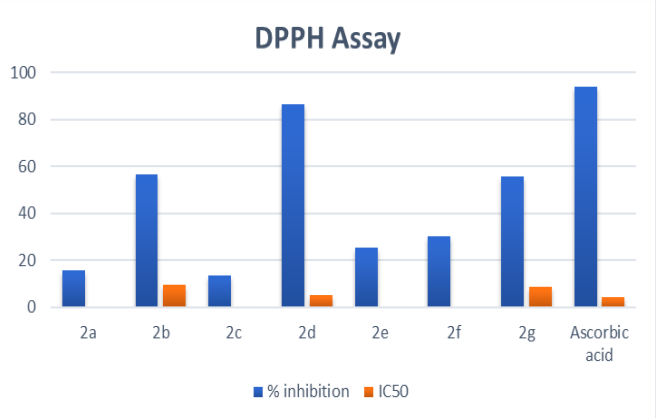
Table 2

DPPH Assay and IC_{50} (µg/ml) Values

Compounds	Scavenging potential (%)	IC50 (µg/ml)
2a	15.87	-
2b	56.7	9.68
2c	13.26	-
2d	86.64	4.96
2e	25.3	-
2f	30.2	-
2g	55.6	8.56
Ascorbic acid	94	4.31

Figure 3

Graphical Representation of DPPH Assay and IC_{50} (µg/ml) Values



Acetylcholinesterase Inhibition Assay

The acetylcholinesterase (AChE) inhibition potential of the synthesized compounds was assessed by the assay described in the section 2.4. All the synthesized compounds exhibited moderate to good acetylcholinesterase inhibitory activities. Four compounds 2g, 2a and 2b showed potent inhibitions of 91.86%, 88.69% and 81.91%with IC_{50} values 3.23, 3.67, 5.23 and 4.87 respectively. As % inhibitions of compounds 2c, 2d, 2e and 2f was below 50% so their IC_{50} values were

not calculated. Donepezil was used as standard with % inhibition of 100% with IC_{50} value 3.04.

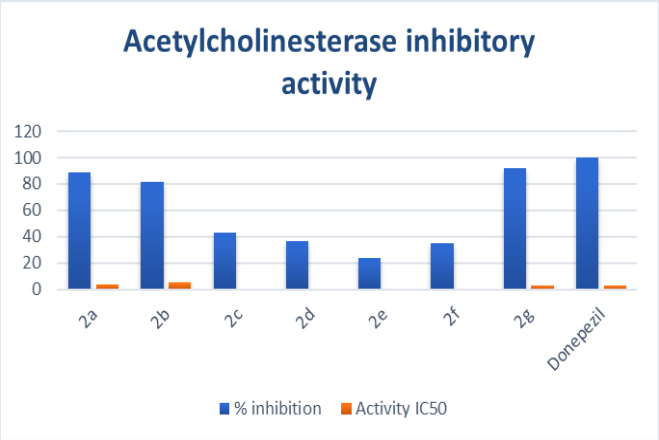
Table 3

Acetylcholinesterase Inhibitory Activity

Compounds	% Inhibition	Activity IC50 (nM±SE)
2a	88.69	3.67 ± 0.092
2b	81.91	5.23 ± 0.072
2c	43.13	-
2d	36.87	-
2e	23.88	-
2f	35.04	-
2g	91.86	3.23 ± 0.093
Donepezil	100	3.04±0.011

Figure 4

Graphical Representation of Acetylcholinesterase Inhibitory Activity



Evaluation of *In-silico* Studies

Acetylcholinesterase PDB ID: 1GQR was used for docking the structure of synthesized ligands as per the methodology given in chapter 2. Highest binding affinity of the individual ligands is given in the Table 4. All the compounds showed good binding affinities while compounds 2e, 2a and 2d exhibited the highest binding affinity with acetylcholinesterase among the synthesized derivative with a value of -10.5 kcal/mol, -10.4 kcal/mol and -10.3 kcal/mol respectively.

Table 4

Binding Affinity of Ligands (2a-h) with Acetylcholinesterase

Protein Target	Ligand	Highest Binding Affinity (Kcal/mol)[a]
Acetylcholinesterase PDB ID: 1GQR	2a	-10.4
	2b	-9.6
	2c	-10.1
	2d	-10.3
	2e	-10.5
	2f	-8.9
	2g	-9.6
	2h	-9.7
	Donepezil	-10.9

Amino acids involved in ligand protein interaction are given in Table 4. In Physostigmine TYR 130 showed conventional hydrogen bond interaction with distance of (2.79, 3.09 Å) and TRP 84 showed pi-stacked interaction with distance of 3.96 Å. All the compounds from 2a to 2h showed conventional hydrogen bond with TYR 121 having distance of (2.59, 3.20 Å), (3.03, 3.20 Å), 2.66 Å, 2.96 Å, 2.95 Å, 1.92 Å, 3.10 Å and 2.98 Å respectively and except the compounds 2e and 2g all other compounds showed pi-pi stacked interaction with TRP 84 having distance of (4.57, 5.23 Å), 5.27 Å, 5.02 Å, (4.83, 4.83 Å), 3.27 Å, (4.29, 4.29 Å). The compound 2e and 2g showed pi-sigma and pi-alkyl interactions with TRP 84 with distance of 5.62 Å and 5.53 Å respectively

Table 5

Distance of Protein Ligands (2a-2h) Interaction with Acetylcholinesterase

Compound	Amino acid involved	Distance(Å)
2a	TYR 121, TRP 84, HIS 440, PHE 330, PHE 331	5.18, (2.59, 3.20), (4.57, 5.23), (4.44, 4.44), 4.81, 2.78, 3.24
2b	TYR 121, TRP 84, PHE 330, PHE 331, ASP 72, PHE 288	3.18, 3.72, (3.03, 3.20), 2.35, 5.08, 3.86, 5.27
2c	TYR 121, TRP 84, PHE 330	2.66, 3.78, 5.34, 5.02
2d	TYR 121, TRP 84, PHE 330, PHE 331, PHE 288, SER 286, ARG 289, ILE 287	2.96, (4.83, 4.83), 4.84, 3.39, 3.18, 3.69, 3.64, 4.96
2e	TYR 121, TRP 84, HIS 440, PHE 330	4.14, 2.95, (4.23, 3.76), (5.24, 4.00), 5.62
2f	TYR 121, TRP 84, TYR 70, SER 122, HIS 440, PHE 331, PHE 330	4.35, 5.14, (4.60, 5.88), 3.27, (5.64, 5.21), 4.31, 1.92, 3.35, 3.17
2g	TYR 121, TRP 84, TYR 334, TRP 279, GLU 199, TYR 130	3.65, 3.34, 5.53, 4.23, 3.69, 3.10, 5.43
2h	TYR 121, TRP 84	2.98, (4.29, 4.29)
Donepezil	TYR 121, TYR 130, TRP 84, PHE 330	3.01, 3.98, 3.96, 3.83, 4.79, (2.79, 3.09)

Figure 5

2a Docking with Acetylcholinesterase

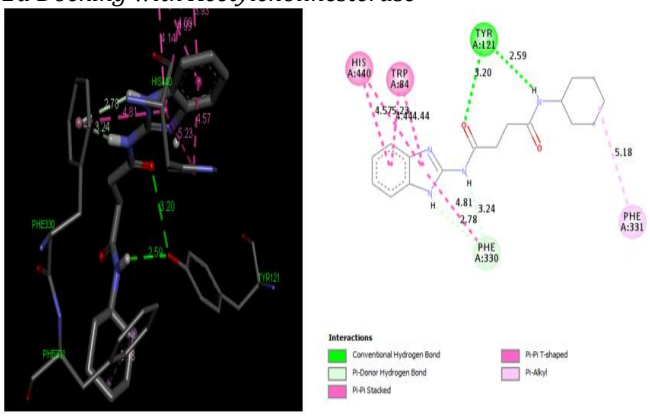


Figure 6

2b Docking with Acetylcholinesterase

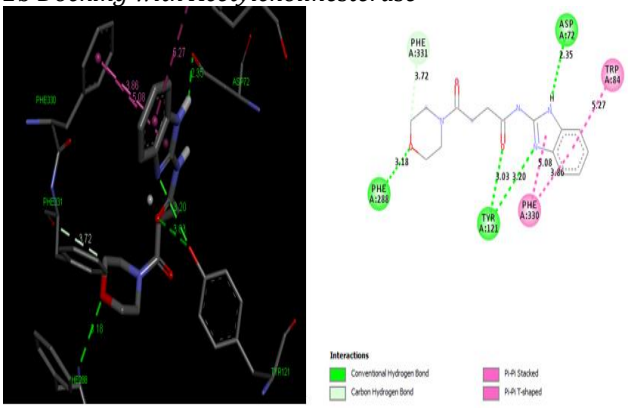


Figure 7
2c Docking with Acetylcholinesterase

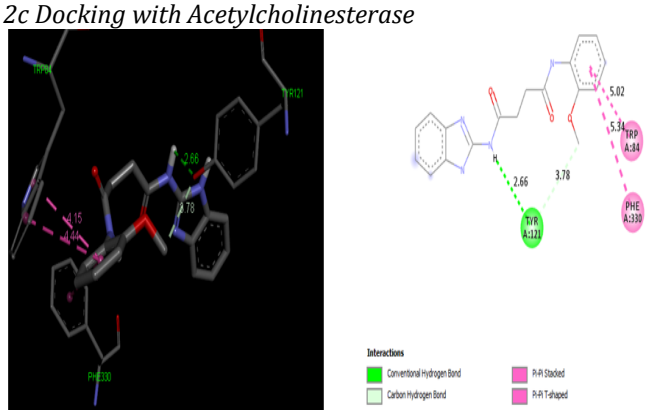


Figure 8
2d Docking with Acetylcholinesterase

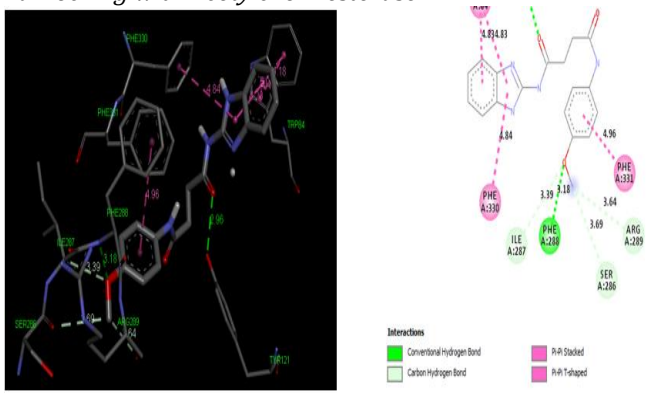


Figure 9
2e Docking with Acetylcholinesterase

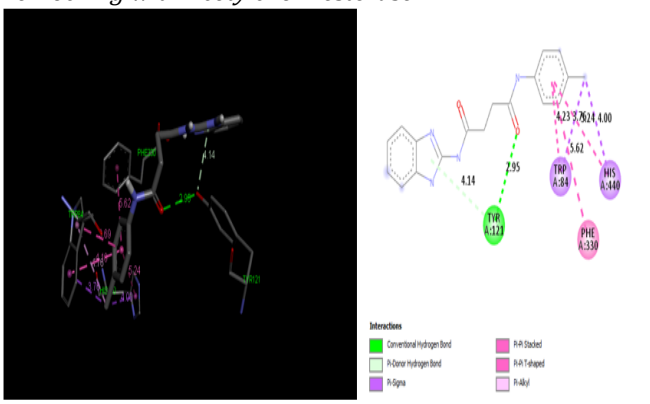


Figure 10
2f Docking with Acetylcholinesterase

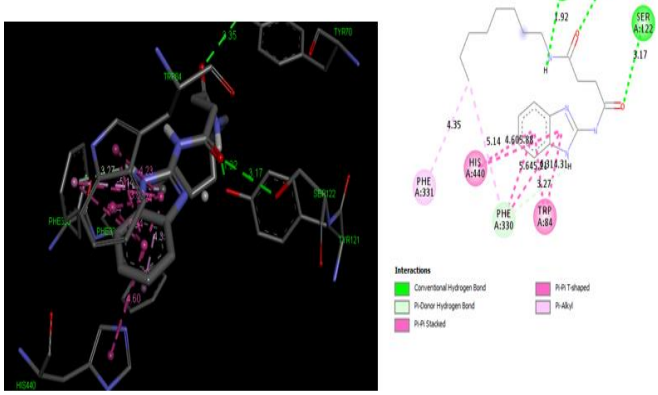


Figure 11
2g Docking with Acetylcholinesterase

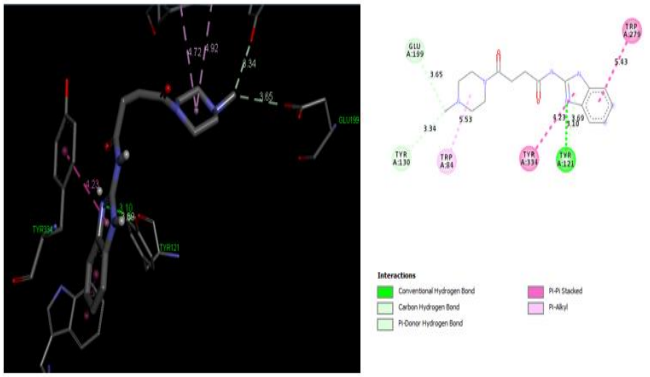


Figure 12
2h Docking with Acetylcholinesterase

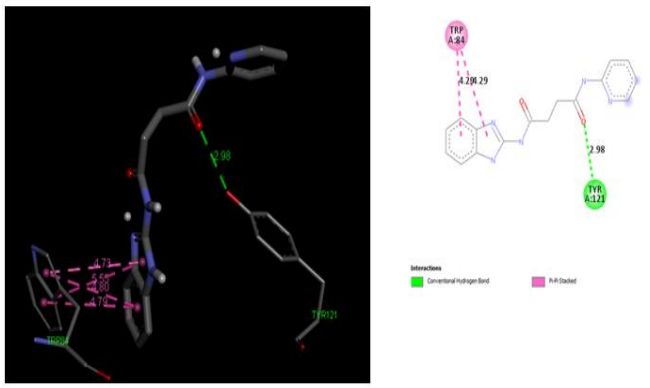
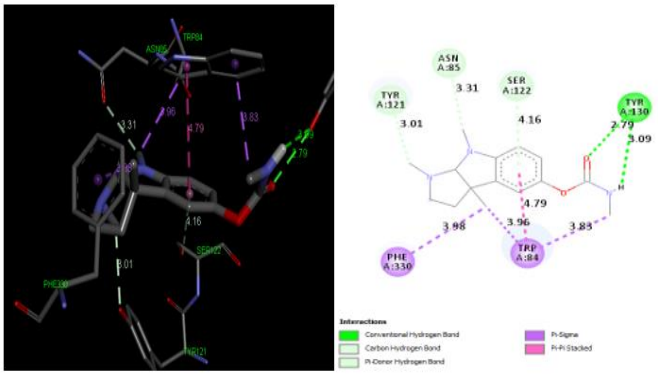


Figure 13
Donepezil Docking with Acetylcholinesterase



DISCUSSION

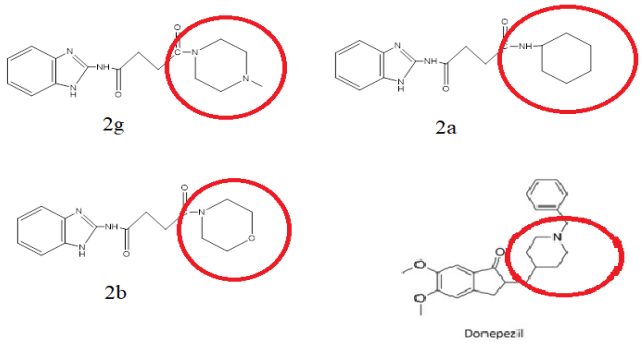
Eight amide derivatives were synthesized and purity of all compounds was checked by TLC using solvent system chloroform: methanol (4:1) and (7:1). Single spot was obtained in each case. The formation of final products was confirmed by IR and ^{1}HNMR spectral data as mentioned earlier. In IR spectra of compounds 2(a-h) N-H stretching's were observed at 2929-3395 cm^{-1}. The starching vibration of amide C=O were observed at 1625-1721 cm^{-1} for all synthesized compounds 2(a-h) indicating the formation of amide bond. Other peaks at 1505-1569 cm^{-1} were observed due to C=C of aromatic moiety. The absence of carboxylic stretching vibration at 1700-1710 cm^{-1}, confirmed the formation of amide derivatives.

Further, ^{1}H-NMR data confirmed the structure of the synthesized derivatives. Singlet of amide proton peak was observed in compounds 2c, 2e and 2f at 7.74-9.64 ppm.

Aromatic protons appeared in the range of 6.15 -8.01 ppm. In 2c a triplet of methoxy protons was observed at 2.76-2.69 ppm. The CH_2 protons of succinic moiety appeared downfield as two triplets in range of 2.26-4.76 ppm in all compounds due to electron withdrawing effect of C=O group attached. In compound 2f Alkyl-H proton appeared as doublet in range of 1.35-1.33 ppm.

The DPPH method was used to test the free radical scavenging potentials of the synthesized compounds and ascorbic acid at different concentrations and the results are depicted in Figure 3. Antioxidants interact with DPPH, a stable nitrogen-centered free radical that exhibits a distinct absorbance peak at 517 nm, reducing it to 1,1-diphenyl-2-picrylhydrazine. The extent of color fading corresponds to the free radical scavenging capacity of the tested compounds. The experimental findings revealed that all synthesized compounds demonstrated antioxidant activity have reduced the DPPH radical in different concentrations. At 40 µg conc, the synthesized compounds and ascorbic acid exhibited 15.87% (2a), 56.7% (2b), 13.26% (2c), 86.64% (2d), 25.3% (2e), 30.2% (2f), 55.6% (2g) and 94.4% (ascorbic acid) free radical scavenging activity. Results indicated that among all the synthesized compounds, the compound 2d was found highly effective having IC_{50} value 4.96 µg/ml.

The acetylcholinesterase (AChE) inhibition potential of the synthesized compounds was assessed by the Ellman's method. Data for their inhibition of acetylcholinesterase (AChE) are reported in Table 3. All the compounds synthesized were found to have acetylcholinesterase inhibitory activity. Four compounds 2g, 2a and 2b showed strong inhibitions as 91.86%, 88.69% and 81.91% with IC50 values 3.23, 3.67 and 5.2 respectively. 2c, 2d, 2e and 2f compounds did not exhibit activity. Compound 2g showed the best acetylcholinesterase inhibitory activity (91.86%), which can be ascribed to the attachment of a methylpiperazine moiety responsible for improved enzyme–ligand interactions by hydrogen bonding and hydrophobic contacts. Compound 2e was found to possess the lowest activity, which could be due to the attachment of a 4-chlorobenzyl substituent reducing its binding efficiency in the active site. These results underscore the importance of acetylcholinesterase inhibition as an integral therapeutic strategy in the treatment of Alzheimer's disease (AD) and point to the role of structural changes in biological activity.



The SAR analysis of the thus synthesized 2-aminobenzimidazole derivatives (2a–2h) provides insightful information regarding the effect of structural diversity on their antioxidant and acetylcholinesterase (AChE) inhibitory activity. The incorporation of amide linkage through succinic anhydride increased molecular conjugation and stability, resulting in enhanced interaction potential inside the catalytic domain of AChE. Overall, derivatives with aromatic substituents showed greater AChE inhibitory activity than those with aliphatic side chains. Interestingly, compound 2e (4-chlorobenzyl) and compound 2d (4-methoxyphenyl) exhibited good binding affinities of −10.5 and −10.3 kcal/mol, respectively, through molecular docking. Electron-donating ($-OCH_3$) and electron-withdrawing (–Cl) groups at the para position of the aromatic ring favored π–π stacking and hydrogen bond interactions with important amino acid residues, Tyr121 and Trp84, involved in catalytic inhibition [22]. Conversely, compound 2g, with a methylpiperazine moiety, had the highest AChE inhibition (91.86%), which may be attributed to its capability of forming extra hydrogen bonds and electrostatic interactions in the enzyme's active pocket. Piperazine ring nitrogen atoms could further increase affinity via hydrogen bonding or dipole stabilization with catalytic residues [23]. Likewise, compound 2a, bearing a cyclohexylamine substituent, was found to be strongly inhibited (88.69%), suggesting sterically bulky groups have the ability to stabilize ligand orientation and enhance hydrophobic interactions with the enzyme surface. In contrast, compounds like 2c (o-anisidine) and 2f (n-octylamine) had weaker antioxidant and inhibitory activities, likely due to restricted π-conjugation and decreased planarity of the molecules, which compromise stacking interaction in the aromatic binding site of AChE. The antioxidant activity was greatest in compound 2d, which contains a methoxy group with ability to donate hydrogen atoms and stabilize free radicals via resonance mechanisms. Such an observation underscores the significance of electron-rich aromatic systems in radical scavenging capacity and overall antioxidant activity.

Molecular docking studies were conducted to understand the binding mode and interaction affinity of the synthesized derivatives with the AChE enzyme (PDB ID: 1GQR). Docking simulations, conducted with AutoDock Vina incorporated into PyRx, were able to accurately predict bindin energies and interaction patterns. The structure of the enzyme was retrieved from the RCSB Protein Data Bank, and pre-processing like the removal of crystallized water molecules and ligands was done using Discovery Studio Visualizer [24]. Hydrogen atoms were added, and the protein was formatted to PDBQT. Ligands were geometry-optimized, and the docking grid was set to encompass the catalytic anionic site (CAS) and the peripheral anionic site (PAS) regions responsible for ligand recognition and catalytic activity. The docking protocol was tested by re-docking the native co-crystallized ligand, which resulted in low RMSD values, validating the correctness of the computational setup. The docking outcomes provided binding energies between −8.9 and −10.5 kcal/mol, reflecting favorable interactions for the series of derivatives. Out of all, 2e (−10.5 kcal/mol), 2a (−10.4 kcal/mol), and 2d (−10.3 kcal/mol) maintained the strongest affinities, very close to that of the reference inhibitor donepezil. Visualization of binding poses indicated that all active ligands formed hydrogen bonds

with Tyr121 and π–π stacking with Trp84, key residues in the AChE catalytic site. They make significant contributions to complex stabilization and increased inhibitory potency. The similar binding orientation of these derivatives with donepezil indicates that they contain crucial pharmacophoric elements necessary for efficacious AChE inhibition.

In summary, the experimental and in-silico results collectively demonstrate that the synthesized 2-aminobenzimidazole derivatives possess dual antioxidant and anti-AChE activities, both integral to neuroprotection in Alzheimer's disease (AD). The structural flexibility and favorable interaction profile of the benzimidazole framework underscore its promise as a multifunctional scaffold for developing next-generation therapeutic candidates targeting both oxidative stress and cholinergic dysfunction associated with AD pathology [25].

CONCLUSION

A new series of eight 2-aminobenzimidazole derivatives (2a–2h) were synthesized through an easy two-step reaction sequence with succinic anhydride and different substituted amines. The molecular structures of the synthesized molecules were ascertained by FTIR and ^{1}H-NMR spectroscopic studies, which ensured the existence of amide linkages and the structural purity of the compounds. Biological studies revealed that some derivatives showed significant antioxidant and acetylcholinesterase (AChE) inhibitory effects. Among them, compound 2d exhibited the highest antioxidant activity (86.64%, IC_{50} = 4.96 µg/mL), due to the presence of an electron-donating methoxy group that increased its free radical scavenging activity. Compound 2g, 2a, and 2b, on the other hand, possessed strong AChE inhibitory activities (91.86%, 88.69%, and 81.91%, respectively), with relative potencies comparable to the standard inhibitor donepezil, highlighting their therapeutic potential as neuroprotective agents. The structure–activity relationship (SAR) analysis revealed that both heterocyclic and aromatic substituents significantly impacted the measured biological activities. Electron-donating substituents enhanced antioxidant activity, while electron-withdrawing or cyclic amine bulky groups increased AChE inhibition by facilitating more hydrophobic and hydrogen-bonding interactions with the active site of the enzyme. Most significantly, the presence of piperazine and cyclohexylamine groups facilitated higher binding stability and optimal molecular orientation in the catalytic pocket.

The molecular docking study also confirmed the experimental results, indicating that 2e (−10.5 kcal/mol), 2a (−10.4 kcal/mol), and 2d (−10.3 kcal/mol) showed the most significant binding affinities to AChE (PDB ID: 1GQR). They developed stable hydrogen bonds with Tyr121 and π–π stacking interactions with Trp84, which are residues found to be most crucial in the inhibition of AChE. Their binding modes were very similar to that of donepezil, indicating these derivatives could be a good structural template for the creation of next-generation inhibitors. Generally, the unification of the synthetic, biological, SAR, and in-silico results is indicative of the potential of 2-aminobenzimidazole derivatives as a multifaceted scaffold with dual antioxidant and cholinesterase inhibitory activity. These molecules are potential candidates for the development of new therapeutics for the prevention of oxidative stress and cholinergic impairment in Alzheimer's disease. In-vivo studies, ADME profiling, and toxicity assessment should be the focus of future studies to ascertain their safety and therapeutic potential.

Declarations

Ethics Approval: It is noted that this investigation did not entail the involvement of either animal subjects or human participants, thereby rendering ethics approval unnecessary.

Consent to Participate: The concept of obtaining consent for participation does not apply to the scope of this study.

Consent for Publication: All the authors have diligently examined and provided their approval for the final version of the manuscript, endorsing its readiness for publication.

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