Design, Synthesis, and biological evaluation of pyrazolo-benzothiazole derivatives as a potential therapeutic agent for the treatment of Alzheimer’s disease

Harish Kumar1,2 · Anju Goyal2 · Navneet Kumar3 · Prabha Garg3

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Abstract
Alzheimer’s disease (AD) is a neurodegenerative age-related brain disease which diminishes learning and memory. Two main cholinesterases (ChEs), first acetylcholinesterase (AChE) and second one butyrylcholinesterase (BChE) are the major targets to treat this disease. In the current study, six novel pyrazolo-benzothiazole analogues were systematically planned, synthesized, and biological tested for their ChEs activities, antioxidant, enzyme kinetic, self-induced Aβ modulation, PAMPA-BBB, and docking and molecular simulation study. Among all derivatives, compound 4a bearing 3,4-dimethoxypyrazolo-benzothiazole was the potential inhibitor of AChE (IC50 = 8.69 ± 0.42 µM), Aβ modulator and good antioxidant property. Docking studies also clearly revealed that compound 4a fit fully into the active sites of hAChE.

Graphical abstract

Keywords Alzheimer’s disease (AD) · Chalcone · Pyrazole · Acetylcholinesterase

Introduction
Alzheimer’s disease (AD) is a multifactorial, age-related neurological brain illness caused by the loss of cholinergic neurons and synapses in the cerebral cortex, cortical and subcortical brain areas [1]. In the year 2020, there will be more than 55 million people living with dementia all over the world. Every 20 years, the population will nearly double, to 78 million in 2030 and 139 million in 2050. Although the exact pathophysiology of AD is not fully clear, literature suggests that low levels of the neurotransmitter acetylcholine (ACh), amyloid beta (Aβ) deposition in the form of Aβ plaque, hyperphosphorylation of tau protein in the form of neurofibrillary tangles (NFTs), increased oxidative stress (OS), and metal-ion dyshomeostasis all interdependently play a significant role in the progression of AD [2, 3]. Acetylcholine (ACh) is a main
cholinergic neurotransmitter that plays an active role in learning and memory [4]. Cholinergic pathways in the CNS are disrupted in AD, and the resulting cholinergic deficit adds to cognitive impairment [5]. The metabolism of ACh involves hydrolysis through acetylcholinesterase (AChE) and butrylcholinesterase (BChE) in synaptic region and which reflects in AD in form of decreased levels of ACh and BCh [6]. Thus, restoration of levels of these neurotransmitters (ACh and BCh) through blocking their reduction and hydrolysis by inhibiting AChE and BChE is a major contributor to provide symptomatic relief in AD (Fig. 1).

The senile plaque accumulation outside neurons (Aβ_{1-42}) and intraneuronal accumulation of microtubule associated Tau protein in form of neurofibrillary tangles (NFTs) are the major hallmarks of AD [7]. Both markers of AD are present many years before the actual initiation of the pathological progression (Fig. 1). The Aβ plaque and NFTs are accumulated majorly in the areas responsible for control and regulation of cognitive and behavioural processes including cortex and hippocampus. Amyloid precursor protein (APP) is an important transmembrane protein which is processed by α, β, and γ secretases in a balanced fashion. The altered activity of these enzymes especially γ secretases leads to production of Aβ_{1-42} in an uncontrolled manner which leads to excessive accumulation and aggregation of this protein. Aβ has self-aggregatory properties and high tendency to get accumulated in form of plaques which tuns highly neurotoxic and propagates AD pathology through their aggregation in form of dimmers and fibrils (senile plaque). These neurotoxic oligomers interact with several synaptic proteins and hampers several neurophysiological processes and neurotransmission in a manner that aggravates other etiological factors too which are responsible for AD progression. Other characteristic etiological contributors to AD include increased oxidative stress and decrease in antioxidant defence. The excessive generation of free radicals leads to extensive cellular damage that not only damages mitochondria, nucleic acids, DNA, and proteins but also alters transcription of genes. The ROS directly promulgates generation of free radicals (H_2O_2, peroxides, hydroxides) and is intricately involved in accumulation of senile plaques and NFTs (Fig. 1) [8].

Fig. 1 Pathophysiology of Alzheimer’s disease (AD)
There is currently no permanent cure for Alzheimer’s disease, and FDA-approved medications for the treatment of the disease are only intended to provide symptomatic relief [9]. Approved medications only provide symptomatic relief or decrease the worsening of symptoms when used alone or in combination, but they do not halt or stop the progression of the disease [10]. The pharmacological agents approved for AD management have some beneficial effects on cognitive, and behavioural symptoms of the disease, however, these agents do not play any role to address basic aspects of the disease [11]. Moreover, these agents are neuro-destructive rather than having neuroprotective effect [12].

Acetylcholinesterase (AChE) inhibitors and N-Methyl-D-aspartic acid or N-Methyl-D-aspartate (NMDA) receptor antagonists are currently available treatment option for Alzheimer’s disease. Donepezil (1), rivastigmine (2), galantamine (3), and tacrine (4, withdrawn from the market) are AChE inhibitors, while memantine (5) is an NMDA receptor antagonist (Fig. 2) [13]. AChE is a serine protease enzyme that hydrolyzes adenosine triphosphate (ACh) into choline and acetic acid [14]. As a result, it is extremely important in cholinergic neurotransmission. The catalytic anionic site (CAS), the oxyanion hole, and the peripheral anionic site are the three parts of AChE’s binding site (PAS) [15]. Next, butrylcholinesterase (BChE), an isomer of ChE that is responsible for the cleavage of ACh, is found in the brain [16]. As the disease advances, the levels of both AChE and BChE fluctuate dramatically [17]. As a result, both enzymes have been investigated as potential neuroprotective and disease-modifying therapies for Alzheimer’s disease. In the last two decades, research on Alzheimer’s disease has yielded new insights into core pathogenic processes such as the accumulation of misfolded proteins Aβ in the form of aggregates, oxidative stress, and increased concentration of metals in the AD brain.

Chalcones (6) are naturally occurring molecules and their synthetic compounds display a variety of biological activities and are precursors of natural flavonoids derivatives which are present in vegetables, fruits and various medicinal plants (Fig. 3) [20]. Natural flavonoids contain OCH₃ and OH groups at different positions on aromatic rings. Both the aromatic rings are linked through a three-carbon α, β-unsaturated carbonyl group with structural diversity that can be achieved by naturally [eg. broussochalcone A (7), xanthohumol (8) and dixanthohumol (9)] and synthetically [21]. They also serve as an important precursor for the synthesis of varieties of heterocyclic systems. Chalcones exhibit wide class of pharmacological activity related to the neurodegenerative disorder including anti-inflammatory, antioxidant, cholinesterase inhibition, and Aβ modulation properties [22]. Sang Z. et al. in year 2019, developed a new series of chalcone derivatives as a potential candidate for the management of AD (10, 11) (Fig. 3). They targeted their developed molecules for their multitargeted properties against AChE, BChE and MAO-A and MAO-B. Wang L et al., in year 2016 also synthesize and biologically evaluated a novel series of chalcone as a potential anti-AD agent (12) (Fig. 3). These finding clearly suggest that chalcone could be used as a carrier linker in the development of new class of multifunctional compounds for the management of AD.

Pyrazole scaffold enormously plays a significant role in medicines and many functional derivatives are commercially available [23]. Pyrazole analogues served as an essential core scaffold in many pharmaceutical drugs and agrochemicals namely mepiprazole (13), celecoxib (14), phenazone (15), ruxolitinib (16), deracoxib (17) etc. These derivatives are readily able to show interactions with...
Numerous enzymes located on the target cells in biological system including cyclooxygenase, α-glycosidase, carbonic anhydrase and acetylcholinesterase [24]. There are several literature reports which clearly suggest that pyrazole derivatives showed potential anti-AD efficacy in-vitro and in-vivo study. Therefore, a series of pyrazole analogues were systematically designed based on multifunctional property. Developed compounds were further biological evaluated for their inhibition of ChEs, enzyme kinetic, antioxidant, Aβ modulation and in-vitro BBB permeability study. Molecular docking and dynamic studies were also depicted to get the detailed into the binding interaction of the developed molecules with enzyme.

Results and discussion

Chemistry

The complete synthetic route of pyrazolo-benzothiazole analogues were presented in Scheme 1. In first step, 4-hydroxyacetophenone was treated with different mono/disubstituted aldehyde in the presence of NaOH and solvent ethanol to afford substituted chalcone as a key intermediate 3a-3c. The 1H-NMR spectra of 3a-3c showed characteristic two doublet (d) peak for E-alkene of chalcone at 7.66–7.80 ppm with coupling constant (J) 15.50 Hz clearly indicate E-configuration for developed intermediates. While second intermediate were prepared by the reaction of substituted 2-aminobenzothiazole with hydrazine hydrate, ultimately lead to the formation of substituted hydrazine derivative (6a-6b). Finally, the desired compounds 4a-4f were obtained by the simple cyclization of intermediate 3a-3c with substituted hydrazino-benzothiazole (6a-6b) in n-butanol at 120 °C. These final compounds have not been previously mentioned in the literature. Their structures analysis was analysed by 1H NMR, and 13C NMR spectroscopy.

Rationale and Design

We started our drug design approach by selecting the chalcone scaffold as a starting point. The chalcone consist of two parts (i) acetophenone fragment and (ii) aldehyde fragment, therefore, impart antioxidant potential to developed analogues. Furthermore, benzothiazole is an important pharmacophore for imparting Aβ modulation property. Therefore, the concept of hybridization was used for the development of novel pyrazolo-benzothiazole derivatives, which is based on the connection of two fragments (chalcone and hydrazino-benzothiazole) via a suitable dihydropyrazole fragment (Fig. 4).
Scheme 1  Synthesis of pyrazolo-benzothiazole analogues 4a-4f. *Reagents and conditions: (i) KOH, EtOH, reflux, 78 °C, 10–12 h, seal tube; 75–85% yield. (ii) n-butanol reflux, 120 °C 10–12 h; 70–80% yield (iii) NH₂NH₂, HCl, Ethylene glycol, heat 150 °C, 10 h, 70–80% yield

Fig. 4 A Design of the multifunctional cholinesterase (ChEs) inhibitors. B Mechanism of pyrazolo-benzothiazole derivatives as potential anti-AD agent.
Drug likeness studies

All the developed derivatives along with their intermediate 3a-3c and 4a-4f were assessed for their drug-likeness property determination (Lipinski’s rule of five) using admetSAR software (Table 1). Molecular weight, logP, no. of hydrogen donors and acceptors and rotatable bond were determined using admetSAR software (Table 1). Lipinski’s points can be summarized as follows.

1. Molecular weight (MW) should be 500 g/mol or less.
2. M logP: (n-octanol/water partition coefficient) should be 5 or less.
3. Topological polar surface area (TPSA) should be less than 140 Å.
4. Number of H-bond acceptors (HBAs) should be 10 or below and number of H-bond donors (HBDs) should be 5 or below.
5. Number of rotatable bonds (RBs) should be 10 or below.

Form the data depicted in Table 1, we can clearly conclude that our developed compound presented good drug likeness profile.

Evaluation of AChE and BChE inhibitory activities

The inhibitory activities of the discovered compounds 3a-3f and 4a-4f against hAChE (human serum) and eqBChE (equine serum) were determined using a modified Ellman colorimetry assay. A well-known medication, donepezil (DNP), was employed as the control. The inhibitory actions of the synthesized analogues against AChE and BChE are listed in Table 2 as percent inhibition and IC50 values. When compared to their chalcone intermediates, all the synthesized analogues displayed good to medium ChE inhibitory activity against AChE and BChE. (3a-3f). Almost all the resulting analogues had significant selectivity for hAChE over eqBChE and showed good inhibitory potency in AChE (IC50 ranging from 8.69 ± 0.42 to 17.32 ± 0.74 μM).

To validate our drug design approach, firstly chalcone analogues (3a-3c) was synthesized and tested for their cholinesterase inhibitory (ChEI) activity. To our interest, as mentioned on Table 2 chalcone analogues (3a-3c) presented % age AChE inhibition (at 20 μM) from 11.36 – 31.14%. This result of the chalcone scaffolds encouraged us to further explore this template. Then, we plan to cyclize chalcone system with hydrazino-benzothiazole to generate compounds 4a-4f and tested them for their ChE inhibitory potency against AChE and BChE. We found that most of the developed analogues presented good to moderate AChE and BChE inhibitory potency in in-vitro experiment. All the synthesized compounds showed higher ChEs inhibitory property compared to their parent chalcone analogues (3a-3c). Among all, 4a showed highest ChEs inhibitory activity with IC50 value of 8.69 ± 0.42 μM against AChE, and 15.36 ± 0.33 μM against BChE. SAR study revealed that mono substituted (−OCH3) pyrazolo-benzothiazole (3e and 3f) showed poorer ChEs activity compared to the disubstituted (−OCH3) pyrazolo-benzothiazole derivatives (IC50, AChE (μM); 8.69 ± 0.42 for 4a, 11.62 ± 0.52 for 4b, 11.23 ± 0.61 for 4c, 17.32 ± 0.74 for 4d, 15.62 ± 0.62 for 4e, and 23.43 ± 1.98% inhibition at 20 μM for 4f). While in case of BChE all the developed compounds showed mixed activity IC50 value from 15.36 ± 0.33 μM to 34.32 ± 1.63% inhibition at 20 μM. It has been observed that introducing additional methoxy (−OCH3) to the benzene ring increases ChE inhibitory activity compared to monosubstituted one (Fig. 5).

AChE inhibition kinetic studies

To investigate the method of enzyme inhibition, enzyme kinetic study was performed with the most potent inhibitor of AChE (i.e., 4a) against hAChE. The categories of inhibition were examined by a Lineweaver–Burk double reciprocal plot with the velocity of the substrate acetylthiocholine iodide (y-axis) and increasing concentrations. The plots (Fig. 6) clearly indicate mixed inhibition pattern of 4a against hAChE. From this study we can conclude that 4a binds to both free AChE and AChE substrate complex.

Evaluation of antioxidant activity

It is apparent from the literature that oxidative stress plays a significant role in the progression of AD [25]. Drugs inhibiting the generation or clearing of the free radicals in the brain would be advantageous for AD treatment. Therefore, the antioxidant activities of the developed molecules were evaluated using DPPH assay. DPPH radical can be used in initial testing of analogues capable of scavenging radical species. For comparison, tocopherol (TP) was used as reference. All the

| Compound Code | MW  | logP | TPSA | HBA  | HBD  | RBs |
|---------------|-----|------|------|------|------|-----|
| 3a            | 284.31 | 2.98 | 55.77 | 4 | 1 | 5 |
| 3b            | 284.31 | 3.19 | 55.77 | 4 | 1 | 5 |
| 3c            | 254.28 | 3.39 | 46.53 | 3 | 1 | 4 |
| 4a            | 445.54 | 5.41 | 67.17 | 6 | 1 | 5 |
| 4b            | 445.54 | 5.38 | 67.19 | 6 | 1 | 5 |
| 4c            | 445.54 | 5.80 | 67.19 | 6 | 1 | 5 |
| 4d            | 445.54 | 5.78 | 67.19 | 6 | 1 | 5 |
| 4e            | 415.52 | 5.79 | 57.96 | 5 | 1 | 4 |
| 4f            | 415.52 | 5.76 | 57.96 | 5 | 1 | 4 |
developed derivatives (4a-4f) presented potent radical scavenging activity compared to the intermediate analogues (3a-3c). All the synthesized compounds showed IC$_{50}$ value from 59.65 ± 1.43 to 91.51 ± 1.09 µM. Among the developed molecules compound 4a exhibited highest DPPH radical scavenging capacity with IC$_{50}$ = 59.65 ± 1.43 µM. This study clearly indicates that developed compounds represent potential antioxidant activity (Supporting figure S1).

### Inhibition of the self-mediated Aβ$_{1-42}$ aggregation

The ChEs inhibitor which bind to PAS region are competent of inhibiting Aβ$_{1-42}$ aggregation. To estimate Aβ modulation property of compound 4a, thioflavin T (ThT) fluorescence experiment was performed. To perform this experiment, Aβ alone, Aβ + DNP (10:10 µM), Aβ + DNP (10:20 µM), Aβ + 4a (10:10 µM), and Aβ + 4a (10:20 µM) was incubated for 48 h at 37 °C. The results were presented as normalized fluorescence intensity (NFI) (Fig. 7). Compound 4a, at a concentration ratio of 10:10 µM and 10:20 µM, exhibited potent Aβ modulation property compared to self-induced Aβ aggregation. These finding clearly suggested that derivative 4a significantly inhibited the Aβ aggregation and had good peripheral anionic site-AChE binding affinity.

### Molecular docking study

The molecular docking was performed using the Glide in extra precise (XP) mode on the crystal structures of AChE with co-crystallized ligand DNP (Friesner et al. 2006). The
residues Trp86, Tyr337, and Phe338 via π–cation interactions (Fig. 8). The protein-ligand contact histogram and protein-ligand contact timeline (Fig. S3) depict that the hydrophobic interactions of residues Trp86, Tyr337, and Phe338 with DNP are stable and consistent. The benzyl moiety attached to piperidine also makes a stable and consistent π–π stacking interaction with the Trp86. The dark color in protein-ligand contact timeline shows the multiple interaction of Trp86 with DNP (Fig. S3). Similarly, the dimethoxyindanone moiety of DNP also interacts with Trp286 and Phe295 via π–π stacking and hydrogen bond respectively (Fig. 8).

The benzothiazole moiety present in the ligand 4a mainly interacts with Tyr337, Trp 86 and His 447 via hydrogen bonds and hydrophobic (π–π interaction) interactions (Fig. 8). The protein-ligand contact histogram and timeline depict that interaction of residues Tyr337 (hydrogen bonds) and Trp86 (π–π interaction) with 4a are prominent, consistent and stable (Figure S3). The phenolic moiety of 4a also makes water mediated hydrogen bond and direct hydrogen bonds with Trp286 and Ser298, respectively (Fig. 8).

Analysis of structural stability, compactness and binding free energy

The radius of gyration and root mean square deviation (RMSD) are important parameters to examine the structural stability of AChE and its complexes with various chemicals (Rg). The root mean square deviation plot of apo-AChE and its complex with the DNP is shown in Fig. 9 (4a). The RMSD changes for both complexes indicate that the simulation has reached a point of convergence. As previously noted in earlier studies, binding of ligands, namely DNP, results in a considerable reduction in the RMSD value [25, 26]. During the last few ns of simulations, the RMSD fluctuation of AChE decreased in the presence of (4a). There is significant decrease in average RMSD value of AChE in presence of both ligands. Figure 9 shows the significant alteration in the Rg of the AChE in presence and in absence of the compounds. The Rg is an main predictor of the protein compactness. The Rg plot (Fig. 9) and average Rg analyzed from the whole trajectory indicate that there are no considerable and significant changes in Rg of AChE in presence of ligand as compared to apo-AChE.

The last 25 ns trajectory was used to compute the binding free energy between AChE and both ligands (DNP and 4a). In Table 4 summarises the final binding free energies & its numerous supporting terms for all simulated complexes. AChE-DNP (DPZ) and AChE-4a complexes had average binding energies was found to be of −64.19 3.7 and −64.324.3 Kcal/mol, respectively.

### Molecular dynamics simulations

#### Interaction of Donepezil and 4a with hAChE

During MD simulation it was observed that the piperidine moiety of DNP mainly interacts with three different grid was described using the co-crystallized ligand DNP. The AChE was re-docked with the co-crystallized ligand. Both co-crystallized and docked donepezil binding sites were compared. It was discovered that the ‘RMSD’ difference between co-crystallized and docked donepezil was 0.657. Table 3 displays the results of molecular docking of all ligands 4a-4f against the AChE. The ligand 4a primarily interacts with Arg296 via hydrogen bonds, although it also interacts with Tyr337 and His447 via π–π stacking interactions. The potent molecule DNP mainly interacts with Phe295, Trp86, Trp286, Tyr337 and one water molecules via hydrogen bonds, π–π interaction, and π–cation interactions.
In-vitro blood-brain barrier permeation assay

For CNS active candidates, blood-brain barrier (BBB) permeation is the main selection criteria. All the developed analogues were tested for their BBB permeation assessment using parallel artificial membrane permeation assay (PAMPA-BBB) (Supporting Fig. S4). The permeation constant of two marketed drugs were used as a positive and negative control for BBB permeability ($Pe$). The $Pe$ values of compounds (4a-4f) are listed in Table 2. Among all the synthesized analogues, compound 4a, $Pe = 8.36 \pm 0.56 \times 10^{-6}$ cm s$^{-1}$ showed highest BBB permeability compared to all derivatives. Moreover, all the synthesized compounds pass BBB in-vitro, with good permeation potentials.

Prediction of ADMET, blood-brain barrier (BBB) penetration properties

Absorption, distribution, metabolism, and excretion (ADME) is one of the major criteria in the development of orally active drug. The admetSAR server (http://lmmd.ecust.edu.cn:8000/predict/) was used to estimate the target compound’s ADMET profile, BBB permeability, and acute toxicity using molecular modelling. Table 5 shows that the developed compound 4a has a fine ADMET profile & potential BBB permeability according to admetSAR.

Conclusion

This study depicted the development of pyrazolo-benzothiazole derivatives as a multifunctional agent for the management of AD. All the developed derivatives were tested for their multifunctional property including ChE inhibition, antioxidant evaluation using DPPH assay, enzyme kinetics study and Aβ modulation study. Among all developed derivatives, 4a containing 3,4-dimethoxy substitution was the most potent AChE inhibitor. SAR study clearly revealed that di-methoxy substituted derivatives showed better activity compared to that of mono-substituted derivatives. DPPH assay clearly revealed that 4a significantly presented free radical scavenging activity. Aβ
modulation experiment showed Aβ anti-aggregation property of 4a. Docking study showed that compound 4a correctly adjusted within the pocket of AChE via strong hydrogen bond and hydrophobic interactions. Finally, the current study clearly indicates that the pyrazolo-benzothiazole hybrids can be a potential core for further development of anti-AD agent (Fig. 10).

**Experimental section**

**Chemistry**

All the solvents used for the synthesis of the developed compounds were dried by distillation techniques before use. All the chemicals and reagents were obtained from the CDH chemicals (India), Qualigen (India), S.D. Fine Chemicals (India) and Finar chemicals (India). The progress of reactions was checked by thin-layer chromatography (TLC) on precoated silica gel 60 F254 (MerckKGaA) and were checked under UV light. Column chromatographic purifications were done using silica gel 60–120 mesh size (Avra synthesis, India). 1H nuclear magnetic resonance (1H-NMR) and 13C NMR spectra were measured on Bruker Advance, 500 MHz spectrometers with tetramethylsilane (TMS) as the internal standard. The NMR solvents used were CDCl3 or DMSO-d6 as indicated. Chemical shifts were measured in ppm and coupling constants (J) were measured in Hz. The following abbreviations are used to describe peak splitting patterns when appropriate: d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, br = broad. Coupling constants J are reported in Hertz (Hz).

**General procedure for synthesis of chalcone (3a-3c)**

Into a stirring solution of 4-hydroxyacetophenone (2) (1.2 g, 8.81 mmol, 1.0 equiv.) in ethanol (20 mL), substituted benzaldehyde (1a-1c) (1.75 g, 10.57 mmol, 1.20 equiv.) were added. Then, KOH (1.48 g, 26.43 mmol, 3.0 mmol) was added to the reaction mixture. The solution was refluxed at 78 °C for 10–12 h. After cooling down to RT, the solution was acidified with dilute HCl and left for 2 h. The obtained precipitate was filtered and washed with the water. The resulting yellow solid was recrystallized using ethanol to yield compound 3a-3c as a solid yellow powder in good to high yield.

**Compound 3a** was prepared according to general procedure mentioned above. 4-hydroxyacetophenone (2) (1.2 g, 8.81 mmol, 1.0 equiv.), 3, 4-dimethoxybenzaldehyde (1a) (1.75 g, 10.57 mmol, 1.2 equiv.), KOH (1.48 g, 26.43 mmol, 3.0 mmol) were mixed in 20 mL ethanol, and refluxed for 10 hr. Yellow solid powder (1.242 g, 81% yield), TLC (EtOAc:Hexane 30:70 v/v), Rf = 0.60. 1H NMR (500 MHz, DMSO-d6): δ 10.46 (bs, 1H, -OH), 8.09 (d, J = 8.75 Hz, 2H, Ar-H), 7.81 (d, J = 15.50 Hz, 1H, -CH=CH-), 7.66 (d, J = 15.50 Hz, 1H, -CH=CH-), 7.52 (d, J = 5.50 Hz, 1H, Ar-H), 7.34 (dd, J1 = 8.5 Hz, J2 = 2.5 Hz, 1H, Ar-H), 6.99 (d, J = 6.50 Hz, 1H, Ar-H), 6.93 (d, J = 8.50 Hz, 2H, Ar-H), 3.86 (s, 3H, -OCH3), 3.80 (s, 3H, -OCH3).

**Compound 3b** was prepared according to general procedure mentioned above. 4-hydroxyacetophenone (2) (1.2 g, 8.81 mmol, 1.0 equiv.), 2, 5-dimethoxybenzaldehyde (1b) (1.75 g, 10.57 mmol, 1.2 equiv.), KOH (1.48 g, 26.43 mmol, 3.0 mmol) were mixed in 20 mL ethanol, and refluxed for 10 hr. Yellow solid powder (1.32 g, 78% yield), TLC (EtOAc:Hexane 35:65 v/v), Rf = 0.55. 1H NMR (500 MHz, DMSO-d6): δ 10.32 (bs, 1H, -OH), 8.08 (d, J = 8.0 Hz, 2H, Ar-H), 7.81 (d, J = 15.0 Hz, 1H, -CH=CH-), 7.66 (d, J = 15.0 Hz, 1H, -CH=CH-), 7.52 (d, J = 5.00 Hz, 1H, Ar-H), 7.34 (dd, J1 = 8.5 Hz, J2 = 2.5 Hz, 1H, Ar-H), 6.99 (d, J = 6.50 Hz, 1H, Ar-H), 6.93 (d, J = 8.50 Hz, 2H, Ar-H), 3.86 (s, 3H, -OCH3), 3.80 (s, 3H, -OCH3).

**Compound 3c** was prepared according to general procedure mentioned above. 4-hydroxyacetophenone (2) (1.2 g, 8.81 mmol, 1.0 equiv.), 4-methoxybenzaldehyde (1c) (1.75 g, 10.57 mmol, 1.2 equiv.), KOH (1.48 g, 26.43 mmol, 3.0 mmol) were mixed in 20 mL ethanol, and refluxed for 10 hr. Yellow solid powder (1.38 g, 85% yield), TLC (EtOAc:Hexane 35:65 v/v), Rf = 0.52. 1H NMR (500 MHz, DMSO-d6): δ 10.31 (bs, 1H, -OH), 8.08 (d, J = 8.0 Hz, 2H, Ar-H), 7.99 (d, J = 16.0 Hz, 1H, -CH=CH-), 7.88 (d, J = 16.0 Hz, 1H, -CH=CH-), 7.53 (d, J = 6.00 Hz, 1H, Ar-H), 7.01–7.00 (m, 2H, Ar-H), 6.92 (d, J = 6.50 Hz, 2H, Ar-H), 3.83 (s, 3H, -OCH3), 3.79 (s, 3H, -OCH3).
(E)-1-(4-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (3c)

Compound 3c was prepared according to general procedure mentioned above. 4-hydroxyacetophene (2) (1.2 g, 8.81 mmol, 1.0 equiv.), 4-methoxybenzaldehyde (1c) (1.42 g, 10.57 mmol, 1.2 equiv.), KOH (1.48 g, 26.43 mmol, 3.0 equiv.) were mixed in 20 mL ethanol, and refluxed for 12 hr. Yellow solid (1.10 g, 76% yield), TLC (EtOAc:Hexane 40:60 v/v), Rf = 0.50. 1H NMR (500 MHz, DMSO-d6): δ 10.24 (bs, 1H, -OH), 8.01 (d, J = 7.5 Hz, 2H, Ar-H), 7.82 (d, J = 15.75 Hz, 1H, -CH = CH-), 7.78 (d, J = 15.75 Hz, 1H, -CH = CH-), 7.53 (d, J = 8.5 Hz, 2H, Ar-H), 7.11–7.07 (m, 2H, Ar-H), 6.98 (d, J = 8.5 Hz, 2H, Ar-H), 3.68 (s, 3H, -OCH3).

General procedure for synthesis of substituted 2-hydrazinobenzothiazole

To a 60 mL seal tube equipped with magnetic stirrer, 4-methylbenzol[d]thiazol-2-amine (1.64 g, 10 mmol, 1.0 equiv.), ethylene glycol (30 ml), and hydrazine hydrate (1.6 mL, 50 mmol, 5 equiv.) were mixed and refluxed for 10 h. White solid crystalline powder (1.8 g, 81% yield), TLC (EtOAc:Hexane 50:50 v/v), Rf = 0.60. 1H NMR (500 MHz, DMSO-d6): δ 8.86 (bs, 1H, -NH), 7.47 (s, 1H, Ar-H), 7.20 (d, J = 7.5 Hz, 1H, Ar-H), 7.01 (d, J = 8.0 Hz, 1H, Ar-H), 6.88 (t, J = 6.5 Hz, 1H, Ar-H), 4.96 (s, 2H, -NH2), 2.50 (s, 3H, -CH3).

2-hydrazinyl-6-methylbenzo[d]thiazole (6a)

Compound was prepared according to general procedure mentioned above. 6-methylbenzo[d]thiazol-2-amine (1.64 g, 10 mmol, 1.0 equiv.), ethylene glycol (30 ml), and hydrazine hydrate (1.6 mL, 50 mmol, 5 equiv.) were mixed and refluxed for 10 hr. White solid powder (1.72, 78% yield), TLC (EtOAc:Hexane 50:50 v/v), Rf = 0.55. 1H NMR (500 MHz, DMSO-d6): δ 9.01 (bs, 1H, -NH), 7.49 (dd, J1 = 7.5 Hz, J2 = 2.5 Hz, 1H, Ar-H), 7.01 (d, J = 8.0 Hz, 1H, Ar-H), 6.88 (t, J = 6.5 Hz, 1H, Ar-H), 4.99 (s, 2H, -NH2), 2.51 (s, 3H, -CH3).

2-hydrazinyl-4-methylbenzo[d]thiazole (6b)

Compound was prepared according to general procedure mentioned above. 4-methylbenzo[d]thiazol-2-amine (1.64 g, 10 mmol, 1.0 equiv.), ethylene glycol (30 ml), and hydrazine hydrate (1.6 mL, 50 mmol, 5 equiv.) were mixed and refluxed for 10 h. White solid crystalline powder (1.8 g, 81% yield), TLC (EtOAc:Hexane 50:50 v/v), Rf = 0.60. 1H NMR (500 MHz, DMSO-d6): δ 8.86 (bs, 1H, -NH), 7.47 (s, 1H, Ar-H), 7.20 (d, J = 7.5 Hz, 1H, Ar-H), 7.01 (d, J = 8.0 Hz, 1H, Ar-H), 6.88 (t, J = 6.5 Hz, 1H, Ar-H), 4.96 (s, 2H, -NH2), 2.50 (s, 3H, -CH3).

General procedure for synthesis of pyrazolo-benzothiazole derivatives

To a 30 mL seal tube equipped with magnetic stirrer, compound 3a-3c (1.0 equiv.), and compound 6a-6b (1.2 equiv.) were refluxed in n-butanol at 120 °C for 10–12 h. The progress of reaction was monitored by TLC (EtOAc:Hxane 1:1). The crude residues were then filter and subjected to column chromatography to get aforementioned product.

Table 4 The average ΔGbind (Kcal/mol) and its contributing energy terms Donepezil and 4a against hAChE calculated from MD trajectories (last 25) ns

| Complex | ΔGbind | ΔGbind | ΔGbind | ΔGbind | ΔGbind | ΔGbind |
|---------|--------|--------|--------|--------|--------|--------|
| AChE_DPZ | -11.99 | 1.71   | -0.47  | -32.73 | -5.57  | 32.11  |
| AChE_4a  | -21.00 | 2.29   | -1.33  | -24.38 | -5.79  | 29.40  |

Table 5 Theoretical prediction of ADMET profile, blood-brain barrier (BBB) penetration of 4a

| Compound | Model                  | Results | Probability |
|----------|------------------------|---------|-------------|
| 4a       | Plasma protein binding | 100%    | 1.08        |
| 4a       | Blood-Brain Barrier    | +       | 0.9765      |
| 4a       | Caco-2 Permeability    | +       | 0.5287      |
| 4a       | Acute oral toxicity    | III     | 0.6006      |
| 4a       | Fish Toxicity          | –       | 0.9466      |
Compound was prepared according to general procedure mentioned above. \((E)-3-(3,4\text{-dimethoxyphenyl})-1-(4\text{-hydroxyphenyl})\text{prop-2-en-1-one} (3a) (0.2 g, 0.703 mmol, 1.0 equiv.) and 2-hydrazinyl-6-methylbenzo[d]thiazole (6a) (0.126 g, 0.703 mmol, 1.0 equiv.) were refluxed in n-butanol at 120 °C for 10–12 h. White solid crystalline powder (0.289 mg, 80% yield), TLC (EtOAc:Hexane 1:1 v/v), \(R_f = 0.60\). 

\[ \text{1H-NMR (500 MHz, DMSO)} \delta \text{ ppm: 7.60 (m, 3H, Ar-H), 7.06 (d, } J = \text{7.3 Hz, 1H, Ar-H), 7.01–6.95 (m, 2H, Ar-H), 6.87–6.80 (m, 3H, Ar-H), 6.67 (d, } J = \text{3.0 Hz, 1H, Ar-H), 5.84 (dd, } J = \text{11.8, 5.3 Hz, 1H, -CH), 3.93 (q, 1H, -CH}_2, 3.78 (s, 3H, -OCH}_3), 3.62 (s, 4H, -OCH}_3, \text{ methylene -H), 2.31 (s, 3H, -CH}_3.} \]

\[ \text{13C-NMR (126 MHz, DMSO)} \delta \text{ ppm: 161.98, 154.87, 153.51, 151.75, 151.17, 130.95, 128.73, 128.62, 126.82, 121.82, 119.06, 116.28, 114.72, 114.30, 113.36, 113.03, 59.48, 56.82, 55.74, 18.11.} \]

4-(5-(3,4-dimethoxyphenyl)-1-(6-methylbenzo[d]thiazol-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)phenol (4a)

4-(5-(3,4-dimethoxyphenyl)-1-(4-methylbenzo[d]thiazol-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)phenol (4b)

Compound was prepared according to general procedure mentioned above. \((E)-3-(3,4\text{-dimethoxyphenyl})-1-(4\text{-hydroxyphenyl})\text{prop-2-en-1-one} (3a) (0.2 g, 0.703 mmol, 1.0 equiv.) and 2-hydrazinyl-4-methylbenzo[d]thiazole (6b) (0.126 g, 0.703 mmol, 1.0 equiv.) were refluxed in n-butanol at 120 °C for 10–12 h. White solid crystalline powder (0.25 mg, 75% yield), TLC (EtOAc:Hexane 1:1 v/v), \(R_f = 0.60\). 

\[ \text{1H NMR (500 MHz, DMSO)} \delta \text{ ppm: 7.60 (dd, } J = \text{17.7, 8.1 Hz, 1H, Ar-H), 7.06 (d, } J = \text{7.3 Hz, 1H, Ar-H), 7.06–6.90 (m, 2H, Ar-H), 6.93–6.75 (m, 3H, Ar-H), 6.67 (d, } J = \text{3.0 Hz, 1H), 5.84 (dd, } J = \text{11.8, 5.3 Hz, 1H), 3.94 (d, } J = \text{11.8 Hz,} \]
2H, -CH$_2$), 3.84 (d, $J = 6.5$ Hz, 3H, -OCH$_3$), 3.62 (s, 3H, -OCH$_3$), 2.31 (s, 3H, -CH$_3$).$^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ ppm: 163.14, 161.19, 147.41, 146.74, 143.04, 130.69, 130.63, 127.80, 121.88, 115.28, 115.11, 114.80, 114.40, 109.92, 62.03, 55.98, 31.93, 29.70.

4-(5-(2,5-dimethoxyphenyl)-1-(6-methylbenzo[d]thiazol-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)phenol (4c) Compound was prepared according to general procedure mentioned above. (E)-3-(2,5-dimethoxyphenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (3b) (0.2 g, 0.703 mmol, 1.0 equiv.) and 2-hydrazinyl-6-methylbenzo[d]thiazole (6a) (0.126 g, 0.703 mmol, 1.0 equiv.) were refluxed in n-butanol at 120 °C for 10–12 h. White solid powder (0.26 mg, 76% yield), TLC (EtOAc:Hexane 1:1 v/v), Rf = 0.50. $^1$H-NMR (500 MHz, DMSO) $\delta$ ppm: 7.69 (d, $J = 7.5$ Hz, 2H, Ar-H), 7.52 (s, 1H, Ar-H), 7.12 (s, 1H, Ar-H), 7.01 (d, $J = 7.5$ Hz, 2H, Ar-H), 6.92 (s, 1H, Ar-H), 6.68–6.62 (m, 2H, Ar-H), 6.57 (d, $J = 3.0$ Hz, 1H, Ar-H), 5.75 (dd, $J = 8.5$, 3.5 Hz, 1H, -CH), 4.01 (t, $J = 6.5$, 1H, -CH$_2$), 3.63 (s, 3H, -OCH$_3$), 3.51 (s, 4H, -OCH$_3$), 2.39 (s, 3H, -CH$_3$). $^{13}$C-NMR (126 MHz, DMSO) $\delta$ ppm: 160.63, 151.36, 149.68, 148.61, 147.38, 145.36, 131.31, 125.18, 121.45, 118.74, 115.64, 112.31, 111.27, 110.32, 110.17, 58.62, 57.21, 55.41, 20.14.

4-(5-(4-methoxyphenyl)-1-(6-methylbenzo[d]thiazol-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)phenol (4e) Compound was prepared according to general procedure mentioned above. (E)-3-(4-methoxyphenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (3c) (0.2 g, 0.703 mmol, 1.0 equiv.) and 2-hydrazinyl-6-methylbenzo[d]thiazole (6a) (0.126 g, 0.703 mmol, 1.0 equiv.) were refluxed in n-butanol at 120 °C for 10–12 h. White solid crystalline powder (0.245 mg, 75% yield), TLC (EtOAc:Hexane 1:1 v/v), Rf = 0.60. $^1$H-NMR (500 MHz, DMSO) $\delta$ ppm: 7.54 (d, $J = 8.5$ Hz, 2H, Ar-H), 7.44 (d, $J = 7.5$ Hz, 2H, Ar-H), 7.05 (d, $J = 7.5$, 2H, Ar-H), 6.94 (s, 1H, Ar-H), 6.66 (d, $J = 7.5$, 2H, Ar-H), 6.47 (d, $J = 7.5$ Hz, 1H, Ar-H), 6.41 (s, 1H, Ar-H), 5.80 (d, $J = 6.5$, 1H, -CH), 4.12 (d, $J = 6.5$, 2H, -CH$_2$), 3.61 (s, 3H, -OCH$_3$), 3.51 (s, 3H, -OCH$_3$), 2.41 (s, 3H, -CH$_3$). $^{13}$C-NMR (126 MHz, DMSO) $\delta$ ppm: 161.32, 158.14, 151.32, 147.52, 144.65, 143.84, 140.66, 137.63, 135.41, 125.62, 118.14, 115.11, 114.21, 113.21, 106.44, 57.32, 56.44, 53.94, 18.65.
4-(5-(4-methoxyphenyl)-1-(4-methylbenzo[d]thiazol-2-yl)-4,5-dihydro-1H-pyrazol-3-yl)phenol (4f)

\[
\begin{align*}
\text{CH}_3 & \\
\text{H}_2\text{CO} & \\
\text{N} & \\
\text{N} & \\
\text{N} & \\
\text{OH} & 
\end{align*}
\]

Compound was prepared according to general procedure mentioned above. \((E)-3-(4\text{-methoxyphenyl})-1-(4\text{-hydroxyphenyl})\text{prop-2-en-1-one} \) \((3e) \) \((0.2 \text{ g}, 0.703 \text{ mmol}, 1.0 \text{ equiv.})\) and \(2\text{-hydrazinyl-4-methylbenzo[d]thiazole} \) \((6b)\) \((0.126 \text{ g}, 0.703 \text{ mmol}, 1.0 \text{ equiv.})\) were refluxed in \(\text{n-butanol} \) at \(120 \degree \text{C} \) for \(10–12 \text{ h} \). White solid powder \((0.29 \text{ mg}, 82\% \text{ yield})\), TLC \((\text{EtOAc:Hexane 1:1 v/v})\), \(R_f\) \(–\text{n-butanol} \) at \(120 \degree \text{C} \) for \(10\text{ min} \). 1H-NMR \((500 \text{ MHz, DMSO})\) \(\delta\) ppm: \(7.61 \text{ (d, } J = 7.5 \text{ Hz, } 2\text{H, Ar-H}), 7.56 \text{ (d, } J = 6.5 \text{ Hz, } 2\text{H, Ar-H}), 6.99 \text{ (d, } J = 8.5, 2\text{H, Ar-H}), 6.91 \text{ (m, } 3\text{H, Ar-H}), 6.40 \text{ (d, } J = 8.5 \text{ Hz, } 1\text{H, Ar-H}), 6.38 \text{ (s, } 1\text{H, Ar-H}), 5.84 \text{ (d, } J = 7.5, 1\text{H, -CH})), 4.21 \text{ (d, } J = 7.0, 2\text{H, -CH}_2), 3.66 \text{ (s, } 3\text{H, -OCH}_3), 3.55 \text{ (s, } 3\text{H, -OCH}_3), 2.44 \text{ (s, } 3\text{H, -CH}_3). 13\text{C-NMR} \((126 \text{ MHz, DMSO})\) \(\delta\) ppm: \(160.32, 159.74, 154.12, 149.74, 147.63, 147.15, 138.15, 133.12, 131.50, 124.32, 116.50, 111.16, 110.74, 109.84, 108.63, 58.62, 55.12, 20.32.

**Biological evaluation**

**Determination of IC\(_{50}\) values**

Cholinesterase inhibitory activity of developed molecules was assessed calorimetrically using modified Ellman method [27]. Briefly, \(50 \mu\text{L} \) of AChE \((0.22 \text{ U/mL})\) and \(10 \mu\text{L} \) of the test or standard compound were incubated in \(96\text{-well plates} \) at room temperature for \(30 \text{ min} \). Furthermore, \(30 \mu\text{L} \) of the substrate viz. ATCI \((15 \text{ mM})\) was added and the solution. After \(30\text{ min} \), \(160 \mu\text{L} \) of DTNB \((1.5 \text{ mM})\) was added to it and the absorbance was measured at \(413 \text{ nm} \) wavelength using a \(96\text{-well microplate reader} \).

**Kinetic characterization of AChE and BChE inhibition**

In order to evaluate the mechanism of action of \(4a\), reciprocal plots of \(1/[V]\) versus \(1/[S]\) were constructed using six different concentrations of the substrate ATCI (from \(0.5, 1.0, 1.5, 2.0, 2.5, \) and \(3.0 \text{ mM} \) for hAChE) by using the modified Ellman method [27]. Briefly, compound \(4a\) \((10 \mu\text{L})\) at different concentrations \((5 \mu\text{M, 10 \muM and 20 \muM})\) was pre-incubated with hAChE \((50 \mu\text{L} \) of \(0.22 \text{ U/mL}) \) at \(RT\) for \(30 \text{ min} \), followed by the addition of \(30 \mu\text{L} \) of the substrate at different concentrations. The kinetic characterization of the hydrolysis of ATCI catalyzed by AChE is done spectrometrically using a \(96\text{-well microplate reader at} 413 \text{ nm}\).

**DPPH radical-scavenging potency**

The DPPH \((2,2\text{-diphenyl-1-picryl-hydrazyl-hydrate})\) free radical scavenging method is a simple and most convenient antioxidant assay based on reduction of DPPH radical. DPPH was purchased for Sigma-Aldrich (Merck, CAS No. 11659-21-1). All the experiments were carried out in biology grade methanol (for poor solubility ethanol can be used). Eight different concentrations \(1, 10, 20, 40, 80, 100, 160, \) and \(200 \mu\text{M} \) of test sample were used. In brief, \(75 \mu\text{L} \) of different concentrations of the test sample were added to a \(96\text{-well plate} \). Then, \(75 \mu\text{L} \) of DPPH \((200 \mu\text{M})\) solution was added to it. Finally, a \(96\text{-well microplate} \) was allowed to stand at \(RT\) for \(30 \text{ min} \), followed by absorbance at \(520 \text{ nm} \) using microplate reader. The radical scavenging capacity was determined using the equation \% radical scavenging activity = [(absorbance of control-absorbance of the test)/ absorbance of control] X 100. All the experiments were performed in triplicate.

**PAMPA-BBB Assay**

Brain permeability prediction of the developed compound was assessed by in-vitro PAMPA-BBB assay reported method from Di et al. [28]. The protocol involved the coating of porcine brain lipid solution dissolved in dodecane \((5 \mu\text{L})\) on filter membrane of donor microplate. The hydrolysis of ATCI catalyzed by AChE is done spectrometrically using a \(96\text{-well microplate reader at} 413 \text{ nm}\).
test compounds in the acceptor plate was calculated by determining the absorbance spectrophotometrically. The experiment was conducted in triplicate.

Inhibition of Aβ Aggregation

Aβ peptide (CAS No. 107761-42-2), molecular biology grade DMSO (CAS No. 67-68-5), Phosphate buffer saline (PBS) was procured from Sigma-Aldrich and HiMedia respectively. Aβ peptide 0.1 mg was dissolved in 100 μL of molecular biology grade DMSO, and aliquot in five different vials. The test compounds were prepared in molecular biology grade DMSO and PBS pH 7.4 (DMSO ≤ 1% w/v final concentration). Two different ratios of Aβ peptide and 4a were evaluated (10:10 μM, and 10:20 μM, respectively).

For self-induced anti-Aβ aggregation assay, the mixture of Aβ peptide (20 μM) Aβ peptide (40 μM) in PBS pH 7.4 in the presence or absence of inhibitor (20 μM, and 40 μM) was incubated (37 °C, 48 h) followed by addition of 100 μM of thioflavin T (10 μM). The fluorescence intensity was measured at excitation (λ ex = 450 nm) and emission (λ emission = 485 nm) wavelengths. The anti-Aβ aggregatory potential was calculated as percentage inhibition following an expression: [100 − (Fi/Fo × 100)]; and NFI = Fi/Fo. The Fi and Fo are the fluorescence intensities in the presence or absence of inhibitor, respectively.

Molecular docking

The 3D crystal structure of hAchE in complex with DPZ (PDB ID-4EY7) was retrieved from the Brookhaven protein data bank [29, 30]. Protein Preparation Wizard of Schrodinger software package (Schrodinger, LLC, New York, NY) was used to prepare proteins. This step includes removal of water beyond 5 Å from the HET group, addition of missing hydrogen, optimization of orientations of hydroxyl and amino groups, assignment of right bond orders, and the determination of ionization of amino acids using Prot Assign utility. The resulting structures were further subjected to restrained minimization with cut off root mean square deviation (RMSD) of 0.3 Å. Finally, the prepared complexes were further used for molecular docking and MD simulation study. All the small molecules were drawn using 2D sketcher and were subjected to ligand preparation using the Lig Prep module of Schrodinger software package (Schrodinger, LLC, New York, NY) [31]. The different possible ionization states for ligands were generated at the physiological pH (7.0 ± 2), and OPLS4 force field was used to minimize the ligands. Finally, docking of all ligands was performed by the Glide module of the Schrodinger software package (Schrodinger, LLC, New York, NY) using standard operating procedures with the extra precision (XP) protocol [32].

Molecular dynamics simulation

All-atom MD simulations were performed using the desmond-v6.6 module of Schrödinger Software Package (Schrödinger, LLC, New York, NY) [33]. The system builder panel was used to prepare the initial systems for MD simulations. The apo-AchE and both docked complexes (AchE-DNP, an AchE-4a) were placed in a cubic box of 1.0 nm size. The boxes were solvated with TIP3P water models and charged systems were neutralized using counter ions (Na+ or Cl− ions) [34]. An ionic strength of 0.15 M was maintained by adding Na+ and Cl− ions to all the systems. Further, the solvated systems were minimized and equilibrated under NPT ensemble using the default protocol of Desmond. It includes a total of nine stages, among which there are two minimization and four short simulations (equilibration phase) steps [35]. All minimized and equilibrated systems were subjected to MD run with periodic boundary conditions in NPT ensemble using OPLS4 force field parameter for 100 ns [36]. During the simulation, the pressure (1 atm) and temperature (300 K) of the systems were maintained by Martyna–Tobias–Klein barostat and Nose–Hoover Chain thermostat, respectively [37–40]. The binding energy between the AchE and ligands (DNP & 4a) was calculated using the built-in script thermal_mmgbsa.py [41, 42]. The binding energy was calculated from the last 25 ns of trajectory at an interval of 50 ps for both systems ((AChE-DNP, an AchE-4a).

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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