Chapter 4
(Experimental)

4.1 Computational studies

All the computational studies were performed using Schrödinger Maestro 2018-4 on HP Z410 3.10 GHz workstation with Kernel Linux based operating system.

4.1.1 Pharmacophore modeling

The structure-based drug designing approach was accomplished through the generation of e-pharmacophore models using protein structures of AChE (4EY7) [Cheung et al. 2012] and BACE-1 (2ZJM) [Yang et al. 2009]. Protein structures were selected based on their cocrystallized ligands bearing *N*-benzylpiperidine nucleus. Briefly, both protein structures were preprocessed using Protein Preparation Wizard and Prime modules to add missing hydrogens atoms, assign bond orders, and fill in the missing side chains and loops. Protein structures were optimized using PROPKA at pH 7.0, and minimized using an OPLS3 force field. The prepared protein structures were used to generate the respective e-pharmacophore models of AChE and BACE-1.

The e-pharmacophore models were generated by the 'Phase' module of the Schrödinger. The e-pharmacophore model was created using receptor-ligand complex with hypothesis settings of maximum four features, a minimum feature-feature distance of 2 Å, and the rest of the parameters were kept as default.

4.1.2 Virtual screening and docking-post processing (DPP)

Maybridge HitFinder database was selected and screened by Phase module using generated e-pharmacophore models and virtual screening (VS) workflow, which comprised of three steps: HTVS, standard precision (SP), and extra precision (XP) docking with the criteria of screening as 30% ligands in each step.

The DPP function was performed to identify common ligands interacting with active site residues of AChE and BACE-1. The pose filter tool was employed, and potential hits were screened based on their interactions with important PAS (Tyr72, Asp74,

Tyr124, Trp286, and Tyr341) and CAS (Ser203 and His447) residues of AChE and aspartate dyad (Asp32 and Asp228) of BACE-1.

4.1.3 MM-GBSA

Molecular mechanics generalized Born surface area (MM-GBSA) analysis of screened ligands was performed to determine the binding free energy of docked ligands into the respective proteins, and the potential top hit was identified. The Prime MM-GBSA module was employed for the pose viewer files of docked complexes with variable surface generalized Born (VSGB) model of solvation to calculate the ΔG binding energy for ligand-protein complexes.

4.1.4 Molecular docking study

Molecular docking study was performed on AChE (PDB code: 4EY7, *Homo sapiens*) and BACE-1 (PDB code: 2ZJM, *Homo sapiens*) to observe the potential noncovalent binding interactions. Initially, active sites of proteins were identified, and grids were generated surrounding the active pocket. The generated grids, and docking parameters were validated by extracting, and redocking the cocrystallized ligands into the respective protein grids of AChE and BACE-1. Superposition tool was used to validate the actual and redocked poses of cocrystallized ligands, and the RMSD values were calculated. Ligands were prepared by using the LigPrep module to generate the different conformers. Glide XP docking was used with default set parameters, and XP visualization function was used to observe the respective binding poses.

4.1.5 Molecular dynamics simulations study

The stability of protein-ligand docked complexes was evaluated by molecular dynamics simulation runs using Desmond on Schrödinger 2018-4. Initially, the system was built by soaking docked complexes in adequate TIP3P water molecules and neutralized by the addition of counter ions. The system was minimized with a maximum of 2000

iterations and a convergence threshold of 1 kcal/mol/Å. Molecular dynamics simulation run was performed, and interval trajectories were recorded accordingly. The NPT ensemble class was set, and the pressure and temperature of the system were maintained to 1.013 bars and 300.0 K, respectively.

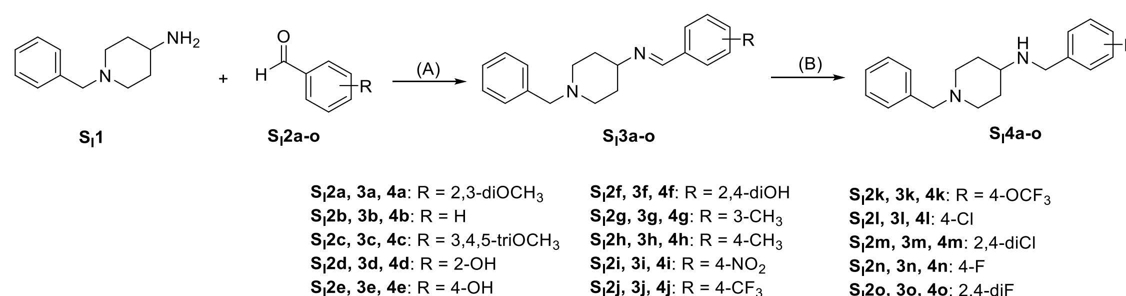
4.2 Synthesis

4.2.1 Chemicals and reagents

All the chemicals and reagents used in the study were procured from commercial sources, and used without further purification.

4.2.2 Series I: N-Benzylpiperidine analogs with substituted phenyl methanimines/ methanamines

The compounds of Series I were synthesized as per the reaction sequence outlined in Scheme 1.



Scheme 1. Synthesis of compounds from Series I (**S_I3a–o, and S_I4a–o**). **Reagents and conditions**: (A) Glacial acetic acid, absolute EtOH, room temperature, 4–6 h; (B) $NaBH_4$, dry MeOH, 0–5 °C, 2–4 h.

*4.2.2.1 General procedure for the synthesis of compounds (**S_I3a–o**)*

To the mixture of compound **S_I1** (0.500 g, 2.630 mmol) in absolute ethanol (10 mL), few drops of glacial acetic acid, and respective aromatic aldehydes (**S_I2a–o**, 1.2 equiv) were added, and the reaction was stirred for 4–6 h at room temperature [Sprung 1940]. The completion of the reaction was monitored by TLC using DCM:MeOH (95:5 v/v) as a mobile phase. The solvent was evaporated *in vacuo*, and the obtained residue was dissolved in DCM and washed with 5% w/v $NaHCO_3$, brine, and passed over anhydrous Na_2SO_4. The organic layer was concentrated under reduced pressure, and the crude

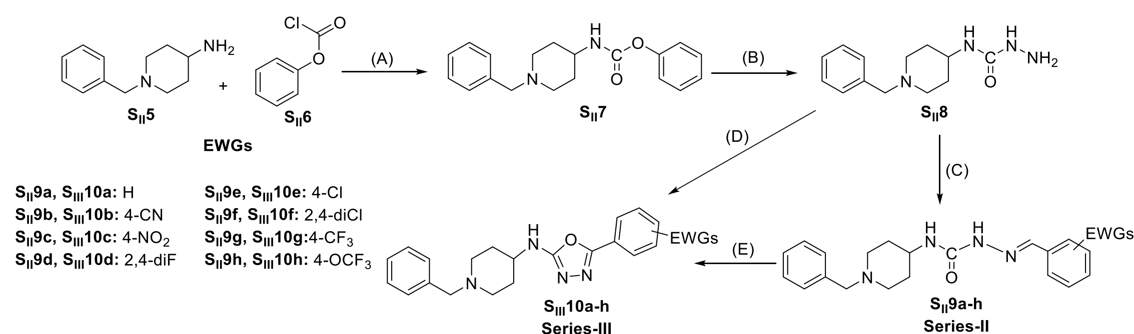
product was further purified by column chromatography (DCM:MeOH) using activated basic aluminum oxide to obtain the pure compounds **S_I3a–o**.

*4.2.2.2 General procedure for the synthesis of compounds **(S_I4a–o)***

Respective methanimine derivatives (**S_I3a–o,** 0.300 g) were stirred in dry methanol (10 mL) for 10 min with an equimolar quantity of $NaBH_4$. The mixture was stirred for 2–4 h and completion of the reaction was monitored by TLC using DCM:MeOH (95:5 v/v) as a mobile phase [Chaikin and Brown 1949]. The obtained residue was dissolved in DCM, washed with water, and passed over anhydrous Na_2SO_4. The organic layer was evaporated under reduced pressure, and the obtained crude product was purified over column chromatography (DCM:MeOH) using activated basic aluminum oxide to afford the pure compounds **S_I4a–o.**

4.2.3 Series II: N-Benzylpiperidine and substituted benzylidenehydrazine-1-carboxamides and Series III: 5-Phenyl-1,3,4-oxadiazoles tethered with —NH linker

The compounds from Series II (**S_{II}9a–h**) and Series III (**S_{III}10a–h**) were synthesized following the sequential reactions mentioned in Scheme 2.



Scheme 2. Synthesis of compounds from Series II (**S_{II}9a–h**) and Series III (**S_{III}10a–h**). **Reagents and conditions:** (A) Pyridine, DCM, 0–5 °C to room temperature, stirring, 4 h; (B) hydrazine hydrate (80% v/v), absolute EtOH, reflux, 6 h; (C) substituted benzaldehydes, glacial acetic acid, absolute EtOH, reflux, 2–4 h; (D) substituted benzoic acids, $POCl_3$, reflux, 8–10 h; (E) diacetoxyiodobenzene, DCM, stirring, room temperature, 4–6 h.

*4.2.3.1 General procedure for the synthesis of compounds (**S_{II}7**)*

To the mixture of compound **S_{II}5** (1.000 g, 5.259 mmol) in dry DCM (20 mL), pyridine (0.541 g, 6.837 mmol) was added. The temperature of the reaction mixture was maintained to 0–5 °C with the addition of phenyl chloroformate (**S_{II}6**, 0.988 g, 6.311 mmol) for 20 min. The reaction was stirred for 4 h at room temperature in a nitrogen environment [Bunnett and Zahler 1951]. The progress of the reaction was monitored by TLC using DCM:MeOH (90:10 v/v). After completion, the reaction mixture was quenched with 5% w/v $NaHCO_3$ solution, washed with brine, and passed over anhydrous Na_2SO_4. The organic layer was concentrated *in vacuo*, and the crude product was further purified by column chromatography (DCM:MeOH) using activated basic aluminum oxide to obtain the pure compound **S_{II}7.**

*4.2.3.2 General procedure for the synthesis of compounds (**S_{II}8**)*

The mixture of compound **S_{II}7** (1.000 g, 3.224 mmol) and hydrazine hydrate (0.807 g, 16.120 mmol) in absolute ethanol (15 mL) was refluxed for 6 h until the completion of the reaction as monitored by TLC using DCM:MeOH (90:10 v/v) [Alves et al. 2015]. The solvent was evaporated *in vacuo*; the obtained residue was taken in diethyl ether and triturated for 15 min. The solid residue was filtered, dried, and collected as the pure compound **S_{II}8**.

*4.2.3.3 General procedure for the synthesis of compounds Series II (**S_{II}9a–h**)*

To the mixture of compound **S_{II}8** (0.500 g, 2.013 mmol, 1 equiv) in absolute ethanol (10 mL), few drops of glacial acetic acid, and respective aromatic aldehydes (1.2 equiv) were added, and the reaction was refluxed for 2–4 h until the completion of reaction as monitored by TLC using DCM:MeOH (90:10 v/v) [Sprung 1940]. The reaction mixture was kept overnight, and the obtained precipitate was filtered, washed, dried, and collected as the pure compounds **S_{II}9a–h**.

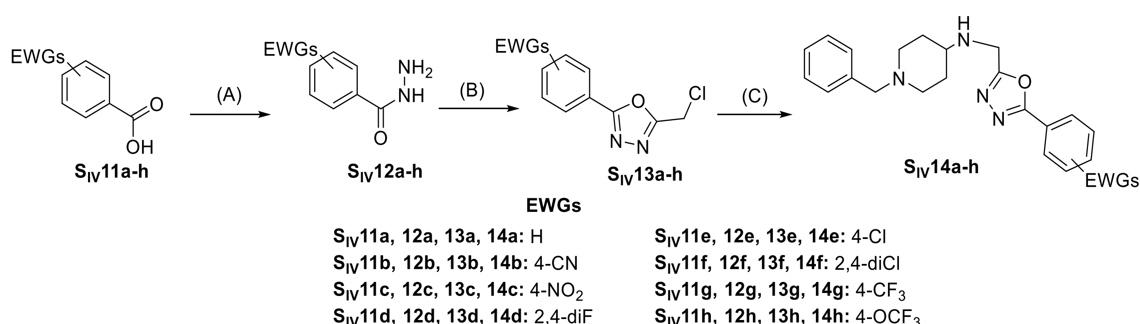
4.2.3.4 General procedure for the synthesis of compounds Series III ***(S_{III}10a–c)***

To the mixture of compound **S_{II}8** (0.500 g, 2.013 mmol, 1 equiv) in $POCl_3$ (10 mL), respective aromatic benzoic acids (1.2 equiv) were added, and the reaction was refluxed for 8–10 h until the completion of reaction as monitored by TLC using DCM:MeOH (90:10 v/v) [Berghot et al. 1992]. The reaction mixture was quenched by pouring it slowly over crushed ice and obtained precipitate was filtered, washed, dried, and further purified by column chromatography (DCM:MeOH) using activated basic aluminum oxide to afford the pure compounds **S_{III}10a–c**.

4.2.3.5 General procedure for the synthesis of compounds Series III ***(S_{III}10d–h)***

To the mixture of respective methanimine derivatives (**S_{II}9d–h**, 0.500 g, 1 equiv) in dry DCM (10 mL), diacetoxyiodobenzene (2.2 equiv) was added, and the reaction mixture was stirred at room temperature for 4–6 h [Prakash et al. 2010]. The completion of the reaction was monitored by TLC using DCM:MeOH (90:10 v/v) as the mobile phase. The organic layer was evaporated *in vacuo* and obtained crude product was purified by column chromatography (DCM:MeOH) using activated basic aluminum oxide to obtain the target compounds **S_{III}10d–h**.

4.2.4 Series IV: N-Benzylpiperidine and substituted 5-phenyl-1,3,4-oxadiazoles tethered with —$NHCH_2$ linker



Scheme 3. Synthesis of compounds from Series IV **(S_{IV}14a–h). Reagents and conditions:** (A) HOBT, EDC, ACN, hydrazine hydrate (80% v/v), room temperature followed with 0–5 °C, stirring, 2–4 h; (B) chloroacetic acid, $POCl_3$, reflux 4–6 h; (C) 4-amino-1-benzylpiperidine (**S_I1**), KI, DMF, room temperature, stirring, 6–8 h.

The compounds from Series IV (**S_{IV}14a–h**) were synthesized following the sequential reactions mentioned in Scheme 3.

*4.2.4.1 General procedure for the synthesis of compounds (**S_{IV}12a–h**)*

To a suspension of corresponding benzoic acid (**S_{IV}11a–h**, 1.000 g, 1 equiv) in acetonitrile (10 mL), 1.2 equiv of HOBT and EDC were added, and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then slowly added into the solution of hydrazine hydrate (2 equiv) and acetonitrile (5 mL) at 0–5 °C. After addition, the reaction mixture was stirred at room temperature, and the completion of the reaction was monitored by TLC using hexane:EtOAc (50:50 v/v). The reaction mixture was quenched in water (15 mL) and extracted with EtOAc (3 × 30 mL). Further, the reaction medium was washed with 5% w/v $NaHCO_3$ solution, brine, and passed over anhydrous Na_2SO_4. The organic layer was concentrated *in vacuo* to obtain the pure compounds **S_{IV}12a–h** [Zhang et al. 2002].

*4.2.4.2 General procedure for the synthesis of compounds (**S_{IV}13a–h**)*

To the mixture of corresponding benzohydrazide (**S_{IV}12a–h**, 0.500 g) in $POCl_3$ (3 mL), chloroacetic acid (1.2 equiv) was added, and the reaction mixture was refluxed for 2–4 h [Berghot et al. 1992]. The completion of the reaction was monitored by TLC using DCM:MeOH (90:10 v/v). The reaction mixture was quenched by pouring it dropwise over crushed ice, and obtained precipitate was filtered, washed, dried, and collected as pure compounds **S_{IV}13a–h**.

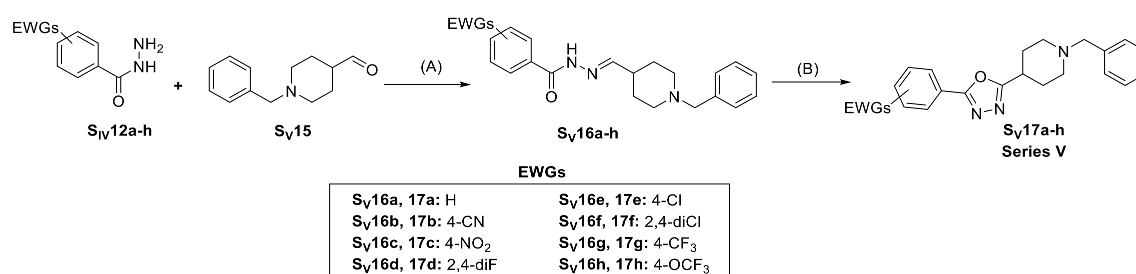
*4.2.4.3 General procedure for the synthesis of compounds Series IV (**S_{IV}14a–h**)*

To a suspension of corresponding oxadiazole intermediate (**S_{IV}13a–h**, 0.500 g, 1 equiv) in DMF (5 mL), potassium iodide (1.5 equiv) was added and stirred for 20 min. Then, 4-amino-1-benzylpiperidine (2 equiv) was added into the reaction mixture and stirred for 6–8 h at room temperature [Bunnett and Zahler 1951]. The completion of the

reaction was monitored by TLC using DCM:MeOH (90:10 v/v). The reaction mixture was quenched by pouring it dropwise over crushed ice, precipitate was filtered, and crude product was purified by column chromatography (DCM:MeOH) using activated basic aluminum oxide to obtain the target compounds **S_{IV}14a–h**.

4.2.5 Series V: N-Benzylpiperidine and substituted 5-phenyl-1,3,4-oxadiazoles tethered without a linker

The compounds from Series V (**S_V17a–h**) were synthesized following the sequential reactions mentioned in Scheme 4.



Scheme 4. Synthesis of compounds from Series V (**S_V17a–h). Reagents and conditions:** (A) glacial acetic acid, absolute EtOH, reflux, 4–6 h; (B) chloramine T, absolute EtOH, room temperature, stirring, 1–2 h (65–75%).

*4.2.5.1 General procedure for the synthesis of compounds **(S_V16a–h and S_V17a–h)***

To the mixture of corresponding benzohydrazide derivative (**S_{IV}12a–h**, 0.500 g, 1 equiv) in absolute ethanol (10 mL), few drops of glacial acetic acid, and *N*-benzylpiperidine-4-carboxaldehyde (**S_V15**, 1 equiv) were added, and the reaction mixture was refluxed for 4–6 h [Sprung 1940]. The reaction mixture was cooled to obtain the methanimine derivatives (**S_V16a–h**), which were *in situ* reacted with chloramine T (2.3 equiv) and stirred at room temperature for 1–2 h [Musad et al. 2011]. The completion of the reaction was monitored by TLC using DCM:MeOH (90:10 v/v). The precipitate was filtered, washed, and purified by column chromatography (DCM:MeOH) using activated basic aluminum oxide to obtain the target compounds **S_V17a–h**.

4.3 Characterization of the synthesized compounds

4.3.1 Melting point

The melting points were determined on a Stuart Melting Point apparatus (SMP10, Barloworld Scientific Ltd., UK) using capillary tubes and reported as uncorrected.

4.3.2 TLC (R_f value)

The TLC is an important technique used to monitor the progress of a reaction qualitatively, and it also ascertains the purity of the substance. R_f values were determined using precoated Merck silica gel 60F254 aluminum sheets (Merck, Germany). The visualization of TLC plates was accomplished using the UV light, iodine vapors, or Dragendorff reagent. R_f value was calculated by the following equation:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

4.3.3 FT-IR

FT-IR spectra were recorded on Alpha ECO-ATR Spectrophotometer (Bruker, USA), and were reported as % Transmittance *vs.* Wavenumber (cm^{-1}) at the scanning range of 4000-667 cm^{-1}. The samples, either solid or liquid, were directly placed on the ATR diamond for analysis.

4.3.4 ^{1}H NMR and ^{13}C NMR

NMR spectra (^{1}H NMR: 500 MHz and ^{13}C NMR: 125 MHz) were recorded on Bruker Avance FT-NMR spectrophotometer (Bruker, USA), and chemical shift values (δ) were recorded with reference to tetramethylsilane (TMS). The minimal required quantity of samples (8-10 mg for ^{1}H NMR and 15-20 mg for ^{13}C NMR) was dissolved in 0.6 mL of solvents (DMSO-d_6 or $CDCl_3$), and spectra were interpreted by MestReNova 6.0.2. The splitting patterns were depicted as singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), doublet of doublets of doublets (ddd), and multiplet (m).

4.3.5 Mass spectra

The mass spectra were recorded on 6410 Triple Quadrupole LC/MS (Agilent, USA) in positive ion mode using electrospray ionization (ESI) source. The mass samples were prepared by dissolving 1-2 mg of test compounds in methanol.

4.3.6 Determination of percentage purity by HPLC

Infinity II 1260 HPLC (Agilent, USA) was used with the Eclipse Plus C8 column using Methanol/Water (90:10 v/v) as the mobile phase at the flow rate of 1 mL/min. The retention time was monitored for 10 min using a photodiode array detector at $\lambda = 270$ nm. %Area mode calculations (Agilent ChemStation Online) determined the percentage purity of all compounds for observed peaks in each chromatogram.

4.4 Biological Evaluation

4.4.1 In vitro studies

4.4.1.1 Cholinesterase (AChE and BChE) inhibition by Ellman assay

Cholinesterase inhibitory potential of synthesized compounds was evaluated spectrometrically using the Ellman method. Cholinesterase specifically catalyzes the hydrolysis of thiolated substrates to produce the thiocholine, which reduces the 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) to yield a yellow color product that can be detected colorimetrically at 412 nm. The stock solution of hAChE (EC No. 3.1.1.7, from human erythrocytes) was dissolved in 20 mM HEPES buffer (pH 8) with Triton X-100 (0.1% v/v). The hBChE (EC No. 3.1.1.8, from human serum) stock solution was prepared in aqueous gelatin solution (0.1% w/v). The five increasing concentrations of inhibitors producing inhibition in the range of at least 20–80% were prepared in DMSO (final concentration ≤1% v/v). Briefly, the mixture of 25 µL hAChE or hBChE (0.25 U/mL) and 10 µL of test compound was preincubated (10 min), followed by addition of 340 µM 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), and 550 µM of respective substrate (ATCI for hAChE; and BTCI for hBChE). The blank readings were taken with

all the components except enzyme to account for the nonenzymatic hydrolysis of substrates. The increased absorbance was monitored at λ = 412 nm at 37 °C for 6 min on a Multimode Microplate Reader (BioTek Synergy H1M, USA). The reaction rates were compared with or without inhibitors, and the percentage inhibition was calculated by the following expression: $[(V_o - V_i)/V_o] \times 100$. The V_i and V_o are the reaction rates with or without inhibitor, respectively. The IC_{50} value for each test compound was determined using nonlinear variable slopes of log (inhibitor) vs normalized response (Graph Pad Prism 5.01). The assay was conducted as three separate experiments in triplicate [Ellman et al. 1961, Peauger et al. 2017b].

4.4.1.2 Enzyme kinetics study

An enzyme kinetics study was performed to determine the type of hAChE/hBChE inhibition. The kinetic parameters K_m and V_{max} were calculated by determining the enzymatic activity at six different concentrations (50–500 µM) of the substrate (acetylthiocholine iodide, ATCI for hAChE and butyrylthiocholine iodide, BTCI for hBChE). Test compounds were used in three different concentration ranges. Each concentration of inhibitor was evaluated with six different concentrations of substrate. The values of V_{max} and K_m were calculated using Michaelis-Menten nonlinear regression kinetics, and the mechanism of enzyme inhibition was determined by Lineweaver-Burk plots [Lineweaver and Burk 1934] using Graph Pad Prism 5.01. Further, the K_i value was determined by Dixon plot as a function of four different concentrations of the inhibitor [Dixon 1972]. The enzyme kinetic assay was performed in three separate experiments.

4.4.1.3 BACE-1 inhibition assay

The BACE-1 inhibitory potential of test compounds was evaluated using the FRET-based BACE-1 activity detection kit (Sigma Aldrich, Catalog No. CS0010). The assay

involved the enhancement of fluorescent signal due to cleavage of the substrate by BACE-1. The kit contains a fluorescent assay buffer, stop solution, assay standard, BACE-1 substrate (7-Methoxycoumarin-4-acetyl-[Asn^{670}, Leu^{671}]- Amyloid β/A4 precursor protein 770 fragment 667-676-(2,4-dinitrophenyl) Lys-Arg-Arg amide trifluoroacetate salt), and BACE-1 enzyme. The inhibitor was used in five different concentrations, to achieve target enzyme inhibition in the range of 20-80%. The fluorescence intensity was monitored immediately after the addition of the BACE-1 enzyme with the wavelength of excitation, and emission was set at 320 nm and 405 nm, respectively. Plates were covered and incubated for 2 h at 37 ºC, and fluorescence intensity was again recorded using Multimode Microplate Reader (BioTek Synergy H1M, USA). All the measurements were performed in triplicate. The percentage inhibition was calculated using the following expression: $[(IF_o–IF_i)/IF_o] \times 100$, where, IF_i and IF_o are the fluorescence intensities obtained in the presence and absence of inhibitor, respectively and the IC_{50} values were calculated using linear regression graph (GraphPad Prism 5.1, GraphPad Software Inc.) [Gurjar et al. 2014, Lisa Prostak et al.].

4.4.1.4 Propidium iodide (PI) displacement assay

PI displacement assay is a useful method to determine the binding of compound to the peripheral site of AChE by competitively displacing the PI. The assay mixture of hAChE (5U) was incubated with or without test compounds (final concentrations 10 µM and 50 µM, 150 µL) for 6 h at 25 °C. Post incubation, PI (final concentration 1 µM, 50 µL) was added to make the final assay volume of 200 µL. After 10 min, fluorescence intensity was measured at excitation and emission wavelength of λ_{ex} = 535 nm and λ_{em} = 595 nm, respectively, using fluorescence plate reader (BioTek Synergy H1M). The percentage inhibition was calculated by the following expression: $100 – (IF_i/IF_0 \times 100)$, where IF_i and IF_0 are the fluorescence intensities with and without inhibitor,

respectively. Each assay was performed in triplicates, as three separate experiments [Peauger et al. 2017a].

4.4.1.5 Parallel artificial membrane permeation assay (PAMPA-BBB)

The *in vitro* brain permeability of the compounds was assessed by PAMPA-BBB assay [Di et al. 2003]. The porcine brain lipid (PBL, 4 μL) (Avanti Polar Lipids, India) with dodecane (20 mg/mL PBL in dodecane) was coated on the bottom porous filter disks of the acceptor microplates. Test compounds were dissolved initially in DMSO, followed by the addition of PBS pH 7.4 to obtain a final dilution of 25 μg/mL. The acceptor and donor plates (Merck Millipore, membrane pore size 0.45 μm) were filled with 200 μL of the final test solution and 200 μL of PBS pH 7.4, respectively. The acceptor plate was further sandwiched over donor plate and incubated for 18 h to allow the diffusion of the test compound from the donor to the acceptor well *via* lipid membrane. Post incubation, the drug concentrations in both the plates were determined spectrophotometrically. The experiment was performed in triplicate. The model was validated using nine commercial drug samples with known permeability, and linear correlation was established using the values of reference [$P_{e(ref)}$] and experimental [$P_{e(exp)}$] permeability [Seth et al. 2018].

4.4.1.6 Aβ aggregation (self- and AChE-induced) inhibition by thioflavin T assay

$A\beta_{1-42}$ (Sigma, India) was dissolved in 1% v/v ammonium hydroxide solution to get the 2000 μM stock solution and was stored at −80 °C. The test compounds were initially dissolved in DMSO, and final dilutions were made in PBS pH 7.4. Different proportions of $A\beta_{1-42}$: inhibitors were tested (10:5, 10:10; and 10:20 μM) by thioflavin T assay [Bolognesi et al. 2007, Kumar et al. 2018, Zha et al. 2015]. All the experiments were performed in triplicate.

For the $A\beta_{1-42}$ self-induced aggregation inhibition experiment, the mixture of $A\beta_{1-42}$ (final dilution 10 μM, 10 μL) with 50 mM PBS pH 7.4 and incubated at 37 °C for 48 h

with or without test compound (5 µM, 10 µM, and 20 µM; 10 µL). Blank assays were performed by using PBS pH 7.4 instead of $A\beta_{1-42}$ with or without inhibitor. The fluorescence intensities were measured with the addition of 50 mM glycine-NaOH buffer (pH 8.0) containing 5 µM thioflavin T at the excitation and emission wavelength of λ_{ex} = 450 nm and λ_{em} = 485 nm, respectively. The percentage inhibition of self-induced aggregation was calculated using the following expression: $100 - (IF_i/IF_0 \times 100)$, where IF_i and IF_0 are the fluorescence intensities with and without inhibitor, respectively after subtracting the values with the blank. The results were also reported as normalized fluorescence intensity with respect to control.

For AChE-induced $A\beta_{1-42}$ aggregation inhibition experiment, the mixture of $A\beta_{1-42}$ (final concentration 10 µM, 2 µL) and AChE from human erythrocytes (final concentration 230 µM, 16 µL) were incubated with or without test compounds (5 µM, 10 µM, and 20 µM; 2 µL) at 37 °C for 48 h. The assay solution was diluted to a final volume of 200 µL with the addition of 50 mM glycine-NaOH buffer (pH 8.0) containing 5 µM thioflavin T. The detection method and calculation were performed similarly to the self-induced aggregation experiment.

4.4.1.7 AFM study

Aβ aggregation inhibition was monitored by AFM analysis using the NT-MDT Ntegra Prima (Russia). Briefly, $A\beta_{1-42}$ (final concentration 10 µM, 20 µL) was incubated in 0.1 M PBS pH 7.4 with or without test compound (final concentration 10 µM, 20 µL) at 37 °C for 7–10 days. 5 µL of the mixture was placed freshly cleaved mica surface using spin coater (302 ×g, 60 s) and dried to remove the excess solvent (1207 ×g, 30 s). AFM measurements were performed on samples at different time points in ambient condition by keeping the probe spring constant between 3.5 N/m and high resolution acoustically driven cantilevers operating at the resonance frequency of 140 kHz in tapping mode

with the scanning rate of 0.5 Hz. The images were visualized at 5 × 5 µm using the Nova Px image analysis software (NT-MDT, Russia) [Harte et al. 2015, Ryu et al. 2008].

4.4.1.8 Neurotoxic liabilities against SH-SY5Y cell lines by MTT assay

The neurotoxic liabilities of test compounds were evaluated against SH-SY5Y neuroblastoma cell lines by MTT assay. Briefly, the cell lines (density 1×10^5 cells/wells) were seeded in 100 µL of medium followed by incubation in 5% CO_2 environment at 37 °C for 24 h. The test compounds in the different concentration range (10, 20, 40, and 80 µM) were added, and cells were incubated for 48 h. Post incubation, 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added, and the cells were incubated for an additional 2 h. The obtained purple-colored formazan was solubilized in 100 µL DMSO. The absorbance was measured at $\lambda = 570$ nm, and % cell viability was calculated [Xu et al. 2018].

4.4.2 In vivo and ex vivo studies

4.4.2.1 Animals

The Wistar rats weighing 200-250 g of either sex were procured from the Institute of Medical Sciences, Banaras Hindu University, Varanasi. Animals were maintained in an environmentally controlled temperature (25 ± 2 °C) and humidity (65 ± 5 %RH) with 12 h light/dark cycles. The animals had free access to commercial feed and water *ad libitum*. The investigation protocol for behavioral studies was duly approved by the Institutional Animal Ethical Committee (No. Dean/2017/CAEC/93).

4.4.2.2 Acute oral toxicity study

Female Wistar rats were used to evaluate the acute oral toxicity of test compounds. The animals were randomly assigned different groups with six animals in each group. The test compounds were suspended in 0.3% w/v Na-CMC and administered orally at the

graded doses (677, 1333, 2000 mg/kg) to the corresponding group of animals [Wang et al. 2015]. After dosing, the animals were monitored continuously for the first 6 h, intermittently for next 24 h, and daily up to 14 days for abnormal or toxic reactions such as tremors, convulsions, salivation, diarrhea, sleep, lacrimation, and feeding behavior [Lu et al. 2013].

4.4.2.3 Scopolamine-induced amnesia model: Y-maze test

The scopolamine-induced model was performed on healthy male Wistar rats. Scopolamine hydrobromide (Sigma Aldrich, India) was dissolved in sterilized normal saline. Test compounds and standard donepezil were suspended in 0.3% w/v Na-CMC solution. The rats were divided into following groups of six animals each: control, scopolamine, donepezil (5 mg/kg, p.o.), Test compounds (2.5, 5, and 10 mg/kg, p.o.). Donepezil and test compounds were orally administered once daily for seven consecutive days to the respective group of animals. Scopolamine-group of rats was administered vehicle (0.3% w/v Na-CMC) only.

On the seventh day of trial 30 min after the treatment, scopolamine hydrobromide (0.5 mg/kg) was injected intraperitoneally to all the animals except the control group. After 5 min of scopolamine injection, immediate spatial working memory was evaluated using three arms Y-maze apparatus. The arms were thoroughly cleaned, wiped with 70% v/v ethanol, and labeled as A, B, and C arms. The rats were placed individually in the center of the maze. The first entry was omitted from the calculation as initially rats were always entered into the arm of facing. The spontaneous alternation behavior, total arm entries, and the number of entries to each arm were recorded for total of 8 min. The maze was cleaned and wiped between each session. An arm entry was considered when a rat had crossed all four paws into the arm. Increased spontaneous alternations, i.e., three consecutive arm entries were considered as an indicator of improvement in

learning and memory. Percentage spontaneous alternations were calculated following the expression: [number of spontaneous alternations/(total arm entries-2)] × 100 [Shidore et al. 2016, Wolf et al. 2016].

4.4.2.4 Ex vivo studies: AChE estimation and antioxidant activity

At the end of the Y-maze test, all the rats of the respective groups were sacrificed, and their brain was isolated. The whole-brain/hippocampus was homogenized in ice-cold PBS (0.1 M, pH 7.4), and centrifuged at 4 °C (10062 ×g, 20 min). The collected supernatant was analyzed for biochemical estimations. Initially, the actual protein concentration of each of the collected supernatant was determined using Folin and Ciocalteu's (F&C) reagent as per the Lowry's method [Lowry et al. 1951] or standard Bradford assay [Bradford 1976].

The AChE level in each of the supernatant was determined by modified Ellman's colorimetric assay. The assay mixture consisted of supernatant (25 μL), PBS pH 7.4 (10 mM, 150 μL), and DTNB (1 mM, 100 μL) was preincubated (37 °C, 10 min). The reaction proceeded with the addition of ATCI (7.5 mM, 20 μL), and reaction rates were determined at wavelength λ = 412 nm at 37 °C for 6 min. The rate of ATCI hydrolysis was estimated as μM of substrate hydrolyzed/min/mg of protein [Ellman et al. 1961, Shrivastava et al. 2019].

To estimate the antioxidant potential of compound, biochemical analyses of oxidative stress biomarkers, i.e., MDA and SOD, were performed. The level of MDA was assessed using the TBARS assay, also termed as lipid peroxidation assay. The assay is based upon the spectrophotometric detection of a red-colored complex of TBARS, formed due to the reaction of MDA and thiobarbituric acid under acidic medium. Briefly, a mixture of each supernatant sample (200 μL) and trichloroacetic acid (10% v/v in 0.1 M HCl, 1 mL) was centrifuged at 4 °C (10 min, 101 ×g). The supernatants

were collected and mixed with an equal volume of thiobarbituric acid (0.67%) and warmed in a water bath for 10 min. After cooling, the absorbance of the assay mixture was spectrophotometrically estimated at λ = 532 nm. The results were calculated as a number of moles of MDA/mg protein [Ohkawa et al. 1979].

The SOD is a key enzyme responsible for the dismutation of free radicals. The assay was performed based on spectrophotometric detection of blue formazan product formed by autoxidation of hydroxylamine hydrochloride (pH 10.2) in the presence of EDTA. The assay mixture consisted of EDTA (100 μM), nitro blue tetrazolium (24 μM), and Na_2CO_3 (50 mM). The above mixture (200 μL) was mixed with 50 μL of supernatant and hydroxylamine hydrochloride. The absorbance was measured at λ = 560 nm, and the results were calculated as SOD units (U/mL)/min/mg protein [Kono 1978].

4.4.2.5 Aβ-induced AD phenotypic model: Morris water maze test

The improvement in cognitive impairment from test compounds was assessed by the Aβ-induced AD phenotypic model in rats [Bagheri et al. 2011]. $A\beta_{1-42}$ (4 μM) solution was dissolved in sterilized saline (0.9% NaCl) and incubated (37 °C, 7 days) to form neurotoxic aggregates. Test compounds (10 mg/kg, p.o.) and donepezil hydrochloride (5 mg/kg, p.o.) were suspended in a vehicle (0.3% Na-CMC). The rats were divided into different groups of six or ten animals each. Group 1: sham (saline); Group 2: model ($A\beta_{1-42}$ + vehicle); Group 3: donepezil + $A\beta_{1-42}$; Group 4: Test compounds + $A\beta_{1-42}$.

Stereotaxic surgery of rats was performed under anesthesia by a combination of intraperitoneal administration of ketamine (90 mg/kg) and xylazine (9 mg/kg). All the rats were individually placed on a stereotaxic instrument with the symmetric mounting of incision bars (–3.3 mm). The scalp was cleaned and wiped with sterilized saline. The burr hole was drilled through the skull, and stereotaxic coordinates were set relative to bregma (–0.5 mm AP, +1.2 mm ML, and –3.2 mm DV) [Colaianna et al. 2010]. The

$A\beta_{1\text{-}42}$ (4 μM, 5 μL) was injected to all the groups of rats except sham group through Hamilton microsyringe (infusion rate: 2 μL/min). The injection lasted for about 2.5 min, and the syringe was left in place for an additional 5 min to prevent further reflux. The same procedure was performed for the sham group of rats, except they were injected with sterilized saline instead of Aβ. Post-operatively, rats were kept in individual cages, and iodine ointment was applied at the surgical site. The rats were given special care until their normal feeding restored. After 7 days of recovery, donepezil and test compounds were administered to the corresponding group of animals for 9 successive days (from 8th to 16th day), while a model group of animals was given vehicle (0.3 % Na-CMC) only.

The improvement in learning and memory was assessed through the Morris water maze test [Morris 1984] during the last five days of treatment. The apparatus consisted of a circular pool (diameter: 121 cm; height: 62 cm; and depth: 32 cm) filled with opaque water (25 ± 2 °C). The pool was designated in four equally sized imaginary quadrants, which were maintained in the same position throughout the test. The task was based on incremental learning and memory by rats to locate the hidden platform submerged 2 cm under the water surface. Each day of the test, two trials were performed to record time to reach the hidden platform (escape latency time, ELT), and a total number of platform crossovers by rats, respectively. Each trial was conducted for 90 s with an intertrial difference of at least 3 h, and all the results were monitored in a soundproof room with the help of a tracking camera [Wang et al. 2015].

4.4.2.6 Western-blot analysis

Immediately after the Morris water maze test, the hippocampal tissue from each experimental group (Part-II) was isolated and homogenized in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and

phosphatase inhibitors followed by sonication for 5 s and centrifugation at 4 °C (10062 ×g, 20 min). The samples containing an equal amount of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto polyvinylidene difluoride membrane and incubated overnight at 4 °C with anti-Aβ_{1-42} (1:1000, CST #2454), anti-BACE-1 (1:1000, CST #5606S), and anti-β-actin (1:500, SC-47778) primary antibodies. The membranes were washed with Tris-buffered saline/Tween 20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, CST #7074). The blots were visualized using DAB and H_2O_2 as substrates. The relative density of each band was calculated with respect to β-actin, and their expression was indicated as of fold change using Quantity One Software (Windows, Bio-Rad).

4.4.2.7 Immunohistochemical analysis

The animals of each experimental group from Part-II were sacrificed and perfused intracardially with ice-cold saline (0.9% w/v, pH 7.2) and 4% ice-cold paraformaldehyde (v/v). Brains were decapitated and stored overnight in 10% w/v paraformaldehyde solution, and further transferred to sucrose solution for cryopreservation. The brain sections of 20 μm thickness were cut coronally at the hippocampus level using a cryomicrotome (Leica, Wetzlar, Germany). The nonspecific binding sites were blocked by PBS containing 10% normal goat serum, 0.3% Triton-X 100, and 1% BSA in PBST. The sections were incubated with anti-Aβ (1:1000) and anti-BACE-1 (1:500) primary antibodies for 18 h at 4 °C. Further, washing was done with PBS and 1% BSA-PBS respectively two times each to remove unbound primary antibodies and then incubated with TRITC-conjugated secondary antibody prepared in 1% BSA-PBS for 1 h at room temperature. Finally, sections were washed three times with PBS and then mounted on slides using poly(vinyl alcohol) mounting medium with

DABCO antifading (Fluka analytical). The images were captured under a fluorescent microscope equipped with Nikon digital camera (Thermo Fisher Scientific). Immunofluorescence was analyzed by ImageJ software (NIH United States).

4.4.3 Pharmacokinetic studies

Pharmacokinetic parameters of test compounds were estimated in healthy male Wistar rats (200-250 g, n = 3). The rats were fasted for 12-16 h and had free access to water. Test compound suspended in 0.3% w/v Na-CMC was administered to animals at a dose of 10 mg/kg, p.o. The animals were anesthetized, and blood samples were collected from retro-orbital plexus at several time points (0, 0.5, 1, 2, 4, 8, 16, 24, 36, 48, 60, and 72 h). All the blood samples were pretreated with sodium heparin to prevent coagulation. Blood samples were centrifuged (6797 ×g, 6 min, 4 °C) to collect the plasma, 100 µL of which was mixed with 200 µL of MeOH to extract the test compound**.** The mixture was further mixed, centrifuged (6797 ×g, 6 min, 4 °C), the organic layer was separated, and evaporated *in vacuo*. The collected residue was dissolved in the mobile phase, and 5 µL of the sample was injected into Infinity II 1260 High-Performance Liquid Chromatography (Agilent, USA) using MeOH: H_2O (85:15) as mobile phase at a flow rate of 1 mL/min. The Eclipse Plus C8 column (Pore size: 95 Å; Particle size: 3.5 µ; Inner diameter: 4.6 mm, Length: 30 mm) was used, and retention time was monitored for 10 min using photodiode array detector at λ = 270 nm. The plasma-drug concentration-time profiling was done, and calculated data were utilized to evaluate the pharmacokinetic parameters, such as T_{max}, C_{max}, AUC, $t_{1/2}$, etc. The extravascular non-compartment model was followed using Kinetica Software. The C_{max} is the concentration at which maximum plasma concentration achieved, and the time at which C_{max} reached, is referred to as T_{max}. While, $t_{1/2}$, i.e., the half-life is the time taken for the C_{max} to reduce to half of the maximal value.