Chapter 4

(Experimental)

4.1 Computational studies

The in silico molecular modeling studies were performed using Schrödinger Maestro 2018-1 software.

4.1.1 Pharmacophore Modeling

An e-pharmacophore hypothesis of structure-based drug design was generated using two co-crystal structures utilized to screen an in-house database. Two e-pharmacophore models for hAChE (PDB code: 4EY7) [Cheung et al. 2012] and hBACE-1 (PDB code: 2ZJM) [Yang et al. 2009] were generated using co-crystal (donepezil and FIM for AChE and BACE-1 respectively) structures of the proteins. Schrodinger's protein preparation wizard module was used to process and prepare the protein, such as to add hydrogen atoms, force field OPLS-2005 was used to allocate the partial charges, and by using the Prime module of the Schrodinger the missing side chains and loops were added. Epik was used at pH 7.0 to create heteroatom states and the water molecules larger than 5 Å were removed from the heteroatoms. Additionally, the PROPKA technique at pH 7.0 was used to minimize the structure of the protein by keeping the convergence threshold RMSD of the atoms at 0.30 Å at constrained minimization. The prepared protein was utilized to generate e-pharmacophore models of both (AChE and BACE-1) proteins.

The Phase module of Schrödinger Maestro suite 2018 was used to generate the e-pharmacophore models. The e-pharmacophore model was generated using receptor-ligand with a maximum four-feature hypothesis, and a minimum 2 Å feature-feature distance and the other parameters were kept as default.

4.1.2 Virtual Screening and Docking-Post processing

The in-house database was screened utilizing the Phase module of Schrödinger Maestro suite 2018. The identified hits were further subjected to virtual screening workflow (vsw). The vsw comprised high throughput virtual screening (HTVS), standard

precision (SP), and extra precision (XP) docking and was set at 30% filtration criteria in every step.

The docking post-processing (DPP) function of the Schrödinger Maestro was used to identify the common ligands interacting with the active site of both enzymes. The interactions of the ligands with the catalytic active site (CAS) region (His447, Glu334, and Ser 203) and the peripheral active site (PAS) region (Tyr124, Tyr72, Tyr341, Trp286, and Asp74) of the AChE and catalytic dyad (Asp32 and Asp228) region of BACE-1 were mainly considered. Finally, the pose filter tool was used to identify the top hits interacting with the active site residues of both enzymes.

4.1.3 Mechanics-Generalized Born Surface Area (MM-GBSA)

The binding free energy (ΔG_{bind}) was calculated as an average of several protein-ligand confirmations. The binding free energy of the protein-ligand complex was calculated based on the $\Delta G_{bind} = G_{complex}-G_{protein}-G_{ligand,}$ where $G_{bind,}$ $G_{complex}$, $G_{protein}$, and G_{ligand} are the binding free energy, free energy for complex, free energy for protein and free energy for ligand respectively. The energies were estimated using the equation ΔE_{MM} + ΔG_{GB} + $\Delta G_{nonpolar}$- TΔS ($\Delta E_{MM\ =gas\text{-}phase}$ interaction, ΔG_{GB}, $\Delta G_{nonpolar}$= polar and nonpolar components of the desolvation and TΔS is the change in conformational entropy). The prime MM/GBSA module of Schrödinger 2018-1 was used to calculate the ΔG_{bind} of the docked complexes of **SD-4** and **SD-6** against hAChE, hBChE, and hBACE-1. The results of MM/GBSA are expressed as ΔG_{bind} in kcal/mol for both the compounds and standards [Sinha and Shrivastava 2013].

4.1.4 Molecular Docking Studies

In silico molecular modeling studies were performed on Schrödinger Maestro 2018-1. The “Glide” module of the Schrödinger was used to perform the molecular dockings of the compounds on the X-ray crystal structure of hAChE (PDB: 4EY7), hBChE (PDB: 4TPK), and hBACE-1 (PDB: 2ZJM). The “Protein Preparation Wizard” module was

used initially to preprocess, refine, and correct the protein structures followed by optimization using the Propka method (at pH 7.0) followed by heavy atom restrained minimization (RMSD 0.30 Å). A grid box of (10 × 10 ×10 $Å^3$) around the co-crystallized ligand surrounding the active site in each protein was generated. To validate the docking protocol, a co-crystallized ligand was extracted and redocked into the corresponding generated grids of the hAChE and hBACE-1. The "LigPrep" module was used to prepare the ligands and finally "Glide XP" & "Glide XP visualizer" module was used for docking studies and to visualize the ligand-protein interaction and docking score respectively.

4.1.5 Molecular Dynamics and Simulation studies

The stability of the compounds against hAChE, hBChE, and hBACE-1 enzymes was estimated using molecular dynamic (MD) simulation. An MD simulation run of 100 ns for each ligand-protein docked complex was performed using Desmond (Schrödinger) on GPU. A virtual TIP3P water environment system was generated around the docked complexes and counter-ions were added to neutralize the system using the system builder module. The system thus generated was minimized to a 1.0 kcal/mol/Å convergence threshold with a maximum of 2000 interactions. Finally, a 100ns MD simulation run for each docked complex was performed to obtain approximately 1000 frames with a 100 ps recording interval and energy at 9.6.

4.1.6 In silico drug-likeness determination

The drug-likeliness properties of the compounds were identified using the QikProp module of the Schrodinger Maestro's 2018-1. Lipinski's rule of five (donor HB < 5, accept HB < 10, mol MW < 500, QPlogPo/w < 5,) was used to predict a number of descriptors, including QPlogBB, SASA (solvent accessible surface area), and others, to assess the drug-likeliness properties in the compounds [Tripathi et al. 2020b].

4.1.7 DFT and Fukui function calculation

The electrostatic properties of the compounds were determined using a Jaguar module for DFT calculations. The docked poses of both compounds were used as input for DFT calculation. To optimize the molecule and to calculate the molecular orbital surface a single point energy module of jaguar with default parameters was used while electron density calculations were performed using B3LYP/6-31G theory. The electrostatic potential surface over the structures was generated to understand the electrophilic and nucleophilic behavior of the compound roughly the Vanderwall surface of the molecules [Hou et al. 2011, Tripathi et al. 2023]. The Fukui function is used in the conceptual DFT to explore the reactive sites of the structure. The Fukui reactivity descriptor examines the molecular site prone to the nucleophilic or electrophilic attack thus predicting properties of the loss or gain of an electron. The $f^{+}j$ refers to gain while $f^{-}j$ to the loss of an electron and is termed as an index of nucleophilic and electrophilic attack respectively and can be calculated as $f^{+}j = q_j(N) - q_j(N-1)$ and $f^{-}j = q_j(N+1) - q_j(N)$, where q_j = atomic charge (NBO) at the j^{th} atomic site and the (N), (N-1) and (N+1) are the total electrons present on the molecule under investigation at neutral, cation and anionic state respectively. The condensed dual descriptor Δf (r) and condensed Fukui functions were calculated based on the NBO (atomic charge) charged distribution as Δf (r) = $f^{+}j - f^{-}j$ *[Dlala et al. 2021].*

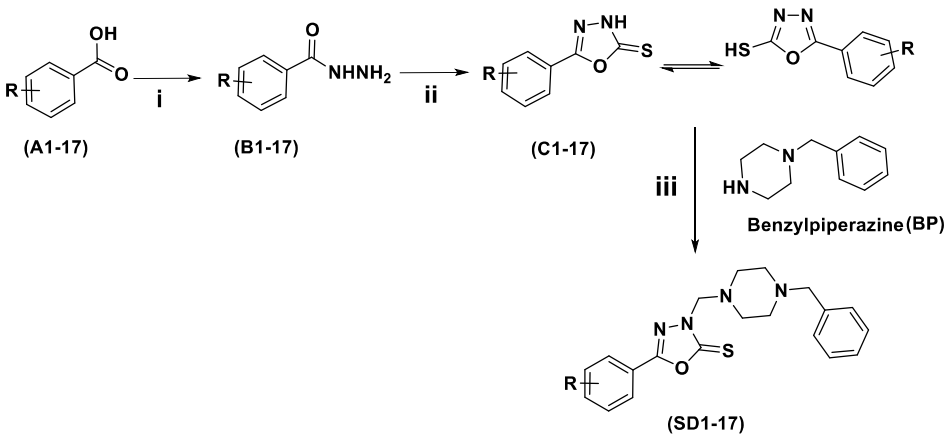
4.2 Synthesis

4.2.1 Chemicals and reagents

All the chemicals and reagents were purchased from approved commercial sourced and used without further purification otherwise stated.

4.2.1.1 Series-I : 3-NH linked benzylpiperazine derivatives of 5-phenyl-1,3,4-oxadiazole 2-thione

The compounds **SD 1-17** were synthesized as per the reaction sequence presented in Scheme 1



Scheme 1. Synthesis of targeted compounds **SD1-17, Reagents, and conditions:-** (i) HOBT, EDC.HCl, Acetonitrile, $NH_2NH_2.2H_2O$, 0-5 °C, 2 h, 78-91% (ii) CS_2, KOH, Ethanol, reflux, 3 h, 85-89% (iii) HCHO, Ethanol, RT, 16 h, 58-78%.

4.2.1.2 General procedure for the synthesis of compound (B1-17, 2, and 2a-q).

To a suspension of benzoic acid and substituted benzoic acids (1 equiv) in CH_3CN, HOBt (1.2 equiv), and EDC.HCl (1.2 equiv) was added at room temperature and the reaction was monitored for total conversion of acid to easter through TLC using hexane: EtOAc (50: 50 v/v). After the full conversion of acid to ester, this suspension was added dropwise to a mixture of hydrazine hydrate (2 equiv) in acetonitrile previously kept at 0-5^{0}C. The reaction was further allowed to be stirred at room temperature and monitored by TLC using hexane: EtOAc (50:50 v/v). After completion, the reaction mixture was quenched with water and extracted with EtOAc (3 × 30 ml) and washed with saturated $NaHCO_3$, brine solution dried over Na_2SO_4, and the solvent was evaporated to dryness to get pure compounds **2**, which was used in the next step without further purification [Sharma et al. 2019b].

4.2.1.3 General procedure for the synthesis of compound (C1-17, 3, and 3a-q).

An appropriate amount of 2 and 2a-q aryl hydrazides (1 equiv) in ethanol was taken into two neck round bottom flasks under an inert atmosphere. Potassium hydroxide (1 equiv)

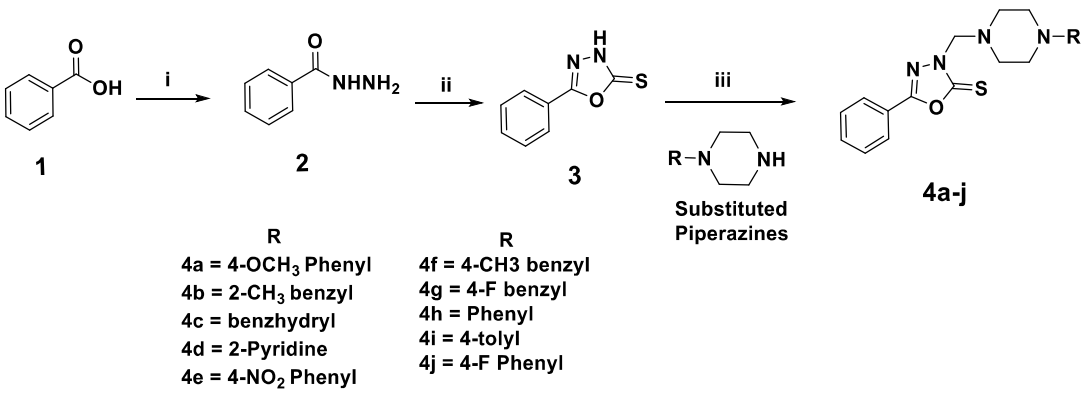
was added to the ethanolic solution and an excess amount of carbon disulfide (2.5 equiv) was added dropwise to it. The reaction mixture was stirred and refluxed for 4-6 hrs and monitored by TLC using hexane: EtOAc (50:50 v/v). After completion of the reaction, the solvent was evaporated under reduced pressure, and then water was added to the mixture followed by the addition of 3 M HCl to adjust the pH 2-3 to get a complete precipitate [Karabanovich et al. 2016, Livani et al. 2018]. The precipitate was further washed with water to obliterate HCl and recrystallized with acetonitrile to obtain pure products **3** and **3a-q**.

4.2.1.4 General procedure for the synthesis of compound (SD 1-17)

To a hot solution of **C1-17** (0.005 moles), in ethanol benzyl piperazine (0.01 mole) and 37%, formaldehyde solution (1.0 mL) was added and the mixture was stirred at room temperature for 5 h. the reaction mixture was allowed to stand overnight and 5ml water was added dropwise for 1 h with continuous stirring. The precipitate formed was filtered and washed with water thrice, dried, and recrystallized further with ethanol to get desired product **SD1-17** [Al-Wahaibi et al. 2021].

4.2.2 Series-II : 3-NH linked substituted piperazine derivatives of 5-phenyl-1,3,4-oxadiazole 2-thione

The compounds **4a-j** were synthesized as per the reaction sequence presented in Scheme 2



Scheme 2. Synthesis of the targeted Compounds **(4a-j), Reagents, and conditions:-** (i)

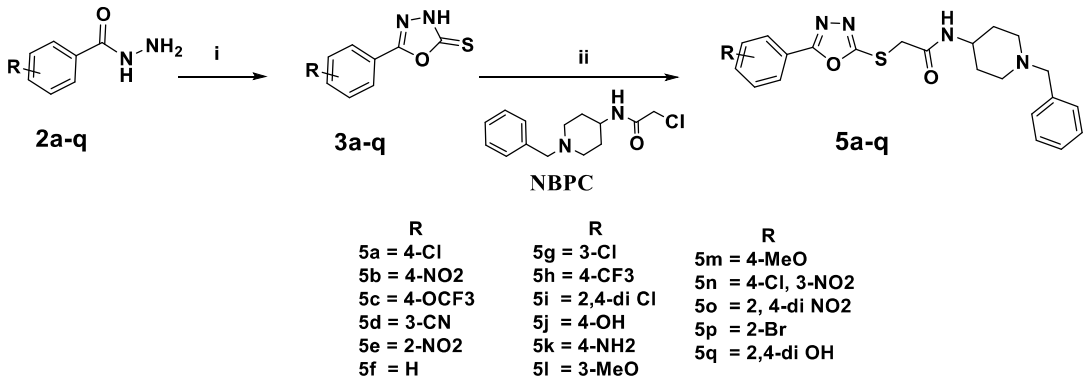
HOBt, EDC.HCl, CH_3CN, $NH_2NH_2.2H_2O$, 0-5 °C, 2 h, 88% (ii) CS_2, KOH, Ethanol, reflux, 3 h, 85% (iii) HCHO, Ethanol, RT, 16 h, 70-76%.

4.2.2.1 General procedure for the synthesis of compound (4a-j)

The appropriate 1-substituted piperazines (1 equiv) and formaldehyde solution 37% (3 equiv) were added to a hot solution of **3** (1 equiv), in ethanol, and the mixture was stirred at room temperature overnight. Water (5 mL) was then added drop-wisely to the reaction mixture with continuous stirring for one hour. The separated precipitate was filtered, washed with water, dried, and crystallized with ethanol to get compounds **4a-j** [Caneschi et al. 2019, Mishra et al. 2017b].

4.2.3 Series-III : 2-thiol linked N-(1-benzylpiperidin-4-yl)-2-chloroacetamide derivatives of 5-phenyl-1,3,4-oxadiazole 2-thione

The compounds **5a-q** were synthesized as per the reaction sequence presented in Scheme 3



Scheme 3. Synthesis of the targeted compounds (5a-q), **Reagents and conditions:-** i) CS_2, KOH, Ethanol, reflux, 3 h, 81-87% (ii) TEA, Ethanol, reflux, 3 h, 61-82%.

4.2.3.1 General procedure for the synthesis of compound (NBPC)

The 1-benzylpiperidine-4-amine (1 equiv) was dissolved in DCM and stirred at 0-5 ^{0}C on an ice bath, chloroacetyl chloride (1.1 equiv) was added dropwise to it and the reaction mixture was further stirred in room temperature for 2 hrs. The reaction mixture

was extracted with DCM (50 ml) and washed thrice with saturated $NaHCO_3$ solution. The solvent was dried over $MgSO_4$ and evaporated at reduced pressure to obtain an off-white solid and was used in the next step without further purification[Berglund et al. 2008].

4.2.3.2 General procedure for the synthesis of compound (5 a-q)

The 1 molar equivalent of compounds **3 a-q** and NBPC in ethanol were refluxed in the presence of triethylamine (1 equiv) for 4 hrs. The reaction progression was monitored with TLC using DCM: MeOH (90:10 v/v). after completion of the reaction, the solvent was evaporated on vacuo and poured into the water with stirring to get precipitate and washed with water [Abd-Ellah et al. 2016, Yang et al. 2022]. The precipitate obtained was further recrystallized with ethanol to get pure compound 5a-q.

4.3 Characterization of the synthesized compounds

4.3.1 Melting point

The melting point implies the relationship between structure and properties. Hence, different compounds tend to have different melting points. It is one of the important criteria to indicate the purity of compounds. 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 UV light, iodine vapors, or Dragendorff reagent.

The R_f value was calculated using the following equation:

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

4.3.3 FT-IR

FT-IR spectra of the compounds were recorded as % Transmittance Vs. Wavenumber (cm^{-1}) at the scanning range of 4000-667 cm^{-1} on Alpha ECO-ATR Spectrophotometer (Bruker, USA).

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

^{1}H NMR (500MHz) and ^{13}C NMR (125MHz) spectra of the synthesized compounds were captured on a Bruker (Avance III HD 500 MHz) spectrophotometer using tetramethylsilane (TMS) as a reference standard in DMSO-*d6* or $CDCl_3$. and spectra were interpreted using MestReNova 6.0.2. The splitting patterns were depicted as singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), the triplet of doublets (td), doublet of doublets of doublets (ddd), and multiplet (m).

4.3.5 Mass spectra

The HRMS spectrums of the compounds were recorded on AB Sciex X500R QTOF mass spectrometer hyphenated with High-performance liquid chromatography (Shimadzu, USA).

4.3.6 Single crystal X-Ray Crystallography

Diffraction data of single crystals were obtained on a Bruker APEX-III photon detector diffractometer at 100 K. Data reduction was piloted following standard procedures via the Bruker software package SAINT [Dudka 2007], and absorption and other systematic error corrections were carried out using SADABS. The structures were solved by direct methods using SHELXS-97 and refined using SHELXL-97. X-Seed was used as the graphical interface for the SHELX program suite. Hydrogen atoms were positioned in the calculated locations using riding models.

4.3.7 HPLC percentage purity determination

The % purity of the compounds was determined through High-performance liquid chromatography (Shimadzu, USA) using 1 ml/min flowrate of ACN: H2O: TFA (95:5:0.1%) as mobile phase in the C18 column.

4.4 Biological Evaluations

4.4.1 Pharmacology (In vitro studies)

4.4.1.1 Human Cholinesterase (hAChE and hBChE) inhibition assay to determine IC50 values

The inhibitory potency of the compounds against hAChE and hBChE was estimated using Ellman's method with slight modification [Ellman et al. 1961]. The hAChE from human erythrocyte (EC.No 3.1.1.7), hBChE from human serum (EC No. 3.1.1.8), acetylthiocholine iodide (ATCI, CAS No. 1866-15-5), butyryl thiocholine iodide (BTCI, CAS No. 1866-16-6), and Ellman's reagent, (5,5'-dithiobis-2-nitrobenzoic acid, DTNB CAS No.69-78-3) were procured from Sigma Aldrich USA. Donepezil and Rivastigmine were used as a standard for hAChE and hBChE inhibition activity, respectively. The hAChE stock solution was prepared in 20 mM HEPES buffer pH 8.0 with Triton X-100 (0,1% v/v), similarly, the hBChE stock solution was prepared in aqueous gelatine (0.1% w/v) solution. The five inhibitor concentrations were prepared in DMSO (≤ 1% in the final concentration) showing at least 20-80% inhibitions. The assay method utilized consisted of the enzyme 50 μL (0.022 U/mL of AChE and 0.06 U/ml hBChE) incubated with 10 μL of the test compounds for 30 min in 96-well plates at room temperature followed by the addition of 30 μL of ATCI (1.5 mM) or BTCI (1.5 mM) for the respective experiments and incubated further for 30 min at room temperature. Finally, the DTNB (0.15 mM) solution 160 μL was added to each well, and after 30 seconds, the absorbance was measured at λ= 412 nm using a 96-well microplate reader (Synergy HT, Bio-Tek instruments, Inc.) for 6 min at 37 °C. The

blank was also prepared by mixing all the components except the enzyme to measure the non-enzymatic hydrolysis of the substrate. The assay was performed in triplicates while each experiment was repeated 2-3 times. The percentage inhibition for each compound was calculated using the equation: [(Ac-Ai)/Ac] × 100, where Ai is the absorbance for hAChE and hBChE in the presence of inhibitor and Ac is the absorbance for hAChE and hBChE in the absence of inhibitor.

4.4.1.2 Enzyme Kinetics study

The Enzyme Kinetics study was performed to evaluate the type of inhibition (hAChE and hBChE) by the compounds. The study was performed with six varying concentrations (ranging from 60-500 μM) of substrates ATCI and BTCI (for AChE and BChE respectively) to that of three varying inhibitor concentrations (0.030, 0.150, and 0.300 μM for hAChE and 0.100, 0.200 and 0.300 μM for hBChE). All three inhibitor concentrations were evaluated against six varying concentrations of substrates and plotted as Lineweaver-Burk double reciprocal plots and reaction rates were prepared to determine the type of hAChE and hBChE inhibition. The Dixon plots for each respective enzyme inhibitor were also generated to determine the dissociation constant (K_i) value. The assay was performed in triplicates [Dixon 1972].

4.4.1.3 BACE-1 Inhibition study

In vitro, BACE-1 inhibition studies of the compounds were evaluated through the FRET-based fluorometric assay method [Sharma et al. 2019a, Sharma et al. 2019b]. The assay was performed on the BACE-1 detection kit (CS00100, Sigma, USA) as per the supplier's given protocol which was based on the BACE-1-derived substrate cleavage that results in increased fluorescence intensity. The assay solution consisted of the substrate (20 μL), fluorescent assay buffer (78 μL), and inhibitor (2 μL). To this solution, BACE-1 enzyme was added and the fluorescence was measured immediately at λ_{ex}= 320 nm and λ_{em}= 405 nm (as excitation and emission wavelengths respectively).

The positive and negative controls were also prepared with and without enzymes and their fluorescence was recorded in the absence of the inhibitors. The negative control was considered as a blank while the positive control and inhibitor were used to determine the percentage inhibition using their fluorescence intensity. The Percentage inhibition was calculated using the formula: 100- ($F_i/F_o \times 100$) where F_i and F_o are the fluorescence intensity with and without inhibitor respectively. The experiment was performed in triplicate and the corresponding IC_{50} values of each compound were determined using GraphPad Prism 5.01 software.

4.4.1.4 Propidium Iodide displacement assay

The ability of the compounds to displace the PI from the *h*AChE binding site was determined through the PI-displacement assay [Peauger et al. 2017]. The *h*AChE (5U) was incubated without and with inhibitor molecules (5 μM, 10 μM, and 20 μM) for 6 h at 25 °C and PI was added. The Fluorescent intensity was measured immediately after the PI addition at λ_{ex}= 535 nm and λ_{em}= 595 nm (as excitation and emission wavelengths respectively). The assay was conducted in triplicates and the % PI displacement by the inhibitors was compared with the change in fluorescent intensity to that of the control (without inhibitor).

4.4.1.5 Parallel artificial membrane permeation (PAMPA) assay

The reported method by Di et al. was adopted to predict the brain permeability of the compounds [Di et al. 2003]. The assay method involves the partitioning of the inhibitor from a donor plate to an acceptor microplate via a membrane filter coated with porcine brain lipid (4 μL, 20 mg/ml dodecane). Both the acceptor and donor microplates were filled with pH 7.4, PBS: EtOH, (7:3 v/v, 200 μL) following the addition of test solution 200 μL (25 μg/ml of the compound to be tested in PBS: EtOH, 7:3 v/v). The acceptor and donor microplate (0.45 μM Merck Millipore) were sandwiched together and incubated for 18 h at 25 °C [Tsinman et al. 2011]. The experiment was conducted in

triplicates and the amount of the test sample increased in the acceptor plate due to diffusion was measured spectrophotometrically on a 96-well microplate plate reader (Synergy HT, Bio-Tek instruments, Inc.).

4.4.1.6 Neuroprotective studies on SH-SY5Y cell line

4.4.1.6.1 Differentiation of SH-SY5Y cell lines

SH-SY5Y cells were differentiated in 6 days into neuron-like cells using the protocol given by Forster et al. During the first phase of differentiation spanning 3 days, cells were exposed to 10 μM of Retinoic Acid (RA) present in Dulbecco's Modified Eagle Medium (DMEM). During the second phase, they were kept in a Neurobasal medium containing Brain-Derived Neurotrophic Factor (BDNF), N-2 supplement, and Glutamine [Singh et al. 2021, Xu et al. 2018].

4.4.1.6.2 MTT assay

Differentiated cells were seeded in a 96-well plate at a density of 10,000 cells per well. Cells were treated with different concentrations of the drug and standard in triplicate for 24 hours. 3- [4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was then added to each well at a working concentration of 0.5 mg/ml and the plate was kept in a CO_2 incubator for 3-4 hours. Media was removed from each well and DMSO was added. The plate was shaken for 30 seconds and absorbance was measured at 570 nm. Percentage viability at different concentrations was calculated relative to the Control group. A similar assay procedure was followed for $A\beta_{1-42}$ cell rescue estimation where the differentiated SH-SY5Y cells were incubated with the $A\beta_{1-42}$ (10 μM) in a 6-well plate for 24 h after which the culture medium was replaced with the medium containing donepezil, compounds (20 μM) and incubated further for 48 and 72 h. MTT assay was performed and the cell viability was determined.

4.4.1.6.3 Effect of drug and standard on the morphology of differentiated SH-SY5Y cells

SH-SY5Y cells in 3 T25 flasks were differentiated into neuron-like cells. After differentiation, cells in the first flask were treated with 20 μM drugs, and cells in the second flask were treated with 20 μM of standard. Cells in the third flask served as controls. After 24 hours of treatment, the morphology of cells in different groups was observed using an Inverted tissue culture microscope. Images were taken at 10X and 20X magnifications using a Sony DSC-W510 Camera.

4.4.1.7 Anti – Aβ aggregation activity

4.4.1.7.1 Thioflavin-T assay

The self and hAChE-induced anti-aggregation potential of compounds were evaluated by thioflavin T assay [Bolognesi et al. 2007, Choubey et al. 2021, Zha et al. 2016]. A 1 mM stock solution of Aβ_{1-42} protein fragment (Caymann, USA) was prepared in DMSO (Sigma, India). Further, the desired concentration of 25 μL was prepared in PBS pH 7.4 from the stock solution. Compounds were prepared in DMSO and PBS pH 7.4 (DMSO $\leq$ 1% w/v). Aβ and inhibitors were evaluated at three different ratios (10:5, 10:10, and 10:20 μM, respectively) in triplicate experiments.

The self-induced Aβ aggregation assay was performed in the presence and absence of the inhibitors (5 μM, 10 μM, and 20 μM concentration) with Aβ (1o μM) in PBS buffer pH 7.4. The mixture was incubated for 48 h at 37 °C and then added thioflavin T (50 μM) in Glycine NaOH buffer pH 8.0. Finally, the fluorescence intensity was measured at λ_{ex}= 450 nm and λ_{em}= 485 nm as excitation and emission wavelength respectively. The equations: [100-($F_i/F_o \times 100$)]; and NFI = F_i/F_o (F_i & F_o fluorescence intensity in the absence and presence of inhibitors) were used to estimate the anti-Aβ aggregation potential in terms of percentage inhibition.

The hAChE-induced Aβ aggregation was measured in the presence and absence of the inhibitor molecules (5 μM, 10 μM, and 20 μM concentration) along with hAChE and A$\beta_{1\text{-}42}$ (1:100) in PBS pH 7.4. The mixture was incubated at 37 °C for 48 h. The thioflavin T (5 μM) prepared in 50 nM glycine NaOH buffer pH 8.0 was added and the fluorescence was measured at λ_{ex}= 450 nm and λ_{em}= 485nm as excitation and emission wavelength respectively. The NFI and percentage inhibition were determined similar to that of the self-induced experiment.

4.4.1.7.2 Confocal Microscopy

Confocal fluorescence imaging was conducted to observe the fluorescence effect of ThT on the formation of aggregates in the presence of compounds **[Gutti et al. 2019b]**. Following incubation of the compounds with Aβ aggregates for 48 h the fluorescent dye (ThT) was added and the sample (blank, Aβ 0h, Aβ 48h (aggregates), Aβ+compound 0h, Aβ+compound 24h, Aβ+compound 48h) was mounted on glass slides using 1,4-diaza- 66icycle[2.2.2]octane (DABCO; Sigma) as the fixing agent and fluorescence images were captured on FITC fluorescent filter cube at 40X magnification using λ= 494 nm and λ= 518 nm as excitation and emission wavelengths respectively.

4.4.1.7.3 AFM study

Compounds were evaluated for anti-aggregation potential with and without inhibitor and were performed on NT-MDT Ntegra prima. Compounds (40 μL, 10μM of each) along with Aβ (40 μL, 10μ) in PBS (0.1 M, pH 7.4) were incubated for 10 days at 37 °C. A 5-10 μL sample was spread on the surface of a clean mica sheet using a spin coater (302 × g 60s) and by drying (1207 × g, 30s) the excess solvent was removed. The samples were analyzed at different time intervals (0, 3, 5, 7, and 10 days) at ambient instrument conditions as probe spring constant (3.5N/m), cantilever operating resonance frequency (140 kHz), and mode-tapping. The images were captured at a 0.5 Hz

scanning rate using Nova Px (NT-MDT, Russia) [Harte et al. 2015, Ryu et al. 2008, Sharma et al. 2019a].

4.4.1.7.4 SEM analysis

Scanning electron microscopy was performed to investigate the surface morphology/microstructures of A$\beta_{1\text{-}42}$ monomers, A$\beta_{1\text{-}42}$ oligomers, and the anti-A$\beta_{1\text{-}42}$ aggregation effects of compounds. The SEM images were captured on the VO - Scanning Electron Microscope MA15 / 18 (Carl ZEISS microscopy LTD, USA) at 20 kV of voltage and magnification of 50 KX. The samples (blank, Aβ aggregates, Aβ + compounds after 48h incubation) were studied by taking 5 μL samples (diluted with PBS) and dried overnight. The glass slides (1×1 cm) coated with samples (Au sputter coating) were further mounted on an aluminum tub with carbon adhesive and coated with Au before being subjected to final imaging [Spitzer et al. 2016].

4.4.2 In-vivo behavioral and ex-vivo studies

4.4.2.1 Animals

The Wistar rats of both the sex and Swiss albino mice (male) were obtained from the central animal facility of the Institute and were kept for 7 days for acclimatization in the institute animal house facility (12:12 h dark/light cycle at 25 ± 2 °C) at the Department of Pharmaceutical Engineering and Technology, IIT-BHU. All the animals were supplied with tap water and commercial food unless otherwise stated. After acclimatization, the animal experiments were performed in accordance with the CPCSEA guidelines, Ministry of Environment Govt. of India, Forest and climate change Govt. of India, and as approved by the central Animal Ethical Committee of the Institute (IIT-BHU (IT(BHU/MAEC2021/001 and Dean/2017/CAEC/93).

4.4.2.2 Acute toxicity studies

Compounds were selected after in vitro findings were evaluated for oral acute toxicity studies on Swiss albino mice (30 ± 2 gm) as per the OECD-423 guidelines. The animals

were segregated into six groups by the random distribution of eight animals in each group. The suspension of the compounds in 0.3 % Na-CMC was administered orally in the animals in a graded dose of (500 mg/kg) of different groups assigned for the study [Shrivastava et al. 2018, Tripathi et al. 2019c]. After administration of the compounds, all the animals were carefully watched for 6 h. for any abnormalities such as seizer, tremors, convulsion, salivation, diarrhea, sleep, etc. The animals were also observed for the next 24 h to 14 days for any abnormalities to occur. After 14 days, the animals were sacrificed and different organs such as kidneys, liver, and heart were examined microscopically for any organ or tissue damage.

4.4.2.3 Scopolamine-induced amnesia models for testing cognition enhancement in rat/mice

4.4.2.3.1 Scopolamine induced Y-maze test

The scopolamine hydrobromide in normal saline was administered intraperitoneally to each treated group after the 7^{th} day of the treatment except the control group. The improvement in instant and short-term memory was analyzed through the Y-maze test. The maze was comprised of three arms of Y-shaped. The rats were independently positioned in the maze's middle. The initial entry was eliminated from the calculations because the rats would frequently enter the arm of facing. The animals were observed carefully for about 8 min for total arm entries and also watched for spontaneous alternations [Wolf et al. 2016]. The results of the Y-maze experiment are expressed using the following equation, percent spontaneous alteration = [number of spontaneous alternations / (total arm entries-2)] ×100 has been utilized for memory improvement score [Jin et al. 2014].

4.4.2.3.2 Elevated Plus maze test

The elevated plus maze test was performed to determine the transfer latency of the animals in different groups [Uniyal et al. 2021]. After 30 min. of the dosing (2.5 mg, 5

mg & 10 mg/kg) to animals on the first-day trial, scopolamine hydrobromide (1 mg/kg) was given intraperitoneally to the animals of all groups except the control group. After 5 min. the experiment was initiated in the maze with two open and two closed arms. The animals (mice) from each group were placed on one end of the open arm facing away from the closed and central platform. The induced time latency of the animals was determined as the time taken by the animals to reach either of the closed arms. The animals were trained with one trial every day and the experiment was conducted for up to three days. The average transfer latency for three days was calculated as the time spent by the animals on both arms (open & closed) by individual animals.

4.4.2.4 Ex Vivo and biochemical analysis in scopolamine-induced model

After In vivo behavioral studies, animals were sacrificed through cervical dislocation from each respective group. The brains were isolated from the skull and the hippocampus and cortex region of the brain were isolated carefully and homogenized with a glass homogenizer and centrifuged (10000 × g, 25 min) at 4 °C in ice-cold PBS (0.1 M, pH 7.4). The supernatants were collected and tested initially for protein concentration using a standard Bradford assay and also used for several biochemical estimations.

The brain hippocampal and cortex supernatant were also examined for the AChE levels by the modified Ellman's colorimetric assay method. The supernatant (50 μL) was initially incubated with ATCI (15 mM of 30 μL) for 30 min at 37 °C followed by the addition of 1.5 mM DTNB (160 μL) and the reaction rates were determined immediately at λ= 412 nm for 6 min at 37 °C. The AChE level was determined as μM of substrate hydrolyzed /min/mg protein.

Biochemical estimation of oxidative stress biomarkers malonaldehyde (MDA) was estimated in the supernatant of the hippocampal and cortex brain. A lipid peroxidation assay (TBRAS assay) was performed to estimate the MDA levels. The formation of

red-colored TBARS as a result of the reaction of thiobarbituric acid with MDA under acetic conditions. The red-colored formed can be detected spectrophotometrically on its corresponding wavelength. The method briefly involves the mixture of trichloroacetic acid (10% v/v in 0.1M HCl, 1 mL) with each supernatant sample (200 μL) followed by centrifugation (10 min, 101 × g) at 4 °C. After centrifugation thiobarbituric acid (0.67%) and the supernatant was mixed in equal volumes and was warmed up to 10 min. After cooling the sample, the absorbance was estimated at λ= 532 on a 96-well microplate reader and the number of moles of MDA/mg protein was calculated as the final result.

The estimation of the ACh in brain homogenate was performed as per the manufacturer's instruction (Krishgen Biosystems, Rat Acetylcholine, ACH GENLISA ELISA) on an ELISA kit, and the amount of ACh was expressed as pg/ml.

The nitrite level in brain homogenates was estimated using the Griess reagent. The Greiss reagent used was prepared freshly by adding 5mL of 0.1% N-(1-Napthyl)ethylenediamine dihydrochloride (NEDD) and 5 mL of 1% sulfanilic acid in 2.5% concentrated phosphoric acid (H_3PO_4), in distilled water. The supernatant (50 μL) was taken into 96-well plates and to it added freshly prepared Greiss reagent (250 μL) and absorbance was taken on a 96-well microplate reader at λ= 546 nm. The Sodium nitrite standard curve was used to determine the nitrite level as mg/mL in protein.

The SOD assay was performed to assess the antioxidant potential of the compound. The assay performed was based on the formation of blue color formazan as a result of autooxidation of hydroxylamine hydrochloride (pH 10.2) by SOD (free radical dismutation enzyme) in the presence of EDTA. The equal proportions (50 µl) of each the brain homogenate (hippocampal) and the hydroxylamine hydrochloride were added to a mixture containing EDTA (100 µM), Na_2CO_3 (50 mM), and the nitro blue tetrazolium (24 µM). After addition, the absorbance was recorded at λ = 560 nm

wavelength, and results are represented as SOD units (U/mL)/min/mg of protein of three independent experiments [Tripathi et al. 2020a].

Reduced glutathione assay (GSH) was conducted by mixing 100 µl of supernatant from hippocampal brain tissue homogenate and 1 ml of sulfosalicylic acid 4% w/v. The precipitate formed after mixing was kept at a temperature of 2–8 °C in a refrigerator for 1 h. The cold precipitate was centrifuged after 1 h in a cold centrifuge (1200g for 15 min at 4 °C). After centrifugation pellet obtained was discarded and the supernatant was utilized further in the next step. The supernatant 100 µl was diluted with 2.7 ml of 0.1 M PBS (pH 8) and to this 200 µl of 0.1 M DTNB (Ellman's reagent) solution was added which resulted in a pale yellow color solution and the absorbance was recorded at λ = 412 nm on a multimode microplate reader (Synergy HT, Bio-Tek Instruments, Inc., USA). Furthermore, by using the molar extinct coefficient 1.36×104 M^{-1} cm^{-1} the calculation was performed and expressed as nmol GSH per mg of protein [Jollow et al. 1974].

4.4.2.5 qRT-PCR analysis of proinflammatory cytokines (TNF-α and IL-β) in scopolamine-induced model

The mRNA expression of proinflammatory cytokines (TNF-α and IL-1β) was estimated using the qRT-PCR technique [Uniyal et al. 2022b]. The RNA was isolated from the hippocampus and cortex region of the brain through the triazole reagent method and quantified using Nanodrop (Thermo) and a cDNA synthesis kit (K1622 Thermo Scientific) was used to prepare the corresponding cDNA primer sequence for TNFα and IL-1β as shown in Table S 4. The qPCR analysis was performed on Rotor-gene Q (Qiagen, Germany) using master mix (15 μL) prepared by mixing of forward primer, reverse primer, Maxima SYBER Green/Fluorescein qPCR Master mix (K0241), nuclease-free water (SigmaW4502) and template DNA [Uniyal et al. 2022a].

4.4.2.6 Aβ-induced Morris Water Maze test

In the $A\beta_{1-42}$ induced ICV rat/mice model the four groups of animals (control, sham, donepezil, and compound) having 6 animals in each group, were used. The sterile 0.9% NaCl solution was used as the vehicle and also used to dissolve the $A\beta_{1-42}$ (Sigma Aldrich, India). The ketamine (90 mg/kg, i.p.) in combination with the xylazine (9 mg/kg, i.p.) cocktail was used for anesthesia. The rats/mice, post-anesthesia, were kept on the stereotaxic equipment after cleaning their scalp with iodine solution, and saline and ear bars were set symmetrically. The skull of the rat brain was drilled using stereotaxic coordinates bregma (-0.5 mm anteroposterior, -3.2 mm dorsoventral with an incision bar set at -3.3 mm and +1.2 mm mediolateral) while, stereotaxic coordinates bregma (-1.0 ± 0.06 mm posterior, 1.8 ± 0.1 mm lateral to the sagittal suture and 2.4 mm dorsoventral) to put a hole in animal brain [Colaianna et al. 2010, Kim et al. 2016]. With the exception of the sham group, all the rats received $A\beta_{1-42}$ (4 μM, 5 μl) injections with a 2 μl/min infusion rate using a Hamilton microsyringe. However, the sham group received only a vehicle. Following a seven-day period of postoperative recuperation, donepezil (5 mg/kg) and compound (10mg/kg) was given orally to each respective group of animals on the eighth day for a total of nine consecutive days i.e., up to the sixteenth day. During the last five days of treatment (12-16th days), the Morris water maze test was performed to determine learning and memory improvements in the experimental animals. The Morris water maze comprised of a circular pool (diameter 121 cm, depth 32 cm, and height 62 cm) filled with water (25 ± 2 °C). The pool was made opaque using TiO_2 to hide the platform 2 cm under the water surface and divided into four equal quadrants. The number of platform crossings and the escape latency time was determined for a period of 90 s, two trials every day with a minimum of 3 h difference. [Morris 1984]

4.4.2.7 Western Blot Analysis

The animals from each respective group were sacrificed after the A*β*-induced Morris water maze test and their brains from skulls were isolated carefully. The isolated brains were homogenized in radioimmunoprecipitation (RIPA) assay buffer supplemented with phosphate and protease inhibitors. The brain homogenate was sonicated and centrifuged at 4 °C (10062 ×g, 20 min) followed by Bradford assay to estimate the protein content. The equal protein content was isolated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein was transferred to polyvinylidene fluoride (PVDF). The membrane was blocked with 8% milk for 2 h and incubated with anti-*β*-actin (1: 500), anti-A*β* (1:1000), APP/A*β* (1:1000), BACE-1 (1:500), tau (1: 1000) and primary antibodies (Alzheimer's disease antibody sampler kit#9784, Cell signaling technology, USA) at 4 °C overnight. The membrane was rinsed with tris-buffered saline/0.01% Tween 20 (TBST) and anti-mouse horseradish peroxide-linked secondary antibodies (CST) were incubated with the membrane for 2 hours at room temperature. after being. The immune complex was detected using an enhanced chemiluminescence substrate (Biorad) and the blots were visualized using ChemiDoc^{TM} (Biorad, Hercules, USA). Finally, quantification of the blots was performed on image *J* software.

4.4.2.8 Immunohistochemistry

The experimental animals of each group were sacrificed and perfused intracardially through an ice-cold saline (pH 7.2) and ice-cold paraformaldehyde (4% v/v). The brains were removed and kept in a 10% formaldehyde solution overnight followed by a different sucrose solution for dehydration and cryopreservation. The hippocampal brain of experimental animals was cut coronally from each group via cryo-microtome (Leica, Wetzlar, Germany) into 20 μ*m* thick sections. Using PBS containing 10% goat serum, 1% BSA, and 0.3% Triton-X in PBST the nonspecific binding sites were blocked. Each

brain hippocampal section was incubated with primary antibodies (anti-BACE-1(1:500) and anti-Aβ (1:1000)) for 18 h at 4 °C followed by washing with PBS and 1% BSA-PBS solutions twice to remove unbounded anti-BACE-1 and anti-Aβ primary antibodies. Finally, the sections were incubated with FITC-conjugated secondary antibodies and incubated further for 1 h at room temperature. The sections were further washed with PBS three times and fixed on the slides using DABCO (Fluka analytical) antifading and poly(vinyl alcohol) as mounting medium. The images of each slide were captured on a Confocal/fluorescent microscope (Carl Zeiss Microscopy, GMBH) and the quantitation was done using ImageJ software (NIH United States) [Sharma et al. 2019b].

4.4.2.9 Brain tissue histopathology

The transcardial perfusion in the Aβ-induced mice was performed with precooled PBS pH 7.4 followed by 4% formaldehyde (in 0.1 mol/L PBS). The isolated brains were transferred to a container having 10% sucrose solution followed by 20% and 40% sucrose solution (24 h with each sucrose solution) for complete dehydration. The brain blocks were prepared using optimal cutting temperature (O.C.T.) embedding medium solution (Fisher Healthcare, 4585, USA), and the sectioning was done on a freezing microtome (Leica Microsystems, Germany). The 15 μM thick sections were collected in 0.01 mol/L PBS. The free-floating brain sections were mounted with poly lysine-coated glass slides washed thrice, and Nissl's staining (0.126 cresyl violet) was applied. After staining the sections were washed with alcohol (70%, 95%, and 100%) followed by xylene thrice. Using a light microscope (MLXi-TR plus from Olympus) the images were captured and the neurons population was counted (neuronal density, Nissl's stained violet neurons) using Image J software and expressed as % of control mice [Mishra and Krishnamurthy 2020].