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 modelling and Phase Screening

The structure-based drug design approach was used and the e-pharmacophore models of hAChE (PDB: 4EY7) and hBACE-1 (PDB: 2ZJM) proteins were created [Cheung et al. 2012, Godemann et al. 2009, Sharma et al. 2019b]. On the basis of their co-crystallized ligands having N-benzylpiperidine nucleus, these proteins were chosen. Briefly, both the protein structures were preprocessed, optimized, and minimized using the Protein Preparation Wizard, PROPKA pH 7.0, and an OPLS3 force field, respectively to add missing hydrogens atoms, assigning bond orders, and filling missing side chains and loops. These prepared proteins were further utilized to create their respective e-pharmacophore models of hAChE and hBACE-1. 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. These pharmacophoric models were utilized for the Phase screening of the ZINC15 database (accessed at https://zinc.docking.org in January 2020) containing 193889 which yielded 1000 hits. The details of the pharmacophore overlay of the identified hit (**ZINC000015441499**) and most promising compound **AV-2** were also discussed.

4.1.2. Virtual Screening and Docking post processing

The Phase module of Schrödinger Maestro suite 2018 was adopted for Virtual screening workflow (vsw), which consists of three steps high throughput virtual screening (HTVS), standard precision (SP), and extra precision (XP) and was set at the filtration criteria of 50%, 50%, and 25% respectively. The potential hits after vsw for AChE and BACE-1 individually were further subjected to pose filtration using DPP function.

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. Two significant binding sites like PAS (Tyr72, Asp74, Tyr124, Trp286, and Tyr341) and CAS (Ser203, Glu334, and His447) were mainly considered for hAChE inhibitory activity while Asp32 and Asp228 dyad residues at the N- and C-terminus interface were considered for hBACE-1 inhibitory activity. 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 prime MM/GBSA module of Schrödinger 2018-1 was used to calculate the binding free energy (ΔG_{bind}) of docked ligand-protein complexes i.e. compound-hAChE/hBACE-1/$A\beta_{42}$ complexes were estimated and compared with the standards [Hou et al. 2011]. This ΔG_{bind} and ΔE were estimated based on the equations given ($\Delta G_{bind} = \Delta E_{MM} + \Delta G_{sol} - T\Delta S$) ($\Delta E_{MM} = E_{complex} - E_{protein} - E_{ligand}$). The ΔG_{sol} represents the binding energy associated with the difference in the GBSA and non-polar solvation energy (determined by solvent-accessible surface area) while $T\Delta S$ represents the temperature and change in entropy. The ΔG_{bind} is expressed in Kcal/mol.

4.1.4. Molecular Docking Studies

The X-ray crystal structures of hAChE (4EY7), hBACE-1 (2ZJM), and hAβ (2BEG) were molecularly docked by compounds using the "Glide" module of Schrödinger Maestro 11.2. [182]. Initially, these crystal structures of the proteins were preprocessed, refined, and corrected by using the Protein Preparation Wizard module Schrödinger Maestro 11.2 [183]. The Propka method (at pH 7) was used to optimize each protein structure, and restrained minimization was applied to heavy atoms with RMSDs of less than 0.30 Å. The co-crystallized ligand's active site was enclosed by the grid box (10×10×10 $Å^3$). The co-crystallized ligand was removed and re-docked into the appropriate protein grids of hAChE, and hBACE-1 to validate the docking techniques and produced grids. However, due to the non-availability of co-crystallized ligands in the 3D NMR crystal structure of protofibril $Aβ_{42}$ (PDB: 2BEG), potential binding sites were identified and explored using the default settings in Schrodinger's SiteMap tool [Halgren 2007, Halgren 2009]. Based on the SiteScore and Dscore of all the binding sites, the best binding site was identified and selected for further molecular modeling studies like molecular docking and dynamic simulation studies [Halgren 2009]. The grid was constructed around the favorable binding location. The "LigPrep" module and the "Glide XP" module have been utilized to prepare the ligands and to conduct docking tests, respectively [182, 186]. The docking scores and binding profile were viewed using the "Glide XP visualizer" program.

4.1.5. Molecular Dynamics and Simulation studies

MD simulations were used to confirm the stability of docked compounds-hAChE/hBACE-1/hAβ complexes by using Desmond [187]. Around the ligand-protein docked complex, a

virtual environment system of TIP3P water was created and counter ions were introduced to the system to neutralize it by using the system builder module. With 2000 maximum iterations, these systems were reduced at the convergence criterion of 1.0 kcal/mol/Å. Each docked complex underwent an MD simulation run for 100 ns while maintaining a trajectory with their default settings to collect about 1000 frames.

4.1.6. ADME drug-likeness analysis

The QikProp module of Schrödinger Maestro was used to identify the drug-likeliness characteristics of the compounds and standard donepezil [Lipinski 2004]. Several descriptors like Lipinski's rule of five, (mol MW < 500, donorHB < 5, accptHB ≤ 10, QPlog Po/w < 5), the other expected parameters like QPlogPo/w (2.0-6.5), QPlogBB (3.0-1.2), and SASA (300–1000) of compounds were predicted and compared them with donepezil.

4.1.7. DFT-based computational studies

Quantum chemical analysis of compounds was performed using DFT based Jaguar module of Maestro 11.2 [190]. The molecular orbital structures, optimization, and electronic density were determined using the B3LYP/6-31G theory of Jaguar single-point energy. The FMO studies were carried out to estimate the HOMO/LUMO energy levels and their gaps. Based on these energy gaps, various global reactivity parameters like ionization potential (I), electron affinity (A), hardness (η), electronegativity (X), electrophilicity (ω), chemical potential (μ), and global softness (σ), were also determined. The electron affinity (A) and ionization potential (I) of compounds were estimated using equations like $A = -E_{LUMO}$, and $I = -E_{HOMO}$. The η and X can be estimated using equations like $\eta = I-A/2$ and $X = I+A/2$. The μ, ω, and σ can be estimated using following equations: $\mu = E_{HOMO} + E_{LUMO}/2$, $\omega = \mu^2/2\eta$,

and σ = 1/2η. The electrostatic surface potential was also generated to elucidate the nucleophilic/electrophilic properties of compounds. DFT-based Fukui function calculations were executed to predict the probable reactive sites of compounds where the probability of electrophilic and nucleophilic attacks takes place. These functions can be represented as $f^+j = qj(N) - qj(N-1)$ and $f^-j = qj(N+1) - qj(N)$, where qj = atomic charge (NBO) at the j^{th} atomic site and the $(N-1)$, (N), and $(N+1)$ were electronic surrounding the compound at cationic, neutral, and anionic states, respectively. The f^+j and f^-j represent the gain and loss of an electron, respectively.[Dlala et al. 2021] Moreover, the pK_a values of all the nitrogen atoms of compounds were predicted Jaguar pK_a module of Maestro 11.2 [Schmidt am Busch and Knapp 2004].

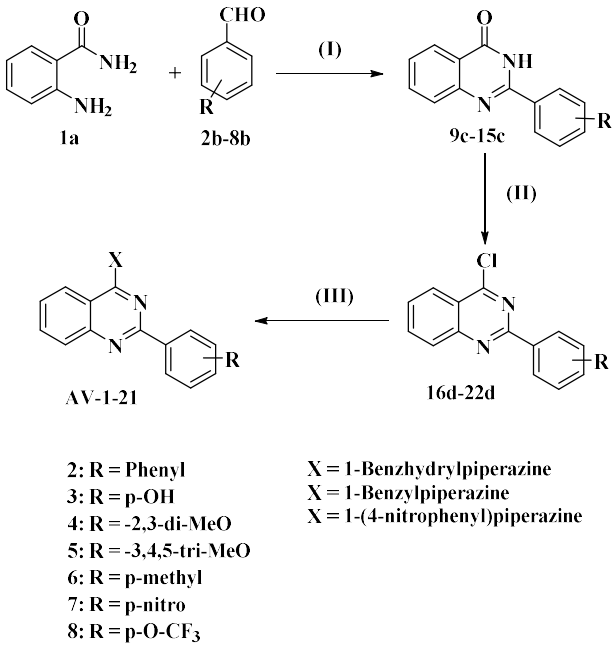
4.2. Synthesis

4.2.1. Chemicals and reagents

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

4.2.2. Synthesis of Series I (AV-1 to AV-21)

The compounds **AV-1** to **AV-21** were synthesized as per the reaction sequence presented in Scheme 1.



Scheme 1. Synthesis of target compounds **AV-1 to AV-21**. **Reagents and conditions:** (I) Substitute aldehydes, Iodine, methanol, 80°C, 4-6 h; (II) $SOCl_2$, dry DCM, DMF, 40°C, 5-6 h; (III) K_2CO_3, dry DMF, 80°C, 4-5 h.

4.2.2.1. General procedure for the synthesis of intermediate 9c-15c

To get the desired product (**9c-15c**), Anthranilamide (1eq.) was reacted and cyclized with substituted aldehyde (1eq.) in presence of iodine (1eq.) which acts as an oxidative catalyst and methanol (10 mL) as a solvent. The reaction mixture was agitated for 4 to 6 h at 80 °C. TLC was used to track the completion of the reaction. The excess solvent from the reaction was evaporated from the mixture under a vacuum using a rotary evaporator once the reaction was completed. The reaction mixture was poured with chilled water, and the resulting precipitates were filtered and dried [Dwivedi et al. 2022]. The compounds (**9c-15c**) were experimentally characterized.

4.2.2.2. General procedure for the synthesis of16d-22d

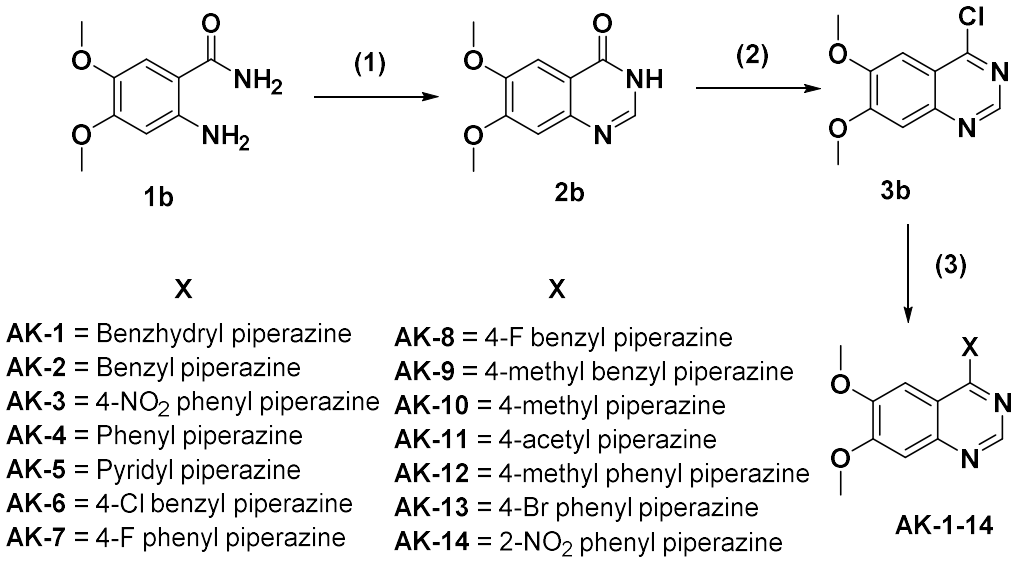
The compounds **9c-15c** (differently substituted quinazolinones) was dissolved in dichloromethane (DCM). The 1.2 eq. thionylchloride ($SOCl_2$) and N, N-dimethylformamide (DMF) were added dropwise in ice-cold conditions to reaction mixture and refluxed at 40 °C for 5 to 6 h. TLC was used to monitor the progress of the reaction [Chakravarty et al. 2002]. After reaction completion, ice was added to the reaction mixture, neutralized by sodium bicarbonate, extracted with chloroform (25 mL × 3), washed with brine, and dried over anhydrous sodium sulfate [Xu and Russu 2013]. The obtained organic layer was subsequently concentrated under a vacuum using a rotatory evaporator to obtain the desired intermediates **16d-22d** [Romer 2009]. All these intermediates **16d-22d** were experimentally characterized well using ^{1}H and ^{13}C NMR spectroscopy.

4.2.2.3. General procedure for the synthesis of AV-1 to AV-21

In a 50 mL reaction vial, intermediates **16d–22d,** K_2CO_3 (2 e.q.), and substituted piperazines (1 e.q.) were dissolved in DMF. The reaction mixture was then heated at 80 °C for 4 h. The progress of the reaction was monitored using TLC [Goldfarb 2014]. After reaction completion, 5 mL of water was put into the mixture, which was then extracted with ethyl acetate (15 mL × 3), washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum using a rotatory evaporator. To obtain the pure products (**AV-1 to AV-21**), the crude product was refined further using column chromatography [Dwivedi et al. 2022].

4.2.3. Synthesis of Series II (AK-1 to AK-14)

The compounds **AK-1** to **AK-14** were synthesized as per the reaction sequence presented in Scheme 2.



Scheme 2. Synthesis of designed derivatives **AK-1** to **AK-14**. **Reagents and conditions:** (1) I_2, CH_3OH, 80°C, 3-5 h; (2) $SOCl_2$, DCM, DMF, 40°C, 5-6 h; (3) Substituted piperazines, K_2CO_3, DMF, 80°C, 3-4 h.

4.2.3.1. General procedure for the synthesis of 2b

Substituted aldehyde (1eq.) was reacted with anthranilamide (1eq.), iodine (1eq.), a catalyst for oxidation, and CH_3OH (10 mL) as a solvent. Up to 3-5 h, the reaction mixture was stirred at 80 °C. To track the reaction completion, TLC was used. After the completion of the reaction, the surplus solvent was removed from the mixture using a rotary evaporator under a vacuum. On adding cooled water to the reaction mixture, the precipitates formed were filtered and dried [Dwivedi et al. 2022].

4.2.3.2. General procedure for the synthesis of 3b

Compound **1** was dissolved in DCM in an ice-cold condition, followed by the addition of thionyl chloride ($SOCl_2$) (1.3 eq), as well as a catalytic quantity of DMF and was refluxed for up to 6 h at 45^oC [Chakravarty et al. 2002]. The reaction completion was checked using TLC. Once the reaction was completed, the reaction mixture was neutralized with ice and $NaHCO_3$, extracted with $CHCl_3$ (30 mL). After extraction, the rotating evaporator was used to concentrate the resulting organic layer under a vacuum [Romer 2009, Xu and Russu 2013].

4.2.3.3. General procedure for the synthesis of AK-1 to AK-14

The intermediate **3**, K_2CO_3 (2.5 eq.), substituted piperazines (1 eq.), and DMF were mixed in RBF and heated at 80 °C for 4 h. The reaction's progression was tracked using TLC. 5 mL of water was added to the mixture after the reaction upon completion, and the reaction mixture was extracted with ethyl acetate (15 ml × 3) and concentrated on the rotatory evaporator. The product was further purified using column chromatography to get pure compounds **(AK-1** to **AK-14)** [Verma et al. 2024].

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. 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.

4.3.4. 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).

4.3.5. ^{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).

4.3.6. 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).

4.3.7. 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.

4.4. Pharmacology

4.4.1. In-vitro studies

4.4.1.1. Cholinesterase inhibition assay (hAChE and hBChE)

Cholinesterase preferentially catalyzes the hydrolytic cleavage of thiolated substrates to form thiocholine, which reacts with the 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) to provide a yellow product that can be identified spectrophotometrically at 412 nm [Kiran et al. 2023]. The Ellman's technique was used with slight modifications to measure the anti-cholinesterase potential of designed compounds spectrometrically [Ellman et al. 1961]. The stock solution of hAChE (≥ 500 units/mg protein, EC No. 3.1.1.7 from human erythrocytes) was procured from Sigma Aldrich and prepared in 20 mM HEPES buffer pH 8, 0.1% Triton X-100. Similarly, the stock solution of hBChE (vial of ≥ 4 units, EC No. 3.1.1.8) (human serum) was obtained from Sigma Aldrich and prepared in 0.1% aqueous solution of gelatin to

obtain the 0.22 and 0.6 U/mL enzyme solutions, respectively. The donepezil and rivastigmine were taken as standard compounds. The stock solutions of designed and reference compounds were initially prepared in DMSO, and five escalating concentrations of inhibitors and standards having less than 1% final concentration of DMSO were used, which were able to cause 20-80% of enzymatic inhibition. Briefly, 50 µL of hAChE or hBChE and 10 µL of the test or standard compound were incubated in 96-well plates at rt for 30 min. After incubation, 30 µL of the substrate (ATCI: 1.5 mM and BTCI: 15 mM) was added and the solution was further incubated for an additional 30 min at RT. Finally, 160 µL of DTNB (for hAChE: 0.15 mM and hBChE: 1.5 mM) was added to it and after 30 s the absorbance was measured at 412 nm wavelength using a 96 well with Multimode Microplate Reader (BioTek Synergy H1M, USA). To determine the rate of non-enzymatic reaction, the blank readings were also taken by analyzing the solution without hAChE or hBChE. The response rates were compared in the presence or absence of inhibitors and the percentage of inhibition resulting from each compound tested were estimated by the following expression: $[V_0\text{-}V_i/V_0] \times 100$. The V_i and V_0 are representing the reaction rates in the presence or absence of inhibitors, respectively. The experiment was carried out in triplicate, and non-linear regression log inhibitor concentration vs normalized response curves was used to graphically determine the IC_{50} values of all the compounds examined (Graph Pad Prism 5.01) [Shrivastava et al. 2022].

4.4.1.2. Enzyme kinetics study

To ascertain the type of hAChE inhibition caused by the most promising compounds, an enzyme kinetics investigation was conducted [Choubey et al. 2021]. By measuring the enzyme activity at six different concentrations of ATCI (final concentration of 50–500 µM),

the kinetic parameters K_m and V_{max} were derived. Three different concentrations of compound (0.03, 0.15, and 0.30 μM) were employed and evaluated with six different concentrations of ATCI. The Michaelis-Menten non-linear regression kinetics was employed to determine the V_{max} and K_m. While Lineweaver-Burk plots demonstrated the type of enzyme inhibition using Graph Pad Prism 5.01 [Lineweaver and Burk 1934]. Further, the Dixon plot was used to determine the K_d value as a function of the three distinct inhibitor concentrations [Dixon 1972]. The enzyme kinetic assay was carried out in triplet.

4.4.1.3. Enzyme reversibility study

The enzyme reversibility study was conducted as per the protocol given in the reported literature [Kumar et al. 2018]. The AChE enzyme was incubated with the compound at concentrations of 10 and 100 times the IC_{50} for 30 min at 37 °C (negative control was performed without inhibitor). The samples were then diluted 100 times with the addition of ATCI substrate to reach final inhibitor concentrations of 0.1 and 1× IC_{50}, respectively. The residual AChE activities were assessed and expressed as mean ± SD (n = 3).

4.4.1.4. BACE-1 inhibition assay

Using the FRET-based BACE-1 activity detection kit, the hBACE-1 inhibitory potential of all the synthesized compounds was determined (Sigma Aldrich, Catalog No. CS0010). The general principle of the FRET BACE-1 assay is that at one end of the FRET protease substrate is a quenching acceptor, and the other is a fluorescence donor. Because of the intramolecular resonance energy transfer to the quenching group, the intrinsic fluorescence of the substrate is drastically decreased. The energy transfer is stopped by enzymatic cleavage, and the donor's complete quantum yield is recovered. The assay was carried out as per the manufacturer's instructions and involved the amplification of fluorescence signal as a result

of the substrate's cleavage by BACE-1. The kit contains fluorescent assay buffer, stop solution, assay standard, BACE-1 substrate, and hBACE-1 enzyme. Different inhibitor concentrations that can inhibit an enzyme between 20-80% were utilized. After the addition of BACE-1 enzyme to the inhibitor, the fluorescence intensity was measured at λ_{ex} = 320 nm and λ_{em} = 405 nm, respectively at zero time and after 2 h incubation at 37 °C using a Multimode Microplate Reader (BioTek Synergy H1M, USA). All the experiments were carried out in triplicate. The following expression $[(IF_o\text{-}IF_i)/IF_o] \times 100$ was utilized to analyze the percentage inhibition, where IF_i and IF_o are the fluorescence intensities obtained in presence or absence of inhibitors and the IC_{50} values were estimated *via* linear regression graph (GraphPad Prism 5.1) [Prostak and Barnea , Waiker et al. 2023b].

4.4.1.5. PAMPA-BBB assay

The PAMPA-BBB assay kit (PMBBB-096) (Krishgen Biosystems) was used to determine the *in-vitro* brain permeability of the compounds [Di et al. 2003]. The bottom porous filter disks of the acceptor microplates were coated with the dodecane containing 4 µL of porcine brain lipid (PBL) (20 mg/mL PBL in dodecane). The targeted compounds were initially solubilized in DMSO, then PBS pH 7.4 was incorporated to make the final concentration of 25 µg/mL. 200 µL of the final test solution and 200 µL of PBS pH 7.4 were placed in the acceptor and donor plates (Merck Millipore, membrane pore size 0.45 mm), respectively [Tsinman et al. 2011]. The donor plate was then placed on top of the acceptor plate and incubated for 18 h to allow the substance to diffuse from the donor to the acceptor well via the lipid membrane. After incubation, spectrophotometric analysis was used to quantify the drug concentrations in both plates were quantified *via* spectroscopically. The experiment was carried out in triplicates.

4.4.1.6. Propidium iodide displacement assay

The PI displacement assay can be used to detect the binding of a molecule to the PAS-AChE by competitively replacing the PI. The targeted compounds (final concentrations of 10 µM and 50 µM, 150 µL) were initially incubated with hAChE (5U) for 6 h at 25 °C. PI (final concentration 1 µM, 50 µL) was incorporated to the mixture after incubation. After 10 min, a fluorescence plate reader was used to measure the fluorescence intensity at λ_{ex} = 535 nm, the excitation wavelength, and λ_{em} = 595 nm, the emission wavelength, respectively (BioTek Synergy H1M). The formula used to determine the percentage inhibition is 100 - (IF_i/IF_0 X 100), where IF_i and IF_0 represent the fluorescence intensity in presence or absence of inhibitors, respectively. Each experiment was conducted in triplicates [Peauger et al. 2017].

4.4.1.7. Aβ inhibition-thioflavin T assay

The anti-Aβ aggregation inhibition of the most active compound was determined by the thioflavin T assay. The structure of thioflavin T consists of a hydrophobic end coupled to a more polar benzothiazole group that contains the polar N and S, and a dimethylamino group attached to a phenyl group. Thioflavin T molecules may form micelles in aqueous solution as a result of this combination of polar and hydrophobic areas, with the positively charged N pointing toward the solvent and hydrophobic interiors. The specific binding of these dyes to amyloid formations results from hydrogen bonds formed between the hydroxyl groups of amyloid structures and the thiazole nitrogen of the dye [Khurana et al. 2005].

The 1% v/v ammonium hydroxide solution was added to the $A\beta_{1-42}$ (cayman, India) to obtain the 1 mM stock solution, which was then kept at -80 °C. Initial dilution of the compound and standard was prepared in DMSO, and further dilutions in PBS pH 7.4. Thioflavin T assay was used to investigate different $A\beta_{1-42}$: inhibitor concentrations (10:5, 10:10, and 10:20

μM). All the experiments were carried out in triplicate [Bolognesi et al. 2007, Jiang et al. 2019, Zha et al. 2016].

For the $A\beta_{1-42}$ self-induced aggregation inhibition experiment, $A\beta_{1-42}$ (final dilution 10 μM, 10 μL) was combined with 50 mM PBS pH 7.4 and incubated at 37 °C for 3 days without inhibitor. Further, compounds and standard curcumin (5 μM, 10 μM, and 20 μM; 10 μL) was added to $A\beta_{1-42}$ on 3rd day and incubated at 37 °C for further 3 days. For blank tests, PBS pH 7.4 was used in place of $A\beta_{1-42}$ with or without an inhibitor. With the addition of 50 mM glycine-NaOH buffer (pH 8.0) containing 5 μM thioflavin T at the excitation and emission wavelengths of λ_{ex} = 450 nm and λ_{em} = 485 nm, respectively, the fluorescence intensities were measured. The following expression was used to calculate the percentage inhibition of self-induced aggregation: $100 - (IF_i/IF_0 \times 100)$, where IF_i and IF_0 are the fluorescence intensities with and without inhibitor after deducting the values from blank, respectively. Additionally, the outcomes were presented as NFI in comparison to the control.

For the AChE-induced $A\beta_{1-42}$ aggregation inhibition experiment, $A\beta_{1-42}$ (final dilution 10 μM, 10 μL) was combined with AChE (final concentration 0.22 U/mL, 10 μL) incubated at 37 °C for 3 days without inhibitor. Further, compounds and standard donepezil (5 μM, 10 μM, and 20 μM; 10 μL) was added to AChE-induced $A\beta_{1-42}$ on 3rd day and incubated at 37 °C for further 3 days. The addition of 50 mM glycine-NaOH buffer (pH 8.0) containing 5 μM thioflavin T and diluted up to a final volume of 200 μL. The calculation and detection procedures were carried out in the same way as the self-induced aggregation experiment.

4.4.1.8. Microscopic (AFM and confocal) studies

By using NT-MDT Ntegra Prima (Russia), the AFM analysis was used to monitor the Aβ aggregation inhibition. The $A\beta_{1-42}$ (final concentration 10 μM, 20 μl) was incubated for 3

days in the absence of an inhibitor at 37 °C in 0.1M PBS pH 7.4. On 3rd day, compounds was incorporated into it and further incubated at the same condition for 3 more days. Additionally, a spin coater (1000 rpm, 60 s) was used to apply 5 µl of the aforementioned mixture on a freshly cleaved mica surface before being dried to remove any leftover solvent (4000 rpm, 15 s). AFM investigations were carried out on samples at 0, 3, and 6 days under ambient conditions. Using the image analysis program Nova Px, the images were shown at a resolution of 5 × 5 µM (NT-MDT, Russia) [Waiker et al. 2023b].

Additionally, the confocal microscopy was also analyzed to observe the morphological characteristics of $A\beta_{1-42}$ in the absence or presence of compounds. The same procedure as described above was also applied here and the images were taken at λ_{ex} = 494 nm and λ_{em} = 518 nm wavelengths on a FITC fluorescent filter [Bhanukiran et al. 2023].

4.4.1.9. Neurotoxicity estimation on differentiated SH-SY5Y cell lines

The MTT assay was used to assess the neurotoxicity of compound on differentiated SH-SY5Y neuroblastoma cell lines. The principle behind the MTT assay is that it is a rapid colorimetric assay based on the cleavage of the tetrazolium ring of MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) by dehydrogenases in active mitochondria of living cells as an estimate of viable cell number. Initially, the SH-SY5Y neuroblastoma cells were cultured in a neuronal induction medium (NIM) comprising of 5% fetal bovine serum (FBS), 10 µM RA, and minimum essential medium (MEM) for 6 days then transferred to NIM without serum. On the 7th day, the cell lines having a density of 1 × 10^3 cells/wells were seeded in 96 well plates and incubated in a humidified condition like 5% CO_2 at 37 °C for 24 h. After that, the culture media was extracted and the compound and donepezil in different concentrations (10, 20, 40, and 80 µM) was incorporated, and cells

were further incubated for the next 24 h. 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 4 h. Finally, the media was extracted out and the observed purple colored formazan was dissolved in DMSO (200 µL). The absorbance was measured at 570 nm, and the results of % cell viability were estimated and represented as mean ± SD of three separate experiments (n=3) [Mishra et al. 2021, Mongre et al. 2020].

4.4.1.10. H_2O_2 induced Neuroprotective estimation on differentiated SH-SY5Y cell lines

The MTT assay was conducted to estimate the hydrogen peroxide (H_2O_2) induced neuroprotective activity of compound on differentiated SH-SY5Y neuroblastoma cell lines. Briefly, the neuroblastoma cells were seeded and incubated for 24 h at 37 °C in 96 well plates. After incubation, compound (5, 10, and 20 µM) was added to the plates and incubated for 3 h at 37 °C. Further, H_2O_2 was incorporated into each well and incubated for the next 24 h at 37 °C. The control contained the cells without any treatment while the positive control contained the H_2O_2 incubated cells. The experiment was carried out three times (n = 3), and the results are presented as changes in cell shape and population as mean ± SD, in comparison to control and H_2O_2 incubated cells [Lingappa et al. 2021].

4.4.1.11. $A\beta_{1-42}$ induced Neuroprotective estimation differentiated SH-SY5Y cell lines

A similar assay procedure was used to estimate the effectiveness of compound on $A\beta_{1-42}$ cell rescue [More and Vince 2012, Singh et al. 2021]. The $A\beta_{1-42}$ (10 µM) was incorporated into the differentiated neuroblastoma SH-SY5Y cells and incubated for 24 h. After incubation, the culture medium was then changed to a medium comprising compound (20 µM) and standard donepezil and then incubated further for the next 48 and 72 h. After incubation, the MTT assay was executed and quantified cell viability.

4.4.2. In-vivo and ex-vivo studies

4.4.2.1. Animals

The Wistar rats weighing 200-250 g and aged 10-12 weeks of either sex were procured from the institute's central facility and acclimatized for 7 days in an ambient conditions like temperature (25 ± 2 C) and relative humidity (65 ± 5 %) with 12 h light/dark cycles at institute animal facility, Department of Pharmaceutical Engineering & Technology, IIT-BHU. The commercial feed and water was continuously provided to these animals. Following acclimatization, the experimental protocol were executed as per following the CPSCEA guidelines for behavioral studies which was duly permitted by the institute's central animal ethical committee, IIT-BHU with IAEC approval number IIT(BHU)/IAEC/2023/020.

4.4.2.2. Acute toxicity studies

As per the *in-vitro* findings, the most promising compounds was examined for its acute oral toxicity on female Wistar rats (group of five in a particular dose) based on the protocol given in the OECD-423 guidelines. Before proceeding with the experiment, the animals were allowed freely access to water and fasted overnight. The dose of 500 mg/kg were prepared in 0.3% w/v Na-CMC and administered accordingly. For up to 14 days, the animals were continuously observed for any odd behaviour and toxic reactions, like, lethargy, diarrhoea, salivation, nervousness, convulsions, etc. Additionally, the animals were also monitored for coma, sleep, and mortality as well.

4.4.2.3. Scopolamine-induced amnesia model for testing cognition enhancement in rats

Healthy male Wistar rats were used for the scopolamine-induced study. In sterilized normal saline, the Scopolamine hydrobromide (Sigma Aldrich, India) was solubilized. The standard donepezil, and compound **AV-2**, were all suspended in a 0.3% w/v Na-CMC solution. The

rat population was split into the following groups, each consisting of six animals: control, scopolamine, donepezil (5 mg/kg, p.o.), and compound **AV-2** (5, 10, 20 mg/kg, p.o.). For seven days, the appropriate group of animals received one oral dose each of donepezil and **AV-2**. Only vehicle (0.3% w/v Na-CMC) was administered to the scopolamine group of rats.

4.4.2.4. Y-maze test

After 30 min of the 7th-day treatment, all the animals except the control group were administered scopolamine hydrobromide (1 mg/kg) intraperitoneally, and spatial working memory was evaluated using the Y-maze apparatus. In the center of the maze, the rats were placed individually and the first entry was left out because the rats preferentially entered into the facing arm. The spontaneous alternation behavior, number of entries to each and all the arms was recorded for up to 5 min. When a rat crossed all four paws into the arm, it was considered to have entered the arm. The three successive arm entries were thought to be a sign of better memory, learning, and hence enhanced spontaneous alterations. The percentage spontaneous alternation was evaluated as per the following expression: {number of spontaneous alternations/(total arm entries-2)} × 100 [Srivastava et al. 2019a, Wolf et al. 2016].

4.4.2.5. Ex-vivo and biochemical analysis

Rats were immediately sacrificed after the Y-maze experiment via cervical dislocation. The hippocampus part of the brains was carefully removed, rinsed with saline (0.9% w/v NaCl), homogenized in 100 mM cold PBS (pH 7.4) using a Teflon-glass homogenizer, and centrifuged at 4 °C (10,000×g for 15 min). After centrifugation, the supernatant was collected and analysed for their protein estimation *via* Bradford assay. Several biochemical parameters were also estimated using the supernatants [Shrivastava et al. 2017].

Primarily, The AChE levels in the hippocampal were determined by modified Ellman's colorimetric method. Initially, the mixture containing ATCI (15 mM, 30µL) and DTNB (1.5 mM, 160 µL) was prepared as Ellman's reagent. After that, the supernatant (50 µL) was mixed with Ellman's reagent, and immediately the reaction rates (rate of increase of absorbance) were estimated spectrophotometrically at 412 nm for up to 6 min. The AChE level was represented as µM of substrate hydrolyzed/min/mg of protein.

The ACh level in the brain homogenate was determined as per the protocol given in Krishgen Biosystems, rat acetylcholine, and ACh GENLISA ELISA kit and was represented as pg/mL.

The thiobarbituric acid reactive substance (TBARS) assay was used to calculate MDA, an oxidative stress biomarker of lipid peroxidation. When MDA reacts with thiobarbituric acid (TBA) in an acidic environment, a complex of TBARS (red color) can be identified spectrophotometrically. According to the assay methodology, 200 µL of each supernatant was combined with 1 mL of 10% cold trichloroacetic acid (TCA) in 0.1M HCl and centrifuged for 10 minutes at 153 × g. The 1 mL of 0.67% TBA was combined with the 1 mL of supernatant. The assay mixture was warmed in a water bath for 10 minutes, and the number of MDA moles per milligram of protein was determined by measuring absorbance at $\lambda = 532$ nm [Srivastava et al. 2019b].

SOD is a crucial enzyme in the transformation of free molecular O_2 or H_2O_2 from O_2 radicals. To create a blue formazan product, which can be detected spectrophotometrically, hydroxylamine hydrochloride was autoxidized with nitro blue tetrazolium (NBT) at pH 10.2 in the presence of EDTA. The assay reagent was made up of 50 mM Na_2CO_3, 24 mM NBT, and 100 mM EDTA. The assay process was started by mixing 50 µL of homogenates with 200 µL of prepared reagent and an aqueous solution of hydroxylamine hydrochloride (50

μL). SOD units were calculated as (U/mL)/min/mg protein and the absorbance was recorded at λ = 560 nm for 10 min [Mishra et al. 2019].

The ROS neutralizing capability of the compound **AV-2** was determined using reduced glutathione (GSH) assay. Briefly, 1 mL of 4% w/v sulfosalicylic acid was mixed with the 100 μL hippocampal brain homogenate and kept aside for 1 h at 2-8 °C for precipitate formation. Afterward, these precipitates were centrifuged at 1200*g* for 15 min at 4 °C. After centrifugation, 100 μL of supernatant was added to the 2.7 mL of 0.1 M PBS (pH 8) with the subsequent addition of 200 μL Ellman's reagent (DTNB, 0.1 M) which resulted in yellow color formation. With the help of a multimode microplate reader (Synergy HT, Bio-Tek Instruments, Inc.), the absorbance was recorded at λ = 412. Lastly, the calculations were carried out using a molar extinct coefficient of 1.36 × 104 M^{-1} cm^{-1}, and results were expressed as nmol of GSH per mg protein [Jollow et al. 1974].

The catalase activity was also estimated by incorporating 50 μL of hippocampal homogenate into 3 mL of 12.5 mM H_2O_2 in 50 mM phosphate buffer pH 7.0. Immediately after introducing H_2O_2, the absorbance was measured at λ = 240 nm for 2 min in each 60-second time interval. The results are represented as mol of H_2O_2 decomposed/min/mg of protein [Youssef et al. 2018].

4.4.2.6. $A\beta_{1-42}$ induced ICV rat model: Morris water maze test

The male Wistar rats weighing 200-250g were taken in the ICV model and categorized in to four distinctive groups having six animals in each group like sham (control), model (Aβ), donepezil (5 mg/kg, p.o.), and compound (20 mg/kg, p.o.). Briefly, rats were individually mounted on stereotaxic equipment after being given the anaesthetics in the combination of ketamine and xylazine at concentrations of 90 mg/kg and 9 mg/kg, respectively via

intraperitoneally. Rat's scalps were washed with iodine solution and saline after the ear bars were symmetrically placed. Stereotaxic coordinates relating to the bregma were set before drilling the hole in the skull (-0.5 mm anterior, +1.2 mm mediolateral, -3.2 mm dorsoventral with incision bar placed at -3.3 mm) [Colaianna et al. 2010].

The $A\beta_{1-42}$ (Sigma Aldrich, India) (5 µL, 4 µM) was diluted in sterile saline and administered by Hamilton microsyringe at a 2 µL/min infusion rate to all rats excluding the sham group (received only vehicle). The needle was left in place after injection for an additional five minutes to stop reflux. All the rats underwent special care following surgery, and iodine ointment was routinely administered to the surgical site until normal behaviour was regained [Bagheri et al. 2011].

The water maze apparatus was a circular pool with dimensions of 121 cm in diameter, 62 cm in height, and 32 cm in depth. TiO_2 was used to make the pool opaque after it had been filled with water (25 ± 2 °C). This water maze was equally divided into four quadrants, one of which had a hidden platform that was 2 cm under the surface of the water. Following seven days of post-operative recuperation, donepezil, and compound were orally delivered to the corresponding group of animals starting on the eighth day and continuing through the sixteenth day. To evaluate learning and memory, the Morris water maze experiment was carried out throughout the final five days of therapy (12-16th days). To find the escape latency time and the number of platform crossings during a period of 90 s, two trials per day were run at an interatrial difference of 3 h [Wang et al. 2015].

4.4.2.7. Western blot studies

After the Aβ-induced Morris water maze test, the animals from each group were sacrificed, and their brains hippocampal were meticulously separated. The extracted hippocampal brains

were homogenized in phosphate and protease inhibitor-infused radioimmunoprecipitation assay (RIPA) buffer. The hippocampus brain homogenate was sonicated and centrifuged at 4 °C (10,062×g, 20 min), and the protein content was then determined using a Bradford assay. Through sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the equal protein content was extracted, and the protein was then transferred to polyvinylidene fluoride. The membrane was incubated with anti-β-actin (1:5000), APP (1:1000), BACE-1 (1:1000), and tau (1:1000) primary antibodies (AD antibody sampler kit#9784, Cell Signalling Technology, USA) at 4 °C overnight after being blocked with 8% milk for 2 h. After that, the membrane had been cleaned with TBST (tris-buffered saline/0.01% Tween 20) and treated for 2 h at room temperature with anti-mouse horseradish peroxide-linked secondary antibodies (CST, USA). The blots were visualized with ChemiDoc (Bio-Rad, Hercules, USA) and an enhanced chemiluminescence substrate (Bio-Rad) for the detection of the immunological complex. The blots were then quantified using the Image J software [Sharma et al. 2019c, Waiker et al. 2023b].

4.4.2.8. Immunohistochemistry analysis

After Aβ-induced Morris water maze test the experimental animals were underwent intercardially perfusion of paraformaldehyde (4% v/v) and cold saline (pH 7.2) and sacrificed. The brains were removed, maintained in a 10% formaldehyde solution overnight, then dehydrated and cryopreserved in a separate sucrose solution. The 20 µm thick slices of the hippocampal brain of respective animals of each group were coronally cut by using cryo-microtome (Leica Wetzlar, Germany). The non-specific binding sites were blocked by using 1% BSA, 10% goat serum, and 0.3% Triton-X in PBST. Each brain hippocampal underwent a primary antibody incubation with anti-BACE-1 (1:400) and anti-Aβ (1:800) for 18 h at 4

°C, followed by two rounds of washing with 1% BSA-PBS to eliminate the unbound primary antibodies. The sections were then subsequently treated for 1 h at room temperature with FITC-conjugated secondary antibodies. After treatment, each section was then given three further PBS washes before being mounted on the slides using poly(vinyl alcohol) and DABCO (Fluka analytical) anti-fading agents. A fluorescent microscope (Olympus) was used to take pictures of each slide, and ImageJ software (NIH, United States) was used for quantification [Sharma et al. 2019c, Waiker et al. 2023b].

4.4.2.9. Brain histopathology

To perform the histopathology on the rat brain tissues, rats were slaughtered immediately after transcardial perfusion with precooled PBS pH 7.4 and 4% formaldehyde (in 0.1 mol/L PBS). For fixation, the brains were isolated from each experimental group and were preserved at 4 °C overnight. Afterward, these brains were shifted to the sucrose solutions having concentrations of 10, 20, and 40 % consecutively after 24 h intervals for complete dehydration. For brain sectioning, the brain sections were cut with the help of a freezing microtome (Leica Microsystems, Germany) and transferred into 0.01 mol/L of PBS after washing three times. These brain sections put on the glass slides were stained *via* Nissls staining (0.125% cresyl violet) and the section was then washed three times with alcohol (70, 95, and 100%), followed by xylene. Finally, the images were obtained using an Olympus MLXi-TR plus light microscope, and neuronal density were counted using the ImageJ program and expressed as a percentage of control rats [Wang et al. 2015].

4.4.2.10. In-vivo blood-brain permeability

The extent of compounds in the brain tissue of Wistar rats following oral administration was checked to assess the compound's permeability to the BBB *in-vivo*. Briefly, the 10 and 20

mg/kg doses of compounds were orally administered for four consecutive days to their respective treatment rat groups (n=3). On the 4th day, the rats were sacrificed and their brain tissue homogenates were prepared in chloroform. The samples were agitated on an oscillating shaker for 10 min and then centrifuged at 2000 g for 10 min to separate the bottom organic layer. The organic phase was subjected to evaporation and residual product was collected in ACN. The 20 µL of the sample was injected into the High-Performance Liquid Chromatography (Shimadzu). The flow rate of the ACN:H_2O:TFA (95:5:0.1) mobile phase was maintained at 1 mL/min and the retention period was observed for 10 min at λ = 270 nm using the Eclipse Plus C8 column and photodiode array detector. The peak area, retention time, and brain drug concentrations were reported [Kumar et al. 2022].

4.4.2.11. Drosophila phenotypic model

4.4.2.11.1. Compound AK-2 toxicity analysis

Wild-type *Drosophila* melanogaster (Oregon R+) flies were utilized in a toxicity assay. Control flies were raised on standard fly food, while the test group was exposed to different concentrations of **AK-2** (1 µM, 10 µM, 50 µM, 100 µM, and 200 µM) in a BOD incubator at 24 ± 1°C. Each concentration was tested in triplicate fly culture vials, with five virgin females and five age-matched male flies in each vial. Parental flies were removed after 5 days, and analysis of F1 progeny continued until the 15 days of their developmental stages.

4.4.2.11.2. Cell Viability in AK-2 in AD flies

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed to evaluate the cell viability of larval gut tissue treated with **AK-2**. Third-instar wild-type larvae were dissected in 1X PBS under ice-cooled conditions. A total of 10 larval guts from each group were selected for the experiment. The control group consisted of 1X PBS-treated gut

tissue, while the test group included gut tissue treated with **AK-2** at concentrations of 1 µM, 5 µM, 10 µM, and 50 µM. The experimental procedure includes triplicate samples.

4.4.2.11.3. Drosophila eye phenotype

Adult F1 flies including wild-type (Oregon R^+), UAS-Aβ42 < ey-GAL4 (AD) AD treated with **AK-2** at 5 µM, 10 µM, and 15 µM were anesthetized using diethyl ether. High-resolution images were captured using a Dewinter trinocular stereo-zoom microscope.

4.4.2.12. Pharmacokinetic studies

The compounds pharmacokinetic parameters were determined in male Wistar rats weighing 200-250 g (n = 3). Initially, the rats were fasted for 14-16 h and allowed freely access to water. After compound **AV-2** administration, blood samples were taken from the retro-orbital plexus of the sedated animals at various times (0.5, 1, 2, 3, 5, 7, 12, 24, and 32 h). To stop the coagulation, sodium heparin was used as a pre-treatment on all of the blood samples. The plasma was collected from these samples after centrifugation (6797 × g, 6 min, 4 °C). The 100 µL of plasma was added to the 200 µL of MeOH to isolate the compound from the mixture. Further, mixing, centrifugation, organic layer separation, and evaporation were done to collect the residual product which was further dissolved in the mobile phase. ACN:H_2O:TFA (95:5:0.1) was used as the mobile phase at a flow rate of 1 mL/min, and 20 µL of the sample was injected into the High-Performance Liquid Chromatography (Shimadzu). The retention period was observed for 10 min at λ = 270 nm using the Eclipse Plus C8 column and photodiode array detector. By utilizing the extravascular non-compartment model's standard calibration curve, the plasma drug concentration of compound **AV-2** was determined [Sharma et al. 2019b].