Chapter 3

(Rationale, Objectives & Plan of Work)

3.1. Rationale and objectives

AD is a multifactorial condition that severely damages the brain's structural and functional integrity and marked by declining neurological functions, memory impairment, cognitive disabilities, and behavioral alterations [Chen et al. 2022]. There are no therapeutic regimens available in the market right now that could halt the disease's progression rather than providing only symptomatic relief [Sharma et al. 2019a]. Current therapies involve the use of FDA-approved medications like AChE inhibitors (AChEIs) (donepezil, rivastigmine, galantamine), and NMDA-receptor antagonist (memantine) which provide symptomatic relief to the patients [Ramrao et al. 2021]. Recently, Aβ directed monoclonal antibodies (aducanumab and lecanmab) have approved by the FDA in the accelerated approval pathway which is still controversial [Swanson et al. 2021, Tampi et al. 2021].

Due to the complex etiology and a multifaceted pathophysiology involved in the AD, the treatment is challenging [Verma et al. 2022]. Various pathophysiological processes including a decrease in acetylcholine (ACh) levels, tau protein hyperphosphorylation, development of neurofibrillary tangles, amyloid beta (Aβ) aggregates formation, hyper-activation of N-methyl D-aspartate (NMDA) receptor, oxidative stress, CREB signaling pathways, biometal dyshomeostasis, pathogenic involvement, and neurovascular dysfunction have been proposed in the past two decades to play a significant role in AD progression.

Drug treatment for this condition is still in its infancy and does not halt the disease's progression. The multi-targeted inhibitors strategy has been used in light of the fact that AD is a multifactorial condition associated with several targets and includes simultaneously targeting various enzymes with a single compound at a time. Thus, the designing of multi-

targeted inhibitors may prove useful for efficaciously halting the spread of the disease rather than just treating symptomatically.

Cholinesterase (ChE) including both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are serine hydrolytic enzymes that involve the catalytic hydrolysis of acetylcholine (ACh) and butyrylcholine (BCh) to choline and acetic acid [Soreq 2012]. Therefore, specific cholinesterase inhibitors have been employed to control its native hydrolytic breakdown and hence increase the lowered ACh levels which may be the crucial target in treating cognitive impairments [Waiker et al. 2023b, Waiker et al. 2023c]. AChE has also been identified to play a significant role in enhancing the Aβ aggregation and its deposition in AD brain [Sharma et al. 2019a]. Aβ is a small peptide produced by the proteolytic breakdown of a large transmembrane protein called amyloid precursor protein (APP). Beta-site APP cleaving enzyme-1 also known as β-secretase-1(BACE-1) is responsible for the generation of insoluble APP fragments which leads to the formation of Aβ plaques [Chen et al. 2017]. Overall, these events are held responsible for neuronal communication disruption, neuritic injury, inflammatory responses, cognitive dysfunctions, and neuronal deficits [Verma et al. 2022]. Aβ accumulation may also result in mitochondrial dysfunction which increases free radicals levels and hence oxidative damage to the brain [Cheignon et al. 2018]. Hence, AChE inhibition is considered to be the most effective strategy for treating cognitive disabilities in AD.

Another crucial target BACE-1, which plays a substantial role in the APP cleavage, Aβ aggregation, and senile plaque formation that leads to oxidative damage to the brain cells. Thus, compounds having BACE-1 inhibitory potency along with Aβ disaggregating

capabilities and strong anti-oxidant properties may retard neuronal damage and hence protect the neurons from oxidative stress [Qu et al. 2021].

3.1.1. Designing of MTDLs (Part-I)

Based on the above discussion, and our search to find out the most promising multi-targeting ligands for AD therapy, the structure-based drug design technique was employed by developing e-pharmacophore models of protein structures (hAChE and hBACE-1). The co-crystallized ligands of both the enzymes (hAChE: donepezil [PDB: 4EY7] and hBACE-1: F1M [PDB: 2ZJM]) were selected for e-pharmacophore model generation. These models were utilized to screen the large database (ZINC15 database) as compared to the previously reported Maybridge database to increase the chances of getting the new scaffold with different pharmacoporic features [Sharma et al. 2019b]. The ZINC15 database undergoes filtrations processes including various computational methods like phase screening, virtual screening (VS), docking-post processing (DPP), and Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) estimation. The pharmacophoric features of the identified hit (ZINC000015441499) and most potent compound **AV-2** were also analysed as the pharmacophoric scaffold (quinazoline) of both the identified hit (ZINC000015441499) and most potent compound **AV-2** is quite different from the earlier study conducted in our lab by the Sharma et al., which is based on the pharmacophoric features of N-benzylpiperidine nucleus [Sharma et al. 2019b]. A series of compounds with better binding properties at the aspartate dyad of hBACE-1, catalytic active site (CAS), and PAS of hAChE was rationally designed using the identified potential hit.

Based on the *in silico* study's findings, it was observed that quinazoline moiety of potential hit might play a significant role in AD therapy *via* inhibiting multiple targets [Rehuman et al.

2021]. Further, a literature survey of some recently reported compounds containing quinazoline moiety was observed which suggested that advanced series with substituted quinazoline derivatives due to its interaction capabilities and binding profile showed promising inhibitory activity against hAChE, hBACE-1, and Aβ aggregation [Haghighijoo et al. 2022, Huang et al. 2009, Mohamed and Rao 2017]. The 2-phenylethan-1-amine of compound **S1** demonstrated to be a suitable moiety capable of attaching with the AChE-PAS by producing a hydrophobic environment. The introduction of chlorine at the C-8 position and isopropyl at the R1 position provides excellent results against $A\beta_{40}$ [Mohamed et al. 2017]. Compound **S2** (4-(benzylamino)quinazoline-2-ol) was also considered as the excellent Aβ aggregation inhibitor ($A\beta_{40}$; IC_{50} = 0.27 μM) and was found to be four times more effective as compared to the standard compound curcumin ($A\beta_{40}$; IC_{50} =3.3 μM) [Mohamed and Rao 2017]. It was observed that the 2^{nd} and 4^{th} position of the quinazoline ring is important to design multi-targeting agents in the treatment of AD. Briefly, lower affinity for AChE was observed when piperazine was bio-isostatically substituted for piperidine, but provides better lipophilicity and inotropic effects with considerable neuroprotective efficacy [Ramrao et al. 2021]. Also, protonation of both hydrogens at piperazine at physiological pH may enhance the binding profile towards the catalytic dyad of the BACE-1 enzyme [Waiker et al. 2023c]. Therefore, various substituted piperazines like 1-(4-nitrophenyl)piperazine, 1-Benzhydrylpiperazine, and 1-Benzylpiperazine were introduced at the 2^{nd} position of quinazoline moiety. The idea behind choosing 1-(4-nitrophenyl)piperazine was that the para-nitro group at the phenyl ring has conjugating electrons between oxygen and nitrogen atoms which may induce polarity in the compound and could show polar interaction with the CAS of hAChE. Meanwhile, the one carbon chain between the piperazine and phenyl i.e.,

benzhydryl and benzyl was introduced based on one of the FDA-approved drugs donepezil which contains benzylic moiety that may provide the optimum length and flexibility to the compounds [Prati et al. 2016]. The new diaryl-imidazole scaffold combined with benzhydryl piperazine i.e. compound **S3** enhanced cognition and memory along with slowing the progression of neurodegeneration [Panek et al. 2018]. Further, a phenyl ring containing various para-substituted electron-withdrawing groups (EWGs) and electron-releasing groups (ERGs) was also introduced at the 4th position of quinazoline to evaluate their binding profile towards the PAS region of hAChE. Thereby, these findings support our theory that a series of compounds that have been specifically synthesized may enhance the inhibitory potential against both hAChE and hBACE-1. The overall designing of part I has been represented in Figure 3.1.

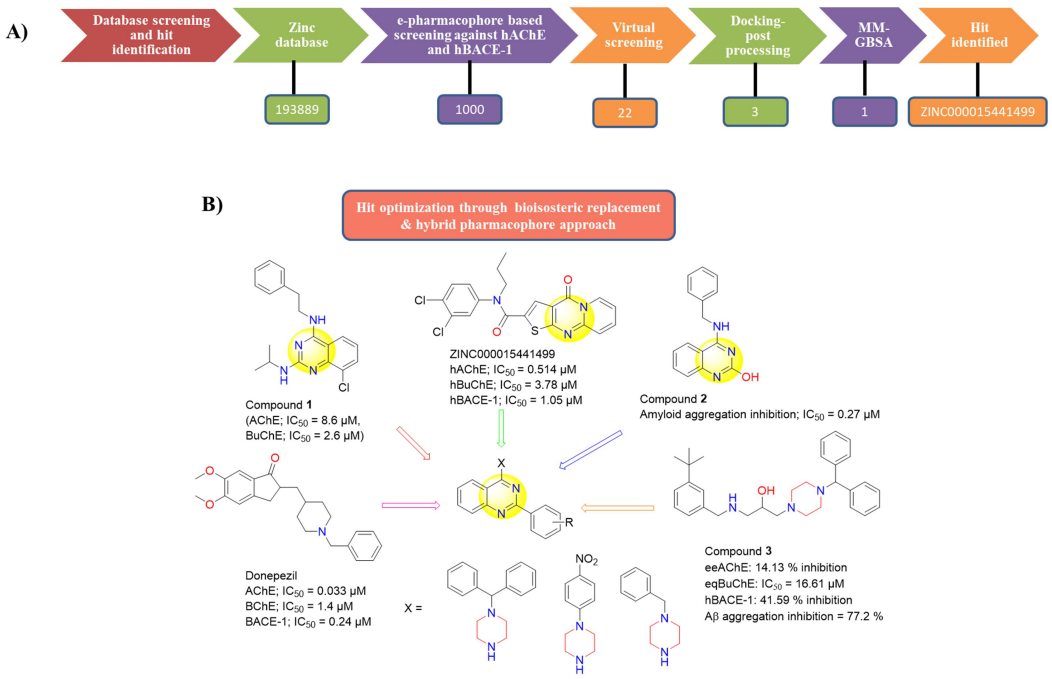

Figure 3.1. Design strategy for compound **AV-1** to **AV-21**

3.1.2. Designing of MTDLs (Part-II)

A series of multi-target directed ligands (MTDLs) was designed based on the bioisosteric replacement and molecular hybridization approaches. In our previous studies, we reported MTDL by tethering substituted piperazines and phenyl rings at the C2 and C4 positions of the quinazoline nucleus, respectively. Amongst them, compound **AV-2** revealed remarkable MTDL activity [Verma et al. 2024]. The MTDL nature of compound **AV-2** has driven us to explore it further. Mohammad et al. reported a compound **2a** bearing 3,4-dimethoxybenzylamino and benzylpiperazine at C2 and C4 positions of quinazoline acting as a dual inhibitor against ChE and Aβ aggregation inhibition (AChE, IC_{50}: 1.2 μM; BChE, IC_{50}: 8.3 μM; and Aβ, IC_{50}: 2.3 μM, respectively). A further modification was done by substituting amino and benzyl amino on C2 and C4 positions of quinazoline (compound **3a**) demonstrating good inhibitory profile against AChE, BChE and $Aβ_{40}/Aβ_{42}$ in the micromolar range (AChE, IC_{50}: 7.5 μM; BChE, IC_{50}: 11.6 μM; and $Aβ_{40}/Aβ_{42}$, IC_{50}: 2.2/8.4 μM, respectively) [Mohamed and Rao 2017]. Wang et al. reported a compound **4a** containing ligustrazine-derived chalcones containing substituted quinazoline which displayed promising inhibitory results and neuro-protective effects (AChE, IC_{50}: 0.10 μM; BChE, IC_{50}: 22.4 μM; % anti-Aβ-aggregation at 10 μM: 26.7, % neuro-protection at 100 μM: 77, respectively) [Wang et al. 2018]. Moreover, previously from our lab Sharma et al. reported a compound **5a** comprising N-benzylpiperidine attached with p-CF_3 substituted 5-phenyl-1,3,4-oxadiazole which displaced a promising inhibitory activity against ChE and BACE-1 (hAChE, IC_{50}: 0.055 μM; hBChE, IC_{50}: 0.186 μM; and hBACE-1, IC_{50}: 0.146 μM) and corroborated the findings with the *in-vivo* and *ex-vivo* studies [Sharma et al. 2019c]. The FDA-approved drug i.e., donepezil is the most commonly prescribed drug for AD therapy but it only provides

symptomatic relief. The SAR of donepezil revealed that the indanone nucleus of donepezil was found to be oriented towards the AChE PAS with an extension of the N-benzylpiperidine ring into the catalytic site of AChE [Mezeiova et al. 2018].

The quinazoline ring was chosen to be the central nucleus for the design of new hybrids based on the preceding explanation and thorough literature studies. It has been identified that the substitution at the C4 position of quinazoline moiety plays a significant role in improving multitargeting inhibitory activity in AD. Therefore, we introduced various substituted piperazines at the C4 position of quinazoline considering bioisosteric replacement for benzylpiperidine ring. Piperazine exhibits enhanced neuroprotective effects and enhanced affinity towards AChE and the aspartate BACE-1 residues due to its bioisosteric equivalency with piperidine and the basic nitrogen atoms, which protonate at physiological pH which significantly increases BBB permeability as a result of acid-base equilibrium [Waiker et al. 2023a]. The methoxy (-OCH_3) group was introduced at the C7 and C8 positions of the quinazoline ring to improve its binding towards the PAS region of AChE as it was present in donepezil [Sharma et al. 2019b]. The overall design strategy is discussed in Figure 3.2.

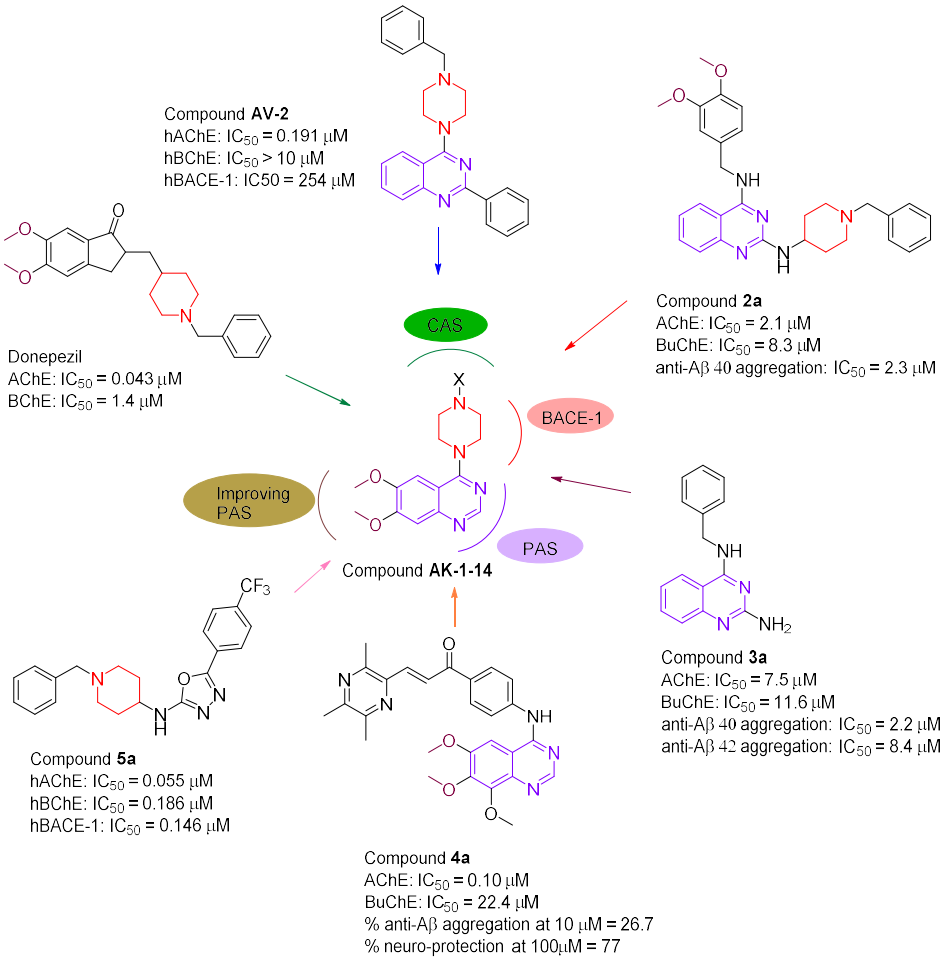

Figure 3.2. Design strategy for compound **AK-1** to **AK-14**

3.2. Plan of Work

3.2.1. In-silico optimization studies

- Protein Preparation and Grid generation
- Pharmacophore modelling
- Structure-based virtual screening workflow (HTVS and VSW)
- Docking-post processing (DPP) and pose filtration
- Molecular Docking Studies

- Molecular Dynamics and simulation
- In-silico drug likeliness
- DFT studies

3.2.2. Synthesis of quinazoline derivatives:

- **Series-I :** 2,4-di-substituted quinazoline derivatives
- **Series-II :** 4-substituted 6,7-dimethoxy quinazoline derivatives

3.2.3. Characterization of synthesized compounds

- Physicochemical characterization including melting point and R_f using TLC
- Estimation of % purity by HPLC
- Structural characterization using state of art techniques ^{1}H NMR, ^{13}C NMR, FT-IR and Mass spectrometry.
- SC-XRD characterization of the most-promising compounds.

3.2.4. In-vitro Biological evaluation:

- Human Cholinesterase (hAChE and hBChE) inhibitory assay using Ellman's method
- Enzyme kinetics study
- BACE-1 inhibition assay
- Propidium iodide displacement assay
- Parallel artificial membrane permeability (PAMPA-BBB) assay

- Anti-Aβ aggregation (self- and AChE-induced) activity by thioflavin T assay and microscopic analysis.
- Neurotoxicity and neuroprotective studies on differentiated SH-SY5Y cell lines using MTT assay

3.2.5. In-vivo and ex-vivo studies

- Oral acute toxicity studies on rat and mice animal models
- *In-vivo* behavioral studies (Y-maze test) on rats models
- *Ex-vivo* biomarker estimation
- Aβ-induced AD phenotypic model (rat/mice)- Morris Water Maze Test
- Western blot analysis
- Immunohistochemical (IHC) analysis
- Drosophila eye-phenotypic AD model
- *In-vivo* BBB permeability estimation

3.2.6. Pharmacokinetic studies

- T_{max}
- C_{max}
- $T_{1/2}$
- MRT
- Bioavailability

3.3. Significance of the studies

Since most AD survivors endure irreversible dementia and a progressive loss of cognitive function, the majority of FDA-approved medications for the treatment of AD only offer symptomatic relief. Although the exact cause of AD is still unknown, a number of factors are thought to play a direct role in its development and progression. These include elevated levels of AChE, deposits of amyloid-beta (Aβ), BACE-1, and over-activation of APP, which promotes Aβ aggregation by forming monomers, protofibrils, annular oligomers, and plaques, which in turn causes oxidative stress and neuronal loss.

Consequently, rather than treating AD symptoms, the multi-targeting single ligand method may be a viable way to defeat the illness. Our hypothesis suggests that a lead candidate that targets several pathways at once may eventually slow the disease's progression and improve the cognitive impairment brought on by the buildup of NFTs and Aβ plaques.