Chapter 3

(Rational, Objectives and Plan of Work)

3.1 Rationale and Objective

The exact cause and treatment of AD are still in their infancy owing to the multifaceted pathophysiology involved in the disease and its progression. Several factors such as lower acetylcholine (ACh) levels [Hensley et al. 1994], increased Acetylcholinesterase (AChE) in the synaptic cleft [Enz 1995], activation of N-methyl-D-aspartate receptor (NMDAR) [Hynd et al. 2004], Central nervous system (CNS) inflammation in response to activated microglial cells and astrocytes [Heneka et al. 2015]. hyperphosphorylated tau and formation of neurofibrillary tangles (NFTs) [Alonso et al. 1996], Oxidative stress [Markesbery 1997], and accumulation of amyloid beta (Aβ) and its aggregates [Hensley et al. 1994] are the hallmarks of AD and its progression.

Widespread efforts are continuously being made in search of a drug for AD [Kaide et al. 2019]. The current treatment regimen for AD therapy involves the use of NMDA receptor antagonist (memantine), and AChE inhibitors (donepezil, rivastigmine, and galantamine) that deliver symptomatic relief with slight cognition and memory improvements in AD. [Zemek et al. 2014]. Recently, Aducanumab (monoclonal antibody) has been approved by FDA as a disease-modifying therapy for AD. Aducanumab is associated with improvements in memory and cognitive impairments linked to Aβ plaques, though it's use is still controversial in AD progression [Yang and Sun 2021].

Several mechanisms and hypotheses have been proposed explaining the progression of AD. However, a precise etiology is still elusive [Rivera-Marrero et al. 2020]. Several hypotheses governing AD progression, amongst which cholinergic transmission dysfunction and formation of Aβ-aggregates have emerged as the widely acceptable pathophysiology for synaptic loss and neurodegeneration [Huang and Mucke 2012, Talesa 2001]. The cholinesterase enzymes (ChE) i.e. AChE and Butyrylcholine esterase (BChE) inhibitors are the most promising therapeutic strategy to halt

proteolytic degradation of the ACh into choline and acetic acid and increase ACh levels in synaptic cleft to regulate cholinergic neurotransmission [Stanciu et al. 2019]. AChE and BChE were also observed to promote Aβ-aggregation and the formation of neocortical Aβ-plaques and neurofibrillary tangles [Mushtaq et al. 2014]. Another most widely acceptable cause of AD is Aβ-aggregation which results in synaptic loss and, ultimately, neuronal cell death. Aβ accumulates in the brain as an insoluble protein in response to beta secretase-1(BACE-1) associated APP cleavage. The accumulation of Aβ results in neuroinflammation and also elevates free radicals production in the mitochondria of the neuronal cell causing oxidative stress.

3.1.1 Designing of Part-I molecular hybrids

To design a new molecular hybrid, 5-phenyl-1,3,4-oxadiazole was selected as a lead scaffold (Figure 3. 1) due to its binding in the AChE-PAS region which was replaced with substituted 5-phenyl-1,3,4-oxadiazole-2-thione. The sulfur atom was introduced in the scaffold as it has an established role in improving the defense mechanism against oxidative stress and reactive oxygen species (ROS) in pathological diseased conditions [Saraf et al. 2022]. The benzylpiperazine (BP) moiety was attached to 3-NH of 1,3,4-oxadiazole-2-thione through a single carbon chain linker with an assumption that it will provide enough flexibility and optimum length to the BP ring to accommodate the AChE-Catalytic anionic site (CAS) and catalytic dyad (Asp32 and Asp228 residues) of the BACE-1 enzyme, respectively. The BP moiety was also introduced owing to the protonation capabilities of its nitrogen atom at the physiological pH that will lead to enhanced BBB permeability, ChE, and BACE-1 inhibitory activity. [Sharma et al. 2019b]. Various EWG and EDG were attached to the 5-phenyl ring to generate a structure-activity relationship (SAR) of the designed compounds against ChE and BACE-1 enzymes.

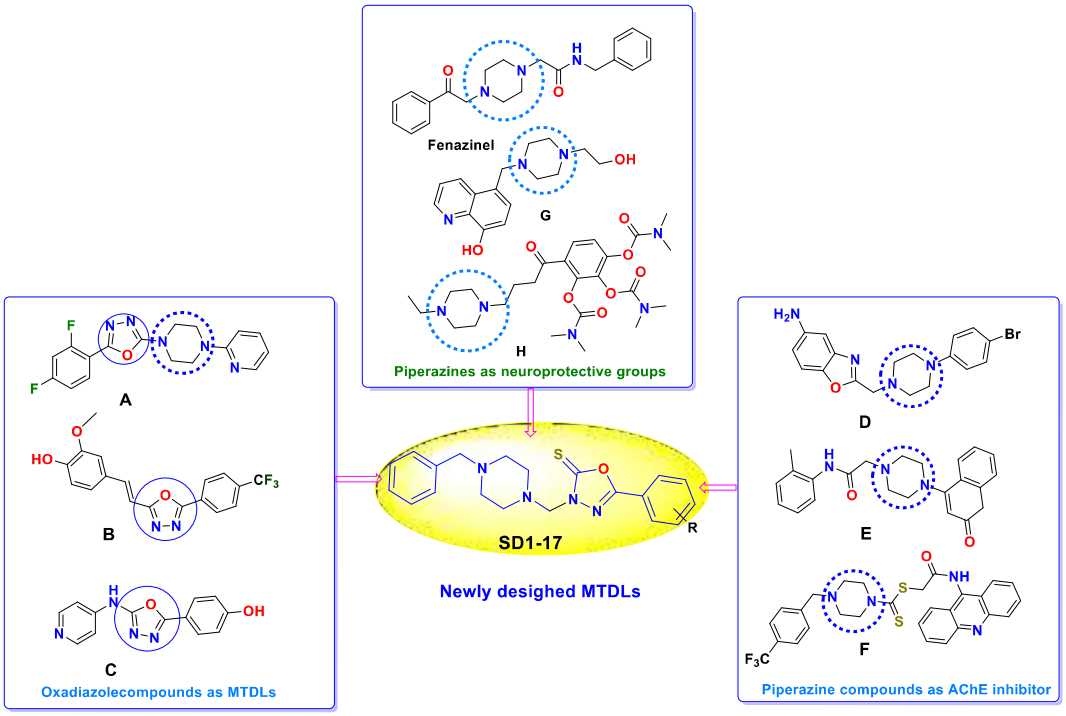

Figure 3. 1. Designing strategy of the present series of compounds **SD1-17** using molecular hybridization approach.

3.1.2 Designing of Part-II molecular hybrids

Using an *in-silico* method screening large datasets of molecules, hit molecular scaffolds were identified. Selections of top candidates were guided by the potential for optimization for AD multitargeted agents [Tripathi et al. 2020b]. 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 (hAChE, PDB code: 4EY7, and hBACE-1, PDB code: 2ZJM) were generated using co-crystal structures. The e-pharmacophore model of hAChE incorporated one hydrogen bond acceptor, two aromatic rings, and two hydrophobic residues, while the hBACE-1 e-pharmacophore model suggested the presence of two hydrogen bond donors, a single positive ionic group, and two aromatic rings as necessary features. The in-house database was screened utilizing the Phase module of Schrödinger Maestro suite 2018 resulting in 379 hits which were further subjected to virtual screening workflow (vsw)

(Figure 3. 2). 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 screening resulted in 10 compounds, from which 5 hits were selected based on their docking scores and interactions 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. All five hits identified through the screening have already been reported as ChEIs and were further optimized to design a new series of compounds (**4a-j** and **5a-q**).

The design considerations were focused mainly on two aspects. The first involved introduction of different substituted piperazines at the 3-NH of the 5-phenyl oxadiazole-2-thione ring via a single alkyl carbon atom to design series **4a-j** where various substituted piperazines were chosen based on the docking and Mechanics-Generalized Born Surface Area (MM-GBSA) scores of the compounds.

The second aspect was to incorporate an N-benzyl piperidine ring, a common feature in both the co-crystallized ligand complex of AChE and BACE-1 (donepezil and F1M) by a one-carbon acetamide spacer appended to 5-phenyl substituted 1,3,4-oxadiazole 2-thiol heterocycle revealing series **5a-q.** The oxadiazole moiety was selected for a new scaffold design since it occurs in all five selected hits obtained after the screening of the in-house database. The identified hits show the presence of an electron-withdrawing group (4-CF_3) and electron-releasing group (4-OH) responsible for the ChE inhibitory activity. Further to improve the binding affinity towards the AChE peripheral active site (AChE-PAS) and to generate the structure-activity relationship (SAR), both EWG and EDG were incorporated into the phenyl ring [Sharma et al. 2019a]. The one-carbon acetamide spacer group was added to the compounds with an electron donor-acceptor pair to interact with the catalytic dyad of the BACE-1 and also to provide enough length

to the molecule to occupy the active gorge. Based on the structural features required to interact with the enzyme's active site, a total of 27 compounds (**4a-j** and **5a-q**) were prioritized for synthesis and evaluation.

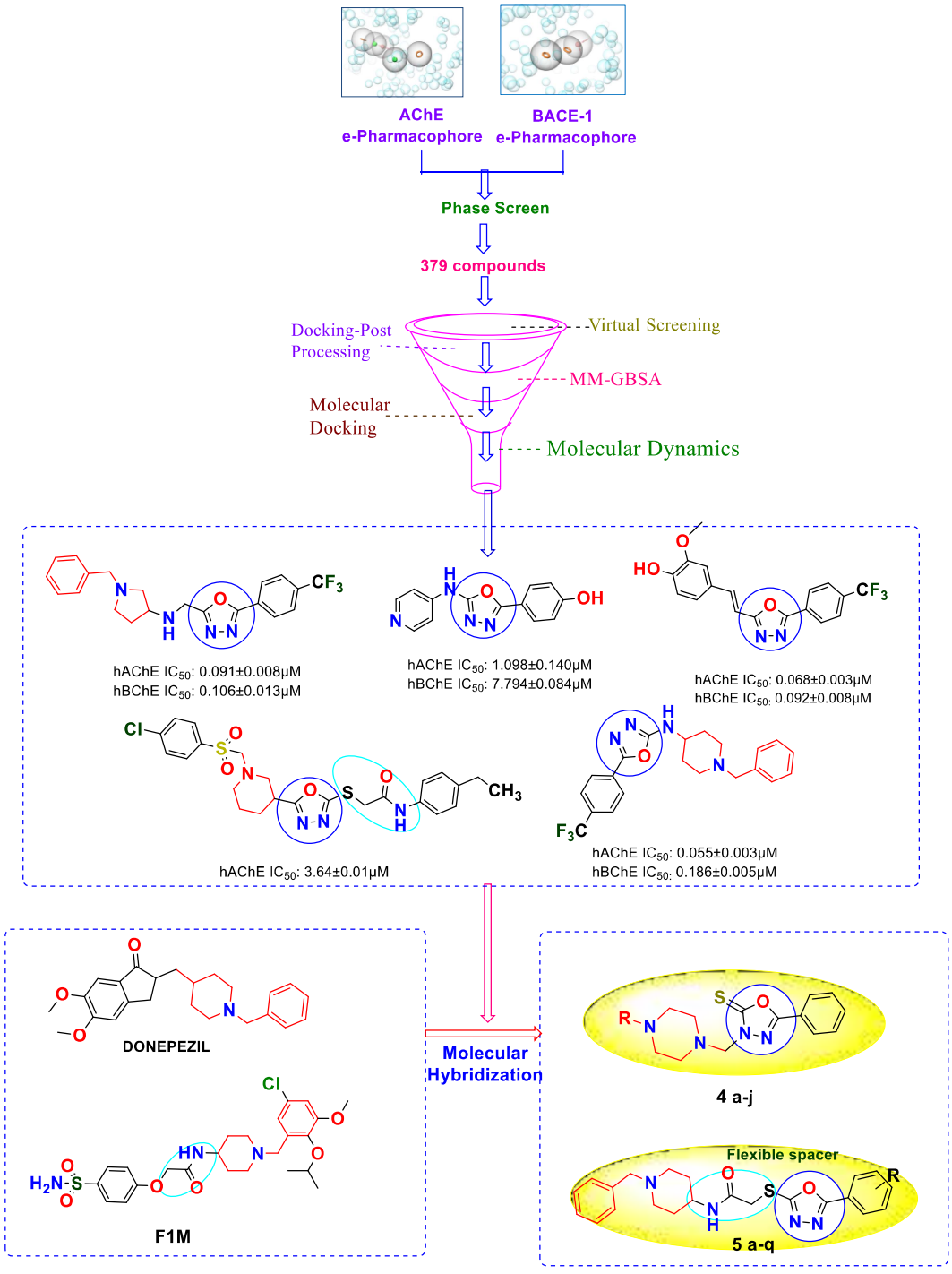

Figure 3. 2 Identified hits and design strategy for the present series of compounds (**4a-j** and **5a-q**)

3.2 Plan of Work

3.2.1 In-silico optimization studies

- Protein Preparation and Grid generation
- 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

3.2.2 Synthesis of 5-phenyl-1,3,4-oxadiazole 2-thione derivatives:

- **Series-I :** 3-NH linked benzylpiperazine derivatives of 5-phenyl-1,3,4-oxadiazole 2-thione
- **Series-I :** 3-NH linked substituted piperazine derivatives of 5-phenyl-1,3,4-oxadiazole 2-thione
- **Series-I :** 2-thiol linked N-(1-benzylpiperidin-4-yl)-2-chloroacetamide derivatives of 5-phenyl-1,3,4-oxadiazole 2-thione

3.2.3 Characterization of synthesized compounds

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

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 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 and elevated plus maze test) on rats/mice models
- Ex vivo biomarker estimation
- Aβ-induced AD phenotypic model (rat/mice)- Morris Water Maze Test
- Western blot analysis
- Immunohistochemical (IHC) analysis

3.3 Significance of the studies

Most of the FDA-approved drugs for the treatment of AD provide only symptomatic relief as the majority of AD survivors experience irreversible dementia and increasing loss of cognition. The AD etiology is still elusive, though several factors are believed to be associated directly with the development and its progression includes increase AChE level, amyloid-beta (Aβ) deposits, BACE-1, and APP over activation which promotes Aβ aggregation via the formation of monomers, protofibrils, annular oligomers, and plaques which leads to oxidative stress and neuronal loss.

Therefore, the multitargeting single ligand approach may be a promising strategy to overcome the disease rather than providing symptomatic relief in AD. Our hypothesis suggests that targeting multiple pathways simultaneously by a lead candidate could eventually reduce the progression of the disease and at the same time will also ameliorate the cognitive deficit caused by the deposition of Aβ plaques and NFTs.