Objective, Rationale, and Plan of Work

Chapter 3

3. Objective, Rationale, and Plan of Work

AD is a complex and progressive neurodegenerative disorder characterized by losing cholinergic neurons in essential brain regions, including the cerebral cortex and subcortical areas. Current therapeutic approaches are primarily centered on alleviating symptoms and decelerating the disorder's progression through the administration of AChE and BChE inhibitors, as well as NMDA receptor antagonists (136). However, these interventions do not target the fundamental pathophysiological processes underlying the disease and fail to provide a definitive cure or prevent its progression.

Considering the multifactorial etiology of AD, which encompasses various underlying mechanisms such as oxidative stress, iron accumulation, and Aβ aggregation, reliance on a single-target approach is likely inadequate. Consequently, there is an urgent need for therapeutic agents with disease-modifying potential that can target these multifaceted pathogenic processes. Researchers have adopted a drug discovery paradigm to develop novel multifunctional agents to address this necessity (137-139). These agents exhibit a combination of pharmacological activities, including AChE/BChE inhibitory action, antioxidant properties, iron-chelating capabilities, and modulation of Aβ aggregation. Relying on single-target drugs often fails to address these interconnected mechanisms, limiting therapeutic efficacy. While combination therapies allow flexible dosing, they can lead to drug-drug interactions, complex regimens, and patient compliance issues (140).

Moreover, the cost of developing multiple drugs, including R&D, preclinical studies, and clinical trials, significantly increases, with estimates often exceeding $2 billion for AD therapies due to high failure rates. In contrast, multifunctional ligands, which can target several pathological pathways simultaneously, offer a streamlined and cost-effective

alternative by reducing the need for multiple clinical trials and simplifying regulatory approval, making them a promising strategy for tackling the multifactorial nature of AD.

3.1. Objective and Rationale

While AChE has traditionally been the primary target for AD treatment, recent developments have highlighted the potential of selective BChE inhibition (26). Studies have shown that BChE activity increases as AD progresses, particularly in regions of the brain associated with cognitive function, while AChE activity decreases (141). This shift suggests selective BChE inhibitors could be more effective in the later stages of AD. Furthermore, selective BChE inhibitors have demonstrated promising *in-vivo* activity in animal models of cognitive deficits, showing potential for improved efficacy and safety profiles compared to traditional AChE inhibitors. In light of these developments, we propose that selective and potent AChE and BChE inhibitors exhibiting multifunctional properties may offer a therapeutic advantage, particularly at different stages of AD (142-144).

Given the significant roles of AChE and BChE in AD pathogenesis, there is an urgent need for compounds capable of precisely inhibiting both enzymes rather than targeting a single enzyme. Additionally, it is imperative for newly developed molecules to target AD's pathological hallmarks, such as oxidative stress, protein aggregation (Aβ), and cell apoptosis.

RIV is a well-known second-generation pseudocholinesterase inhibitor that can selectively cross BBB and irreversibly inhibit BChE. It is recognized as a potent inhibitor of BChE and a moderate inhibitor of AChE. Rivastigmine (RIV) comprises carbamate moiety and a basic amine side chain. Evidence from several literature shows that the carbamate fragment of RIV is the pharmacophore for ChE activity (145, 146). RIV is reported to be a potent inhibitor of BChE without addressing the key hallmarks of oxidative stress, Aβ and tau protein aggregation, and inflammation associated with the disease (145, 147, 148). RIV acts

via carbomylation of the enzyme's active site serine residue. The covalent inhibitors suffer from several limitations (148).

In AD, AChE, BChE, and aforesaid pathological hallmarks play a key role in the onset and progression of the disease. Therefore, multi-targeted ligands (MTDL) have been designed to achieve competitive inhibitors targeting not only BChE but also inhibit AChE along with antioxidant effects, anti-Aβ aggregation tau protein aggregation modulation, and neuroprotective benefits.

Given the fact that the aromatic amino acid residues such as Tyr70, Tyr121, Trp279, Ser203, His447, and Glu334 play a key role in peripheral and catalytic sites of AChE. Interestingly, these features also play an important role in improving its affinity for the hydrophobic regions of Aβ (149, 150). Intriguingly, RIV lacks a balance of aromatic hydrophobic features; therefore, we decided to replace the amine functionality on the right-hand side (**Figure 3.1**) in the design of the first series of molecules with aromatic or heterocyclic features to investigate its dual role in ChE inhibition and Aβ/tau aggregation modulation. Additionally, hydroxylamines (amidoximes) are well-known for their antioxidant properties (151-153). Based on this, we replaced RIV's left-side amine functional group with amidoximes to enhance antioxidant activity while achieving a better balance between hydrophobicity and hydrophilicity.

Furthermore, we replaced the carbamate group of RIV with a urea functionality. Urea forms a stronger bond than carbamate and is less susceptible to attack by serine residues, which is expected to produce reversible inhibitors rather than covalent inhibitors. This strategic molecular design aims to create multifunctional molecules with potent anti-ChE activity and enhanced antioxidant properties. These molecules are also anticipated to effectively modulate the aggregation of key proteins ($A\beta_{1-42}$ and Tau), thereby offering neuroprotection in the context of diseases.

Inflammasomes are complexes of proteins within the innate immune system that recognize patterns associated with pathogens and trigger inflammatory responses. These complexes act as sensors that identify potential threats and initiate a cascade of events leading to the production of inflammatory cytokines, which are important for fighting infections. The inflammasome is composed of several core components, including nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), absent in melanoma 2 (AIM2)-like receptors, pyrin receptors, and an enzymatic component known as caspase 1. NLRP3 inflammasome activation can lead to increased amyloid β deposition in the microglial cells, thus leading to AD development. Recent research has explored the role of targeting NLRP3 inflammasome in activated microglia. Studies have depicted that inhibiting the expression of NLRP3 in the activated microglia has shown a prominent strategy in halting the progression of neuroinflammation and AD.

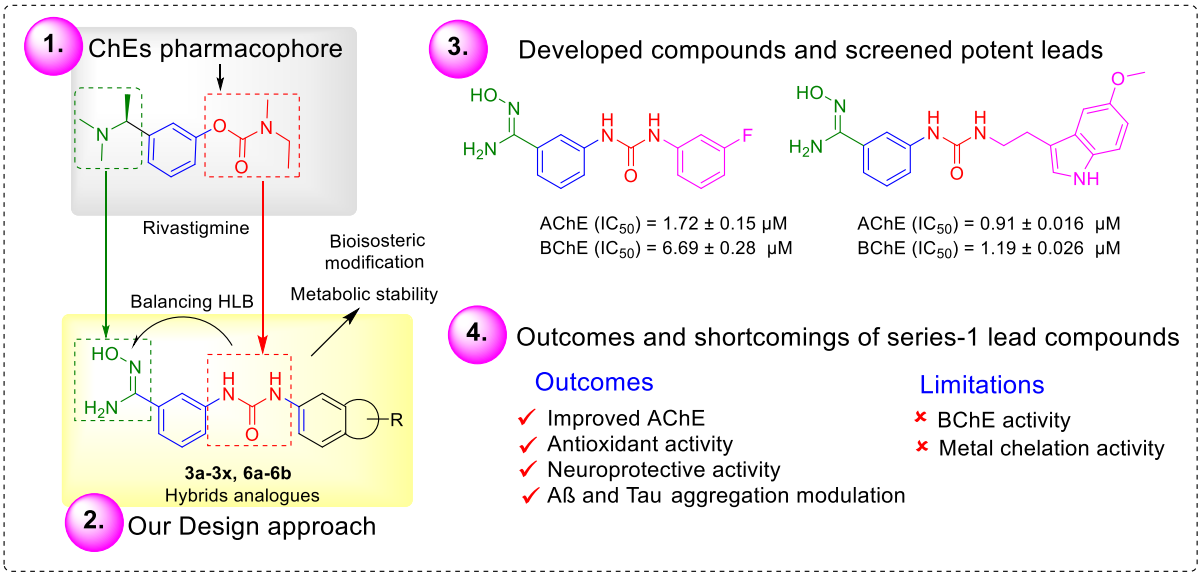

Figure 3.1: The rationale of 1st series of design molecules.

We successfully achieved these desired activities in our first series of designed compounds, including AChE inhibition, antioxidant properties, and modulation of Aβ and tau aggregation. However, despite these promising outcomes, the enhancement of BChE inhibition was not as significant compared to RIV. This highlights a need for further

optimization to improve BChE inhibitory activity while maintaining the other beneficial effects.

RIV is a pseudo-irreversible inhibitor of BChE (IC_{50}= 91 ± 0.40 nM) and a weak inhibitor of AChE (IC_{50}= 6630 ± 0.76 nM), providing only symptomatic relief (154). Given the key role played by both AChE and BChE in AD pathogenesis. RIV is a potent inhibitor of only BChE. Along with this, oxidative stress, inflammation, and Aβ play a significant role in AD neurodegeneration, while RIV is unable to target these critical hallmarks of AD. Therefore, to address the drawbacks of RIV, we have developed mechanism-based multifunctional cholinesterase inhibitors.

We reviewed additional literature on carbamate-based inhibitors to address the limitations observed in our first series. Intriguingly, there are only a few mechanism-based multifunctional molecules reported in the literature, including the development of new carbamate-based selective *h*BChE inhibitors (**JMC-3c**) with two components:(i) the part of the carbamate that is transferred to the catalytically active Ser198 of *h*BChE and is, therefore, mainly responsible for the duration of action in case of additional interaction with an allosteric site, and (ii) the part also referred to as “carrier” on which the high selectivity over *h*AChE described by Decker et al. Also, the carbamate and its hydrolysis product have shown an antioxidant effect (27, 155). Drawing inspiration from Decker *et al*.'s approach, we incorporated these insights into the design of our second series of compounds. This series aimed to develop potent BChE and AChE inhibitors (117, 156) with added therapeutic benefits, including strong antioxidant properties, modulation of Aβ aggregation, and neuroprotective effects. By integrating these features, the second series was intended to serve as disease-modifying therapeutic interventions in managing AD, offering a more comprehensive approach to targeting multiple pathological aspects of the disease.

Our previous study reported **6e** (**Figure 1**), a tryptamine template-based series of molecules that exhibit favorable tolerance towards AChE/BChE and can confer capacity antioxidant and Aβ aggregation modulation properties (156, 157). Literature evidence has shown that the indole moiety of tryptamine exhibits considerable promise as a fragment for advancing targeted anti-amyloid agents (117). In our current design of a series of novel molecules, we have replaced the N-ethyl-N-methylamine moiety of RIV with substituted tryptamine, which is well tolerated by the AChE/BChE enzymes in the first series, to investigate the impact of substituents on binding interactions with the enzyme and their multifunctional properties (158). We hypothesize that these designed molecules will mechanistically carbamoylate the AChE/BChE enzymes, thereby preventing the hydrolysis of ACh. Also, the carbamate and its hydrolysis product have shown an antioxidant effect Aβ aggregation modulation properties.

In our present study, we further explored the effects of the chirality of dimethylaminoethylphenol fragments on enzyme inhibition activity. We started with the racemic version, 3-(1-(dimethylamino)ethyl)phenol fragment of RIV. Among the fragments mentioned above, chloro and hydroxy tryptamine analogs, i.e., **14d** and **14e**, exhibited the most potent inhibition of the target enzyme. Further, selected compounds were synthesized utilizing the S-configuration of the RIV fragment ((*S*)-3-(1-(dimethylamino)ethyl)phenol), resulting in the production of compounds **15a-15e**. Among the developed molecules, the 5-hydroxy (5-OH) and 5-chloro (5-Cl) analogs exhibited the highest potency, displaying approximately a two-fold activity enhancement, further confirming our earlier observation. Based on the enzyme inhibition studies, we selected lead compound **15e** and synthesized the corresponding *R*-configuration-generating of **16e**. As anticipated, all the compounds demonstrated significant inhibitory activity against the enzymes.

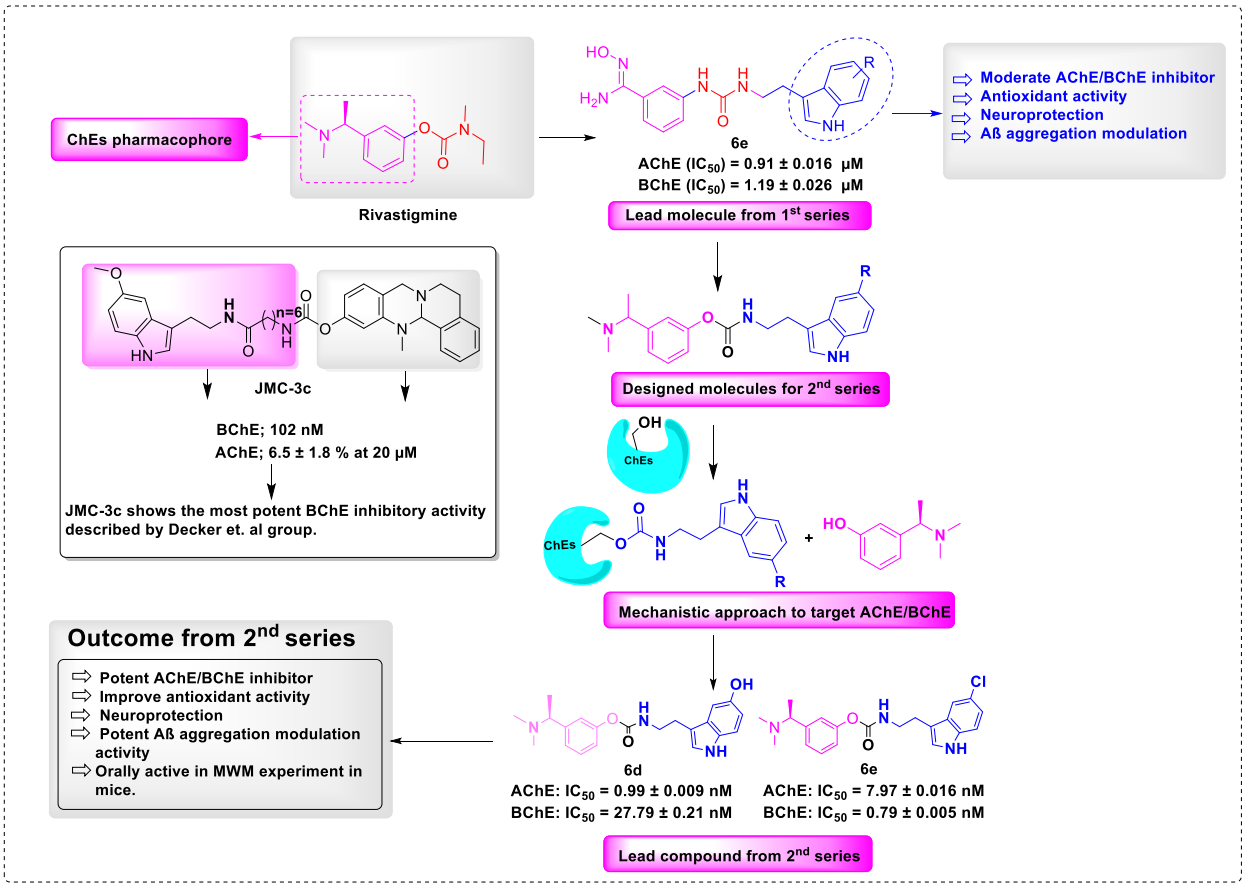

Figure 3.2: The rationale of 2nd series of designed molecules.

3.2. Plan of Work

It is divided into the following headings:

1. **Design, synthesis and characterization of novel urea and carbamate derivatives.**
2. ***In-vitro* biological evaluation.**

- ChEs inhibition studies
- Antioxidant property evaluation
- Enzyme kinetics
- Reversibility studies against AChE and BChE
- Metal chelation study
- Aβ aggregation modulation study
- *In-vitro* blood-brain barrier permeation

3. ***In silico* studies.**

- Molecular docking studies
- Molecular dynamics simulation studies

4. ***In-vitro* cell-based experiments.**

- Cytotoxicity studies
- Neuroprotection studies
- Inhibition of NLRP3 Inflammasome in HMC-3 cell line

5. **Evaluation of toxicity and neuroprotection ability of 3q, 6e, and RIV in AD *drosophila model***

- Mitochondrial ROS and oxidative stress
- Cellular ROS and oxidative stress

6. ***In-vivo* evaluation of the efficacy in AD models.**

- Morris Water Maze Test
- Y-Maze Test

7. ***Ex-vivo* evaluation of neurochemical parameters.**

- Estimation of AChE level
- Estimation of BChE level
- Estimation of superoxide dismutase (SOD) level
- Estimation of Malondialdehyde (MDA) level
- Estimation of Catalase (CAT) level

Figure 3.3: Overview of the designed study.