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. 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 amyloid-beta (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. 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. 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

Earlier we reported **EJMC-4e,** a FA derivative exhibiting moderate activity against AChE and BChE (**Figure** 4.1). However, **EJMC-4e** exhibits weak antioxidant and metal chelation properties. Also, there is scope to further improve upon its AChE and BChE inhibitory property. Therefore, a series of structural modifications were designed to improve the cholinesterase inhibition, antioxidant activity, and metal chelation ability of the compound **EJMC-4e**. The study first examined (**Scheme 1**) the impact of linker length by introducing spacers of different linker lengths (3-5 linkers) between the left-side FA moiety and the right-side amine. Once the optimal linker length was determined, the effect of the Michael acceptor concept (**Scheme 2**) was tested by replacing FA with 3-(4-hydroxy-3methoyphenyl) propanoic acid in compound **EJMC-4e**. We also replaced the distal phenyl ring with indole moiety to evaluate its potential against cholinesterase enzymes. We also explored the effect of hydroxy and methoxy groups on the phenyl ring of FA on enzyme inhibition activity. The results clearly indicated that the double bond, methoxy, and hydroxy groups are necessary for enzyme inhibition. Based on these findings, the FA fragment remained constant while modifications were introduced on the other side of the molecule. Considering the radical-scavenging properties of phenols, we incorporated the hydroxyl groups at different positions on the phenyl ring and assessed whether these modifications would also improve anticholinesterase activities. The pyridine moiety is known for its anticholinesterase and metal chelation capabilities. Therefore, m-anisidine in **EJMC-4e** was replaced with pyridine moieties

(**Scheme 3**) to assess its impact on AChE/BChE inhibitory activity. We also explore biphenyl and naphthyl rings (**Scheme 3**) for their potential to enhance anticholinesterase activity.

Given the previous findings on the well-tolerated behavior of the quinoline moiety against AChE/BChE, we replaced the m-anisidine moiety of **EJMC-4e** with the quinoline moiety (**Scheme 3**) to evaluate its impact on enzyme activities. The choice of the 8-hydroxyquinoline moiety for further investigation was based on its established role in metal chelation and modulation of Aβ, as reported in the literature. Therefore, the 8-hydroxyquinoline moiety was a promising pharmacophore for potential AD agents. Thus, we introduced 8-hydroxyquinoline moiety to assess its impact on enzyme inhibition activities. The aim is to enhance understanding of the optimal structural features for improved anticholinesterase activity, antioxidant properties, Aβ aggregation, and metal chelation activity. We identified **12o** as a lead molecule based on its *in-vitro* enzyme inhibition assay performance. This systematic, iterative approach allows for the comprehensive optimization of **EJMC-4e**, elucidating structural features that enhance cholinesterase inhibition, antioxidant activity, metal chelation, and other multifunctional properties.

In a previous study, we reported the synthesis of FA glycinamide derivatives EJMC **4a-4t** as a multifunctional ligand for targeting AD [147]. Considering the potential of the piperazine ring in developing CNS-active compounds [151-154], we also synthesized and reported piperazine containing FA derivatives **EJMC-10a-10g** [147]. Intriguingly, piperazine-bearing molecules were turned out to be weak inhibitors of AChE and BChE. We further carried out a SAR study by introducing an amide linker between the phenyl ring and piperazine moiety located on the distal part of the earlier identified molecules (**EJMC-10a-10g**) to developed molecules **BMC-3a-3p** (AChE = 30 to 49 % and BChE = < 20 µM)[150]. Interestingly, the developed molecule

BMC-3l (BChE = 14.85 ± 0.37 µM) exhibited better inhibition properties towards BChE over **EJMC-10c** (BChE = 27.66 ± 0.39 %). However, there was scope to further improve upon its AChE and BChE inhibitory properties along with antioxidant and metal chelation properties. So, to further improve upon the multifunctional properties of FA-piperazine-based molecules and given the critical role played by presence of the amide linker towards AChE inhibition in **EJMC-4a-4t** (AChE IC_{50} = 9 to 30 µM and BChE = 19 to 45 %), here we developed a novel series of molecules by introducing an amide linker between ferulic acid-piperazine and the distal phenyl ring present in **EJMC-10c**. We started with the phenyl substitution and synthesized a series of molecules (**Scheme 4**), followed by an evaluation of cholinesterase inhibition activity. These compounds exhibited better activity against AChE. Intriguingly, the developed molecules were found to be weak inhibitors of BChE (Table 4.5). Given the well-tolerated behavior of quinoline towards AChE and BChE in **EJMC-7b** (IC_{50}, (µM) AChE, 4.89 ± 0.37, and BChE, 14.32 ± 0.04), we next replaced the distal phenyl ring with different positions of quinoline ring (**Scheme 5**) to develop a series of novel molecules. The primary objective of replacing phenyl with quinoline was to target the more significant binding site of BChE [155]. It is noteworthy to observe the substantial improvement in BChE activity in the developed compounds (Table 4.5). Given the importance of metals in AD progression, our next goal was to introduce metal chelator, Aβ modulator, and antioxidant enhancer fragment. Earlier researchers have identified 8-hydroxy-quinoline (8-HQ) derivatives, such as **clioquinol**, **PBT-1033, HLA-20, BMC-4g, and BMC-5b (Figure 4.20),** as potential candidates for targeting metal chelation, regulating Aβ, and boosting antioxidant effect [156-160]. Therefore, we decided to replace the quinoline ring with an 8-HQ ring to synthesize an 8-HQ derivative. To further achieve improvement in AChE/BChE inhibitor property, based on

our earlier observation of well-tolerated behavior of the benzyl group present in **BMC-3a-3p** (IC_{50} < 20 µM), we decided to introduce 8-HQ benzyl moiety to synthesize (**Scheme 6**) compound **24a** to improve the AChE/BChE inhibition, antioxidant property and multifunctional properties of these molecules

3.2 Plan of Work

It is divided into the following headings:

1. **Design, synthesis and characterization of novel FA 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. ***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

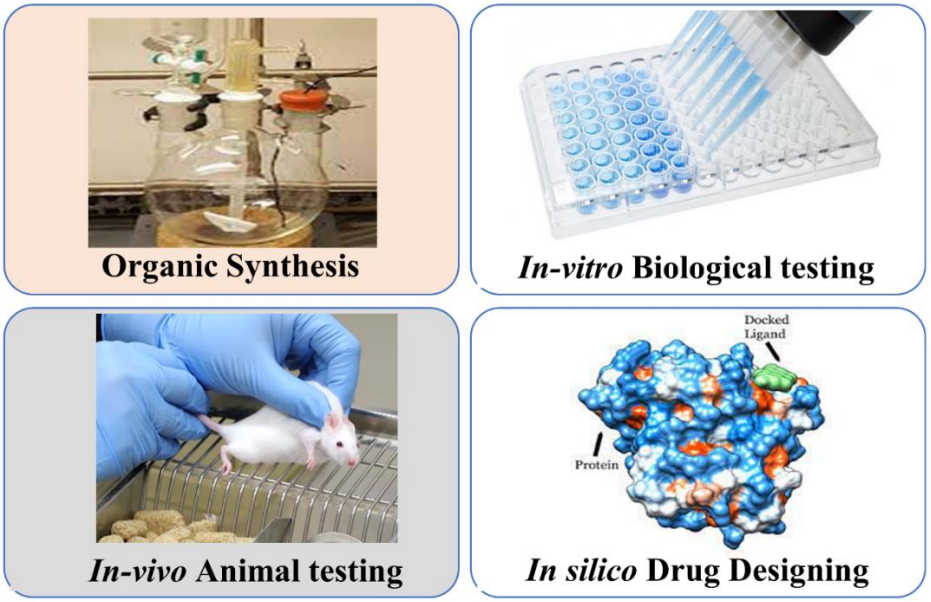
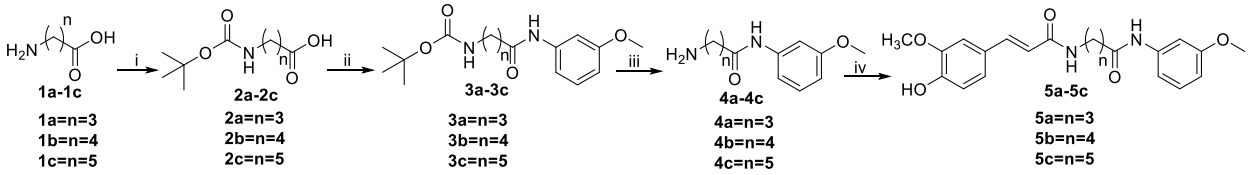


Figure 3.1: Overview of the designed study.

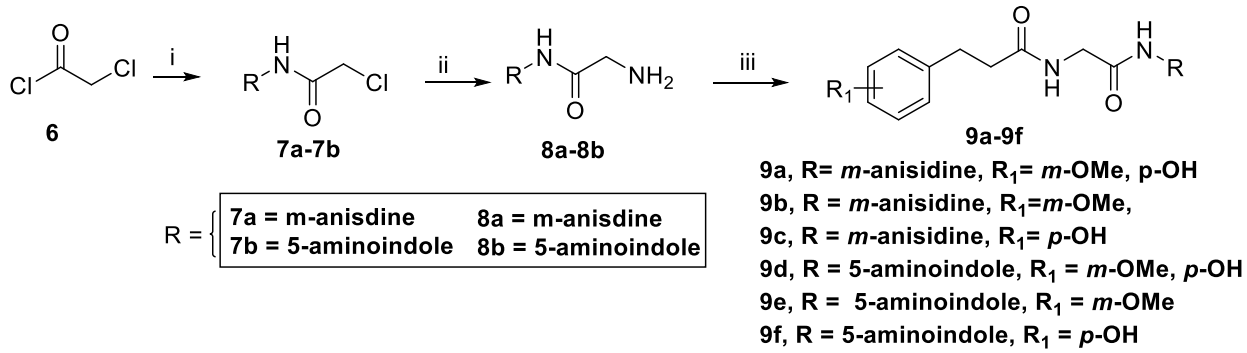
3.3: Synthetic schemes for the synthesis of ferulic acid derivatives

Compounds **5a**–**5c** were synthesized according to the reactions described in **scheme 1**.



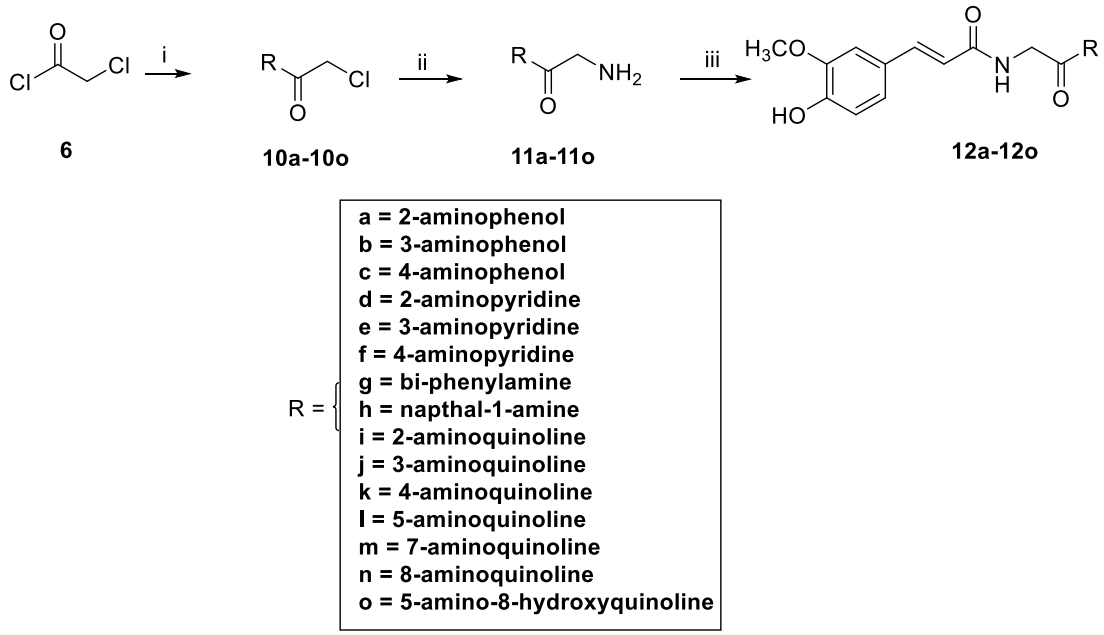
Scheme 1. Synthesis of ferulic acid-amide derivatives **5a-5c**. Reagents and conditions: (i) **Boc**-anhydride- THF-H_2O, rt, 4 hr, (ii) m-anisidine, EDCI, HOBt, DIPEA, THF, rt, overnight, 70-75%. (iii) DCM, TFA, rt, 2 hr., 85-90% (iv) FA, EDCI, HOBt, DIPEA, THF, rt, overnight, 60-65%.

Compounds **9a-9f** were synthesized according to the reactions described in **Scheme 2**.



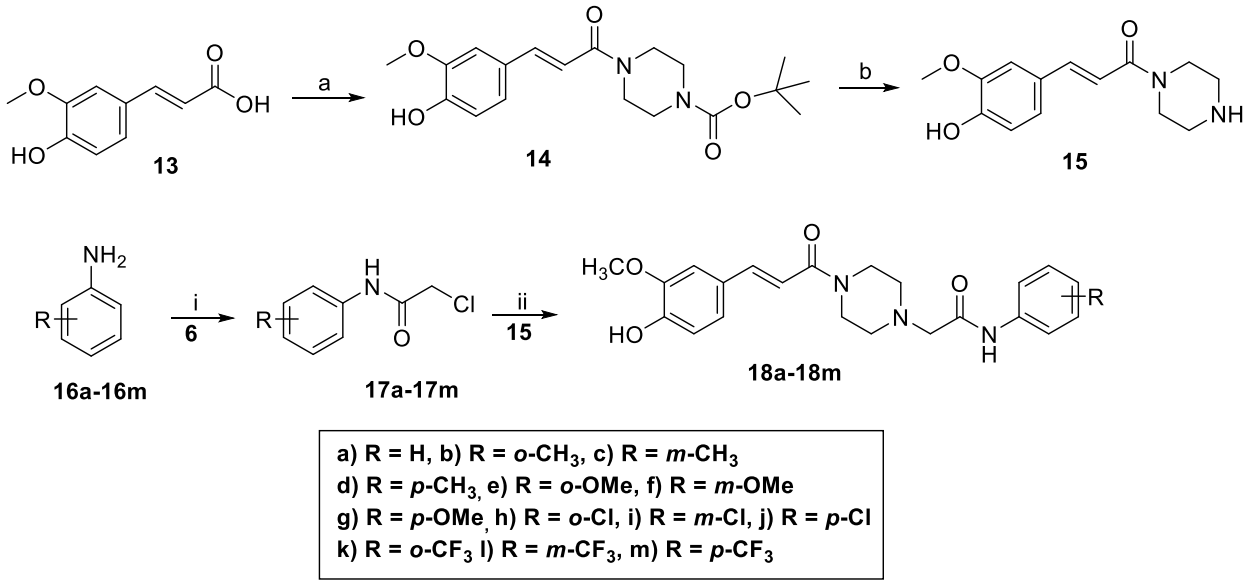
Scheme 2: Synthetic scheme for the synthesis of target compound **9a-9f**. Reagents and conditions: (i) m-anisidine/5-amino indole, K_2CO_3, DCM, 0º C to rt, 2 hr. 90-95%. (ii) Excess of ammonia reflux, 60º C, 6 hr, 85-90%. (iii) Various substituted phenyl propanoic acid, EDCI, HOBt, DIPEA, DCM, rt, 70-75%.

Compounds **12a**–**12o** were synthesized according to the reactions described in **Scheme 3**.



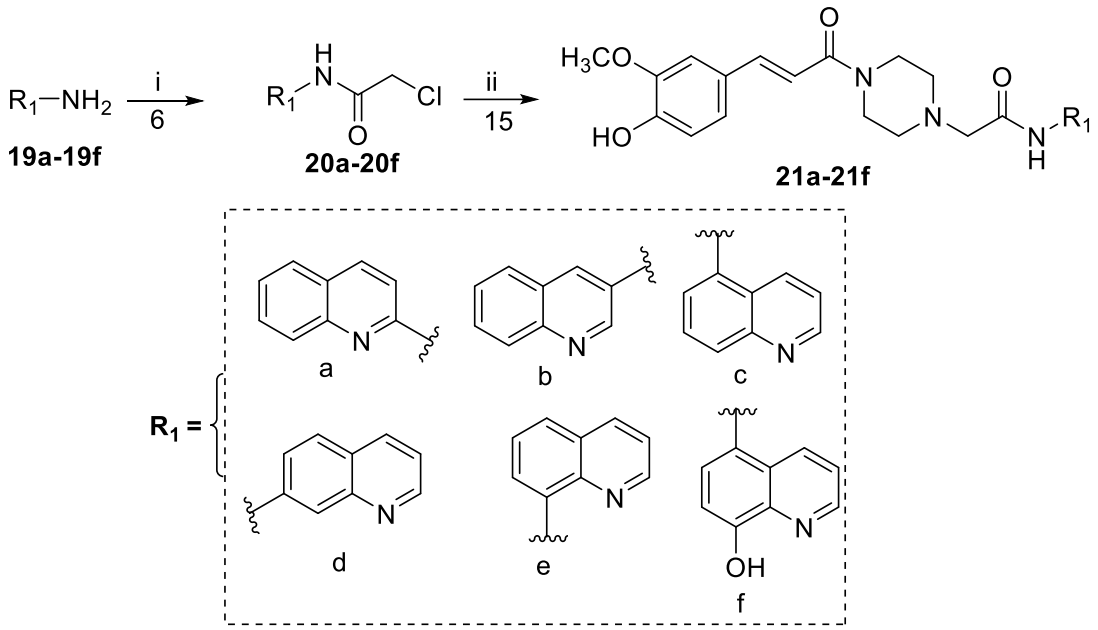
Scheme 3. Synthesis of ferulic acid derivatives **12a-12o**. Reagents and conditions: (i) K_2CO_3, DCM, 0 °C, 2 hr, 90-95%; (ii) Excess NH_3, solution, 60 °C, 6 hr, 80-85%; (iii) FA, EDCI, HOBt, DIPEA, THF, rt, overnight, 60-65%.

Compounds **18a**–**18m** were synthesized according to the reactions described in **Scheme 4**.



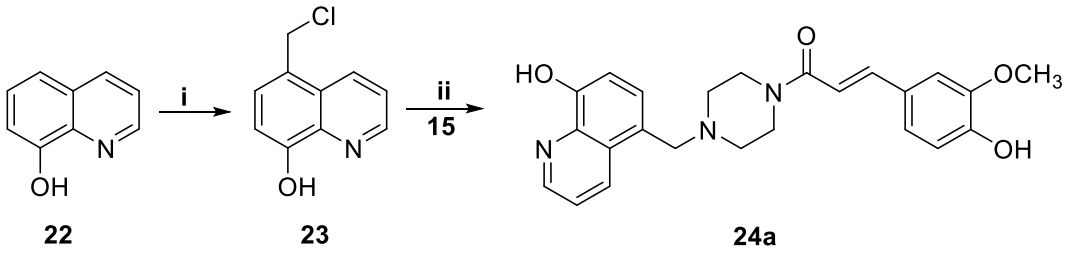
Scheme 4. Synthesis of ferulic acid-piperazine derivatives **18a**-**18m**. Reagents and conditions: (a) EDCI, HOBt, DIPEA, DCM, rt, overnight, 75-85%. (b) TFA, DCM Rt 2hr. i) Chloroacetyl chloride (6), K_2CO_3, DCM, 0 °C, 2 h, 90-95%; (iii) FAPIP (15), K_2CO_3, ethanol, reflux, 4 hr, 60-65%.

Compounds **21a**–**21f** were synthesized according to the reactions described in **Scheme 5**.



Scheme 5. Synthesis of ferulic acid-quinoline derivatives **21a-21f**. Reagents and conditions: (i) K_2CO_3, DCM, chloroacetyl chloride (6), 0 °C, 2 hr, 90-95%, (ii) FAPIP (15), K_2CO_3, Ethanol, reflux, 6 hr, 60-65%.

The second series of compound **24a** was synthesized following the reaction outlined in **scheme 6**.



Scheme 6: Synthetic route of target compound **24a**. Reagents and conditions: (i) HCHO, HCl gas, 70-80% (ii) FAPIP (15), K_2CO_3, Ethanol, reflux for 6 hr, 60-65%.