Abstract

Design, Synthesis, And Biological Evaluation of Mechanism Based Potent AChE And BChE Inhibitors Exhibiting Multifunctional Properties For The Management of Alzheimer's Disease

Alzheimer's disease (AD) is a complex and progressive neurodegenerative disorder characterized by the loss of 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. By targeting multiple pathological pathways involved in AD progression, these multifunctional agents hold promise as more effective disease-modifying treatments compared to existing options. This strategy is considered a promising avenue for the development of drugs that offer a more comprehensive and impactful response to the complexity of AD.

Rivastigmine (RIV), a second-generation pseudo-irreversible cholinesterase inhibitor, is a widely used therapeutic agent for AD. However, it exhibits several limitations that restrict its efficacy as

a comprehensive treatment. RIV shows selective inhibition of butyrylcholinesterase (BChE; IC_{50} = 91 ± 0.40 nM) while only moderately inhibiting acetylcholinesterase (AChE; IC_{50} = 6630 ± 0.76 nM), making it less effective in the earlier stages of AD where AChE activity predominates. Additionally, RIV provides symptomatic relief without targeting the pathological hallmarks of AD, such as oxidative stress, amyloid beta (Aβ) and tau protein aggregation, inflammation, and neurodegeneration. Its covalent mechanism of action through carbomylation of the enzyme's active site serine residue further limits its safety profile by causing pseudo-irreversible inhibition and potential off-target effects. To address these drawbacks, we designed two series of multifunctional molecules to enhance therapeutic efficacy.

The first series of molecules focused on improving RIV's limitations by replacing its amine group with aromatic and heterocyclic moieties to enhance interactions with AChE and BChE while modulating Aβ and tau aggregation. The carbamate moiety of RIV was replaced with amidoxime to enhance antioxidant properties and achieve a better balance between hydrophobicity and hydrophilicity. This series showed significant improvement in AChE inhibition, antioxidant properties, and modulation of Aβ and tau aggregation, but BChE inhibition remained suboptimal compared to RIV. In the second series, insights from the literature on carbamate-based selective BChE inhibitors were utilized to design potent inhibitors with additional therapeutic benefits. Substituted tryptamine fragments replaced the N-ethyl-N-methylamine group of RIV to improve multifunctional activity, including inhibition of AChE and BChE, antioxidant effects, and modulation of Aβ aggregation. Chirality was optimized by synthesizing the molecules' racemic and enantiomeric versions, with S-configured analogs exhibiting superior potency. Compounds such as **15e** and **16e** demonstrated enhanced inhibitory activity and antioxidant and neuroprotective properties, offering a disease-modifying therapeutic approach. These rationally

designed molecules aim to overcome the limitations of RIV and provide a comprehensive strategy to target multiple pathological aspects of AD.

Summary of the work

In this study, we designed and synthesized novel diarylurea-hydroxyamidine analogs as multifunctional agents for treating AD. These compounds were evaluated as potential ChEs (*h*AChE and *eq*BChE) inhibitors. Among the synthesized compounds, compounds **3q** and **6e** exhibited the most potent AChE inhibition with (**3q** IC_{50}: 1.72 ± 0.15 μM, **6e** IC_{50}: 0.91 ± 0.016 μM), and BChE inhibition (**3q** IC_{50}: 6.69 ± 0.28 μM, **6e** IC_{50}: 1.19 ± 0.026 μM respectively). 3q and 6e were further evaluated for enzyme kinetics study among the synthesized compounds. The data from the enzyme kinetic study proved that **3q** and **6e** showed mixed inhibition of AChE and competitive inhibition of BChE. Significantly, compounds **3q** and **6e** exhibited remarkable antioxidant activity (IC_{50} 16.15 ± 1.05 & 15.17 ± 0.07 μM, for 3q & 6e, respectively) in the DPPH assay among all tested molecules. PAMPA experiments indicated that compounds **3q** and **6e** exhibited permeability (Pe) values exceeding 4, suggesting their potential to cross the blood-brain barrier and effectively target brain sites. Interestingly, **3q** and **6e** could effectively inhibit self-induced full-length tau and $A\beta_{1\text{-}42}$ aggregation. **3q** and **6e** were found to curtail microglial cell proliferation, mitigating the damage arising from LPS and ATP-induced ROS and MMP. In addition, acute toxicity tests in mice showed that **3q** and **6e** had no toxicity at the dose of 2000 mg/kg. The **3q** and **6e** inhibited NLRP3 inflammasome and reduced microglial cell proliferation in response to LPS and ATP-induced ROS and MMP. It also demonstrated the ability to reverse scopolamine-induced amnesia by enhancing spatial and cognitive memory in the AD mice model. Additionally, compared to the scopolamine treatment group, these compounds upregulated

neuroprotective biomarkers, including BDNF and TRKB. The above results indicate that compounds 3q and 6e could be new multi-target-directed ligands for treating AD.

We further extend the SAR and lead to developing a new series of compounds derived from the rivastigmine fragment template **15d and 15e**, explicitly focusing on enhancing anti-cholinesterase, antioxidant, and metal chelation properties. In summary, we designed and synthesized novel tryptamine-rivastigmine hybrid analogs as multifunctional agents for treating AD. These compounds were evaluated as potential ChEs (*h*AChE and *eq*BChE) inhibitors. Among the synthesized compounds, compounds **15d** and **15e** as the lead molecules with a potent inhibitor against AChE (**15d**, IC_{50}**:** 0.99 ± 0.009 nM and **15e**, IC_{50}: 7.97 ± 0.016 nM and BChE (**15d**, IC_{50}: 27.79 ± 0.21 nM and **15e**, IC_{50}: 0.79 ± 0.005 nM respectively), compared to the marketed drug Riv (AChE, IC_{50}: 6630 ± 0.76 nM, BChE IC_{50} = 91 ± 0.40 nM, respectively). Compound 6e exhibited remarkable radical scavenging activity in the DPPH assay (IC_{50}: 22.91 ± 1.73 µM) compared to rivastigmine (% radical scavenging activity: 3.71 ± 0.09 at 200 µM) among all tested molecules. PAMPA experiments indicated that compounds **15d** and **15e** exhibited permeability (Pe) values exceeding 4, suggesting their potential to cross the blood-brain barrier and effectively target brain sites. Lead molecules **15d** and **15e** demonstrated the capability to counteract oxidative stress and amyloid-induced neuronal death in SH-SY5Y cells. Interestingly, **15d** and **15e** could inhibit self-induced full-length $A\beta_{1-42}$ aggregation. In addition, acute toxicity tests in mice showed that **15d** and **15e** had no toxicity at the dose of 175 mg/kg. It also demonstrated the ability to reverse scopolamine-induced amnesia by enhancing spatial and cognitive memory in the AD mice model at doses as low as 0.3 and 0.5 mg/kg. Additionally, compared to the scopolamine treatment group, these compounds upregulated neuroprotective biomarkers, including BDNF and TRKB. The

above results indicate that compounds **15d** and **15e** could be new multi-target-directed ligands for treating AD.

List of Publications

1. Shankar G, Kumar P, Rai S, Ghosh A, Varma T, Wani MA, Kumar S, Mandloi U, Singh GK, Garg P, Kulkarni O, Srikrishna S, Kumar S, Modi G. Discovery of novel hybrid tryptamine-RIV molecules as potent AChE and BChE inhibitors exhibiting multifunctional properties for the management of Alzheimer's disease. Eur J Med Chem. 2024 Nov 27; 283: 117066. doi: 10.1016/j.ejmech.2024.117066.

2. **Shankar G,** Praveen Kumar C, Yadav M, Ghosh A, Panda SR, Banerjee A, Tiwari A, Rai S, Kumar S, Garg P, Naidu VGM, Kulkarni O, Modi G. Discovery of novel substituted (Z)-N'-hydroxy-3-(3-phenylureido)benzimidamide derivatives as multifunctional molecules targeting pathological hallmarks of Alzheimer's disease. Eur J Med Chem. 2024 Dec 15; 280:116959. doi: 10.1016/j.ejmech.2024.116959.

3. Kumar, J., **Shankar, G,** Kumar, S. *et al.* Design, synthesis and biological evaluation of novel piperic acid and benzylpiperazine hybrid molecules for improvement of memory impairment via cholinesterase inhibitory activity. *Chem. Pap.* (2024). https://doi.org/10.1007/s11696-024-03787-7.

4. Kumar J, **Shankar G**, Kumar S, Thomas J, Singh N, Srikrishna S, Satija J, Krishnamurthy S, Modi G, Mishra SK. Extraction, isolation, synthesis, and biological evaluation of novel piperic acid derivatives for the treatment of Alzheimer's disease. Mol Divers. 2024 Jun;28(3):1439-1458. doi: 10.1007/s11030-023-10667-x.

5. Singh G, **Shankar G,** Panda SR, Kumar S, Rai S, Verma H, Kumar P, Nayak PK, Naidu

VGM, Srikrishna S, Kumar S, Modi G. Design, Synthesis, and Biological Evaluation of Ferulic Acid Template-Based Novel Multifunctional Ligands Targeting NLRP3 Inflammasome for the Management of Alzheimer's Disease. **ACS Chem Neurosci.** 2024 Apr 3;15(7):1388-1414**.** https://doi.org/10.1021/acschemneuro.3c00679.

6. Venkatesh R, **Shankar G,** Narayanan AC, Modi G, Sabiah S, Kandasamy J. Multicomponent Synthesis of S-Benzyl Dithiocarbamates from para-Quinone Methides and Their Biological Evaluation for the Treatment of Alzheimer's Disease. J Org Chem. 2022 May 20;87(10):6730-6741. doi: 10.1021/acs.joc.2c00423.

7. Singh YP, Kumar N, Priya K, Chauhan BS, **Shankar G**, Kumar S, Singh GK, Srikrishna S, Garg P, Singh G, Rai G, Modi G. Exploration of Neuroprotective Properties of a Naturally Inspired Multifunctional Molecule (F24) against Oxidative Stress and Amyloid β Induced Neurotoxicity in Alzheimer's Disease Models. **ACS Chem Neurosci.** 2022 Jan 5;13(1):27-42. https://doi.org/10.1021/acschemneuro.1c00443.

8. Singh YP, **Shankar G**, Jahan S, Singh G, Kumar N, Barik A, Upadhyay P, Singh L, Kamble K, Singh GK, Tiwari S, Garg P, Gupta S, Modi G. Further SAR studies on natural template based neuroprotective molecules for the treatment of Alzheimer's disease. **Bioorg Med Chem.** 2021 Sep 15;46:116385. doi: 10.1016/j.bmc.2021.116385.

9. Singh YP, Tej GNVC, Pandey A, Priya K, Pandey P, **Shankar G,** Nayak PK, Rai G, Chittiboyina AG, Doerksen RJ, Vishwakarma S, Modi G. Design, synthesis and biological evaluation of novel naturally-inspired multifunctional molecules for the management of Alzheimer's disease. Eur J Med Chem. 2020 Jul 15;198:112257. doi: 10.1016/j.ejmech.2020.112257.

Manuscript Under Revision

10. Himanshu Rai, Rishabh Singh, **Shankar G,** Sanskriti Rai, Prabhat Kumar, Aishwarya S. Nilakhe, Neha Singh, Poonam Bhadoria, Venkatnarayan Ramanathan, Gourav Singh, Sarika Gupta, Sairam Krishnamurthy, Saripella Srikrishna, Saroj Kumar, Gyan Modi. Discovery of novel NIRF theranostic probes targeting amyloid-β fibrils and cholinesterases in Alzheimer's disease models. **Nature Communication (Under revision).**

Manuscript Under Preparation

11. Evaluation of neuroprotection properties of (Z)-N-hydroxy-3-(3-phenylureido) benzimidamide (EJMC-3e and 6q) in oxidative stress and Amyloid in Alzheimer's disease models.

PATENT GRANTED

1. A natural template-based anticholinesterase inhibitors and antioxidants for the treatment Alzheimer's disease. Gyan Modi, Yash Pal Singh, **Shankar G**, Gourav Singh, Atanu Barik, Lovejit Singh. Patent #202111016470.
2. A multifunctional diaryl ureas-hydroxyamidine based compounds for the treatment of Alzheimer's disease. Gyan Modi, Yash Pal Singh, C. Praveen Kumar, Meenu Yadav, **Shankar G**, Gourav Singh, Saroj Kumar, S. Srikrishna. Patent #202111001482.

PATENT FILED

1. Discovery of novel tryptamine-rivastigmine analogs as potent AChE and BChE inhibitors. **Shankar G,** Gyan Modi, Sunil Kumar. Patent No. #202411081240.

2. An Imaging Probe for Detection of Key Biomarkers in Alzheimer Disease. Gyan Modi**,** Himanshu Rai, Brijesh Singh, Saroj Kumar, Rishab Singh, Sarika Gupta, Sairam Krishnamurthy, **Gauri Shankar**, S. Srikrishna.ICMR-202211027649. DATE OF FILLING: May 13, 2022

SEMINARS & WORKSHOPS ATTENDED:

1. Participated in a workshop on "Biological Evaluation of Brain Targeting Molecules" Held at **IIT BHU**, November 10-12, 2022.

2. Presented poster at 48th Annual Conference of Indian Immunology Society, Infections, Vaccines & Immuno-Innovations for Human Health, held at Human Genetics Department **BHU** July 8-9, 2022.

3. Participated in a sponsored Workshop on the topic "Role of Artificial Intelligence and Machine Learning in Drug Discovery, held at the Institute of Pharmacy, Harishchandra PG College, **Varanasi,** December 12-17, 2022.

4. Presented Poster 2nd National Conference on CONTEMPORARY FACETS IN ORGANIC SYNTHESIS (CFOS-2022) in partnership with Royal Society of Chemistry, held at **IIT Roorkee**, December 01-04, 2022.