ABSTRACT

Discovery and Characterization of Small Molecule Inhibitors of Choline Acetyltransferase

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Introduction

Neurodegenerative diseases are a collective group of pathological conditions negatively affecting the central and peripheral nervous systems, leading to the progressive loss of neuronal structures and its function causing neuronal cell death. Alzheimer's Disease (AD) is one of the most predominant neurodegenerative diseases which accounts for 60 to 80 % cases of dementia, affecting the cerebral cortex and hippocampus of the brain. A common denominator in such neurodegenerative diseases is the degeneration of the neuronal cholinergic system. Such neurodegenerative disease alone constitutes major challenges and tremendous unmet needs, in term of effective tools for clinical and/or research purposes. This is perhaps one of the reasons why despite the intensive search for the past half century only symptomatic treatments are currently available. Nonetheless, AD is a complex multifactorial disease, making it highly challenging to find a cure.

Cholinergic hypotheses in AD, is linked with the decline in the key cholinergic neurotransmitter, acetylcholine. Choline Acetyltransferase (ChAT) is the enzyme responsible for the biosynthesis of acetylcholine, a neurotransmitter involved in memory, learning, and other cognitive processes. The maintenance of ChAT expressions is essential for proper neuronal function and overall health of the brain and the body, and disruption in its expression or function can have detrimental effects on both mental abilities and neurotransmitter balance and motor controls. Thereby, it is one of the potential targets for development of biomarkers for monitoring the health of the cholinergic neurons in the central and peripheral nervous system for early-stage diagnosis. In addition, detecting early bio-signature changes of AD should help to halt the progression by suitable therapeutics.

One of the major challenges for targeting ChAT is that very few ligands for this enzyme is known. Most of these are ChAT inhibitors, containing primarily naphthyl-vinylpyridine derivatives, stilbazole derivatives, alkylaminoethyl esters and α-NETA, with the drawbacks like lack of selectivity, irreversible binding and poor blood brain barrier (BBB) permeability. Overall, discovery of novel and selective ligands for ChAT is of utmost importance for providing us with tools that can increase our understanding and accelerating research on early pathological events affecting the diverse cholinergic neurons, as well as for aiding in early phase diagnosis of the onset of a spectrum of neurodegenerative disorders, in which cholinergic dysfunction is one of the key feature of the disease, such as AD, Lewy body disorder (LBD, including Parkinson's dementia), Down's syndrome and ALS. In the following studies we aim to identify selective and potent inhibitors of ChAT keeping the above

shortcomings in mind to overcome the same. Thus, such novel hit molecules hold the potential to be further explored using SAR studies to develop a potent lead compound possessing high affinity, selectivity and optimal physicochemical properties along with good blood brain barrier (BBB) permeability which can be used as molecular probes in positron emission tomography (PET) imaging for mapping the ChAT distribution in the brain for the early phase diagnosis of the health of cholinergic neurons in neurodegenerative diseases like AD.

Study 1: Proton pump inhibitors (PPIs) have revolutionised the management of stomach acid suppression in patients with gastro-oesophageal reflux disease. However, evidence suggests that Long-term PPI use may increase the risk of developing AD. In previous report from our lab, we presented findings on the inhibitory effects of PPIs on ChAT. Here in this study, our objective was to understand the mechanism of binding of PPIs in the ChAT binding tunnel, we have employed a series of computational tools, namely molecular docking and classical molecular dynamics to gain a mechanistic understanding of the molecular interactions between PPIs and the binding pocket of ChAT. Enabling the elucidation of protein-ligand complexes binding interactions, conformational stability, and dynamic evolution within a time frame of 200 nanoseconds. Further, the binding free energies for the complexes under investigation were calculated using Molecular Mechanics Poisson-Boltzmann Surface Area. The findings indicate that the PPI's have comparable or greater binding affinity to the ChAT catalytic tunnel in comparison to the standard compound α-NETA. Additionally, it was observed that the pyridine ring of the PPI's predominantly interacts with the catalytic residue HIS324. Moreover, the free energy landscape analysis showed that the folding process was linear, and the residue interaction network analysis provided insight into the roles of various amino acid residues in stabilization of the PPIs in the ChAT binding pocket. As a major factor for the onset of Alzheimer's disease is linked to cholinergic dysfunction, our previous and the present findings give clear insight into the PPI's interaction with ChAT.

Study 2: In this study, we have explored the novel piperidine scaffold of the previously identified hit compound B4 from our lab with an objective to simplify the structure to improve its selectivity towards ChAT and also possess better blood brain barrier permeability (BBB), synthesizing 60 novel derivatives. The compounds were tested in-vitro for their ChAT inhibitory activity as well as selectivity by screening them against Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE). Which led to the identification of a potent hit compound A-1, on further evaluation cytotoxicity analyses indicated no toxicity and it was found to have good aqueous solubility we proceeded with testing it for in-vivo

pharmacokinetics and brain kinetics which suggested that the compound has optimum pharmacokinetic parameters along with good BBB permeability. Moreover, in-silico including molecular docking and molecular dynamics simulation of 200 ns supported the favourable interaction of the compound with the HIS324 amino acid residue and formed a stable complex during the simulation.

Study 3: Based on the success of our previous studies our aim was to explore large chemical space to identify novel hits. Here, we have successfully utilised structure-based virtual screening approach to screen a VITAS-M small molecule library containing ~1.4 million compounds by using a structure-based virtual screen protocol based on MPI-Vina. Identifying 46 top performing hits displaying prominent interaction with the catalytic residue His324 which is necessary for inhibitory activity of ligands. The compounds were procured and were then subjected to rigorous in vitro characterization which led to the identification of two novel, selective and potent ChAT inhibitors V6 (STK161404) and V15 (STK306932). The compounds showed good aqueous solubility and cytotoxicity analyses indicated no toxicity. We also performed a 200 ns molecular dynamics simulation, which revealed the intricate interaction dynamics for V6 and V15 with ChAT binding pocket. the Tanimoto similarity analysis indicated the novelty and structural diversity of the hits.

Study 4: Traditional virtual screening protocols has a lot of limitations given the current rate of chemical database expansion exceeding billions, demanding much faster screening protocols. Motivated from the great success of our study 3 here we have tapped into the power of Artificial Intelligence to explore the domain of an ultra-large compound library. Deep Docking (DD) is one such platform that leverages the power of DNN-based virtual screening, empowering researchers to dock billions of molecules in a speedy, yet explicit manner. Here, we have screened 1.3 billion compound library from ZINC20 database, identifying the best performing hits. Where a subset of the compound's library is sampled and docked using MPI-Vina and then a QSAR model is built upon the resultant docking scores obtained with the 2D fingerprints of the sampled compounds, which is then used to predict the docking scores of the rest of the compounds in the library. The process is repeated for 'n' number of iterations until the library has been reduced to manageable size. During our runs the first iteration gave ~116 million hits out of 1.3 billion compounds, the second iteration gave ~3.7 million hits and the final third iteration gave 168447 hits from which further refinement using pharmacokinetic pre filters and manual docking pose examination for its interaction with the catalytic residue HIS324 of the ChAT, gave us the top 5 compounds CPD1 (ZINC001329075107), CPD2

(ZINC000685404542), CPD3 (ZINC001258168283), CPD4 (ZINC000102819103) and CPD5 (ZINC001255982897) as potential ChAT inhibitors. Furthermore, to understand the dynamic behaviour of the compounds in the ChAT binding tunnel we performed molecular dynamics simulations for 200 ns on the complexes, which suggested that the formed complexes are stable and are compatible with the ChAT binding tunnel. We further extended our analysis to identify the per residue H-bond interactions taking place where it was observed that the compounds were forming H-bond interactions with the catalytic residue HIS324 throughout the simulation, which indicated the compounds can inhibit ChAT. The discovery of novel ChAT inhibitors will enable researchers to develop new probes that can be used as novel theranostic agents against cancer and as an early-stage diagnostics for onset of AD, for timely therapeutic intervention to halt the further progression of AD.

Conclusion

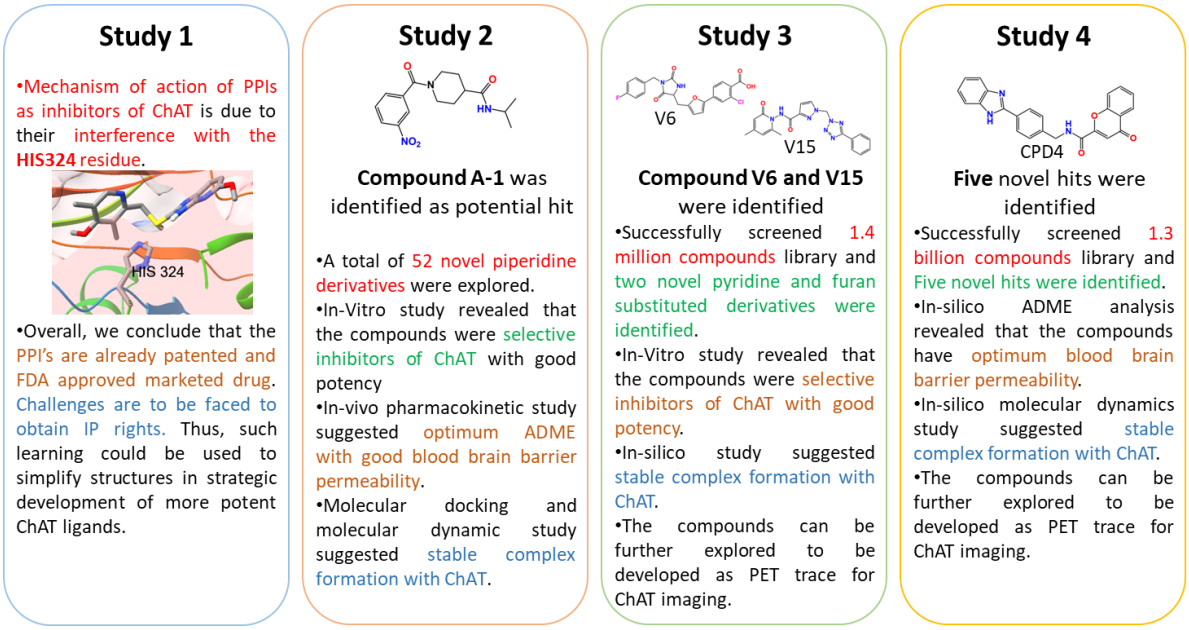

Fig 1. Overall summary of the research work carried out in the thesis.

In conclusion, we have primarily focused on the discovery of selective and potent ChAT inhibitors, having optimum pharmacokinetics and BBB permeability (**Fig .1**). We deduced the binding mechanistic of how PPIs inhibit the ChAT protein. Based on the learnings we synthesized 52 compounds simplifying the piperidine scaffold of previously identified potent inhibitor B4. In-vitro studies suggested compound A1 to be most potent and selective ChAT inhibitor of the series and in-vivo Pharmacokinetic and brain kinetic studies suggested the compound has optimum pharmacokinetic parameters along with good BBB permeability. Further more we have performed virtual screening of a large library of 1.4 million compounds

using structure based virtual screening which led us to the identification of two novel, potent and selective inhibitors of ChAT namely V6 and V15, when tested in-vitro. Motivated from the great success we further harnessed the power of AI to screen a much bigger chemical space of an ultra-large library of 1.3 billion compounds which resulted in the identification of 5 hit compounds CPD1, CPD2, CPD3, CPD4 and CPD5 as potent inhibitors of ChAT. Overall, such novel hit molecules along with our previously reported compounds hold the potential to be further explored using SAR studies to develop a potent lead compound possessing high affinity, selectivity and optimal physicochemical properties which can be used as molecular probes in PET imaging for mapping the ChAT distribution in the brain for the early phase diagnosis of the health of cholinergic neurons in neurodegenerative diseases like AD.