CHAPTER 6

CONCLUSION & FUTURE SCOPE

Protein folding is a critical process for any protein to perform its biological functions. Protein synthesis, attaining correct structure and degradation must be critically regulated to keep the biological processes, go on constitutively. There are cell quality control systems in place which oversee this entire process. Despite tight regulations, a protein can sometimes fail to attain the native structure and can form partially folded states. Chaperones assist misfolded / partially folded proteins fold in native conformation. If this chaperone mediated folding mechanism fails, proteins are degraded by ubiquitinylation and subsequent proteolysis. Under certain circumstances like ageing, mutations, and metabolic diseases, the cell quality control can fail to convert the partially folded state to the native state, in which case the protein becomes kinetically trapped in local energy minima as misfolded structures. Over time, accumulating such misfolded proteins without proper clearance can start accumulating and forming amorphous aggregates and, subsequently, oligomeric intermediates. Deposition and lack of clearance of these oligomeric intermediates can ultimately form stable amyloid fibrils associated with various neurodegenerative and pathological conditions.

Protein misfolding and aggregation is not only a great intellectual challenge but also of excellent therapeutics importance. Protein misfolding is associated with various pathological conditions in humans, including neurodegenerative diseases like Alzheimer's (AD), Parkinson's (PD), and prion-related protein misfolding diseases. Drug discovery processes for protein

misfolding diseases are still a tremendous scientific challenge as the protein folding process is still an enigma, and the underlying processes are still poorly understood. Protein amyloids usually form stable β-sheet-rich structures, as reported in most cases. Amyloid fibrils represent a very stable class of structures in terms of thermodynamics. Among various schools of the hypothesis, the toxicity of oligomeric intermediates is well established and accepted compared to matured fibrils. Currently, therapeutic development for protein misfolding diseases focuses on these oligomeric intermediates.

We have utilized extensive computational methods like molecular docking and simulation to analyse the interactions of various nucleosides, nitrogenous bases and nucleotides with aggregation-prone zones of model amyloid protein, *i.e.*, Hen egg While Lysozyme (HEWL). We further analyzed the binding interactions and molecular docking statistics to identify the best binding molecules. The hypothesis for this work mainly revolved around the fact that interaction of various intracellular metabolites with aggregation-prone zones within the protein would prevent intracellular interactions within these regions, which could drive the protein away from aggregation-mediated pathways under stress conditions. We have identified a few small intracellular metabolites like nucleosides and nitrogenous bases with the best docking parameters within the aggregation-prone regions of HEWL. The selected metabolites show a good binding affinity and potential anti-amyloid properties. The encouraging results with few compounds prompted us to test the hypothesis experimentally. A general mechanism of the hypothesis presenting the computational study is shown in Figure 6.1.

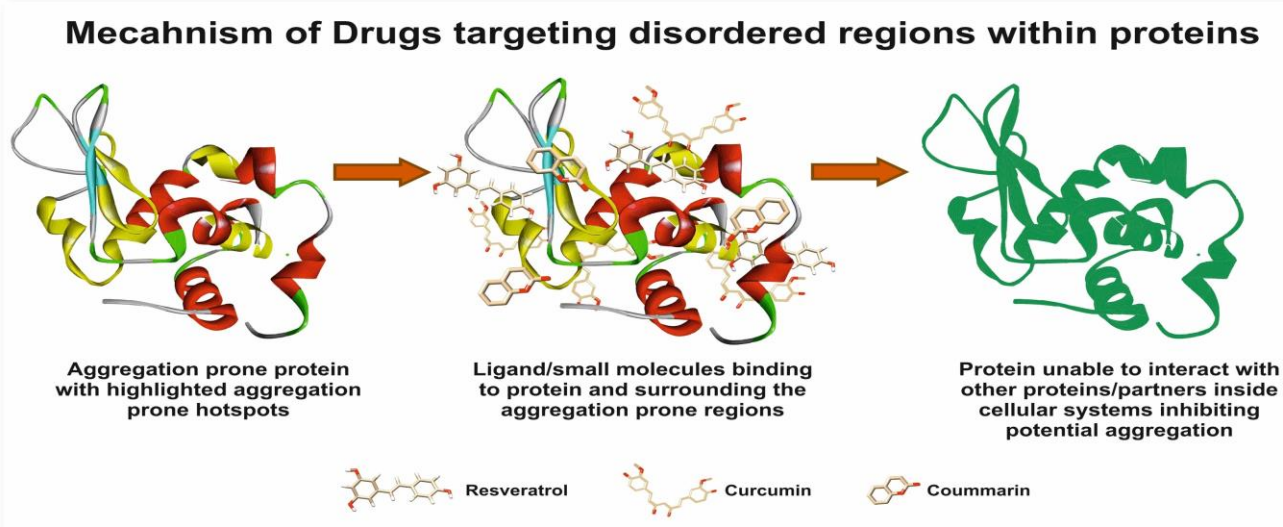

Fig 6.1 Mechanism of Drugs targeting IDRs: The principle behind the potential binding of small molecules within disordered regions within the protein and their action of preventing further notorious interactions of the protein-based on which we performed our computational study

Subsequently, we examined the effect of the best computationally identified compounds for anti-amyloid properties using HEWL amyloid as a model system. HEWL amyloid is well characterized at pH 12.2 and widely reported in the literature. We have also characterized HEWL amyloid at near physiological pH, *i.e.,* 7.4. Our results showed that amyloid fibrillation is more pronounced in near physiological pH than at pH 12.2. Further, we also report a lower formation of oligomers and, subsequently, matured fibrils of HEWL in the presence of both nitrogenous bases (Cytosine, Guanine, Thymine and Uracil), nucleosides (Adenosine and Guanosine) in a time-dependent manner. We also validated the results using supporting data like aggregation index, dynamic light scattering (DLS) studies and atomic force microscopy (AFM) imaging. We also performed and interpreted the RMSD, RMSF, SASA, and secondary structure analyses through molecular dynamics and simulation of the selected holoprotein complexes after binding these compounds with amyloid-prone regions. We concluded that the selected nucleosides and nitrogenous bases potentially suppress (slow down) primary nucleation processes under these experimental conditions (Figure 6.2).

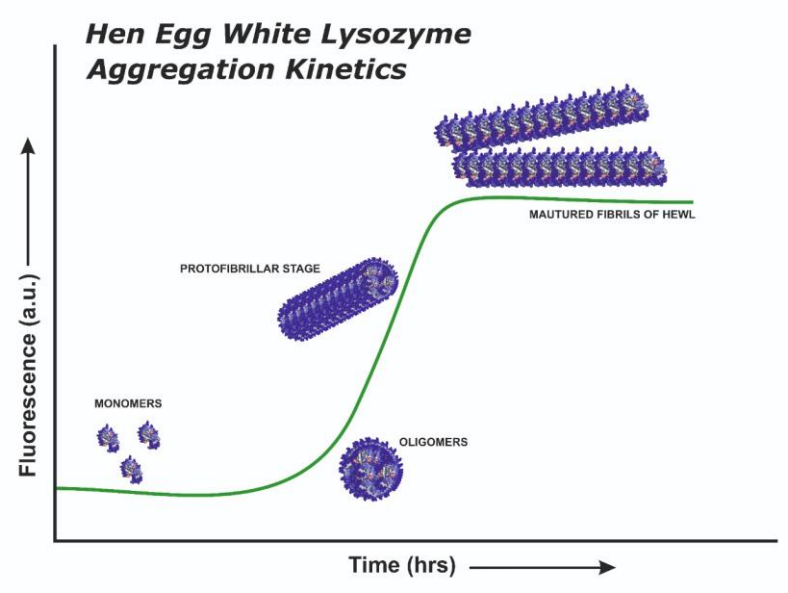

Fig 6.2 Protein aggregation phases: In the current chapter, we have focused on the impact of the ligands on the monomeric stage under aggregating conditions, which resulted in slowing down the appearance of the oligomeric and amyloid fibrillar stages

Furthermore, we have also explored the impact of these metabolites on the preformed early oligomeric state of HEWL. We attempted to unravel the HEWL amyloid kinetics in near physiological pH and determine the critical microscopic stages of HEWL aggregation. The HEWL aggregation followed a Saturation Elongation and Fragmentation model. We report that upon adding ligands on preformed HEWL amyloids, there is a sharp increase in the Thioflavin T intensity of the samples indicating the rapid formation of matured fibrils upon adding the chosen ligands. The observation was repeated at three different time points. Our AFM analyses also supplemented this. On the contrary, our turbidity and UV scattering analyses showed a decreasing trend, which could be explained by the overall reduction of scattering intensity due to large aggregates. We further elucidated using the Thioflavin T kinetics assay that in the presence of the metabolites, the HEWL aggregation half-time is shortened compared to HEWL aggregation without metabolites. Further, there is an increase in combined nucleation and elongation rate. Considering these two factors, we concluded that the selected metabolites enhance and promote fibrillation processes at this stage. Although in seeded reactions where we had anticipated quickening up of overall reaction and shortening of lag phase, the addition of ligands along with preformed seeds to

HEWL monomers showed varied results. Adenosine and Cytosine did not decrease lag time compared to control samples. On the contrary, Guanine, Guanosine and Thymine increased overall lag time. This important observation indicates that the metabolites modify the lag phase period in aggregation kinetics, depending much upon the stage of protein aggregation they encounter in solution.

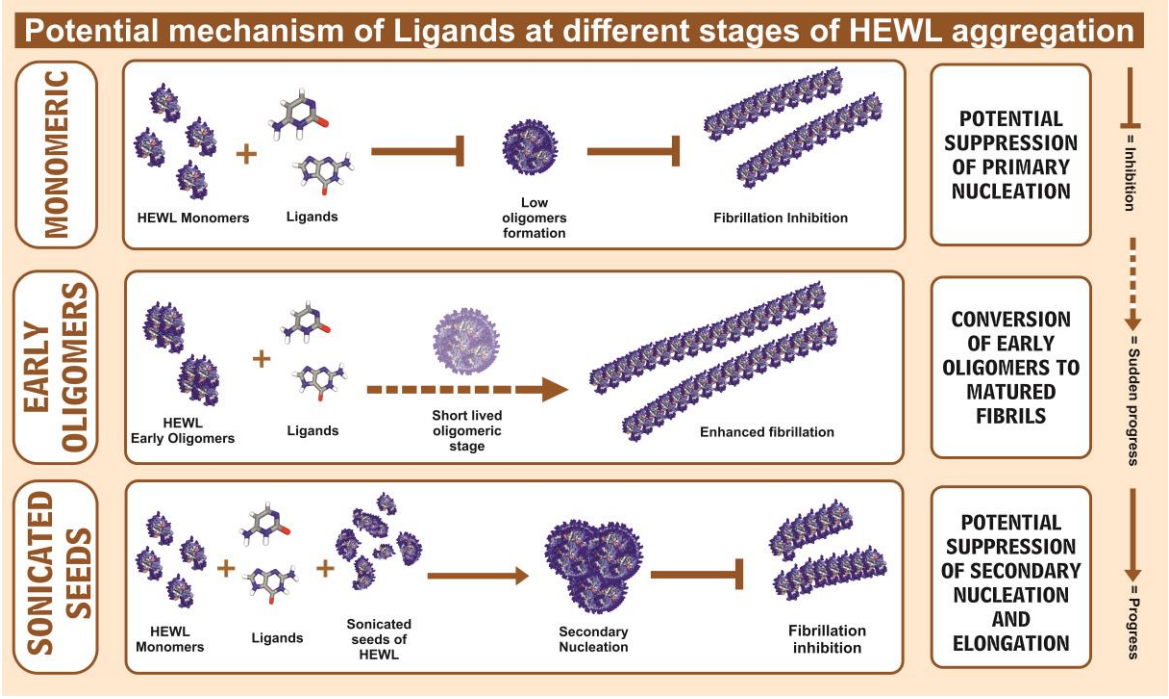

Fig 6.3 Possible mechanism of action of selected metabolites at various stages of HEWL aggregation: The highlight of the study shows that the metabolites are capable of diverse mechanisms depending on the aggregation stage. This insight could impact their use as therapeutics.

Since it is now established that protein aggregation remains the critical process behind the occurrence of notorious forms of protein aggregation diseases, some additional *in vivo* factors also direct the overall pathogenicity of the diseased state. In the last chapter of the thesis, we have used *in silico* methods to decipher potential novel inhibitors of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) by drug repurposing strategy, which are vital enzymes identified as factors aggravating the pathophysiology of Alzheimer's Disease (AD). We performed molecular docking in critical domains of these two enzymes with some of the recently approved drugs by FDA for various other neurological and psychiatric diseases. Subsequently, we employed molecular dynamics (MD) simulations for further insights. We report Brexpiprazole and Deutetrabenazine as

potential BuChE and AChE inhibitors by their high binding affinity and capacity to interact with critical residues of the oxyanion hole and active catalytic site (CAS), indicating a possible dual mechanism of enzyme inhibition. The results are encouraging for other studies *in vivo* as potential enzyme inhibitors for AD treatment. Few key inhibitor molecules with their interaction with the enzyme is shown in Figure 6.4

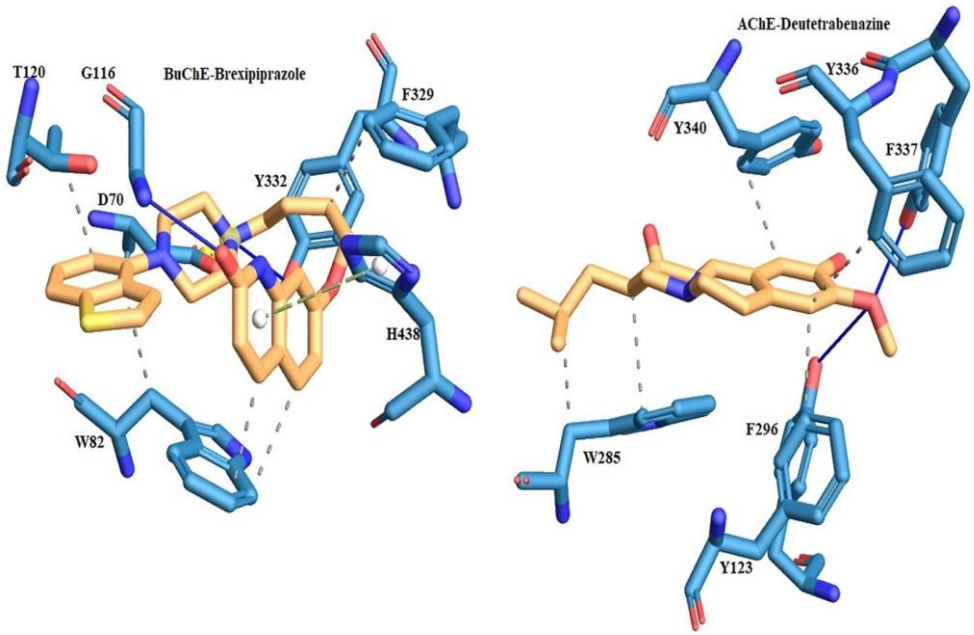

Fig 6.4 3D diagrams of interaction between FDA-approved drugs and critical enzymes in Alzheimer's Disease: (A) Brexipiprazole and **(B)** Deutetrabenazine. The drugs could be potential cholinesterase inhibitors

The results from this thesis open up some critical areas for a better understanding of protein aggregation, especially in correlation with loss in the homeostasis of intracellular metabolites. The work depicted here also gives us insights into potential mechanisms of small ligands, how they could act as protein aggregation modifiers, and areas where more useful therapeutics could be developed. The primary significant findings of the study and various potential areas of research are highlighted in Figure 6.5.

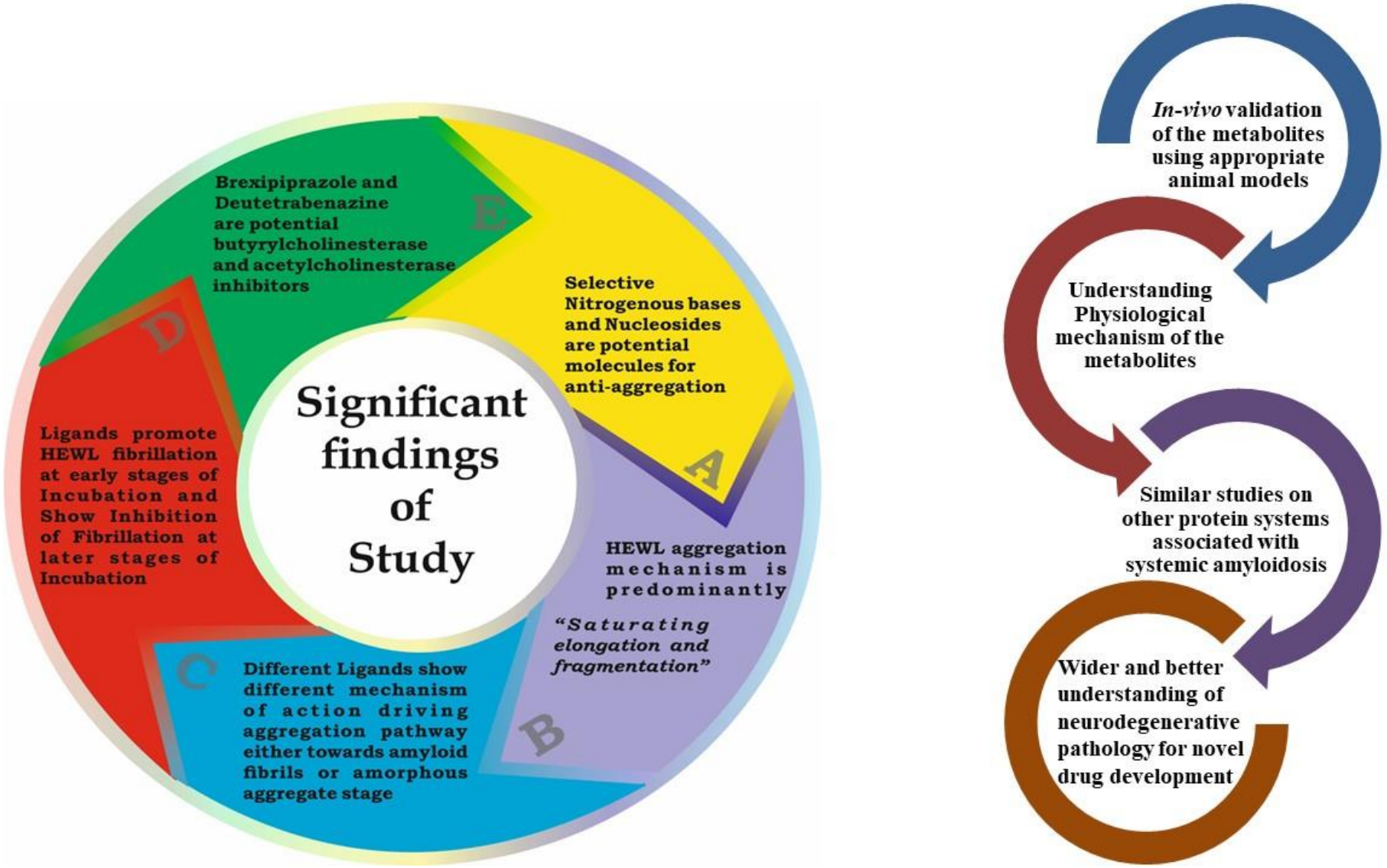

Fig 6.5- Significant findings of the current study and future scope of research in bridging the connection between the role of intracellular metabolites and proteinopathies