Extended abstract

Peptides have emerged as an important class of biomolecules, which have found various therapeutics and diagnostic applications owing to their properties such as high target specificity, biocompatibility and the presence of pre-organised structure. In the past decade, peptide-based drugs have captivated an overall share of 10% in the pharmaceutical sector amounting to 40 billion dollars. There are currently 74 FDA approved peptide-based drugs in the market, and several others are in different phases of clinical trials. Despite the beneficial properties of peptides as prospective therapeutic agents, the major challenges remain such as their limited bioavailability, proteolytic stability and chemical stability. To counter these problems, several approaches are being used such as the introduction of molecular rigidity by forming disulphide cross-linkages, backbone modifications, non-canonical amino acids and D-amino acids are adopted. Present work details the efficient generation of peptide-based inhibitors targeting tau protein aggregation and Sonic Hedgehog/patched interaction. This dissertation explores the following approaches:

1. Novel deprotection strategy for difficult peptide synthesis

In Solid Phase Peptide Synthesis (SPPS), contamination with deletion sequences which often co-elute with the target peptide continues to be a major challenge as these impurities can significantly affect the target peptide's properties. Here, we report an efficient Fmoc-deprotection solution containing piperazine and DBU which can cause complete removal of Fmoc group in less than a minute. This combination rivals piperidine in speediness as revealed by kinetic studies. We demonstrate the efficiency of piperazine/DBU solution by synthesizing polyAla stretch with a significant reduction of deletion products occurring due to partial Fmoc-deprotection. We verify the utility of the deprotection solution by successfully synthesizing four

aggregation-prone difficult peptide sequences. We further demonstrate that this combination can also be used to synthesize aspartimide and epimerization prone sequences when supplemented with 1% formic acid and is compatible with 2-chlorotrityl chloride resin. We conclude that piperazine/DBU can be used as a safer and effective alternative to piperidine in Fmoc-SPPS.

2. Effect of peptide length on the modulation of tau protein aggregation

The microtubule-associated protein tau is an intrinsically disordered protein which forms insoluble β-sheet rich assemblies in the human brain. Tau is implicated in several neurodegenerative disorders collectively known as tauopathies, where the most common tauopathy is Alzheimer's disease (AD) which is known to affect over 45 million people worldwide. Till date, there are no disease-modifying therapies available for AD. In an AD patient's brain, tau protein undergoes abnormal post-translational modifications (PTMs) which allow it to self-assemble, resulting in the formation and deposition of Paired Helical Filaments (PHFs) and Neurofibrillary Tangles (NFTs). It is believed that tau protein can aggregate via the template-based conversion to form amyloids and may plausibly be a representative of prion-like protein. However, the precise mechanism of tau aggregation and molecular factors responsible for triggering the conformational conversion of unstructured tau monomers to form ordered assemblies remain elusive. Tau aggregation can be modulated by both intrinsic and extrinsic factors such as mutations in the *MAPT gene*, the concentration of protein, temperature, pH, salts, shear force, polyanions, PTMs and cross-seeding by other amyloidogenic proteins/ peptides. In the present study, we have evaluated the modulatory effect of the length and residue dependent short tau-derived peptides on the *in vitro* aggregation of full-length tau protein. Four short peptide variants were synthesized using SPPS: Tetra (IVYK), Hexa (VQIVYK), Octa (SVQIVYKP) Deca (GSVQIVYKPV). Complementary *in vitro* experiments demonstrated that

tetrapeptide enhanced the tau protein aggregation, hexapeptide acted as complete inhibitor, whereas octa and deca peptides acted as a partial inhibitor of tau amyloid aggregation, respectively. Computational analysis of tetra and hexapeptides with R2 domain of tau protein (model dimer system) suggests that the addition of these peptide to tau system plausibly altered the structural conformation of the tau monomers in two different ways: (i) The tetrapeptide influenced the formation of intermolecular hydrogen bonds between the two tau monomers and increased the propensity of β-bridge formation, promoting aggregation (ii) hexapeptide promoted intramolecular hydrogen bonds within the tau monomers to form alpha-helix, thus facilitating aggregation inhibition.

3. Knottin based grafted peptides as Tau protein aggregation inhibitors

AD is the major form of dementia affecting over 45 million people worldwide. Till date, there are no disease-modifying therapies available for AD. Since the self-assembly of tau protein is closely related to AD and the initial stages of aggregation appear likely as the rate-limiting step, intervening in this process with aggregation-modulating agents might be an attractive strategy for AD therapeutics. Previously, few small molecules and peptide-based molecules have been reported to inhibit tau aggregation and disassemble pre-formed assemblies into non-toxic species. To this end, herein, we have rationally designed and synthesized potential knottin-based peptide inhibitors, which can perform targeted inhibition of tau aggregation. Knottins are cysteine know miniproteins with characterstic disulphide bonding which helps in providing proteolytic, thermal and enzymatic stability to the synthsiszed knottin based peptides. Knottins have previouslyknown to cross the blood-brain-barrier and hence we can use this property to our advantage for designing peptide based inhibitors for AD. Among the three synthesized peptide inhibitors, i.e., VQIVYK-knottin, VQIINK-knottin and Combo-knottin, VQIVYK-knottin was

the most efficient peptide to inhibit tau-derived PHF6 hexapeptide aggregation as well as full-length tau protein aggregation in a concentration-dependent manner. The results were further validated using complementary biophysical characterization techniques. Cell culture studies revealed that VQIVYK-knottin peptide inhibitor was non-toxic to live cells and could permeabilize the cell membrane. Our findings signify the effectiveness of VQIVYK-knottin inhibitor towards tau aggregation and highlight the design to synthesize novel disease-modifying drugs for AD treatment.

4. Cysteine cross-linked macrocycle inhibitors for Hedgehog signaling pathway

Hedgehog signalling cascade is an important signalling pathway involved in embryonic development and organogenesis. In the case of aberrant expression, it can lead to the development of colon, lung and pancreatic cancers. The key player of the hedgehog signalling pathway is sonic hedgehog protein (Shh), which interacts with another protein named Patched to activate the downstream signalling cascade. In this work, we focus on the development of a macrocyclic peptide inhibitor against Sonic Hedgehog and patched interaction. The design is based on the specific binding site sequence of Hedgehog-interacting protein (HHIP) which competes with Patched protein for its binding site on Shh. We identified a 17 amino-acid long peptide sequence and subjected it to alkylation using several linkers reported previously. This reaction resulted in the formation of a bridge between the side-chains of two cysteine residues present in the sequence. The cysteine cross-linking provided molecular rigidity and resulted in several-fold improvement in the dissociation constant (Kd). Further, based on structural assessment of the native peptide, we replaced the canonical amino acid residues with non-canonical amino acids and subsequently generated a pool of peptides with unnatural amino acids and cross-linked cysteines. Using this approach, an optimized peptide named Biotin-M5St-DNle

was obtained that binds to Shh with a Kd of 900 nM, corresponding to multi-fold improvement in affinity when compared to the parent peptide. This work introduces a new strategy to design potent peptide inhibitors against Shh/patched interaction.