ABSTRACT

ABSTRACT

Neurodegeneration, an age-related disorder, occurs as a progressive loss of neurons or from the neuronal failure to transmit signals. Neurodegenerative diseases (NDs), a group of neuronal dysfunctions, principally affect the neurons in the CNS and ultimately result in the defect of specific brain functions such as cognition, memory, and movement. The epidemiology of the NDs surges with aged people. It contributes approximately 12% of the total deaths globally. As a result, WHO has declared NDs to be the second leading cause of death worldwide. The most common NDs are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Amyotrophic lateral sclerosis (ALS). Decades of research evidenced the hallmarks of the NDs, including aberrant proteostasis, pathological protein aggregation, synaptic and neuronal network dysfunction, DNA and RNA defects, altered energy inflammation, homeostasis, and neuronal cell death. PD is the second most frequent ND, followed by AD. Despite groundbreaking research, physicians have no effective treatment or medications available to stop, slow, or prevent the ill effects of NDs.

Neurotrophins are a group of endogenous soluble proteins with similar structures and functions, which profoundly affect neuronal development in vertebrates. The neurotrophin molecules contain nerve growth factor (NGF), brain-derived neurotrophic factors (BDNF), neurotrophin 4/5 (NT-4/5), and neurotrophin-3 (NT-3). The mechanism of neuritogenesis involves binding neurotrophin molecules to the transmembrane receptors belonging to the tyrosine kinase receptor (Trk) family. After binding neurotrophin molecules to their respective Trk receptors, neurotrophins promote their dimerization and autophosphorylation of the intracellular tyrosine residues of the receptor, which activates cascades of signaling pathways. These signaling pathways resulted in neuronal proliferation, survival, and differentiation. Because of the prominent role of neurotrophin molecules in neuronal development, endogenous administration of the exogenous neurotrophin molecule (such as NGF and BDNF) can be used as the drug prototype to treat NDs. Despite the high pharmacotherapeutic potential of neurotrophin, their poor pharmacokinetic potential, inability to permeate through the blood-brain barrier (BBB), large molecular weight, adverse side effects, and short half-life have limited their therapeutic applications.

Peptide mimetics is an apparent way to challenge the disadvantages of natural peptides. To overcome the limitations associated with using endogenous neurotrophin molecules for treating NDs, scientists have tried since the 90s to develop low molecular weight neurotrophin mimetics with improved pharmacological efficacy and without any adverse side effects. To date, two neurotrophin mimetics (LM11A-31 and D3) are undergoing clinical trials, two (GK-2 and 7,8-dihydroxyflavone) have completed preclinical studies, and one (GSB-106) is at the stage of advanced pharmacological studies.

Venoms are intriguing sources of unique molecules that are being enhanced in evolution and also have unique characteristics such as low molecular mass, pharmacological activity, stability and high potency, and selectivity and affinity in mammalian systems for many targets. Animal venom has, therefore, a remarkable ability to generate therapeutic agents, and several venom toxins have been clinically applied and used as templates for drug design. NGF, a prominent member of the neurotrophin family, is one of the non-enzymatic intriguing proteins found in snake venoms. The proteomic analysis from our lab has shown that venoms of India's 'Big Four' venomous snakes contain several isoforms of NGF, however, in a small proportion. Previous studies from our lab have isolated and characterized neurotrophin molecules from Indian snake venoms, which exhibit neuritogenesis potency in rat pheochromocytoma (PC-12) cells. However, drug development from a native snake venom toxin has various limitations. The neurotrophin molecules in snake venom make up only about 0.02% of the total venom composition, meaning they are found in extremely low abundance. These molecules are vital for nerve cell development and repair, and their unique properties have attracted interest for potential therapeutic use. However, directly extracting neurotrophin from snake venom as a therapeutic agent would lead to a significant depletion of the venom supply. Given the already scarce presence of neurotrophins, large-scale extraction could result in a shortage of venom, impacting not only medical applications but also the ecological balance, as venom is essential for the snake's survival. Therefore, alternative methods such as synthetic production or bioengineering of neurotrophins may be necessary to avoid depleting natural venom sources. The purification and characterization of the neurotrophin molecule from snake venom is costly and tedious.

Therefore, we have designed two low molecular weight peptide mimetics (trideca-neuropeptide, TNP; heptadeca-neuropeptide, HNP) inspired by the snake venom neurotrophin molecules. These two synthetic custom peptides (CPs) were selective to the TrkA receptor (TrkA) determined by computational in silico analysis. In silico results were validated by an *in vitro* binding study of the FITC-conjugated CPs to PC-12 cell TrkA receptors. Pre-treatment of PC-12 cells with TNP and HNP induced neuritogenesis and significantly reduced the paraquat (PT)-induced cellular toxicity, the release of lactate dehydrogenase from the cell cytoplasm, production of intracellular ROS, restored the level of antioxidants, prevented alteration of mitochondrial transmembrane potential ($\Delta\Psi m$) and adenosine triphosphate (ATP) production, and inhibited cellular apoptosis. These peptides lack *in vitro* cytotoxicity, hemolytic activity, and platelet-modulating properties and do not interfere with the blood coagulation system. Functional proteomic analyses demonstrated the reversal of PT-induced upregulated and downregulated metabolic pathway genes in PC-12 cells that were pre-treated with HNP and revealed the metabolic pathways regulated by HNP to induce neuritogenesis and confer protection against PT-induced neuronal damage in PC-12. The quantitative RT-PCR analysis confirmed that the PT-induced increased and decreased expression of critical pro-apoptotic and anti-apoptotic genes had been restored in the PC-12 cells pre-treated with the CPs. A network gene expression profile was proposed to elucidate the molecular interactions among the regulatory proteins for HNP to salvage the PT-induced damage. Our results show how peptides can protect against PT-induced oxidative stress, mitochondrial dysfunction, and cellular death and suggest new opportunities for developing neuroprotective drugs.

The *in vivo* protective mechanisms of the two low molecular mass (~1.4 kDa) novel CPs (TNP and HNP) against PT-induced neurodegenerative dysfunction in the *Caenorhabditis elegans* model were also deciphered. CPs prevent the PT binding to the nerve ring adjacent to the pharynx in *C. elegans* (N2 strain) by stable and high-affinity binding to the tyrosine-protein kinase receptor CAM-1, resulting in significant inhibition of PT-induced toxicity by reducing enhanced reactive oxygen species production, mitochondrial membrane depolarization, and chemosensory dysfunction. The CPs inhibited PT-induced dopaminergic (DAergic) neuron degeneration and alpha-synuclein aggregation, the hallmarks of Parkinson's Disease, in transgenic BZ555 and NL5901

strains of *C. elegans*. The qRT-PCR, transcriptomic, and functional proteomics analyses unanimously elucidated that CPs restored PT-mediated oxidative stress, apoptosis, and neuronal damage in *C. elegans* by inhibiting increased expression of the skn-1 downstream pathway genes. A network of gene expression profiles showed the molecular interactions among the regulatory proteins to salvage the PT-induced damage by the neuroprotective peptides. Further, CPs (10 mg/kg, parental route) did not show toxicity or induce inflammatory mediators in the mice model.

MicroRNAs (miRNAs) are small non-coding RNAs that control different biological processes, viz. proliferation, differentiation, development, and apoptosis through translational inhibition and mRNA degradation. In this study, we aimed to decode the role of miRNAs in the neuritogenesis and neuroprotection activity of snake-venom-inspired CP (HNP) in the model organism *C. elegans*. We compared the miRNA expression profiles of the CPs-treated *C. elegans* versus mouse 2.5S-NGF-treated ones (positive control). This study is the first report that showed the upregulation and downregulation of miRNAs in the mouse 2.5S/HNP-treated *C. elegans*. The potential miRNAs involved in the neuroprotection against PT-induced toxicity in the *C. elegans* PD model were studied. Functional characterization of its target genes regulating the different pathways was investigated. Mir-4813-3p was highly downregulated with the PT treatment, which involves the clearance of alpha-synuclein protein, and upregulated with the HNP/mouse 2.5S-NGF treatment. Two novel miRNAs, cel-miR-57-3p and cel-miR-8207-3p, were drastically upregulated with the PT treatment and significantly downregulated when pre-treated with the HNP/mouse 2.5S-NGF, indicating their roles in the protection against PT-toxicity in *C. elegans*.

For easy understanding, this thesis is structured into the following six chapters-

Chapter I: This chapter introduces neurodegenerative disorders (NDs), the second most common cause of death worldwide. This chapter also briefly introduces clinically meaningful NDs, their pathophysiology, current treatment, and associated challenges and limitations. We have briefly discussed several mechanisms allied with neurodegeneration that are concerned with the progression and pathogenesis of NDs. This chapter also introduced neurotrophin molecule and its role in treating NDs. The aim and objectives of the present study are also described in this chapter.

Chapter II: This chapter reviews the published literature on limitations associated with the therapeutic application and delivery of neurotrophic molecules for treating NDs around the globe. This chapter also reviews the advantages of neurotrophin mimetic molecules over parent neurotrophins as a drug prototype to treat NDs.

Chapter III: This chapter enlists the chemicals and consumables used in the study and the methods and protocols employed for performing various experiments.

Chapter IV, V, and VI: These chapters include results and discussions, and the content of each chapter is briefly discussed below:

Chapter IV depicts the in silico and *in vitro* binding of custom peptides (CPs) to the mammalian TrkA receptor. The binding of custom peptides does not ensure the therapeutic potential of our designed CPs; therefore, we determine the neurite outgrowth and differentiation potential of the CPs in PC-12 cells. Further, the finding suggests the neuroprotective potential of the CPs against the paraquat (PT)-induced Parkinson's disease (PD) model in PC- 12 cells by thwarting excessive ROS production, oxidative stress, MMP, and premature apoptotic death. Moreover, this chapter provides a greater understanding of the altered expression of proteins involved in the neurodegeneration pathways in the PT-induced PD model of PC-12 cells and how the expression of our custom peptides can be used in treatments by qRT-PCR and quantitative proteomic analysis.

Chapter V: After confirming the exclusive binding of TNP and HNP to the TrkA receptor, we were puzzled to understand the binding region of custom peptides in *C. elegans* because it lacks Trk receptors. Therefore, this chapter confirms the binding of CPs to the tyrosine-protein kinase receptor CAM-1 receptor (TrkA receptor homolog) present at the nerve ring region of *C. elegans*. This study also focuses on the neuroprotective potential of the CPs against the PT-induced PD model of *C. elegans* by thwarting excessive ROS production, MMP, and premature apoptotic death in a higher *in vivo* wild-type N2 *C. elegans* model. Moreover, this chapter includes that CPs protect PT-induced DAergic neuron degeneration in BZ555 worms, loss of chemotaxis behavior, and diminished α-synuclein accumulation in NL5901 worms. Furthermore, qRT-PCR, transcriptomics, and proteomics studies unveiled the in-depth mechanism of

neuroprotective activity of custom peptides in the PT-induced PD model of *C. elegans* N2 worms.

Chapter VI: This chapter focuses on studying expression profiles of the microRNAs (miRNA) in the neurotrophic and neuroprotective potential of the CPs/Mouse 2.5S-NGF against the PT-induced PD model of *C. elegans*. The acute toxicity of CPs tested in mice models showed that the peptide is devoid of toxicity in mice.

Chapter VII: This chapter presents the conclusion of this study and visualizes the future prospective of the study's findings.

In this study, our findings provide a fundamental basis for developing new agents that specifically target neurodegenerative diseases, especially PD, and encourage future, in-depth investigations through *in vivo*, pharmacokinetic, and pharmacodynamic studies to establish safe drug prototypes.