CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Synthetic custom peptides

Custom peptides trideca-neuropeptide (TNP, NH2-GGDRCSGGKVGGK-COOH), heptadeca-neuropeptide (HNP, NH2-HGGKGIGKGGTGGAGVG-COOH), a scrambled peptide (NH2-KGGDRCSGGAVGVK-COOH) and fluorescein isothiocyanate (FITC)-peptides synthesized by S Biochem, Thrissur, Kerala, India. Sequences of these peptides are shown in Chapter IV.

3.1.2 Cell lines, cell culture reagents, and cell culture plastic wares

The rat adrenal phaeochromocytoma (PC-12) cell line (initial passage number 8) (CRL-1721™) was procured from the American Type Culture Collection (ATCC), USA. Rat myoblast or myogenic cells (L6) (initial passage number 18), human breast adenocarcinoma cells (MDA-MB-231) (initial passage number 16), and Michigan Cancer Foundation-7 (MCF7) cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. The specification sheets provided by NCCS and ATCC stated the authenticity of the microbial contamination-free cell lines we procured from them. Each cell line was subjected to 5-6 or more passages during the experimental plan.

Dulbecco's Modified Eagle Medium (DMEM), Eagle's Minimum Essential Medium (MEM), trypsin, and heat-inactivated fetal bovine serum (FBS) were purchased from GibcoTM (USA). Cell culture accessories and sterile plastic plates were procured from Thermo Fisher Scientific, USA.

3.1.3 *Caenorhabditis elegans* strains and *Escherichia coli*

C. elegans Bristol wild-type strain N2, NL5901, BZ555, the bacterial control food *E. coli* OP50 (OP50), were purchased from Caenorhabditis Genetics Centre (CGC), University of Minnesota, USA. CAM-1(ak37) mutant strain was a kind gift from Dr. Kavita Babu, Associate Professor, Indian Institute of Science, Banglore.

3.1.4 Neurotrophic factors and fine chemicals

The nerve growth factor-2.5S from the murine submaxillary gland, fluorescein isothiocyanate (FITC), Thiazolyl blue tetrazolium bromide (for MTT assay), and protease inhibitor cocktail was procured from Sigma-Aldrich, Missouri, USA. All chemicals were

of analytical grade from Sigma, Merck, HiMedia, Invitrogen, and Thermo Fisher Scientific USA. The Annexin V-FITC / propidium iodide (PI) apoptosis detection kit was procured from Cell Signaling Technology (CST, 6592), the MitoProbeTM JC-1 assay kit and the CyQUANT™ LDH cytotoxicity assay kit was purchased from Invitrogen (M34152), from CST. RNAeasy mini kit was purchased from Invitrogen, Waltham, MA, USA, and the cDNA synthesis kit was procured from Thermo Scientific, Waltham, USA.

3.2 Methods

3.2.1 To study the interaction of synthetic Custom Peptides with mammalian TrkA receptor and TrkA homolog in *C. elegans* by computational (*in silico*) analysis.

3.2.1.1 Predicting the binding affinity of synthetic peptides to different domains of human TrkA receptors by *in silico* analysis

The peptides' cheminformatic parameters such as mass, isoelectric point, net charge, hydrophobicity, etc., were computed using the PepDraw server [1]. The peptide sequences were converted to PDB structures, and their dynamics and conformations were studied using molecular dynamic simulations for 100 ns. Simulations were carried out in GROMACS v2022.2 with the CHARMM36 (v 2021) force field. The periodic boundary was a dodecahedron with a 1.5 Å buffer region. Peptide structures were first minimized in a vacuum using 5000 steps of the steepest descent algorithm. The system was solvated using the transferable intermolecular potential with a three-point water model (TIP3P), and 0.15 M sodium chloride ions were added till neutralized. The system was equilibrated using NVT (canonical) and NPT (isobaric-isothermal) ensembles in 300 K and 1.0133 bar. Temperature coupling was done using the v-rescale (Berendsen) method, and pressure coupling was done using c-rescale [2] and integrated using a leapfrog algorithm. Before the production MD run, the position restraints were released following three NPT equilibration runs. The trajectory was studied for root mean square deviation (RMSD), radius of gyration, hydrogen bonding, secondary structure of the peptides, and thermodynamic properties. From the trajectory frames, an average structure of the peptide was derived in a PDB format and used in the subsequent analysis.

A molecular docking study was conducted to predict the binding affinity of custom peptides and a scrambled peptide with the TrkA receptor. Crystal structures of human TrkA (UniProtKB - P04629) domains were obtained from Protein Data Bank (PDB) with

entry viz. 2IFG (extracellular domain), and 5JFX (cytoplasmic domain). The structures were prepared using the protein preparation wizard of the Schrodinger suite (Schrodinger Inc. USA). Briefly, water and hetero atoms were removed, and missing residues and side chains were filled and corrected for alternate positions of the residues. The docking used a peptide dock module with the molecular mechanics/generalized born surface area (MM-GBSA) scoring method. The receptor grid was generated from centroid residues so that the grid box could occupy the whole protein domain. The peptide sequences were then added individually to output the ten best poses for each domain.

Molecular dynamic simulations were conducted using Schrodinger Desmond (Academic version, v.2022). The simulation system comprised the docked peptide complex, solvent, and salts. The TIP3P water model was used as a solvent within an orthorhombic boundary condition with a 10 Å buffer region. NaCl (0.15 M) was added to the system and neutralized using the required number of Na^+ or Cl^- ions. The system was equilibrated with NVT and NPT ensembles at 300 K and 1 bar. The reversible reference system propagator algorithms (RESPA) were used as an integrator (at 2 fs time-steps) of Newtonian dynamics with a column cut-off radius of 1.5 Å. A Nose Hoover chain thermostat and Martyna Tobias Klein barostat were used with isotropic coupling at 1 ps and 2 ps relaxation times. Relaxation of the system was done using the default, which provided a relaxation protocol followed by a production MD run for 300 ns. The energy and trajectory were recorded at every 0.5 ps and analyzed using the simulation interaction diagram module of Schrodinger Maestro. The binding free energies of the simulation trajectory complexes were calculated using the MM-GBSA method.

3.2.1.2 Predicting the binding affinity of synthetic peptides to different receptors in *C. elegans* by *in silico* analysis

The custom peptides were initially developed to target the Human NTRKA. To investigate the binding of these custom peptides, the scrambled peptide, and mouse-NGF, a homologous *C. elegans* protein was used. Human NTRKA protein sequence was obtained from UniProt (ID: P04629), and homologous proteins in *C. elegans* were identified using BLAST search. The structural and functional information about the homologous protein found by BLAST search was retrieved from UniProt. 3D structures of different protein domains in *C. elegans*. TRK (CAM-1) were manually modeled using template-based homology modeling and Alpha Fold Prediction. Furthermore, the binding

sites of these domains were predicted in PrankWeb 3 server [3]. Similarly, other proteins were selected based on the neuroprotective pathways from previous studies [4]. The proteins with available 3D structures in PDB were preferably taken. However, the unavailable 3D structures were modeled using homology modeling. Mouse NGF structure was deduced from PDB (ID 1BET) and prepared for protein-protein interactions with CAM-1 immunoglobulin (IG) and frizzled (FZ) domains model.

The peptides were processed by molecular dynamics simulation using our previously described method [4], and different properties, such as secondary structure and beta-hairpin loop formation, were analyzed. Subsequently, all the proteins were processed for peptide docking by adding hydrogens, filling missing side chains and residues, and restraining minimization. The peptide docking was carried out in the CABS Dock server [5]. The stability of the docked complexes was further analyzed by molecular dynamic simulation. The simulation was conducted in Schrodinger Desmond (academic version v2022) with an OPLS4 force field. The simulation system was prepared with orthorhombic boundary condition with 10 Å buffer region and solvated with TIP3P water model, followed by the addition of 0.15 M sodium chloride. The system was equilibrated at 300 K and 1 bar using NVT and NPT ensembles. Nose Hoover chain thermostat and Martyna Tobias Klein barostat were used with isotropic coupling at 1 ps and 2 ps relaxation time. The system was relaxed using default options provided by Desmond, and finally, production MD was carried out for 100 ns. The binding free energy of the complexes was calculated using the MM-GBSA method.

3.2.2 To study the *in vitro* mechanism of neuritogenesis and neuroprotective role, of custom peptides in pheochromocytoma of the rat adrenal medulla (PC-12) cells

3.2.2.1 Solid-phase synthesis and biophysical characterization of the custom peptides and the scrambled peptide

The solid-phase peptide synthesis was done using Fmoc protocols for the esterification of amino acid to the wang resin followed by a coupling reaction [6]. The peptide was then detached from the resin and isolated using cold diethyl ether. The purity and molecular mass of the peptides were ascertained by mass spectrometry analysis in a single quadrupole mass spectrometer (Shimadzu LC-MS 2020, Japan) via positive electrospray ionization (ESI). The biophysical properties of the peptides were then calculated in an

online peptide property calculator (https://pepcalc.com/). The secondary structures of the peptides were also determined by circular dichroism (CD) spectroscopy (Jasco J715 spectropolarimeter, Japan) following our previously described procedure [7]. CDPRO CLUSTER software was used to determine the secondary structures of the peptides.

3.2.2.2 Cell growth and maintenance

Mammalian cells, viz. PC-12, L6, and MDA-MB-231 were grown and maintained with a complete medium comprising of DMEM containing 1% antibiotic solution (GIBCO™) and 10% heat-inactivated FBS (GIBCO™) and in 75 cm^2 tissue culture flasks at 37°C in a humidified incubator with 5% CO_2. MCF-7 cells were maintained in MEM containing 10% FBS and 0.01 mg/mL insulin. The cells were re-suspended in a medium every 2-3 days and subcultured after reaching confluence [8-10].

3.2.2.3 Assessing the binding of FITC-conjugated custom peptides to TrkA receptor-expressing mammalian cells

Custom peptides and the scrambled peptide were synthesized by S Biochem, Thrissur, Kerala, India, following the previously described protocol with slight modifications [8]. Fresh FITC reagent was prepared (1 mg/mL) in DMSO, added to the resin, and incubated in the dark for 2 h at 4°C. The resin was washed with dimethylformide; standard peptide cleavage protocol was followed. Spectrofluorometric interactions between the FITC-conjugated peptides and TrkA receptors were determined in mammalian cells expressing TrkA receptors, such as PC-12, MCF7, and MDA-MB-231 cells, and in non-TrkA receptor-expressing L6 cells (negative control) following the procedures that were previously described [8-10].

The PC-12, MCF7, MDA-MB-231, and L6 cells (1×10^4 cells per well in 96-well plates) were incubated with the FITC-conjugated peptides (2 µg/mL) at different time intervals (from 0 to 360 min) at 37°C in a humidified incubator with 5% CO_2 (in triplicates). Cells were washed with 1X phosphate-buffered saline (PBS), pH 7.4, three times and then fixed in 4% formaldehyde-PBS solution (pH 7.2) for 15 min at room temperature (~23°C). The fluorescence intensities were recorded at 519 nm (ʎem~519 nm; [excitation wavelength was 488 nm (ʎex ~488 nm)] using a multimode plate reader (Varioskan Flash, Thermo Scientific, USA). The fluorescence intensity of cells treated with 0.1% dimethyl sulfoxide (DMSO) was used as a baseline, to which other values were compared.

Similarly, the PC-12 cells (1 x 10^4 cells) were incubated with graded concentrations (40 nM to 800 nM) of FITC-conjugated peptides at 37°C in a humidified incubator with 5% CO_2 (in triplicates). The fluorescence intensities were recorded at 519 nm using a multimode plate reader (Varioskan Flash, Thermo Scientific, USA). A hyperbola curve was plotted for change in λmax (Δ λmax) against the concentrations (nM) of custom peptides with PC-12 cells expressing TrkA receptor, and the equilibrium dissociation constant (K_d) value was determined using GraphPad Prism 8.1.1 software.

In another study, the interaction between FITC-conjugated peptide and TrkA receptors was confirmed by fluorescence microscopic image analysis in MCF7 and MDA-MB-231 cells and non-TrkA receptor-expressing L6 cells (negative control) as previously described [9]. The cells were incubated with FITC-peptides with MCF7, MDA-MB-231, and L6 (negative control) cells with or without TrkA receptor inhibitor K252a (100 nM) for 120 min (TNP) and 30 min (HNP) at 37°C in CO_2 incubator. After incubation, cells were washed with 1X PBS (pH 7.4) and fixed with 4% formaldehyde. Then, the cells were mounted on a cover slip and photographed under a fluorescent microscope (Olympus IX 83, Japan) at 40X magnification attached to a CCD camera.

3.2.2.4 Assessing the *in vitro* cell cytotoxicity of the custom peptides

For the cell viability assay, MCF7 cells, PC-12 cells, and myoblast cells isolated from L6 cells (0.1 x 10^5) were grown in 96-well plates at 37°C, 5% CO2, as described by us [11]. Different concentrations of the custom peptides (500 ng/mL to 2 µg/mL) were then added to the wells (in triplicates), and cell viability was assessed after 24 h of incubation by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based method [11]. The release of lactate dehydrogenase (LDH) from the treated cells was compared to control cells. The custom peptide-induced cytotoxicity, if any, was expressed as percent cell viability, which was determined by the viability percentage of control cells (control cells were considered to be 100% viable) and LDH released from the triton-X-treated cells (assumed to be 100% enzyme release) [11]. According to the instruction manual, cellular LDH release was measured using the CYQUANT™ LDH cytotoxicity assay kit (Invitrogen, C20300) [12].

3.2.2.5 *In vitro* effect of custom peptides on the mammalian hematological system

For the plasma clotting assay, goat blood was collected from a slaughterhouse in 3.8% trisodium citrate (9:1). The citrated blood was centrifuged at 4,300 rpm for 15 min at 4°C. The yellowish supernatant (the platelet-poor plasma, PPP) was used within 4 h to determine the plasma clotting activity [7,13]. One unit of pro-coagulant (or anticoagulant) activity was defined as a decrease (or increase) of 1 s in the clotting time of the PPP that was incubated with test samples (5 µg/mL), in comparison to the control PPP (1X PBS, pH 7.4).

The hemolytic activity of goat blood erythrocytes was assayed as described previously [7,13]. Two milliliters of a 5% red blood cells suspension (goat blood) were incubated with each custom peptide (1 µg/mL) / 1X PBS (negative control) / Triton X-100 (20 µL) (positive control) at 37ºC for 3 h. The reaction mixture was centrifuged at 4,000 rpm for 10 mins, and 200 µL of the supernatant was put into a 96-well microtiter plate. The absorbance at 540 nm was measured, and the percent hemolytic activity was calculated as stated previously [7,13].

As described previously, platelet-rich plasma (PRP) was isolated from citrated goat blood [7,13]. For assessing the platelet aggregation / de-aggregation properties of the custom peptides, the peptides (TNP, HNP, 1 µg/mL) were mixed with the PRP, and the absorbance at 540 nm was measured for 30 min at 30 sec intervals in a spectrophotometer (Multiskan GO, Thermo Scientific, USA). Percent aggregation / de-aggregation of the platelets was calculated for the treated samples compared to the control.

3.2.2.6 Assessing the neuritogenesis properties of custom peptides in PC-12 cells and the effects of chemical inhibitors on their neuritogenesis potency

For the neurite outgrowth bioassay, PC-12 cells were used as an *in vitro* model [6,8-10]. PC-12 cells are clonal cells derived from stem cells of rat pheochromocytoma expressing TrkA receptors and $p75^{NTR}$, and thus, are recommended as a functional model system for neuronal differentiation [8-10,14]. Our previous study adopted the neuritogenesis assay [8-10]. Cells (1.0×10^5 cells/well) were seeded in six-well plates (in triplicates) and kept overnight at 37°C in an incubator with 5% CO_2 to allow the cells to adhere to the surface. The next day, the complete medium (DMEM) containing 100 ng/mL mouse 2.5S-NGF (positive control) / custom peptide (12.5 ng/mL to 100 ng/mL) / 1X PBS, pH 7.4 (negative

control) was added to the wells and the plates were incubated at 37°C in 5% CO_2 for 14 days. The old media was replenished with fresh media at intervals of 72 h. Neurite outgrowth of the PC-12 cells was visualized under a phase-contrast microscope (Olympus IX 83, Japan) at 20x magnification. The percentage of neurite-bearing cells and the average neurite length (in µm) were determined using MOTIC IMAGE PLUS 3.0 software. Cells were considered to be differentiated if they had at least one neurite with a length equal to or greater than the diameter of the cell body [6,8-10].

Cells were pre-incubated with a chemical inhibitor, such as TrkA receptor inhibitor K252a (100 nM) / inhibitor of phosphatidylinositol 3-kinase (PI3K) stimulation of protein kinase B (Akt) signaling pathways (PI3K/AKT), pathway LY294002 (30 nM) / inhibitor of mitogen-activated protein kinase (MAPK), pathway U0126 (10 nM), or anti-TrkA, TrkB, and anti-TrkC antibody (1:1000 in DMEM) for 1 h. Cells were then treated with or without the addition of 100 ng/mL (~71 nM) custom peptide/mouse 2.5 S-NGF (positive control, 100 ng/mL) / 1X PBS (control), incubated for 14 days as above. The average length of neurite outgrowth and the percentage of neurite-bearing cells were determined [6,8-10].

3.2.2.7 Assessing the protective role of custom peptides against the PT-induced cytotoxicity in PC-12 cells

The cytotoxicity assay was performed as previously described [8-11]. In brief, PC-12 cells at a density of 1×10^4 cells/well were placed in a 96-well plate and then incubated in 5% CO_2 to facilitate the attachment of the cells to the well. The cells were treated with various concentrations (1- 30 mM) of PT or growth medium (control), and incubated at 37 °C in 5% CO_2 for 24 h. After 24 h, PT-induced cytotoxicity was determined by the MTT-based cytotoxicity assay [11]. A standard curve was generated from each paraquat concentration, and the IC_{50} values were calculated from the regression analysis of growth curves of PC-12 cells in the presence of PT. The protective role of TNP, HNP, and scrambled peptide against PT-induced cytotoxicity (IC_{50} value was determined as 10 mM) was determined by the MTT-based cytotoxicity assay [11]. Cells were pre-incubated / post-treated / co-treated with custom peptides (HNP / TNP) / mouse 2.5S-NGF (positive control) in an increasing concentration-dependent (12.5, 25, 100, 250 and 500 ng/mL; equivalent to 18 nM to 357 nM) and time-dependent (0.5, 1, 2, 4, and 6 h) manner. The

percent cell viability for each group was calculated after 24 h of PT incubation, and each experiment was repeated thrice.

Assessing the protective role of custom peptides and their optimum dosages against the PT-induced cytotoxicity in PC-12 cells was also confirmed by quantitating the release of marker enzyme LDH from the treated cells. PC-12 cells were dispensed into a 96-well plate at a density of 1 X 10^4 cells/well. Cells were pre-treated with different concentrations (25, 100, 250, and 500 ng/mL; equivalent to 36 nM to 357 nM) of custom peptide HNP / TNP / NGF (100 ng/mL) as a positive control/control (triton X-treated) for 1 h (optimum time derived from the previous experiment) and followed by 10 mM PT (IC_{50} value) treatment for 24 h (in triplicates).

3.2.2.8 Flow cytometry analysis to determine PT-induced apoptotic cell death and its protection by custom peptides

Annexin-V binding and PI uptake studies determined apoptotic and necrotic cells. Annexin V has a high affinity for phosphatidylserine, which is translocated from the internal to the external surface of the cell membrane when PC-12 cells undergo apoptosis. Necrotic cells absorb PI due to the higher permeability of their damaged cell walls.

Briefly, PC-12 cells were seeded on a 24-well culture plate, pre-treated with 100 ng/mL (~ 71 nM) custom peptide (HNP and TNP) for 1 h, which was followed by the PT (10 mM) treatment for 24 h before being harvested. Cells were washed three times using PBS and centrifuged at 1200 rpm for 5 min, with the concentration adjusted to 1 X 10^5 cells/well [15]. Pellets were re-suspended in the 1X binding buffer, following the instructions of the manufacturer, and then incubated for 5 min with Annexin V-FITC and PI at 4°C in proportions mentioned in the Annexin V-FITC early apoptosis kit (Cell Signaling Technology, CST-6592). Fluorescence intensity was analyzed with flow cytometry (BD-FACS-Melody, USA, Melody multi-color analyzer cum high-speed cell sorter). Normal cells without any treatment were used as a control for fluorescence compensation adjustment to reduce the spectral overlap and establish the location of the cross gate.

3.2.2.9 Determination of the effect of custom peptides in inhibiting the reactive oxygen species (ROS) production in PT-treated PC-12 cells by spectrofluorometric assay and flow cytometry analysis

The ROS level in the PC-12 cells post-PT treatment was determined using the fluorogenic probe 2',7'-dichlorofluorescein-diacetate (H_2DCFDA) according to the procedure of Mukherjee et al. (2015) [11,16]. Briefly, PC-12 cells (1 X 10^6) at 70% confluence were cultured in 24-well plates and kept overnight. Cells were pre-treated with different concentrations of custom peptides (12.5 ng/mL to 500 ng/mL, equivalent to 18 nM to 357 nM) / Vitamin C (10 µg/mL, positive control) / PBS for 1 h, followed by treatment with PT (10 mM) for 12 h. Media was aspired out, cells were washed with PBS, and incubated with 10 µM H_2DCFDA in the dark for 30 min at 37°C. The cells were washed twice with 200 µL PBS and incubated with 1% Triton X-100 (170 µL). The reaction was stopped by adding DMSO (130 µL). Cells were scraped and transferred to the black plate. Absorbance was measured at excitation and emission wavelengths of 480 and 530 nm using a microplate reader (in triplicates). The ROS production by PT-treated cells was considered 100% (baseline), compared to other activities.

In another set of experiments, intracellular ROS levels generated post-PT exposure in PC-12 cells were measured by flow cytometry (BD-FACS-Melody, USA) analysis using H_2DCFDA, according to the procedure described by Mukherjee et al. (2015) [11]. Briefly, PC-12 cells were pre-treated with custom peptides (HNP and TNP) at different concentrations (12.5 ng/mL to 500 ng/mL, equivalent to 18 nM to 357 nM) and Vitamin C (10000 ng/mL as the positive control) for 1 h, which was followed by PT incubation for 12 h. The adhesive and non-adherent cells were collected and washed twice with PBS (pH 7.4). The cells were then incubated with 10 µM H_2DCFDA at 37°C for 30 min in the dark, then washed twice with chilled PBS. The fluorescence intensities of 2`,7`-dichlorodihydrofluorescein (DCF) produced by intracellular ROS were analyzed by flow cytometry with excitation and emission at 480 and 530 nm, respectively.

3.2.2.10 Determination of the effect of custom peptides on reducing the PT-induced depolarization of mitochondrial membrane potential

Changes in the mitochondrial transmembrane potential were determined with the Mito Probe™ JC-1 assay kit (Invitrogen, USA, Catalog number M34152) protocol [17]. PC-

12 cells were pre-treated with 100 ng/mL (~71 nM) of a custom peptide (HNP/ TNP) for 1 h, which was followed by the PT (10 mM) treatment for 10 h at 37°C, in 5% CO_2 (in triplicates). After the treatment, cells were washed three times, incubated with 1 µg/mL JC-1 dye, and incubated at 37°C in a humidified CO_2 incubator for 4 h. The PC-12 cells were washed three times, and images were captured at excitation and emission wavelengths of 490 nm and 530 nm, respectively, to see the green fluorescence of JC-1 monomers. For the red fluorescence J-aggregates, excitation and emission wavelengths were set at 525 nm and 590 nm under a confocal microscope (TCS SPE, Leica, Wetzlar, Germany). The fluorescence intensities were measured using Image J software. Changes in the mitochondrial transmembrane potential were also analyzed by flow cytometry with the same JC-1 dye, as described in the previous section (Section 2.11). The carbonyl cyanide m-chlorophenyl hydrazone (CCCP, mitochondrial uncoupler) was used as a positive control for flow cytometry analysis.

3.2.2.11 Determination of the effect of custom peptides on the restoration of PT-induced cellular and nuclear morphological changes in PC-12 cells

To study the cellular and nuclear morphological changes induced by PT, 1 mL of 1 X 10^6 PC-12 cells was seeded in a 24-well plate and allowed to adhere overnight at 37°C. PT-induced nuclear damage in the PC-12 cells was observed by 4′,6-diamidino-2-phenylindole (DAPI) staining [11,18]. After the pre-treatment mentioned above with custom peptides (100 ng/mL, equivalent to ~71 nM), the cells were collected, washed in PBS, and then fixed in 1% formaldehyde (in PBS) for 30 min at room temperature. After washing with 1X PBS, the cells were suspended in DMEM media and incubated with 5 µL of DAPI (1 µg/mL) for 30 min at 37°C. The cells were washed with PBS, placed onto a glass slide, and observed under a fluorescence microscope at 40X magnification. The percentage of dead cells was calculated in four randomly selected microscopic fields, and the changes in cellular and nuclear morphology were compared to the untreated cells (control).

3.2.2.12 DPPH free radical scavenging activity of custom peptides *in vitro* condition

DPPH radical-scavenging activity of custom peptides was measured [19]. Briefly, in a 96-well plate, 100 µL peptide sample (25 ng/mL – 250 ng/mL) / vitamin C (10000 ng/mL, or 10 µg/mL, positive control) was uniformly mixed with 100 µL of 0.2 mM DPPH

radical solution (dissolved with 95% ethanol) and incubated at room temperature for 30 min. Instantly, the absorbance of the solution was measured at 517 nm by a microplate reader (Varioskan Flash, Thermo Scientific, USA). The percentage of DPPH free radical-scavenging activity was determined as follows:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \left[1 - \frac{A1-A2}{A0}\right] * 100$$

where A0 signifies the absorbance of 95% ethanol (100 µL) (v/v) mixed with DPPH radical solution (100 µL) at 517 nm; A1 shows the absorbance of the peptide (100 µL) with DPPH radical solution (100 µL), A2 denotes the absorbance of peptide solution (100 µL) with 95% ethanol (100 µL). This experiment was performed in triplicates to ensure reproducibility.

3.2.2.13 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis to determine the expression levels of pro- and anti-apoptotic genes in the PT-treated PC-12 cells and the effect of pre-treatment of custom peptides

The procedure for qRT-PCR analysis of stress-related gene expression was adopted from our previous studies [17,20]. PC-12 cells were subjected to different treatments at 37 ºC: (a) 1X PBS (control) treated PC-12 cells (CT); (b) 10 mM PT (PT) treatment for 12 h; (c) pre-treatment with 100 ng/mL (~71 nM) custom peptide (TNP or HNP) or mouse 2.5S-NGF (positive control) (NGF) for 1 h followed by 10 mM PT treatment (PTNP, PHNP, and PNGF) for 12 h. Total RNA was isolated with a pure link RNA mini kit (Invitrogen, USA; Cat- 12183018A), and the purity and concentration (A260 / A280) of the RNA were measured using a Nanodrop 2000C spectrophotometer (Thermo Scientific, USA). Reverse transcription was performed from 1 µg of the total RNA using a verso cDNA synthesis kit (Thermo Scientific, USA; Cat: AB-1453/A) according to the manufacturer's protocol. The reaction was performed at 42 ºC for 30 min and ended by incubation at 85 ºC for 5 min. The qRT-PCR was done with SYBR Green (Bio-Rad, USA) to quantify the expression of pro-apoptotic genes, such as caspase-3, B-cell lymphoma 2 (bcl-2), and heat shock protein function (hsp-70) (in triplicates) in a CFX96 Touch Real-Time PCR detection system (Bio-RAD, USA). The relative expression of every gene was analyzed by normalizing it with the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene using the $2-\Delta\Delta Ct$ method under identical experimental

conditions. The experiment was repeated three times to ensure reproducibility. The sequence of primers used in this experiment [21-24] is shown in Table 3.1.

Table 3.1 List of oligonucleotide primer sequences for the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis in PC-12 cells.

Gene	**Orientation**	**Primer sequence (5'→3')**	**Product size (bp)**	**Criteria for RT PCR**	**References**
GAPDH	Forward	GGTGAAGGTCGGTGTGAACG	232	Hold at 95 °C for 3 min, followed by 40 cycles of 95o C for 30 s, 58o C for 45 s, and 72o C for 30 s.	[21]
	Reverse	CTCGCTCCTGGAAGATGGTG			
Bcl-2	Forward	TGGGATGCCTTTGTGGAACT	153		[21]
	Reverse	GCAGGTTTGTCGACCTCACT			
Bax	Forward	GGTTTCATCCAGGATCGAGA	151		[21]
	Reverse	TCCTCTGCAGCTCCATATTGC			
Bid	Forward	AGCTACACAGCTTGTGCCAT	186		[21]
	Reverse	CAGCTCGTCTTCGAGGTCTG			
HSP-70	Forward	GAGACAACCCAGGCACCCAA	223		[22]
	Reverse	CCACCCCGAGATCCCCTAAAAA			
Cytochrome-C	Forward	GAAGAAGGGAGAAAGGGCAGA	302		[24]
	Reverse	CGGGGGCTGTCCAACAAA			
Caspase-3	Forward	TGACTGGAAAGCCGAAACT	147		[24]
	Reverse	GGGTGCGGTAGAGTAAGCAT			

3.2.2.14 Quantitative proteomic analysis comparing the expression of global proteins for PC-12 cells exposed to PT and cells pre-treated with custom peptides followed by the PT treatment

Quantitative proteomics was used to identify and categorize PT-transformed proteins and compare their restoration by custom peptides in pre-treated PC-12 cells [10]. For comparing the differential expression of cellular proteins in treated and control cells, PC-12 cells were subjected to the following treatments (at 37°C):

(a) 1X PBS (control)-treated PC-12 cells (CT); (b) 10 mM PT (PT) treatment for 1 h; (c) pre-treatment with 100 ng/mL (~71 nM) custom peptide for 1 h followed by 10 mM PT

treatment (PHNP) for 24 h; and (d) treatment with 100 ng/mL (~71 nM) of custom peptide (HNP) for 1 h. Differentially treated PC-12 cells were collected and lysed separately in a RIPA buffer containing proteinase K inhibitor for 30 min on ice.

The tissue lysate was sonicated and centrifuged at 13,000 rpm for 20 min at 4°C, and the protein concentration was estimated [25]. Briefly, 30 µg of the protein from the treated PC-12 cells was subjected to reduction with 10 mM dithiothreitol (DTT) and alkylation with 50 mM iodoacetamide (in the dark), which was followed by incubation with sequencing-grade trypsin (50 ng/µL in 25 mM ammonium bicarbonate comprising 10% acetonitrile) for 18 h at 37°C [8-10,26,27]. The digested peptides were desalted and concentrated using ZipTip (Millipore, USA), and then re-suspended in 20 µL of 0.1% (v/v) formic acid in 2% acetonitrile. The 1 µL samples were exposed to nano-ultra-performance liquid chromatography-tandem mass spectrometer (UHPLC-MS/MS) analysis. ESI (nano-spray) was used as the ion source, collision-induced dissociation (y and b ions) was used as the fragmentation mode, FT-ICR/Orbitrap was used as the MS scan mode, with an MS/MS scan range of 500 to 2000 m/z being used as the linear ion trap. Double or triple-charged ions were selected for the collision-induced dissociation (CID) MS/MS analysis.

For the data analysis, MS data protein was identified, quantitated, and analyzed using the Peak studio from the Sequest HT database search engine with a 1% false discovery rate (FDR) and two missed cleavage cut-off standards. The database search comprised all entries from the *Rattus norvegicus* (Taxonomy ID: 10116) UniProt reference proteome database. Total protein level analysis was executed using 10 parts per million precursor ion tolerance. The product-ion tolerance used for the data evaluation was 0.05 Da. Oxidation of methionine residues (+15.995 Da) was kept as a variable amendment, whereas cysteine carbamidomethylation (+57.021 Da) was kept as a static modification. Peptide spectra match (PSMs) was adjusted to have an FDR of 0.01. Relative abundances were calculated from the spectral intensity of the respective protein, and the fold change values of the relative abundances were also calculated. Protein-protein interaction (PPI) network analysis was carried out with Cytoscape 3.9.1 (download freely available at https://cytoscape.org/download.html).

3.2.3 To study the *in vivo* neuroprotective mechanism of custom peptides in *C. elegans*

3.2.3.1 Maintenance of *C. elegans* strain

Wild-type Bristol strain N2 was used as an *in vivo* model for determining PT-induced oxidative stress and its protection by the custom peptides. The transgenic BZ555 (Pdat-1::gfp) strain of *C. elegans* has DAergic neurons expressing GFP, which was used for studying PT-induced neurodegeneration and protection by the custom peptide. A Transgenic NL5901 strain of *C. elegans* (Punc-54: *α*-synuclein:: YFP+unc-119; expressing human *α*-synuclein protein tagged YFP in the muscles) was used to measure the deposition of toxic *α*-synuclein protein after custom peptides treatment. *C. elegans* (N2), BZ555, CAM-1(ak37), and NL5901 strains were grown on nematode growth medium (NGM) plates at 20 ˚C in a bio-incubator, and *E. coli* strain OP50 was provided as a food source.

Synchronization was performed using the standard procedure of the alkaline hypochlorite method described previously [28] with some modifications. The NGM plates with adequate eggs were washed with M9 buffer and collected into a 15 mL conical centrifuge tube. A mixture of sodium hypochlorite (1 mL) and 5 N NaOH (0.5 mL) was added to the 15 mL tube containing worms in 3.5 mL M9 buffer. The tubes were then agitated for 2 min and centrifuged at 7500 rpm at room temperature to settle the eggs. The supernatant was discarded, and the pellet was washed two times with M9 buffer. Then 0.1 mL of M9 buffer was added to the pellet, transferred to the unseeded NGM plate, and incubated at 20°C after adding *E. coli* OP50 to the NGM plate.

3.2.3.2 Determination of *in vivo* binding of FITC-conjugated custom peptides to *C. elegans* (CAM-1 mutant and N2 strains)

The peptides were conjugated with FITC following the procedure Islam et al. described (2020) [8]. The binding assay was performed with the synchronized wild-type L4 larvae of N2 worms and CAM-1 mutant; 10 worms for each treatment were then collected in a 1.5 mL tube and washed multiple times with M9 buffer to remove the *E. coli* contamination. The N2 worms were then treated with FITC-conjugated peptides (TNP and HNP) for 1 to 4 h and the CAM-1 mutant worm for 2 h at room temperature, followed by washing the worms with M9 buffer (to remove adherent *E. coli*) and transferred onto

3% (w/v) agarose pads on a glass slide for confocal microscopy study (TCS SPE, Leica, Wetzlar, Germany). The excitation wavelength was set at 488 nm (ʎex ~488 nm), and the emission fluorescence signal was recorded at 519 nm (ʎem~519 nm) to detect the binding of peptides to *C. elegans*. The image was then quantified using Image J software.

The following experiment determined whether the peptide binds to a site where PT binds. The worms were divided into different groups:

1. Only FITC (50 µg/mL) treated N2 worms for 2 h.
2. FITC-custom peptide (HNP, 50 µg/mL or 35.7 µM) and FITC-scrambled peptide (50 µg/mL or 38.5 µM) treated N2 worms for 2 h.
3. FITC-conjugated custom peptide (HNP, 50 µg/mL) pre-treated N2 worms for 2 h, followed by PT (10 mM) treatment for 1 h.
4. PT (10 mM) treatment for 1 h followed by FITC-conjugated custom peptide (HNP, 50 µg/mL) treatment for 2 h in N2 worms.
5. FITC-conjugated custom peptide co-treated N2 worms with different concentrations of PT (5 mM, 7.5 mM, 10 mM) were observed under a confocal microscope.
6. FITC-conjugated peptides (50 µg/mL) treated with CAM-1 mutant strain for 2 h. The signals were recorded at an excitation (ʎex) and emission wavelength (ʎem) of ~498 nm and ~519 nm respectively and quantified using Image J software.

3.2.3.3 Assessment of the protective role of custom peptides and mouse-NGF against PT-induced toxicity in *C. elegans* (N2 and CAM-1 mutant strain)

Custom peptides and mouse-NGF' *in vivo* protective activity was determined against the PT-induced oxidative stress in the *C. elegans* N2 and CAM-1 mutant strain [29]. Synchronized L4-stage adult N2 worms were pre-incubated with different concentrations (12.5 µg/mL to 100 µg/mL; equivalent to 9.6 to 76.9 µM) of custom peptides. Mouse 2.5S-NGF (50 µg/mL), vitamin C (100 µg/mL), and quercetin (50 µg/mL) were used as a positive control, whereas scrambled peptide, which did not show binding with CAM-1 receptor was used as a negative control. The N2 larvae were pre-treated with them for 2 h followed by PT (10mM)-treatment for 1 h. In an additional set of experiments, the CAM-1 mutant was pre-incubated with 50 µg/mL of custom peptides (HNP / TNP) and

mouse NGF 2.5S for 2 hours, followed by PT treatment for 1 hour, to demonstrate the binding of custom peptide and mouse NGF 2.5S (at optimal dose and timing) to the CAM-1 receptor.

In another set of experiments, N2 worms were pre-incubated with custom peptides and quercetin (50 µg/mL, positive control) for 2, 12, and 24. For 24 h of pre-incubation study, the custom peptides were added at 12 h of pre-incubation. The pre-treated worms were then transferred into the wells of 96-well plates (20 worms per well in triplicates; approximately 60 individuals for each group). The worms were provided with *E. coli* OP50 (as a food source) and treated with 10 mM PT for 24 h [17,30], whereas, in control wells, 1X PBS was added. 'Worms' percentage (%) survival was scored visually using a stereo-zoom microscope after 24 h. Worms that did not show movement after light exposure and gentle tapping were considered dead.

In another set of experiments, the N2 worms were treated with 10 mM PT for 1 h, followed by post-treatment with various concentrations (12.5 µg/mL to 100 µg/mL; equivalent to 9.6 to 76.9 µM) of custom peptide (HNP / TNP) and vitamin C (100 µg/mL) /quercetin (50 µg/mL, positive control)/ mouse 2.5 S NGF (50 µg/mL)/ NGM buffer with OP50 (control) for 2 h and 24 h respectively at 20 °C. The survival of N2 worms was determined, as stated above. This experiment was performed in triplicate to ensure reproducibility.

3.2.3.4 PT-induced loss of chemotaxis behavior in *C. elegans* and its restoration by treatment with custom peptides

The chemotaxis/memory learning assay was done as described by [31] with slight alterations. Synchronized wild-type N2 strain of *C. elegans* (L4 stage) was subjected to the following treatment conditions at 20 °C:

(i) untreated (control), (ii) 10 mM PT-treatment for 1 h, (iii) PT (10 mM)-treatment for 1 h followed by treatment with custom peptides HNP and TNP (50 µg/mL ~38 µM) for 2 h/ vitamin C (100 µg/mL) for 24 h, (iv) custom peptide (HNP / TNP, 50 µg/mL) pre-treatment for 2 h/ vitamin C(100 µg/mL) pre-treatment for 24 h followed by PT (10 mM) treatment for 1 h, and (v) treatment with only custom peptides (50 µg/mL) for 2 h.

The concentration and incubation time of the custom peptides used in this experiment were determined from the previous experiments (described in sections 4.3 and 4.4). The treated and untreated (control) worms were then collected in 60 mm NGM plates, and their chemotaxis behavior was determined below [31].

The plates were divided into two halves; one side was marked as "attractant" (A), and the other half was marked as "control" (C). For this experiment, 0.1% benzaldehyde dissolved in 100% ethanol was considered an odorant for the test sample, and 100% ethanol was an odorant for the control sample. On the "A" side, 2.5 µL of 0.5 M sodium azide containing 2.5 µL of odorant was added, whereas 2.5 µL of 100% ethanol containing 2.5 µL sodium azide was added on the opposite "C" side (Fig 3.1). Immediately after the addition, 20 worms were transferred to the center of the plate. This experiment was performed in triplicates to ensure reproducibility. The assay plates were then scored for the number of worms in each quadrant after 1 h. The CI was calculated as follows:

Number of worms at the "A" quadrant ---------- (T)

Number of worms at the "C" quadrant ---------- (C)

Total number of worms taken on the plate ---------- (N)

Therefore, $CI = (T - C)/N$

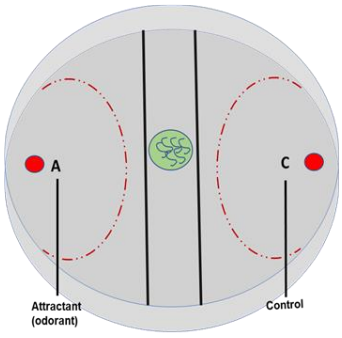

Fig 3.1 The schematic diagram represents the experimental design of the chemotaxis assay to calculate the chemotaxis index (CI).

3.2.3.5 Determination of the effect of custom peptides in inhibiting the ROS production in PT-treated *C. elegans*

The ROS level in *C. elegans* (N2 and CAM-1 mutant) post-PT treatment was determined using the fluorescent probe chloromethyl '2',7'-dichlorofluorescein-diacetate (CM-

H_2DCFDA) by following the procedure of Kumar et al. [17]. Briefly, the N2 worms were synchronized, as stated above. Worms were washed and re-suspended in 1X PBS containing 50 µg/mL peptide (HNP / TNP). The mouse 2.5-S NGF (50 µg/mL) and vitamin C (100 µg/mL) were positive controls. The worms were then shaken on a tube rotator (ROTOSPINTM, Tarson, India) for 2 h with custom peptides and 24 h with positive controls at 50 rpm, washed with 1X PBS, and further re-suspended in PBS containing PT (10 mM) /1X PBS (control) and incubated for 1 h at 23 °C. The worms were washed with PBS and re-suspended in 200 µL PBS. The worms were freeze-cracked and sonicated for 10 s. The worm lysate was then kept on ice for 30 minutes, centrifuged at 13,500 rpm for 30 minutes, and the supernatant was transferred to another tube.

After protein estimation (Pierce™ BCA Protein Assay Kit), 25 µg of protein from each group was taken and made up the final volume of 50 µL with 1X PBS. For determining the PT-induced ROS generation, 50 µL protein extract containing 25 µg of each group was mixed with 100 µL of 50 µM of CM-H_2DCFDA in PBS and incubated for 4 h at 37 °C. Finally, fluorescence was measured in a multimode fluorescence plate reader (excitation at 485 nm and emission at 535 nm). A hundred worms per treatment group were used to measure ROS production. This experiment was performed in triplicates to ensure reproducibility.

The confocal microscopic analysis also quantified the *in vivo* cytoplasmic ROS level in another set of experiments. For this experiment, ten worms of each wild type and CAM-1 mutant strain were divided into three groups. They were treated as–(i) worms pre-treated with peptides (50 µg/mL) for 2 h and mouse NGF (50 µg/mL)/ vitamin C (100 µg/mL, positive control) for 24 h followed by PT (10 mM) treatment for 1 h, and (ii) only PT (10 mM) treated worms for 1 h, and (iii) worms incubated with 1X PBS (control) for 3 h. Each group of N2 and CAM-1 mutant worm was then incubated with 50 µM of CM-H_2DCFDA at 37°C for 4 h, visualized under a confocal microscope (TCS SPE, Leica, Wetzlar, Germany). Images were photographed at 40X with a CCD camera, and the fluorescence intensity was determined and quantitated using Image J software. The experiments were carried out in triplicates to ensure reproducibility.

3.2.3.6 Determination of the effect of custom peptides on reducing the PT-induced depolarization of mitochondrial membrane potential

Mitoprobe JC-1 assay kit (Invitrogen) was used to monitor mitochondrial transmembrane potential using the Mito Probe™ JC-1 Assay Kit protocol. The L4-stage young adult of each N2 and CAM-1 mutant worm were divided into three groups- (i) pre-incubated with 50 µg/mL peptides for 2 h and vitamin C for 24 h, mixed well by shaking on a tube rotator for 2 h at 50 rpm followed by the treatment with PT (10 mM) for 1 h at 20 °C, (ii) 1X PBS (control) treated worms for 3 h, (iii) only PT (10 mM) treated worms for 1h at 20 °C. After the treatment, worms were washed three times, incubated with 1 µg/mL JC-1 dye, and incubated at room temperature for 4 h. The experiments were repeated in triplicates to ensure reproducibility. The worms were washed three times, and the images were captured at excitation and emission wavelengths of 490 nm and 530 nm, respectively, for green fluorescence JC-1 monomers and at excitation and emission wavelengths of 525 nm and 590 nm for red fluorescence J-aggregates under a confocal microscope. The fluorescence intensity was measured using Image J software.

3.2.3.7 Quantitative analysis of the effect of custom peptides on PT-induced dopaminergic (DAergic) neurodegeneration

The transgenic BZ555 (P^{dat-1}::gfp) strain of *C. elegans* has specifically DAergic neurons expressing GFP, to which PT could induce degeneration [32,33]. Briefly, the L4 stage nematode of the BZ555 strain was washed with PBS and collected in a 1.5 mL centrifuge tube containing M9 buffer. The worms were treated under four different conditions- (i) 1X PBS (control), (ii) 10 mM PT-treated worms for 1 h, (iii) PT (10 mM)-treatment for 1 h followed by treatment with custom peptide (HNP / TNP, 50 µg/mL) for 2 h, and (iv) custom peptides treatment for 2 h and vitamin C (100 µg/mL) for 24 h followed by PT (10 mM) treatment for 1 h. The worms were observed under a confocal microscope and photographed, and the fluorescent images were then analyzed using Image J software. The experiment was repeated three times to ensure reproducibility.

3.2.3.8 Quantitative analysis of the effect of custom peptides on preventing the *α*-synuclein accumulation in the NL5901 strain of *C. elegans*

NL5901 worms were used to observe the aggregation of the *α*-synuclein protein after being treated with custom peptides (50 µg/mL), as stated previously [34]. Briefly, synchronized L4 stage nematodes were incubated with 50 µg/mL custom peptides for 12 h and 100 µg/mL vitamin C for 24 h at 20 °C. After treatment, worms were washed two times with M9 buffer and transferred onto a 3 % agarose pad slide containing 100 mM

sodium azide. The fluorescence intensity of the accumulated *α*-synuclein was observed under a microscope, and the live worms were also observed under a confocal microscope. The fluorescent and confocal images were photographed and analyzed using Image J software. The experiment was repeated three times to ensure reproducibility.

3.2.3.9 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis to determine the effect of pre-treatment of *C. elegans* with custom peptides on the PT-induced stress-related gene expression

The procedure for qRT-PCR analysis of stress-related gene expression is adopted from our previous studies [17,20]. Approximately 400-500 L4-stage adult worms were collected in a 1.5 mL centrifuge tube and washed multiple times to remove the adhering bacteria. Further, the worms were centrifuged, the supernatant was aspirated, and the pellet was re-suspended in 1 mL of 1X PBS containing 50 µg/mL peptide (HNP / TNP) and 100 µg/mL vitamin C (positive control) and shaken on a tube rotator for 2 h and 24 h respectively at 50 rpm. The worms (pellet) were then washed, and the pellet was re-suspended in 1 mL of 1X PBS containing 10 mM PT and incubated for 1 h at 20 °C. Then, the worms were washed, total RNA was isolated using an RNA isolation kit (Invitrogen, USA), and RNA quality was determined by measuring absorbance at 260/280 nm in a nanodrop spectrophotometer. The cDNA was described from mRNA as described previously [4]. The sequence of the primers used for qRT-PCR [17] is shown in Table 3.2. The relative expression of every gene was analyzed by normalizing it with the expression of the *act-1* housekeeping gene using the $2^{-\Delta\Delta Ct}$ method under identical experimental conditions. The experiment was repeated three times to ensure reproducibility.

Table 3.2: List of oligonucleotide primer sets used for qRT-PCR analysis in *C. elegans*.

Sequence name	Sequence	No. of Bases
sek-1_F	GCCGATGGAAAGTGGTTTTA	20
sek-1_R	TAAACGGCATCGCCAATAAT	20
pmk-1_F	CCGACTCCACGAGAAGGATA	20
pmk-1_R	AGCGAGTACATTCAGCAGCA	20
skn-1b_F	CTCTCTTCTGGCATCCTCTACCA	23
skn-1b_R	CCGACTCCACGAGAAGGATA	20
pmk-1_F	AGCGAGTACATTCAGCAGCA	20
pmk-1_R	TTCTTGGATTCTTCTTCTTGTTCGT	25
act-1_F	GCTGGACGTGATCTTACTGATTACC	25

act-1_R	GTAGCAGAGCTTCTCCTTGATGTC	24
sod-1_F	CGTAGGCGATCTAGGAAATGTG	22
sod-1_R	AACAACCATAGATCGGCCAACG	22
sod-3_F	TTCAAAGGAGCTGATGGACACT	22
sod-3_R	AAGTGGGACCATTCCTTCCAA	21
trx-1_F	TCCAACACTTTTTGACGCAG	20
trx-1_R	CAAGATGATGCCGACTTTCA	20
ser-1_F	AAGAGCCAGTCGCCAGAAC	19
ser-1_R	GTGGTTGATGCCTCTGTCGT	20
hsp-16.1_F	GCAGAGGCTCTCCATCTGAA	20
hsp-16.1_R	GCTTGAACTGCGAGACATTG	20
hsp-16.2_F	CTATTTCCGTCCAGCTCAAC	20
hsp-16.2_R	TTTGTTCAACGGGCGCTTGC	20
hsp-60_F	CTATGGGCCCAAAAGGAAGAAACGTG	26
hsp-60_R	GGATTTCGCGACGGTGACTCCGTCC	25
hsp-70_F	GAAAGGTTGAAATCCTCGCGAACTC	25
hsp-70_R	TCCGGATTACGAGCGGCTTGATCTT	25
ctl-1_F	CGGATACCGTACTCGTGATGA	21
ctl-1_R	CCAAACAGCCACCCAAATCA	20
ctl-2_F	TCCGTGACCCTATCCACTTC	20
ctl-2_R	TGGGATCCGTATCCATTCAT	20
gst-4_F	GATGCTCGTGCTCTTGCTG	19
gst-4_R	CCGAATTGTTCTCCATCGAC	20
gst-6_F	GGACAAGACTTCGAGGACAAC	21
gst-6_R	AACTGACGAGCCAAGTAACG	20
gst-10_F	AAGAGATTGTGCAGACTGGAG	21
gst-10_R	AGAACATGTCGAGGAAGGTTG	21
ced-3_F	ACGGGAGATCGTGAAAGC	18
ced-3_R	AGAGTTGGCGGATGAAGG	18
ced-4_F	AGTCACTCGCAATGGCTCT	19
ced-4_R	GCTGATGAACGACGGAAT	18
ced-9_F	AAAGGCACAGAGCCCACC	18
ced-9_R	CGTTCCCATAACTCGCATC	19

3.2.3.10 Transcriptomic analysis to study the gene expression in *C. elegans* when treated with PT vs. pre-treatment with custom peptides followed by treatment with PT

Synchronized L4 stage wild-type N2 strain of *C. elegans* was subjected to the following treatments at 20 °C: (a) CT group, (b) PT group, (c) PHNP, (d) HNP group. Total RNA extraction was done from the treatments of C. *elegans* using the Trizol Method [10]. Extracted RNA was subjected to DNase treatment per manufacturer protocol using New England Biolabs (NEB) (Cat# M0303L kit). The concentration and quality of RNA were measured using a Qubit fluorometer. Further, the quality of preparation was also

examined by checking the RNA integrity number (RIN) using BioAnalyser from Agilent. The samples with RIN value ≥ 7 were subjected to the RNA library preparation.

Per the manufacturer's protocol, transcriptomic mRNA libraries were prepared using NEB (cat# E7770L kit) as previously described [35]. The libraries were subjected to quantification using Qubit and Bioanalyser from Agilent. Sequencing done on an Illumina 150 bp PE platform was outsourced (Biokart India Pvt Ltd, Banglore, India).

The quality of reads obtained from the libraries was assessed using Fastqc (v0.11.8), followed by eliminating adapters / inferior-quality reads using Trim Galore (v 0.6.7). Trinity (v v2.13.2) was used for *de novo* transcriptome assembly. The redundancy of the primary assembly was removed and validated using cd-hit and RNA-Seq by Expectation-Maximization (RSEM), respectively.

The validated transcripts are subjected to Gene Ontology (GO) analysis, a Database for Annotation, Visualization, and Integrated Discovery (DAVID), and respective databases. Raw read count files were obtained by using tools viz RSEM (v v1.3.3) / Spliced Transcripts Alignment to a Reference (STAR) (v v2.7.10a) or Salmon (v v1.6.0)/ Binary Alignment Map (BAM) & Sequence Alignment Map (SAM) (v v1.14)/ Picard (v v2.18.7). For a comparative analysis, the Interactive Gene Expression Analysis Kit for Microarray & RNA-seq Data (iGEAK) (v1.0a) tool was used, which is an R (v3.3.2) and JavaScript-based open-source desktop application with the Shiny platform. Further, Voom /edgeR/limma analysis was performed to analyze differentially expressed genes (DEG). The data generation and analysis were performed at Biokart India Pvt Ltd, Bangalore, India.

3.2.3.11 Quantitative proteomics analysis to compare the expression of global proteins between *C. elegans* pre-treated with custom peptides followed by PT and *C. elegans* treated only with PT

For comparison of differential expression of the cellular proteins, the worms were subjected to the following treatments at room temperature: (a) CT group (untreated), (b) PT group (treated with PT), (c) PHNP group (pre-treated with HNP for 2h followed by PT treatment for 1h, and (d) HNP group (only HNP treatment. Approximately 400-500 L4-stage adult worms from each group were collected in a 1.5 mL centrifuge tube and washed multiple times to remove the adhering bacteria. Further, the worms were

centrifuged, the supernatant was aspirated, and the pellet was re-suspended in 1 mL of 1X PBS. The proteins were extracted separately from the above-mentioned treated groups using RIPA lysis buffer containing proteinase K inhibitor for 30 min on ice, followed by sonication and centrifugation.

The extracted proteins were quantified, and 30 µg of the protein from each treatment group was subjected to ESI LC/MS-MS analysis as described in our previous study [10]. Briefly, the proteins are reduced (10 mM DTT) and alkylated (50 mM iodoacetamide), followed by subjected to digestion with sequencing-grade trypsin for 18 h at 37 °C. The tryptic-digested peptides were desalted, concentrated, and subject to nano-UHPLC, followed by LC/MS-MS analysis. For ionization and fragmentation nano spray electrospray positive ionization (ESI) and collision-induced dissociation were used, respectively. MS and MS/MS scans (ranging from 500 to 2000 m/z) were carried out in FT-ICR/Orbitrap and linear ion trap, respectively. Only double or triple-charged ions were selected for collision-induced dissociation (CID) MS/MS analysis.

The raw data obtained from MS/MS analysis was analyzed in Peak studio, and the parameter for identifying proteins was adopted from our previous study [4]. The relative abundances were calculated from the spectral intensity of the respective protein, and fold change values of the relative abundances were also calculated.

3.2.4 Study the micro-RNA expression profile in mouse 2.5 S-NGF and custom peptides-treated *C. elegans*

3.2.4.1 Sequencing of miRNA to study the global miRNA expression profile and comparison between the PT group and PHNP group of *C. elegans*

Synchronized L4 stage wild-type N2 strain of *C. elegans* was subjected to the following treatments at 20 °C: (a) 1X PBS treated worms (CT group), (b) 10 mM paraquat treatment for 1 h (PT group), (c) 50 µg/mL custom peptide (HNP) pre-treatment for 2 h followed by 10 mM paraquat incubation for 1 h (PHNP group), (d) treatment with 50 µg/mL custom peptide HNP for 2 h (HNP group), (e) 50 µg/mL mouse 2.5 S-NGF pre-treatment for 2 h followed by 10 mM paraquat incubation for 1 h (PHNGF group), (f) treatment with 50 µg/mL mouse 2.5 S-NGF for 2 h (NGF group).

Total RNA extraction and quantification was done using RNeasy Plant Mini Kit (Qiagen) as described in section 3.2.3.10. Extracted RNA was subjected to DNase treatment per

manufacturer protocol using New England Biolabs (NEB) (Cat# M0303L kit). The concentration and quality of RNA were measured using a Qubit fluorometer. Further, the RNA quality was also analyzed by checking the RNA integrity number (RIN) using BioAnalyser from Agilent. The samples with RIN value ≥ 7 were subjected to the RNA library preparation.

Transcriptomic mRNA libraries were prepared using QIAseq miRNA Library Kit, as per the manufacturer's protocol, where the qualified RNA is subjected to either poly-A capture or ribodepletion. This is followed by RNA fragmentation, 3' Ligation, 5' Ligation, cDNA Cleanup, adapter, barcode ligation, size selection, and amplification. These libraries were subjected to quantification using Qubit and Bioanalyser from Agilent. Sequencing done on an Illumina 150 bp PE platform was outsourced (Biokart India Pvt Ltd, Banglore, India).

Total reads generated from the libraries were subjected to quality control using Fastqc (v0.11.8), followed by removing adapters / low-quality reads using Trim Galore (v 0.6.7). The de novo transcriptome assembly is performed by Trinity (v v2.13.2). The primary assembly is subjected to redundancy removal and validation using cd-hit and RNA-Seq by Expectation-Maximization (RSEM). The validated transcripts are subjected to Gene Ontology (GO) analysis—database for Annotation, Visualization, and Integrated Discovery (DAVID) and respective databases. RSEM (v v1.3.3) / Spliced Transcripts Alignment to a Reference (STAR) (v v2.7.10a) or Salmon (v v1.6.0)/ Binary Alignment Map (BAM) & Sequence Alignment Map (SAM) (v v1.14)/ Picard (v v2.18.7) tools were used for obtaining a raw read count file. For a comparative analysis, the Interactive Gene Expression Analysis Kit for Microarray & RNA-seq Data (iGEAK) (v1.0a) tool was used, which is an R (v3.3.2) and JavaScript-based open-source desktop application with the Shiny platform. The raw read counts were subjected to Voom /edgeR/limma analysis, and this data was used to perform differentially expressed genes (DEG) analysis with the up and down-regulated genes. MiRTarBase predicted the target genes of miRNAs and the function of those target genes was analyzed through the Panther database. The data generation and analysis were performed at Biokart India Pvt Ltd, Bangalore, India.

3.2.4.2 Determination of acute *in vivo* toxicity and biochemical changes post-treatment of the peptides in a Swiss albino mice model

Acute *in vivo* toxicity of custom peptides was assessed in Swiss albino mice (18-20 g) following OECD guidelines. Ethical permission was approved by the animal ethics committee of IASST (IASST/IAEC/2022/10). For *in vivo* toxicity evaluation, custom peptides (TNP: HNP, 1:1 w/w) were dissolved in 0.2 mL of 1X PBS (pH-7.4) and injected intravenously (10 mg/kg body weight, *i.v.*) into the Swiss albino mice (n=6) (70). The control mice group received only 0.2 mL of 1X PBS (pH-7.4) (placebo). The treated mice were continuously monitored at intervals of 6 h up to 24 h of injection for any behavioral alteration or death. The mice were sacrificed after 24 h post-treatment of custom peptides, and blood was collected instantly by cardiac puncture. Plasma was isolated from the blood (control/treated group) by centrifuging at 4500 rpm for 20 min at 4°C, and the biochemical parameter was investigated viz. SGOT, ALKP, SGPT, BUN, glucose content, creatinine, cholesterol, bilirubin, and albumin level using BeneSphera C61 semi-automatic biochemistry analyzer.

To study possible custom peptides (1:1)-induced morphological changes, the heart, liver, brain, kidney, liver, ovary, and testis of each control and treated group of mice were dissected after 24 h of treatment. Tissues were fixed in 10% buffered formaldehyde, dehydrated, and embedded in paraffin. The slides were observed under light microscopic (Zeiss Axiolab A1) after hematoxylin-eosin (H/E) staining (70). Serum levels of proinflammatory cytokines (TNF-α, IL-6, IL-1β) were determined by a commercial immunoassay kit (Quantikine® HS Immunoassay kit, biotech R and D systems) and followed manufacturer instructions.

3.2.5 Statistical analysis

All the data were denoted as mean ± standard deviation (SD) of independent triplicates. Significant differences between the test and control were analyzed using the student's t-test in Sigma Plot 11.0 for Windows (version 10.0). The significance of difference was examined for more than two groups using one-way variance analysis (ANOVA) in GraphPad Prism software. The p-value ≤ 0.05 was considered statistically significant. The fold change of miRNA expression was calculated by averaging the reads of the control, and each corresponding miRNA was normalized for individual treatment groups concerning the control before converting into $\log_2$ values. miRNAs without reads were removed from consequent analyses in each biological replicate.

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