SUPPLEMENTARY INFORMATION

CONFORMATION AND CYTOTOXICITY OF A TETRAPEPTIDE CONSTELLATED WITH ALTERNATIVE D- AND L-PROLINE

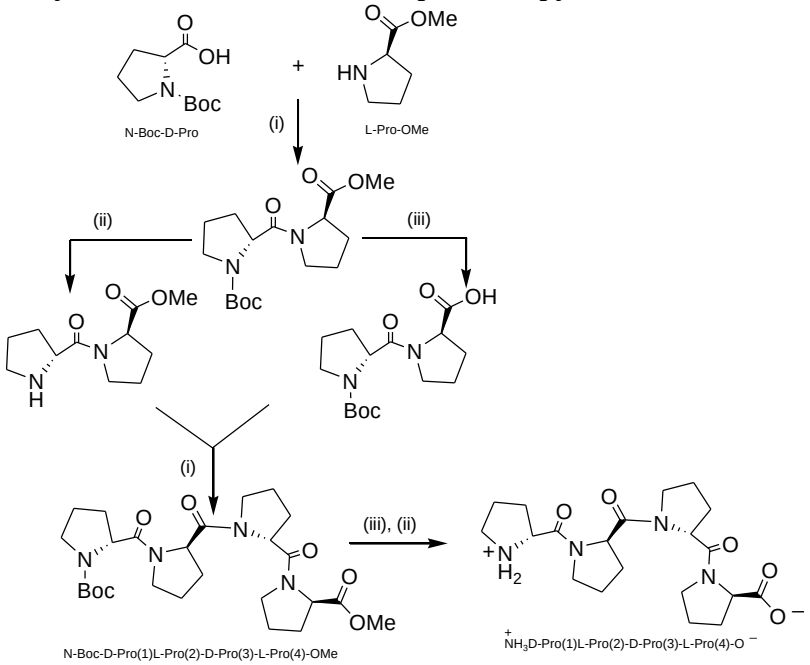
Biswadip Banerji*, Sumit Kumar Pramanik, Uttam Pal, Nakul Chandra Maiti.

Materials and Methods

All chemicals were purchased from Across Organics, and used without further purification unless otherwise stated. Solvents were freshly distilled by standard procedures prior to use. Flash chromatography was performed on silica gel (Merk, 100-200 mesh) with the indicated eluant. All ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker 300 MHz spectrometer. For 1H NMR, tetramethylsilane (TMS) served as internal standard ($\delta = 0$) and data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and coupling constant(s) in Hz. Mass spectra were obtained on a Jeol MS station 700.

Synthesis of tetrapeptide

The tetrapeptide was synthesized using conventional solution phase methodology. The N-terminus was protected by the Boc-group and C-terminus was protected as a methyl ester. Couplings were mediated by 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride/Hydroxybenzotriazole (EDC.HCl/HOBt). The deprotection of methyl ester was performed by using the saponification (aq LiOH) method and the Boc group was removed by Trifluoroacetic acid (TFA). The final tetrapeptide was fully characterized by ^{1}H-NMR, HRMS and IR spectroscopy.



Conditions: (i) EDC.HCl, HOBT, Triethyl amine, DMF, room temperature; (ii) TFA, DCM, 0^{o}C; (iii) LiOH, THF-MeOH-H_2O(3:3:1).

Characterization Data:

NH_3^+D-Pro(1)L-Pro(2)-D-Pro(3)-L-Pro(4)-O^-

^{1}H NMR (300 MHz, $CDCl_3$): δ (in ppm) 1.43-1.46 (10 H, m), 1.97-1.86 (6 H, m), 2.35-2.28 (4 H, m), 3.76-3.38 (8 H, m), 4.46-4.37 (1 H, m)11.42 (1 H, s); HRMS (FAB+): (M+H)/z Calcd for $C_{20}H_{31}N_4O_5$ $(M+H)^+$ 407.2289, Found: m/z 407.2280.

CD spectroscopy

CD spectra (190-400 nm) were acquired at 200 nm/min scan speed, with 1 nm bandwidth on a Jasco J-810 spectrometer using a 10 mm quartz cell. Peptide stock solutions (2mg/ml) were prepared in water and appropriately diluted during CD measurements. Each displayed CD curve (spectrum) was an average of two scans and baseline corrected.

FT-IR

The FT-IR spectra of the samples were recorded on a JASCO FT-IR 4200 spectrometer using (KBr) disc technique. The peptide was mixed with KBr in a clean glass pestle and and compressed to obtain a pellet. The spectra were recorded from 400-4000 cm^{-1}. Background spectra were obtained with KBr pellet for each sample. JASCO software was used for data processing.

AFM Sample Preparation and Imaging

10µl of the peptide sample(3 mg dissolved in 1 mL water) were deposited onto freshly cleaved muscovite Ruby mica sheet (ASTM V1 Grade Ruby Mica from MICAFAB, Chennai) for 5-10 minutes , Mica sheets are basically negatively charged so the peptide molecule binds strongly on the mica surface, after 5 min dry the sample by using vacuum dryer. Sometimes the sample was gently washed with 0.5ml MilliQ water to remove the molecule that were not firmly attached to the mica and dry the sample as mentioned above.
AAC mode AFM was performed using a Pico plus 5500 AFM (Agilent Technologies USA) with a piezoscanner maximum range of 9µm. Micro fabricated silicon cantilevers of 225µm in length with a nominal spring force constant of 21-98 N/m were used from Nano sensors. Cantilever oscillation frequency was tuned into resonance frequency. The cantilever resonance frequency was 150-300 kHz. The images (512 by 512 pixels) were captured with a scan size of between 0.5 and 5 µm at the scan speed rate of 0.5 rpm . Images were processed by flatten using Pico view software (Molecular Imaging Corporation, USA). All the images were presented in this report derived from the original data. Length, height and width were measured manually using Pico view software.

TEM sample preparation and imaging

For TEM imaging, the peptide sample in a concentration of 3mg/mL was placed on a 300-mesh carbon coated copper grid (Allied Scientific Product, USA) (5µl, 10-15 min) and the excess samples were removed cautiously by tissue paper. It was finally dried and the images were recorded on Tecnai G2 Spirit Bio TWIN (Type: FP5018/40) at an acceleration voltage of 80 kV.

The Differential interference contrast (DIC) image :
For differential interference contrast (DIC) image the aqueous solution of the tetra-peptide in a concentration of 3mg/mL was observed by using Laser based Confocal microscope (model NIKON – AIR, Japan with 60X objective using NIS-AR software).

Computational model calculation

Structure of the tetra-peptide was drawn on Schrodinger Maestro molecular modeling environment. Geometry was optimized using simulated annealing algorithm with the help of Desmond molecular dynamics simulations software tool. Simulation was run for 4.8 ns in SPC water environment in an orthorhombic periodic boundary condition. The model system was relaxed before simulation. Number of particle (N), and volume of system (V) in the ensemble were constant and the system had a well defined temperature (T). The molecule was cooked at a high temperature and then slowly cooled down to 300K. The process was repeated several times. The lowest energy conformation at 300K was chosen for further analysis. Three of such peptide fragments were joined head to end to form a duodeca peptide. It was relaxed in vacuo with steepest descent and then conjugate gradient algorithm in UFF force field.

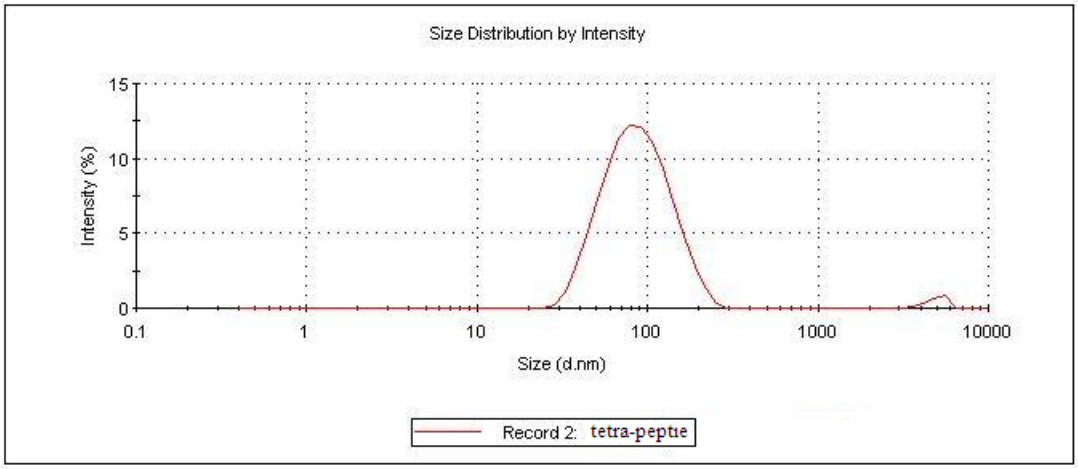
Cell culture

Neuroblast cells, HEK 293 and human hepatocellular carcinoma cell line Hep G2 were procured from the National Centre for Cell Sciences (NCCS, Pune, India) and were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin and gentamicin). Cells were cultured at 37^{o}C in 95% air and 5% CO_2 humidified incubators. Hep G2 cells were seeded at a density of 10^5/well plated in 96 well plates. Cells were typically grown to 60–70% confluence, rinsed in phosphate-buffered saline (PBS) and placed into serum-free medium overnight prior to treatments. After overnight incubation, the Hep G2, HEK 293 cells, and neuroblast cells were treated with this tetra peptide separately at the concentration of 10µM . After 48 hours the medium was removed and 50 µl of fresh medium was added along with 10 µl of MTT (5mg/ml). MTT solution was slowly removed after 4 hours and the purple crystals were solubilised in 1.4 ml of DMSO. The absorbance was measured at test wavelength of 550 nm in Elisa Plate Reader.

Size analysis by dynamic light scattering(Zetasizer Nano,Malvern Instruments):

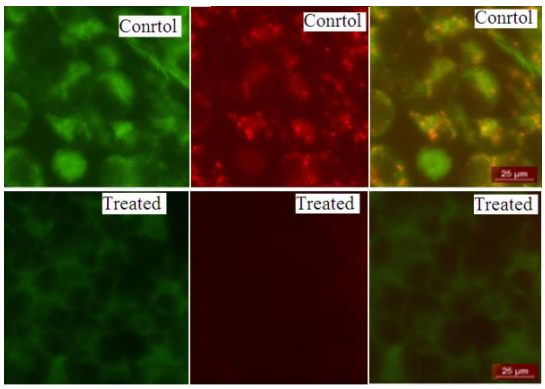
Particle size analysis of this tetrapeptide in aqueous solution by using dynamic light scattering (Zetasizer Nano,Malvern Instruments) showed the mean particle size of nearly 93.26 nm.

		Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm): 79.31	Peak 1:	93.26	98.0	40.97
PdI: 0.231	Peak 2:	4894	2.0	675.6
Intercept: 0.933	Peak 3:	0.000	0.0	0.000
Result quality : Good				



JC-1 staining of HEK 293 cells:

Here the cells were treated with the tetra-peptide for overnight then stained with JC-1 dye for checking mitochondrial health. The cells were then studied and imaged under a fluorescence microscope. Cells with proper mitochondrial health shows punctuated red staining along with green scattered staining, whereas the cells with mitochondrial dysfunction shows only scattered green staining without any red puncta.

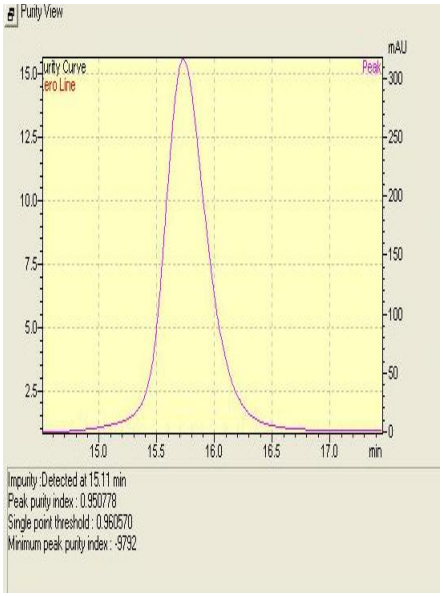


Here the first, second row shows control cells and cells treated with tetra-peptide. First column represents green fluorescent monomer of JC-1, second column represents reddish orange fluorescence of J-aggregate and third column shows merged images. Scale bar represents 25 μm.

HPLC Studies:

The purity of this tetrapeptide was done HPLC method and the purity is 97.92%. Here acetonitrile and water (2:3) was used as elutamt.

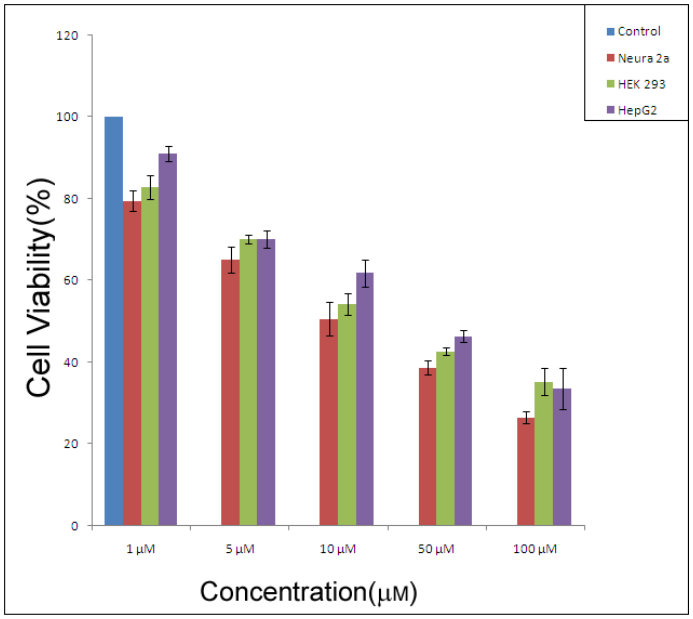
The chromatogram is :



The intensity chart of caspase 3:

						ROI		
FileName	Time (ms)	Z Pos	X Pos	Y Pos	ROI ID	Area	Mean Intens	Max Intensity
Control	0	5885.8	7198	-3685.8	1	9867.096	516890	255
Tetra Pept	0	5879.73	6593.7	-3421.1	1	9846.539	795839	255

Cytotoxicity studies of this tetrapeptide at different concentration:



Cytotoxicity studies of this tetrapeptide at different concentration against three different cell lines.

The IC50 values of this tetrapeptide for Neura 2a, HEK 293 and Hep G2 are 10.1 µM, 11 µM, and 14 µM respectively.

Table 1. FT-IR peak positions of the tetrapeptide

Sl. no.	Peak position	Normal mode
1	3406.64	O-H (3200-3650, var, broad when H-bonded) N-H (3300-3500, wk, 1° and 2° amines) C-H (3300, str, sharp) Carboxylic OH (2500-3300, str, very broad)
2	2924.52	CH_3,CH_2,CH stretch (2850-3000; str) =CH (3020-3100; med, sharp) CH (arenes) (3030; var, may be several bands) Carboxylic OH (2500-3300, str, very broad)
3	2857.02	
4	2360.44	C=C (2100-2250, from air)
5	1731.76	C=O overtone or free conformation
6	1649.8	Amide I (extended helix)
7	1457.92	CH_2, CH_3 deformation
8	1301.72	CH_2 deformation
9	1186.01	C-O (970-1250, str) C-N (1000-1250, med)