Electronic Supplementary Information†

Designed Hybrid Anticancer Nuclear Localized Peptide Inhibits Aggressive Cancer Cell Proliferation

Prasenjit Mondal,[#a,b‡] Saswat Mohapatra,[#a,b] Debmalya Bhunia,[#a] Prabir Kumar Gharai,[a] Nabanita Mukherjee,[b] Varsha Gupta,[a] Satyajit Ghosh,[b] and Surajit Ghosh[a,b,c*]

a. *Organic and Medicinal Chemistry and Structural Biology and Bioinformatics Division, CSIR-Indian Institute of Chemical Biology, Kolkata 700 032, West Bengal, India.*
b. *Academy of Scientific and Innovative Research (AcSIR), Ghaziabad 201002, India.*
c. *Department of Bioscience & Bioengineering, Indian Institute of Technology Jodhpur, NH-62, Nagaur Road, Karwar, Rajasthan 342037, India.*

 # These authors have contributed equally to this work

‡Present Address: Department of neurology, Massachusetts general hospital, Harvard Medical School, 114, 16th street, Charlestown, MA-02129, USA

** Corresponding author, Email: sghosh@iitj.ac.in*

Experimental Section

Materials: Fmoc-amino acids and Fmoc-Rink Amide AM resin were purchased from Novabiochem. O-(Benzotriazol-1-yl)-*N, N, N′, N′*-tetramethyluronium hexafluorophosphate (HBTU), Piperidine, Diisopropylethylamine (DIPEA), Diethyl ether, Dimethyl sulfoxide (DMSO), Methanol, Thioanisole and Trifluoroacetic acid (TFA) were purchased from Spectrochem. Phenol, Dichloromethane (DCM), Ethanedithiol (EDT), and *N, N′*-Dimethylformamide (DMF) were procured from Merck. Triton X-100 was brought from SRL. *N, N′*- Diisopropylcarbodiimide (DIC), 5(6)-Carboxyfluorescein, 5-diphenyltetrazolium bromide (MTT), 4′, 6- diamidino-2-phenylindole dihydrochloride (DAPI), Doxorubicin hydrochloride, dulbecco's modified eagle's medium (DMEM), trypsin-EDTA solution, DMSO for cell culture and formaldehyde solution (molecular biology grade) were procured from Sigma Aldrich. Neutravidin and fetal bovine serum (FBS) were purchased from Invitrogen. Annexin V and Propidium iodide (PI) apoptosis detection kit was bought from Santa Cruz Biotechnology. Bisbenzimide H 33258 (Hoechst) was purchased from Calbiochem. Cover glass bottom dishes were purchased from SPL. For purification, we used Shimadzu HPLC system equipped with C-18 reverse phase column. Peptides were lyophilized in Vertis 4K freeze drier after column purification. HPLC grade water and Acetonitrile were purchased from J. T. Baker. All the chemicals were used without further purification.

Cell culture: U937, K562 cells were procured from National Centre for Cell Science (NCCS) Pune, India. 5 % CO_2 incubator and 37 ^oC temperature was maintained to cultivate cells using DMEM containing fetal bovine serum (FBS) (10 %), kanamycin sulphate (110 mg/L), penicillin (50 units/mL) and streptomycin (50 µg/mL) in our lab. Trypsin-EDTA (1X) solution was used for cell detachment during cell splitting.

Molecular docking: Autodock-Vina software version 1.1.2 was used for blind docking [1]. 50×62×48 affinity grid box was centred on the receptor cyclin dependent kinase (CDK4) (PDB ID: 3G33) [2] for docking with p16 peptide.

Methods

Synthesis of p16, nuclear localized sequence (NLS) and p16-NLS peptides along with carboxy-fluorescein attachment: 300 mg of wang resin swelled for overnight in DMF-DCM (1:1) solvent. Five equivalents of excess fmoc protected amino acids were coupled successively followed by deprotection using 20% piperidine in CEM microwave peptide synthesizer (Liberty 1). Coupling and deprotection steps were maintained for eight and five min respectively. *N, N′*-Diisopropylethylamine (DIPEA) and HBTU were used as an activator base and activator respectively. DMF was used as solvent. After that, peptide attached resin was washed by DMF and DCM solvent. Synthesized peptides were cleaved by standard resin cleavage cocktail solution containing 92.5% trifluoroacetic acid (TFA), 2.5% milli Q water, 2.5% EDT and 2.5% phenol. Cleavage solution containing peptide containing resin kept on

automated shaker (Labnet international) for 3 h. Then, TFA was removed from the filtrate by nitrogen flow. Cold diethyl ether was added gradually to the remaining filtrate to ensure complete precipitation and then was separated out by centrifugation. Peptides were purified by using C-18 reverse phase HPLC column and expected masses were confirmed by MALDI-TOF spectrometry. Similarly, covalently attached fluorescein tagged peptides were synthesized and purified by above mentioned procedure.

Fluorescence microscopy: Fluorescein attached p16 and p16-NLS (5 μM) were treated on K562 cells for 4 h. After that, treated cells were observed under inverted fluorescence microscope with a 40× objective (Olympus IX83 equipped with ANDOR iXON3 camera).

Apoptosis study: U937 and K562 cells (~5 × 10^5 cells/mL) were harvested overnight in a 6-well plate and treated with either 45 μM of p16-NLS or p16 separately for 24 h. After that, cells were suspended having 100 μL solution of binding buffer contained with Propidium iodide (PI) and annexin V and incubated at 37 ºC for 15 min. Emission of annexin V and PI has been analysed by using FITC and PI channels of BD LSRFortessa™ flow cytometer using emission filters at 530 and 610 nm respectively. Cells in Q1, Q2 and Q4 are considered as necrotic, early and late apoptosis. Q3 quadrant cells are considered as healthy cell population.

Cell cycle study: Cell cycle study has been performed by treatment with either p16 or p16-NLS of 45 μM for 24 h. Next, cells were incubated with PI (100 μg/mL) and RNase (10 μg/mL) for 45 min at 37 ºC temperature. After treatment, U937 and K562 cells were fixed with 70% ethanol at 20 ºC for overnight. Finally, cell cycle analysis was performed using PI channels of BD LSRFortessa™ flow cytometer having emission filters at 610 nm.

Cellular uptake study for microscopic imaging: K562 cells (~ 2×10^3) were seeded in DMEM medium containing 10% fetal bovine serum (FBS) on confocal disc for overnight prior to treatment. K562 cells were treated with fluorescein attached p16 and p16-NLS (5 μM) in DMEM containing media and incubated for 4 h (DMSO concentration maintained 0.4%). Next, 4% formaldehyde in PBS buffer was added for 30 min to fix the cell in each cover glass. Next, formaldehyde solution was removed and washed with PBS buffer. Nucleus was stained with Hoechst 33258 (1 μg/mL) for 1 h. Finally, Hoechst 33258 solution was removed and washed by PBS buffer for three times. Thus, each confocal disc was ready for the microscopic imaging. Cell imaging was performed by an Andor spinning disc confocal microscope with 40X objective (Olympus) equipped with Andor iXon 3897 EMCCD camera in bright field, 488 and 405 nm wavelength light.

Flow cytometry for cellular uptake: U937 and K562 cells were cultured in a 6-well plate at density of ~5 × 10^5 cells each well prior to 24 h of treatment. Cells were treated with fluorescein attached p16 and p16-NLS (5 μM) for 4 h. After that, cells were washed with phosphate buffer and trypsinized. Comparative cellular uptake of p16 and p16-NLS has been performed and analyzed by FACS (E_x - 488 and E_m -500 to 600 nm).

Cellular internalization study using FACS: Cellular internalization mechanism of fluorescein-p16 and fluorescein-p16-NLS treated cells has been analysed using previously

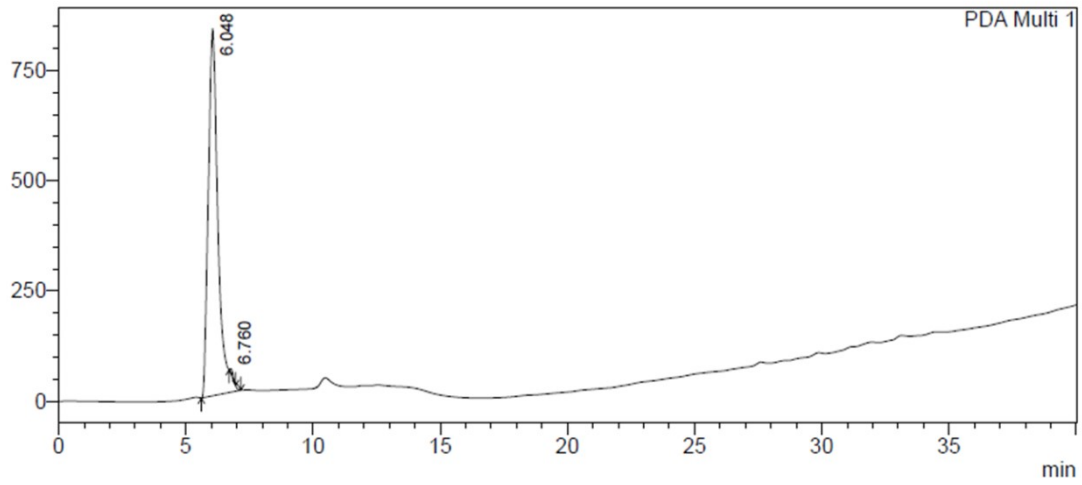
described method [4]. In short, seeded K562 cells (1×10^6) were detached and collected in a suspension containing serum free DMEM (colourless) culture medium. After that, cells were incubated at 37 °C and 4 °C for 60 min separately. Next, these cell suspensions were treated with both fluorescein attached peptides separately of final concentration 5 µM and incubated at 37 °C or 4 °C for 4 h separately. After that, the cell suspension was centrifuged to remove excess fluorescein-p16 and fluorescein-p16-NLS from the solution and resuspended in DMEM culture medium containing trypsin (1 mg/mL) and incubated for 15 min. Cells were washed with serum free DMEM (colorless) culture medium and fluorescent signal was analysed using 488 nm channels of BD LSRFortessa™ flow cytometer with an emission filter at 530 nm.

References:

1. O. Trott and A. J. Olson, *J. Comput. Chem.* 2010, **31**, 455.
2. T. Takaki, A. Echalier, N.R. Brown, T. Hunt, J.A. Endicott, M.E. Noble, *Proc Natl Acad Sci U S A*, 2009, 106, 4171-4176
3. W. Jeong, Y. Lim, Bioconjugate Chem., 2014, 25, 1996.
4. (a) D. Bhunia, S. Mohapatra, P. Kurkute, S. Ghosh, B. Jana, P. Mondal, A. Saha, G. Das and S. Ghosh, *Chem. Commun.*, 2016, 52, 12657. (b) D. Bhunia, A. Saha, A. Adak, G. Das and S. Ghosh, *RSC Adv.*, 2016, 6, 113487.

Electronic Supplementary figures

(a)



PDA Ch1 220nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.048	21882002	832417	99.579	98.902
2	6.760	92427	9240	0.421	1.098
Total		21974429	841656	100.000	100.000

(b)

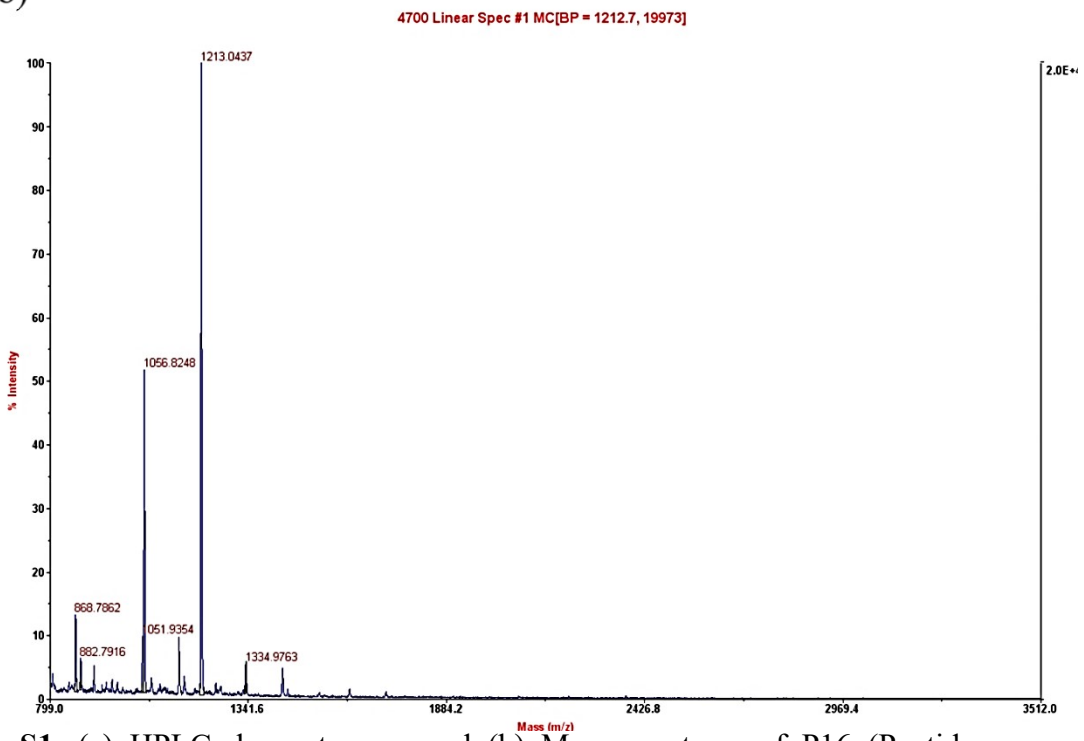


Figure S1. (a) HPLC-chromatogram and (b) Mass spectrum of P16 (Peptide sequence: FLDTLVVLHR; Expected Mass (M) 1212 Da, Observed: 1213.04 $[M+H]^+$).

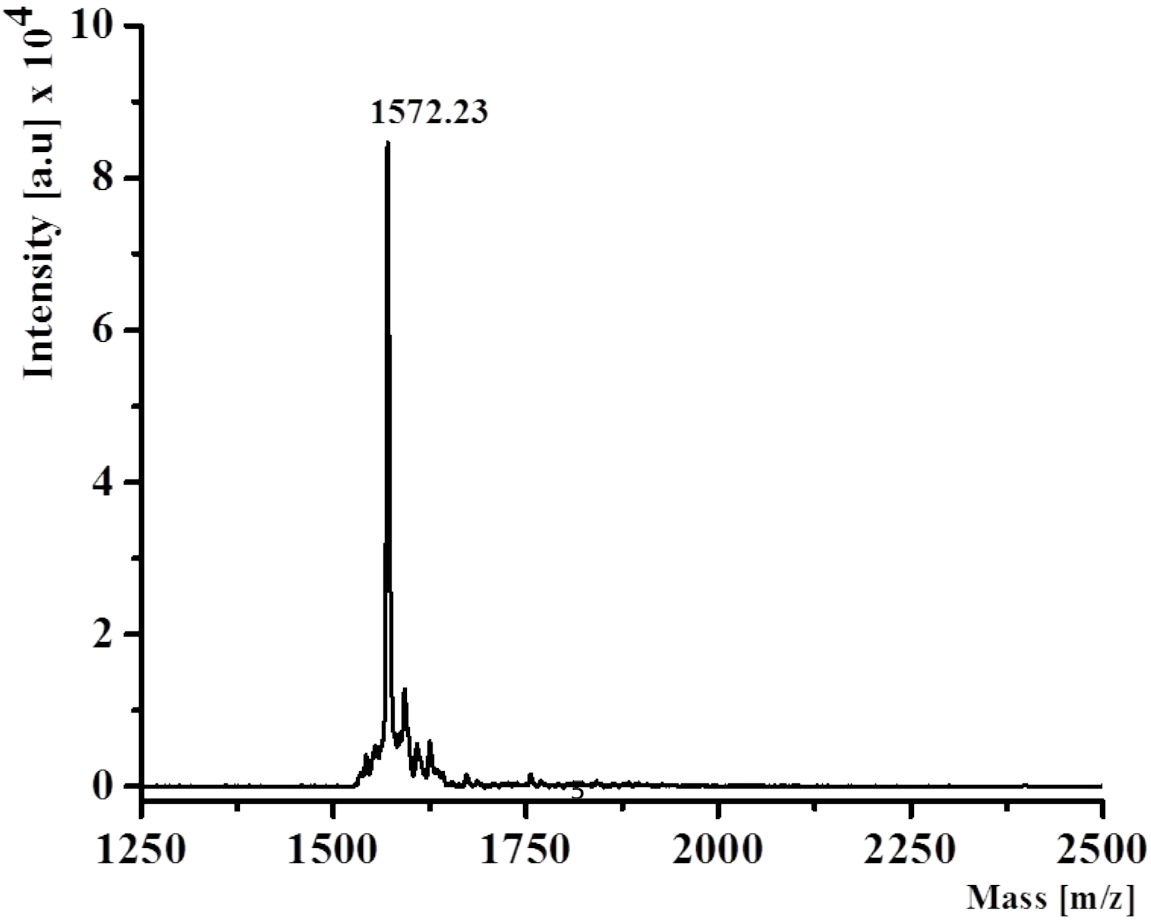


Figure S2. Mass spectrum of Fluorescein-p16 (Expected Mass (M) 1570 Da, Observed: 1572.23 $[M+2H]^{+}$).

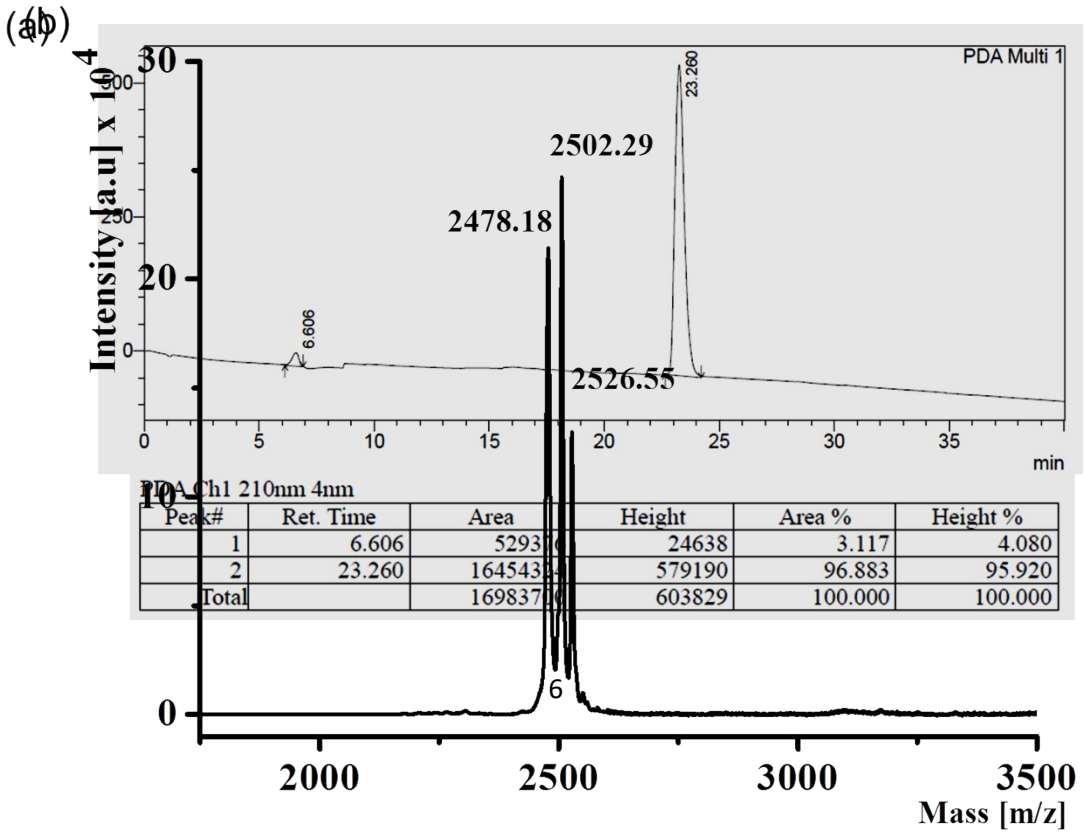


Figure S3. (a) HPLC-chromatogram and (b) Mass spectrum of p16-NLS (Peptide sequence: FLDTLVVLHR-VQRKRQKLMP; Expected Mass (M) 2478 Da, Observed: 2478.18 [M], 2502.29 $[M+Na+H]^+$, and 2526.55 $[M+2Na+2H]^+$).

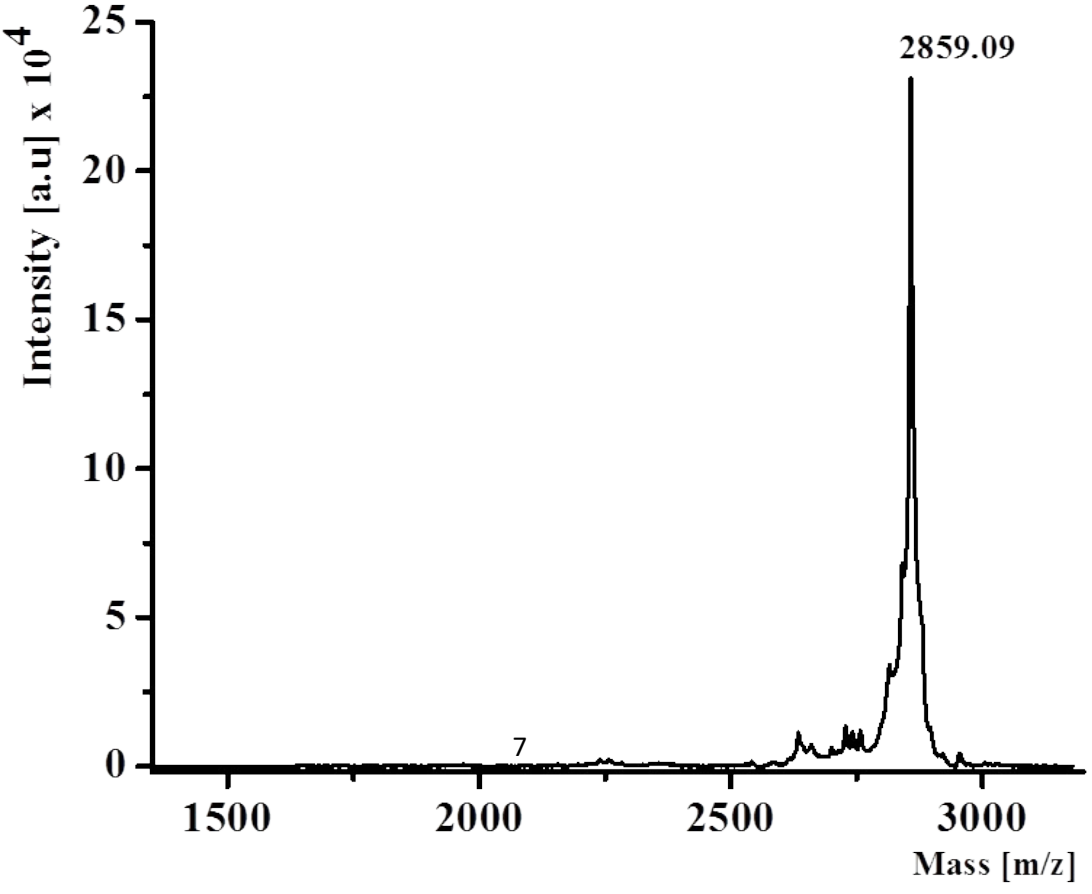
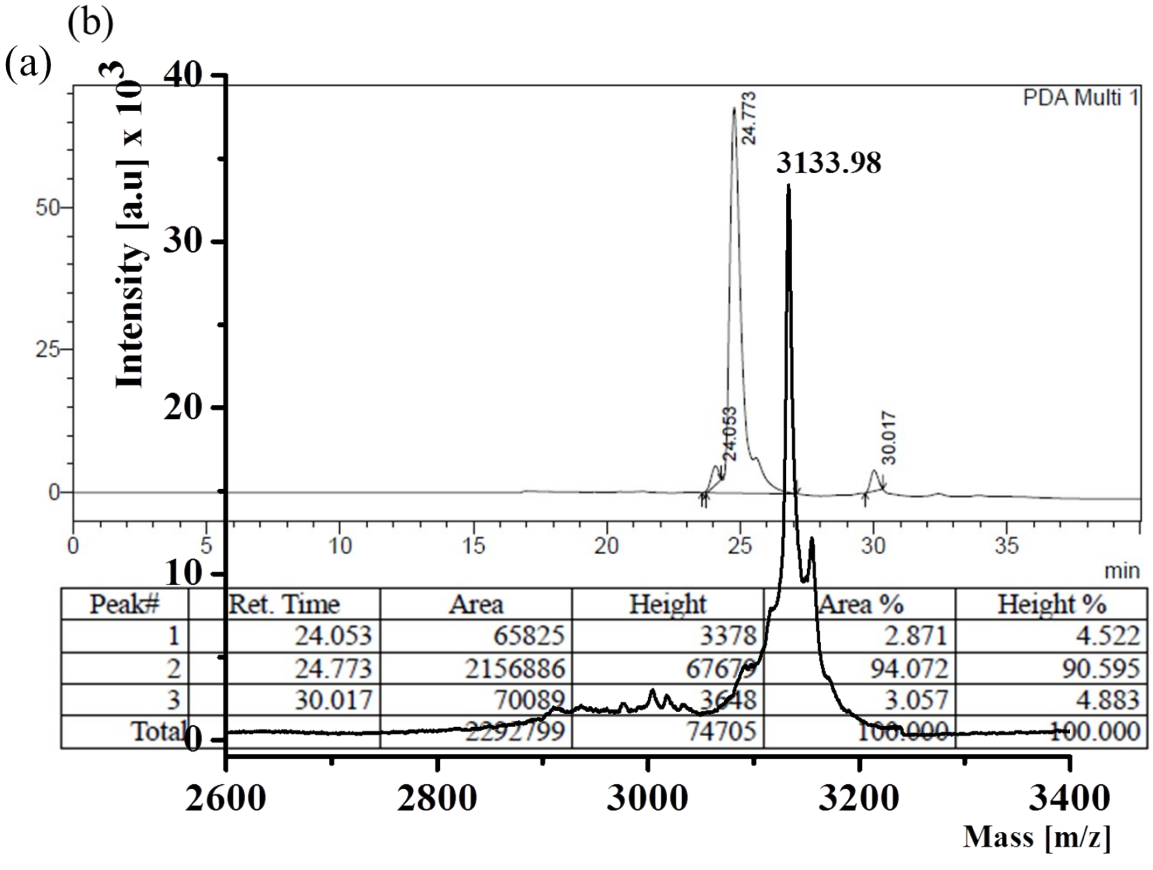


Figure S4. Mass spectrum of Fluorescein-p16-NLS (Expected Mass (M) 2836 Da, Observed: 2859.09 $[M+Na]^+$).



Peak#	Ret. Time	Area	Height	Area %	Height %
1	24.053	65825	3378	2.871	4.522
2	24.773	2156886	67679	94.072	90.595
3	30.017	70089	3648	3.057	4.883
Total		2292799	74705	100.000	100.000

Figure S5. (a) HPLC-chromatogram and (b) Mass spectrum of AVPI-FK-FLDTLVVLHR-VQRKRQKLMP (Expected Mass (M) 3132.9 Da, Observed: 3133.98 $[M+H]^+$)

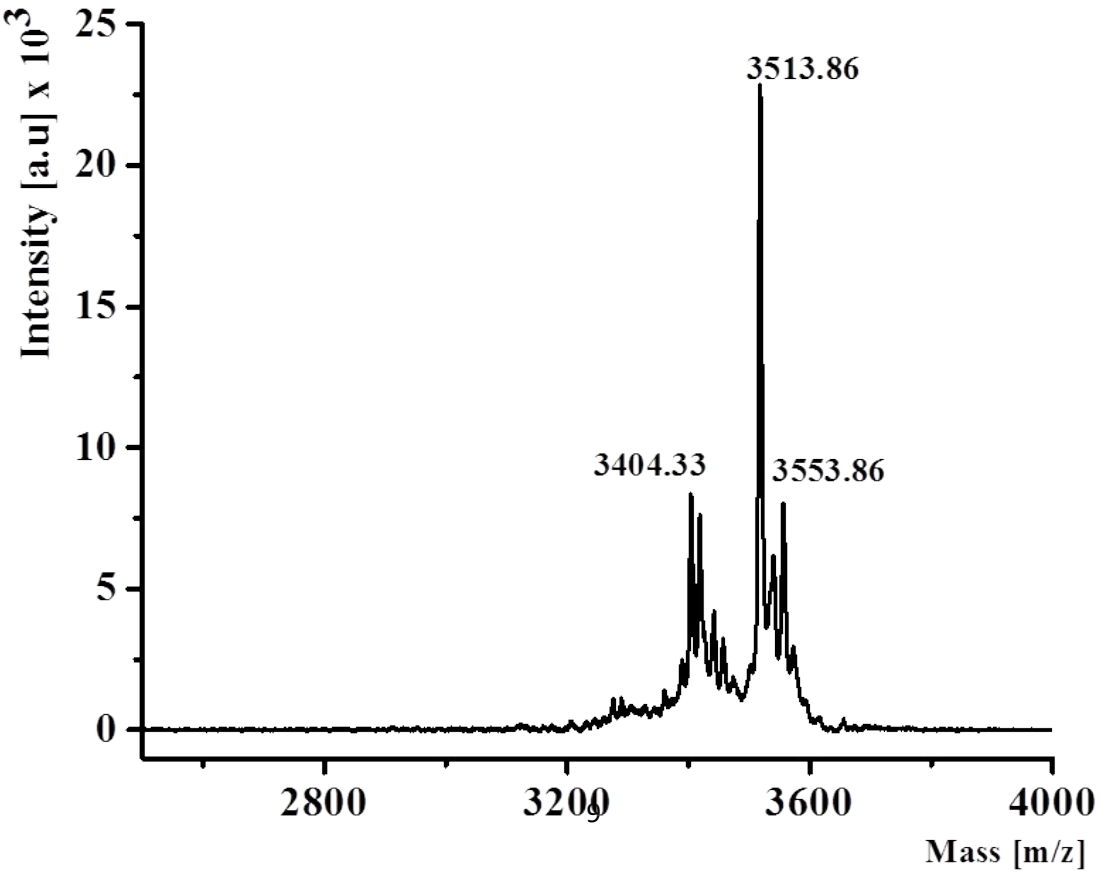


Figure S6. Mass spectrum of Fluorescein-AVPI-p16-NLS (Expected Mass (M) 3491 Da, Observed: 3513.86 $[M+Na]^+$, 3553.86 [M+Na+K]+).

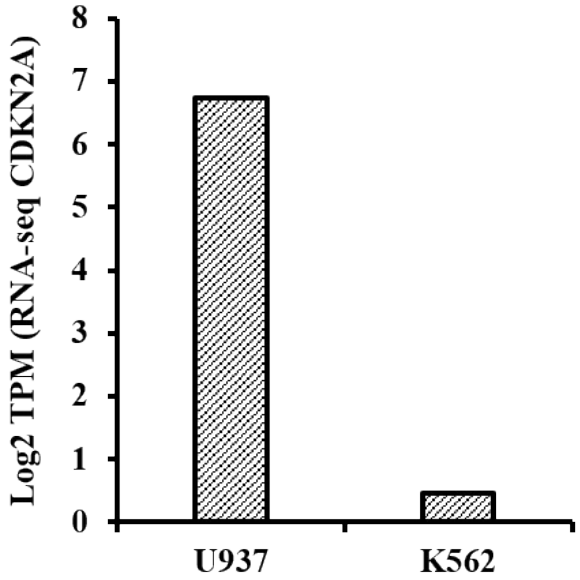


Figure S7. Histogram based on the meta-analysis of the RNAseq data for p16 expression in two cell lines (U937 and K562). Overexpression of p16/CDKN2A is there in U937 cell compared to K562 (Ref. CDKN2A (cyclin dependent kinase inhibitor 2A) expression 21Q4 Public database of DepMap).

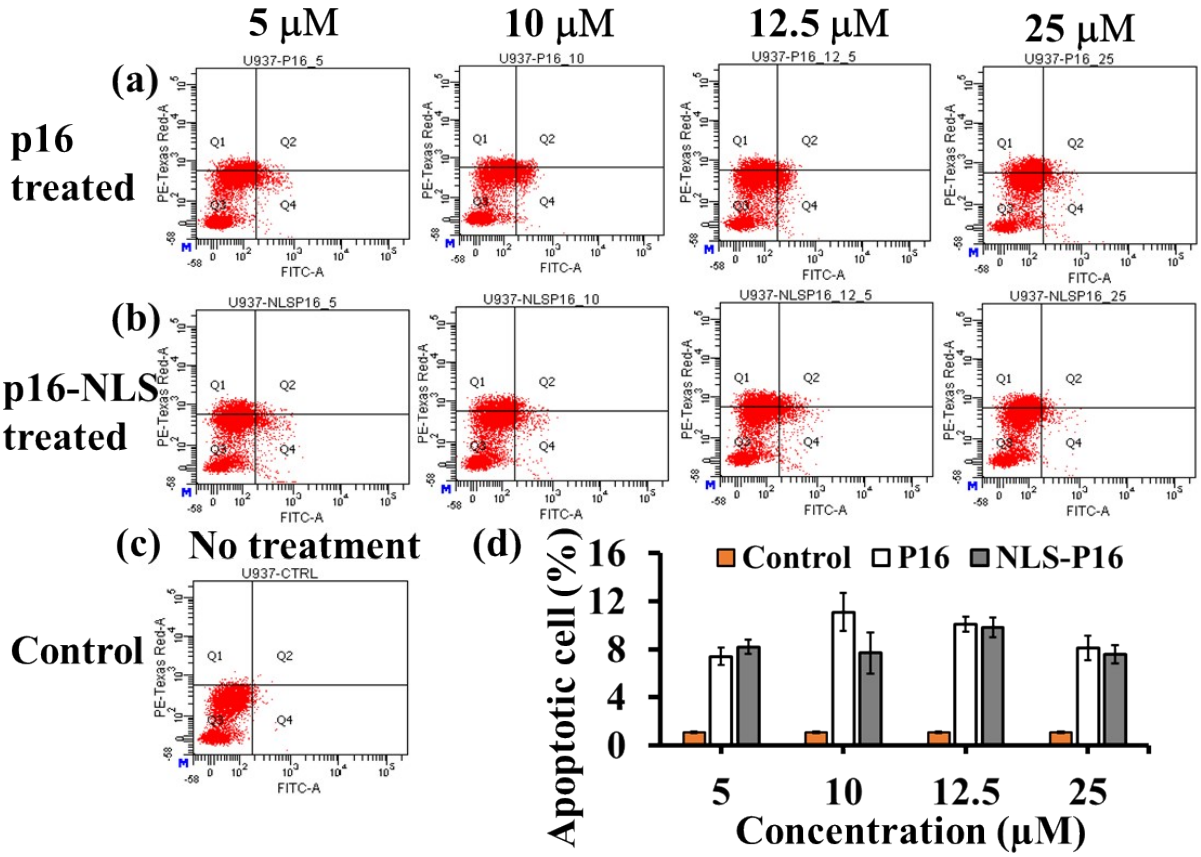


Figure S8. FACS apoptosis study performed in U397 cells upon treatment with p16 (a) and p16-NLS (b) at different concentration with untreated control cells (c). (d) Quantification of this study indicates no significant difference in apoptotic cell (%) after treatment with p16 and p16-NLS peptide in U937 cell.

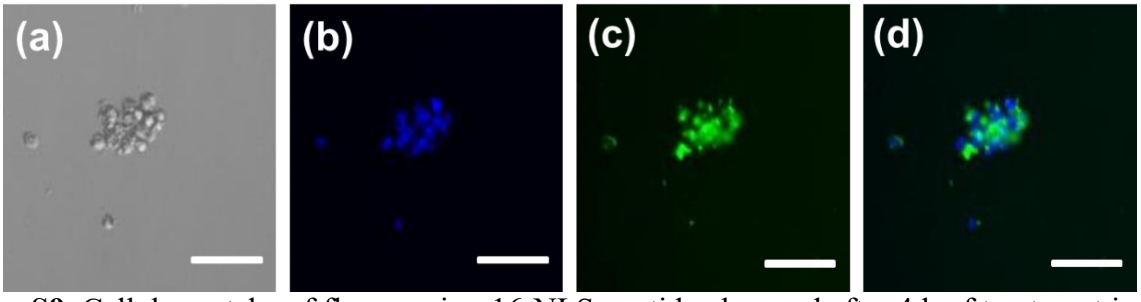


Figure S9. Cellular uptake of fluorescein-p16-NLS peptide observed after 4 h of treatment in K562 cells, scale bars correspond to 100 µm.

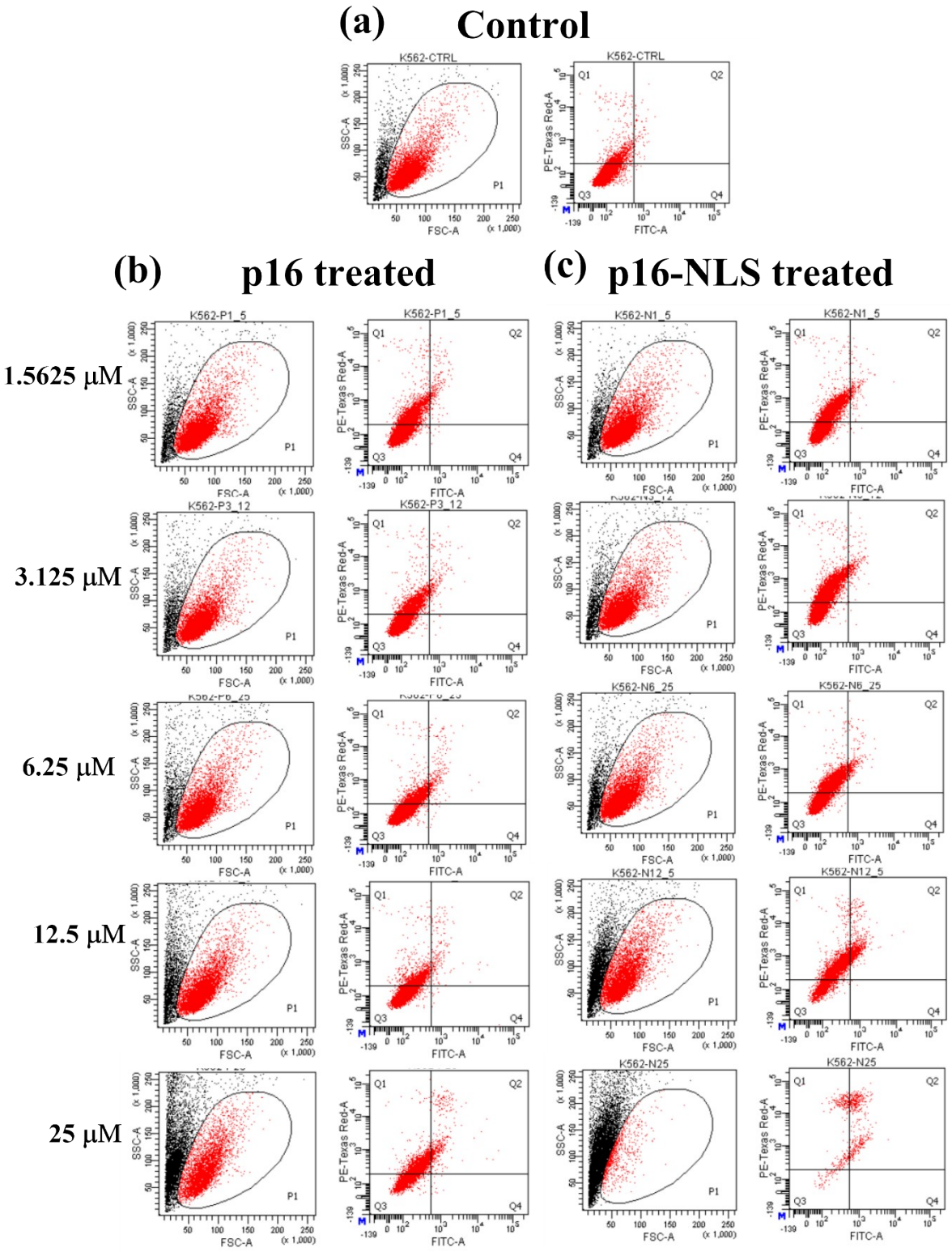

(a) Control
K562-CTRL
SSC-A
FSC-A
P1
PE-Texas Red-A
FITC-A
Q1
Q2
Q3
Q4
(b) p16 treated
(c) p16-NLS treated
1.5625 μM
3.125 μM
6.25 μM
12.5 μM
25 μM
K562-P1_5
K562-P3_12
K562-N1_5
K562-N6_25
K562-N12_5
K562-N25

Figure S10. Apoptosis study performed by FACS. K562 cells treated with (a) Untreated control (b) p16 (c) p16-NLS.

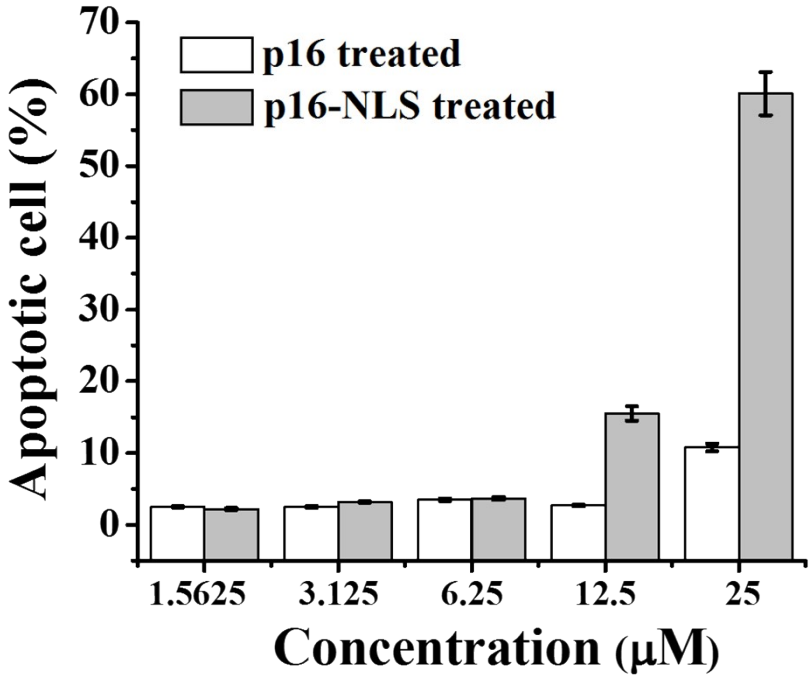


Figure S11. Bar diagram exhibits quantitatively that p16-NLS causes higher apoptotic death of K562 cells at higher concentration.