Electronic Supplementary Information (ESI)

Intra-Mitochondrial Self-Assembly to Overcome the Intracellular Enzymatic Degradation of L-peptides.

M. T. Jeena[a], Seokyoung Lee[b], Ayan Kumar Barui[a], Seongeon Jin[a], Yuri Cho[b,c], Suk-Won Hwang[c], Sehoon Kim[b,c]*, Ja-Hyoung Ryu[a]*

[a] Department of Chemistry, Ulsan National Institute of Science and Technology, Ulsan 44919, Republic of Korea.

[b] Center for theragnosis, Korea Institute of Science and Technology, Seoul 02792, Republic of Korea.

[c]KU-KIST Graduate School of Converging Science and Technology, Korea University, Seoul 02841, Republic of Korea.

1. Experimental Procedures

1.1. Materials. All peptides were synthesized manually on a 0.25 μM scale. The products were purified by high-performance liquid chromatography (HPLC, Agilent Technologies, USA) with a C-18 reverse column in ACN/water mixture. Amino acids were purchased from Bead Tech, Korea and APEX Bio, Houston, USA. The tetramethyl rhodamine methyl ester (TMRM) was purchased from Santa Cruz Biotechnologies, Korea. The successful synthesis of peptides was confirmed with matrix-assisted laser desorption/ionization (MALDI-TOF/TOF, Ultraflex III). The confocal laser scanning microscopy images were taken with LSM 780.The TEM images were obtained using a BioTEM system (JEM 1400).

1.2. Synthesis of Peptide. The peptides FFK/ffk were synthesized by the standard 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis on a 0.25 mmol scale. The synthesized FFK/ffk peptides were treated with 1-pyrene carboxylic acid (500 μmol) and O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) (500 μmol) in the presence of diisopropyl ethyl amine (DIPEA, 500 μmol) and allowed to stir at room temperature for 24 h in DMF. The resin was collected by filtration and washed with dimethylformamide (DMF) to remove unreacted chemicals. The product was cleaved from the resin with cleavage cocktail (TFA/ Water/Tri isopropyl amine mixture (9.5:0.5:0.5)), and products were precipitated in cold ether, purified by HPLC, and confirmed by mass analysis using MALDI-TOF/TOF. To achieve the triphenyl phosphonium (TPP) conjugation, synthesized peptide (0.02 mmol) was treated with 1-hexyl triphenylphosphonium bromide salt (0.04 mmol) with triethyl amine (0.02 mmol) in DMF and allowed to stir for 12 h at room temperature. The pure product was collected using HPLC, freeze-dried, and used for further studies. The peptide was obtained with 90 % yield.

1.3. Cell Culture. Human cancer cells were obtained from ATCC and cultured in DMEM containing 10% fetal bovine serum (FBS; Life Technologies) and 1% penicillin/streptomycin (Life Technologies) at 37 °C in a humidified atmosphere of 10% CO2. Fluorescence measurements were obtained using a plate reader (Tecan Infinite Series, Germany) by setting the excitation wavelength at 565 nm and monitoring emission from the 96-well plates at 590 nm. The 96-well Nunc (Thermo Fisher Scientific Inc.) plates were seeded with cells at a density

of 5 × 103 cells per well, and the cells were allowed to settle for 24 h with incubation at 37 °C under 5% CO2 in the respective growth medium (e.g., RPMI 1640, DMEM, or L-15).

1.4. In vitro cell viability. For cytotoxicity analysis, 5000 cells were seeded in 96 well plate. After 24 h, the cells were incubated with 10, 20, 30 and 40 μM Mito-FF and Mito-ff from the stocke solutions of 1, 2, 3 and 4 mM in DMSO. The control group were incubated with 1% DMSO. The cell viability was measured at 24 h using the Alamar blue assay, with each data point measured in triplicate.

1.5. In vivo tumor therapy. Experiments with mice were performed according to the guidelines offered by Korea Institute of Science and Technology (KIST). For animal experiments, BALB/c nude mice (male, ∼20 g bodyweight, 5 weeks of age; Orient Bio Inc., Korea) were anaesthetized with intraperitoneal injection of a mixture of zoletil-rompun. Tumors were established by subcutaneous injection of HT-29 (human colorectal adenocarcinoma) cells (1.0 × 107 cells) into the mice. After tumor establishment (70−100 mm^3 of volume, ∼3 weeks), the peptides (1 and 10 mg/kg) in 60 μL of PBS (pH7.4) containing 10% of DMSO were peritumorally injected around the tumor tissues for tumor treatment. The control group were injected with 10% DMSO in PBS without peptide. This operation was repeated every 2 days for 2 weeks, 7 times in total. The tumor volumes were measured and calculated as a × b2 /2, where a and b are the largest and smallest diameters, respectively. To validate in vivo toxicity of the peptides, the bodyweight of mice was monitored during the treatment period.

1.6. Enzymatic Stability Analysis. To analyze the stability of Mito-FF/Mito-ff against digestive enzymes, 100 μM of Mito-FF and Mito-ff solutions were prepared in PBS. 100 μL of 100 μM of each solution was mixed with 50 μL of 1 mg/mL of trypsin, chymotrypsin and proteinase K and incubated at 37 °C. The solutions were analyzed by HPLC (analytical, C-18 column, ACN-water eluent) after 12 and 24 h of incubation time. In each time, the untreated control (peptide solutions with no chymotrypsin treatment) was injected for reference. The analytical HPLC peaks were plotted in origin pro 2015, and the area under the peak was obtained for each plot. The % of sample retained in the solution was calculated by comparing with respective control samples.

1.7. Stability analysis against mitochondrial enzyme. To analysis the stability of Mito-FF and Mito-ff in presence of mitochondrial enzyme, we have isolated the mitochondria using the mitochondria isolation kit according to the procedure provided by manufacture's protocol (Thermo scientific). The isolated mitochondria immediately treated with 10 μM of Mito-FF/Mito-ff in 1 mL of PBS at 37 °C in the cell culture incubator. After 1 h, the mitochondria were collected by centrifuge to remove the un-internalized Mito-FF/Mito-ff. The mitochondrial pellet were incubated in the incubator for another 12 h. After the desired time period, the membrane of the mitochondria were destroyed using RIPA buffer: MeOH mixture (1:1) and kept for lysis. The lysate were analyzed by HPLC to observe the peaks for Mito-FF or Mito-ff.

1.8. Mass spectra of cell lysate. HeLa cells were grown in 24 well plate. After 24 h the cells were incubated with 20 μM of Mito-FF and Mito-ff for 24 h. After 24 h, the cells were washed with PBS and collected in a 1 mL falcon tube. 1:1 mixture of RIPA buffer: MeOH mixture is added to break the membrane and lysis the cells. The cell lysate were analyzed by MALDI spectra to observe the peak for Mito-FF and Mito-ff.

1.9. Determination of amount of Mito-FF/Mito-ff inside the mitochondria. In order to determine the amount of Mito-FF and Mito-ff present inside the cell, we used fluorescence assay. 10 μM of Mito-FF and Mito-ff is incubated with HeLa cells for 12 h. 12 h is sufficient time period for the complete uptake, mitochondrial localization and the degradation to occur if any. After 12 h of incubation with HeLa cells, the cells washed well and collected. Cell number in each triplicate sample were determined by cell counter. The cell were made to pellet and lysed with 1:1 (500 μL) MeOH: Lysis buffer. After the lysis, the solution were analyzed with fluorescence emission spectra (Ex = 343 nm). A calibration plot is made prior to the experiment for both Mito-FF and Mito-ff. The intensity of emission of Mito-FF and Mito-ff is compared with the calibration plot and calculated the amount of Mito-FF and Mito-ff present per cell (assuming all compounds are in the mitochondria) after 12 h of incubation.

1.10. Analysis of mitochondrial membrane depolarization. Mitochondrial membrane depolarization is analyzed by both TMRM and JC-1 dye. To observe mitochondrial depolarization by confocal imaging, HeLa cells were seeded in confocal dish. After 24 h, the cells were incubated with 40 μM of Mito-FF and Mito-ff at different time point to obtain a final time of incubation 6 h, 5 h, 3 h and 1 h. TMRM labeled control were prepared in each time point. After the incubation time, the cells were washed and observed in confocal. To analysis the mitochondrial membrane depolarization by FACS, the same procedure is repeated, while cells were grown in 24 well plate. After the incubation time, the cells were washed, trypsinized and analyzed by FACS. HeLa cells were incubated with Mito-FF/ff of 40 μM concentration at pre-designed interval for the final measurement at 6 h, 5 h, 3 h and 1 h. The Mito-FF/ff incubated HeLa cells and control HeLa cells were labelled with JC-1 dye for 30 min analyzed the fluorescence by confocal microscopy after washing.

1.11. Procedure for western blot analysis. Western blot analysis was carried out in HeLa cells treated with Mito ff and Mito FF following published report.[1-2] In brief, cells were first seeded in 6 well tissue culture plate and upon reaching 70% confluency, the cells were incubated with 20 μM of Mito-FF and Mito-ff for 24 h. The cells were then lysed employing RIPA buffer, followed by centrifugation of the lysed cell suspension at 10,000 rpm, 4 °C for 15 min to obtain the supernatant cell lysate. The protein concentration of the cell lysate was quantified by Bradford assay. Next, electrophoretic separation of 40 μg proteins corresponding to each treatment was performed on 12% denaturing SDS-PAGE and the proteins were then blotted in PVDF membrane. Blocking of the membrane was carried out for around 2 h at room temperature using 5% nonfat dry milk in TBST solution, followed by subsequent incubation with the primary antibodies (Cytochrome C: abcam, ab133504 and β-actin: ab6276, abcam) for overnight at 4 °C and secondary antibodies (goat anti-rabbit IgG-ALP: ab97048, abcam or goat anti-mouse IgG-ALP: ab97020, abcam) for around 1 h at room temperature. BCIP/NBT reagent was used to develop the immunoblot in dark condition, followed by its quantification employing Image J analysis software.

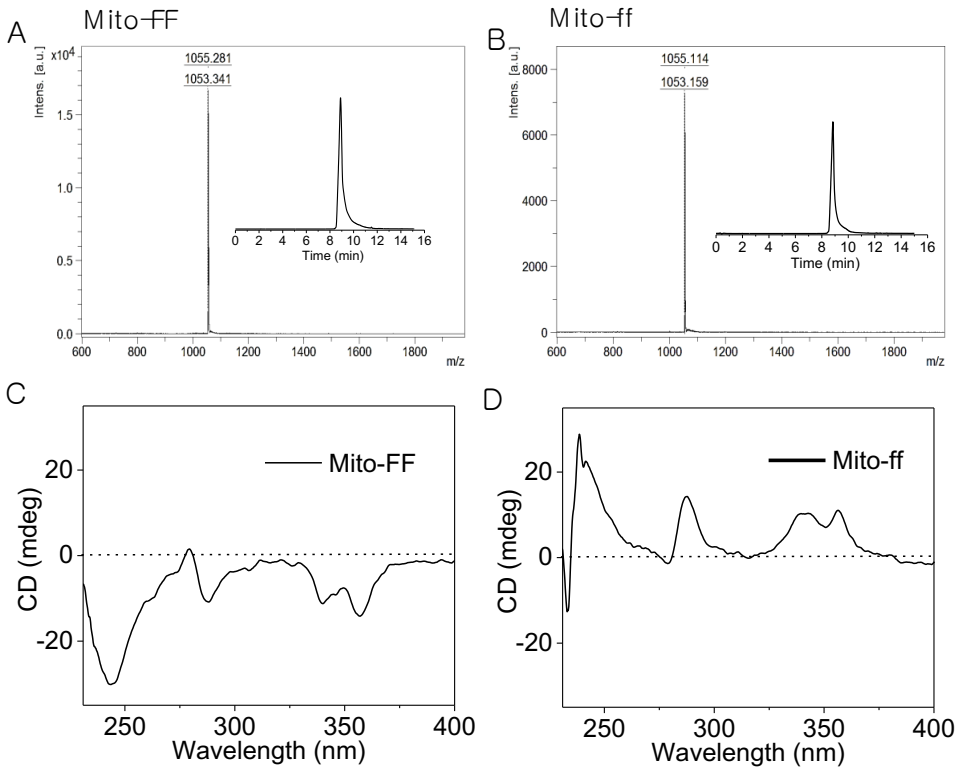


Fig. S1. Mass analysis and HPLC trace of A) Mito-FF and B) Mito-ff. CD spectra analysis of C) Mito-FF and D) Mito-ff for a concentration of 100 µM.

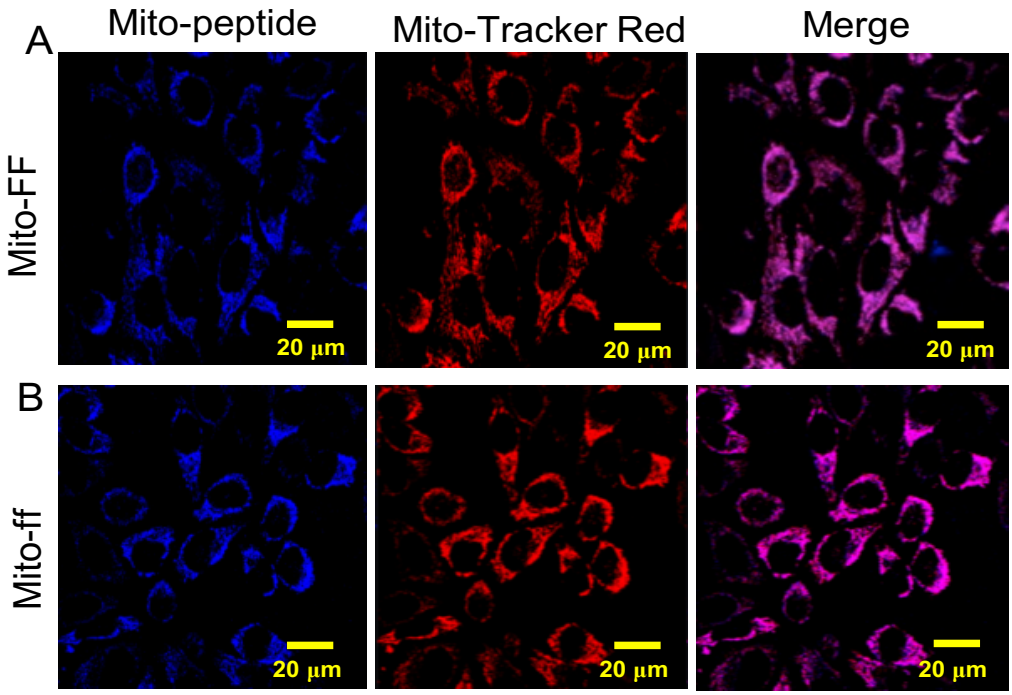


Fig. S2. Confocal images showing the mitochondrial localization of A) Mito-FF and B) Mito-ff after incubation with HeLa cells for a time period of 6 h at 20 µM concentration.

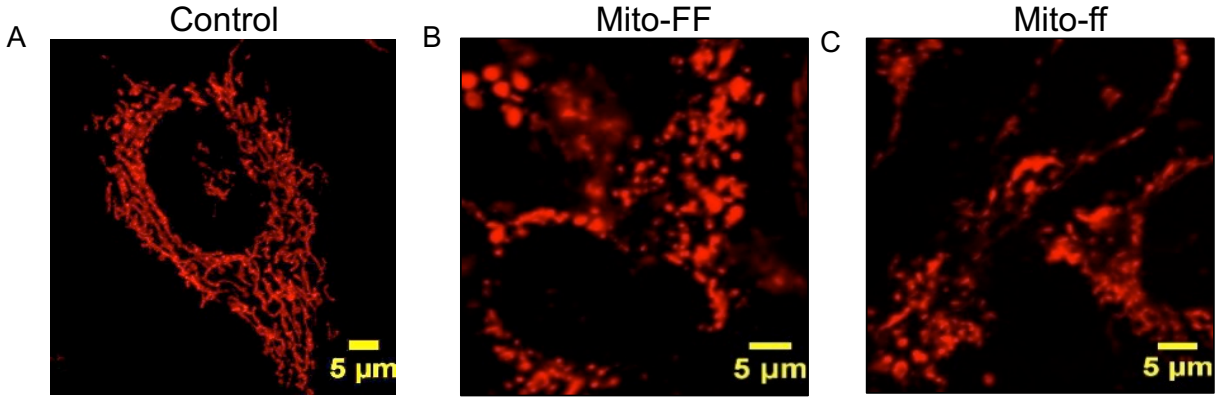


Fig. S3. Untreated mitochondria of the HeLa cells showing elongated and healthy mitochondria compared with the Mito-peptide treated mitochondria of the HeLa cells.

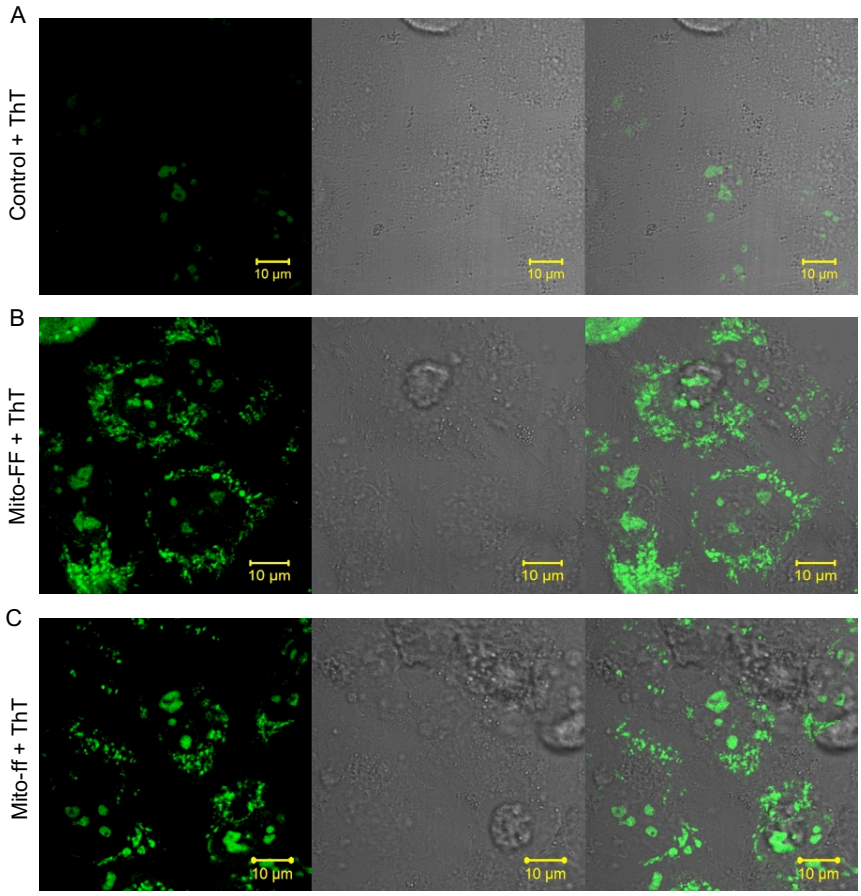


Fig. S4. The confocal images of Thioflavin T labelled HeLa cells after treating with A) control B) Mito-FF and C)Mito-ff that shows indication of fiber formation after treating with Mito-FF and Mito-ff .

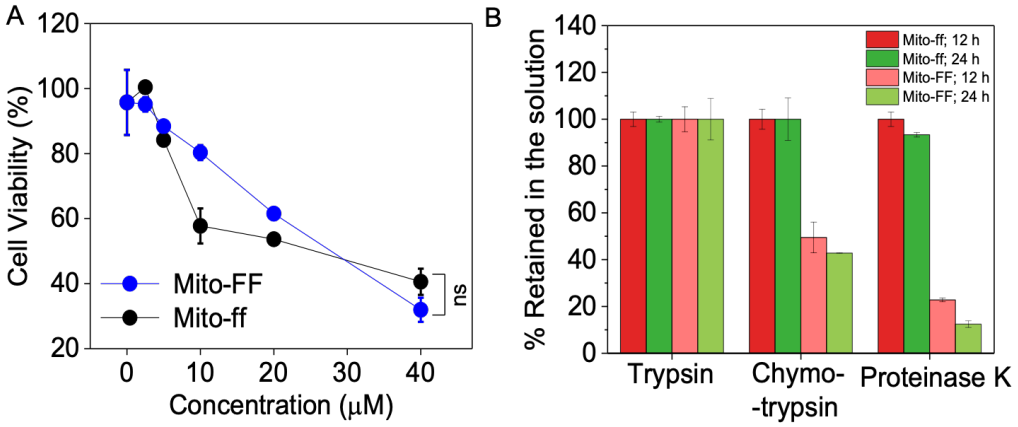
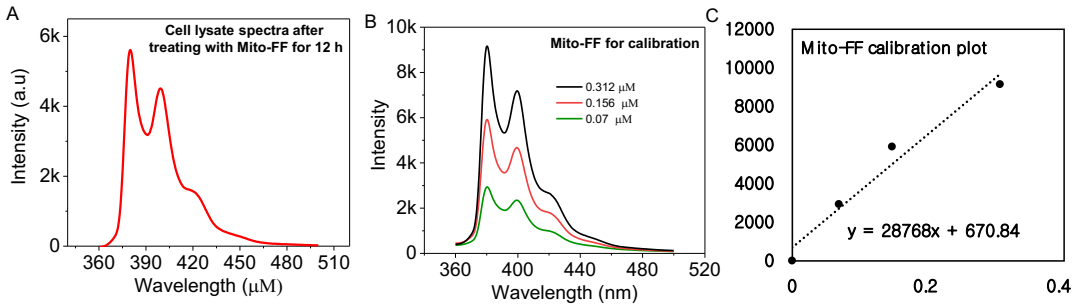
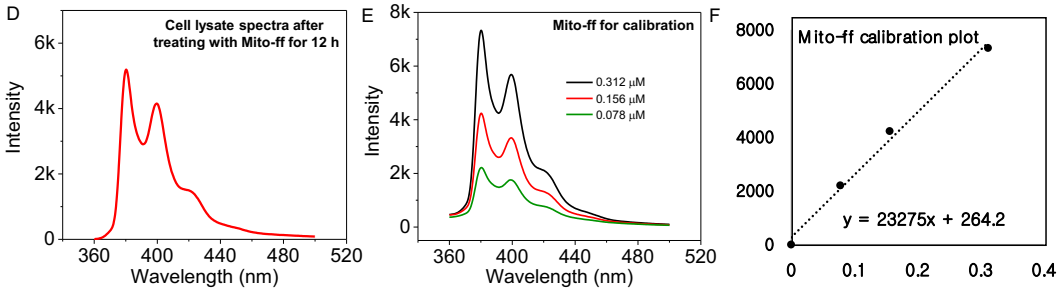


Fig. S5. A) Cell cytotoxicity of Mito-FF/Mito-ff against SCC-7 cells for a time period of 24 h incubation showing similar toxicity for both D- and L- form. Data represent mean ± SD from three independent experiment. ns, not significant, shows no significant difference between toxicity induced by Mito-FF and Mito-ff. P-value is calculated to be 0.16602 B) Enzymatic stability of Mito-FF/Mito-ff against different digestive enzymes.



- Calculated amount of Mito-FF present per cell after 12 h of incubation = $0.89*10^{-9} \pm 0.23$ μM



- Calculated amount of Mito-ff present per cell after 12 h of incubation = $0.885*10^{-9} \pm 0.05$ μM

Fig. S6. Quantification of the amount of Mito-FF and Mito-ff inside the cell. A) Emission spectra of Mito-FF from the cell lysate in 1:1 mixture of MeOH : RIPA, B) emission spectra of Mito-FF at different concentration for the calibration in 1:1 mixture of MeOH : RIPA, C) calibration plot of Mito-FF, D) emission spectra of Mito-ff from the cell lysate in 1:1 mixture of MeOH : RIPA, E) emission spectra of Mito-ff at different concentration for the calibration in 1:1 mixture of MeOH : RIPA, F) calibration plot of Mito-ff.

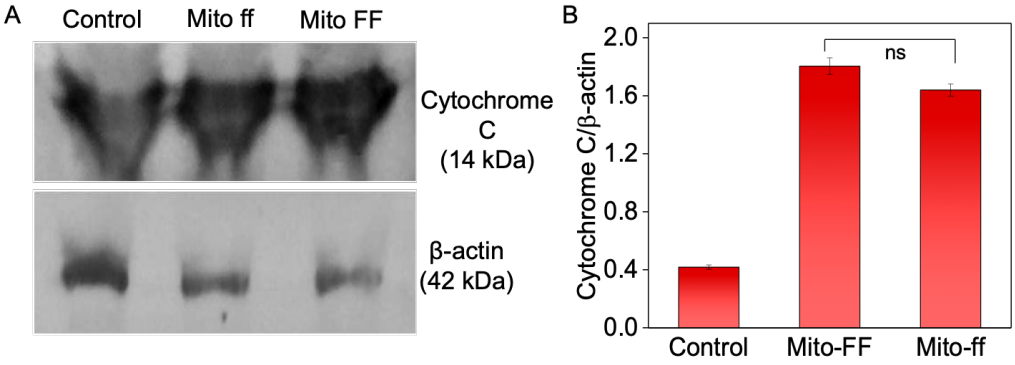


Fig. S7. A) Cell cytotoxicity of Mito-FF/Mito-ff against SCC-7 cells for a time period of 24 h incubation showing similar toxicity for both D- and L- form. Data represent mean ± SD from three independent experiment. ns, not significant, shows no significant difference between toxicity induced by Mito-FF and Mito-ff. P-value is calculated to be 0.16602 B) Enzymatic stability of Mito-FF/Mito-ff against different digestive enzymes.

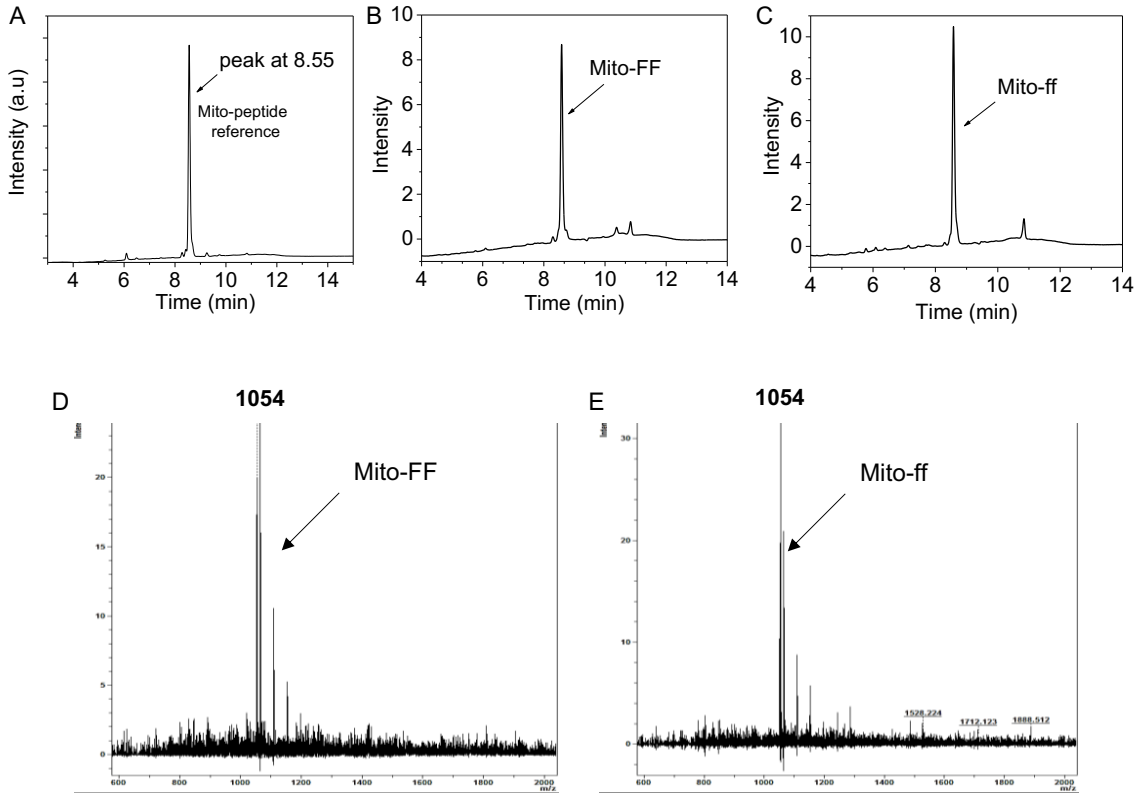


Fig. S8. Stability analysis of Mito-FF and Mito-ff after incubating with isolated mitochondria. The HPLC analysis of A) Mito-peptide for the reference showing the peak at 8.55 min. B) Mito-FF and C) Mito-ff after incubating with isolated mitochondria for 12 h showing the presence of both Mito-FF and Mito-FF of almost equal intensity at 8.55 min suggesting that the peptide does not undergo degradation once internalized inside the mitochondria. MALDI analysis of cell lysate after treating with D) Mito-FF and E) Mito-ff showing presence of both compounds.

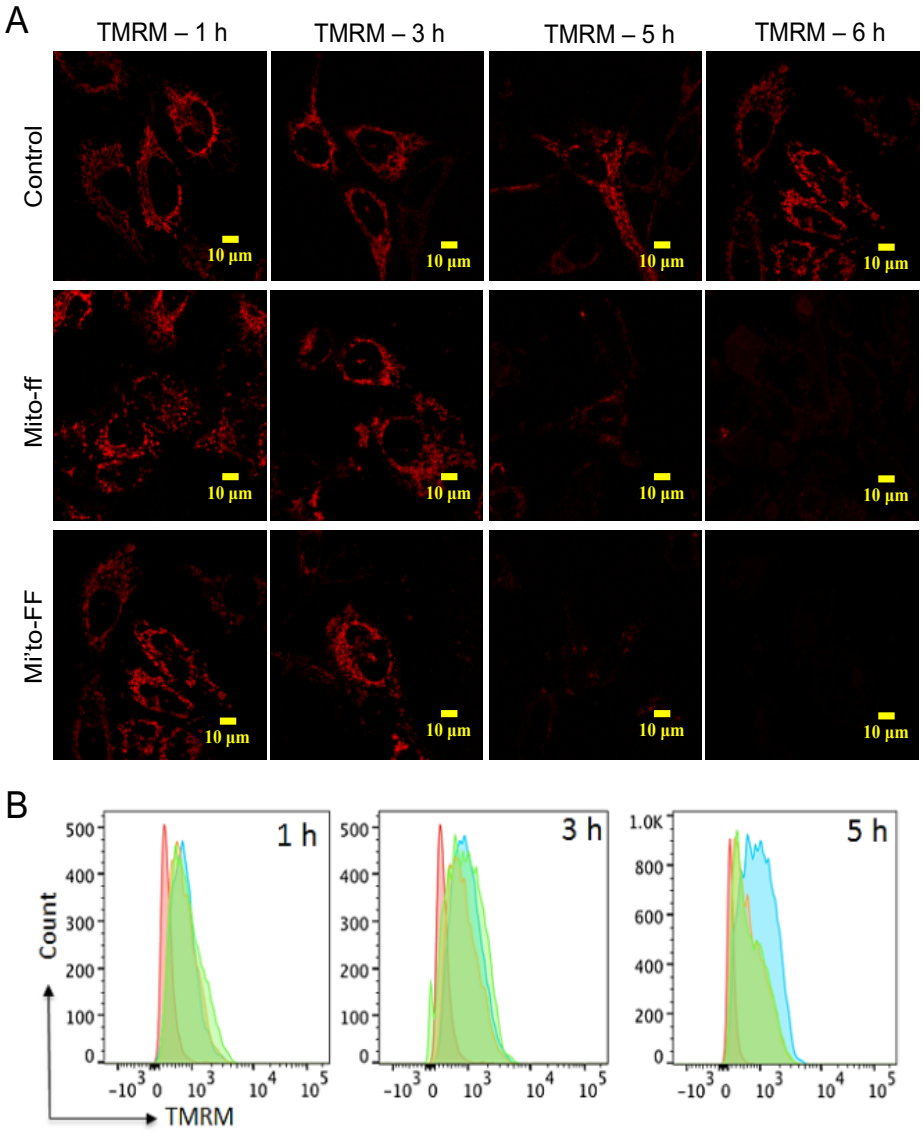


Fig. S9. A) Time dependent analysis of mitochondria membrane depolarization using membrane staining dye TMRM in HeLa cells after staining with 40 µM of Mito-FF/Mito-ff until 6 h (The high concentration of 40 µM is employed for faster action as TMRM may diffuse to cytoplasm for longer time of incubation in mitochondria). B) Time dependent flow cytometric analysis for the mitochondria membrane depolarization using membrane staining dye TMRM showing decrease in the TMRM fluorescence after 5 h of incubation with 40 µM of Mito-FF and Mito-ff (Color code: red-cell control, blue-control cells labelled with TMRM, green-Mito-FF treated sample, orange-Mito-ff treated sample). Within 1 h, both peptides induced mitochondrial fragmentation; however, no depolarization occurred until 3 h. After 5 h, both D- and L-isomers began depolarizing the mitochondria, which entirely depolarized at 6 h. The obtained results suggest that both D- and L-isomers induce mitochondrial damage at the same time point. As seen in Fig. S9A, this process occurs rapidly. Further, fluorescence-activated cell sorting (FACS) is conducted to quantitatively confirm the mitochondrial depolarization induced by Mito-FF/Mito-ff. The FACS analysis supported the data obtained from the confocal images. The FACS analysis have not showed any difference in the fluorescence intensity compared with the TMRM control until 1 h and 3 h. However, after 5 h,

the fluorescence decreased for the peptide treated sample indicating that the depolarization of the mitochondria membrane occurred (Fig. S9B).

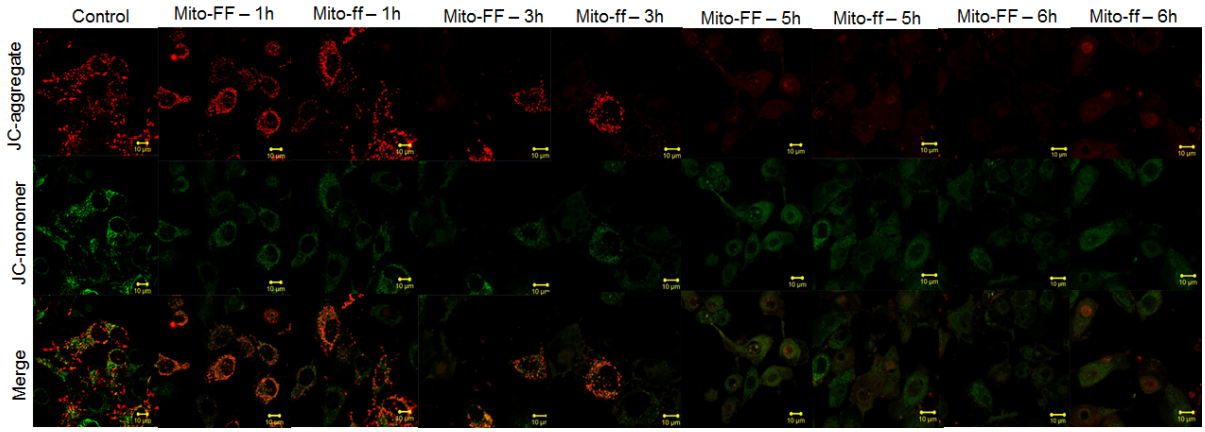


Fig. S10. Time dependent analysis of mitochondrial damage analyzed by JC-1 dye after treating with 40 μM of Mito-FF/ff. The analysis showed that after 1 h of incubation time, neither Mito-FF nor Mito-ff showed a change in the red emission. After 3 h of incubation with Mito-FF/Mito-ff, the red emission starts reducing. After 5 and 6 h, the red emission disappeared indicating that the mitochondrial membrane potential drop down. HeLa cells were incubated with 40 μM of Mito-FF/Mito-ff at pre-designed time interval to take images at 6 h, 5 h, 3 h and 1 h. The Mito-FF/Mito-ff incubated HeLa cells and control HeLa cells were labelled with JC-1 dye and analysed the fluorescence by confocal microscopy. The control HeLa cells showed both red and green fluorescence of JC-1 aggregate and monomer respectively. After 1 h of incubation time, both the molecule have not showed any change in the red and green fluorescence emission, while the red emission starts fading after 3 h of incubation. After 5 and 6 h, the red emission disappeared completely indicating the drop down of mitochondrial membrane potential (Fig. S10).

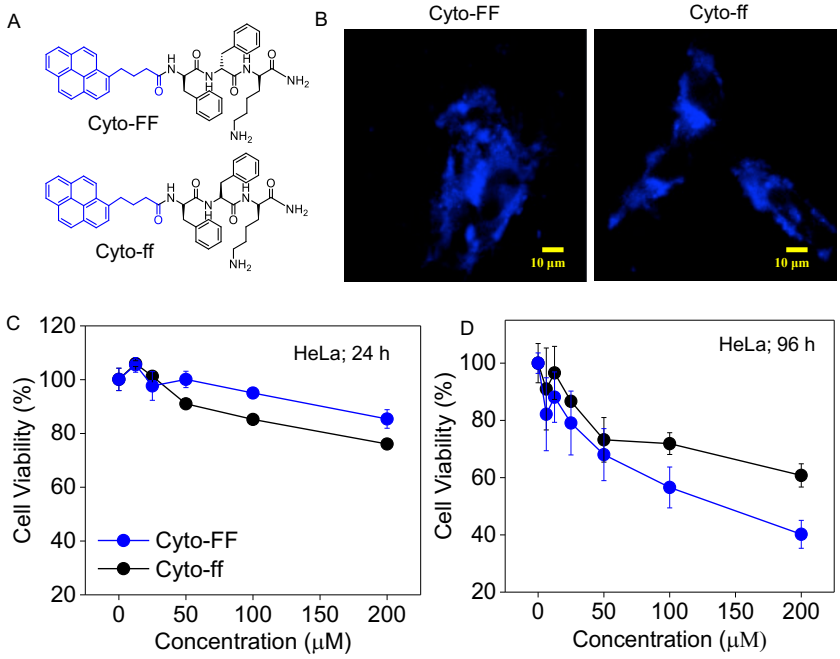


Fig. S11. A) Molecular design of Cyto-FF and Cyto-ff. B) Confocal images showing the

internalization of Cyto-FF/Cyto-ff in the cytoplasm and showing no co-localization with the Mitotracker Red FM. C) The cellular cytotoxicity of Cyto-FF/Cyto-ff in the HeLa cells for 24 h D) for 96 h of incubation. The cytotoxicity analysis of Cyto-FF/Cyto-ff after 24 h revealed that 75% and 85% of the cells were viable following treatment with 200 µM of Cyto-FF/Cyto-ff. When the incubation period was extended to 96 h, 38% and 60% viability was observed for Cyto-ff and Cyto-FF, respectively (Fig. S12C-D). Hence, based on the results obtained in our study, it can be concluded that the similar activity for the D- and L-peptides is specific and occurs when the peptides self-assemble intra-mitochondrially. In addition, the working concentration of the cytoplasm-targeting peptides Cyto-FF/Cyto-ff is ~10 times higher than that of the mitochondria-targeting self-assembling peptide Mito-FF/Mito-ff, suggesting higher efficiency of the mitochondria-targeting amphiphilic peptide.

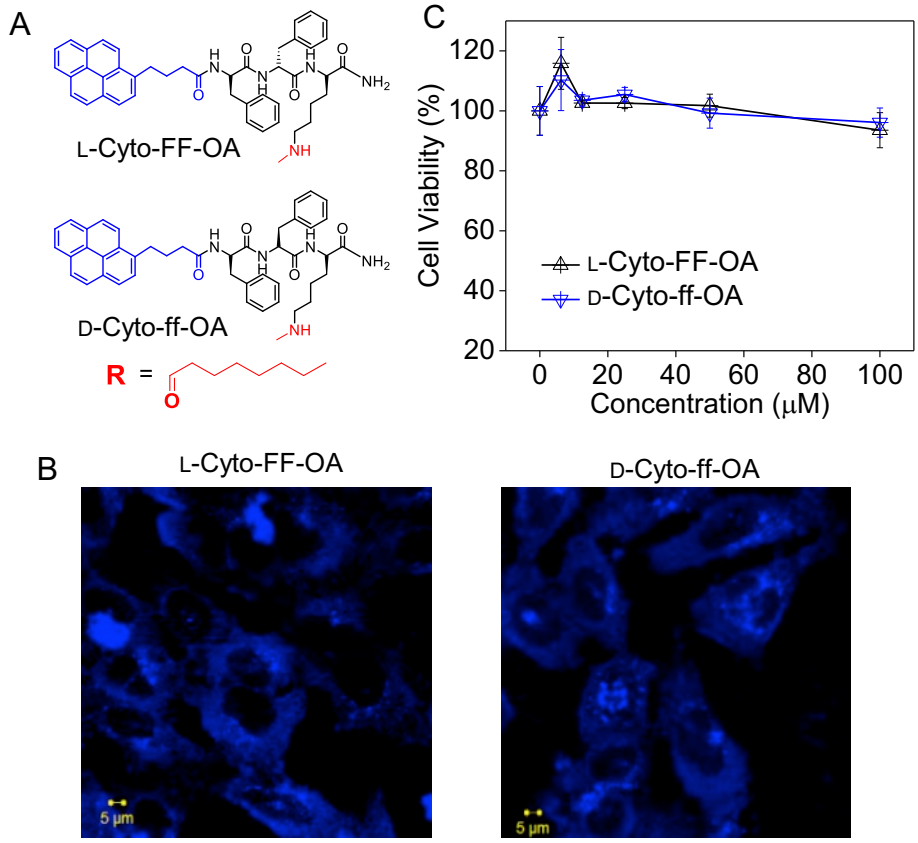


Fig. S12. Molecular design of L-Cyto-FF-OA and D-Cyto-ff-OA. B) Confocal images showing the internalization of L-Cyto-FF-OA/ D-Cyto-ff-OA in the cytoplasm. C) The cellular cytotoxicity of L-Cyto-FF-OA/ D-Cyto-ff-OA in the HeLa cells for 24 h.

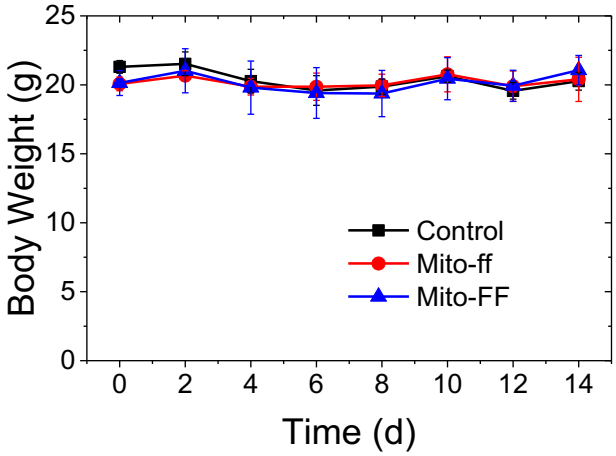


Fig. S13. Body weight loss after treating with our peptide showing there is no fetal toxicity induced by our peptide.

Reference.

1. A. K. Barui, S. K. Nethi, and C. R. Patra. *J. Mater. Chem. B,* 2017, **5**, 3391-3403.

2. P. Nagababu, A. K. Barui, B. Thulasiram, C. S. Devi, S. Satyanarayana, C. R. Patra, and B. Sreedhar. *J. Med. Chem.* 2015, **58**, 5226-5241.