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Spatial Targeting of Bcl-2 on Endoplasmic Reticulum and Mitochondria in Cancer Cells by Lipid Nanoparticles

Shalini Pandey,[a] Sohan Patil,[a] Nirmalya Ballav,[a] Sudipta Basu*[b]

a. Department of Chemistry, Indian Institute of Science Education and Research (IISER)-Pune, Dr. Homi Bhabha Road, Pashan, Pune, Maharashtra, 411008, India.

b. Discipline of Chemistry, Indian Institute of Technology (IIT)-Gandhinagar, Palaj, Gandhinagar, Gujarat, 382355, India.

Corresponding author: sudipta.basu@iitgn.ac.in

MATERIAL AND METHODS

Materials

All the chemicals were purchased from commercial suppliers unless otherwise noted. Obatoclax mesylate was purchased from Selleck Chemicals. Reactions were performed in the oven-dried glassware with or without inert gas. Analytical thin-layer chromatography (TLC) was performed using pre-coated silica gel aluminium sheets 60 F254 bought from EMD Millipore Laboratories. Compounds were purified by column chromatography using silica gel 100-200 mesh as the stationary phase. 1H, 13C, 31P spectra were recorded on a Bruker Avance III HD Ascend 9.4 Tesla/400 MHz with autosampler and/or Jeol 9.4 Tesla/400 MHz with autosampler spectrometer. Chemical shifts are mentioned in parts per million (ppm) and referred to residual protons on the corresponding deuterated solvent. UV- Visible spectra was recorded on Shimadzu. DMEM media and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from HiMedia. Nunc® Lab-Tek® II chambered coverglass, and Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich. MitoTracker® Green, ER Tracker Green and LysoTracker Green DND-26 were purchased from Invitrogen. AnnexinV- APC and 7-AAD staining Kit was purchased from Biolegend. Flow Cytometry analysis was recorded on BD- Accuri. Western blot analysis was performed on Las ImageQuant 400.

Methods

Synthesis of N-(2-aminoethyl)oleamide (2). Ethylene diamine was conjugated to oleic acid via amide coupling according to the reported procedure[1].

Synthesis of N-(2-((5-dimethylaminonapthalene)-1-sulfonamido)ethyl)oleamide (4). To a solution of N-(2-aminoethyl)oleamide (1 eq.) in DCM was added dansyl chloride (1 eq.) and trimethylamine (2.2 eq.). The reaction mixture was allowed to stir overnight under nitrogen atmosphere. Progress of the reaction was monitored through TLC. On completion, the reaction mixture was diluted with DCM and washed with brine. Organic layer was collected, dried over sodium sulphate and evaporated. Residue obtained was purified by column chromatography (35% ethyl acetate in hexane) in 45% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ=8.56 (d, *J* = 8 Hz, 1H), 8.22 (dd, J = 2Hz, 4 Hz, 2H), 7.58 (m, 2H), 7.20 (d, *J* = 8 Hz, 1H), 5.82 (m, 1H), 5.5 (s, 1H), 5.35 (m, 2H), 3.29-3.26 (m, 2H), 3.02 (q, *J* = 4 Hz, 8Hz, 2H), 2.89 (q, J = 4 Hz, 8 Hz, 6H), 2.00 (m, 6H), 1.41-1.25 (m, 22H), 2H), 0.86 (m , 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 174.33, 152.28, 139.42, 134.48, 130.82, 130.15, 130.09, 129.89, 129.64, 128.72, 123.33, 118.72, 115.42, 114.21, 77.16, 45.56, 32.08, 32.06, 30.39, 29.85, 29.81, 29.68, 29.47, 29.40, 29.31, 27.39, 27.35, 25.66, 22.84, 14.27

Synthesis of (5-((2-oleamidoethyl)amino)-5-oxopentyl)triphenylphosphonium bromide (6). To a solution of N-(2-aminoethyl)oleamide (1 eq.) at 0^{o}C in DCM under nitrogen atmosphere was added HBTU and DIPEA. The reaction mixture was allowed to stir for 15 minutes and then (4-carboxybutyl)triphenylphosphonium bromide (1.2 eq.) was added.

Reaction was then allowed to stir overnight. Progress of the reaction was monitored through TLC. On completion, the reaction mixture was diluted with DCM and washed with brine. Organic layer was collected, dried over sodium sulphate and evaporated. A sticky residue was obtained which was further washed with pentane to get the pure compound in 75% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71 (t, *J* = 32.2 Hz, 15H), 6.92 (s, 1H), 6.73 (s, 1H), 5.31 (t, *J* = 5.4 Hz, 2H), 3.27 (s, 3H), 3.17 (s, 2H), 2.88 (d, *J* = 66.7 Hz, 2H), 2.31 (s, 2H), 2.16 (t, *J* = 7.1 Hz, 2H), 2.03 – 1.93 (m, 4H), 1.87 (s, 2H), 1.69 (s, 2H), 1.55 (s, 2H), 1.25 (s, 22H), 0.86 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 174.33, 173.17, 135.43, 133.45, 133.36, 130.79, 130.67, 129.95, 118.32, 117.40, 39.81, 39.63, 36.49, 34.43, 31.97, 29.85, 29.60, 29.39, 29.24, 27.30, 26.09, 25.93, 25.84, 22.75, 21.64, 21.54, 21.50, 14.20.

^{31}P NMR (162 MHz, $CDCl_3$): δ 24.4

Synthesis of nanoparticles. 0.5 mg of oleic acid conjugate, 0.5 mg of obatoclax, 1 mg of L-α-phosphatidylcholine (PC) and 0.1 mg of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polythylene glycol)2000] (DSPE-PEG) were dissolved in 5 mL of DCM- Methanol mixture. Solvent was then slowly evaporated into a thin and uniform film with the help of rotary evaporator. Once the film is thoroughly dried, it was hydrated with 1 mL of distilled water for 60 minutes at 60^{o}C, accompanied with sonications after every 15 minutes. It was then passed through Sephadex G-25 column and extruded through 200 nm Whatmann polycarbonate membrane to obtain sub 200 nm particles. The nanoparticles were then stored at 4^{o}C for further use.

Determination of shape, size and morphology. The shape, size and morphology of the nanoparticles was determined using light scattering and electron microscopy techniques like field-emission scanning electron microscopy (FESEM) and atomic force microscopy (AFM). Samples were prepared according to the previously reported procedures.

Quantification of drug loading in the nanoparticles. Loading of obatoclax in the nanoparticles was determined at λ_{max}= 488 nm by UV-Vis spectroscopy using the previously reported method.

$$\text{Drug Loading Efficiency (\%)} = \frac{Amount\ of\ drug\ loaded\ in\ nanoparticle}{Amount\ of\ drug\ used} X\ 100$$

$$Weight\ \%\ of\ Obatoclax = \frac{\text{Weight of obatoclax in nanoparticles}}{\text{Weight of solid nanoparticles}}$$

Cell Viability assay. 100 µL of 5000 cells in DMEM media were seeded per well in 96-well microtiter plate and incubated for 16 h in a 5% CO_2 incubator at 37^{o}C. Cells were treated with different concentrations of the nanoparticles and incubated for 24 h and 48 h. After the said time points, media was removed and 100 µL of MTT in DMEM (0.5 mg/mL) was added. After 4h, 50 µL of solubilisation buffer (10%SDS in 0.01 N HCl) was added to dissolve the formazan crystals and left for incubation overnight. Absorbance was then recorded at 570 nm on Perkin Elmer Ensight.

Cellular Internalization. 2 x 10^4 cells were seeded in a 8-well LabTek chamber. Cells were incubated with the ER and mitochondria targeted nanoparticles for the mentioned time points. Cells were washed with cold PBS and costained with ER Tracker Green and MitoTracker green respectively and incubated for 20 mins. Cells were then washed and visualised using CLSM, Zeiss LSM 700. Similar method was adopted for monitoring the localization of nanoparticles in lysosomes using LysoTracker Green DND-26.

DCFH-DA assay. 2 x 10^4 cells were seeded in a 8-well LabTek slide. Cells were treated with the nanoparticles for 24 h. Post incubation cells were treated with DCFH-DA for 15 min. Cells were then washed thrice and visualized by CLSM.

Calcein AM assay. 2 x 10^4 cells were seeded in a LabTek slide (8-well). Cells were treated with the nanoparticles. Post 24 h incubation calcein AM (1 μM) and 1mM $CoCl_2$ were added into the cells followed by imaging by CLSM.

Flow Cytometry analysis. 2 x 10^6 cells were seeded in a 6-well plate and treated with the nanoparticles for 24 h. The cells were then trypsinized and washed with PBS. Suspended cells were then incubated with APC Annexin V and 7-AAD according to the manufacturer's protocol. Apoptotic and necrotic cells were then quantified using BD Accuri C6 flow cytometer.

Western Blot analysis. 1x 10^6 cells were seeded in a 6-well plate and treated with the nanoparticles for 24 h. Cells were then lysed. SDS-PAGE was used to separate the different proteins.

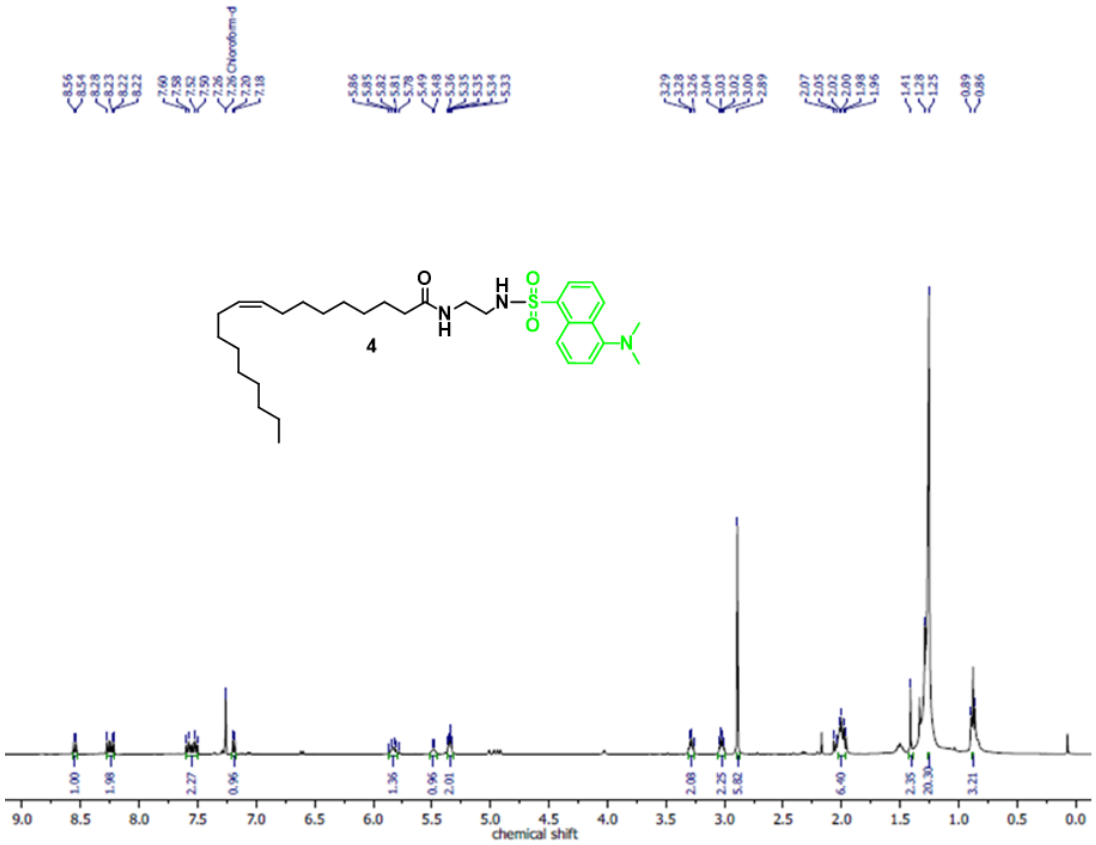

Fig. S1: ^{1}H NMR spectrum of compound 4 in $CDCl_3$ at 400 MHz.

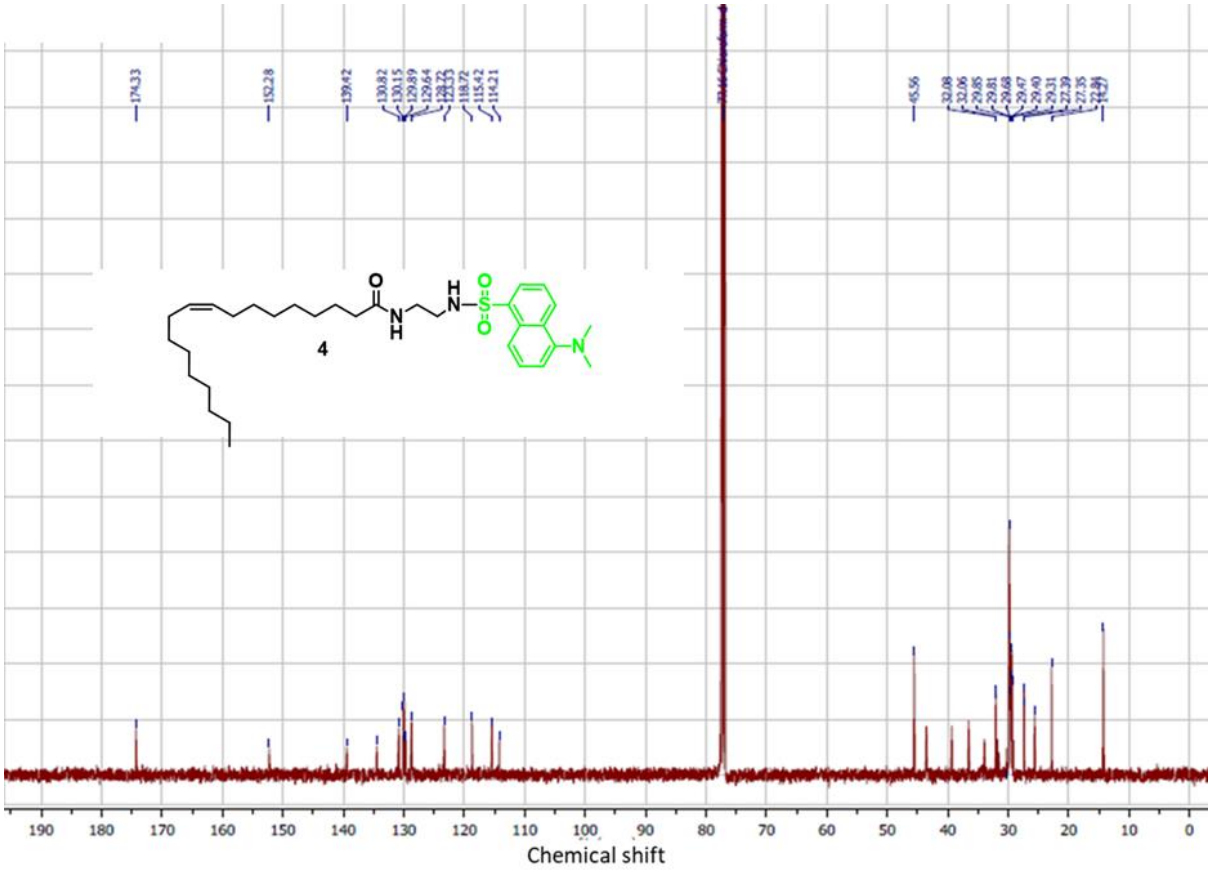

Fig. S2: ^{13}C NMR spectrum of compound 4 in $CDCl_3$ at 100 MHz.

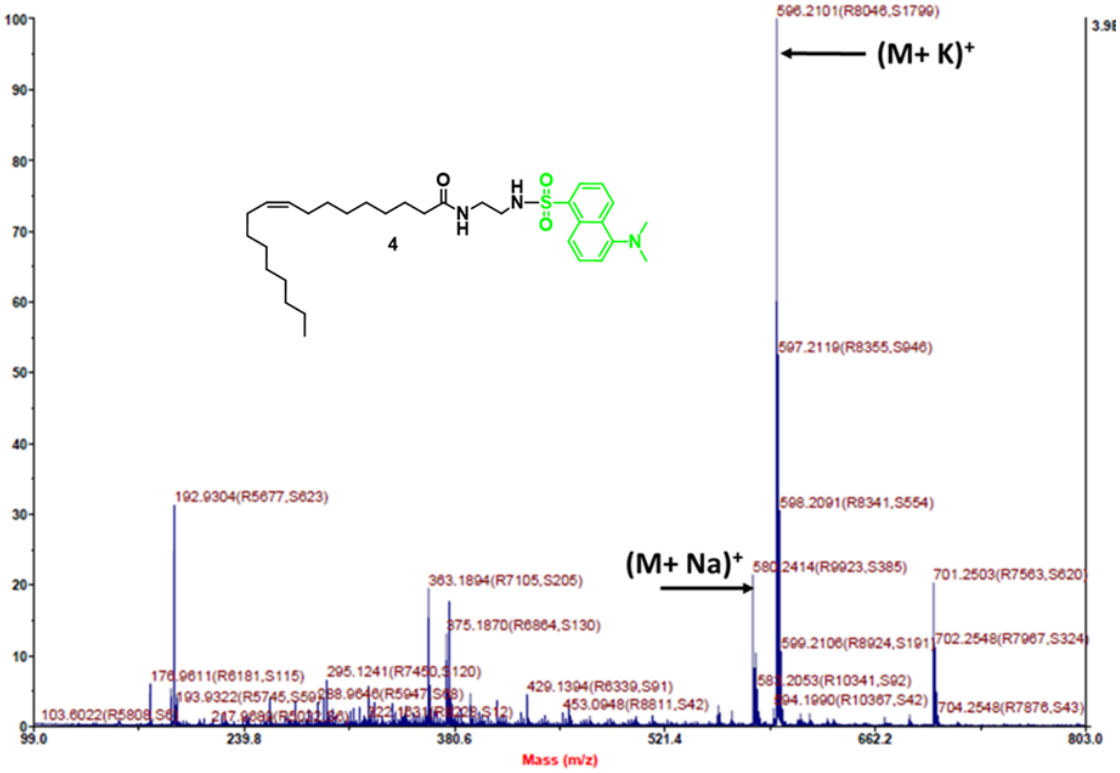

Fig. S3: MALDI- TOF spectrum of Compound 4.

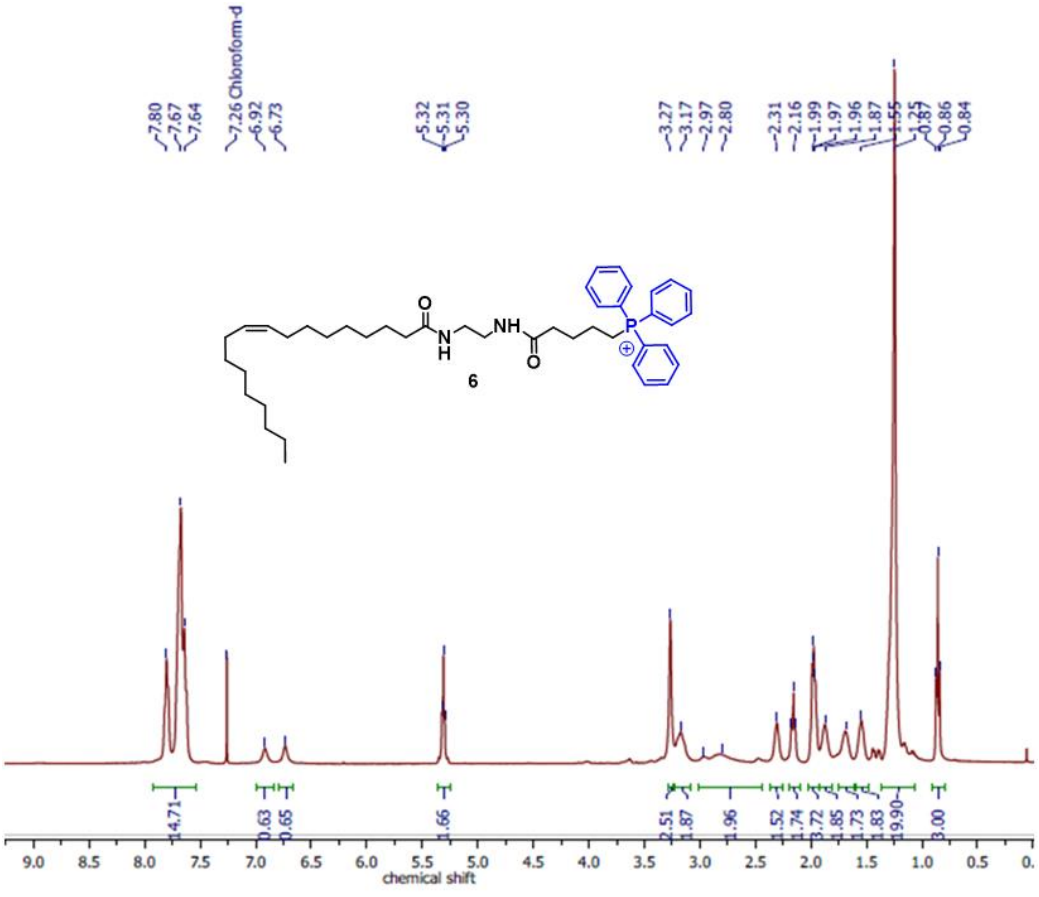

Fig. S4: ^{1}H NMR spectrum of compound 6 in $CDCl_3$ at 400 MHz.

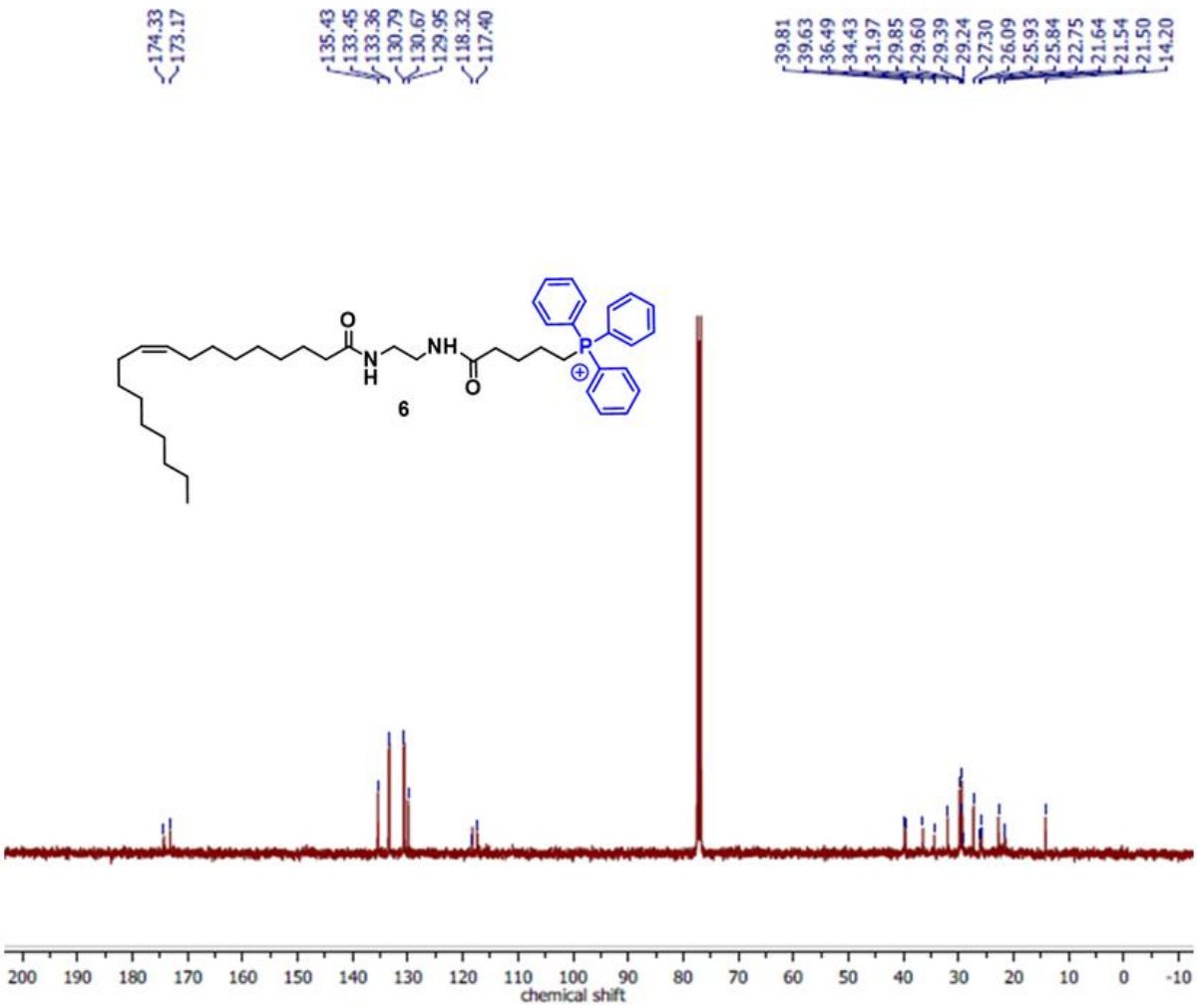

Fig. S5: ^{13}C NMR spectrum of compound 6 in $CDCl_3$ at 100 MHz.

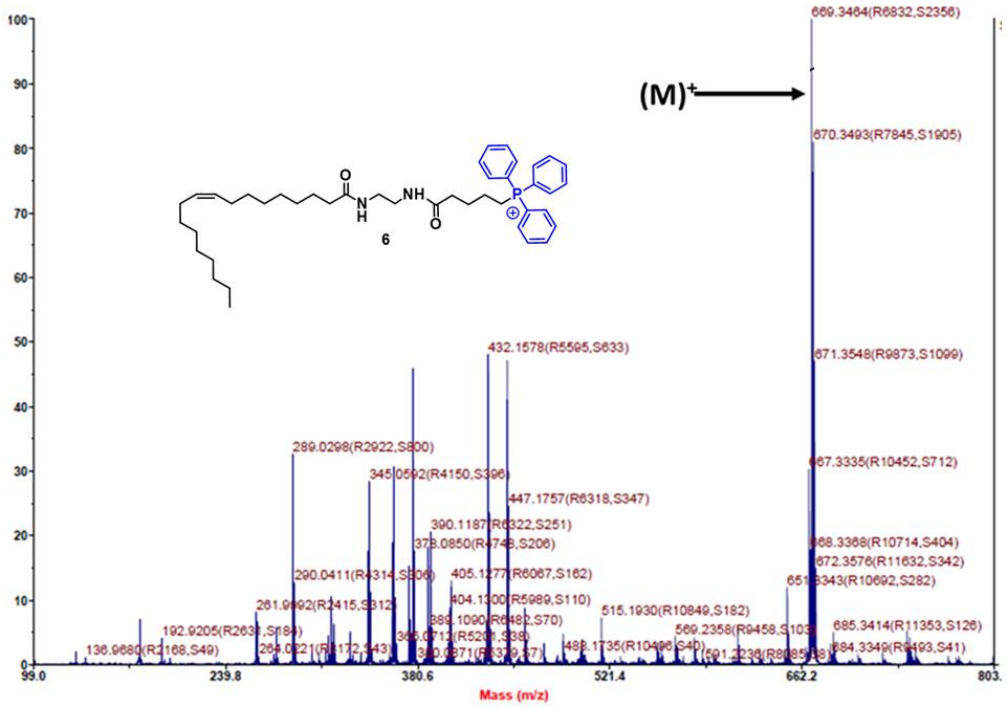

Fig. S6: MALDI- TOF spectrum of compound 6.

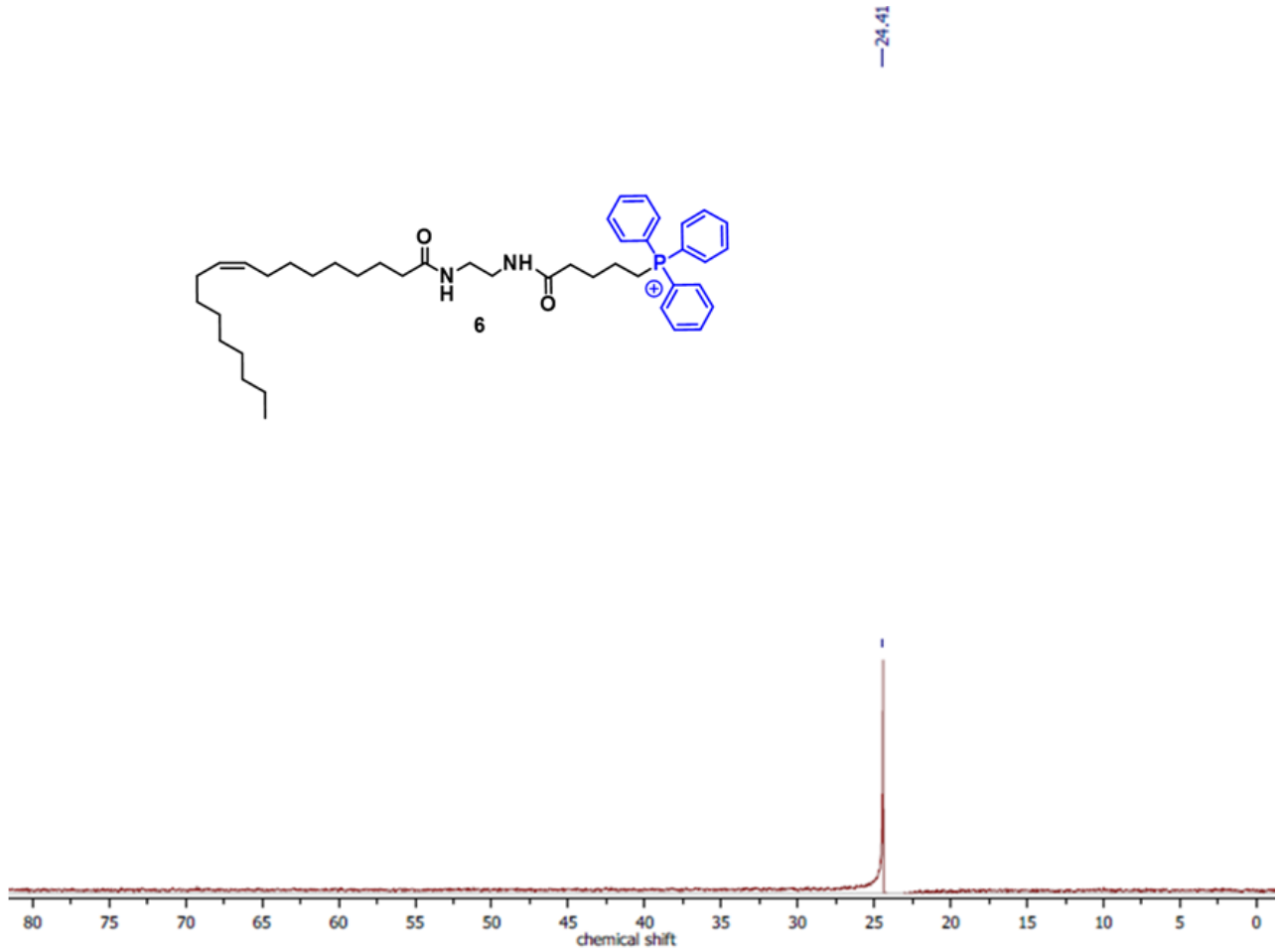

Fig. S7: ^{31}P NMR spectrum of compound 6 in $CDCl_3$.

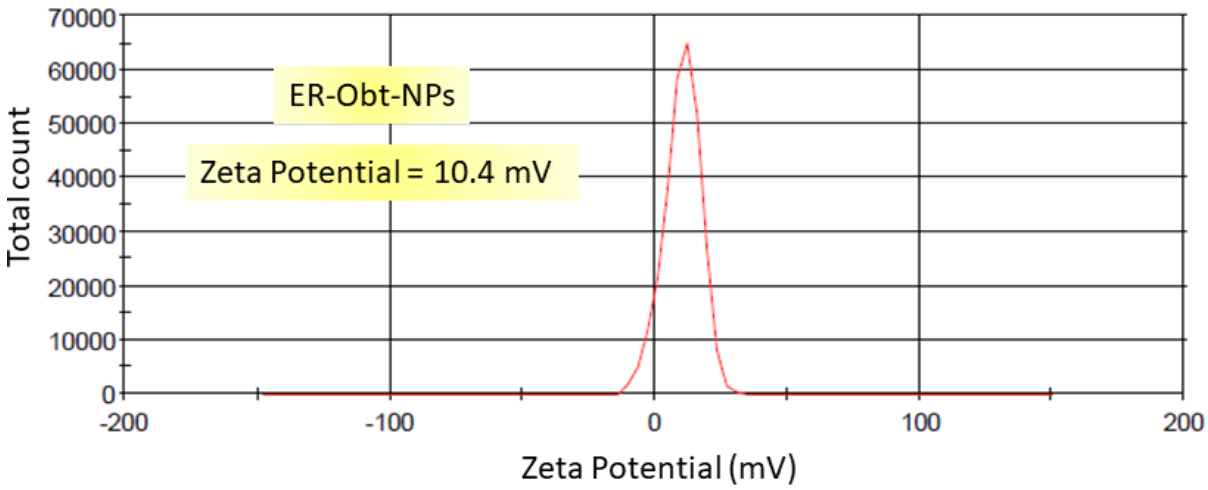

Fig. S8: Zeta Potential of ER-Obt-NPs by DLS.

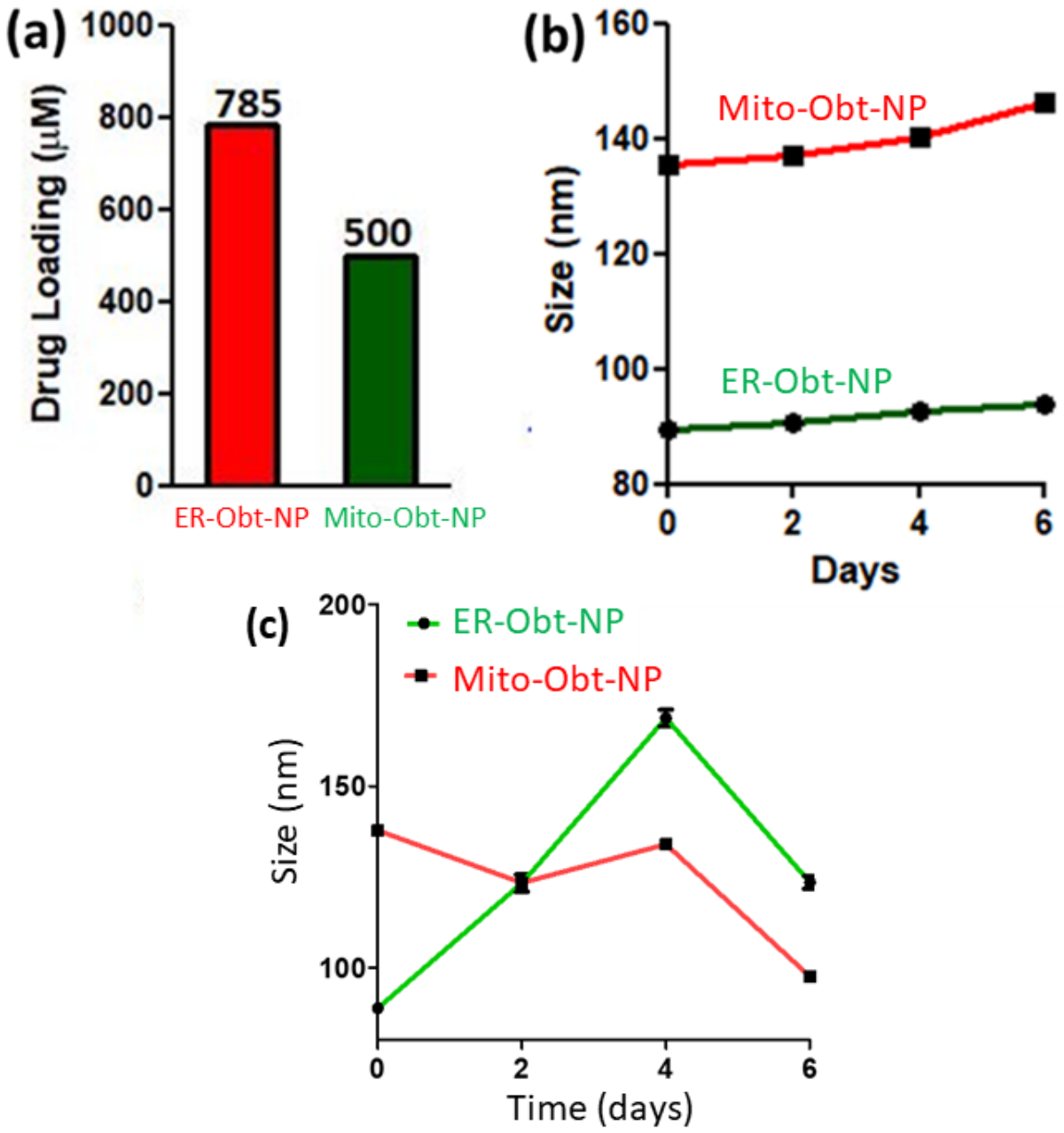

Fig. S9: (a) Drug loading of ER-Obt-NP and Mito-Obt-NP calculated at λ_{max}= 488 nm using UV- Vis spectroscopy. (b,c) Stability of the nanoparticles in PBS and in DMEM cell culture media with 10% FBS at 37^{o}C respectively.

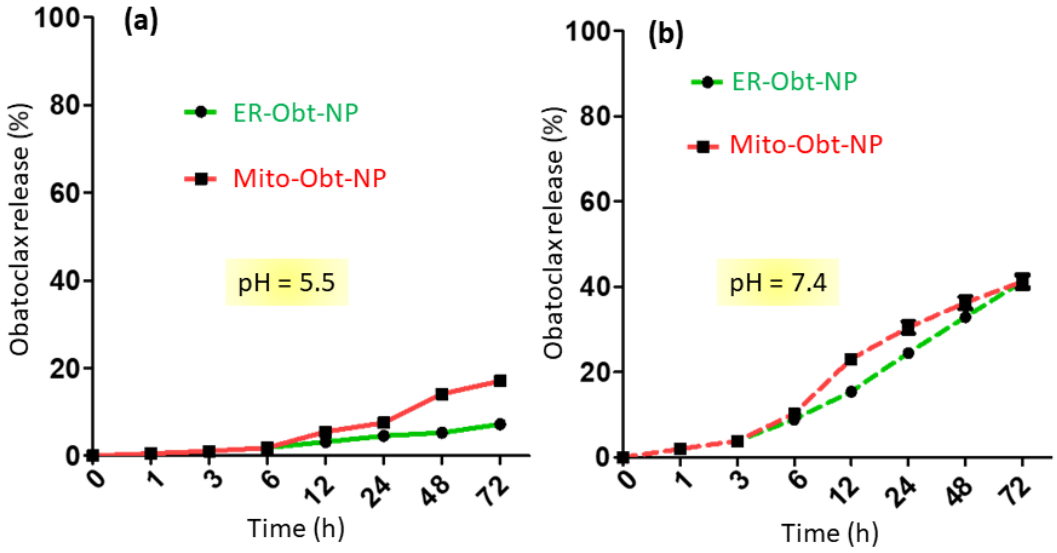

Fig. S10: (a,b) Release of obatoclax from ER-Obt-NPs and Mito-Obt-NPs at pH = 5.5 and pH = 7.4 over 72h respectively.

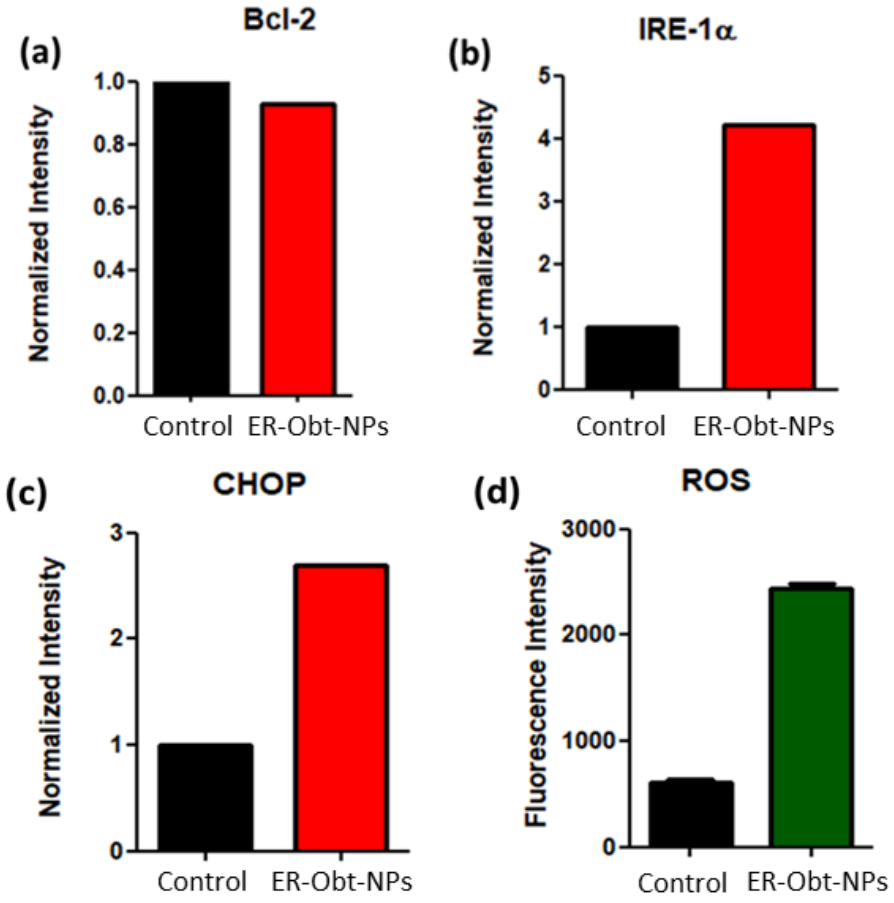

Fig. S11: Quantification of (a) Bcl-2 (b) IRE1-α (c) CHOP from western blot analysis after treatment of HeLa cells with ER-Obt-NPs (d) Confocal microscopy-based quantification of green fluorescence intensity after incubation with H2DCFDA in ER-Obt-NPs treated HeLa cells.

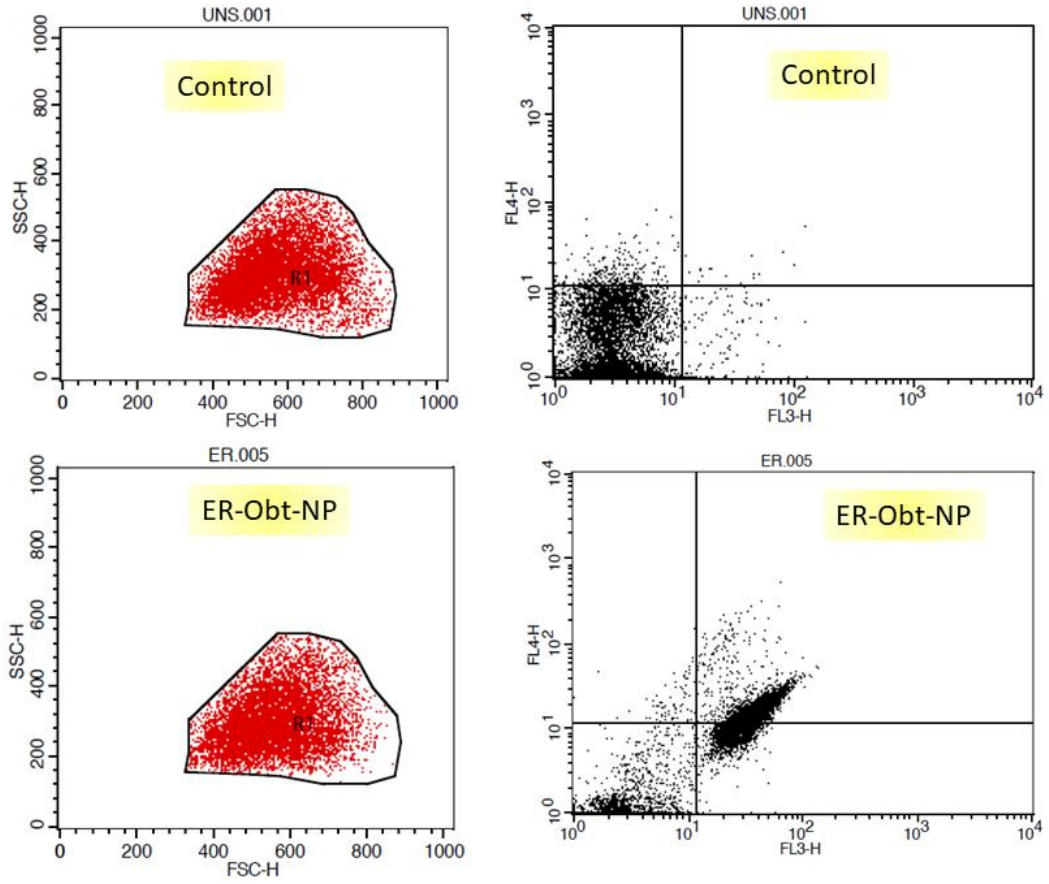

Fig. S12: SSC vs FSC plots obtained from flow cytometry analysis of HeLa cells treated with control and ER-Obt-NPs for 24h.

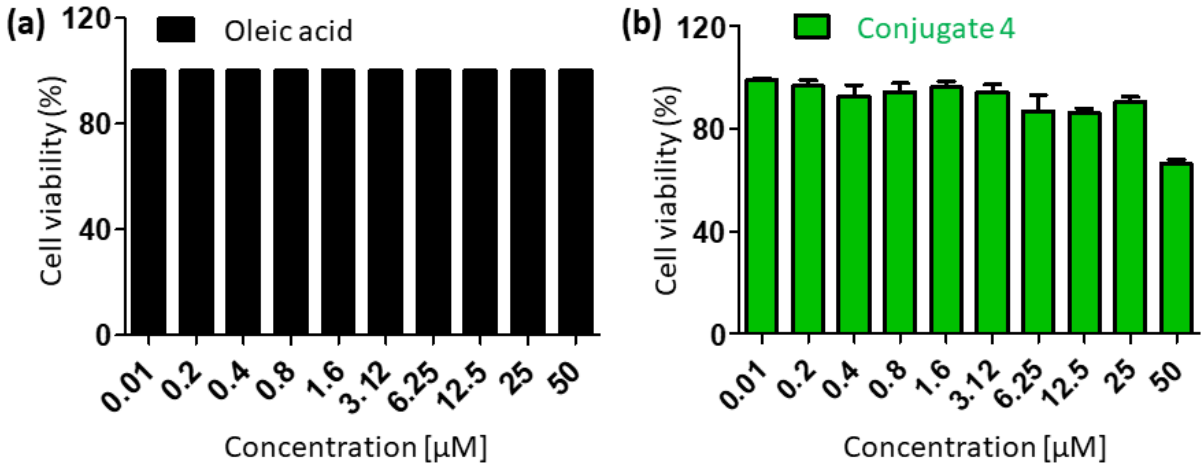

Fig. S13: (a,b) Viability of HeLa cells after treatment with oleic acid and oleic acid-dansyl conjugate (4) in a dose dependent manner over 48h, determined by MTT assay.

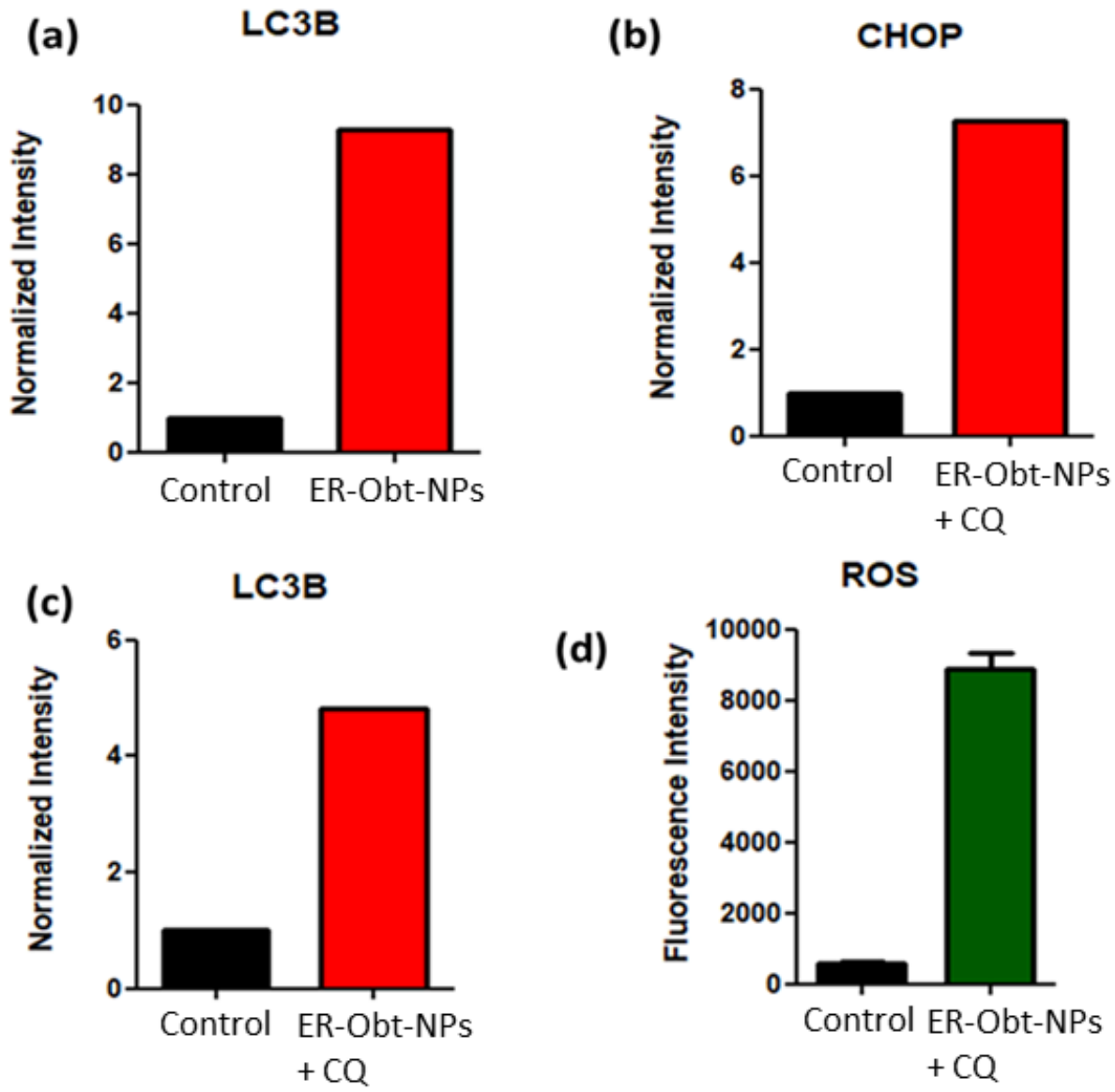

Fig. S14: Quantification of (a) LC3B from western blot analysis after treatment with ER-obt-NPs for 24 h in HeLa cells. (b) CHOP and (c) LC3B from western blot analysis after treatment of HeLa cells with ER-Obt-NPs and chloroquine (d) Confocal microscopy-based quantification of green fluorescence intensity after incubation with H2DCFDA in ER-Obt-NPs and chloroquine treated HeLa cells.

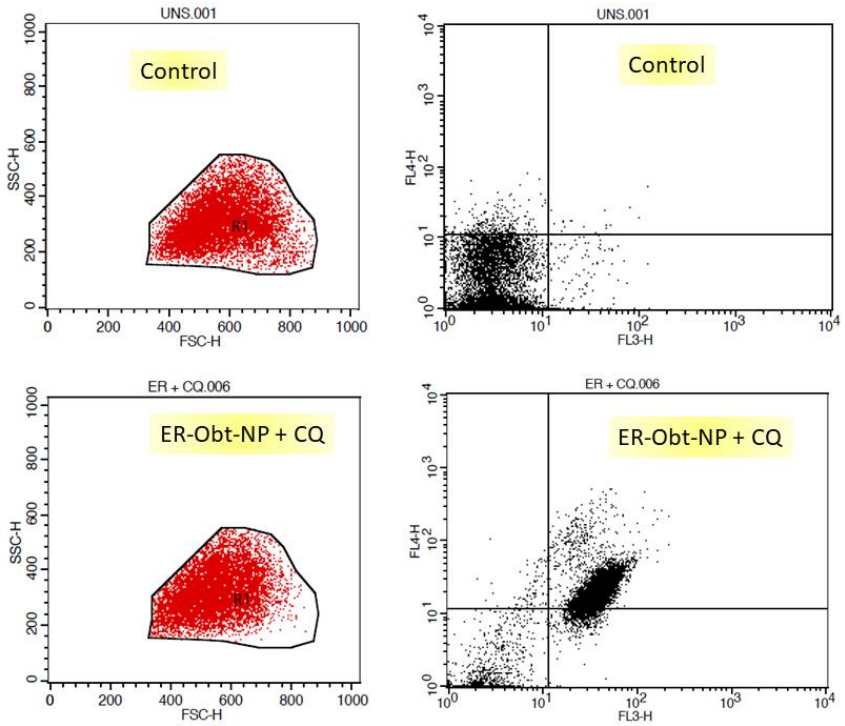

Fig. S15: SSC vs FSC plots obtained from flow cytometry analysis of HeLa cells treated with control and ER-Obt-NPs + CQ as combination therapy for 24h.

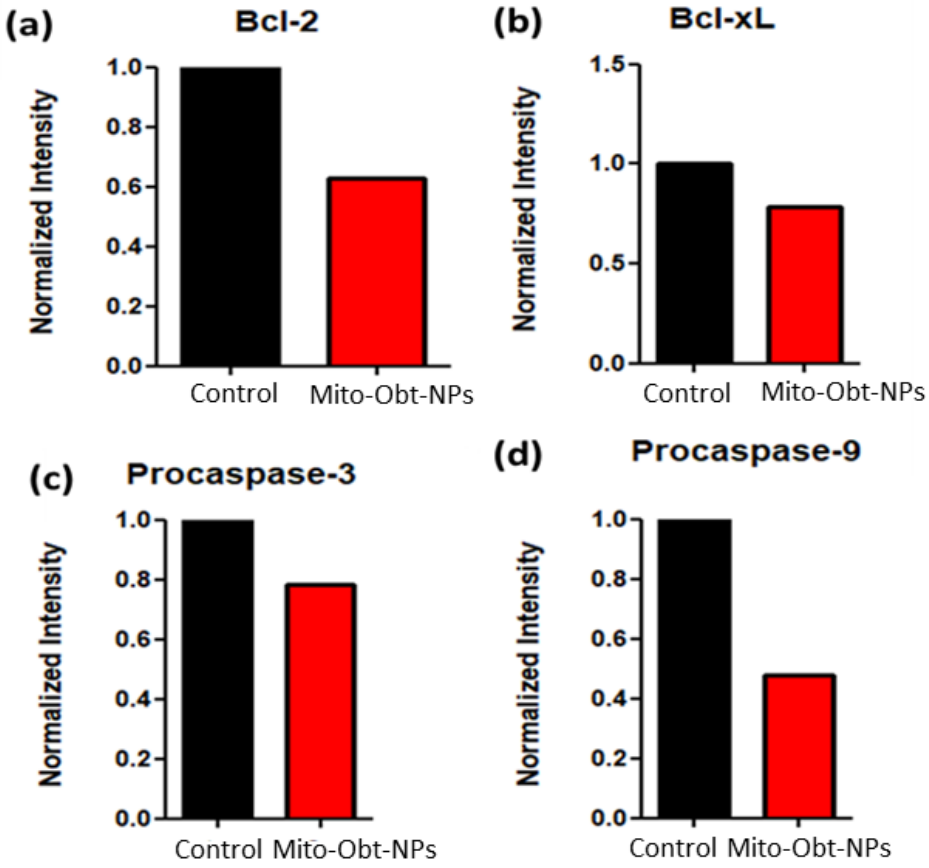

Fig. S16: Quantification of (a) Bcl-2 (b) Bcl-xL (c) Procaspase-3 (d) Procaspase-9 from western blot analysis after treatment of HeLa cells with Mito-Obt-NPs.

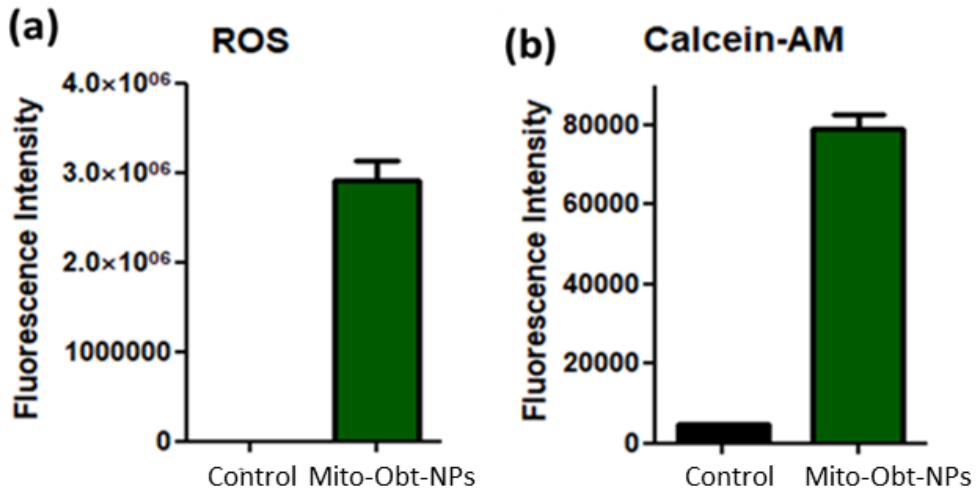

Fig. S17: Confocal microscopy-based quantification of green fluorescence intensity after incubation with (a) H2DCFDA and (b) Calcein-AM in Mito-Obt-NPs treated NPs in HeLa cells.

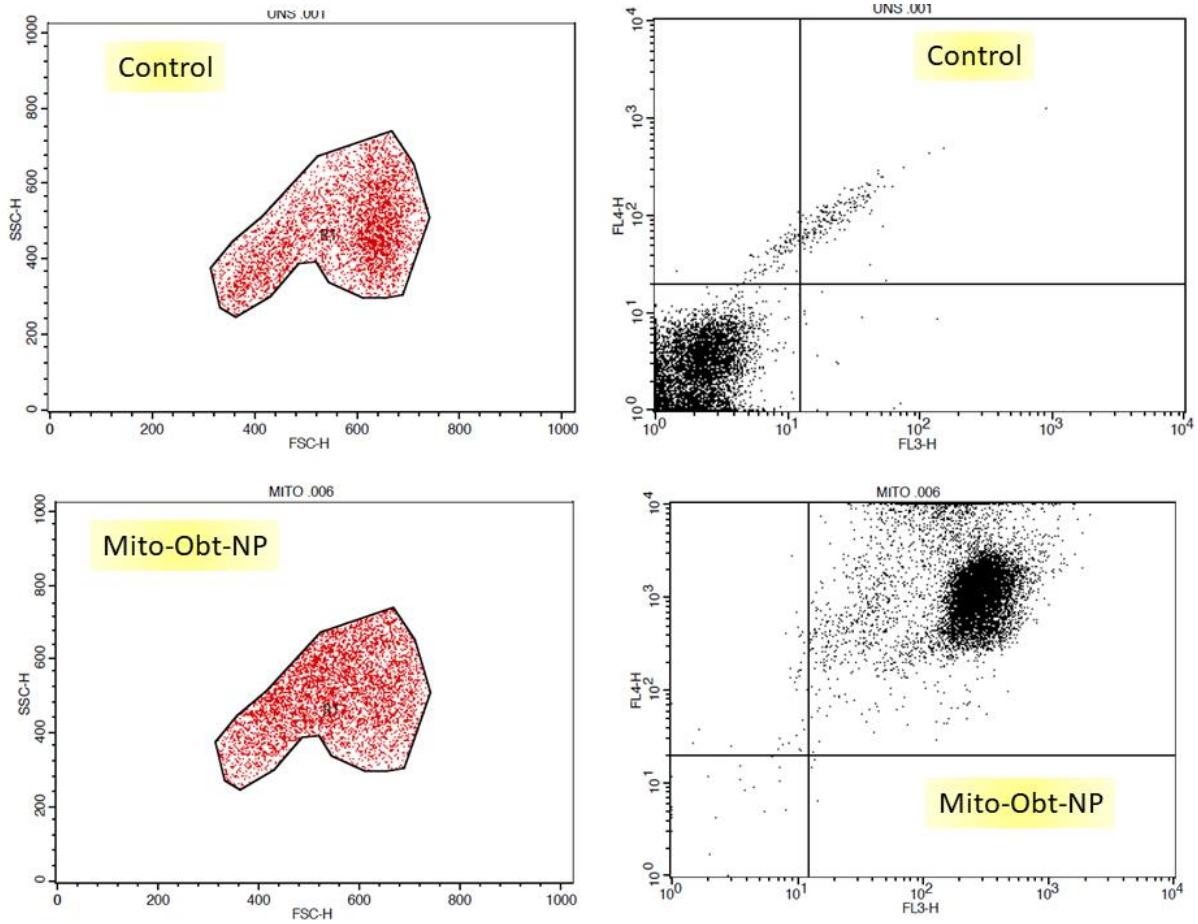

Fig. S18: SSC vs FSC plots obtained from flow cytometry analysis of HeLa cells treated with control and Mito-Obt-NPs for 24h.

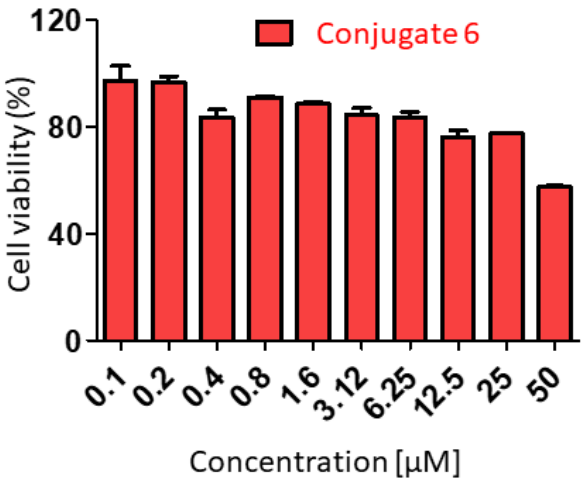

Fig. S19: Viability of HeLa cells after treatment with oleic acid-TPP conjugate (6) in a dose dependent manner over 48h, determined by MTT assay.

Treatment Time		3 h	6 h	24 h
Image Channels		C1 (green) C3 (red)	C1 (green) C3 (red)	C1 (green) C3 (red)
Pearsons' Correlation Coeffecient		0.937	0.964	0.906
Manders Coeffecients	M1 (fraction of C1 overlapping C3)	0.824	0.9182	0.913
	M2 (fraction of C3 overlapping C1)	0.7084	0.8284	1.00

Table S1: Quantification of co-localization of ER-Obt-NPs in ER of HeLa cells at 3 h, 6 h and 24 h from CLSM.

Treatment Time		1 h	3 h	6 h
Image Channels		C2 (green) C3 (red)	C2 (green) C3 (red)	C2 (green) C3 (red)
Pearsons' Correlation Coeffecient		0.2253	0.2381	0.1574
Manders Coeffecients	M1 (fraction of C2 overlapping C3)	0.363	0.5237	0.5331
	M2 (fraction of C3 overlapping C2)	0.5787	0.7227	0.6743

Table S2: Quantification of co-localization of ER-Obt-NPs in lysosomes of HeLa cells at 1 h, 3 h and 6 h from CLSM.

Treatment Time		3 h	6 h	24 h
Image Channels		C1 (green) C2 (red)	C1 (green) C2 (red)	C1 (green) C2 (red)
Pearsons' Correlation Coeffecient		0.8097	0.7663	0.7214
Manders Coeffecients	M1 (fraction of C1 overlapping C3)	0.949	0.891	0.8403
	M2 (fraction of C3 overlapping C1)	0.9292	0.8619	0.4751

Table S3: Quantification of co-localization of Mito-Obt-NPs in mitochondria of HeLa cells at 3 h and 6 h and 24 h from CLSM.

References

1. S. Palvai , P. More , N. Mapara , J. Nagraj , R. Chowdhury and S. Basu , *ChemNanoMat*, 2016, **2** , 201 —211