Design of novel microtubule targeted peptide vesicle for delivering different anticancer drugs

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Materials and Methods:

Reagents: All Fmoc-amino acids, HBTU and Fmoc-Rink Amide AM resin were purchased from Novabiochem. Pipyridine, Diisopropylethylamine (DIPEA) 1-Hydroxybenzotriazole (HOBT), Pyridine, Ether, Dimethylsulfoxide (DMSO), Methanol and Trifluoroacetic acid were purchased from Spectrochem. Phenol, Dichloromethane (DCM), Ethanedithiol (EDT), Hydrogen peroxide (30% solution), Acetone, Dichloromethane and N, N′-Dimethylformamide (DMF) were purchased from Merck. TritonX-100 was purchased from SRL. N, N′- Diisopropylcarbodiimide (DIC), 5(6)-Carboxyfluorescence (FITC), 5(6)-Carboxytetramethylrhodamine (TAMRA), 5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), Doxorubicin hydrochloride, curcumin, dulbecco's modified eagle's medium (DMEM), MES, trypsin-EDTA solution, DMSO for cell culture and formaldehyde solution (molecular biology grade) were purchased from Sigma Aldrich. Penicillin-Streptomycin, neutravidin, Alexafluor568-carboxylic acid succinimidyl ester and fetal bovine serum (FBS) were purchased from Invitrogen. Anti-alpha Tubulin (EP1332Y) antibody and Goat polyclonal anti-Rabbit IgG H&L (Cy3.5 ®) were purchased from Abcam. For purification, we used Simadzu HPLC system with Symmetry C-18 (Waters) semi preparative reverse phase column. Pure product was 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.

Methods:

Peptide Synthesis: K_3NQ (NH_2-KKKNAPVSIPQ-$CONH_2$) and E_3NQ (NH_2-EEENAPVSIPQ-$CONH_2$) were synthesized by solid phase peptide synthesis method in CEM-LIBERTY1 automated microwave peptide synthesizer using Rink Amide AM resin. Deprotection of Fmoc group is carried out by 20% v/v piperidine in DMF. HBTU (5 equiv.) and DIPEA (10 equiv.) FITC-K_3NQ and TAMRA-E_3NQ peptides were synthesized through amide coupling reaction taking FITC and TAMRA (5 equiv. each) with K_3NQ and E_3NQ respectively. For this coupling step, HBTU (5 equiv.) and DIPEA (10 equiv.) are used as coupling reagent and base respectively. After the synthesis, peptides were cleaved from the resin with a mixture of TFA, EDT, H_2O, Phenol 94:2.0:2.0:2.0 (v/v/v/v) at room temperature for 2 h and precipitated

in cold ether. The peptides were lyophilized and purified by Reverse Phase HPLC (Simadzu) and characterized by MALDI Mass spectroscopy.

MD Simulation: For MD simulation, two peptides (K_3NQ and E_3NQ) were kept at the centre of cubic box solvated by 2970 Simple Point Charge (SPC) water model in 4.5 nm cubic box. GROMACS 4.5.5 package was used for simulation study.[1] Gromos 9653a6 force field was applied for peptides. Periodic boundary conditions were applied in all three directions. Cut-off radii were set at 0.9 nm for electrostatic interactions and 1.4 nm for Lennard-Jones interactions. Long-range electrostatics interactions were tested using Particle-Mesh Ewald (PME) method.[2] Simulation was performed at time step of 2 fs. The first phase involved the simulating for 500 ps under a constant volume (NVT) ensemble. Protein and non-protein atoms were coupled to separate coupling baths and temperature maintained to 310 K using V-rescale coupling method. Following NVT equilibration, 1 ns of constant-pressure (NPT) equilibration was performed using Parrinello-Rahman coupling method. Relaxation time of 0.1 ps and 1ps were used for NVT and NPT respectively. Then production run was performed for 20 ns. LINCS algorithm was used to constrain bond lengths.[3]

Initially, V7, S8 and I9 residues of K_3NQ and V7, S8, I9 of E_3NQ interact by H-bonding at 6 ns of simulation. In addition, K1 residue of K_3NQ and E1 of E_3NQ start interacting using H-bonding after 10 ns to 20 ns of simulation and results into β-sheet conformation.

Docking: Autodock-Vina software version 1.1.2 was used for blind docking.[4] 98x60x64 affinity grid box was centred on the receptor tubulin (1Z2B)[5] for docking with E_3NQ and K_3NQ peptides.

Fourier Transform Infrared Spectroscopy: Freshly prepared peptides (K_3NQ and E_3NQ) and their mixture were incubated for four days, lyophilized. Then FT-IR spectroscopic analysis was performed in a Perkin-Elmer Spectrum 100 FT-IR spectrometer using KBr pellets. Spectra of these pellets were recorded and collected of 5 times scan with the speed of 0.2 cm/s at a resolution of 1.6 cm^{-1} in the Perkin-Elmer Spectrum 100 series spectrometer. Then data plotting was performed in the LiTaO3 detector. Each time background correction was performed to eliminate interference from air (or any other parameters).

Förster Resonance Energy Transfer (FRET) experiment: In order to know the binding site of two peptide K_3NQ and E_3NQ with tubulin we have performed FRET[6] study which helps us to know the distance between two interacting partners using two fluorophore probe.[7, 8] In FRET study, non-radiative energy is transferred from the excited donor molecule to the ground state acceptor molecule. By choosing appropriate FRET pair we can study the interaction between two macromolecules without disturbing their physical property. From, steady state emission intensity we can get a qualitative idea about the efficiency of energy transfer of two peptides K_3NQ and E_3NQ from the donor (Colchicine-tubulin complex at 355 nm) to acceptor (FITC-K_3NQ at 488 nm) and donor (Colchicine-tubulin complex at 355 nm) to acceptor (FITC-E_3NQ at 488 nm) respectively.

We have studied the steady state FRET experiment between FITC-K_3NQ and FITC-E_3NQ to determine how strong these two peptide interacting with each other and also the distance between the two fluorophores tagged peptides. The loss of donor fluorescence intensity in presence of an acceptor fluor is often quantitated as FRET efficiency.

Förster distance (R_0) between Colchicine-tubulin complex and FITC-E_3NQ peptide is calculated to be ~ 29.5 ± 1 Å from the spectral overlap graph of these two donor-acceptor pair.

Förster distance $R_0 = 0.211[\kappa^2 n^{-4} \Phi(\lambda)\, D(\lambda)]^{1/6}$

Where, κ is the orientation factor for the relative geometries of donor and acceptor, n is the refractive 2 index of the medium, ϕ is the fluorescence quantum yield, D(λ) is the fluorescence intensity of the donor, $J(\lambda)$ is the integrated overlap between the donor emission and acceptor absorption spectra[9].

The efficiency of FRET is given by the Equation,

$$\varepsilon_{FRET} = \frac{I_A}{I_A + I_D}$$

In our FRET studies, as obtained from Figure 3a, acceptor intensity I_A= 0.41, donor intensity I_D= 1, thus the FRET efficiency (ε_{FRET}) = 0.3.

Now the distance (R_{DA}) between Colchicine-tubulin complex and FITC-E_3NQ peptides is calculated by the following equation.

$$R_{DA} = R_0\left(\frac{1-\varepsilon_{FRET}}{\varepsilon_{FRET}}\right)^{1/6}$$

R_{DA} is calculated to be ~ 32.4±2 Å.

We have again studied steady state FRET experiment taking Colchicine-tubulin complex and FITC-K_3NQ.

Förster distance (R_0) between Colchicine-tubulin complex and FITC-K_3NQ peptide is calculated to be ~ 29.5 ± 1 Å from the spectral overlap graph of these two donor-acceptor pair.

Förster distance $R_0 = 0.211[\kappa^2 n^{-4} \phi(\lambda)\, D(\lambda)]^{1/6}$

Where, κ is the orientation factor for the relative geometries of donor and acceptor, n is the refractive 2 index of the medium, ϕ is the fluorescence quantum yield, D(λ) is the fluorescence intensity of the donor, $J(\lambda)$ is the integrated overlap between the donor emission and acceptor absorption spectra.

The efficiency of FRET is given by the Equation,

$$\varepsilon_{FRET} = \frac{I_A}{I_A + I_D}$$

In our FRET studies, as obtained from Figure 3b, acceptor intensity I_A= 0.23, donor intensity I_D= 1 thus the FRET efficiency (ε_{FRET}) = 0.187

Now, the distance (R_{DA}) between Colchicine-tubulin complex and FITC-K_3NQ peptides is calculated by the following equation.

$$R_{DA} = R_0\left(\frac{1-\varepsilon_{FRET}}{\varepsilon_{FRET}}\right)^{1/6}$$

R_{DA} is calculated to be ~ 37.6 ± 2 Å.

The value is quite close to the Förster distance (R_0) which means that they are interacting and efficient FRET is occurring between FITC-E_3NQ and TAMRA-K_3NQ peptides.

Fluorescence microscopy: 5 μL incubated (4 days at 37 ^{o}C) sample (from the mixture of both FITC-K_3NQ and TAMRA-E_3NQ peptides at concentration of 500 μM) was taken and placed on a cleaned glass slide and dried in desiccators. Next, the glass slide was observed under inverted fluorescence microscope (NIKON Ti-U) in 100× magnification using ANDOR iXON3 camera. Fluorescence microscopic image reveals nice hollow peptide vesicles, which is abbreviated in this manuscript as microtubule targeted peptide vesicles "**MTPV**".

Atomic Force Microscopy: 5 μL incubated (4 days at 37 ^{o}C) solution of the sample (from the mixture of both K_3NQ and E_3NQ peptides at concentration of 500 μM) was taken and placed on a clear glass slide and dried in desiccators. Then, the glass slide was observed under Nanosurf C3000 Controller Atomic Force Microscopy in dynamic mode.

Dynamic Light Scattering (DLS) study to characterize the vesicles size: In order to characterize the size of the peptide vesicles we have performed the DLS study. The DLS study was examined for both the peptide vesicles (MTPV) and drug loaded MTPV at concentration of 250 μM. The experiment was done in Malvern particle size analyser (Model no. ZEN 3690 ZETASIZER NANO ZS 90).

Drug loading in the peptide vesicles: Initially, 1 mL solution of either DOX (200 μM) or PI (500 μM) was added with freshly prepared peptides i.e. K_3NQ and E_3NQ (500 μM in 1:1 ratio). The resulting solution was mixed uniformly with sonication for five minutes. Then it was kept in 37 ^{o}C incubation for four days. Similarly, CUR loaded MTPV has been prepared following above method with some modifications. Here, CUR aqueous solution was initially prepared by dissolving CUR (2 mg/mL) in 1% aqueous DMSO solution followed by multiple centrifugations at higher centrifugal force. Then K_3NQ and E_3NQ (500 μM in 1:1 ratio) was dissolved in CUR aqueous solution collected from the supernatant of centrifugation followed by 4 days of incubation at 37^{o}C.

FITC-K_3NQ, E_3NAP and Dox were co-incubated for the preparation of doxorubicin loaded MTPV. FITC-MTPV and doxorubicin show green and red fluorescence respectively. Similarly, K_3NQ, TAMRA-E_3NQ and curcumin were co-incubated for preparation of curcumin loaded MTPV. TAMRA-MTPV and curcumin show red and green fluorescence respectively.

Gel filtration of drug loaded MTPV from free drug: Purification of drug loaded MTPV from the free drug was performed by gel filtration as reported before.[10] First a uniform sepharose bed was prepared using Sepharose 4B. Then it was washed with PBS (Phosphate Buffer Saline) for several times. After that the incubated sample was added drop wise on this bed. The sample was adsorbed on the bed. Then, PBS was added and the different fractions were collected. The fractions were analysed with UV using their characterization absorbance wave length (Peptide: 220 nm; DOX: 480 nm; CUR: 425 nm and PI: 493 nm). Only the desired fraction (drug loaded MTPV) was collected and concentration of loaded molecule (drugs) was measured.

Time dependent drug release study: A small fraction (15 μL) of the sample was taken in cuvette and a control reading was recorded. After that 2% Triton X-100 was added and immediately a reading was recorded in Quanta Master Spectrofluorometer (QM-40). Due to addition of Triton X, higher fluorescence intensity was observed than control. Then, after different time intervals readings were recorded with enhanced intensity till the saturation was reached. The drug release profile was calculated from following equation:

% drug release = $[(F-F_0)/F_f]*100$

Where, F_0 = Maximum value of the control.

F_f = Maximum value of the final reading.

p^H dependent drug release study: Two stock solutions of drug loaded MTPV in two different buffer at pH 7.4 and pH 5.4 were prepared at 1 μM concentration. Next, immediately fluorescence was measured of these two stock solutions (at an excitation of 425 nm and emission from 450 nm to 650 nm for CUR, at an excitation of 480 nm and emission from 550 to 750 nm). After that fluorescence dataset was measured at a certain interval until saturation was reached. The drug release profile was calculated from the following equation.

% drug release = $[(F-F_0)/F_f]*100$

Where, F_0 = Maximum value of the control.

F_f = Maximum value of the final reading.

Determination of binding affinity of MTPV to the tubulin by fluorescence intensity quenching study of intrinsic Tryptophan residue of tubulin: MTPV, which bind to the tubulin, quenched the intrinsic Tryptophan fluorescence intensity of tubulin. So, the intrinsic Tryptophan fluorescence intensity of tubulin was measured in presence of different concentration of peptide vesicles. From that, binding constant was calculated using a modified Stern-Volmer equation. 20 μM of tubulin was mixed with different concentration of peptide vesicles in BRB80 buffer in ice and the fluorescence emission spectra was recorded from 310 to 450 nm exciting the sample at 295 nm at 4 °C in Quanta Master Spectrofluorometer (QM-40) in addition with peltier for controlling the temperature.

Drug release study from MTPV upon contact with tubulin by fluorescence measurement: A stock solution of 1 μM CUR loaded MTPV was prepared. Next, fluorescence graph of CUR loaded MTPV was measured at an excitation of 425 nm and at an emission range of 450 to 650 nm, which was considered as control. After that the stock solution was mixed with 10 μL (200 μM) tubulin and a fluorescence graph of this solution was taken. Here, the fluorescence graph of tubulin was also measured as control.

Disruption of MTPV upon contact with tubulin monitored by fluorescence microscopy: Two solutions were prepared one was containing MTPV and another was containing MTPV in the presence of tubulin after 30 min incubations at 37 ºC. Next we have placed 5 μL samples of these two solutions on microscopic glass slides and observed under fluorescence microscope. We have found nice peptide vesicles in absence of tubulin but it disappears in presence of tubulin.

Cell culture: HeLa and U87GM cell lines were purchased from National Centre for Cell Science (NCCS) Pune, India. Cells were cultured in 5% CO_2 humidified atmosphere containing incubator at 37 ^oC using dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum, kanamycin sulfate (110 mg/L), penicillin (50 units/mL), streptomycin (50 μg/mL).

Cellular uptake studies: Cellular uptake of K_3NQ, E_3NQ and MTPV were studied in HeLa cell line. The cells were seeded at a density of 5,000 cells per cover glass bottom dish one day prior to the treatment. Then the cells were treated with these samples at a concentration of 2 μg/mL in DMEM medium for 4 hour. Nucleus was counter stained with hoechst33258.

Cellular delivery of DOX, CUR and PI loaded MTPV: HeLa cells were harvested at a density of 5,000 cells per cover glass bottom dish before treatment. Then cells were treated with 1 μM DOX-MTPV for 4 h. Nucleus was counterstained with Hoechst 33258. After incubation cells were washed properly and live cell imaging was performed using Nikon fluorescent inverted microscope using 561 nm and 405 nm

wavelength filter. Similarly, CUR delivery has been analysed using above described method. Here, cells were treated with 1.25 μM CUR-MTPV and incubated for 4 h. Then live cell imaging has been carried out using Nikon fluorescent inverted microscope in DIC mode and using 488 nm wavelength filter.

In case of cellular uptake of PI, we have performed flow cytometric analysis. HeLa cells (5×10^6 cells/mL) were first taken in a suspension containing serum free media and incubated with PI-MTPV for 30 min. Then cells were washed to remove excess PI in solution and suspended in warmed serum free culture media followed by analysed in flow cytometer (BD LSRFortessa™). Without treatment control was taken to nullify cellular auto-fluorescence in PI channel.

LysoTracker study of MTPV: HeLa cells were seeded at a density of 10,000 cells per well one day before the treatment. Then the cells were treated with 20 μM of FITC attached MTPV for four hours. Cells were washed two times with colorless media and cells were treated with LysoTracker red for 15 minutes. Then cells were washed with colorless DMEM media and images were taken under Nikon fluorescent inverted microscope. Similarly, we have treated the cells with TAMRA tagged MTPV and LysoTraker green and followed above described method.

Co-localization of MTPV with tubulin/microtubules in U87GM cells: U87GM cells were seeded in a cover glass bottom dish for 24 hours. 20 μM of FITC-MTPV were used to treat the seeded cells. In each case, incubation period was 4h. Then, cells were fixed with 4% paraformaldehyde solution and permeabilized with 0.1% Triton X-100. Microtubules were stained using monoclonal anti-α-tubulin IgG (1:300) and either Cy3.5 pre-absorbed goat anti-rabbit IgG (1:600). Nucleus was stained with Hoechst 33258 (1 μg/mL). Nikon Ti-U eclipse fluorescence microscope was used for imaging in bright field, 488, 405 and 561 nm wavelength fluorescent filters.

Cytotoxicity study: Cytotoxicity of doxorubicin and curcumin loaded MTPV in HeLa and U87GM cancer cells respectively was evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction. The cells were seeded at a density of 10000 cells per well in a 96-well plate one day prior to the treatment. Then HeLa cells were treated with different concentration DOX (0.5 μM, 1 μM, 2 μM, 4μM) loaded MTPV in DMEM medium containing 10% FBS for 24 h and compared with free DOX. Similarly, U87GM cells were treated with different CUR (0.312 μM, 0.625 μM, 1.25 μM, 2.5μM) loaded MTPV. Following the termination of experiment, cells were washed and promptly assayed for viability using MTT. Results were expressed as percent viability = [(A550 (treated cells)-background) / (A550 (untreated cells)-background)] x 100. Similarly, cytotoxicity of individual and combination of MTPV-DOX and MTPV-CUR has been evaluated in U87GM following above described method. Drug reduction index (DRI) was evaluated for combination of DOX-MTPV and CUR-MTPV using CompuSyn software. Combination effect of DOX-MTPV and CUR-MTPV has been analyzed by the combination index (CI) using Chou-Talalay method using following formula.[11]

$$\mathbf{CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 = 1/(DRI)_1 + 1/(DRI)_2}$$

Where, $(D)_1$ and $(D)_2$ represent the concentration of DOX-MTPV and CUR-MTPV respectively, in combination having particular effect. $(Dx)_1$ and $(Dx)_2$ represents the concentration of DOX-MTPV and CUR- MTPV respectively administrated individually having similar effect as $(D)_1$ and $(D)_2$.

Growth inhibition analysis of 3D tumor spheroidal cultures: The Multicellular Tumor Spheroidal culture of HeLa cell and U87GM cells were performed using non adhesive culture system. In brief, tumor spheroid of HeLa and U87GM cells have been generated using liquid overlay or non-adherent method as described before[12]. In brief, 2D monolayer cells were detached, transferred into the 35 mm dish, previously coated with 1% agarose and incubated in 37°C till 3D spheroid formation. Morphological

structures of spheroid were captured in bright field using inverted Olympus microscope equipped with EMCCD camera and represented as day one. Then the spheroids were divided in groups such as without treatment group as control and different treatment groups such as, only DOX (2μM), DOX-MTPV (2μM) CUR (1.25 μM) and CUR-MTPV (1.25 μM). After that spheroid morphology was assessed for upto day 7 using inverted Olympus microscope. Volume of the sphere was analyzed using following formula as described before. **Volume = 0.5 × Length (major axis) × Width^2 (minor axis)**

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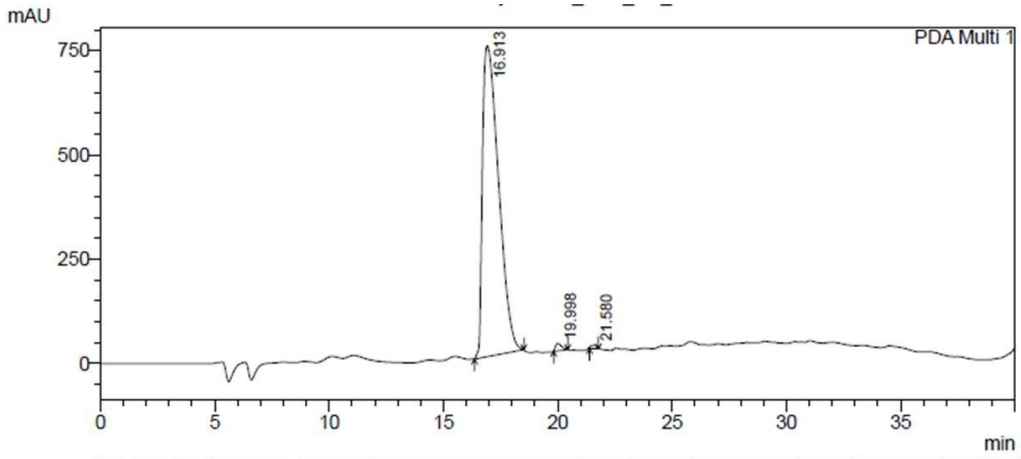
Supplementary Figures



Peak#	Ret. Time	Area	Height	Area %	Height %
1	16.913	36443101	745816	98.681	96.553
2	19.998	336225	17713	0.910	2.293
3	21.580	151014	8916	0.409	1.154
Total		36930340	772445	100.000	100.000

Figure S1: HPLC chromatogram of K_3NQ peptide.

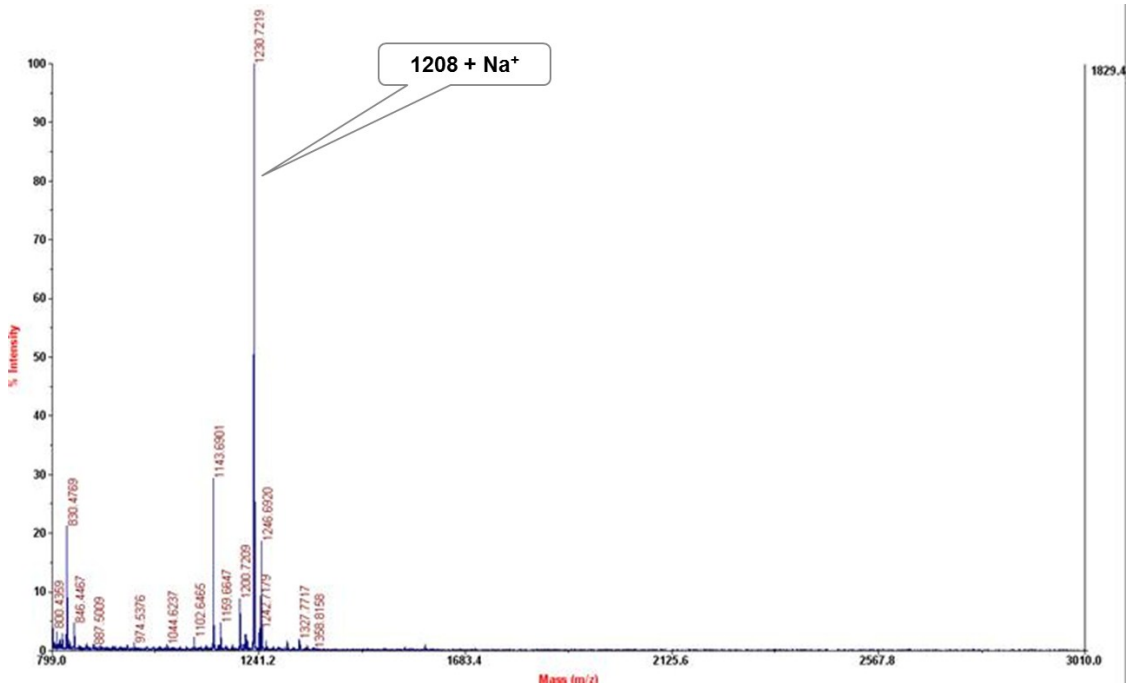
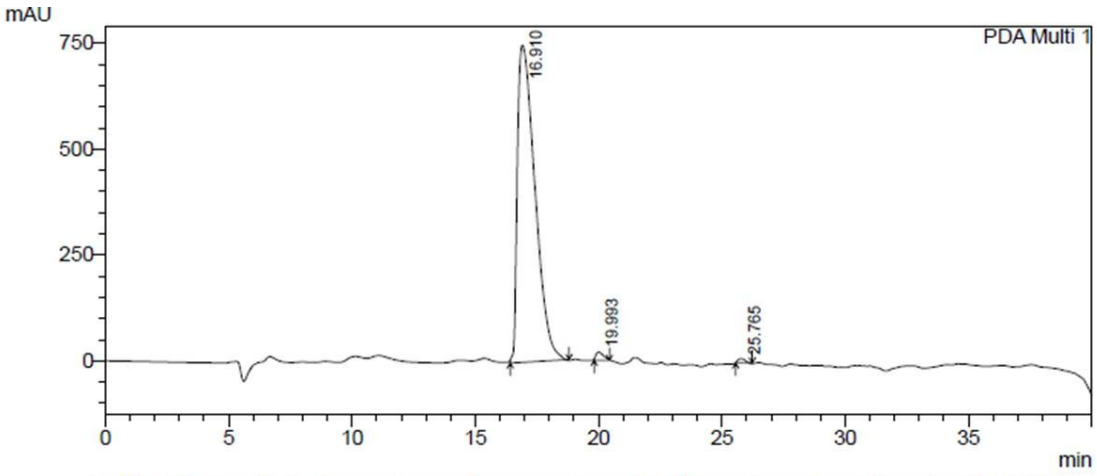


Figure S2: MALDI-TOF mass spectra of K_3NQ peptide.



Peak#	Ret. Time	Area	Height	Area %	Height %
1	16.910	37075776	748424	98.315	96.084
2	19.993	431575	20344	1.144	2.612
3	25.765	203784	10162	0.540	1.305
Total		37711135	778930	100.000	100.000

Figure S3: HPLC chromatogram of FITC-K_3NQ peptide.

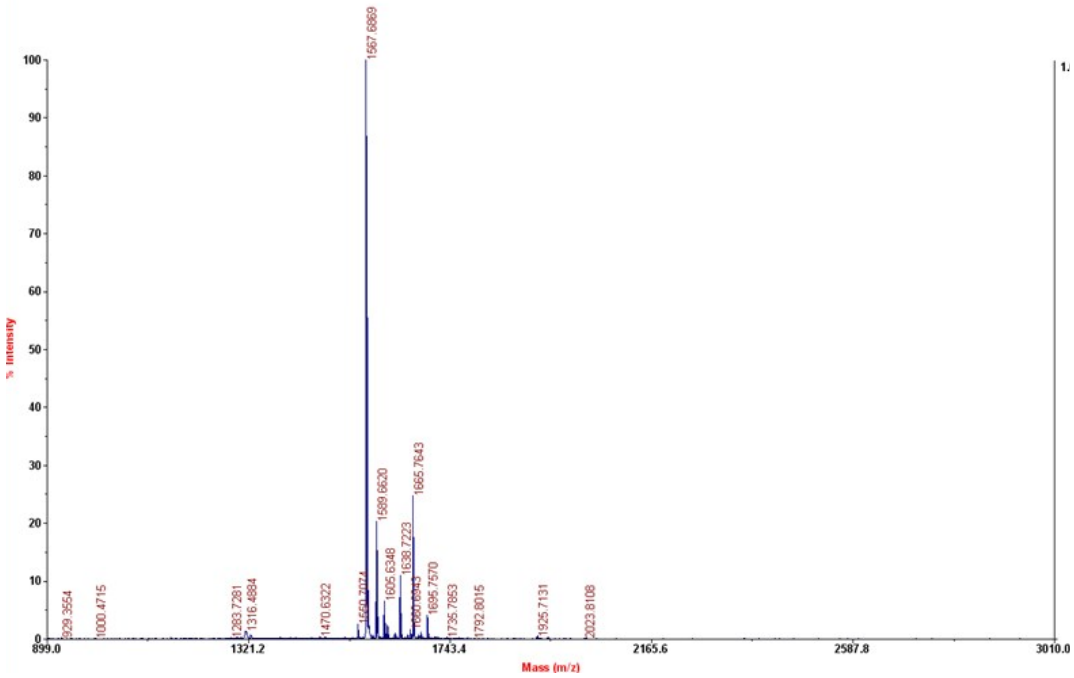


Figure S4: MALDI-TOF mass spectra of FITC-K_3NQ peptide.

Figure S5: HPLC chromatogram of E_3NQ peptide.

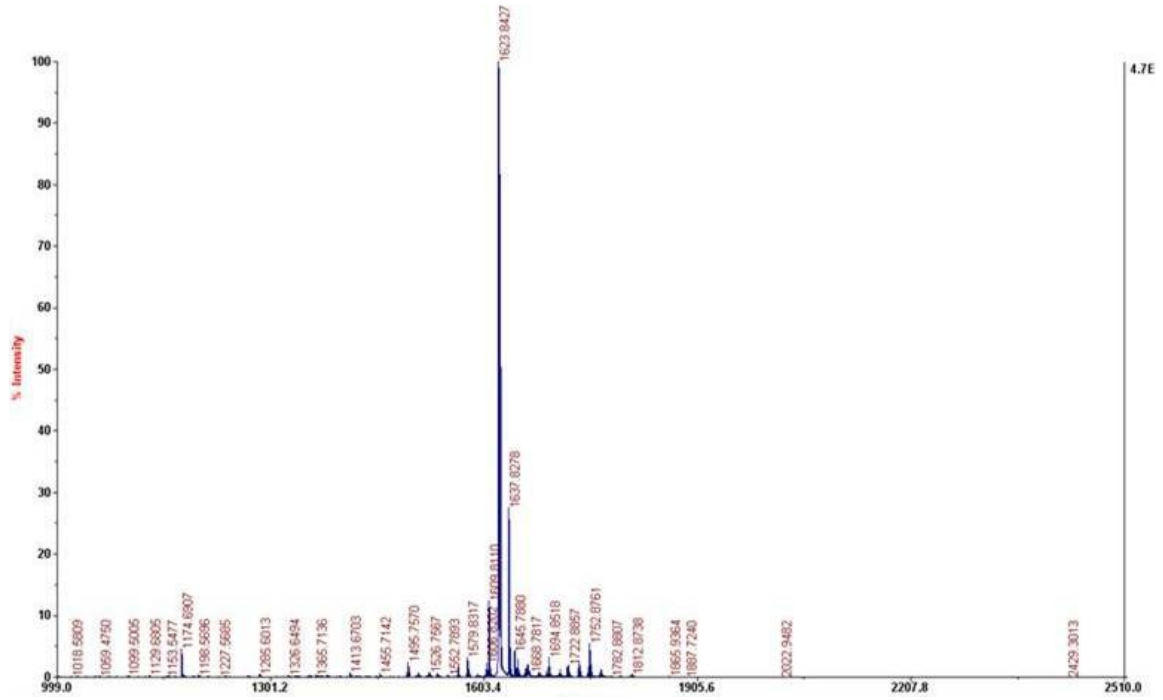
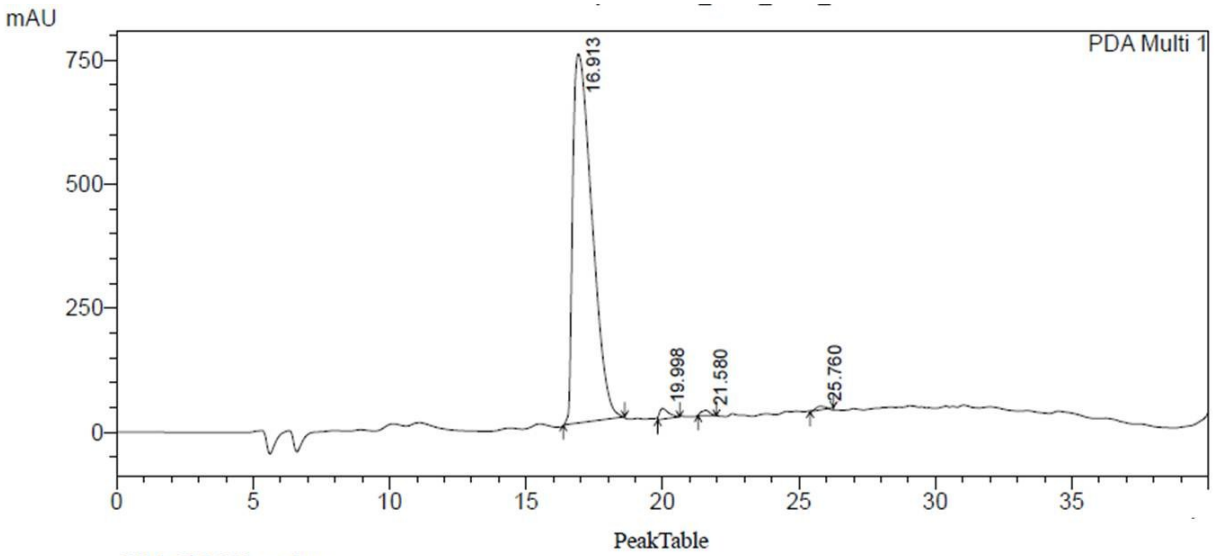


Figure S6: MALDI-TOF mass spectra of E_3NQ peptide.



PDA Ch1 210nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	16.913	36351060	743456	97.647	94.907
2	19.998	495878	21654	1.332	2.764
3	21.580	242237	10973	0.651	1.401
4	25.760	137715	7268	0.370	0.928
Total		37226890	783351	100.000	100.000

Figure S7: HPLC chromatogram of TAMRA-E_3NQ peptide.

Figure S8: MALDI-TOF mass spectra of TAMRA-E_3NQ peptide.

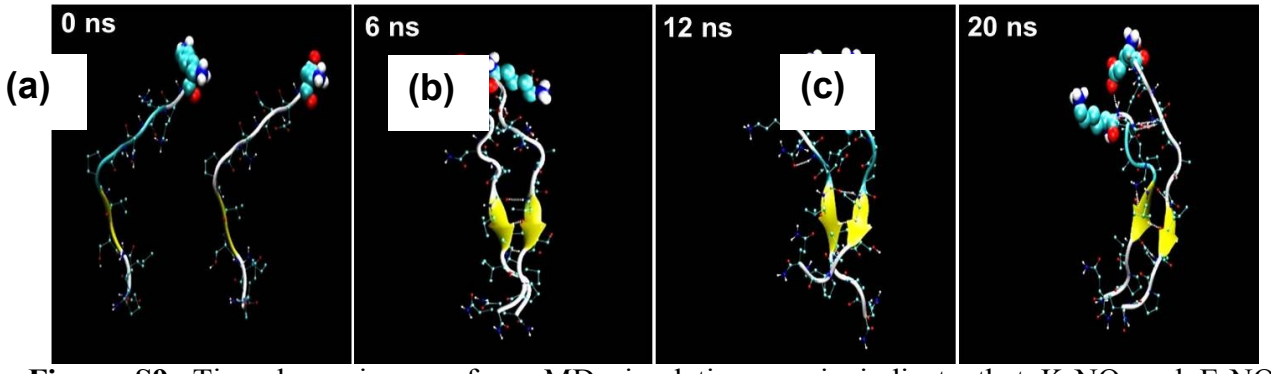


Figure S9. Time lapse images from MD simulation movie indicate that K_3NQ and E_3NQ peptides slowly transform to parallel β-sheet rich structure.

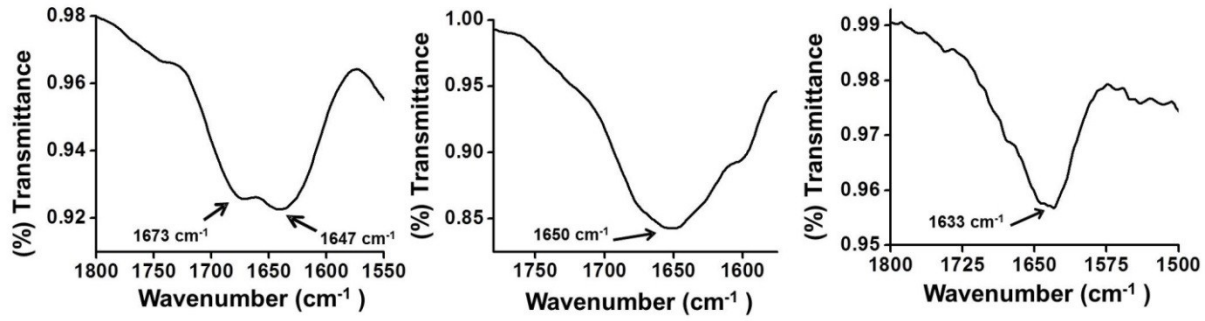


Figure S10: FT-IR Spectra of (a) K_3NQ peptide, (b) E_3NQ peptide and (c) the mixture of these two peptides i.e. MTPV after 4 days of incubation.

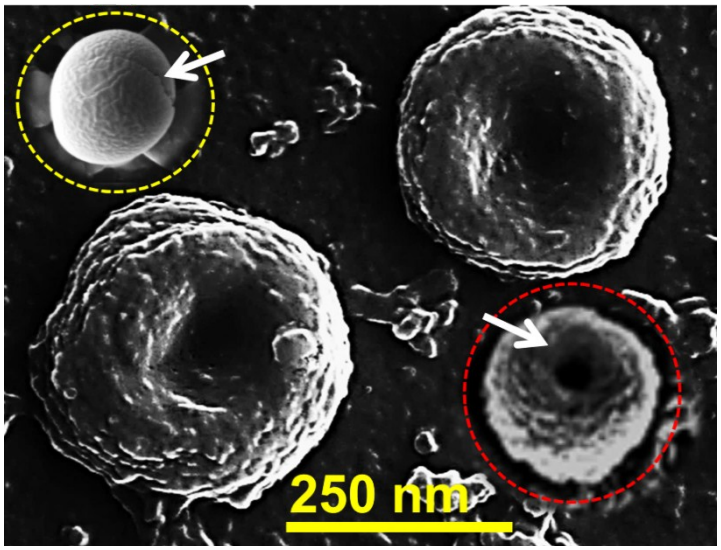


Figure S11: Characterization of the MTPV by Scanning electron microscope (SEM). White arrow indicates hollowness of the MTPV.

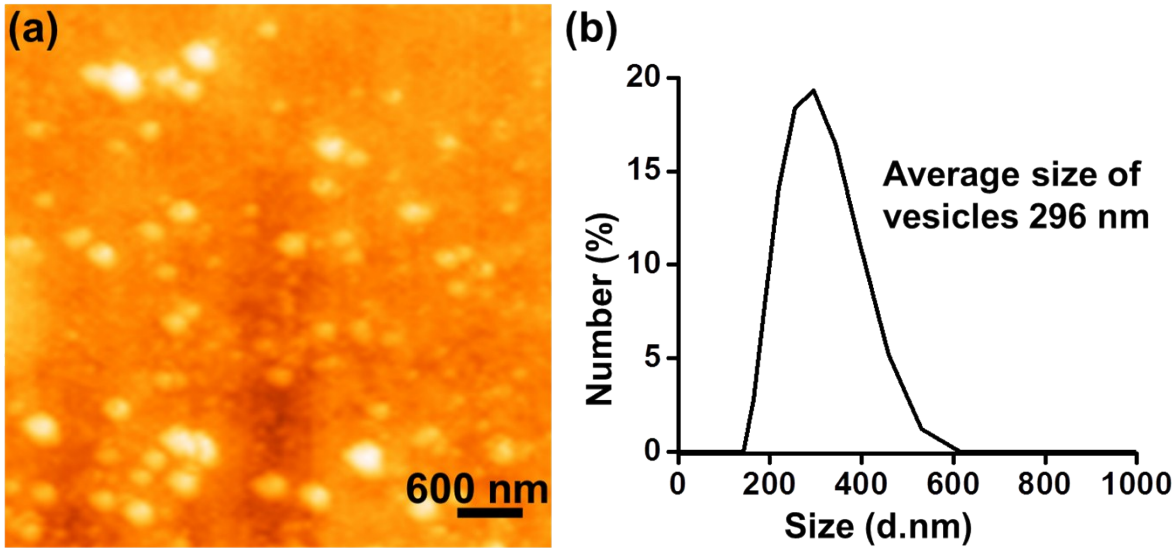


Figure S12: Characterization of the MTPV (a) AFM image of MTPV. (b) DLS experiment reveals average size of MTPV.

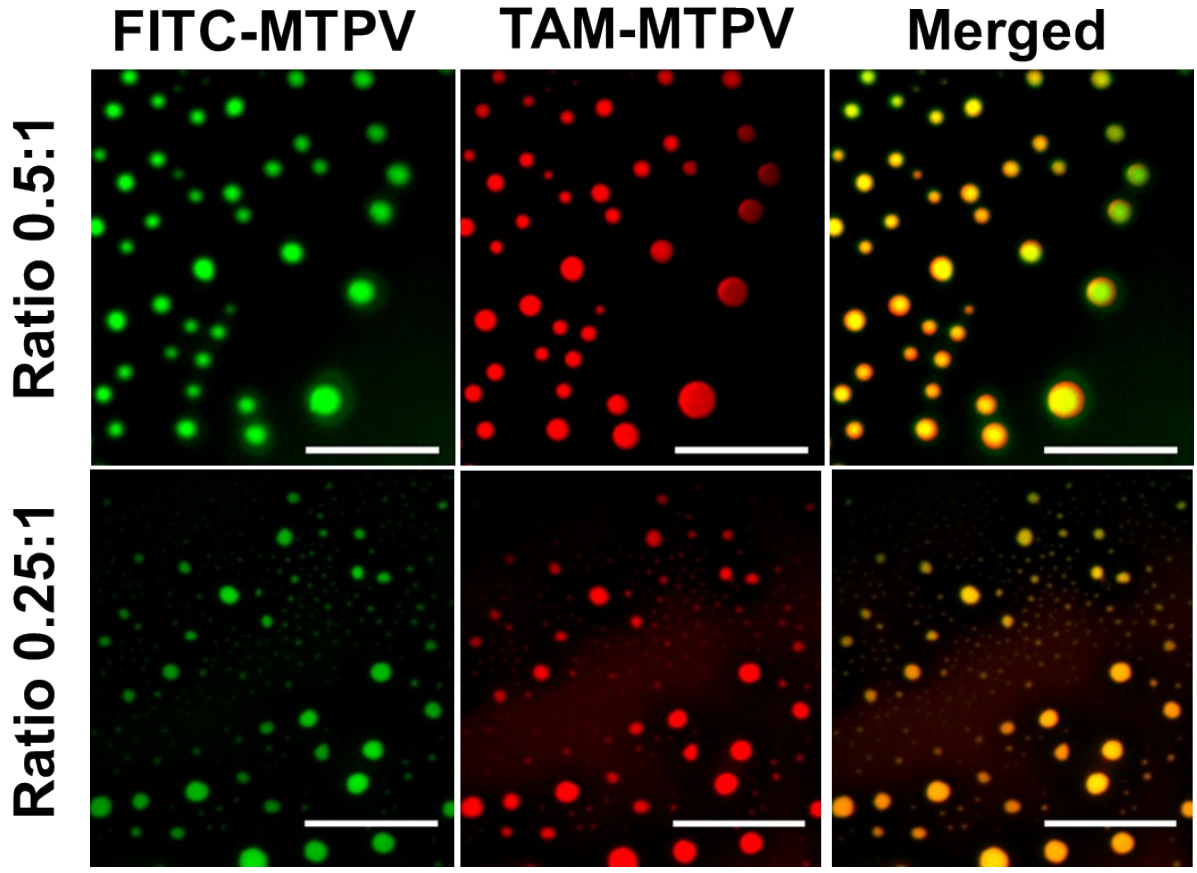
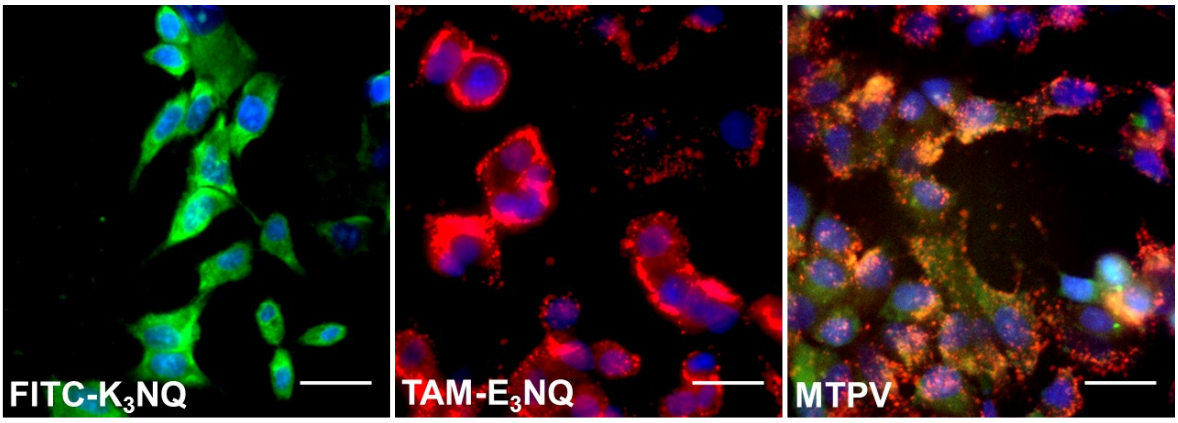


Figure S13: CLSM study of the MTPV in different stoichiometric ratios. Scale bars correspond to 1 μm.

Figure S14: Zeta potential measurement of MTPV in 1:1 ratio.



			Mean (mV):	Area (%)	Width (mV):
Zeta Potential (mV):	30.6	Peak 1:	31.0	100.0	7.65
Zeta SD (mV):	14.5	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm):	0.910	Peak 3:	0.00	0.0	0.00

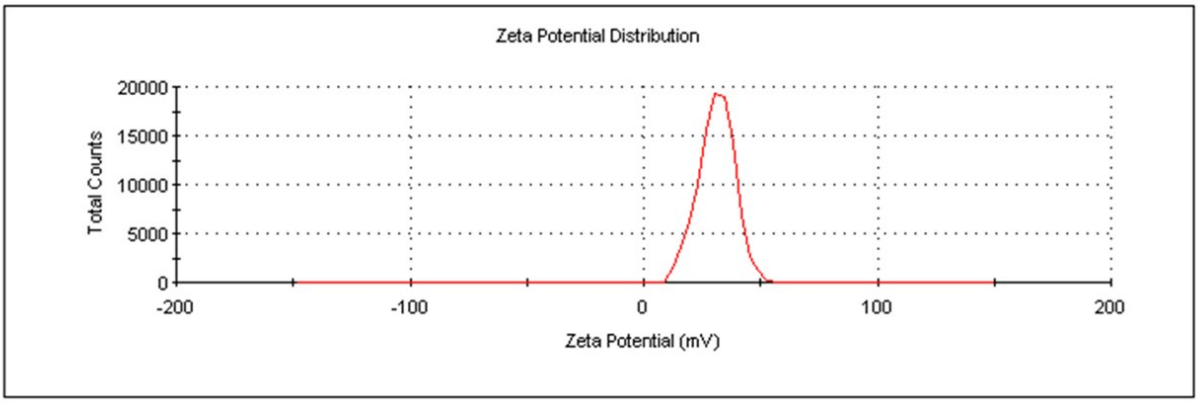


Figure S15: Cellular uptake of FITC-K_3NQ, TAMRA-E_3NQ, MTPV in HeLa cell. Scale bars correspond to 20 μm.

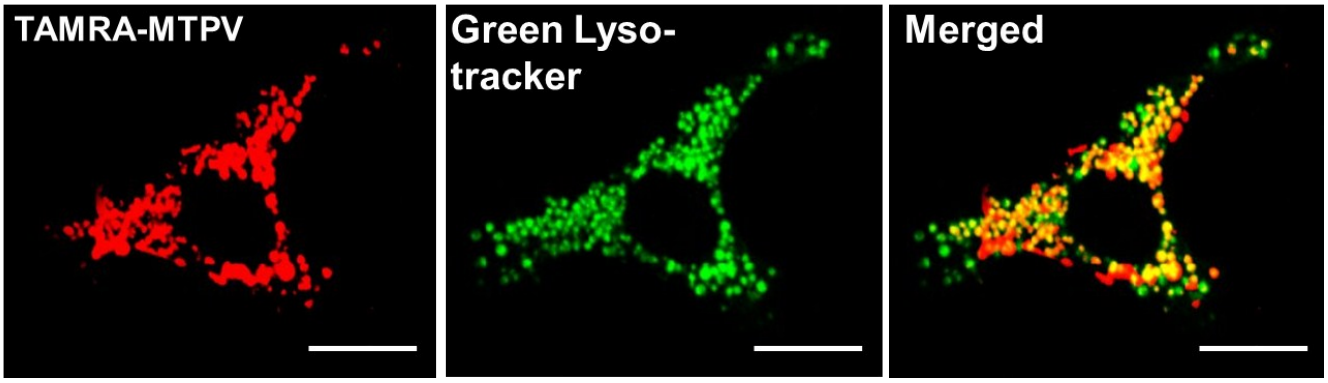


Figure S16: Co-localization of TAMRA-MTPV with green Lyso-Tracker in HeLa cells. Scale bars correspond to 20 μm.

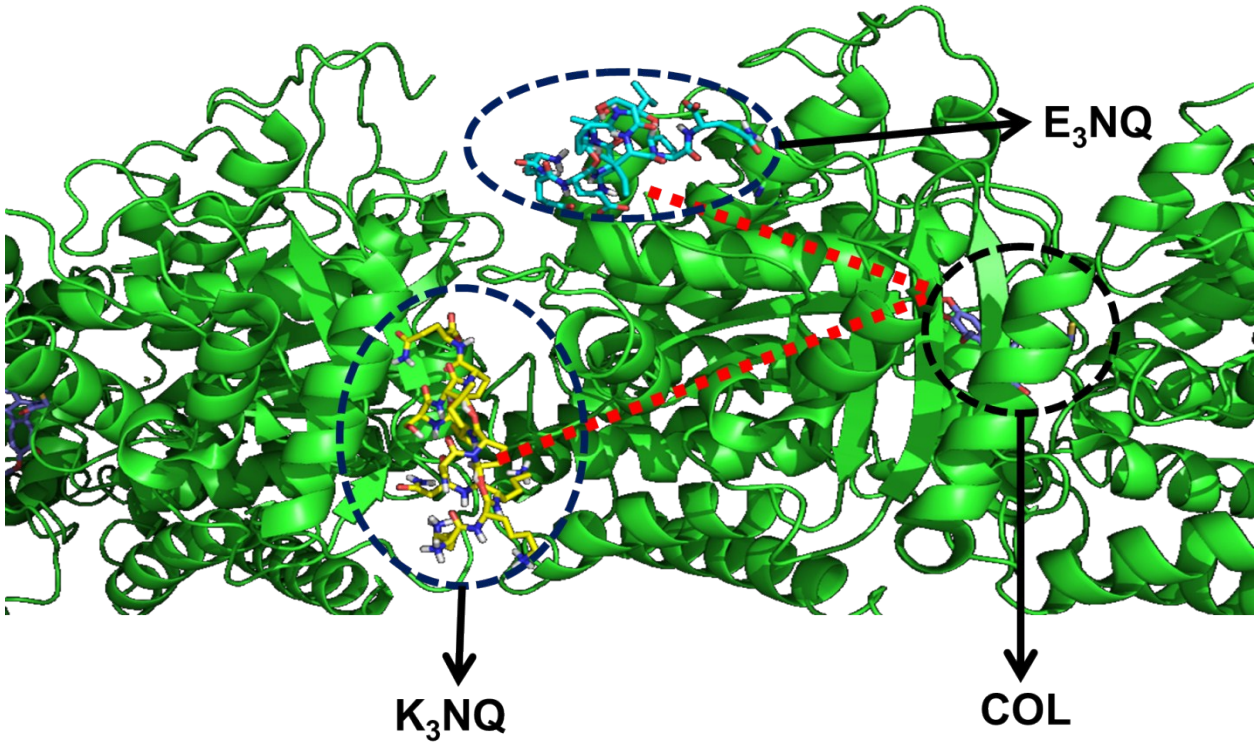


Figure S17: Molecular docking image represents binding of E_3NQ and K_3NQ at taxol site and vinblastine (vinca) site of tubulin respectively. The calculated distance of E_3NQ and K_3NQ from colchicine site is 32.1 Å and 41.3 Å respectively, which suggests that E_3NQ and K_3NQ bind nearly to the taxol and vinca site of tubulin.

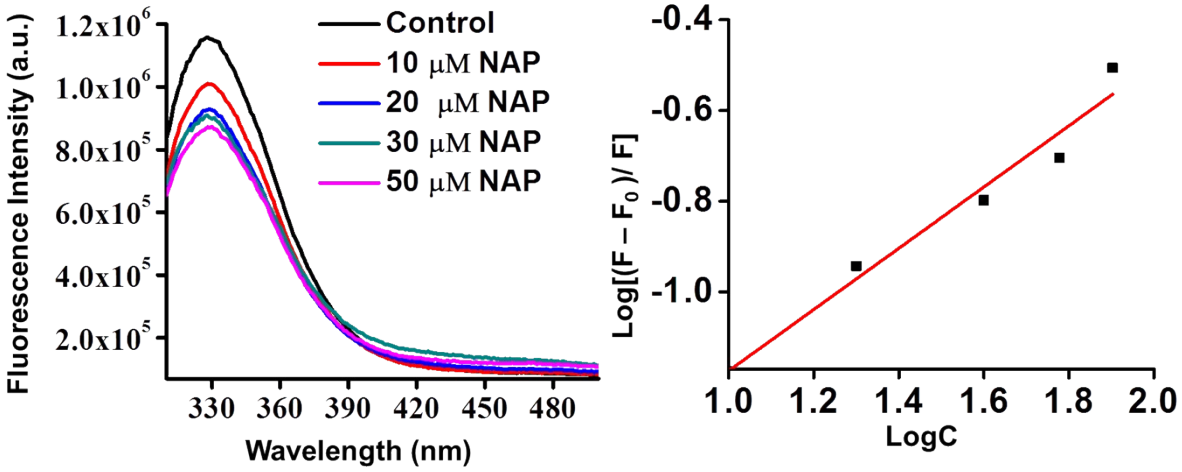


Figure S18: Intrinsic tryptophan quenching of tubulin by MTPV.

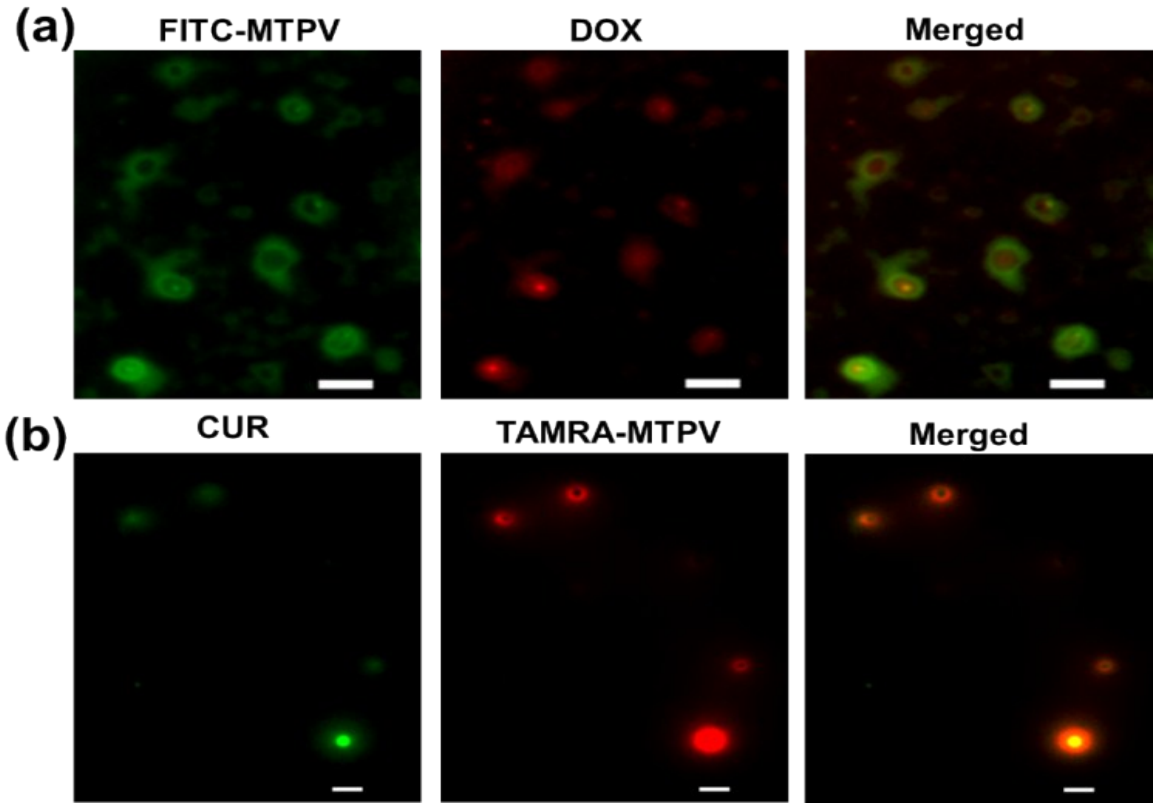


Figure S19: Fluorescence images represent that green coloured FITC-MTPV encapsulates red fluorescent DOX (a). Fluorescence images represent that red coloured TAMRA-MTPV vesicle encapsulates green fluorescent CUR (b). Scale bars correspond to 400 nm.

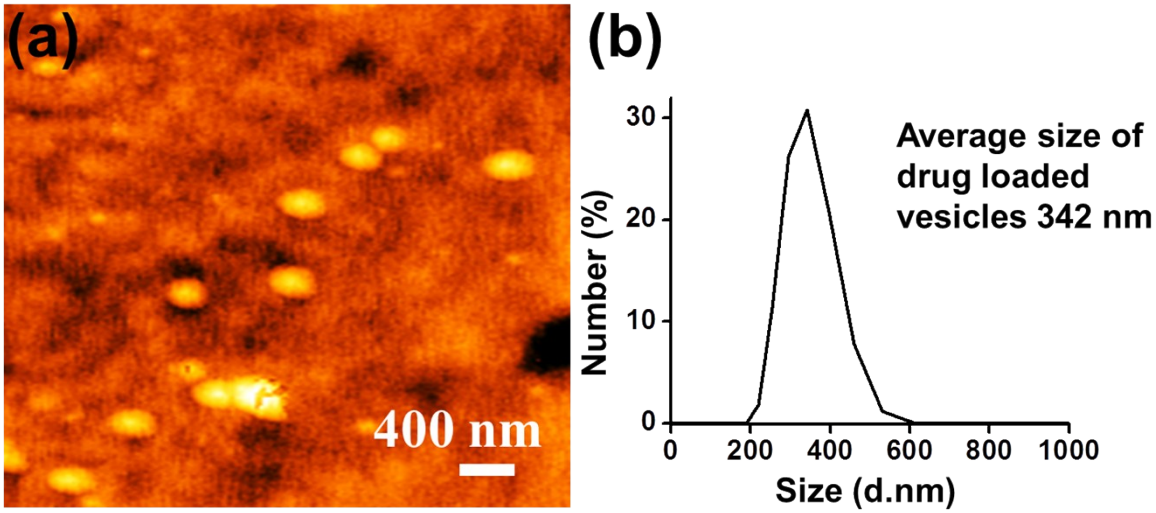


Figure S20: Characterization of the drug loaded MTPV (a) AFM image of DOX loaded MTPV. (b) DLS experiments reveals average size of DOX loaded MTPV.

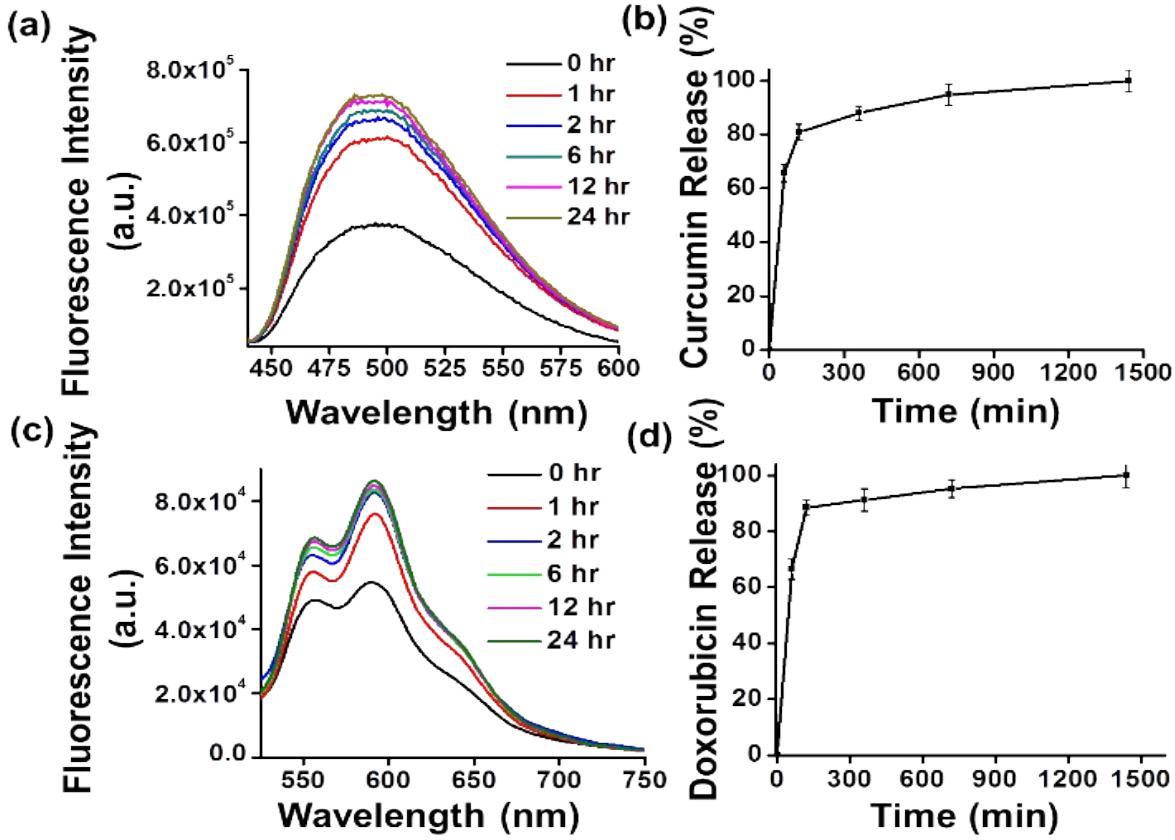


Figure 21: (a) Time dependent release of curcumin from curcumin loaded MTPV up-to 24 hour in presence of 2% TritonX-100, (b) its' release kinetics, (c) time dependent release of doxorubicin from doxorubicin loaded MTPV up-to 24 hours, (d) it's release kinetics.

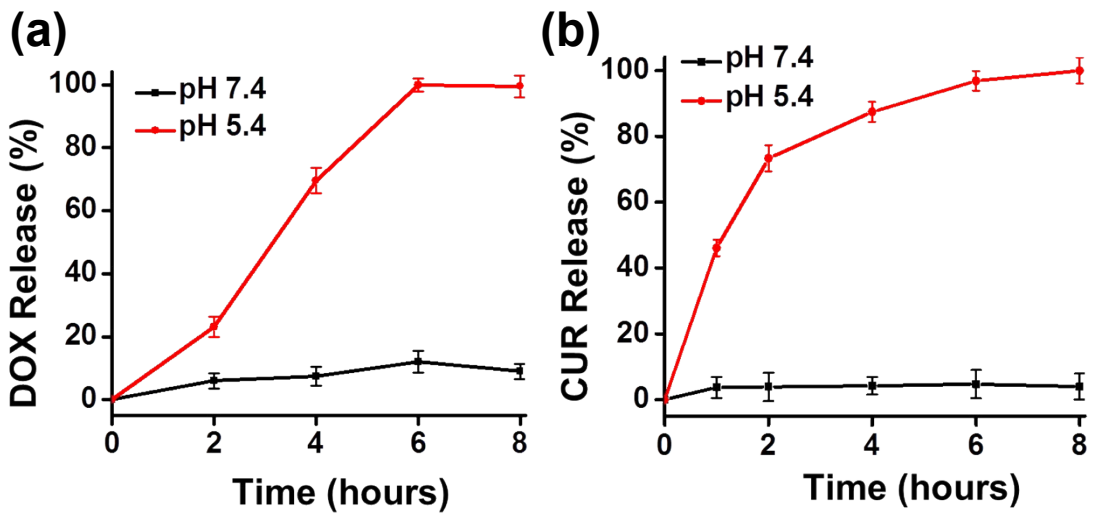


Figure S22: pH dependent release kinetics study of (a) doxorubicin loaded MTPV and (b) curcumin loaded MTPV at two different pH.

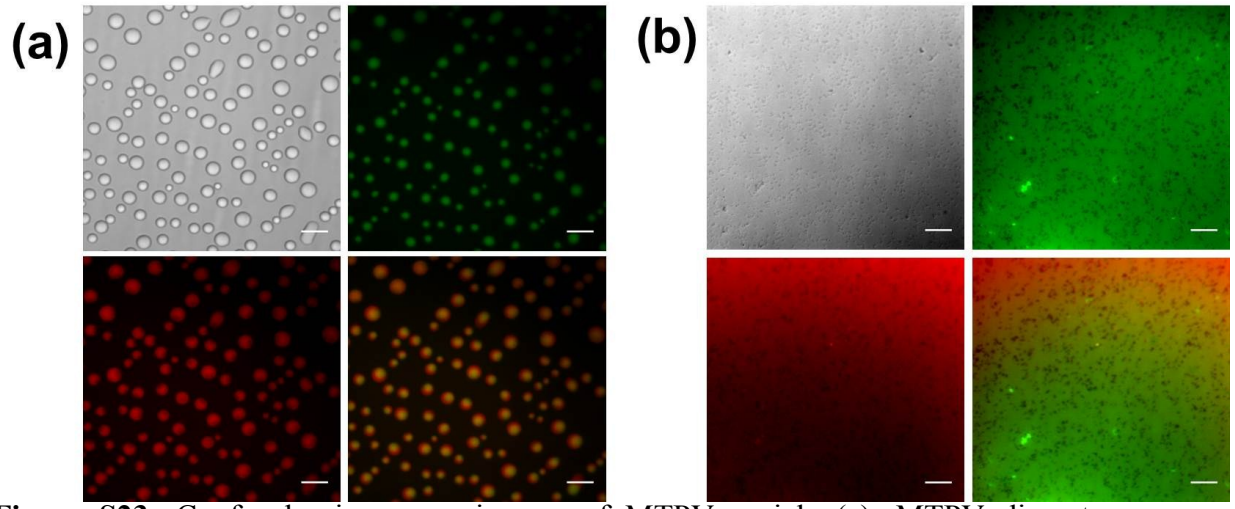


Figure S23: Confocal microscopy images of MTPV vesicle (a), MTPV disrupts upon co-incubation with tubulin at 37 ºC (b). Scale bars correspond to 1 μm.

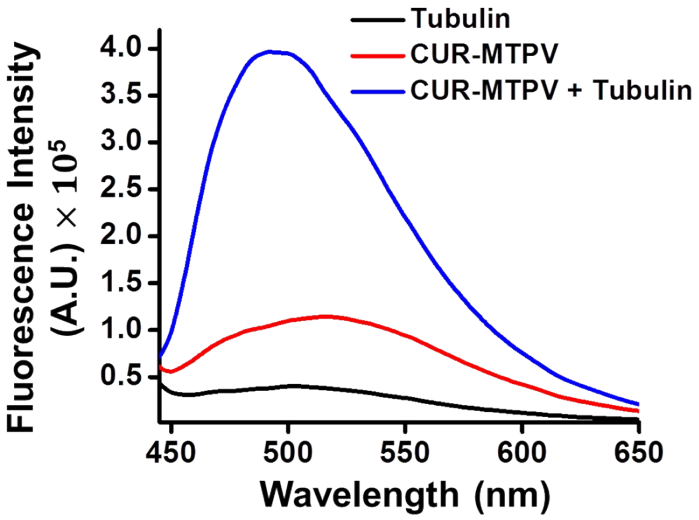


Figure S24: Fluorimetric curcumin release study from curcumin loaded MTPV in presence of tubulin.

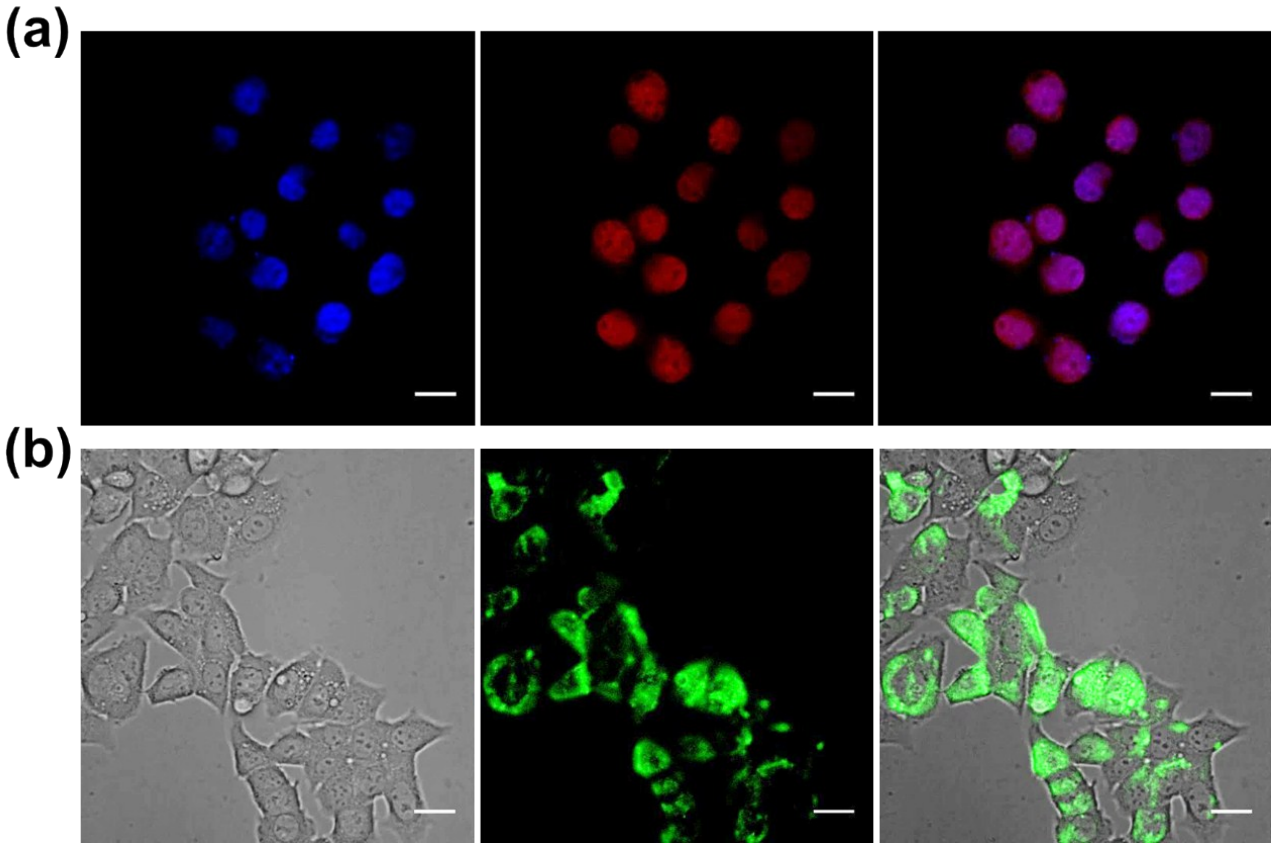


Figure S25: Fluorescent microscopic image represents intracellular delivery of (a) DOX in HeLa cells treated with DOX-MTPV and (b) CUR in U87GM cancer cells treated with CUR-MTPV. Scale bars correspond to 20 µm.

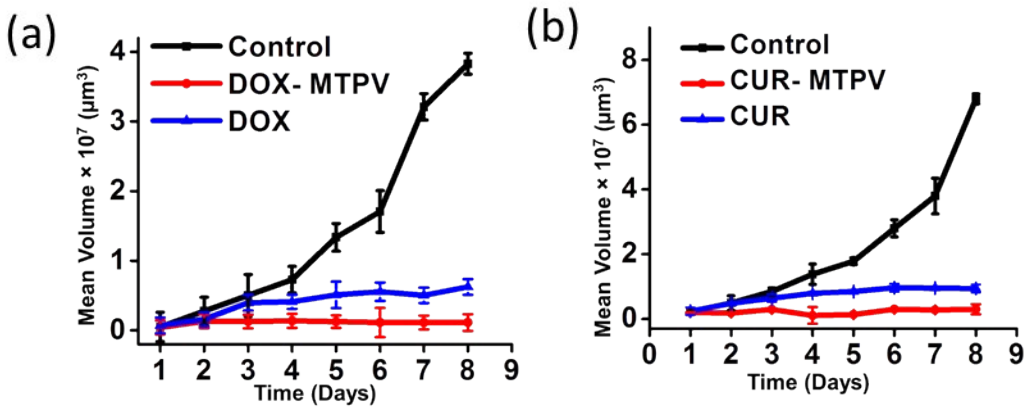


Figure S26: Line graphs represent spheroid growth inhibition experiment in HeLa spheroid after treatment with DOX-MTPV (red line) and in comparison to DOX alone (blue line) and untreated spheroid (black line) (a) and in U87GM spheroid after treatment with CUR-MTPV (red line) in comparison to CUR alone (blue line) and untreated spheroid (black line) (b).