Supporting Information

Conjugation of Paclitaxel to Hybrid Peptide Carrier and Biological Evaluation in Jurkat and A549 cancer cell lines

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1. Experimental Procedures and Characterization

1.1 Peptides and Modification Procedures

Peptides were purchased from the Tufts University Core Facility (solid support synthesis and HPLC purification) unmodified or modified with fluorescein isothiocyanate (FITC; excitation wavelength (EX) = 494 nm, emission wavelength (EM) = 521 nm) at the N-terminus via a β-alanine–glycine–glycine linker. Both terminals were blocked; N-terminus by acylation and C-terminus by amidation. Peptide COL-CPP was modified with paclitaxel via primary amine of lysine in position 14.

2'-Succinyl-Paclitaxel

Following Deutsch's method, 25.1 mg (0.029 mmol) of paclitaxel and 37.5 mg (0.37 mmol) succinic anhydride were added to 0.6 mL pyridine in a round-bottomed flask and stirred for 3 hours at room temperature. The solvent was removed in vacuo, and the residue was washed with 1 mL of water and dried again. The precipitate was then dissolved in acetone. Ice water was added dropwise to the solution to induce crystallization (white crystals, Yield: 98%). Paclitaxel was modified with succinic anhydride at 2'-OH and confirmed using NMR spectroscopy (Bruker Ascend^{TM} 400 MHz NMR) with the presence of the multiple peaks at 2.61-2.68 identified to be the succinyl linker. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.13 [s, $^{16}CH_3$], 1.23 [s, $^{15}CH_3$], 1.68 [s, $^{18}CH_3$], 1.92 [s, $^{17}CH_3$], 2.22 [s, OAc], 2.44 [s, OAc], 2.52-2.78 [m, $^{2'}$C-OOC-***CH_2-CH_2***-COOH], 3.80 [d, *J* = 7.0 Hz, ^{3}CH], 4.20 [d, *J* = 8.3 Hz, $^{19}CH_2$], 4.31 [d, *J* = 8.3 Hz, $^{19}CH_2$], 4.43 [dd, *J* = 10.9, 6.60 Hz, ^{7}CH], 4.97 [d, *J* = 7.5 Hz, ^{9}CH], 5.54 [d, *J* = 3.4 Hz, $^{2'}$CH], 5.68 [d, *J* = 7.1 Hz, ^{2}CH], 5.99 [dd, *J* = 9.3, 3.2 Hz, $^{3'}$CH], 6.24 [t, *J* = 9.2 Hz, ^{13}CH], 6.29 [s, ^{10}CH], 7.02 [d, *J* = 9.3 Hz, NH], 7.25 [s, 3'-Ph], 7.39-7.61 [m, 3'-NBz , 2-OBz], 7.77 [d, *J* = 7.1 Hz, 3'-NBz], 8.13 [d, *J* = 7.1 Hz, 2-OBz].

^{13}C NMR ($CDCl_3$, 400 MHz) δ 203.79, 174.4 [$^{2'}$C-OOC-CH_2-CH_2-***C***OOH], 171.2 [$^{2'}$C-OO***C***-CH_2-CH_2-COOH], 171.0, 169.9, 168.7, 167.2, 167.0, 166.0, 142.7, 136.8, 133.7, 133.5, 132.7, 132.0, 130.2, 129.2, 129.1, 128.7, 128.6, 127.2, 126.5, 81.1, 79.1, 75.6, 58.5, 52.7, 45.6, 43.1, 35.5, 28.9 [$^{2'}$C-OOC-***CH_2-CH_2***-COOH], 26.8, 22.1, 20.8, 14.8, 9.6.

ESI-MS (Advion expression CMS) of 2'-Succinyl-Paclitaxel calcd $C_{51}H_{55}NO_{17}$ $[M]^+$ 953.35, found 953.5.

Conjugation of 2'-Succinyl-Paclitaxel to COL-CPP peptide:

10.32 mg (0.011 mmol) of succinyl paclitaxel was dissolved in 200 µL DMF. 100 µL of this solution (containing 5.16 mg/ 0.0055 mmol) was pipetted into a round-bottomed flask and 28.4 µL (0.16 mmol) of N, N-Diisopropylethylamine was added to the flask. Then 16.09 mg (0.0005 mmol) COL-CPP peptide was dissolved in 50 µL DMF and added to the mixture in, followed by 33.1 mg (0.087 mmol) HATU. The reaction was stirred at 50 °C for 4 hours. Then the mixture was diluted with 2 mL of water and extracted with 5 mL ethyl acetate three times. The aqueous phase was obtained and underwent dialysis at a MW cutoff of 1 kDa against water for 72 hours. The dialyzed compound was lyophilized for 48 hours and yielded an off white fluffy product. The final product had a 20% yield.

The conjugation was confirmed with MS-ESI: PTX-COL-CPP ($C_{190}H_{270}N_{48}O_{58}$; Calc. Mass: 4151.97) peaks found was fragment of Succinyl-COL-CPP ($C_{143}H_{221}N_{47}O_{45;}$ Calc. Mass: 3316.64): $[M]^{3+}$ found: 1106.4 *m/z* Calc. Mass: 1105.54 *m/z*, $[M]^{4+}$ found: 829.8 Calc. Mass: 829.16 *m/z.*

1.2 Solubility Assay

Solubility studies were conducted using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) for the following compounds: COL-CPP, COL-CPP-PTX. For each compound a solution of known concentration was prepared in distilled water. The UV absorbance was measured for each dilution at 230 nm for COL-CPP and 225nm for COL-CPP-PTX. A calibration cure was then constructed for both compounds. Next, saturated samples were made for each compound in water. The supernatants were then analyzed by measuring absorbance at the same wavelength as calibration: at 230nm for COL-CPP and 225nm for COL-CPP-PTX. The supernatant's concentrations were calculated from the calibration cure, followed by the calculation of both compounds solubility.

1.3 Circular Dichroism Spectroscopy

Circular dichroism (CD) measurements were performed using a JASCO J-810 spectropolarimeter (JASCO Inc., Easton, MD, USA) equipped with a Peltier temperature control system containing a quartz cell (path length 0.2 cm). Prior to each measurement, the peptides were thermally annealed: peptide solutions in water (2×10^{-4} M) were pre-heated at 80 °C for 5 min and slowly cooled to 4 °C, then incubated for 24 h. The peptide solutions were transferred to a CD cell and equilibrated for 30 min at 37 °C. A scan speed of 50 nm/min was used, and four scans per sample were acquired. A reference spectrum containing deionized water was subtracted from the final peptide spectrum.

Thermal unfolding curves were obtained by monitoring the decrease in ellipticity in a 25–80 °C temperature range (dependent on the peptide) at a wavelength where the CD spectra show a positive maximum (224 nm) at a heating rate of 10 °C/h. The derivative of the plotted unfolding curve was calculated using the JASCO Spectra Manager II software (JASCO Inc.). The

minimum of the derivative indicates the steepest slope of the unfolding process and determines the helix-to-coil transition temperature (T_m) under the described conditions. All experiments were performed in duplicate or triplicate.

1.4 Cell culture

All cell lines were purchased from ATCC and cultured according to provided protocols. Briefly, cell cultures were grown in 10 mL of corresponding cell nutrient medium, and incubated 37°C for 3 weeks in a humidified 5% CO_2 atmosphere. Jurkat cells, Clone E6-1 human leukemia, were cultured in Rosewell Park Memorial Institute Medium (RPMI, Mediatech Inc.). A549 cells, epithelial lung carcinoma, were cultured in F-12K (Kaighn's) Medium (ATCC). FaDu cells, hypopharyngeal squamous carcinoma, were cultured in Eagle's Minimum Essential Medium (EMEM, Mediatech Inc.). All cultured cells were supplemented with 10% fetal bovine serum (Cellgrow)and 1% penicillin streptomycin-glutamine (Fisher Scientific). Passages 3–10 were used for all experiments.

1.5 IC50 Assay

To measure IC50 a CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) from Promega was utilized for all cell lines. For adherent cell lines, A549 and Fadu cells Phosphate-Buffered Saline (PBS) and trypsin were used to induce detachment. In a 96-well plate, cells were seeded at a density of 2.5 x 10^5 cells per well. A549 cells required an additional step of centrifugation and dissolving with medium in order to properly distribute the cells into the plate. Subsequently, concentrations (1 nM – 50 uM) of the stock solution of PTX-COL-CPP were added to the plate and incubated for 48 hours. Next, MTT dye was added to each well and

incubated at 37°C for 4 hours. This followed with the addition of solubilizing solution and an overnight incubation period at 37°C. The plate was read through photometric scans at 570 nm using the Thermo Scientific Varioskan Flash Reader. From the absorbance readings, the average absorbance of each concentration and cell fraction was calculated. Kaleidagraph was used for statistical analysis of data and IC_{50} was determined from curve fitting (sigmoidal) parameters. The sigmoidal line fit equation is defined as:

$$y = x_{\max} + \frac{(x_{\min -} x_{\max})}{(1+10^{(\log(IC_{50})-c)*m)})}$$

where c is the constant and m is the slope.

1.6 Confocal Microscopy

Cells were imaged using a confocal micro-scope (Olympus FluoView 1000). Cells (A549, FaDu) were seeded at a density of 50,000 cells/mL in complete media in 35-mm Petri dishes with No. 1.5 coverslip as a bottom (MatTek Corporation, Ashland, MA, USA) and incubated overnight. Cells were incubated with FITC-COL-CPP in PBS (0.1–100 μM) for 30 min. After incubation, cells were washed three times with fresh PBS. The cells were imaged immediately following treatment. Jurkat cells (about 1.5 x 104 cells/mL) were counted ,placed in separate centrifuge tubes (Eppendorf) and centrifuged for 5 minutes at 1,000 rpm. Each pellet was resuspended and washed with 500μL PBS. The cells were centrifuged again (5 min @ 1,000 rpm) and each pellet of cells were resuspended in varying concentrations of FITC-CPP-COL for 1 hour at 37°C. After incubation Jurkat cell suspensions were centrifuged (5 min @ 1,000 rpm) and the pellets were washed in 500μL PBS. This step was conducted twice. The cells were pelleted, resuspended in 200μL of media (RPMI), and placed in 35-mm Petri dishes with No. 1.5 coverslip as a bottom (MatTek Corporation, Ashland, MA, USA). The cells were immediately imaged after treatment.

Cellular localization of internalized FITC-COL-CPP was determined after 1 hour incubation at 37°C with A545, FaDu and Jurkat cells using anti-late endosomal/lysosomal marker: Lysosome Associated Membrane Protein 1 (LAMP1) (D2D11) XP rabbit monoclonal antibody (1:100), and Alexa Fluor 594 conjugated anti-rabbit IgG (1:200) (Cell Signaling Technology, Inc., Danvers, MA). The cells were imaged with Olympus FluoView 1000 confocal microscope and colocalization analysis was performed using Image J (NIH)."

1.7 Flow Cytometry

Cells were grown to 90% confluency and incubated with peptides for 30 min at 15 or 37 °C according to procedure for confocal microscopy outlined above. After the incubation period, cells were washed three times with cold PBS, trypsinized (adherent cells only), and then resuspended in PBS containing 0.1% Bovine Serum Albumin. Cells (10-80k /sample) were analyzed using flow cytometry (Cell Lab Quanta SC-MPL, Beckman Coulter, Inc., Brea, CA, USA) to quantify the cellular uptake of the FITC-COL-CPP.

2. Supplemental Figures

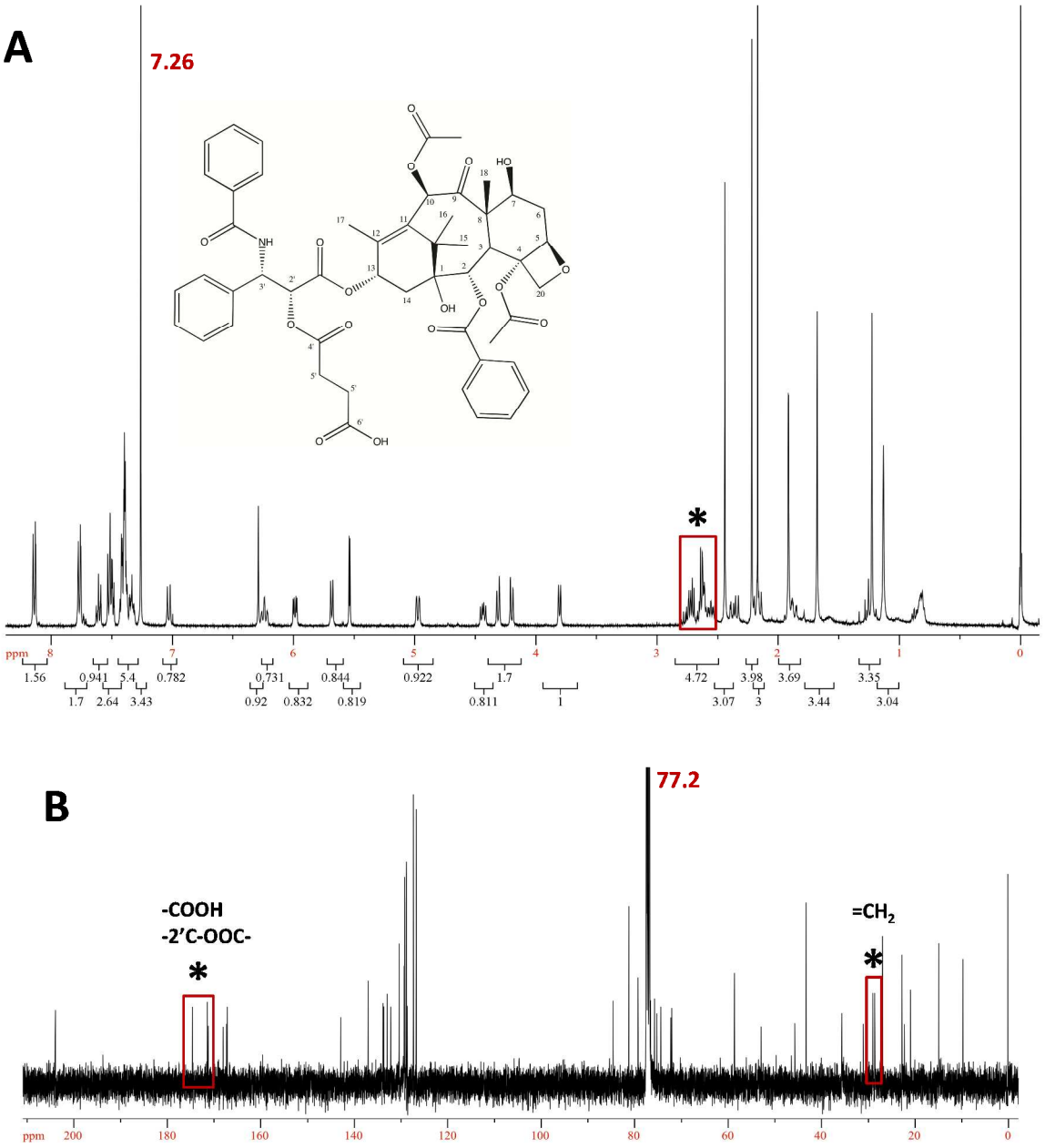


Figure S1. HNMR (A) and CNMR (B) Spectra of 2'-Succinyl-Paclitaxel in $CDCl_3$. Solvent peak is indicated in red. Peaks indicated with the star represent signal from succinyl linker. The numbers on 2'-Succinyl-Paclitaxel correspond to peaks listed in experimental section.

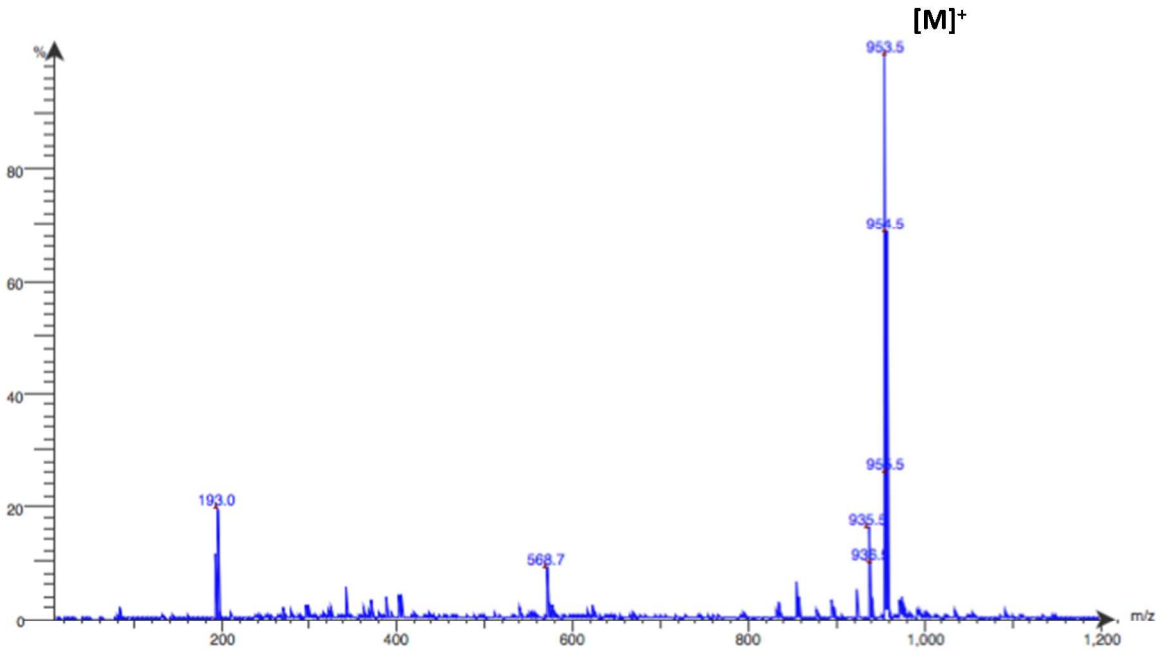


Figure S2. ESI-MS Spectrum of 2'-Succinyl-Paclitaxel

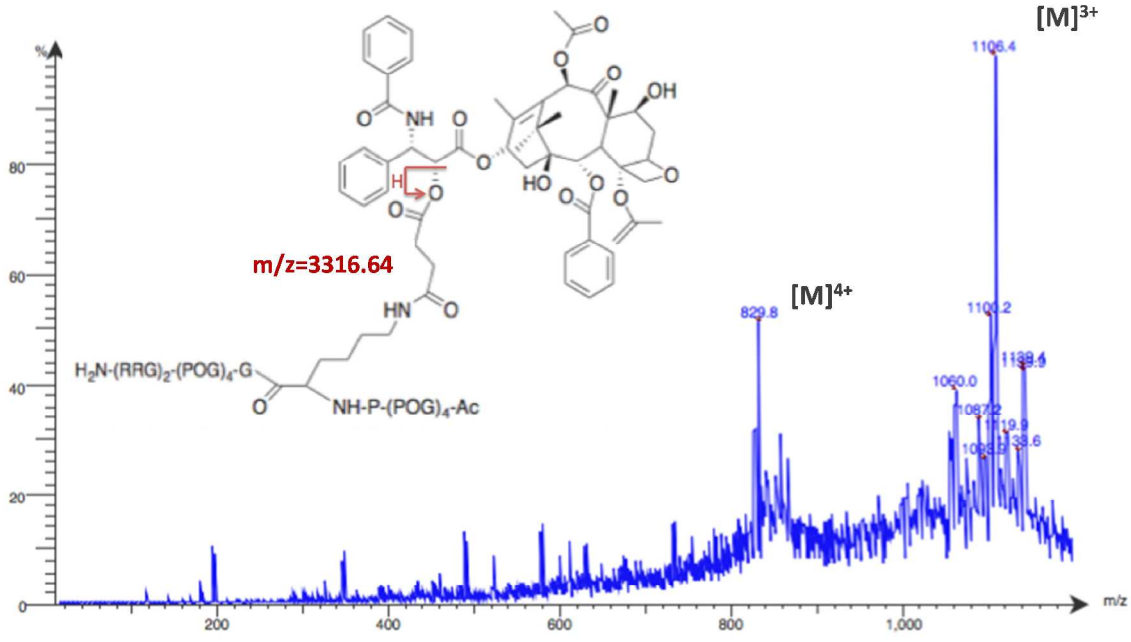


Figure S3. ESI-MS Spectrum of PTX-COL-CPP with indicated fragmentation.

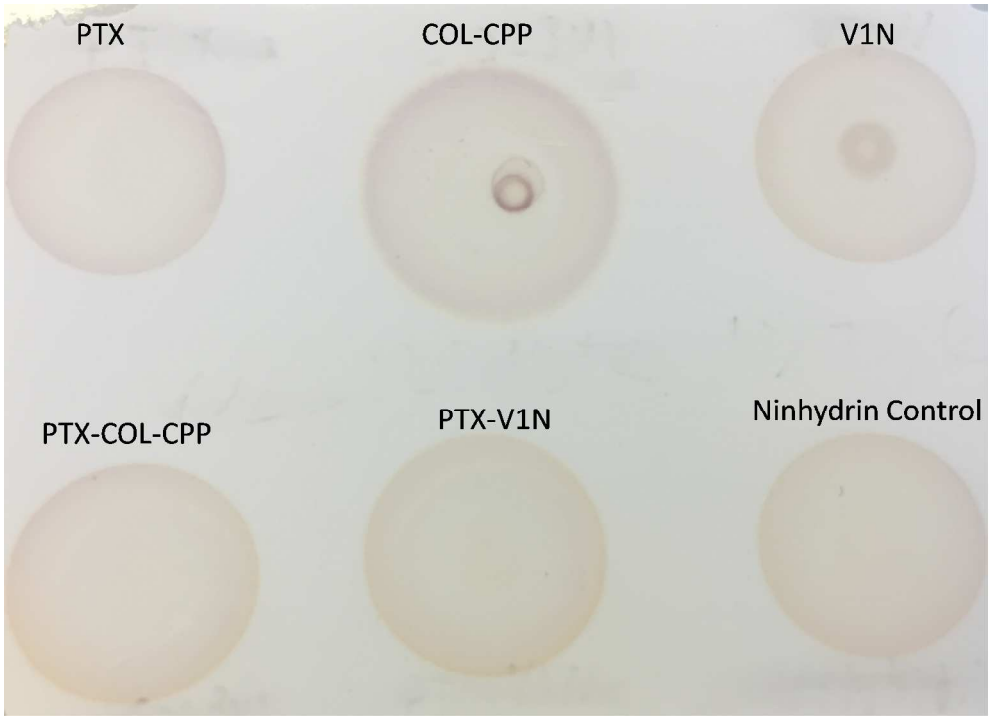


Figure S4. PTX-COL-CPP reaction with ninhydrin. The absence of a dark spot on the PTX-COL-CPP spot indicates that the reaction went to completion and there were no unreacted primary amine groups (lysine) left in the final product.

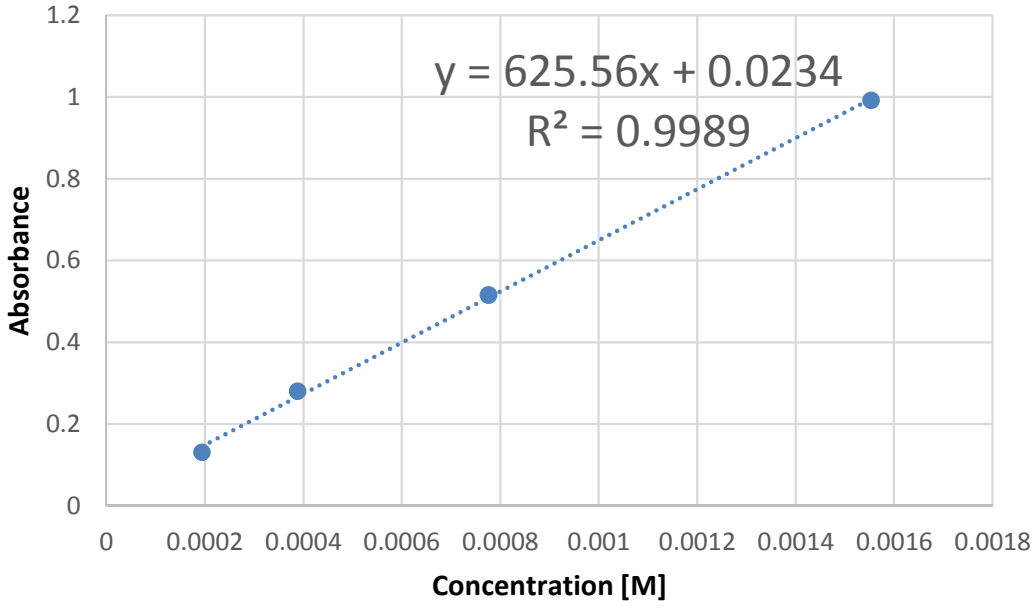


Figure S5. Solubility of COL-CPP measure as absorbance at 230nm.

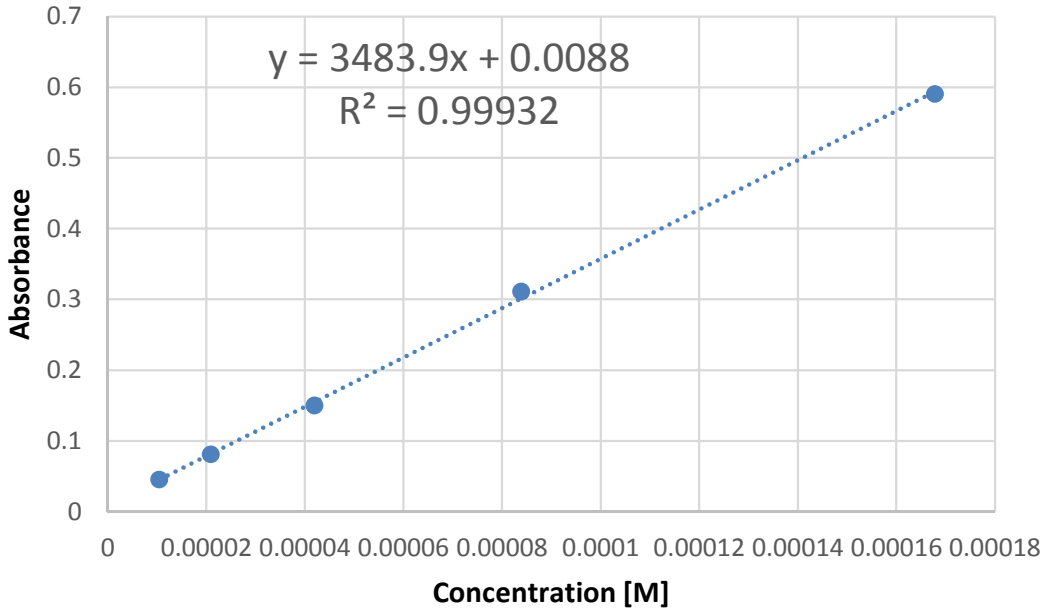
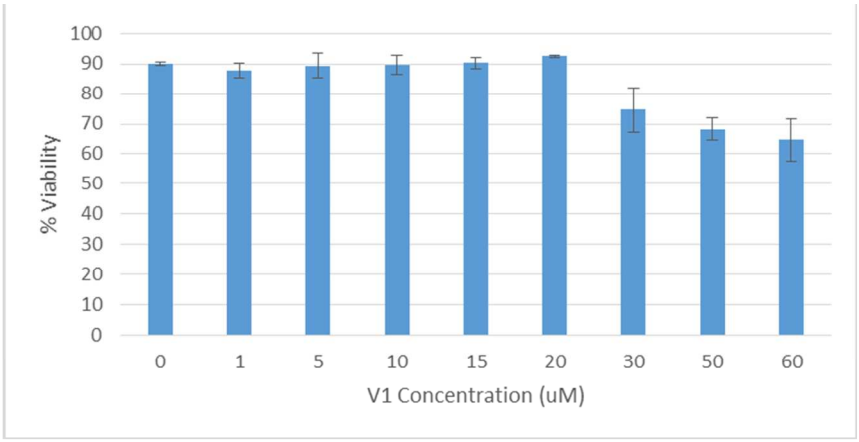
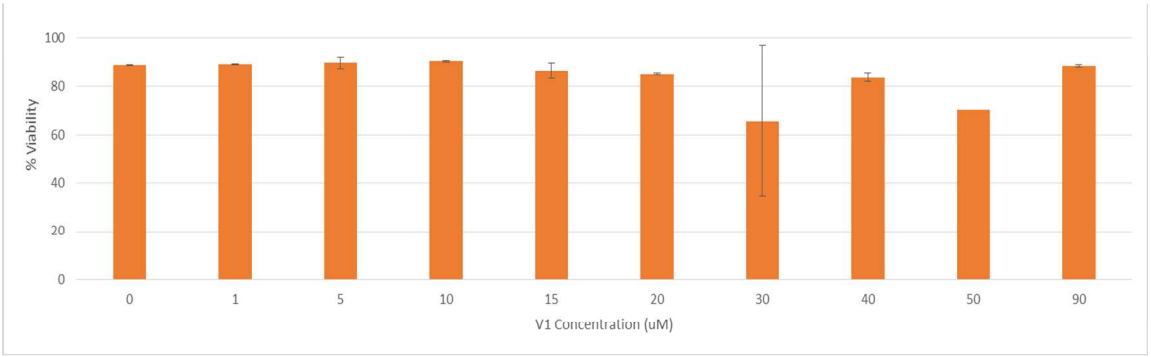


Figure S6. Solubility of PTX-COL-CPP measure as absorbance at 230nm.





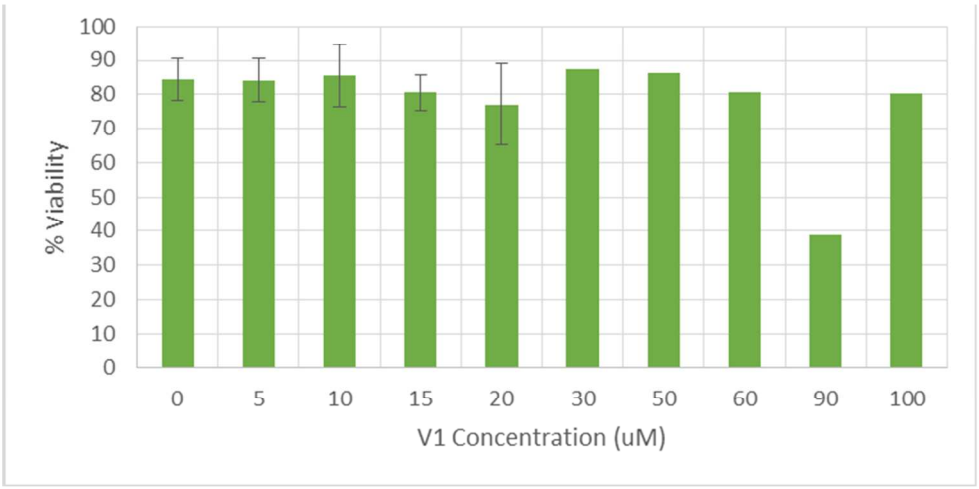


Figure S7. Viability study of Jurkat (blue), A549 (orange) and FaDu (green) cells incubated (1h) with FITC-COL-CPP.

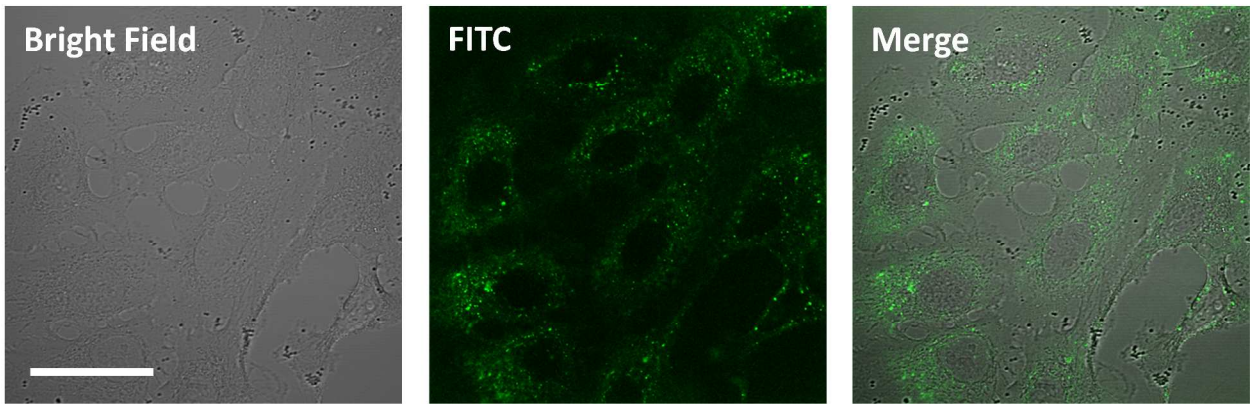


Figure S8. Confocal microscopy images of A549 cells incubated for 30 min with FITC-COL-CPP (15 μM) at 37 °C. Mag 60X. Bar represents 20 μm. No Hoechst dye.