Supporting information for *Chemical Communications*

Co-delivery of small-interfering RNA using a camptothecin prodrug as the carrier

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Materials

Tetrahydrofuran **(**THF), dimethyl sulfoxide (DMSO), triethylamine (TEA), acryloyl chloride, methanol, Tris-Borate-EDTA (TBE) buffer, phosphate-buffered saline (PBS), and DL-dithiothreitol (DTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Camptothecin (CPT) was from Chem Impex International Inc (Wood dale, IL, USA). Nile red was from Acros Organics (Morris Plains, NJ, USA). The peptide, NH_2-RRRRRHHHHHC-COOH (R5H5), was designed by us and synthesized by GenScript (Piscataway, NJ, USA) at > 95% purity. UltraPure™ Agarose-1000, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, non-essential amino acids, sodium pyruvate, L-glutamine, Trypsin-EDTA, nuclease-free water, ProLong® Gold Antifade Reagent, TRIzol® Reagent, PureLink® RNA Mini kit, SYBR® Select Master Mix, High Capacity cDNA Reverse Transcription kit, and Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay kit were all purchased from Life Technologies (Carlsbad, CA, USA). RQ1 RNase-Free DNase was bought from Promega (Madison, WI, USA). Label IT® siRNA Tracker Intracellular Localization kit (TM-Rhodamine) was purchased from Mirus (MirusBio, WI, USA). The sequence of the siRNA targeted to *MAP3K7* was from a previously published article [17] and the non-targeting control siRNA were designed by us. siRNA was synthesized by Integrated DNA Technologies (Coralville, IA). The primers (PrimeTime® qPCR Assays) of *MAP3K7*, *GAPDH* and *18SrRNA* genes were ordered from Integrated DNA Technologies (Coralville, IA, USA). Human MDA-MB-231 breast cancer cells were donated by Dr. Hossein Tavana at the University of Akron.

Synthesis of CPTssR5H5

To a solution of CPT (300 mg, 0.86 mmol), 3-(2-Pyridyl)-dithiopropionic acid (646 mg, 3.0 mmol) in anhydrous dichloromethane (50 mL) was added 4-dimethylaminopyridine (DMAP, 404 mg, 3.3 mmol) and EDC (634 mg, 3.3 mmol). The solution was stirred at room temperature for 16 hours. Then the solution was filtered and concentrated by rotary evaporator. Product was purified by column chromatography as a yellowish powder. (Yield: 60%) The structure was confirmed with ^{1}H NMR. ^{1}H NMR (300 MHz) δ 8.44 (m, 1H), 8.38 (m, 1H), 8.21 (m, 1H), 7.94 (m, 1H), 7.83 (m, 1H), 7.67 (m, 2H), 7.44 (m, 1H), 7.25 (m, 1H), 7.02 (m, 1H), 5.55 (m, 2H), 5.28 (s, 2H), 3.54-3.84 (m, 4H), 2.22 (m, 2H), 0.98 (t, 3H, J = 7.6 Hz). CTPssPy (34 mg, 0.062 mmol) and R5H5C (50 mg, 0.031 mmol) was dissolved in 2 mL of DMF. The mixture was

stirred at room temperature for 24 hours. Conjugated product CPTssR5H5 was purified by reverse Phase HPLC as a yellowish powder. The purity and structure were confirmed with both HPLC (Figure S1) and ^{1}H NMR (Figure S2).

Determination of the critical micelle concentration (CMC) of CPTssR5H5

A series of CPTssR5H5 solutions containing 100 μM nile red were prepared following the procedure in Y. Shen's paper with minor changes.[4b] Briefly, 15.9 μL of nile red solution (1 mg mL^{-1} in methanol) was added to a series of vials and the solvent was evaporated, then the appropriate volumes of CPTssR5H5 solutions (1 mg mL^{-1} in H_2O) and H_2O were added to obtain CPTssR5H5 concentrations ranging from 2 to 500 μg mL^{-1} at a total final volume of 500 μL. The mixed solutions were stirred at room temperature overnight, and then excited at 605 nm and the emission spectra were recorded from 620 to 700 nm using a PerkinElmer LS45 fluorescence spectrometer (Waltham, MA, USA). The CMC value was obtained from the intersection of the tangents to the two linear portions of the graph of the fluorescence intensity as a function of CPTssR5H5 concentration.

Preparation of CPTssR5H5/siRNA complexes

Firstly, CPTssR5H5 and siRNA were dissolved in nuclease-free water to make a stock solution at 10 mg mL^{-1} and 20 μM, respectively. Then the CPTssR5H5/siRNA complexes were formed by mixing equal volume of CPTssR5H5 and siRNA solutions at various N/P ratios, followed by incubation at room temperature for 20 minutes to allow complete electrostatic interaction between CPT-conjugate and siRNA. Here, the N/P ratio is the molar ratio of arginine residue (N) in the CPT-conjugate to phosphate group (P) in the siRNA molecule.

Size and zeta-potential measurements

Particle size and zeta-potential were measured by a Malvern Nano-ZS Zetasizer (UK). Before the measurement, an aliquot of 40 μL of each sample was diluted in 850 μL H_2O. The size measurements were performed in disposable sizing cuvettes at a laser wavelength of 633 nm and a scattering angle of 173 °, while the zeta-potential measurements were performed in disposable zeta-potential cells. All measurements were carried out at room temperature. Each parameter was measured in triplicate and mean value was calculated.

Transmission electron microscopy (TEM)

TEM imaging of CPTssR5H5 was performed by using a JEOL 2010 high resolution scanning TEM (Peabody, MA, USA). An aliquot of 10 μL solution (0.1 mg mL^{-1} in H_2O) was deposited

onto ultra-thin carbon film/holey carbon 400 mesh copper grids (Ted Pella, Inc., Redding, CA, USA). After 2 minutes, excess liquid of the sample was removed with a filter paper and further died for 2 minutes. Then 10 μL of 2 % (w/v) uranyl acetate solution was added onto the TEM grid as a negative staining agent and stained for 1 min. Again, excess solution was removed using a filter paper to result in a thin film on the grid that was allowed to air dry before imaging.

Cryo-TEM

Cryo-TEM image of CPTssR5H5/siRNA at the N/P of 45 was obtained by a FEI Technai G2 F20 microscope (FEI, Hillsboro, OR) at voltage of 200 kV. Briefly, a droplet of 3 μL complex solution was pipetted onto a carbon-coated copper TEM grid (400-mesh) loaded into an FEI Vitrobot apparatus (Mark IV). The grid was automatically blotted and plunged into liquid ethane cooled by liquid nitrogen. The vitrified samples were transferred to a Gatan ±70° Model 626.DH holder in a cryo-transfer stage immersed in liquid nitrogen. During the imaging, the cryo-holder was kept below -170 °C to prevent sublimation of vitreous solvent.

Circular Dichroism (CD) spectropolarimetry

A stock solution of CPTssR5H5 (10 mg/mL in H_2O) was diluted into 0.1 mg/ml in PBS buffer (pH7.5), Tris buffer (pH7.5) and Citric buffer (pH5), respectively. And the samples were analyzed on a Jasco 715 CD spectropolarimeter (JACSO, Tokyo, Japan) in a 1.0 or 0.1 cm quartz cell at room temperature. Each sample was scanned from 190 to 250 nm with a band width of 1 nm, and the spectra were recorded and corrected for the blank sample.

Gel retardation assay

A gel retardation assay was conducted to evaluate siRNA condensation of CPTssR5H5. The CPT-conjugate/siRNA complex solutions were prepared at N/P ratios from 0 to 50, following the procedure mentioned above. An aliquot (20 μL, 10 pmol siRNA) of each solution was mixed with 4 μL of gel loading dye, orange (New England Biolabs, Ipswich, MA, USA) and loaded in an agarose gel (4 %), followed by electrophoresis at 120 V for 40 minutes. The gel was stained in ethidium bromide (0.5 μg mL^{-1}) for 30 minutes and destained with water for 15 minutes. Then the gel was visualized and documented using a UVP BioDoc-It imaging system (Upland, CA, USA).

Stability of CPTssR5H5/siRNA complexes under reducing conditions

A reducing reagent DTT was used to evaluate the disassociation of CPTssR5H5/siRNA complexes upon reducing environment due to the breakage of disulfide bond in CPTssR5H5.

CPTssR5H5/siRNA complexes at N/P ratios of 30 and 45 were formulated as described above and incubated with or without DTT (25 mM) at 37 °C for 1 hour. The reaction mixtures were analyzed by electrophoresis, following the same protocol as mentioned above. The siRNA amount released from the complexes was visualized by a UVP BioDoc-It imaging system (Upland, CA, USA).

Intracellular localization of CPTssR5H5/siRNA complexes

Intracellular localization of CPTssR5H5/siRNA complexes in MDA-MB-231 cell line was studied by an Olympus FLUOVIEW FV1000 confocal laser scanning microscopy (CLSM) (Japan). MDA-MB-231 cells were seeded on a Nalgene Lab-Tek™ II 8-well chamber slide (Waltham, MA, USA) at a density of 10, 000 cells/well and cultured for 24 hours. Then the medium was replaced with 180 μL of fresh medium, and 20 μL of the complex solutions (CPTssR5H5 or Lipofectamine 2000 complexed with siRNA) containing 20 pmol Rhodamine-labeled siRNA was subsequently added into each well. CPTssR5H5/siRNA complexes were formed at the N/P ratio of 45, while Lipofectamine 2000/siRNA complexes were formed according to manufacturer's instruction. After 6-hour incubation, the medium was removed and cells were washed with cold PBS three times. The cells were fixed in 3.7% paraformaldehyde for 30 minutes. Then the cells were washed with PBS three times, and incubated for 30 minutes in 100 μL of Hoechst 33342 (2 μg mL^{-1}) for each well. For microscopy imaging, the chamber slide was mounted with Prolong Gold Antifade solution and covered by glass slip. The slide was then examined by differential interference contrast (DIC) microscopy and CLSM with excitation at 405 and 559 nm for Hoechst 33342 and TM-Rhodamine, respectively. The images were recorded and processed with Olympus FV10-ASW software.

Gene expression

The expression level of *MAP3K7* gene in MDA-MB-231 cell line was analyzed by quantitative reverse transcription (RT)-PCR. Cells were plated (100000 cells per well) on 6-well plates and cultured for 24 hours. Then old medium was replaced with 1.8 mL of fresh medium and 200 μL of various drug formulations (CPTssR5H5 with MAP3K7 siRNA or NC siRNA at N/P ratios of 30 and 45, Lipofectamine 2000 with MAP3K7 siRNA or NC siRNA). After 48 hours' incubation, cells were treated with TRIzol® Reagent, and RNA was isolated using a PureLink® RNA Mini kit. First strand cDNA was synthesized by a High Capacity cDNA Reverse Transcription kit with 400 ng of total cellular RNA from which genome DNA has been removed by RQ1 RNase-Free

DNase. Then a PCR reaction using the synthesized cDNA as template was carried out by a Bio-Rad CFX96 Real-Time Detection System (Hercules, CA, USA). The PCR data was analyzed using qbase+ software (Belgium).

Cytotoxicity

The cytotoxicity of CPTssR5H5/siRNA complexes was evaluated against MDA-MB-231cells using Vybrant® MTT Cell Proliferation Assay. Cells were seeding on 96 well-plates at the density of 5000 cells per well. After incubation for 24 hours, the old medium was replaced with 90 μL of fresh growth medium and 10 μL of various concentrations of drugs (CPTssR5H5 with MAP3K7 siRNA or NC siRNA at N/P ratios of 30 and 45) were added to each well. Lipofectamine 2000/siRNA complexes were prepared according to the manufacturer's protocol and used as positive control. After incubation at 37 ºC for 48 hours, the medium was replaced with fresh one (100 μL) and 10 μL of MTT stock solution (5 mg mL^{-1} in PBS), and the cells were incubated for 4 hours at 37 ºC. Finally, the medium was removed and 150 μL of DMSO was added to each well to dissolve the purple formazan crystals. The absorbance was measured at 570 nm using a Tecan Infinite 200 microplate reader (Switzerland). The cytotoxicity test was performed in 8 replicates of each sample. The cells without any treatment were used as a control (100% cell viability), and the cell viability was expressed as a percentage of the control.

Apoptosis

The analysis of apoptosis induced by various drug solutions in MDA-MB-231 cell line was examined under CLSM using Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay. Cells were plated (15000 cells per well) on an 8-well chamber slide. After 24-hour incubation, cells were treated with following formulations: control (fresh medium); free CPT; Lipofectamine/MAP3K7 siRNA; CPTssR5H5/NC siRNA and CPTssR5H5/MAP3K7 siRNA. Then cells were incubated for 48 hours before TUNEL assay. The procedure followed the manufacturer's protocol.

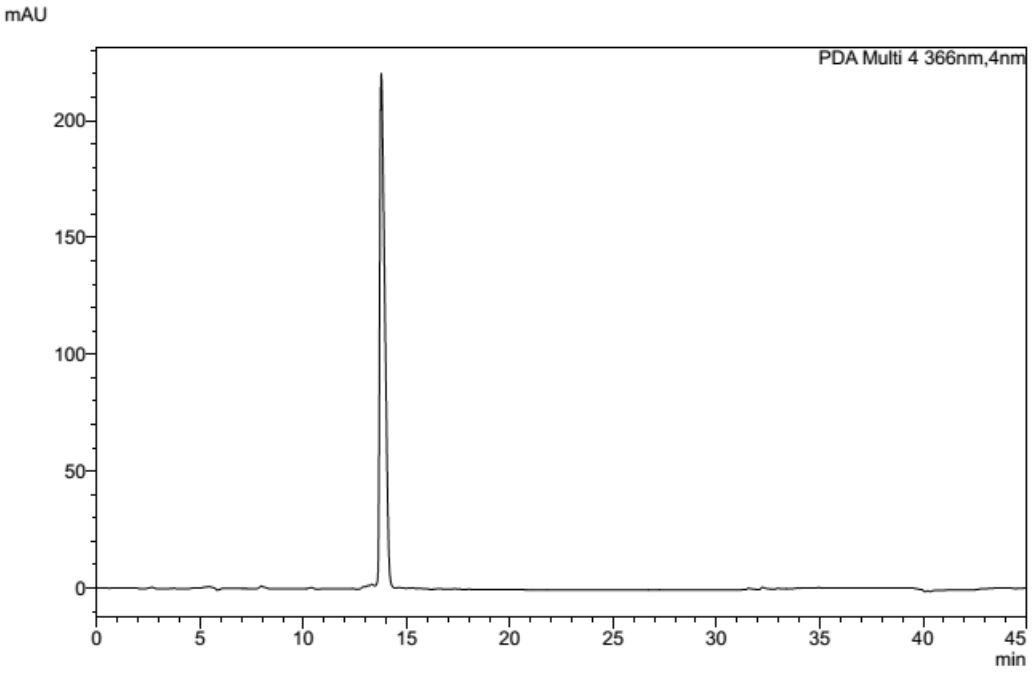


Figure S1. Analytical HPLC trace of CPTssR5H5.

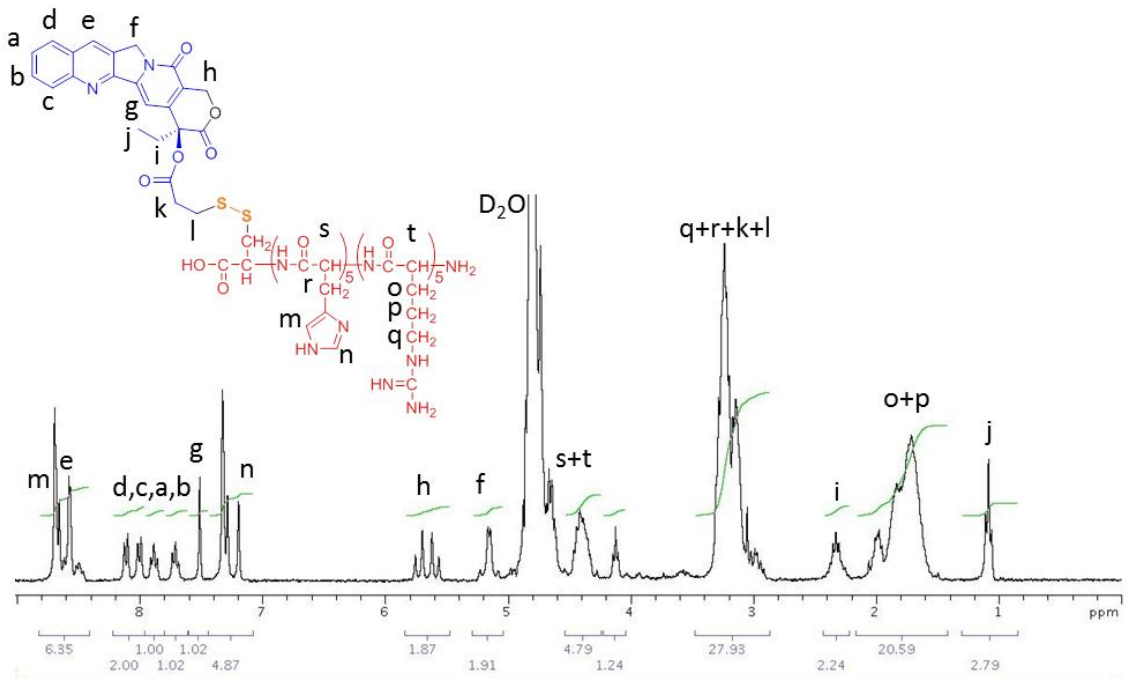


Figure S2. 300MHz ^{1}H NMR spectra of CPTssR5H5 (bottom) in D_2O.

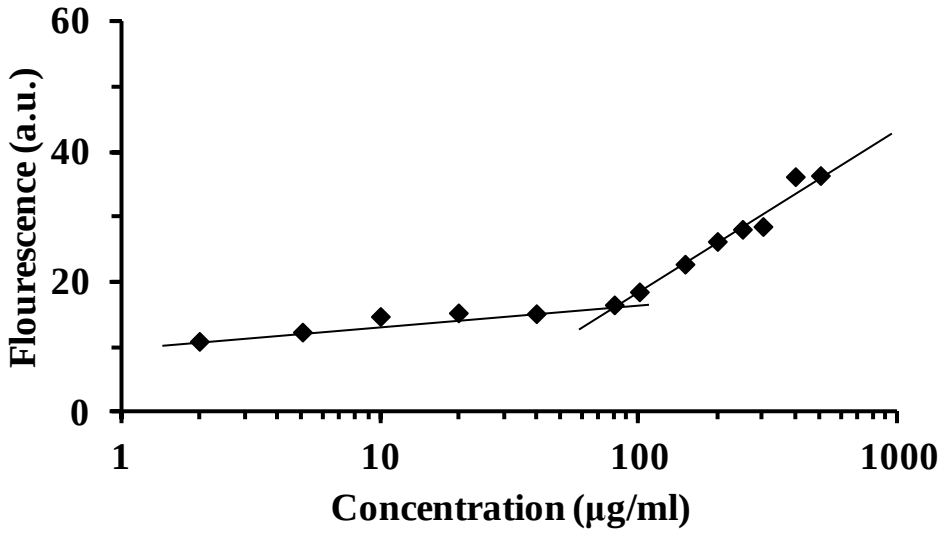


Figure S3. The fluorescence intensity of nile red as a function of the concentration of CPTssR5H5.

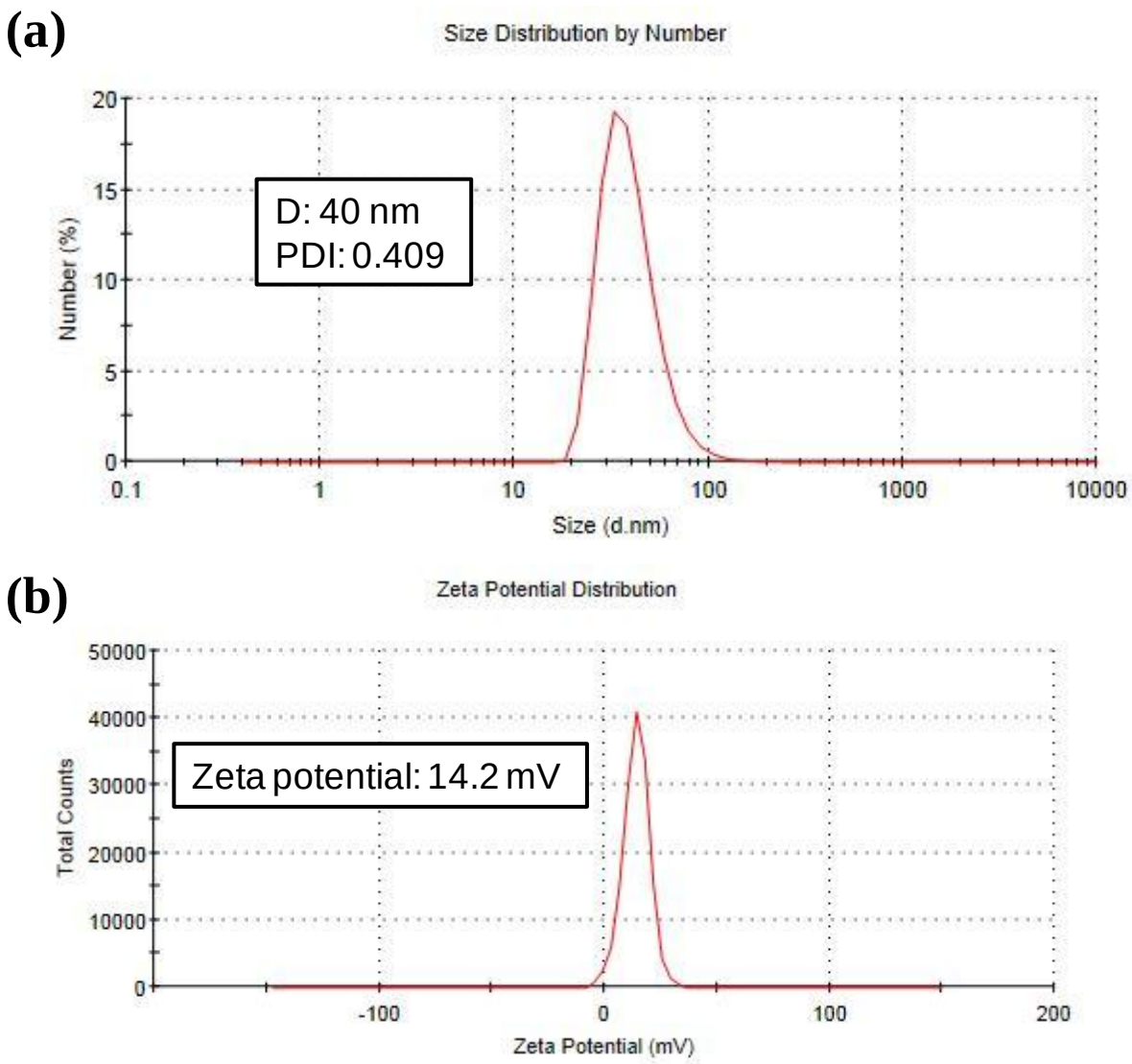


Figure S4. The size (a) and zeta potential (b) of CPTssR5H5 vesicles measured by DLS.

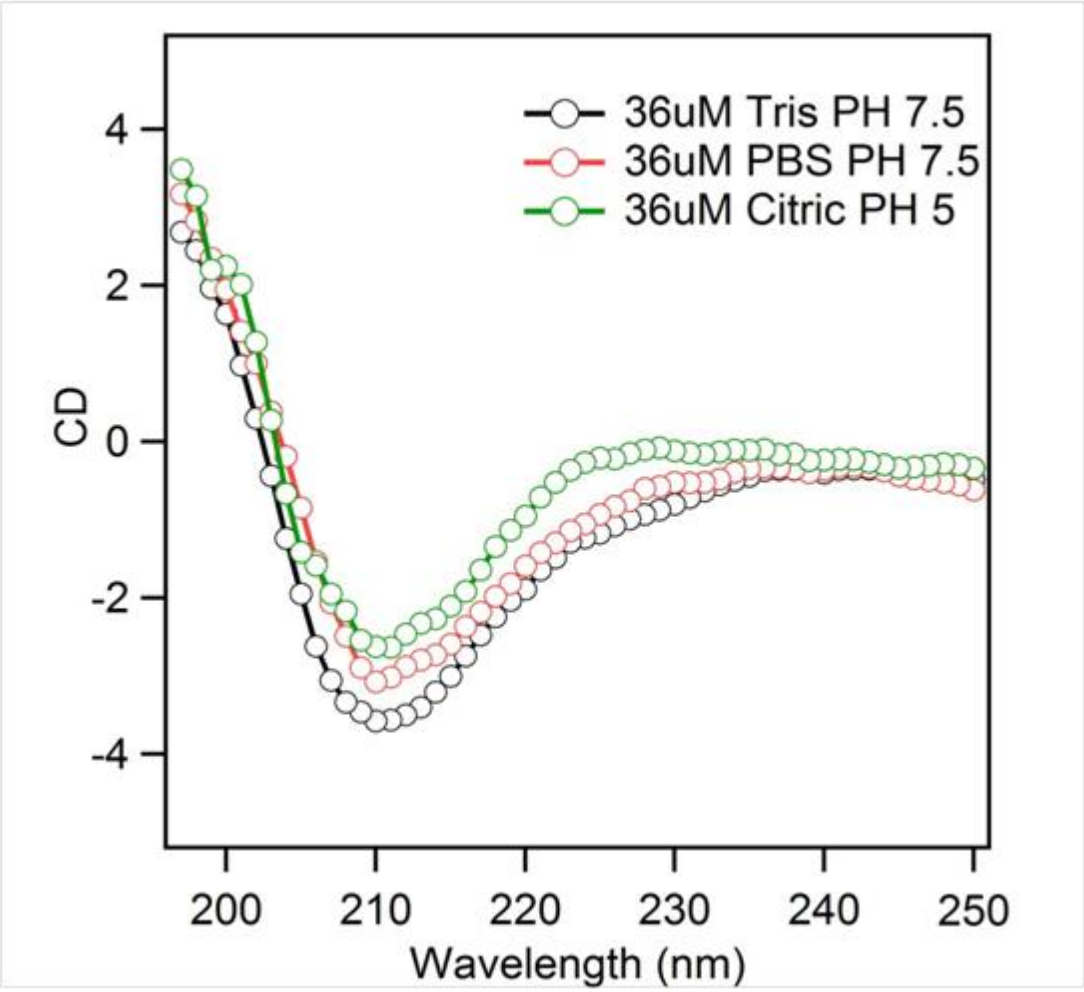


Figure S5. Circular dichroism spectra of CPTssR5H5 in different buffers at room temperature.

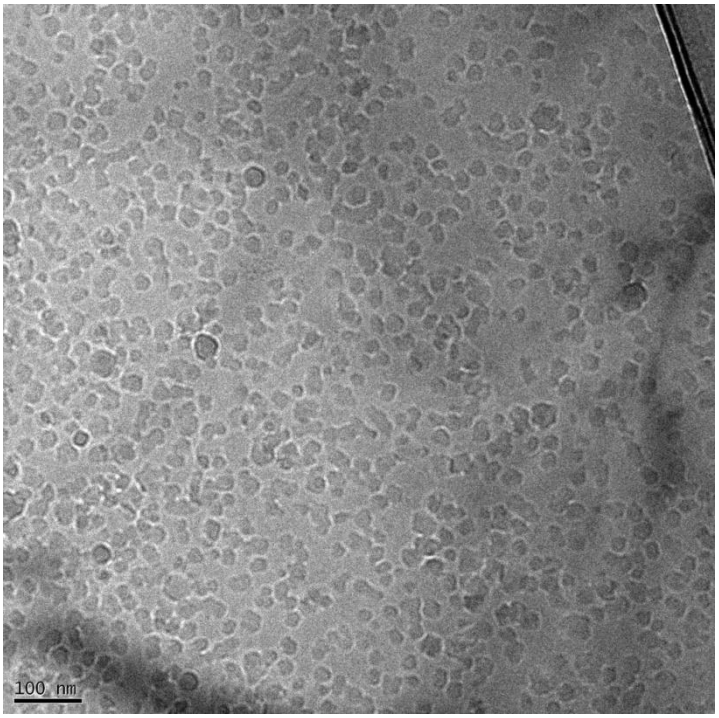


Figure S6. Cryo-TEM image of CPTssR5H5/siRNA complexes formed at the N/P ratio of 45 (scale bar of 100 nm)**.**

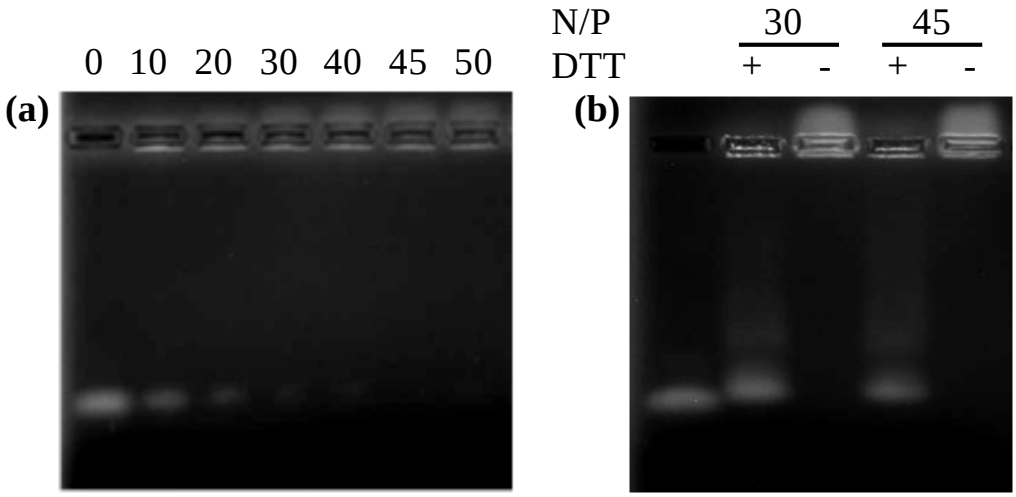
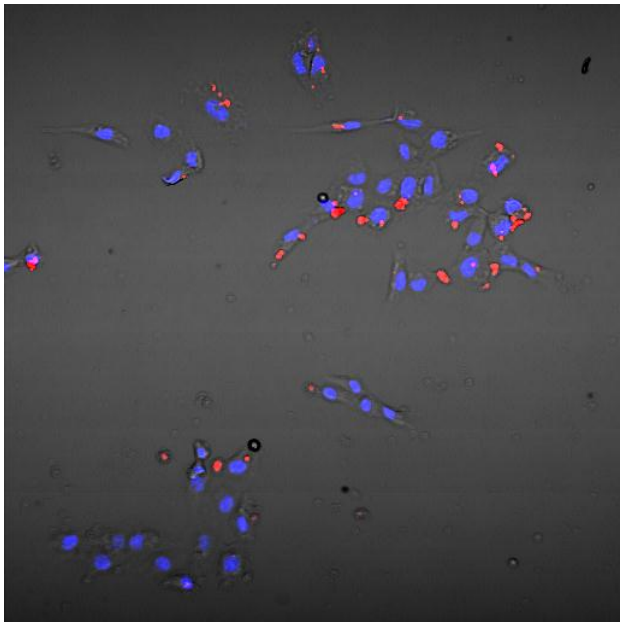


Figure S7. (a) Electrophoretic mobility of siRNA complex with CPTssR5H5 at different N/P ratios. (b) Decomplexation of CPTssR5H5/siRNA following incubation with or without DTT (25 mM). Left lane: naked siRNA.

Figure S8. Cellular internalization of Lipofectamine2000 complexed with MAP3K7 siRNA after



6-hour incubaiton**.** The siRNA was stained by TM-Rhodamine (red) and cell nuclei were stained by Hoechst 33342 (blue).

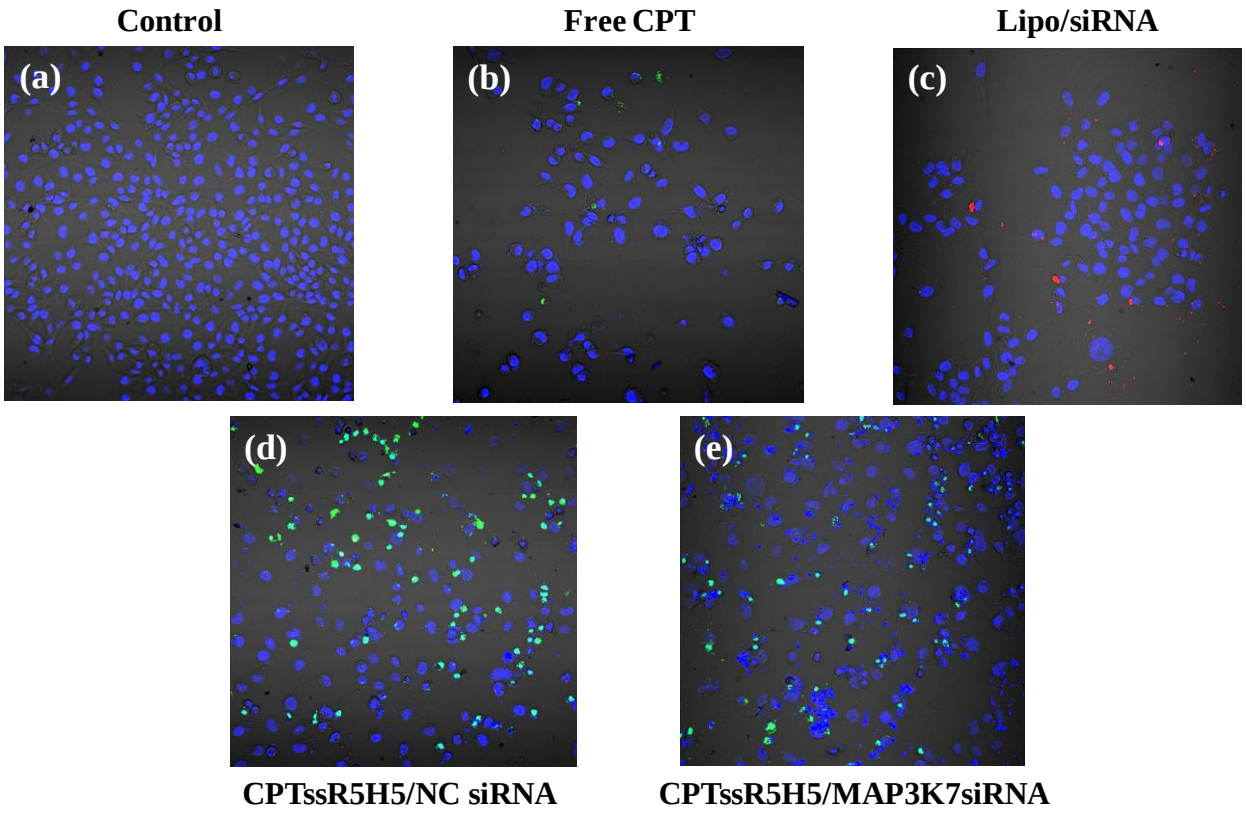


Figure S9. Apoptosis induced by different drug formulations after 48-hour incubation. The siRNA was stained by TM-Rhodamine (red), cell nuclei were stained by Hoechst 33342 (blue) and TUNEL-stained cells (green).