Supplementary information for

Multistage rocket: integrational design of prodrug-based siRNA delivery system for sequential release enhanced antitumor efficacy

Qian Jiang, [†, a, b] Xiaobing Chen, [†, a] Hong Liang, [a] Yu Nie, [*, a] Rongrong Jin, [a] Matthias Barz, [c] Dong Yue, [a] and Zhongwei Gu[*, d]

a. National Engineering Research Center for Biomaterials, Sichuan University, Chengdu, 610064, P. R. China. E-mail: nie_yu@scu.edu.cn

b. Department of Pharmacy, The Second People's Hospital of Chengdu, Chengdu 610017, P. R. China.

c. Institute of Organic Chemistry, Johannes Gutenberg-University Mainz Duesbergweg 10-14, 55099 Mainz, Germany.

d. College of Materials Science and Engineering, Nanjing Tech University, 30 South Puzhu Road, Nanjing 211816, P. R. China. E-mail: zwgu1006@hotmail.com

* Corresponding authors

† Equal contribution to the manuscript as co-first authors

Acronyms

RLS **lipopeptides with a 2nd generation dendritic arginine as cationic segment**

RSC **a general designation of the integrated peptide-camptothecin prodrug**

$R_{2/3}SC$ **the integrated peptide-camptothecin prodrug with a 2nd or 3rd generation dendritic arginine**

R_2SC **the integrated peptide-camptothecin prodrug with a 2nd generation dendritic arginine**

R_3SC **the integrated peptide-camptothecin prodrug with a 3rd generation dendritic arginine**

1. Experimental Section

1.1. Materials

Di-tert-butyl dicarbonate ($(Boc)_2O$), 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), and succinic anhydride (SUC) were purchased from Aladdin (China), cystamine dihydrochloride was obtained from J&K (China) and Boc-Lys(Boc)-OH, H-Lys-OMe·2HCl, Boc-Arg(pbf)-OH, N1-(ethylimino)methylene)-N3,N3-dimethylpropane-1,3-diamine (EDC), N,N,N',N'-tetramethyl-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole hydrate (HOBT) were from GL Biochem (Shanghai). N,N-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA) were obtained from Astatech (China) and reduced glutathione (GSH) was from Sigma-Aldrich (USA). N,N-Dimethyl formamide (DMF) and dichloromethane were distilled or vacuum-distilled before use. Lipofectamine™ 2000 reagent, Dulbecco's modified

Eagle's medium with high glucose (DMEM-HG) and fetal bovine serum (FBS) were purchased from Life Technologies Corporation (Gibco®, USA). The human cervical epithelial carcinoma cell line (Hela) was obtained from Shanghai Institutes for Biological Sciences (China). The special Hela cells (GFP-Hela), which can express green fluorescent protein, were donated by Professor Tong from State Key Laboratory of Biotherapy. A BCA protein assay kit was purchased from Pierce (USA). LysoTracker® Green DND-22 was obtained from Life Technologies Corporation (USA). Antibodies against human polo-like kinase 1 (PLK1) was purchased from Cell Signaling Technology, Inc. (USA). Antibodies against β-actin, goat anti-rabbit IgG-HRP antibody, goat anti-mouse IgG-HRP antibody, Annexin V-FITC/PI apoptosis detection kit and one step TUNEL apoptosis assay kit were purchased from Beyotime Biotechnology (China). siRNAs targeting human PLK1 (siPLK1) (sense strand, 5'-UGAAGAAGAUCACCCUCCUUAdTdT-3' and antisense strand, 5'-UAAGGAGGGUGAUCUUCUUCAdTdT-3'), siRNAs targeted to the GFP (sense strand, 5'-GGCUACGUCCAGGAGCGCACC-3' and antisense strand, 5'-UGCGCUCCUGGACGUAGCCUU-3') and scrambled siRNA (siNonsense) (sense strand, 5'-UUCUCCGAACGUGUCACGUdTdT-3' and antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3') were supplied by Shanghai GenePharma Co. Ltd. (China). Fluorescein-tagged siRNA (Cy5-siRNA) was synthesized by modification of the 5'-end of the sense strand of the scrambled siRNA with fluorescein. Ki67 rabbit monoclonal antibody was purchased from Servicebio (China).

1.2. Chemical Experiments

1.2.1. Synthesis of disulfide bond modified camptothecin prodrug (compound 1, Figure S1)

Camptothecin (CPT, 348.4 mg, 1.0 mmol) and succinic anhydride (SUC, 300.3 mg, 3.0 mmol) were added to a 100 mL round-bottom flask and suspended with 30 mL of dichloromethane. 0.44 mL of DBU (3.0 mmol) was slowly dropped into the mixture at 0°C. And then the reaction was stirred for 4 h at room temperature and stopped by 20 mL of water addition. 1% HCl solution was used to acidize the mixture. The yellow precipitate was collected and washed with 1% aqueous HCl solution and water thrice, and recrystallized with methanol. The product was obtained as a yellow green powder (CPT-COOH, 440 mg) and characterized by nuclear magnetic resonance (NMR) spectrometry and electrospray ionization mass spectrometry (ESI-MS).

Boc-Cys-NH_2 was the single Boc-protected cysteine, which was obtained as follows. Cystamine dihydrochloride was first desalinated by NaOH. It (3.2 mmol, 0.32 g) was dissolved in 100 mL methanol together with some TEA (3.2 mmol, 0.32 g) and cooled to 0 °C (ice bath). $(Boc)_2O$ was dissolved in 20 mL methanol and added into the mixture. After 30 min, the ice bath was removed and the reaction was kept at room temperature overnight. And then, the solution was concentrated to remove the methanol, and the resultant residue was resolved in dichloromethane and washed with saturated sodium carbonate. The collected organic layer was concentrated under reduced pressure to obtain Boc- Cys-NH_2.

To a solution of CPT-COOH (440 mg, 0.1 mmol), Boc-Cys-NH_2 (500 mg, 0.2 mmol), HOBT (54 mg, 0.4 mmol) and HBTU (151 mg, 0.4 mmol) in anhydrous N,N-dimethylformamide (DMF, 50

mL) was added DIPEA (62 mg, 0.5 mmol). The mixture was stirred at room temperature for 48 h, filtered and concentrated by rotary evaporator. The resultant residue was purified by column chromatography as a yellowish powder, redissolved in trifluoroacetic acid (TFA, 50 mL) and dichloromethane (DCM, 20 mL) mixture for 24h-deprotection with stirring at room temperature. The solvent was concentrated by rotary evaporation and ether precipitation to give a white powder. (compound 1, 60% yield).

1.2.2. Synthesis of the peptide-prodrug conjugation: camptothecin prodrug with 2nd or 3rd generation dendritic arginine ($R_{2/3}SC$, Figure S1)

The synthesis of compound 2 and compound 3 was according to our previous work.[1, 2]

In a typical experiment for the 3rd generation dendritic arginine (compound 4), compound 3 (0.5 g, 1.2 mmol), Boc-Arg(pbf)-OH (3.2 g, 6.0 mmol), HOBT (0.8 g, 6.0 mmol) and HBTU (2.3 g, 6.0 mmol) were dissolved in 30 mL of anhydrous DMF. DIPEA (2.3 g, 3.0 mmol) was added dropwise into the reaction system at 0 °C and stirred at room temperature under N_2 for 30 min. 48 h later, after washing with saturated sodium bicarbonate, diluted hydrochloric acid and saturated brine, the collected organic layer was dried with $MgSO_4$ overnight and concentrated under reduced pressure. The resultant residue was purified by column chromatography (silica gel, DCM/ MeOH = 10:1) as white powder, and the methoxy group was removed by NaOH to obtain compound 4. (75% yield).

Under nitrogen atmosphere, compound 4 (1.5 g, 0.6 mmol), compound 1 (0.3 g, 0.5 mmol), HBTU (0.2 g, 0.6 mmol), and HOBT (0.08 g, 0.6 mmol) were weighed and

dissolved in anhydrous DMF (25 mL). After addition of DIPEA (0.4 mL, 1.2 mmol), the solution was stirred in an ice bath for 30 min and at 25 °C for 48 h. Organic solvents were removed by a rotary evaporator. The residue was redissolved in DCM and washed with saturated sodium bicarbonate, diluted hydrochloric acid, and saturated brine solution. The collected organic compound was dried with $MgSO_4$ and concentrated under reduced pressure. The crude products was obtained by column chromatography (silica gel, DCM/MeOH = 8: 1) as white powder. The N-tert-butoxycarbonyl group was removed by the same method as described in *section 1.2.1.* to obtain R_3SC as an off-white solid in 64% yield, and then was characterized by nuclear magnetic resonance (NMR) spectrometry and matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF MS).

As described in Figure S1, the synthesis of R_2SC was similar to R_3SC with an off-white powder in 71% yield, and then was characterized by nuclear magnetic resonance (NMR) spectrometry and matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF MS).

1.3. Assembly and characterization of $R_{2/3}SC$ assemblies

These camptothecin prodrug-based assemblies ($R_{2/3}SC$) were prepared by the injection method as reported previously[3] with some modifications. In brief, appropriate amphiphilic molecules ($R_{2/3}SC$) were dissolved in about 10 μL of methanol, and injected into 1 mL of a fast stirring HBG solution (20 mM HEPES pH 7.4, 5% glucose). Cationic assemblies were formed spontaneously in the aqueous medium.

The cationic assemblies were diluted with HBG to a final concentration of 1 mg/mL for particle size and zeta potential measurement by a Zetasizer Nano-ZS (Malvern Instruments, U.K.) at 25 °C. Moreover, appropriate amount of the sample dispersion was dropped on a 100 mesh copper grid, and then put into a desiccator for morphology observation by transmission electron microscopy (TEM, JM-1011, JEOL).

1.4. Physicochemical characterization of peptide-prodrug assemblies ($R_{2/3}$SC) and gene complexes

1.4.1. Assembly and characterization of $R_{2/3}$SC-siRNA complexes

siRNA complexes were prepared by mixing siRNA (20 μM) and cationic assemblies (1 mg/mL) solution gently at different N/P ratios (in the range of 10-40) in HBG buffer and incubated at room temperature for 30 min before use. Size and zeta potential were measured at a final siRNA concentration of 3 μg/mL using a Zetasizer Nano-ZS (Malvern Instruments, U.K.).

Gel retardation assay was used for siRNA compaction ability evaluation of cationic assemblies with different N/P ratios (0-30). All samples containing with 100 ng of siRNA were loaded onto 1% agarose gel for electrophoresis (60 V, 1 h). The gel was stained with ethidium bromide and analyzed on the Molecular Imager ChemiDoc XRS+ (Bio-Rad, USA) to visualize the location of siRNA bands.

1.4.2. Redox responsiveness of the cationic complexes

To evaluate the stability of $R_{2/3}$SC assemblies and $R_{2/3}$SC/siRNA complexes under reductive conditions, the solution (1 mg/mL) were incubated with 5 mM GSH at 37 ^{o}C. The changes of particle size and zeta potential were measured by Zetasizer Nano-ZS

(Malvern Instruments, U.K.) at indicated time points. Furthermore, the environment stimulated release of siRNA from complexes in the presence of reducer (GSH) was estimated. siRNA complexes at different N/P ratios were incubated with 5 mM GSH for 2 h at 37 °C. The resulting mixture was electrophoresed on the agarose gel electrophoresis. Naked siRNA and $R_{2/3}SC$/siRNA complexes incubated without reducer were used as control.

1.4.3. Release of camptothecin (CPT) from cationic assemblies

The *in vitro* release of CPT was performed in these assemblies with different conditions in a time-course procedure. CPT was isolated by dialysis bag (MWCO 1000, Millipore) in medium with 5 mM GSH or 1 μM esterase or a mix of the two above at 37 °C. The CPT concentration in solution was detected using high performance liquid chromatography (HPLC) equipped with a UV detector and a C18 column at 25 °C (Shimadzu, Japan). The mobile phase was methanol and water (volume ratio 60: 40, Sigma, HPLC grade) at a flow rate of 1.0 mL/min and a UV detector at 254 nm wavelength. The free CPT was determined based on the peak area at the retention time of 3.9 min according to the calibration curve. The release percentage of CPT was calculated using the formula:

Release percentage (%) = $W_1/W_0 \times 100\%$

where W_1 was the weight of CPT in solution, W_0 was the weight of total CPT in assemblies.

1.5. *In vitro* study on cells

1.5.1. In vitro gene silencing efficiency

GFP-Hela cells (1 ×10^5) were seeded in 96-well plates and incubated at 37 °C in 5% CO_2 for 24 h to reach about 70% confluence. Various complexes containing siGFP were added to 0.1 mL of DMEM medium with 10% FBS, and incubated with the cells for 48 h. Qualitative evaluation of GFP knocking-down for N/P ratio optimization was evaluated by an inverted fluorescence microscope (Leica, Germany), and the semi-quantitative assay was calculated by Image-Pro Plus 6.0 software. In order to investigate *in vitro* functional gene silencing efficiency, Hela cells (5×10^5) were seeded in 6-well plates for attachment. The transfection formulations containing siPLK1 was added with new medium containing 10% FBS, followed by incubating for 24 h (for mRNA isolation) or 48 h (for protein extraction). The cellular levels of PLK1 mRNA and protein were assessed using quantitative real-time PCR (qRT-PCR) and western blot (WB), respectively. Lipofectamine 2000 (Lipo, Invitrogen, Carlsbad, CA) was used according to the manufacturer's guidelines as controls, while untreated cells was the blank group. Besides, cells treated with equal CPT (8 μM) and similar siPLK1 complexes (RLS/siPLK1) were as nonsequential release group (RLS/siPLK1+CPT).

1.5.2. Cellular uptake and intracellular tracking study of complexes

To further discern the effect of different generation dendrimers on cellular uptake and efficient intracellular trafficking of camptothecin prodrug-based siRNA delivery systems, intracellular localization of $R_{2/3}$SC/siRNA complexes in Hela cells was investigated using confocal laser scanning microscopy (CLSM). Hela cells (1 ×10^5 cells/dish) were seeded into 35-mm glass-bottom culture dishes and incubated for overnight. Then, cells were replaced with fresh medium (with 10% serum) containing

various Cy5-labelled complexes for 2 and 4 h at 37 °C. At prearranged time intervals, cells were rinsed twice with cold PBS to remove non-ingested complexes, stained with Lyso Tracker Green (75 nM) and washed with cold PBS for three times. Finally, the cells were counterstained with Hoechst 33242 (10 mg/ml) for 15 min and observed by CLSM.

1.5.3. In vitro cytotoxicity and cell apoptosis measurement

The cytotoxicity of $R_{2/3}$SC/siRNA complexes was evaluated against Hela cells using CCK-8 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 100 μL of fresh growth medium containing various complexes or drug (Lipo/siPLK1, CPT, RLS/siPLK1+CPT, $R_{2/3}$SC with siPLK1 or siN.C.) were added to each well. After incubation at 37 ºC for 72 hours, the medium was replaced with fresh one (90 μL) and 10 μL of CCK-8 (Dojindo Molecular Technologies, Japan), and the cells were incubated for 2 hours at 37 ºC. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, model 550, USA). The cytotoxicity test was performed in 5 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.

For apoptosis analysis, Hela cells (5×10^5) cultured in 6-wells plates were treated with the above-mentioned complexes at the siRNA of 2 μg or free CPT dose of 8 μM. After 48 h of treatment, apoptosis cells were detected on BD Calibur flow cytometry (BD, USA) using the Annexin V-FITC Apoptosis Detection Kit I (Beyotime Biotechnology, China). Besides, apoptosis cells were detected on flow cytometry and

CLSM at 450-500 nm by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) cell apoptosis detection kit (Beyotime, China).

1.6 Animal Experiment

Healthy BALB/c nude mice with body weight of 18-20 g were maintained in a pathogen-free environment in accordance with NIH guidance and had access to sterilized food and distilled water. 1×10^7 Hela cells in 100 μL of PBS were inoculated subcutaneously into the right flank region of BALB/c nude mice to establish a xenograft tumor model. Tumor size was measured using a vernier caliper across its longest (L) and shortest (W) diameters, and tumor volume was calculated using V [mm^3] =1/2 × LW^2. When the tumor reached 150-200 mm^3, the nude mice were randomly divided into four groups (n = 6), which received R_2SC/siPLK1, RLS/siPLK1+CPT or CPT complexes treatments via intratumoral injection every other day. The dose of complexes with siPLK1 of each injection was fixed at 1 mg/kg, and the concentration of free CPT was equal to the concentration of CPT for prodrug-based complexes corresponding to siRNA concentration. After observation for 12 days, mice were sacrificed, and tumor tissues were excised 48 h after the last treatment after the last intratumoral injection. For PLK1 protein analysis, a piece of tumor tissues was lysed in 100 μL tissue lysis buffer. The lysates were incubated on ice for a total of 30 min and vortexed every 5 min. The lysates were centrifuged for 10 min at 12 000g. Proteins were then detected by Western blot analyses as described above. Furthermore, the residual tumor tissues were fixed with 4% paraformaldehyde overnight at 4 °C and embedded in paraffin for analysis. Tissue sections (6 μm) were stained with

hematoxylin/eosin (H&E). The TUNEL assay was applied to further detect the cell apoptosis in tumor tissues according to the manufacturer's instructions. The expression levels of Ki-67 in tumor tissues after different treatments were conducted by immunohistochemical staining. All sections were examined on the inversion fluorescence microscope (Leica DMI4000B).

1.7 Statistical Analysis

Data are presented as means with standard deviations (SD) of at least five independent samples, and each measurement was performed in triplicate. Statistical analysis was determined by analysis of variance tests (ANOVA) using the software of Microsoft Excel 2007. Data sets were compared using two-tailed, unpaired t tests. A p value of < 0.05 was considered to be statistically significant.

Reference

1. Q. Jiang, D. Yue, Y. Nie, X. Xu, Y. He, S. Zhang, E. Wagner and Z. Gu, *Molecular Pharmaceutics*, 2016, **13**, 1809-1821.
2. Q. Jiang, Y. Nie, X. Chen, Y. He, D. Yue and Z. Gu, *Advanced Functional Materials*, 2017.
3. Y. Nie, M. Gunther, Z. Gu and E. Wagner, *Biomaterials*, 2011, **32**, 858-869.

2. Results

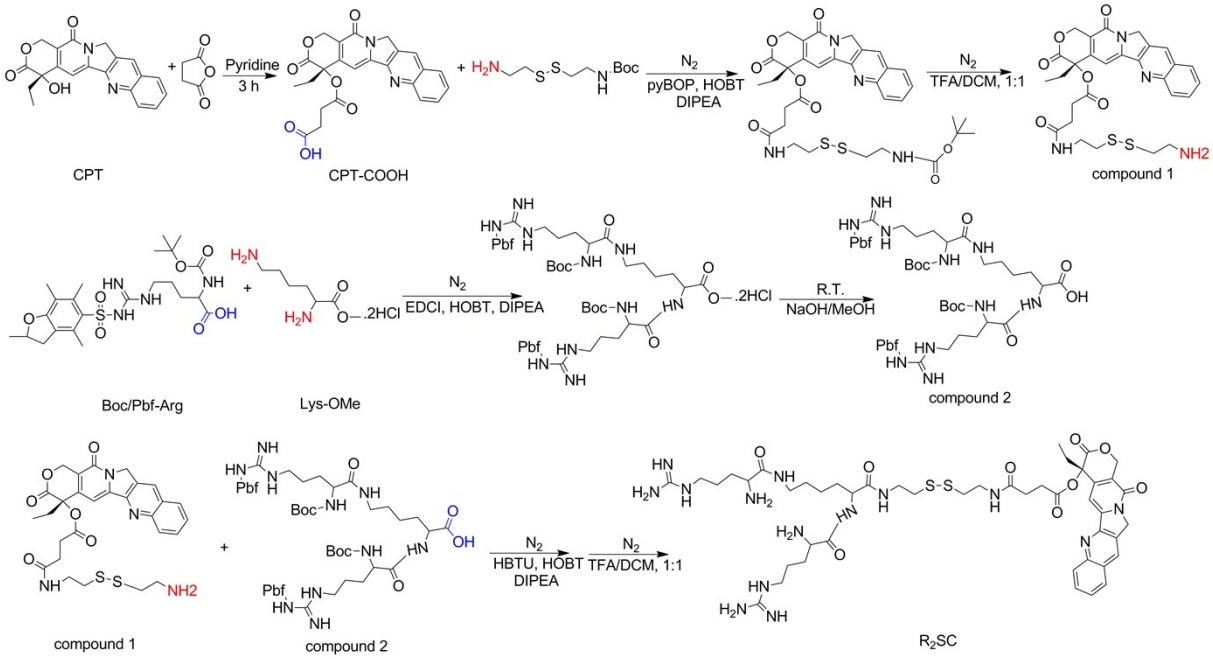

Figure S1. Synthesis process of disulfide bond containing dendritic arginine-camptothecin conjugation (R_2SC) as three-stage step rocket.

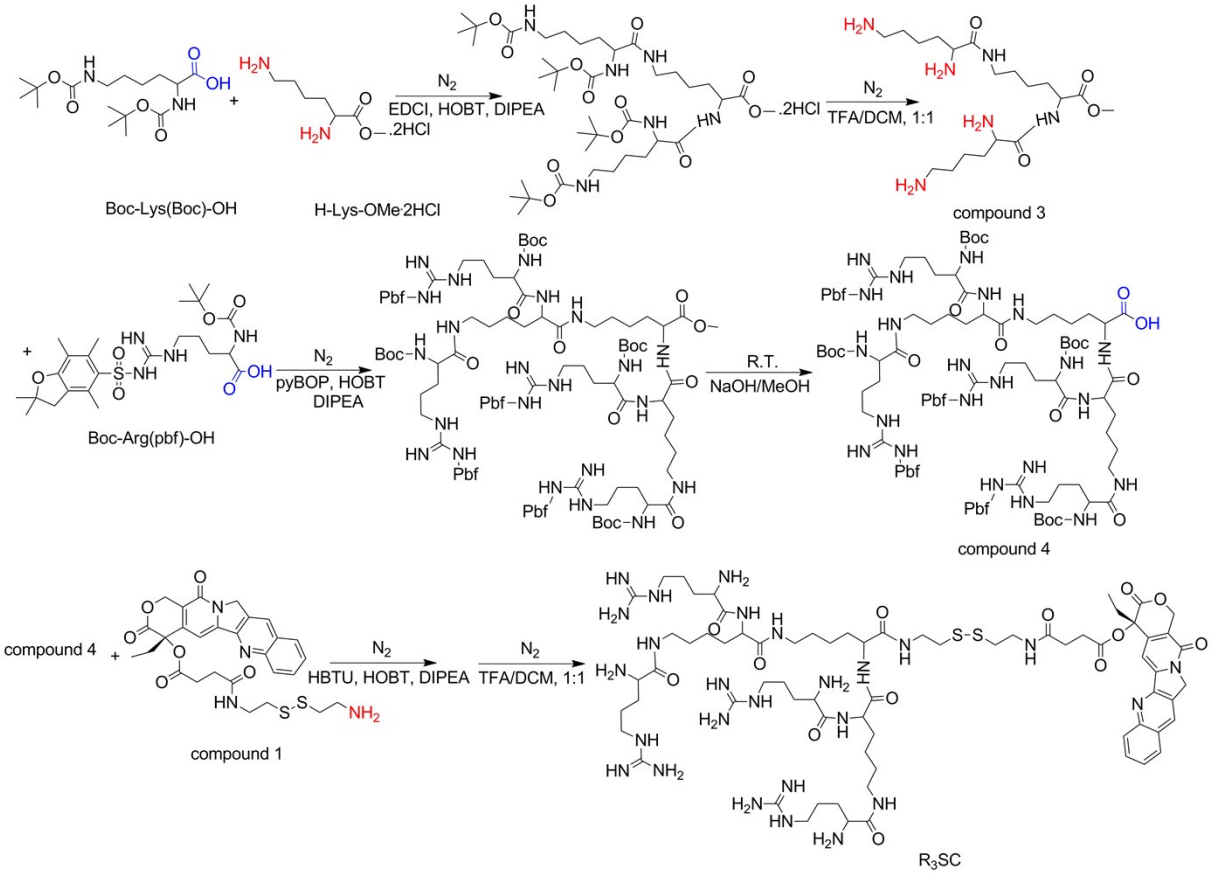

Figure S2. Synthesis process of disulfide bond containing dendritic arginine-camptothecin conjugation (R_3SC) as three-stage step rocket.

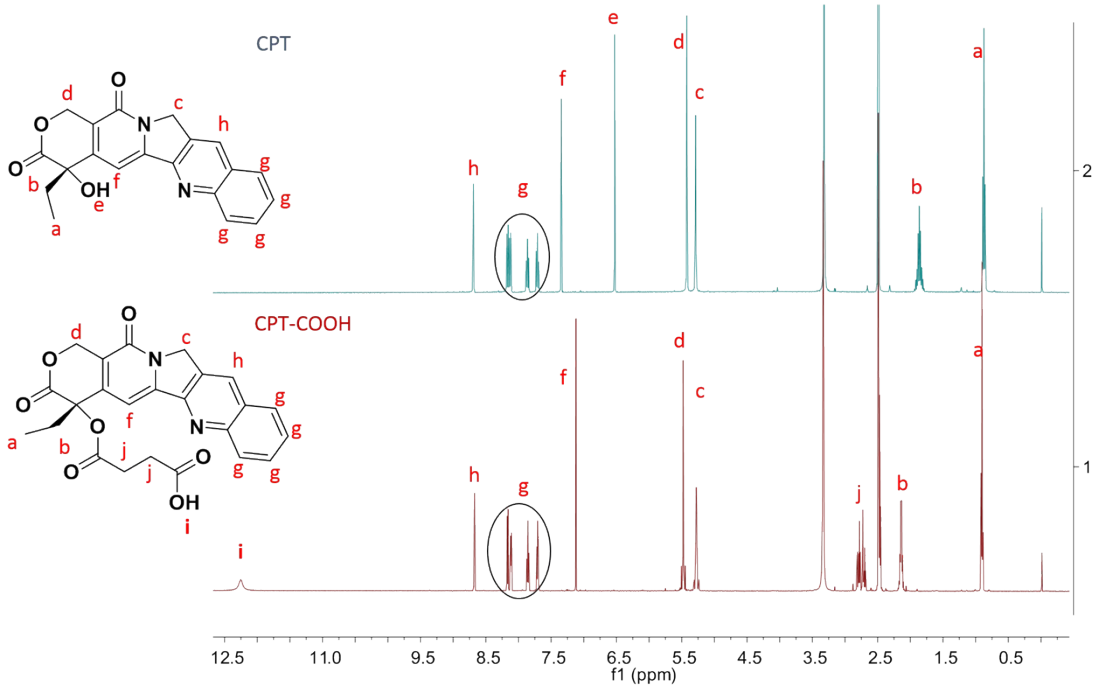

Figure S3. ^{1}H-NMR of camptothecin and carboxyl modified camptothecin (CPT-COOH).

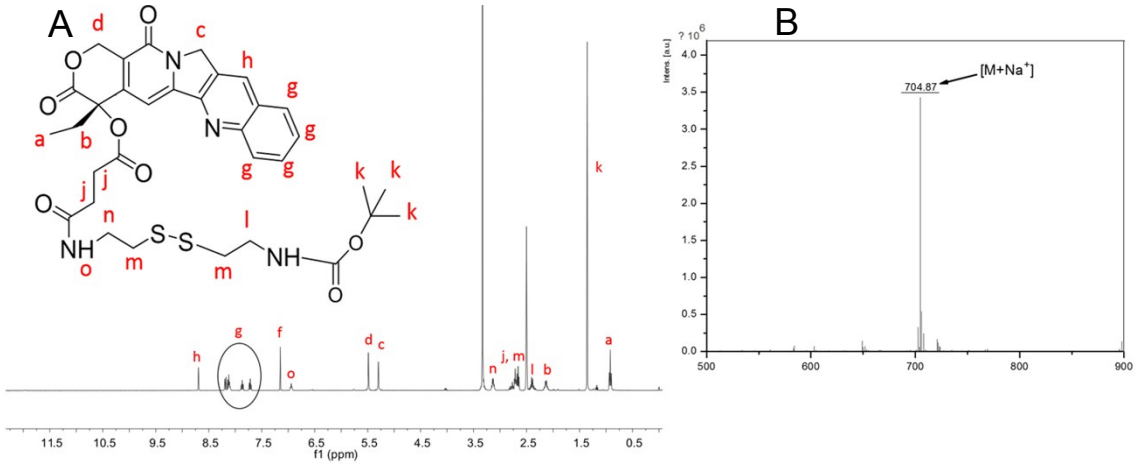

Figure S4. ^{1}H-NMR (A) and MALDI-TOF MS (B) results of disulfide bond modified camptothecin.

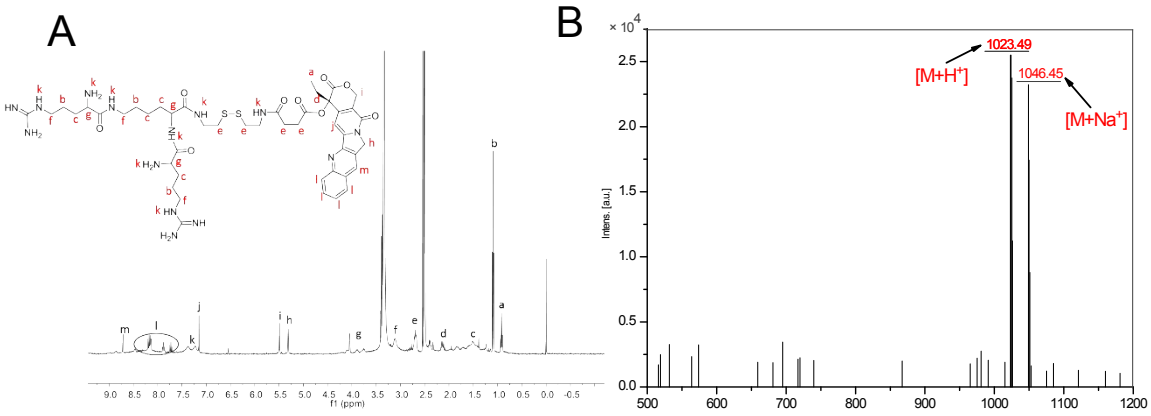

Figure S5. Chemical characterization (^{1}H-NMR spectrum and MALDI-TOF-MS) of disulfide bond containing dendritic arginine-camptothecin prodrug conjugation as three-stage rocket. (A) ^{1}H-NMR spectrum of R_2SC. (B) ESI-MS of R_2SC.

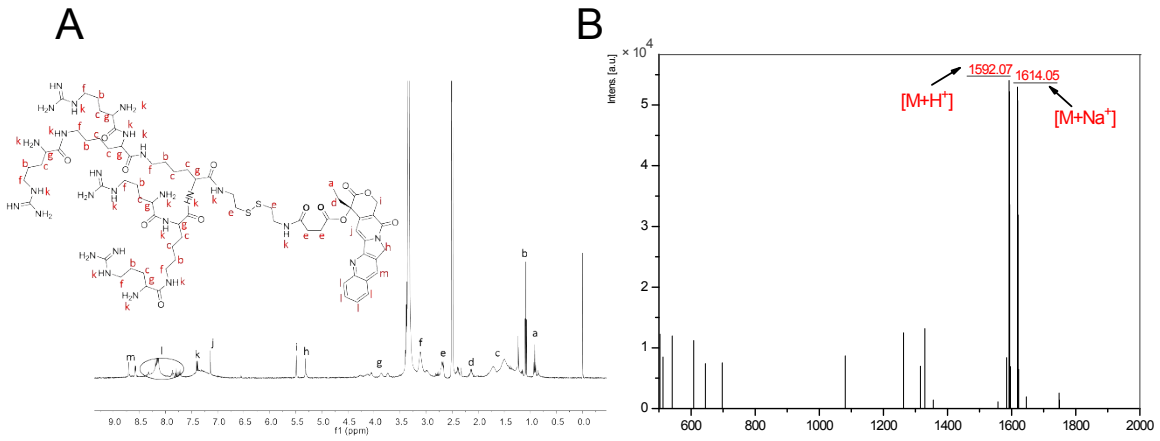

Figure S6. Chemical characterization (^{1}H-NMR spectrum and MALDI-TOF-MS) of disulfide bond containing dendritic arginine-camptothecin prodrug conjugation as three-stage rocket. (A) ^{1}H-NMR spectrum of R_3SC. (B). ESI-MS of R_3SC.

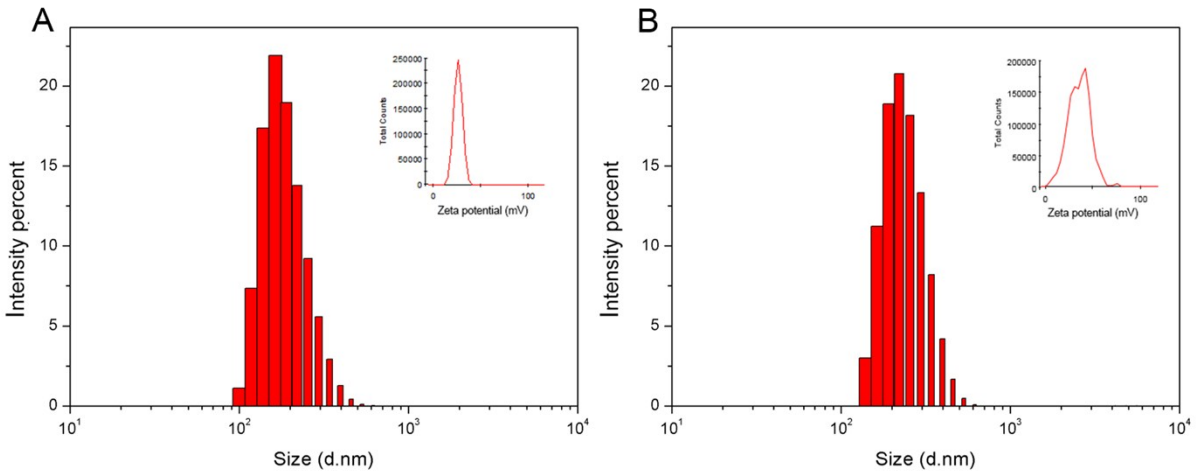

Figure S7. The size distribution, zeta potential of dendritic arginine-camptothecin conjugation R_2SC (A) and R_3SC (B) assemblies.

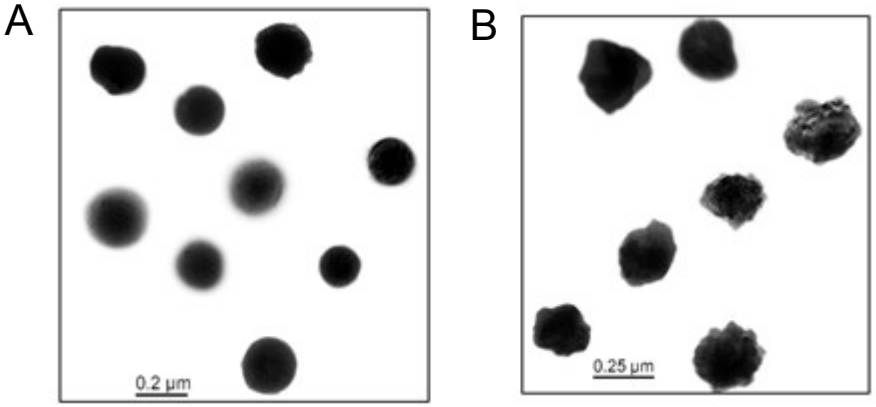

Figure S8. Transmission electron microscope (TEM) image of dendritic arginine-camptothecin conjugation R_2SC (A) and R_3SC (B) assemblies.

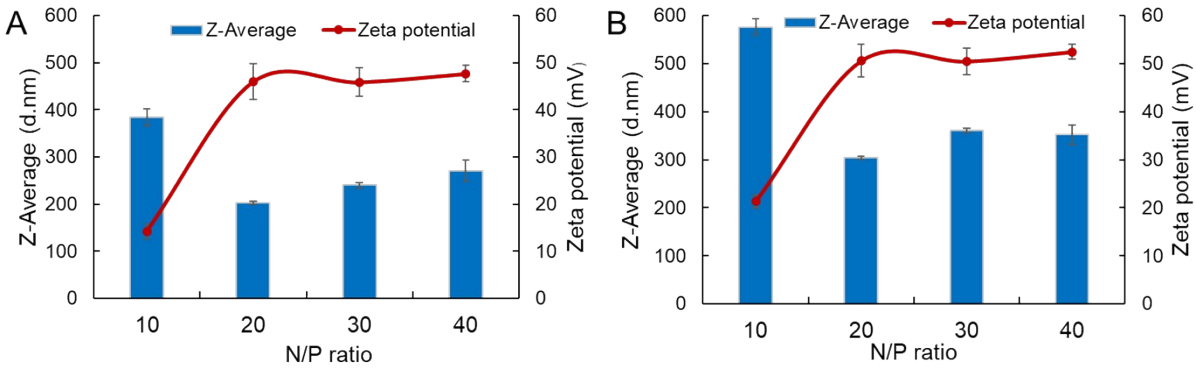

Figure S9. Size distribution and zeta potential of R_2SC/siRNA (A) and R_3SC /siRNA (B) complexes at different N/P ratios.

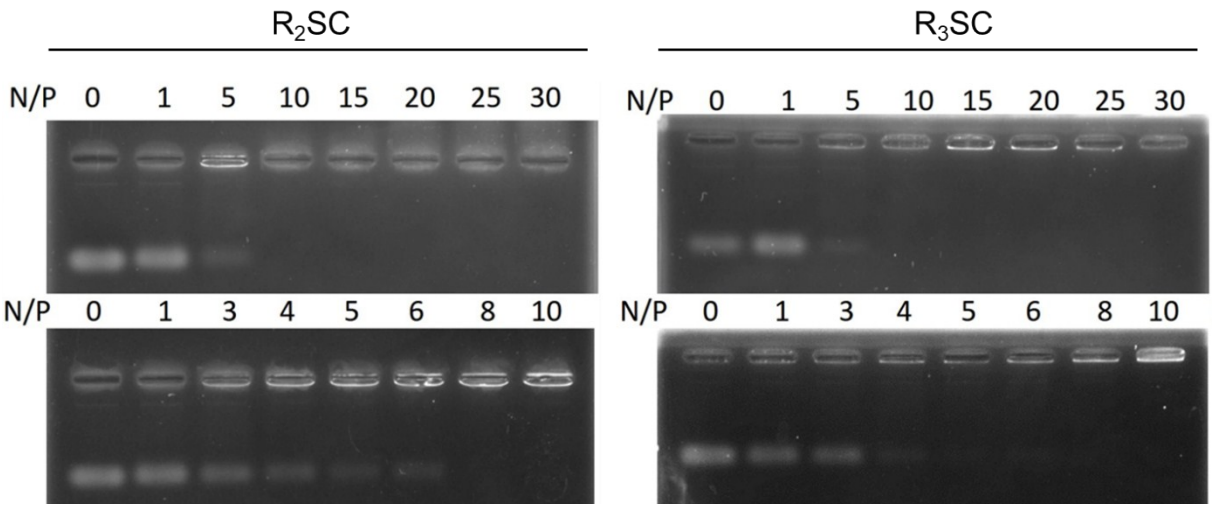

Figure S10. Agarose gel retardation assay of R_2SC/siRNA (A) and R_3SC/siRNA complexes (B) at different N/P ratios.

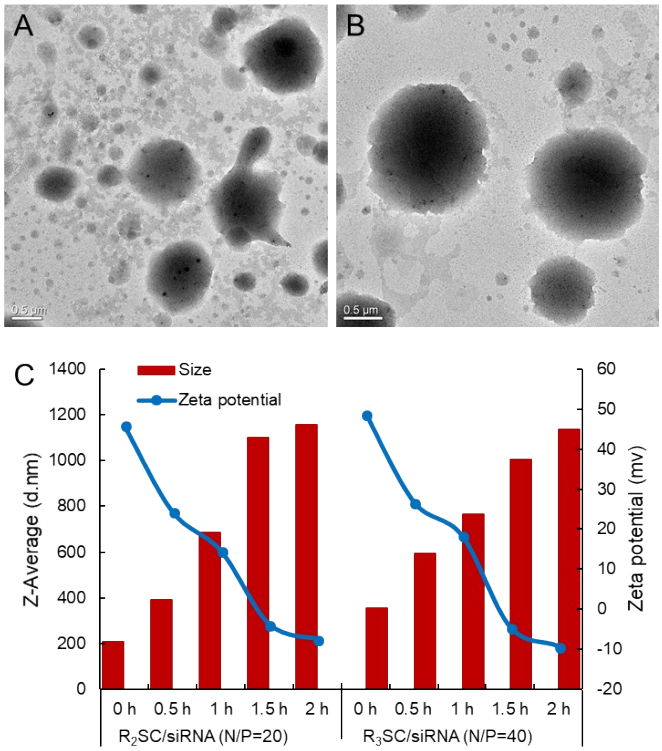

Figure S11. Transmission electron microscope (TEM) image of R_2SC assemblies after incubation with GSH for 1 (A) and 2 h (B). (C) Size distribution and zeta potential of $R_{2/3}SC$/siRNA with 5 mM GSH.

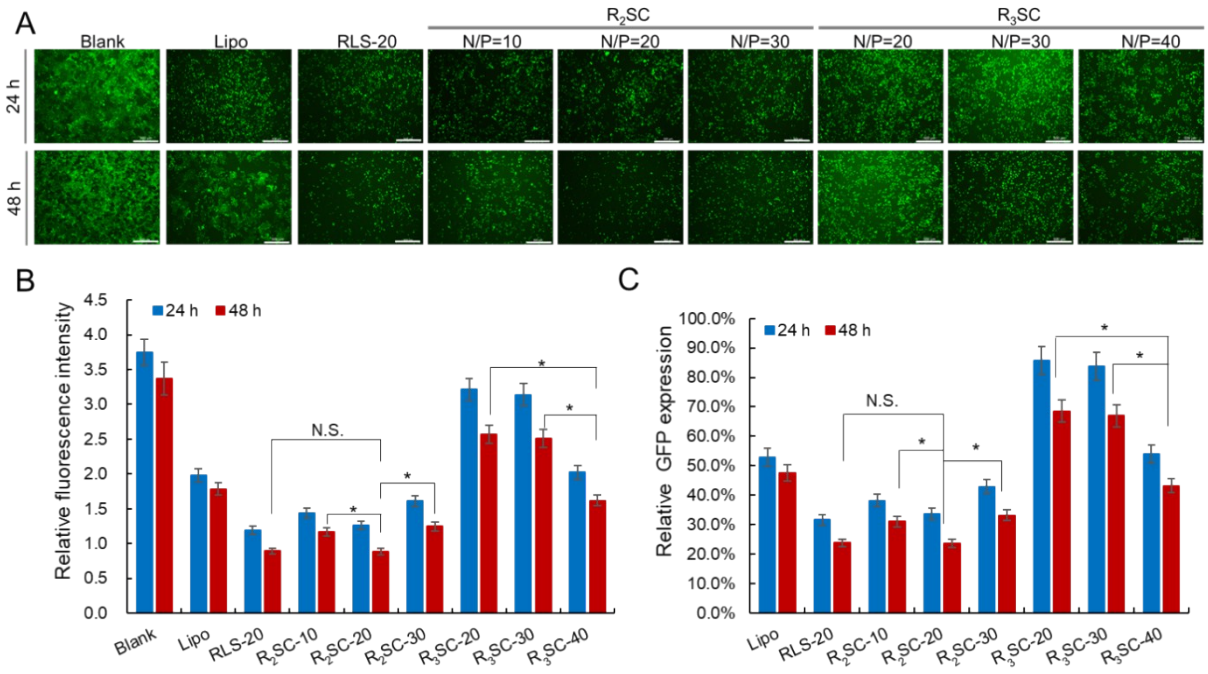

Figure S12. Silence target GFP expression in GFP-Hela cell by $R_{2/3}SC$/siGFP complexes at different N/P ratios with 10% FBS. (A). The fluorescent images of GFP-Hela cells treated with various siGFP complexes. (B). The semi-quantitative analysis of relative fluorescence intensity by Image-Pro Plus 6.0 software. The relative fluorescence intensity was calculated by dividing the total intensity of green fluorescence (siRNA) to the total cell numbers. (C). Relative GFP expression in various group. The GFP-expression level of Blank cells was set as 100 %. *$p < 0.05$, N.S. not significant.

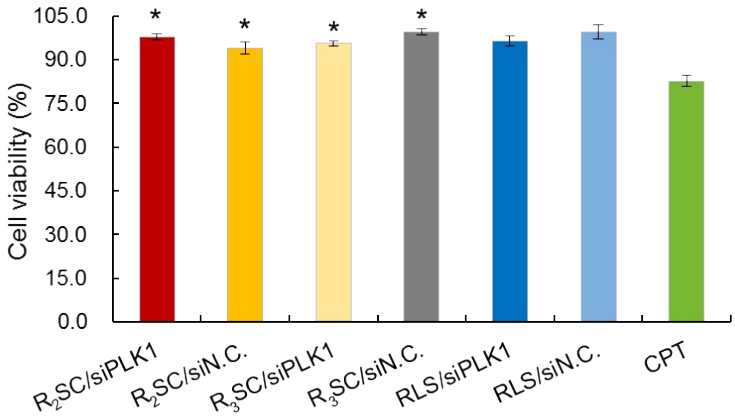

Figure S13. *In vitro* cytotoxicity of different complexes on Hela cells after 24 h incubation. The concentration of free CPT was equal to the concentration of CPT for prodrug-based complexes corresponding to siRNA concentration. Data represent mean ± S.D. * $p < 0.05$ vs. RLS/siN.C..

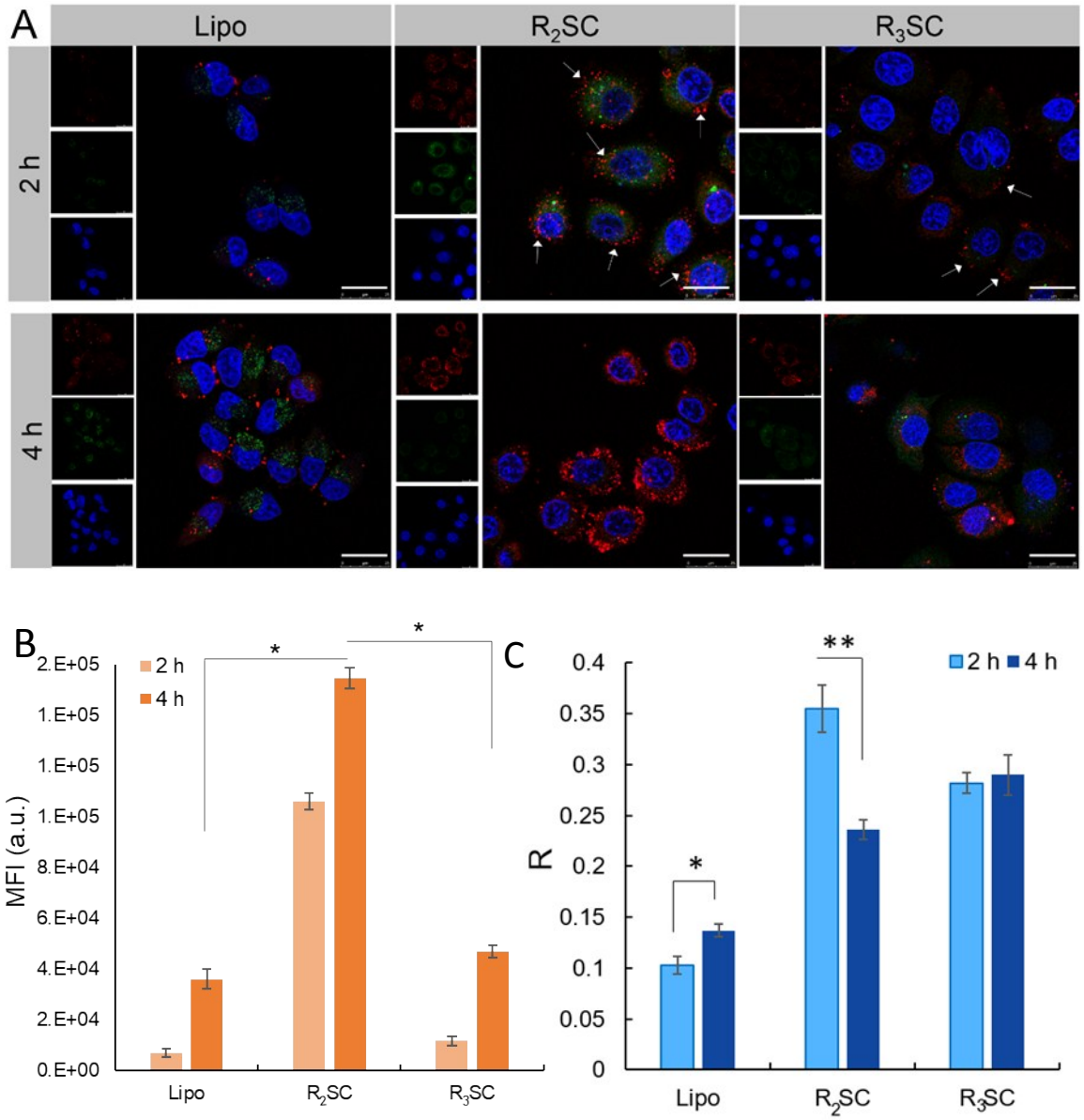

Figure S14. (A) Confocal laser scanning microscopy (CLSM) images of the cellular distribution of different complexes with Cy5-siRNA (red) in Hela cells after 2 and 4 h of incubation at 37°C in the presence of 10% serum. The red fluorescence represents Cy5-labelled siRNA cluster, green ones represents Lysotracker Green-stained lysosomes and the blue represents Hoechst 33342-stained nucleus. (B). Semi-quantitative evaluation of the cellular internalization/uptake was assessed by mean fluorescent intensity (MFI) comparison of red fluorescence. (C). Co-localization ratio (R) of fluorescence intensity of Cy5-siRNA and Lysotracker Green was qualified with CLSM. (Scale bar: 25 μm). * $p < 0.05$, ** $p < 0.01$.

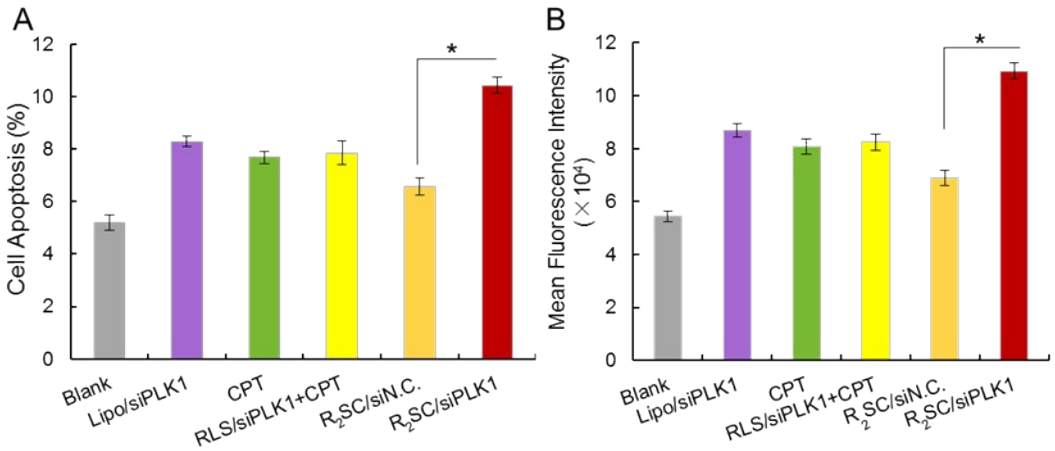

Figure S15. The percentage of apoptotic cells (A) and mean fluorescence intensity (B) of Hela cells incubated with various complexes. The apoptosis cells were stained by TUNEL cell apoptosis detection kit and detected on flow cytometry. *$p < 0.05$.

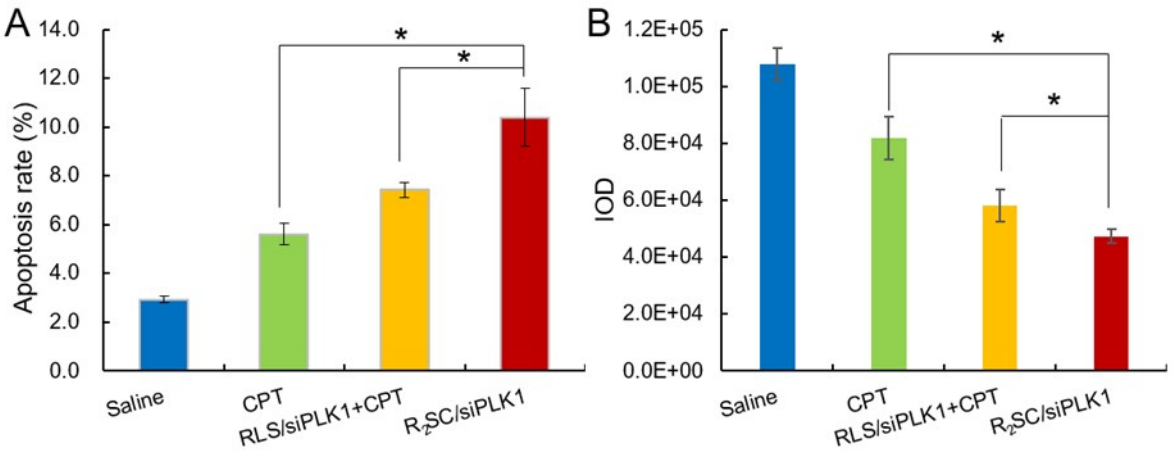

Figure S16. Semi-quantitative assay of TUNEL (A) and Ki-67 (B) immunohistochemical staining of tumors by Image-Pro Plus 6.0 software. *$p < 0.05$.