Supporting information

Development of a novel sialic acid-conjugated camptothecin prodrug for enhanced cancer chemotherapy

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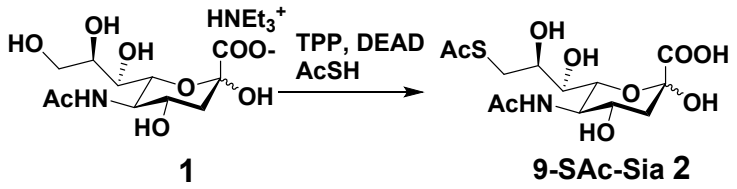
Materials

All chemicals were reagent grade and were used as received from the manufacturer unless otherwise noted. S-(+)-Camptothecin, triphosgene, diethyl azodicarboxylate (DEAD) and 2-(2-pyridinyldithio) ethanol were purchased from Shanghai Macklin. Thioacetic acid and triphenylphosphine (TPP) were purchased from J&K Scientific Ltd. Gel filtration chromatography was performed using a column (100 cm × 2.5 cm) packed with BioGel P-2 Fine resins (Bio-Rad, Hercules, CA). 4T1, H22 and EL4 cells were obtained from the American Culture Collection (ATCC) and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), penicillin (100 U mL^{-1})/streptomycin (100 μg mL^{-1}), L-glutamine (2 mM), and sodium pyruvate (1 mM). These cells were cultured at 37 ℃ in a 5% CO_2/air incubator. Confocal microscopic images were performed on Leica SP8 using the following filters: λex@405 nm and λem@410−450 nm for CPT; λex@488 nm and λem@500−530 nm for MitoTracker Green and JC-10 (green fluorescence); λex@543 nm and λem@555−600 nm for JC-10 (red fluorescence). The BALB/c, KM (Chinese Kun Ming) and C57BL/6 female mice aged 6−8 weeks were provided from the Laboratory Animal Center of Shandong University. All animal experiments were conducted by the guidelines of the Animal Care and Use Committee of Shandong University.

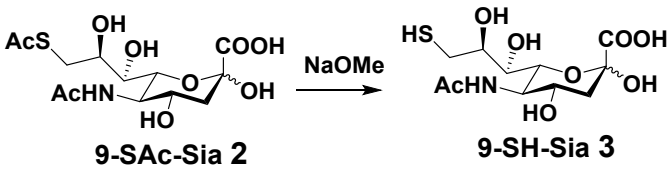
Synthesis of 9-SAc-Sia



1 **9-SAc-Sia 2**

DEAD (4.67 mL, 30 mmol) was added dropwise to THF (100 mL) containing TPP (7.869 g, 30 mmol) and stirred for 10 min at 0 °C to obtain the TPP/DEAD adduct. Meanwhile, the triethylamine salt form of Sia **1** (3.081 g, 10 mmol) was dissolved in anhydrous DMF (250 mL) containing 2.8 mL of thioacetic acid. Then, the DMF solution was added to the preformed TPP/DEAD adduct in THF. After reacting for 3 h at room temperature (RT), methanol was dropped to terminate the reaction. The solvent was removed by rotary evaporation. The crude product was extracted with EtOAc/Et_2O/H_2O (1:1:1). The aqueous layer was concentrated and purified by silica gel column chromatography (DCM/MeOH, 5:2) to obtain 9-SAc-Sia **2** (2.010 g, 55%).

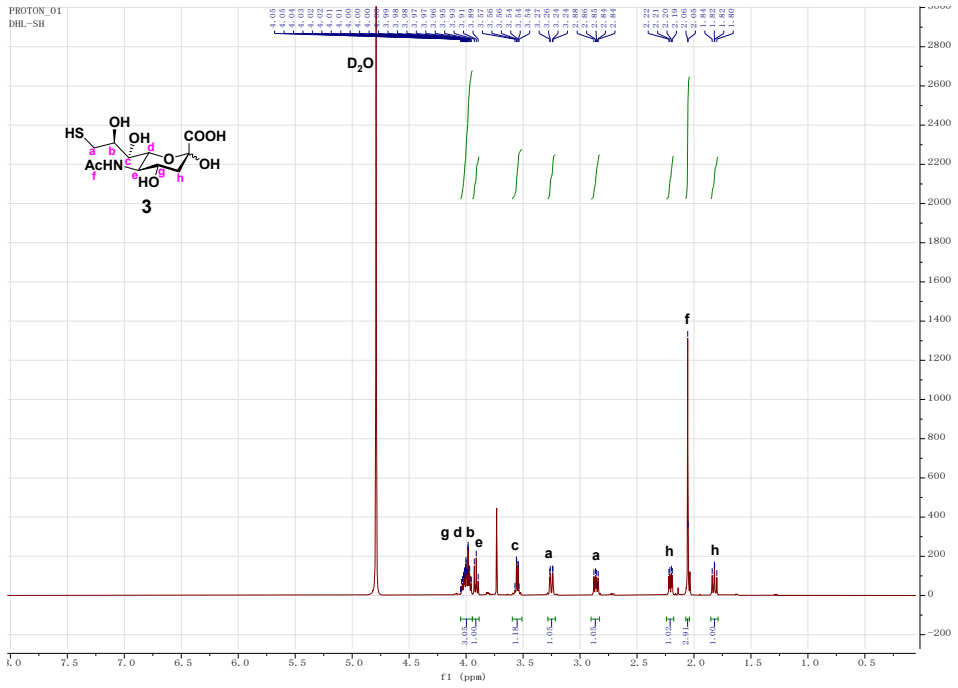
Synthesis of 9-SH-Sia 3



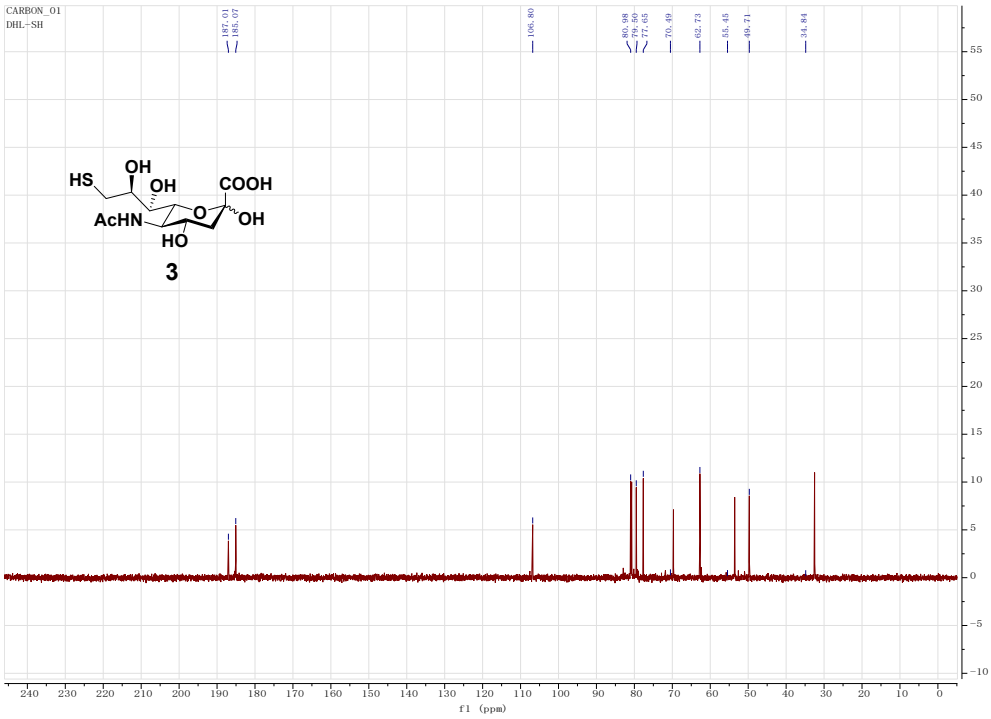
9-SAc-Sia 2 9-SH-Sia 3

9-SAc-Sia **2** (500 mg, 1.486 mmol) was dissolved in MeOH (10 mL), followed by the addition of 1 M NaOMe in MeOH (1 mL) under oxygen-free conditions in an ice bath. The solution was kept for 2 h at 0 ℃, then neutralized with Amberlite IRC-120 (H) and filtered. After the solvent was removed by a rotary evaporator, 5 mL of water was added to dissolve the sample and freeze-dried to obtain 450 mg of 9-SH-Sia **3** (yield: 93 %). ^{1}H NMR (600 MHz, D_2O) δ 4.05 − 3.95 (m, 3H), 3.91 (t, *J* = 10.2 Hz, 1H), 3.55 (dd, *J* = 8.2, 1.2 Hz, 1H), 3.25 (dd, *J* = 14.2, 2.9 Hz, 1H), 2.86 (dd, *J* = 14.2, 8.6 Hz, 1H), 2.20 (dd, *J* = 12.9, 4.9 Hz, 1H), 2.06 (s, 3H), 1.82 (dd, *J* = 13.0, 11.5 Hz, 1H). ^{13}C NMR (150 MHz, D_2O) δ 187.01, 185.07, 106.80, 80.98, 79.50, 77.65, 70.49, 62.73, 55.45, 49.71, 34.84. HRMS (ESI) m/z calcd for $C_{11}H_{19}NO_8S$ $[M-H]^-$ calcd 324.0759, found 324.0736.

(a)



(b)



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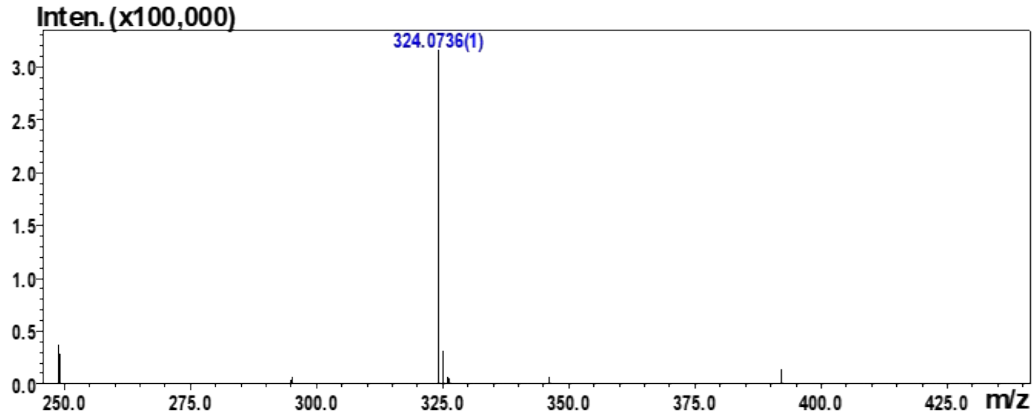
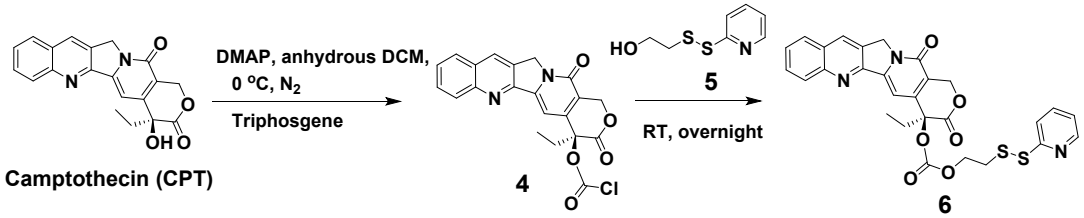


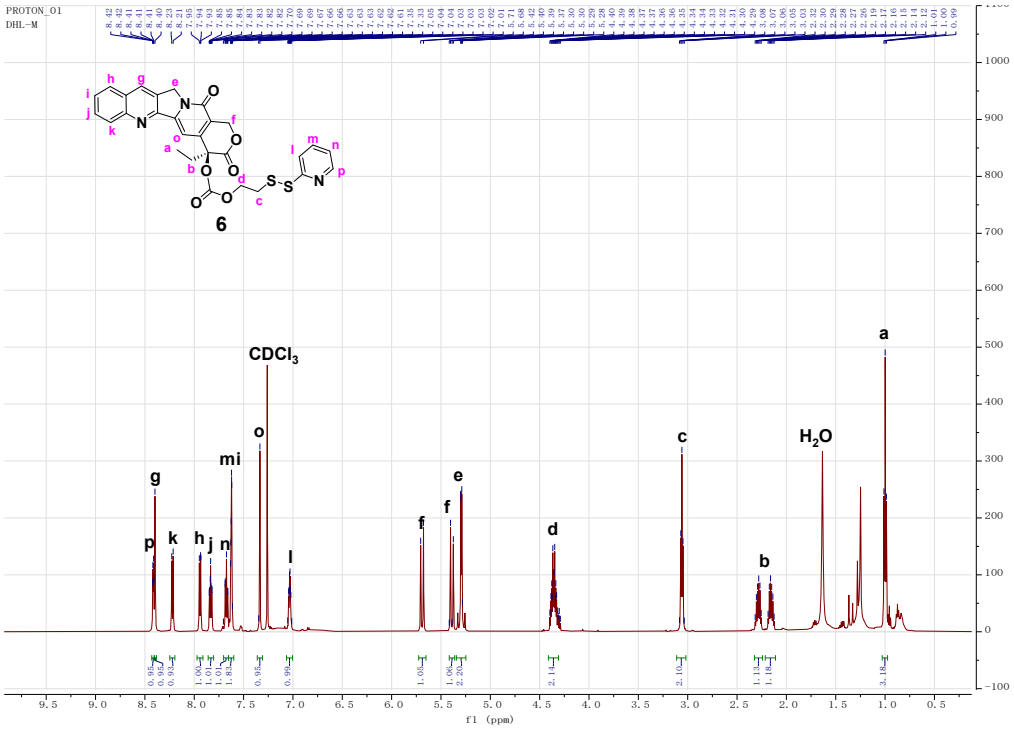
Fig. S1 (a) ^{1}H-NMR spectra recorded in D_2O for 9-SH-Sia **3**, (b) ^{13}C-NMR spectra recorded in D_2O for 9-SH-Sia **3**, and (c) HRMS of 9-SH-Sia **3**.

Synthesis of CPT pyridyldithioethyl carbonate (6)



CPT (500 mg) and DMAP (351 mg) were dissolved in anhydrous DCM (10 mL) under nitrogen protection at 0 °C, followed by the addition of triphosgene (149 mg) in anhydrous DCM. After stirring at 0 °C for 20 min, CPT acid chloride (**4**) was obtained, which was directly used for the next step of the reaction. Next, to the solution of **4** was added 2-(2-pyridinyldithio) ethanol **5** (269 mg). The reaction was performed overnight at RT. Finally, a rotary evaporator removed the solvent, and the crude product was purified by silica gel chromatography (DCM/MeOH, 120∶1) to obtain **6** with a yield of 65%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.42 (dd, J = 4.6, 1.6 Hz, 1H), 8.40 (s, 1H), 8.22 (d, J = 8.5 Hz, 1H), 7.93 (m, 1H), 7.83 (ddd, J = 8.3, 6.7, 1.4 Hz, 1H), 7.70 − 7.65 (m, 1H), 7.63 − 7.61 (m, 2H), 7.33 (s, 1H), 7.03 (td, J = 5.2, 2.9 Hz, 1H), 5.69 (d, J = 17.1 Hz, 1H), 5.39 (d, J = 17.1 Hz, 1H), 5.31 − 5.27 (m, 2H), 4.41 − 4.28 (m, 2H), 3.06 (t, J = 6.6 Hz, 2H), 2.29 (dq, J = 14.8, 7.4 Hz, 1H), 2.15 (dq, J = 14.8, 7.5 Hz, 1H), 1.00 (t, J = 7.5 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.05, 160.54, 156.63, 153.52, 152.40, 149.83, 148.62, 146.62, 145.66, 137.84, 132.11, 130.87, 130.29, 128.56, 128.32, 128.31, 128.24, 121.04, 120.42, 118.94, 96.13, 80.47, 67.22, 66.52, 50.13, 36.60, 32.51, 6.86. HRMS (ESI) m/z calcd for $C_{28}H_{23}N_3O_6S_2$ $[M+H]^+$ 562.1101, found 562.1125.

a)



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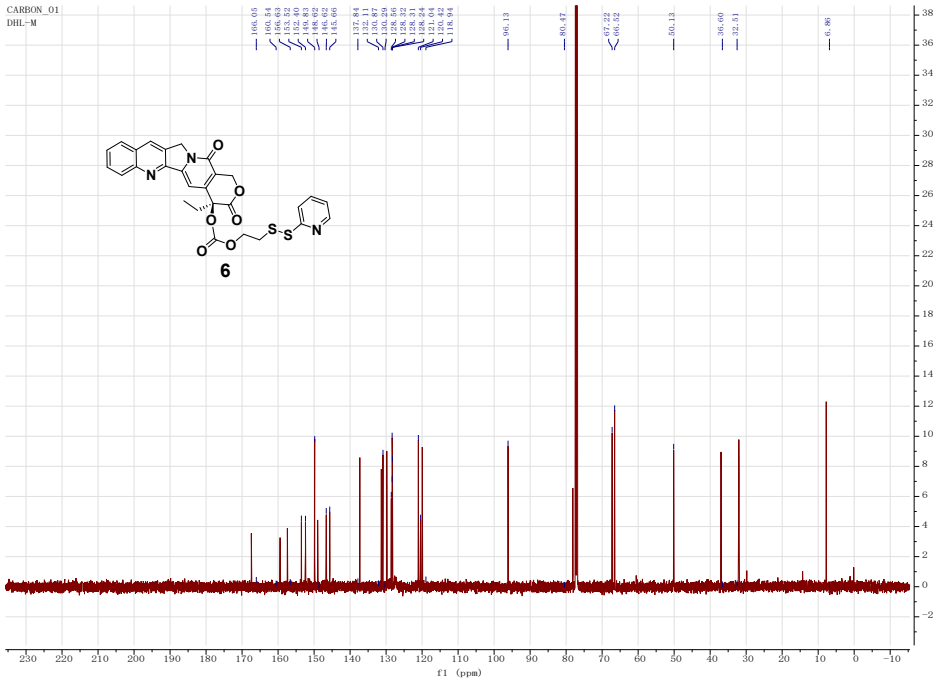

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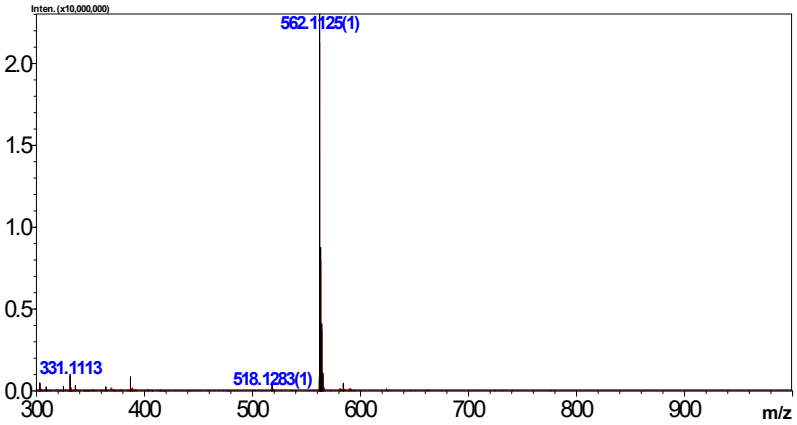
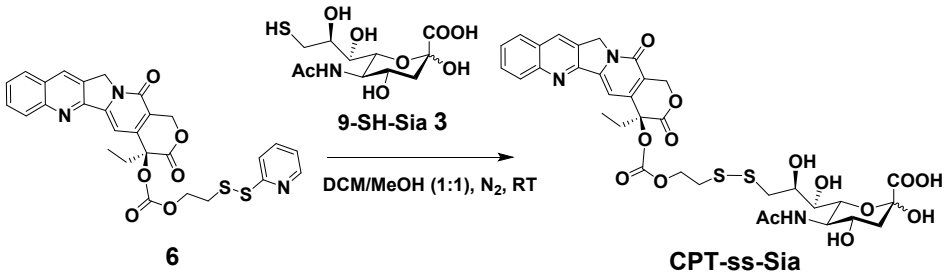


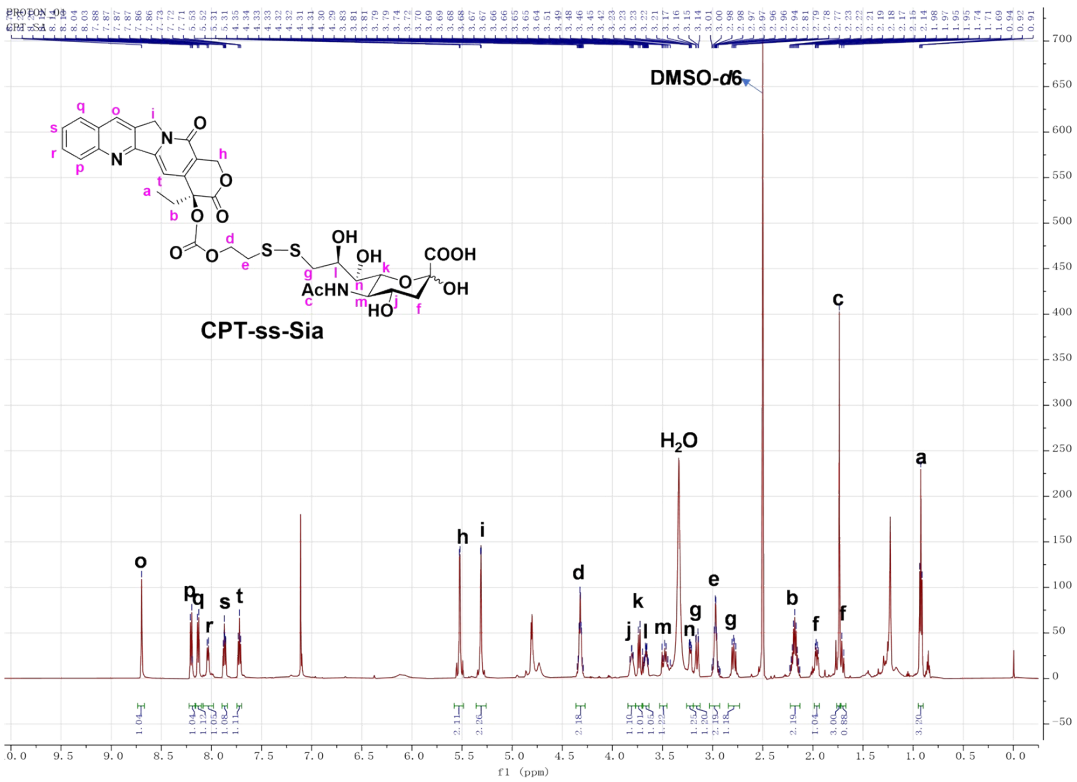
Fig. S2 (a) ^{1}H-NMR spectra recorded in $CDCl_3$ for **6**, (b) ^{13}C-NMR spectra recorded in $CDCl_3$ for **6**, and (c) HRMS of **6**.

Synthesis of CPT-ss-Sia



100 mg of **6** (0.18 mmol) was dissolved in anhydrous DCM/MeOH (v/v, 2:1), followed by the addition of 9-SH-Sia **3** (87.77 mg, 0.27 mmol). After stirring overnight at RT, the reaction solution was concentrated and purified by flash column chromatography (DCM/MeOH, 10:1→1:1), affording CPT-ss-Sia as a white solid (95 mg, yield: 68%). ^{1}H NMR (600 MHz, DMSO-*d*6) δ 8.70 (s, 1H), 8.20 (d, *J* = 8.5 Hz, 1H), 8.13 (d, *J* = 8.1 Hz, 1H), 8.04 (d, *J* = 8.3 Hz, 1H), 7.90 − 7.84 (m, 1H), 7.72 (t, *J* = 7.5 Hz, 1H), 5.52 (d, *J* = 4.1 Hz, 2H), 5.31 (d, *J* = 2.7 Hz, 2H), 4.36 − 4.29 (m, 2H), 3.81 (m, 1H), 3.73 (d, *J* = 10.5 Hz, 1H), 3.69 − 3.63 (m, 1H), 3.54 − 3.41 (m, 1H), 3.24 − 3.20 (m, 1H), 3.15 (dd, *J* = 13.4, 2.4 Hz, 1H), 2.97 (td, *J* = 6.1, 3.1 Hz, 2H), 2.79 (dd, *J* = 13.2, 9.3 Hz, 1H), 2.24 − 2.12 (m, 2H), 1.96 (dd, *J* = 13.1, 5.1 Hz, 1H), 1.74 (s, 3H), 1.70 (d, *J* = 12.2 Hz, 1H), 0.92 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-*d*6) δ 172.26, 171.77, 167.56, 156.95, 153.26, 152.70, 148.38, 146.68, 145.23, 132.04, 130.91, 130.26, 129.48, 128.96, 128.46, 128.21, 119.57, 95.21, 94.99, 78.35, 71.74, 70.53, 68.32, 66.89, 66.48, 66.07, 53.49, 50.78, 45.81, 36.16, 30.69, 29.46, 22.82, 8.01. HRMS (ESI) m/z calcd for $C_{34}H_{37}N_3O_{14}S_2$ $[M+H]^+$ calcd 776.1790, found 776.1805; $[M+Na]^+$ calcd 798.1609, found 798.1605.

(a)



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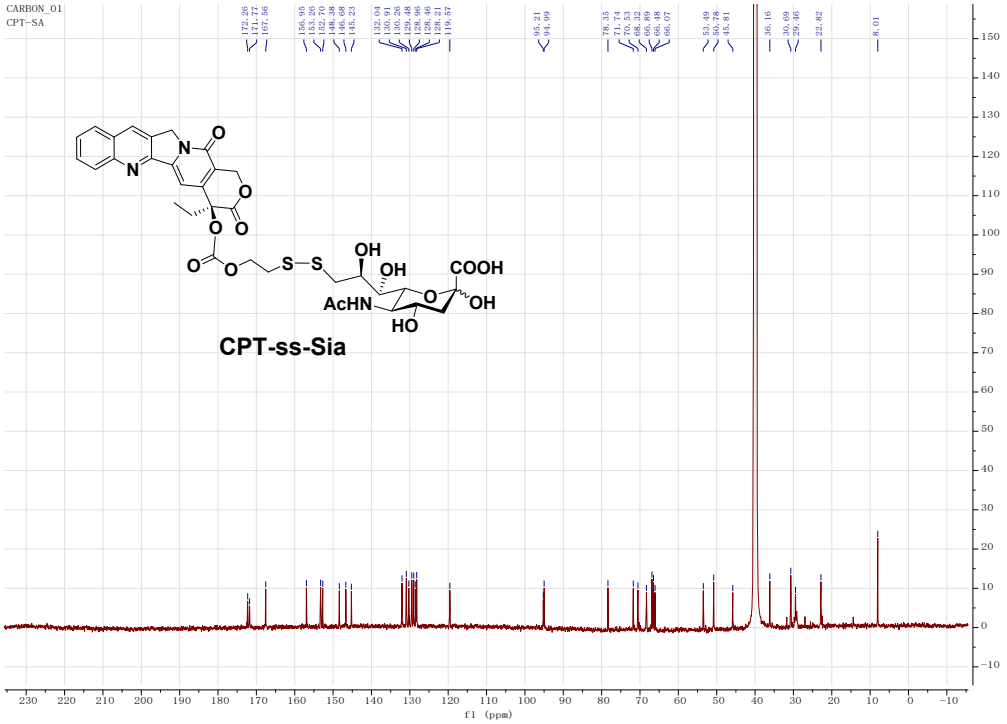

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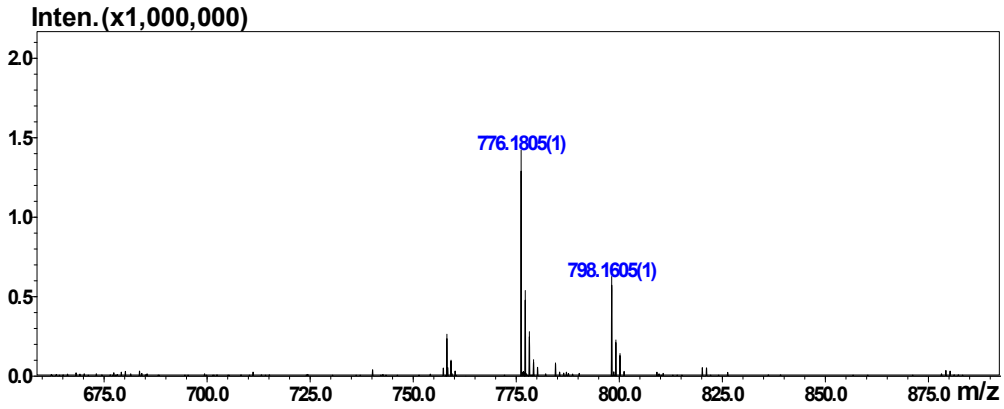

Fig. S3 (a) ^{1}H-NMR spectra recorded in DMSO-*d*6 for CPT-ss-Sia, (b) ^{13}C-NMR spectra recorded in DMSO-*d*6 for CPT-ss-Sia, and (c) HRMS of CPT-ss-Sia.

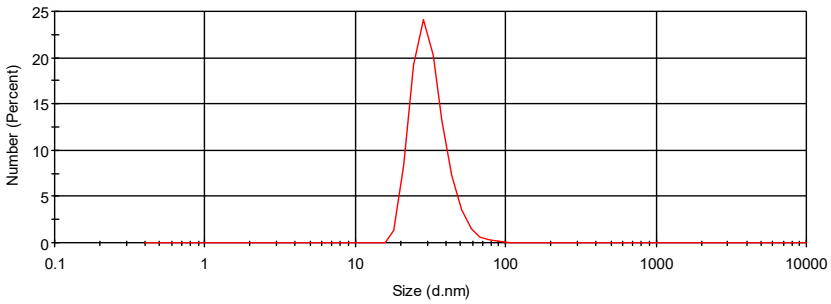

Fig. S4 Dynamic light scattering (DLS) analysis of CPT-ss-Sia in water. The result shows that the average diameter of CPT-ss-Sia is 35 nm.

Stability of lactone in physiological conditions

CPT and CPT-ss-Sia (1 mg mL^{-1}) were dissolved in DMSO. The solution was then diluted to 5 μg mL^{-1} with PBS at pH 7.4 and kept at 37 ℃. At the indicated time, the solution (10 μL) was analyzed by high-performance liquid chromatography (HPLC). The proportion of lactone hydrolysis was determined by comparing the HPLC peak area of the lactone form and the carboxylate form.

Hemolysis test of CPT and CPT-ss-Sia

Collect fresh blood from the mouse saphenous vein to heparin tubes. The red blood cells (RBCs) were obtained after centrifugation at 1000 rpm for 5 min. The RBCs were then washed with PBS and resuspended in PBS to a concentration of 2×10^7 cells/200 μL. To the RBCs were added CPT or CPT-ss-Sia. After incubating at 5% CO_2, 37 °C for 1−4 h, the RBCs were centrifuged at 12000 rpm for 5 min. The absorbance of the resulting supernatant was measured at 541 nm using a microplate reader. All samples were tested in triplicate.

Intracellular localization of CPT-ss-Sia

4T1 cells were seeded into laser confocal cell culture dishes at a density of 5×10^5 cells per dish and incubated in an incubator (5% CO_2) at 37 ℃ for 12 h. Then the cells were incubated with CPT or CPT-ss-Sia (20 μM). 5 h later, the cell medium was removed, and the cells were washed 3 times with PBS, then stained with Mitotracker Green for 30 min. After washing with cold PBS, the cells were imaged by a confocal laser fluorescence microscopy.

Evaluation of mitochondrial dysfunction

4T1 cells were seeded in cell culture dishes (5×10^5 cells) with an incubator at 37 ℃ with 5% CO_2 overnight. CPT-ss-Sia (20 μM) was added to the corresponding confocal dish. After 5 h of incubation, the medium was removed. The resulting cells were washed with PBS and stained with JC-10, a mitochondrial membrane potential fluorescent probe. After staining for 30 min, the cells were washed with PBS and imaged by a confocal laser fluorescence microscopy.

Cytotoxicity experiments

Cells (4T1, H22 and EL4) were seeded in 96-well plates at 5×10^3 per well and incubated overnight. Then, CPT or CPT-ss-Sia (0.001、0.005、0.01、0.05、0.1、0.2、0.4、0.8、1、2、4 μg) were added to the corresponding wells. After 18 h of incubation, the medium and residue CPT or CPT-ss-Sia were removed. Notably, suspension cells should be centrifuged before the extraction of the medium. Next, to each well was added fresh medium and 10 μL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (5 mg mL^{-1}). After incubating for 4 h, the cell medium containing MTT was removed, and 100 μL of dimethyl sulfoxide (DMSO) was added to each well. The 96-well plate was gently shaken for 10 min to ensure that the formazan was fully dissolved. The absorbance was measured at 570 nm using a microplate reader. All samples were tested in triplicate.

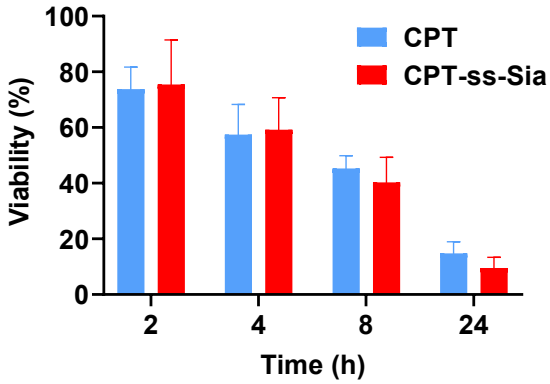

Fig. S5 Cytotoxicity of CPT and CPT-ss-Sia to 4T1 cells. 4T1 cells were incubated with CPT or CPT-ss-Sia (20 μM) for 2−24 h. The cytotoxicity of CPT and CPT-ss-Sia on 4T1 cells was tested by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Pharmacokinetics studies

CPT and CPT-ss-Sia (with the same amount of CPT, 4.1 mg kg^{-1}) were respectively injected into BALB/c mice. At the indicated times, 80 μL of mouse blood was collected into heparin tubes and centrifuged to get plasma. Each part of plasma was added with excess DTT solution and reacted for 1 h at RT. 0.1 M HCl and 8 volumes of acetonitrile were then added to make the protein precipitation. After that, the protein precipitation was removed by centrifugation at 12,000 rpm for 20 min, and HPLC was used to analyze the supernatant.

Tumor challenge studies

On day 0, 4T1, H22 and EL4 tumor cells (1×10^6) were injected subcutaneously into BALB/c, KM and C57BL/6 mice, respectively. Then, for 4T1 tumor challenge, on days 7 and 9, the BALB/c mice were intravenously injected with PBS, CPT, or CPT-ss-Sia (with the same amount of CPT, 4.1 mg kg^{-1}) *via* the tail vein. For H22 tumor challenge, on days 5 and 7, the KM mice were intravenously injected with PBS, CPT, or CPT-ss-Sia with the same amount of CPT (2.4 mg kg^{-1}) through the tail vein. For EL4 tumor challenge, on days 8 and 10, the C57BL/6 mice were intravenously injected with PBS, CPT, or CPT-ss-Sia (with the same amount of CPT, 4.1 mg kg^{-1}) through the tail vein. The body weights and the tumors were recorded daily. Tumor volume (mm^3) calculation formula: 1/2 (length × width × height). After the treatment, the mice were euthanized, and the tumors of the mice were dissected and weighted. Notably, after 4T1 tumor challenge in BALB/c mice, their organs were collected, fixed in paraformaldehyde, and analyzed by hematoxylin and eosin (H&E) staining.

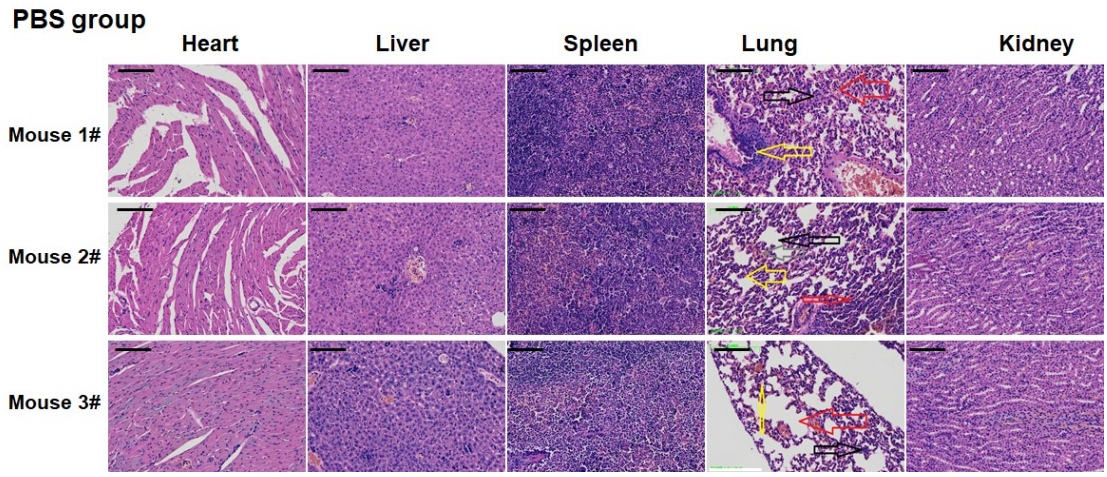

Fig. S6 Histological analysis of mouse organs treated with PBS. The mouse organs are from the 4T1 tumor challenge study mentioned in **Fig. 9**. The mouse organs were harvested, sectioned and stained with hematoxylin/eosin. Scale bars: 100 μm. The analysis was as follows:

Mouse 1-3#: The myocardium was not abnormal; slight bleeding was observed in the myocardial interstitium; the hepatic lobular structure was normal; hepatic interstitial intra-lymphocyte infiltration and neutrophil infiltration were observed in the manifold area; no apparent abnormalities in spleen tissues; interstitial bleeding in renal.

Mouse 1#: Bleeding in the alveolar cavity (red arrow), increased lung interstitial fibrous tissue (black arrow), neutrophil infiltration in the lung interstitium (yellow arrow).

Mouse 2#: Bleeding in the alveolar cavity (gray arrow), increased fibrous tissue of the lung (yellow arrow), neutrophil infiltration in the interstitium of the lung (red arrow), destruction of part of the alveolar wall, alveolar fusion, emphysema formation (black arrow).

Mouse 3#: Bleeding in the alveolar cavity (yellow arrow), neutrophil infiltration in the interstitium of the lung (black arrow), destruction of part of the alveolar wall, alveolar fusion, emphysema formation (red arrow).

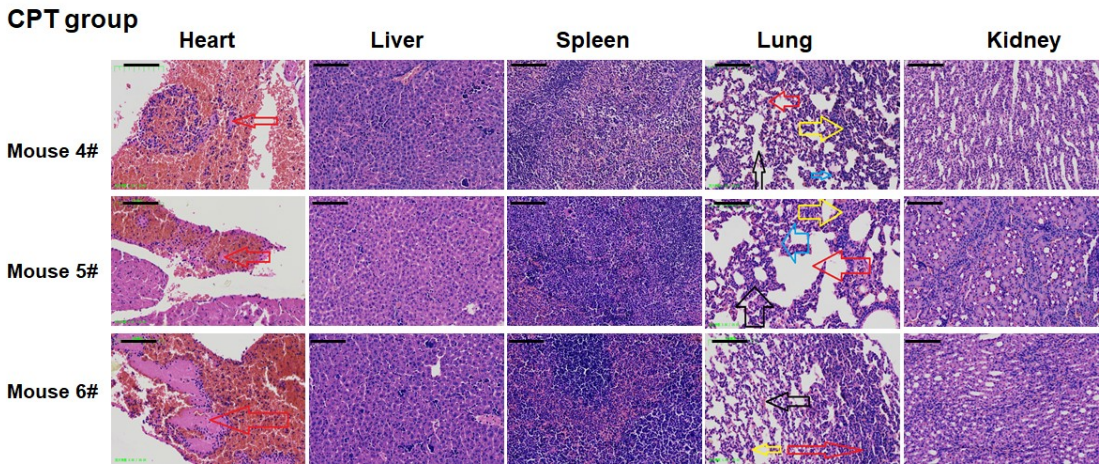

Fig. S7 Histological analysis of mouse organs treated with CPT. The mouse organs are from the 4T1 tumor challenge study mentioned in **Fig. 9**. The mouse organs were harvested, sectioned and stained with hematoxylin/eosin. Scale bars: 100 μm. The analysis was as follows:

Mouse 4-6#: The myocardium was not abnormal; slight bleeding was observed in the myocardial interstitium; *thrombosis* was observed in the heart chambers (red arrow); the hepatic lobular structure was normal; hepatic interstitial intra-lymphocyte infiltration and neutrophil infiltration was observed in the manifold area; no apparent abnormalities in spleen tissues; interstitial bleeding in renal.

Mouse 4#: Bleeding in the alveolar cavity (red arrow), increased fibrous tissue in the lung (blue arrow), neutrophil infiltration in the interstitium (yellow arrow), destruction of part of the alveolar wall, alveolar fusion, emphysema formation (black arrow).

Mouse 5#: Bleeding in the alveolar cavity (blue arrow), increased fibrous tissue of the lung (yellow arrow), neutrophil infiltration in the interstitium (black arrow), destruction of part of the alveolar wall, alveolar fusion, emphysema formation (red arrow).

Mouse 6#: Bleeding in alveolar space (black arrow), increased interstitial fibrous tissue (red arrow), neutrophil infiltration in interstitial lung (yellow arrow).

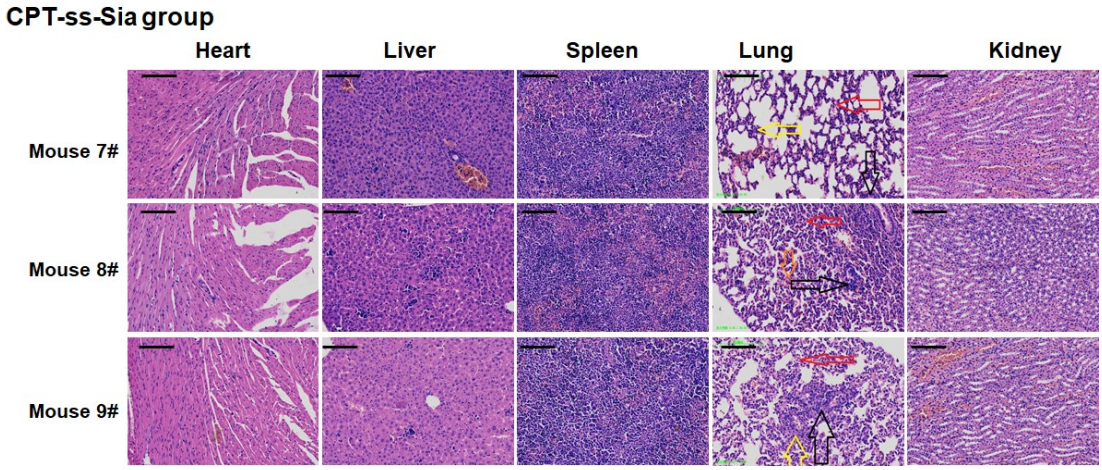

Fig. S8 Histological analysis of mouse organs treated with CPT-ss-Sia. The mouse organs are from the 4T1 tumor challenge study mentioned in **Fig. 9**. The mouse organs were harvested, sectioned and stained with hematoxylin/eosin. Scale bars: 100 μm. The analysis was as follows:

Mouse 7-9#: The myocardium was not abnormal; slight bleeding was observed in the myocardial interstitium; the hepatic lobular structure was normal; hepatic interstitial intra-lymphocyte infiltration and neutrophil infiltration were observed in the manifold area; no apparent abnormalities in spleen tissues; interstitial bleeding in renal.

Mouse 7#: Bleeding in alveolar space (red arrow), increased fibrous tissue in the lung (black arrow), neutrophil infiltration in interstitial lung (yellow arrow).

Mouse 8#: Bleeding in alveolar cavity (orange arrow), increased fibrous tissue in the lung (red arrow), neutrophil infiltration in interstitial lung (black arrow).

Mouse 9#: Bleeding in alveolar cavity (red arrow), increased interstitial fibrous tissue (yellow arrow), neutrophil infiltration in interstitium (black arrow).

Notably, no thrombosis appeared in the CPT-ss-Sia treatment group. In addition, there was no partial alveolar wall destruction, alveolar fusion, or emphysema formation, proving that CPT-ss-Sia treatment significantly reduced lesions.