Supplementary materials

An esterase-activatable prodrug formulated liposome strategy: potentiating the anticancer therapeutic efficacy and drug safety

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The authors declare no potential conflicts of interest.

Figures

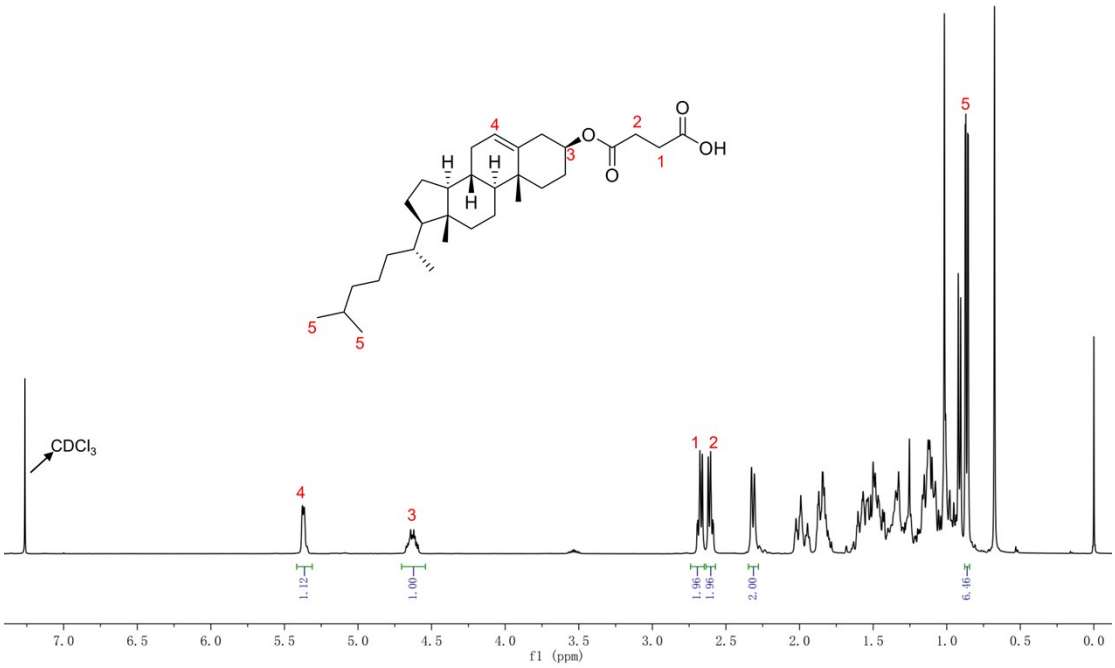


Figure S1. ^{1}H NMR spectrum of Cholesteryl hemisuccinate in $CDCl_3$.

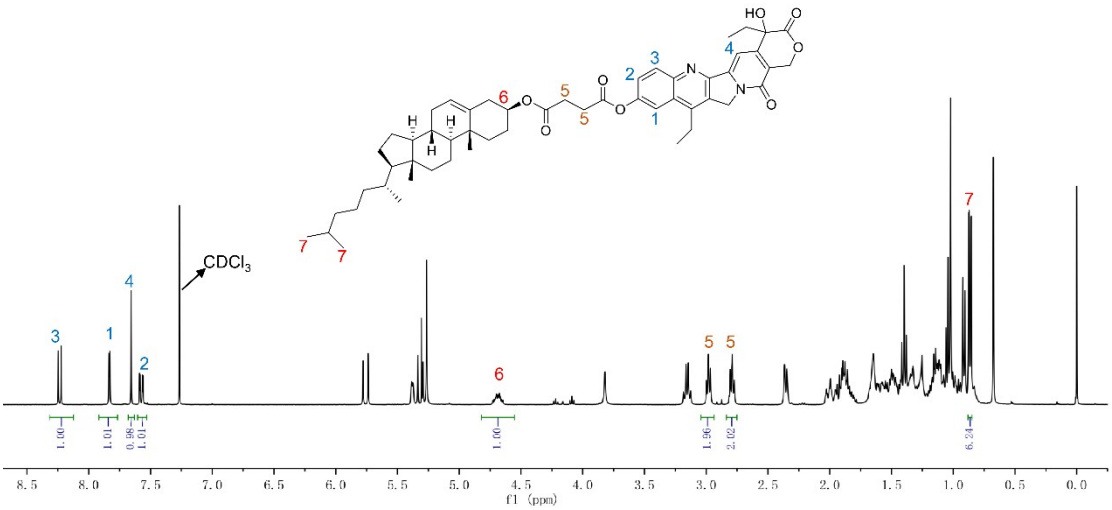


Figure S2. ^{1}H NMR spectrum of SN38 based prodrug conjugate (**Chol-SN38**) in $CDCl_3$.

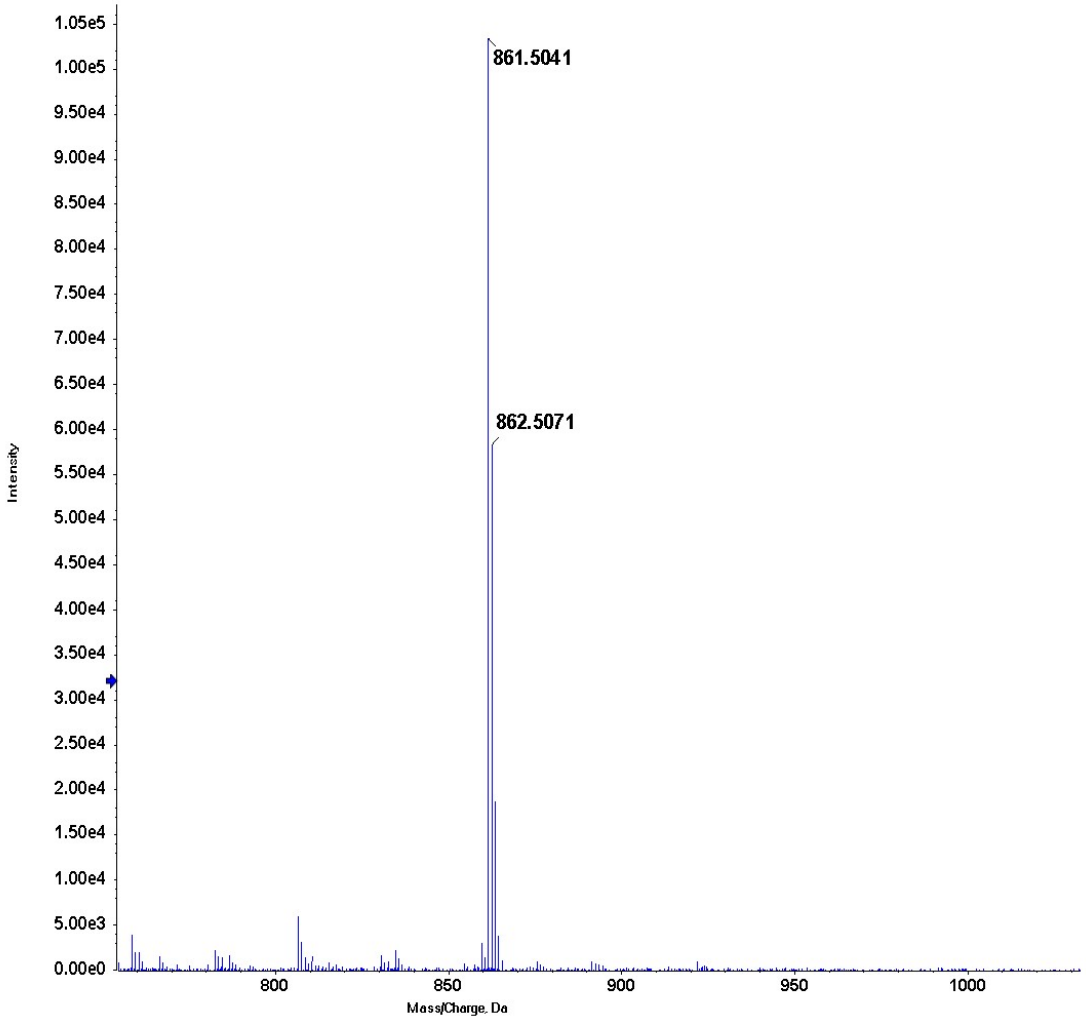


Figure S3. Mass spectrum of **Chol-SN38**.

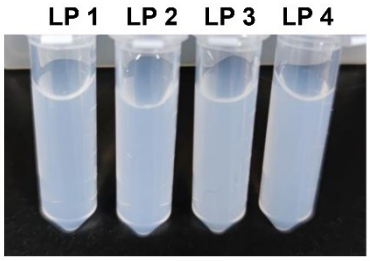


Figure S4. With different molar ratio of **Chol-SN38** verses cholesterol, the prepared liposomes **LP 1**, **LP 2**, **LP 3** and **LP 4** were presented.

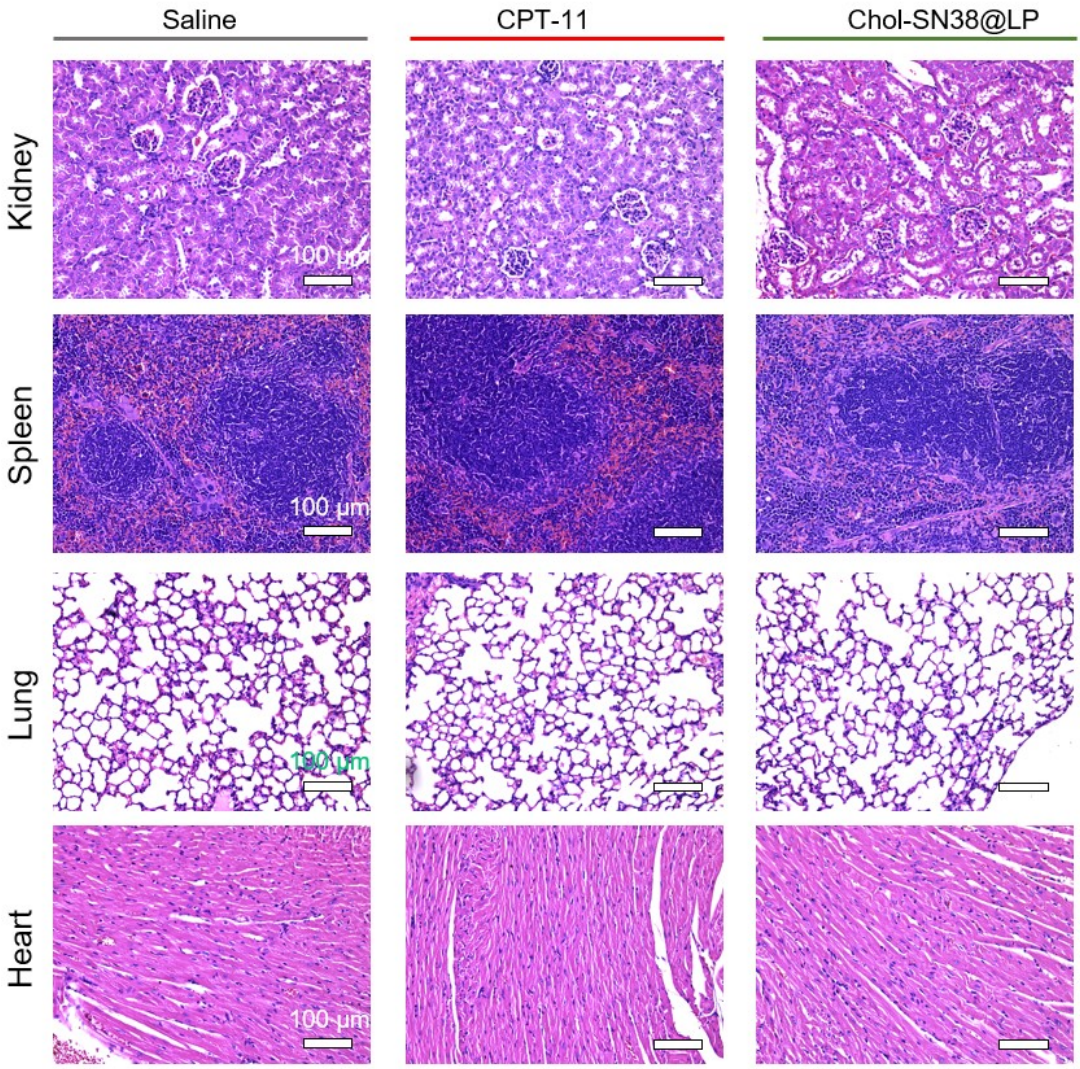
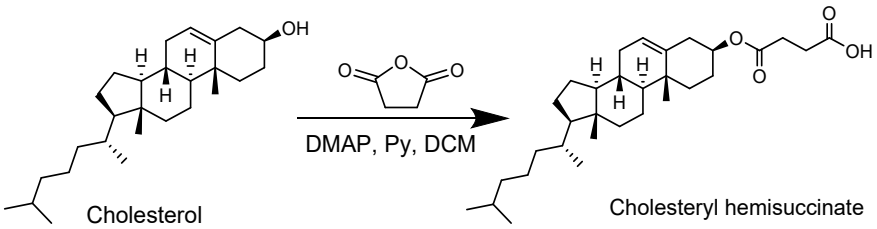


Figure S5. H&E staining images of organs extracted from mice at 7 days post administration of CPT-11 and **Chol-SN38@LP** (15 mg/kg, SN38 equiv.). The mice receiving saline were included as healthy control.

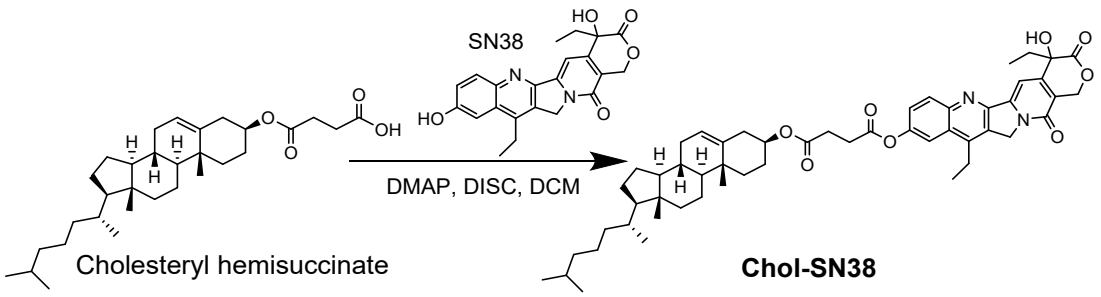
Methods

Synthesis of cholesteryl hemisuccinate: Cholesterol (300 mg, 0.776 mmol) and succinic anhydride (233 mg, 2.328 mmol) were dissolved in 1 mL of anhydrous dichloromethane (DCM) and 3 mL anhydrous pyridine. The catalyst 4-Dimethylaminopyridine (DMAP, 9.46 mg, 0.078 mmol) were added to the reaction. The mixture was stirred overnight at 45 °C before washed with 0.1 M HCl, saturated $NaHCO_3$ and brine. The solution was dried by evaporation and residue was purified by TLC (DCM: MeOH = 15:1) to obtain the product cholesteryl hemisuccinate (352 mg, 93.2%).



^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (d, J = 4.5 Hz, 1H), 4.70 – 4.54 (m, 1H), 2.68 (dd, J = 9.9, 4.0 Hz, 2H), 2.60 (dd, J = 9.9, 4.1 Hz, 2H), 2.30 (t, J = 9.3 Hz, 2H), 0.86 (dd, J = 6.6, 1.8 Hz, 6H).

Synthesis of Cholesterol-SN38 (Chol-SN38) conjugate: To synthesis esterase sensitive compound Cholesterol-SN38 conjugate, we added the above synthesized cholesteryl hemisuccinate (310 mg, 0.637 mmol) and SN38 (250 mg, 0.637 mmol) into anhydrous DCM. The catalyst DMAP (37 mg, 0.306 mmol) and 1,3-diisopropylcarbodiimide (DISC, 121 mg, 0.956 mmol) were added into the solution. The mixture was refluxed and stirred overnight at 45 °C. Then the mixture was washed with 5% citric acid, saturated Na_2CO_3 and brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporation. The crude residue was purified by flash column chromatography on silica gel to get **Chol-SN38** conjugate (444 mg, 83.1%).



^{1}H NMR (400 MHz, CDCl3) δ 5.37 (d, J = 4.5 Hz, 1H), 4.70 – 4.54 (m, 1H), 2.68 (dd, J = 9.9, 4.0 Hz, 2H), 2.60 (dd, J = 9.9, 4.1 Hz, 2H), 2.30 (t, J = 9.3 Hz, 2H), 0.86 (dd, J = 6.6, 1.8 Hz, 6H).

Size and zeta potential characterization: The liposomes were diluted with DI water to the concentration of 0.01 mg/mL (SN38 equivalent concentration) for measuring the hydrodynamic diameter (D_H), polydispersity index (PDI), and zeta potential by Dynamic Light Scattering (DLS) using Malvern Nano-ZS 90 (Malvern Instruments, Malvern, UK). Each sample was tested for three times.

Transmission electron microscopy (TEM) analysis: The morphological characteristics of four liposomes were observed by TEM (Philips, 120 kV, Netherlands). The liposomes were diluted to the concentration of 0.02 mg/mL (SN38 equivalent concentration). Then the samples were dropping onto 300-mesh copper grid and stained with 1% uranyl acetate solution for TEM observation.

Encapsulation efficiency (EE): Liposomes were centrifuged at 6,000 g for 5 min to get precipitates for determining encapsulation efficiency (EE). The precipitates were recovered and dissolved in DMSO. Then the solvents were analyzed by UV-Vis spectrometer (Shimadzu, UV-2700). The EE of liposomes were calculated with the following formula:

$$EE\ (\%) = W_{\text{drug in liposomes}} / W_{\text{initial drug added}} \times 100$$

Colloidal stability of prodrug formulated liposomes: We measured the colloidal stability of the liposomes **LP 1** (termed **Chol-SN38@LP**) against deionized (DI) water and PBS (pH=7.4) containing 20% FBS. In brief, 0.1 mg/mL liposomes (SN38 equivalent concentration) were diluted by those media to the final concentration of 0.01 mg/mL. The diluted liposomes were stored at room

temperature for measuring D_H by DLS at predetermined time intervals within a week.

Cytotoxicity study using Cell Counting Kit-8 (CCK-8) Assay: In evaluating the cytotoxicity of **Chol-SN38@LP**, cells were seeded in 96 well plates (3000-5000 cells per well) and incubated at 37 °C overnight for attachment. Serial concentrations of CPT-11, free SN38 or **Chol-SN38@LP** were administered to cells for 72 h. At the end of incubation period, the cells medium was removed and incubated with diluted CCK-8 solution (#40158, MedChemExpress, USA) for 1 h. The cell absorbances were measured using a microplate reader (Multiskan FC, Thermo Scientific). The experiments were repeated three times.

Cell Proliferation Study by EdU Test: The cell proliferation rates were analyzed by Cell-Light™ EdU Apollo567 *In Vitro* Kit (C10310-1, RIBOBIO, China). A549 cells were seeded in 48-well plate. After drug administration of CPT-11 (6 μM), free SN38 (6 μM) or **Chol-SN38@LP** (6 μM, at SN38 equivalent) for 24 h, EdU (5-ethynyl-2′-deoxyuridine) was first added to each well and incubated for 2 h at 37 °C. The cells were next fixed with 4% formaldehyde for 20 min and permeabilized with 0.5% Triton-X100 for 20 min. Later on, azide-labeled Apollo® 567 were added to cells for another 0.5 h incubation at room temperature. Next, cell nuclei were counterstained with Hoechst 33342 for 0.5 h. The cells were observed under fluorescence microscope (AxioCam MR, R3, Zeiss, Germany).

Flow cytometry (FCM) analysis of cell cycle and apoptosis: For cell cycle and apoptosis analysis, A549 cells were seeded in 6-well plate and incubated for 24 h. For apoptosis analysis, A549 cells were administrated with CPT-11 (4 μM), free SN38 (4 μM) or **Chol-SN38@LP** (4 μM, at SN38 equivalent) for 48 h before resuspended in 500 μL 1 × binding buffer with 4 μL of FITC Annexin V and 4 μL of PI. After incubation for 15 min, the samples were analyzed by FCM (Deflex, Beckman Coulter, USA). Cells for cell cycle distribution analysis were administrated with CPT-11 (50 nM), free SN38 (50 nM) and **Chol-SN38@LP** (50 nM, at SN38 equivalent) for 48 h before resuspension and fixed with 75% alcohol at -20 °C overnight. Then the cells were stained with 500 μL PI (50 μg/mL) for 30 min. The samples were analyzed using a CytoFlex LX flow cytometer. FCM was repeated for at least 3 times.