

Materials and methods

1.1 Materials

With the purity of over 98%, 7-Ethyl-10-hydroxylcamptothecin (SN38) and irinotecan hydrochloride (CPT-11) were purchased from Meilun Biotechnology Co., LTD (Dalian, China). All other commercially obtained chemical reagents were analytical grade or better.

1.2 Preparation of Nanocrystals-SN38 (NCsS)

10 mg of SN38 and 1 mL ammonium hydroxide were added in 3 mL DI water. The reaction was kept stirring at room temperature and away from light, until turning to transparent solution, which means that all the lactone forms of SN38 transformed to the carboxylate acid (CA-SN38). Next, the transparent solution was diluted to 0.5 ~ 1 mg/mL with DI water and freeze-dried to induce the carboxylate acid of SN38 converting into active lactone form. During the freeze drying, the nanocrystals were reconstructed and took the form of fluffy powders. The freeze-drying powders could be dispersible in 5% glucose or water for injection.

1.3 ¹H NMR Spectra

The ¹H NMR spectra, recorded by Varian INOVA spectrometer at 400 MHz in DMSO d₆, was applied to confirm that the NCs were completely permuted with lactone form of SN38. Chemical shifts were displayed as δ value (ppm) against the internal standard compound – Tetramethylsilane (TMS). Also, the coupling constants (J) were marked as hertz (HZ). Multiplicities were recorded as singlet (s), doublet (d), triplet (t) and multiplet (m).

1.4 High Performance Liquid Chromatography (HPLC)

The qualitative and quantitative detection of SN38 and CPT-11 were realized by an Agilent HPLC system (Agilent 1260 Infinity, Agilent technology, USA), including an Agilent quat pump, an Agilent variable wavelength detector and a C₁₈ reversed-phase analytical column (250mm × 4.5 mm, 5 μm, Scienhome, China)¹. 36 mM sodium dihydrogen phosphate, 4 mM sodium 1-heptanesulfonate (pH = 3.0, adjusted with phosphoric acid solution) and acetonitrile (65 : 35) were applied as the mobile phase,

with a flow rate of 1 mL/min and a column temperature of 35 °C. The UV detector was set at 380 nm.

1.5 pH-dependent dynamic equilibrium

1 mg/mL SN38 or CPT-11 stock solution dissolved with DMSO was diluted to 5 µg/mL with 10 mM buffer solution at varying pH values, including 5.0, 6.0, 7.0, 8.0 and 9.0. Samples were placed at 37 °C for 2 h to achieve the equilibrium between lactone and carboxylate acid form,² and analyzed by HPLC to characterize the ratio of the closed-lactone at different pH values.

1.6 HPLC tandem triple-quad mass spectrometry (LC–MS/MS)

The quantitative detection of SN38 and CPT-11 were realized by a LC-MS/MS (Agilent 6410B, Agilent technology, USA)³, including an Agilent binary pump and an ODS analytical column (50 × 4.6 mm, 1.8 µm, Diamonsil). 10mM ammonium acetate (0.1% formic acid, pH = 3) and acetonitrile (65:35) were applied as the mobile phase, with a flow rate of 0.4 mL/min and an injection volume of 1 µL. The retention time of CPT-11 and SN38 were 0.84 and 1.31 min respectively. Multiple reaction monitoring (MRM) positive mode was set to analyze CPT-11 and SN38. The parameters of mass spectrometer were applied as following settings: CPT-11 transition (m/z), 587.3→167.1, collision energy, 44 eV; SN38 transition (m/z), 393.3→349.1, collision energy, 24 eV; gas temperature, 350 °C; nebulizer pressure, 35 psi and spray voltage, 4000 V.

1.7 Characterization of NCsS

Size and Zeta Potentials. The particle size, size distribution and Zeta potentials of NCs were measured by Dynamic Light Scattering (DLS, Zetasizer Nano ZS90, Malvern, UK) at 25 °C.

Morphology. By using field emission scanning electron microscope (JSM – 7500F, JEOL, Japan), the Scanning Electron Microscope (SEM) image of NCs was gained at 15 keV working voltage. The Transmission Electron Microscope (TEM) imaging of NCsS was performed on an H-600 TEM (Hitachi, Japan) with negative staining by 2% phosphotungstic acid.

Crystalline Form. Powder X-ray Diffraction (PXRD) analyses were applied to reveal the formation mechanism of NCs from 10° to 70° with a minimum step size 2θ of

0.001° at a detecting speed of 0.045° 2θ/min. To analyze crystalline states, SN38 powder and freeze-dried NCsS powder were included.

1.8 Cell lines

The 4T1 cells⁴ were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin streptomycin combination, in a humidified atmosphere of 5% CO₂ at 37 °C.

1.9 Cytotoxicity assays

The cytotoxicity of free CPT-11, free SN38 and NCsS were determined by 3- (4, 5-Dimethylthiazol-2-yl) - 2, 5- diphenyltetrazolium bromide assay (MTT assay) in 4T1 cells. Briefly, cells were seeded in 96 well plates at a density of 5000 cells per well and incubated for 12 h. The medium was replaced by 100 µL medium with serial dilutions of CPT-11, SN38 or NCsS (CPT-11 from 10 µg/mL to 100 µg/mL and SN38 from 0.1 µg/mL to 10 µg/mL) and were incubated for 24 h. Next, cells were treated with 100 µL MTT solution (0.5 µg/mL in PBS) for 4 h at 37 °C. Then, the medium was discarded and 100 µL DMSO was added to dissolve the formazan crystal formed by viable cells. The absorbance at 470 nm was recorded by a microplate reader (Tecan spark 10M, China). Cells treated with fresh RPMI-1640 medium were set as control. Cell viability (%) was calculated in accordance with the following equation:

$$\text{Cell viability (\%)} = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100\%.$$

1.10 Cellular uptake study

The 4T1 cells were seeded in 6 well plates at a density of 5×10^5 cells per well and incubated for 12 h. Next, 4T1 cells were incubated with free CPT-11, SN38 or NCsS at an isomolar concentration of 13 nM for 1, 2 or 4 h, in a humidified atmosphere of 5% CO₂ at 37 °C. Then, the cells were washed with cold PBS for 3 times and obtained by centrifuging at 2000 rpm for 3 min. And 0.3 mL DI water was added to resuspend centrifuged cells. Finally, the cells suspension was lysed in the freezing and thawing cycle for 5 times to release the intracellular uptake drugs. The lysed cell suspension (100 µL) and organic solvent mixture (200 µL, methanol/acetonitrile, 1:1 and 0.5% ethylic acid) were mixed. After vortex mixing for 5 min, the supernatant was obtained

by twice centrifuging at 13, 500 rpm for 15 min and analyzed by LC-MS/MS. Meanwhile, 20 μ L lysed cell suspension was applied to detect the total protein content by BCA assay reagent kit (Thermo Fisher Scientific, USA). The results were represented by the amount of CPT-11, SN38 or NCsS (nmol) in per milligram protein.

1.11 Confocal laser scanning microscopy (CLSM)

The intracellular distribution of free SN38 and NCsS were studied in 4T1 cells by the CLSM assay. Cells were seeded in confocal dishes at a density of 1×10^5 cells per well. After 12 h, the original medium was removed and medium containing 13 nM of free SN38 or NCsS was added. After incubation for 2 h, culture medium was removed, and cells were rinsed with PBS for 3 times. The lysosomes were stained with Lyso-Tracker Red (KGMP006, KeyGEN BioTECH, China) for 1 h. After being fixed with 4% (w/v) paraformaldehyde operation solution for 20 min, the cell nuclei were stained with DAPI for 15 min. Then cells were washed with PBS for 3 times and were observed with CLSM (LSM-800, ZEISS, Germany), with emission wavelength of 454 nm for DAPI, 530 nm for SN38, and 590 nm for Lyso-Tracker Red.

1.12 Animals and BALB/c mice bearing 4T1 tumors

Healthy male Sprague-Dawley rats and female BALB/c mice (6 weeks old) were purchased from Institute of Laboratory Animals of Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (Chengdu, China). All the animal experiments presented were approved of the Institutional Animal Care and Ethic Committee of Sichuan University in accordance with the Chinese guidelines for the care and use of laboratory animals.

4T1 cells were orthotopically injected into the right underarm mammary fat pad at a density of 1×10^5 cells per BALB/c mouse in cold PBS^{4, 5}. The volume of tumor was measured by a digital caliper every other day, and was calculated by the following equation: $V = 0.5 \times \text{length} \times \text{width}^2$.

1.13 Pharmacokinetics

The pharmacokinetics of CPT-11 and NCsS were evaluated on male SD rats (n = 5). Irinotecan hydrochloride (CPT-11), a prodrug of SN38, is widely applied in treating many kinds of carcinoma. According to previous reports^{3, 6}, it was dissolved in 5%

dextrose at pH 6.0 (1 mg/mL) for injection. The free CPT-11 solution or NCsS was administered intravenously at an equivalent dosage of 5.6 mg/kg SN38. At planned time points after tail vein injection (0.17, 0.33, 0.50, 1, 2, 4, 8 and 12h), 100 μ L of blood was collected from infraorbital venous plexus. Organic solvent mixture (200 μ L, methanol/acetonitrile, 1:1 and 0.5% ethylic acid) was added and eddied for 5 min. The supernatant was acquired by twice centrifugation at 13, 500 rpm for 15 min and analyzed by LC-MS/MS.

1.14 Biodistribution

The tumor-bearing mice were randomly divided into two groups (n = 20) with intravenously injection of free CPT-11 solution or NCsS at an equivalent dosage of 10 mg/kg SN38, when tumor volume reached approximately 300 mm³. Mice were sacrificed at planned time points after injection (1, 4, 8 and 24 h, n = 5). Major organs (heart, liver, spleen, lung and kidney) and tumor were collected, washed with normal saline solution, dried with filter paper, weighed and homogenized with double volume of PBS (mL/g). Samples were disposed in accordance with the pharmacokinetics. The concentrations of drug in different organs were detected by LC-MS/MS.

The penetration of free SN38 or NCsS were evaluated by immunofluorescence staining. SN38 was dissolved in a mixed solvent, which was composed of dimethyl sulfoxide, polyethylene glycol 400, ethanol and 5% glucose (3:3:2:2, in volume) for injection. The tumor-bearing mice were tail intravenously injected with free SN38 solution or NCsS at an equivalent dosage of 10 mg/kg SN38. Mice were sacrificed 8 h after injection and tumors were collected. The tumor sections were prepared and stained with CD31 (EPR3094, Alexa Fluor® 647, Thermo Fisher Scientific, USA) to mark the tumor vessel by the reported method.⁷ The nuclei were stained with DAPI and sections were observed with the CLSM on the uniform conditions, with an emission wavelength of 454 nm for DAPI, 530 nm for SN38, and 668 nm for CD31.

1.15 In vivo antitumor efficacy

The tumor-bearing mice were randomly divided into three groups (n = 5) and were tail intravenously injected with normal saline solution (NS), free CPT-11 solution or NCsS at an equivalent dosage of 10 mg/kg SN38, when tumor volume reached 50 ~ 100 mm³. The dosage regimen was 5 injections with each in every 3 days. The weight

and tumor size of mice were recorded every other day. On the 22nd day, major organs including heart, liver, spleen, lung, kidney and intestinal segments (containing duodenum, jejunum, ileum and colon) were collected for haematoxylin-eosin (H&E) staining to evaluate the toxicity of different treatments. Tumors were collected, rinsed with normal saline solution, dried with filter paper, weighed, stained with H&E and TUNEL staining. Tumor growth inhibition (TGI) was calculated as the following

$$\text{TGI (\%)} = \frac{\bar{W}_{\text{NS}} - W_{\text{test}}}{\bar{W}_{\text{NS}}} \times 100\%.$$

equation: Images were obtained with microscope (BX53, Olympus, Japan) and digital color camera system (DP73, Olympus, Japan).

1.16 Systemic toxicity

Healthy female BALB/c mice (6 weeks old) were randomly divided into three groups (n = 5) and were tail intravenously injected with normal saline solution (Saline), free CPT-11 solution or NCsS at an equivalent dosage of 10 mg/kg SN38. The dosage regimen was injection on every 3 days for five times. The weight of mice was recorded every other day, until sacrificed at the 13rd day. Major organs including heart, liver, spleen, lung, kidney and intestinal segments (containing duodenum, jejunum, ileum and colon) were collected for haematoxylin-eosin (H&E) staining to evaluate the toxicity of different treatment. To evaluate the liver injury in different groups, serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were measured using the automatic analysis system with ALT and AST standard kits (Cobas C 311, Roche Diagnostics, Germany).

1.17 Statistical analysis

All experiments were conducted at least three times. Data were presented as mean \pm SD. Comparisons among groups were performed by Graphpad Prism 7.0 (GraphPad Software Inc, California, USA). Differences were assessed by one-way ANOVA. A *P* value of <0.05 was considered statistically significant.

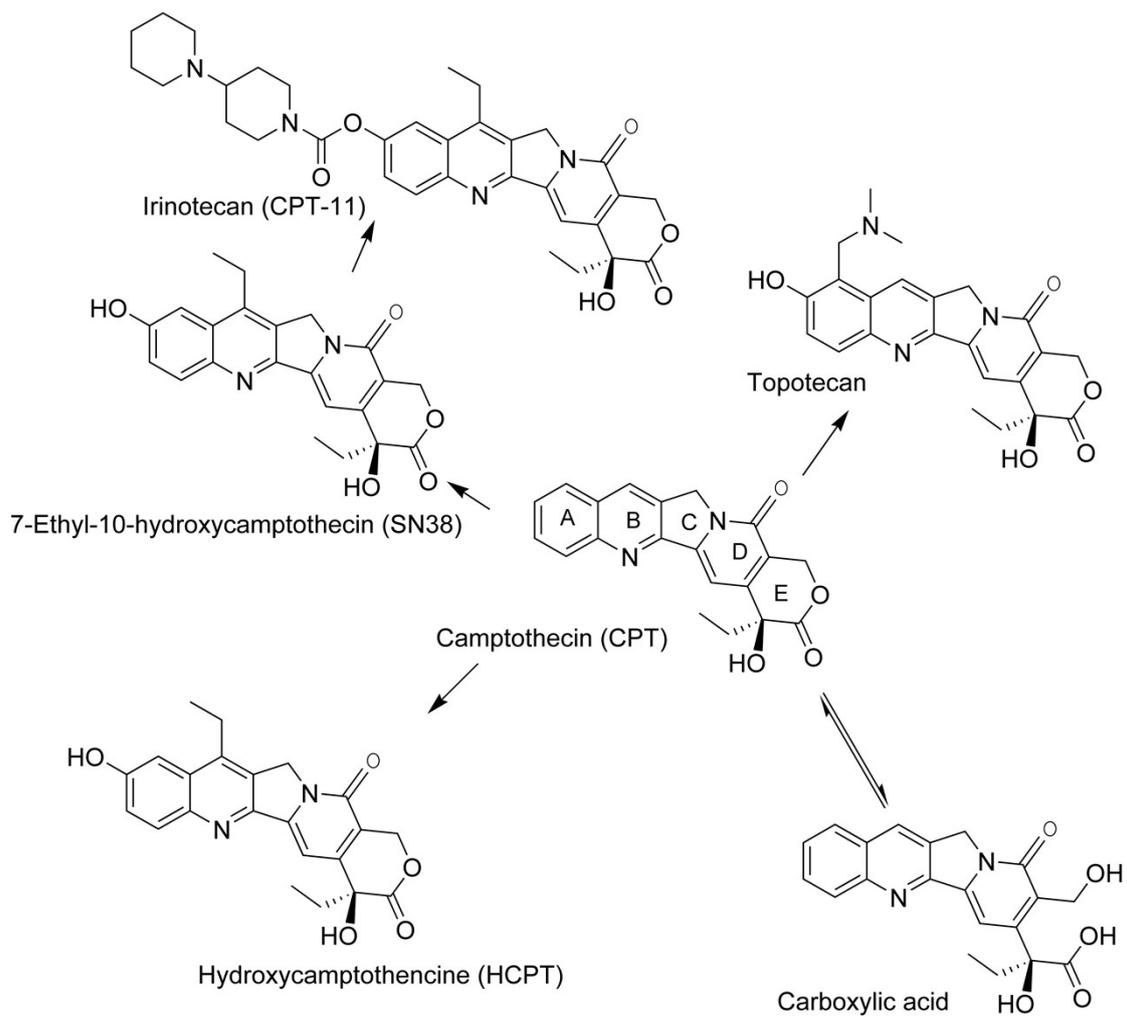


Fig. S1 Camptothecin analogues.

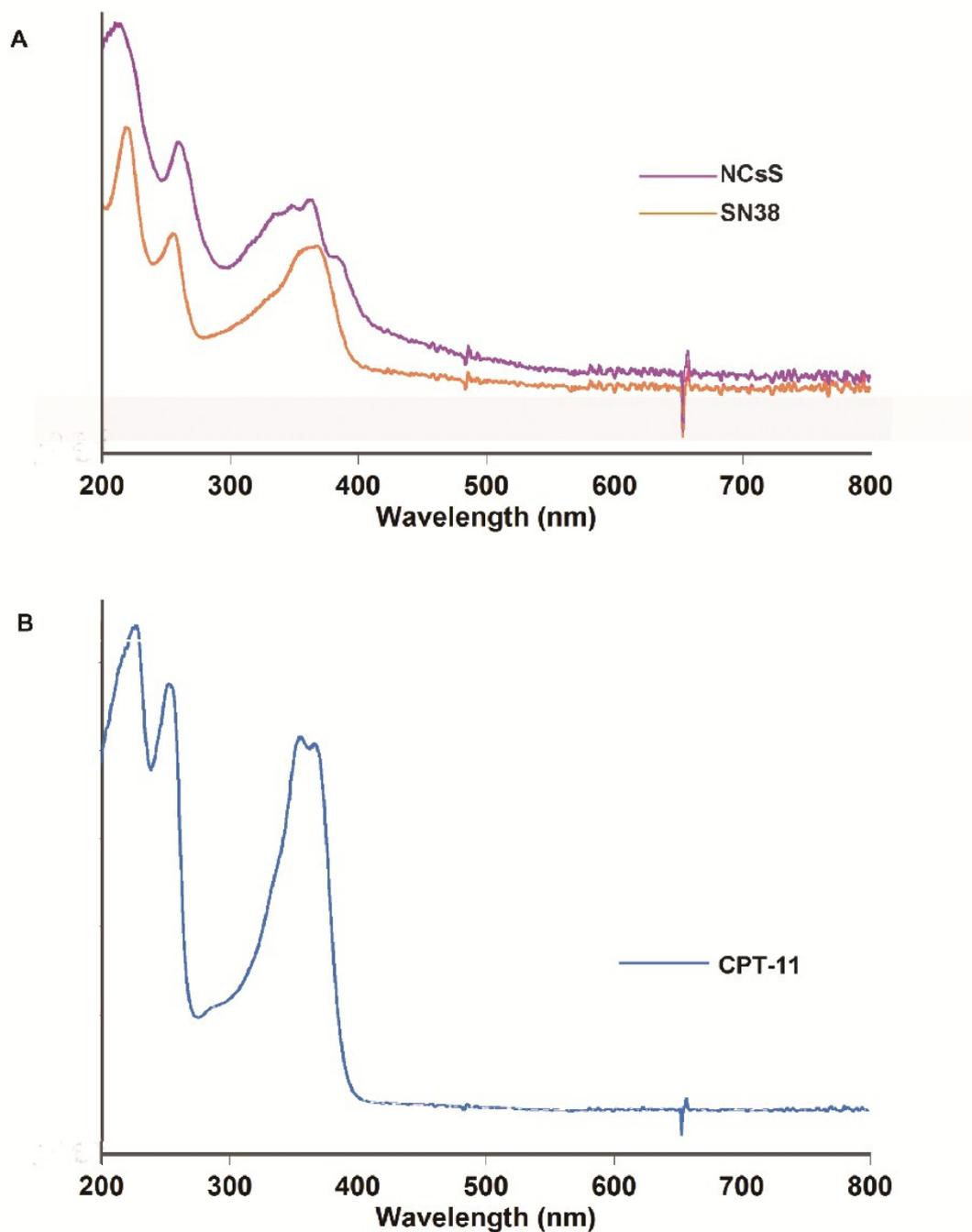


Fig. S2 UV-spectra of NCsS, SN38 (A) and CPT-11 (B). SN38 or CPT-11 stock solutions and NCsS solution were diluted by deionized water to about 5 $\mu\text{g}/\text{mL}$ and recorded by a UV-Visible Spectrometer (Cary 100 Conc, Varian, USA).

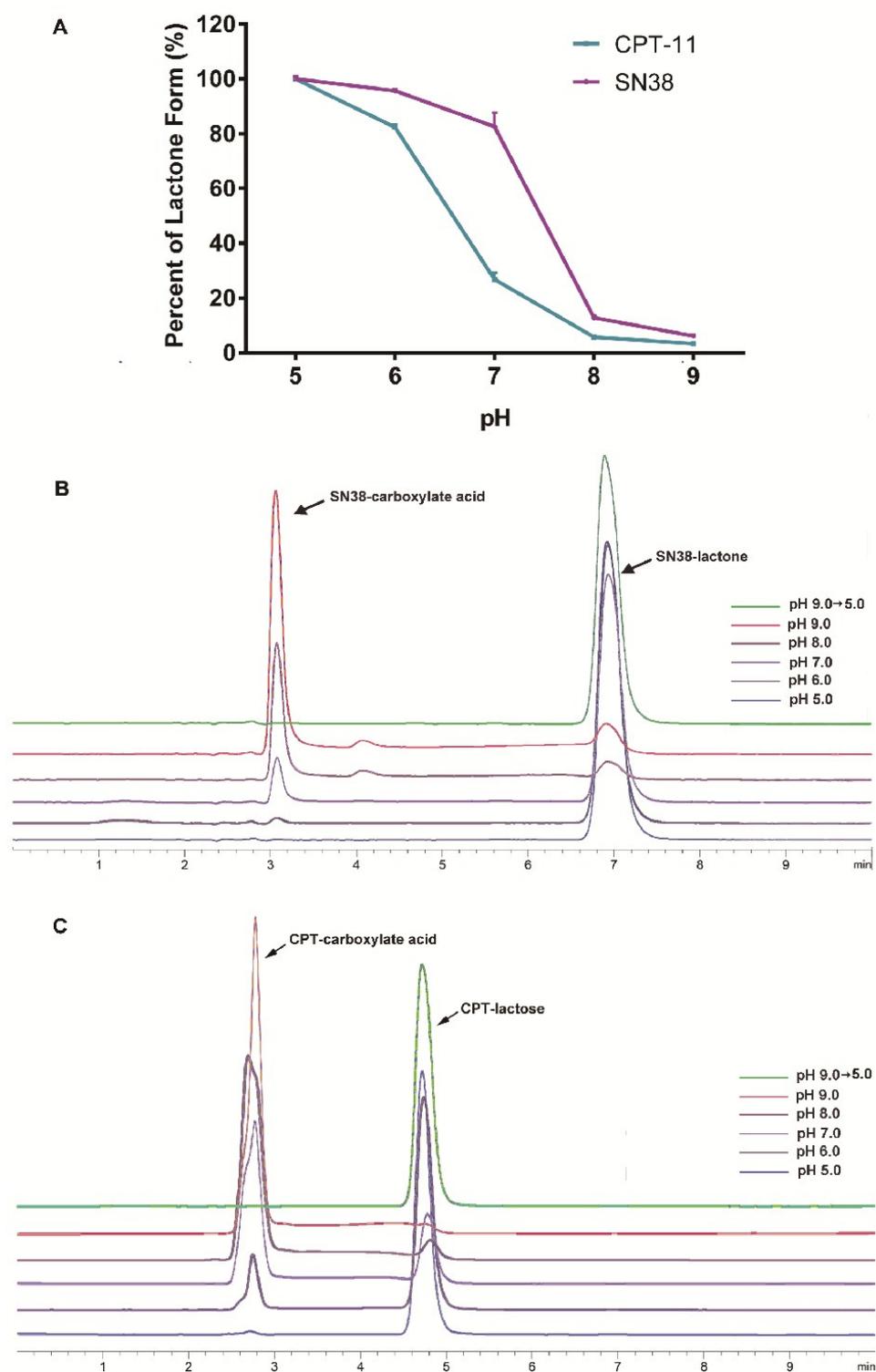


Fig. S3 pH-dependent dynamic equilibrium of SN38 and CPT-11. A: Ratios of lactone form between the chemical equilibrium of SN38 or CPT-11 on varying pH ($n = 3$). The dynamic equilibrium between lactone form and carboxylate acid form of SN38 (B) and CPT-11 (C) monitored by HPLC.

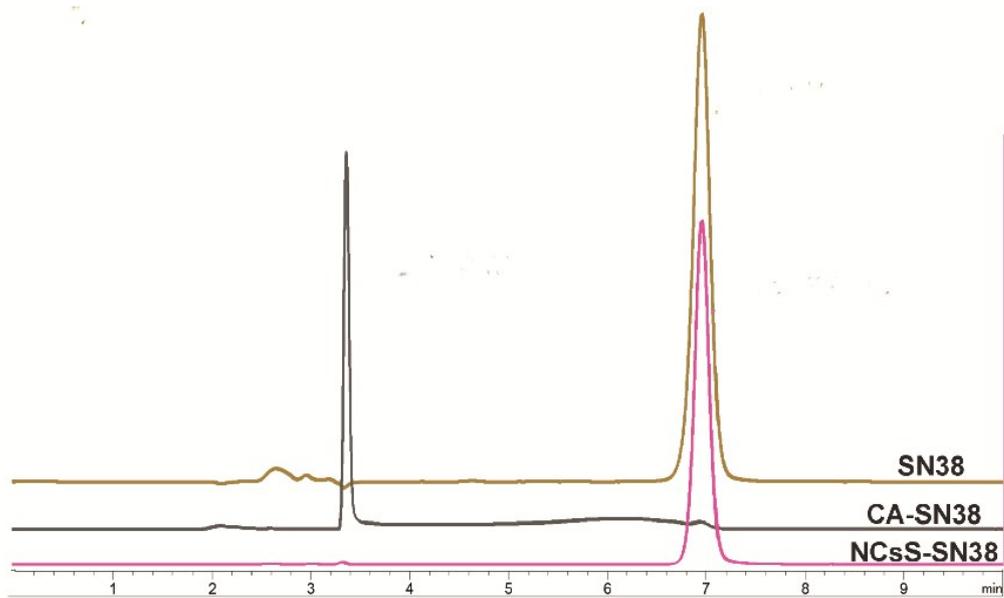


Fig. S4 Characterization of free SN38, reaction product (CA-SN38) and pharmaceutical product (NCsS-SN38) with HPLC (6.97, 3.42 and 6.97 min, respectively).



Fig. S5 5 mg NCsS powder (left) and SN38 (right) with the same weight.

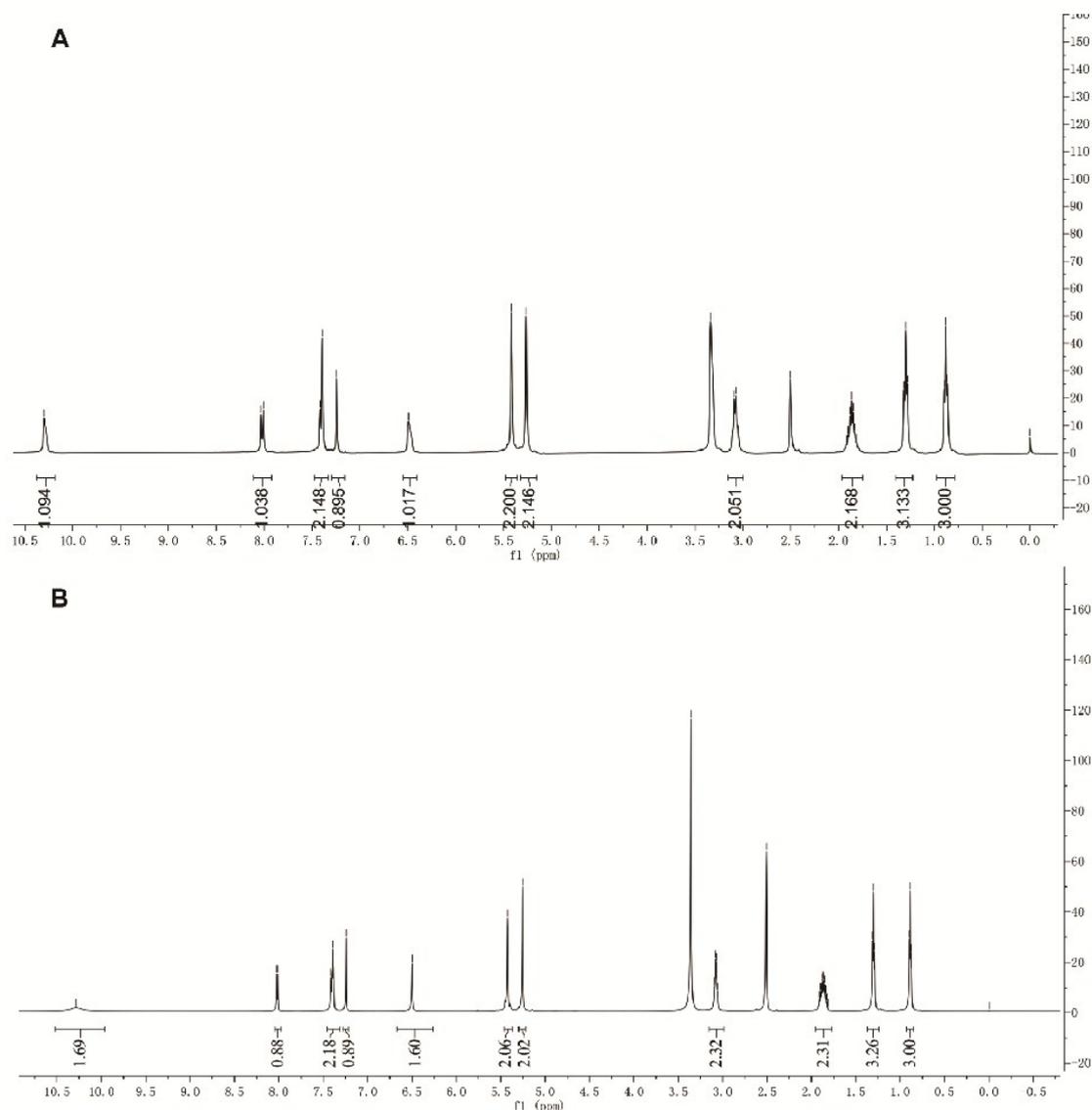


Fig. S6 ^1H NMR spectra of SN38 (A) and NCsS (B) ($\text{DMSO-}d_6$, 400MHz). A: δ 10.30(s, 1H), 8.02(d, $J = 8.4\text{Hz}$, 1H), 7.42-7.39(m, 2H), 7.25(s, 1H), 6.49(s, 1H), 5.42(s, 2H), 5.27(s, 2H), 3.08(d, $J = 7.2\text{Hz}$, 2H), 1.92-1.81(m, 2H), 1.30(t, $J = 7.2\text{Hz}$, 3H), 0.88(t, $J = 7.2\text{Hz}$, 3H). B: δ 10.29 (brs, 1H), 8.02 (d, $J = 6.0\text{Hz}$, 1H), 7.41-7.39 (m, 2H), 7.24 (s, 1H), 6.50 (s, 1H), 5.42(s, 2H), 5.25 (s, 2H), 3.09-3.07 (m, 2H), 1.88-1.85 (m, 2H), 1.28 (t, $J = 7.2\text{Hz}$, 3H), 0.88 (t, $J = 7.2\text{Hz}$, 3H).

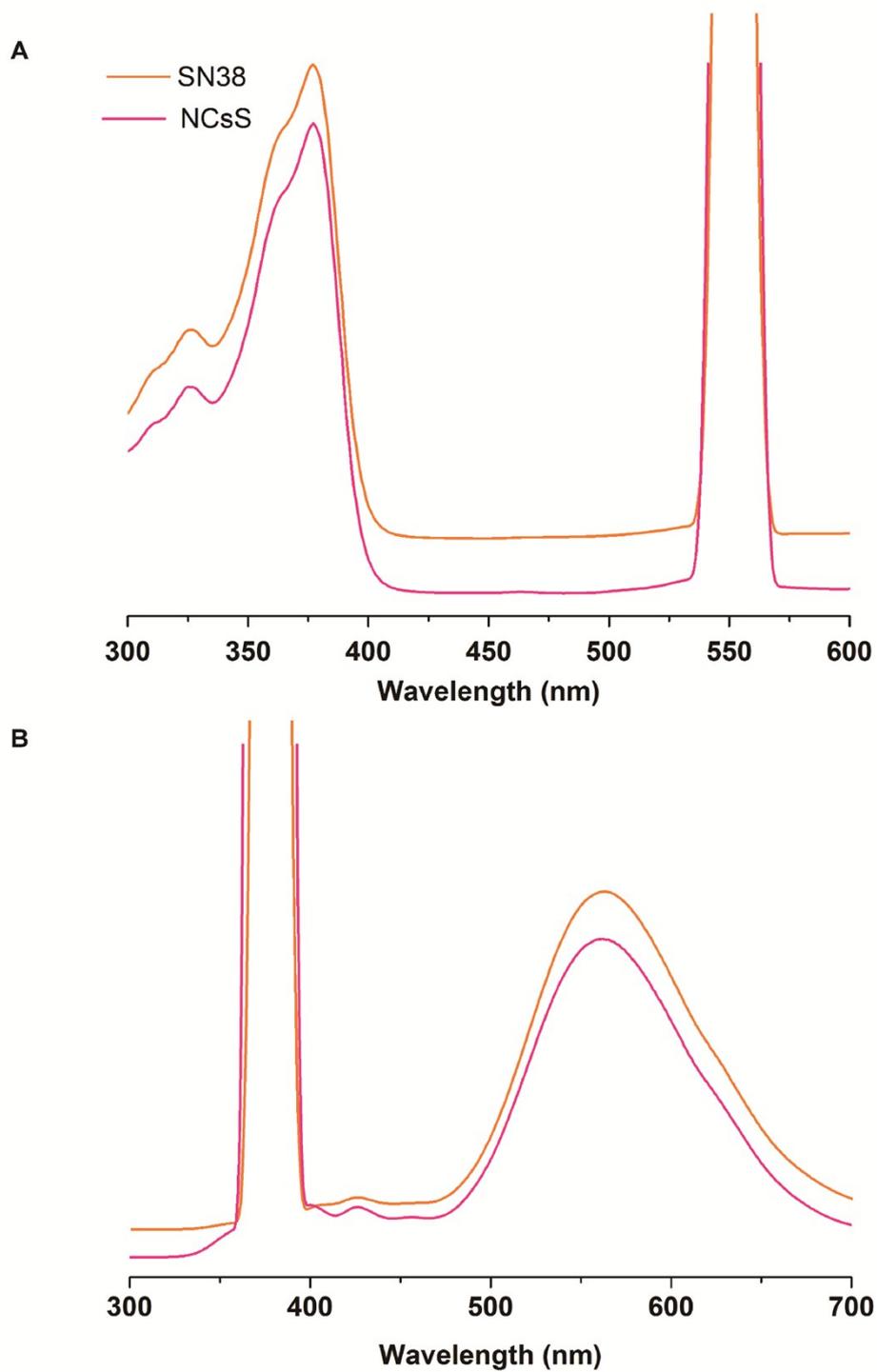


Fig. S7 Fluorescence excitation (A) and emission (B) spectra of SN38 (yellow) and NCsS (carmine) recorded by a fluorescence spectrophotometer (RF-6000, Shimadzu, Japan), with an emission and excitation wavelength of 550 nm and 380 nm respectively .

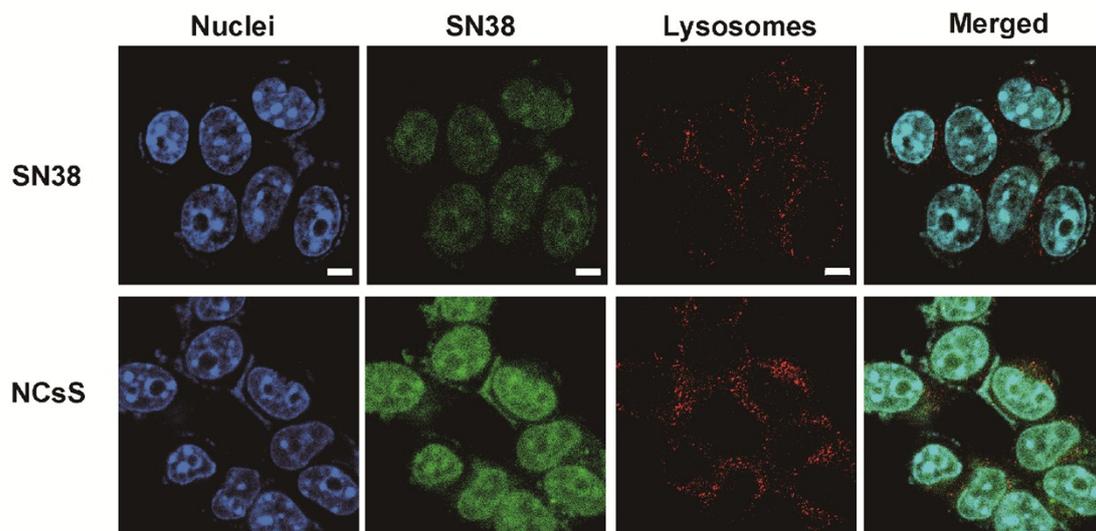


Fig. S8 CLSM images of 4T1 cells incubated with free SN38 and NCsS for 2 h. The nuclei were stained with DAPI (blue), SN38 fluorescence was shown in green and lysosomes were stained with Lyso – tracker in red. The scale bars represent 5 μm .

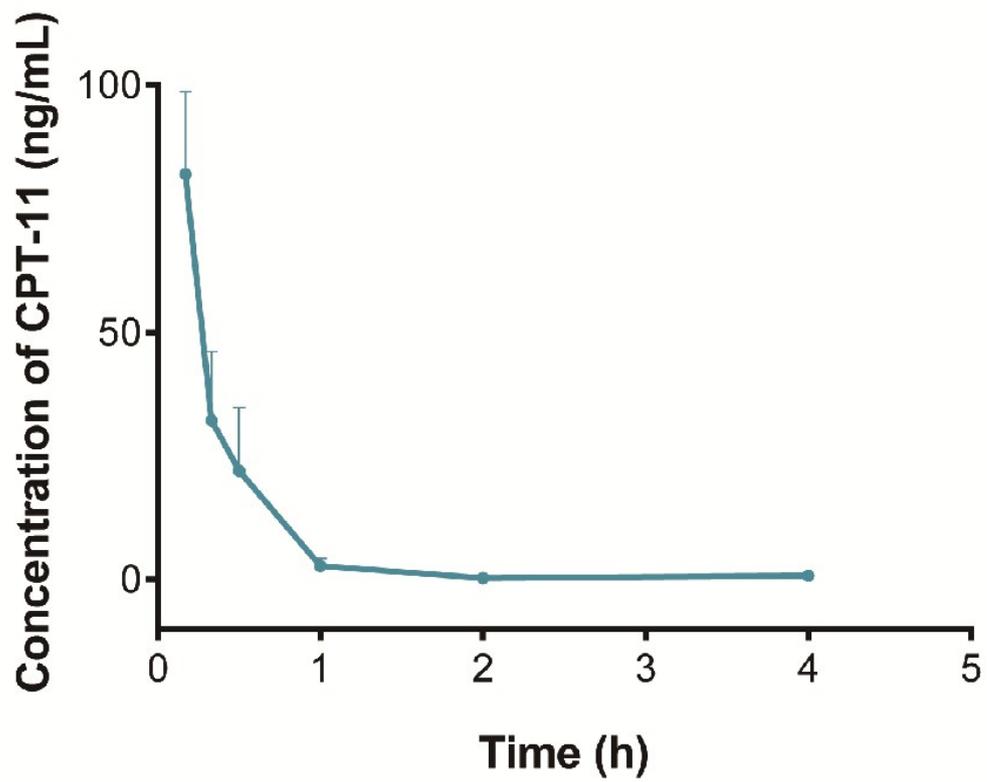


Fig. S9 The concentration of CPT-11 and time curves of injected CPT-11 in SD rats. Data represent mean \pm SD, n = 5.

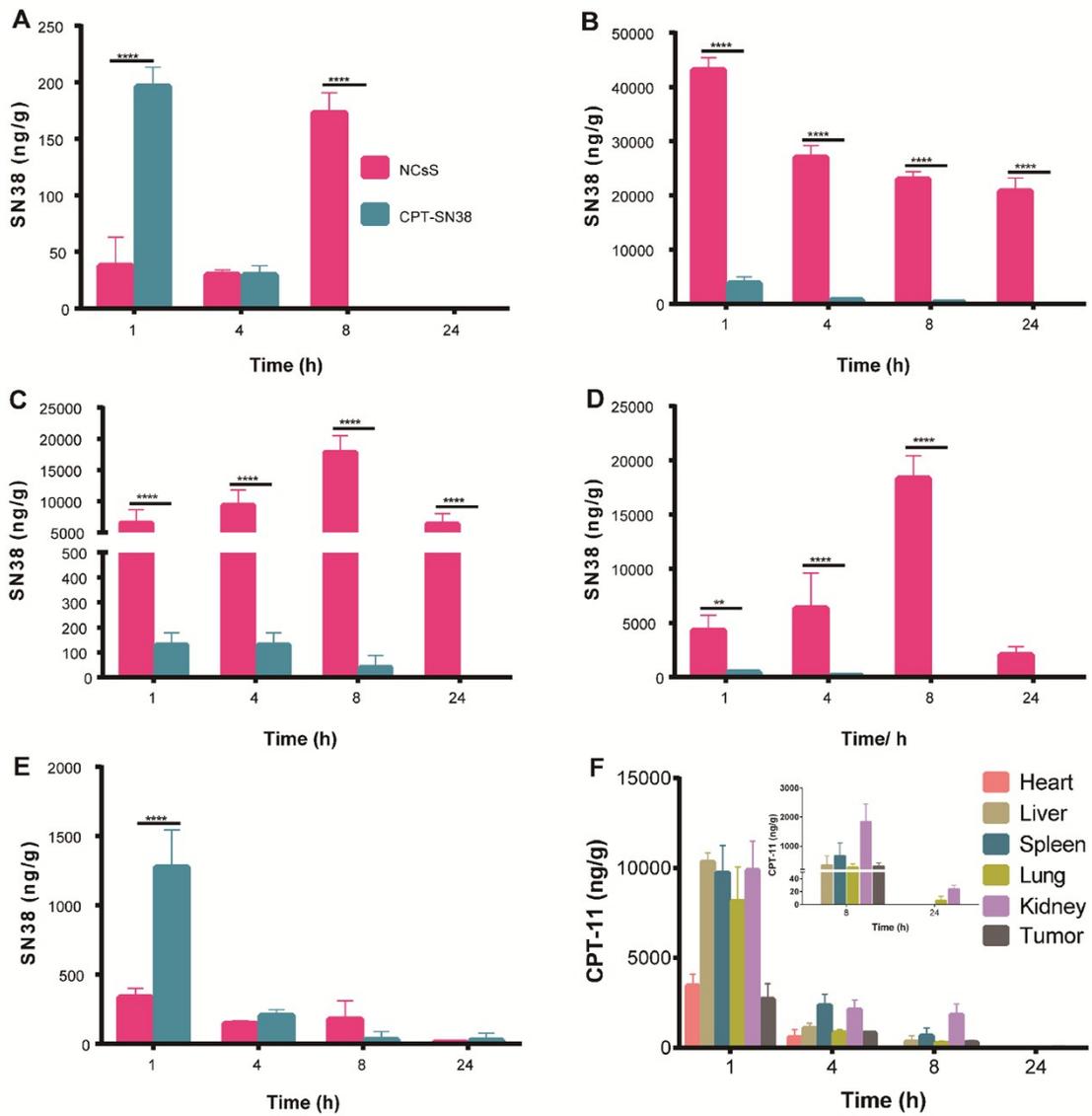


Fig. S10 Biodistribution of NCsS or SN38 converted from injected free CPT-11 (CPT-SN38) in major organs, including heart (A), liver (B), spleen (C), lung (D), kidney (E); (F): The concentration of CPT-11 in major organs 1, 4, 8 and 24 h after injection of free CPT-11. Data represent mean \pm SD, n = 5. **, p < 0.01, compared with the free CPT-11 group.

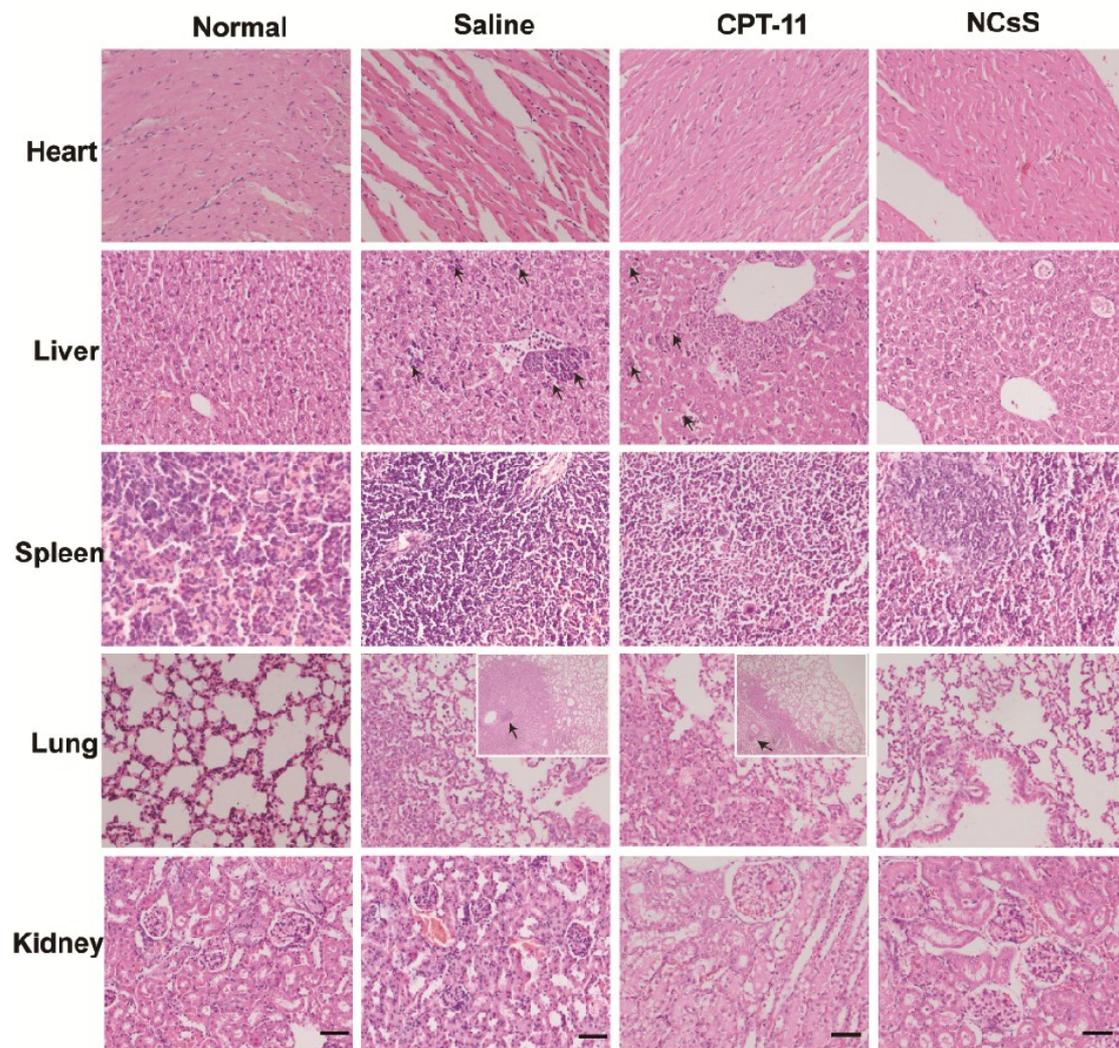


Fig. S11 The histological analysis on major organs of tumor-bearing mice from differently treated groups to evaluate the antitumor efficacy and toxicity. The scale bars represent 50 μ m.

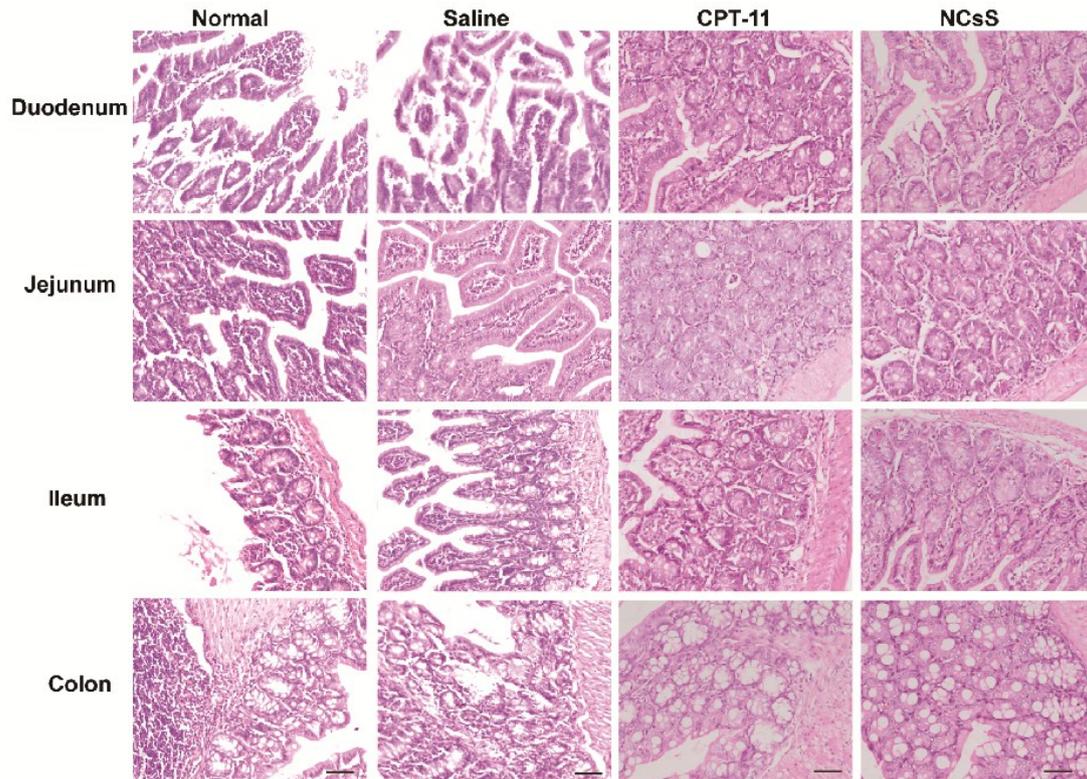


Fig. S12 The histological analysis on major intestinal segments of tumor-bearing mice administrated with different treatments to evaluate the toxicity. The scale bars represent 50 µm.

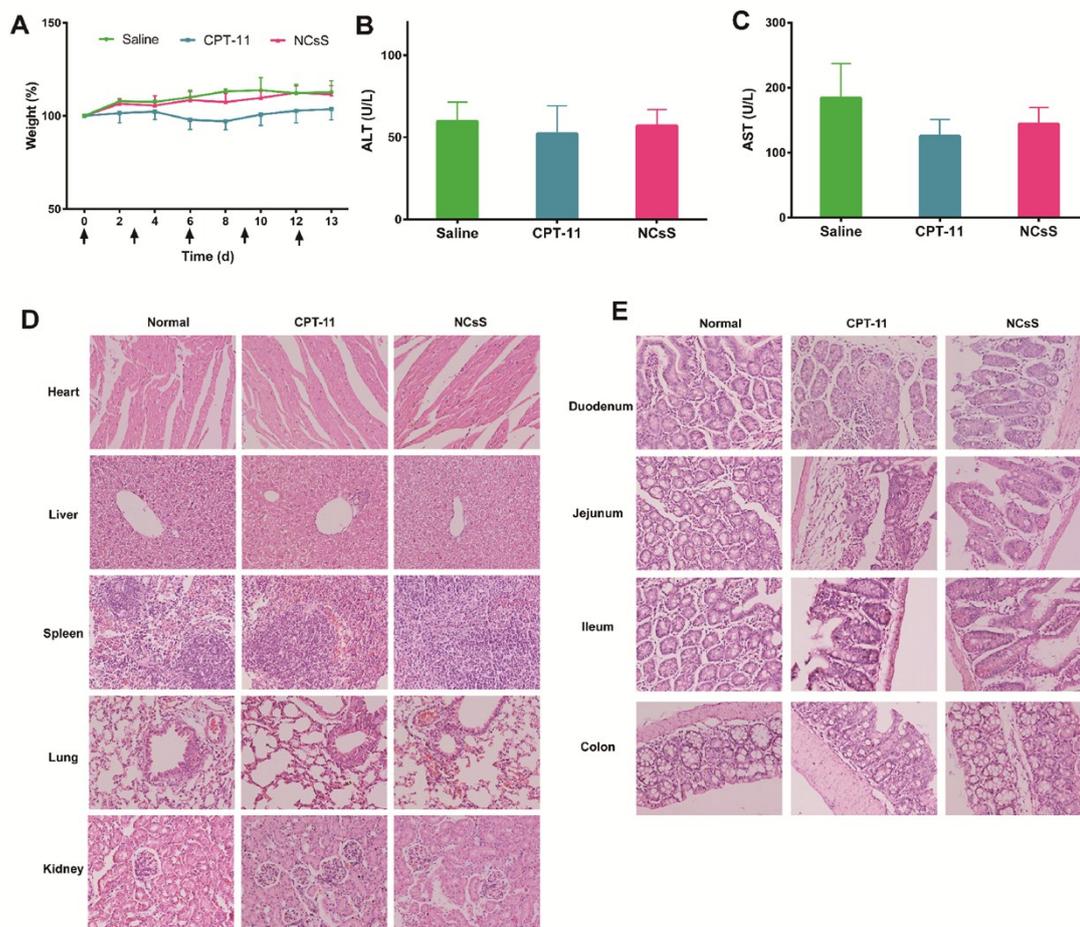


Fig. S13 Systemic toxicity evaluation. (A) Body weight shift of health BALB/c mice treated with free CPT-11, NCsS or saline. ALT (B) and AST (C) levels in the serum of the mice determined at 24 h after the fifth administration. (D) The histological analysis on major organs of mice from differently treated groups. (E) The histological analysis on major intestinal segments of mice administrated with different treatment.

References

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