Critical roles of linker length in determining the chemical and self-assembly stability of SN38 homodimeric nanoprodrugs

Authors: Xin Wang[a,b,1], Tian Liu[a,1], Yuetong Huang[a], Fudan Dong[c], Lingxiao Li[a], Jiaxuan Song[a], Shiyi Zuo[a], Zhengyang Zhu[b], Ken-ichiro Kamei[a], Zhonggui He[a], Bingjun Sun[a]*, Jin Sun[a]*

[1]Both authors contributed equally

Affiliations:

[a]Wuya College of Innovation, Shenyang Pharmaceutical University, Shenyang 110016, China.

[b]Department of Radiology, Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School, Nanjing 210008, China

[c]Henan Provincial People's Hospital, Zhengzhou 450003, China

***Corresponding Authors:**

Bingjun Sun, Ph.D. Jin Sun, Ph.D.

Mailing address: Department of Pharmaceutics, Wuya College of Innovation, Shenyang Pharmaceutical University, Shenyang 110016, China.

Tel: +86-024-23986325; Fax: +86-024-23986325.

E-mail: sunbingjun_spy@sina.com; sunjin@syphu.edu.cn.

Experimental Section

Materials

SN38 were purchased from Jingzhu Bio-Technology Co., Ltd. (Nanjing, China). 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDCI), 4-dimethylaminopyrideine (DMAP), 2,2'-dithiodiglycolic acid, 3,3'-dithiodipropionic acid, 4,4'-dithiobisbutyric acid and coumarin 6 were obtained from Aladdin Biochemical Technology Co. Ltd. (Shanghai, China). DSPE-PEG2k was supplied by Shanghai Advanced Vehicle Technology Pharmaceutical Ltd. (Shanghai, China). Dithiothreitol (DTT), bovine serum albumin (BSA), DAPI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 1,1'- dioctadecyl-3,3,3',3'-tetramethyindotricarbocyanine iodide (DiR) were purchased from Meilun Biotechnology Co. Ltd. (Dalian, China). Roswell park memorial institute (RPMI-1640) and Dulbecco's modified eagle medium (DMEM) were purchased from Gibco (Beijing, China). Fetal bovine serum (FBS) was supplied by Hyclone (Beijing, China). 96-well plate, 24-well plate and 12-well plate were obtained from NEST Biotechnology. Hoechst 33342 was purchased from BD Biosciences (USA). Histone H2AX rabbit mAB and Cy3 Goat Anti-Rabbit IgG (H+L) were obtained from ABclonal Technology Co., Ltd (Wuhan, China). TUNEL apoptosis detection kit and Ki67 cell proliferation kit were supplied by were supplied from Beijing Solarbio Science & Technology Co., Ltd.

Synthesis of SN38 homodimeric prodrugs

Diacid linker (2,2'-Dithiodiglycolic Acid, 3,3'-Dithiodipropionic Acid, 4,4'-Dithiobisbutyric acid, 1 mmol), EDCI (191.7 mg, 1 mmol) and DMAP (6.11 mg, 0.05 mmol) was dissolved in 20 mL chloroform. SN38 (784.8 mg, 2 mmol) was dissolved in 5 mL chloroform and added to reaction system. The reaction system was stirred under a nitrogen atmosphere at room temperature. After 12 h, another EDCI (191.7 mg, 1 mmol) and DMAP (61.1 mg, 0.5 mmol) were added for reacting 24 h at 25 °C. The reaction process was monitored by thin layer chromatography. The product was

purified by high performance liquid chromatography (HPLC) (acetonitrile: water = 70: 30, 1 ‰ formic acid).

The molecular mass of the prodrug was confirmed by high-resolution mass spectrometry and Bruker 400M AVIII (solvent: $CDCl_3$; standard sample: tetramethylsilane; repetitions: 16; sample weight: 5 mg). The purity of the sample was determined by high performance liquid chromatography (HPLC).

Chemical stability of SN38 homodimeric prodrugs

An equal amount of the prodrugs (α-SN38-SS-SN38, β-SN38-SS-SN38 or γ-SN38-SS-SN38) were dissolved in DMF, respectively. The peak area (0 h) of prodrug was recorded by HPLC. Samples were left at room temperature for 9 hours. At different time points, the peak area of prodrug was detected by HPLC, and the relative purity was calculated by comparing with the peak areas of the prodrugs at 0 h.

Preparation, characterization and self-assembly stability of SN38 HDPNs

One step nanoprecipitation method was used to explore the impact of linker length on the self-assembly of SN38 homodimeric prodrug. Briefly, the SN38 prodrugs were dissolved in DMF and added dropwise into pure water under stirring (1000 r/min). After 5 minutes, DMF was removed by ultrafiltration centrifuge tubes (3000 rpm). The morphology of the SN38 HDPNs was observed with transmission electron microscopy (TEM, JEOL, Japan). The zeta potential and mean diameter of SN38 HDPNs and DSPE-PEG_{2K} were obtained by Zetasizer (Nano ZS, Malvern Co., UK). DSPE-PEG2k (20%, w/w) was utilized to prepare PEGylated prodrug nanoassemblies following the same procedure. Dye-labeled HDPNs were prepared similarly by co-assembling prodrugs, DSPE-PEG_{2K} and DiR or coumarin-6.

Self-assembly mechanism and molecular dynamics simulations

Briefly, Gaussian view 5 was used to construct β-SN38-SS-SN38 and γ-SN38-SS-SN38 molecules. After the stable optimized structure was obtained by calculation, five groups of composite systems consisting of 40 monomers were constructed using the Packmol program, and then molecular dynamics simulations were carried out for the systems. The HF/STO-3G method was used to fit the RESP charge parameters of the molecules based on the antechamber program, and then the GAFF force field

parameters were constructed. Using the TIP3P water box model, the side length of the water box was set to 1 nm. A 5000-step energy minimization was performed first, followed by short-term (100 ps) simulations under the NVT and NPT ensembles, respectively, resulting in a 50-ns equilibrium simulation. The cutoff radius was set to 8.0 nm, the time step was set to 2 fs, and the temperature and pressure were set to 300 K and 1 bar, respectively. Configurations were saved every 10 ps for subsequent analysis. All molecular dynamics simulations were based on the Gromacs2019 program. Analysis of hydrogen bonding, hydrophobic and sulfur bonding interactions was performed using the Discovery Studio 4.5 program.

Drug loading

The drug loading (100%) $=MW_{SN38}/MW_{prodrug}\times(M_{prodrug}/(M_{prodrug}+M_{DSPE\text{-}PEG2K}))\times EE\%$. MW, M and EE% refer to molecular weight, weight and encapsulation efficiency respectively. The EE% was calculated by (1-the amount of unencapsulated prodrug/the amount of total prodrug) × 100%. In brief, 1 mL of prodrug nanoassemblies (1 mg/mL) were put into an ultrafiltration centrifuge tube, and centrifuged at 4000 rpm for 20 min. Then, 20 μL of the filtrate was mixed with 180 μL of acetonitrile to dissolve prodrug completely to determine the peak area of prodrugs by HPLC. The peak area was regarded as A1. In the meantime, 20 μL of the prodrug nanoassemblies was mixed with 180 μL of acetonitrile to disintegrate nanoassemblies to determine the peak area of prodrugs by HPLC. The peak area was regarded as A2. The encapsulation efficiency was obtained by (1-A1/A2 ×100%).

Redox-responsive release

PBS (pH 7.4, containing 30% absolute ethanol) buffer containing different concentrations of hydrogen peroxide (H_2O_2, a commonly used ROS substitute) or dithiothreitol (DTT, a substitute for glutathione) [9] was used as the release medium to investigate the drug release behavior of the SN38 HDPNs. The specific operation was as follows: 1 mL of SN38 HDPNs (200 μg equivalent to SN38) was added to 30 mL of release medium, and then placed in a constant temperature shaker at 37 °C with the shaking speed at 100 r/min. 200 μL was sampled at a preset time point, and the content of free SN38 was determined by HPLC (column: 250 mm × 4.6 mm

COSMOSIL®5C18-PAQ; mobile phase: 70% acetonitrile/30% water; flow rate: 1 mL/min; wavelength: 254 nm, injection volume: 10 μL.).

Cell culture

Mouse mammary carcinoma cells (4T1), mouse colon cancer cells (CT26), and human normal liver cells (L02) were provided by the cell bank of Chinese Academy of Sciences (Beijing, China). 4T1 cells, CT26 cells and L02 cells were cultured in Gibco 1640 medium containing 10% FBS and 1% antibiotics. All cells were cultured in incubator (37 °C, 5% CO2). The culture medium was replaced regularly.

Cell uptake, cytotoxicity assays and DNA damage

The 4T1 cells were seeded into a 24-well plate pre-placed with glass dishes (5 × 104 cells/well), and cultured for 24 h. Then, the culture medium was discarded, and 200 μL of coumarin-6 solution or the coumarin-6-labeled prodrug nanoassemblies diluted with fresh culture medium was added (250 ng/mL equal to coumarin-6). Subsequently, the cells were cultured for another 0.5 h or 2 h. After that, cold PBS (pH 7.4) was added to stop cell uptake. The cells were washed three times with PBS (pH 7.4), fixed with 4% paraformaldehyde for 15 min. Then, the cells were stained with Hoechst-33323. Finally, the samples were observed using confocal microscopy. For quantitative analysis, 4T1 cells were cultured into 12-well plates (105 cells/well) for 24 h with the procedure same as above.

The cytotoxicity of SN38 and HDPNs on 4T1, CT26, and L02 cells was investigated by MTT method, and the median inhibitory concentration (IC50) and tumor cell selectivity index (SI) were calculated. In brief, the cell suspension was diluted with fresh culture medium to a concentration of 2 × 104 cells/mL, then 100 μL of the diluted cell suspension was inoculated into a 96-well plate. After 24 h, the old culture medium was replaced with different concentrations of SN38 or HDPNs diluted with fresh culture medium. The cells added pure culture medium were used as the control group, and the wells without cells were the zero-adjusted wells (n = 3). After 48 h, 35 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Then, the medium in plates was poured off, and 180 μL of DMSO was added into each well to dissolve the formazan. Absorbance was measured at a wavelength of 490 nm. The

median inhibitory concentration (IC50) was calculated using Graph Pad Prism 8. The tumor selective index (SI) value was calculated by dividing the IC50 values of L02 cell lines by the IC50 values of tumor cell lines.

γH2AX was regarded as a biomarker for formation of DNA double-strand breaks [1]. Accordingly, 4T1 cells were seeded into 24-well plates pre-placed with glass dishes and cultured for 24 h. 4T1 cells were treated with SN38 solution or HDPNs with a concentration of 5 × 10-8 M equivalent to SN38. Then, the cells were fixed with 4% paraformaldehyde for 15 min. After that, the cells were washed with cold PBS for three times and incubated with anti-γH2AX primary antibodies for 24 h at 4 °C. Finally, Cy3-labeled Goat Anti-Rabbit IgG secondary antibodies were added in dark conditions and the nuclei were stained with Hoechst-33323. The prepared samples were observed by CLSM (TCS SP2/AOBS, LEICA, Germany). The quantitative analysis of the immunofluorescence was performed by Image J software (n = 3)

Animal studies

Live subject statement: Our research obtained ethical approval from the Institutional Animal Ethical Care Committee (IAEC) of Shenyang Pharmaceutical University. All the animals were provided by the Animal Centre of Shenyang Pharmaceutical University. All the animal protocols were performed in line with the Guidelines for the Care and Use of Laboratory Animals of Shenyang Pharmaceutical University (Male Sprague-Dawley rats (200-250 g): Grant No. SYPHU-IACUC-C2020-8-24-202; female Balb/C (18-22 g): Grant No. SYPHU-IACUC-C2021-1-13-103.). The institutional guidelines followed the law of Certification and Accreditation Administration of the People' s Republic of China (RB/T 173-2018). The IACUC registration number of Institute of Shenyang Pharmaceutical University is SYXK 2018-0009.

Animals were placed at a standard clean room with temperature kept at 25 °C and a 12 h light/dark cycle. Standard food and water can be freely obtained. Cervical dislocation under deep anesthesia was used for euthanasia.

Hemolysis assay**:** 5 mL of fresh blood was withdrawn and centrifuged for 5 min at 1600 rpm to obtain the blood cells. In addition, the blood cells were washed with saline

and centrifuged several times, until the supernatant was colorless. Then, a 2% blood cell suspension was prepared by mixing 3.2 mL of saline with 0.8 mL of blood cells. After that, the 2% blood cell suspension was incubated with saline (negative control sample), prodrug nanoassemblies or Triton X-100 (positive control sample) at 37 °C for 2 h, respectively. Finally, the samples were centrifuged at 3000 rpm for 10 min to observe whether the prodrug nanoassemblies were hemolytic. Furthermore, the absorbance of supernatant was measured at 530 nm with a multifunctional microplate reader. And, the hemolysis percentage (HP%) was calculated by (As-An)/(Ap-An) ×100%. As, An and Ap respectively refer to the absorbance of the sample, negative control sample and positive control sample.

In vivo pharmacokinetic study: SD rats were randomly divided into 3 groups (n = 5) and fasted for 12 h before administration. Then SN38, β-SN38-SS-SN38 NPs and γ-SN38-SS-SN38 NPs (2.5 mg/kg equivalent to SN38) were injected via tail vein, respectively. At the pre-set time point, the orbital blood was drawn into a tube pre-coated with heparin, then centrifuged at 13,000 rpm for 5 min to collect the plasma. The content of SN38 in the plasma was determined by UPLC-MS-MS.

In vivo biodistribution: 100 μL of 4T1 cell suspension (5 × 107 cells/mL) was injected subcutaneously into the right back of Balb/C mice. When tumors grew to about 400 mm3, tumor-bearing mice were randomly divided into 3 groups (n = 9). DiR solution or DiR-labeled HDPNs (1 mg/kg equivalent to SN38) were injected into mice via tail vein. Then at 4 h, 12 h and 24 h after administration, three mice in each group were sacrificed, and the heart, liver, spleen, lung, kidney and tumor tissues were isolated to determine the fluorescence intensity using noninvasive optical imaging system (IVIS) spectrum (n = 3 for each group). In addition, the content of free SN38 in tumors were certified using UPLC-MS-MS. Briefly, the SN38 or HDPNs (5 mg/kg equal to SN38) were injected into the tail vein (n = 3 for each group) when tumors grew to about 400 mm3. At 4 h, 12 h and 24 h after injection, the tumors were isolated and weighed for content analysis.

In vivo antitumor efficacy and safety evaluation: 100 μL of 4T1 cell suspension with a concentration of 5×10^7 cells/mL was inoculated into the right back of BALB/c mice

by subcutaneous injection. When the tumor volume grew to about 100 mm3, the tumor-bearing mice were randomly divided into 4 groups (n = 5), and were injected with saline, SN38 solution, β-SN38-SS-SN38 NPs or γ-SN38-SS-SN38 NPs via tail vein (2.5 mg/kg equal to SN38), respectively. Dosing was given every other day for a total of 5 doses. The long and short diameters of the tumors and the body weight of the mice were measured and recorded daily, and the tumor volume was calculated. The calculation method of tumor volume is: long diameter × short diameter × short diameter × 0.5. The mice were sacrificed one day after the last administration, and the tumor tissue was isolated, weighed and photographed. The tumor burden (tumor weight/ body weight × 100%) was calculated. The tumor tissue was fixed with 4% paraformaldehyde for later TUNEL assay and Ki67 assay. Fluorescence images were quantitatively analyzed with Image J software.

One day after the last administration, the orbital blood of the mice was collected for blood routine examination. And centrifugation was utilized to acquire the serum for the evaluation of hepatic and renal function. The isolated heart, liver, spleen, lung, kidney and tumor tissues were washed with normal saline, and stored in 4% paraformaldehyde for later H&E staining and photographing.

Statistical Analysis

The data were displayed as mean value ± standard deviation. Statistical comparisons between groups were analyzed with two-tailed Student's t-test. Statistical significance was considered at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

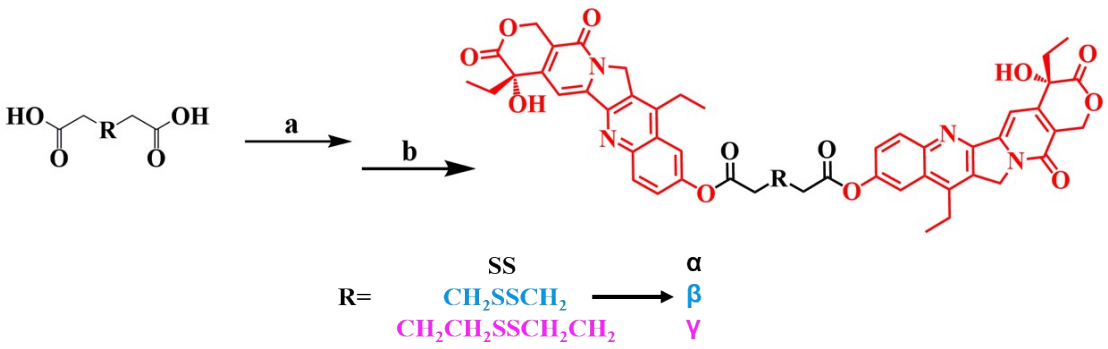

Figure S1. The synthetic route of disulfide bond-bridged SN38 homodimeric prodrugs. (a) EDCI, DMAP, 25℃, 12 h; (b) EDCI, DMAP, 25℃, 24 h.

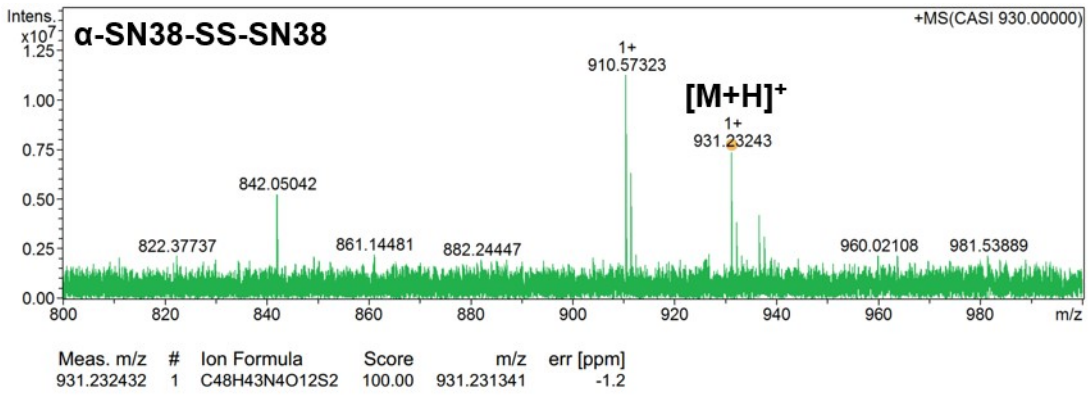

Figure S2. The mass spectrum of α-SN38-SS-SN38.

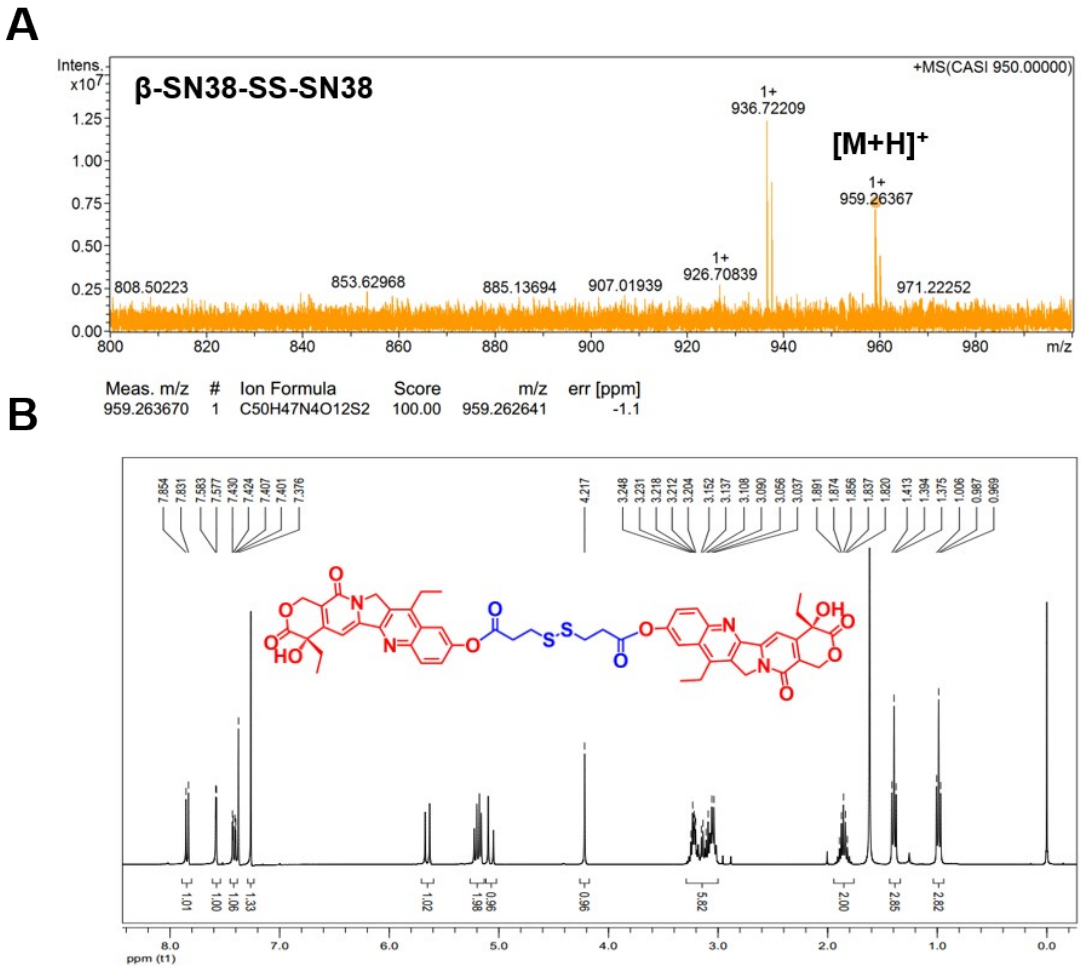

Figure S3. (A) Mass spectrum of β-SN38-SS-SN38. (B) ^{1}H NMR spectrum of β-SN38-SS-SN38.

^{1}H NMR (400 MHz, *CDCl$_3$*) ppm 7.84 (d, 2H, Ar-H, J = 9.14 Hz), 7.58 (d, 2H, Ar-H, J = 2.38 Hz), 7.42 (dd, 2H, Ar-H, J = 9.14, 2.40 Hz), 7.26 (s, 2H, Ar-H), 5.65 (d, 2H, 22-CH_2-αH, J = 16.38 Hz), 5.19 (dd, 4H, 5-CH_2, J = 17.55, 8.12 Hz), 5.07 (d, 2H, 22-CH_2-βH, J = 18.75 Hz), 3.29-3.00 (m, 12H, $CH_2\underline{CH_2}SS\underline{CH_2}CH_2$, 23-$CH_2$), 1.86 (td, 4H, 18-$CH_2$, J = 14.28, 7.02 Hz), 1.39 (t, 6H, 24-CH_3, J = 7.66 Hz), 0.99 (t, 6H, 29-CH_3, J = 7.38 Hz).

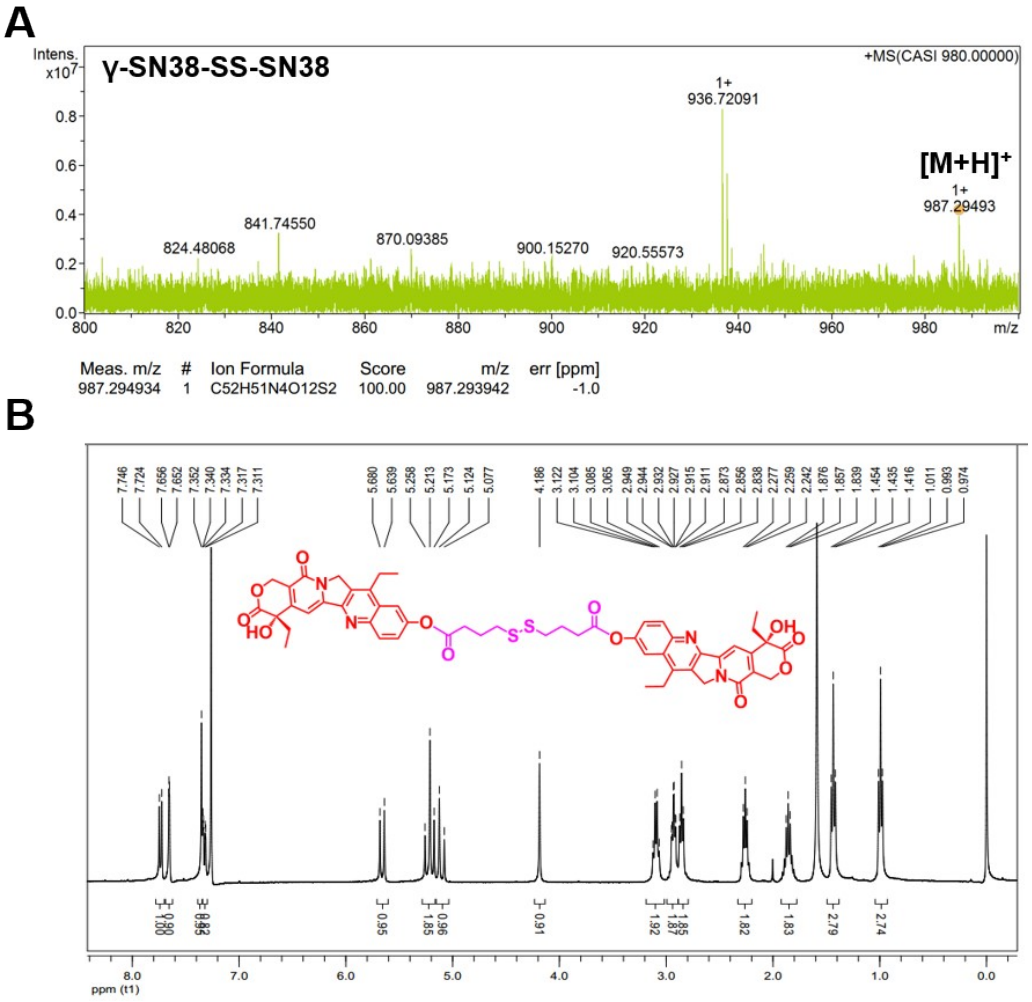

Figure S4. (A) Mass spectrum of γ-SN38-SS-SN38. (B) ^{1}H NMR spectrum of γ-SN38-SS-SN38.

^{1}H NMR (400 MHz, *CDCl$_3$*) ppm 7.74 (d, 2H, Ar-H, J = 9.13 Hz), 7.65 (d, 2H, Ar-H, J = 1.97 Hz), 7.35 (s, 2H, Ar-H), 7.33 (dd, 2H, Ar-H, J = 9.20, 2.14 Hz), 5.66 (d, 2H, 22-CH_2-αH, J = 16.39 Hz), 5.21 (t, 4H, 5-CH_2, J = 17.00 Hz), 5.10 (d, 2H, 22-CH_2-βH, J = 18.70 Hz), 3.09 (d, 4H, $CH_2CH_2\underline{CH_2}SS\underline{CH_2}CH_2CH_2$, J = 7.72 Hz), 2.93 (dt, 4H, $CH_2\underline{CH_2}CH_2SSCH_2\underline{CH_2}CH_2$, J = 6.63, 1.73 Hz), 2.86 (t, 4H, $\underline{CH_2}CH_2CH_2SSCH_2CH_2\underline{CH_2}$, J = 6.89 Hz), 2.33-2.20 (m, 4H, 23-CH_2), 1.92-1.78 (m, 4H, 18-CH_2), 1.43 (t, 6H, J = 7.59 Hz), 0.99 (t, 6H, J = 7.34 Hz).

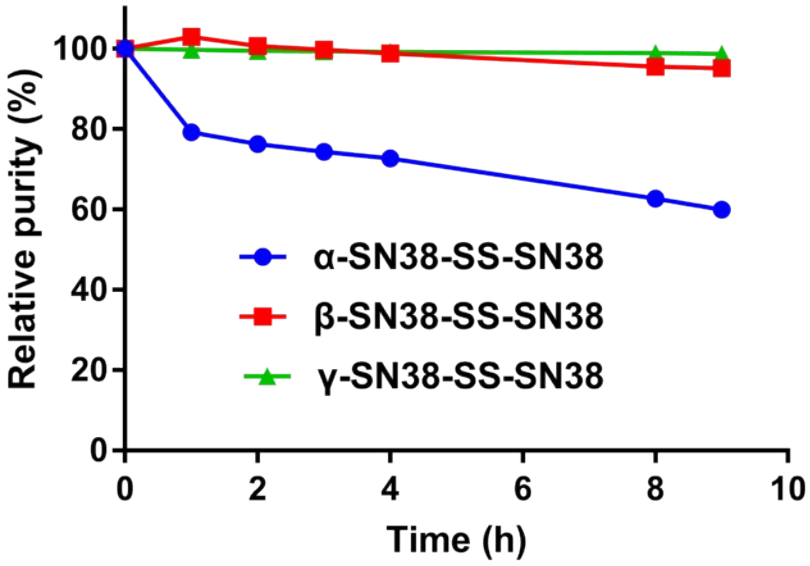

Figure S5. The relative purity of three prodrugs at room temperature.

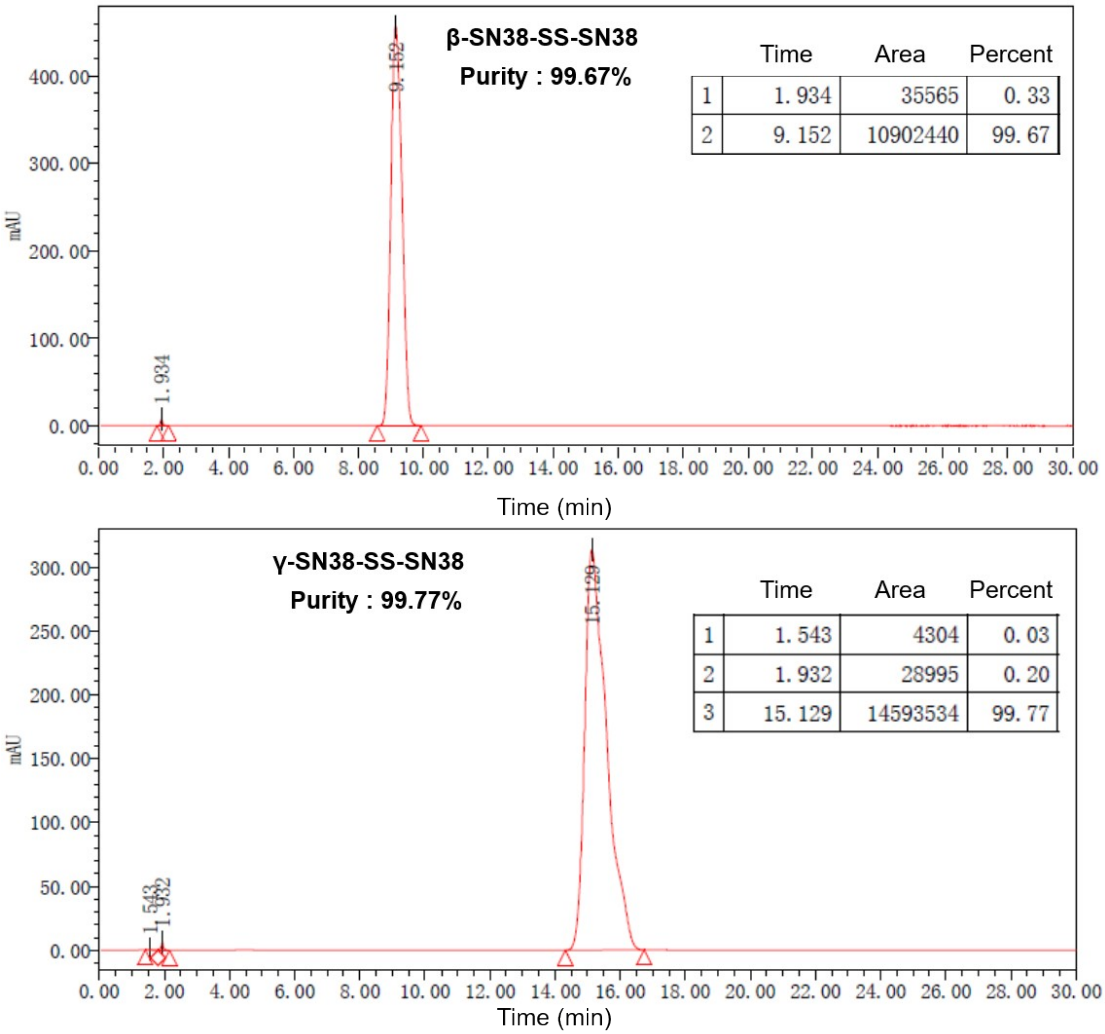

Figure S6. The purity of β-SN38-SS-SN38 and γ-SN38-SS-SN38.

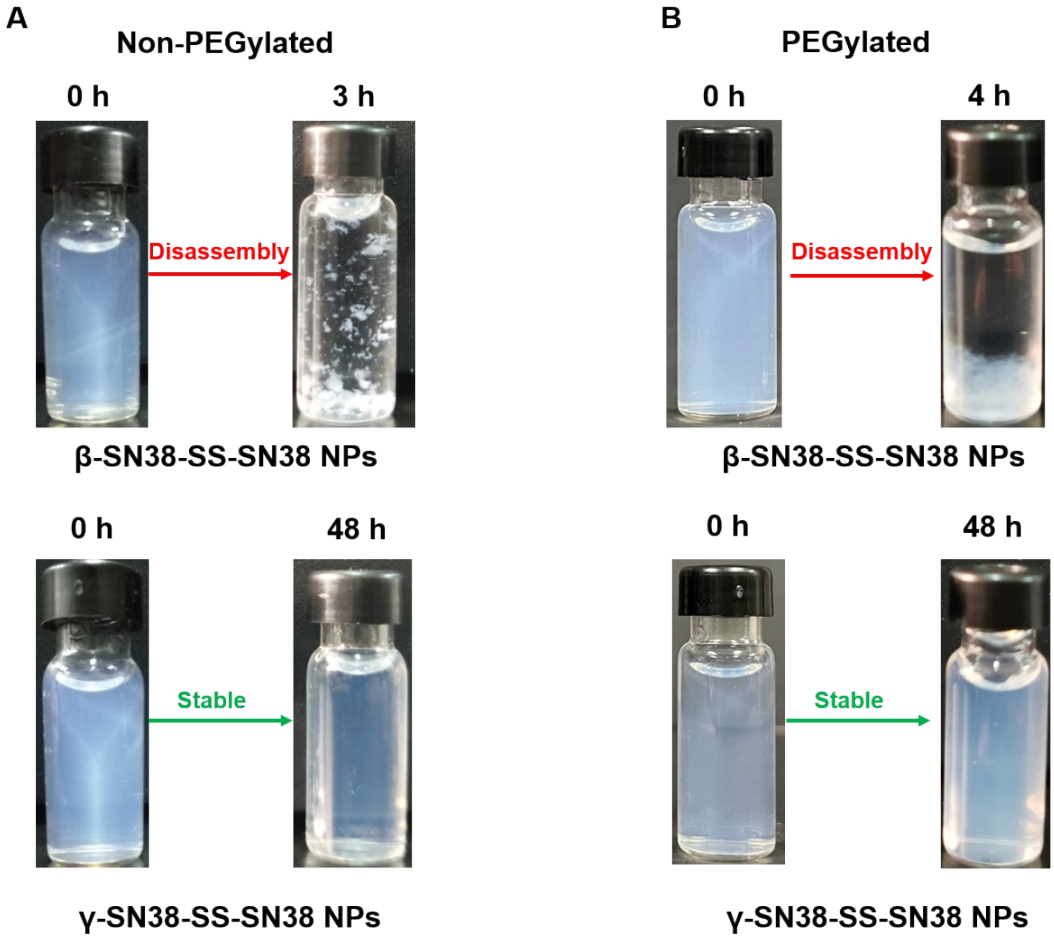

Figure S7. The image of (A) non-PEGylated and (B) PEGylated HDPNs stored for different time at room temperature.

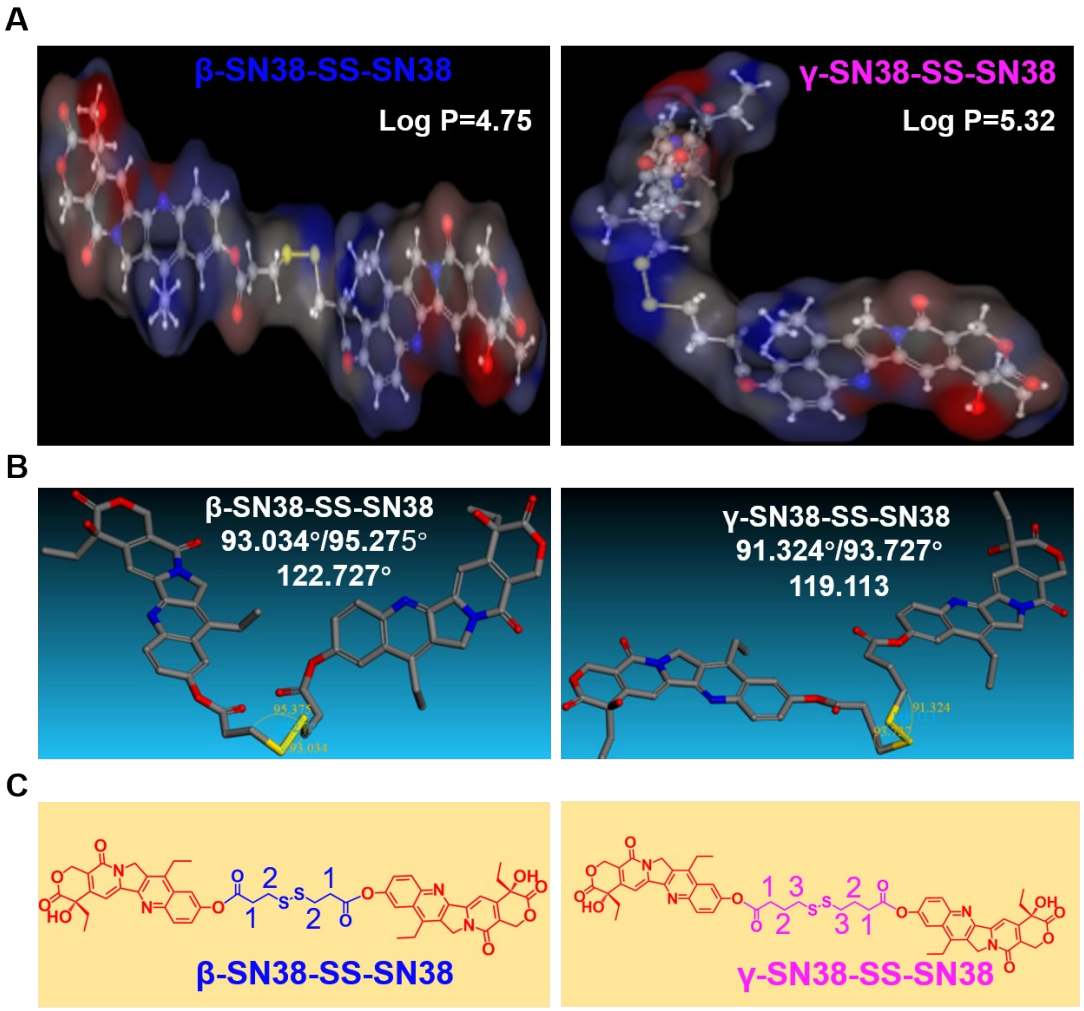

Figure S8. (A) The Log P of β-SN38-SS-SN38 and γ-SN38-SS-SN38. (B) The bond angles and dihedral angle of -SS- in β-SN38-SS-SN38 and γ-SN38-SS-SN38. (C) The chemical structure of β-SN38-SS-SN38 and γ-SN38-SS-SN38.

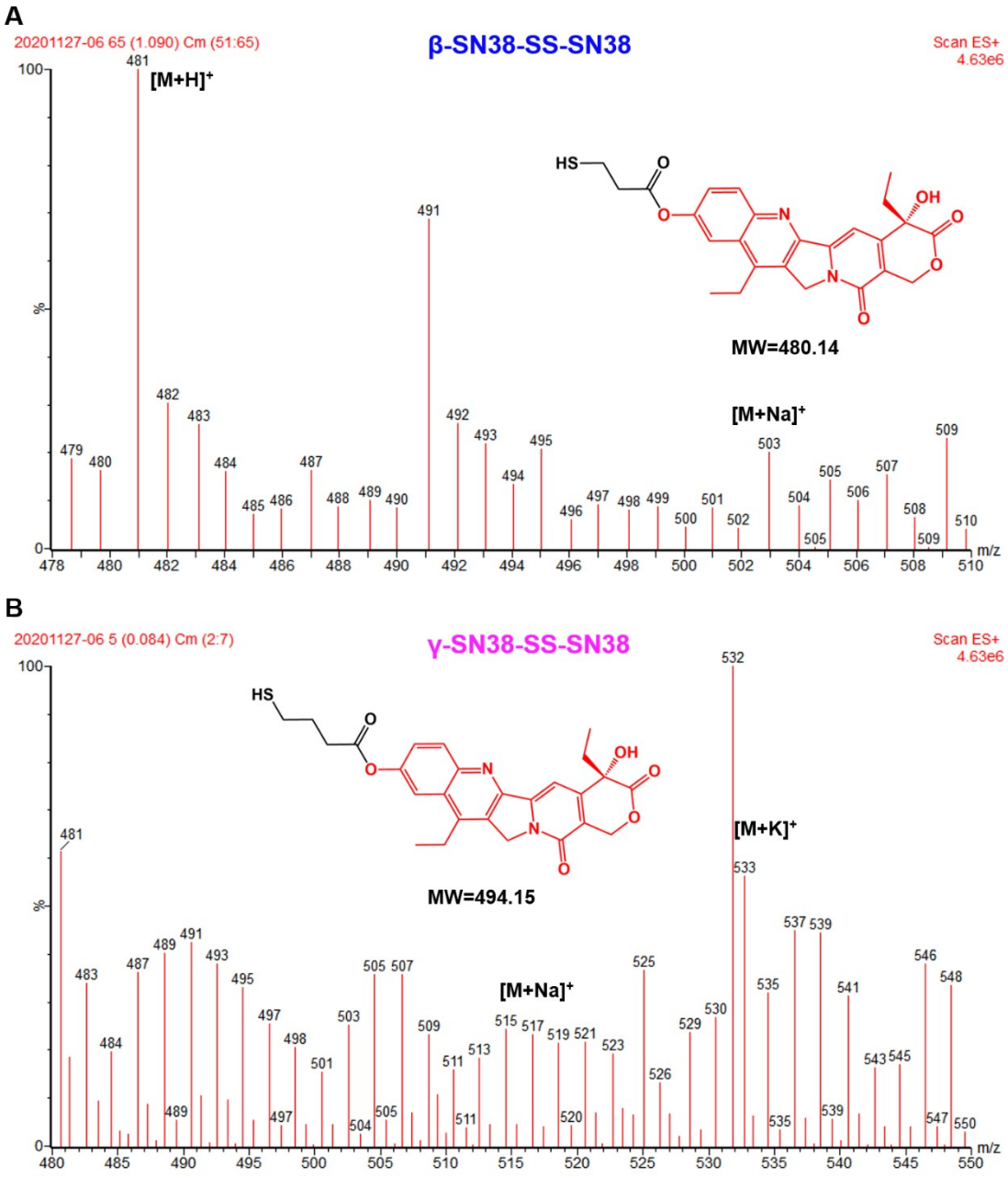

Figure S9. Mass spectra of (A) β-SN38-SS-SN38 NPs and (B) γ-SN38-SS-SN38 NPs after incubated with DTT containing release media.

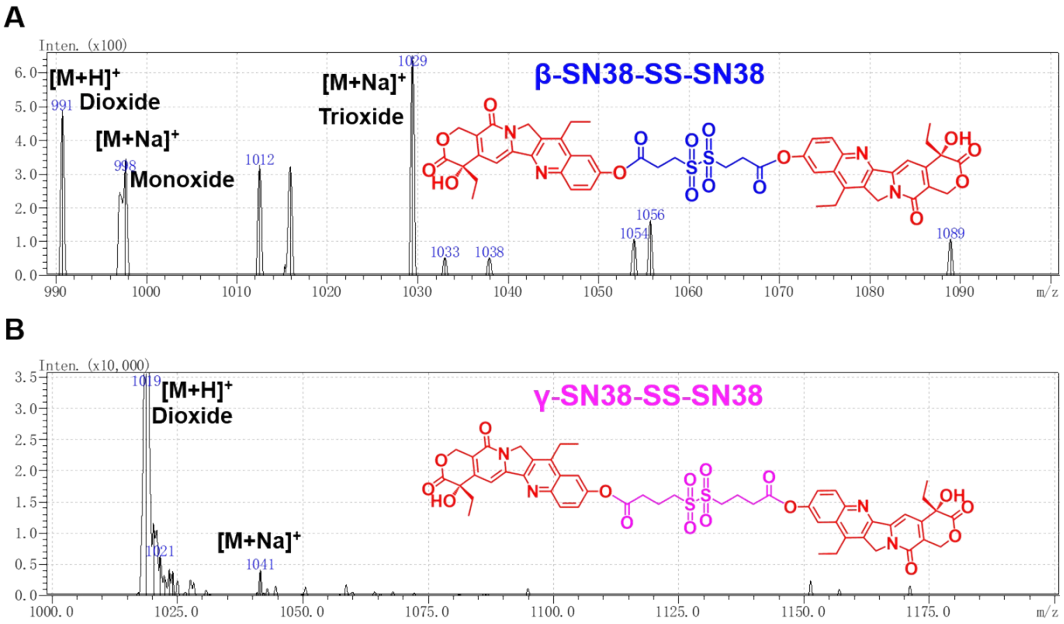

Figure S10. Mass spectra of (A) β-SN38-SS-SN38 NPs and (B) γ-SN38-SS-SN38 NPs after incubated with H_2O_2 containing release media.

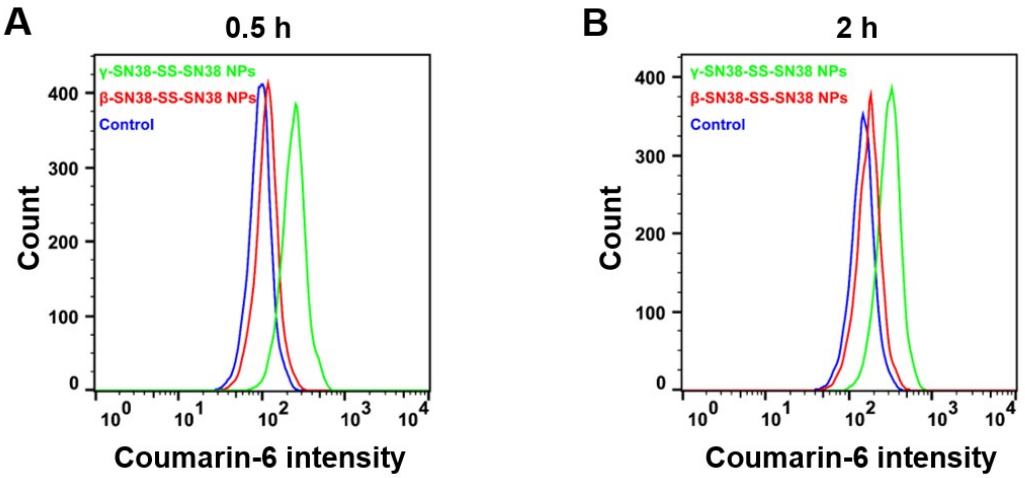

Figure S11. Flow cytometry images of 4T1 cells treated with coumarin-6 solution or coumarin-6-loaded SN38 HDPNs at 0.5 h (A) and (B) 2 h.

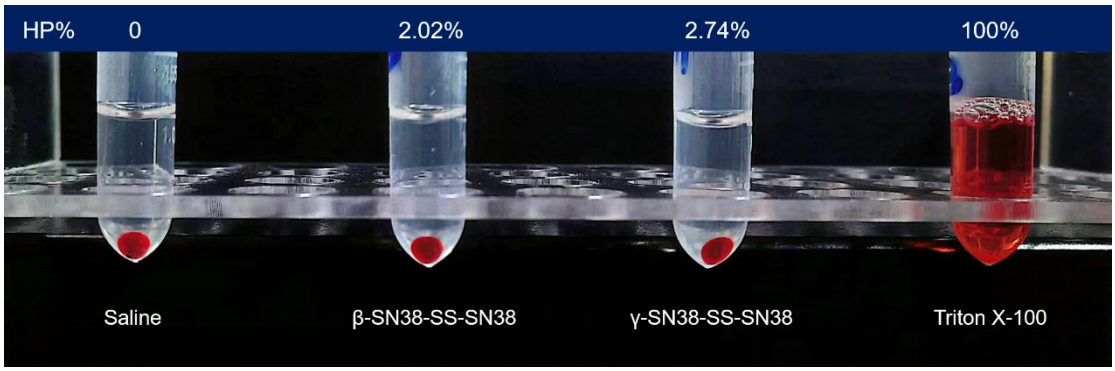

Figure S12. Hemolysis of the prodrug nanoassemblies, HP% represents hemolysis percentage.

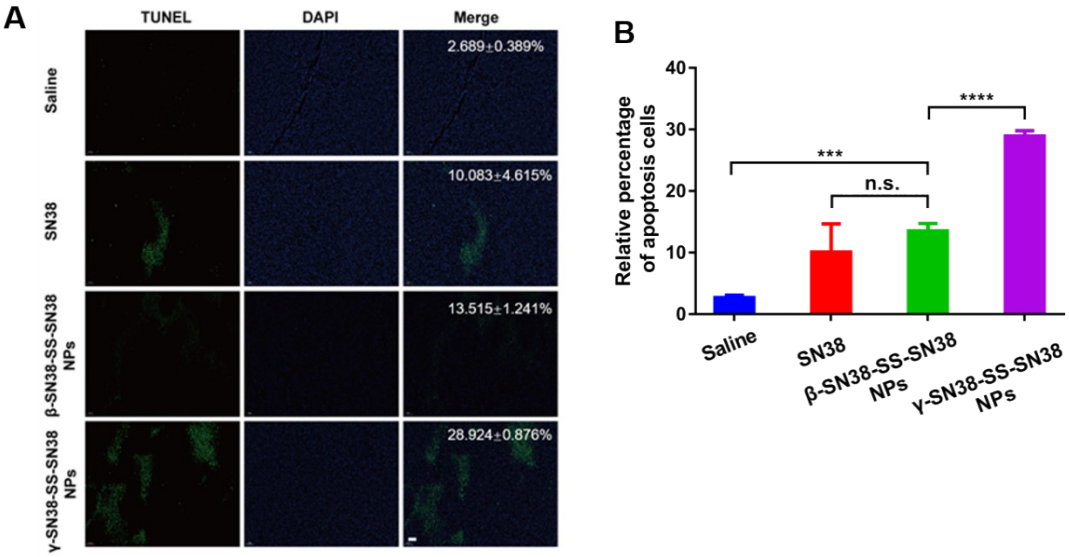

Figure S13. (A) TUNEL assay. 4T1 tumor sections were prepared after the last treatment. Scale bar represents 100 μm. (B) Quantification of the relative area (%) of apoptosis cells. *** $P < 0.001$, **** $P < 0.0001$. Data are presented as mean ± SD (n=3).

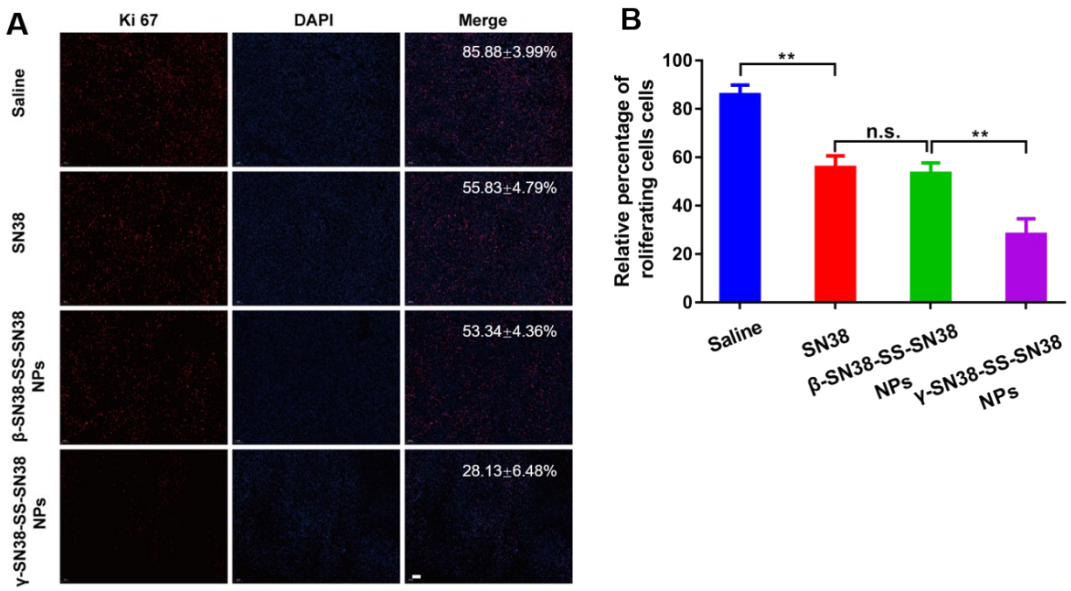

Figure S14. (A) Ki 67 assay. 4T1 tumor sections were prepared after the last treatment. Scale bar represents 100 μm. (B) Quantification of the relative area (%) of proliferating cells. ** $P < 0.01$, Data are presented as mean ± SD (n=3).

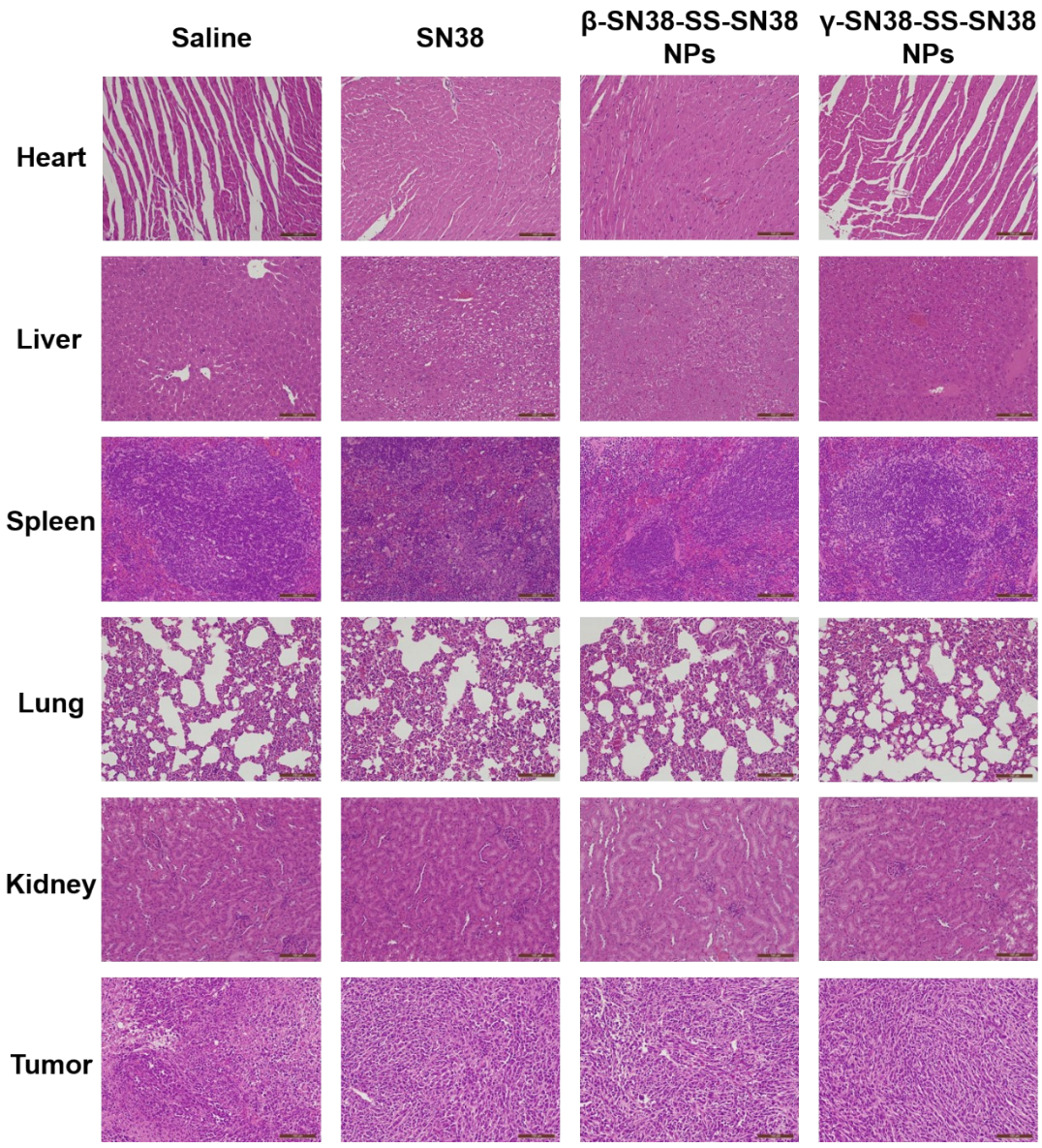

Figure S15. H&E staining of the major organs and tumor. Scale bar represents 100 μm.

Supplementary Tables

Table S1. Characterization of non-PEGylated prodrug nanoassemblies (n=3).

Nanoassemblies	Size (nm)	PDI	Zeta (mV)	DL (w/w)
β-SN38-SS-SN38 NPs	114.3±0.12	0.139±0.04	-4.64±0.25	81.86%
γ-SN38-SS-SN38 NPs	91.3±7.27	0.172±0.10	-7.03±0.70	79.53%

Table S2. Characterization of PEGylated prodrug nanoassemblies (n=3).

Nanoassemblies	Size (nm)	PDI	Zeta (mV)	DL (w/w)
β-SN38-SS-SN38 NPs	98.9±1.76	0.193±0.09	-19.7±0.35	65.5%
γ-SN38-SS-SN38 NPs	97.7±0.93	0.092±0.01	-21.5±0.62	63.7%
DSPE-PEG$_{2k}$	——	——	-19.5±1.85	——

Table S3. IC_{50} values (nmol L^{-1}) of SN38 and HDPNs against tumor cell lines and normal cell line (n=3).

Cell lines	SN38	β-SN38-SS-SN38 NPs	γ-SN38-SS-SN38 NPs
4T1	16.4	6.9	5.2
CT26	64.98	48.27	34.91
L02	1426	1544	1151

Table S4. The selectivity index (SI) of prodrug nanoassemblies between normal cells and tumor cells after being incubated for 48 h.

Cell lines	SN38	β-SN38-SS-SN38 NPs	γ-SN38-SS-SN38 NPs
4T1	86.95	223.76	221.35
CT26	21.94	31.99	32.98

Table S5. Pharmacokinetic parameters of Taxol and prodrug nanoassemblies.

Formulations	Determined	$AUC_{0\text{-}24h}$ [a)]	C_{max} [b)]
SN38	SN38	0.335±0.063	0.70±0.149
β-SN38-SS-SN38 NPs	SN38	0.504±0.161	2.889±0.924
γ-SN38-SS-SN38 NPs	SN38	1.079±0.211	5.269±1.019

a) Area under curve (nmol h mL^{-1}). b) Peak plasma concentration (nmol h mL^{-1}). Data are presented as mean ± SD (n=5).

Reference:

[1] R. J. Kinders, M. Hollingshead, S. Lawrence, J. Ji, B. Tabb, W. M. Bonner, Y. Pommier, L. Rubinstein, Y. A. Evrard, R. E. Parchment, J. Tomaszewski and J. H. Doroshow, Clin Cancer Res, 2010, 16, 5447-5457.