Supporting Information

Prodrugs Forming Multifunctional Supramolecular Hydrogels for Dual Cancer Drug Delivery

Wei Ha, [†] Jing Yu, [†] Xin-yue Song, [†] Zhi-jun Zhang, [‡] Ying-qian Liu, [‡] Yan-ping Shi[†,*]

[†] Key Laboratory of Chemistry of Northwestern Plant Resources of CAS and Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, People's Republic of China. E-mail: shiyp@licp.cas.cn

[‡] School of Pharmacy, Lanzhou University, Lanzhou 730000, People's Republic of China

Material: Methoxypoly(ethylene glycol) (mPEG MW=2000) was purchased from Sigma-Aldrich without further purification. α-cyclodextrin (α-CD) and 4-(dimethylamino) pyridine (DMAP) was purchased from Aladdin Chemical Co. (China). Camptothecin (CPT) was purchased from Sichuan Jiangyuan Natural Products Co. (China). 5-fluorouracil (5-FU) was kindly given by Lanzhou University. N,N'-Diisopropylcarbodiimide (DIC) were purchased from Shanghai GL biochem Ltd. (China). Other reagents were analytical pure and used directly without further purification.

Measurements: ^{1}H and ^{13}C NMR were measured on a Bruker AVANCE III-400 spectrometers. The chemical shifts of ^{1}H NMR are expressed in parts per million downfield relative to the internal tetramethylsilane (δ = 0 ppm) or chloroform (δ = 7.26 ppm). The crystalline changes of the hydrogels were confirmed by X-ray

diffraction measurements, which were performed by using Cu-Kα irradiation with PHILP X'Pert PRO. The transmission electron microscopy (TEM) image was measured on Jeol JEM-1200EX at an accelerating voltage of 100 KV. The rheological behavior of the hydrogels was investigated by a ZNN-D6 rotational rheometer. For the Scanning electron microscopy (SEM) observations, the specimens were freeze-dried under vacuum and ground to fine powder. The powder were placed on conducting glue and coated with gold vapor and then analyzed on a JSM-5600LV electron microscope.

Synthesis of CPT-PEG Prodrug: Equivalent of mPEG (Mn=2000, 10.0 g, 5 mmol) and 2 equivalents of succinic anhydride (1.0 g, 10 mmol) (dried over P_2O_5 under high vacuum) were dissolved in 25 mL anhydrous dichloromethane and refluxed for 3 days under nitrogen with 2 equivalents of pyridine (0.8 mL, 10 mmol) as catalyst. After evaporation of the solvent, the crude product was dissolved in water. Unreacted succinic anhydride was eliminated by washing with a mixture of ethyl acetate and hexane (v:v =1:1) for several times. The aqueous phase was extracted by $CHCl_3$, and all organic layers were combined followed by drying over anhydrous $MgSO_4$ overnight. The filtrate was concentrated and recrystallized from diethyl ether to give 8.6 g of white solid, carboxyl-functionalized mPEG (mPEG-COOH, 82.4% yield).

mPEG-COOH (2.1 g, 1 mmol) was dissolved in 10 mL of anhydrous dichloromethane at room temperature. The solution was cooled to 0℃ and DIC (170 μL, 1 mmol), DMAP (122 mg, 1 mmol) and camptothecin (348 mg, 1

mmol) were added successively, and stirred for 2 h at 0℃. The reaction mixture was allowed to warm to room temperature and left for 16 h. After filtration, the filtrate was washed with 0.1 N HCl, dried with anhydrous $MgSO_4$ and evaporated under reduced pressure to yield the crude product as a light yellow solid. An analytical sample of CPT-PEG was prepared by flash chromatography (silica, $CHCl_3$/MeOH 20:1) to afford product as a light yellow solid (1.9 g, 78.8% yield): ^{1}H-NMR (400MHz, $CDCl_3$): δ 8.39 (1H, s, H-7), 8.21 (1H, d, J=8.4Hz, H-12), 7.93 (1H, d, J=7.2Hz, H-9), 7.82 (1H, t, J=7.2Hz, H-10), 7.66 (1H, t, J=7.2Hz, H-11), 5.52 (2H, ABq, J=16.8Hz, H-17), 5.27 (2H, s, H-5), 4.22 (4H, s), 3.87-3.79 (2H, m), 3.63 (323H, br, mPEG), 3.36 (6H, s, mPEG-OCH3), 2.83 (2H, s, mPEG), 2.73-2.66 (4H, m, mPEG), 2.14 (2H, m, H-19), 0.97 (3H, t, J=7.6Hz, H-18)。 ^{13}C-NMR (100MHz, $CDCl_3$): δ 7.68, 23.60, 28.93, 31.81, 42.02, 50.00, 59.11, 64.06, 65.91, 67.08, 69.02, 70.63, 71.99, 76.29, 96.27, 120.08, 128.07, 128.22, 128.30, 128.55, 129.68, 130.70, 131.25, 145.93, 146.31, 148.91, 152.46, 157.40, 167.42, 171.35, 171.80.

Critical micelle concentration (CMC) of CPT-PEG prodrug: The critical micelle concentration (CMC) was determined as our previous work. Pyrene was used as a fluorescence probe. The concentration of CPT-PEG prodrug was varied from 0.0001 to 1 mg/mL and the the concentration of pyrene was fixed at 1.0 μM. Fluorescent spectra were measured using fluorescence spectrophotometer with a slit width of 10.0 and 2.5 nm for excitation and emission. For fluorescence emission spectra, excitation wavelength was set at 339 nm, and for fluorescence excitation spectra, the emission

wavelength was set at 390 nm. The CMC was obtained as the intersenction of the tangents to the two linear portions of the graph of the fluorescence intensity as a function of CPT-PEG concentration (Figure S1).

Formation of Hydrogels: The general protocol for the hydrogel formation is as follows: An aqueous solution of α-CD (90.0 mg/mL) was added to an aqueous solution of CPT-PEG. Various concentrations of CPT-PEG were used to formulate different hydrogels. For all samples, the solution was mixed thoroughly by sonication for 5 min followed by incubation at room temperature for 72 h before measurements. For encapsulating 5-FU, α-CD (90.0 mg) and 5.0 mg 5-FU was added to 1.0 mL aqueous solution of CPT-PEG (20.0 mg/mL), the solution was mixed thoroughly by sonication for 5 min followed by incubation at room temperature for 72 h before measurements.

In vitro release kinetics studies: 90.0 mg α-CD and 5.0 mg 5-FU was added into 1.0 mL CPT-PEG prodrug PBS solution (20.0 mg/mL). The solution was added into a 1.5-mL cuvette. The solution was mixed thoroughly by sonication for 5 min followed by incubation at 37℃ for 72 h, allowing the mixture to form a viscous hydrogel. The cuvette was placed upside-down in a test tube with 30.0 mL of PBS and incubated in a 37℃ water bath. The PBS was changed in determined intervals of time. The concentrations of the 5-FU and CPT-PEG prodrug released from hydrogels were determined using a Agilent 1260 high performance liquid chromatography. Chromatographic separation was performed on an Agilent ZORBAX SB-C18 column

(4.6×150 mm, 5 μm) at 30℃ with methanol and 0.1% phosphoric acid aqueous solutions (75:25, v/v) as mobile phase at a flow rate of 1.0 mL/min. A wavelength of 372 nm was used to detect CPT-PEG, and 265 nm to 5-FU. The typical HPLC results are shown in Figure S3. The concentration of CPT-PEG and 5-FU was calculated based on the equation for calibration curve. The release of CPT from the CPT-PEG nanoparticles was analyzed by a dialysis method. CPT-PEG solution (1.0 mL) at pH 7.4 (CPT-PEG at 20.0 mg/mL) was loaded into a dialysis bag (MWCO 3500). The dialysis bag was then immersed in 30.0 mL of PBS buffer (pH 7.4) at 37 ℃. PBS medium (5.0 mL) was withdrawn at timed intervals and replaced by 5.0 mL fresh PBS to maintain submersed conditions. The CPT concentration was determined by HPLC (Figure S4).

In vitro anticancer activities: The anticancer activities of the CPT hydrogels loading 5-FU was evaluated using the methyl tetrazolium (MTT) assay. A total of 115 mg (containing 3 mg CPT and 5 mg 5-FU) of freeze-fried powder sample of hydrogel were put into 5 mL of RPMI 1640 cell culture medium in a 50 mL centrifuge tube, and then placed in a shaker incubator (37℃, 60 rpm) for 2 days. After that, the media were filtered with 0.22 μm sterile filter into a sterile container and stored in a refrigerator at 4℃ before use.

A549, HepG2 cells were separately seeded in a 96 well culture plate at a density of 8000 cells per well and cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum at 37℃ in a humidified environment of 5 % CO_2 for 1 day.

Thereafter, the cells were incubated with CPT, 5-FU and the extracted leached medium from the hydrogel at varing concentrations for 72 h. Then, the RPMI 1640 medium was aspirated and replaced with 100 μL fresh medium. 24 h later, 15 μL of 5 mg/mL MTT solution was added to each well and incubated for further 4 h. The medium solution was then replaced with 150 μL DMSO to dissolve the MTT-formazan that was generated by live cells, and the plate was shaken for 30 min to produce a homogeneous coloured solution. Absorbance was read at 570 nm on a microplate reader. The relative cell viability (%) was expressed as a percentage of that of the control culture. The release experiments were carried out six times. The results presented are the average data.

Median-effect principle for dose-effect analysis and the combination index studies: The multiple drug effect analysis based on the median-effect principle was used to examine drug interactions. This involves plotting dose effect curves for each agent and for multiple diluted, fixed ratio combinations of agents using the median effect equation:

$$f_a / f_u = (D / D_m)^m$$

In this equation, D *i*s the dose, D_m is the dose required for 50% effect (e. g. 50% inhibition of cell growth), f_a is the fraction effected by D, f_u is the unaffected fraction, (1-f_a), and m is the coefficient of sigmoidicity of the dose-effect curve; The dose-effect curve is plotted using a logarithmic conversion of this equation to:

$\log^{f_a/f_u} = m\log^{(D)} - m\log^{(D_m)}$ for the median-effect plot: $x = \log^{(D)}$ versus $y = \log^{f_a/f_u}$, which determines the m (slope) and D_m (anti-log of x intercept) values. A combination index (CI) is then determined with the classic isobologram equation of Chou-Talalay:

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

Where $(D_x)_1$ is the dose of agent 1 (CPT) required to produce×percentage effect alone and $(D)_1$ is the dose of agent 1 required to produce the same×percentage effect in combination with $(D)_2$. Similarly, $(D_x)_2$ is the dose of agent 2 (5-FU) required to produce×percentage effect alone and $(D)_2$ is the dose required to produce the same effect in combination with $(D)_1$. The denominators of the CI equation above, $(D_x)_1$ and $(D_x)_2$ can be determined by $D_x = D_m\left[f_a/(1-f_a)\right]^{1/m}$. Different values of CI may be obtained for solving the equation for different values of f_a. CI values of <1 indicate synergy, >1 indicate antagonism and =1 indicates additive effect.

Table S1. Dose-effect relationship parameters for CPT and 5-FU in cancer model

Cell type	CPT			5-FU			(3:5) CPT:5-FU		
	D_m (μg/mL)	*Linear equation*	*r*	D_m	*Linear equation*	*r*	D_m	*Linear equation*	*r*
A549	5.41	Y=2.0175x-1.4787	0.99	6.21	Y=0.8226X-0.6522	0.98	2.25+3.75	Y=0.7485X-0.5822	0.98
HepG2	4.97	Y=1.2644X-0.8801	0.99	7.38	Y=1.666X-1.4460	0.95	1.49+2.50	Y=0.9024X-0.5414	0.97

Shape (sigmoidicity) and conformity of dose-effect curve (linear correlation coefficient) are represented by *Dm*, linear equation, *r*, respectively, where D_m is the antilog of *x*-intercept in μg/mL, *r* is the linear correlation coefficient of the median-effect plot.

Table S2. Interaction of CPT and 5-FU combinations in cells at different stage of carcinogenesis: combination indices at different effect levels

Cell type	Combination index (*CI*) at:								
	$f_{a0.1}$	$f_{a0.2}$	$f_{a0.3}$	$f_{a0.4}$	$f_{a0.5}$	$f_{a0.6}$	$f_{a0.7}$	$f_{a0.8}$	$f_{a0.9}$
A549	0.53	0.64	0.75	0.87	1.02	1.22	1.52	2.05	3.42
Hep G2	0.26	0.36	0.45	0.54	0.64	0.76	0.91	1.15	1.63

CI value <1, =1, >1 indicates synergism, additive effect, and antagonism, respectively. f_a is the fraction effected.

The *CI* values were calculated by the Chou-Talalay method based on the median-effect equation and the classic isobologram equation.

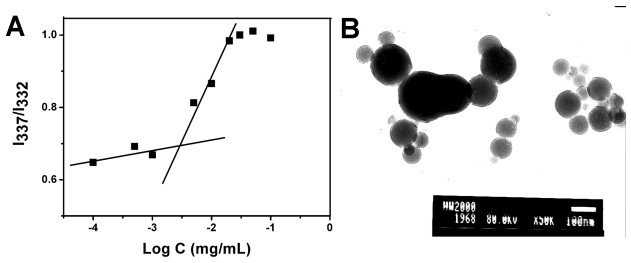

Figure S1. (A) Plot of the intensity ratio I_{337}/I_{332} (from pyrene excitation spectra) as a function of log concentration of CPT-PEG. (B) The TEM image of CPT-PEG nanoparticles.

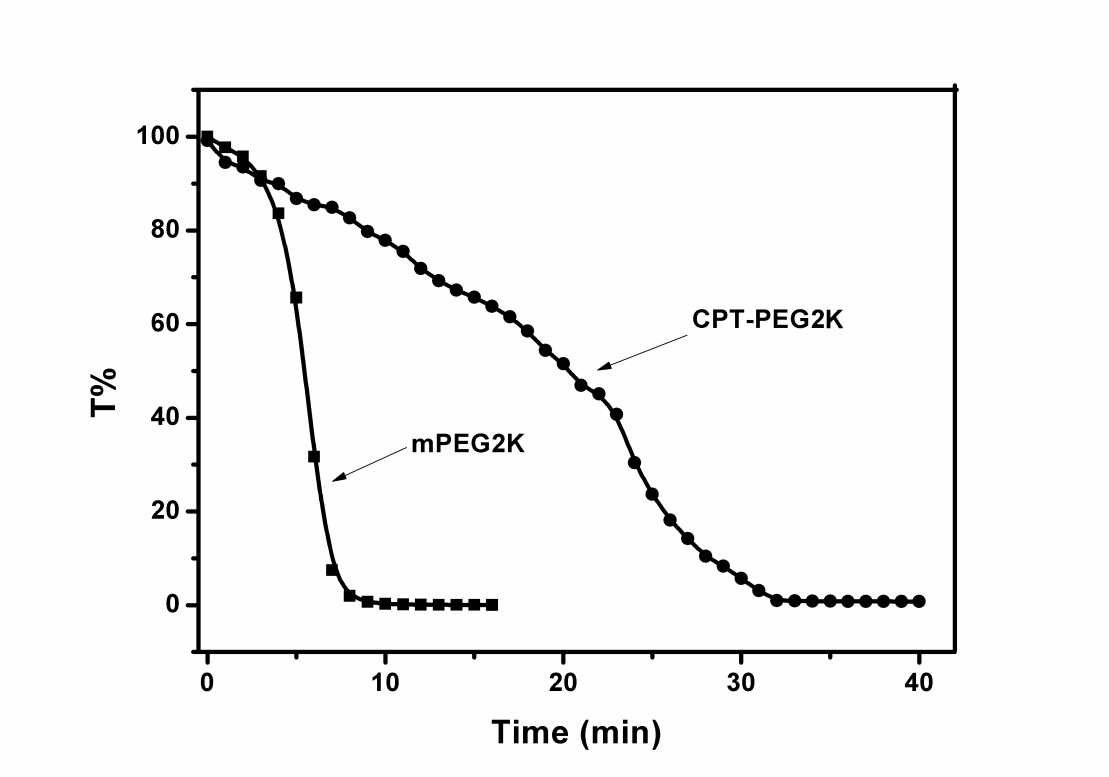

Figure S2. Evolution curves of optical transparency versus time after the addition of α-CD solution to mPEG2K and CPT-PEG2K, without stirring at room temperature. For all samples, the concentration of the polymers is 10 mg/mL, and the ratio or the ethylene glycol units to α-CD is 2.

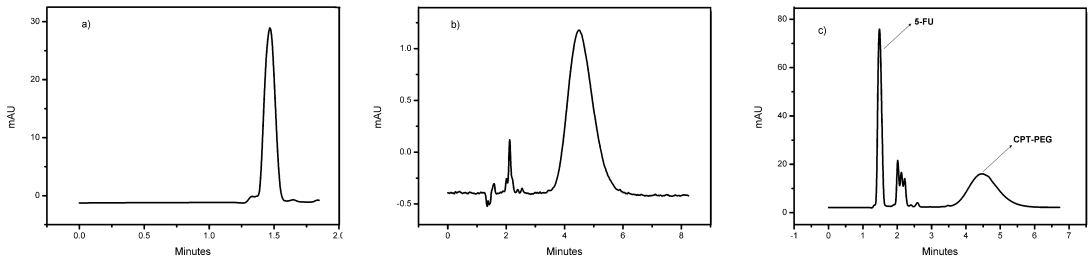

Figure S3. The HPLC curves of pure 5-FU (a), CPT-PEG (b), and the release sample of 5-FU loaded CPT-PEG hydrogel for 98 h (c).

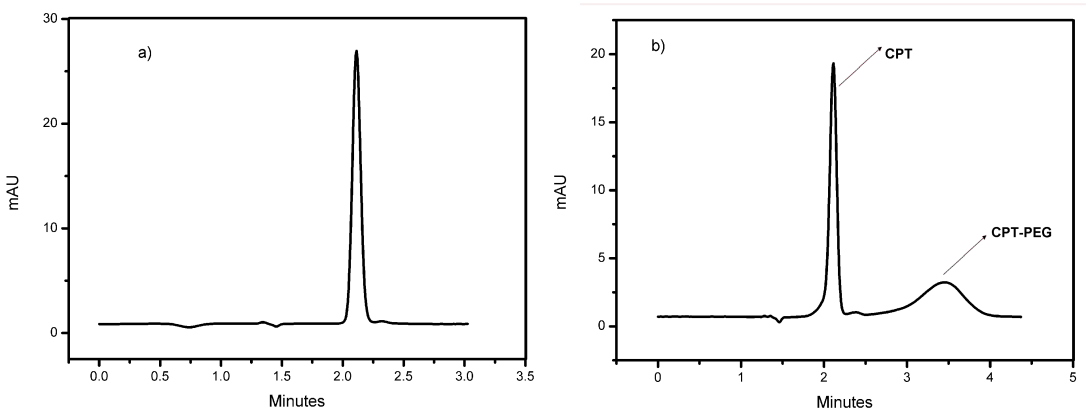

Figure S4. The HPLC curves of pure CPT (a), and the release sample of CPT-PEG nanoparticles (b).