Supporting Information

Colon-targeted podophyllotoxin nanoprodrug: synthesis, characterization, and supramolecular hydrogel formation for drug combination

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1. General Information

1.1 Chemicals. Monomethoxy polyethylene glycol with a molecular weight of 1900 was purchased from Alfa Aesar. Podophyllotoxin and 4'-Demethylepipodophyllotoxin was obtained from Nanjing Spring & Autumn Biological Engineering Co., Ltd (Nanjing, China). 5-Fluorouracil (5-FU) was purchased from Shanghai Shaoyuan Co. 4, 4'-Azodianiline was purchased from HEOWNS Biochem. Technology Co., Ltd (Tianjing, China). α-cyclodextrin (α-CD, purity ≥ 98.0%) were purchased from Aladdin Chemical Co. Ltd. Lipase from candida and Proteinase K was purchased from Sigma-Aldrich. α-Amylase was purchased from novozymes (Tianjing, China). The lung fibroblasts of guinea pig (CHL) and kidney cells of African green monkey (Vero) were obtained from Northwest Minzu University (Lanzhou, China), Live/Dead viability assay kit was purchased from Abnova Company. Dichloromethane (analytical reagent) was used after drying through CaH_2. All other reagents and solvents were of reagent grade or purified according to standard methods before use. Ultrapure water was obtained by a water purification system, which was purchased from Shanghai Laikie Instrument Co., Ltd.

1.2 Materials Characterization. NMR spectra were obtained on a Bruker AVANCE III-400 spectrometers using $CDCl_3$ as the solvent. The morphologies of PEG-S-S-CPT prodrug micells were investigated on an FEI-Tecnai G2 Transmission Electron Microscope (TEM). The morphologies of self-assembled supramolecular hydrogel were investigated and analyzed on a JSM-5600LV electron microscope after the samples were freeze-dried and coated with gold vapor. The X-ray diffraction measurements of hydrogels were performed by a PHILP X'Pert PRO, using Cu Kα (λ = 1.542 Å) irradiation (40 kV, 40 mA) in the range of 2θ=5-80°. UPLC-MS/MS were investigated by an ultra-high performance liquid chromatography mass spectrometry (Waters ACQUITY UPLC MS/MS, USA). HPLC was performed on an Waters 1525 HPLC system installed with a C18 column (BDS HYPERSIL, 5 μm, 250 mm × 4.6 mm) and a photodiode array detector. Fluorescence images on cells were obtained on

an inverted microscope (Olympus IX73+DP73 Tokyo, Japan). Imaging mass spectra were performed an imaging mass spectrometer (iMScope TRIO Shimadzu, Kyoto, Japan). Data were collected at 30 μm intervals. For each tissue pixel, the surface was laser-irradiated with 100 shots (1000 Hz repetition rate, 25 μm laser diameter, 50 laser intensity). The detector voltage of the microchannel plate detector was 1.985 kV, while the sample voltage was 3.5 kV.

1.3 Enzyme-Triggered Hydrogel Gel-Sol Transition

The self-assembled hydrogel (1 mL) formed by prodrug (20 mg) and α-CD (100 mg) were placed in the bottom vials and sealed by caps. Then, 300 μL of proteinase K, lipase (10 mg/mL), amylase (10 mg/mL) and $Na_2S_2O_4$ solution (10 mg/mL) were added into the vials, respectively. The vials were incubated at 37 ^oC in a water bath and taken out 30 min later for observation. The gel-sol transition behavior can be monitored visually by inverting the vials for 1.0 min to observe whether the hydrogels flowed.

1.4 Biocompatibility Evaluation of Prodrug Hydrogel

The MRC-5 and Vero cells were seeded in a 96 well culture plate at a density of 5000 cells mL^{-1}. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified environment of 5% CO_2 for 1 day and incubated with various concentrations of prodrug hydrogel (0.01, 0.1, 1, 3 and 5 mg/mL, dissolved in cell culture medium) for 3 days, respectively. The cell viability were determined by a MTT assay according to the generally procedures and the relative cell viability (%) was expressed as a percentage of that of the control culture.

For Live-Dead assay, Vero cells were seeded in a 24-well plate (1×10^6 cells per well) and incubated for one day. The old media were changed with the media containing the 1 mg mL^{-1} of prodrug hydrogel. The media were removed and 0.5 mL of a mixture of Live-Dye and propidium iodide (PI) was added to each well after 24 h

of incubation. Both fluorochromes can be excited at 488 nm but emit green (λ_{Em} = 518 nm, live cells) and red fluorescence (λ_{Em} = 615 nm, dead cells), respectively. Then, the image of the stained cells was acquired by an inverted microscope.

1.5 Animals

All animal experiments were approved by the Experimental Animal Use and Care Committee, Lanzhou University and in accordance with the principles of laboratory animal operation regulation. The Chinese Kunming mice (8-20 g) and Sprague-Dawley rats (200-210 g) were purchased from Lanzhou University.

1.6 Preparation of rat cecal contents

This experiment has been approved by the ethics committee. A deoxygenated solution of PBS buffer (10 mM, pH 7.4) was prepared by bubbling nitrogen for 15 min at 0 °C. Male Sprague-Dawley rats were sacrificed and the cecum was dissected in the absence of oxygen. The cecal contents were immediately placed into the previously prepared deoxygenated PBS buffer to make a 10% w/v suspension with bubbling nitrogen for 10 min. The solution was centrifuged at 4000 rpm/min for 10 min to remove debris. The centrifuged solution was directly use to the next experiments.

1.7 HPLC and UPLC-MS/MS conditions

HPLC was performed on an Waters 1525 HPLC system installed with a photodiode array detector. Chromatographic separation was performed on BDS HYPERSIL C18 column (5 μm, 250 mm × 4.6 mm). For PBS and enzyme samples, the methanol and 0.1% triethylamine aqueous solutions (90:10, v/v) was used as a mobile phase at a flow rate of 1.0 mL/min while methanol and 0.1% triethylamine aqueous solutions (75:25, v/v) was used as a mobile phase for $Na_2S_2O_4$ samples. A wavelength of 259.6 nm was used to detect PEG-N=N-AP and AdP, and 265 nm to detect 5-FU.

The UPLC experiments were carried out on a Waters ACQUITY UPLC system. The separation of AdP were achieved on a UPLC C18 column (2.1×50 mm, 1.8 μm). The gradient elution was used with A (acetonitrile) and B (water, ammonium acetate,

5 mM) as mobile phase at a flow rate of 0.3 mL/min: 0-1.5 min, 98% B; 1.5-2.5 min, 98-30% B; 2.5-3.5 min, 30% B; 3.5-4.0 min, 30-98% B; 4.0-5.5 min, 98% B. The column temperature was maintained at 35 °C and the injection volume was 1 μL each time. The detection and identifications of AdP was performed on a Waters ACQUITY tandem triple quadrupole mass spectrometer (TQD) equipped with a Z-Spray multi-mode electrospray ionization/atmospheric pressure chemical ionization (ESI/APCI) ion source. The MS/MS analysis conditions were optimized as follows: source temperature, 110 °C; desolvation temperature, 350 °C; The flow rates of desolvation gas (nitrogen 99.999%), 600 L/h; the flow rates of cone gas (argon 99.999%), 50 L/h. The data acquisition and the system control were performed using a MassLynx 4.1 work station (Waters, USA).

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

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

Where $(D_x)_1$ is the dose of agent 1(AdP) 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 $D_x = D_m[f_a/(1-fa)]^{1/m}$. 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 AdP and 5-FU in cancer model

Cell type	AdP			5-FU			(1:1) AdP:5-FU		
	D_m (μM)	*Linear equation*	R^2	D_m	*Linear equation*	R^2	D_m	*Linear equation*	R^2
HepG2	91.47	y = 0.5695x - 1.117	0.93	46.17	y = 0.4562x - 0.7593	0.92	7.25+7.25	y = 0.9043x - 1.0501	0.96

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 μM, *r* is the linear correlation coefficient of the median-effect plot.

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

Cell type	Combination index (*CI*) at:								
	$f_{a0.1}$	$f_{a0.2}$	$f_{a0.3}$	$f_{a0.4}$	$f_{a0.5}$	$f_{a0.6}$	$f_{a0.7}$	$f_{a0.8}$	$f_{a0.9}$
HepG2	2.04	0.90	0.53	0.35	0.24	0.16	0.11	0.07	0.03

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

2. Supplementary Figures S1-14

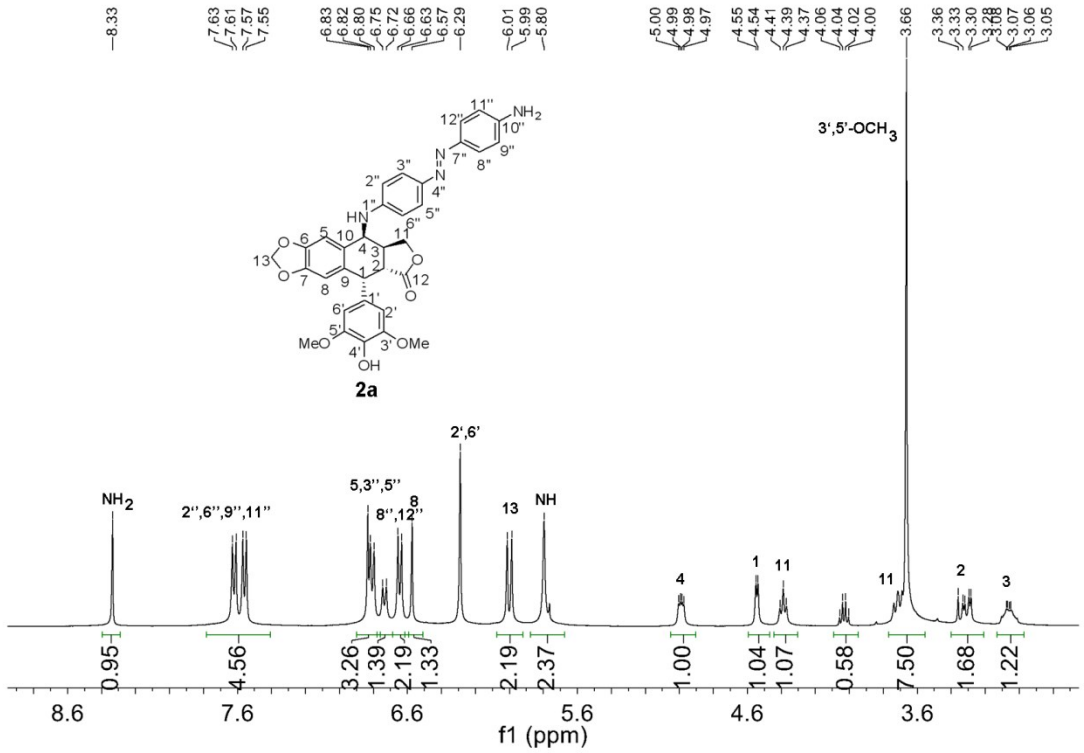

Figure S1. ^{1}H NMR (400MHz, d_6-DMSO) spectrum of compound 2a.

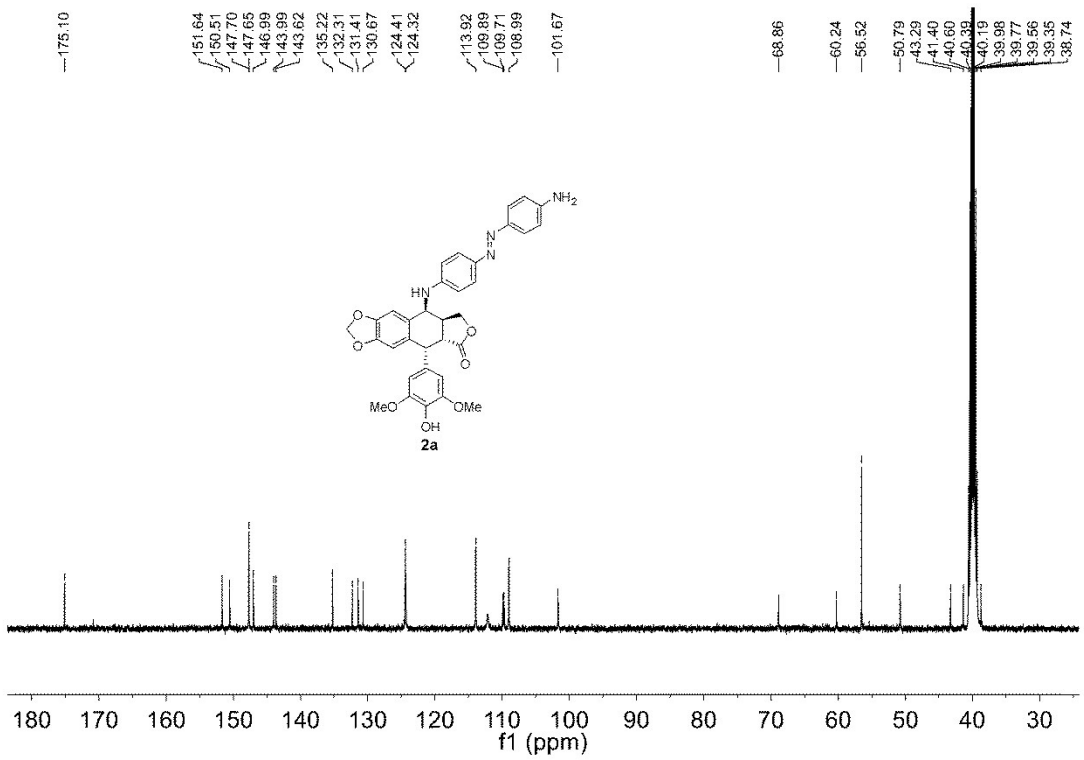

Figure S2. ^{13}C NMR (100MHz, d_6-DMSO) spectrum of compound 2a.

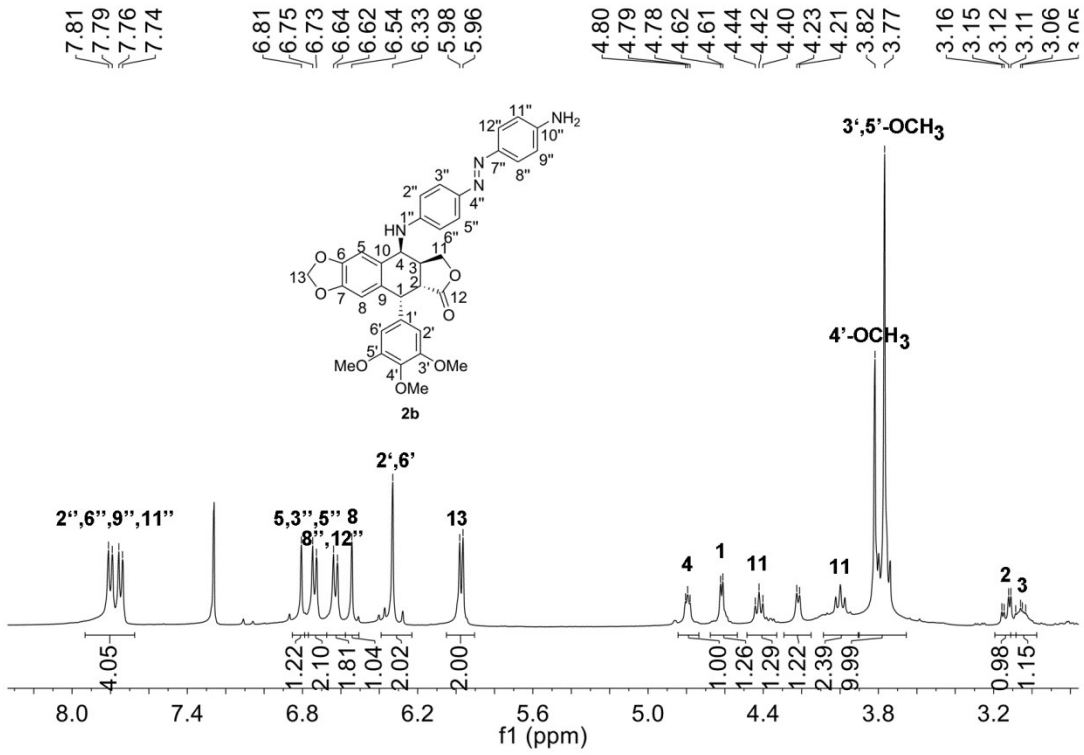

Figure S3. ^{1}H NMR (400MHz, $CDCl_3$) spectrum of compound 2b.

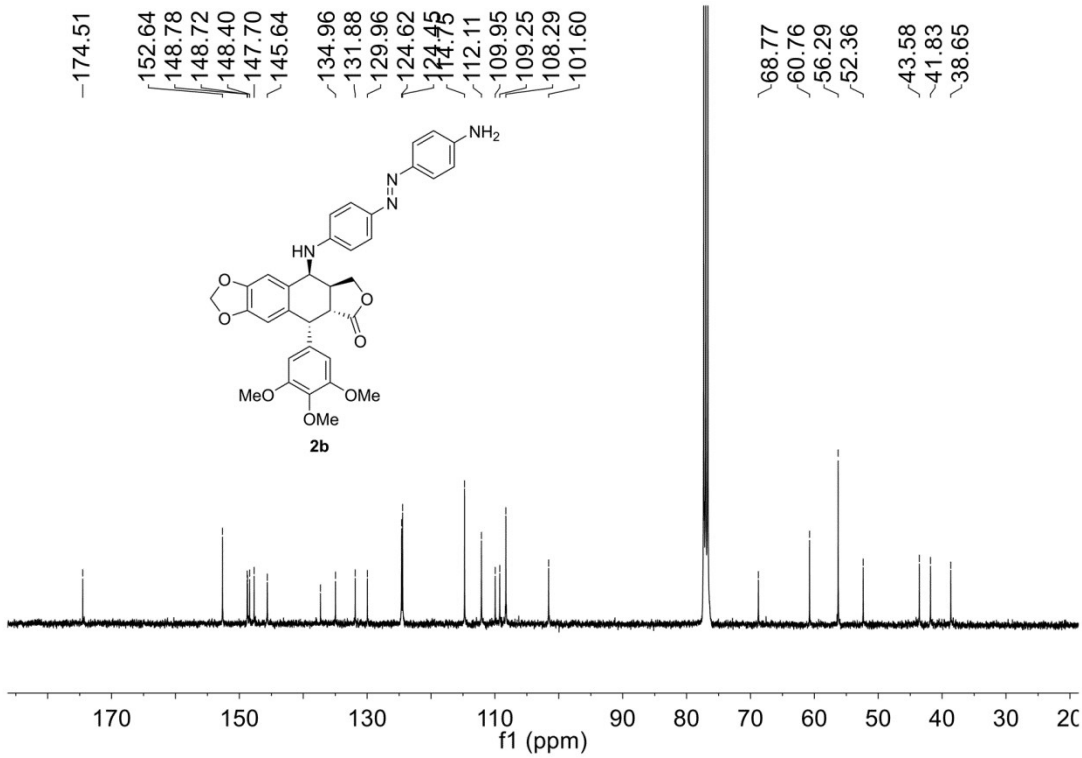

Figure S4. ^{13}C NMR (100MHz, $CDCl_3$) spectrum of compound 2b.

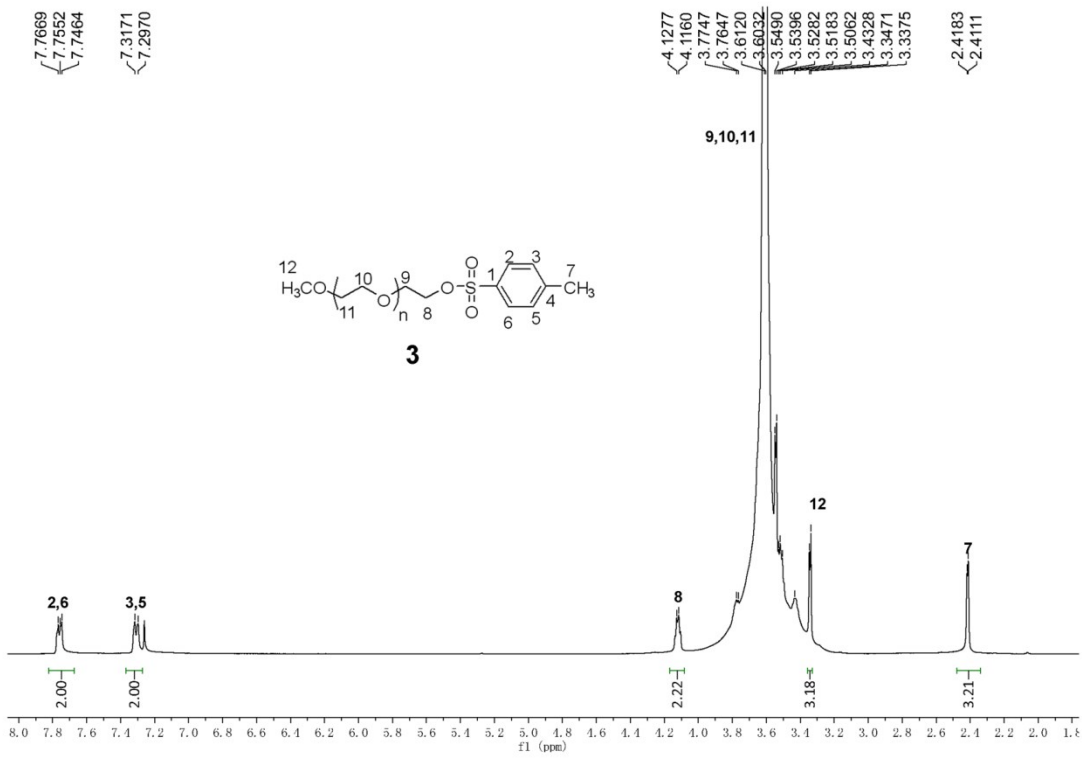

Figure S5. ^{1}H NMR (400MHz, $CDCl_3$) spectrum of compound 3.

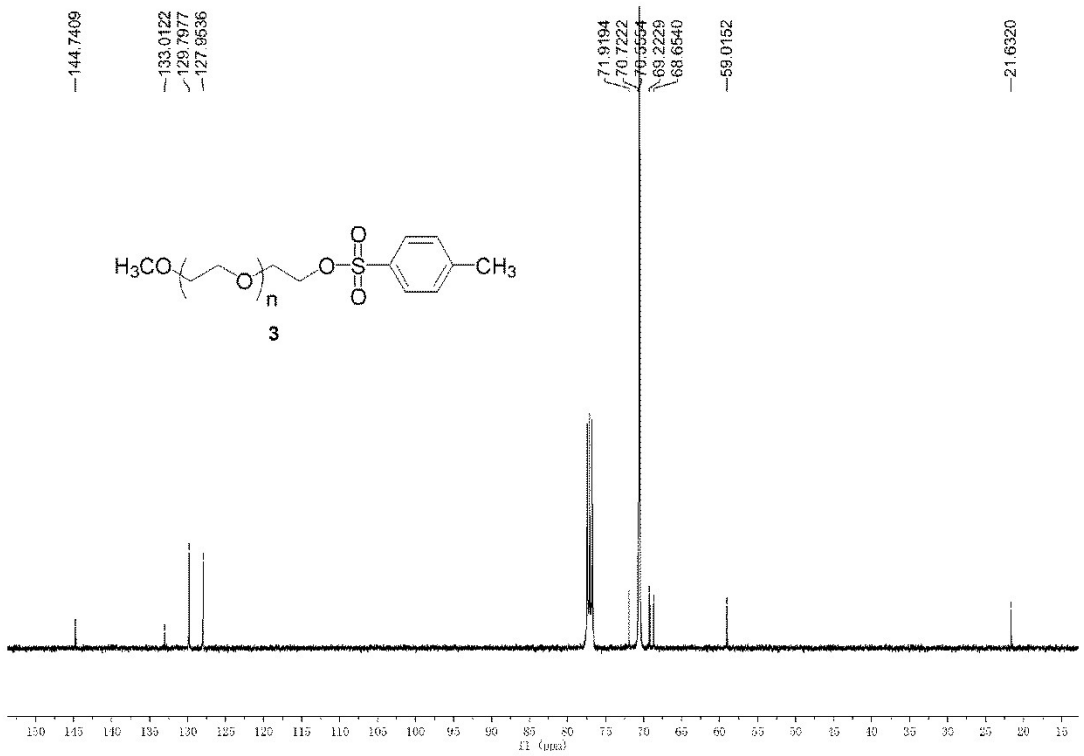

Figure S6. ^{13}C NMR (100MHz, $CDCl_3$) spectrum of compound 3

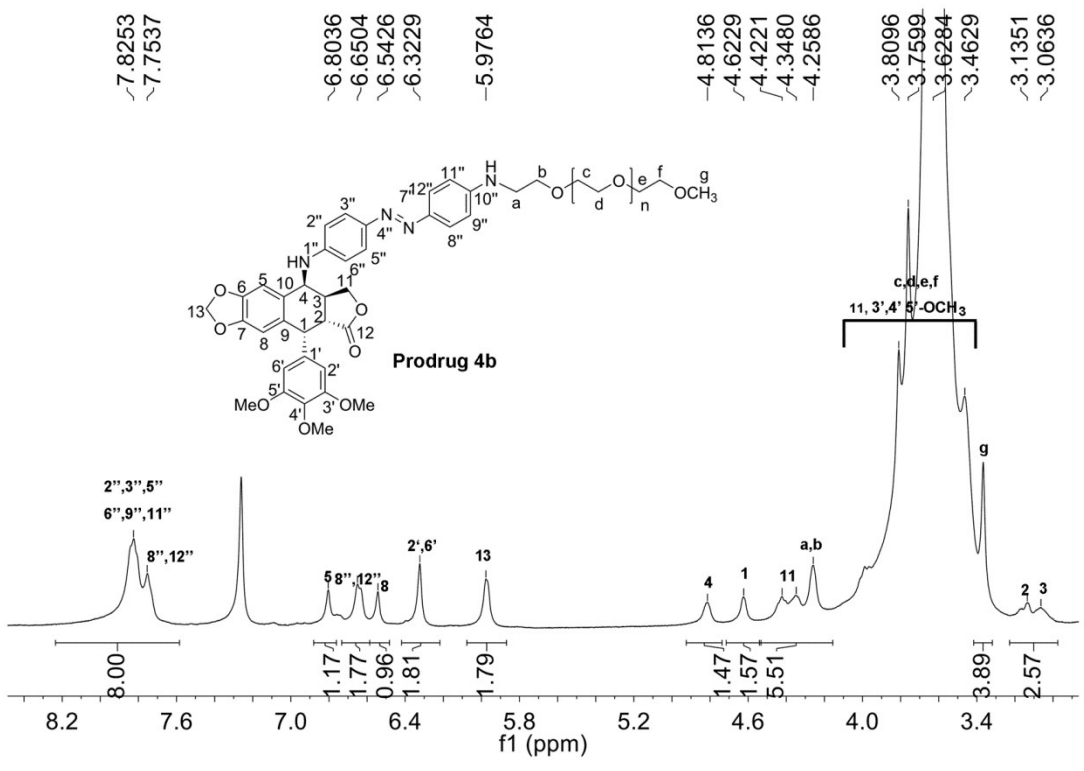

Figure S7. ^{1}H NMR (400MHz, $CDCl_3$) spectrum of prodrug 4b.

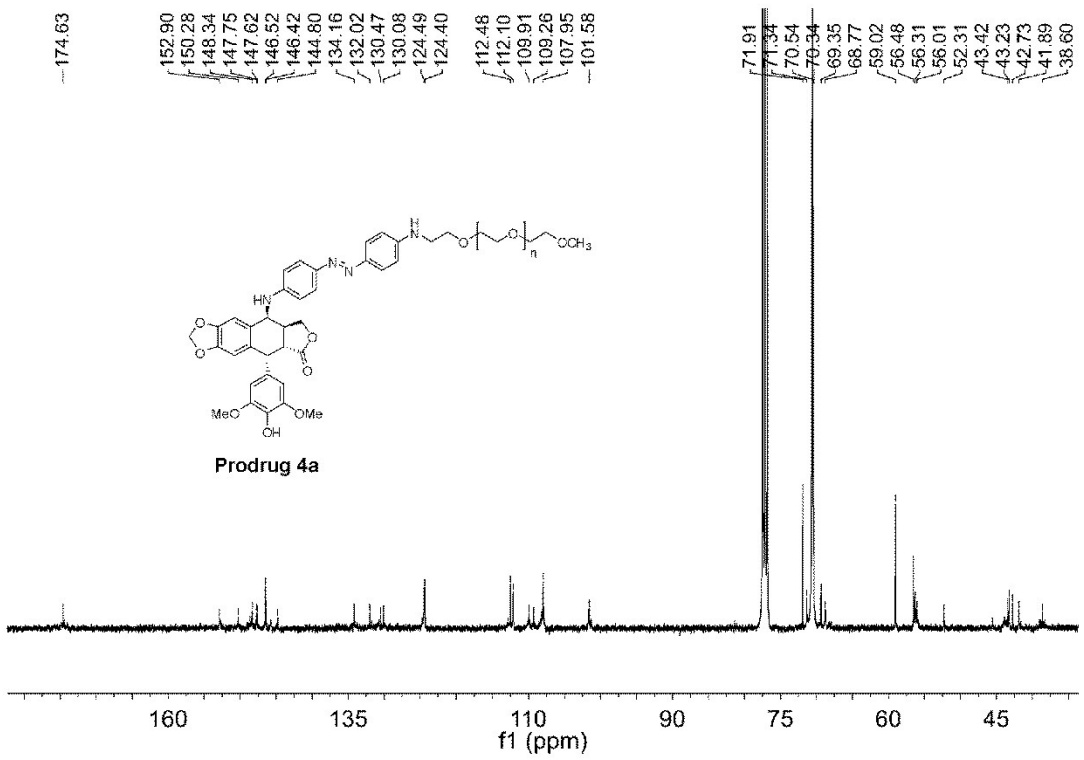

Figure S8. ^{13}C NMR (100MHz, $CDCl_3$) spectrum of prodrug 4a.

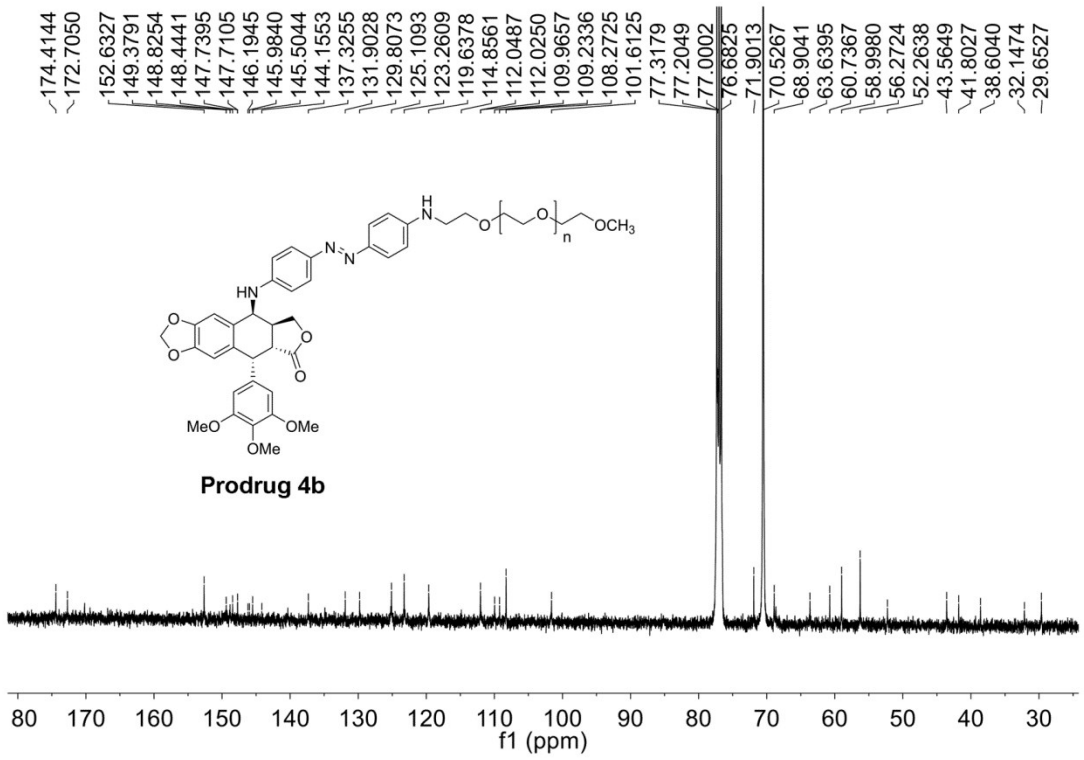

Figure S9. ^{13}C NMR (100MHz, $CDCl_3$) spectrum of prodrug 4b.

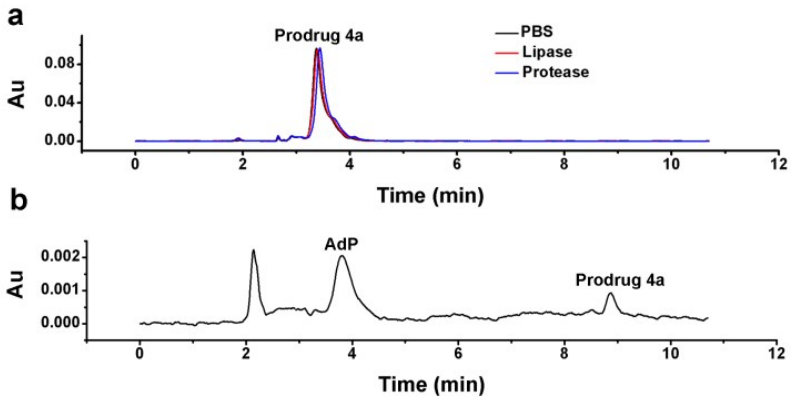

Figure S10. (a) HPLC curves of prodrug 4a upon the addition of PBS, lipase and protease. (b) HPLC curves of prodrug 4a upon the addition of $Na_2S_2O_4$.

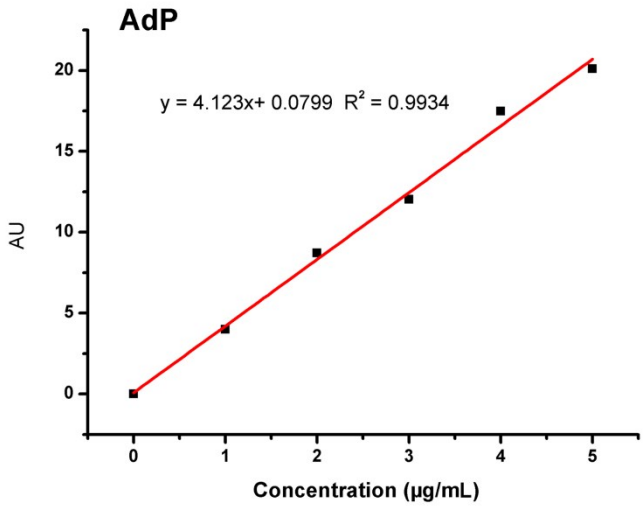

Figure S11. Calibration curve of AdP established by UPLC-MS/MS.

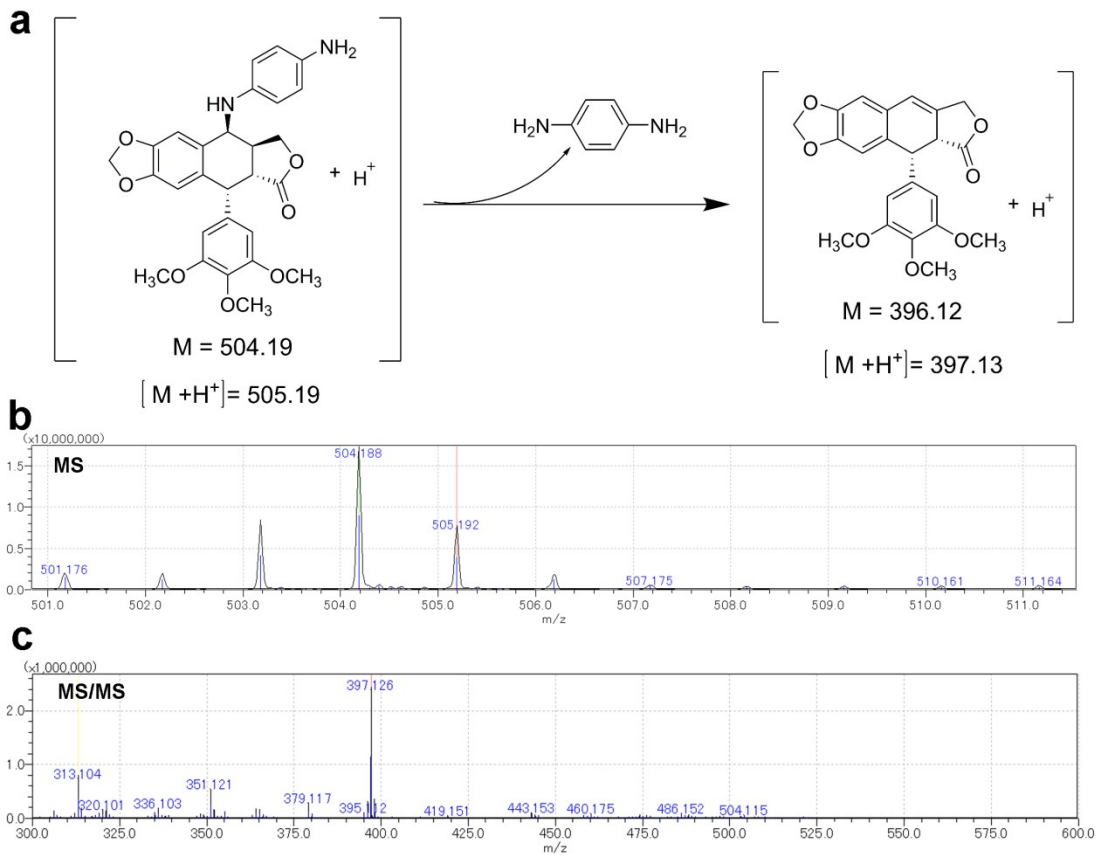

Figure S12. (a) The mechanism of mass spectral fragmentation (*m/z* 397.13) of podophyllotoxin derivates released from prdrug 4b. Positive ion MS (b) and MS/MS (c) spectra.

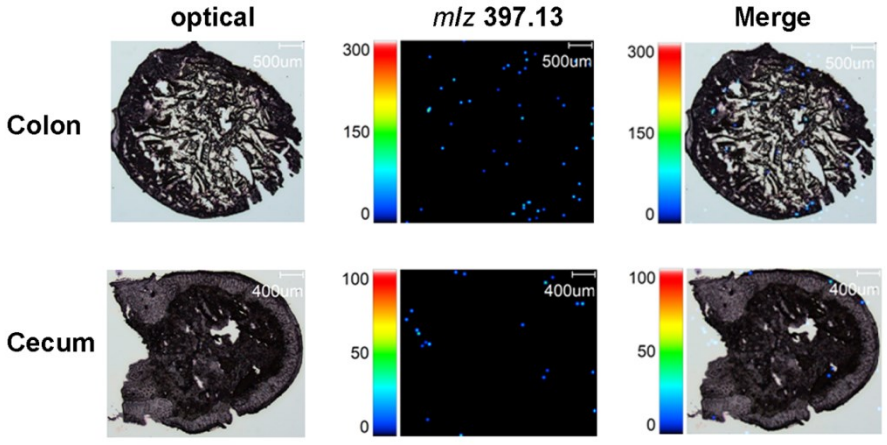

Figure S13. Imaging mass spectrometry of mice colon and cecum tissue at 16 h after gavage with PBS.

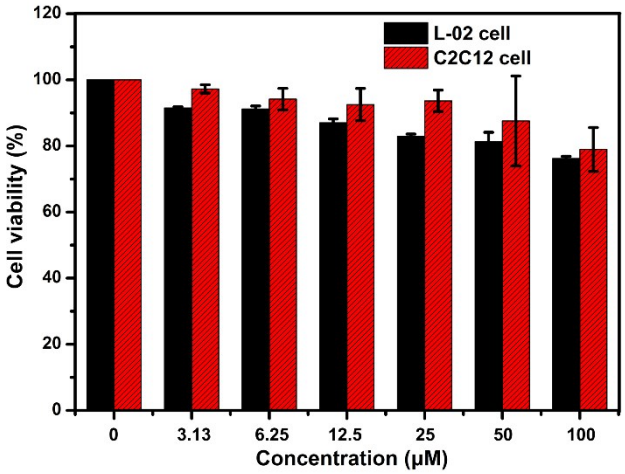

Figure S14. The cell viability of prodrug against L-02 and C2C12 cells using MTT assay.