Electronic Supplementary Information

Construction of drug-drug conjugate supramolecular nanocarriers based on water-soluble pillar[6]arene for combination chemotherapy

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1. General information and experimental procedures

1) General information:

All reactions were performed in air atmosphere unless otherwise stated. The commercially available reagents were used as supplied without further purification. Chloroform and THF were dried according to procedures described in the literatures, and other solvents were used as received without further purification unless otherwise stated. Column chromatography was performed with silica gel (200 – 300 mesh) produced by Qingdao Marine Chemical Factory, Qingdao (China). All yields were given as isolated yields. NMR spectra were recorded on a Bruker DPX 400 MHz spectrometer with internal standard tetramethylsilane (TMS) and solvent signals as internal references at 298 K, and the chemical shifts (δ) were expressed in ppm and J values were given in Hz. Low-resolution electrospray ionization mass spectra (LR-ESI-MS) were obtained on Finnigan Mat TSQ 7000 instruments. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded on an Agilent 6540Q-TOF LCMS equipped with an electrospray ionization (ESI) probe operating in positive-ion mode with direct infusion. Dynamic light scattering (DLS) measurements were carried out on a Brookhaven BI-9000AT system (Brookhaven Instruments Corporation, USA), using a 200-mW polarized laser source (λ = 514 nm). The UV-Vis absorption spectra were measured on a Perkin Elmer Lambda 35 UV-Vis Spectrometer. Transmission electron microscope (TEM) investigations were carried out on CM 200 FEG instrument (Phillips Company). Atomic Force Microscope (AFM) images were obtained in tapping mode by using a NanoDrive Controller with an Innova scanning probe microscope (Veeco Germany, Mannheim) and N-type silicon cantilever (AC 160TS OLYMPUS) at a scan rate of about 5 μm/s, the type of the

AFM probe is AFM CMCL-AC160TS-R3 (R tip = 7 nm). Samples were prepared by spin coating (35 rps) the sample solutions on freshly cleaved mica (Plano) for 10 min.

2) Experimental procedure:

Fabrication of WP6⊃G supramolecular prodrug nanoparticles. WP6⊃G nanoparticles were prepared as follows: CPT-Cb guest **G** (1.0 mg) was dissolved in acetone (120 μL), then a stock solution of **WP6** (*C* = 1 mM) was prepared. Firstly, **WP6** stock solution (60 μL) was added to a volumetric flask (5 mL), and then water (3 mL) was added to dilute the **WP6** solution, finally, **G** (30 μL) was quickly injected to the above solution. The ultimate concentrations of **G** and **WP6** were 0.1 and 0.02 mM, respectively. After standing overnight, the prepared **WP6⊃G** nanoparticles were purified by dialysis (molecular weight cutoff 10 000) in distilled water for several times until the water outside the dialysis tube exhibited negligible CPT absorption.

The encapsulation efficiency and loading content of anticancer drugs were calculated by the following equation:

$$\text{Encapsulation efficiency (\%)} = (m_{CPT} + m_{Cb} / m_{CPT\text{-}Cb}) \times 100$$

$$\text{Loading content (wt\%)} = (m_{CPT} + m_{Cb} / m_{CPT\text{-}Cb} + m_{WP6}) \times 100$$

where m_{CPT} and m_{Cb} are mass of the two kinds of anticancer drugs encapsulated into the nanoparticles respectively, $m_{CPT\text{-}Cb}$ and m_{WP6} are mass of the CPT-Cb (**G**) and **WP6** added, respectively.

GSH-responsive behaviors of the supramolecular prodrug nanoparticles. In a typical GSH-induced release experiment, a certain amount of GSH solution was added into 10 mL of the **WP6⊃G** nanoparticle solution, the final GSH concentration was 2 mM, 5 mM, and 10 mM, respectively. At selected time intervals, 20 μL of the release media was taken out for measuring the released CPT concentrations by HPLC.

A nearly 100% release of CPT from **WP6⊃G** nanoparticles was obtained by adding Triton X-100 to the nanoparticle solution.

***In vitro* cell cytotoxicity assay.** The relative *in vitro* cytotoxicities of **WP6**, **G**, and **WP6⊃G** prodrug nanoparticles against MRC-5 cells (normal human fetal lung fibroblast cell line) and MCF-7 cells (human breast adenocarcinoma cell line) were assessed by using the MTT assay (MRC-5 and MCF-7 cell lines were purchased from Jiangsu KeyGEN BioTECH Corp., Ltd.). The operation process was as follows: the cells were digested, counted and seeded into 96-well plates at a density of 5×10^4 cells per well in 200 μL of Dulbecco's modified Eagle's medium (DMEM) (for MCF-7cells) or Minimum Essential Medium (MEM) (for MRC-5 cells) containing 10% fetal bovine serum (Gibco), supplemented with 50 U·mL^{-1} penicillin and 50 U·mL^{-1} streptomycin (Hyclone), and cultured in 5% CO_2 at 37 ℃ for 24 h. Then, the original medium was removed and 200 μL of fresh culture medium containing different concentrations of **WP6**, **G**, and **WP6⊃G** prodrug nanoparticles was added into each well, respectively, then the cells were further incubated for 24 h. Subsequently, 20 μL of MTT solution (5 mg/mL) was added into each well and incubated for another 4 h. After that, the medium containing MTT was removed, and dimethyl sulfoxide (DMSO, 150 μL) was added to each well to dissolve the MTT formazan crystals. Finally, the plates were shaken for 30 min, and the absorbance of formazan product was measured at 570 nm by a microplate reader (Thermo Scientific). Untreated cells in medium were used as the blank control. All experiments were carried out with five replicates. The cytotoxicity was expressed as the percentage of the cell viability relative to the blank control.

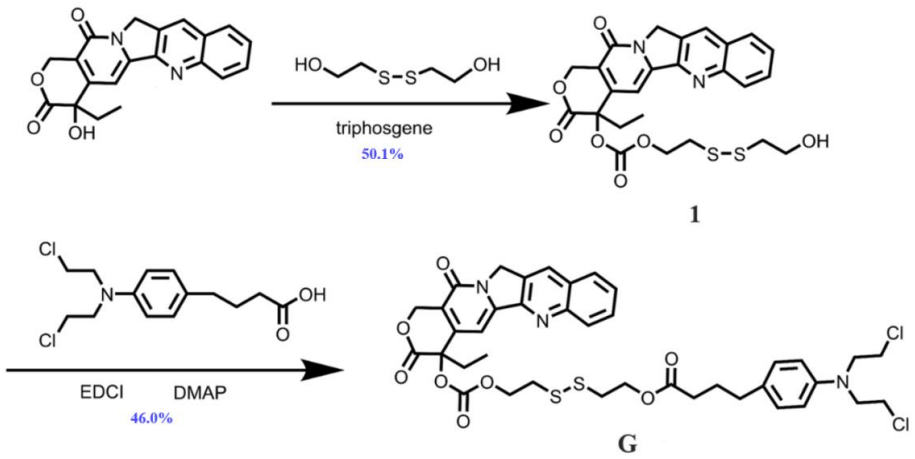
Cellular uptake and intracellular localization observed by CLSM. The cellular uptake and intracellular localization of CPT were examined in MCF-7 cell line. First,

MCF-7 cells were seeded in 8-well chamber slides with 200 μL of complete DMEM culture medium and grown for 24 h before treatment. Then **WP6⊃G** nanoparticles with a concentration of 6 μM were added to the cell incubator and continued to incubate for 1 h and 4 h, respectively. Then the LysoTracker Red DND-99 (Thermo Fisher, USA) was added to the medium with a final concentration of 50 nM and incubated for 1 h to label lysosomes. Finally, after removing the culture medium and washing with PBS thrice, the cells were fixed with 4% paraformaldehyde and investigated by fluorescence microscopy (LSM710, CarlZeiss). The fluorescence characteristic of CPT was used to directly monitor the localization of **WP6⊃G** prodrug vesilces without utilizing additional dye.

Flow cytometric analysis. MCF-7 cells were seeded in 6-well plates at a density of 5.0×10^5 cells per well in DMEM culture medium (1 mL), cultured for 24 h, and then the culture medium was removed. **WP6⊃G** vesicles dissolved in DMEM culture medium at a final concentration of 6 μM were added into the wells and the cells were incubated at 37 °C for 24 and 48 h, respectively. MCF-7 cells without incubation with **WP6⊃G** vesicles were used as a control. After incubation for 24 and 48 h, the cell solutions were centrifuged at 2000 rpm for 5 min. Culture medium was removed and the cells were washed with cold PBS twice. After removal of the supernatants, the cells were resuspended in binding buffer (500 μL). The apoptotic cells were determined by staining using an Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol: Annexin V-FITC (5 μL) was added to the cell suspensions, after incubation in the dark for 15 min, cell apoptosis was detected by flow cytometry at specific wavelengths (Ex=488 nm; Em=530 nm).

2. Synthesis of CPT-Cb guest G

The synthetic procedures for CPT-Cb guest **G** was shown in **Scheme S1**.



Scheme S1. Synthetic route for CPT-Cb guest **G**.

Synthesis of compound **1**

Triphosgene (0.22 g, 0.74 mmol) and DMAP (0.90 g, 7.4 mmol) were dissolved in anhydrous chloroform (60 mL). The mixture was stirred for 0.5 h under nitrogen atmosphere. Then, camptothecin (0.70 g, 2.0 mmol) was added into the reaction mixture in three successive batches. After stirred for another 3 h at room temperature, 2-hydroxyethyl disulfide (3.1 g, 20 mmol) dissolved in THF (15 mL) was added to the reaction mixture slowly. The resulting mixture was stirred at room temperature for 12 h. After removal of the solvent under reduced pressure, the crude product was purified by recrystallization to give compound **1** as a pale yellow solid (0.59 g, 11.2 mmol, 70.1 %). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): δ 8.54 (s, 1H), 8.43 (d, J = 8.7 Hz, 1H), 8.01 (d, J = 8.1 Hz, 1H), 7.93 (m, 1H), 7.75 (m, 1H), 5.72 (d, J = 17.4 Hz, 1H), 5.38 (m, 3H), 4.40 (t, J = 6.4 Hz, 2H), 3.90 (td, J = 6.3, 3.1 Hz, 2H), 2.93 (m, 4H), 2.23 (ddd, J = 48.7, 14.0, 7.5 Hz, 2H), 1.03 (t, J = 7.5 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, 298 K) δ (ppm): 173.0, 167.5, 157.0, 152.7, 146.7, 145.1, 132.1, 130.3, 129.5, 129.0, 128.5, 128.2, 119.6, 97.2, 94.8, 78.3, 72.8, 66.9, 66.8, 59.7, 50.8, 41.5, 36.6, 30.8, 8.0.

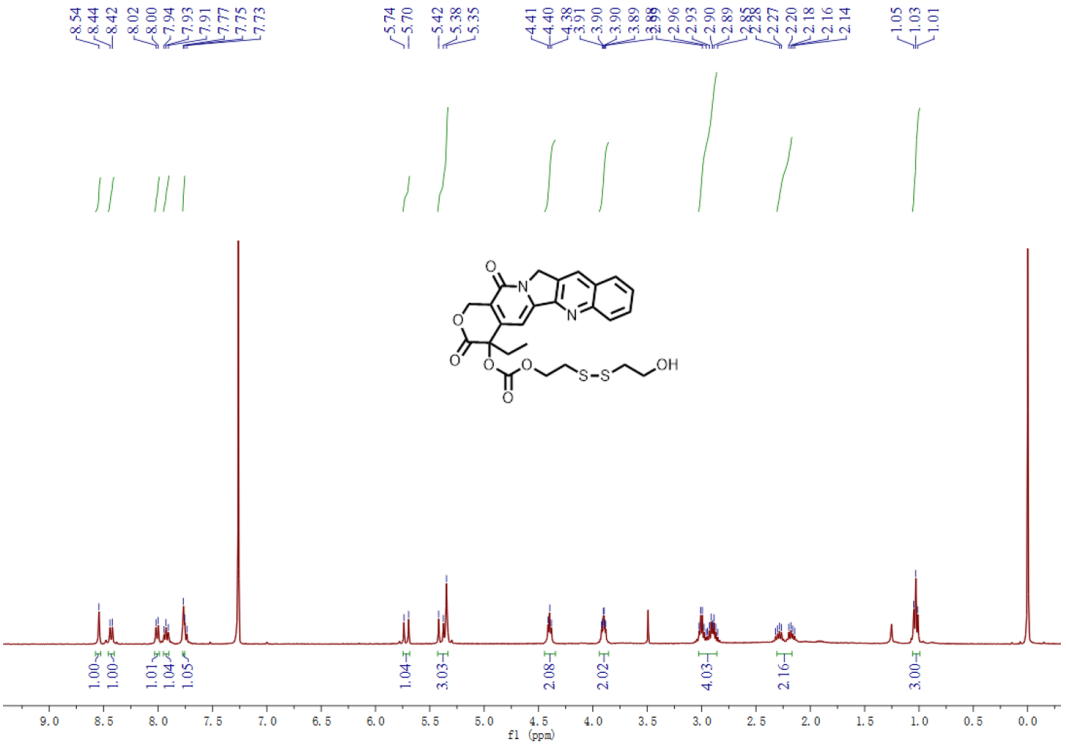


Fig. S1 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of compound **1**.

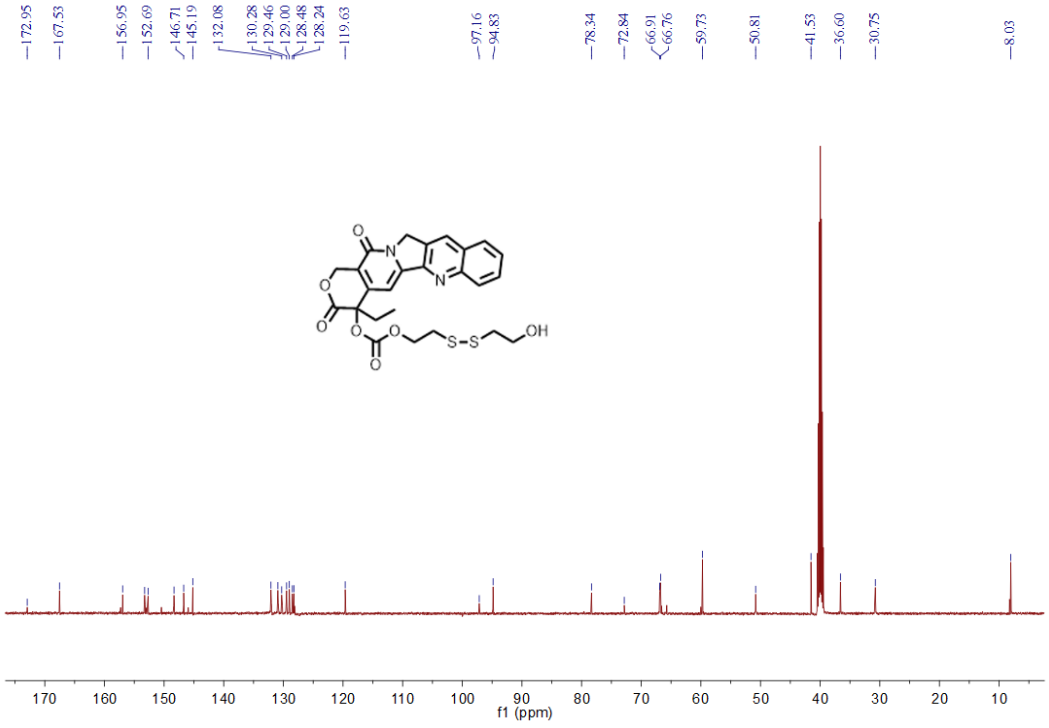


Fig. S2 ^{13}C NMR spectrum (100 MHz, DMSO-d_6, 298 K) of compound **1**.

Synthesis of compound **G**

A mixture of chlorambucil (0.14 g, 0.45 mmol) and EDCI (0.095 g, 0.49 mmol) in 10 mL of DMF was stirred under argon atomosphere for 0.5 h at room temperature. Then compound **1** (0.20g, 0.38 mmol) and DMAP (0.01g, 0.076mmol) were added into the above solution. The resulting mixture was stirred at room temperature for 12 h. After removal of the solvent under reduced pressure, the crude product was purified by silica gel chromatography using dichloromethane/ethyl acetate (*v/v*, 1:1) as the eluent to afford compound **G** as a pale yellow power (0.14 g, 0.17 mmol, 46 %). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): 8.41 (s, 1H), 8.24 (d, J = 8.5 Hz, 1H), 7.96 (s, 1H), 7.85 (t, J = 7.3 Hz, 1H), 7.68 (t, J = 7.3 Hz, 1H), 7.37 (s, 1H), 7.05 (d, J = 8.4 Hz, 2H), 6.64 (d, J = 8.4 Hz, 2H), 5.70 (d, J = 17.2 Hz, 1H), 5.39 (d, J = 17.2 Hz, 1H), 5.30 (s, 2H), 4.37 (m, 2H), 4.26 (t, J = 6.4 Hz, 2H), 3.69 (t, J = 6.7 Hz, 4H), 3.62 (t, J = 6.3 Hz, 4H), 2.94 (t, J = 6.9 Hz, 2H), 2.88 (t, J = 6.4 Hz, 2H), 2.53 (t, J = 7.5 Hz, 2H), 2.30 (d, J = 7.5 Hz, 2H), 2.17 (m, 2H), 1.87 (m, 2H), 1.01 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K) δ (ppm): 173.3, 167.3, 157.3, 152.2, 148.8, 146.2, 145.6, 144.0, 129.7, 129.6, 128.5, 128.2, 120.3, 112.4, 96.1, 78.0, 67.1, 66.6, 62.0, 53.7, 50.0, 40.4, 33.9, 31.9, 26.6, 7.7. HR-ESI-MS: m/z calcd $[M + Na]^+$ 836.1610, found 836.1602.

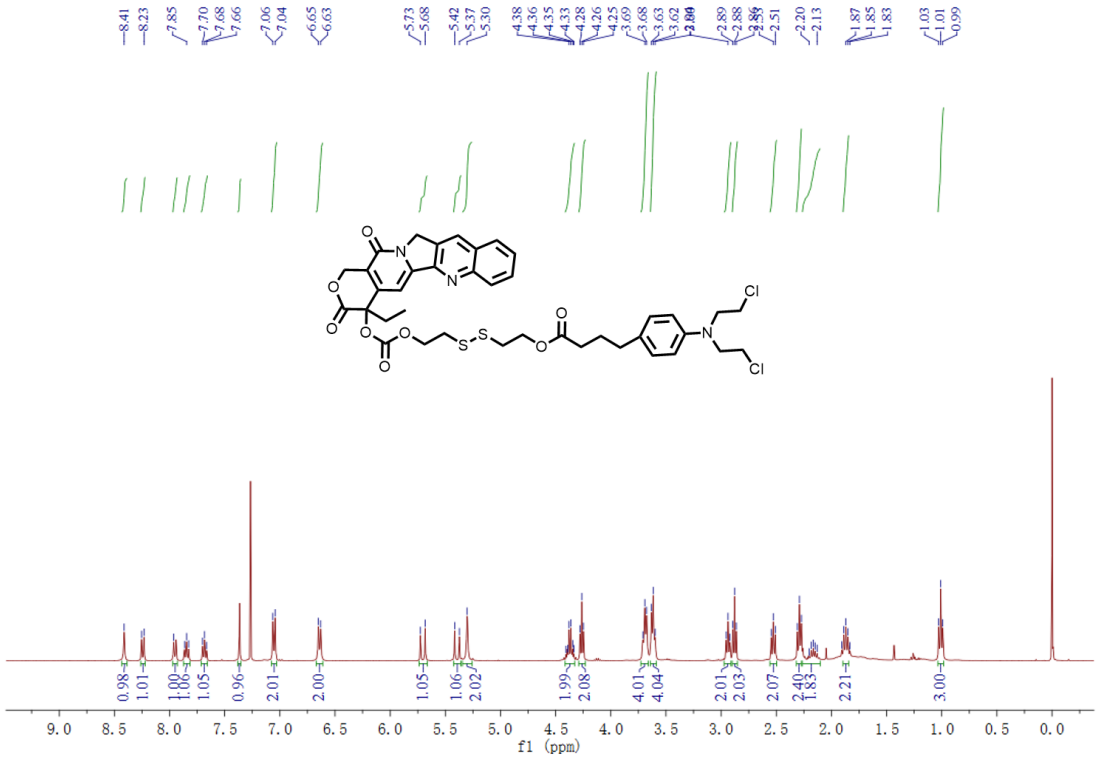


Fig. S3 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of compound **G**.

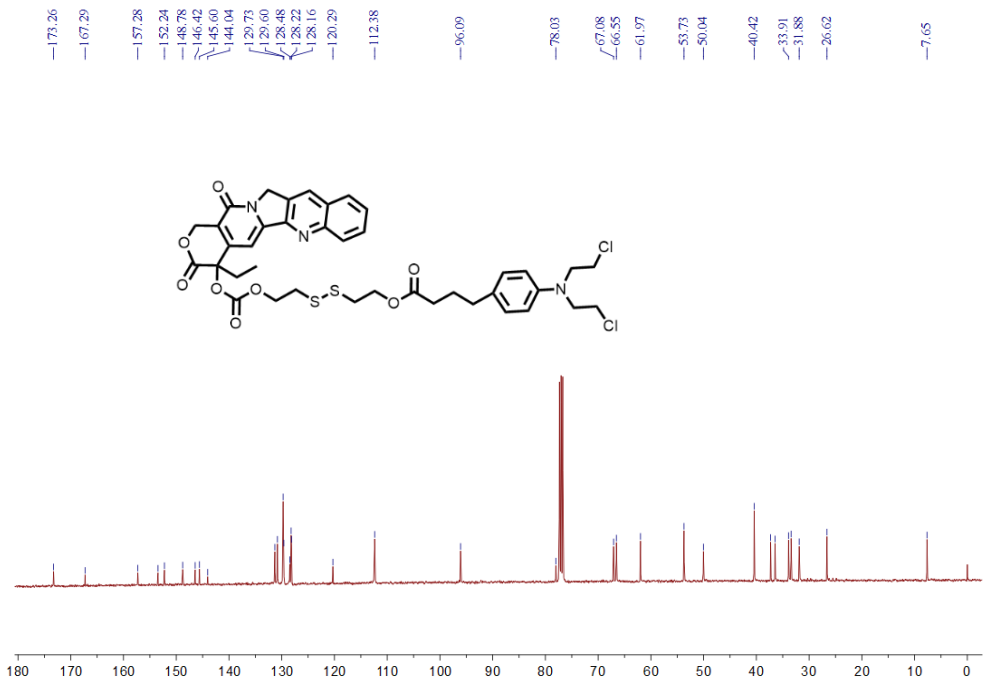


Fig. S4 ^{13}C NMR spectrum (100 MHz, $CDCl_3$, 298 K) of **G**.

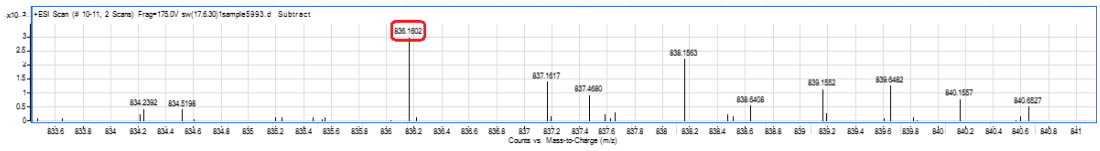


Fig. S5 HR-ESI-MS spectrum of **G**.

3. Host-guest complexation of WP6 and Cb

Due to the poor solubility of guest **G** in water, a model compound Cb was selected for investigating the host-guest interactions between **WP6** and **G** by using ^{1}H NMR spectroscopy. As shown in Fig. S6, when 1.0 equiv. of **WP6** was added to the aqueous solution of Cb, all the resonance signals of Cb (H_a-H_g) shifted upfield remarkably, and the signals of H_c and H_d became broad due to the shielding effects of the electron-rich cavities of **WP6** toward Cb. Meanwhile, the protons of **WP6** (H_1-H_3) exhibited slight downfield chemical shift changes probably because the complexation between **WP6** and Cb led to the decrease of the electron density for these protons and their rotation was limited. The above results suggested that Cb was fully threaded into the hydrophobic cavity of **WP6** to form a pseudorotaxane structure. Moreover, 2D NOESY experiment further indicated the conformation of such a host-guest inclusion complex, from which NOE correlation signals between protons H_1 of **WP6** and H_e, H_f,

H_g of Cb could be clearly observed, confirming that compound Cb was located in the cavity of **WP6**.

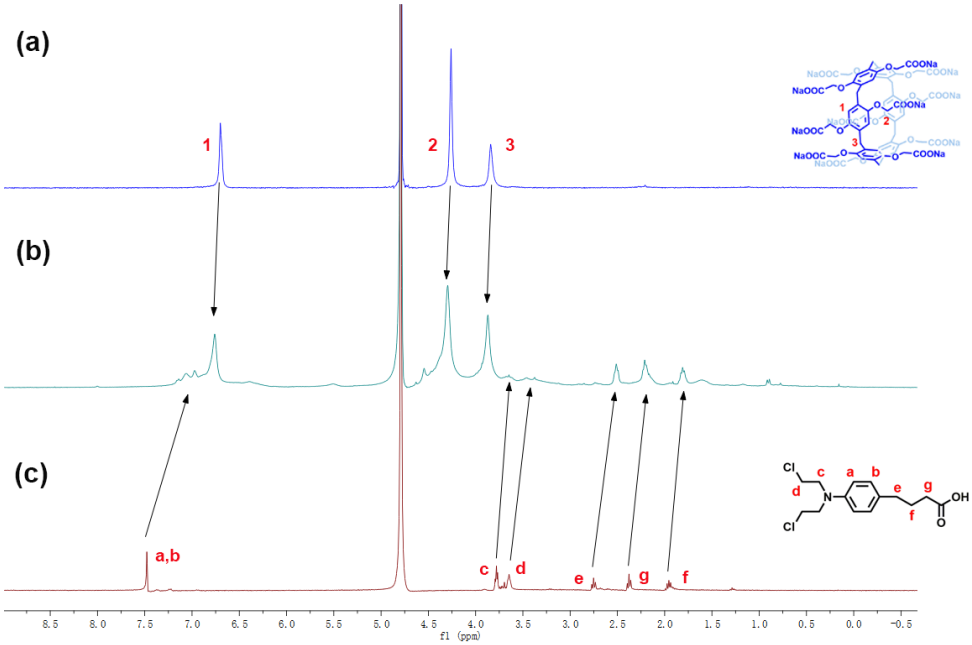


Fig. S6 ^{1}H NMR (400 MHz, D_2O, 298 K) spectra: (a) Cb (1.0 mM), (b) Cb (1.0 mM) and **WP6** (1.0 mM), (c) **WP6** (1.0 mM).

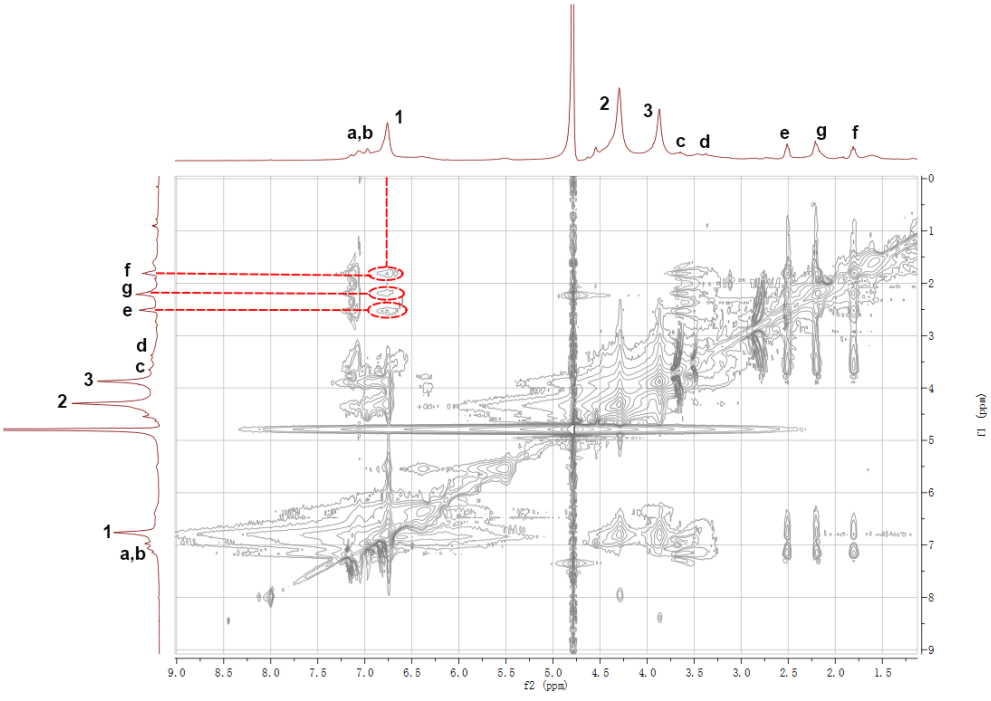


Fig. S7 2D NOESY spectrum of **WP6** and Cb (400 MHz, D_2O, 298 K) ([**WP6**] = 1.0 mM, [**Cb**] = 1.0 mM).

4. Determination of the best molar ratio of WP6 and G leading to aggregation

Since the addition of different amounts of **WP6** to the **G** solution led to obvious changes of opalescence intensity in aqueous solution, therefore, it was necessary to determine the best molar ratio between **WP6** and **G** for constructing supramolecular aggregates, which was determined by optical transmittance experiments. As shown in Fig. S8, the best molar ratio of 10:1 ([**G**]/[**WP6**]) for the formation of supramolecular aggregates was observed at the inflection point.

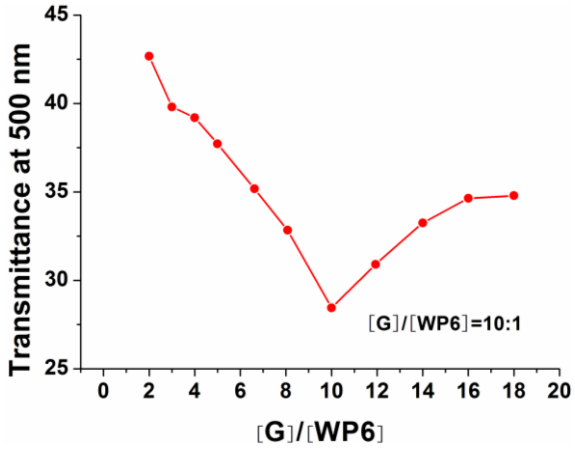


Fig. S8 Dependence of the relative optical transmittance at 500 nm on the **WP6** concentration with a fixed concentration of **G** (0.1 mM) at 25 ℃.

5. Critical aggregation concentration (CAC) determination of WP6⊃G

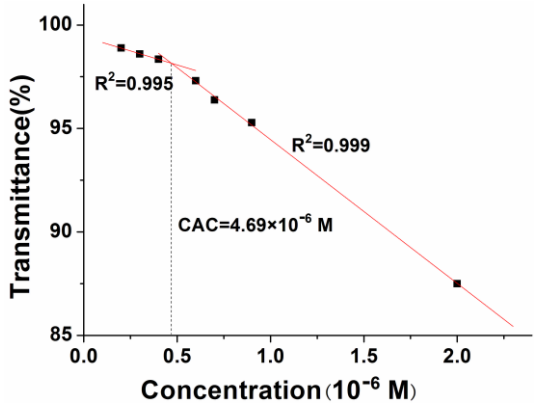


Fig. S9 Concentration-dependent optical transmittance at 500 nm of the **WP6⊃G** solutions with fixed molar ratio of [**WP6**]/[**G**] =1/5.

6. Stablity of WP6⊃G prodrug nanoparticles in water, PBS, and 50% serum

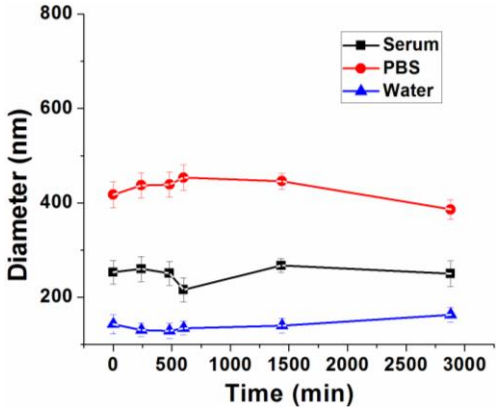


Fig. S10 Time-dependent size changes of **WP6⊃G** nanoparticles under different conditions.

7. TEM images of WP6⊃G aggregates under GSH-stimulus

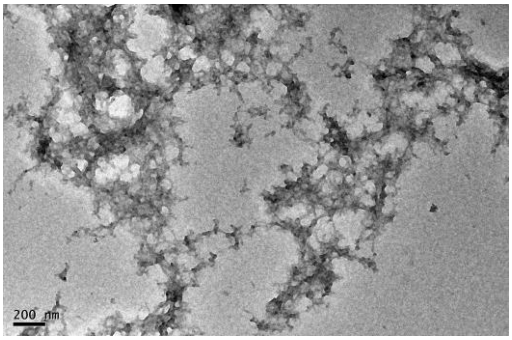


Fig. S11 TEM images of **WP6⊃G** aggregates in the presence of GSH (5.0 mM).

8. Fluorescence images of WP6⊃G aggregates under GSH-stimulus

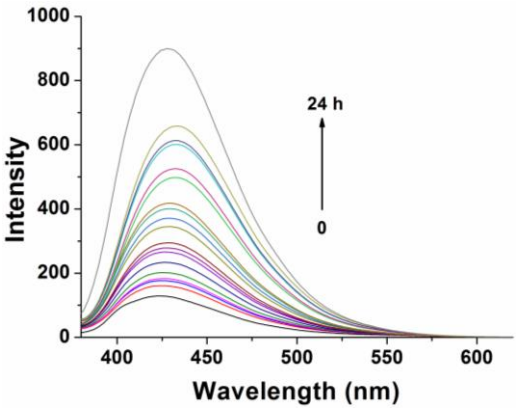


Fig. S12 Changes in fluorescence intensity after treated with GSH (5 mM) at selected time intervals.

9. HPLC traces under GSH-stimulus

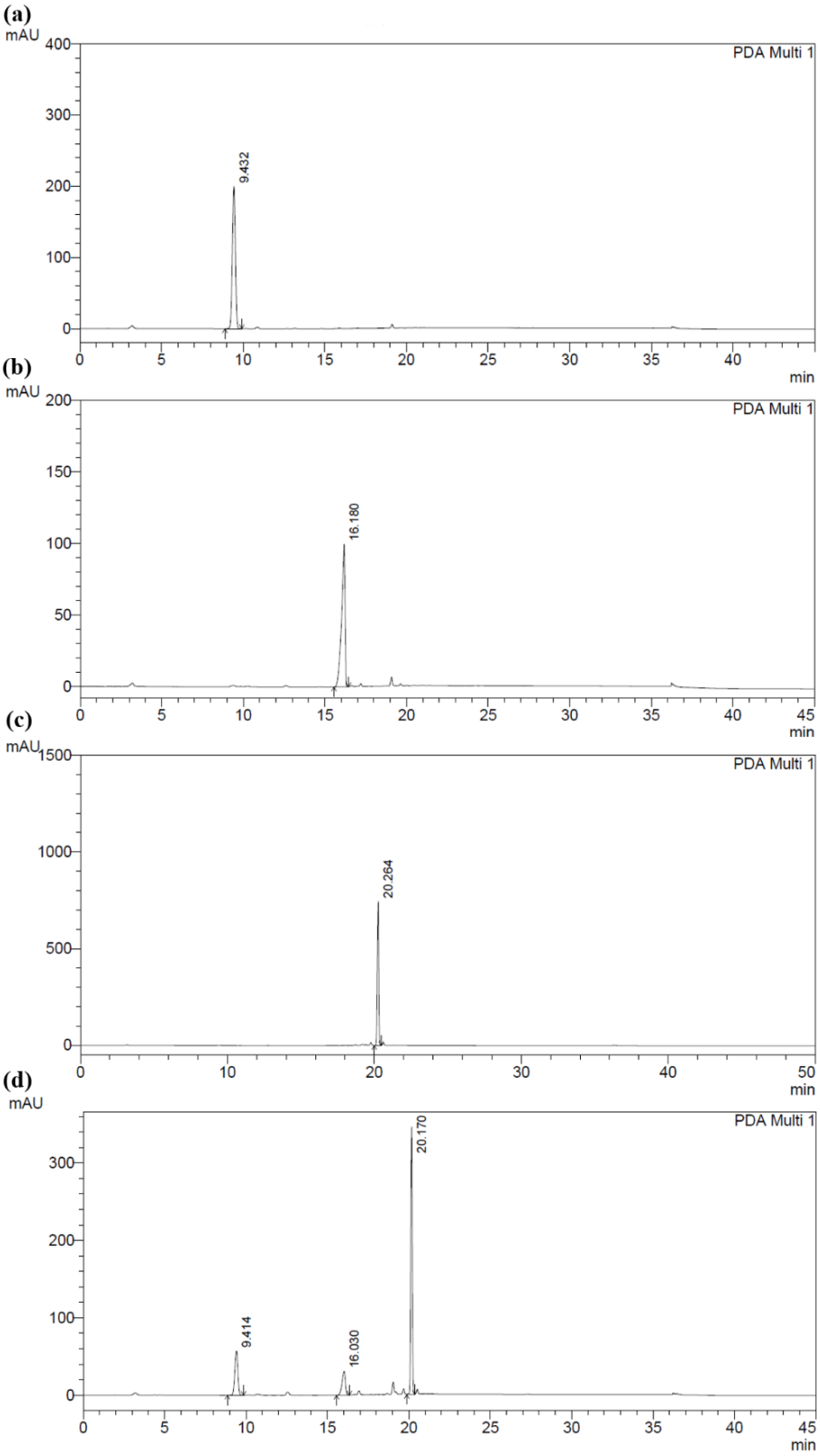


Fig. S13 HPLC traces of (a) CPT, (b) Cb, (c) **G**, and (d) drug release after treated with GSH (5 mM) for 2 h. Standard CPT was used as reference.

10. HPLC conditions

In the HPLC experiments, 20 μL sample was injected into the column (Thermo Syncronis C18 reversed-phase column) each time, and was detected by UV-absorption detector at 254 nm. The composition of the gradient eluent was shown as below.

Time (min)	Flow (mL/min)	MeCN	H_2O
0	1.0	30%	70%
10	1.0	100%	0%
30	1.0	100%	0%