Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

A DT-diaphorase responsive theranostic prodrug for diagnosis, drug release monitoring and therapy

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Experimental

Reagents and Materials: 4-Nitrobenzyl alcohol, t-butyldimethylchlorosilane (TBSCl), imidazole, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloricde (EDC), Pd/C, ammonium formate, 4-dimethylaminopyridine (DMAP), triphosgene were purchased from Aladdin Reagents. Glutathione (GSH), cysteine (Cys), homocysteine (Hcy), DL-dithiothreitol (DTT), ascorbic acid (AA), glycine (Gly), arginine (Arg), phenylalanine (Phe), lysine (Lys), DT-diaphorase (lyophilized powder, recombinant, expressed in *E. coli*), *β*-nicotinamide adenine dinucleotide reduced disodium salt (NADH), sodium borohydride, boric acid, chloride salts of metal ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, Cu^{2+}), camptothecin (CPT), 4-acetamidobenzaldehyde were purchased from Sigma-Aldrich. Analytical grade reagent dichloromethane (DCM) was dried with CaH_2 and distilled under nitrogen atmosphere. Analytical grade reagent N, N-dimethyl-formamide (DMF) was dried with CaH_2 and vacuum distilled. Ethanol, ethyl acetate, petroleum ether, methanol, tetrahydrofuran were analytical grade solvents and used without further purification. The water used throughout the experiments was the triple-distilled water which was further treated by ion exchange columns and then by a Milli-Q water purification system.

Synthesis of 1: TBSCl (4.4 g, 29.4 mmol) was added to a solution of 4-nitrobenzylic alcohol (3 g, 19.6mmol) and imidazole (2.67 g, 39.2mmol) in anhydrous DMF (20 mL). After stirring for 3 h at room temperature, the mixture was diluted with ethyl acetate (200 mL), washed with water (3×100 mL), dried over anhydrous Na_2SO_4. After the solvent was removed, the crude product was purified by column chromatography on silica gel (petroleum ether: ethyl acetate = 20: 1 in v/v) to afford compound **1** (yield: 95%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$, ppm) δ 8.20 (d, *J* = 8.5 Hz, 2H), 7.49 (d, *J* = 8.4 Hz, 2H), 4.83 (s, 2H), 0.96 (s, 9H), 0.13 (s, 6H).
Synthesis of 2: Pd/C (10%, 0.5 g) and ammonium formate (1.72 g) were added to a solution of compound **1**(1.66 g, 6.2 mmol) in methanol (30 mL). After the solution was stirred for 4 h at room temperature, the catalyst was filtered off, then the filtrate was evaporated and the residue was purified by column chromatography on silica gel (petroleum ether: ethyl acetate = 4: 1 in v/v) to afford compound **2** (yield: 89%) as a light yellow oil.
Synthesis of 3: Compound **3** was synthesized according to literature procedures.[1]
Synthesis of 4: Under nitrogen atmosphere, compound **2** (400 mg, 1.69 mmol) was dissolved in anhydrous DMF (8 mL) and anhydrous pyridine (5 mL), then EDC (390mg, 2 mmol) was added. After stirring for 10 min, compound **3** (508 mg, 2 mmol) was added. The resultant mixture was stirred for 36 h at room temperature. The reaction solution was then diluted with ethyl acetate (250 mL), washed with water (3×150 mL), dried over anhydrous Na_2SO_4. After the solvent was removed, the crude product was purified by column chromatography on silicagel (petroleum ether: dichloromethane = form 1: 1 to 1:3 in v/v) to furnish compound **4** (yield: 65%) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$, ppm) δ7.35 (d, *J* = 8.4 Hz, 2H), 7.23 (d, *J* =

8.3 Hz, 2H), 7.11 (s, 1H), 4.67 (s, 2H), 3.01 (s, 2H), 2.15 (s, 3H), 1.95 (s, 6H), 1.49 (s, 6H), 0.92 (s, 9H), 0.07 (s, 6H). MS(ESI): m/z 492.18 $[M+Na]^+$.

Synthesis of 5: Compound **4** (200 mg, 0.43mmol) was dissolved in 10 ml DCM. To this solution, HCl (1.25 M in MeOH, 10 ml) was added dropwise. The mixture was stirred for 30 min, and it was diluted with DCM (150 mL), washed with saturated $NaHCO_3$ (100 mL), saturated NaCl (100 mL), dried over anhydrous Na_2SO_4.The solvent was removed under reduced pressure and the residue was purified by column chromatography on silicagel (petroleum ether: ethyl acetate = 1: 1 in v/v) to afford compound **5** (yield: 88%) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$, ppm) δ7.38 (d, *J* = 8.2 Hz, 1H), 7.27 (d, *J* = 8.1 Hz, 2H), 7.20 (s, 1H), 4.62 (s, 2H), 3.00 (s, 2H), 2.15 (s, 3H), 1.94 (s, 6H), 1.49 (s, 6H). MS(ESI): m/z 377.51 $[M+Na]^+$.

Synthesis of 6 (the prodrug): Under nitrogen atmosphere, CPT (97mg, 0.279mmol) and DMAP (103 mg, 0.836 mmol) were suspended in 8 mL DCM. Triphosgene (34mg, 0.114mmol) was added and stirred for 30 min at room temperature. Then compound **5** (100mg, 0.282mmol) in 3 mL DCM was added. The reaction mixture was stirred overnight. The reaction solution was diluted with ethyl acetate (50 mL) and washed with water (50 mL) and saturated NaCl solution (50 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the resultant crude product was purified by column chromatography on silicagel (petroleum ether: ethyl acetate = 1: 5 in v/v) to afford the prodrug (yield: 82%) as a yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$, ppm) δ8.42 (s, 1H), 8.25 (d, *J* = 8.5 Hz, 1H), 7.97 (d, *J* = 8.1 Hz, 1H), 7.87 (t, *J* = 7.7 Hz, 1H), 7.70 (t, *J* = 7.9 Hz, 1H), 7.35 (d, *J* = 8.3 Hz, 2H), 7.32 (s, 1H), 7.25 (d, *J* = 8.2 Hz, 2H), 7.09 (s, 1H), 5.69 (d, *J* = 17.1 Hz, 1H), 5.39 (d, *J* = 17.1 Hz, 1H), 5.29 (s, 2H), 5.10 (d, *J* = 12.1 Hz, 1H), 5.01 (d, *J* = 12.1 Hz, 1H), 2.93 (s, 2H), 2.32 – 2.22 (m, 2H), 2.14 (s, 3H), 1.93 (s, 6H), 1.46 (s, 6H), 0.99 (t, 3H). MS(ESI): m/z 752.45 $[M+Na]^+$.

Synthesis of 7: Compound **7** was synthesized according to literature procedures.[2] A mixture of 4-acetamidobenzaldehyde (326 mg, 2 mmol), $NaBH_4$ (228 mg, 6 mmol), and boric acid, (372 mg, 6 mmol) was ground with an agate mortar and pestle until TLC showed complete disappearance of the starting material. The mixture was quenched with 1 N HCl solution, followed by extraction with ethyl acetate, dried over anhydrous Na_2SO_4. After the solvent was removed, the crude product was purified by column chromatography on silica gel (petroleum ether: ethyl acetate = 1: 5 in v/v) to afford compound **7** (yield: 90%) as a white solid. ^{1}H NMR (600 MHz, DMSO-d6, ppm) δ 9.89 (s, 1H), 7.52 (d, *J* = 8.5 Hz, 2H), 7.22 (d, *J* = 8.4 Hz, 2H), 5.09 (brs, 1H), 4.42 (s, 2H), 2.03 (s, 3H). MS(ESI): m/z 186.58 $[M+Na]^+$.

Synthesis of CPA (the control compound): Under nitrogen atmosphere, CPT (88mg, 0.253mmol) and DMAP (93 mg, 0.761 mmol) were suspended in 7 mL DCM. Triphosgene (30mg, 0.101mmol) was added and stirred for 30 min at room temperature. Then compound **7** (50mg, 0.303mmol) in 3 mL tetrahydrofuran was added. The reaction mixture was stirred overnight. The reaction solution was diluted with ethyl acetate (100 mL) and washed with water (100 mL) and saturated NaCl solution (100 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the resultant crude product was purified by column

chromatography on silicagel (ethyl acetate) to afford the prodrug (yield: 89%) as a white solid. ^{1}H NMR (600 MHz, DMSO-d6, ppm) δ 9.90 (s, 1H), 8.69 (s, 1H), 8.18 (d, *J* = 8.5 Hz, 1H), 8.14 (d, *J* = 7.7 Hz, 1H), 7.87 (t, *J* = 8.3 Hz, 1H), 7.73 (t, *J* = 7.5 Hz, 1H), 7.51 (d, *J* = 8.4 Hz, 2H), 7.27 (d, *J* = 8.5 Hz, 2H), 7.00 (s, 1H), 5.52 (d, *J* = 2.9 Hz, 2H), 5.30 (s, 2H), 5.09 (dd, *J* = 29.2, 12.1 Hz, 2H), 2.23 – 2.10 (m, 2H), 1.98 (s, 3H), 0.91 (t, *J* = 7.4 Hz, 3H). MS(ESI): m/z 561.46 $[M+Na]^{+}$.

Cell culture: A549 cells (human nonsmall cell lung cancer) and L929 cells (murine aneuploid fibrosarcoma cells) were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 ℃ under a humidified atmosphere containing 5% CO_2.

Cell imaging: A549 and L929 cells were incubated in RPMI1640 medium supplemented with 10% Fetal Bovine Serum. One day before imaging, cells were passaged and plated on polylysine-coated cell culture glass slides inside 30-mm glass culture dishes and allowed to grow to 50–70% confluence. Cells on glass slides were washed with RPMI1640, and re-incubated in RPMI1640 medium containing the prodrug (10 μM) at 37℃ under 5% CO_2 for 0.5 h or 2 h. Afterwards, the culture dishes were washed with PBS, then glass slides were taken out, washed with PBS for three times and then imaged on an Olympus IX71 inverted fluorescence microscope equipped with a DP72 color CCD.

Cell viability assay: The viability of A549 and L929cells exposed to prodrug were assessed by MTT assay. The cells were seeded in 96-well plates at the cell population of ca. 5000 cells/well. After 24 h of incubation at 37 ℃, cells were washed with PBS buffer, then the PBS was replaced with fresh medium containing serial dilutions of prodrug from 0.1 to 80 μM. The cells without the treatment were used as control. The cells were grown for another 48 h. The wells were washed with PBS buffer, then RPMI 1640 medium containing 0.5 mg/mL MTT was added and incubated for another 4 h. The medium was carefully removed and 150 μL of DMSO was added to dissolve the precipitates and the absorbance was recorded with a Thermo MK3 ELISA reader at 570 nm. As for the assays, for each independent experiment, the assays were performed in six replicates. And the statistical mean and standard deviation were used to estimate the cell viability.

Apoptosis Analysis by Annexin V-FITC and Propidium Iodide (PI) Double Staining: A549 and L929 cells were plated with 1.0 $\times 10^{6}$ cells/dish in 35 mm culture dish at 37 ℃ for 12 h. After incubation with the prodrug or CPT for 24 h, the treated cells were washed, trypsinized and centrifuged. The cells were collected and re-suspended in 400 μL of Annexin V Binding buffer provided with the AnnexinV-FITC kit, and 5 μL Annexin V-FITC and 10 μL PI were added. Furthermore, the stained cells were incubated at room temperature for 15 min in the dark, and approximate 10,000 cells were analyzed using flow cytometry on a Beckman Coulter Epics XL equipped with a single 488 nm argon laser and the data were analyzed using the Beckman Coulter EXPO 32 software.

Cellular Uptake Measured by Flow Cytometry: The cellular uptake of the prodrug was measured by flow cytometry. A549 cells were seeded onto six-well plates at 2 × 10^{5} cells/mL and allowed to culture for 24 h before treatment. Cells were exposed to

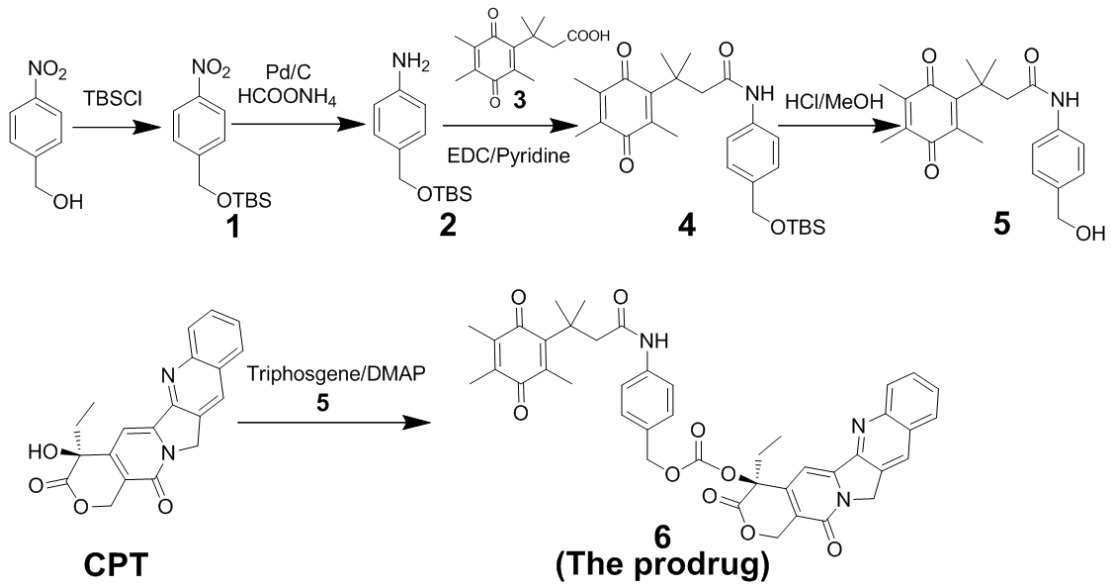
the prodrugs incubated for 0.5 h, 2 h at a final concentration of 10 μM. The treated cells were washed, trypsinized and centrifuged. The harvested cells were then resuspended in PBS. Each sample was analyzed on a BD Accuri C6 flow cytometer using a 370 nm solid-state laser and the signals were collected in the FL4 channel. Data were collected from 10000 gated events and analyzed with BD Accuri C6 Software program.

Measurements: ^{1}H NMR spectra were recorded on a BrukerAvance600 MHz NMRspectrometer. Mass spectra were obtained through a Bruker Esquire HCT Plus masss pectrometer. UV-vis spectra were recorded on a Hitachi U-3010 UV-vis spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4600 fluorescence spectrophotometer. Fluorescence images were obtained using an Olympus IX 71 with a DP72 color CCD.

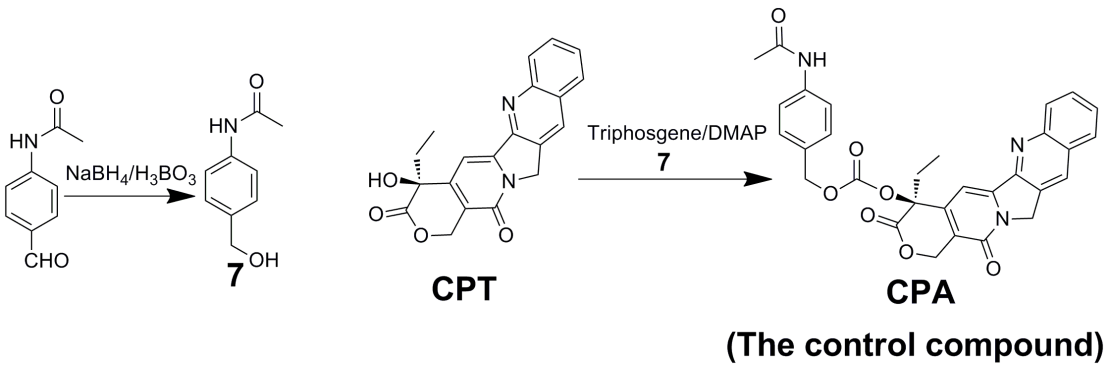
Reference:

1. L. A. Carpino, S. A. Triolo and R. A. Berglund, *J. Org. Chem.*, 1989, **54,** 3303.

2. B. T. Cho, S. K. Kang, M. S. Kim, S. R. Ryu and D. K. An, *Tetrahedron*, 2006, **62,** 8164.



Scheme S1. Synthetic route for the prodrug.



Scheme S2. Synthetic route for the control drug (CPA).

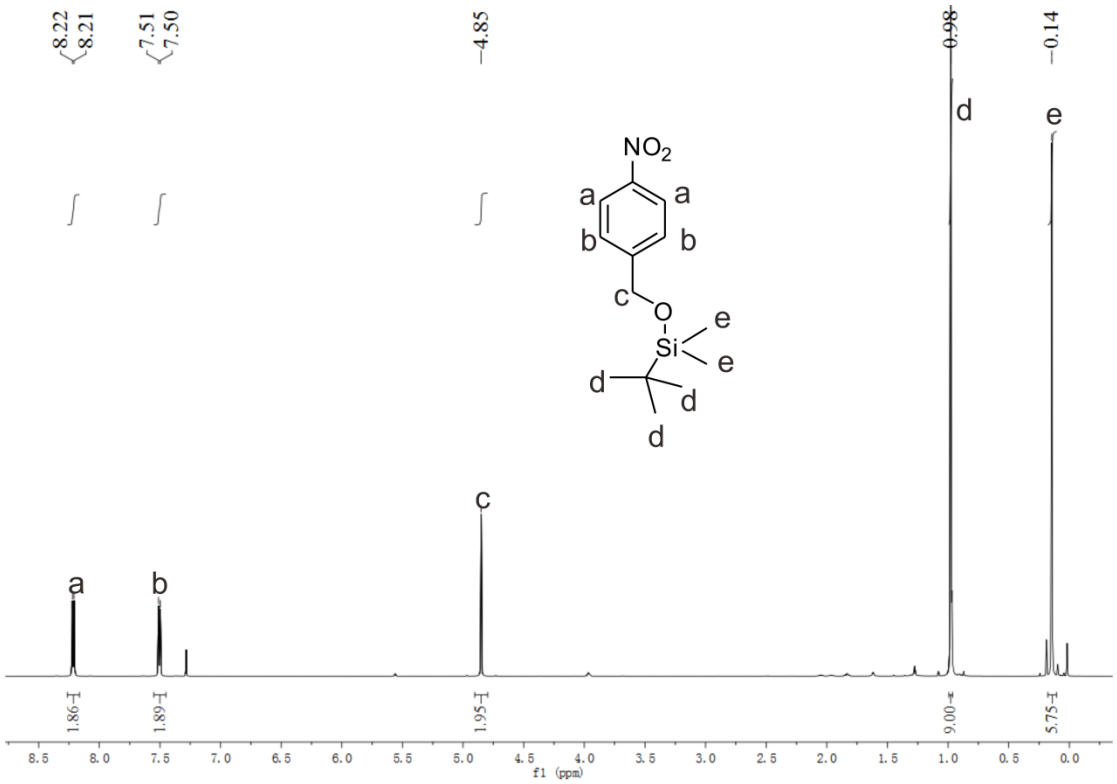


Fig. S1. ^{1}HNMR spectrum (in $CDCl_3$) of **1**.

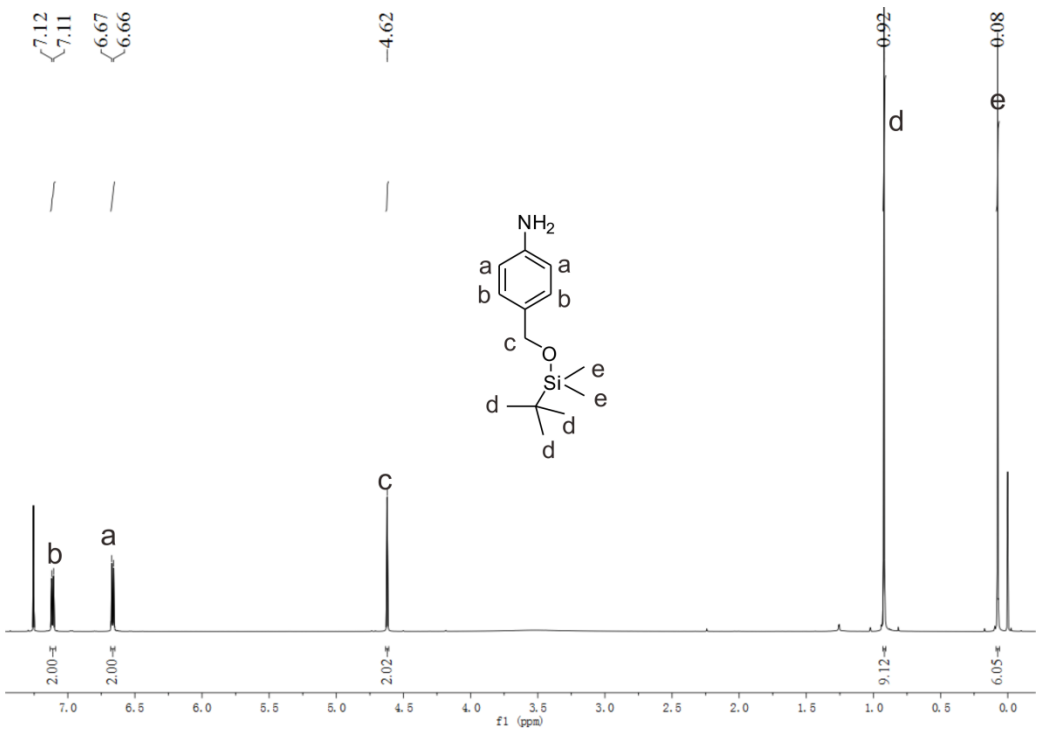


Fig. S2. ^{1}HNMR spectrum (in $CDCl_3$) of **2**.

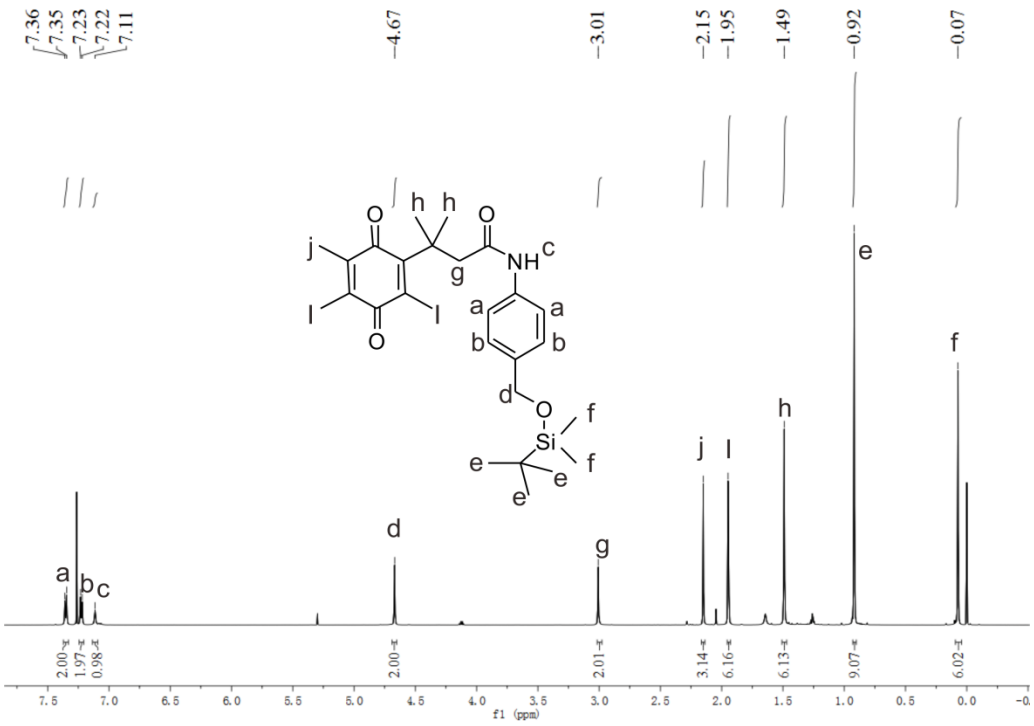


Fig. S3. ^{1}HNMR spectrum (in $CDCl_3$) of **4**.

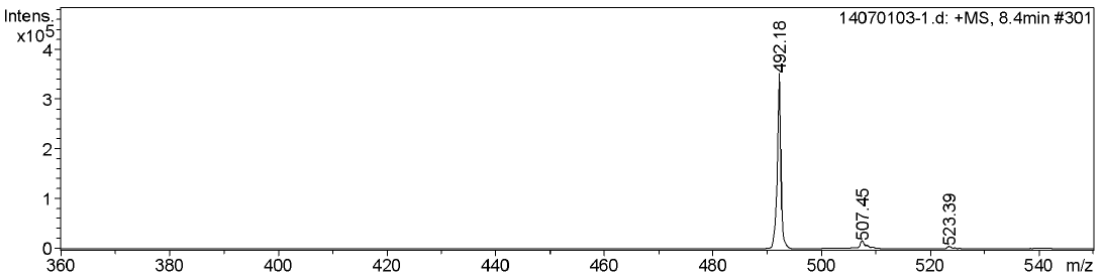


Fig. S4. Mass spectrum of **4**. MS(ESI): m/z 492.18 $[M+Na]^+$

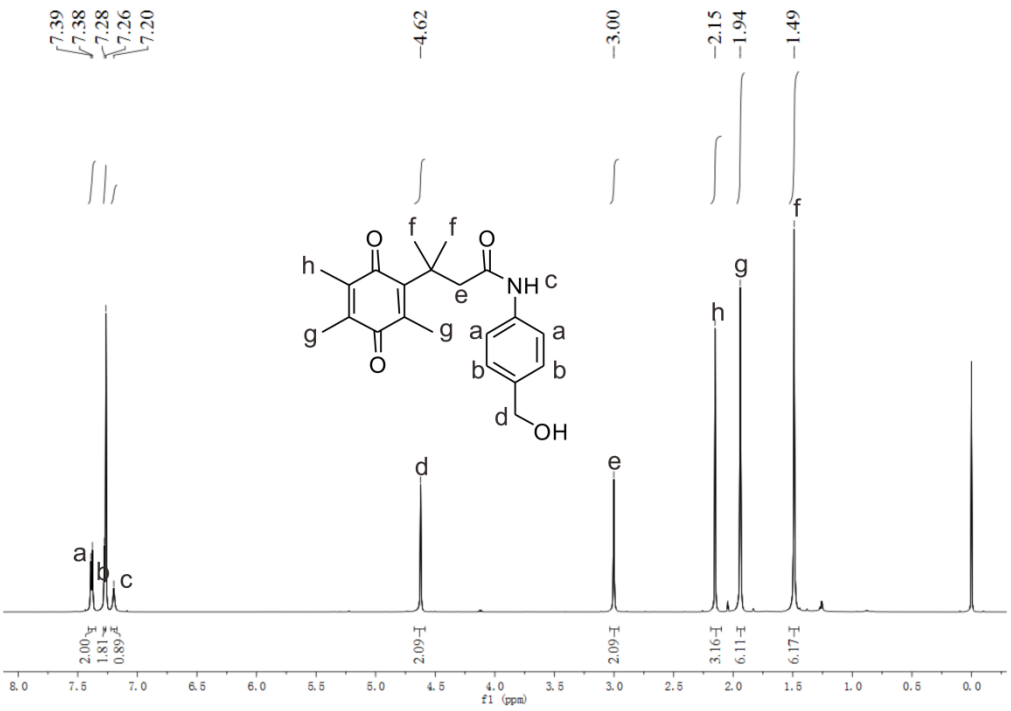


Fig. S5. ^{1}HNMR spectrum (in $CDCl_3$) of **5**.

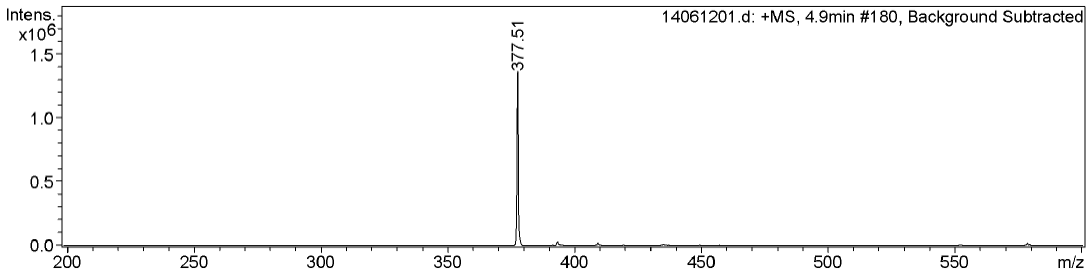


Fig. S6. Mass spectrum of **5**. MS(ESI): m/z 377.51 $[M+Na]^+$.

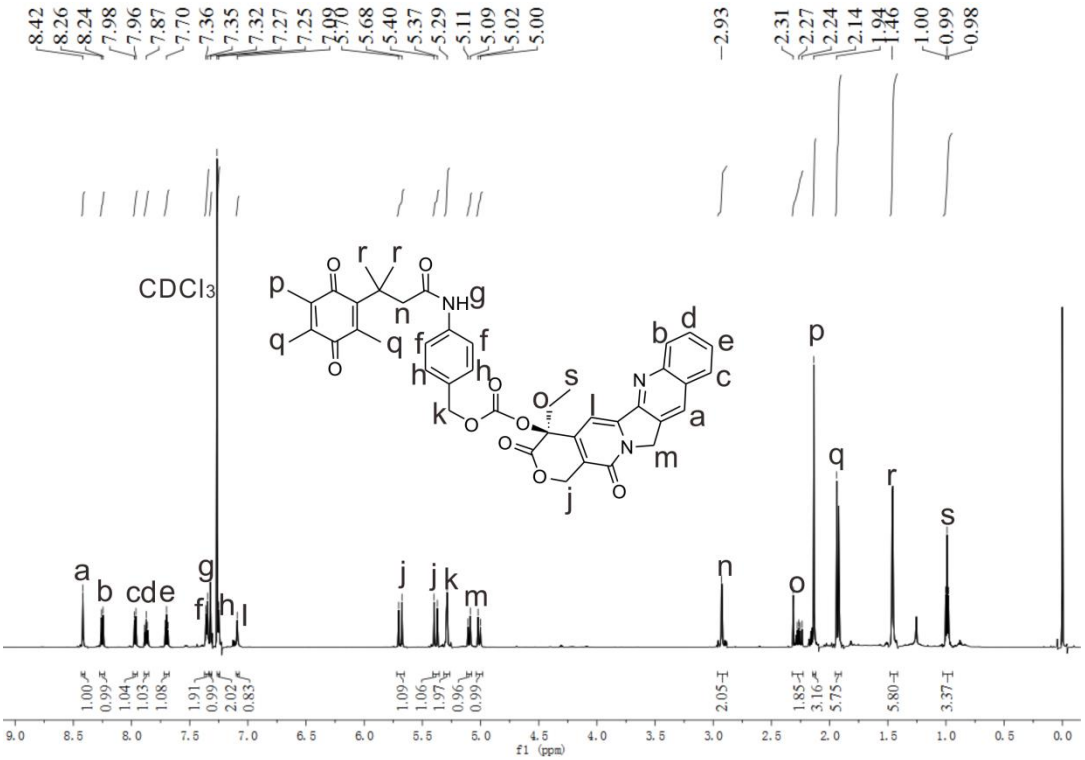


Fig. S7. ^{1}HNMR spectrum of (in $CDCl_3$) the **prodrug**.

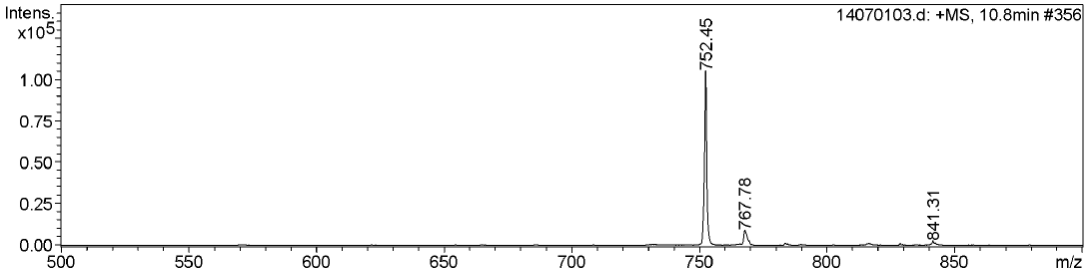


Fig. S8. Mass spectrum of the **prodrug**. MS(ESI): m/z 752.45 $[M+Na]^{+}$.

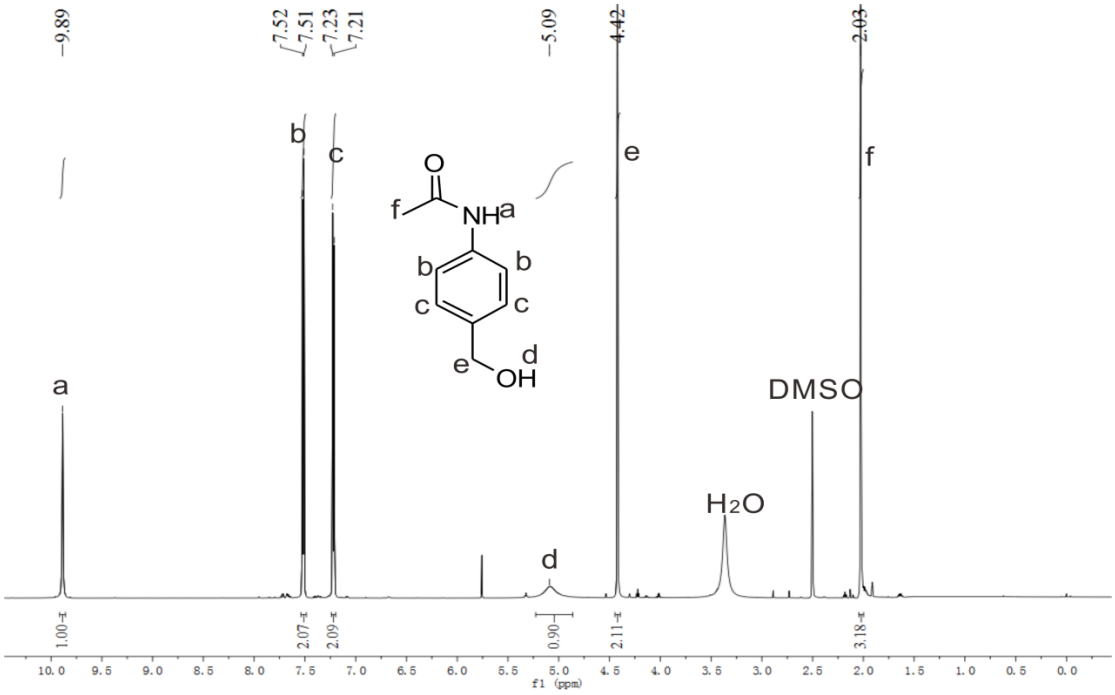


Fig. S9. [1]HNMR spectrum (in DMSO-d6) of **7**.

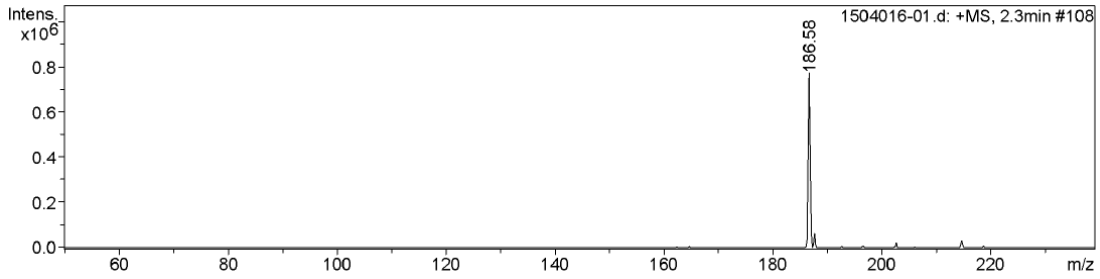


Fig. S10. Mass spectrum of **7**. MS(ESI): m/z 186.58 $[M+Na]^+$.

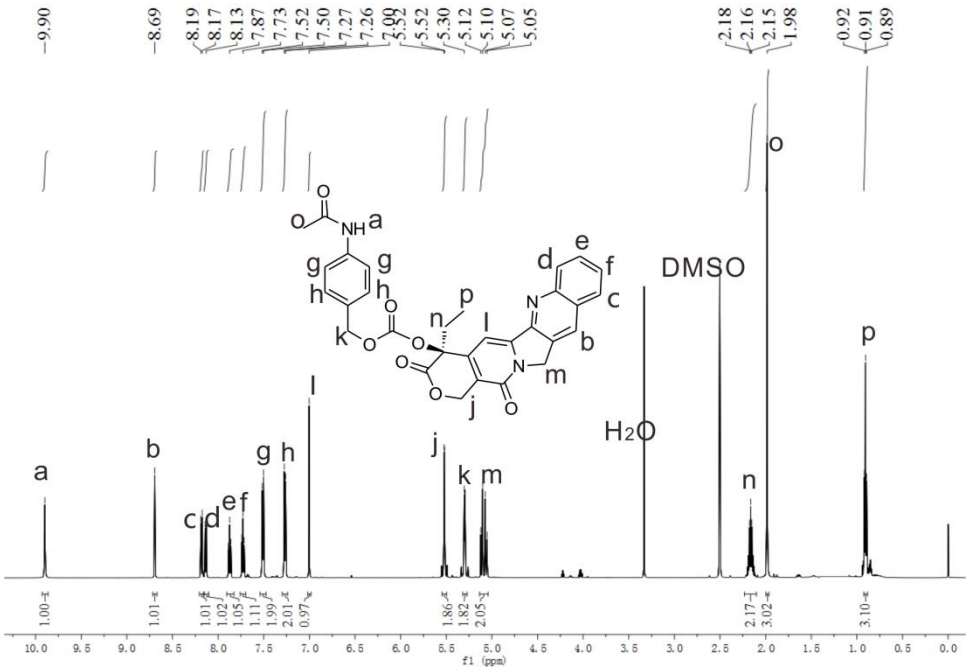


Fig. S11. [1]HNMR spectrum (in DMSO-d6) of **CPA**.

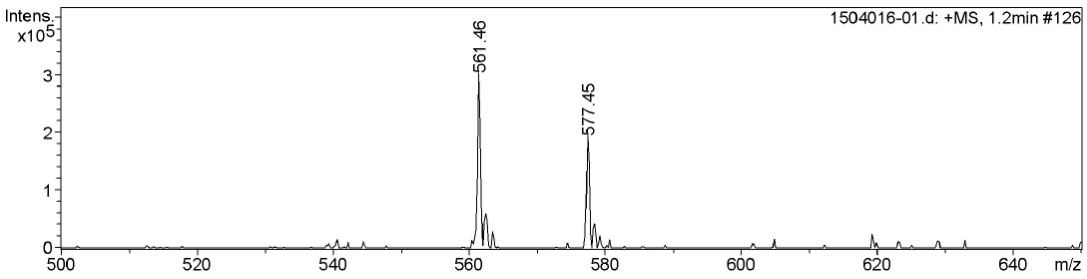


Fig. S12. Mass spectrum of CPA. MS(ESI): m/z 561.46 $[M+Na]^+$; 577.45 $[M+K]^+$.

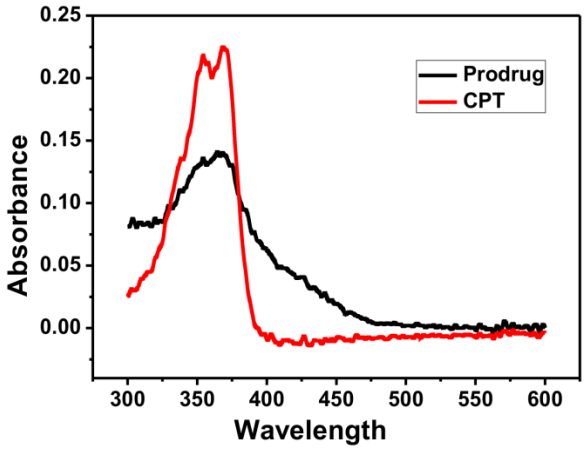


Fig. S13. Absorption spectra of the prodrug and CPT (5μM) in pH 7.4 PBS buffered water solution (containing 1% DMSO).

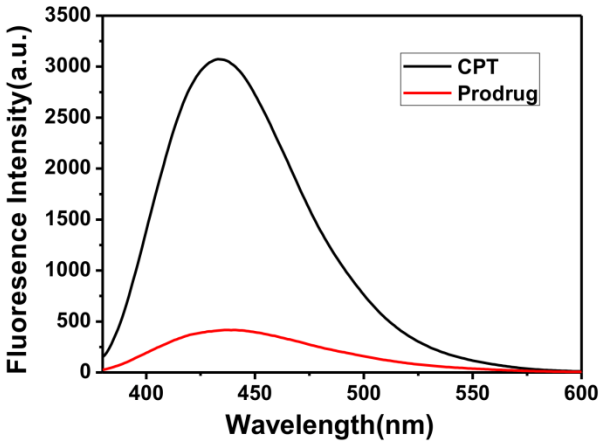


Fig. S14. Fluorescence spectra of the prodrug and CPT (5 μM) in pH 7.4 PBS buffered water solution (containing 1% DMSO).

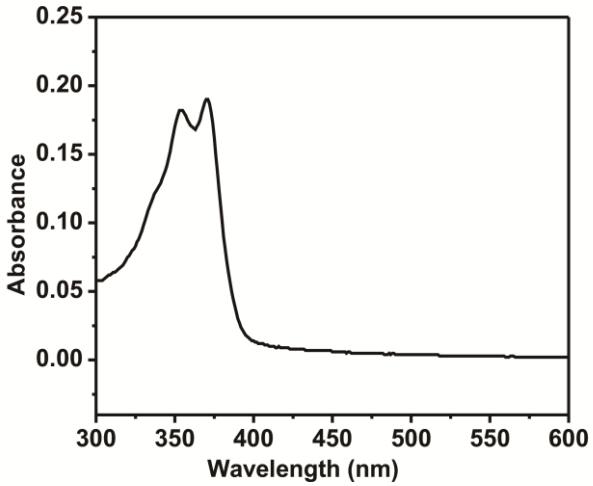


Fig. S15. Absorption spectrum of CPA (5μM) in pH 7.4 PBS buffered water solution (containing 1% DMSO)

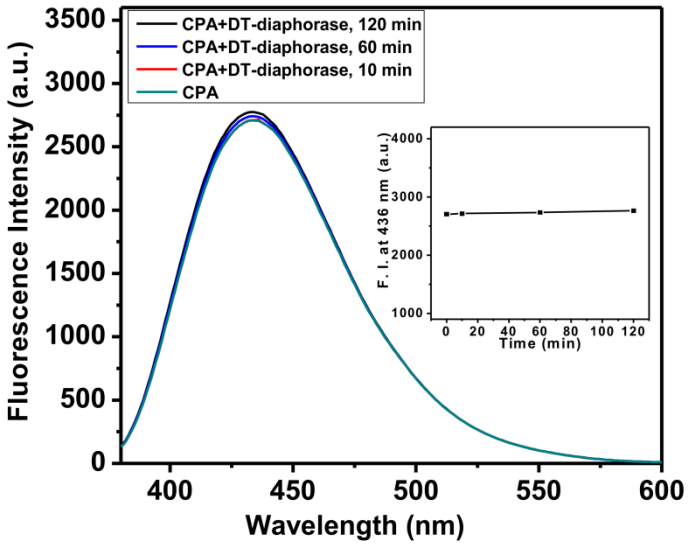


Fig. S16. Time-dependent fluorescence spectra of CPA (5 μM) in the absence or presence of DT-diaphorase (20 μg/mL). Data were obtained at 37 ℃ in phosphate buffer (pH 7.4, 10mM) containing 1% (v/v) DMSO in the presence of cofactor NADH (100 μM). Inset: fluorescence intensity at 436 nm at varied time periods upon addition of enzymes. Excitation wavelength: 365 nm.

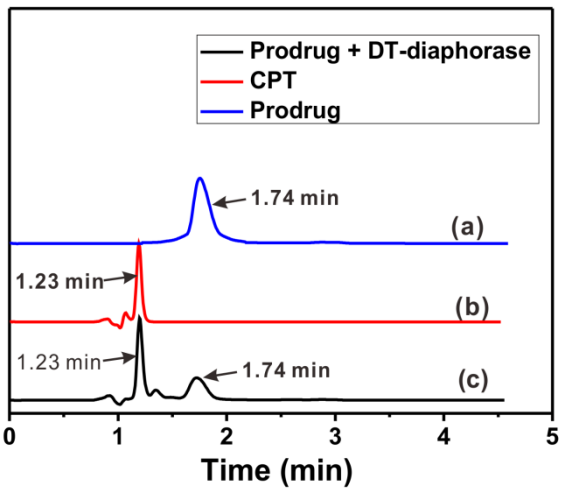


Fig. S17. Typical HPLC chromatogram of the prodrug (a), CPT (b) and prodrug incubated with DT-diaphorase (20 μg/mL) for 60 min (c). Peaks in the chromatograms were detected by monitoring the absorption at 365 nm. The mobile phase was 75/25 acetonitrile/water at a flow rate of 1.0 mL/min.

The prodrug and CPT give rise to a peak at 1.74 and 1.23 min respectively, in HPLC chromatogram. For the prodrug, upon treatment with DT-diaphorase (20 μg/mL), the peak intensity at 1.74 min (corresponding to the prodrug) decreases and a new strong peak emerges at 1.23 min which well matches that for CPT.

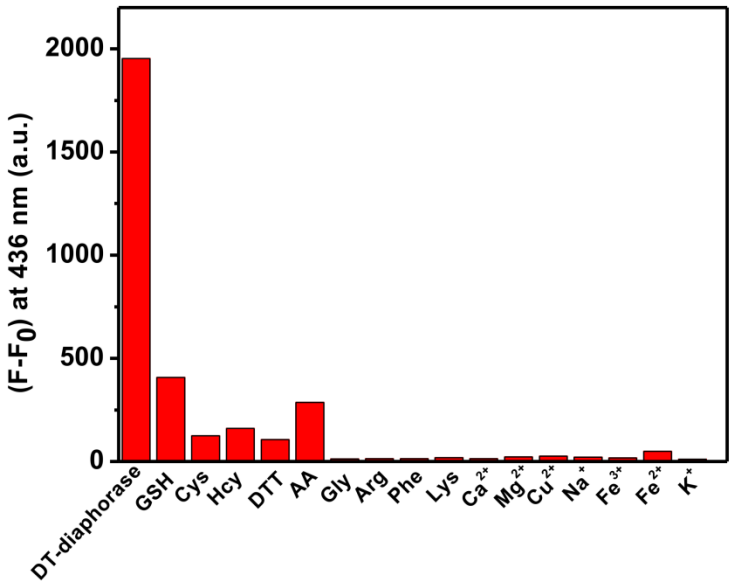


Fig. S18. Fluorescence intensity for the prodrug (5 μM) at 436 nm in phosphate buffer (pH 7.4, 10 mM, containing 1% (v/v) DMSO) in the presence of various relevant species for 60 min. Excitation wavelength: 365 nm. (20 μg/mL for DT-diaphorase, 5 mM for GSH, Gly, Arg, Phe and Lys, 1 mM for others)

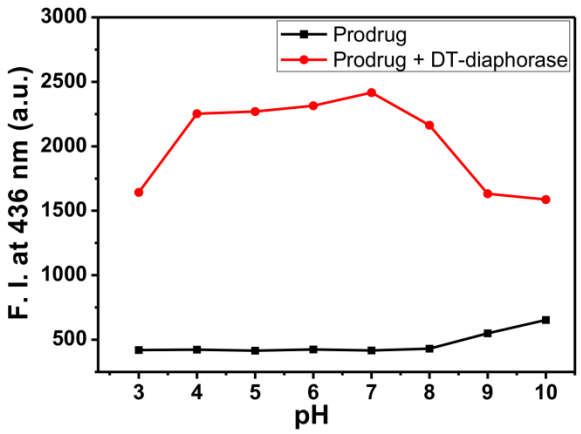


Fig. S19. Fluorescence intensity at 436 nm of the prodrug (5μM) as a function of pH value in the absence or presence of DT-diaphorase (20 μg/mL) in phosphate buffer (pH 7.4, 10mM, containing 1% (v/v) DMSO). Each point was recorded after exposure to DT-diaphorase for 60 min at 37 ℃. Excitation wavelength: 365 nm.

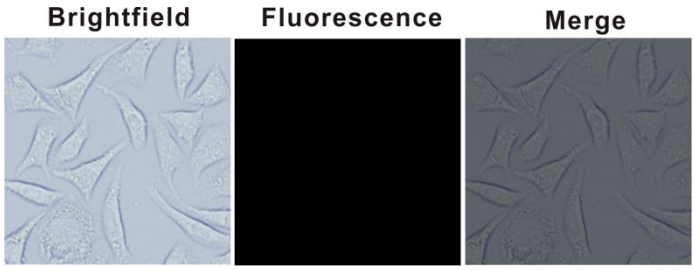


Fig. S20. Fluorescence images of the prodrug in L929 cells. The cells were incubated in PBS buffer (pH 7.4, 10 mM) with prodrug (10 μM) for 2 h.

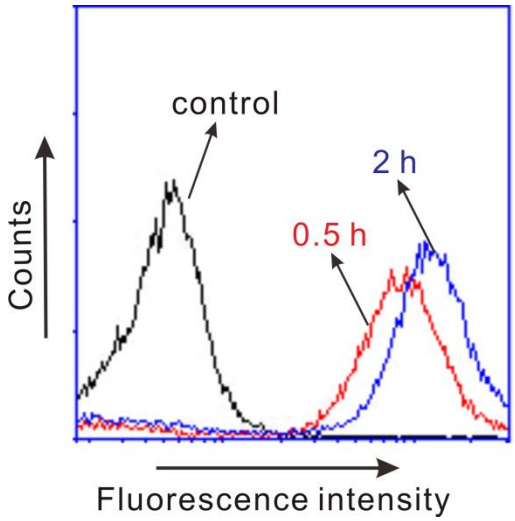


Fig. S21. Flow cytometry profiles for A549 cells in the absence (the control) and presence of the prodrug for 0.5 or 2 h.

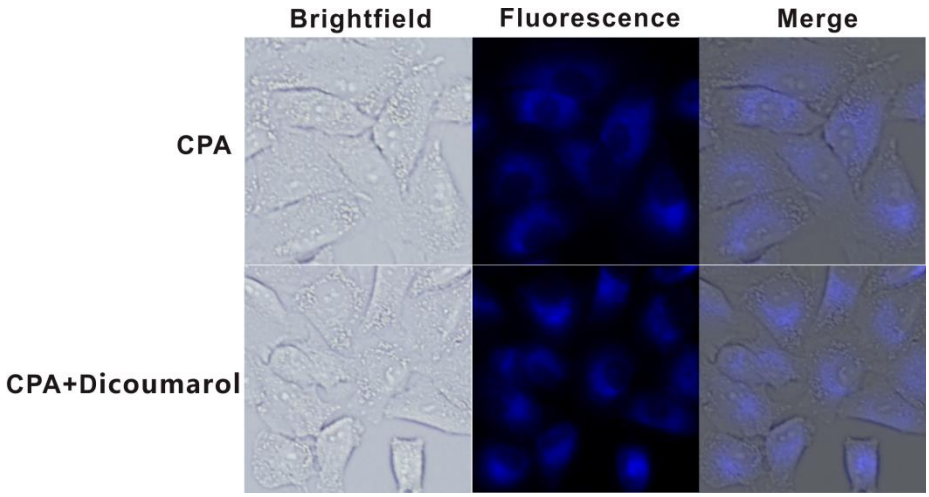


Fig. S22. Fluorescence images of the CPA in A549 cells in the absence and presence of dicoumarol. The cells were incubated in PBS buffer (pH 7.4, 10 mM) with CPA (10 μM) for 2 h. For the inhibition experiment, A549 cells were pretreated with dicoumarol (20μM) for 30 min, then CPA (10 μM) was added and incubated for 2 h.

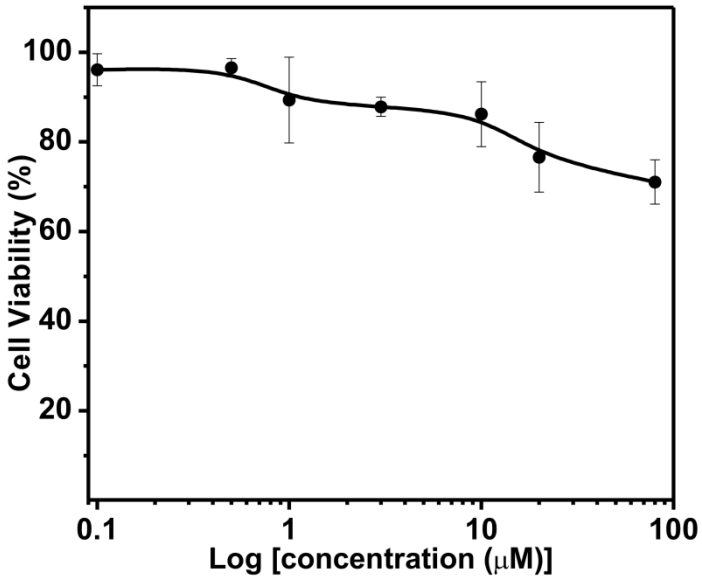


Fig. S23. Cell viability profiles for A549 cell line treated with the CPA of varied concentrations.