

Supporting Information

Hierarchical Targeted Delivery of Lonidamine and Camptothecin Based on the Ultra-Rapid pH/GSH Response Nanoparticles for Synergistic Chemotherapy

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1. Materials and instrumentation

Materials. Unless otherwise stated, all chemicals and reagents were obtained commercially, and were used without further purification. All solvents used in spectra test systems were chromatographically pure and aqueous solutions were freshly prepared using ultrapure water from Thermo Scientific Smart 2 Pure 6 UV/UF. Triphenylphosphine (TPP), CuBr (99%), 2-bromoethylamine hydrobromide, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), 4-Dimethylaminopyridine(DMAP), *N,N',N'',N'''*-pentamethyldiethylenetriamine (PMDETA, 99%), 3-bromo-1-propyne (98%), and pyridinium p-toluenesulfonate (PPTS, 98%), poly(ethylene glycol) (PEG, Mn=2000 Da), α -bromoisobutyryl bromide (98%), and 2,2'-dithiodiethanol ($\geq 90\%$) were purchased from Aladdin reagent (Shanghai, China) and used as received.

Instrumentation. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker DMX-400 with chemical shifts expressed in parts per million (in deuteriochloroform, Me_4Si as an internal standard. Fluorescence spectra and UV-vis absorption spectra were recorded using a Hitachi F-7000 fluorescence spectrometer and a Hitachi PharmaSpec UV-1900 UV-visible spectrophotometer. The confocal laser scanning microscopy (CLSM) was performed on a ZEISS LSM 780 confocal laser scanning microscope. The cells lines were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The HepG2 cells were cultured in DMEM with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10 kU

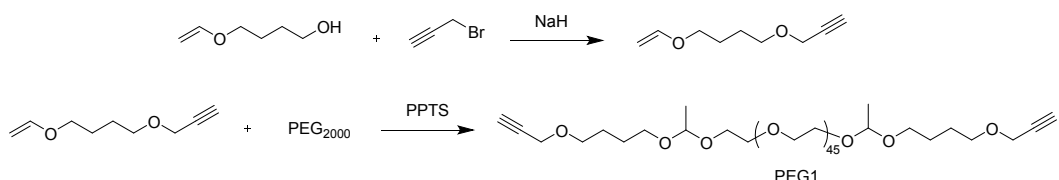
mL⁻¹) at 37 °C in a humidified atmosphere containing 5% CO₂. Some experiments and methods, including cell culture, gel permeation chromatography (GPC), and dynamic light scattering (DLS) were carried out following previously reported procedures.

2. Synthesis

Synthesis of 4-propargyloxybutylvinyl ether. In a 250 mL round bottom flask, 4-hydroxybutylene ether (60 mmol) was added and dissolved in anhydrous THF (100 ml). Then, NaH (66 mmol) was add slowly under the stirring in ice bath. After 1 h, propargyl bromide (66 mmol) was add and stirred for 12 h at room temperature. Water (5 ml) was uesd to quench the reaction drop by drop. After the filtrate was decompressed and concentrated, ethyl acetate (50 mL) was added to dissolve the residue and washed with water (50 mL×2). The organic phase was collected, dried with anhydrous MgSO₄ and filtered. The filtrate was concentrated, and the residue was separated by column chromatography (petroleum ether/ethyl acetate = 8/1, v/v) to obtain 4-propargyloxybutylvinyl ether, which was a yellow liquid in 75% yield.

Synthesis of alkyne end-functional poly(ethylene glycol) containing acetal groups (PEG1). PEG1 were synthesized as described in our previous works.¹ The reaction was performed according to a procedure similar tothe literature. Poly(ethylene glycol) (PEG₂₀₀₀, 2 mmol) and PPTS (0.2 mmol) were dissolved in 15 mL dry DCM. Then, 4-propargyloxybutylvinyl ether (10 mmol) was added dropwise at 0 °C under an argon atmosphere. After stirring for 6 h at 0 °C, the reaction was terminated by adding 5 wt% of an aqueous Na₂CO₃ solution (12 mL). The quenched reaction mixture wasdiluted with another 20 mL DCM and washed with 10 mL water followed by 20 mL brine. Each aqueous layer was further extracted with DCM. The combined solution was dried over MgSO₄ and evaporated under reduced pressure. The crude

product was redissolved in DCM (10 mL) and precipitated into 250 mL cold hexane. The precipitate was further washed with a large amount of hexane and dried under vacuum to yield a white powder in 90% yield.



Scheme S1. Synthesis of **PEG1**.

Synthesis of 2-Hydroxyethyl-2'-(bromoisobutyryl)ethyl Disulfide (HO-ss-iBuBr).

Triethylamine (25 mmol) and 2,2'-dithiodiethanol (30 mol) were dissolved in 100 mL of dry THF in ice bath. α -Bromoisobutyryl bromide (20 mol) dissolved in 40 mL of THF, was added dropwise into the solution. The solution was stirred in ice bath for 1 h and at room temperature for 16 h. The resulting mixture was filtered and removed THF by rotary evaporation, dichloromethane was added and the solution was washed by 3% HCl solution for three times, 2% NaOH solution for three times and water for three times. The organic phase was collected and dried over magnesium sulfate anhydrous for 12 h. The crude solution was concentrated by rotary evaporation and purified by column chromatography(4:1 petroleum ether–ethyl acetate) resulting in a yellow powder in 51.8% yield.

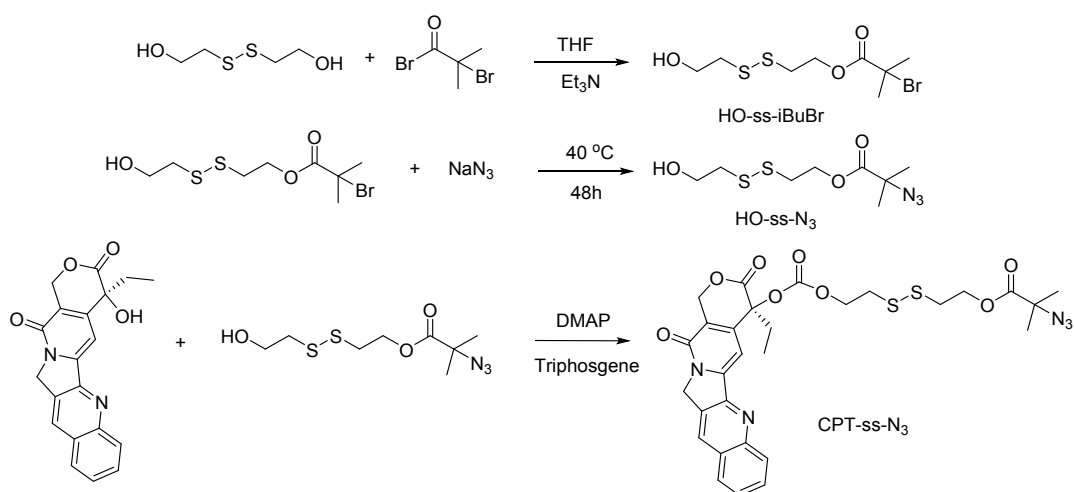
Synthesis of 2,2'-dithiobis[1-(2-azido-2-methylpropionyloxy) ethane] (HO-ss-N₃).

HO-ss-iBuBr (10 mmol) and NaN₃ (30 mmol) were dissolved in 100 mL of DMF in one 100 mL of flask. The reaction was performed at 40 °C for 48 h. The mixture was passed through a short column of basic Al₂O₃ and DMF was evaporated. The crude product was dissolved in 100 mL of water and extracted with 200 mL CH₂Cl₂ for

three times. The organic layer was dried over anhydrous Na_2SO_4 for 12 h and the filtrate was dried by rotary evaporation. Finally, the product was dried under vacuum at 25 °C for 24 h to give a yellow viscous liquid in 93.6% yield

Synthesis of GSH-responsive and clickable CPT derivative (CPT-ss- N_3).

CPT-ss- N_3 were synthesized as described in our previous works.² CPT (2.87 mmol) and DMAP (8.65 mmol) were suspended in 150 mL of dry DCM in ice bath under a argon atmosphere. Triphosgene (0.96 mmol) was added and the mixture was stirred for 30 min. After that, **HO-ss- N_3** (4.26 mmol) in 15 mL of dry DCM was added dropwise into the reaction. The reaction was further stirred at 25 °C for 16 h. After filtration and the removal of the solvents by rotary evaporation, the residue was diluted with 100 mL of DCM and washed with water for once, 1.0 M HCl solution for twice, and brine for three times, respectively. The organic layer was collected and dried over anhydrous Na_2SO_4 for 12 h. The filtrate was concentrated and the crude product was purified by silica column chromatography (4:1 DCM–ethyl acetate) resulting in a yellow powder in 38.5% yield.

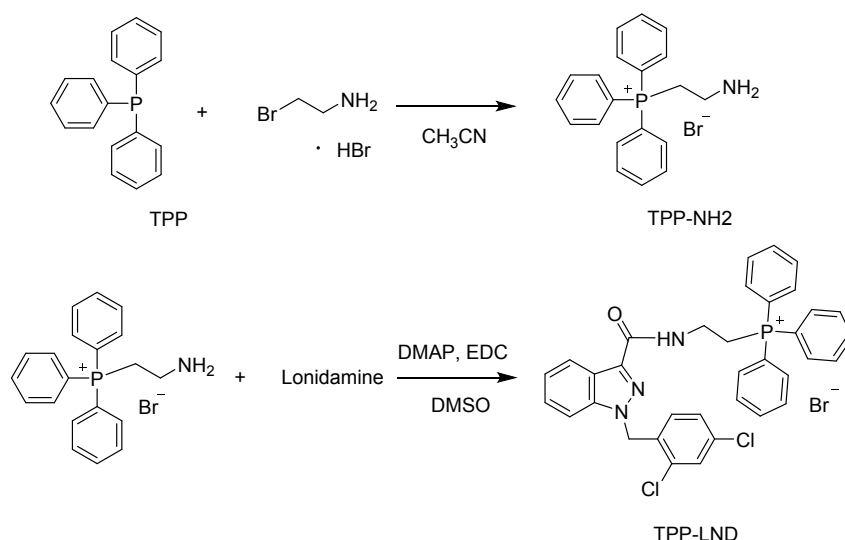


Scheme S2. Synthesis of **CPT-ss- N_3** .

Synthesis of CPT Prodrugs. **PEG1** (5 mmol) and **CPT-ss-N₃** (5 mmol) were added to a Schlenk tube. Then, 10 mL dry DCM was added to the tube *via* injection under argon. CuBr (0.4 equiv. with respect to azide groups) and PMDETA (0.4 equiv. with respect to azide groups) were subsequently transferred to the reaction mixture. The reaction product was washed with a large amount of ethyl ether to remove the **PEG1** and **CPT-ss-N₃** and was subsequently purified three times using a mixture of hexane and chloroform (3:1, v/v) after reaction at room temperature for 24 h. The products (**CPT Prodrugs**) were dried under a vacuum, and resulting in a brown solid in 93% yield.

Synthesis of TPP-NH₂. 2-bromoethylamine hydrobromide (10 mmol) and **TPP** (10 mmol) in CH₃CN (40 mL), stirred, and refluxed for 24 h. Then the reaction mixture was evaporated, and the rest of the crystals were dissolved in a minimal amount of H₂O; the aqueous solution was adjusted with NaOH (2 mol/L) to pH 11.0. Thereafter, the water was evaporated and the amine was extracted with MeOH. After NaBr was filtered, 2.41 g (yield, 40%) of **TPP-NH₂** precipitated from the methanolic filtrate triturated with diethyl ether. This method was performed as described previously.

Synthesis of TPP-LND³. Lonidamine (3 mmol), 4-dimethylaminopyridine (DMAP, 3 mmol), and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC, 3 mmol) were dissolved in anhydrous DMSO (20 mL) and stirred for 6 h. **TPP-NH₂**/CH₃CN solution was added next and the solution stirred for 72 h. After that, the solution was extracted with a mixed solution of methylene chloride and ultrapure water (methylenechloride:ultrapure water=5:1) and then washed with saturated saltwater, and then a certain amount of anhydrous Na₂SO₄ was added, standing overnight. Next, after filtration and evaporation of the filtrate, the remaining crystals were dissolved in a lot of water and freeze-dried at -80 °C to afford of TPP-LND in 35% yield.



Scheme S3. Synthesis of TPP-LND.

3. Experimental methods

Preparation of blank (CPT NPs) and TPP-LND loaded NPs (TL-CPT NPs). 20 mg **CPT Prodrugs** with or without 5 mg or 10 mg TPP-LND were dissolved in 2 mL THF. Then, 10 mL of H₂O was added dropwise to the solution. After stirring at room temperature for 12 h, the solution was filtered with a 0.45 μm filter to obtain micelles. The loading of TPP-LND was quantified *via* UV-vis. The drug loading content (DLC) and the drug loading efficiency (DLE) were calculated according to the following formula 1.

$$\text{DLC (wt\%)} = (\text{weight of loaded drug}) / (\text{weight of loaded drug and polymer}) \times 100\%$$

$$\text{DLE (wt\%)} = (\text{weight of loaded drug}) / (\text{weight of drug in feed}) \times 100\% \quad \text{formula 1}$$

In vitro CPT and TPP-LND synergetic release. The CPT and TPP-LND release experiments were investigated in PBS at pH 7.4 with 2 μM GSH, at pH 5.8 with 2 μM GSH, at pH 7.4 with 10 mM GSH, and at pH 5.8 with 10 mM GSH. 0.5 mL of

TL-CPT NPs was dissolved in 2.0 mL of the above solutions and immediately placed into dialysis tubes (MWCO 500-1000 Da). After that, 45 mL of the above solutions was added to each dialysis tube at 37 °C under constant shaking at 100 rpm. At predetermined time intervals, 20 µL of the internal solutions were diluted to 1 mL with DMSO. Finally, the amount of released CPT and TPP-LND was analyzed via fluorescence spectrometry and UV-vis, respectively. 8 h later, the changes in morphology were monitored by TEM and the change of molecular weight measured by GPC. The data of drug release are shown as the mean \pm SD based on triplicate independent measurements.

Cell uptake mechanism of TL-CPT NPs. HepG2 cells were cultured for 24 h in a 24-well plate. Further study the mechanism of different modifications, uptake inhibitors including cytochalasin D (10 µg/mL), genistein (10 µg/mL), chlorpromazine (10 µg/mL) and 4 °C were cultured for 30 min. The control group did not undergo any treatment. Then, **TL-CPT NPs** with the final concentration of TPP-LND was 2.5 mg/L were co-cultured into each group for another 4 h. The cells were washed by PBS for thrice and treated with trypsin. Finally, the cells were centrifuged (1500 rpm, 5 min) thrice and resuspended in 0.5 mL of PBS. The cell uptake of micelles were analyzed using a flow cytometer (BD FACSCelesta).

Intracellular localization and lysosome permeabilization. Cells were seeded at 5×10^4 cells per well for 12 h, and each well was incubated with free TPP-LND and CPT (**TL+CPT**) or **TL-CPT NPs** for different time and the final concentration of TPP-LND was 2.5 mg/L. For nuclei co-localization, the cells were cocultured with NucRedTM Live 647 (2 drops) for additional 15 min. For lysosome permeabilization, the cells were cocultured with Acridine Orange ($5 \mu\text{g mL}^{-1}$) for additional 15 min at 37 °C. Then, the cells were imaged on CLSM.

Cell mitochondria isolation. Cells were cultured in a 35 mm culture dish for 24 h. Then, 5 mL **TL-CPT NPs** with the final concentration of TPP-LND was 2.5 mg/L were co-cultured into culture dish for another 4 h. Mitochondria were isolated from cells by Cell Mitochondrial Isolation Kit (Beyotime Biotechnology). CPT and TPP-LND for endocytosis of cells and mitochondria was measured by fluorescence spectrometry and UV-vis, respectively. The relative uptake percentage by calculating based on formula 2.

$$\text{Relative uptake percentage (\%)} = (\text{weight of TPP-LND in mitochondria}) / (\text{weight of TPP-LND in feed}) \times 100\% \quad \text{formula 2}$$

Cell viability assay. A cytotoxicity assay to evaluate the effects of the TPP-LND, **CPT-NPs**, TL+CPT and **TL-CPT NPs** were performed using a MTS Reduction Assay. The HepG2 cells were seeded in 96-well plates for 12 h and were then incubated with DOX at concentrations ranging from 0.63 to 10 mg/L. The micelles were removed after 24 h. For MTS assay, 20 μ L MTS solution in 80 μ L PBS was added to each well for incubating another 45 min. For ATP Assay, CellTiter-Glo® Luminescent Assay was added to each well for incubating another 10 min. The absorbance (MTS Reduction Assay) or luminescent (CellTiter-Glo® Luminescent Assay) was measured using a microplate reader. Cell viability (%) was evaluated according to the manufacturer's instructions as follows: cell viability = $(\text{OD}_{\text{treated}}/\text{OD}_{\text{control}}) \times 100\%$. All experiments were performed in triplicate.

Mitochondrial membrane potential depolarization. Cells were seeded at 5×10^4 cells per well for 12 h, the culture media were then replaced and treated with TL+CPT or **TL-CPT NPs** with the final concentration of **TL** was 2.5 mg/L. The control experiment was blank culture medium. After 4 and 12 h incubation, the culture

medium was removed, the cells were washed with PBS two times, and then the cells were stained with JC-1 for 30 min under 5% CO₂ at 37 °C. Cell images were collected on a CLSM.

Expression of caspase-3. HepG2 cells were cultured for 24 h in a 6-well plate. the culture media were then replaced and treated with TL+CPT or **TL-CPT NPs** with the final concentration of TPP-LND was 2.5 mg/L. After 4 and 12 h incubation, the culture medium was removed, the cells were washed with PBS two times, and then the cells were treated by Caspase 3 Activity Assay Kit (Beyotime Biotechnology). The expression of caspase-3 was measured using a microplate reader (Tecan Spark).

Detection of ROS content in cells. Cells were seeded at 5×10⁴ cells per well for 12 h, the culture media were then replaced by 1 mL fresh DMEM containing the following samples: TL+CPT or **TL-CPT NPs** with the final concentration of TPP-LND was 2.5 mg/L. The control experiment was blank culture medium. After 4 and 12 h incubation, the culture medium was removed, the cells were washed with PBS two times, and then the cells were stained with DCFH-DA for 20 min under 5% CO₂ at 37 °C. Cell images were collected on a CLSM.

Cell apoptosis detection of TL-CPT NPs. HepG2 cells were cultured for 24 h in a 24-well plate. Then, TPP-LND, **CPT-NPs**, TL+CPT and **TL-CPT NPs** with the final concentration of TPP-LND was 2.5 mg/L were co-cultured into each group for another 12 h. The control group did not undergo any treatment. The cells were washed by PBS for thrice and treated by Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology). The cell apoptosis were analyzed using a flow cytometer (BD Accuri C6).

Morphological observation of mitochondria. Cells were cultured for 24 h in a 10 cm culture dish, and treated with TL+CPT or **TL-CPT NPs** with the final concentration of TPP-LND was 2.5 mg/L. After 12 h, cells were collected by centrifugation after digestion, prefixed with a 2.5% glutaraldehyde, then the cells was postfixed in 1% osmium tetroxide, dehydrated in series acetone, infiltrated in Epox 812 for a longer, and embeded. The semithin sections were stained with methylene blue and Ultrathin sections were cut with diamond knife, stained with uranyl acetate and lead citrate. Sections were examined with JEM-1400Flash Transmission Electron Microscope.

Determination of cytotoxicity and drug permeability in 3D spheroids.

HepG2 cell suspensions were diluted in DMEM containing 0.24% (w/v) methylcellulose at a density of approximately 2×10^5 cells mL⁻¹. Then, 20 μ L droplets of diluted cells were placed onto the lids of cell culture plates. The 3D spheroids had formed and were transferred to agarose-coated 96-well plates after 24 h. Then, the spheroids were incubated for another 36 h before harvesting for drug treatment experiments using **TL-CPT NPs** for 12 h, the spheroids were washed with DMEM and treated by LIVE/DEAD® Cell Imaging Kit (Thermo Fisher Scientific Inc.). The 3D spheroids was observed by CLSM, which captured the images at excitation wavelengths of 405 nm for CPT (blue), 488 nm for Live cells (green) and 546 nm for Dead cells (red).

Statistical analysis. Unless otherwise stated, mean \pm SD values based on triplicate independent measurements were used for the expression of data. Statistical analyses of data were performed using the Student's t-test. Differences of $P < 0.05$ were considered statistically significant.

Table S1. Basic characteristics of **CPT NPs**, **25% TL-CPT NPs** and **50% TL-CPT NPs**.

Sample	Size ^a (nm)	Zeta potential ^a (mV)	DLC (%)	DLE (%)
CPT NPs	26.35	-6.86	19.4 ^b	-
25% TL-CPT NPs	40.93	+1.06	71.1±0.5 ^c	15.1±0.1 ^c
50% TL-CPT NPs	62.18	+5.86	57.8±0.5 ^c	22.4±0.1 ^c

^a The size and zeta potential of nanoparticles were determined by DLS. ^b The DLC of **CPT NPs** was determined using ¹H NMR spectra. ^c The DLC and DLE of **TL-CPT NPs** were determined using fluorescence spectrometer, n=3.

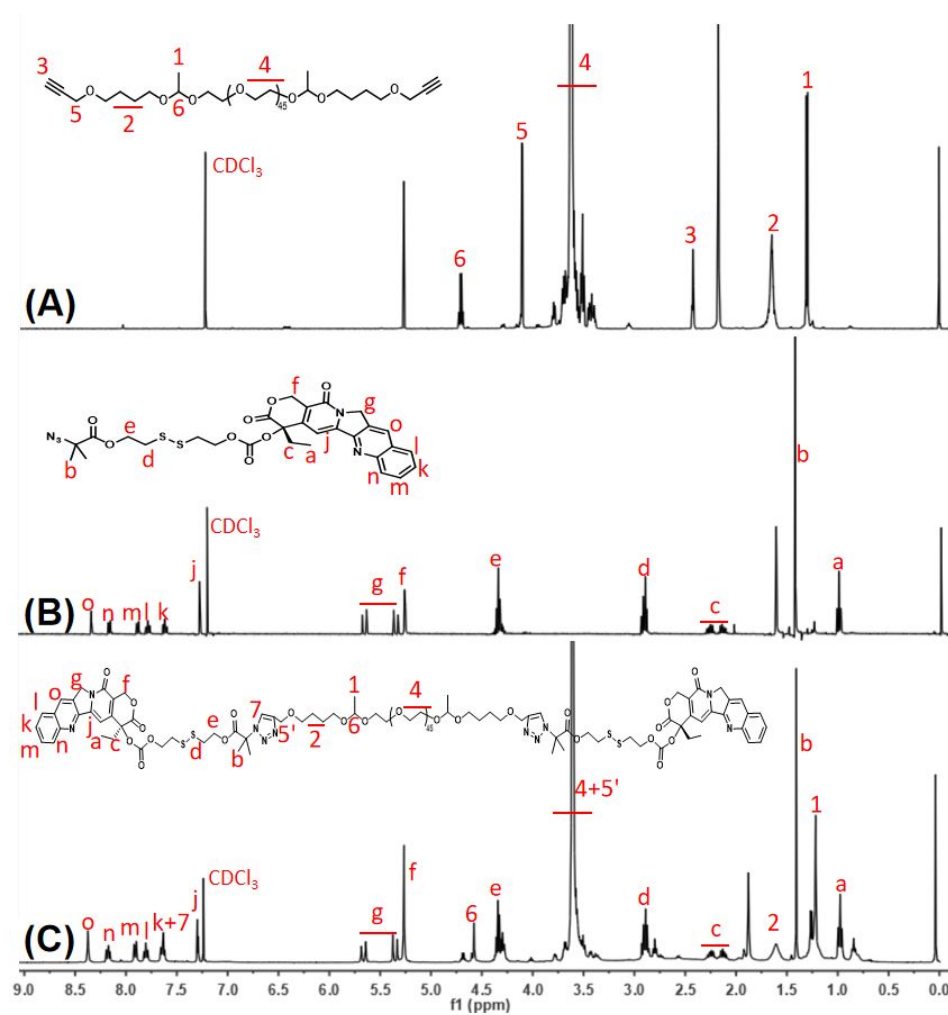


Figure S1. ^1H NMR spectra of (A) PEG1, (B) CPT-ss- N_3 (C) CPT Prodrugs in CDCl_3 .

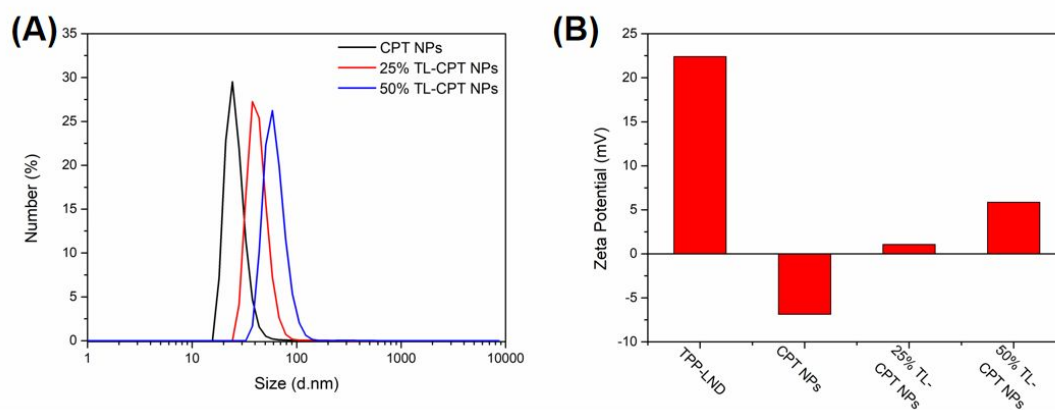


Figure S2. (A) Size and (B) zeta potential of TPP-LND, CPT NPs, 25% TL-CPT

NPs and 50% TL-CPT NPs in PBS.

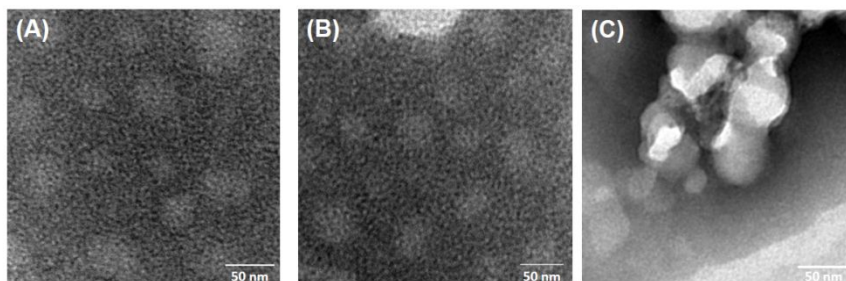


Figure S3. TEM micrographs of **TL-CPT NPs** under PBS of (A) pH 7.4 for 0 h, (B) pH 7.4 with 2 μM GSH and (C) pH 5.8 with 10 mM GSH for 8 h. The scale bar is 50 nm.

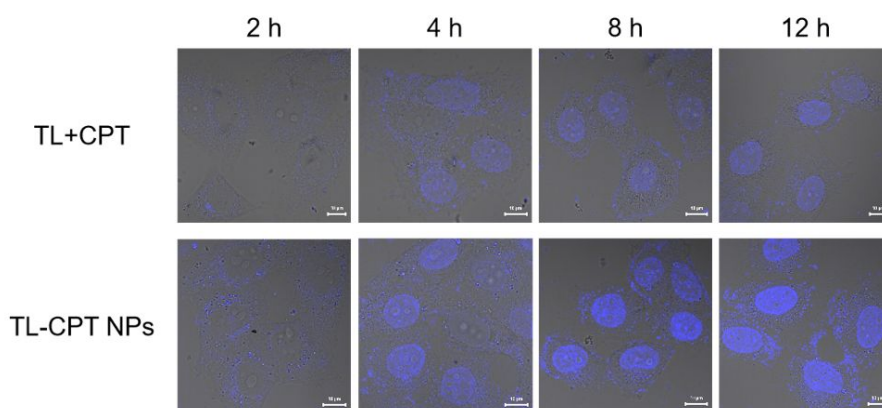


Figure S4. CLSM images of HepG2 cells incubated with **TL+CPT** and **TL-CPT NPs** for different time. The scale bar is 10 μm.

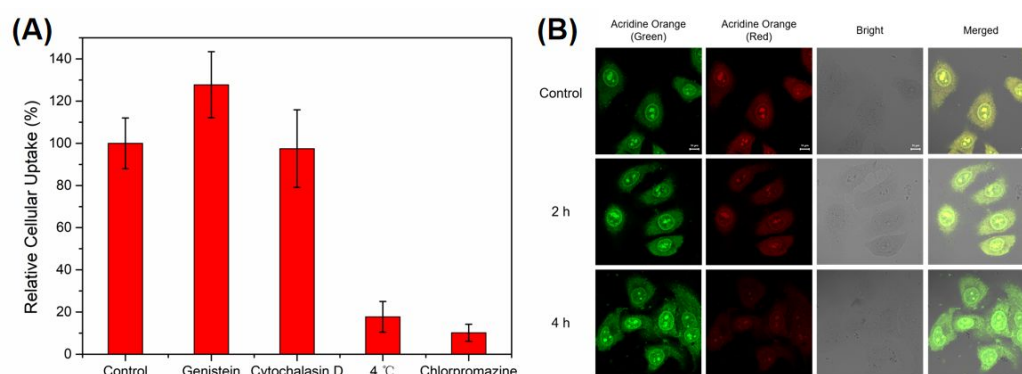


Figure S5. (A) Relative cellular uptake of TL-CPT NPs in the presence of various endocytic inhibitors. (B) Acridine Orange assay in HepG2 cells after treatment with TL-CPT NPs for different time. The scale bar is 10 μm.

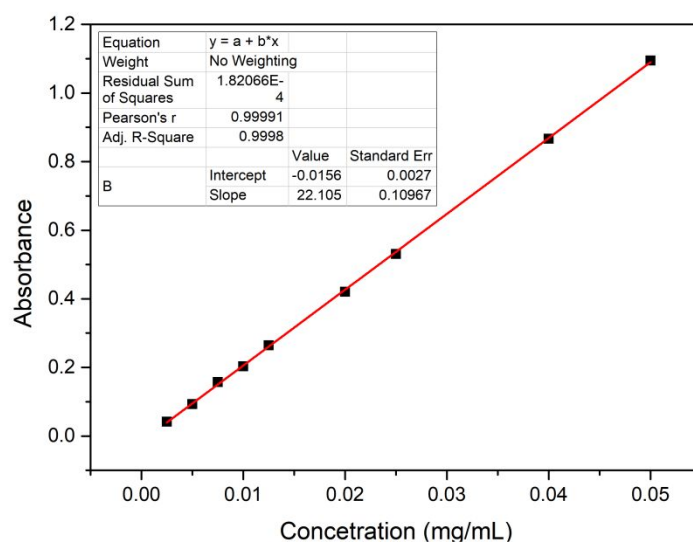


Figure S6. UV absorption curves of TPP-LND in different concentration and standard curve formula (insert) at 300 nm measured by UV-vis in DMSO.

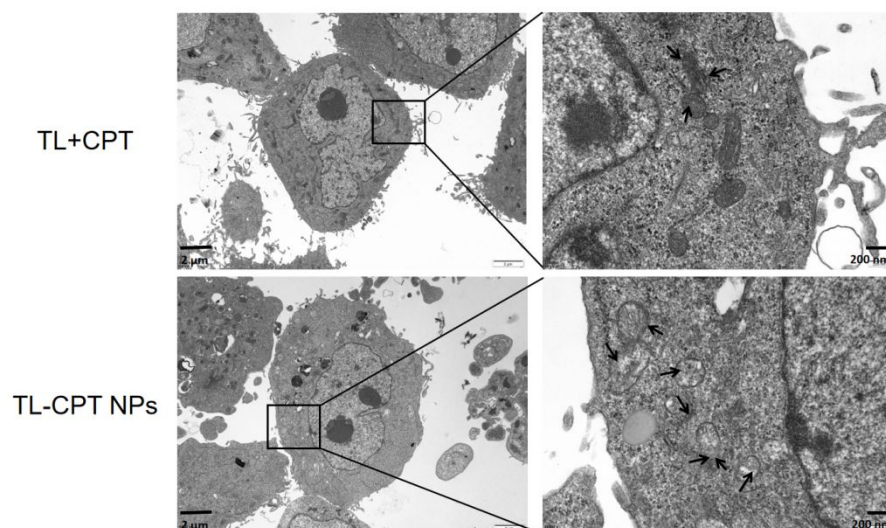


Figure S7. TEM images of cell mitochondria after incubating with TL+CPT and TL-CPT NPs for 12 h.

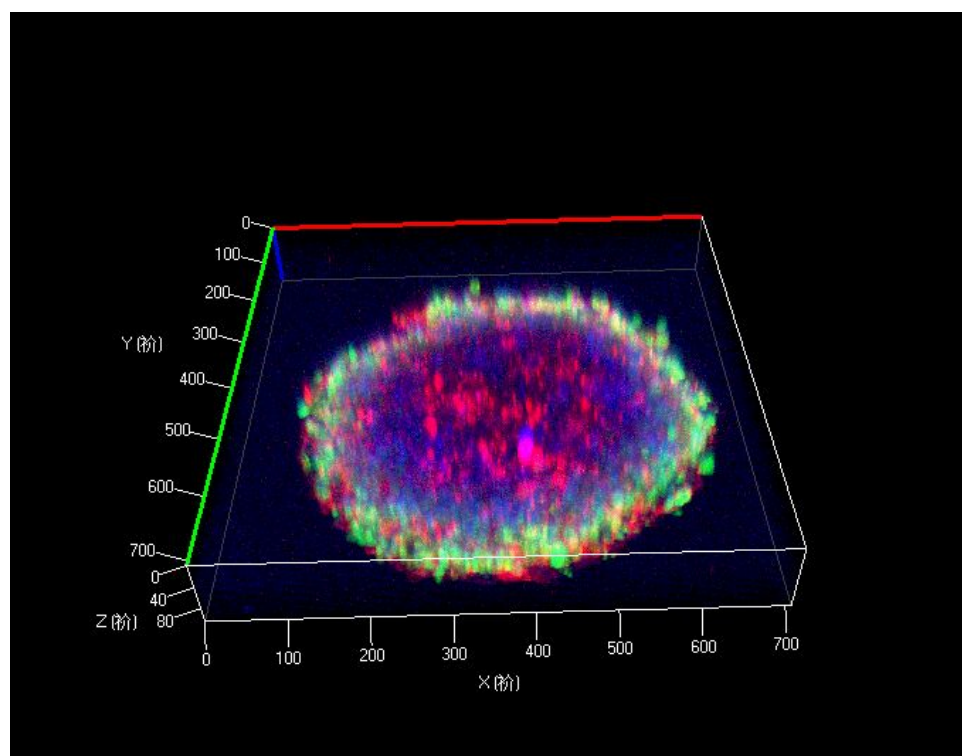


Figure S8. 3D reconstruction image of 3D spheroids after incubating with TL-CPT NPs for 12 h and staining with LIVE/DEAD® Cell Imaging Kit.

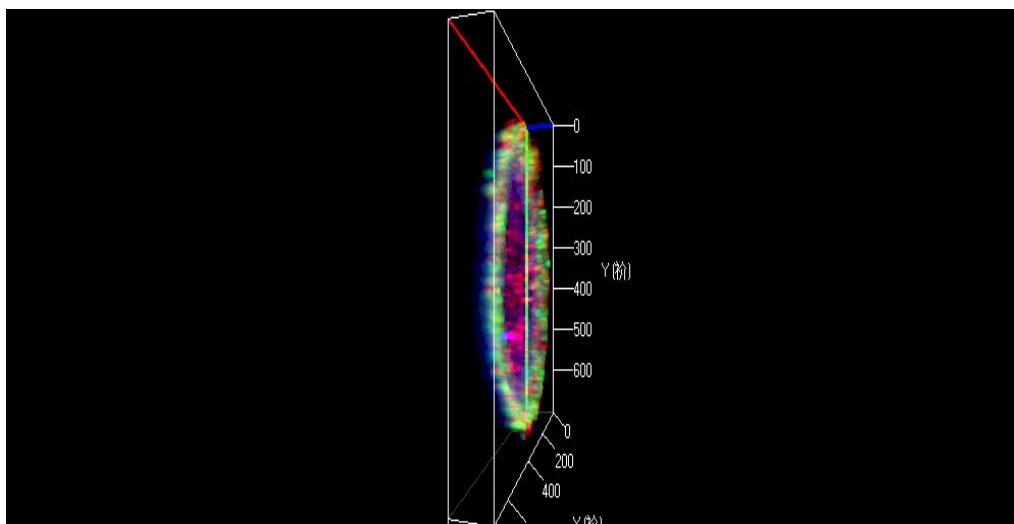


Figure S9. Image video of 3D spheroids after incubating with **TL-CPT NPs** for 12 h and staining with LIVE/DEAD® Cell Imaging Kit.

Reference

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2. Wu, W. X.; Wang, N.; Liu, B. Y.; Deng, Q. F.; Yu, X. Q., Lipase-Catalyzed Synthesis of Azido-Functionalized Aliphatic Polyesters towards Acid-Degradable Amphiphilic Graft Copolymers. *Soft Matter* **2014**, 10, 1199-1213.
3. Liu, Y. Q.; Zhang, X. J.; Zhou, M. J.; Nan, X. Y.; Chen, X. F.; Zhang, X. H., Mitochondrial-Targeting Lonidamine-Doxorubicin Nanoparticles for Synergistic Chemotherapy to Conquer Drug Resistance. *ACS Appl. Mater. Interfaces* **2017**, 9, 43498-43507.

