Supplementary Information

For

Dual-Drug Backboned Polyprodrug with a Predefined Drug Combination for Synergistic Chemotherapy

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Experimental Section

1. Materials

Mitoxantrone (MTO) was purchased from Dalian Meilun Biotechnology (Dalian, China). Curcumin (CUR) was purchased from Arocss. 2,2'-Dithiodiethanol were purchased from Aladdin. 11-Chloro-1,1'-di-n-propyl-3,3,3',3'-tetramethyl-10,12-trimethyleneindatricarbocyanine iodide (IR-780) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Carboxylic acid functionalized methoxyl polyethylene glycol ($mPEG_{45}$-COOH) was purchased from JenKen Co. LTD. Triphosgene, 2,2'-dithiodiethanol, *N*, *N*'-dicyclohexylcarbodiimide (DCC), di-tert-butyl-pyrocarbonate (BOC_2O), 4-dimethylaminopyridine (DMAP), trifluoroacetic acid (TFA), glutathione (GSH) were purchased from ENERGY (Shanghai, China). Tetrahydrofuran (THF), *N*, *N*-dimethylformamide (DMF), dichloromethane (DCM), triethylamine (TEA), dimethyl sulfoxide (DMSO), methanol, acetonitrile, acetic acid, potassium carbonate (K_2CO_3), sodium carbonate (Na_2CO_3) were purchased from China National Pharmaceutical Group Corporation. Dulbecco's modifed eagle's medium (DMEM), trypsin-EDTA and penicillin−streptomycin were obtained from Gibco BRL (Eggenstein, Germany). Fetal bovine serum (FBS) was obtained from ExCell Biology, Inc (Shanghai, China). Hoechst 33342 were purchased from Life Technologies. P-Glycoprotein Monoclonal Antibody (F4) was purchased from Invitrogen.

NMR spectra were measured on a Bruker ARX 400 NMR spectrometer. Particle size and size distribution were carried out in aqueous solution using a Malvern ZS90 dynamic light scattering instrument (Malvern Instruments Ltd., England). The absorption spectra were measured on a UV-3802 spectrophotometer (UNICO). Confocal images were acquired by confocal microscope (CLSM, Nikon Ti-E A1, Japan). Photoluminescence (PL) spectra were measured on a Shimadzu RF-6000 spectrofluorometer (Shimadzu UV-2600, Japan). Absorbance and fluorescence intensity were measured by a multifunctional micropore detection

board analysis system (Biotek Cytation5, BioTek, United States).

2. Synthesis of BocMTO.

In a typical procedure, MTO (0.50 g, 1.13 mmol) and TEA (5 mL) were dissolved in 100 mL of methanol. After stirring in ice-salt bath for 1 h, BOC_2O (0.87 g, 4 mmol) dissolved in 10 mL of THF was added dropwise. The mixture was then allowed to stir at room temperature for 10 h. After removal of the solvent, the residue was dissolved in 50 mL of ethyl acetate and washed with saturated K_2CO_3 solution. After drying over Na_2SO_4 and then removal of the solvent, the BocMTO was purified by silica gel column chromatography using a mixture of hexane, ethyl acetate and methanol in the volume ratio of 10:5:1, to yield the product BocMTO as a black solid (0.65 g, 89%). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 13.54 (s, 2H), 10.60 (s, 2H), 7.59 (d, J = 26.1 Hz, 3H), 7.15 (s, 2H), 3.68 (s, 4H), 3.49 (q, J = 6.0 Hz, 8H), 3.31-3.19 (m, 4H), 1.99 (s, 1H), 1.34 (d, J = 32.8 Hz, 18H).

3. Synthesis of P(BocMTO-ss-CUR).

2,2'-Dithiodiethanol (100 mg, 0.65 mmol), DMAP (282 mg, 2.3 mmol) and triphosgene (69 mg, 0.23 mmol) were dissolved in dry DCM (10 mL) and the mixture was stirred for 1 h under a nitrogen atmosphere. Subsequently, BocMTO (168 mg, 0.26 mmol) and CUR (144 mg, 0.39 mmol) dissolved in 5 mL of DCM was added and the mixture was stirred for 48 h. Thereafter, the mixture was concentrated, and cold diethyl ether was added. After placing in ice for 6 h, the precipitation was collected through centrifugation and then washed with 40 mL of methanol thrice. The precipitation was dissolved in 10 mL of THF and further purified by gel permeation chromatography (Bio-Beads^{TM} S-X1 Support). Drying in vacuum for 24 h, the product P(BocMTO-ss-CUR) was obtained as green solid (124 mg, 31%).

4. Synthesis of PEG-P(BocMTO-ss-CUR).

P(BocMTO-ss-CUR) (100 mg), DCC (50 mg, 0.243 mmol), DMAP (5mg, 0.041 mmol) and $mPEG_{45}$-COOH was dissolved in 15 mL of dry DCM, the mixture was stirred for 24 h. The crude product was dissolved in DMF and then transferred to dialysis tube (MWCO 3500). After dialyzed against DMF for 48 h followed by deionized water for 24 h, the PEG-P(BocMTO-ss-CUR) was obtained after freeze-drying under vacuum. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 13.54 (s, 2H), 10.60 (s, 2H), 7.59 (d, J = 26.1 Hz, 3H), 7.15 (s, 2H), 3.68 (s, 4H), 3.49 (q, J = 6.0 Hz, 8H), 3.31-3.19 (m, 4H), 1.99 (s, 1H), 1.34 (d, J = 32.8 Hz, 18H).

5. Synthesis of PEG-P(MTO-ss-CUR)

To remove the BOC protected group, the PEG-P(BocMTO-ss-CUR) was dissolved in 5% TFA/DCM (v/v). After stirring at room temperature for 30 min, excess TEA was added to neutralize TFA and the mixture was concentrated by rotary evaporator. The residue was dissolved in DMF and then transferred to dialysis tube (MWCO 3500). After dialyzed against DMF for 48 h followed by deionized water for 24 h, the final polymer PEG-P(MTO-ss-CUR) was collected after freeze-drying under vacuum (80 mg, 53%).

6. Synthesis of PEG-P(MTO-ss-).

The synthesis of the control polymer PEG-P(MTO-ss-) with single drug of MTO was similar to the method described above by changing the feed of “BocMTO (168 mg, 0.26 mmol) and CUR (144 mg, 0.39 mmol)” to “BocMTO (419 mg, 0.65 mmol)” with a yield of 33.8%. The molecular weight distribution of PEG-P(MTO-ss-) is 1.28 determined by GPC. ^{1}H NMR (400 MHz, Chloroform-*d*): δ= 4.51–4.32 (m, 4H), 3.64 (s, 180H), 3.38 (s, 6H), 2.92 (s, 5H), 1.57–1.32 (m, 18H).

7. Synthesis of PEG-P(CUR-ss-).

The synthesis of the control polymer PEG-P(CUR-ss-) with single drug of CUR was similar

as the method described above by changing the Feed of "BocMTO (168 mg, 0.26 mmol) and CUR (144 mg, 0.39 mmol)" into CUR (239.2 mg, 0.65 mmol) with a yield of 45.1%. The molecular weight distribution is 1.33 determined by GPC. ^{1}H NMR (400 MHz, Chloroform-*d*): δ= 7.61 (d, J = 16.5 Hz, 9H), 7.14 (s, 20H), 4.57–4.30 (m, 24H), 3.98–3.78 (m, 25H), 3.64 (d, J = 1.6 Hz, 180H), 3.18–2.86 (m, 25H).

8. Cell culture and tumor model

Human breast cancer cell line MCF-7 cells and human breast cancer doxorubicin-resistant cell line MCF-7/ADR cells were cultured in DMEM medium with 10% FBS, 1% penicillin-streptomycin and 1 μg mL^{-1} DOX. Cells were cultured in 5% CO_2 and 21% O_2 incubator at 37 °C. Female BALB/c nude mice (20 ± 2 g, 5-6 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China). MCF-7/ADR cells (5 × 10^6) were injected into the right mammary fat pads to establish an orthotopic MCF-7/ADR tumor model. After the tumor volumes reached to 100 mm^3, the mice were used for subsequent experiments. At the end of the experiments, all mice were killed by CO_2 inhalation. All animal experiments were approved by the Ethics Committee of the South China University of Science and Technology.

9. Preparation of the sensitive polyprodrug nanoparticles ($SNP_{MTO/CUR}$, SNP_{CUR}, SNP_{MTO})

The NPs were prepared using the classic nanoprecipitation method. Under stirring (1000 rpm), 2 mL of PEG-P(MTO-ss-CUR) solution (20 mg mL^{-1} in DMSO) was added dropwise into 10 mL of deionized water. Subsequently, the NPs dispersion was transferred to dialysis tube (MWCO 3500), dialyzed against deionized water for 24 h to remove DMSO, the NPs were finally prepared, denoted as $SNP_{MTO/CUR}$ (dual-drug MTO&CUR nanoparticle). Similarly, the SNP_{MTO} (single-drug MTO nanoparticle) and SNP_{CUR} (single-drug CUR nanoparticle) were prepared by changing the PEG-P(MTO-ss-CUR) to PEG-P(MTO-ss), and PEG-P(CUR-ss-), respectively.

10. Preparation of the control encapsulated dual-drug loading nanoparticles (ENP_{MTO}, ENP_{CUR}, $ENP_{MTO/CUR}$)

The control encapsulated dual-drug nanoparticles ($ENP_{MTO/CUR}$) were prepared using the nanoprecipitation method. Under stirring (1000 rpm), the mixture of poly(ethylene glycol)-*block*-poly(lactide-co-glycolide) (PEG_{5k}-*b*-$PLGA_{8k}$, 40.0 mg), CUR (2.0 mg), MTO (2.15 mg) in 2 mL of DMSO was added dropwise into 10 mL of deionized water. Subsequently, the formed NPs dispersion was transferred to dialysis tube (MWCO 3500), dialyzed against deionized water for 24 h to remove DMSO, the $ENP_{MTO/CUR}$ were finally prepared. HPLC analysis was used to confirm that the drug loading ratio of MTO to CUR is the predefined 2:3 in $ENP_{MTO/CUR}$. The control encapsulated single-drug nanoparticles (ENP_{MTO}, ENP_{CUR}) were prepared by similar method.

11. Investigation of reduction-responsive behavior of PEG-P(MTO-ss-CUR)

5 mg of $SNP_{MTO/CUR}$ was dispersed in 1 mL of PBS containing 10 mM GSH, placing at room temperature for 12 h before freeze-drying. 0.5 mL of DMF was added, followed by centrifugal precipitation. The molecular weight distribution of the polymer in the supernatant was examined by Gel Permeation Chromatography (GPC). The drugs release and activation were examined by High Performance Liquid Chromatography (HPLC). The HPLC isocratic mobile phase consisted of acetonitrile and acetic acid solution (5%, v/v) in volume ratio of 75:25, and the UV/vis detector was set at 420 nm and 660 nm for CUR and MTO analysis, respectively.

12. Particle size and stability of sensitive polyprodrug nanoparticles

The particle size was measured by dynamic light scattering (DLS) and the stability of the NPs in the PBS supplemented with 10% FBS was investigated by monitoring changes in particle size.

13. Fluorescence recover of $SNP_{MTO/CUR}$ in the presence of GSH

The $SNP_{MTO/CUR}$ and SNP_{CUR} were prepared as the above method, and the concentration of CUR was adjusted to the same concentration of 10 μg mL^{-1}, then the fluorescence was measured at 420 nm excitation; then GSH (10 mM) was added to the solution and the fluorescence was measured with the same parameters at certain intervals.

14. *In vitro* MTO&CUR release

$SNP_{MTO/CUR}$ and $ENP_{MTO/CUR}$ were prepared according to the method described above and the drug concentration was adjusted to 0.5 mg mL^{-1}. To quantitatively determine the release of MTO/CUR, 1 mL of the NPs solution was taken in the dialysis membrane tube (MWCO 3.5 kDa) and was immersed in 25 mL of PBS containing different concentrations of GSH (0, 1 and 10 mM) and 0.05% Tween-100, in a shaking water bath at 37 °C. At predetermined time points, the external buffer was collected, which was replaced with equal volume of phosphate. The collected release medium was freeze-dried and dissolved in DMSO. Absorption at 420 nm and 660 nm are detected by Synergy HT multi-mode microplate reader. The amount of MTO&CUR was calculated according to the standard curve.

The cumulative MTO&CUR release was calculated as follows:

Cumulative release (%) = (M_t/M) × 100

Where M_t is the amount of MTO&CUR released from NPs at time t;

M is the amount of MTO/CUR in the NPs.

15. *In vitro* cytotoxicity

The MCF-7/ADR cells were seeded in a 96-well plate at a density of 5×10^3 cells/well. After the incubation in 100 μL of DMEM medium containing 10% FBS for 24 h, a fixed amount of NPs (or drug) dispersed in 100 μL of fresh medium was added and the cells were allowed to incubate for another 48 h or 72 h, replacing the medium with 100 μL of PBS containing MTT (0.5 mg

mL^{-1}), incubating for another 4 h, and replacing the PBS with 100 μL DMSO, shaking for 30 min. Absorption at 570 nm was measured by Synergy HT multi-mode microplate reader. The average value of five independent experiments was collected and the cell viability was calculated as follows:

Cell viability (%) = (Ab treated / Ab control) × 100

where Ab control is the absorption obtained in the absence of the NPs and the Ab treated is absorption obtained in the presence of the NPs.

CI values, defined as the sum of the two ratios of the median effect doses (D_1 , D_2) of each drug alone to the median effect doses (D_{m1}, D_{m2}) of each drug in the combination, were plotted against drug effect levels (IC_x values) at various cell viability points.

16. Intracellular MTO&CUR release

MCF-7/ADR cells were seeded in 24-well plates at a density of 10^5 cells/well and incubated overnight. The cells were washed with PBS and treated with NPs in culture medium with equivalent MTO/CUR at a concentration of 5 μg mL^{-1}. Cells were incubated at 37 °C for different periods of time, washed twice with cold PBS, and lysed in deionized water containing 1% Triton X-100 at ultrasound for 1 h with three freeze-thaw cycles. The cell lysates were then freeze dried and dissolved in acetonitrile. MTO/CUR concentrations in cell lysates were measured by HPLC analyses and normalized to the total cellular protein content of the cells, which was determined by the BCA Protein Assay Kit (Pierce, Rock-ford, IL).

17. *In vitro* cellular uptake

The cellular uptake of $SNP_{MTO/CUR}$ was investigated by confocal laser scanning microscope (CLSM). Briefly, MCF-7/ADR cells were seeded into a 35 mm glass-bottom dishes at a density of 1 × 10^5 cells and incubated overnight. The cells were treated with $SNP_{MTO/CUR}$ (1 μg mL^{-1} of MTO). After incubating for 1, 2, 4, 8 and 12 h, the cells were washed with PBS, stained with

Hoechst 33342 and observed by CLSM.

The quantitative evaluation of cellular uptake was evaluated by flow cytometry analysis. Briefly, MCF-7/ADR cells were seeded in 24-well plates at a density of 2×10^5 cells per well in DMEM medium. After 24 h incubation, the culture medium was replaced with fresh medium containing $SNP_{MTO/CUR}$ (1 μg mL^{-1} of MTO). After incubating for 1, 2, 4, 8 and 12 h, the medium was removed, and the cells were washed with PBS for three times. All cells were trypsinized and 0.5 mL of cell suspension was taken to quantify the internalization by flow cytometry.

18. Immunofluorescence of P-glycoprotein

The P-glycoprotein (P-gp) expression suppression ability of $SNP_{MTO/CUR}$ was investigated by immunofluorescence method. Briefly, MCF-7/ADR cells were seed into 24-well plates at a density of 5×10^4 cells and incubated overnight. The cells were treated with free MTO (6 μM), free CUR (4 μM) and $SNP_{MTO/CUR}$ (6 μM MTO and 4 μM CUR) for 6 h. The cells were continuously incubated for 24 h after replacing the medium with fresh DMEM containing 10% FBS. The cells were fixed by 4% paraformaldehyde and then sealed by 5% bovine serum albumin (BSA) for 4 h. After removing the sealing solution, the cells were incubated with P-glycoprotein first antibody overnight at 4 ℃. After washing with PBS, the P-glycoprotein second antibody conjugated with Alexa Fluro 488, was diluted 500 times and incubated with cells for 3 h at room temperature. Removing the second antibody solution, the cells were then stained with DAPI for 10 min and observed by CLSM.

19. *In vivo* imaging and bio-distribution study

The *in vivo* biodistribution of $ENP_{MTO/CUR}$, SNP_{MTO}&SNP_{CUR}, $SNP_{MTO/CUR}$ loaded with IR-780 were examined using MCF-7/ADR tumor bearing Balb/c nude mouse model. To establish the orthotopic tumor model, each nude mouse was injected with 5×10^6 of MCF-7/ADR cells on the right mammary gland. After tumor volume reached 200 mm^3, the tumor-bearing mice were

randomly grouped, and intravenously injected with 200 μL of $SNP_{MTO/CUR}$ at IR780 dose of 1.5 mg kg^{-1}. The body fluorescence images were collected at 24 h after injection using in vivo Xtreme (Bruker, German). The mice were sacrificed at 24 h after administration for *ex vivo* examining the IR780 distribution in major organs.

20. Antitumor efficacy and safety evaluation

Balb/c nude mice (20-25 g) bearing MCF-7/ADR tumor under breast were used as animal modal to investigate the antitumor efficacy *in vivo*. When tumors grew to about 100 mm^3 in size, 7 days after inoculation of the cancer cells, the mice were randomly divided into 4 groups and were given three injections of drugs intravenously on day 0, 3 and 6, with the day of the first injection counted as day 0. The tumor size and body weight were then monitored every two days for two weeks. The recipes for the 4 groups were as follows: (a) Saline (b) $ENP_{MTO/CUR}$ (5.00 mg MTO/kg, 6.03 mg CUR/kg), (c) SNP_{MTO} (5.00 mg MTO/kg) plus SNP_{CUR} (6.03 mg CUR/kg), (d) $SNP_{MTO/CUR}$ (5.00 mg MTO/kg, 6.03 mg CUR/kg). All groups of (b), (c), and (d) have the same drug loading ratio according to the design (molar ratio of MTO:CUR=2:3). All the mice were administrated three consecutive injections and the tumor growth was monitored by measuring perpendicular diameters and tumor volume was calculated as follows:

$V = W^2 \times L/2$

where W and L are the shortest and longest diameters, respectively.

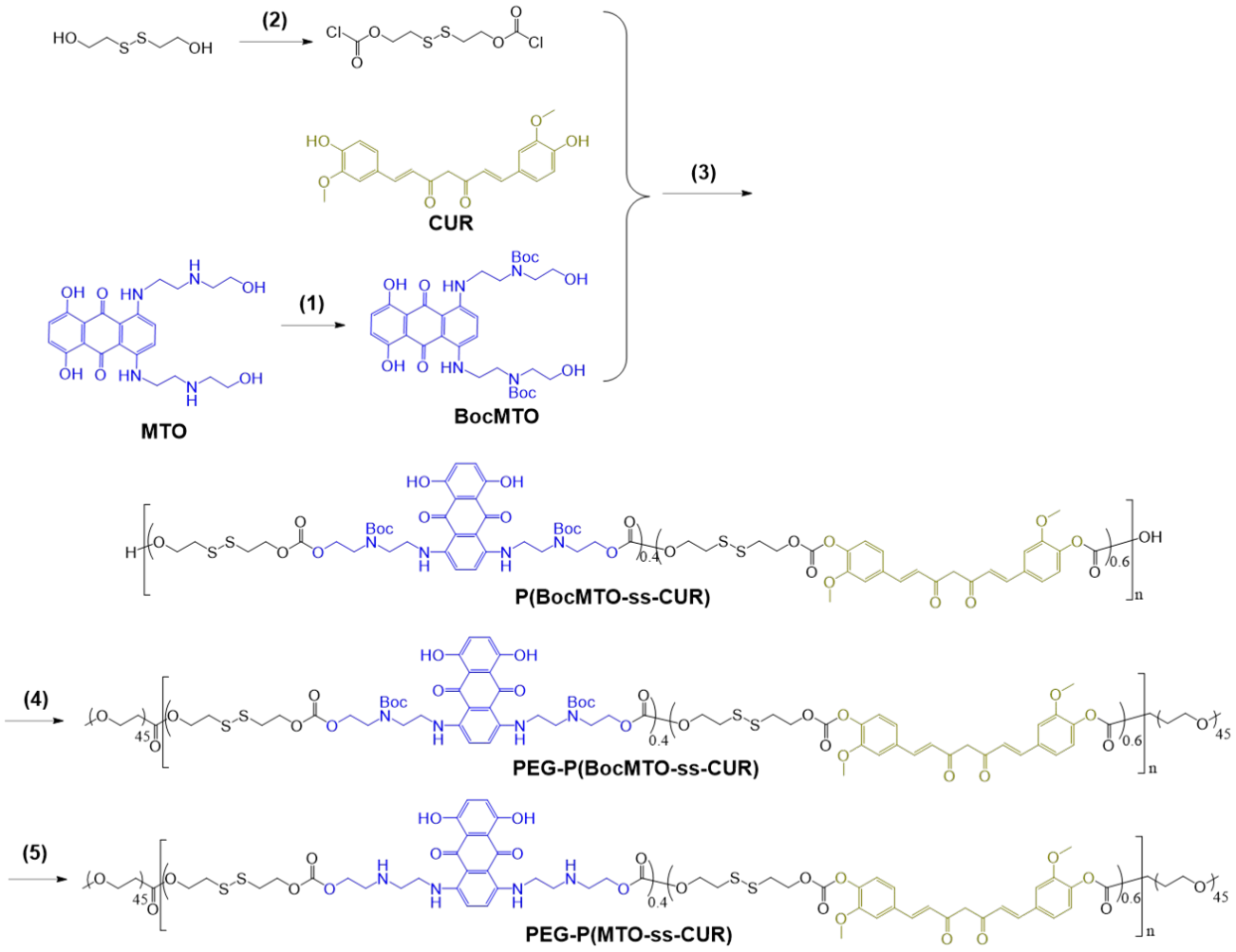
At day 12, mice were sacrificed, and tumor was excised to intuitionally evaluate the tumor inhibition. In order to investigate the safety of formulations, main organs (heart, liver, spleen, lung, kidney) and tumor were collected, fixed in 4% paraformaldehyde solution, and then embedded in paraffin, sliced and stained with hematoxylin and eosin (H&E) to evaluate potential toxicity for main organs.

21. Release ratio in tumor tissue

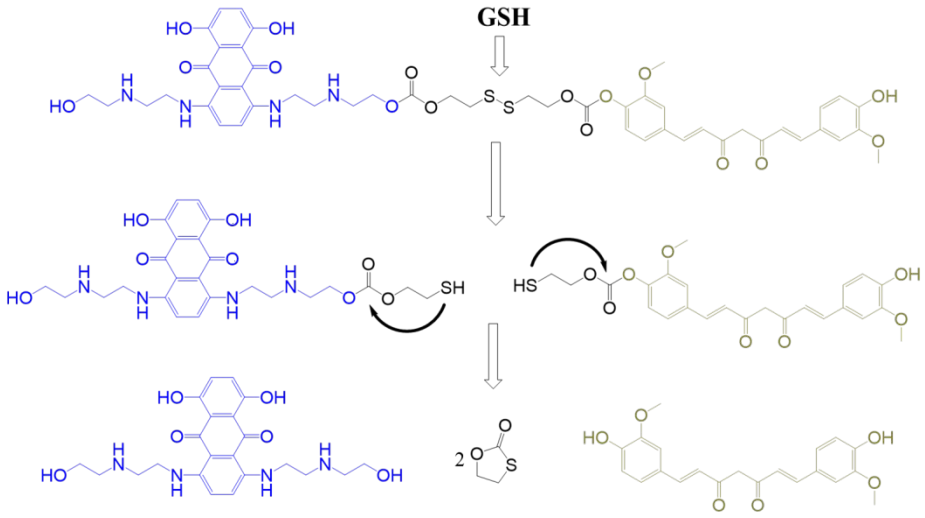
Balb/c nude mice (20-25 g) bearing MCF-7/ADR tumor were used as animal modal to investigate drug release ratio in tumor tissue. When tumors grew to about 500 mm^3 in size, mice were randomly divided into 3 groups (n=3) and were given injections of drugs intravenously. The recipes for the 3 groups were as follows: (b) $ENP_{MTO/CUR}$ (5.00 mg MTO/kg, 6.03 mg CUR/kg), (c) SNP_{MTO} (5.00 mg MTO/kg) plus SNP_{CUR} (6.03 mg CUR/kg), (d) $SNP_{MTO/CUR}$ (5.00 mg MTO/kg, 6.03 mg CUR/kg). All groups have same drug loading ratio according to the design (molar ratio of MTO:CUR=2:3). Two days later after injections, the mice were sacrificed and the tumor tissue (about 1.0 g) were excised and homogenated in PBS solution. The supernatant was obtained after homogenation and centrifugation at 15000 rpm for 2 h. After the supernatant was freeze-dried for 48 h, 1 mL of acetonitrile was used to extract MTO and CUR. The content of free MTO and CUR were detected by HPLC.

22. Statistical Analysis.

All the data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to assess the significance of the difference. Statistical significance was set at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$



Scheme S1. Synthetic procedures of **PEG-P(MTO-ss-CUR).** (1) BOC_2O, TEA, CH_3OH; (2) Triphosgene, DMAP, DCM; (3) DMAP, DCM; (4) $mPEG_{45}$-COOH, DCC, DMAP, DCM; (5) TFA, DCM.



Scheme S2. GSH responsive degradation of **PEG-P(MTO-ss-CUR).**

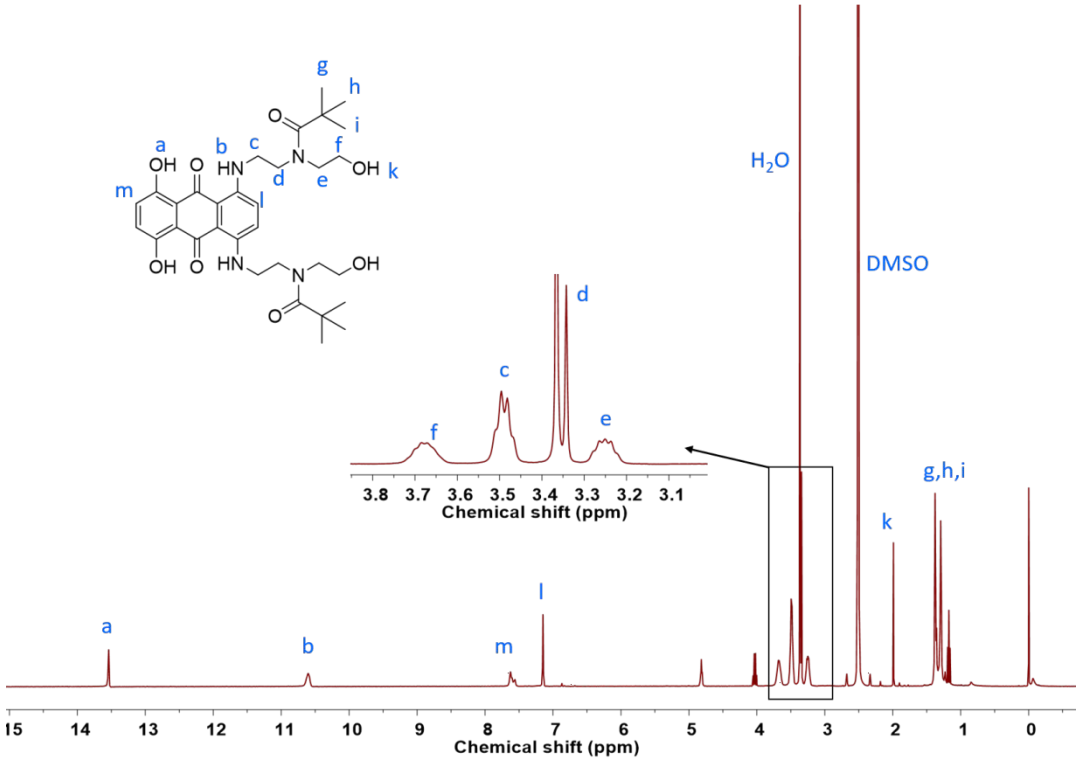


Figure S1. ^{1}H NMR spectrum of BocMTO.

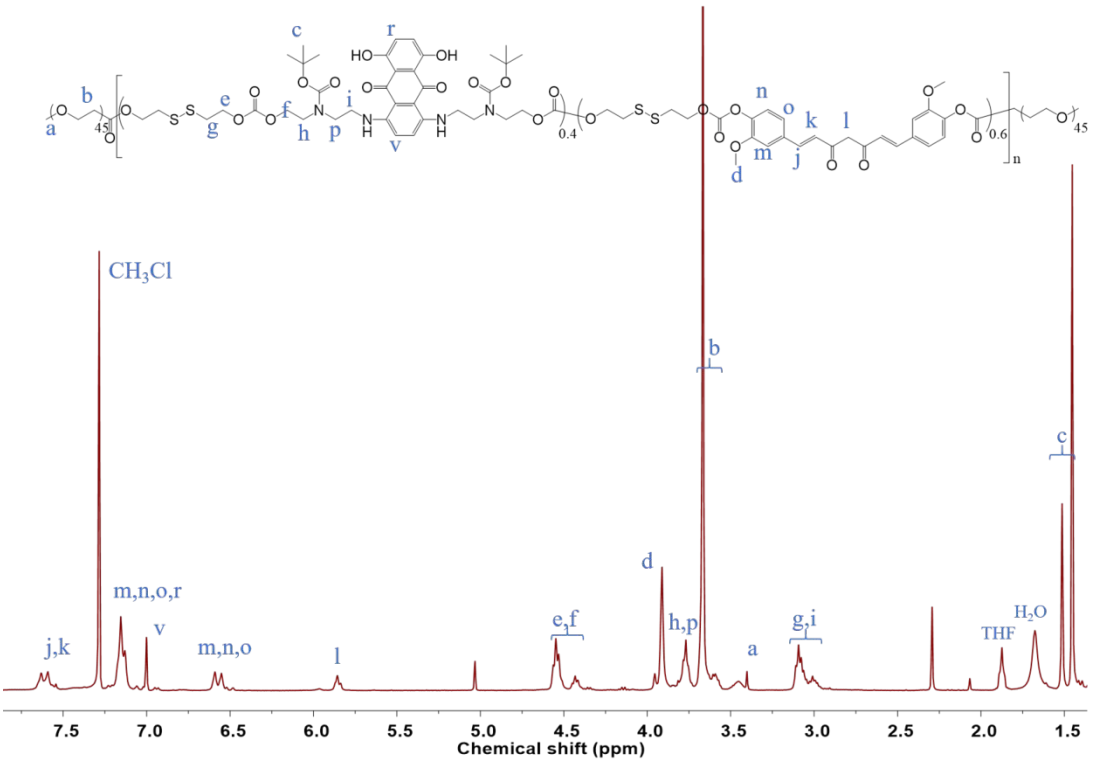


Figure S2. ^{1}H NMR spectrum of the polymer PEG-P(BocMTO-ss-CUR).

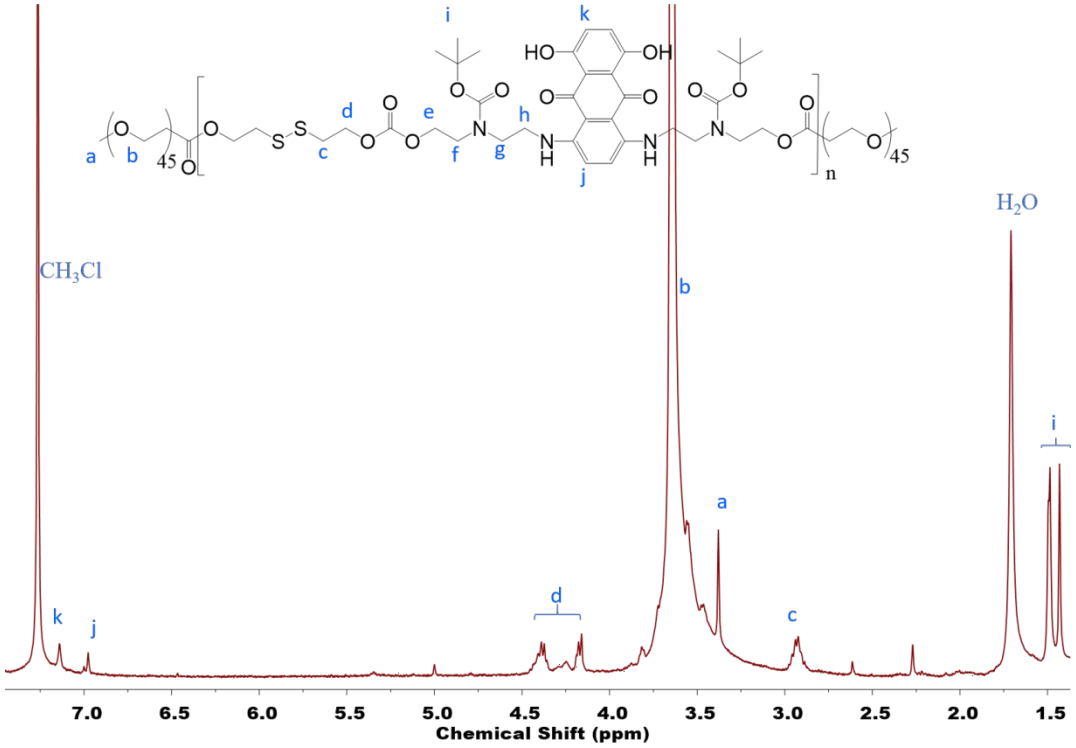


Figure S3. ^{1}H NMR spectrum of the polymer PEG-P(BocMTO-ss).

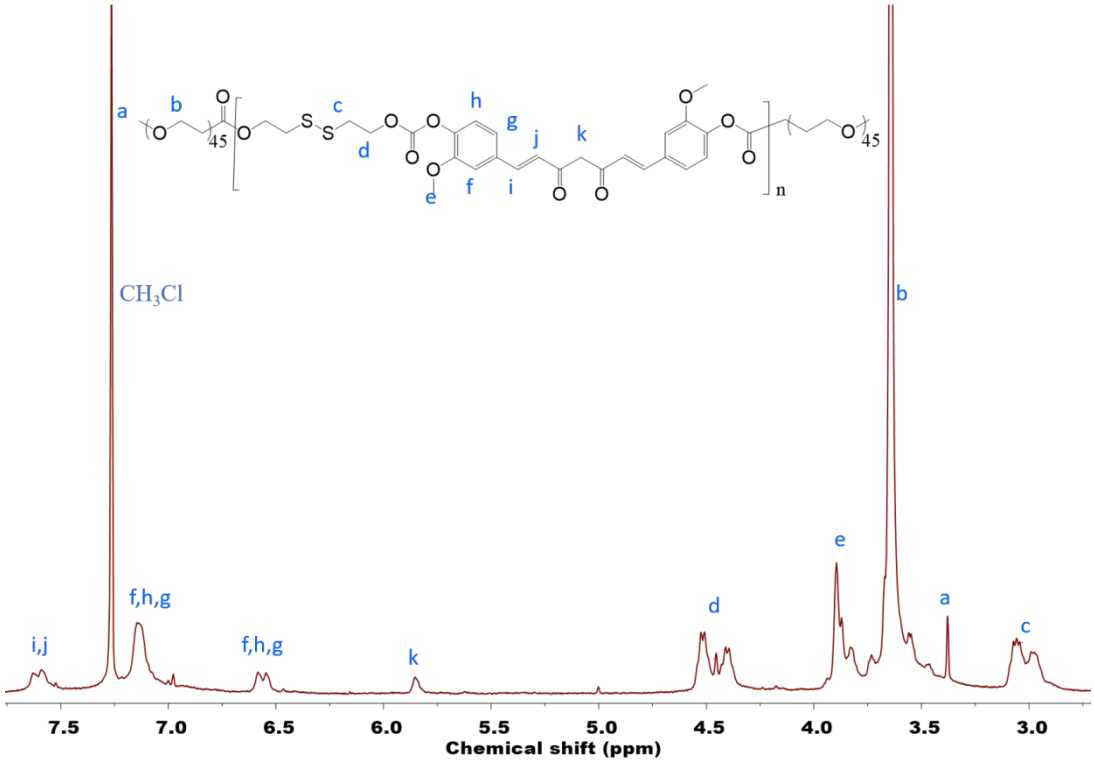


Figure S4. ^{1}H NMR spectrum of the polymer PEG-P(CUR-ss).

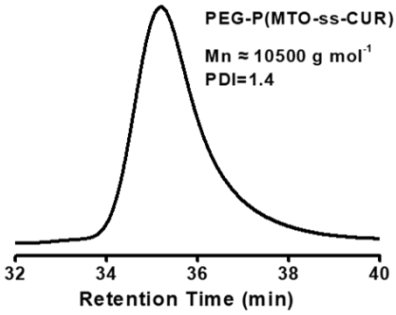


Figure S5. GPC profiles of PEG-P(MTO-ss-CUR)

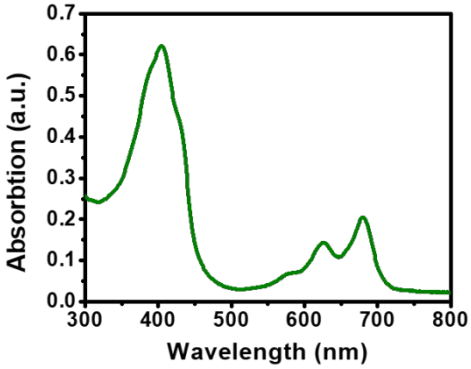


Figure S6. UV/vis absorption spectra of PEG-P(MTO-ss-CUR) in DMSO.

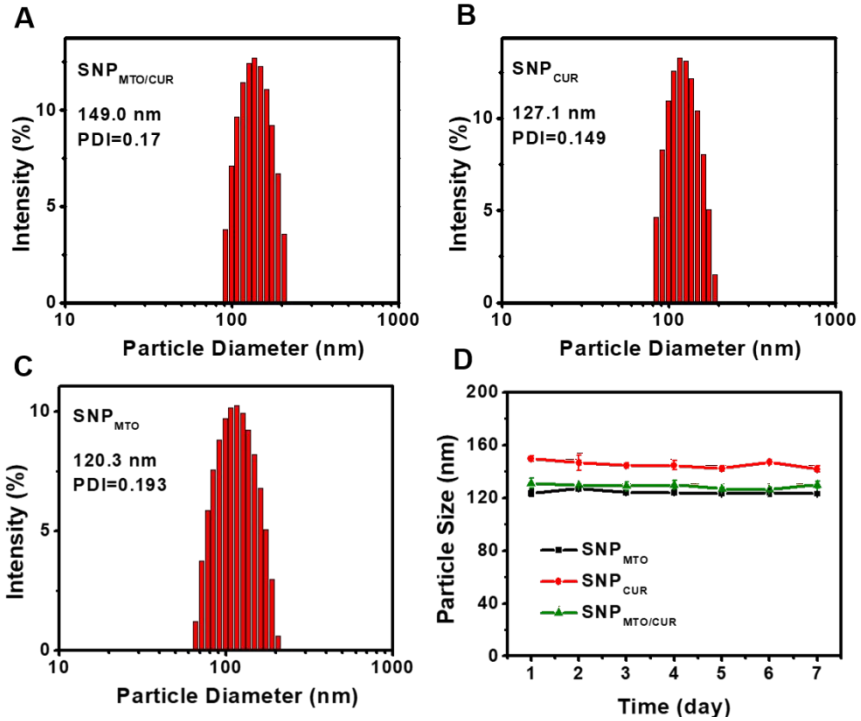


Figure S7. Particle size distribution of (A) $SNP_{MTO/CUR}$, (B) SNP_{CUR}, and (C) SNP_{MTO}. (D) Stability of nanoparticles studied by DLS.

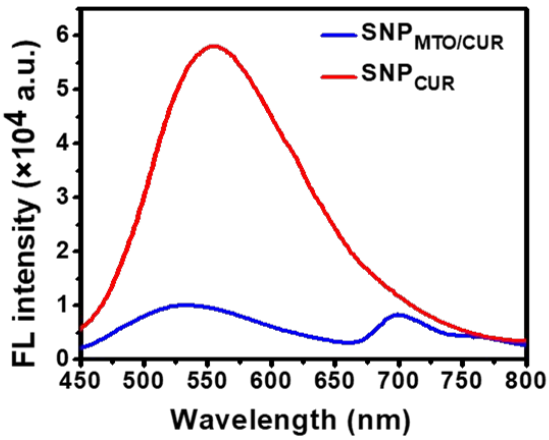


Figure S8. Fluorescence spectra of SNP_{CUR} and $SNP_{MTO/CUR}$ with excitation wavelength at 420 nm.

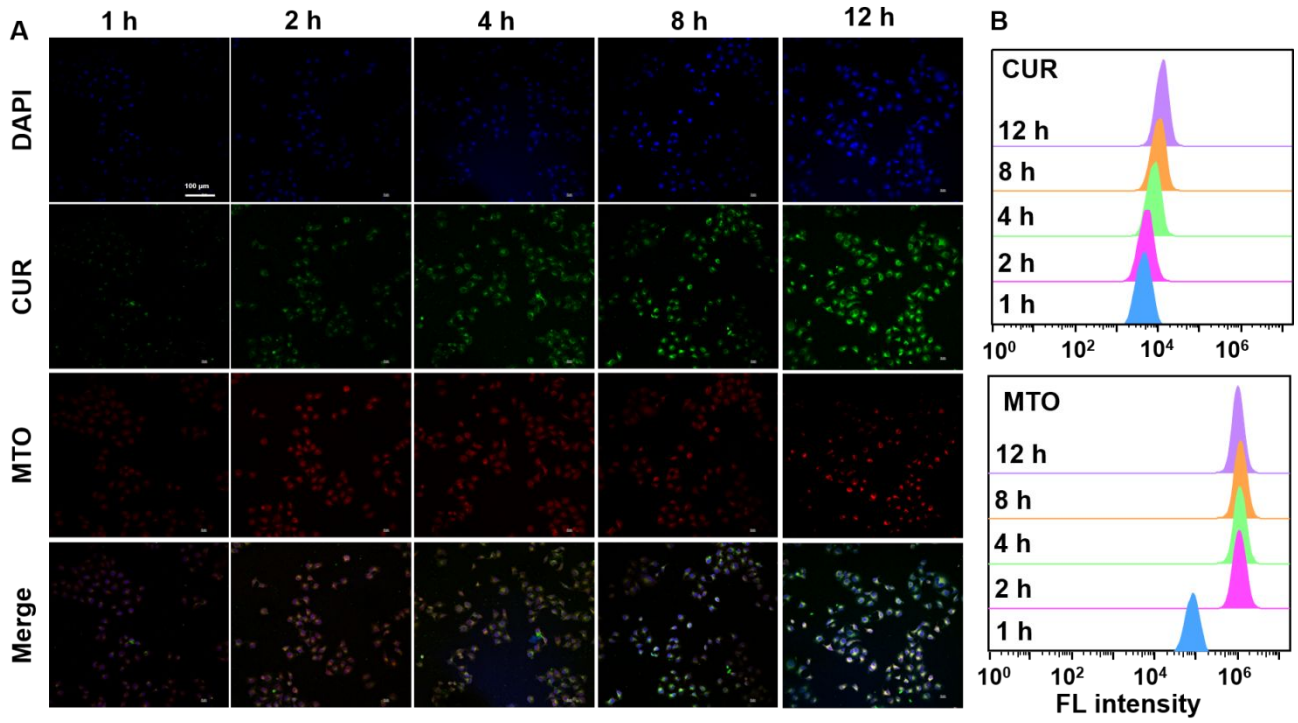


Figure S9. (A) Confocal images of MCF-7/ADR cells incubated with $SNP_{MTO/CUR}$ for different time. The blue fluorescence is from DAPI, the green fluorescence is from CUR, the red fluorescence is from MTO. (B) Cellular uptake of $SNP_{MTO/CUR}$ in MCF-7/ADR cells studied by flow cytometry.

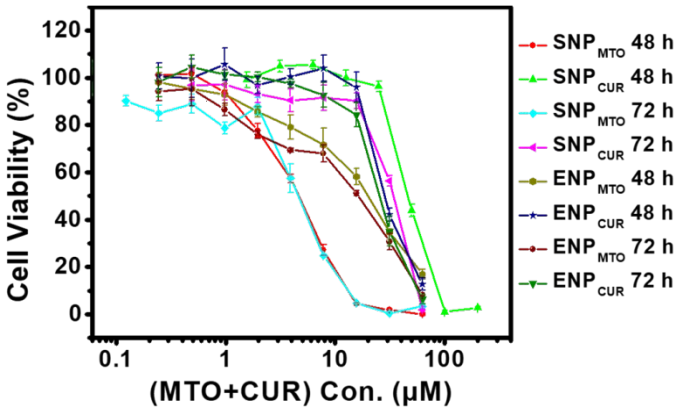


Figure S10. *In vitro* dose-effect profiles of nanoparticles SNP_{MTO}, SNP_{CUR}, ENP_{MTO}, ENP_{CUR} to MCF-7/ADR cells with incubate time of 48 h or 72 h.

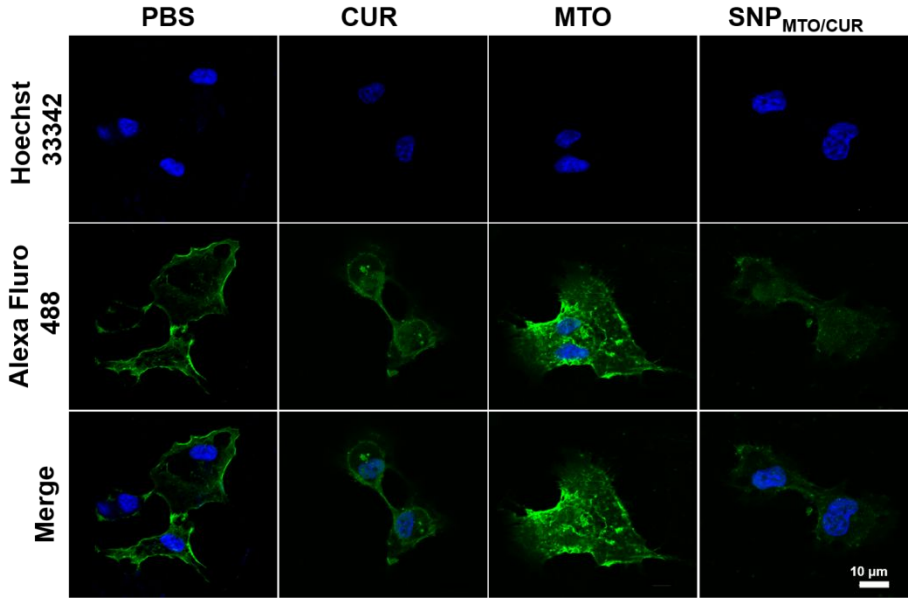


Figure S11. P-gp expression detected by immunofluorescence after treating MCF-7/ADR cells with SNP_{CUR}, SNP_{MTO} or $SNP_{MTO/CUR}$. The blue fluorescence is from Hoechst 33342, the green fluorescence is from Alexa Fluro® 488.

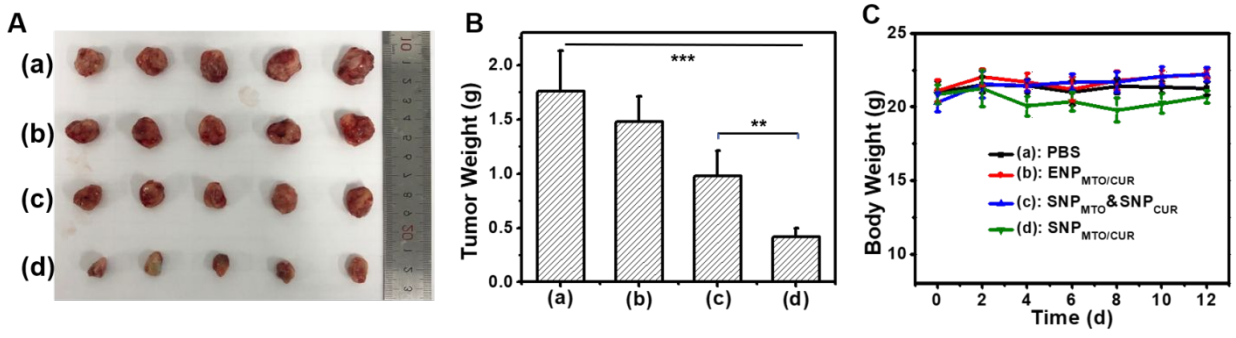


Figure S12. (A) Photos of excised tumors after different treatments. (B) Quantitative analysis of tumor weight after different treatments. (C) Body weight changes of MCF-7/ADR tumor-bearing mice with different treatments. group: (a) PBS, (b) $ENP_{MTO/CUR}$, (c) SNP_{MTO}&SNP_{CUR} and (d) $SNP_{MTO/CUR}$. (Statistical significance was set at *P < 0.05, **P < 0.01 and ***P < 0.001)

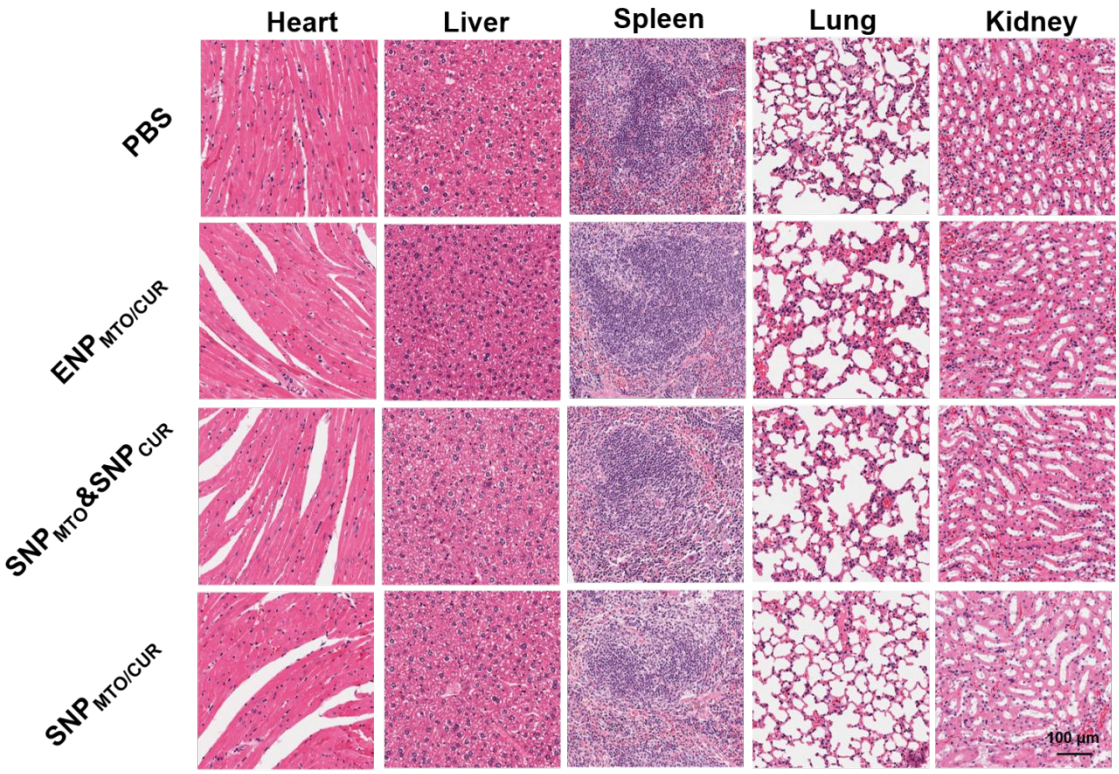


Figure S13. H&E analysis of main organs after different treatments.

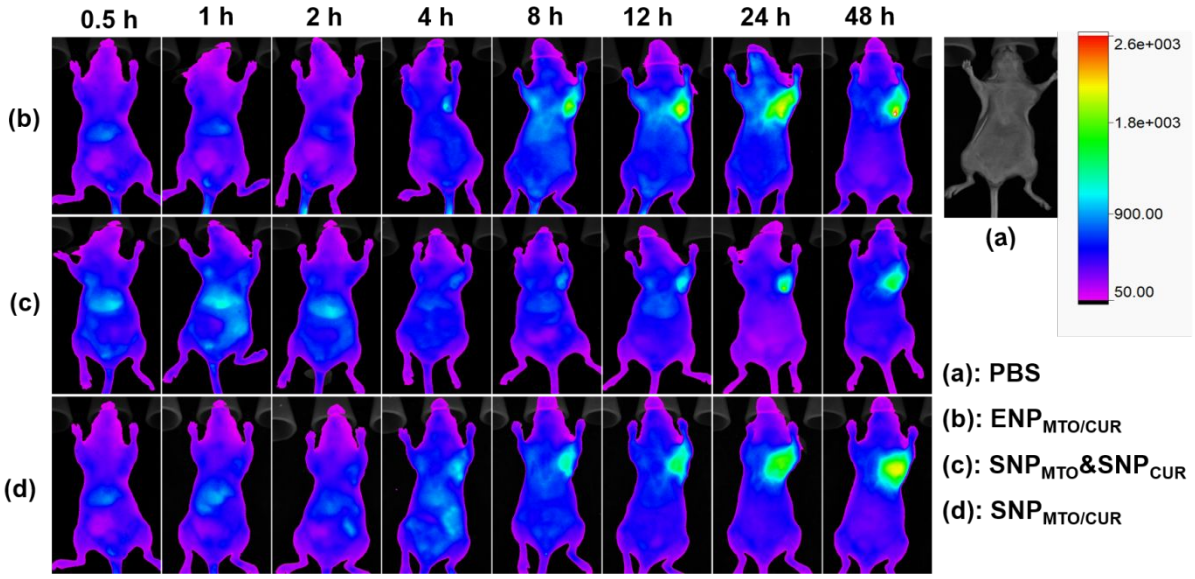


Figure S14. *In vivo* fluorescence images of MCF-7/ADR tumor-bearing nude mouse after intravenous injection of (a) PBS, (b) IR780 loaded $ENP_{MTO/CUR}$, (c) IR780 loaded SNP_{MTO}&SNP_{CUR} and (d) IR780 loaded $SNP_{MTO/CUR}$ after 0.5, 1, 2, 4, 8, 12, 24 and 48 h.