Supporting Information

Synthetic Dimeric-drug Phospholipid: A Versatile Liposomal Platform for Cancer Therapy

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Materials and Methods

1.1 Materials

Unless otherwise noted, chemical regents and solvents of analytical-grade quality were acquired from the domestic suppliers and used without any further purification. Podophyllotoxin (PODO, 98%) and L-*α*-Glycerophosphocholine (GPC, 99%) were obtained from DB Technology Co., Ltd. (Shanghai, China). N'-carbonyldiimidazole (CDI, 99%), 4-(Dimethylamino)pyridine (DMAP, 98%) and adipic anhydride (AA, 98%) were provided from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 98%) and Triethylamine (TEA) were purchased from J&K Scientific Co., Ltd. (Shanghai, China). Biochemical reagents including Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), methyl tetrazolium (MTT), lipophilic Cy5.5, Lysotracker LysoGreen and 4',6-diamidino-2-phenylindole (DAPI) were purchased from KeyGEN BioTECH Co., Ltd. (Nanjing, China).

1.2 Measurements

Monitoring reactions was performed on thin layer chromatography (TLC) and visualized with UV light at 254 nm. The purity of di-PODO-GPC prodrug was analyzed by high-performance liquid chromatography (HPLC, Agilent 1100, CA) which is equipped with an analytical column (ZORBAX SB-C18, 4.6×150 mm). High-resolution electrospray ionization mass spectrometry (ESI-MS) was used to record the molecular mass of di-PODO-GPC prodrug with a LTQ-Orbitrap mass spectrometer (100-3200 m/z, Thermo Electron, Germany). ^{1}H-NMR and ^{13}C-NMR spectra were operated on a DPX 600 MHz spectrometer (Bruker, Germany) while TMS was used as internal standard in solvent of $CDCl_3$ or DMSO-d_6.

1.3 Synthesis of di-PODO-GPC Prodrug

As exhibited in Figure 1, di-PODO-GPC prodrug was synthesized through a two-

step coupling reaction and the experimental process and structure characterization were detailed in the following manner.

Synthesis of PODO-AA compound: adipic anhydride (0.49 g/3.88mmol) was mixed with a solution of PODO (0.4 g/ 0.97 mmol) and DMAP (0.06 g/ 0.48 mmol) in anhydrous CH_2Cl_2 with 2 mL of distilled TEA, stirred and allowed to react for 4 h at room temperature. After diluted with CH_2Cl_2 (30 mL) and washed with HCl (0.1 M, 20 mL×3), the synthesized product was purified by silica gel column chromatography eluting with CH_2Cl_2/CH_3OH (15:1, v/v) to afford PODO-AA (0.48 g, purity 95.3%) as a white solid. ^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.76 (d, *J* = 5.1 Hz, 1H, H-8), 6.57 (s, 1H, H-5), 6.41 (d, *J* = 5.1 Hz, 2H, H-2',6'), 6.00 (t, *J* = 9.7 Hz, 2H, H-13), 5.92 (d, *J* = 9.2 Hz, 1H, H-4), 4.63 (d, *J* = 4.3 Hz, 1H, H-1), 4.46-4.11 (m, 2H, H-11), 3.83 (m, 9H, -OCH_3), 2.95 (m, 1H, H-3), 2.48 (m, 1H, H-2), 2.27 (m, 4H, H-14,17), 1.62 (m, 4H, H-15,16). ESI-MS m/z: calculated for $C_{28}H_{30}O_{11}$ $[M-H]^-$, 541.18; found 541.18 $[M-H]^-$, 1083.37 $[2M-H]^-$.

Synthesis of di-PODO-GPC prodrug: to a solution of PODO-AA (0.5 g/0.92 mmol) in 15 mL of dried CH_2Cl_2, the activating reagent of CDI (0.22 g/1.38 mmol) was added and proceeded to stir for 2 h at room temperature. After that, DBU (0.21 g/1.38 mmol) and GPC (0.11g /0.42 mmol) suspended in DMSO were further added and reacted overnight at 45 °C. The resulting products were precipitated in diethyl ether (40 mL × 3) acidified with 2 mL of glacial CH_3COOH and then, purified by silica gel column chromatography using CH_2Cl_2/CH_3OH (solvent A: CH_2Cl_2:CH_3OH, 15:1; solvent B: CH_2Cl_2:CH_3OH:H_2O 65:25:4) as the gradient elution. Finally, di-PODO-GPC prodrug were acquired by evaporating to yield the white solid of 0.47 g (purity 97.8%). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 6.84 (s, 2H, H-8,8'), 6.58 (s, 4H, H-2'',2''',6'',6'''), 6.54 (s, 2H, H-5,5'), 6.00 (s, 4H, H-13, 13'), 5.68 (d, *J* = 5.4 Hz, 2H, H-4,4'), 5.06 (m, 1H, H-19), 4.45 (m, 4H, H-11,11'), 4.27 (dd, *J* = 16.3, 6.3 Hz, 2H, H-21), 4.09 (m, 2H, H-20), 3.69 (m, 18H, -OCH_3), 3.13 (m, -$N(CH_3)_3$), 2.33 – 2.14 (m, 4H, H-2,2',14,14'), 1.47 (m, 4H, H-15,15',16,16'). ^{13}C-NMR (600 MHz, DMSO-d_6): δ 176.26, 174.55, 174.36, 173.10, 154.47, 148.43, 138.56, 136.57, 131.20, 128.52, 111.43, 109.28, 107.87, 101.78, 69.60, 69.29, 68.50, 68.20, 66.81, 64.25, 60.59, 56.79, 54.72, 44.06, 43.76, 40.32, 34.09, 23.23. ESI-MS m/z: calculated for $C_{64}H_{76}NO_{26}P$ $[M+H]^+$, 1306.44; found 1306.45 $[M+H]^+$, 1328.43 $[M+Na]^+$.

1.4 Liposomes Preparation

The developed di-PODO-GPC liposomes were obtained by conventional lipid film hydration method. In Brief, di-PODO-GPC prodrug (5 mg) was dissolved in a mixture of $CHCl_3$/CH_3OH (3:1, v/v) solution and evaporated under vacuum. The formed thin film was hydrated with 10 mL of PBS (pH 7.4) buffer at 50 °C for 1 h. Afterwards, the liposomal suspension was subjected to sonication by a DA301 supersonic probe (Shunmatech, Shanghai) and further homogenized three times

through mini-Lipid Extruder™ (Vancouver, BC, Canada) which is furnished with 220 nm aseptic membrane. The final concentration of di-PODO-GPC prodrug was determined by HPLC, prior to characterizations. The fixed drug loading content (DLC) was calculated according to the molecular weight as follows:

$$DLC\% = 2 \times M_{PODO}/M_{di-PODO-GPC} \times 100$$

Whereas, M_{PODO} and $M_{di\text{-}PODO\text{-}GPC}$ indicate the molecular mass of PODO and di-PODO-GPC prodrug, respectively.

For *in vitro* cellular internalization study, di-PODO-GPC liposomes labelled by Cy5.5 at a molar ratio of 30:1 (prodrug : Cy5.5) were similarly prepared through re-hydration, sonication and homogenization as illustrated above.

1.5 Physicochemical Characterizations

The morphology of di-PODO-GPC liposomes were observed by FEI Tecnai G2 F20 transmission electron microscopy (TEM). Briefly, 5 μL of liposomes was dropped onto a 300-mesh copper grids following by air-dried, stained with phosphomolybdic acid (2%, w/v) of and then, recorded at 200 kV voltage in vacuum. Cryogenic electron microscopy (Cryo-EM) was further used to detect the typical nanostructure of phospholipid bilayer. In experimental procedure, liposomal solution (10 μL) was applied onto the charged carbon grids and plunge-frozed in ethane at -170 °C using FEI Vitrobot. After set the accelerated voltage as 200 kV and temperature -180 °C, electronic images were collected with Gatan Ultrascan camera (4k × 4 k, FEI Company) under bright-field mode. Particle size, zeta-potential and size distribution of liposomes were analyzed by dynamic light scattering (DLS) and operated at back-scattering detection of 173° via Zetasizer NanoZS90 (Malvern, UK). Moreover, time-dependant changes in average sizes of di-PODO-GPC liposomes were further monitored at 37 °C by DLS, as determinded their serum-stabilities *in vitro*.

1.6 *In vitro* Drug Release

Release of parent PODO was performed in simulated pHs (7.4 or 5.0) by dialysis (MWCO 3500) method for 36 h at 37 °C. Typically, 4 mL of di-PODO-GPC liposomes (0.5 mg/mL) was dialyzed against release media (50 mL, pH 7.4 containing or not 10% FBS and pH 5.0) with 0.1% Tween-80 in a bath shaker. After taken the release medium (5 mL) at the prearranged intervals, the released PODO was determined by HPLC eluting with isocratic acetonitrile/H_2O (35:65, v/v, 0.1 % TFA) in 12 min. Flowrate: 1.0 mL/min. Temp.: 25 °C. Detection wavelength: 254 nm.

1.7 Hemolysis Analysis

Whole blood as a gift from affiliated hospital of Yantai University was stored in heparinized tube and × 1000 g centrifuged for 20 min at 4 °C. The obtained hemocyte was diluted to 2% erythrocyte suspension prior to next experiments. High, medium

and low concentrations of di-PODO-GPC liposomes (500 μg/mL, 100 μg/mL and 10 μg/mL) were hatched with 2% erythrocyte suspension at 37 °C for 4 h in an incubator. Subsequently, samples were centrifuged for 10 min at 1000 g and the absorbance at 540 nm was measured using UV/Vis spectrometer (Shimad, Japan). Deionized water and normal saline (0.9%) were chosen as positive control with 100% hemolysis and negative control with 0% hemolysis, respectively. A% hemolysis ratio (< 5%) was considered as nontoxic. Percent hemolysis rate was calculated using the standard formula as:

$$Hemolysis\ Rate\ (\%) = \frac{Ae - An}{Ap - An} \times 100\%$$

Ae means the absorbance of experimental groups, wherein, Ap and An are the absorbance of the respective positive and negative control groups.

1.8 Cell Culture

In the experiment, tested cells (MCF-7, HeLa and HepG-2 cells) were provided from Chinese Academy of Sciences Shanghai Institute of Cell Bank (Shanghai, China) and cultured in DMEM supplemented with 10% FBS, 100 unit/mL of penicillin and 100 μg/mL of streptomycin. Cells were incubated in humidified atmosphere with 5% CO_2 at 37 °C and after reached to approximately 80% confluency, the cells were trypsinized and passaged before tests.

1.9 Cellular Internalization Assays

The cellular internalization behavior of di-PODO-GPC versus MCF-7 cells was investigated by confocal laser scanning microscopy (CLSM, Leica, Germany) while the fluorescence dye Cy5.5 was selected as a fluorescent probe. 1.0×10^5 per well of MCF-7 cells was seeded into 3.5 cm-confocal dishes for 12 h incubation in 2 mL of DMEM medium. After removed the culture medium, equivalent 5 μM of free Cy5.5 and Cy5.5-loaded liposomes were added for further 2 h incubation. the cells were then washed thrice with PBS (pH 7.4), fixed with 4 (w/v)% paraformaldehyde and staining with DAPI (100 μM in PBS). Finally, the fluorescence micrographs were observed by CLSM.

Further, *in vitro* cellular association and uptake of di-PODO-GPC liposomes was further quantitatively in MCF-7 cells by the method of HPLC analysis. Briefly, 5×10^5 MCF-7 cells/well were seeded into a standard 6-well culture plates and cultured until confluency in DMEM media. Confluent cell monolayers were treated with free PODO and di-PODO-GPC liposomes at the equivalent dosage of 100 μg/mL for various periods (30 to 60 min). Thereafter, MCF-7 cells were lysed and collected for analysis of total protein content using BCA method as well as the amount of equivalent PODO in cells by HPLC (Agilent 1100, CA) method. The percentage of cellular uptake was calculated as a ratio of per PODO mg total cellular protein.

1.10 MTT Assays

Cytotoxicity of di-PODO-GPC liposomes *in vitro* was measured by MTT assays in HeLa, MCF-7 and HepG-2 cells and parent PODO was used as the positive control. In brief, 1×10^4 cells/well of HeLa cells (MCF-7 or HepG-2 cells) was seeded in 96-well plate. After 12 h incubation, cells were treated with serial concentrations of parent PODO and di-PODO-GPC liposomes ranged from 5 μM to 25 μM in medium and further cultured for 24 h. Then, MTT stock solution (20 μL , 5 mg/mL in PBS) was added into each well, followed replacement by 150 μL DMSO after 4 h incubation at °C in the darkness. The absorbance of each sample was checked at the wavelengh of 570 nm by a Microplater Reader. The percentage of cell viability was presented as comparison with the cells incubated in DMEM medium while tested in sixtuplicates and performed in quartets.

To further check whether di-PODO-GPC liposomes exhibited their cytotoxic functions in MCF-7 cells through pH-mediated PODO release, chloroquine as the inhibitor of intralysosomal degradation was introduced to test the effect of lower pH (4.5~5.5) on cell growth. In the following experiment, MCF-7 cells were pretreated with chloroquine (10 mM) for 60 min at 37 °C and after that, di-PODO-GPC liposomes at the equivalent dosage of 25 μM were added for further 5 h and 10 h, respectively. The liposomes without pretreatment of chloroquine (0 mM) was used as a control. Similarly, cell viability of the designed di-PODO-GPC liposomes was analyzed by MTT assay as described above.

1.11 Animals and Tumor Model

Study protocols on animal care and handing procedures were approved by the ethics committee of Yantai University. BALB/c nude mice (female, 6-week-old, 16-18 g) were provided from Shandong Luye Pharmaceutical Co., Ltd. (Yantai, China). Mice were maintained in a SPF environment under a 12-h light/dark cycle. For mice tumor model, 1×10^7 MCF-7 cells (100 μL) was subcutaneously injected in the right flank region and according to the [(tumor length) × (tumor width)^2 /2] formula, the tumor volume was allowed to grow 100-150 mm^3 before test.

1.12 *In vivo* Biodistribution

MCF-7 tumor-bearing BALB/c nude mice (100-150 mm^3) were intravenously injected via tail vein with parent PODO and di-PODO-GPC liposomes at an equivalent dose of 10 mg PODO/kg. The tissues including heart, liver, spleen, lung, kidney and tumor were collected after drug administration (n = 4 at each time point) for the predetermined time-points. Samples were washed by saline (× 3), weighted and homogenized, following the extraction in 2 mL of methanol. Completed post-processing of tissue samples, the amount of liposomes and parent PODO was measured by HPLC method, which was obtained from standard curves previously acquired by analysis of tissues samples containing known amounts of parent PODO and di-PODO-GPC liposomes.

1.13 *In vivo* Efficacy

MCF-7 tumor-bearing nude mice were randomly divided into three groups (n = 4) and treated intravenously with PBS solution, parent PODO and di-PODO-GPC liposomes (10 mg PODO/kg) via the tail vein every two days. The tumor length and width and body weight of mice were measured during the period of 24 days. In the end, mice were sacrified and tumors in different treatment groups were extirpated, weighted and photographed. Tumors inhibition ratio (IR%) was determined based on the following equation:

$$Inhibition\ ratio\ (IR\%) = \frac{W_{PBS} - W_{tested}}{W_{PBS}} \times 100\%$$

Where, W_{PBS} represents the mean tumor weight of PBS control and W_{tested} is that of drug-administered group.

Tumors were then executed to embed in paraffin for histological analysis. The sliced tumor was sectioned into 5 mm and affixed on the glass slides. After double stained by hematoxylin and eosin (H&E), the sections were observated using a light microscopy.

1.14 Statistical Analysis

Numerical results were presented as mean ± S.D. Comparison between different groups was determined using the one-way Anova tests by Graphpad Prism software and P less than 0.05 was regarded as the statistical significance.

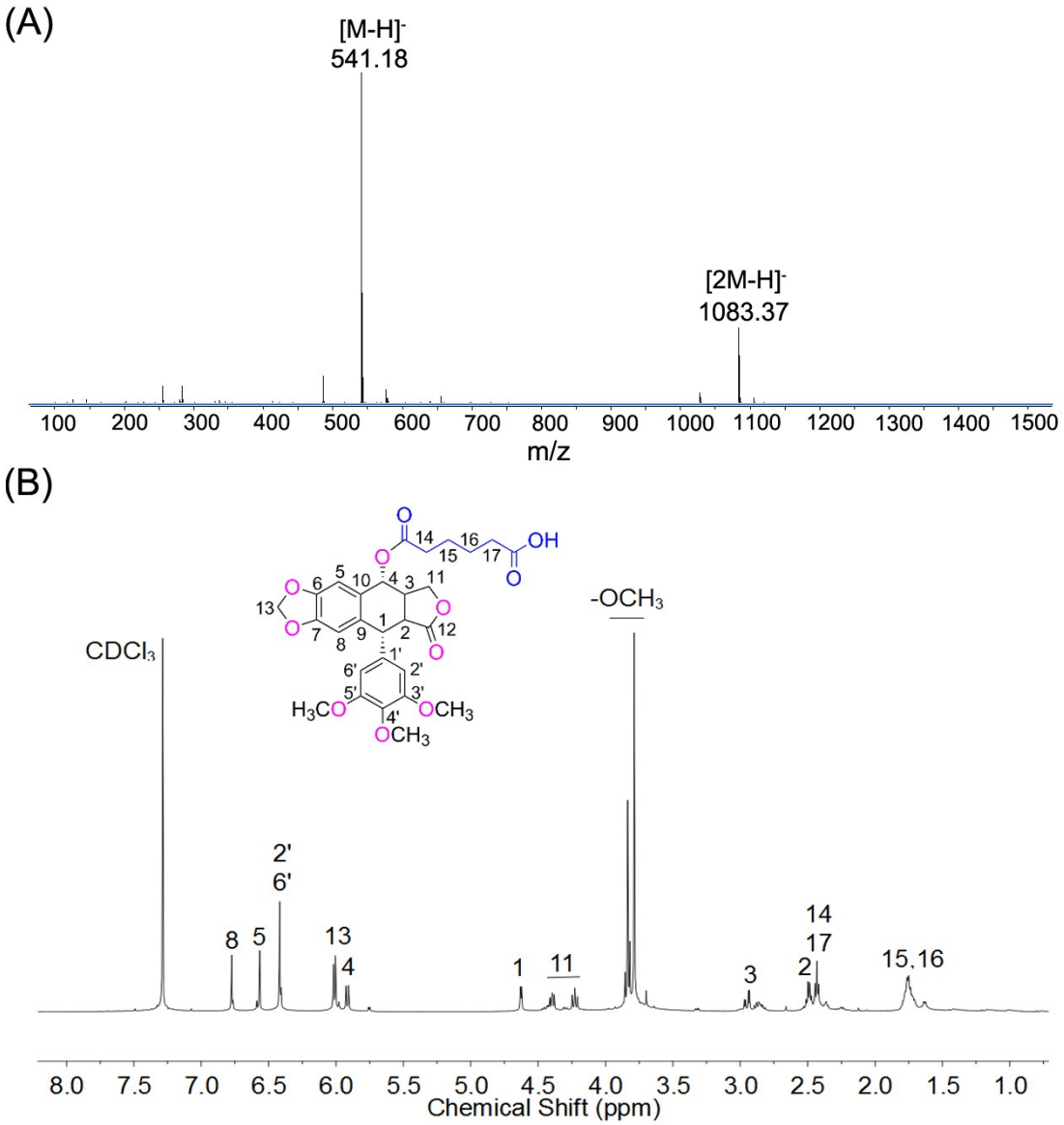

Figure S1. (A) ESI-MS and (B) ^{1}H-NMR (600 MHz, $CDCl_3$) spectra of PODO-AA compound.

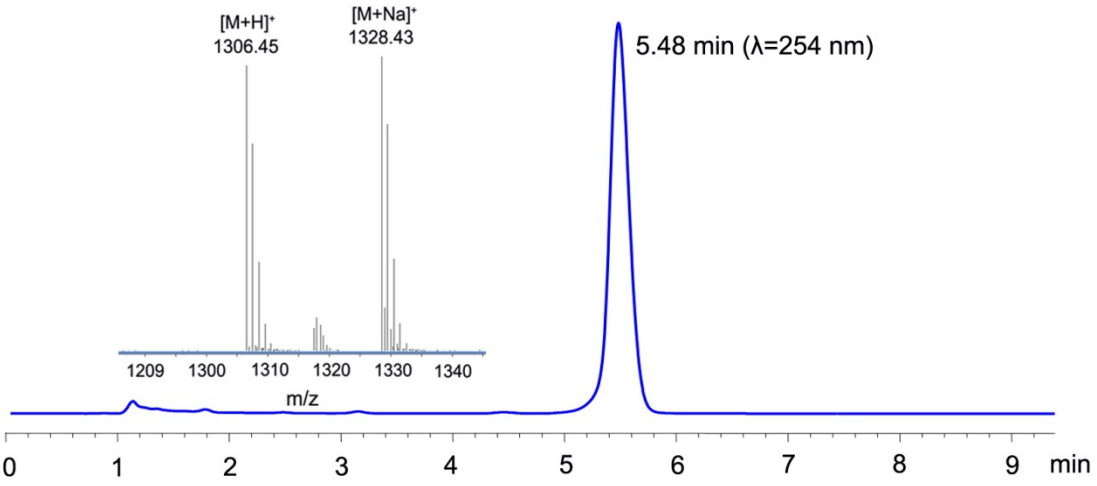

Figure S2. Purity of di-PODO-GPC prodrug analyzed by HPLC that eluted with 42% acetonitrile in water (0.1% TFA) in 12 min (λ = 254 nm). Inset: ESI-MS spectra of di-PODO-GPC prodrug.

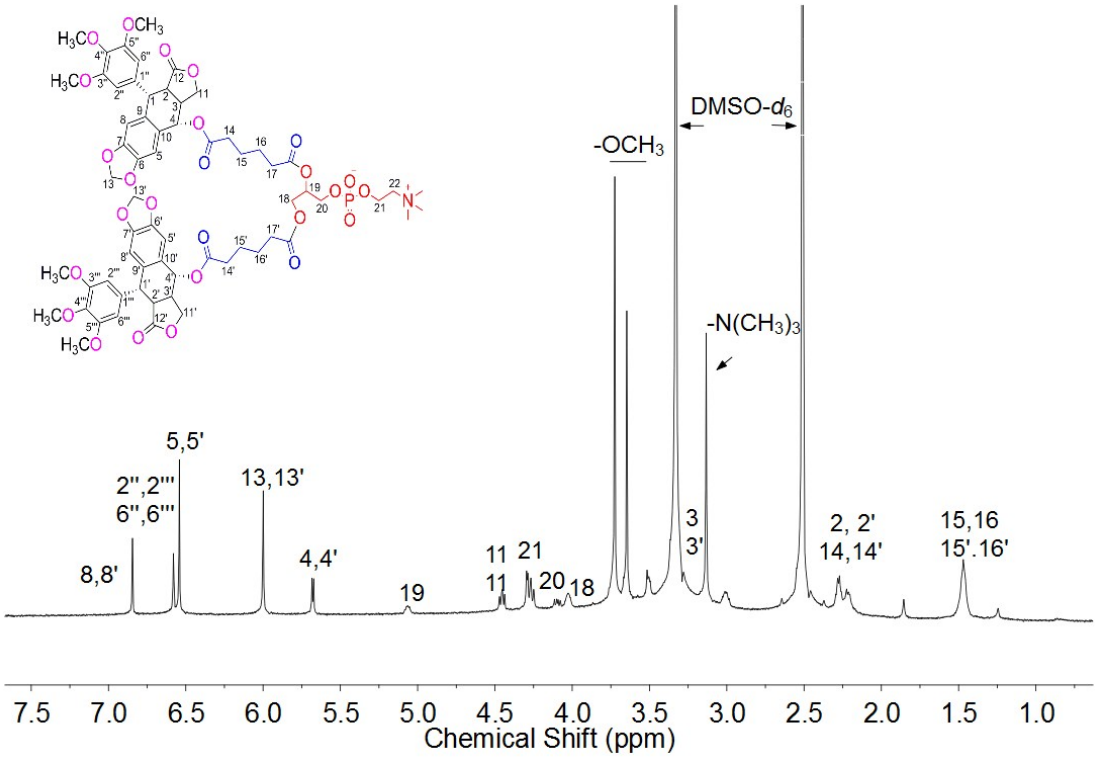

Figure S3. ^{1}H-NMR (600 MHz, DMSO-d_6) spectra of di-PODO-GPC prodrug.

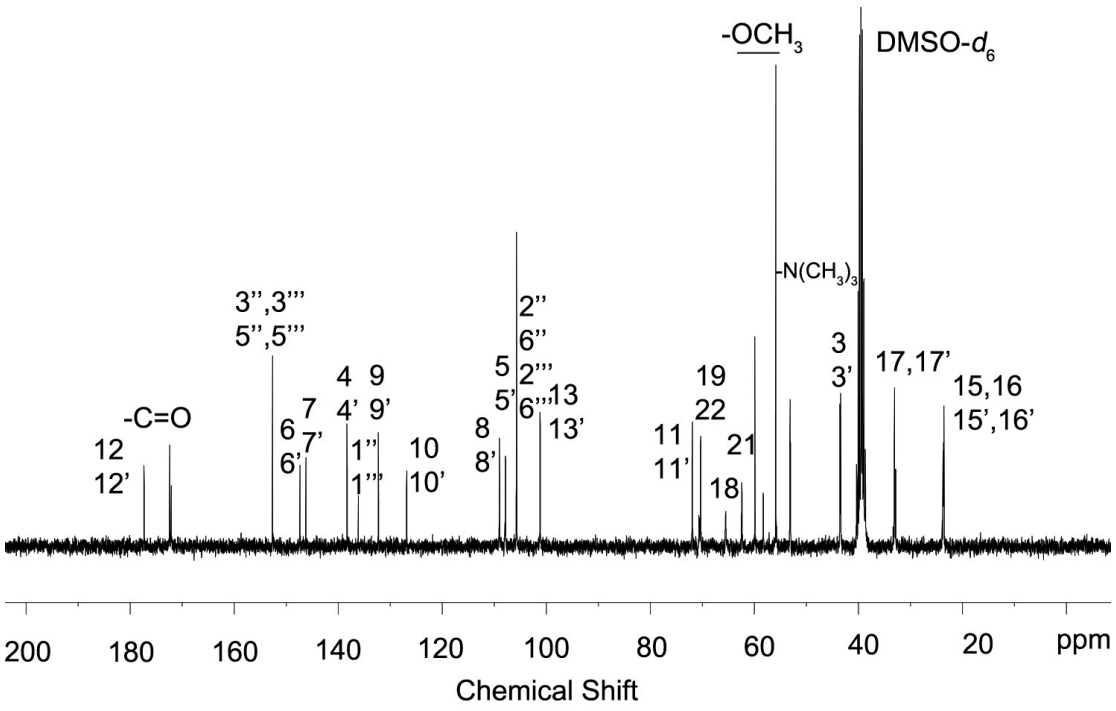

Figure S4. ^{13}C-NMR (600 MHz, DMSO-d_6) spectra of di-PODO-GPC prodrug.

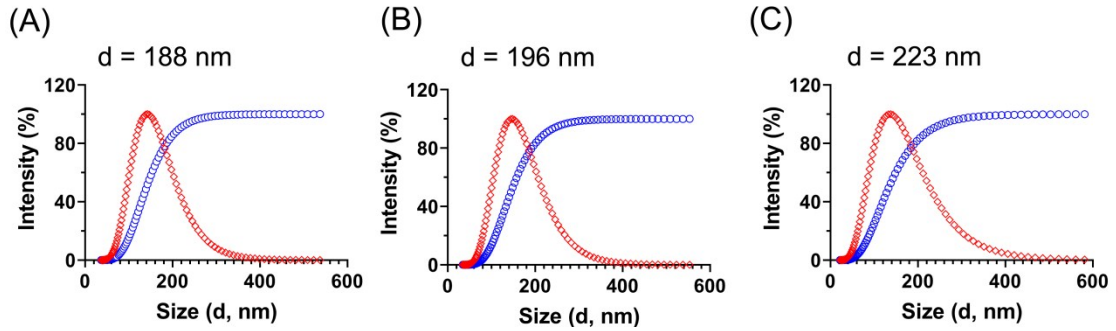

Figure S5. Diameter changes of di-PODO-GPC liposomes in PBS (pH 7.4) with 10% FBS at 37 °C after (A) 2 h, (B) 6 h and (C) 12 h of incubation.

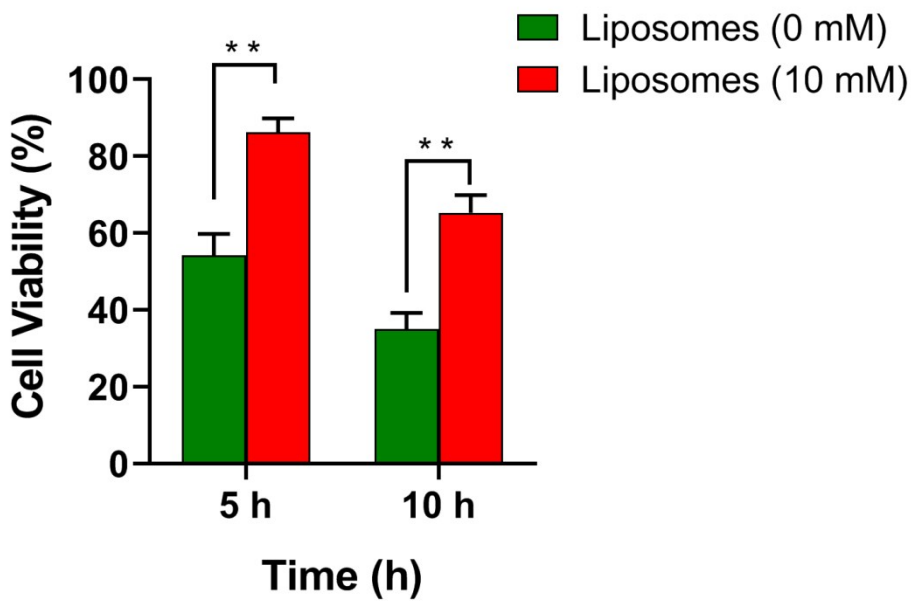

Figure S6. *In vitro* pH-mediated cytotoxicity of MCF-7 cells treated with di-PODO-GPC liposomes at a dosage of 25 μM for 5 h and 10 h incubation. Data are exhibited as average ± SD (n = 4). Where, **P < 0.01.

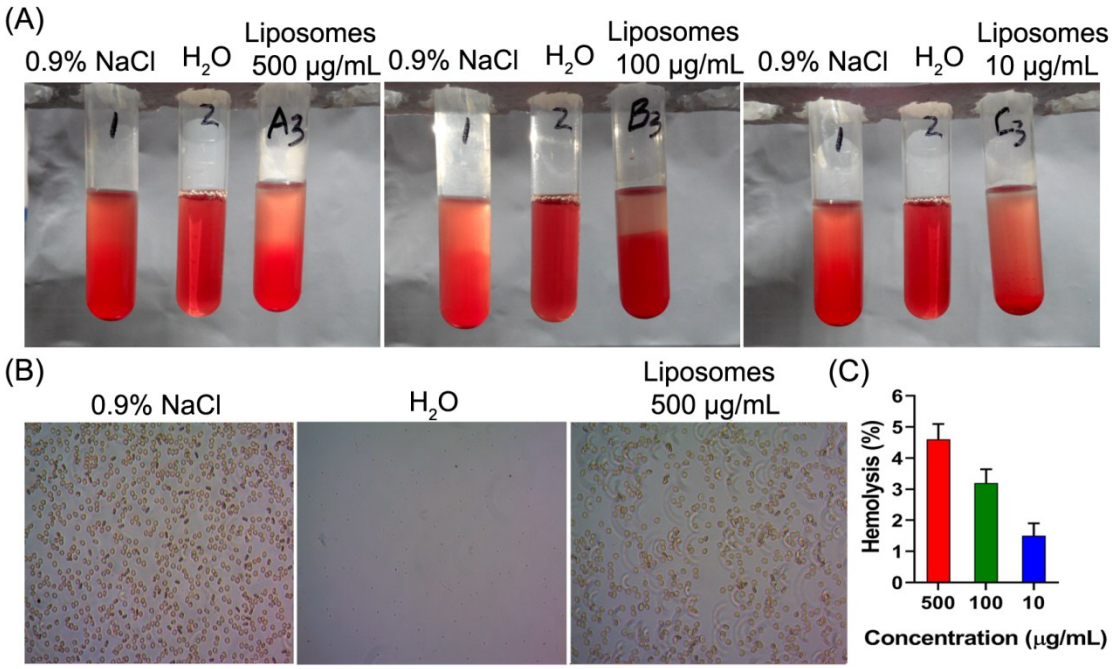

Figure S7. Hemolysis of di-PODO-GPC liposomes: (A) Images of tubes containing red blood cells (RBCs) incubated with different concentrations of liposomes (before centrifugation). 0.9% saline (0% hemolysis) and distilled water (100% hemolysis) was set as negative and positive controls; (B) Micrograph of RBCs exposed to 500 μg/mL di-PODO-GPC liposomes after incubated at 37 °C for 4 h; (C) Hemolysis rate (%) of tested liposomes and the value less than 5% was considered safe for *in vivo* administration.

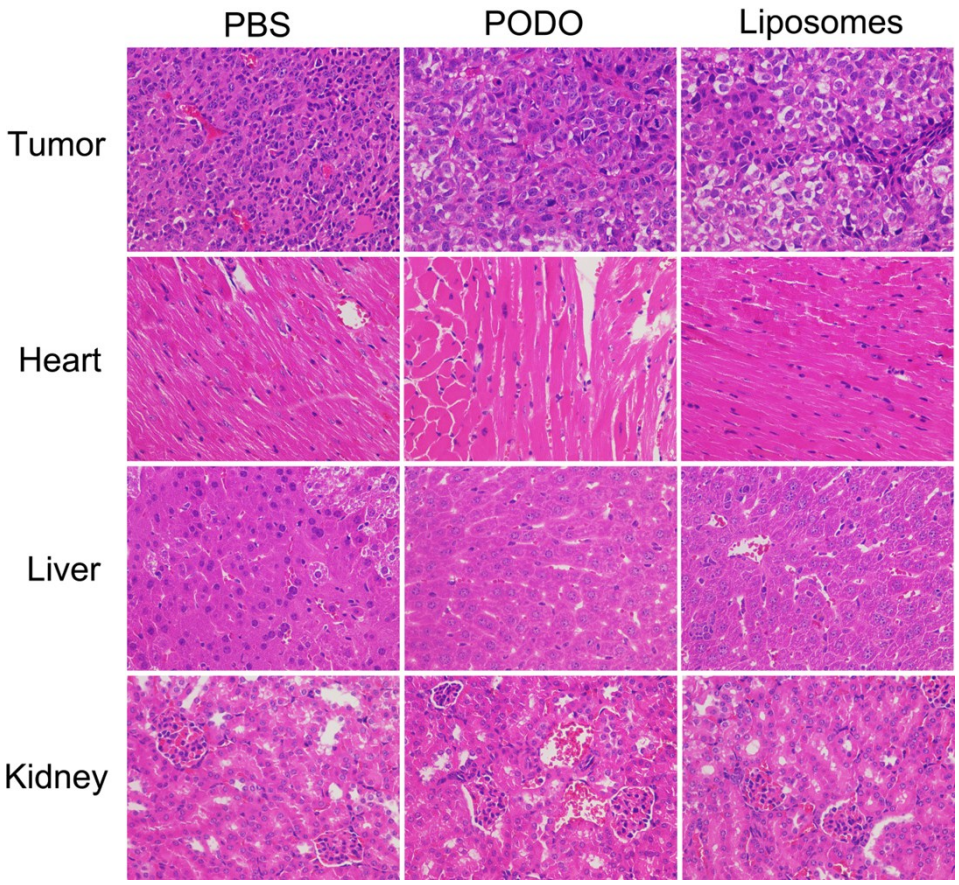

Figure S8. H&E analysis of heart, liver and kidney after intravenous injection of PBS (pH 7.4) solution, parent PODO and liposomes (× 400).

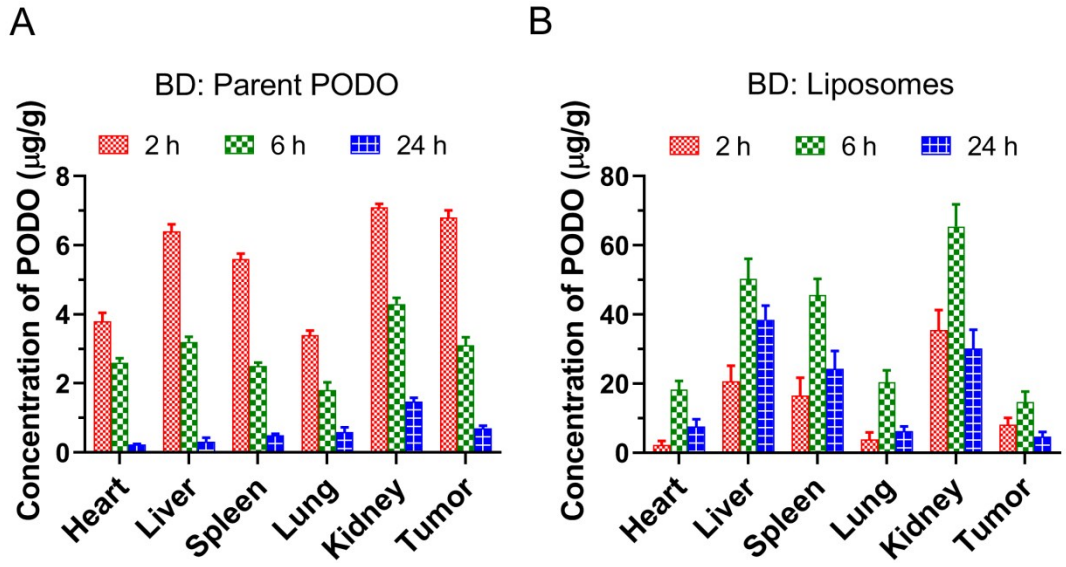

Figure S9. Tissues biodistribution of (A) parent PODO solution and (B) di-PODO-GPC liposomes after i.v. administration at the equivalent dose of 10 mg PODO/kg in MCF-7 tumor-bearing nude mice. Data are presented as mean ± standard error (n = 4).