Supporting Information

Targeting Peptide Functionalized Liposomes towards Aminopeptidase N for Precise Tumor Diagnosis and Therapy

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1. Materials and methods

1.1 Materials

Tentagel Resin was purchased from Rapp Polymere (Germany, loading 0.35 mmol/g). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, Wang resin, and 2-(1*H*-benzotriazole-1-*yl*)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) were purchased from GL Biochem (China). Trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), fluorescein isothiocyanate (FITC), Hoechst 33342, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) and streptavidin coated magnetic beads (1 μm) were from Sigma-Aldrich (USA). N-methyl morpholine (NMM), piperidine and N, N'-dimethylformamide (DMF) were all from Beijing Chemical Plant (China). 1,2-Ethanedithiol (EDT) was from Alfa Aesar (USA). Triisopropylsilane (Tips) was from Acros Organics (USA). Cyanogen bromide (CNBr) was from J&K Chemical (China).

1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol)$_{2000}$]-maleimide (DSPE-PEG$_{2000}$-MAL) was purchased from Nanosoft Biotechnology LLC (USA). Soybean phosphatidylcholine (SPC, AR) and cholesterol (CHOL, AR) were purchased from A.V.T. Pharmaceutical Co., Ltd.. Doxorubicin hydrochloride (DOX) was supplied by Hisun Pharmaceutical Co. Ltd (Zhejiang, China).

SKOV3 cells (hum anovarian carcinoma cell line), HepG2 cells (hepatoma cell line) and 293T cells (Human embryonic kidney) were purchased from Cell Resource Center, Chinese Academy of Medical Sciences (China). Cell culture medium and fetal bovine serum were from WisentInc (Multicell, WisentInc, St. Bruno, Quebec, Canada) Culture dishes and plates were from Corning (Corning, New York, USA). SKOV3 cells were maintained McCoy's 5A Media with 10% fetal bovine serum and 1% penicillin. HepG2 and 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% penicillin. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 ºC.

1.2 Construction and synthesis of the OBOC peptide library towards APN

The details about synthessis process of the OBOC peptide library was shown in Scheme S1. Fmoc strategy SPPS (solid phase peptide synthesis) was employed for synthesis of the OBOC library. Tentagel Resin (loading 0.35 mmol/g) was used as the solid phase support. The peptide library was constructed with the sequence of X_1 X_2 X_3 X_4 X_5 X_6 in which X_1 represents either Phe, Tyr, Ala,or Leu residues. X_2 and X_3 represent Val, Glu, IIe, or Lys residues and X_{4-6} represent Asp, Ary, Gly, His, or Tyr residu. In the library. During the OBOC library synthesis, solid support beads were split equally in each cycle and different amino acids were added, separately. All the synthesis process of peptides were carried out in dehydrous DMF. In the deprotection step, 20% (v/v) piperidine in DMF was used to remove the Fmoc group and the deprotection time was 10 min. During the coupling step, the HBTU (4 mM) and Fmoc-amino acid (4 mM) were dissolved in DMF containing NMM (0.4 mM). The coupling time was 30 min. Qualitative Fmoc deprotection was confirmed by ninhydrin test (ninhydrin, phenol, VC 1:1:1 v/v). It means that amino acid coupling process was carried out in the "split" step while the deprotection process was carried out in "pool" step. All the above experiments were carried out in the solid phase peptide synthesis vessels with sieves in it. With magnetic conjugation assay, OBOC peptide beads could be trapped by APN protein-biotin-streptavidin labelling magnetic beads-assist screening at the magnetic field. An integrated lab-on-chip system performed the whole peptide screening process: positive peptide isolation, single bead trapping, and *in situ* sequencing.

1.3 SPRi detection of the affinity peptide towards APN

SPRi analysis was performed on a Plexera PlexArray® HT system (Plexera LLC, Bothell, WA) using bare gold SPRi chips (Nanocapture® gold chips, with a gold layer of 47.5 nm thickness). APN protein was adsorbed onto the gold chip surface and then incubated in 4 °C overnight in a humid box. The SPRi chip was washed and blocked using 5% (m/v) non-fat milk in PBS overnight before use. The SPRi analysis procedure follows the following cycle of injections: running buffer (PBST, baseline stabilization); sample (four or five concentrations of the protein, binding); running buffer (PBST, washing); and 0.5% (vol/vol) H_3PO_4 in deionized water (regeneration). Peptide (LN) was diluted with PBST to concentrations of 73 nmol/mL, 36 nmol/mL, 18 nmol/mL and 9 nmol/mL. Real-time binding signal were recorded and analyzed by PlexArray HT system.

1.4 Synthesis of LN peptides

Positive peptide (LN) was de novo synthesized. peptides were synthesized by solid-phase methods using a standard Fmoc-Chemistry. Wang Resin (Rapp Polymere, Germany, loading 0.35 mmol/g) was used as the solid phase support. All the synthesis process of peptides was carried out in dehydrous DMF. In the deprotection step, 20% (v/v) piperidine in DMF was used to remove the Fmoc group and the deprotection time was 10 min. During the coupling step, the HBTU (4 mM) and Fmoc-amino acid (4 mM) were dissolved in DMF containing NMM (0.4 mM). The coupling time was 30 min. Qualitative Fmoc deprotection was confirmed by a ninhydrin test (ninhydrin, phenol, VC 1:1:1 v/v). After elongation, cleavage reagents (92.5% TFA: 2.5% water: 2.5% EDT :2.5% Tips, v/v) were introduced into the vessel to cleave the side chain protecting group of each residue for 30 min in ice bath and then at room temperature for another 3 h. After peptides separated from the Wang resin, the mixture above were evaporated by vacuum rotary to remove the TFA. The crude peptides were then precipitated in cold anhydrous diethyl ether, collected by centrifuge and dried under vacuum. The peptides were purified by preparative reversed-phase high performance liquid chromatography (HPLC) with a preparative reversed-phase Inertsil C18 HPLC column (ODS-3, 5 μm, 20 × 250 mm). A linear gradient of acetonitrile/water with 0.1% TFA respectively from 5/95 (v/v) to 70/30 (v/v) during18 min，then 70/30 (v/v) to 90/10 (v/v) in 4min and in this flow continue 1 min ,next return to 5%/95% (v/v) tilled for 3 min was used as the mobile phase. The separation was performed with a flow rate of 1 mL/min and the monitoring wavelength was 220 nm using a UV detector (Waters 2535Q). The purified peptides were determined by MALDI-TOF-MS using Microflex LRF system (Bruker Daltonics, Germany). MALDI-TOF-MS analysis was performed on a Bruker ULTRAFLEXTREME mass spectrometer equipped with a nitrogen laser (wavelength 337 nm, laser pulse uration 3 ns) with laser pulse energy between 0 and 100 μJ per pulse.

1.5 LN as probes for cancer cells imaging *in vitro*.

For SKOV3, HepG2 and 293T cells, approximately 1×10^5 mL^{-1} cells were seeded into culture dishes and cultured overnight. FITC-labeled peptide (LN-FITC) was dissolved in cold PBS at a concentration of 5.0×10^{-5} M. The cells were incubated with FITC-labeled peptide solution (200 μL, with Hoechst 33342 (10 μg/mL,200 μL) in the dark for 30 min at 4 °C. Finally, the cells were washed for three times with cold PBS. Confocal fluorescence imaging was performed on an Olympus FV1000-IX81 confocal laser scanning microscope (CLSM). A FV5-LAMAR 488 nm laser was the excitation source for FITC throughout the experiment, and emission was collected between 520 and 620 nm. Hoechst 33342 was excited at 50 mW (ex: 405 nm, em: 472 nm). The objective lens used for imaging was a UPLSAPO 63× oil-immersion objective (Olympus).

1.6 Synthesis of LN-PEG$_{2000}$-DSPE

LN-PEG$_{2000}$-DSPE was synthesized by conjugation of LN with DSPE-PEG$_{2000}$-MAL. Briefly, LN and DSPE-PEG$_{2000}$-MAL (1:1, w/w) were dissolved in deionized water (total concentration: 14 mg/mL) , gently stirring at room temperature for 24 h continually. We used HPLC to monitor the reaction. The peak of peptide was decreased along with the time. The reaction mixture were purified by dialysis (cut-off M.W. 3500 Da), and then lyophilized. The product was analyzed by MALDI-TOF-MS.

1.7 Preparation and characterization of liposomes

Liposomes loading DOX were prepared by thin film dispersion method. LN-LS-DOX was prepared by mixing of soy phospholipids (SPC), cholesterol (CHO), LN-PEG$_{2000}$-MAL and DOX (SPC: CHO: LN-PEG$_{2000}$-DSPE: DOX 8: 1: 1: 1 w/w/w/w). Briefly, DOX (1 mg) was dissolved in 1 mL methanol at room temperature and mixed with LN-PEG$_{2000}$-DSPE (1 mg), cholesterol (1 mg), and soy phospholipids (8 mg) in 6 mL dichloromethane/methanol (v/v 2:1). The solvent was removed by vacuum rotary evaporation to form a dry drug-containing lipid film. The dried film was hydrated with phosphate buffer saline (PBS) at 60 °C for 30 min, then sonicated for 15 min by bath type sonicator. Finally, the liposome suspension was eluted by 0.22 μm sterile hyperfiltration membrane. With the same method, LS-DOX was prepared.

Particle size (hydration diameter) and zeta potential of liposomes were determined by dynamic light scattering (DLS) using a Zetasizer 5000 (Malvern Instruments, Malvern, Worcestershire, U.K.). The morphology of LN-LS-DOX and LS-DOX were determined using HITACHI Transmission Electron Microscope (HT7700 TEM, Japan) with 120 kV acceleration voltage. The process was described as following:

Put 6 μL liposome suspension onto carbon membrane support copper mesh for 2 min, and then moved away the residual liposome suspension. When the membrane was dried, 6 μL negative staining solution (1% uranyl acetate) was added to the membrane for 3 min. Following, remove the negative staining solution, and dry the membrane in the air. Finally, the samples were observed by TEM.

1.8 Encapsulation efficiency detection of the liposomes

The standard curve of DOX concentration was measured with serial dilutions: 15, 7.55, 3.625, 1.863, 0.981 μg/mL (DOX dissolved in PBS). Fluorescence intensities of DOX solutions were measured at excitation wavelength of 480 nm and emission wavelength of 560-590 nm. Standard curve of DOX concentration with a linear regression coefficient (R^2) about 0.9977 was obtained. Next, the encapsulation efficiency (EE) of DOX in liposomes was also measured using following method. Briefly, unloaded DOX of liposome suspension was centrifuged by ultrafiltration centrifugal tube at 50000 rpm for 30 min. The amount of unloaded DOX in the outer tube was determined by measuring the fluorescence intensity. The total amount of DOX in the system is sum of the amount of DOX loaded in each liposome ($W_{\text{encapsulation}}$) and unloaded DOX (W_{unloaded}). Encapsulation efficiency (EE) was calculated according to the formula:

$$EE = (W_{\text{encapsulation}} / W_{\text{total}}) \times 100\%$$

1.9 *In vitro* release study

The required quantity of DOX loaded liposomes were transferred into a dialysis bag (cut-off M.W. 3500). 1mL free DOX , LS-DOX and LN-LS-DOX were placed into the dialysis bags separately. Then the dialysis bags were introduced into 40 mL PBS buffer solution with 0.5% (v/v) Tween-80. And then the release systems were gently shaken at 37 °C. To estimate the amount of drug release, the fluorescence intensity of

drug in the release medium at each sampling point was measured by infinite M200 microplate reader (Tecan, Durham, USA).

2.0 Confocal fluorescence imaging of cancer cells

For HepG2 and 293T cells, approximately 1×10^5 mL^{-1} cells were seeded into 35 mm microscope dishes and cultured overnight. LN-LS-DOX and LS-DOX were dissolved in cell culture medium to 20 μg/mL. HepG2 and 293T cells were incubated with Hoechst 33342 (nucleus indicator, 10 μg/mL, 200 μL) at 37 °C for 15 min, then incubated with LN-LS-DOX or LS-DOX at 37 °C for 15 min, separately. Finally, the cells were washed three times with cold PBS. Confocal fluorescence imaging was performed on an Olympus FV1000-IX81 confocal laser scanning microscope (CLSM).

we monitored the endocytosis and release effect of LN-LS-DOX and LS-DOX through CLSM observation. HepG2 cells were seeded into 35 mm microscope dishes and cultured overnight. Cells were incubated with Hoechst 33342 (10 μg/mL, 200 μL) for 15 min first. Then cells were incubated with LN-LS-DOX or LS-DOX (20 μg/mL, 200 μL) for 10 min, 15 min, 30 min, 60 min or 120 min at 37 °C. Cells were washed with PBS for three times and observed by confocal laser scanning microscope.

To investigate cell binding specificity of HE2/TAT-LS-DOX towards SKBR3, the competition experiment was carried out. After SKBR3 cells were treated with the anti-HER2 antibody (the recommended dilution of 1:20) for 1h, the cells were incubated with Hoechst 33342 (10 μg/mL, 200 μL) and HE2/TAT-LS-DOX (15 μg/mL, 200 μL) for 15min at 37 °C. Finally, the cells were washed three times with cold PBS. Confocal fluorescence imaging was performed on an Olympus FV1000-IX81 confocal laser scanning microscope (CLSM).

2.0 Inhibition ratio of cells studies

In DOX concentration-dependent experiment, HepG2 cells were seeded at 1×10^3 cells per well in 96-well plates, and pre-incubated for 24 h, then incubated with LN-LS-DOX, LS-DOX or free DOX for 24 h at doxorubicin concentrations ranging from 0.001, 0.01, 0.1, 1, 10, and 100 μg/mL (200 μL). In time-dependent experiment, HepG cells were seeded at a density of 1×10^3 cells per well in flat bottom 96-well plates and incubated for 24 h. Thereafter,LN-LS-DOX, LS-DOX or free DOX (100 μg/mL, 200 μL) were added in the wells of the plates and then exposed for 2, 4, 6, 8, 10 or 24h. Then medium was replaced with 100 μL 0.5 mg/mL MTT and after 3 h the MTT solution was replaced with 150 μL DMSO solution. Untreated cells in medium were used as control. The absorbance was measured at 570 nm with a reference wavelength of 630 nm using an Infinite M200 microplate reader (Tecan, Durham, USA). All experiments were carried out with four replicates.

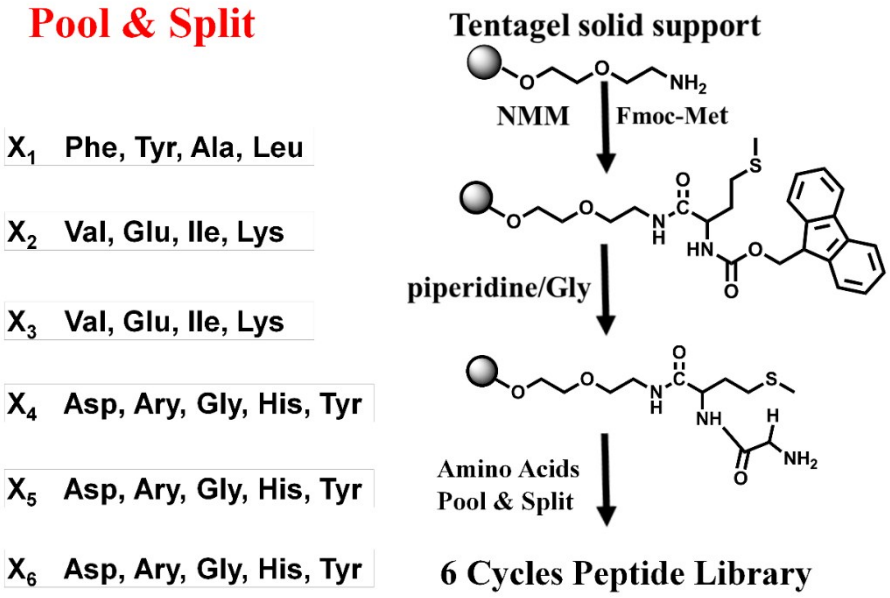
2.1 *In vivo* biodistribution of LN-LS

Female BALB/c nude mice about 19 g were purchased from Vital River Laboratory Animal Center (Beijing, China), and kept under specific pathogen-free conditions with free access to standard food and water. All the animal experiments were conducted in compliance with the guide for the care and use of laboratory animals of Beijing University Animal Study Committee's requirements. The xenograft tumors were established by subcutaneously (S.C.) injection of 1×10^7/mL HepG2 cells (150μL) to the right hind leg of the approximate 6 week-old female BALB/c nude mice. 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbonyaineiodide (Dir) with emission maxima centered at 748 nm were purchased from Invitrogen. Tumor growth was measured periodically and until diameters of the tumors reached to grow to about 7 mm (14 weeks old on arrival). Mice were injected via the caudal vein with, LS-Dir and LN-LS-Dir (200 μL) at dose corresponding to 1 μg/mL Dir. PBS was served as control. Near-infrared imaging was carried out after 8 h by Maestro *in vivo* spectrum

imaging system (Cambridge Research & Instrumentation, Woburn, MA). Then, organs were excised for *ex vivo* fluorescence imaging. All images were taken with ten-second exposure time to ensure consistency in the data.

in vivo tumor suppression studies were carried out to examine the toxicity and tumor inhibition efficiency of LN-LS-DOX and LS-DOX. Mice were injected intravenously with PBS (control group), LN-LS-DOX and LS-DOX (200μL) at a dose corresponding to 6mg/kg of DOX (n=4). Administration was carried out on the first, second, fourth, fifth, seventh, and eighth days. Successive observation was performed in the next 6 days. After the whole assay, tumors were excised. The weights and tumor sizes were recorded daily at the same time. Tumor sizes were measured by a vernier caliper. Tumor volume was calculated by the formula (L×W2)/2. L is for the longest and W is the shortest in tumor diameters (mm). For all the treatment, free DOX treating group caused significant drease in body weight changes count compared with LN-LS-DOX group (Fig. S8). Therefore, LN-LS-DOX might reduce the toxicity of DOX to normal tissues. Furthermore, the heart, liver, spleen, lung, kidney and tumors were used for histopathology analysis. To evaluate levels of necrosis, we stained sections with H&E and analyzed them by light microscope.

3.0 Supplementary figures



Scheme S1 Synthesis process of the OBOC peptide library towards LN

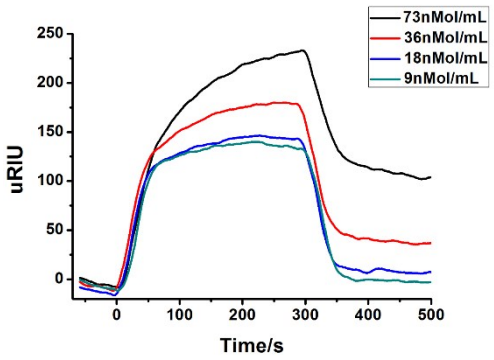


Fig. S1 SPRi detection for specific binding capability of LN with APN protein.

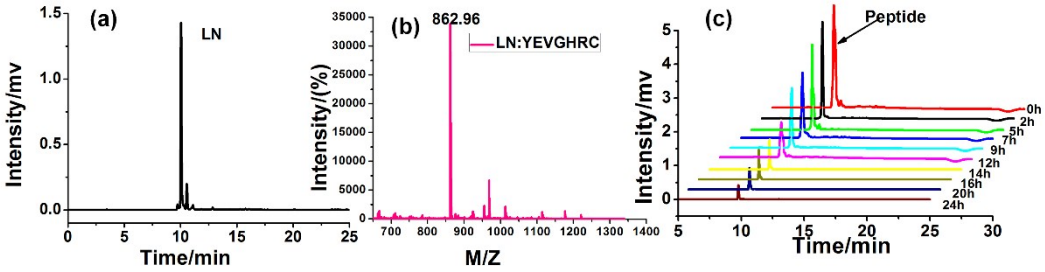


Fig. S2 (a) Purity identification for LN by HPLC. (b) Mass spectrum of LN detected by MALDI-TOF-MS. (c) Monitoring of the conjugation of LN with DSPE-PEG$_{2000}$-MAL by HPLC.

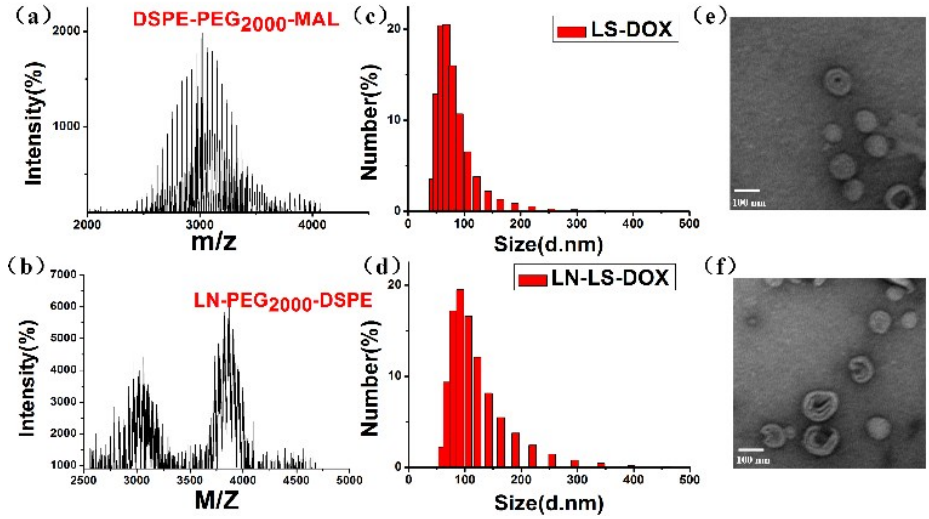


Fig. S3 (a) MALDI-TOF-MS detection of DSPE-PEG2000-MAL. (b) MALDI-TOF-MS detection of LN-PEG2000-DSPE. (c) Hydrodynamic size distribution of LS-DOX. (d) Hydrodynamic size distribution of LN-LS-DOX. (e) Morphology of LS-DOX. (f) Morphology of LN-LS-DOX.

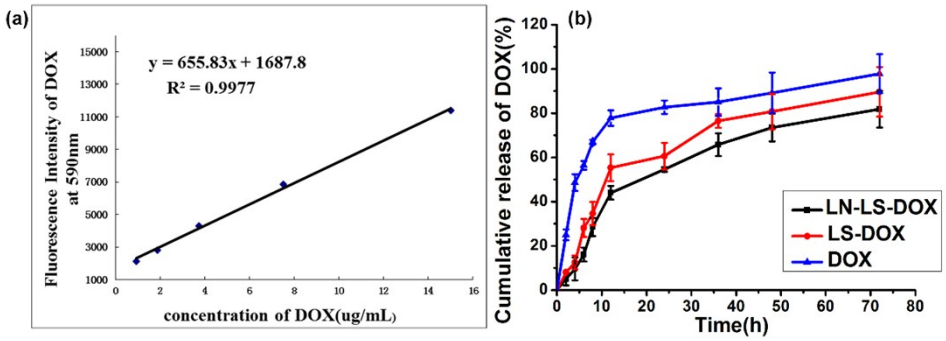


Fig. S4 (a) Standard fluorescence intensity curve of DOX in PBS. (b) In vitro release of DOX from LN-LS-DOX, LS-DOX and free DOX (n=3).

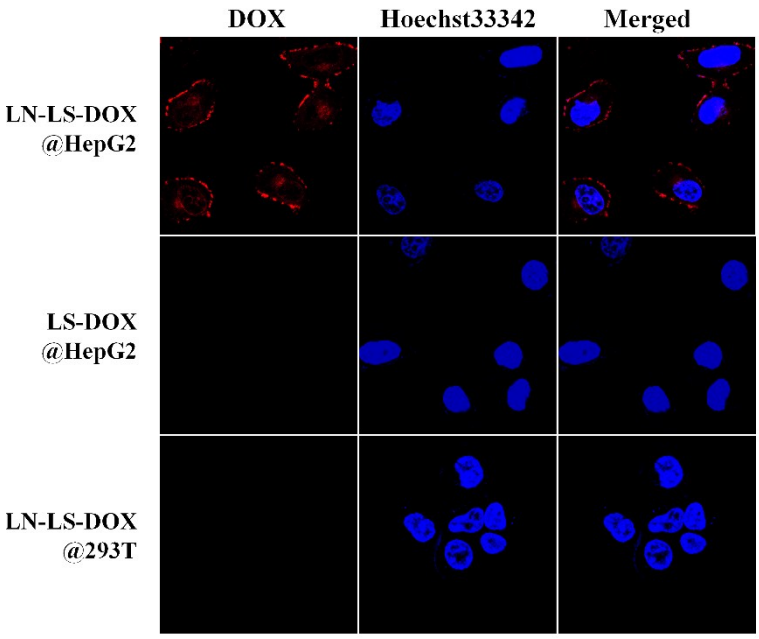


Fig. S5 CLSM images of cells incubated with LN-LS-DOX or LS-DOX for 30 min. (a) 293T cells incubated with LN-LS-DOX. (b) HepG2 cells incubated with LS-DOX. (c) HepG2 cells incubated with LN-LS-DOX.

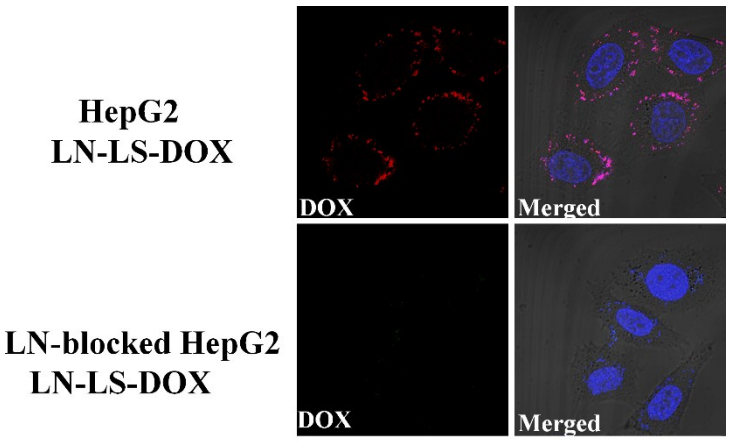


Fig. S6 LN-blocked HePG2 cells and HePG2 cells incubated with LN-LS-DOX.

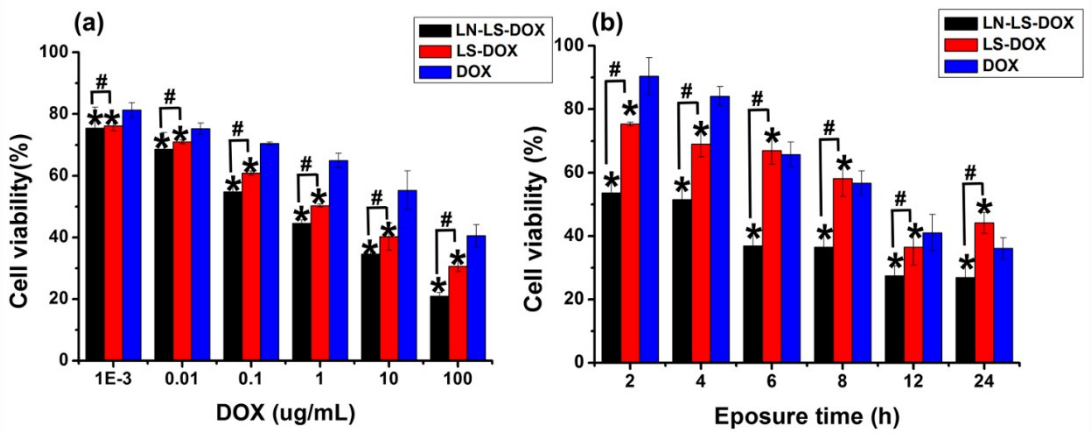


Fig. S7 (a) MTT assay for HepG2 cells incubated with different DOX formulations at different concentrations. (b) MTT assay for HepG2 cells incubated with different DOX formulations for different times (Data were presented as mean ± SD (n = 3). * $P < 0.05$ versus DOX group, $^{\#}$ $P < 0.05$ versus LS-DOX group).

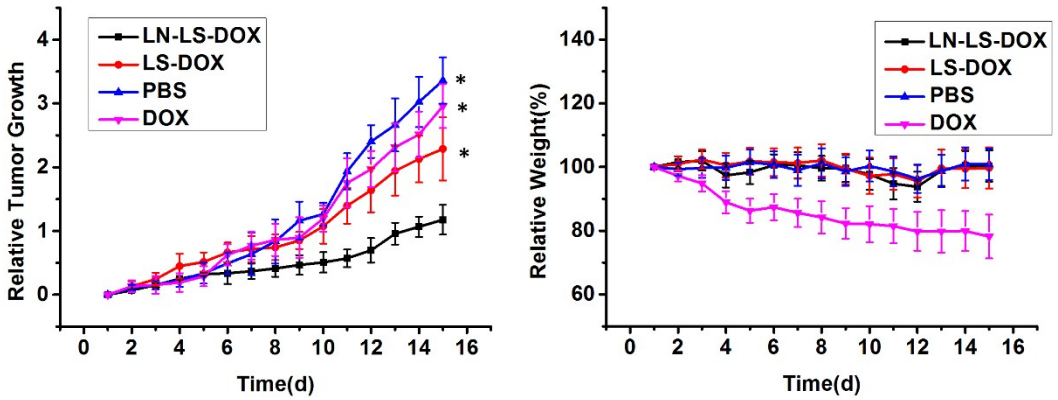


Fig. S8 Antitumor activity of different treatment groups in HepG2 tumor-bearing mice. Mice were injected with saline, free DOX, LS-DOX, and LN-LS-DOX. Relative tumor volume=tumor volume/primary tumor volume. Relative weight (%)=body weight/primary body weight.