Supplementary data

Sialic Acid-Conjugate Modified Liposomes Targeting Neutrophils for Improved Tumour Therapy

1. Experimental procedures

1.1 Stability studies of DOX-loaded liposomes

For long-term stability measurements, prepared liposomes (DOX-CL and DOX-SAL) were stored in tightly closed amber colored glass vials at 4 °C for three months and periodically removed for routine analysis. Any changes in particle size and encapsulation efficiency (*EE*%) were evaluated.

For serum stability measurements, DOX-loaded liposomes were suspended in PBS (pH 7.4) with 10% FBS at 37 °C. At predetermined time points, the size of the nano-liposome was monitored and the percentage of DOX release from liposomes was defined as follows: $\%\ release = (F_t - F_i) / (F_f - F_i) \times 100$. where F_i and F_t mean the initial and intermediary fluorescence intensities of DOX in liposomes suspensions at the Ex/Em 470/580 nm, respectively. F_f is the total fluorescence intensity of suspensions after the addition of Triton X-100 (final concentration, 0.25%).

1.2 Identification of L-selectin expression on PBN membranes

After PBNs were obtained from tumor-bearing mice and normal mice, they were seeded on coverslips in 6-well plates (2×10^5 cells per well). The cells were cultured for 1 h in FBS-free medium, and stained with FITC-labelled anti-CD62L antibody (250 $ng \cdot mL^{-1}$, BioLegend) at 4 °C for 20 min. After staining, PBNs were washed with cold PBS, fixed with 4% paraformaldehyde, and counterstained with 50 μM DAPI prior to visualization with a confocal laser scanning microscope (CLSM; Nikon C2 Confocal, Tokyo, Japan).

1.3 Cellular uptake of liposomal DOX by PBNs *in vitro*

The loading capacity of liposomal DOX in PBNs has been quantified by using a

microplate reader. Briefly, PBNs (1×10^6 cells/mL) were incubated with DOX-CL and DOX-SAL at different DOX concentrations (2.5, 5, 10, 20 and 40 $\mu g \cdot mL^{-1}$) for 30 min, or incubated with DOX-CL and DOX-SAL (10 $\mu g \cdot mL^{-1}$) for different times (10, 20, 30, 40 and 50 min). PBNs with internalized DOX-loaded liposomes (DOX-CL/PBNs and DOX-SAL/PBNs) were obtained after washing with ice-cold PBS thrice. To quantify the amount of DOX in PBNs, cell lysis buffer was used to disrupt PBNs and release DOX. Subsequently, cell lysate was achieved and centrifuged at 10,000 g for 5 min, the supernatant was mixed with methanol (200 μL), vortexed for 5 min, and centrifuged at 10,000 g for 10 min. A microplate reader was used to quantify the mean fluorescence intensity of DOX in the supernatant (200 μL) at the Ex/Em 470/580 nm.

1.4 The physiological functions of PBNs with internalized DOX preparations

The inflammation-responsive expression of CD11b and superoxide-anion production were evaluated by a flow cytometry (FCM). Briefly, PBNs (1×10^6 cells/mL) were cultured with free DOX, DOX-CL, or DOX-SAL (DOX, 10 $\mu g \cdot mL^{-1}$) for 30 min to obtain PBNs with internalized DOX preparations (DOX-S/PBNs, DOX-CL/PBNs and DOX-SAL/PBNs). Afterwards, PBNs (2×10^5 cells per well) with or without DOX were cultured in RMPI 1640 medium with or without 10 nM fMLP for 1 h. Followed by washing with ice-cold PBS twice, PBNs with or without DOX were stained with PE-conjugated CD11b antibody (BioLegend) or dihydroethidium (Beyotime) for 0.5 h.

The fluorescence intensity was determined using flow cytometry.

1.5 Intercellular transport of DiR-labeled liposomes from PBNs to tumor cells

PBNs (1×10^6 cells/mL) were cultivated with DiR-CL or DiR -SAL (DiR, 2 $\mu g \cdot mL^{-1}$) for 30 min to obtain PBNs with internalized DiR-labeled liposomes. S180 tumor cells (2×10^5 cells per well) were seeded in confocal dishes and cultivated overnight. PBNs with internalized DiR-CL or DiR -SAL were added to the seeded tumor cells and co-incubated for 4 h or 8 h. After incubation, cells were staining with PI (5 μg/mL) for 30 min to observe the formation of NETs, followed by counterstaining

with FITC-Gr-1 antibody (250 ng·mL^{-1}) for PBNs and Hoechst 33342 for cellular nuclei to visualize under a CLSM.

1.6 Cellular uptake of DiR-labeled liposomes by PBNs *in vivo*

Flow cytometry was used to investigate the role of circulating leukocytes, mainly PBNs, in tumor targeted drug delivery. Briefly, tumor-bearing mice were intravenously injected with DiR-SAL and DiR-CL (DiR, 1.0 mg kg^{-1}). Whole blood specimens were taken from mice orbital sinus after 5 min, 15 min and 30 min. RBC Lysis Buffer (eBioscience, San Diego,c CA) were used to lyse red blood cells, and the obtained leukocyte suspension was washed with PBS. Then PBNs were separated from total leukocyte by flow cytometry based on cellular size and granularity in the dot plot of forward scatter versus side scatter. The internalization efficiency of PBNs with fluorescent liposomes were measured by calculating DiR positive percentage of PBNs with flow cytometry.

1.7 Neutrophils Depletion experiment

the mice were i.p. injected with 20 mg/kg of Ultra-LEAF™ Purified anti-mouse Gr-1 antibody (500 μL of PBS) to deplete neutrophils. 24 h after the injection, the percentage of PBNs in blood was evaluated. Briefly, about 0.5 mL of blood was collected and treated with 3 mL of RBC lysis buffer for 3 min in ice-water bath. After centrifugation, the obtained peripheral blood leukocytes were re-suspended in PBS and incubated with FITC-Gr-1 antibody (250 ng·mL^{-1}) for 20 min to label PBNs. Finally, cells were washed with cold PBS, fixed with 4% paraformaldehyde and analyzed using a FAC Sort flow cytometry to examine the proportion of PBNs in peripheral blood leukocytes.

The amount of DiR-CL and DiR-SAL in tumors of mice with or without neutrophils depletion was further quantified. At 24 h post-injection of Gr-1 antibody, DiR-CL and DiR-SAL were intravenously administered to tumor-bearing mice. After 1 h, the tumors were harvested and homogenized in normal saline. Tissue homogenate (100 μL) was mixed with 900 μL ethanol, vortexed for 5 min, and centrifuged at 10,000

g for 10 min. A microplate reader was used to determine the measure the concentration of DiR in the supernatant at the Ex/Em 750/790 nm.

1.8 Pharmacokinetics studies

In vivo circulation studies were performed on male Kunming mice. In brief, various DOX preparations (DOX dose, 5 mg/kg) were intravenously injected into male Kunming mice and the mice were sacrificed at predetermined times after injection to collect blood samples. Plasma was obtained by centrifugation for 10 min at 4500 rpm, and then mixed with 900 μL methanol/water (50:50, *v*/*v*) containing 0.3 mol/L hydrochloric acid by a vortex and centrifuged for 10 min at 10,000 rpm to extracted DOX. A Microplate reader was used to measure the concentration of DOX at the Ex/Em 470/580 nm. The elimination half-life ($T_{1/2}$) and area under the drug concentration–time curve (AUC_{0-t}) values were calculated by DAS 2.0 software to investigate the *in vivo* circulation studies.

2. Supplementary Figures

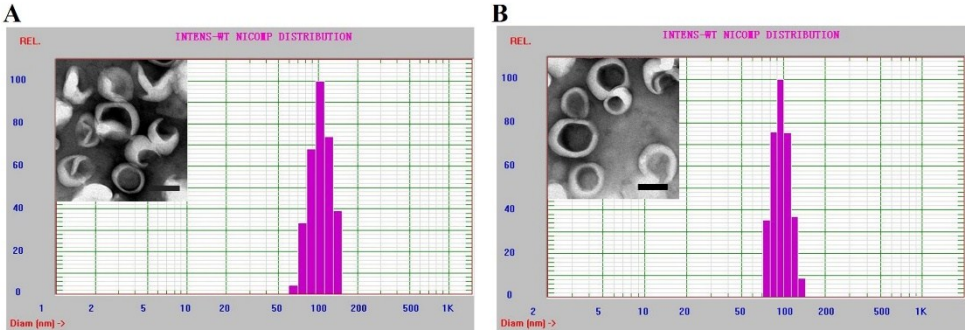

Fig. S1. Histogram of particle-size distribution of DOX-CL (A) and DOX-SAL (B) obtained by dynamic light scattering measurements. Inset: the transmission electronic microscope image of DOX-CL (A) and DOX-SAL (B). Scale bar, 100 nm.

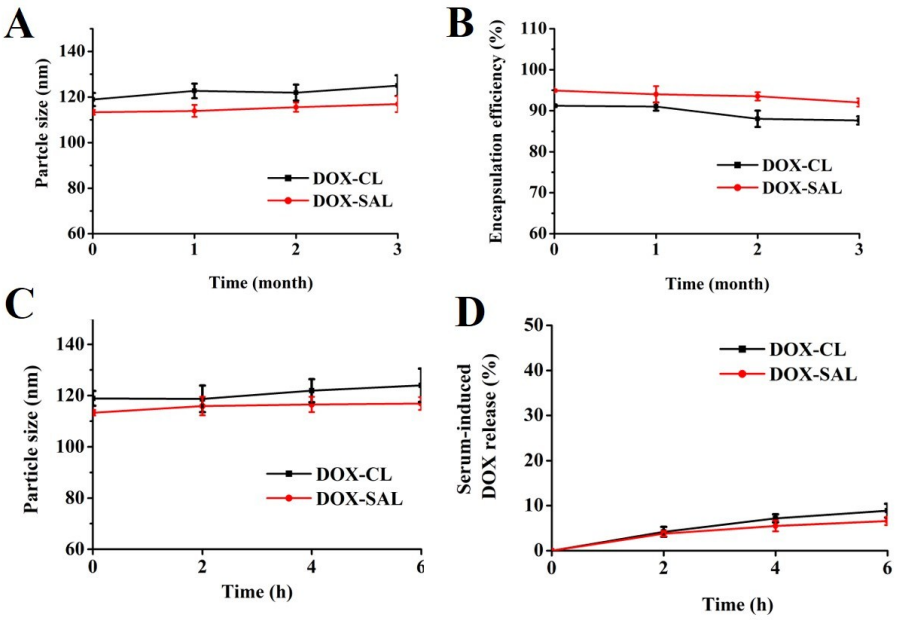

Fig. S2. The long-term stability and serum stability of liposomal DOX. (A) Particle size and (B) *EE*% changes of DOX-load liposomes during 3 months storage at 4 °C. (A) Particle size and (B) DOX release% of DOX-load liposomes after incubation at 37 °C in PBS (pH 7.4) with 10% FBS. Data is shown as mean ± S.D. (n = 3).

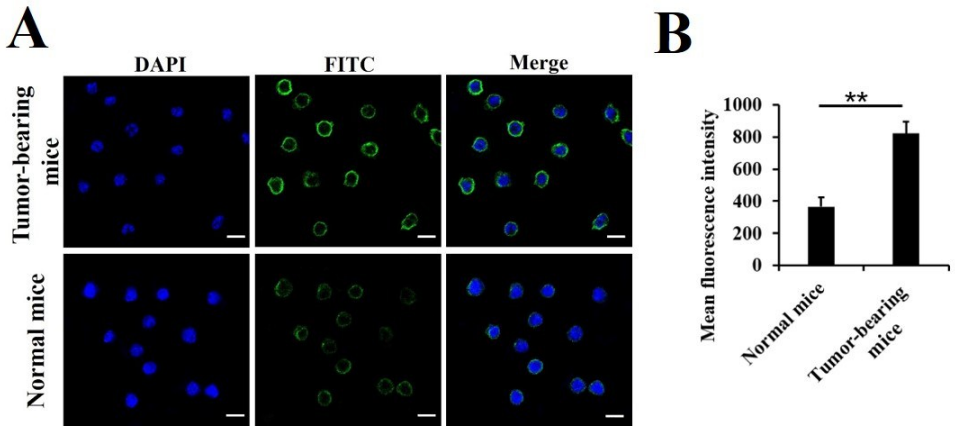

Fig. S3. (B) L-selectin expression on the membranes of PBNs isolated from S180 tumor-bearing mice and normal mice. Cells were stained with FITC-CD62L for L-selectin (green) and DAPI for nuclei (blue), then observed by using a CLSM. Scale bar = 10 μm. (C) The mean fluorescence intensity of FITC-CD62L in PBNs. $^{**}P < 0.01$. Data are expressed as mean ± S.D. (n = 3).

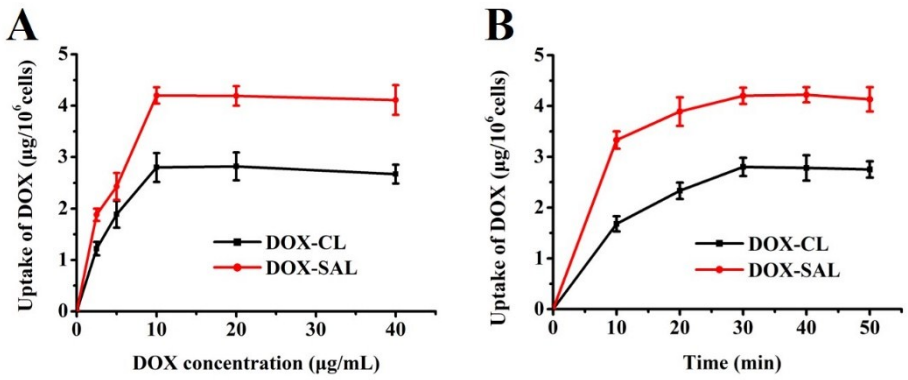

Fig. S4. Cellular uptake of DOX-CL and DOX-SAL by PBNs. (A) Quantity of DOX in PBNs after PBNs (1×10^6 cells/mL) were incubated with DOX-CL and DOX-SAL at different concentrations of DOX for 30 min. (B) Quantity of DOX in PBNs after PBNs (1×10^6 cells/mL) were incubated with DOX-CL and DOX-SAL (10 μg/mL) for different times. The data are presented as the mean ± S.D. (n = 3).

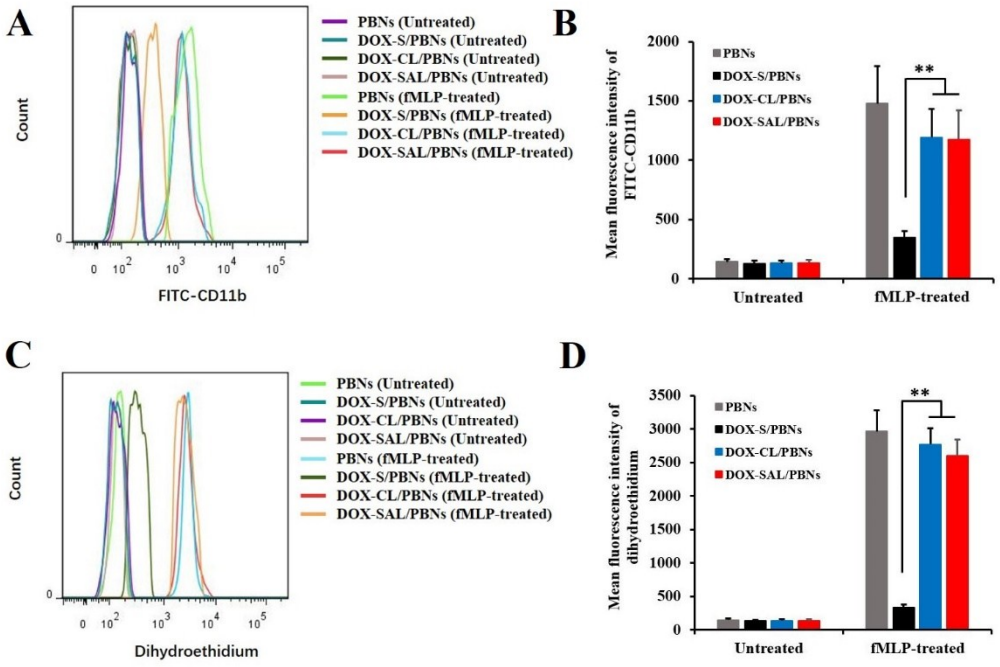

Fig. S5. (A) The expression level of CD11b on the cell membrane of PBNs with internalized DOX formulations in the absence or presence of fMLP (10 nM) for 1 h. CD11b was stained with FITC-conjugated CD11b antibody. (B) Quantification of the CD11b expression on PBNs. (C) Superoxide anion generation of PBNs with internalized DOX formulations in the absence or presence of fMLP. Superoxide anion were stained with dihydroethidium. (D) Quantification of the superoxide anion generation by PBNs. $^{**}P < 0.01$. Data are expressed as mean ± S.D. (n = 3).

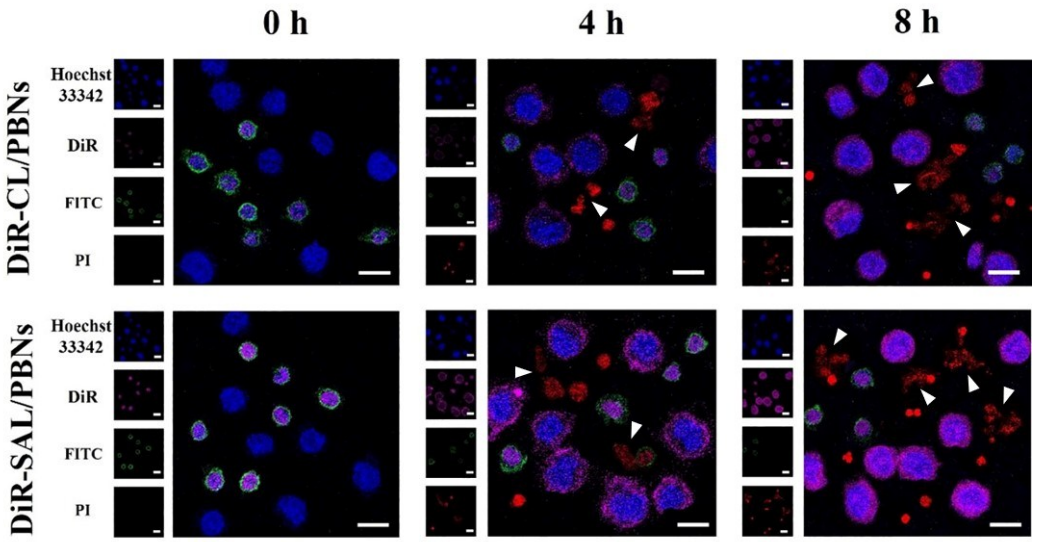

Fig. S6. CLSM images of S180 cells after incubation with PBNs internalizing DiR-labeled liposomes over time. The nuclei of cells were stained with Hoechst 33342 (blue), the PBNs were labelled with FITC-Gr-1 (green) and the released DNA segments were stained with PI (red). The merged image is the overlay of three individual images. The arrows indicate fibrous extracellular DNA released by PBNs after incubation with S180 tumor cells. Scale bar, 10 μm.

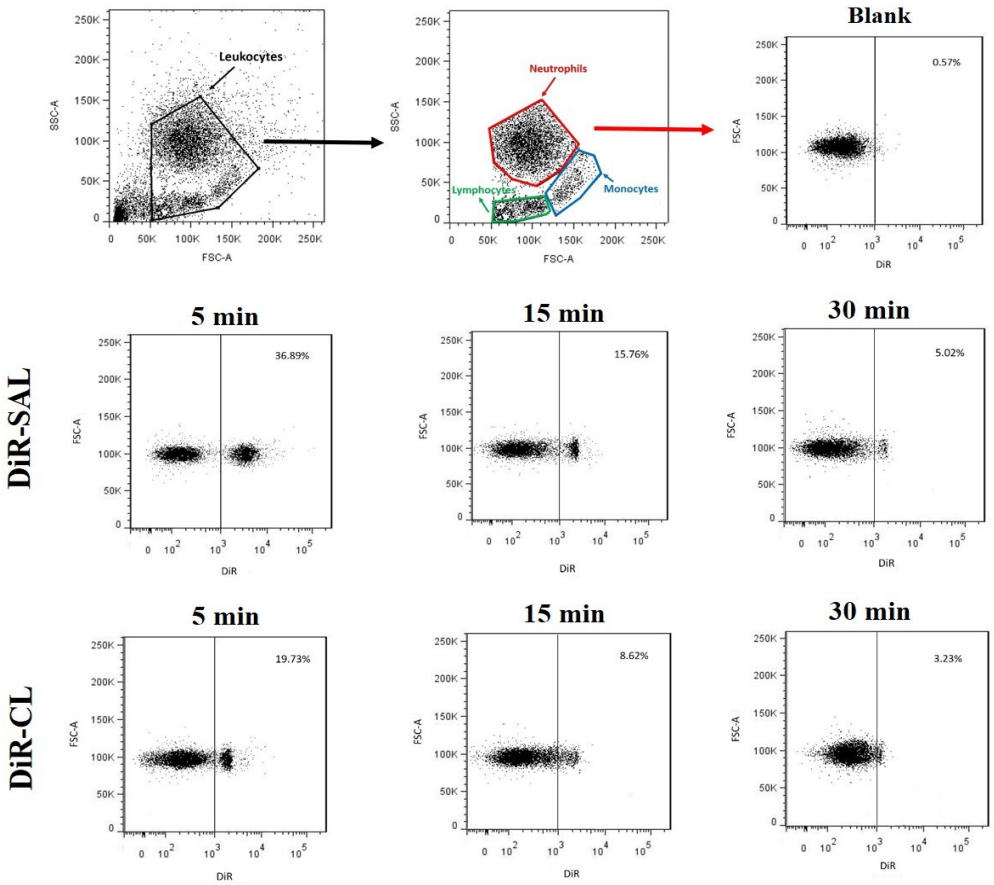

Fig. S7. The classification of leukocytes and the DiR-labeled liposomes uptake by

PBNs at different times using flow cytometry.

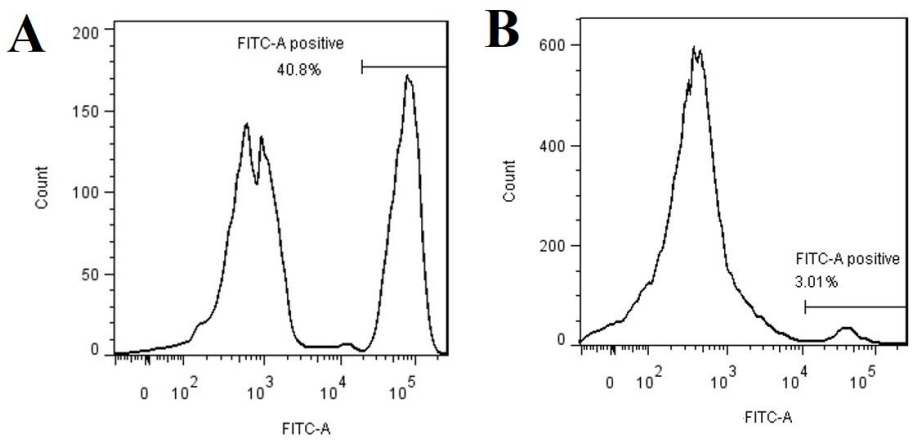

Fig. S8. Percentage of PBNs in peripheral blood leukocytes 24 h after i.p. injection of PBS (A) or 20 mg/kg of anti-Gr-1 antibody (B). After the blood was treated with RBC lysis buffer, PBNs were labelled by FITC-Gr-1 antibody and analyzed using a FAC Sort flow cytometry. (n=3).

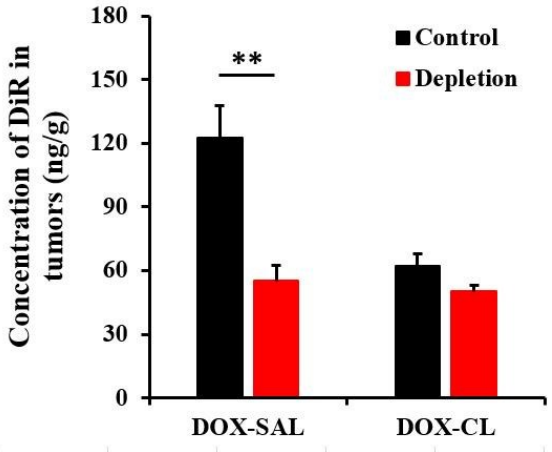

Fig. S9. Quantification of the concentration of DiR in collected tumors. At 24 h after i.p. injection of PBS or 20 mg/kg of anti-Gr-1 antibody, mice were i.v. administered DiR-labeled liposomes and the tumors were extracted from the mice at 1 h after injection. $^{**}P < 0.01$. The data are expressed as the mean ± S.D. (n = 3).

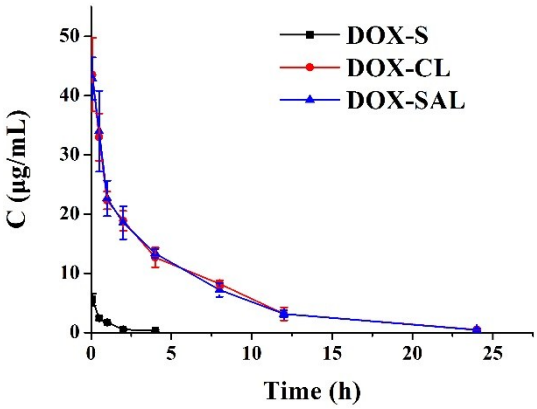

Fig. S10. Pharmacokinetic behavior of different DOX preparations in male Kunming mice. The data are expressed as mean ± S.D (n = 3).

3. Supplementary table

Table S1 Composition and characterization of liposomal DOX (n = 3).

Preparation	Liposome composition (*n*/*n*)	Zeta potential (mV)	Size (nm)	PD1	Encapsulation efficiency (%)
DOX-CL	HSPC/CH (55:45)	-11.4 ± 2.7	118.9 ± 2.9	0.235 ± 0.039	91.2 ± 0.3
DOX-SAL	HSPC/CH/SA-ODC (50:45:5)	-34.8 ± 1.2	113.3 ± 1.1	0.198 ± 0.045	94.9 ± 0.3

Table S2. IC_{50} values of DOX-CL/PBNs and DOX-SAL/PBNsr to S180 tumor cells (n = 3).

DOX-CL/PBNs	DOX-SAL/PBNs
1.90 ± 0.06	1.64 ± 0.05**

Table S3. Main pharmacokinetic parameters of different DOX formulations in mice. Each value represents as mean ± S.D. (n = 3).

Treatment group	AUC_{0-t} (μg/mL·h)	$T_{1/2}$
DOX-S	5.186 ± 0.823	0.636 ± 0.181
DOX-CL	172.37 ± 16.90	4.115 ± 0.306
DOX-SAL	170.21 ± 6.664	3.932 ± 0.648