Supplementary Materials

Chemotherapy priming of the pancreatic tumor microenvironment promotes delivery and anti-metastasis efficacy of intravenous low-molecular-weight heparin-coated lipid-siRNA complex

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Preparation of PTX-Lip

PTX-Lip was prepared by cholesterol, SPC, and DSPE-PEG2000-OMe (molar ratio=33:62:5) using the thin film hydration method. PTX was added to the lipid organic solution prior to the solvent evaporation. The film was hydrated in 1 mL phosphate buffer saline (PBS, pH=7.4) and sonicated as above. PTX-Lip was purified before use by centrifugation (9300 rcf, 20 min) to remove unloaded PTX.

Binding efficiency of LH

The binding efficiency of LH was determined by measuring the free LH using the toluidine blue assay. LH-Lip was divided into two equal portions. Free LH was separated from lipoplex by ultrafiltration at 9300 rcf for 30 min with an ultrafiltration tube (Mw = 10 kDa, Millipore, USA) at predetermined time points (0, 2, 4, 8, and 24 h). Total LH was measured without ultrafiltration. Binding efficiency of LH was calculated as follows: Binding efficiency (%) = $(LH_{total}-LH_{free})/LH_{total}$

The drug/siRNA release profiles of LH-coated lipoplex

In vitro PTX and siRNA release study was performed with a dialysis method. For PTX release study, salicylate sodium (pH 7.4, 1M) was used as the release media. PTX-loaded liposomes or free PTX (dissolved in ethanol-Cremophor ELP35 mixture, v/v = 1:1) were placed into dialysis tubes (Mw = 10 kDa) and incubated at 37 °C with gently oscillating for 24 h. At predetermined time points, 0.1 mL release media was sampled and analyzed by HPLC. For siRNA release study, PBS (pH 7.4) was used as the release media. FAM-siRNA loaded liposomes or free FAM-siRNA were placed into dialysis tubes (Mw = 10 kDa) and incubated at 37 °C with gently oscillating for 72 h. At predetermined time points, 0.1 mL release media was sampled and analyzed by a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA) at 488 nm.

Serum nuclease degradation of siRNA

Electrophoretic mobility shift assay with agarose gel electrophoresis was used to evaluate the protection of siRNA against serum nuclease degradation. LH-coated Lipid–siCTL complex, free siCTL plus free LH, and free siCTL (25 μg/mL siRNA) were incubated at 37 °C in 50% FBS for 0, 30 and 60 min. The agarose gel was stained with GoldView (Solarbio) before electrophoresis and visualized on a Bio-Rad ChemiDoc MP System (Bio-Rad).

Sizes and zeta potentials variations

The lipoplex was mixed with fetal bovine serum (FBS) or mouse serum (v/v = 1:1) and then incubated at 37 °C. The sizes and zeta potentials were measured at predetermined time points (0, 5, 10 and 30 min) using Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK).

Hemolysis assay

For the hemolysis study, red blood cells (RBCs) were obtained from mouse blood after centrifugation and washed with PBS. The RBC suspension (2%, v/v) was mixed with lipoplex under different concentration (100, 150, 300, 500 and 1000 μM) at 37 °C for 1 h. After the incubation, the supernatant was separated after centrifugation at 800 rcf for 10 min and subjected to measurement by a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA) at 540 nm. Samples incubated with 1% Triton X-100 served as the positive control and samples incubated with PBS as the negative control respectively. The degree of hemolysis was calculated as follows:

$$\text{Hemolysis (\%)} = (A - A_0)/(A_s - A_0)$$

Where A, A_s and A_0 represented the absorbance of samples incubated with lipoplex, positive control and negative control, respectively.

Cell culture

Human pancreatic cancer (BXPC-3) cell lines were cultured in RPMI-1640 medium at 37 °C in a 5% CO_2 humidified environment incubator (Thermo Fisher Scientific, USA). Human normal hepatocytes (LO2) cell lines and mouse embryo fibroblast (NIH-3T3) cell lines were cultured in DMEM medium under the same condition. Both media contained 10% FBS (10099-141, Gibco, Thermo Fisher Scientific), 100 U/mL penicillin, and 100 mg/ml streptomycin.

Cell cycle

BXPC-3 Cells were seeded and pretreated with PTX-Lip (0, 0.1, 0.3, 0.6 and 1 μg/mL concentration of PTX) for 24 h as described above. Then the cells were trypsinized, and centrifuged at 400 rcf for 5 min and fixed in 70% ethanol overnight. After removing ethanol, samples were washed twice with PBS, and then incubated with the staining solution (RNase A: PI = 1: 9) for 30 min. Cell cycle was evaluated by a flow cytometry (Cytomics™ FC 500, Beckman Coulter, Miami, FL, USA).

The subcellular localization

BXPC-3 cells were seeded onto 6-well plate as described above. After 1.5 h incubation of

Cy5-labeled siRNA loaded liposomes (siRNA 50 nM), the cells were washed with serum free medium and treated with Lyso-Tracker green (50 nM) for 30 min. Then the cells were washed with PBS for three times, and fixed with 4% (m/v) paraformaldehyde and stained with DAPI (0.5 μg/mL). A confocal microscope (CLSM) (FV1000, Olympus, Tokyo, Japan) was used to observe the cells.

Western blotting assay

The expression levels of fibroblast growth factor 2 (FGF2) on LO2 and BXPC-3 cells were measured using western blotting. Polyvinylidene fluoride (PVDF) films were incubated with rabbit anti-mouse GAPDH or FGF2 primary antibodies (1:2000). After 24 h, the membranes were incubated with HRP-labeled goat anti-rabbit secondary antibodies (1:5000) and detected by Immobilon Western HRP Substrate (Millipore, Billerica, MA, USA) on Bio-Rad ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA, USA).

Western blotting was used for evaluating the expression level of BCL-2 in BXPC-3 cells and cytochrome C in mitochondria. By the end of the incubation, the cells were harvested and lysed by Cell lysis buffer (Beyotime, China). For isolating the mitochondria of BXPC-3 cells, the cell mitochondria isolation kit (Beyotime, China) was used. Polyvinylidene fluoride (PVDF) films were incubated with anti-rabbit GAPDH (1:5000), BCL-2 (1:1000) or cytochrome C (1:1000) primary antibodies, respectively. After 24 h, the membranes were incubated with HRP-labeled secondary antibodies (1:5000) and detected by Bio-Rad ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

To test the relationship between concentration of siBCL-2 and silencing efficiency, BXPC-3 cells was treated with different concentration of siBCL-2 (0-500 nM at final concentration) loaded LH-Lip, and the BCL-2 mRNA was measured using qRT-PCR. The total RNA was extracted, transcribed into cDNA, and subjected to qRT-PCR using target-specific primers for BCL-2. The BCL-2 primer sequences were: forward: 5′ –GACTTCGCCGAGATGTCCAG–3′; reverse: 5′ –GAACTCAAAGAAGGCCACAATC–3′.

Cellular apoptosis assay and cytotoxicity study

The analysis of apoptosis induced by different lipoplex was measured using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen biotech, China). BXPC-3 cells and LO2 cells were

seeded onto 6-well plate, and N-Lip/siBCL-2 and LH-Lip/siBCL-2 were added for 2 h (siRNA 50 nM). By the end of the treatment, cells were washed twice with PBS, suspended in 300 μL binding buffer. 5 μL Annexin V-FITC and 5 μL PI were added into tube for 15 min, and the cells were measured by flow cytometry (Cytomics™ FC 500, Beckman Coulter, Miami, FL, USA).

MTT assay was used for evaluating the cytotoxicity of different formulations. BXPC-3 cells and LO2 cells were seeded onto 96-well plate in the density of 2×10^3. PBS, N-Lip, LH-Lip and free LH (20 μL) were added into plate for 24 h. By the end of the incubation, 20 μL MTT solution (5 mg/mL) was added into each well and cells were further incubated for 4 h. Then the media was removed and cells were dissolved by 150 μL dimethyl sulfoxide. The samples were measured by a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm.

For evaluating the therapeutic effect of PTX and BCL-2 in vitro, PTX-Lip (0.3 μg/mL) was added into plate for incubation of 24 h. Then the media was removed and PBS, LH-Lip/siCTL and LH-Lip/siBCL-2 were added for further incubation of 24 h. The samples were processed as described above.

Tumor spheroid uptake

To prepare the tumor three-dimensional spheroids, BXPC-3 cells were seeded onto 96-well plate coated with 2% (w/v) low melting point agarose in the density of 2×10^3. After 3 days, the diameter of tumor spheroids was approximately 300 nm. And the uniform spheroids were selected and incubated with PTX-Lip (0, 0.1, 0.3, 0.6 and 1 μg/mL concentration of PTX) for 24 h, then DID-labeled LH-Lip/siCTL was added at a DID concentration of 10 μg/mL for 4 h. The spheroids were washed and subjected to CLSM analysis (LSM800, Carl Zeiss, Germany).

To compare the uptake of different formulation after pretreated with PTX-Lip (0.3 μg/mL), Cy5-labeled siRNA-loaded liposomes or Cy5-labeled free siRNA (siRNA 50 nM) were added for incubation for 4 h.

Transwell model of tumor *in vitro*

Transwell chambers were used to establish pancreatic cancer model in vitro. NIH-3T3 cells were seeded onto the upper chamber in the density of 1×10^5, and BXPC-3 cells were seeded onto the under 6-well plate in the density of 5×10^5. After 24 h, PTX-Lip (0.3 μg/mL) was added into upper chamber for another 24 h. Then the media was removed and CFPE-labeled lipoplex was added at the final CFPE concentration of 2 μg/mL for 4h. By the end of the incubation, the cells

were detected by flow cytometry (Cytomics™ FC 500, Beckman Coulter, Miami, FL, USA).

Subcutaneous tumor model

The BXPC-3 subcutaneous tumor model was established by subcutaneous injection of a suspension of 1×10^6 BXPC-3 cells (100 μL) into the right back of 4-week-old female nude mice. Nude mice were purchased from Chengdu Dashuo Biological Institute (Chengdu, China). All the animal experiments were approved by the specialty committee on the ethics of animal experimentation of Sichuan University. Tumors were allowed to grow to about 50 mm^3 (tumor volume = length×$width^2$×0.52) before treatment.

Evans Blue assay

Evans Blue assay was used to measure the tumor permeability quantitatively. By the end of injection of PTX formulations, mice were injected with 0.2 mL of Evans Blue (6 mg/kg) through tail vein. After 24 h, the mice were euthanized and the tumor tissues were homogenized by formamide and incubated for 48 h at 37 °C. Then, the samples were centrifugated at 9300 rcf for 10 min. Absorbance was measured using UV–Vis spectrophotometer (Varian, USA) at 620 nm to determine the concentration of Evans Blue.

The mechanism of tumor priming

After the injection of PBS and PTX-Lip (50 mg/m^2) for three times, the tumor-bearing mice were euthanized and tumors were collected. Tumor histology was performed after TUNEL, alpha-smooth muscle actin (α-SMA), and Masson staining.

Wound healing assay

BXPC-3 cells were seeded onto 12-well plate 5×10^5 for incubation of 24 h. A wound was scratched with a sterile pipette tip, and the cells were washed with PBS. After pretreated with or without PTX-Lip (0.3 μg/mL) for 24 h, PBS, LH-Lip/siBCL-2, N-Lip/siBCL-2, and free LH were added for another 24 h. Images of wound were captured using optical microscopy (DM2000 LED, Lecia, Germany). The quantitative image analysis of wound healing rates was performed using Image J software (1.46r, National Institutes of Health, USA).

In vitro inhibitory effect on cell invasion

BXPC-3 cells were seeded onto upper chamber of a 24-well plate pre-coated with 50 μL of Matrigel (pore size: 8 μm) and treated as above. By the end of incubation, the cells were stained by 0.2% crystal violet and washed three times. Images of invasion were captured using optical

microscopy (DM2000 LED, Lecia, Germany). After capturing, 33% acetic acid was added to dissolve crystal violet. Absorbance was measured using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm.

Interactions of tumor cells with platelets

Platelet adherence assays was used to evaluate the interactions between platelets and tumor cells. Briefly, BXPC-3 cells were seeded onto 6-well plate overnight. Platelets were obtained from mice and labeled with calcein-AM (5 μM). After pretreated with or without PTX-Lip (0.3 μg/mL) for 24 h, PBS, LH-Lip/siBCL-2, N-Lip/siBCL-2, and free LH with calcein-labeled platelets were added for 15 min. Then the cells were washed twice and dissolved by 200 μL dimethyl sulfoxide. The samples were detected using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA) at 490 nm. For CLSM analysis, BXPC-3 cells were stained with DID (5 μM) by the end of incubation.

Expression levels of N-cadherin and E-cadherin were performed by Western blotting assays. By the end of incubation as described above, PVDF films were incubated with anti-rabbit GAPDH (1:5000), N-cadherin (1:1000) or E-cadherin (1:1000) primary antibodies, respectively. After 24 h, the membranes were incubated with HRP-labeled secondary antibodies (1:5000) and detected by Bio-Rad ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA, USA).

Confocal microscopy of actin cytoskeleton

To investigate the effect of tumor cell actin cytoskeletal arrangement after incubated with different formulations. BXPC-3 cells were pretreated with or without PTX-Lip (0.3 μg/mL) for 24 h, then PBS, LH-Lip/siBCL-2, N-Lip/siBCL-2, and free LH were added for 12 h. F-actin of the cells were stained using an ActinRed kit (KeyGen biotech, China), followed by nuclei staining with DAPI for 5 min. The coverslips were imaged by CLSM (LSM800, Carl Zeiss, Germany).

Bio-distribution study in vivo

For bio-distribution study of PTX-Lip, fluorescence dyes (DID) loaded PEGlayted liposomes (PEG-Lip) were injected into subcutaneous tumor-bearing mice. At predetermined time points, mice were imaged using the IVIS Spectrum system (Caliper Life Sciences, Hopkinton, MA, USA) and euthanized. Their blood was collected and mice were suffered heart perfusion using PBS and 4% paraformaldehyde. Then organs were collected and imaged using the IVIS Spectrum system (Caliper Life Sciences, Hopkinton, MA, USA).

For bio-distribution study of lipoplex, subcutaneous tumor-bearing mice were pretreated with or without PTX-Lip (50 mg/m^2). After 24 h, Cy5-labeled free siRNA or Cy5-labeled siRNA-loaded liposomes (siRNA 0.35 mg/kg per dose) were injected. Then, the mice were imaged using the IVIS Spectrum system (Caliper Life Sciences, Hopkinton, MA, USA) and euthanized via heart perfusion using PBS and 4% paraformaldehyde. Their organs were collected and imaged using the IVIS Spectrum system (Caliper Life Sciences, Hopkinton, MA, USA).

Figures:

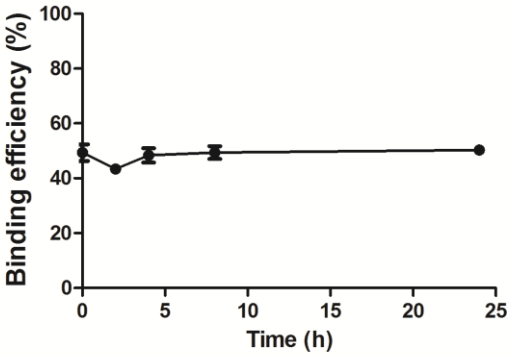

Figure S1. The LH binding efficiency of LH-Lip/siBCL-2 in 24 h

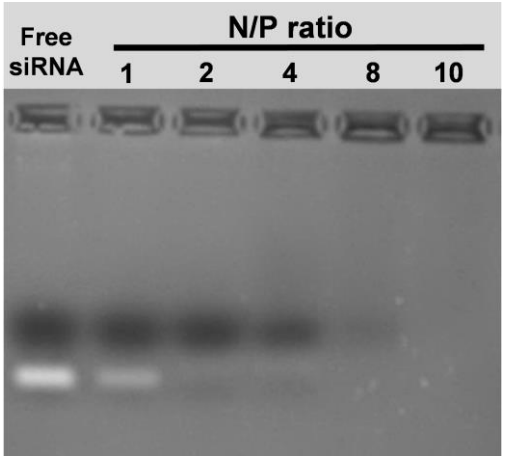

Figure S2. The ability of N-Lip to form self-assembled complexes with siRNA.

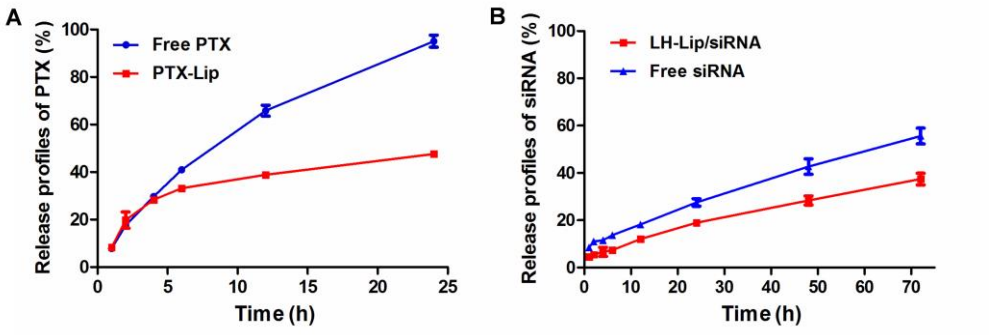

Figure S3. The release profiles of **(A)** PTX and **(B)** siRNA *in vitro*.

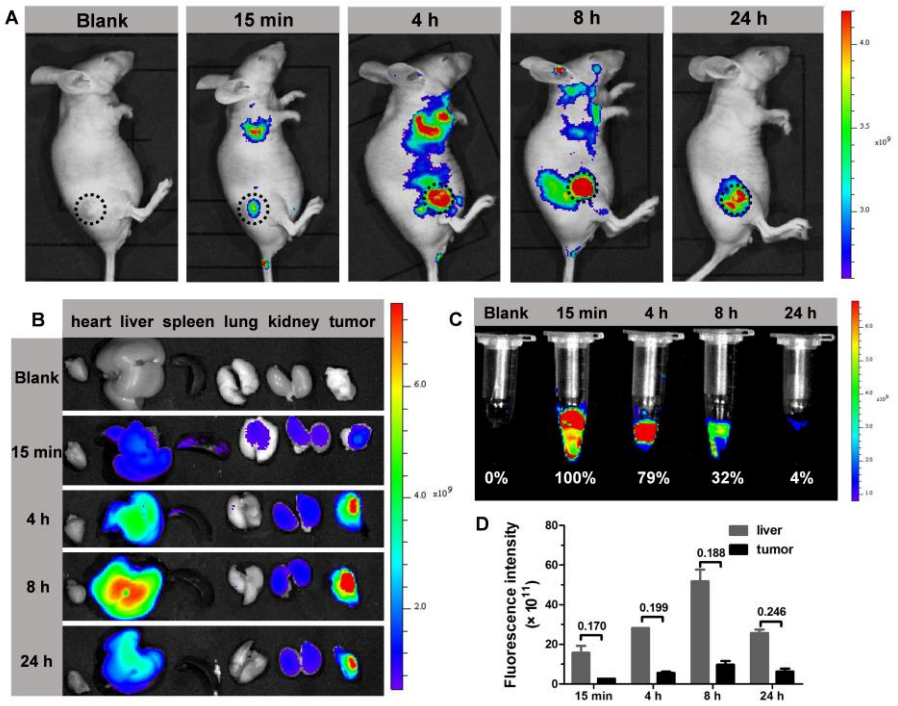

Figure S4. (A) *In vivo* images of tumor-bearing mice after injection of DID-loaded PEG-Lip at different time points. **(B)** *Ex vivo* images of organs and tumor. **(C)** The circulation time of DID-loaded PEG-Lip in blood. **(D)** Semiqualitative study of fluorescence intensity in the liver and tumor from the ex vivo images (n = 3). Average tumor-to-liver ratios were calculated at different time points.

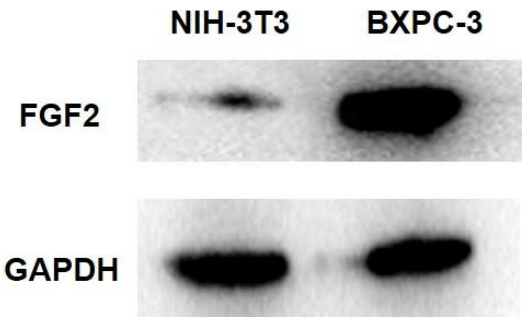

Figure S5. The expression of FGF2 on NIH-3T3 cells and BXPC-3 cells detecting by western blotting.

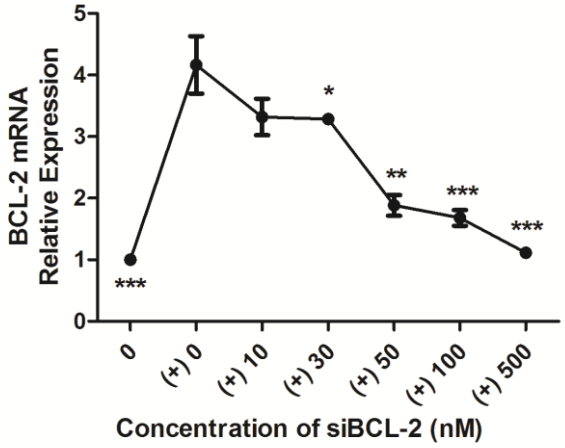

Figure S6. The expression of BCL-2 mRNA treated with different concentration of siBCL-2 loaded LH-Lip. (+) represents incubation with PTX-Lip (0.3 μg/mL) before treatment. (n = 3) *P < 0.05, **P < 0.01, ***P < 0.005 vs. the (+) 0 group.

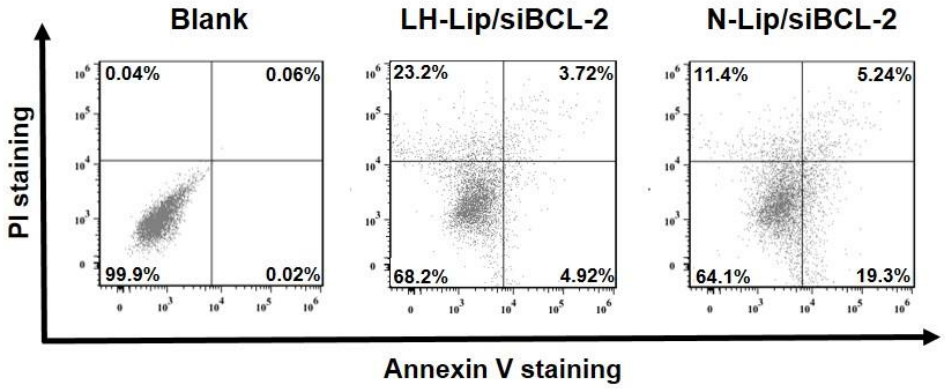

Figure S7. The apoptosis study of LO2 cells after incubation with PBS, LH-Lip/siBCL-2, and N-Lip/siBCL-2.

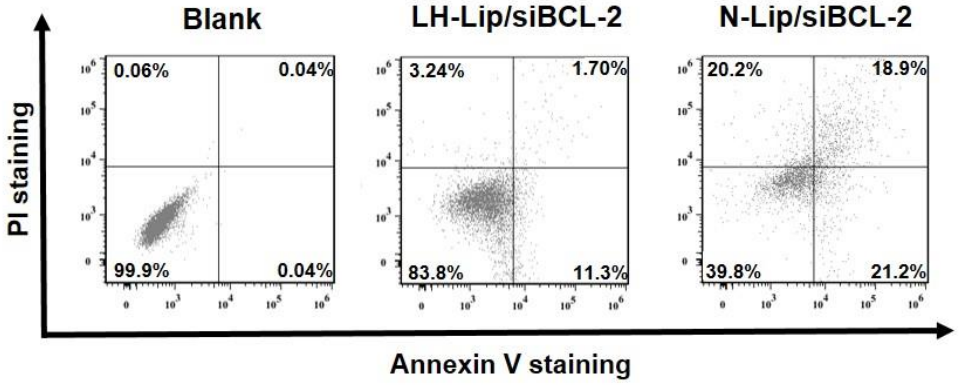

Figure S8. The apoptosis study of BXPC-3 cells after incubation with PBS, LH-Lip/siBCL-2, and

N-Lip/siBCL-2.

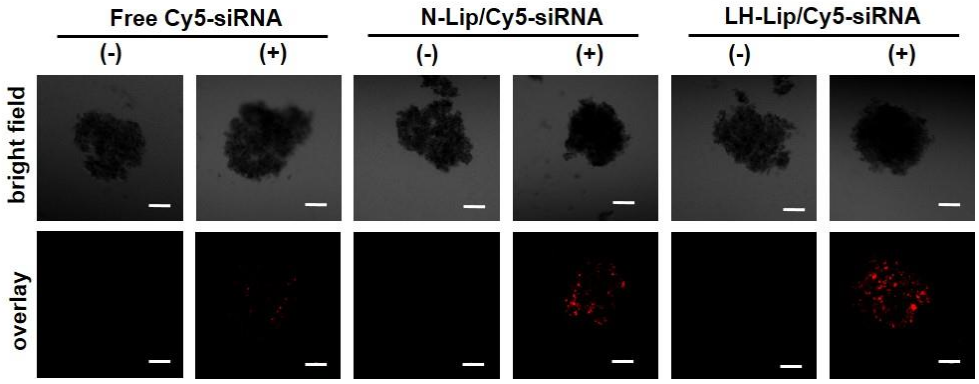

Figure S9. Penetration of Cy5-siRNA into the BXPC-3 tumor spheroids after incubation with free Cy5-siRNA, N-Lip/Cy5-siRNA and LH-Lip/siRNA for 4 h, respectively. (+) and (-) represent incubation with or without PTX-Lip (0.3 μg/mL) before treatment, respectively. Scale bar, 150 μm.

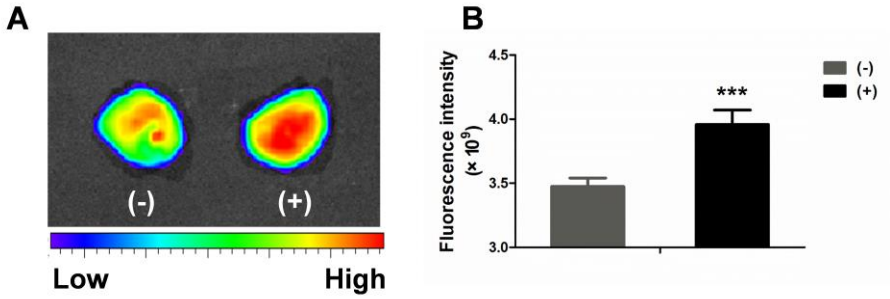

Figure S10. (A) *Ex vivo* images of tumors after injection of DID-labeled LH-Lip for 24 h. **(B)** Semiqualitative study of fluorescence intensity in tumors from the *ex vivo* images. ***P < 0.005. (+) and (-) represent injection of PTX-Lip (50 mg/m^2) before treatment or not, respectively.

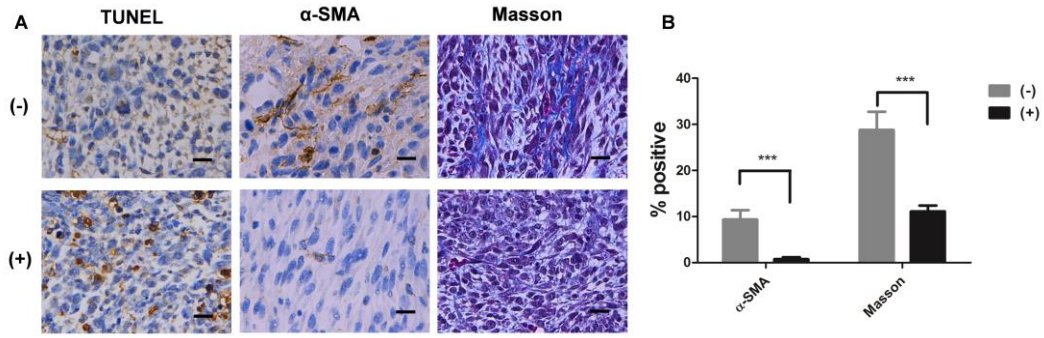

Figure S11. (A) TUNEL staining (apoptotic and necrotic cells shown in brown), α-SMA staining

(myofibroblasts shown in brown) and Masson staining (collagenous fibers shown in blue) of tumor tissues. Scale bar, 20 μm. **(B)** The quantitative image analysis of positive rates on α-SMA and masson stained tumor sections. Mean ± SD (5 microscopic fields per tumor, 5 mice per group). ***P < 0.005 vs. other groups. (+) and (-) represent injection of PTX-Lip (50 mg/m^2) before treatment or not, respectively.

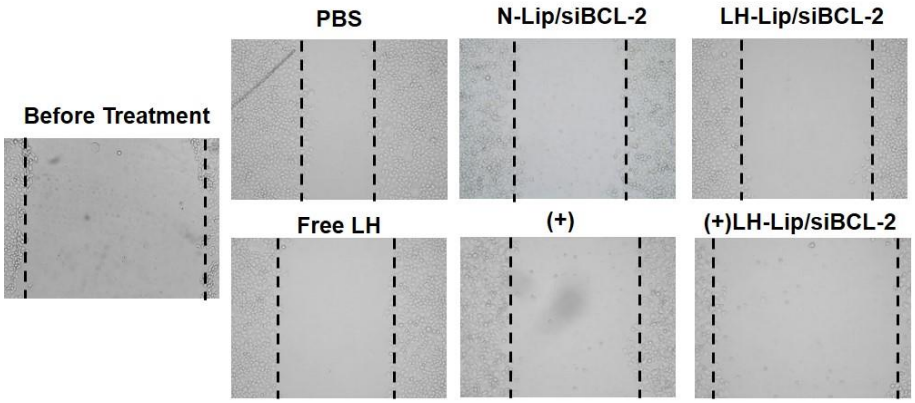

Figure S12. Images showing the inhibition of different preparations on BXPC-3 cell migration in wound healing assay. (+) represents incubation with PTX-Lip (0.3 μg/mL) for 24 h before treatment.

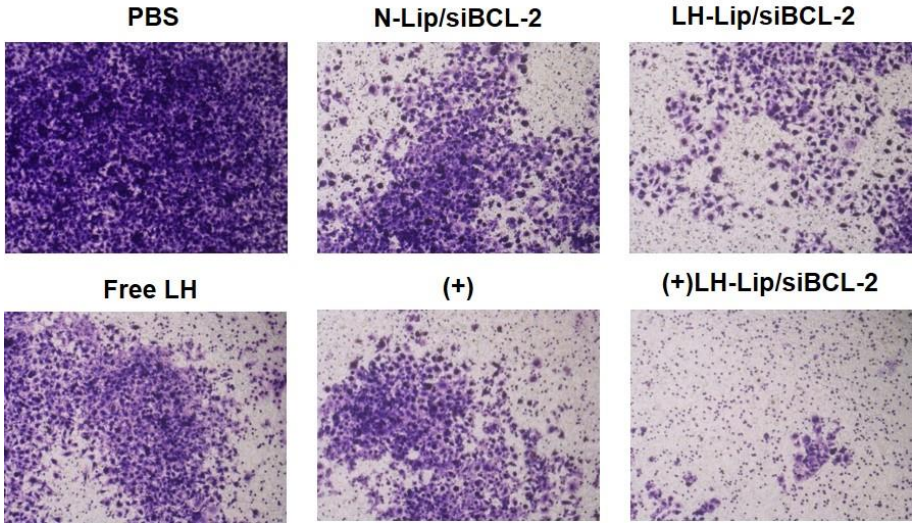

Figure S13. Images showing the inhibition of different formulations on BXPC-3 cell invasion using transwell chambers. (+) represents incubation with PTX-Lip (0.3 μg/mL) for 24 h before treatment.

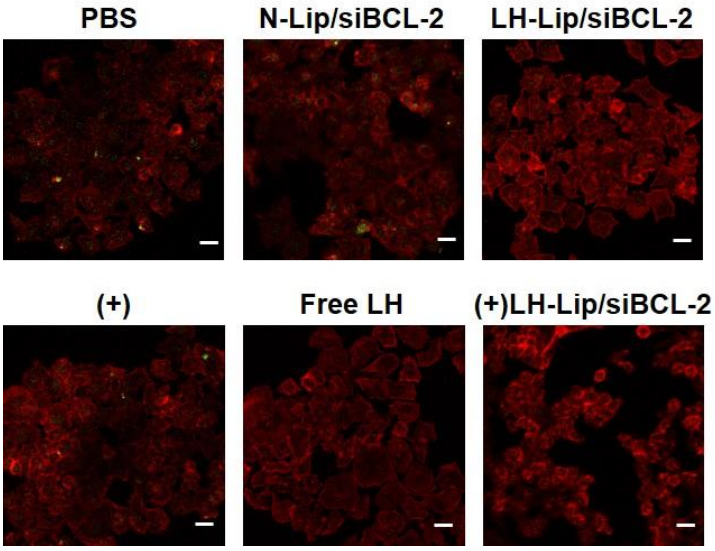

Figure S14. CLSM images of the adhesion of platelets on BXPC-3 cells after incubation with different formulations. BXPC-3 cells were stained with ActinRed (red), and platelets were stained with calcein-AM (green). Scale bar, 20 μm.

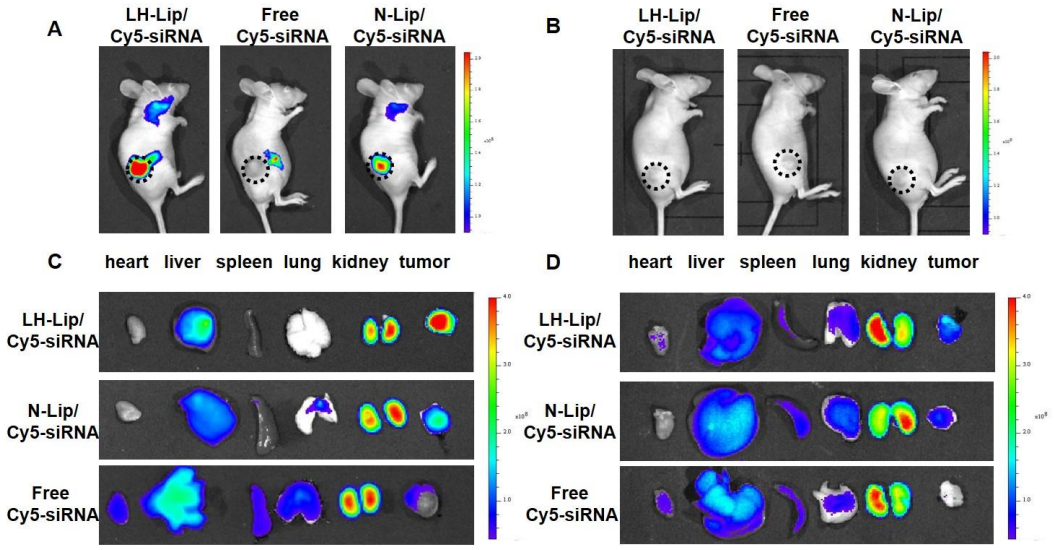

Figure S15. *In vivo* images of tumor-bearing mice after treated with different formulations for 24 h. Mice were pretreated **(A)** with or **(B)** without PTX-Lip before the treatment. Ex vivo images of organs and tumor after in vivo imaging. Mice were pretreated **(C)** with or **(D)** without PTX-Lip before the treatment.

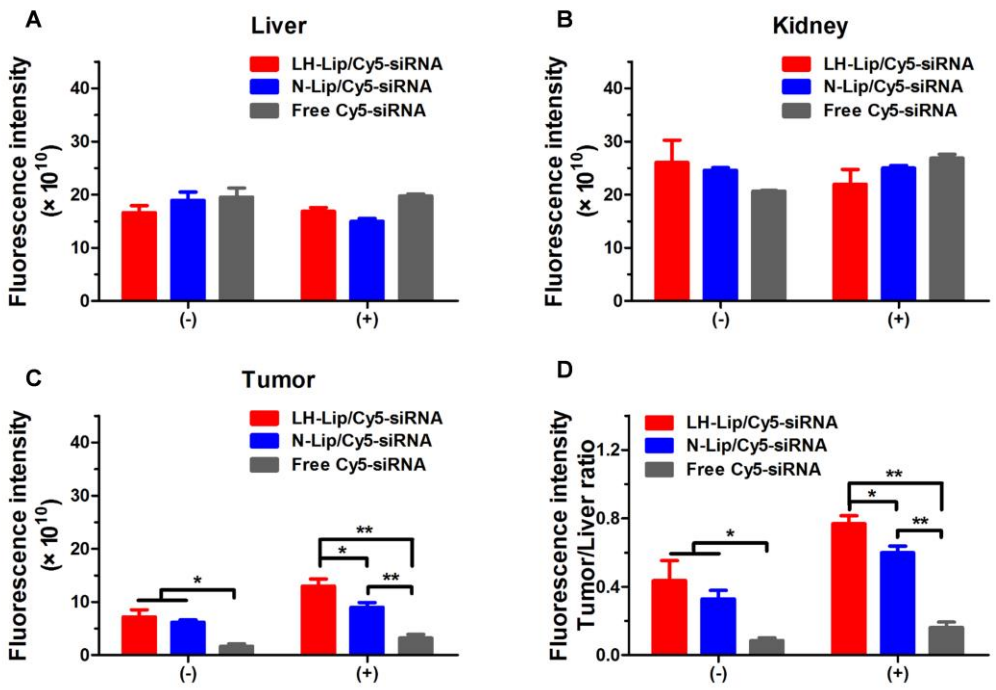

Figure S16. Semiqualitative study of fluorescence intensity in the **(A)** liver, **(B)** kidney and **(C)** tumor from the ex vivo images (n = 3). **(D)** Tumor-to-liver ratios were calculated from different groups. *P < 0.05, **P < 0.01. (+) and (-) represent injection of PTX-Lip (50 mg/m^2) or not, respectively.

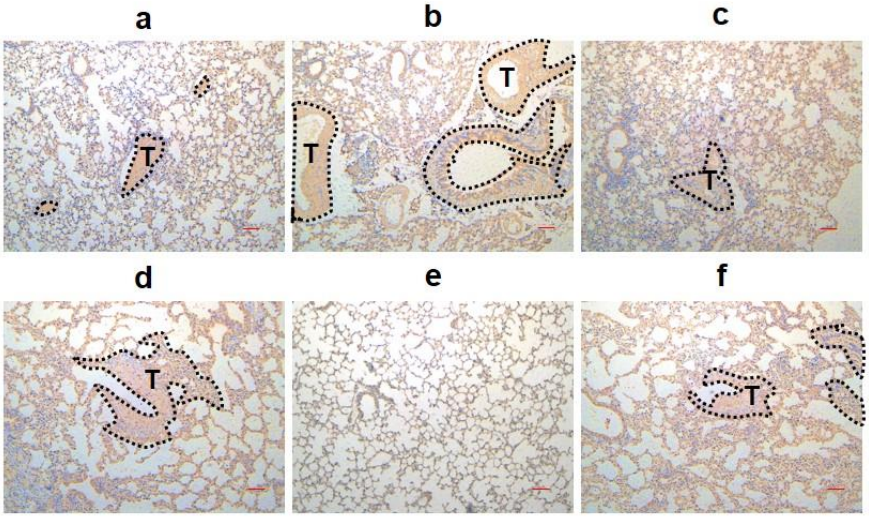

Figure S17. Ki67 staining (proliferous cells shown in brown) of lung tissues. Dashed lines mean metastasis loci. Scale bar, 100 μm. a = (+) LH-Lip/siCTL, b = (+) PBS, c = LH-Lip/siBCL-2, d = (+) N-Lip/siBCL-2, e = (+) LH-Lip/siBCL-2, f = PBS. (+) represents injection of PTX-Lip (50 mg/m^2) before treatment.