

Supporting information for

ATN-161 Peptide Functionalized Reversibly Crosslinked Polymersomes Mediate Targeted Doxorubicin Delivery into Melanoma-Bearing C57BL/6 Mice

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Materials

Methoxy poly(ethylene glycol) (MeO-PEG-OH, $M_n = 5.0$ kg/mol, Sigma) and α -amino, ω -hydroxyl poly(ethylene glycol) (NH₂-PEG-OH, $M_n = 7.5$ kg/mol, Beijing JenKem Technology) were dried by azeotropic distillation from anhydrous toluene. Trimethylene carbonate (TMC, Jinan Daigang Biomaterial Co., Ltd.) was purified by recrystallization in anhydrous ethyl acetate. Dichloromethane (DCM) was dried using a PS-MD-4 PureSolv solvent purification system (Innovative Technology, USA). Doxorubicin hydrochloride (DOX·HCl, > 99%, Beijing Zhong Shuo Pharmaceutical Technology Development Co., Ltd.), pegylated liposomal doxorubicin (DOX-LPs, LIBOD[®], Shanghai Fudan-zhangjiang Bio-Pharmaceutical Co., Ltd.), PHSCNK peptide (ATN, > 98%, Shanghai GL Biochem Ltd.), squaric acid diethyl ester (SADE, 98%, J&K), zinc bis[bis(trimethylsilyl)amide] (97%, Sigma), L-glutathione (GSH, 99%, Roche), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Beyotime Biotechnology Co., Ltd.), and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Beyotime Biotechnology Co., Ltd.) were used without further treatment. Highly metastatic murine melanoma cells (B16F10) were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. All the other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. and used as received. Dithiolane trimethylene carbonate (DTC) and PEG-P(TMC-DTC) with an M_n (¹H NMR) = 30.9 kg/mol and $\bar{D} = 1.30$ were synthesized according to our previous reports.^{1, 2} SAE-PEG-OH was readily obtained by treating NH₂-PEG-OH ($M_n = 7.5$ kg/mol) with squaric acid diethyl ester (SADE) at neutral pH similar to previous reports.³⁻⁵

Characterizations

¹H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz and an Agilent 600 DirectDrive 2 operating at 600 MHz using deuterated chloroform (CDCl₃) or deuterated dimethylsulfoxide (DMSO-*d*₆) as a solvent. The chemical shifts were

calibrated against residual solvent signals. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatography (GPC) equipped with two linear PLgel columns (500 Å and Mixed C) following a guard column and a differential refractive-index detector (RI 2414). The measurements were performed using DMF containing 0.05 mol/L LiBr as the eluent at a flow rate of 0.8 mL/min at 40 °C and a series of narrow poly(methyl methacrylate)s standards for the calibration of the columns. The size of polymersomes was determined using dynamic light scattering (DLS) at 25 °C using Zetasizer Nano-ZS (Malvern Instruments) equipped with a 633 nm He-Ne laser using back-scattering detection. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 µL of 1 mg/mL polymersome suspension on the copper grid followed by staining with 1 wt.% phosphotungstic acid. Fluorescence spectra were recorded using a FLS920 fluorescence spectrometer. Confocal Laser Scanning Microscopy (CLSM) images were taken on a Leica TCS SP5. Flow cytometry was conducted on a flow cytometer (BD FACS Calibur, USA). MTT assays were quantified by measuring the absorbance at a wavelength of 490 nm using a microplate reader (Thermo Multiskan FC).

Determination of ATN substitution degree (DS) using TNBS method

ATN substitution degree (DS) of the copolymer was determined using TNBS method. Briefly, the reaction mixture of ATN and SAE-PEG-P(TMC-DTC) was dialyzed (MWCO: 3500 Da) against 400 mL of pure water for 24 h. To determine the amount of ATN in the dialysis medium using TNBS method, the dialysis medium was concentrated to 3.0 mL and 100 µL was withdrawn and diluted with 2.9 mL of borate buffer (pH 9.2, 10 mM). Then excess picrylsulfonic acid solution (TNBS, 5 wt.%, 20 µL) was added and incubated at 37 °C for 4 h before measuring UV absorbance at 424 nm. The unconjugated ATN was determined based on a calibration curve obtained from ATN of known concentrations in borate buffer. The

conjugated ATN could be calculated by subtracting the unconjugated ATN from the feed. The final ATN substitution degree (DS) was determined to be *ca.* 98.4%.

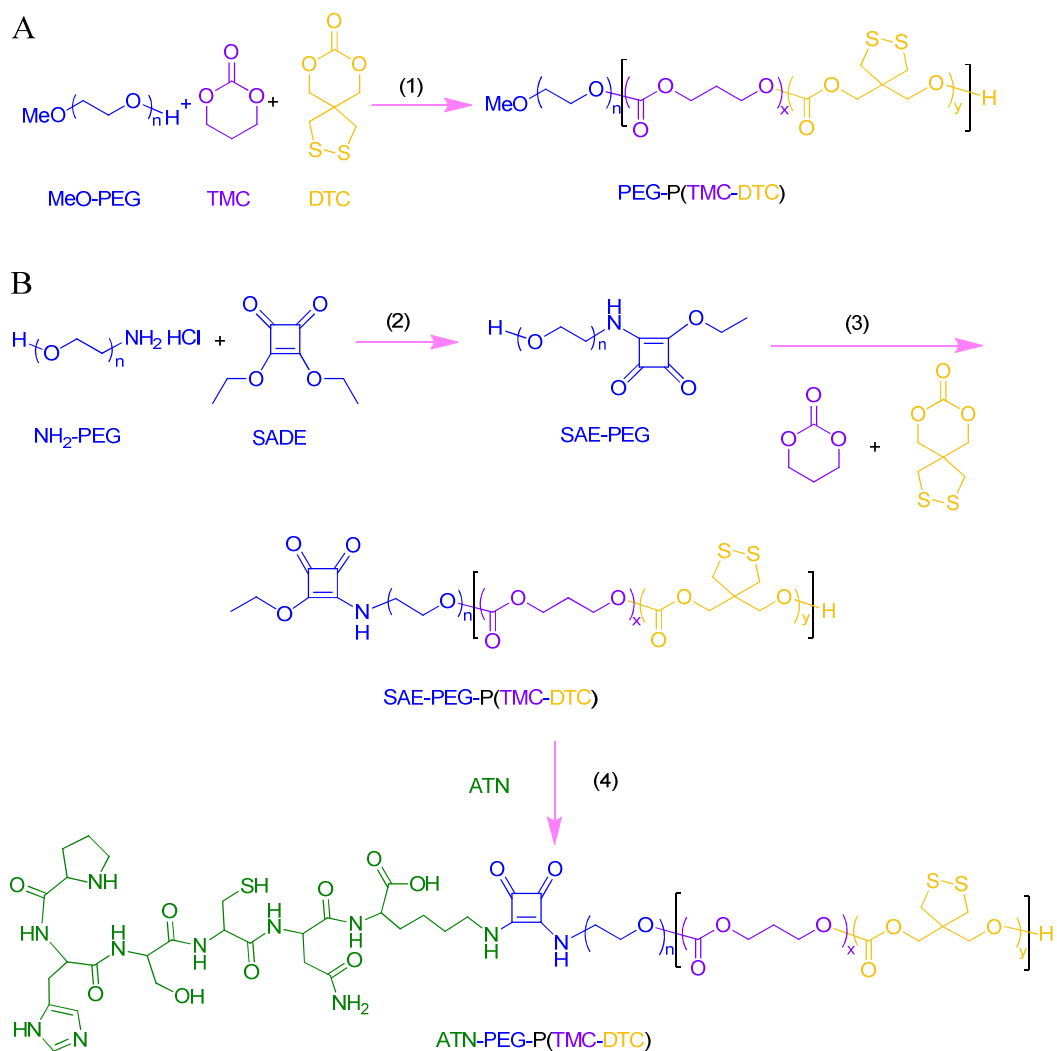
The preparation of DOX-ATN/SCID-Ps and *in vitro* drug release

A pH-gradient method was used to load DOX·HCl into polymersomes. 100 μ L of DMF solution (10 mg/mL) containing ATN-PEG-P(TMC-DTC) and PEG-P(TMC-DTC) at a predetermined weight ratio was added into 900 μ L of citrate buffer (10 mM, pH_{in} = 4) to form polymersomes. After 4 h incubation saturated Na₂HPO₄ solution was added into the dispersion to adjust pH_{out} to 7.15, 7.80 or 8.35 in order to build up a pH gradient across the polymersome membrane. DOX·HCl (5 mg/mL) was added immediately at a theoretical drug loading content (TDLC) of 10, 15 and 20 wt.% under stirring at 37 °C. The dispersion was stirred for 12 h. Extensive dialysis (MWCO: 3500 Da) against PB for 24 h at r.t. was applied to remove excess drug. To determine the drug loading content (DLC) and drug loading efficiency (DLE), an aliquot (100 μ L) of DOX-ATN/SCID-Ps dispersion was taken and dissolved in 2.9 mL of DMF followed by fluorometry measurement (ex. 480 nm, em. 560) based on a calibration curve obtained from DOX·HCl of known concentrations. The DLC and DLE were calculated according to the following formula:

$$\text{DLC (wt.\%)} = (\text{weight of loaded drug} / \text{total weight of loaded drug and polymer}) \times 100$$

$$\text{DLE (\%)} = (\text{weight of loaded drug} / \text{weight of drug in feed}) \times 100$$

The *in vitro* DOX·HCl release from ATN/SCID-Ps and SCID-Ps was investigated using a dialysis tube (MWCO 12 kDa) at 37 °C. Briefly, 0.5 mL of DOX-ATN/SCID-Ps or DOX-SCID-Ps at 50 mg/L was dialyzed against 25 mL of PB (10 mM, pH 7.4) or PB (10 mM, pH 7.4) containing 10 mM GSH. At desired time intervals, 5 mL of release media were taken out and replenished with an equal volume of fresh media. The drug released was quantified using fluorometry. The release experiments were conducted in triplicate, and the results presented were the average data with standard deviations.



Scheme S1. Synthesis of PEG-P(TMC-DTC) (A) and ATN-PEG-P(TMC-DTC) (B). Conditions: (1) Zinc bis[bis(trimethylsilyl)amide], DCM, 40 °C, 24 h; (2) Triethylamine, water/ethanol = 1/1, r.t., 16 h; (3) Zinc bis[bis(trimethylsilyl)amide], DCM, 40 °C, 24 h; (4) DMSO/borate buffer = 1/3, r.t., 48 h.

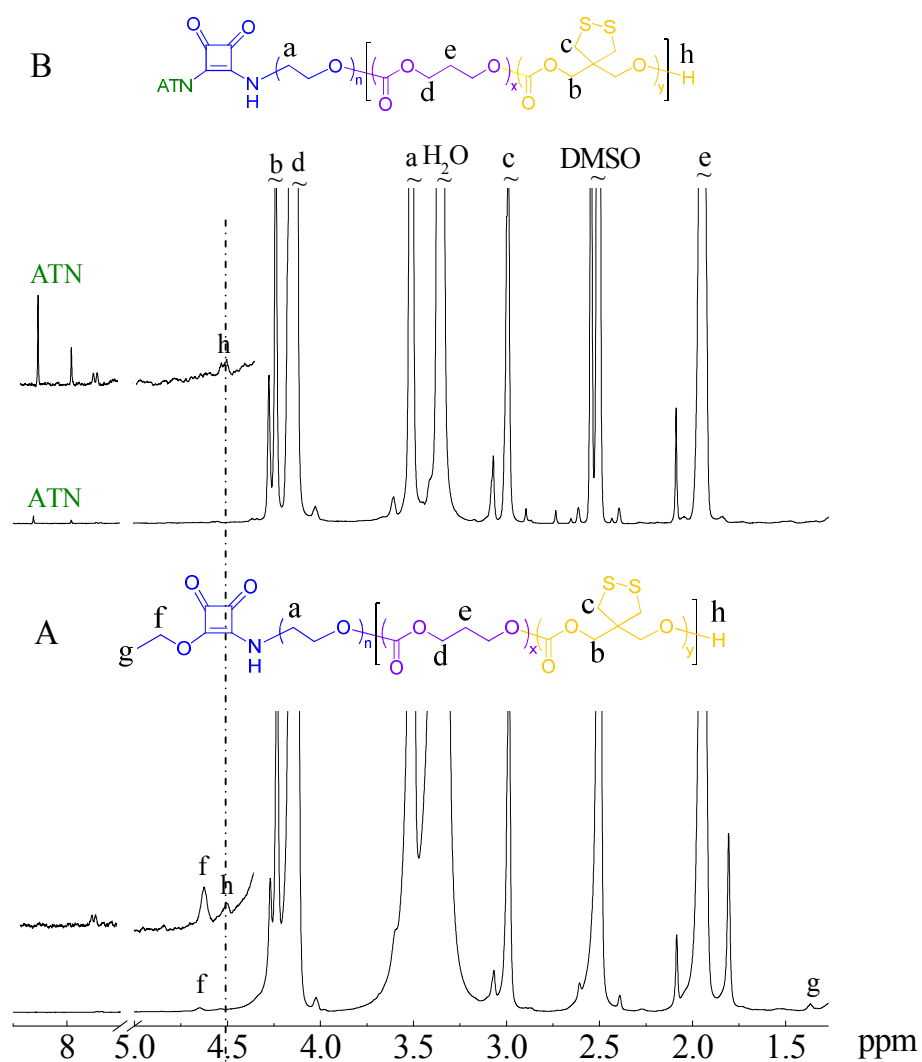


Figure S1. ^1H NMR spectra (600 MHz, $\text{DMSO-}d_6$) of SAE-PEG-P(TMC-DTC) (A) and ATN-PEG-P(TMC-DTC) (B).

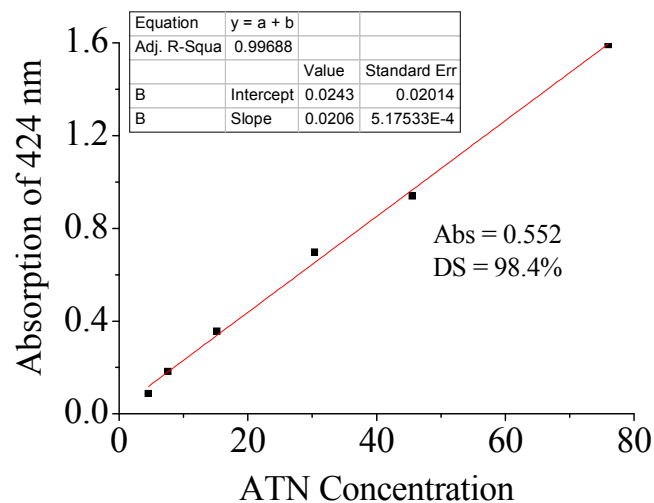


Figure S2. The calibration curve of ATN with known concentrations by UV spectra (424 nm).

Table S1. Characteristics of DOX-SCID-Ps (1 mg/mL)

Theoretical DLC (wt.%)	pH _{out} ^a	Size(nm) ^b	PDI ^b	DLC ^c (wt.%)	DLE ^c (%)
10	7.80	128±3	0.14±0.03	4.8	48.1
15	7.80	126±3	0.16±0.02	9.6	63.7
20	7.15	136±4	0.17±0.02	5.1	25.6
20	7.80	149±5	0.18±0.02	15.5	77.3
20	8.35	195±4	0.22±0.01	19.8	99.1

^a pH_{in} = 4.0, pH_{out} was adjusted to different pH values;

^b Determined by DLS in PB (pH 7.4, 10 mM);

^c Measured by fluorometry.

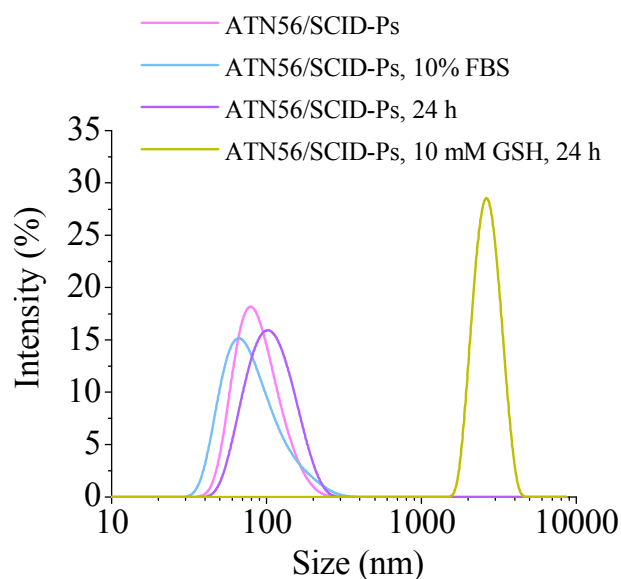


Figure S3. Stability of ATN56/SCID-Ps in cell culture medium containing 10% FBS and in response to 10 mM GSH in PB (pH 7.4, 10 mM) at 37 °C.

Table S2. Summary of tumor-to-normal tissue (T/N) distribution ratios of different formulations at 6 h after *i.v.* injection. Data are presented as mean \pm SD (n = 3).

Entry	Heart	Liver	Spleen	Lung	Kidney
ATN/SCID-Ps	2.53 \pm 0.57	0.58 \pm 0.06	1.70 \pm 0.52	2.24 \pm 0.58	1.01 \pm 0.17
SCID-Ps	0.98 \pm 0.22	0.17 \pm 0.01	0.55 \pm 0.17	0.77 \pm 0.18	0.43 \pm 0.04
DOX-LPs	0.49 \pm 0.14	0.13 \pm 0.04	0.31 \pm 0.07	0.43 \pm 0.22	0.22 \pm 0.02

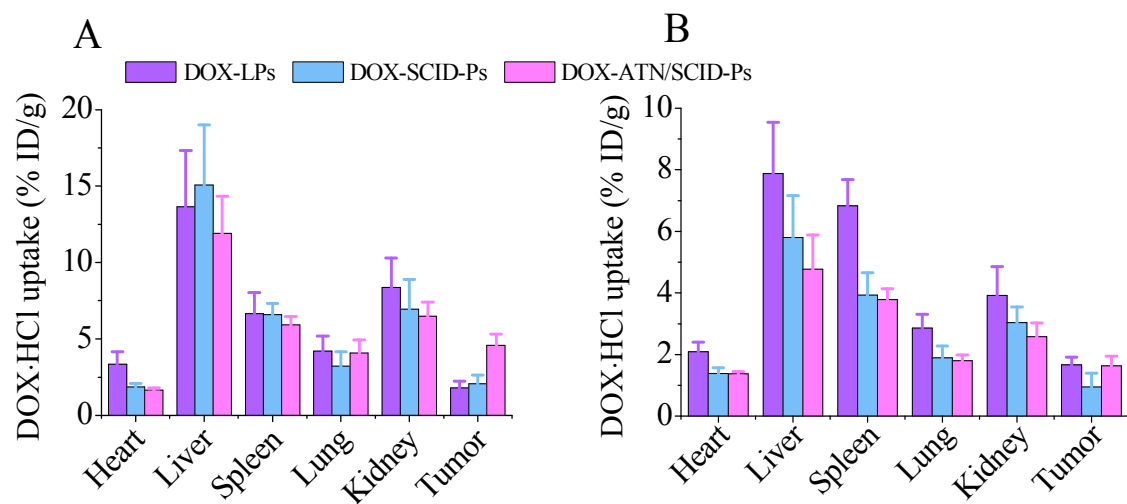


Figure S4. The *in vivo* biodistribution of DOX-ATN/SCID-Ps, SCID-Ps and DOX-LPs in C57BL/6 mice bearing B16F10 xenografts at 10 h (A) or 24 h (B) post-injection at 10 mg DOX·HCl/kg (n = 3).

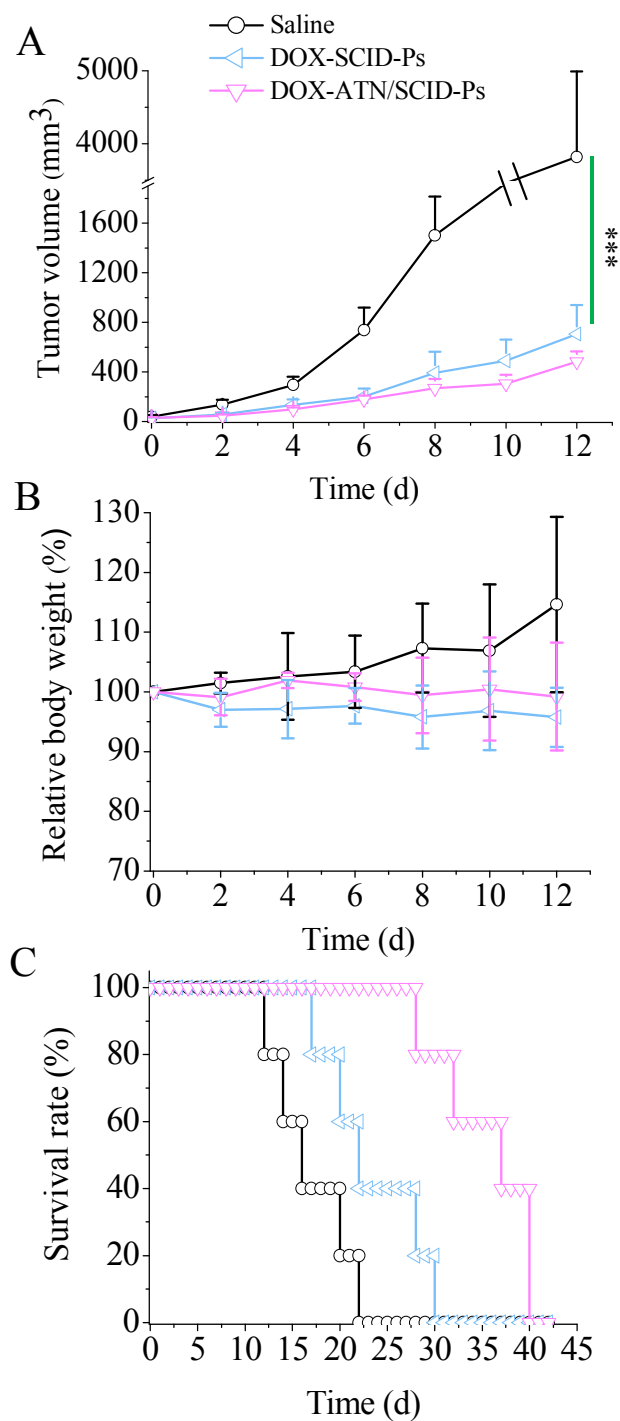


Figure S5. *In vivo* antitumor activity of DOX-ATN/SCID-Ps and DOX-SCID-Ps DOX-LPs in B16F10 tumor-bearing C57BL/6 mice after a single i.v. injection at 100 mg/kg. PBS was used as a control. (A) Tumor volume change of the mice (n = 6). (B) Relative body weight change of mice within 12 days (n = 6). (C) Survival rates of mice (n = 5). One-way ANOVA with Tukey multiple comparison tests, ***p < 0.001.

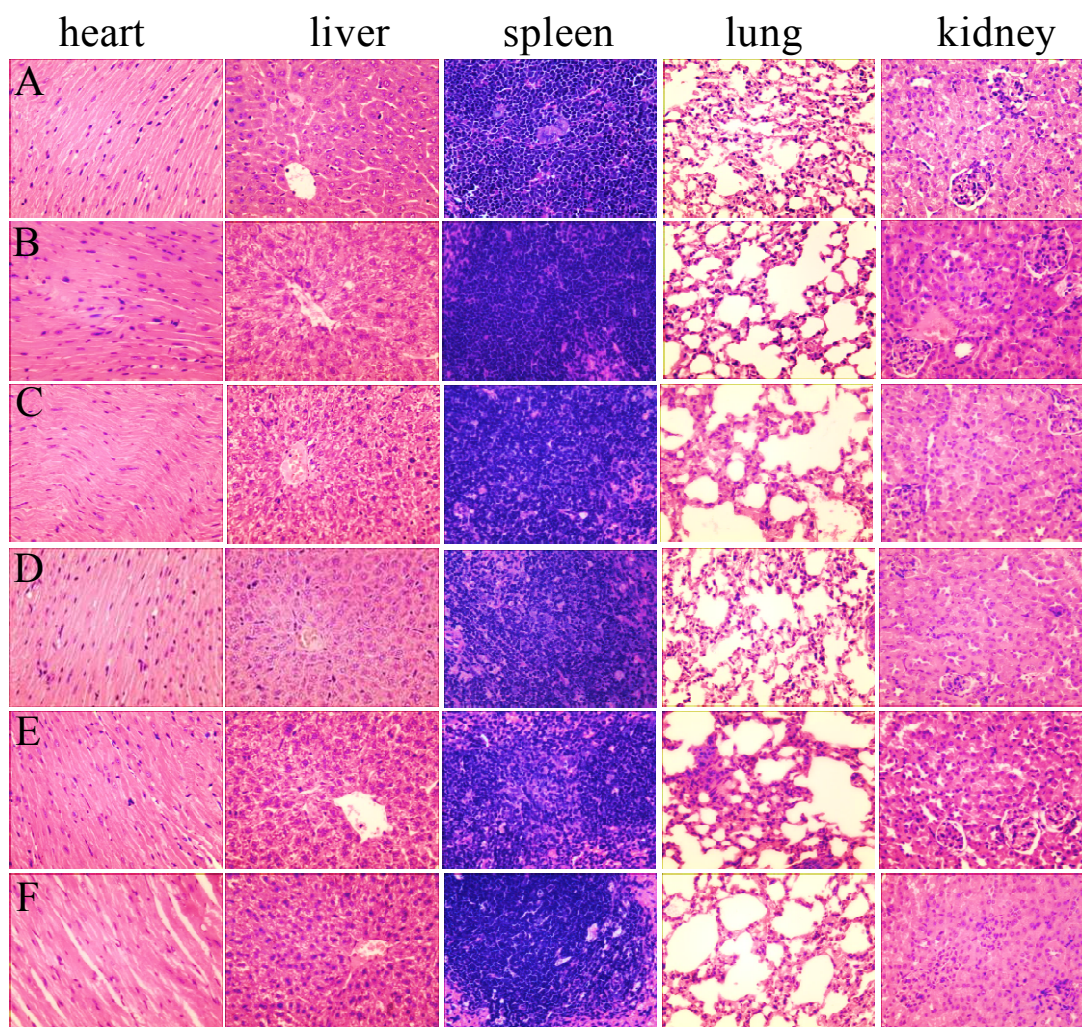


Figure S6. Microscopic images (40 ×) of H & E stained sections of major organs excised from B16F10 tumor bearing mice following 12 day treatment with saline (A), DOX-SCID-Ps/M at 20 mg/kg (B), DOX-SCID-Ps/S at 100 mg/kg (C), DOX-ATN/SCID-Ps/M at 20 mg/kg (D), DOX-ATN/SCID-Ps/S at 100 mg/kg (E), and DOX-LPs at 10 mg/kg (F).

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