Supporting Information

for

Co-delivery of All-trans-retinoic Acid Enhances Anti-metastasis Effect of Albumin-Bound Paclitaxel Nanoparticles

22 August 2024

Note added after first publication: This supplementary information file replaces that originally published on 05 December 2016. The original version of the supplementary information contained incorrect data in Fig. S3. This has been corrected in this updated version. Please see the separate correction notice for details. The correction of the figure does not affect the conclusions of the article.

Experimental Details

Cell culture: 4T1 cell line we purchased from Cell Bank of Shanghai, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM containing 10% FBS at 37 ℃ in a humidified and 5% CO_2 incubator.

Animals: Balb/c nude mice (5 weeks old) were purchased from the Shanghai slack laboratory animal Co., LTD (Shanghai, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use Committee.

Determination of particle size and size distribution: The size distribution of drug loaded HSA nanoparticles in PBS were measured by a Malvern Zetasizer Nano ZS90 with a He-Ne laser (633 nm) at 90 ℃collecting optics. The data were analyzed by Malvern Dispersion Technology Program 4.20. The stability of nanoparticles was evaluated in DMEM containing 10% FBS by monitoring the diameter at 37 ℃ over one week.

HPLC analysis of drug loading content and encapsulation efficiency: The drug loading content (DLC) and drug loading efficiency (DLE) were analyzed using a high-performance liquid chromatography (HPLC) on an Agilent 1200 system equipped with a C18 column (250×4.6 mm, 5 μm). HPLC profiles were recorded on UV detection at 227 nm for PTX and 340 nm for ATRA. 0.1% CF_3COOH in H_2O (A) and CH_3CN /H_2O (B) were used as eluents in a flow rate of 1.0 mL/min. Linear gradient was used from 55% B (0-5 min) to 80% B in 5-10 min. Drugs loaded in HSA nanoparticles were extracted using acetonitrile by vortex for 10 min and ultrasonication for 20 min. HSA nanoparticles were removed by centrifugation at 16000 rpm for 10 min, and the supernatant was collected for HPLC analysis. DLC and DLE were calculated according to following equations:

$$\text{DLC \%} = \frac{\text{Weight of PTX or ATRA in the nanoparticles}}{\text{Weight of the drug loaded nanoparticle}} \times 100\%$$

$$\text{DLE \%} = \frac{\text{Weight of PTX or ATRA in the nanoparticles}}{\text{weight of PTX or ATRA in feeding}} \times 100\%$$

***In vitro* drug release of drug-loaded nanoparticles:** The drug release from nanoparticles was analyzed by dialysis against the release medium (PBS pH 7.4, containing 10% ethanol). 2 mL of nanoparticles in the dialysis tubing (MWCO 14 kDa) was immersed in 15 mL of the release medium at 37 ℃. The release medium was replaced with fresh media at each predetermined time. The drug concentration of each samples were measured by HPLC as above.

Real-time Quantitative PCR: 4T1 cells were seeded at a density of 1×10^6 cells per well onto 6-well tissue culture plates in complete DMEM culture medium. After 12 h incubation, cells were treated with various formulations of PTX and/or ATRA and incubated for 24 h. The total RNA in the cells was then collected using RNAiso Plus (TaKaRa, Dalian, China). 500 ng of total RNA was transcribed into cDNA using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). The mRNA levels of Oct4, Nanog and Sox2 were measure using real-time quantitative PCR (qPCR) on a LightCycler® 480 SYBR

Green I Master (Roche Applied Science, Indianapolis). The mRNA levels were normalized against the housekeeping gene GAPDH. The qPCR starting with 10 min of preincubation at 95 ℃ followed by 40 amplification cycles. Sox2, Oct4 and Nanog mRNA levels were finally normalized to cells with PBS treatment alone. The following primers were used in the analysis:

Sox2 forward: 5'-GCTGGACTGCGAACTGGAGAAG-3';
Sox2 reverse: 5'-TTGCGTTAATTTGGATGGGATTGGTG-3';
Oct4 forward: 5'-GTGTGAGGTGGAGTCTGGAG-3'
Oct4 reverse: 5'-GAGTAGAGTGTGGTGAAGTGG-3';
Nanog forward: 5'-CTTGGTGTGTTAGTGTATTTGTCTTAG-3';
Nanog reverse 5'-CTGGTCCTTCTGTCTCATCCTC-3';
GAPDH forward: 5'-AAGGTGGTGAAGCAGGCATC-3';
GAPDH reverse 5'-GAAGGTGGAAGAGTGGGAGTTG-3'.

Materials: Human serum albumin (HSA) was purchased from Solarbio Biotech Co., Ltd. (Beijing, China). All-trans retinoic acid (ATRA) and paclitaxel (PTX) were purchased from D&B Chemical Technology Co., Ltd. (Shanghai, China). Trypsin-EDTA and fetal bovine serum (FBS) were obtained from Gibco-BRL (Burlington, Canada). Dulbecco's high glucose modified eagles medium (DMEM) was purchased from HyClone (Logan, UT). Gelatin (from porcine skin, Type A) was purchased from Sigma-Aldrich (St. Louis, MO). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) and crystal violet were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Ultra-purified water was prepared using a Milli-Q Synthesis System (Millipore, Bedford, MA). All other solvents and reagents were used as received.

Preparation of ATRA- and PTX-loaded nanoparticles: PTX- and ATRA-loaded HSA nanoparticles were prepared by a self-assembly method modified from the literature method.[1, 2] 5 mg of HSA was dissolved in 2.5 mL of ultra-purified water and 17.5 μL of β-ME or GSH (to a final concentration 50 mM) was added into the solution. The mixture was incubated at 37°C for 10 minutes. PTX and ATRA were dissolved in DMSO in 20 mg/mL, 100 μL PTX or ATRA solution was slowly added into the HSA solution under stirring. Then the mixture was incubated at 37 ℃ for 30 minutes in water bath. The resulting product was dialyzed (MWCO 14 kDa) against PBS for 12 h to remove β-ME, GSH, DMSO and free drugs. The resulting drug-loaded nanoparticles were denoted as PTX-NPs, ATRA-NPs or PTX/ATRA-NPs, indicating the HSA nanoparticles encapsulated with PTX, or ATRA, or PTX and ATRA together, respectively.

***In vitro* cytotoxicity assays:** 4T1 cells were seeded in 96-well plates at 4000 cells/well in 100 μL of DMEM containing 10% FBS. Cells were incubated in a humidified and 5% CO_2 incubator at 37 ℃ for 12 h for cell adhesion. The culture medium was replaced with 100 μL fresh medium containing free drugs or drug-loaded nanoparticles. The cells were further incubated for 72 h, and then the medium was replaced with fresh culture medium and the MTT solution was added. The cells were incubated for another 4 h to allow viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Finally, 100 μL of lysis buffer was added to wells and cells were incubated for another 4 h at 37 ℃. The absorbance was measured at 490 nm using a Bio-Rad 680 microplate reader. The IC_{50} values were calculated using GraphPad Prism software (version 6.01) based on data from three parallel experiments.

***In vitro* transwell assay:** The migration and invasion of cells were accessed using transwell assays. For migration assays, 1×10^5 cells were plated in the top chamber with a non-coated membrane (24-well insert, 8 mm pore size). For invasion assays, 2×10^5 cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert, 8 mm pore size). In both assays, cells were plated in medium without serum, pre-incubated with free drugs or drug-loaded nanoparticles for 24 h. Serum supplemented medium was used as a chemoattractant in the lower chamber. After 24 h incubation, cells that did not cross the pores (non-migration or non-invasion cells) were removed by a cotton swab. Cells on the lower surface of the membrane were stained with 0.1% crystal violet solution and were counted by microscopic examination. Then the dye in the migrating and invading cells on the lower surface of the membrane were dissolved using 10% acetic acid for 10 min and the absorbance of the solution was measured at 550 nm.

Gelatin zymography: 4T1 cells were seeded at a density of 1×10^6 cells per well onto 6-well tissue culture plates in complete DMEM culture medium. After 12 h, the medium was replaced by 2 mL of serum-free DMEM per plate with free drugs or drug-loaded nanoparticles. After 24 h of incubation, the media was collected and centrifuged to remove the cell debris. The centrifuged medium was used for the gelatin zymography assay. 40 μL of conditioned media was mixed with the sample buffer and electrophoresed on 10% denaturing SDS polyacrylamide gels containing 1 mg/mL of gelatin. The gel was washed four times for 20 min in renaturation buffer solution (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5% Triton X-100, pH=7.6). Then it was washed two times for 20 min in washing buffer solution (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM

$ZnCl_2$, pH=7.6). Finally, it was incubated for 48 h at 37 ℃ in incubation buffer solution (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 150 mM NaCl, 0.02% Brij-35, pH=7.6). After that, the gel was stained with Coomassie Brilliant Blue R-250 and then destained with water.

***In vivo* antitumor efficacy:** For *in vivo* antitumor experiment, 3×10^5 4T1 cells were injected to the right mammary gland of female nude mice. Tumors were allowed to grow to a volume of 100-200 mm^3. The mice bearing metastatic 4T1 breast cancer were randomly assigned to 5 groups (n=5) for the treatment of PBS, PTX-NPs, ATRA-NPs, PTX-NPs + ATRA-NPs or PTX/ATRA-NPs. Drugs were administrated through tail vein injection every other days for 11 times in a dosage of 1.5 mg/kg PTX or 5.4 mg/kg ATRA. Tumor growth was monitored by measuring the perpendicular diameter of the tumor using calipers every two days. The tumor volume was calculated according to the formula: tumor volume (mm^3) = $0.5\times length\times width^2$. Animals were sacrificed for humane reasons and the lung tissues were immediately excised for image and H&E staining.

Reference:
1. D. Ding, X. Tang, X. Cao, J. Wu, A. Yuan, Q. Qiao, J. Pan and Y. Hu, *Aaps Pharmscitech*, 2014, **15**, 213-222.
2. W. Wang, Y. Huang, S. Zhao, T. Shao and Y. Cheng, *Chem. Commun.*, 2013, **49**, 2234-2236.

Table S1. Dynamic light scattering characterization and drug loading efficiency of HSA nanoparticles.

PTX/ATRA (w/w)		Diameter[a] (nm)	PDI	DLE[b] (%)		DLC[c] (%)	
Feeding	Resultant			PTX	ATRA	PTX	ATRA
1:0	1:0	164.8±3.5	0.08	72.5	0.0	22.5	0.0
0:1	0:1	222.3±9.7	0.23	0.0	95.0	0.0	27.5
1:2	1:3.8	173.5±3.0	0.17	48.5	94.0	4.9	19.0
1:4	1:7.7	172.3±5.1	0.21	46.9	90.6	2.8	21.8
1:6	1:11.1	181.2±7.2	0.22	50.0	90.1	2.1	23.2

[a] hydrodynamic diameters.
[b] DLE: drug loading efficiency.
[c] DLC: drug loading content.

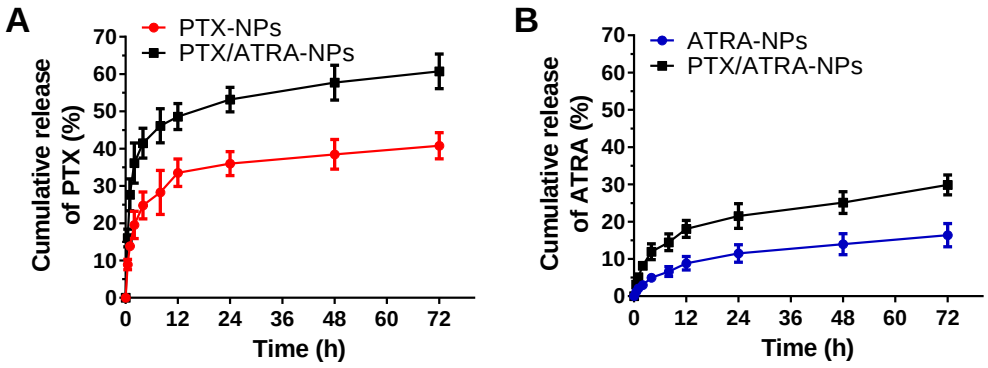

Figure S1. *In vitro* drug release kinetics. (A) Release of PTX from PTX-NPs and PTX/ATRA-NPs; (B) release of ATRA from ATRA-NPs and PTX/ATRA-NPs. The data were obtained by dialysis of the drug loaded HSA nanoparticles against PBS buffer (pH=7.4) containing 10% ethanol. Drug concentration was measured based the integration of peaks on HPLC profiles. Error bars denote standard deviations of thrice independent experiments.

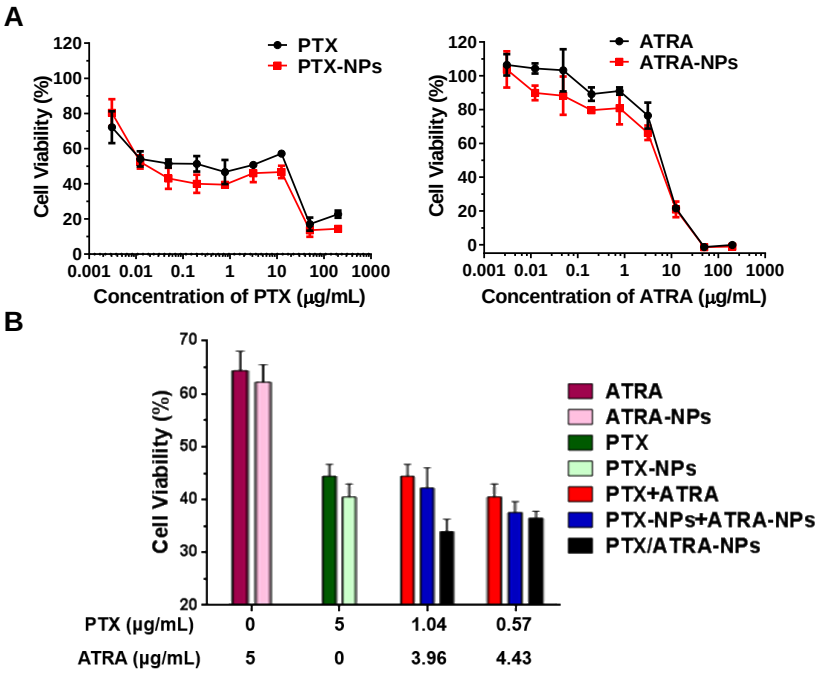

Figure S2. (A) The inhibition of cell proliferation by PTX, ATRA, PTX-loaded or ATRA-loaded nanoparticles. (B) The combination effect of PTX/ATRA and in co-delivery nanoparticles. The assay was performed on 4T1 cells with the treatment of the drug loaded-nanoparticles for 72 h. Error bars denote standard deviations of thrice independent experiments.

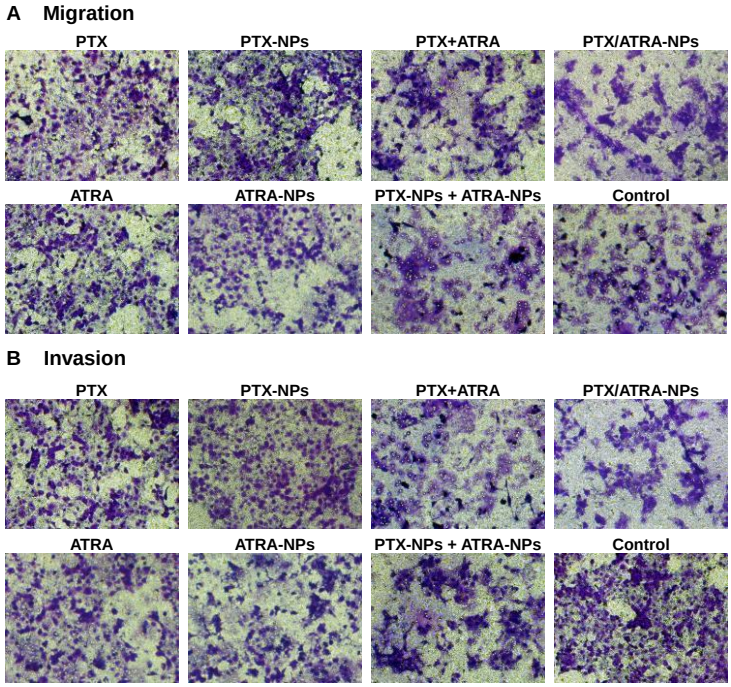

Figure S3. Migration and invasion of cancer cells measured using transwell assay. Representative fields of cells migration (A) and invasion (B). 4T1 cells were plated in top chambers in the medium without serum, pre-incubated for 24 h with drugs equivalent to 0.05 μg/mL PTX or/and 0.19 μg/mL ATRA. Medium supplemented with serum was used as a chemoattractant in lower chambers. The cells that migrated or invaded were stained with 0.1% crystal violet solution and visualized by microscopy.

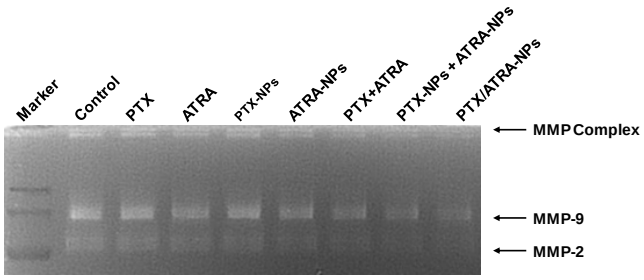

Figure S4. The activity of MMPs analyzed using gelatin zymography. 4T1 cells were cultured in 10 cm dish with DMEM containing 10% FBS. 4T1 cells with serum-free DMEM were treated with equivalent doses of PTX (0.05 μg/mL) and/or ATRA (0.19 μg/mL) in different formulations for 24 h.