Supporting Information for
ORIGINAL ARTICLE

Orange-derived extracellular vesicles nanodrugs for efficient treatment of ovarian cancer assisted by transcytosis effect

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1. Supporting methods

1.1. Stability of DN and DN@OEV

OEV, DN and DN@OEV were solved into 50% fetal bovine serum (FBS) in PBS buffer and incubated at 37 °C. At selected time points, the size distribution of different samples was detected using DLS. Measurements of the different groups were taken in triplicate and the results were analyzed.

1.2. Drug release

The release profile of DOX from DN and DN@OEV was conducted under PBS with pH 7.4 and pH 6.5, respectively. Briefly, the DNs and DN@OEV dissolved in PBS buffer were sealed in a dialysis membrane (MWCO 3500), and then immersed in 100 mL of PBS with gently shaking at 37 °C. At selected time points, the dialyzate of DOX was analyzed by UV–vis spectrometer and added to an equal volume of fresh

buffer. The drug release studies were performed in triplicate for each of the samples.

1.3. Detection of bioactive compounds of OEV

To assay vitamin C concentration in OEV, OEV were added to 1% oxalic acid buffer solution, ultrasonically extracted for 30 min, and filtered through a 0.45 μm microporous membrane to obtain the filtrate. The gradient concentration of vitamin C standard substance was prepared, and the sample solution and standard substance were detected by Shimadzu high performance liquid chromatography (HPLC) system (Tokyo, Japan). The standard curve was drawn according to the concentration and peak area of standard substance, and the sample concentration was calculated. To assay vitamin E in OEV, OEV were added to 20 mL of *n*-hexane solution, extracted by ultrasonic for 30 min, centrifuged to take the supernatant and evaporated under a gentle stream of nitrogen. The residue was dissolved in 1 mL of ethanol and filtered through a 0.45 μm microporous membrane to obtain the filtrate. The sample solution and standard substance were detected by HPLC. To determine hesperidin in OEV, the OEV were added to methanol solution, ultrasonically extracted for 30 min, and filtered through a 0.45 μm microporous membrane to obtain the filtrate. The sample solution and standard substance were detected by HPLC according to the method described above.

1.4. Western blotting

Cells, OEV and DN@OEV were extracted for total proteins using RIPA lysis buffer containing 1 mmol/L EDTA (P0013D, Beyotime Biotechnology, China) supplemented with protease inhibitors (P1006, Beyotime Biotechnology, China). The concentration of total proteins was determined using BCA protein quantification assay kit. Equal quantities of protein were separated by 5%–10% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). The PVDF membranes were blocked for 1 h and incubated with primary antibody overnight at 4 °C, followed by incubation with HRP-conjugated anti-rabbit secondary antibodies for 1 h. The signals

were detected using a Bio-Rad ChemiDoc™ MP Imaging System (Bio-Rad, USA). Western blottings were quantified using Image Lab software.

1.5. Cytotoxicity assay *in vitro*.

SKOV3 and 4T-1 cells were seeded in a 96-well plates at a density of 3×10^3 cells/well and incubated for 12 h. After that, the culture medium was replaced with fresh medium containing different concentrations of free DOX, DN and DN@OEV, and OEV (equvialent dose of DN@OEV) and co-incubated for 48 h. The medium was then replaced with fresh solution (100 μL medium plus 10 μL of MTT solution). After 4 h of incubation, the medium was removed and the wells were added to DMSO to dissolve the crystals formed by living cells, and then plates were measured by a microplate reader at 490 nm (Synergy2, Bio-Tek, USA). Cell viability was calculated as a percentage of the absorbance to that of the control experiment.

1.6. Histopathological evaluation.

The main organs (heart, liver, spleen, lung, kidney, and intestine) and primary tumors from different groups were fixed with 4% PFA for 48 h. Then, all tissues were embedded in paraffin wax, cut into sections, stained with hematoxylin and eosin (H&E) or used for immunohistochemical (IHC) analysis of Ki67 and CD34 expression. For IHC assay, antigen retrieval was conducted using 10 mmol/L citrate buffer (pH 6.0), and endogenous peroxidase was blocked by 3% hydrogen peroxide solution. Detection was performed with DAB working solution (GeneTech, China). The nuclei were counter-stained by haematoxylin. The tissues were observed by using an optical microscope (Nikon, Japan).

2. Supporting tables

Table S1 Loading weight percentage of DOX and cRGD in DN.

Component in DN	Loading weight percentage (%, *w*/*w*)
cRGD peptide	4.14 ± 0.19
DOX	12.32 ± 0.75

Data are presented as means ± SD, *n*=3.

Table S2 Loading weight of DOX and cRGD in per μg OEV in DN@OEV

Component in DN@OEV	Loading weight (μg in per μg of OEV)
cRGD peptide in DN@OEV	0.431 ± 0.05
DOX in DN@OEV	4.14 ± 0.19
cRGD peptide in cRGD-OEV	0.5 ± 0.03

Data are presented as means ± SD, *n*=3.

Table S3 Loading efficiency of DN and DN@OEV

Sample	Loading efficiency (%)		
	DOX	cRGD	DN
DN	39.78±0.72	35.21±0.43	–
DN@OEV	53.45±1.21	52.13±2.03	54.61±3.65

Data are presented as means ± SD, *n*=3. –, not applicable.

Table S4 The half maximal inhibitory concentration (IC_{50}) values of SKOV3 and 4T-1 cells.

IC_{50} (μg/mL)	DOX	DN	DN@OEV
SKOV3	0.14 ± 0.06	0.14 ± 0.05	0.06 ± 0.01
4T-1	0.05± 0.01	0.04 ± 0.0.1	0.04 ± 0.01

Data are presented as means ± SD, *n*=5.

3. Supporting figures

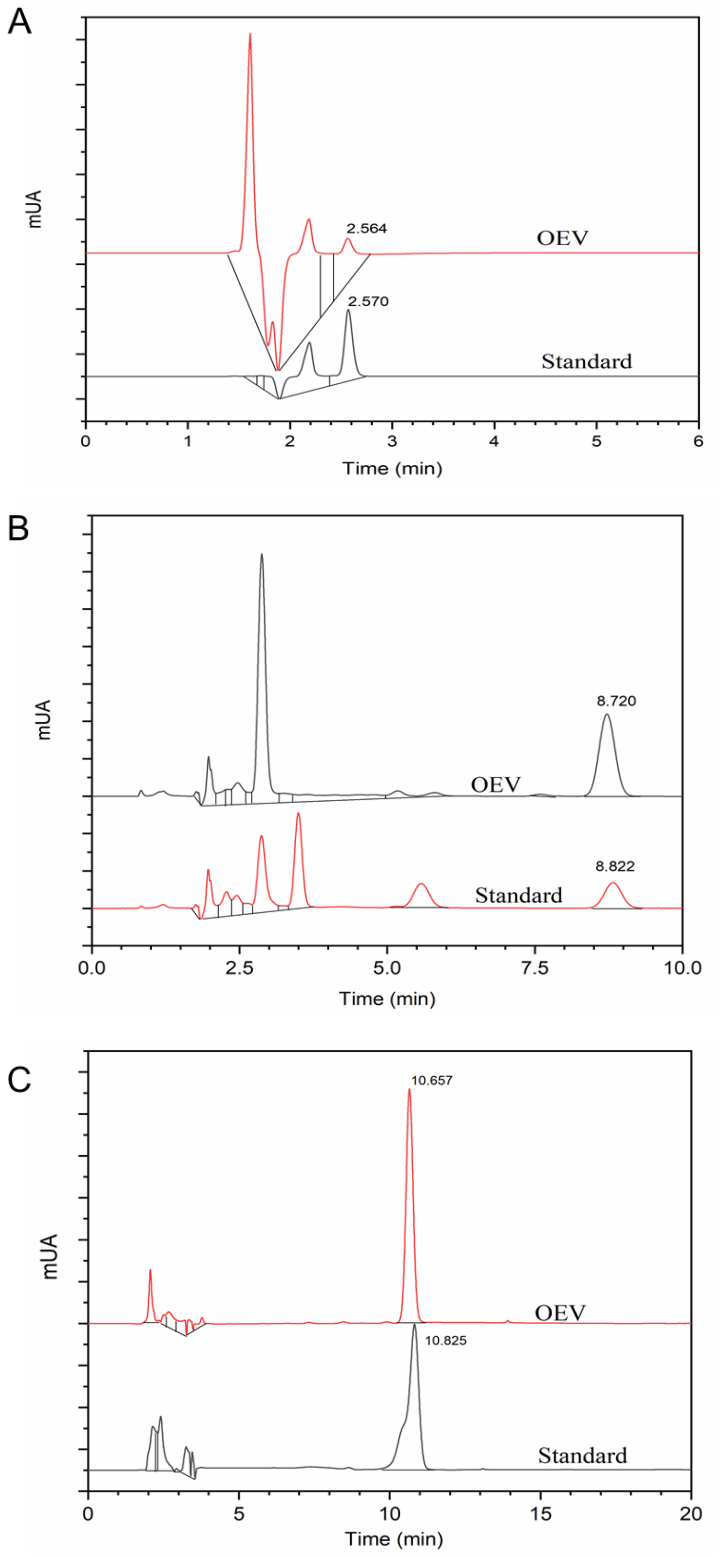

Figure S1 The detection of (A) vitamin C (B) vitamin E and (C) hesperidin in OEV using HPLC method.

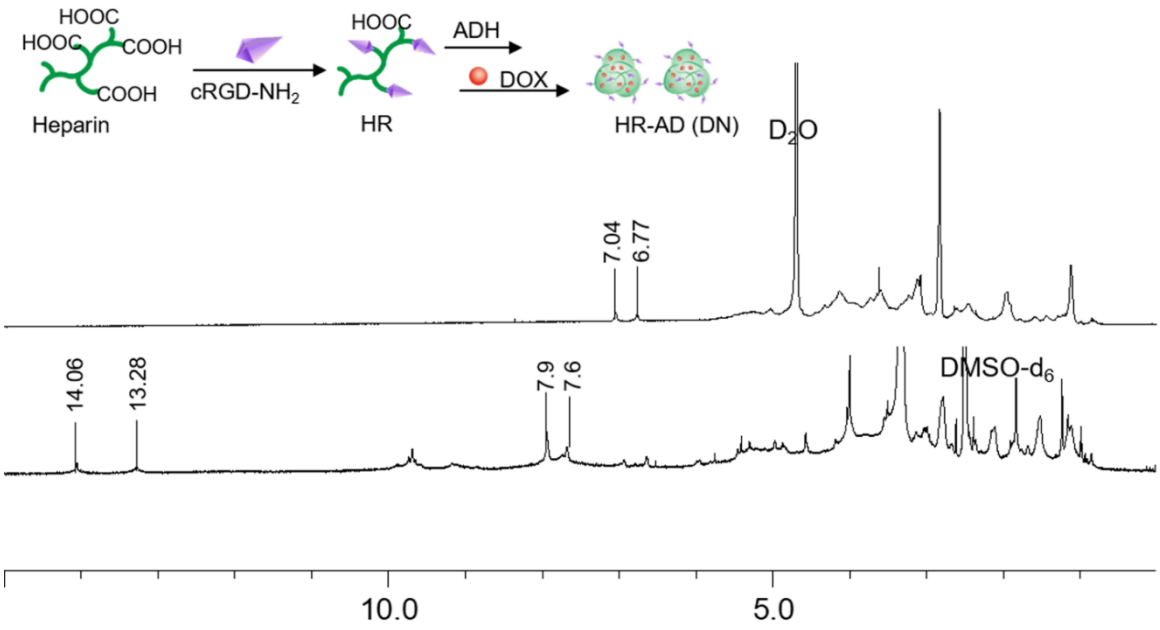

Figure S2 ^{1}H NMR spectra of DN in D_2O and DMSO-d_6 solvents.

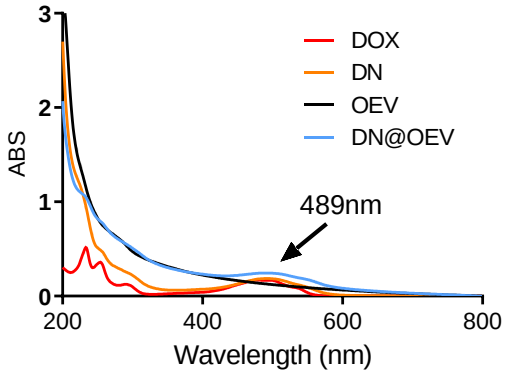

Figure S3 The UV spectrum of DOX, DN, OEV and DN@OEV in water solution.

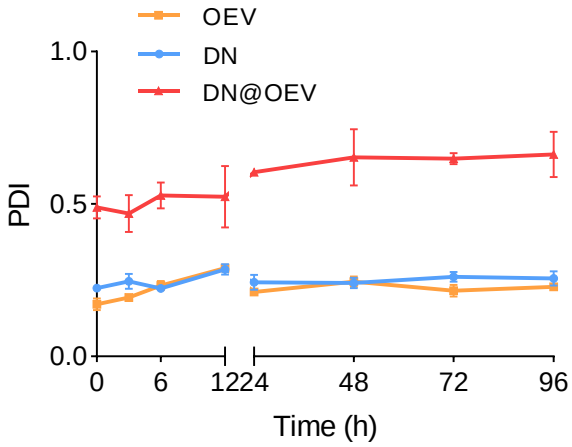

Figure S4 The stability of DN, OEV, and DN@OEV in 10% FBS determined by DLS. The data are shown as mean ± SD (n = 3).

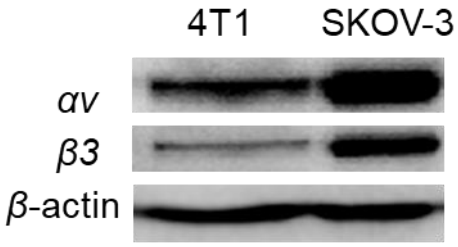

Figure S5 The expression of integrin α_v and β_3 expression in 4T-1 and SKOV3 cells detected by Western blotting.

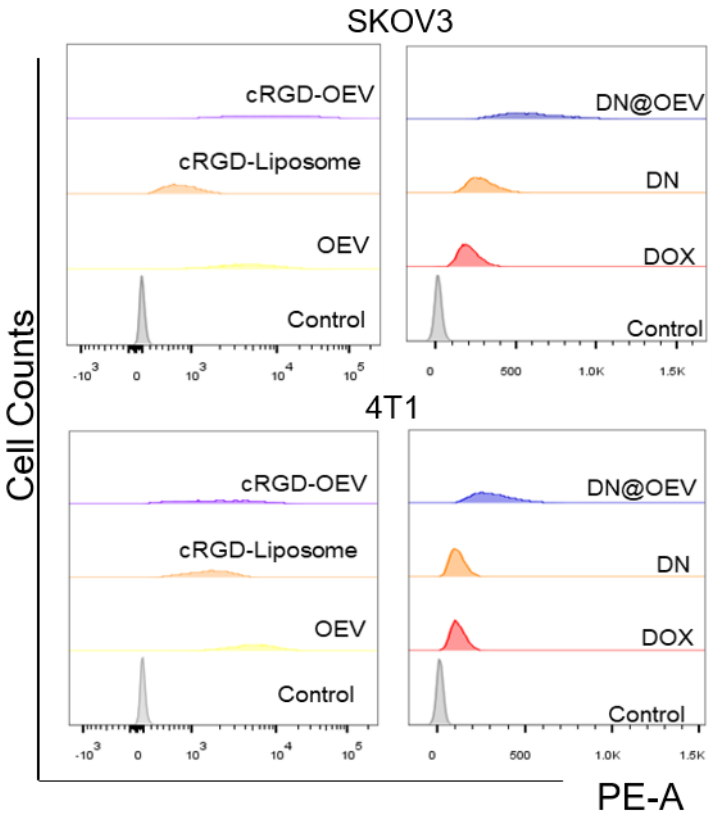

Figure S6 Flow cytometry analysis of cellular uptake of PKH26-OEV, PKH26-cRGD-OEV, DOX, DN and DN@OEV treated with SKOV3 and 4T-1 cells for 4 h. The data are shown as mean ± SD (*n*=3).

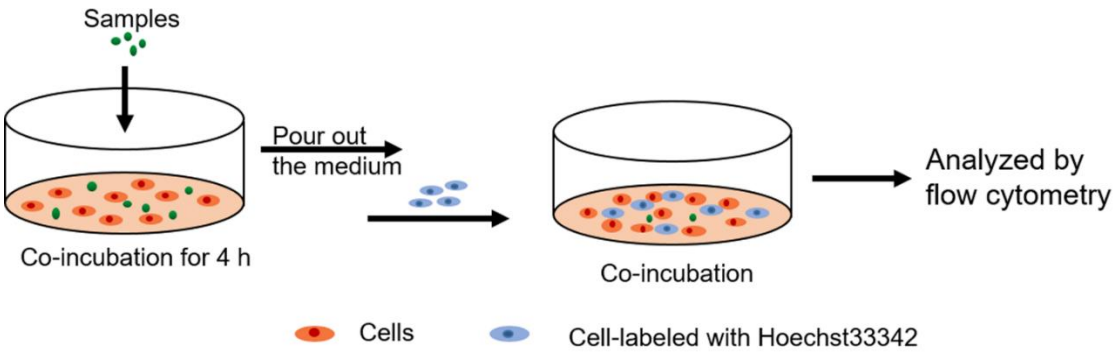

Figure S7 Protocol of transcytosis experiments in the cells and Hochest-labeled cells treated with samples. Flow cytometry detection of transcytosis effect of PKH26-OEV and PKH26-cRGD-OEV co-incubated with SKOV3 and 4T-1 cells for 4 h and then added Hochest-labeled SKOV3 and Hochest-labeled 4T-1 to cells for incubation with different time.

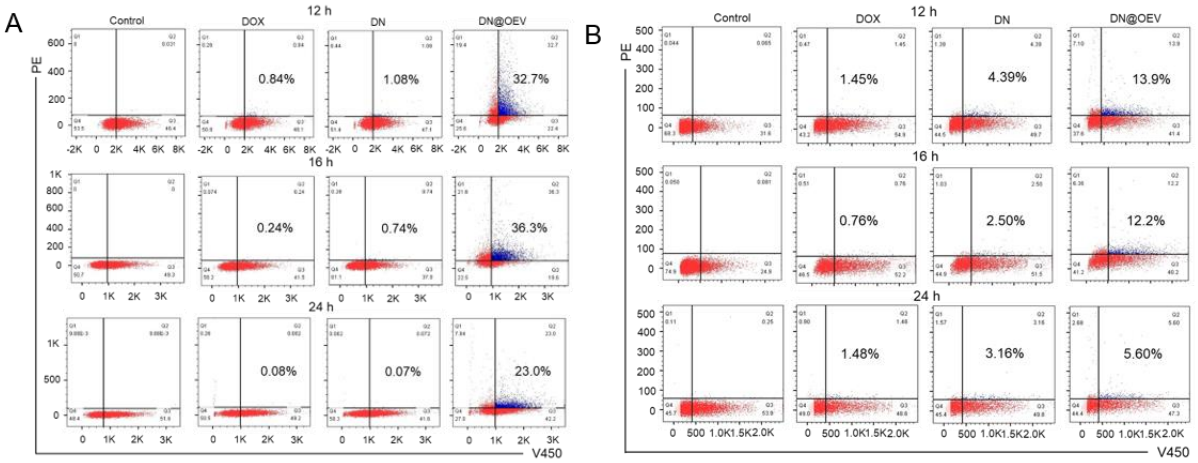

Figure S8 Flow cytometry detection of transcytosis effect of DOX, DN and DN@OEV co-incubated with (A) SKOV3 and (B) 4T-1 cells for 4 h and then added Hochest-labeled SKOV3 and Hochest-labeled 4T-1 to cells for incubation with 12, 16 and 24 h.

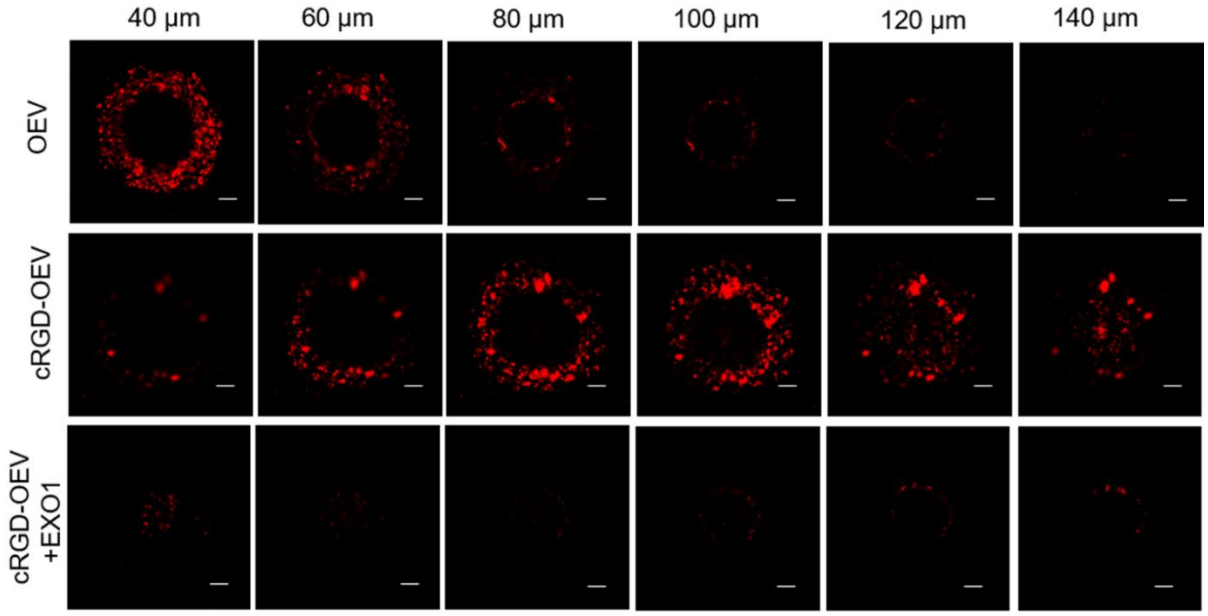

Figure S9 LSCM observation of the penetration ability of PKH26-OEV and PKH26-cRGD-OEV with or without the exocytosis inhibitor Exo1 in LN229 MCSs. Scale bar: 100 μm.

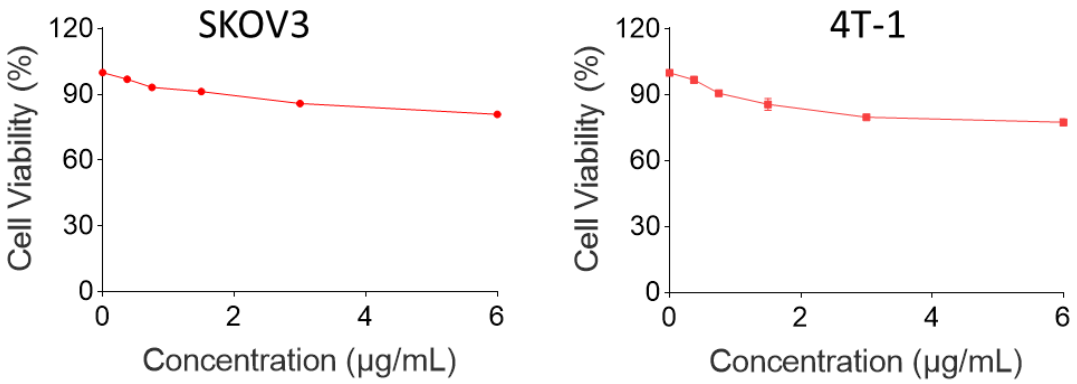

Figure S10 The cytotoxicity of OEV in SKOV3 and 4T-1 cells using MTT assay. The data are shown as mean ± SD (*n* = 3).

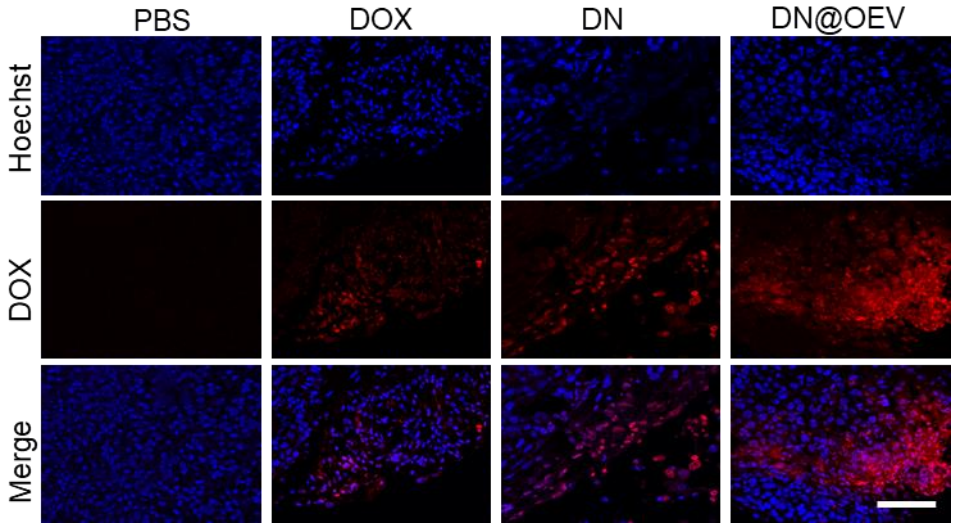

Figure S11 Accumulation of DOX, DN and DN@OEV in tumor section of bearing SKOV3 ovarian cancer nude mice after intraperitoneal injection. Scale bar: 50 μm.

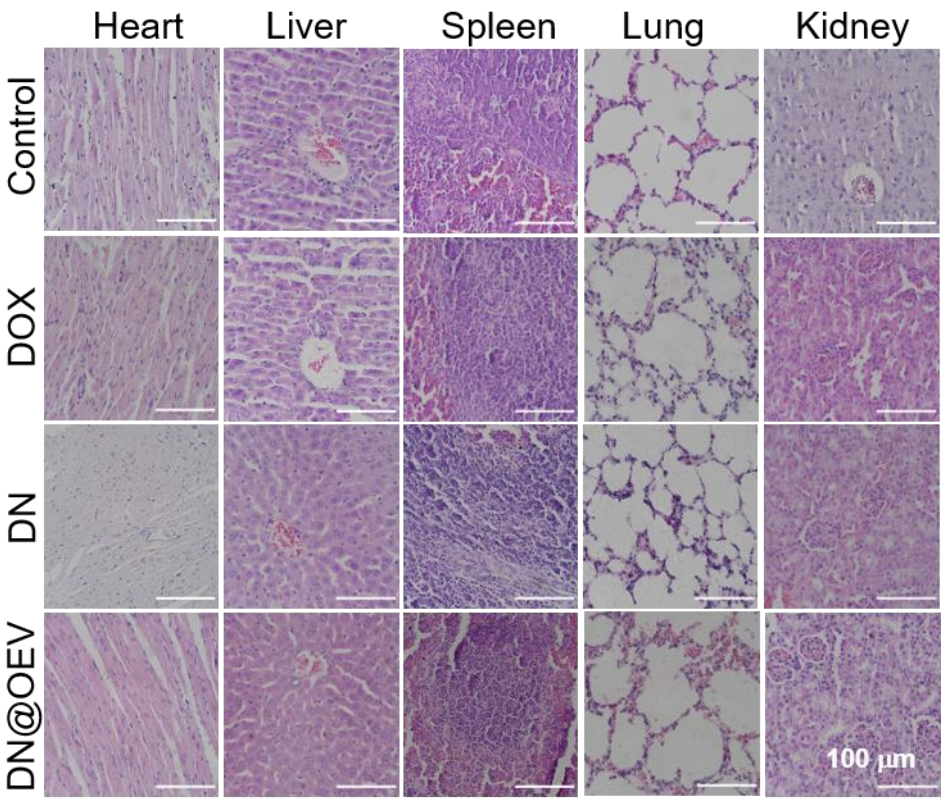

Figure S12 Histological sections of major organs in SKOV3-bearing nude mice in different treatment groups stained with hematoxylin and eosin. The major organs (including heart, liver, spleen, lung and kidney) of SKOV3 tumor-bearing nude mice in different treatment groups were harvested after the mice died and the histological sections of organ tissues were stained by H&E and no acute organ injury was found. Scale bar: 100 μm.

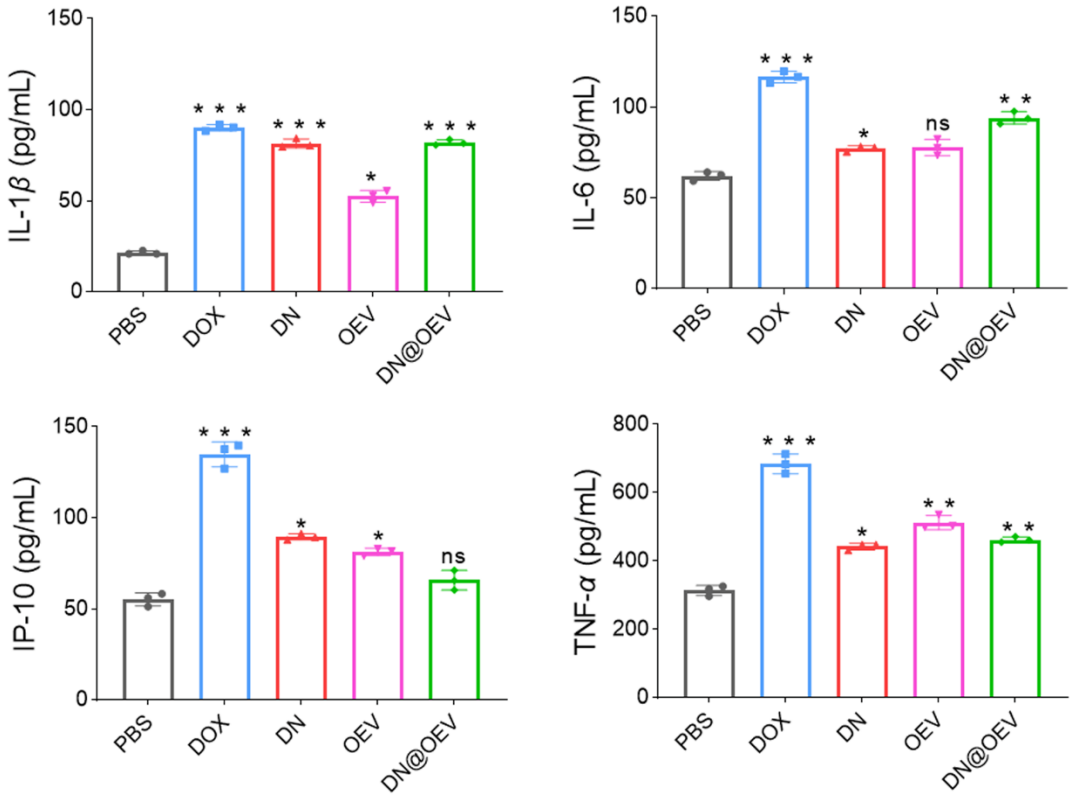

Figure S13 Cytokine concentrations (IL-1β, IL-6, IP-10 and TNF-α) of C57BL/6 mice serum after administration of PBS, DOX, DN and DN@OEV (3 mg/kg DOX), OEV (10 mg/kg EV equivalently to DN@OEV) measured by ELISA. Results are represented as mean ± SD (n=3). *P < 0.05, **P < 0.01, and ***P < 0.001. *ns*, not significant.