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Supporting Information

DTX@VTX NPs Synergy PD-L1 Immune Checkpoint Nanoinhibitor to Reshape Immunosuppressive Tumor Microenvironment for Enhancing Chemo-Immunotherapy

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Supporting methods

Materials: The BMS-1, BMS-202, BMS-1166, Docetaxel and Motolimod compounds were purchased from MedChemExpress (USA). The BMS-8 compound was ordered from Chemical Book (USA). Other reagents were ordered from Sigma-Aldrich (USA). The anti-PD-L1 antibody. (ab238697) used for immunohistochemistry and anti-CD8 antibody (ab33786) used for immunofluorescence were obtained from Abcam (USA). All fluorescein-conjugated antibodies used for immunofluorescence imaging and flow cytometry were ordered from eBioscience (USA). Various cytokine kits were purchased from eBioscience and Abcam (USA). TUNEL kits were order from Dalian Meilun Biotechnology (China).

Cell Lines and Animals: 4T1 murine breast tumor cells were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and 100 U/mL penicillin G sodium and streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. BALB/c mice (6-8 weeks old) were obtained from Beijing Experimental Animal Research Center. All animal experiments were completed at the Animal Experiment Center of the Institute of Radiation Medicine (Tianjin, China) and were approved by China's Committee for Research and Animal Ethics in compliance with the law for experimental animals.

Calculation of tumor volumes and tumour inhibition ratio (TIR): Tumor volumes were measured with a digital caliper and calculated according to the formula:

$$V = 1/2 \times L \times W^2$$

here, W is width of tumour and L is length of tumour

Tumour inhibition ratio (TIR) is calculated according to formula:

$$TIR\ (\%) = (Wc - Wt)/Wc \times 100\%$$

here, Wc and Wt are the average tumor weight of mice in the control and therapy groups, respectively.

In vitro cytotoxicity of small molecule nanoinhibitors: 4T1 cells were seeded in 96-well plates at a density of 2.5×10^3 cells per well for 24 h, and then BMS-1, BMS-8, BMS-202, and BMS-1166 NPs were added into preset wells and incubated for another 24 h. Finally, cell viability was detected using MTT assay according to normal method.

Release of model drugs at pH 6.5: DTX and VTX cumulative releases in vitro were carried out with dialysis method and monitored via UV-vis spectrophotometry. DTX@VTX NP dispersion was piped into a dialysis membrane (MW cutoff = 3.5kDa) and then dialyzed against 40 mL of PBS solution (0.1% Tween 80) at pH 6.5 in the dark. Next, 1 mL of the dialysate was collected and monitored by UV−vis spectrophotometry. Then, 1 mL of the dialysate was poured back into PBS solution to maintain the initial volume.

Analysis of cytokine secretion in tumors: To determine cytokine concentrations in tumors, those tumors were excised and homogenized in a protein extraction buffer containing a protease inhibitor (1 mL). The cytokine concentrations in the tumor were analyzed by ELISA according to the manufacturer's instructions and determined as pg/mg of protein.

Supporting data

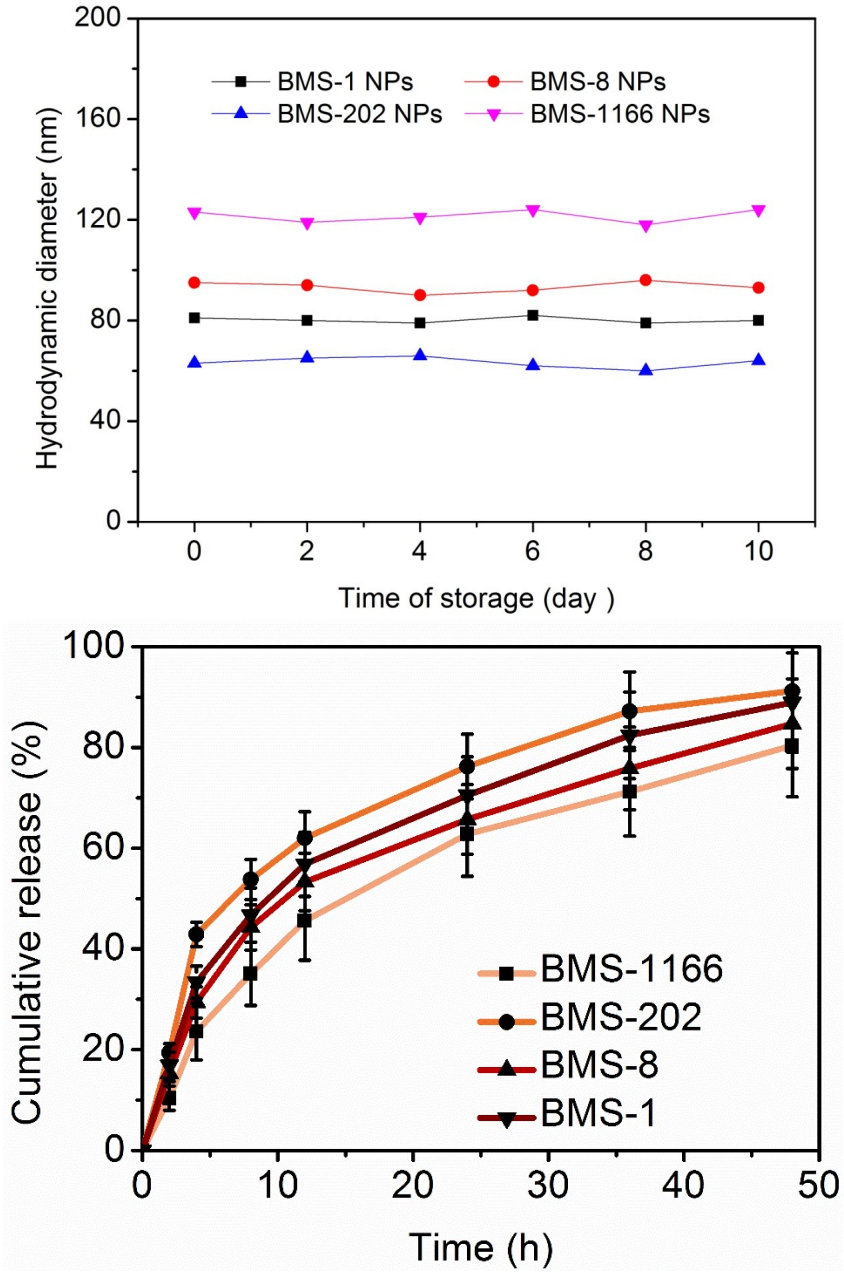

Figure S1 Evaluation of storage stability of the small molecule nanoinhibitors including BMS-1, BMS-8, BMS-202 and BMS-1166 NPs by DLS. The average hydrodynamic diameters of each of the NPs remained unchanged over 10 days of storage, indicating that these NPs have good stability (top). Release profiles of BMS-1, BMS-8, BMS-202 and BMS-1166 NPs in PBS at pH=6.5 *in vitro* (bottom). Data are expressed as the means ± s.d. (n = 3).

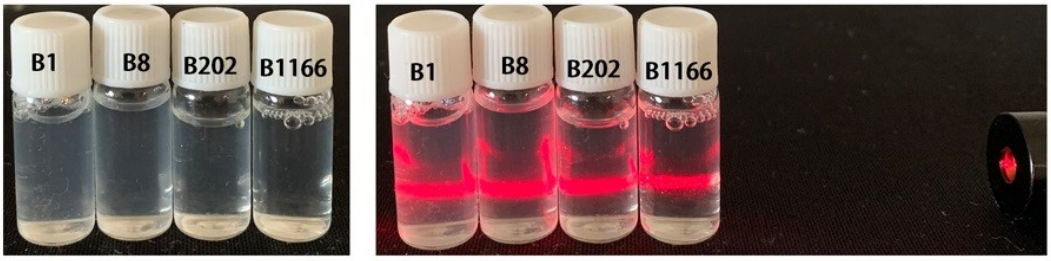

Figure S2 Photographs of BMS-1, BMS-8, BMS-202 and BMS-1166 NP dispersions under room light and a beam of red laser shine**.** All NP dispersions show Tyndall effect, indicating that these small molecule nanoinhibitors are indeed nanoparticles.

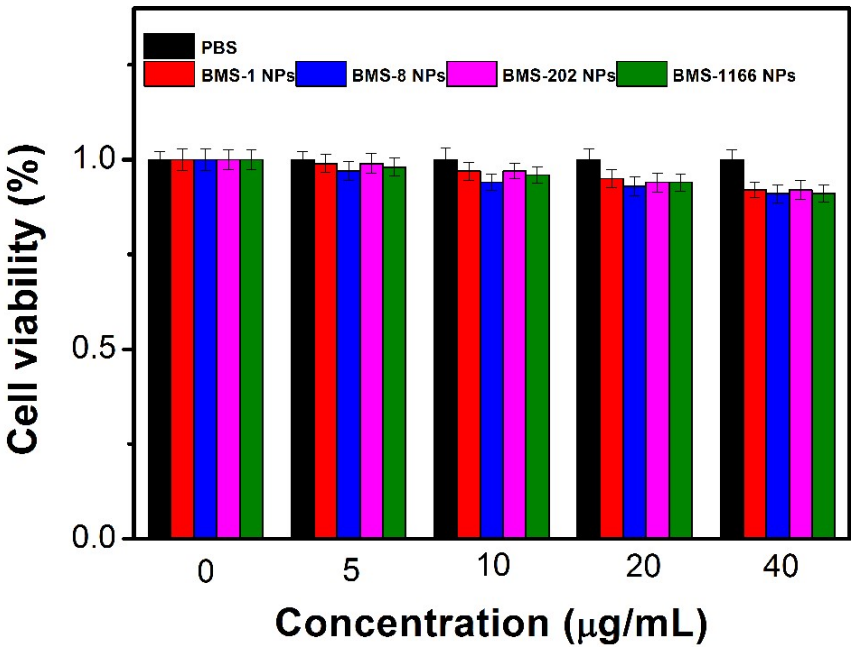

Figure S3 In vitro cytotoxicities of small molecule nanoinhibitors including BMS-1, BMS-8, BMS-202 and BMS-1166 NPs. MTT test results indicated that all BMS NPs have over 90% cell viability, indicating that these small molecule nanoinhibitors were biocompatible and did not cause toxicity.

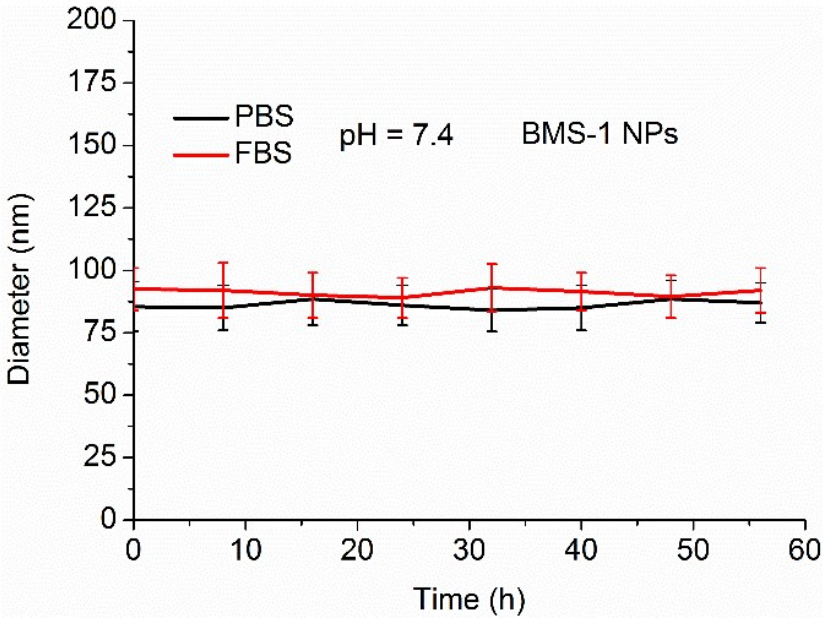

Figure S4 Stability of BMS-1 NPs in PBS and FBS by DLS at pH=7.4.

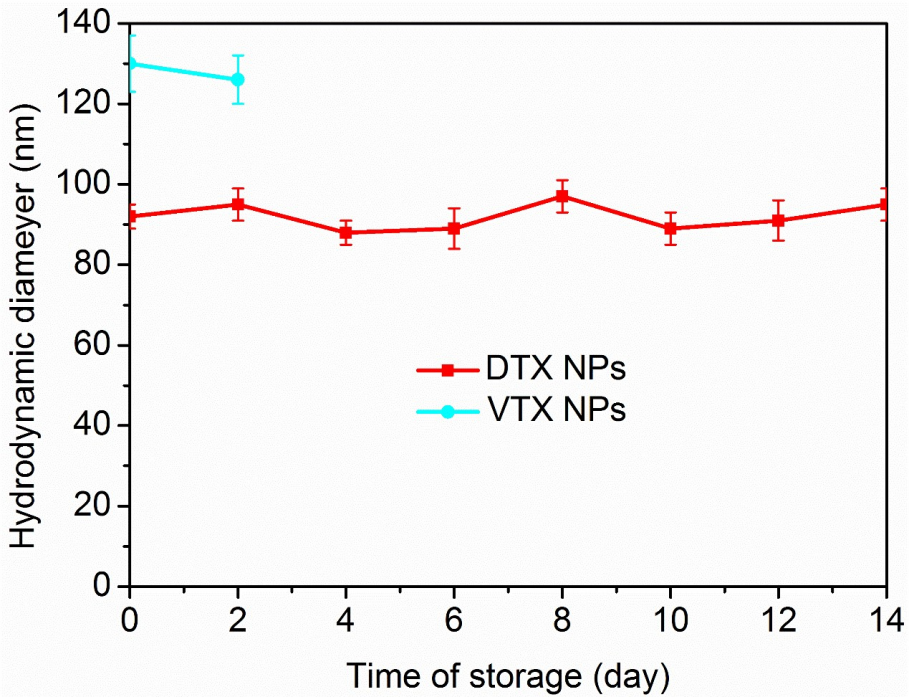

Figure S5 Storage stability of VTX and DTX NPs. These data indicate that DTX NPs are stabler than VTX NPs are unstable.

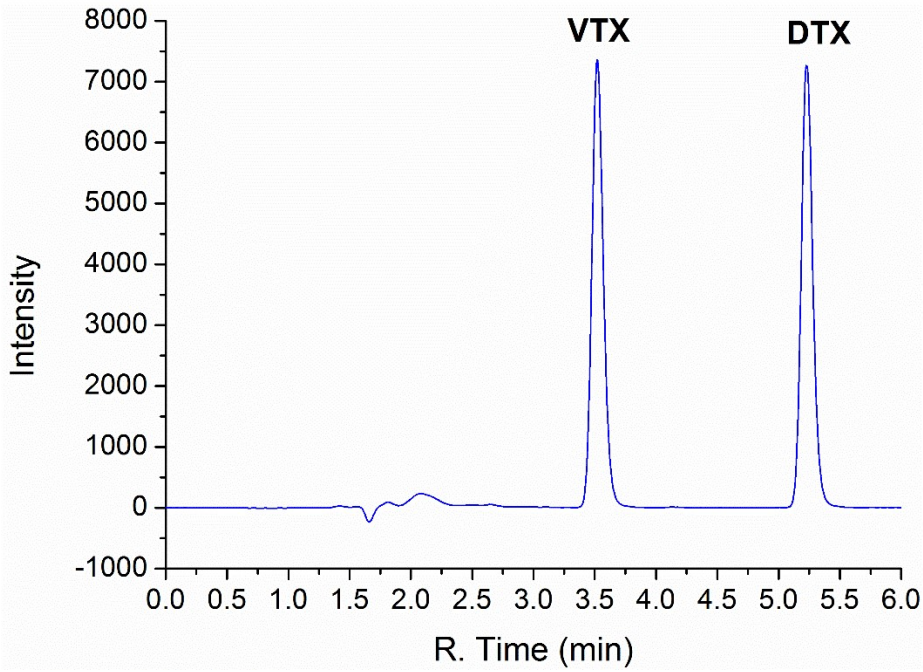

Figure S6 HPLC spectrogram of DTX@VTX NPs.

The measurement conditions are shown as follows:

Mobile phase: 0.1% phosphoric acid aqueous solution as mobile phase A and acetonitrile as Mobile phase B;

Detection wavelength: 210 nm;

Flow rate: 1.5mL/min;

Injection volume: 5μL;

Time	Mobile phase A (%)	Mobile phase B (%)
0	90	10
6	10	90
12	0	100
12.1	90	10
15	90	10

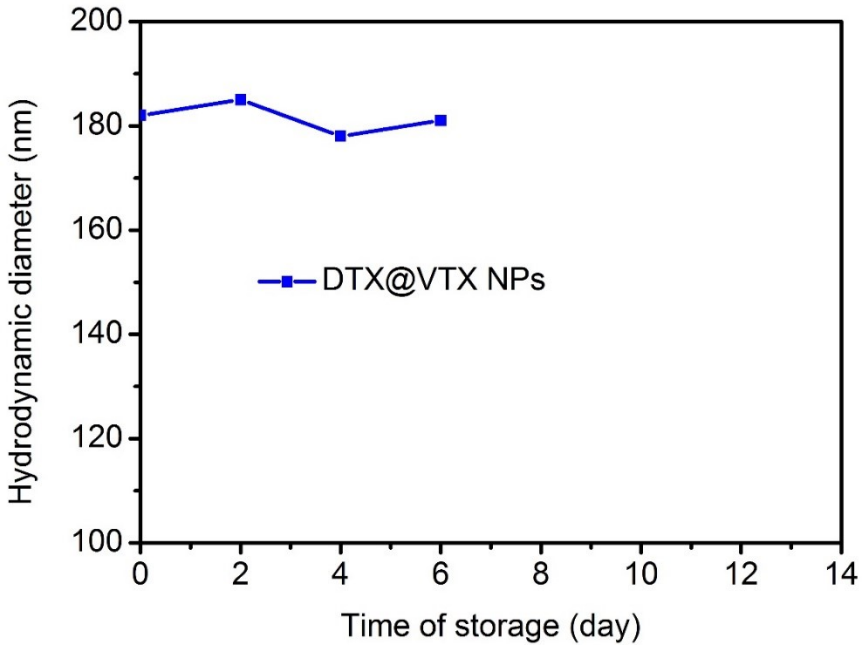

Figure S7 Storage stability of DTX@VTX NPs. The data indicate that DTX@VTX NPs was stable within six days.

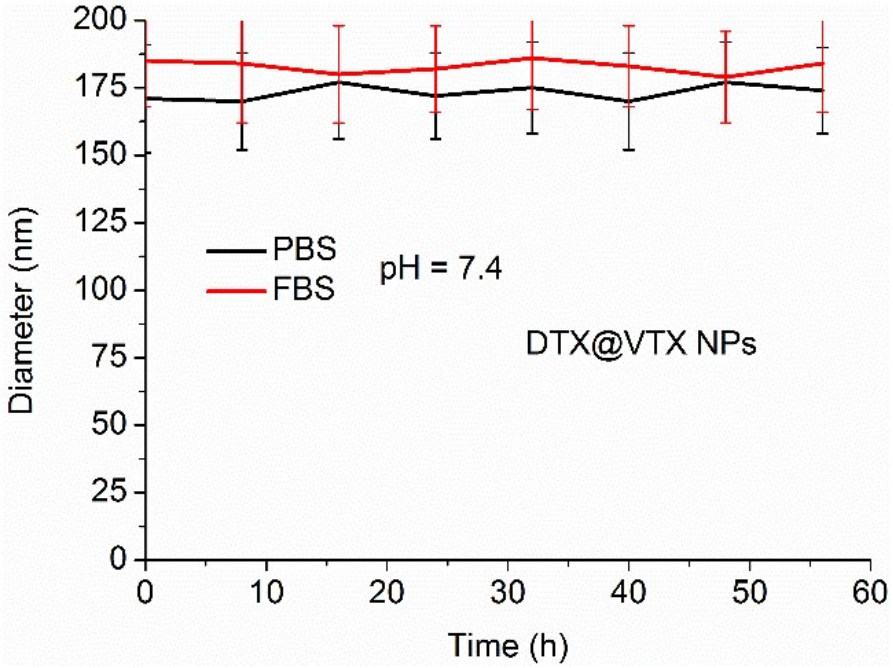

Figure S8 Stability of DTX@VTX NPs in PBS and FBS by DLS at pH=7.4.

Table S1 data analyzed from Fig. 3b using Image J (software)

Group	Pre-treatment stained area (%)	Post-treatment stained area (%)	Reduced stained area (%)
PBS	**15.72**	**16.23**	**- 0.51**
BMS-1166 NPs	**11.49**	**5.86**	**5.63**
BMS-202 NPs	**16.73**	**9.09**	**7.64**
BMS-8 NPs	**17.00**	**8.08**	**8.92**
BMS-1 NPs	**23.15**	**6.67**	**16.48**