Electronic Supplementary Material (ESI) for ChemComm.

Engineering a multi-target therapy nanoplatform against tumor growth and metastasis via a novel NSAID-Pt(IV) prodrug

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Experimental Section

Materials. Cisplatin (CDDP) was purchased from Shandong Boyuan Pharmaceutical Co., Ltd. Bendazac, N,N-dimethylformamide (DMF) and Dimethyl sulfoxide (DMSO) were purchased from J&K Scientific Ltd. Bovine serum albumin (BSA) was obtained from Dingguo Changsheng Biotechnology Co., Ltd. Triethylamine (TEA), hydrogen peroxide solution (30 wt % in H_2O), and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), DAPI, RNase A, propidium iodide (PI), Annexin V-FITC/PI Apoptosis Detection Kit, and Mouse VEGF ELISA Kit were bought from Beijing Solarbio Technology Co., Ltd. γH2AX antibody and ALexa Fluor 488 antibody were bought from Proteintech Group, Inc. Antibodies related to western blot experiment (anti-COX-2, anti-Bcl-2, anti-E-cadherin) were bought from Cell Signaling Technology. APC anti-mouse CD3, PE anti-mouse CD8a, and FITC anti-mouse CD4 were bought from BioLegend, Inc. Lyso-Tracker Green was bought from Beyotime Biotechnology Co., Ltd.

Cell lines and Animals. Human non-small cell lung cancer cell line (A549), human cervical cancer cell line (HeLa), human breast cancer cell line (MCF-7), human pharyngeal squamous carcinoma cell line (Fadu) and mouse breast cancer cell line (4T1) were all obtained from National infrastructure of cell line resource in Chinese Academy of Sciences. A549, HeLa and 4T1 cells were cultured with the final 1640

medium (1640 medium with 1% penicillin-streptomycin solutions and 10% fetal bovine serum). MCF-7 and Fadu cells were cultured with the final DMEM medium (DMEM medium with 1% penicillin-streptomycin solutions and 10% fetal bovine serum). Both of them were cultured at 37 °C in 5.0% CO_2. Female Balb/C mice at 6-8 weeks were bought from Beijing Sibeifu Biotechnology Co., Ltd.

Synthesis of $Pt(NH_3)_4(OH)_2$. Hydrogen peroxide (30 wt%, 2.0 mL) was added dropwise to a round bottom flask containing CDDP (200 mg, 0.67 mmol). The mixture was reacted at 75 °C for 5 h. The bright yellow solution was collected at 4 °C in the dark to obtain crystals, which was recovered and washed with cold water, ethanol and etherto. The light yellow solid ($Pt(NH_3)_4(OH)_2$) was dried. Yield: 84.7%.

Synthesis of Ben-Pt(IV). $Pt(NH_3)_4(OH)_2$ (200 mg, 0.60 mmol) was dissolved in DMF. Bendazac (508 mg, 1.80 mmol), TEA (260 μL, 1.80 mmol) and TBTU (576 mg, 1.80 mmol) were added to the $Pt(NH_3)_4(OH)_2$ solution. The reaction was reacted at room temperature for 24 h in dark with stirring under N_2 to obtain a clear yellow solution. Then, 20 mL of ice water was added to the reaction solution to obtain a yellow solid. Finally, the crude product was purified by a silica gel column with dichloromethane/methanol to obtain a yellow solid after drying. Yield: 53.7%. ^{1}H-NMR (Bruker Avance 400 spectrometer), ^{13}C-NMR (Bruker Avance 400 spectrometer) and ESI-MS (ABSCIEX API 4000) were used to characterize products.

Preparation of BSA-Coated Ben-Pt(IV) nanoparticles (BSA@Ben-Pt(IV) NPs). Ben-Pt(IV) (2 mg) was dissolved in DMF (0.2 mL), and BSA was dissolved in distilled water (2.0 mL). Next, Ben-Pt(IV) solution was dropped into different weights

of BSA (BSA/Ben-Pt(IV), 0:1, 1:1, 2:1, 3:1, w/w) slowly under stirring at 1000 rpm. 10 min later, the mixture solution was stirred (1000 rpm, 10 min) to obtained the soild, which was washed by water twice and then redistributed in water (2 mL). The BSA@Ben-Pt(IV) NPs solutions with different prescription were tested by Zeta potential and appearance of Ben-Pt(IV) to choose the appropriate amount of BSA.

Characterization of BSA@Ben-Pt(IV) NPs. Morphology of BSA@Ben-Pt(IV) NPs was observed by transmission electron microscopy (TEM, JEM-200CX). Size distribution and Zeta potential were tested by Zetasizer Nano ZS90 (Malvern Instrument). Stability of BSA@Ben-Pt(IV) NPs was characterized by change of appearance and size. The hemolysis rate was determined according to the general method.

In vitro **cytotoxicity.** A549, HeLa, MCF-7, Fadu and 4T1 cells were seeded in 96-well plates (200 μL, 5×10^3 cells/well) overnight. Then, cells were treated with bendazac, CDDP, bendazac/CDDP mixture, Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs (0.16-20.0 μM). After 48 h, MTT (10 μL, 5 mg/mL) was added into each well and then incubated for 4 h. Next, the medium was removed, and DMSO (100 μL) was employed to dissolve the formazan crystals. Each sample was measured via the microplate reader at 570 nm. The IC50 values were calculated via GraphPad Prism 7.0 software. The cell inhibition ratio was determined by the following formula.

$$\text{Cell inhibition ratio}\,(\%) = \left(\frac{\text{OD}_{treated} - \text{OD}_{blank}}{\text{OD}_{control} - \text{OD}_{blank}}\right) \times 100\%$$

Where $OD_{control}$, $OD_{treated}$ and OD_{blank} referred to the optical density of the control, blank and sample group, separately. (*ACS Appl. Mater. Interfaces* **2019**, 11, 32633)

Cellular uptake and DNA platination. 4T1 cells were seeded in 6-well plates (2000 μL, 1 × 10^6 cells/well) overnight. Then, cells were treated with CDDP, Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs for 3 h, 6 h or 9 h. After that, the cells were washed via PBS and then collected. The Pt level in 4T1 cells was tested by ICP-MS after standardized pre-processing. (*Eur. J. Med. Chem.* **2018**, 157, 1292)

Intracellular release. 4T1 cells were seeded in 6-well plates (2000 μL, 5 × 10^5 cells/well) overnight. Then, cells were treated with Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs. 4 h later, the cells were washed via PBS and then ground to obtain cell extract. Methanol was employed precipitate protein. The supernatant was collected and then analyzed through a high performance liquid chromatograph (HPLC, Shimadzu) with a mixture of acetonitrile and water (70:30, v/v) and 0.1% acetic acid as mobile phase. In addition, bendazac and Ben-Pt(IV) standard were analyzed by HPLC as control. (*Eur. J. Med. Chem.* **2019**, 167, 377)

Subcellular localization. 4T1 cells were seeded in 6-well plates (2000 μL, 5 × 10^5 cells/well) overnight. Then, cells were treated with free dyes or BSA@dye NPs (5.0 μM). The preparation process of BSA@dye was similar to that of BSA-coated Ben-Pt(IV) nanoparticles. 24 h later, the cells were washed via PBS and stained with Lyso-Tracker Green and DAPI following the instructions, respectively. The Confocal microscope was employed to observe subcellular localization. (*ACS Appl. Mater. Interfaces* **2019**, 11, 42904)

Apoptosis analysis. 4T1 cells were seeded in 6-well plates (2000 μL, 1 × 10^6 cells/well) overnight. Then, cells were treated with different samples (2.0 μM). After

48 h, the cells were washed via PBS and then re-suspended with binding buffer. Annexin V-FITC (5 μL) and PI (10 μL) were employed to stain cells. 15 min later, the cells were detected by Flow cytometry (BD FACS Aria). (*Chem. Commun.* **2021**, 57, 8993)

Wound healing assay. 4T1 cells were seeded in 12-well plates (1000 μL, 5×10^5 cells/well) overnight. Scratches on the mono-layer were created and washed via PBS. Then, cells were treated with different complexes (2.0 μM). After 24 h, the cells were washed via PBS and observed under the microscope. (*J. Control. Release* **2021**, 340, 282)

DNA damage analysis. 4T1 cells were seeded in 6-well plates (2000 μL, 5×10^5 cells/well) overnight. Then, cells were treated with different complexes (2.0 μM). After 24 h, the cells were washed via PBS and fixed via 4% paraformaldehyde. Next, the cells were incubated with primary antibody (γH2AX antibody) for 12 h, and then incubated with Alexa 488-conjugated secondary antibody (ALexa Fluor 488 antibody) for 2 h. Finally, the cells were stained with DAPI for 15 minutes and observed under the Confocal microscope. In addition, 4T1 cells were seeded in 6-well plates (2000 μL, 5×10^5 cells/well) overnight. After being treated with different complexes (2.0 μM) for 24 h, cells were collected and stained for cell cycle analysis via flow cytometry (BD FACS Aria) (*Eur. J. Med. Chem.* **2019**, 167, 377).

Western blot analysis. 4T1 cells were seeded in 6-well plates (2000 μL, 2×10^5 cells/well). Then, cells were treated with PBS, bendazac, CDDP, bendazac/CDDP mixture, Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs (2.0 μM) for 24 h. Next, cells were

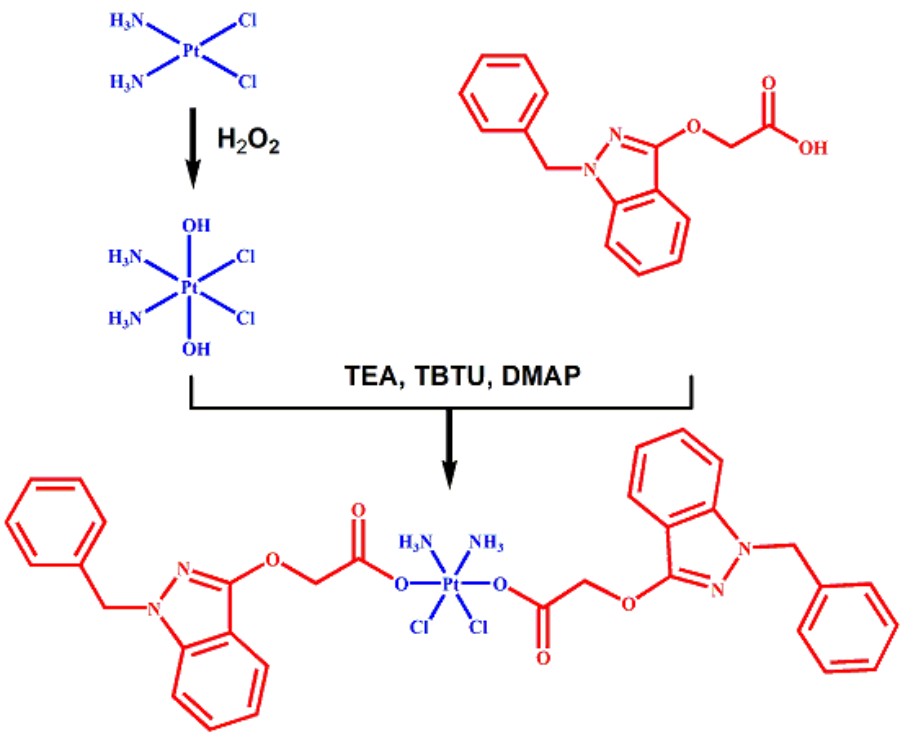
washed with cold PBS and lysed via RIPA lysis buffers. The proteins were extracted after grinding and centrifuging. Expression of COX-2, Bcl-2, E-cad and GAPDH (internal reference protein) were analyzed by western blot following protocols. (*Eur. J. Med. Chem.* **2019**, 167, 377)

***In vivo* anti-tumor activity**. Animal experiments were approved by Medical Ethics Committee of Shandong Second Provincial General Hospital and carried out according to the Health Guide for the Care and Use of Laboratory the Animals of National Institutes. Female Balb/C mice at 6-8 weeks were injected subcutaneously with 1×10^6 4T1 cells in the right buttock. When the tumor volume ($0.5 \times \text{length} \times \text{width}^2$) grew to 50 to 100 mm^3, tumor-bearing mice were randomly divided into six groups (n = 11). The mice were intravenous injected with 1) NS, 2) bendazac, 3) CDDP, 4) bendazac/CDDP mixture, 5) Ben-Pt(IV) or 6) BSA@Ben-Pt(IV) NPs every three days with dose at 2.5 mg CDDP/kg, respectively. The body weight and tumor volume were measured every 2 days for 15 days. Afterwards, mice were sacrificed. The tumor and organs were excised and assessed by hematoxylin and eosin (H&E) stained and ICP-MS. Moreover, three mice in each group were fed sequentially. 20 days later, the mice were sacrificed to observe the lung metastasis. (*J. Med. Chem.* **2019**, 62, 4543)

***In vivo* anti-tumor mechanism analysis**. The tumor-draining lymph nodes (TDLNs), tumors and spleens collected from sacrificed mice were used to analyze the immune response by immunofluorescence or flow cytometry after stained with different antibodies. The blood was used to analyze the VEGF expression. (*ACS Appl. Mater.*

Interfaces **2019**, 11, 32633)

Statistical analysis. GraphPad Prism (7.0) was employed to evaluate statistical significance. Statistical significance: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.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Pt
H3N
Cl
H2O2
OH
H3N
Cl
Pt
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N
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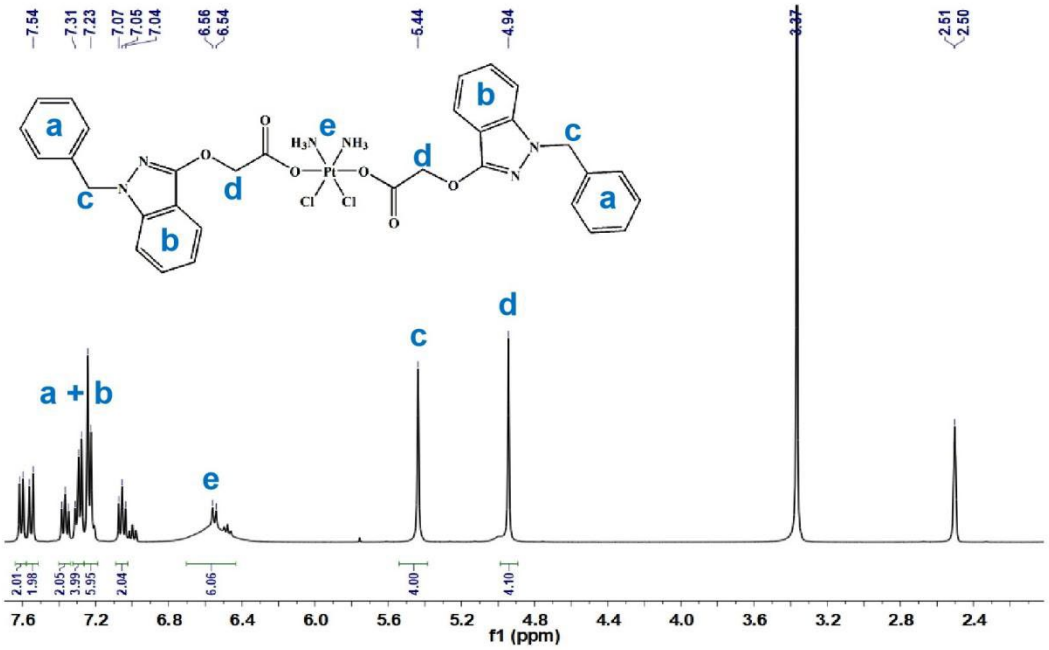

Figure S2. ^{1}H-NMR spectrum of Ben-Pt(IV) in DMSO-*d*$_6$ (Bruker Avance 400 spectrometer).

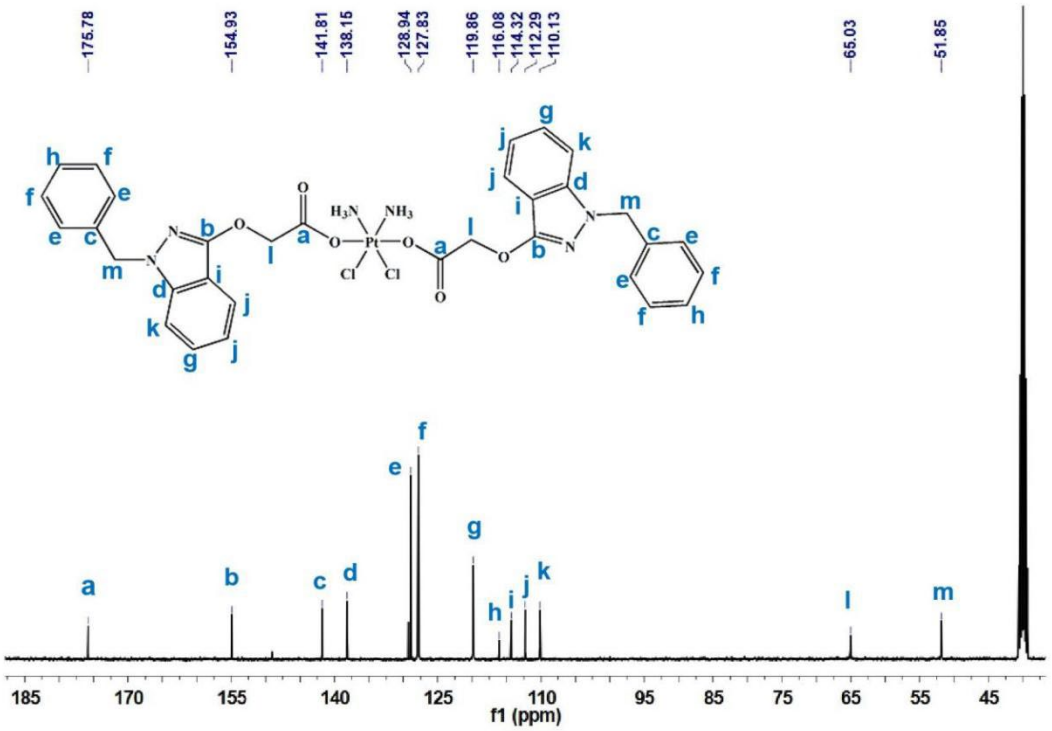

Figure S3. ^{13}C-NMR spectrum of Ben-Pt(IV) in DMSO-*d*$_6$ (Bruker Avance 400 spectrometer).

^{13}C-NMR (101 MHz, DMSO-*d*$_6$, δ): 175.78 (a-C), 154.93 (b-C), 141.81 (c-C), 138.16 (d-C), 128.94 (e-C), 127.83 (f-C), 119.86 (g-C), 116.09 (h-C), 114.32 (i-C), 112.29 (j-C), 110.14 (k-C), 65.03 (l-C), 51.85 (m-C).

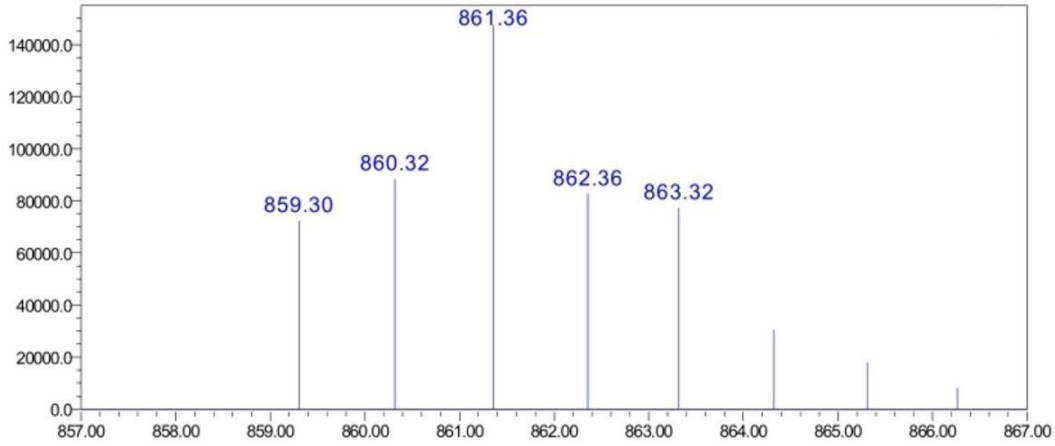

Figure S4. ESI-MS spectrum of Ben-Pt(IV) in methanol (ABSCIEX API 4000) .

MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{32}H_{32}Cl_2N_6O_6Pt$, 861.63; found 861.36.

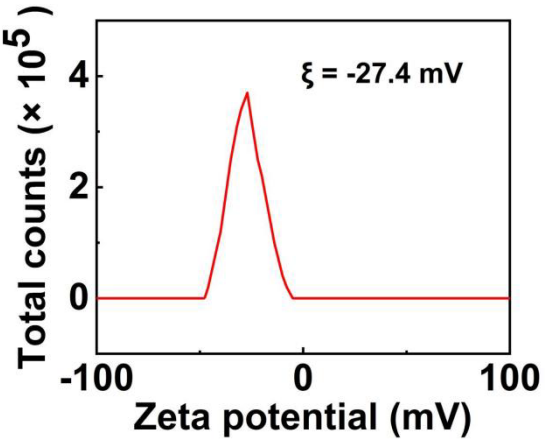

Figure S5. The Zeta potential of BSA@Ben-Pt(IV) NPs.

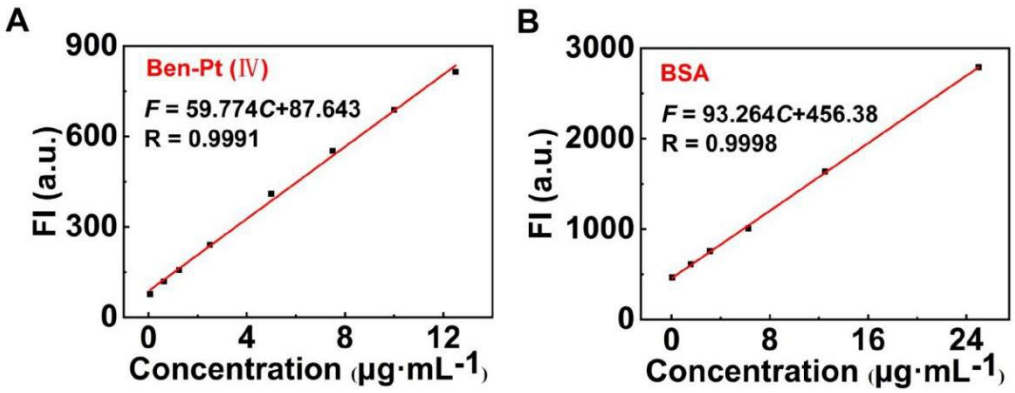

Figure S6. Standard curves of Ben-Pt(IV) and BSA.

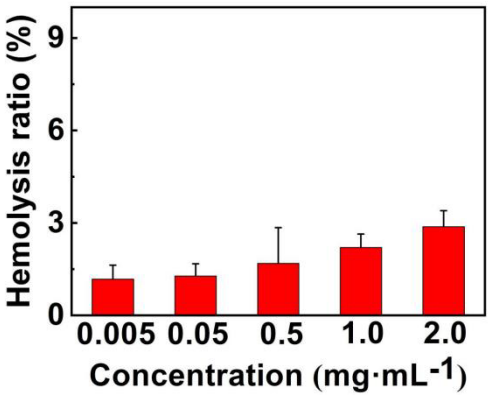

Figure S7. The hemolysis ratios of BSA@Ben-Pt(IV) NPs.

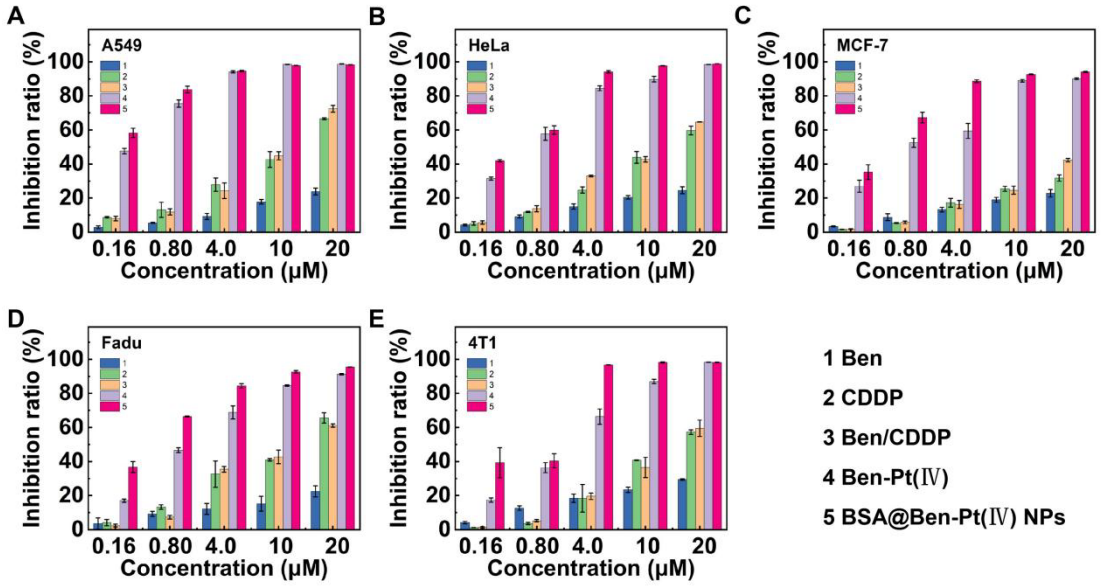

Figure S8. Cell inhibition ratio of Ben, CDDP, Ben/CDDP, Ben-Pt(IV) and BSA@Ben-Pt(IV) NPs of (A) A549 cells, (B) HeLa cells, (C) MCF-7 cells, (D) Fadu cells, and (E) 4T1 cells at different concentrations for 48 h.

Table S1. Inhibitory effect (IC50 values) of Ben, CDDP, Ben/CDDP, Ben-Pt(IV) and BSA@Ben-Pt(IV) NPs on different cells for 48 h.

	A549 (μM)	HeLa (μM)	MCF-7 (μM)	Fadu (μM)	4T1 (μM)
Ben	201.57±25.09	320.74±57.00	370.60±20.81	580.90±125.44	169.86±15.38
CDDP	11.94±1.98	14.57±0.65	55.97±8.60	11.22±1.18	14.44±0.30
Ben/CDDP	10.22±0.60	11.1±0.21	34.73±0.98	11.61±0.57	15.29±0.38
Ben-Pt(IV)	0.19±0.01	0.47±0.03	0.85±0.10	1.13±0.05	1.29±0.06
BSA@Ben-Pt(IV) NPs	0.10±0.02	0.30±0.02	0.34±0.07	0.34±0.03	0.45±0.07

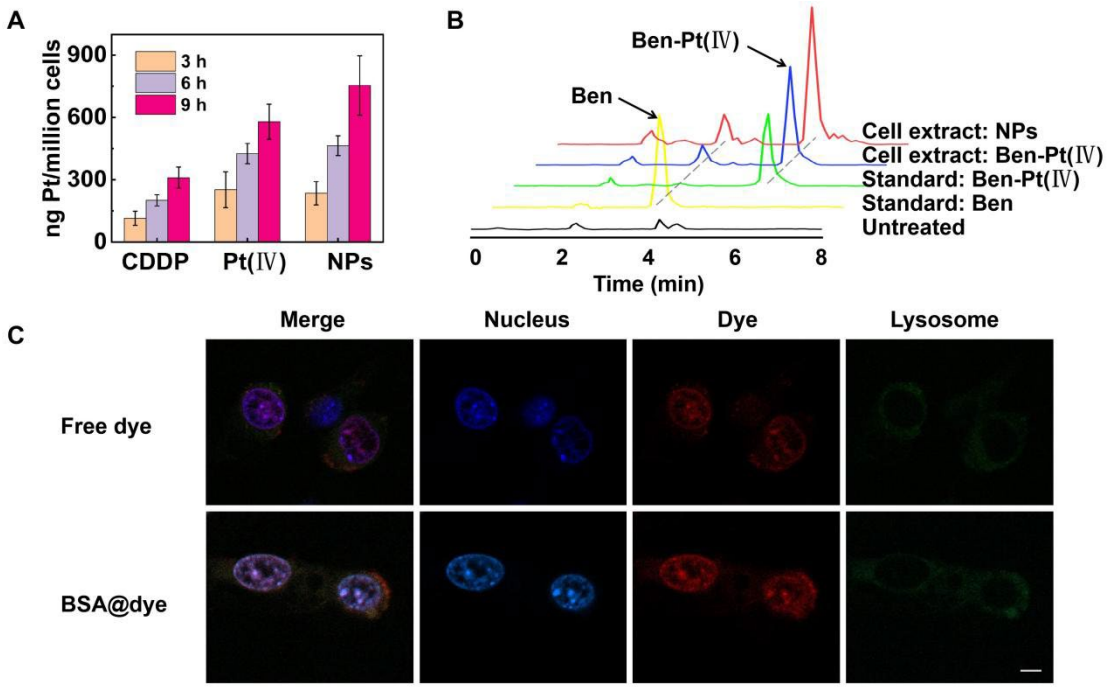

Figure S9. (A) The cellular uptake behavior: The accumulation of Pt in 4T1 cells treated with CDDP, Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs at 2.0 μM for 3 h, 6 h or 9 h, respectively. (B) The drug release behavior: HPLC profiles of 4T1 cells extract after being treated with Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs at 2.0 μM for 4 h. (C) The LSCM images to show subcellular localization of free dye and BSA@bye in 4T1 cells after 24 h co-incubation at 5.0 μM. The scale bar was 10 μm.

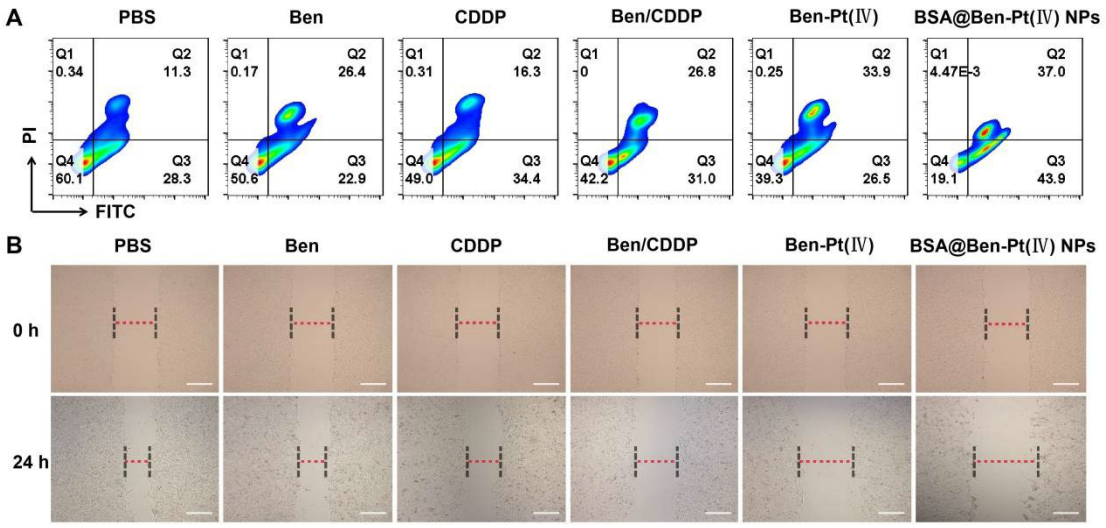

Figure S10. (A) The image of apoptosis induced by PBS, Ben, CDDP, Ben/CDDP, Ben-Pt(IV) and BSA@Ben-Pt(IV) NPs at 2.0 μM for 48 h in 4T1 cells via Annexin V-FITC/PI staining. (B) Migration inhibition induced by PBS, Ben, CDDP, Ben/CDDP, Ben-Pt(IV) and BSA@Ben-Pt(IV) NPs at 2.0 μM for 24 h in 4T1 cells via wound-healing assay. The scale bars were 200 μm.

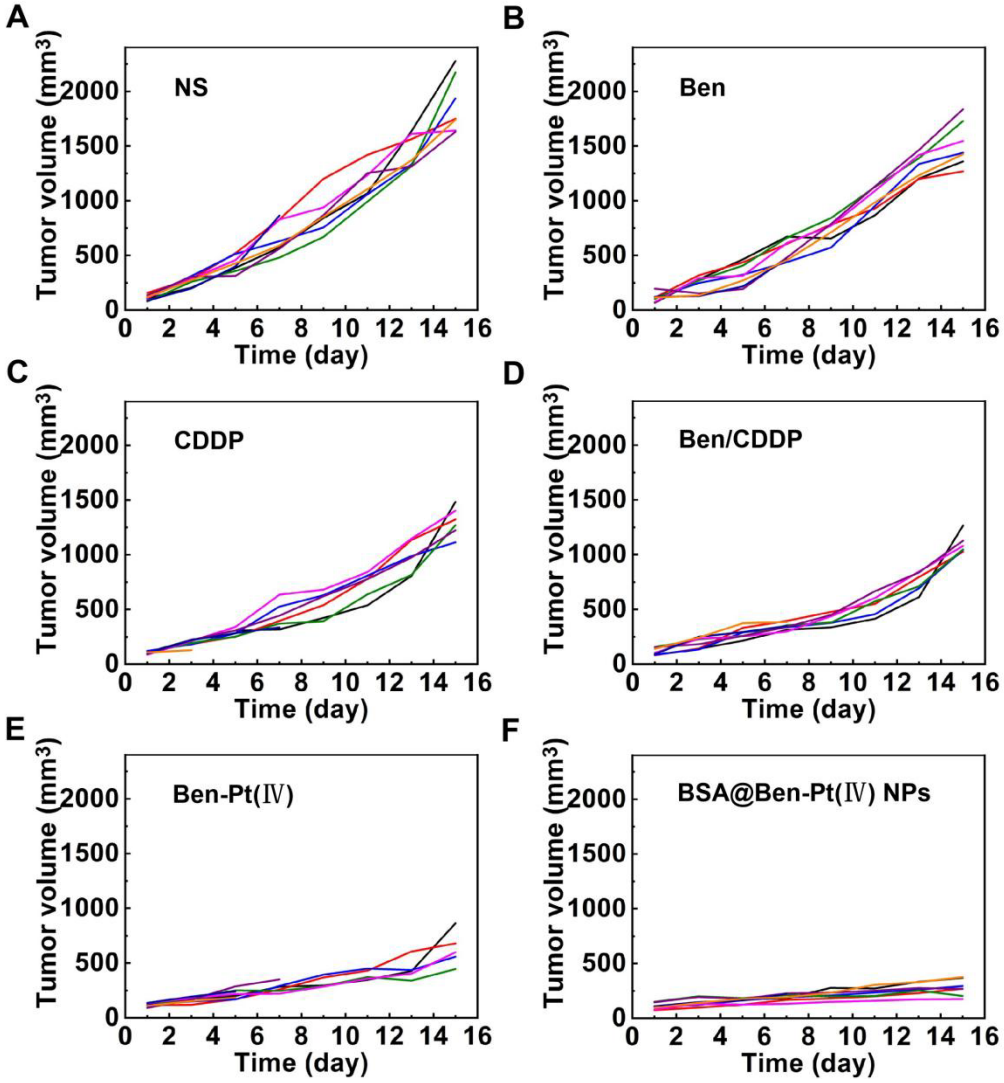

Figure S11. Change of tumor volume of each mouse treated by NS, Ben, CDDP, Ben/CDDP, Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs. (n = 8)

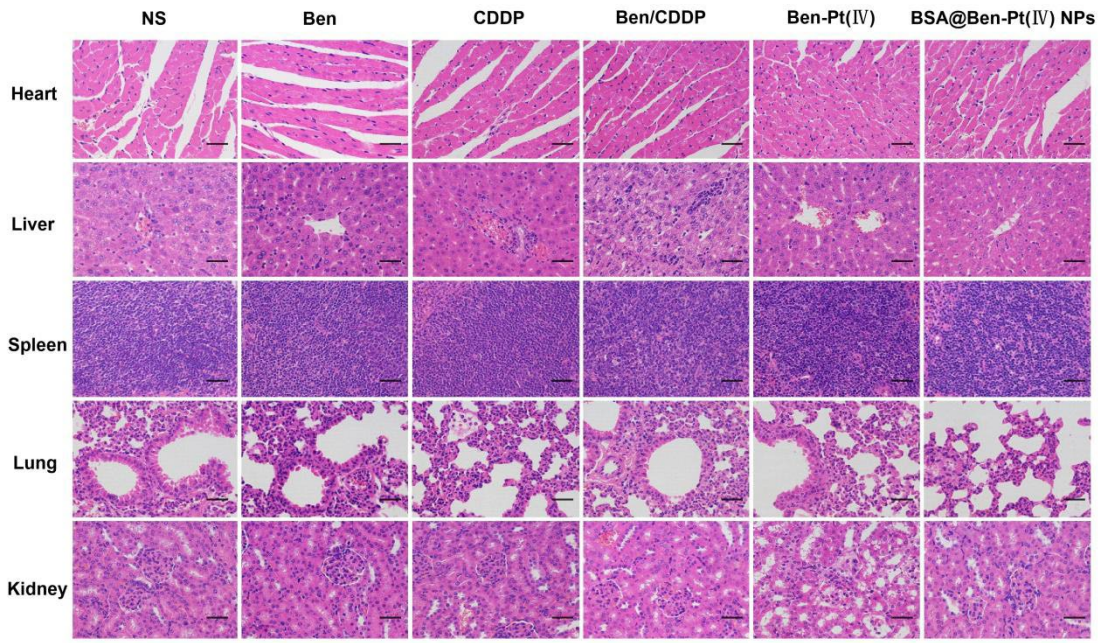

Figure S12. H&E stained images of main organs in mice treated by NS, Ben, CDDP, Ben/CDDP, Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs. The scale bars were 50 μm.

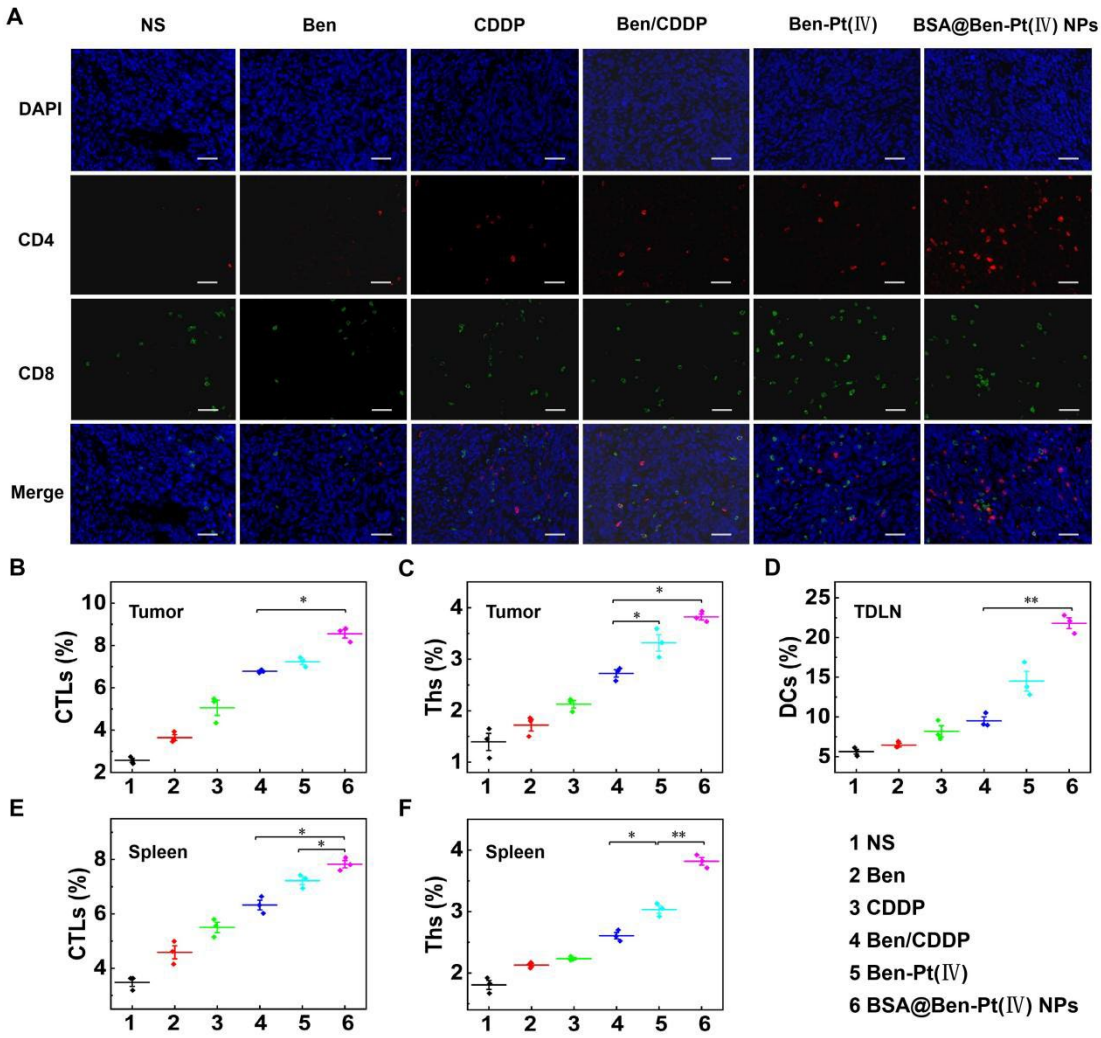

Figure S13. (A) The immunofluorescence stained images of tumors in mice treated by NS, Ben, CDDP, Ben/CDDP, Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs. Nucleus: blue color, CD4: red color, CD8: green color. The scale bars were 40 μm. The proportions of (B) CTLs and (C) Ths in lymphocytes of the same weight of tumors; The proportions of (D) mDCs in TDLNs; The proportions of (E) CTLs and (F) Ths in lymphocytes of spleens in mice treated by NS, Ben, CDDP, Ben/CDDP, Ben-Pt(IV) or BSA@Ben-Pt(IV) NPs.

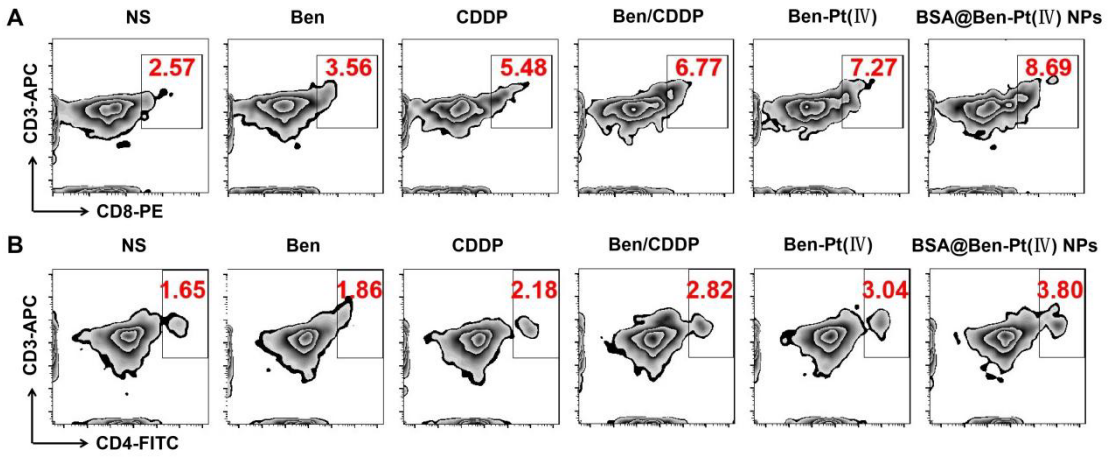

Figure S14. Flow cytometric analyses of the proportions of (A) CTLs ($CD3^+ CD8^+$) and (B) Ths ($CD3^+ CD4^+$) in lymphocytes of tumors after different treatment.

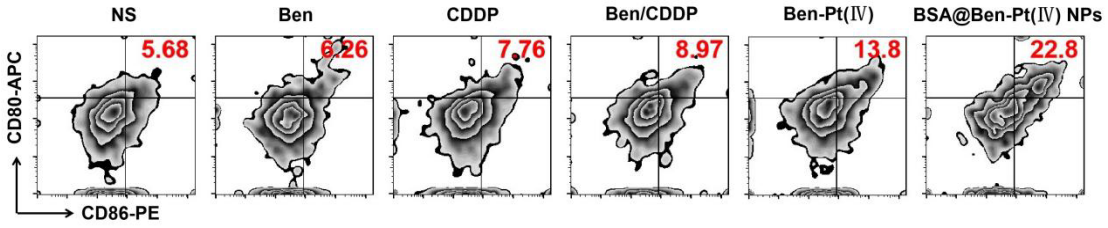

Figure S15. Flow cytometric analyses of the proportions of mDCs ($CD80^+$ $CD86^+$) in lymph nodes after different treatment.

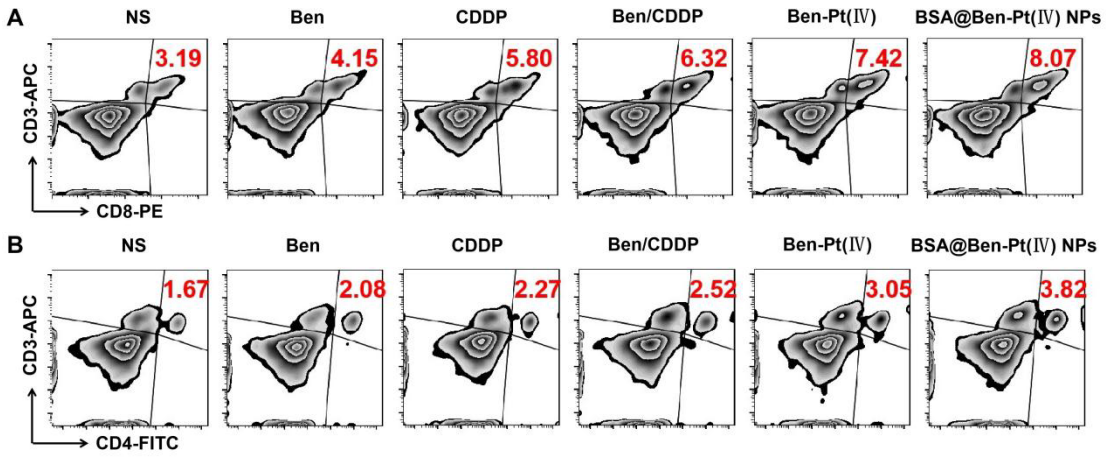

Figure S16. Flow cytometric analyses of the proportions of (A) CTLs ($CD3^+ CD8^+$) and (B) Ths ($CD3^+ CD4^+$) in spleens after different treatment.

Raw Western Blot Data