Supplementary Information

Nano-assembly of Ursolic Acid with Platinum Prodrug Overcomes Multiple Deactivation Pathways in Platinum-Resistant Ovarian Cancer

Yupeng Wang,[a] Zhijian Luo,[b] Dongfang Zhou,[b] Xuefeng Wang,[c] Jianjun Chen,*[b] Shipeng Gong*[a,d] Zhiqiang Yu,*[b]

[a.] Department of Pharmacy, Shunde Hospital, Southern Medical University (The First People's Hospital of Shunde Foshan), Foshan 528300, P. R. China.

[b.] A School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, P. R. China.

[c.] Department of Obstetrics and Gynecology, The Third Affiliated Hospital of Southern Medical University, Guangzhou, 510515, P. R. China.

[d.] Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, P. R. China.

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Materials.

Ursolic acid (99%), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC HCL), L-lysine diisocyanate and sodium ascorbate (NaVc) were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Chemical Company, China. Poly(ethylene glycol) methyl ether amine ($mPEG_{1k}$-NH_2) was purchased from Creative PEGWorks. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Rabbit Bax, BCL-2, Caspase-3, Cyto-3, Nrf2, HO-1, NQO1, and GST antibody were purchased from Bioss Biotechnology Co., Ltd. Beijing, China. The mitochondrial membrane potential assay kit (with JC-1) and Annexin V-FITC/PI kit were purchased from Beyotime (Shanghai, China) and used according to the protocols provided by the manufacturers. Dimethyl sulfoxide (DMSO) and chloroform were dried over calcium hydride for 7 days before distillation.

Cell Lines and Animals.

A2780 (Human ovarian cancer cell) and A2780/DDP (cisplatin resistance human ovarian cancer cell) cells were bought from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. A2780 and A2780/DDP cells were cultured in DMEM medium containing 10% FBS and 50 ng Pt/mL. Female BALB/c nude mice (6-8 week old, 16-18 g) were purchased from Jilin University (Changchun, China) and were housed in an SPF room. All mice received required care conditions throughout the experiments. All animal experiments were approved by the local institution review board and performed according to the Guidelines of the Committee on Animal Use and Care of Southern Medical University.

Instrumentation.

NMR spectra (^{1}H NMR) were detected with a Bruker NMR spectrometer (400 MHz) at 25 ℃. Fourier transform infrared spectra (FT-IR) were measured on Bruker Vertex 70 spectrameter. Mass spectroscopy (ESI-MS) was detected by a Quattro Premier XE system (Waters) with an electrospray interface (ESI) equipment. The platinum contents were calculated with an inductively coupled plasma massspectrometry (ICP-MS, Thermoscientific, USA). Transmission electron microscopy (TEM) imaging was taken on an electron microscope (JEOL JEM-1011). Diameters were recorded by a Brookhaven 90Plus size analyzer. UV-visible electronic absorption spectra were measured on a Varian Cary 300 UV-visible spectrophotometer in 1 cm path-length cuvettes. Cellular and histological fluorescence images were observed on confocal laser scanning microscope (CLSM) imaging system (Olympus FV1000, Zeiss, Japan). Flow cytometry analysis was analyzed by a BD FACS-CaliburTM flow cytometer. Histological section imaging was observed with an optical microscope (Nikon TE2000U).

Synthesis of the PEG-UA Conjugate.

UA (474 mg, 0.6 mmol), EDC (230 mg, 1.2 mmol), and NHS (69.2 mg, 0.6 mmol) were added to anhydrous DMSO (10 mL) with argon protection and stirred in the ice

bath for 1 h. Then PEG_{1k}-NH_2 (400 mg, 0.4 mmol) was mixed with the above DMSO solution and stirred at room temperature for 12 h. Then the solution was dialyzed (MWCO = 1000) against deionized water to remove DMSO and unreacted UA, and then freeze-dried. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): 3.4-3.6 (m, -O-C*H*$_2$-CH_2-O-), 5.12 (H, m, -CH_2-C*H*-C-) (Figure S1). IR: cm^{-1} 1793 (-COOH), 1103 (-CH_2-O-CH_2-) (Figure 1B).

Synthesis of Pt(IV)-NCO Complex.

Synthesis of Pt(IV): $Pt(NH_3)_2(OH)_2Cl_2$ was synthesized according to the reported procedures.[33] Briefly, cisplatin was oxidized with 30% H_2O_2 in water at room temperature for 24 h in the dark. The $Pt(NH_3)_2(OH)_2Cl_2$ product was then isolated and dried in vacuum.

Synthesis of Pt(IV)-NCO: A solution of Pt(IV) (100 mg, 0.3 mmol) and L-lysine diisocyanate (67.86 mg, 0.3 mmol) in anhydrous DMSO (10 mL) was stirred at 40 ℃ for 12 h. Then the solution was precipitated in ethyl ether, and the white precipitate was dried. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): 2.85 (2H, m, -C*H*$_2$-NCO), 3.85 (H, m, -C*H*-NH-), 4.06 (2H, m, -C*H*$_2$-CH_3), 6.60 (6H, s, -NH_3) (Figure S1). IR: cm^{-1} 1670 (-C=O-NH-), 2275 (R-N=C=O), 3330 (-NH-R) (Figure 1B); ESI-MS: (negative mode) m/z 560.4 [M - H]$^-$ (Figure S2).

Synthesis of the Pt(IV)-UA-PEG.

A solution of $mPEG_{1k}$-UA (257 mg, 0.18 mmol) and Pt(IV)-NCO complex (100 mg, 0.18 mmol) in anhydrous DMSO (10 mL) was stirred at 40 ℃ for 12 h. After removed DMSO by vacuum distillation, methanol (5 mL) was added and the clear yellow solution was dropped into an excess of diethyl ether, and the precipitate finally dried in vacuum. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): 3.4-3.6 (m, -O-C*H*$_2$-C*H*$_2$-O-), 5.2 (H, m, -CH_2-C*H*-C-), 6.60 (6H, s, -NH_3) (Figure 1A); IR: cm^{-1} 1670 (-C=O-NH-), 3330 (-NH-R), 1103 (-CH_2-O-CH_2-) (Figure 1B).

Preparation and Characterization of Pt(IV)-UA NPs.

Pt(IV)-UA NPs were obtained by a nanoprecipitation method. In brief, Pt(IV)-UA-PEG (40 mg) was dissolved in DMSO (4 mL) and then the DMSO solution was dropwise added into water (20 mL) under stirring to get a micellar solution. The solution was dialyzed (MWCO = 1,000) against deionized water to remove DMSO and then freeze-dried. The nanoparticle size was measured using DLS. The morphology and size were observed from TEM (0.5 mg freeze-dried samples of Pt(IV)-UA NPs was redissolve in 2 mL water. Then the sample was dripped onto the copper mesh (Gilder Grids), and then observed with TEM). The Pt content of NPs was determined by ICP-MS.

Combination index analysis of Pt and UA

Combination index (CI) was calculated with the equation as below:

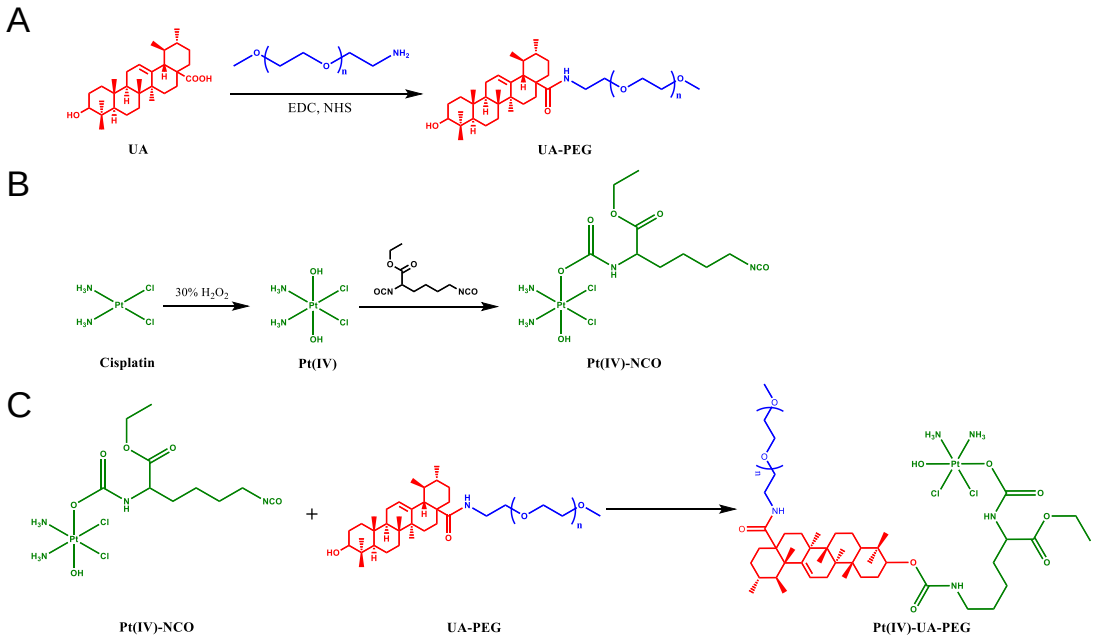
$$\mathrm{CI} = \frac{\mathrm{Ca}_{\mathrm{a,x}}}{\mathrm{IC}_{\mathrm{x,a}}} + \frac{\mathrm{Ca}_{\mathrm{b,x}}}{\mathrm{IC}_{\mathrm{x,b}}}$$

$C_{a,x}$ and $C_{b,x}$ are the concentrations of drug A (Pt) and drug B (UA) used in combination to achieve x% drug effect. $IC_{x,a}$ and $IC_{x,b}$ are the concentrations for P(IV) NPs and UA NPs to achieve the same effect, respectively. The CI values lower than, equal to, and higher than 1 denote synergism, additivity, and antagonism, respectively.

Resistance factor (RF) was calculated with the equation as below:

$$RF = \frac{IC_{50,a}}{IC_{50,b}}$$

$IC_{50,a}$ and $IC_{50,b}$ are the concentrations the concentrations for P(IV)-UA NPs to achieve 50% drug effect for cancer cells and cisplatin-resistant cancer cells.



Scheme S1. Synthetic procedures of Pt(IV)-UA-PEG.

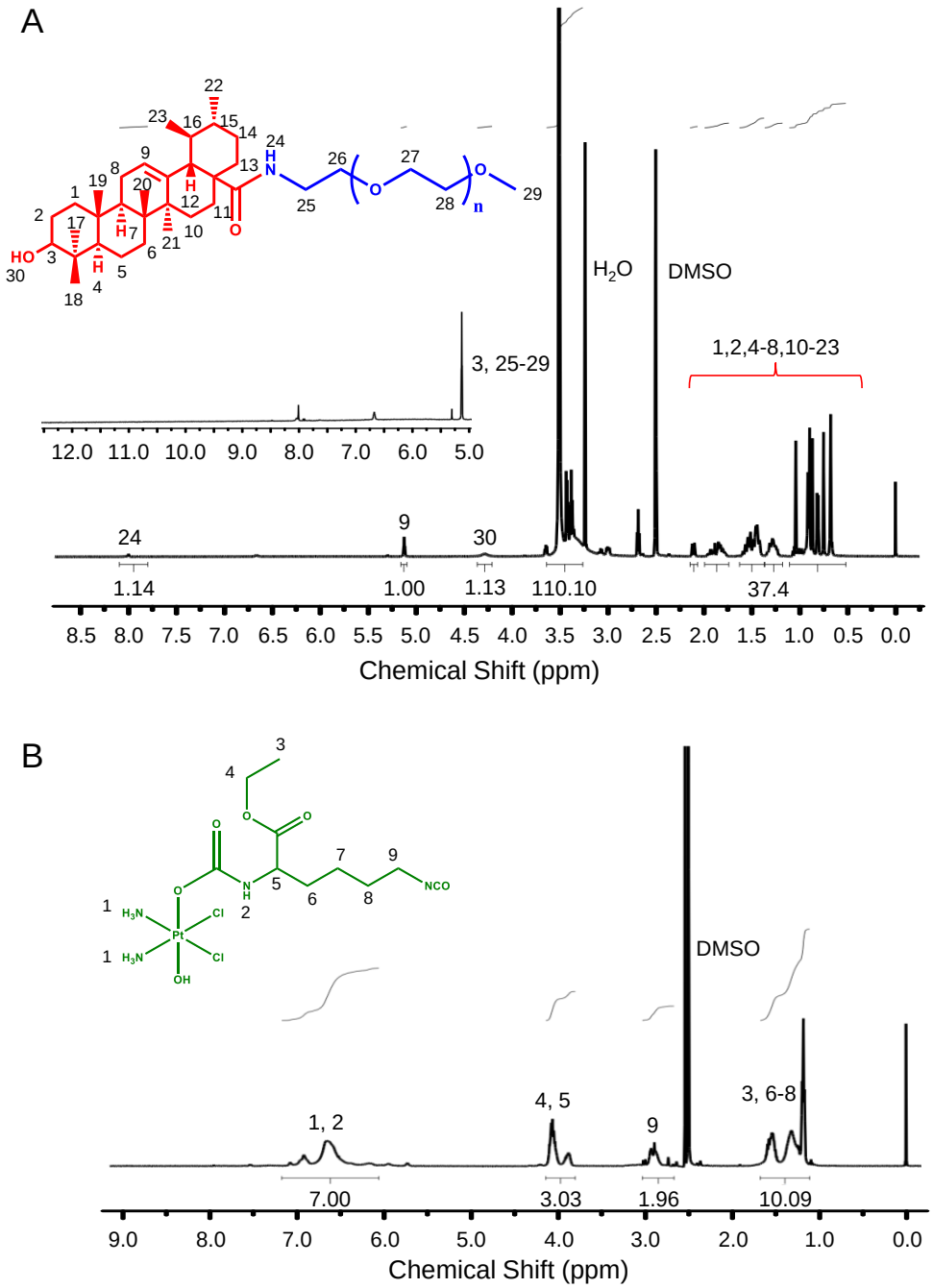


Figure S1. The ^{1}H NMR spectra (400 MHz, room temperature) of (A) UA-PEG (DMSO-*d*6). and (B) Pt(IV)-NCO (DMSO-*d*6).

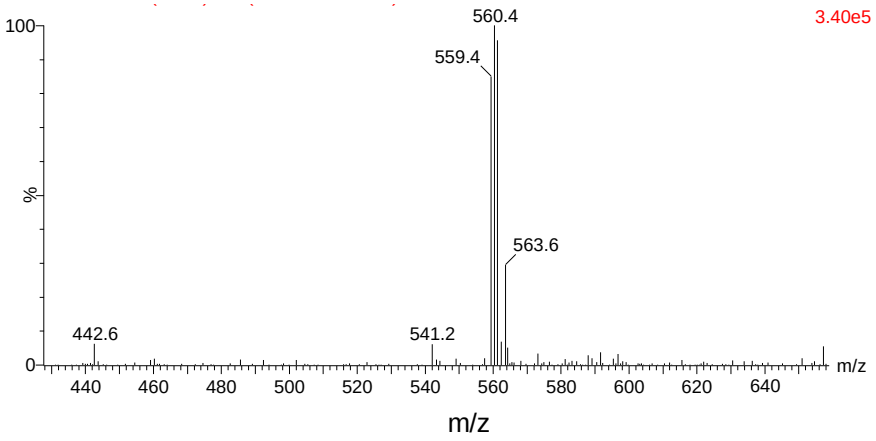


Figure S2. Characterization of Pt(IV)-NCO by ESI-MS (negative mode). The simulated major peak in (A) at m/z = 560.4 could be attributed to its molecular ion of Pt(IV)-NCO.

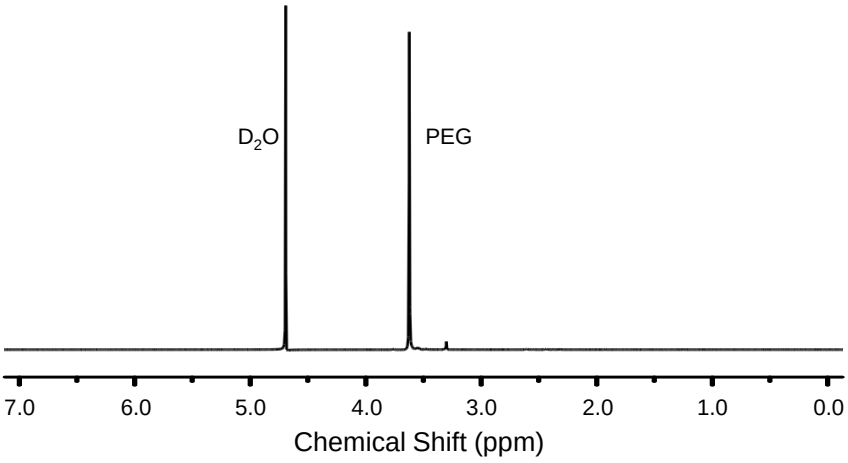


Figure S3. The ^{1}H NMR spectra (400 MHz, room temperature) of Pt-UA NPs (D_2O-d6).

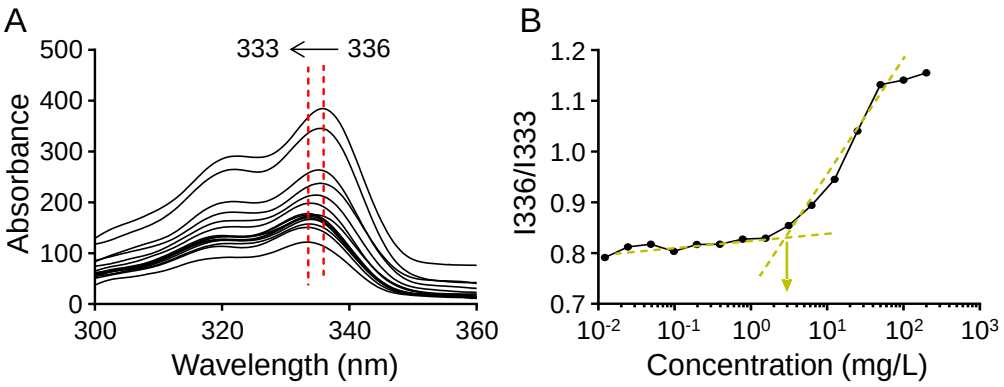


Figure S4. The determination of CAC for Pt(IV)-UA-PEG conjugates. (A) Excitation spectra of pyrene in aqueous solution of various concentrations (200 mg/L to 0.012 mg/L) of Pt(IV)-UA-PEG; (B) The CAC was calculate from Plot of I336/I333 vs. concentration of Pt(IV)-UA-PEG (3 mg/L). The low CAC ensuring the mechanical stability of self-assembly nanoparticles to a certain extent under dilution conditions.

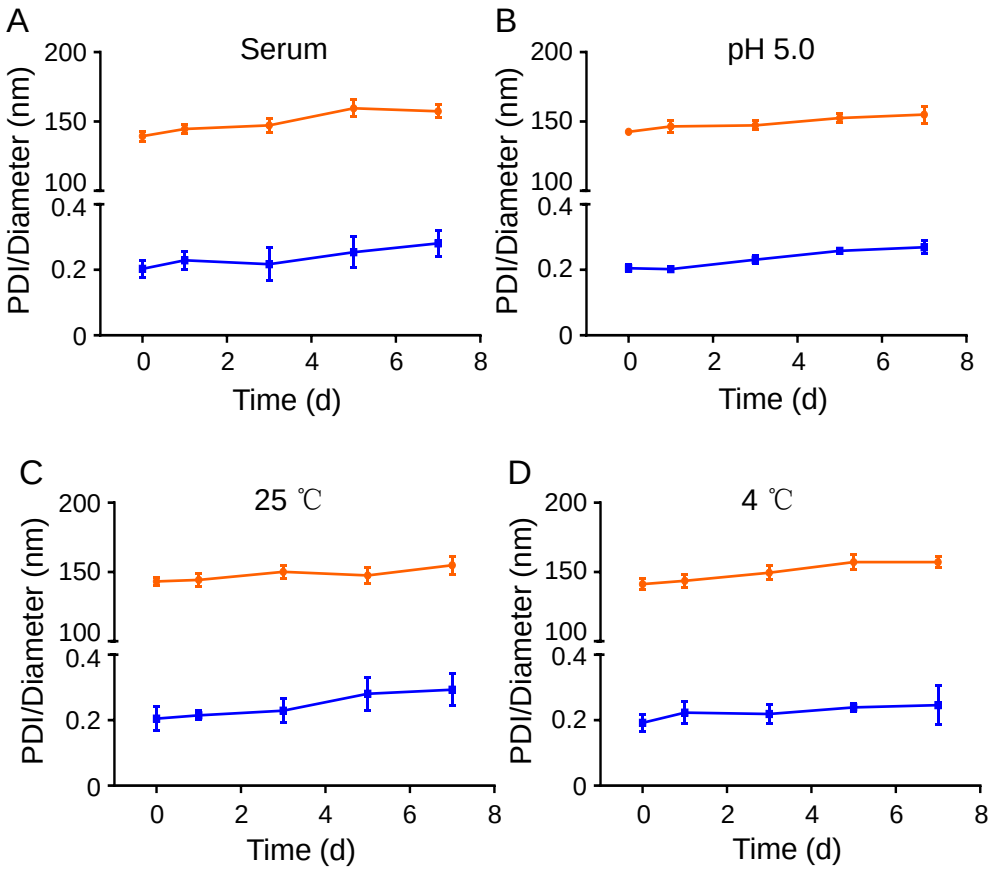


Figure S5. The particle size and PDI changes of Pt(IV)-UA NPs in the presence of (A) serum and (B) PBS (pH 5.0, 0.01 M) at 37℃, and PBS (pH 7.4, 0.01 M) at (C) 25℃ or (D) 4 ℃ over a week.

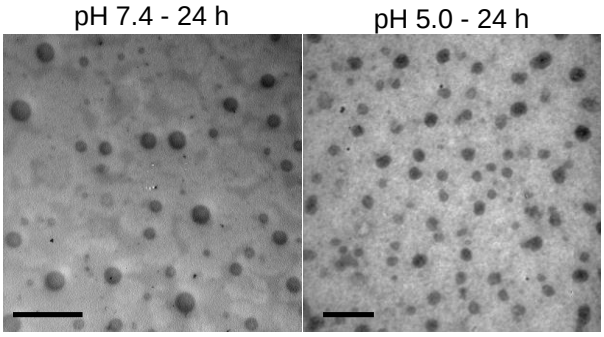


Figure S6. TEM images of Pt(IV)-UA NPs with time in the presence of PBS (10% FBS, 0.01 M) at pH 7.4 and pH 5.0 at 37 ℃. Non-aggregation of Pt(IV)-UA NPs was observed by TEM images in an aqueous environment over 24 h. Results showed that Pt(IV)-UA NPs was stable in mimic blood environment and beneficial to long circulation.

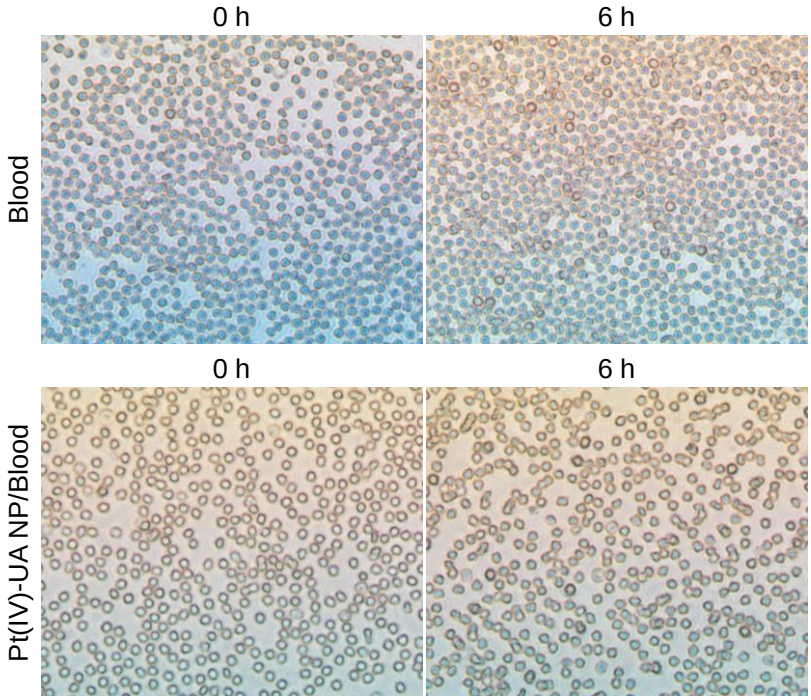


Figure S7. Blood cell morphology of blood and Pt(IV)-UA NPs/blood mixture solution after incubation for 6h.

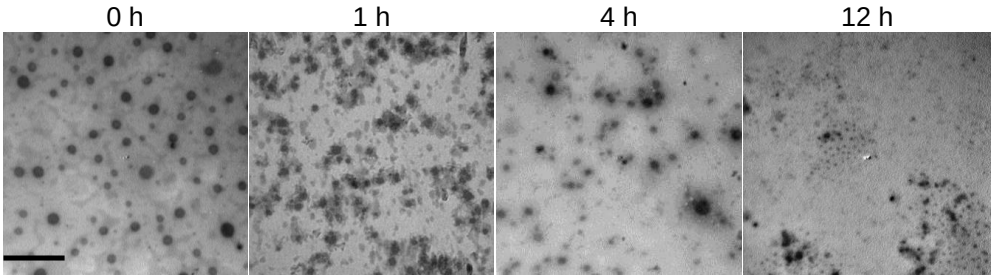


Figure S8. TEM images of Pt(IV)-UA NPs in response to sodium ascorbate (SA, 5 mM) for 0, 1, 4, and 12 h. Scale bar = 1 μm.

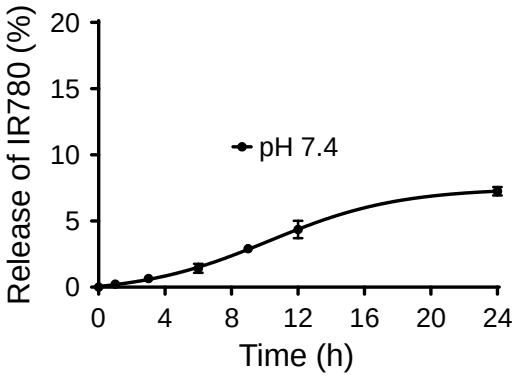


Figure S9. IR780 release profiles of Pt(IV)-UA@IR780 NPs in DMEM at 37°C over 24 h.

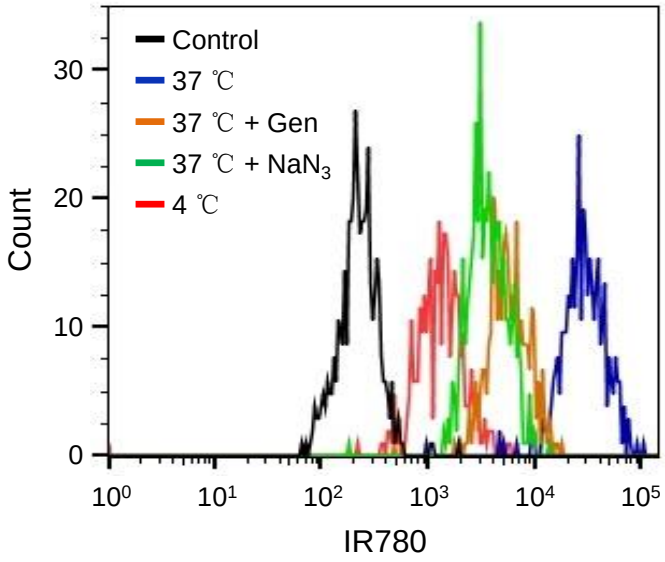


Figure S10. Flow cytometry quantitative analysis of A2780/DDP cells pretreated with competitive genistein or NaN_3 after P(IV)-UA@IR780 NPs incubation (50 μM Pt) for 10 h in the dark at 37 and 4 ℃. Scale bar = 20 μm.

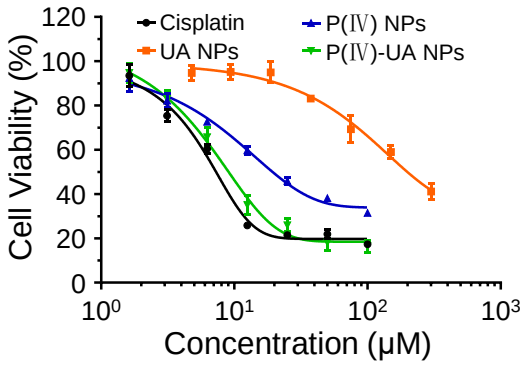


Figure S11. Viability curves of A2780 cells after treatment cisplatin, UA NPs, P(IV) NPs and P(IV)-UA NPs for 72 h.

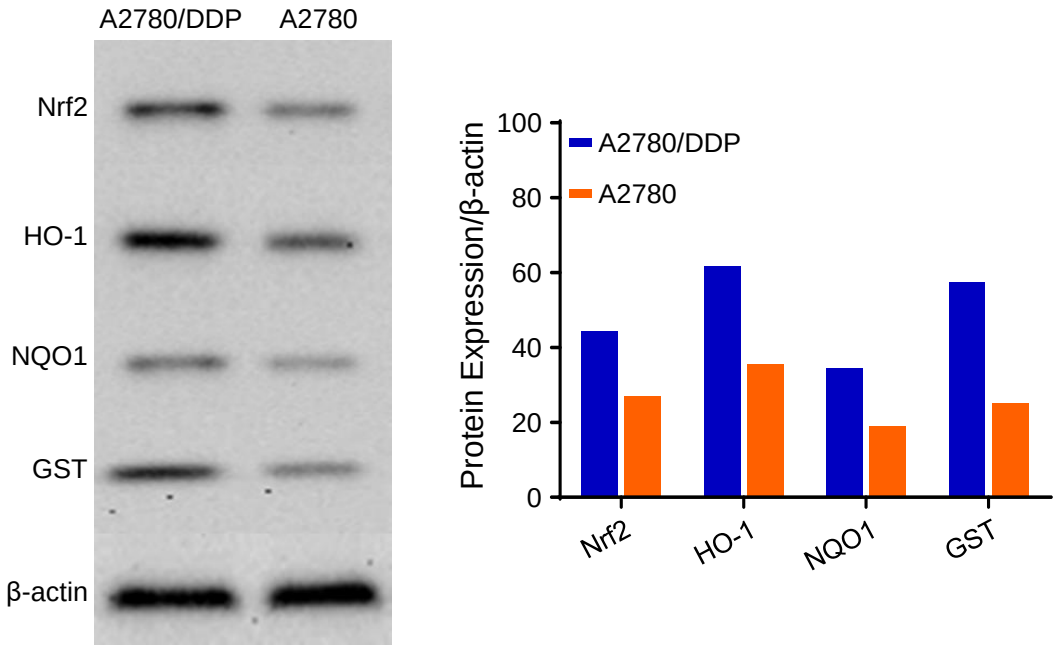


Figure S12. Quantified Nrf2 and its downstream target genes levels of A2780 and A2780/DDP cells according to western blot images using ImageJ software.

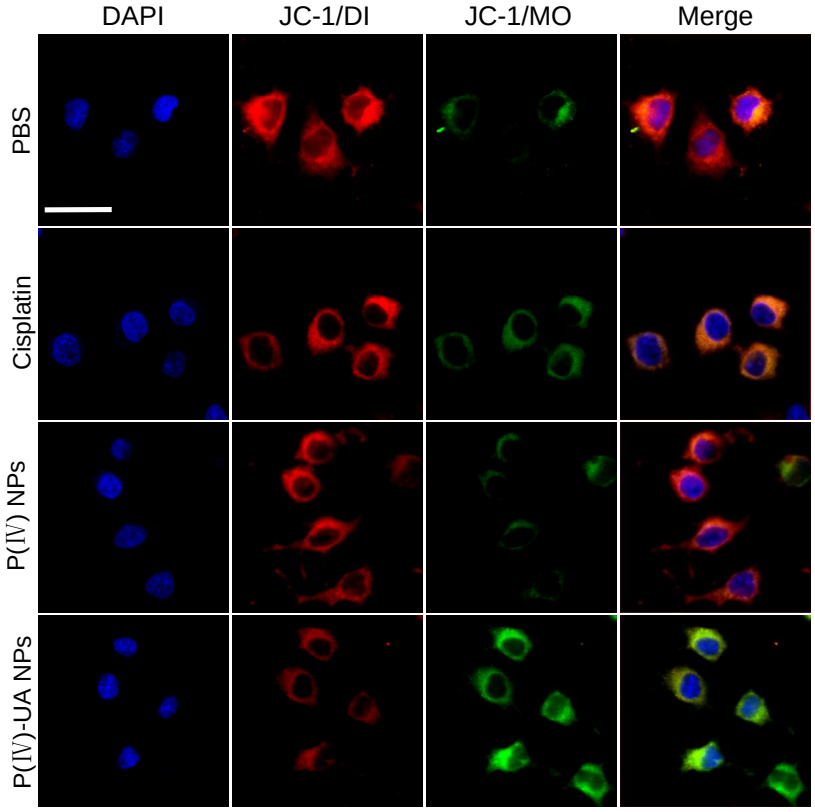


Figure S13. Mitochondrial membrane hyperpolarization as detected by the JC-1 probe. Scale bar = 20 μm.

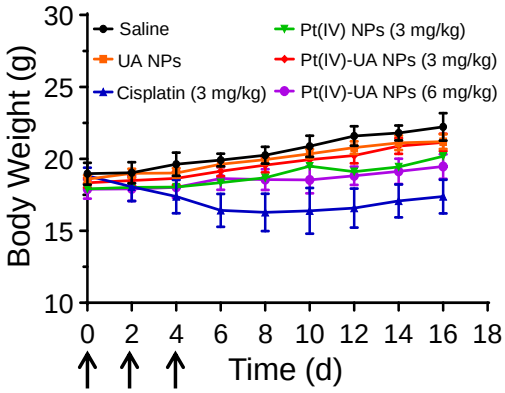


Figure S14. Body weight of the mice in different groups after treatment (the arrows represent the time points of tail vein injection after tumor inoculation).

Table S1. IC_{50} values of cisplatin, UA NPs, Pt(IV) NPs, and P(IV)-UA NPs against A2780 and A2780/DDP cells for 72 h.

		Cisplatin	UA NPs	P(IV) NPs	P(IV)-UA NPs
IC_{50}	A2780	8.319	205.7	24.88	10.31
	A2780/DDP	61.76	284.3	131.4	40.04

Table S2. Survival rate of nude mice after administered intravenously with different doses of cisplatin, UA NPs, Pt(IV) NPs, and Pt(IV)-UA NPs within 7 days (5 mice in each group).

		Cisplatin	UA NPs	Pt(IV) NPs	P(IV)-UA NPs
Survival rate (%)	3 mg/kg	100	100	100	100
	6 mg/kg	20	100	80	100
	9 mg/kg	0	100	20	60