

Supplementary Materials

Reprogramming axial ligands facilitates the self-assembly of a platinum(IV) prodrug: overcoming drug resistance and safer *in vivo* delivery of cisplatin

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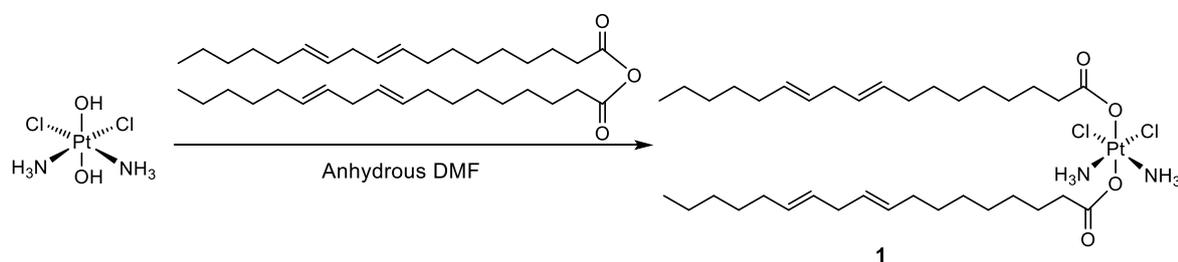
[‡] *These authors contributed equally to this work.*

Materials and Methods for organic synthesis

Cisplatin and Linoleic anhydride were purchased from Tokyo Chemical Industry Co (Shanghai, China). Pluronic[®] F127 was purchased from Sigma-Aldrich (Shanghai, China). Hydrogen Peroxide was purchased from Tianjin Yongda Chemical Reagent Co, (Tianjin, China). All other compounds and solvents were purchased from J&K Chemical (Shanghai, China).

All reactions were performed in a dry atmosphere. Thin layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ pre-coated aluminium sheets (Merck) and visualized by fluorescence quenching. Chromatographic purification was accomplished using flash column chromatography on silica gel (neutral, Qingdao Haiyang Chemical Co., Ltd). ¹H NMR (400 MHz) spectra and ¹³C NMR (100 MHz) were recorded in DMSO-*d*₆ or CDCl₃ on a Bruker 400 spectrometer and calibrated to the residual solvent peak or tetramethylsilane (= 0 ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, dt = double triplet, br = broad. High-resolution mass spectrometry (HRMS)-ESI was recorded on AB TripleTOF 5600+ System (AB SCIEX, Framingham, USA). Reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out on a Hitachi Chromaster 5000 system equipped with a YMC-Pack ODS-A column (5 μm, 250 × 4.6 mm) at a flow rate of 1.0 mL/min. UV detection for Pt(IV) derivatives was at 220 nm. All HPLC runs used linear gradients of acetonitrile (solvent A) and water (solvent B) containing 0.1% trifluoroacetic acid (TFA).

Synthesis of Pt(IV) prodrug **1**



A solution of *c,c,t*-[Pt(NH₃)₂Cl₂(OH)₂] (100 mg, 0.3 mmol) and linoleic anhydride (325 mg, 0.6 mmol) in 2 mL of anhydrous *N,N*-dimethylformamide (DMF) was stirred at 75 °C for 5 h. After removing the solvent, the residue was dissolved in dichloromethane (DCM) and washed with water and brine. The organic layer was dried over anhydrous Na₂SO₄, filtered,

and evaporated under vacuum. The residue was purified by flash column chromatography on silica gel (DCM:MeOH =100:1 to give the product as a yellow solid (153 mg, 58 %).

^1H NMR (400 MHz, chloroform-*d*) δ 5.77 (s, 6H), 5.35 (qd, $J = 11.8, 11.3, 5.6$ Hz, 8H), 2.77 (t, $J = 6.5$ Hz, 4H), 2.45 (t, $J = 7.7$ Hz, 4H), 2.09 – 1.99 (m, 8H), 1.57 (t, $J = 7.4$ Hz, 4H), 1.35 – 1.27 (m, 28H), 0.89 (t, $J = 6.7$ Hz, 6H).

^{13}C NMR (100 MHz, chloroform-*d*) δ 181.40, 181.40, 130.27, 130.27, 130.03, 130.03, 128.09, 128.09, 127.90, 127.90, 31.52, 31.52, 29.65, 29.65, 29.35, 29.35, 29.35, 29.35, 29.26, 29.26, 29.21, 29.21, 29.19, 29.19, 27.21, 27.21, 27.21, 27.21, 25.92, 25.92, 25.63, 25.63, 22.57, 22.57, 14.08, 14.08.

HRMS: calcd for $[\text{C}_{36}\text{H}_{68}\text{Cl}_2\text{N}_2\text{O}_4\text{Pt}]^+ [\text{M}]^+ = 857.4204$; obsd: 857.4249.

Anal. Calcd for $\text{C}_{36}\text{H}_{68}\text{Cl}_2\text{N}_2\text{O}_4\text{Pt} \cdot 2\text{DMSO}$: C, 47.33; H, 7.94; N, 2.76. Found: C, 48.68; H, 7.62; N, 2.67.

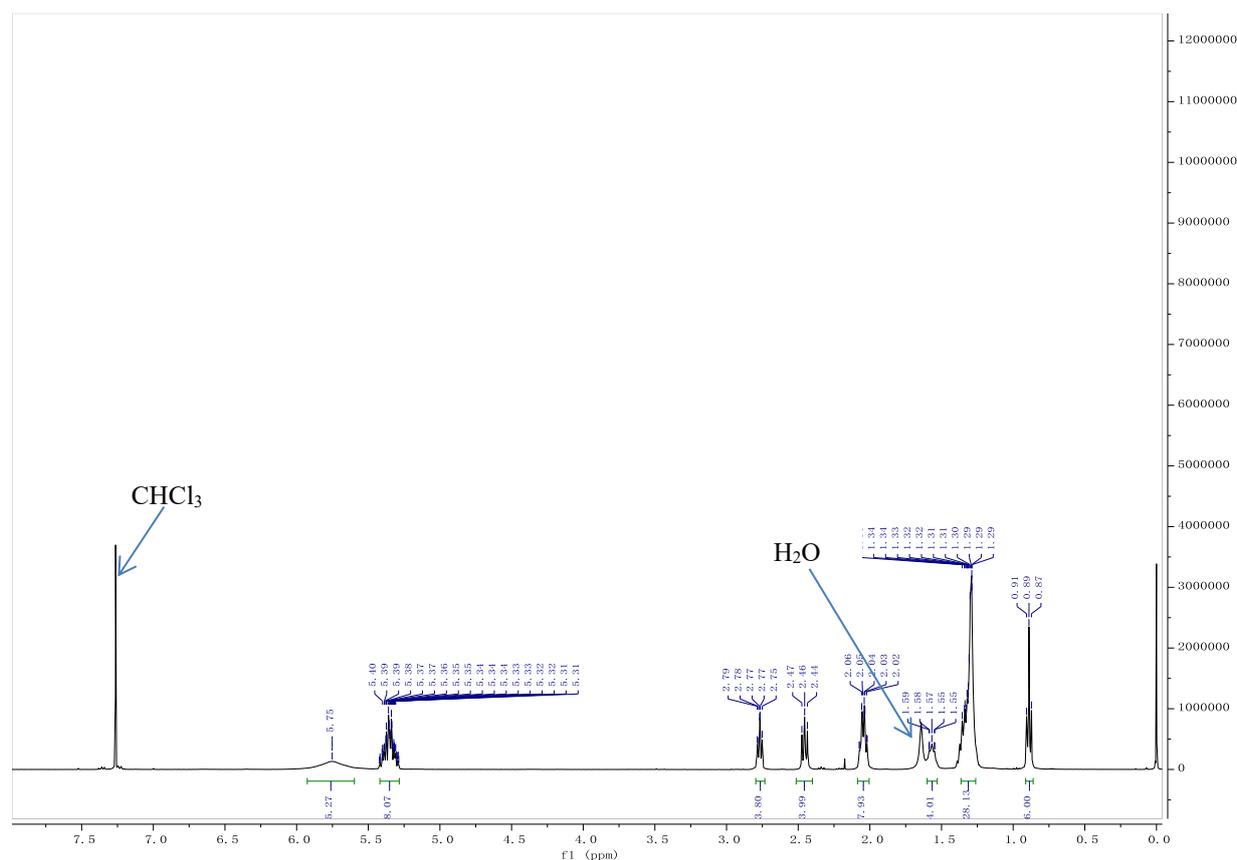


Figure S1. ^1H NMR spectrum of Pt(IV) prodrug 1 in CDCl_3 .

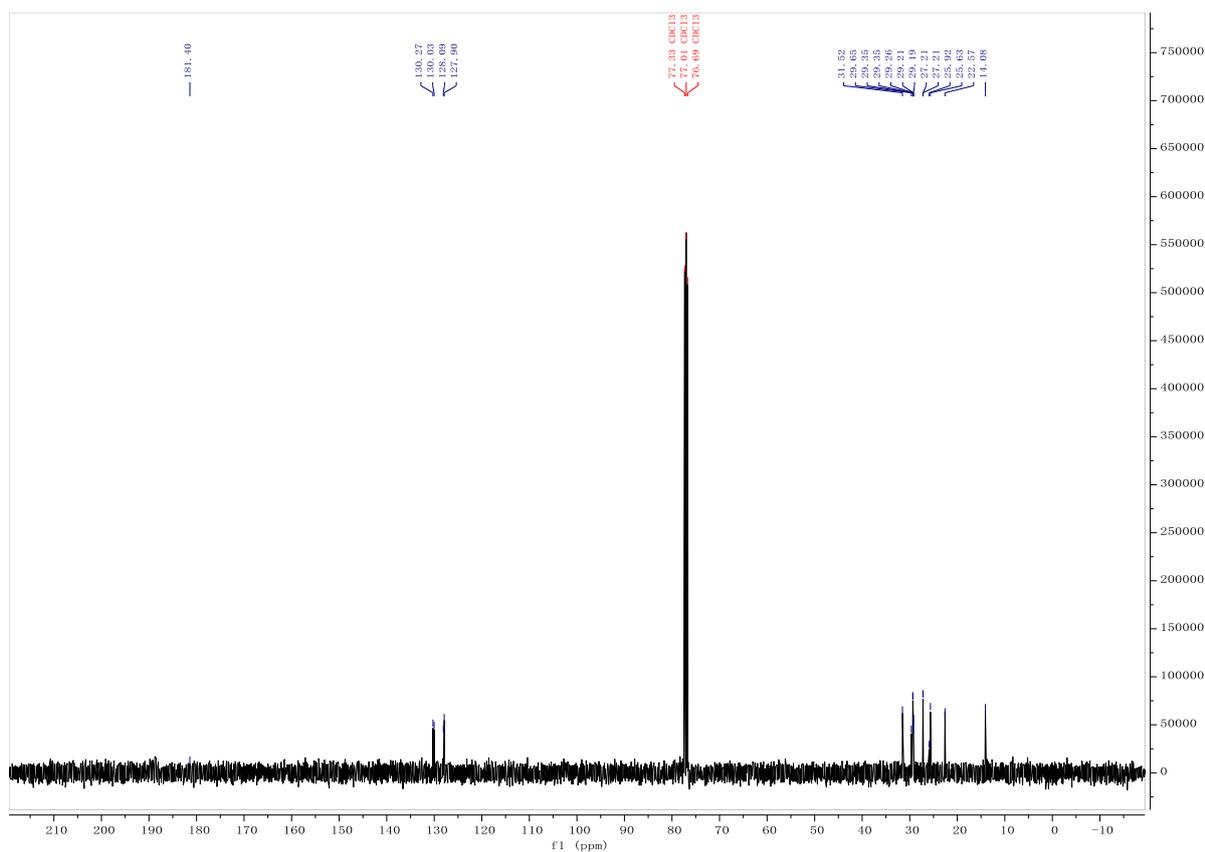


Figure S2. ^{13}C NMR spectrum of Pt(IV) prodrug **1** in CDCl_3 .

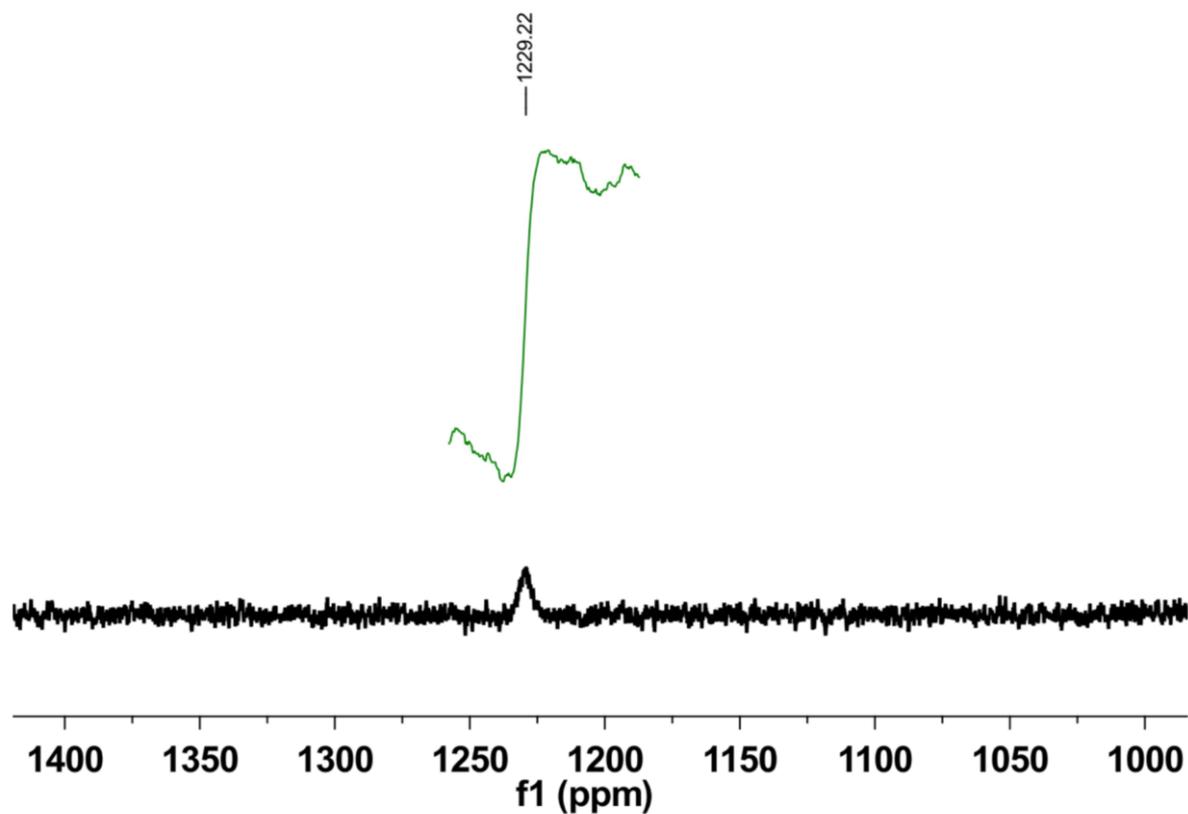


Figure S3. ^{195}Pt NMR spectrum of Pt(IV) prodrug **1** in $\text{DMSO-}d_6$.

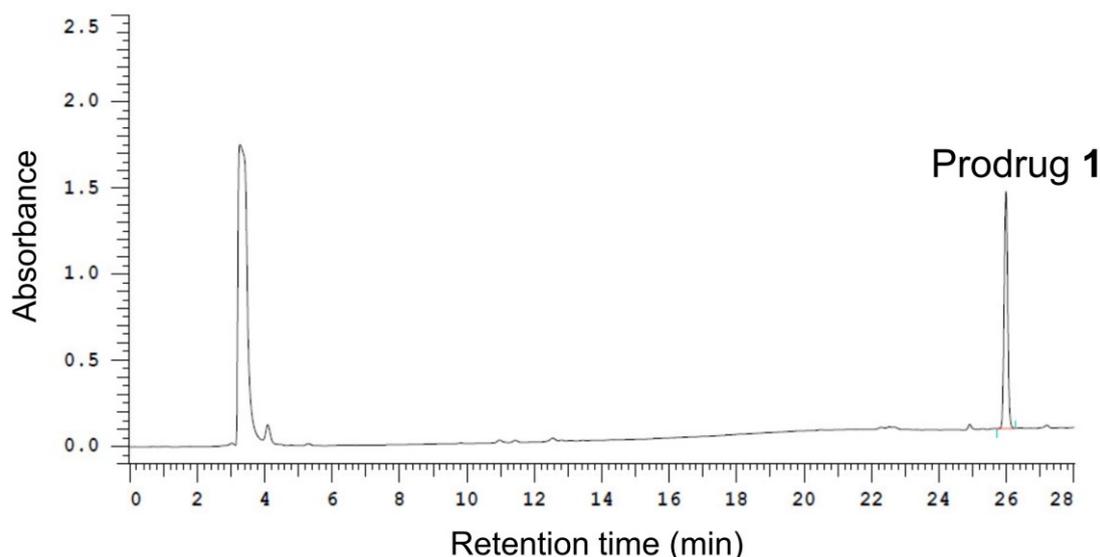
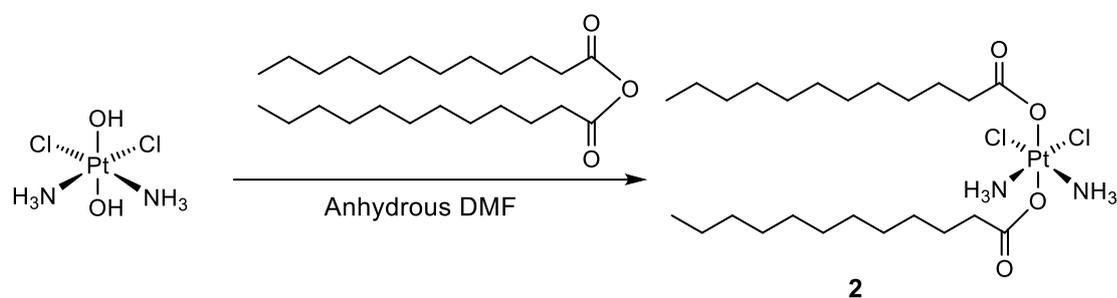


Figure S4. RP-HPLC chromatogram of Pt(IV) prodrug **1**. The purified **1** was subjected to analytical HPLC using a C8 reverse-phase column (5 μm , 250 mm \times 4.6 mm). A gradient of 50-100% acetonitrile in water within 25 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at a wavelength of 220 nm.

Synthesis of Pt(IV) prodrug **2**



A solution of *c,c,t*-[Pt(NH₃)₂Cl₂(OH)₂] (300 mg, 0.9 mmol) and lauric anhydride (723 mg, 1.89 mmol) in 5 mL of anhydrous DMF was stirred at 50 °C for 12 h. After removing most of DMF solvent, the mixture product was precipitated by addition of deionized H₂O and washed with ether. The residue was further dried under reduced pressure, giving compound **2** as a white solid (503 mg, 80 %).

¹H NMR (400 MHz, DMSO-*d*₆) δ 6.52 (s, 6H), 2.20 (t, *J* = 7.5 Hz, 4H), 1.48 – 1.39 (m, 4H), 1.24 (s, 32H), 0.88 – 0.83 (m, 6H).

¹³C NMR (100 MHz, DMSO-*d*₆) δ 181.38, 181.38, 36.18, 36.18, 31.77, 31.77, 29.53, 29.53, 29.51, 29.51, 29.45, 29.45, 29.40, 29.40, 29.19, 29.19, 29.11, 29.11, 25.93, 25.93, 22.57, 22.57, 14.43, 14.43.

HRMS: calcd for $[C_{24}H_{52}Cl_2N_2O_4Pt]^+ [M]^+ = 697.2952$; obsd: 697.3011.

Anal. Calcd for $C_{24}H_{52}Cl_2N_2O_4Pt$: C, 41.26; H, 7.50; N, 4.01. Found: C, 41.70; H, 7.47; N, 3.85.

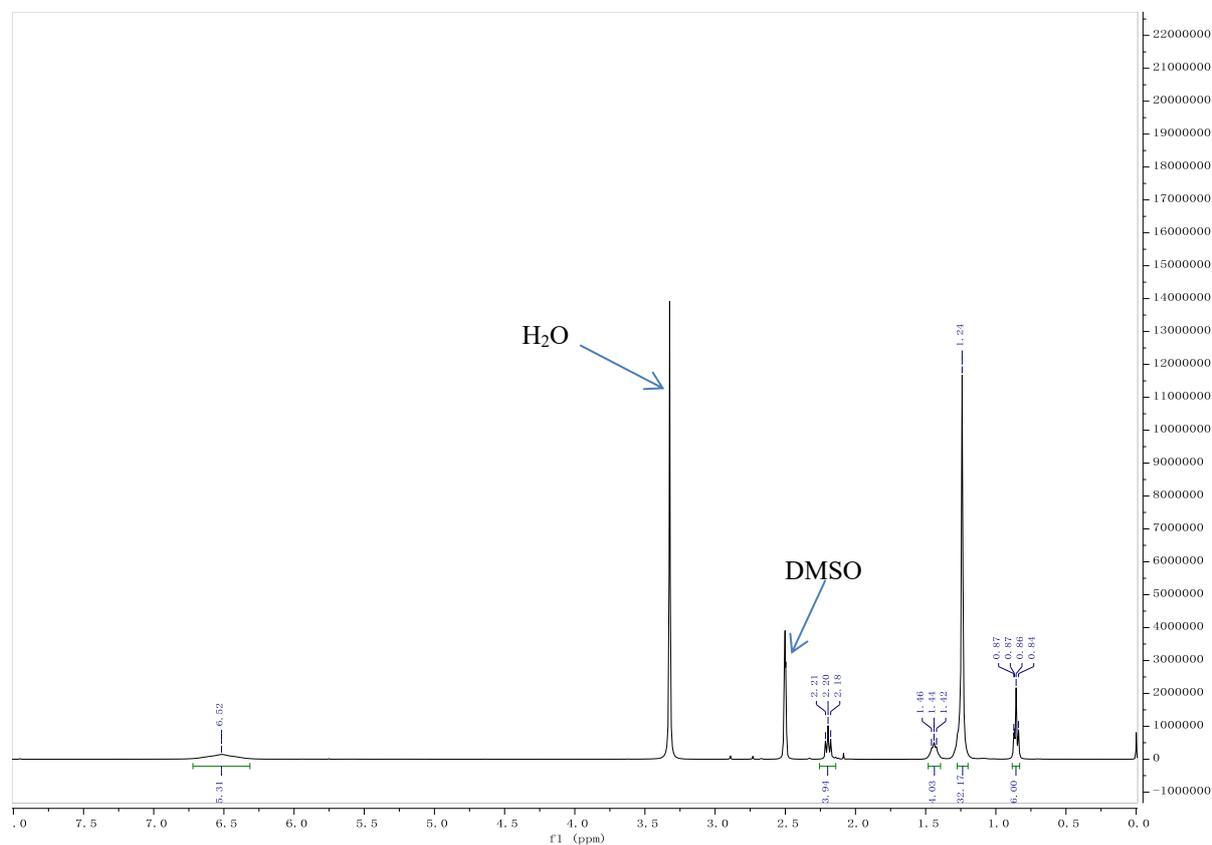


Figure S5. 1H NMR spectrum of Pt(IV) prodrug **2** in $DMSO-d_6$.

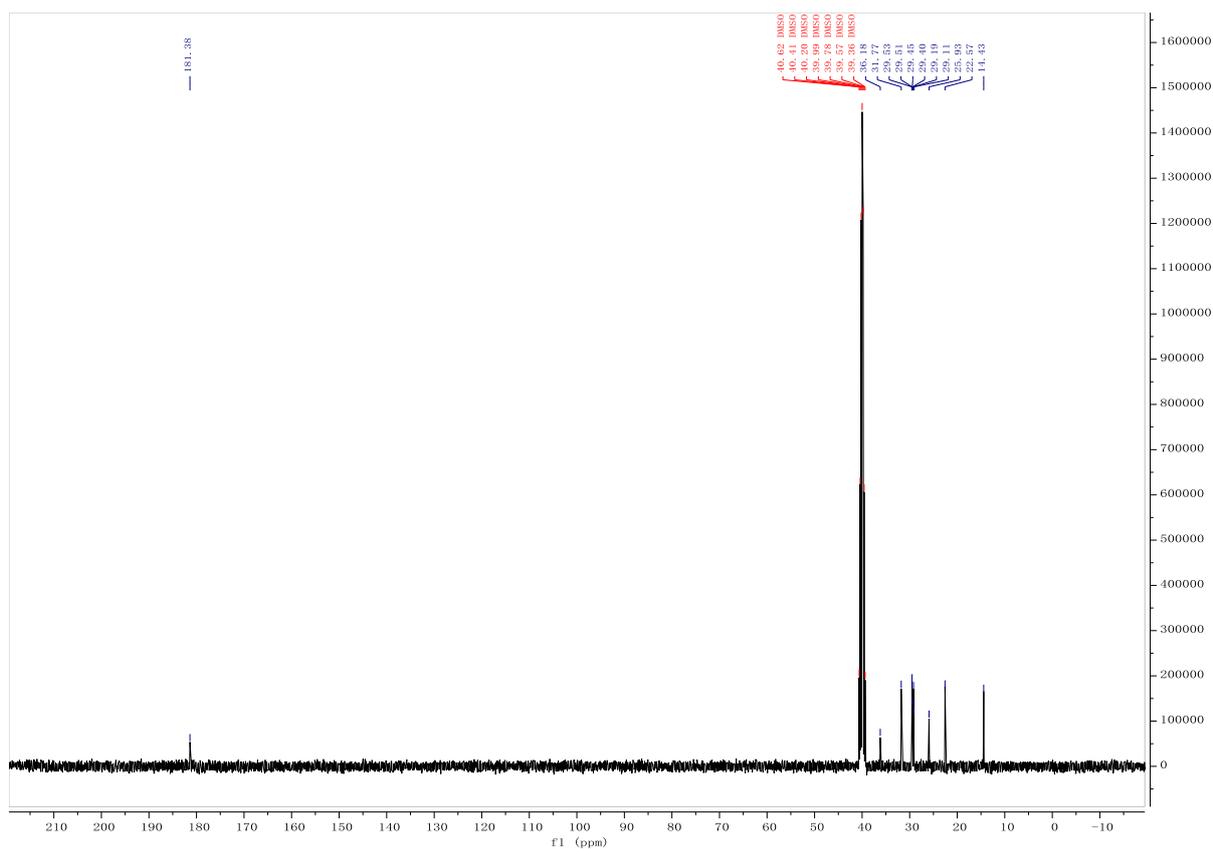


Figure S6. ^{13}C NMR spectrum of Pt(IV) prodrug **2** in $\text{DMSO-}d_6$.

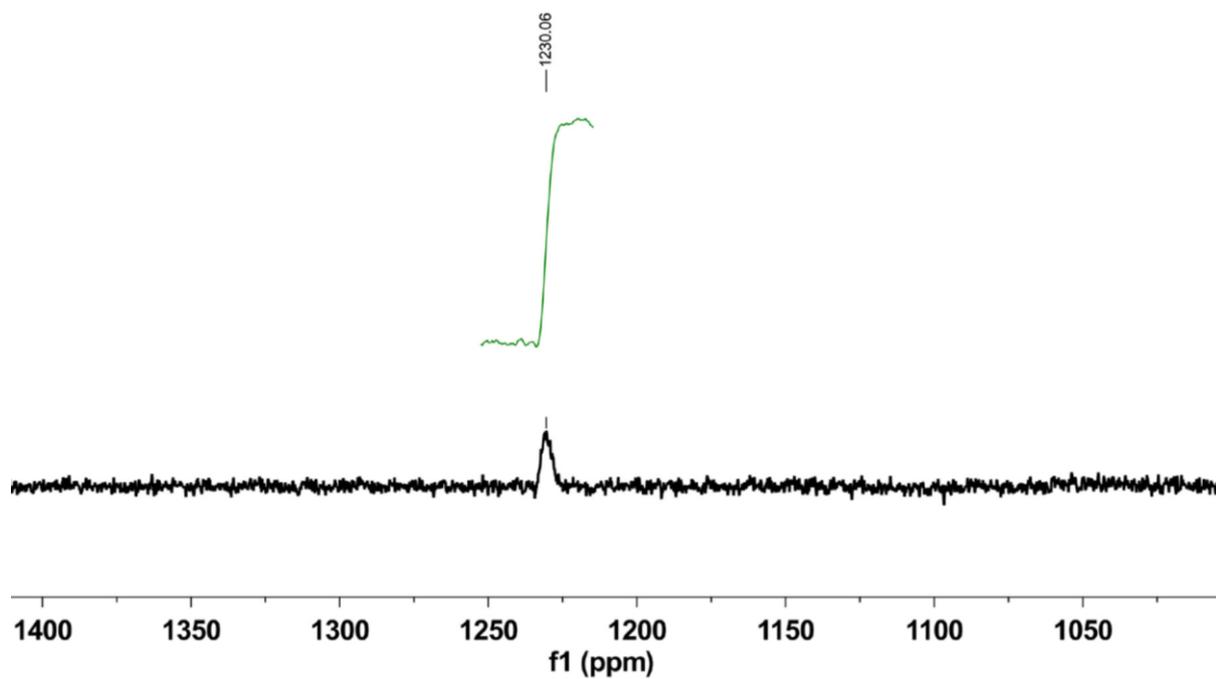


Figure S7. ^{195}Pt NMR spectrum of Pt(IV) prodrug **2** in $\text{DMSO-}d_6$.

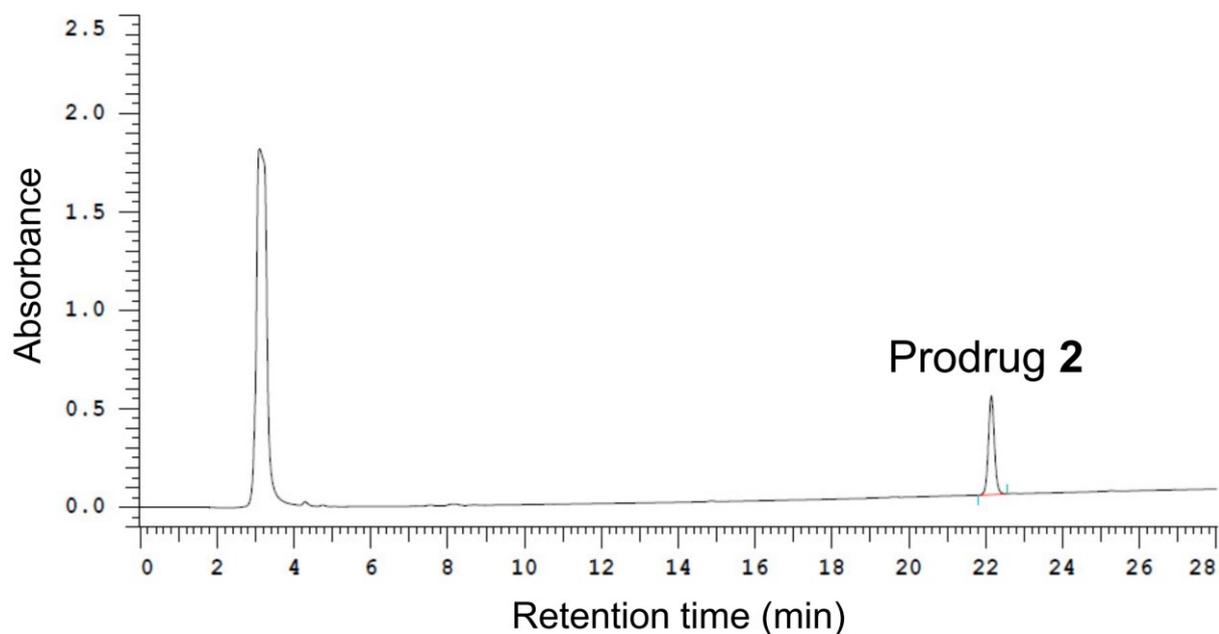
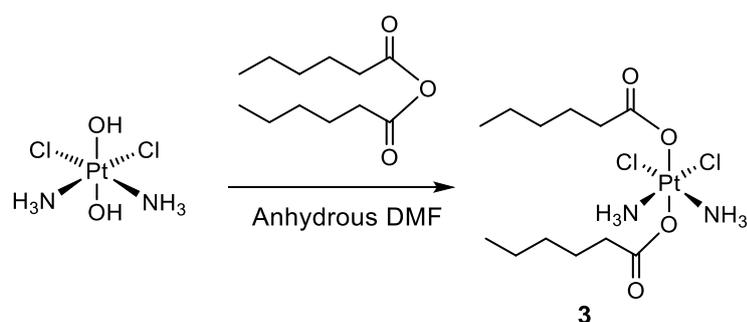


Figure S8. RP-HPLC chromatogram of Pt(IV) prodrug **2**. The purified **2** was subjected to analytical HPLC using a C8 reverse-phase column (5 μm , 250 mm \times 4.6 mm). A gradient of 50-100% acetonitrile in water within 25 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at a wavelength of 220 nm.

Synthesis of Pt(IV) prodrug **3**



A solution of *c,c,t*-[Pt(NH₃)₂Cl₂(OH)₂] (300 mg, 0.9 mmol) and hexanoic anhydride (422 mg, 1.89 mmol) in 5 mL of anhydrous DMF was stirred at 50 °C for 12 h. After removing most of DMF solvent, the mixture product was precipitated by addition of deionized H₂O and washed with ether. The residue was further dried under reduced pressure, giving compound **3** as a white solid (286 mg, 60 %).

¹H NMR (400 MHz, DMSO-*d*₆) δ 6.52 (s, 6H), 2.20 (t, *J* = 7.5 Hz, 4H), 1.45 (dd, *J* = 9.0, 5.7 Hz, 4H), 1.26 (dhept, *J* = 7.6, 3.4 Hz, 8H), 0.91 – 0.82 (m, 6H).

^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ 181.38, 181.38, 36.13, 36.13, 31.31, 31.31, 25.58, 25.58, 22.42, 22.42, 14.35, 14.35.

HRMS: calcd for $[\text{C}_{12}\text{H}_{28}\text{Cl}_2\text{N}_2\text{O}_4\text{Pt}]^+ [\text{M}]^+ = 529.1074$; obsd: 529.1136.

Anal. Calcd for $\text{C}_{12}\text{H}_{28}\text{Cl}_2\text{N}_2\text{O}_4\text{Pt}$: C, 27.18; H, 5.32; N, 5.28. Found: C, 27.10; H, 5.32; N, 5.23.

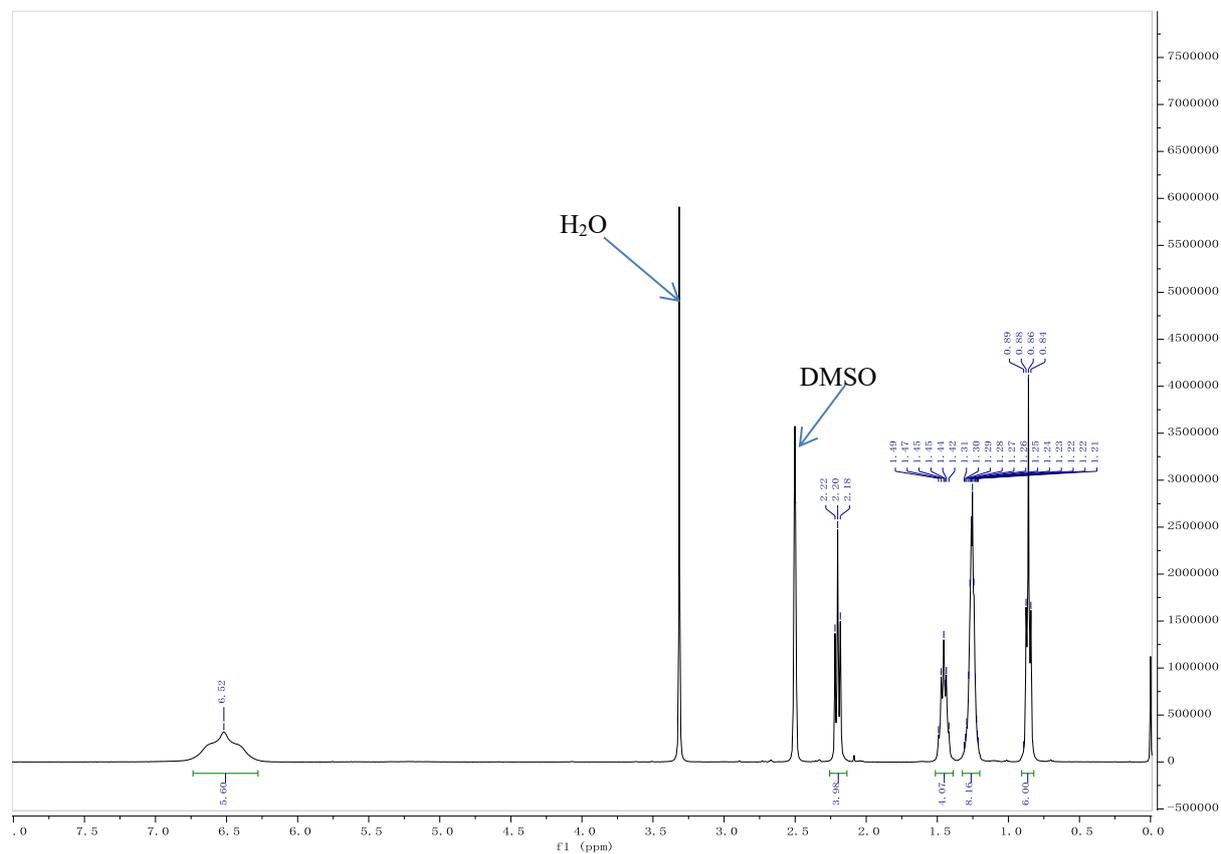


Figure S9. ^1H NMR spectrum of Pt(IV) prodrug **3** in $\text{DMSO-}d_6$.

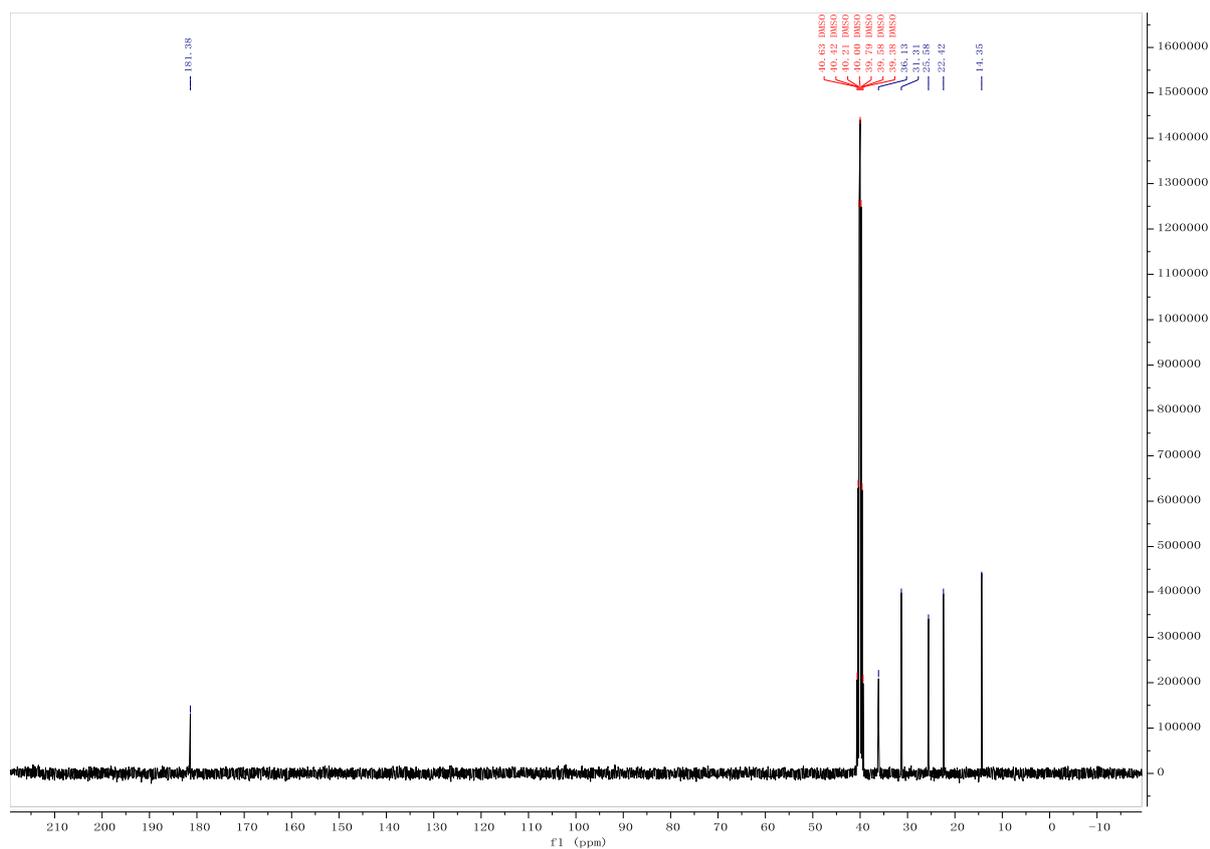


Figure S10. ^{13}C NMR spectrum of Pt(IV) prodrug **3** in $\text{DMSO-}d_6$.

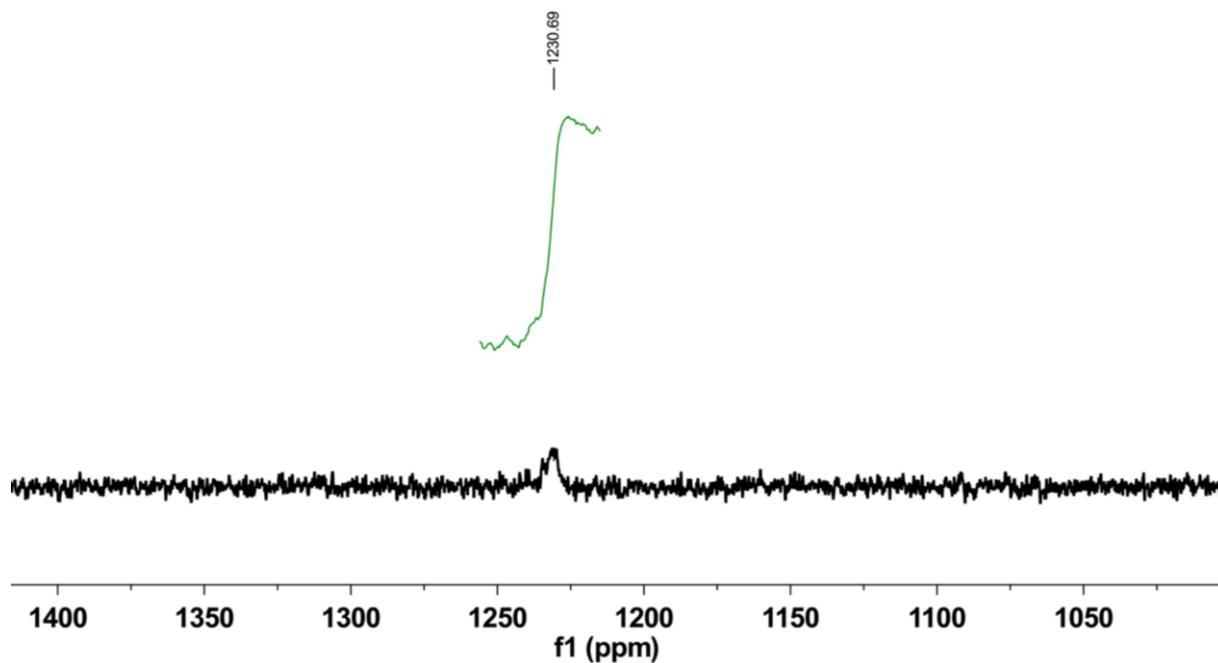


Figure S11. ^{195}Pt NMR spectrum of Pt(IV) prodrug **3** in $\text{DMSO-}d_6$.

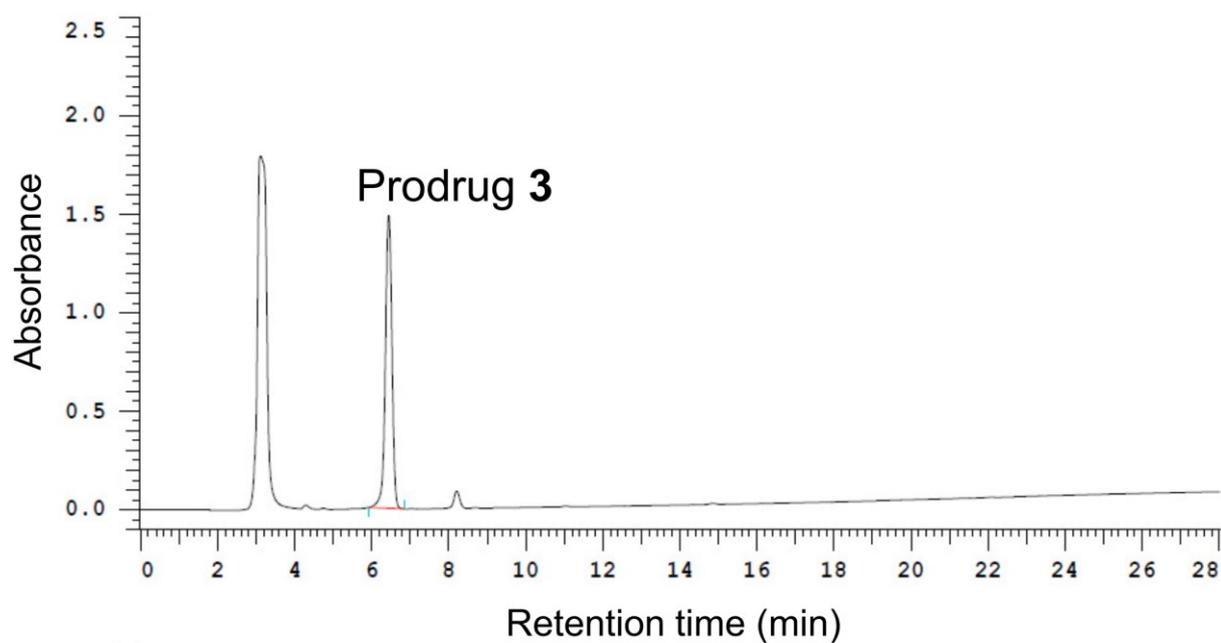


Figure S12. RP-HPLC chromatogram of Pt(IV) prodrug **3**. The purified **3** was subjected to analytical HPLC using a C8 reverse-phase column (5 μm , 250 mm \times 4.6 mm). A gradient of 50-100% acetonitrile in water within 25 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at a wavelength of 220 nm.

Table S1. *In vitro* cytotoxicity of free cisplatin and Pt(IV)-NP against A549 and A549cisR cells after 72 h of incubation (expressed as IC₅₀±SD in μM).

Cell lines	IC ₅₀ (μM) ^{[a][b]}		
	Free cisplatin ^[c]	Pt(IV)-NP	FI ^[d]
A549	3.38±0.37	2.93±0.22	1.2
A549cisR	10.02±1.60	1.71±0.20	5.9
RF ^[d]	2.96	0.58	-
A2780	3.24±0.17	0.67±0.04	4.8
A2780cisR	11.48±1.15	1.32±0.22	8.7
RF ^[d]	3.54	1.97	-

[a] The IC₅₀ values were determined in the MTT assay.

[b] The IC₅₀ is defined as the concentration of compound that inhibits cell proliferation by 50%. Lower values indicate higher cancer cell toxicity.

[c] Cisplatin was dissolved in DMF for the MTT assay.

[c] The FI (fold increase) is defined as IC₅₀(cisplatin)/IC₅₀(Pt(IV)-NP).

[d] The RF (resistance factor) is defined as the IC₅₀ values in cisplatin-resistant cells divided by the IC₅₀ values in cisplatin-sensitive cells.

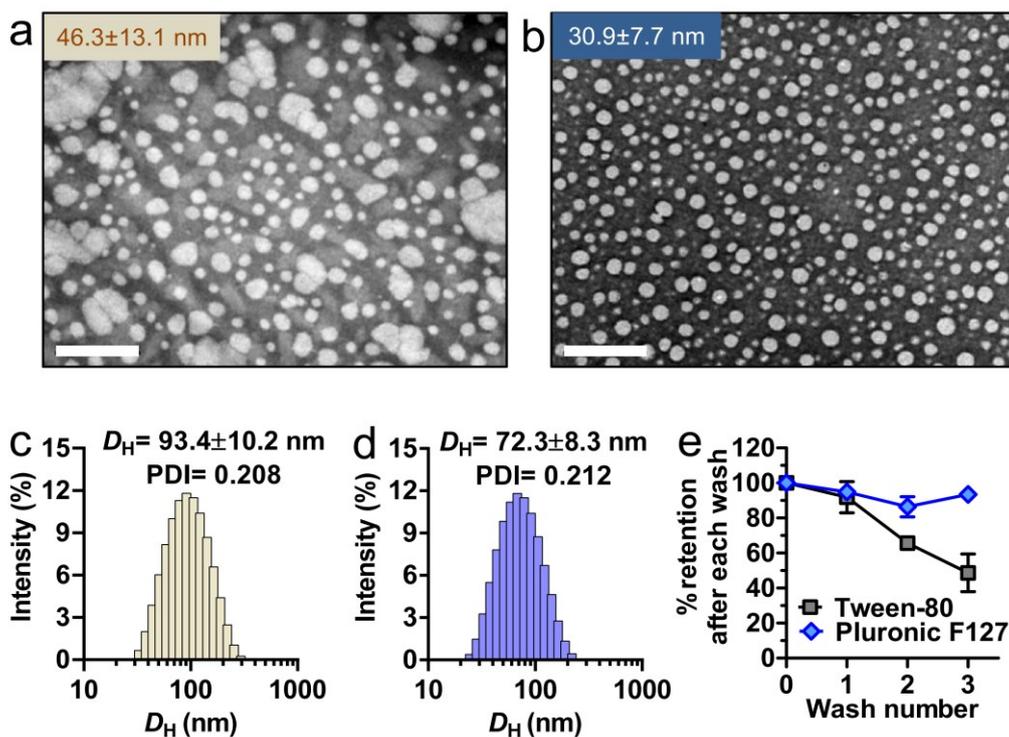


Figure S13. Characterization of Pt(IV) **1**-assembled NPs and PEGylated NPs using the temperature-sensitive triblock copolymer surface cloaking. TEM images for Pt(IV) **1**-assembled NPs (a) and Pt(IV)-NP (b) in water. Scale bars, 200 nm. The distribution of hydrodynamic diameters (D_H) of **1**-assembled NPs (c) and Pt(IV)-NP (d) analyzed by DLS. (e) Retentate amounts of hydrophobic prodrug after solubilization in the indicated surfactants (10% w/v) and repeated centrifugal filtration at 4°C.

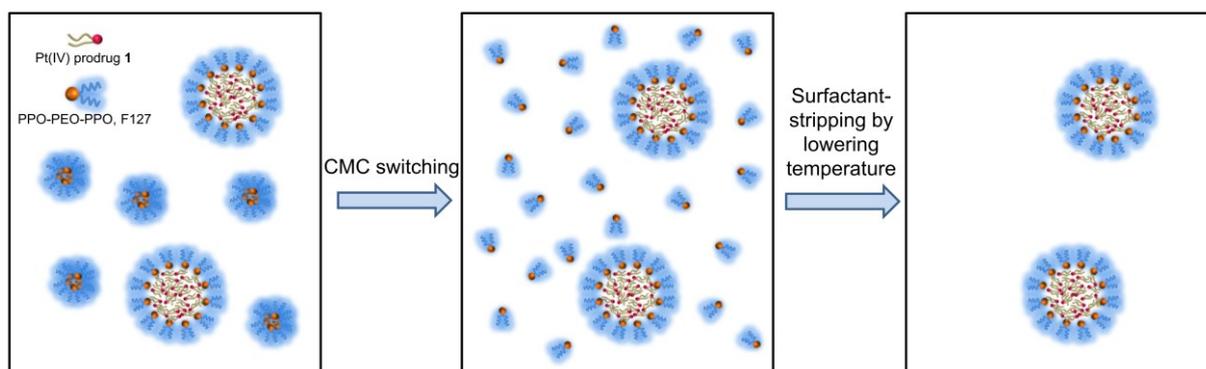


Figure S14. Schematic illustration of preparation of Pt(IV) prodrug **1**-formulated, F127-coated nanoparticle (Pt(IV)-NP) by low-temperature surfactant stripping. The F127 hydrophobic segment, and the F127 hydrophilic segment are shown in brown and blue, respectively.

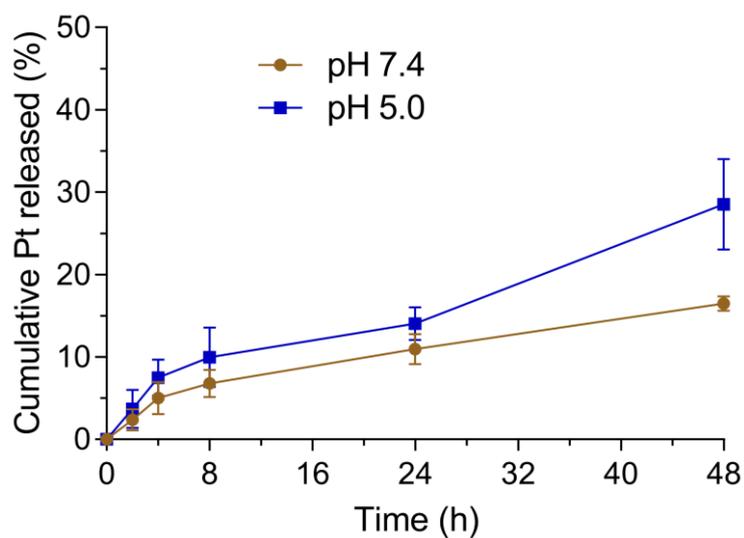


Figure S15. Platinum drug release profiles from Pt(IV)-NP against PBS buffer.

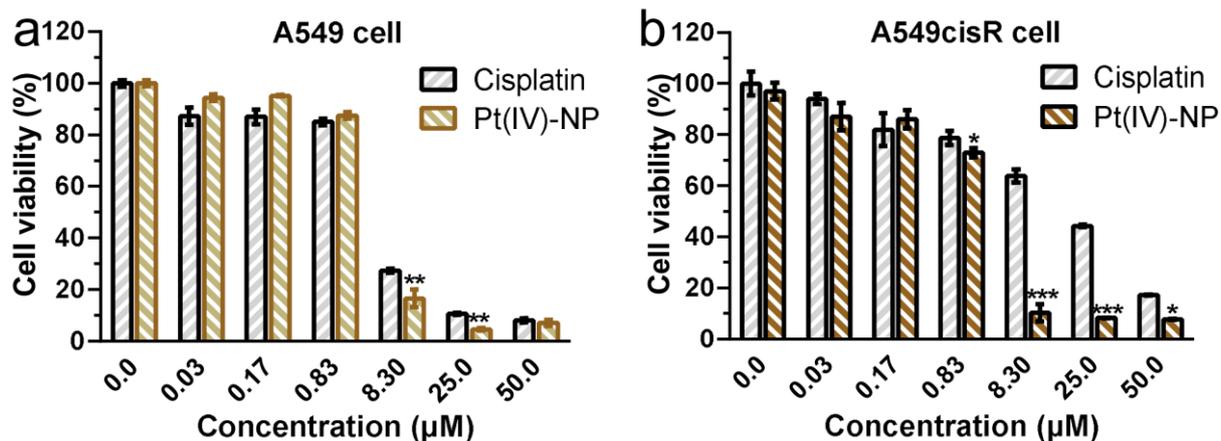


Figure S16. *In vitro* cytotoxicity of cisplatin (dissolved in DMF) and Pt(IV)-NP in human lung cancer A549 cells (a) and cisplatin-resistant A549 cells (A549cisR) (b).

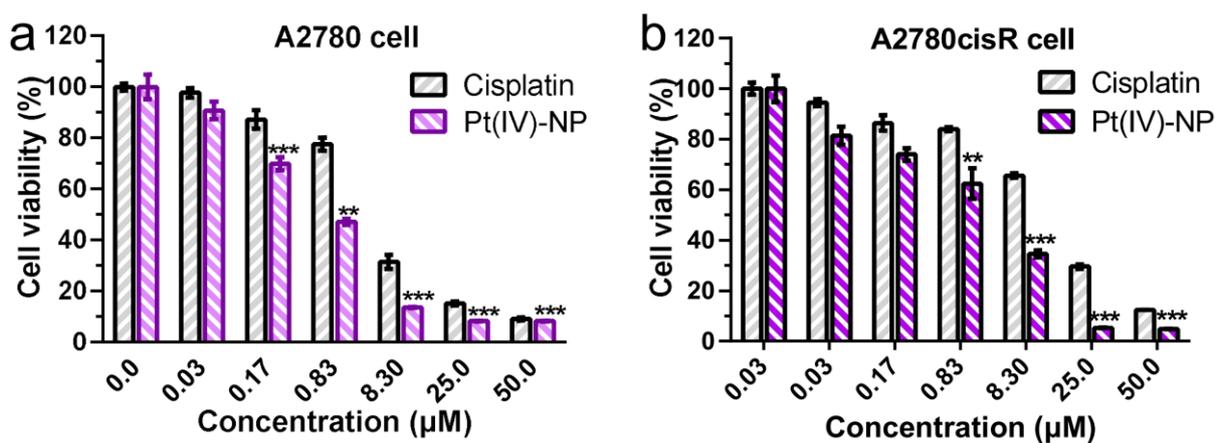


Figure S17. *In vitro* cytotoxicity of cisplatin (dissolved in DMF) and Pt(IV)-NP in human ovarian carcinoma A2780 cells (a) and cisplatin-resistant A2780 cells (A2780cisR) (b).

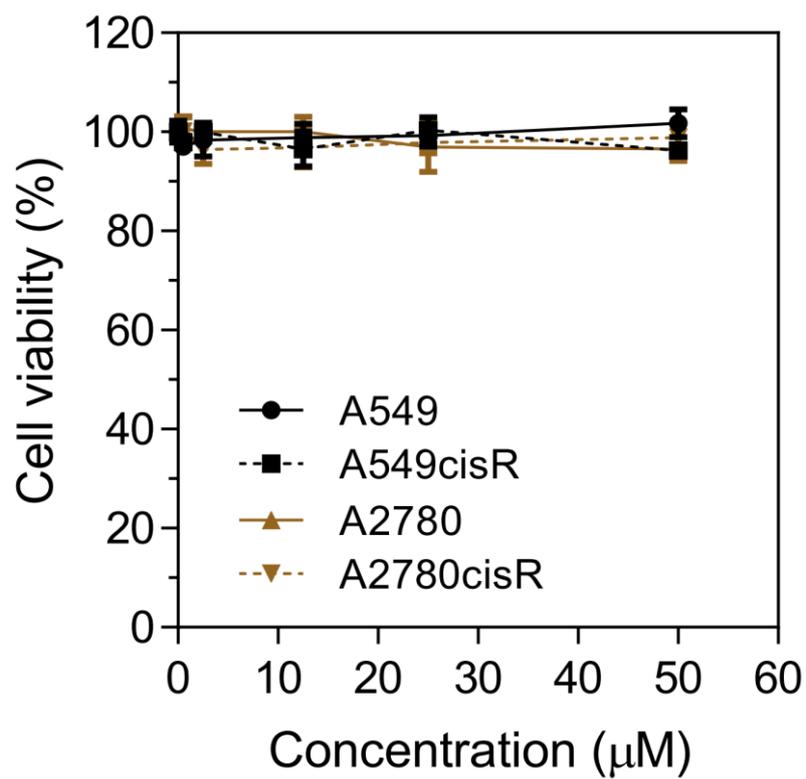


Figure S18. *In vitro* cytotoxicity of linoleic acid (LA) in four cell lines. The cells were treated with LA for 72 h, and the cell viability was determined by the MTT assay.

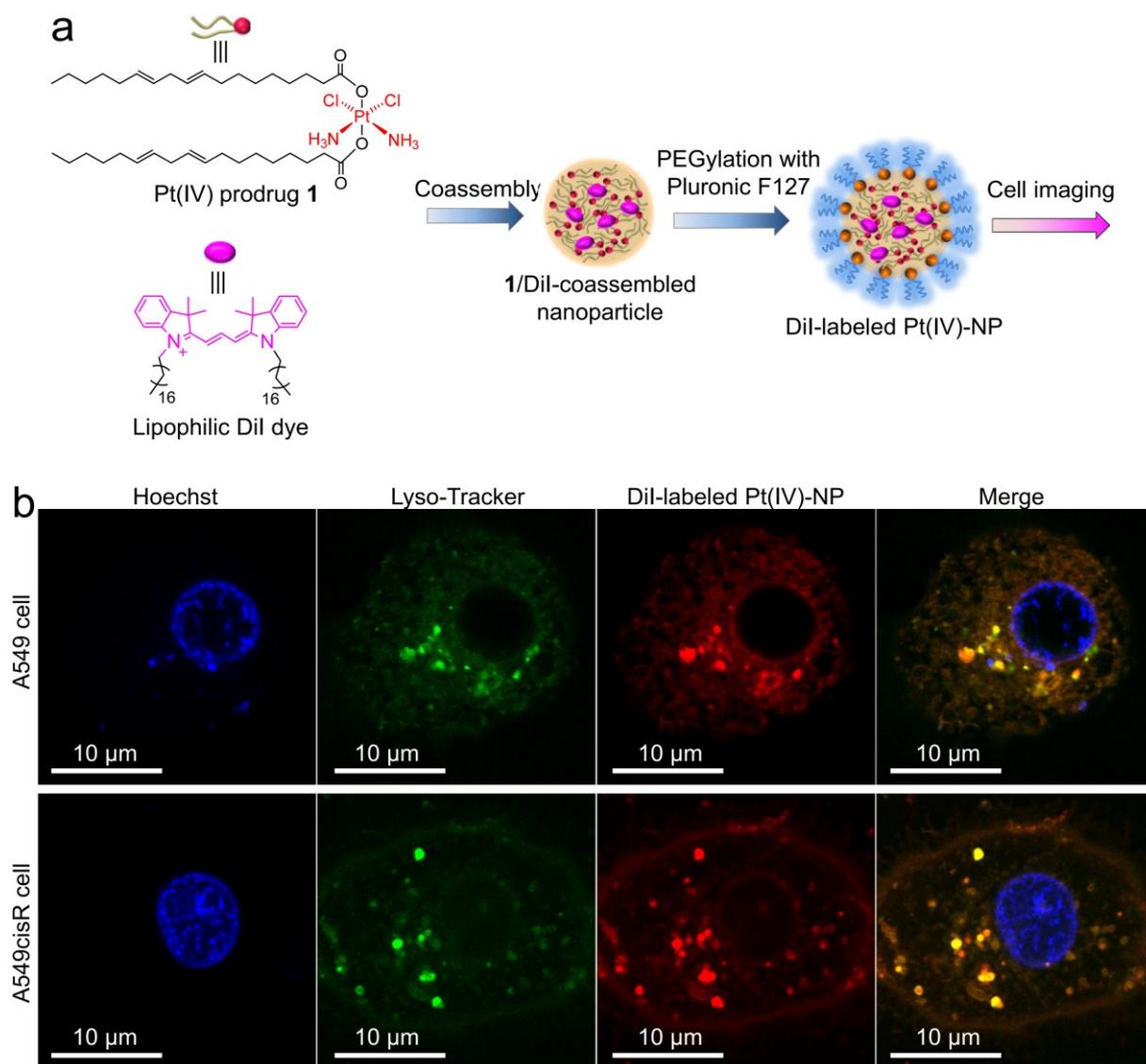


Figure S19. a) Schematic illustration of co-assembling lipophilic DiI dye with Pt(IV) prodrug **1** to form DiI-labeled Pt(IV)-NP for the *in vitro* uptake and intracellular tracking of Pt(IV)-NP. b) Representative confocal laser scanning fluorescence microscopy (CLSM) images of A549 and A549cisR cells that were treated with DiI-labeled Pt(IV)-NP (1 μ M DiI) for 2 h. The nuclei and the endosomes/lysosomes were stained with Hoechst 33342 (blue) and lysotracker DND-26 (green), respectively.

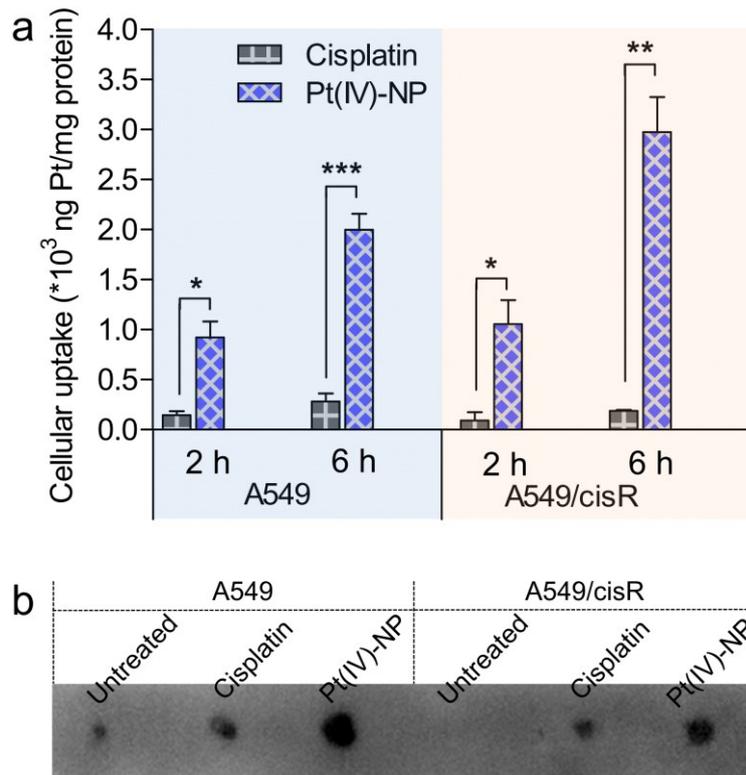


Figure S20. a) Intracellular accumulation of Pt(IV)-NP and cisplatin in A549 and A549cisR cells. Cells were treated with drugs at a concentration of 30 μ M. b) Dot blot analysis indicated that Pt(IV)-NP caused substantially higher level of cisplatin-DNA adducts than cisplatin in both tested cell lines.

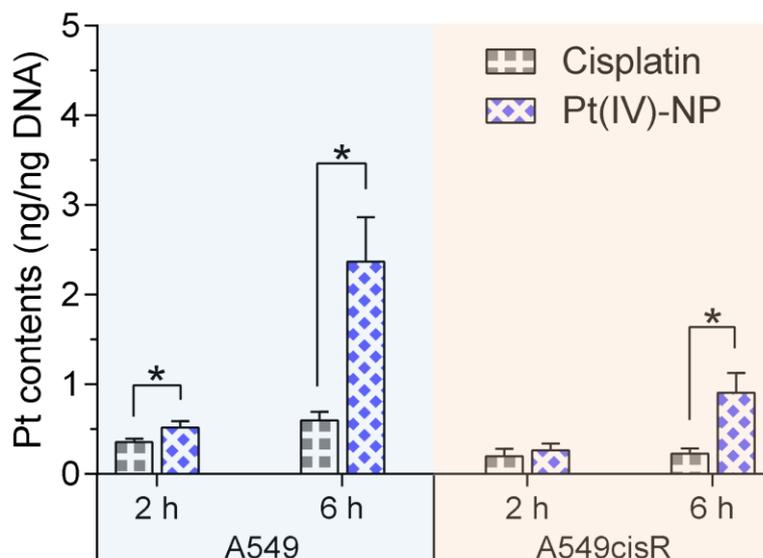


Figure S21. Intracellular DNA platinumation upon the treatment with cisplatin and Pt(IV)-NP in A549 and A549cisR cells. Cells were treated with drugs at a concentration of 60 μ M.

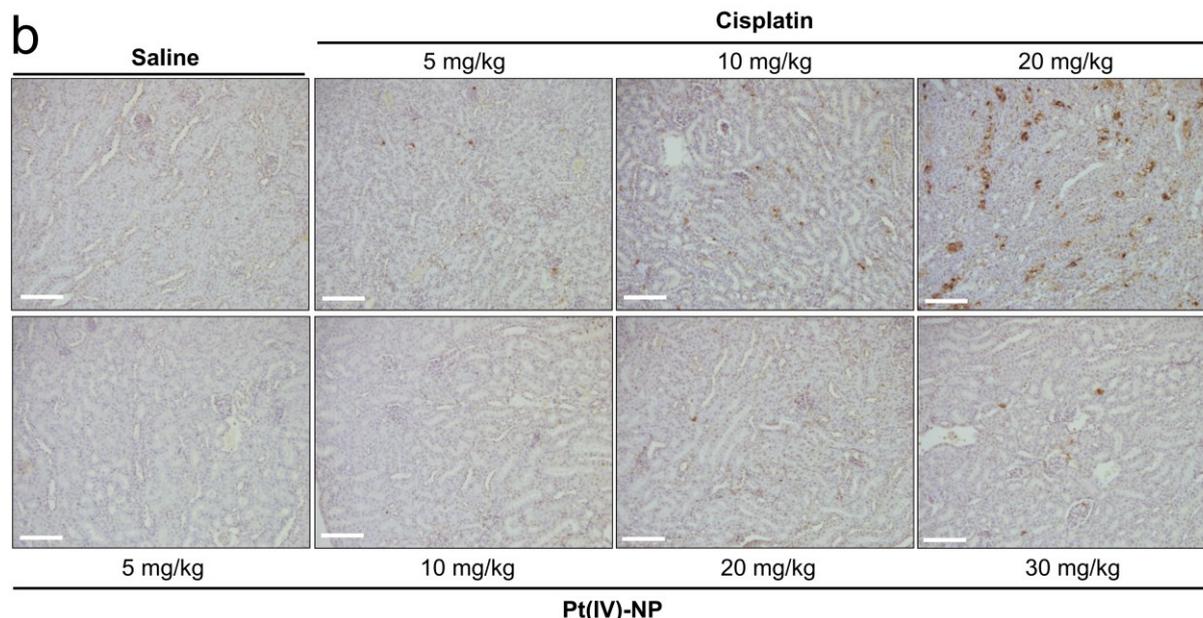
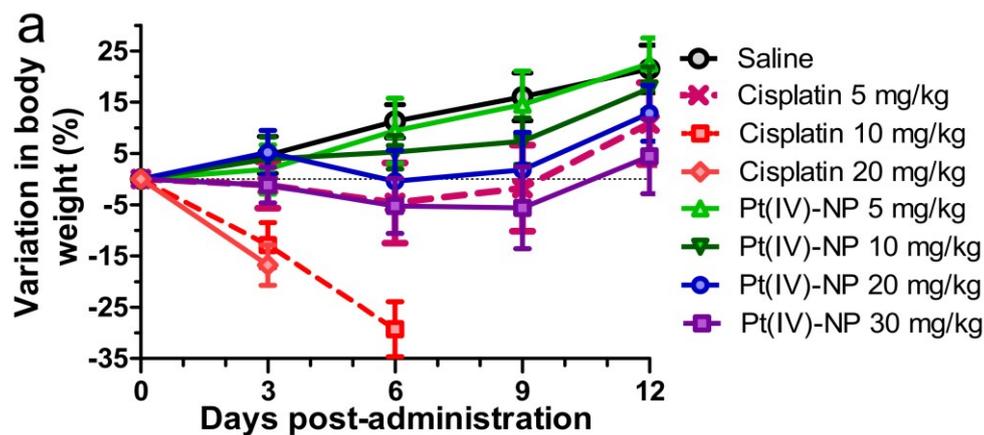


Figure S22. The *in vivo* toxicity of Pt(IV)-NP was evaluated. **a)** Body weight changes were observed for 12 days after intravenous administration of the drug formulations (at cisplatin-equivalent doses). **b)** Representative images of kidney cross-sections after TUNEL analysis.

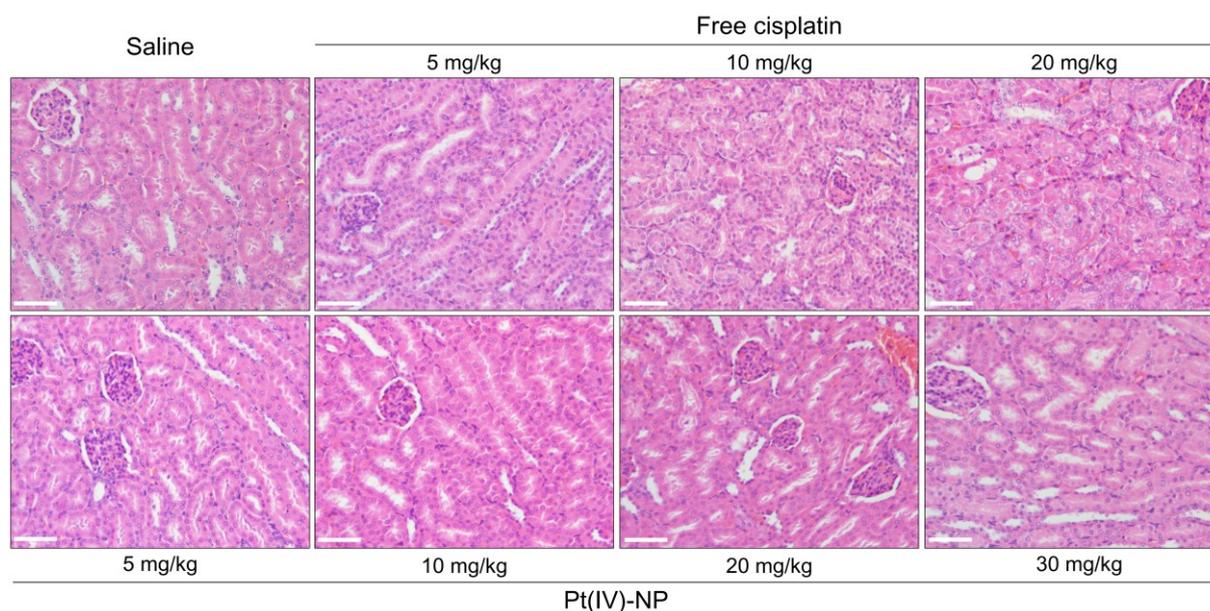


Figure S23. Histological analysis of kidneys of healthy ICR mice. The issues were excised from mice on day 6 after two intravenous injections of Pt(IV)-NP (i.e., Pt(IV)-NP at cisplatin-equivalent dose) at days 0 and 3. Saline and cisplatin were used as controls. The kidney sections were stained with H&E. Scale bars: 50 μ m

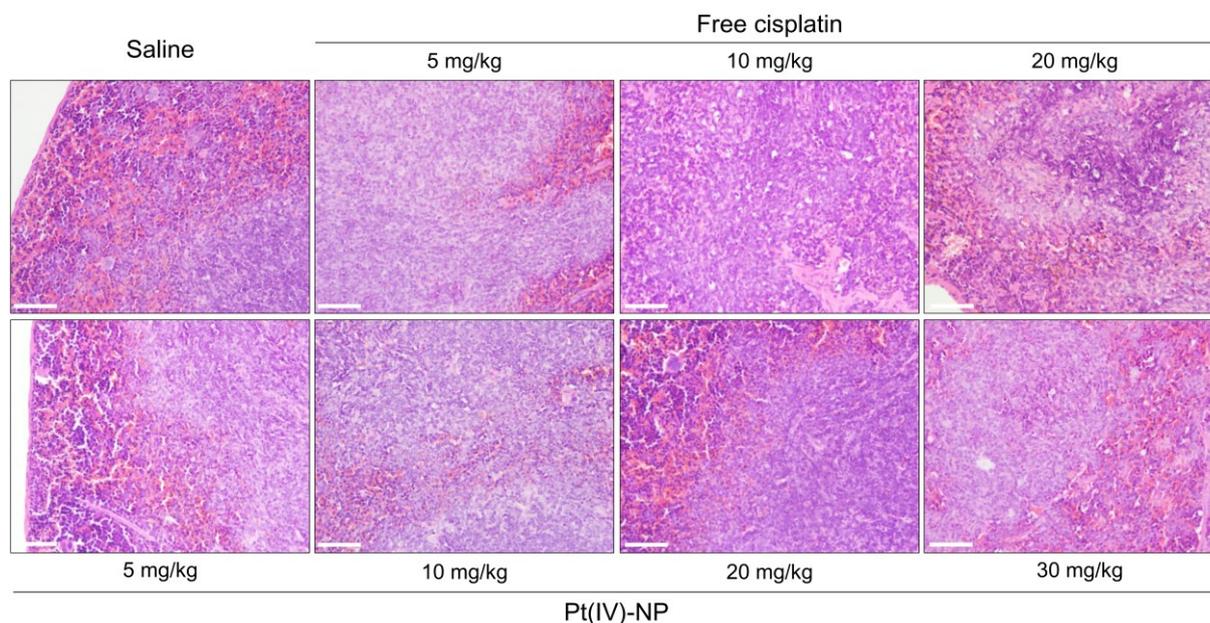


Figure S24. Histological analysis of spleens of healthy ICR mice. The issues were excised from mice on day 6 after two intravenous injections of Pt(IV)-NP (i.e., Pt(IV)-NP at cisplatin-equivalent dose) at days 0 and 3. Saline and cisplatin were used as controls. The kidney sections were stained with H&E. Scale bars: 50 μ m.

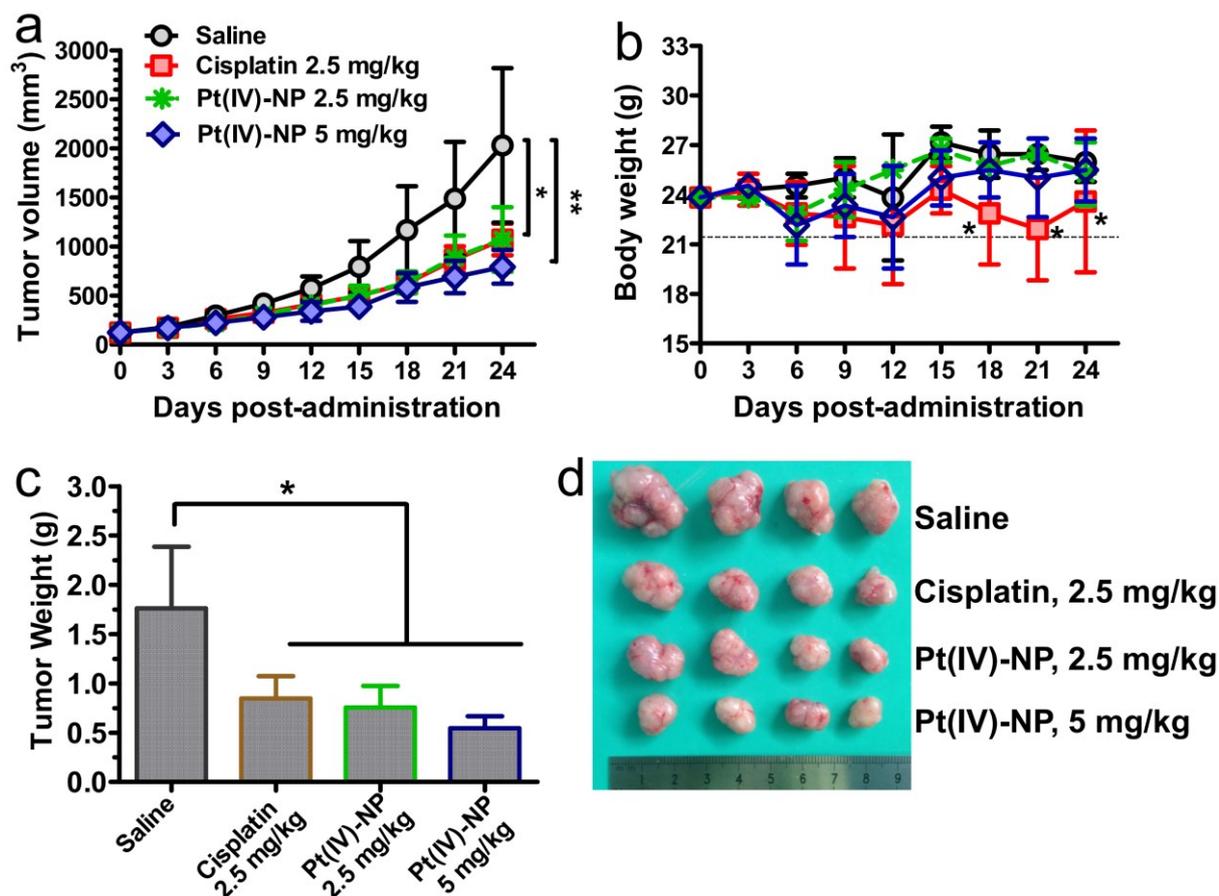


Figure S25. a) Evaluation of *In vivo* antitumor efficacy in a human lung cancer A549 cell-derived xenograft mouse model. A solution of Pt(IV)-NP (at doses of 2.5 and 5 mg/kg) was intravenously injected *via* the tail vein for three times on day 0, 3, and 6. Saline and cisplatin (2.5 and 5 mg/kg) were intravenously injected as reference. Unfortunately, the mice receiving cisplatin at 5 mg/kg died after three injections. The data are presented as the means \pm SD (n = 7); * p < 0.05, ** p < 0.01. b) Body weight changes of the mice receiving various drug formulations. c) Tumor weights at the end point of the study and d) representative images of the excised tumors.

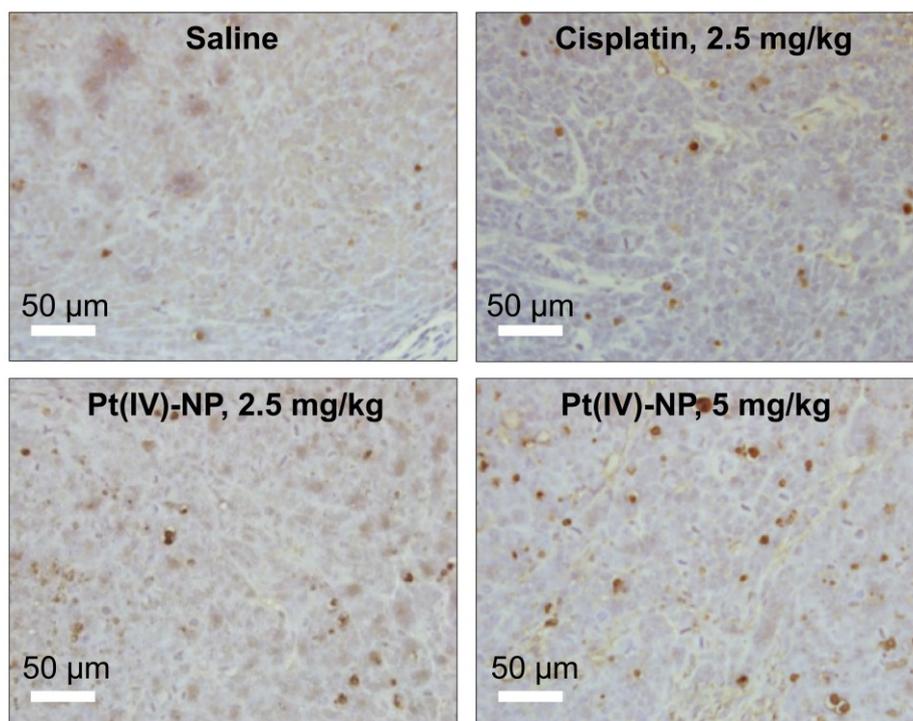


Figure S26. Representative TUNEL analysis of the excised tumors.

Methods

Preparation of Pt(IV) prodrug 1-formulated nanoparticles (Pt(IV)-NP)

1 mg Pt(IV) prodrug 1 (cisplatin equivalent) was dissolved in 1 mL DCM and added dropwise to a 10 mL 10% (w/v) aqueous solution of Pluronic F127 with stirring. After then, DCM was evaporated under vacuum at room temperature. The remaining solution was centrifuged at 4000×g for 5 min to remove aggregates. To remove excess free F127, 10 mL of supernatant was cooled to 4°C and then subjected to filtration using a centrifugal filter (10 kDa cutoff, Amicon Ultra-15, Millipore) at 4°C until ≈200 μL solution was retained. This washing procedure was repeated three times to remove excess unincorporated Pluronic F127 *via* critical micelle concentration (CMC) switching. Finally, the concentration of Pt(IV) prodrug 1 was determined by analytical HPLC.

Determination of drug retention after repeated washing procedure

For the washing curve in Figure 2e, drug retention in Pluronic F127 or Tween-80 was determined by HPLC analysis. Briefly, Pt(IV) 1-formulated nanoparticles (Pt(IV)-NP) were prepared according the above protocol. After each wash, the solution samples (200 μL) were diluted with acetonitrile (200 μL) and were then subjected to HPLC analysis. The amounts of Pt(IV) 1 in nanoparticles was compared with the initially added amounts. For determination of Pt(IV) 1 after Tween-80 formulation, we used the same protocol. Drug retention after each wash (%) = (amount of drug retained/amount of drug fed initially) × 100.

Analytical RP-HPLC was carried out on a Hitachi Chromaster 5000 system equipped with a C18 ODS reverse-phase column (5 μm, 250 × 4.6 mm, YMC Co., Ltd., Kyoto, Japan) at a flow rate of 1.0 mL/min. UV detection was performed at 220 nm. All runs used linear gradients of 50%–100% acetonitrile in water as the mobile phase.

Determination of drug encapsulation efficiency (EE) and drug loading (DL) capacity

After the preparation of prodrug-formulated Pt(IV)-NP, the solution was centrifuged at 6,000 g for 5 min to remove precipitates. To determine the EE values, the supernatants were recovered, and the amounts of prodrugs were determined by analytical HPLC. On the other hand, to quantify drug loading capacity, the supernatant was freeze-dried. The freeze-dried samples were weighed and dissolved in acetonitrile and sonicated for 10 min. Thereafter, the drug amounts in samples were measured by HPLC, with pure prodrug as the standard. The EE and DL values were calculated using the following equations (1) and (2):

$$EE (\%) = W_{\text{drug in NP}}/W_{\text{initial drug added}} \times 100 \quad (1)$$

$$DL (\%) = W_{\text{drug}} / (W_{\text{drug}} + W_{\text{matrix}}) \times 100 \quad (2)$$

Size characterization

The hydrodynamic diameters (D_H) of prodrug **1**-assembled bare NPs and PEGylated NP (Pt(IV)-NP) were measured by dynamic light scattering (DLS) on a Malvern NanoZS90 instrument (Malvern Instruments, Malvern, UK) at 25 °C.

Transmission Electron Microscopy (TEM) analysis

TECNAL 10 (Philips) was used to obtain TEM images, operating at an acceleration voltage of 80 kV. The NPs with a concentration of 0.3 mg/mL (cisplatin equivalent) was placed onto a 300-mesh copper grid coated with carbon. Approximately 5 min after deposition, the surface water was removed with filter paper and then air-dried. Positive staining was performed using a 2 wt % aqueous uranyl acetate solution.

***In vitro* drug release kinetics.**

To quantify the platinum drug release kinetics from Pt(IV)-NP, a dialysis method was used. Briefly, 3 mL of NP solutions containing a 0.1 mg/mL cisplatin-equivalent concentration were loaded into a dialysis tube (Spectrum, molecular weight cutoff of 7 kD) and dialyzed against 20 mL of PBS supplemented with 0.3% polysorbate 80 (pH 7.4 or 5.0). The dialysis tubes were continuously stirred in an orbital shaking water bath and 150 rpm at 37°C. At predetermined times, 1 mL of solution was taken from the release buffer while concurrently adding the same volume of PBS supplemented with 0.3% polysorbate 80. The platinum contents in samples were determined by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer, NexION 350X).

Cell Culture

The cisplatin sensitive human cancer cell lines A549 (lung cancer) and A2780 (ovarian cancer), cisplatin resistant A549 (A549cisR) and A2780 (A2780cisR) cells were kindly provided by the School of Pharmaceutical Sciences, Southern Medical University (China).^[1] A549 and A549cisR cells were maintained in PRMI 1640 medium. A2780 and A2780cisR cells were maintained in PRMI 1640 medium. All media were supplemented with 10% (v/v) heat-inactivated fetal calf serum (FBS) (56°C, 30 min). Cells were maintained in a humid atmosphere at 37 °C with 5% CO₂.

Cytotoxicity study using MTT assay

Cells were seeded in 96-well plates (4000-5000 cells per well) and incubated at 37°C for 24 h. The cells were added by serial dilution of free cisplatin in DMF and Pt(IV)-NP and then incubated at 37°C for 72 h. At the end of the incubation period, each well was added by 30 µL MTT solution (5 mg/mL in PBS). After further incubation at 37 °C for 4 h, the MTT solution was removed from the wells, and the purple MTT-formazan crystals were dissolved by addition of DMSO (100 µL). The absorbance in each individual well was determined at 490 nm using a microplate reader (Multiskan FC, Thermo Scientific). The relative percentage of the untreated cells was used to represent 100% cell viability, and the concentrations of various drugs required to inhibit cell growth by 50% (IC₅₀) were extrapolated from the dose-response curves.

Quantification of platinum in cell lysates

A549 and A549cisR cells were seeded in 6- well plates at a density of 1.5×10^5 per well and incubated at 37°C for 24 h. Then the cells were treated with cisplatin and Pt(IV)-NP with a final concentration of 30 µM (cisplatin equivalence) and incubated at 37°C for 2 h or 6 h. The cells were washed with cold PBS three times, trypsinized, and centrifuged at 1000×g for 3 min to afford cell pellets. Subsequently, 100 µL cell lysis buffer (pH 7.6, 25 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% Deoxycholic acid) containing 1% protease inhibitor cocktail set III (Novagen) was added to each sample to obtain the cell lysis solution. 80 µL cell lysate was diluted with 4 mL DI water and then the samples were subjected to ICP-MS for measurement of platinum contents. Another 20 µL cell lysate was used to determine the protein content using bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, USA).

Quantification of DNA platination

A549 and A549cisR cells were seeded in 6- well plates at a density of 1.5×10^5 per well and incubated at 37°C for 24 h. The cells were treated with cisplatin and Pt (IV)-NP with a final concentration of 60 µM (cisplatin equivalence) and incubated at 37°C for 2 h or 6 h. After then, the cells were washed with PBS three times, and added Dzap reagent (Sangon Biotech, China) to extract DNA. DNA content was determined by Nanodrop 2000. Finally, 50 µL DNA solution was diluted with 3 mL DI water and the samples were subjected to ICP-MS for measurement of Pt-DNA adducts.

Fluorescence imaging of cellular uptake of Pt(IV)-NP

The lipophilic dye, 1,1'-Dioctadecyl-3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) (ThermoFisher Scientific), was used to label Pt(IV)-NP for studying cellular uptake and

intracellular trafficking. The procedure follows the protocol for the preparation of Pt(IV)-NP. Briefly, DiI (0.1 mg) and Pt(IV) prodrug **1** (1 mg, cisplatin equivalence) were dissolved in DCM, and added dropwise to a 10 mL 10% (w/v) aqueous solution of Pluronic F127 with stirring. After then, DCM was evaporated under vacuum at room temperature. The remaining solution was centrifuged at $4000\times g$ for 5 min to remove aggregates. To remove excess free F127, 10 mL of supernatant was cooled to 4°C and then subjected to filtration using a centrifugal filter (10 kDa cutoff, Amicon Ultra-15, Millipore) at 4°C until $\approx 200\ \mu\text{L}$ solution was retained. This washing procedure was repeated three times to remove excess unincorporated Pluronic F127 *via* critical micelle concentration (CMC) switching. Finally, the concentration of DiI was determined using a UV-vis spectrometer (Shimadzu, UV-2700).

A549 and A549cisR cells were plated in glass-bottom culture dish (Nest Biotechnology) at a density of 1.5×10^5 per well and incubated at 37°C for 24 h. Then the cells were treated with and DiI-labeled Pt(IV)-NP with a final concentration of $1\ \mu\text{M}$ DiI and incubated at 37°C for 2 h. After washed with cold PBS, the lysosomes and nuclei were stained with lysotracker green DND-26 (Invitrogen, USA) and Hoechst 33342 (Invitrogen, USA), respectively. Finally, fluorescence imaging was performed using CLSM (Olympus, IX81-FV1000).

Dot blotting

A549 and A549cisR cells were seeded in 10 cm culture dish at a density of 5×10^6 and incubated at 37°C for 24 h. The cells were treated with cisplatin and Pt (IV)-NP with a final concentration of $60\ \mu\text{M}$ (cisplatin equivalent) and incubated at 37°C . After 8 h, the cells were washed with cold PBS three times, and added Dzap reagent (Sangon Biotech, China) to extract DNA. The DNA concentration was quantified by Nanodrop 2000. $5\ \mu\text{g}$ DNA were spotted on Amersham Hybond TM-N^+ membrane (GE Healthcare, USA) and air dry for 10 min. The membranes were blocked with 5% skim milk for 2 h at room temperature and incubated with Anti-Cisplatin modified DNA antibody (ab103261, 1:1000 dilution by 5% skim milk, Abcam, USA) for 1 h at room temperature. After washing with PBST three times, goat anti-rat IgG H&L polyclonal (HRP) (ab97057, 1:5000 dilution by 5% skim milk, Abcam, USA) was used as the secondary antibody for incubation at room temperature for 1 h. Signals were detected with Chemiluminescent HRP Substrate (Millipore Corporation, USA) by chemiluminescent analyzer (Bio-Rad, USA).

Animal Studies

Mice (5 weeks old) used in animal studies were purchased from Shanghai Experimental Animal Centre, Chinese Academy of Science. All animal experiments were performed in

compliance with the guidelines of the Zhejiang University Committee for Animal Use and Care. Zhejiang University Committee approved the animal experiments in this study. They were housed under aseptic conditions and provided with an autoclaved rodent diet and sterile water.

***In vivo* toxicity**

Healthy ICR mice (4-5 weeks old) were randomized into 10 groups (n = 10, five males and five females in each group) and intravenously injected with different doses of Pt(IV)-NP solutions (200 μ L) and cisplatin solution (Hospira Australia Pty Ltd) every three days for three times. Saline was used as control. Cisplatin injection were administered at doses of 5, 10, 20 mg/kg. Pt(IV)-NP were administered at doses of 5, 10, 20, 30 mg/kg (cisplatin equivalents). The body weight changes of mice were monitored.

After receiving two injections of saline, cisplatin and Pt(IV)-NP, two mice in each group were randomly selected and sacrificed by CO₂ inhalation. The major organs such as kidneys and spleens were collected and fixed with 4% formaldehyde. After then, the tissues were embedded in paraffin and sectioned into 5- μ m-thick slices. These slices were stained with hematoxylin and eosin (H&E, Sigma). For the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, the dewaxed and rehydrated kidney sections were incubated with proteinase K for 15 minutes at 37°C, rinsed with PBS twice, and rinsed with the TUNEL *In Situ* Cell Death Detection Kit according to the manufacturer's protocol (Sigma-Aldrich). The TUNEL-stained cells were counterstained with DAB (DAKO) and visualized by optical microscopy in 10 random fields for each group.

***In vivo* antitumor activity**

Human lung cancer A549 cells were grown to 80% confluence in 90 mm tissue culture dishes. After harvesting, cells were suspended in PBS at 4°C to a final concentration of 2.5×10^7 cells/mL. Mice were subcutaneously injected with 200 μ L of cell suspension containing 5×10^6 cells. After the tumors reached ~ 130 mm³ in volume at day 14 after implantation, the animals were randomized into five groups (n = 7 per group).

The mice were intravenously injected with a solution of Pt(IV)-NP (200 μ L) at doses of 2.5 and 5 mg/kg (cisplatin equivalent) every three days for three times. Cisplatin at doses of 2.5 and 5 mg/kg and saline were injected as control. Tumor growth and body weights were monitored and recorded. The length (*L*) and width (*W*) of tumors were measured with calipers and tumor volume was calculated using the following formula: $V = (L \times W^2)/2$, with *W* being

smaller than *L*. Mice were sacrificed by CO₂ inhalation when their tumors reached the 2000 mm³ endpoint value or after 24 days.

For the TUNEL assay, the dewaxed and rehydrated tumor sections were incubated with proteinase K for 15 minutes at 37°C, rinsed with PBS twice, and rinsed with the TUNEL *In Situ* Cell Death Detection Kit according to the manufacturer's protocol (Sigma-Aldrich). The TUNEL-stained cells were counterstained with DAB (DAKO) and visualized by optical microscopy in 10 random fields for each group.

Statistical analysis

All quantitative data are presented as the means \pm SD. The significance of the compared measurements was evaluated using two-tailed unpaired Student's *t*-test. A *p*-value of less than 0.05 was considered significant, while a *p*-value of less than 0.01 was considered highly significant.

Reference:

1. Q. Chen, Y. Yang, X. Lin, W. Ma, G. Chen, W. Li, X. Wang and Z. Yu, *Chem. Commun.*, **2018**, *54*, 5369-5372.