A Spermine-Conjugated Lipophilic Pt(IV) Prodrug Designed to Eliminate Cancer Stem Cells in Ovarian Cancer

Morgan Stilgenbauer,[a] Amarasooriya M. D. S. Jayawardhana,[a] Payel Datta,[a] Zhizhou Yue,[a] Michael Gray, [a] Frederick Nielsen,[a] David J. Bowers,[a] Haihua Xiao, [b] and Yao-Rong Zheng [*a]

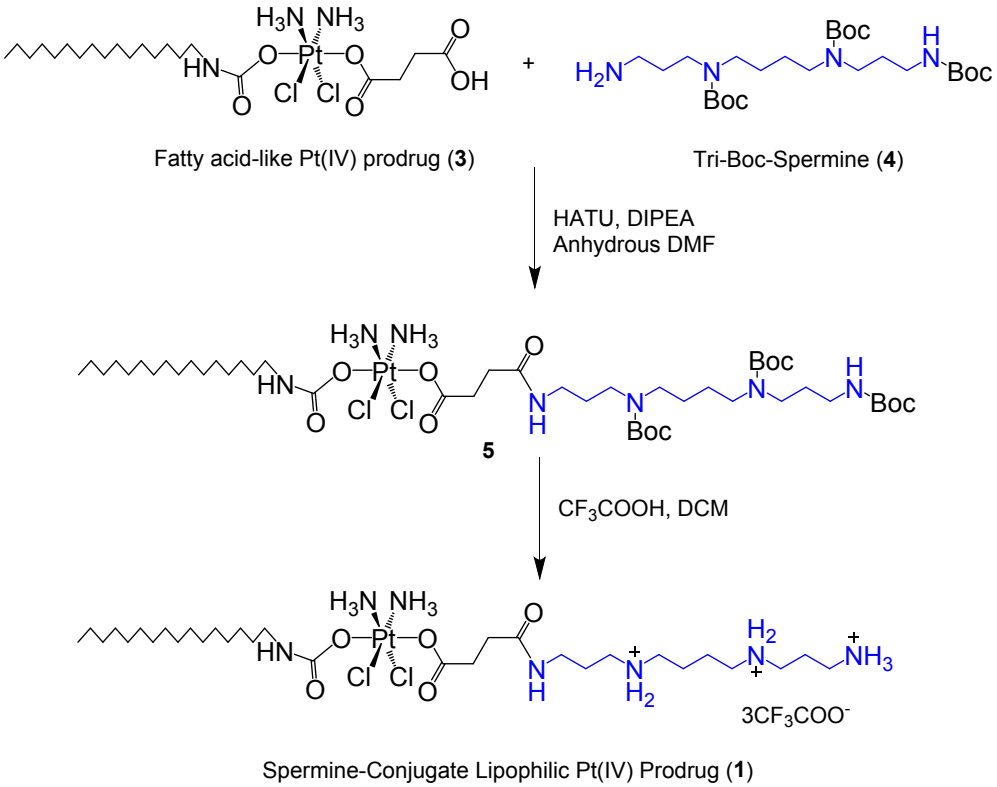
a. Department of Chemistry and Biochemistry, Kent State University, 236 Integrated Sciences Building, Kent, Ohio 44242.

b. Beijing National Laboratory for Molecular Sciences, State Key Laboratory of Polymer Physics and Chemistry, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, P. R. China.

* Corresponding author. Email: yzheng7@kent.edu

Experimental Section

General information. Compound **3** was prepared according the reported literature.[1] The chemical reagents used in this study were purchased from major chemical distributors including Sigma Aldrich, Acros Organic, Alfa Aesar, TCI America, and Matrix Scientific. A Bruker 400 NMR was used for NMR data acquisition (Frequency: 400 M Hz for ^{1}HNMR; 100MHz for ^{13}CNMR) and the plots were generated by TOPSPIN 3.2 software. Chemical shifts in ^{1}H and ^{13}C{^{1}H} NMR spectra were internally referenced to solvent signals (^{1}H NMR: DMSO at δ = 2.50 ppm; methanol at δ = 3.31 ppm; ^{13}C NMR: DMSO at δ = 40.45 ppm).; Coupling constants J were reported in hertz (Hz). The abbreviations s, d, t, q and m were used for singlet, doublet, triplet, quarlet and multiplet, respectively. For compound **1**, the high resolution mass spectra of created ions were recorded on an Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany). Mass spectra were recorded in the positive ionization mode with a scan range of 50–700 m/z, a mass resolving power setting of 140,000, and an automatic gain control (AGC) target value of 1×10^{6} ions. To ensure very high mass accuracy (found to be better than 1 ppm) the instrument was mass calibrated daily and a lock mass of m/z 371.10124, due to polysiloxane, was used throughout. Analytical HPLC was conducted on an Agilent 1100 system using C18 reverse-phase columns (Hypersil GOLD, 100 mm × 3 mm, 5 μm). Graphite furnace atomic absorption spectroscopic (GFAAS) measurements were taken on a PerkinElmer PinAAcle 900Z spectrometer. Fluorescence images were acquired using an Olympus Fluoview FV1000 with PlanApo 60x/1.42 oil-immersion objective lens. Images were processed and intensities were quantified with ImageJ software (NIH). Flow cytometry was carried out on a BD Bioscience Accuri C6 flow cytometer. ATP luminescence assays were performed on a Molecular Devices SpectraMax M4 Multi-Mode Microplate Reader. Cell counting assays were performed on a BioTek Cytation 5 plate reader.



Scheme S1. Synthetic route for preparing the spermine-conjugated lipophilic Pt(IV) prodrug (**1**).

*Synthesis of Tri-Boc-Spermine (**4**)*:

The synthetic route was modified based on the reported protocol.[2] Spermine (1.22 g, 6 mmol) was dissolved in dry methanol (distilled from Na/Benzophenone) (120 mL), cooled to -78 °C. At -78 °C, CF_3COOEt (854 mg, 6.0 mmol) in dry methanol (20 mL) was added dropwise. The reaction was stirred at -78 °C for another hour. Then the -78 °C cooling bath was removed, and stirred for another hour (Finally, the inert temperature slowly increased to around r.t.). The reaction mixture was cooled to 0 °C, then Boc anhydride (5.50 mL, 5.25 g, 24 mmol) was added during 5 min and the reaction mixture was slowly warmed to r.t. and stirred at r.t. for 24h (The process was monitored via TLC, 50% EtOAc/Hexanes, Ninhydrin). The reaction mixture was concentrated to dryness, retreated with DCM 200 mL and water 100 mL, separated, the aqueous layer was extracted with DCM for three times. The combined organic layers was washed with water (100 mL x 5) and brine, dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The crude product was purified by column chromatography on silica gel (gradient elution from ethyl acetate/hexane = 0:100 to 30:70) to give the desired full protected intermediated compound as colorless oil (1.50 g, 40 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ:1.436 (s, 27H, *Boc*), 1.468 (m, 6H, $NH_2CH_2CH_2CH_2NHBoc$, $NHBocCH_2(CH_2)_2CH_2NHBoc$), 2.60 (m, 4H, $NH_2CH_2CH_2CH_2NHBoc$, $NHBocCH_2CH_2CH_2NHBoc$), 2.727 (m, 10H, $NH_2CH_2CH_2CH_2NHBoc$, $NHBocCH_2(CH_2)_2CH_2NHBoc$, $NHBocCH_2CH_2CH_2NHBoc$), 4.685 (NH_2), 5.287 (N*H*Boc).

*Synthesis of the Spermine-Conjugated Lipophilic Pt(IV) prodrug (**1**)*:

The fatty acid-like Pt(IV) prodrug (**3**, 51 mg, 72 μmol), HATU (27 mg, 72 μmol), was added into 1.4 ml anhydrous DMF, stirred for 1 h, and then, Tri-Boc-Spermine (**4**, 45 mg, 90 μmol)

in 0.7 ml anhydrous DMF was added under stirring. After 1 h at r.t., DIPEA (13 μL) was added. The reaction mixture was stirred for 16 h at r.t.. A total of 7 ml brine was added to the reaction mixture. Then, the precipitation was collected by centrifuge and washed with water (2 mLx2). The crude product (**5**) was purified by column chromatography on silica gel (eluent CH_3OH/ DCM 0% to 2%) to give the colorless oil of **5**. Thereafter, the oil (58 mg) was stirred in a mixture of DCM (2.7 ml) and TFA (0.3 ml) for 3.5 h at r.t.. The TFA salt of **1** was obtained after removing the solvent by rotavap and drying under vacuum overnight. Yield: 68 mg, (77%). ^{1}HNMR (400 M Hz, Methanol-d_4) : δ: 0.882 (t, $J_{H\text{-}H}$ =6.8Hz, 3H, CH_3), 1.286 (m, 28H, $CH_2(CH_2)_{14}CH_2$), 1.802 (m, 8H, $CONHCH_2CH_2CH_2NH_2$, $NH_2CH_2(CH_2)_2CH_2NH_2$, $NH_2CH_2CH_2CH_2NH_3$), 2.451 (m, 4H, succinate), 3.026 (m, 14H, $CH_2(CH_2)_{14}CH_2NHCO$, $CONHCH_2CH_2CH_2NH_2$, $NH_2CH_2(CH_2)_2CH_2NH_2$, $NH_2CH_2CH_2CH_2NH_3$). ^{13}C NMR (500 MHz, DMSO-d_6): δ: 180.3, 172.7, 164.3, 46.6, 45.0, 44.4, 40.5, 40.0, 39.4, 31.7, 30.3, 29.5, 29.4, 29.2, 27.4, 26.9, 26.5, 26.2, 24.3, 22.5, 14.4. HRMS (positive mode) for $[C_{31}H_{70}Cl_2N_7O_5Pt]^+$: calcd : 885.4460, obsd: 885.4453. Purity: 97% determined by HPLC.

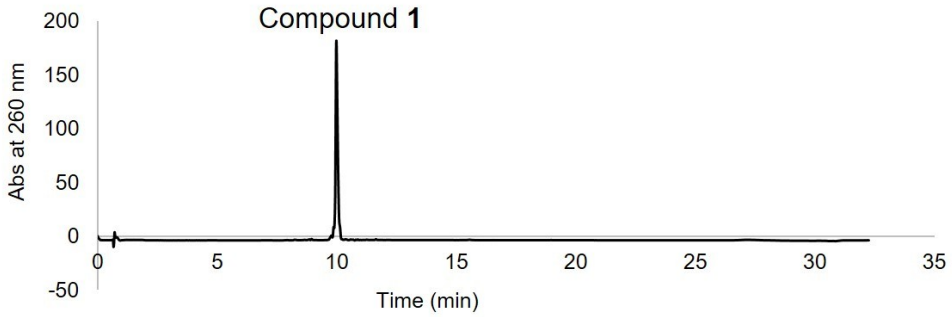


Fig S1. HPLC trace of Compound **1**. Gradient: 0 min 5% B, 5 min 5% B, 10 min 85% B, 15 min 90% B, 20 min 98% B, 30 min 5% B (Solvent A is 0.1% TFA aqueous solution and B is acetonitrile).

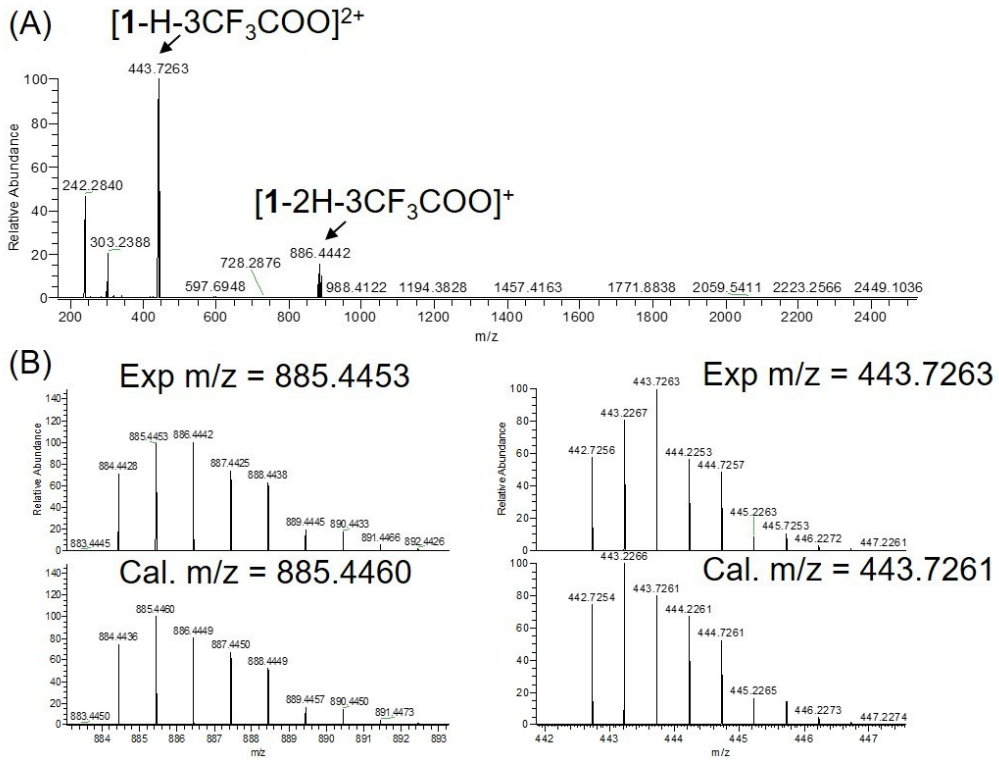


Fig S2. HR-MS spectra of Compound **1**.

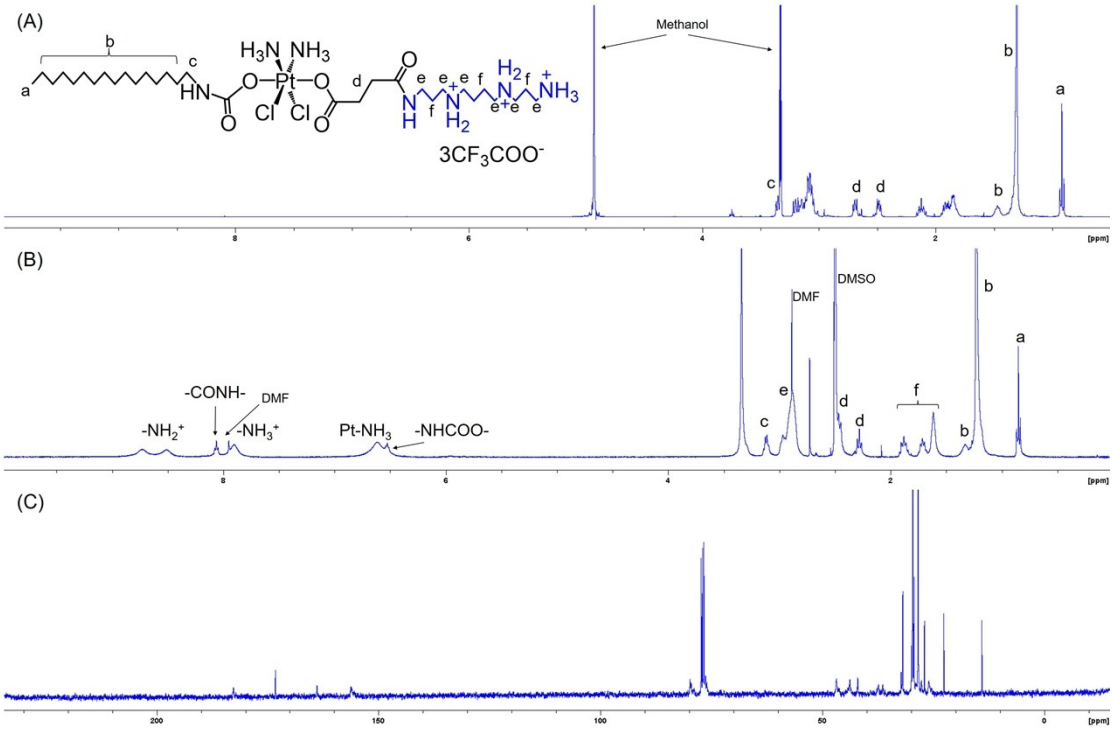
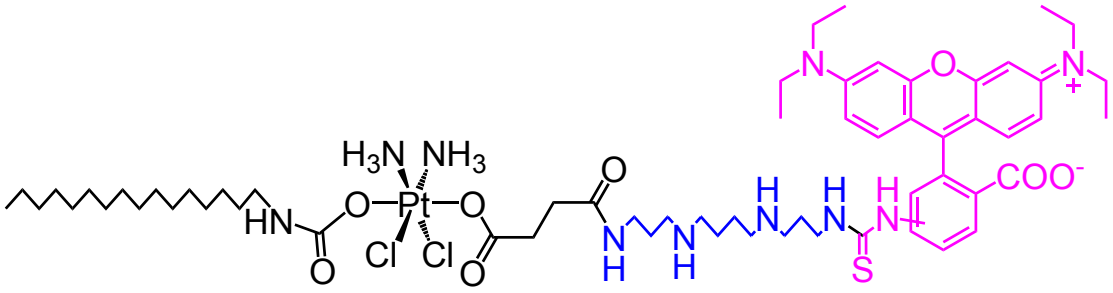


Fig S3. ^{1}H in methanol-d_4 (A), ^{1}H in DMSO-d_6 (B) and ^{13}C in DMSO-d_6 (B) NMR spectra of Compound **1**.

*Labeling the Pt(IV) Prodrug (**1**) with Rhodamine B (**1-RhB**)*:



1-RhB

To a 0.2 mL anhydrous DMF solution of **1** (36.9 mg, 30 μmol) was added 15 μL TEA (10.8 mg, 107.5 μmol). The mixture was stirred at r.t. for 1 h. followed by addition of rhodamine B isothiocyanate (mixed isomers) (18.6 mg, 30 μmol) under stirring and finally stirred at r.t. for 21 h to give a purple solution which was concentrated to give a purple oil residue. The residue was washed with 3 x 3 mL ether until removal of free rhodamine B isothiocyanate confirmed by TLC. The resulting solid was dried in vacuum overnight. Yield: 25.8 mg (89%). ^{1}H NMR (400 MHz, DMSO-d_6): δ: 0.907 (t, $J_{H\text{-}H}$ = 6.8 Hz, 3H, -$CH_2(CH_2)_{14}CH_3$), 1.270 (m, 40H, -$CH_2(CH_2)_{14}CH_3$, RhB-CH_2CH_3), 1.813 (m, 8H, $CONHCH_2CH_2CH_2NH_2$, $NH_2CH_2(CH_2)_2CH_2NH_2$, $NH_2CH_2CH_2CH_2NHCS$), 2.479 (m, 4H, succinate), 3.297 (m, 20H, $CH_2(CH_2)_{14}CH_2NHCO$, $CONHCH_2CH_2CH_2NH_2$, $NH_2CH_2(CH_2)_2CH_2NH_2$, $NH_2CH_2CH_2CH_2NHCS$, RhB-CH_2CH_3), 6.9 (m, 9H, RhB-aromatic H). HRMS (positive mode) for $[C_{60}H_{100}Cl_2N_{10}O_8SPt]^{2+}$: calcd : 693.3233, obsd: 693.3228.

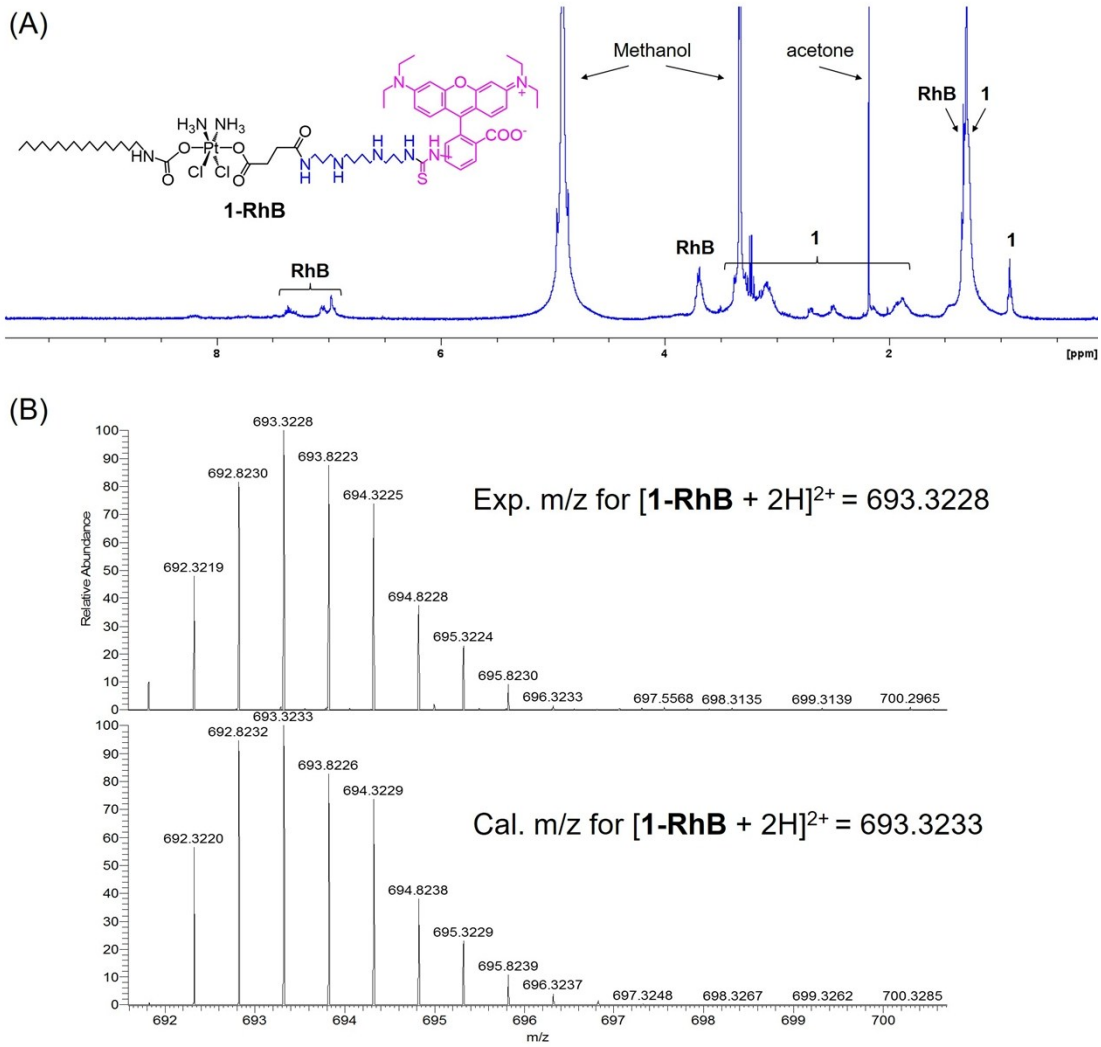


Fig S4. ^{1}H NMR spectrum in methanol-d_4 (A) and HR-MS of the product of labeling **1** with **RhB**.

Cell culture. A2780 and A2780cis cell lines were purchased from Sigma-Aldrich, and cultured in RPMI 1640 with L-glutamine (Corning) supplemented with 10% FBS (Atlanta Biologicals) and 1% Penicillin-Streptomycin (Corning). SKOV-3, MDA-MB-231, A549 cell lines were obtained via American Type Culture Collection, and cultured in DMEM 1 g/L glucose, with L-glutamine & sodium pyruvate (Corning) supplemented with 10% FBS and 1% Penicillin-Streptomycin (Corning). All cell lines were cultured at 37°C under an atmosphere containing 5% CO_2. Cells were passaged upon reaching 70–80% confluence by trypsinization and split in a 1:5 ratio.

Measurements of Cytosolic/Mitochondrial Platinum Contents. SKOV-3 cells were seeded on a 6-well microplate and incubated at 37 ^{0}C 5% CO_2 overnight. The medium was then aspirated and replaced with 2 mL of fresh medium. The cells were then treated with either 150 μM cisplatin or 5 μM **1** or **1-RhB** and left to incubate for 6 h. Cells were then washed with PBS (1 mL) and harvested by trypsinization (1 mL). Cytosolic and mitochondrial fractions were isolated using the Thermo Scientific™ Mitochondria Isolation Kit for Mammalian Cells. The mitochondrial fraction was then dissolved in 100 μL 65% nitric acid

and were shaken at 400 rpm on a Eppendorf ThermoMixer™ F1.5 at R.T. overnight while the cytosolic fraction was maintained at -20 ^{0}C. Next, the fractions were diluted 10x in water and the platinum content was analyzed using GFAAS. All experiments were performed in triplicate.

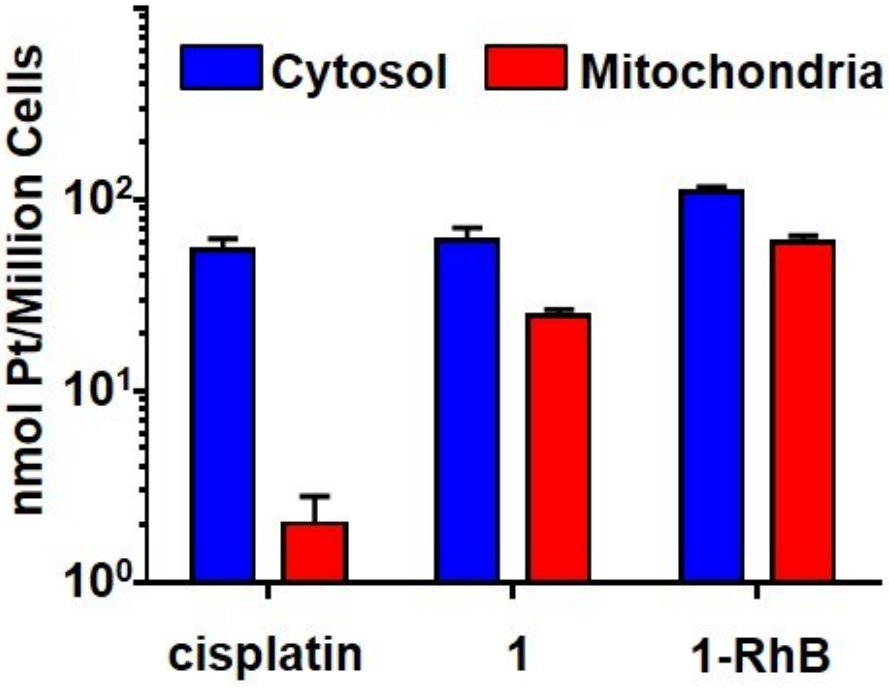


Fig S5. Cytosolic and mitochondrial Pt contents of SKOV3 cells treated with cisplatin (150 μM), **1** (5 μM), and **1-RhB** (5 μM).

Fluorescence imaging studies. MDA-MB-231 cells were seeded in an imaging disk (MatTek) with 2 mL of complete medium. The cells were incubated in medium containing 10 μM **1-RhB**, MitoTracker Green FM (Invitrogen), and Hoechst (Sigma-Aldrich) for 30 minutes. After incubation, the cell medium was aspirated, and the cells were washed 3 times with 1 mL of PBS. A volume of 2 mL of fresh medium was added to the imaging dish for imaging by confocal fluorescence microscopy. Cells were visualized using Olympus Fluoview FV1000 and PlanApo 60x/1.42 oil-immersion objective lens. Images were processed were calculated using Image J software.

MitoSOX™ Red Flow Cytometric Analysis. SKOV3 cells were seeded in 2 mL of cell medium at a cell density of 5x10^4 cells/mL in 10 mm petri dishes and incubated at 37 ^{o}C, 5% CO_2 overnight. Cells were then treated with 60 μM cisplatin and 10 μM **1** for 24 h. The medium was aspirated, and cells were washed with 1 mL PBS. Then, the cells were incubated with 5 μM MitoSOX reagent (Thermofisher) in fresh medium for 60 min at 37 °C in the dark. The cells were trypsinized and collected. The cell pellet was washed twice with 1 mL PBS with 0.5% BSA. The cells were then resuspended with PBS with 0.5% BSA to 10^5 cells/mL and analyzed with BD Accuri C6 Flow Cytometer at 10,000 events.

Mitostatus™ Flow Cytometric Analysis. SKOV3 cells were seeded at a cell density of $5x10^4$ cells/ mL in 5 mL of medium in 6-well plate. After incubating at 37 °C, 5% CO_2 overnight, cells were treated with 60 μM cisplatin and 10 μM **1** for 24 h. The medium was removed, cells were washed 2 times with 1 mL PBS, and 5 mL medium was added to each well. The cells were stained with 200 nM MitoStatus reagent (BD Biosciences) in the dark at 37 °C for 30 min. The stain-containing medium was aspirated, and the cells were washed twice with 1 mL PBS. The cells were trypsinized, collected, and washed with 1 mL PBS. The cell pellet was then resuspended in PBS to 10^5 cells/ mL and analyzed via with BD Accuri C6 Flow Cytometer at 10,000 events.

ATP Luminescence Assays. A total of 8000 SKOV3 cells ($8x10^4$ cells/mL) were seeded in a 96-well microplates. After overnight incubation, cisplatin or **1** was added at concentrations from 0 to 20 μM and incubated for additional 24 h. The medium was then removed and 50 μL of Nucleotide Releasing Buffer (Biovision) was added to the cells. After 10-min shaking, 50 μL of solution was transferred to a luminometer plate. A volume of 100 μL of the reaction mixture containing ATP Monitoring Enzyme and Nucleotide Releasing Buffer was added to the cell solution, and the luminescence was read after 2 min. All experiments were performed in triplicate.

Flow cytometric analysis of phosphorylation of H2AX (γH2AX). A2780cis cells were incubated with or without the test compounds (Pt concentration was 10 μM in both **1** and cisplatin) for 24 h at 37 °C. Cells were harvested from adherent cultures by trypsinization. Following centrifugation at 1500 rpm for 5 min, cells were washed with PBS. All samples were resuspended in BD fixation/Permeabilization solution (250 μL) for 20 minutes at 4 ℃. Then the supernatant was removed, the samples were resuspended with BD Perm/Wash Buffer (1 mL) and centrifuged. Added 10% (in volume) of BD Alexa 647-anti γH2AX antibody solution and incubated cells in dark for 60 minutes at room temperature. The cells were resuspended with PBS (200 μL) and analyzed by Accuri flow cytometer.

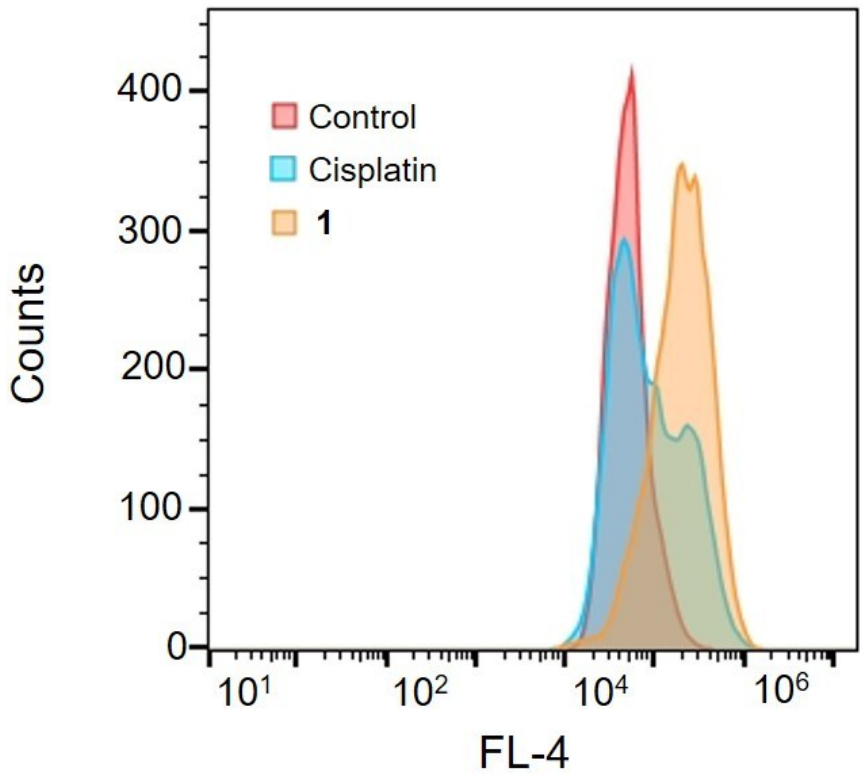


Fig S6. Flow cytometric analysis of phosphorylation of H2AX (γH2AX) of A2780cis cells treated with cisplatin and **1** (10 μM) for 24 h.

Flow Cytometric Analysis of Biomarkers of CSCs. SKOV3 cells were trypsinized, collected, and resuspended in complete CSC medium to a cell concentration of 10–20x10^4 cells/mL. Cells were then seeded into a Corning Costar ultra-low attachment 6-well plate. After 24 h, cells were treated with 1.0 μM **1** or 10 μM cisplatin. After 72 h of incubation, SKOV3 spheroids were harvested from ULT 6-well plates and collected via centrifuge. The cells were trypsinized. Cell pellets were collected and washed with 1 mL PBS. After centrifugation, the PBS was aspirated, and the cells were re-suspended in 100 μL of PBS with 0.5% BSA. Antibodies were added to measure CSC biomarkers CD44-FITC, CD117-PE, CD133-APC (eBioscience) for 1 h at room temperature. The cells were washed with 1 mL PBS with 0.5% BSA and then resuspended to 10^5 cells/ mL. The cells were then analyzed with BD Accuri C6 Flow Cytometer at 10,000 events.

Spheroid-Formation Assays. SKOV3 cells were collected and re-suspended in complete CSC medium to a cell density of 10–20x10^4 cells/mL. A total of 5 mL of the cell solution was added into ULT 6-well microplates. After overnight incubation, 2.5 μM **1** or 5.0 μM cisplatin was added to microplates. After 72 h of incubation, spheroids were harvest from ULT 6-well plates and trypsinized. A cell solution was prepared at 10^3 cells/ mL, and 200 μL was added to each well of an ULT 96-well plate. The cells were incubated for 72 h. Tumor spheroids were then counted under an optical microscope, and representative images were taken. All experiments were performed in triplicate.

MTT Assays. Cytotoxicity profiles of **1** and cisplatin against different cell lines (A549, A2780, A2780cis, MDA-MB-231 and SKOV-3) were evaluated by the MTT assays. A volume of 100 µL of a RPMI or DMEM containing 2 x 10^4 cells/mL was seeded in a 96-well plate. The plate was incubated for 24 h to allow for adherence of cells. A volume of 50 µL of RPMI or DMEM with various concentrations of Pt compounds was added to each well of the microplates. After 72 h, a volume of 30 µL of 5.0 mg/mL MTT (Alfa Aesar) in PBS was added to each well of the microplates. After 2-4 h, the medium was aspirated, and 200 µL of DMSO was added to each well. The plates were shaken gently on a shaker at r.t. for 5 minutes. Then, the absorbance of purple formazan was recorded at 562 nm with a BioTek ELx800 plate reader. IC_{50} values were determined using Origin software. All experiments were performed in triplicate.

Cell Counting Assays. Cytotoxicity profiles of **1** and cisplatin against A2780cis were evaluated by the cell counting assays. A volume of 100 µL of RPMI containing 2 x 10^4 cells/mL was seeded in a 96-well plate. The plate was incubated for 24 h to allow for adherence of cells. A volume of 50 µL of RPMI with various concentrations of Pt compounds was added to each well of the microplates. After 72 h, a volume of 100 µL fresh medium was added to each well of the microplates, and the cell confluence was recorded with a BioTek Cytation 5 plate reader. IC_{50} values were determined using Origin software. All experiments were performed in triplicate.

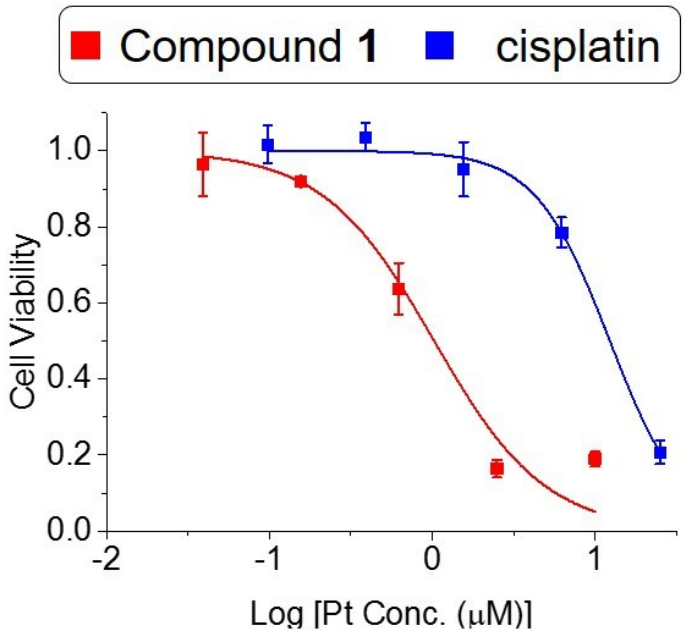


Fig S7. Kill curves of Compound **1** and cisplatin against A2780cis determined by cell counting assays: IC_{50} (**1**) = 0.88 ± 0.18 µM and IC_{50} (cisplatin) = 11.5 ± 0.72 µM.

References:

1. Zheng, Y. -R.; Suntharalingam, K.; Johnstone, T. C.; Yoo, H.; Lin, W.; Brooks, J. G.; Lippard, S. J. *J. Am. Chem. Soc.* **2014**, *136*, 8790−8798.

2. Mueller, L. K.; Kaps, L.; Schuppan, D.; Brose, A.; Chai, W.; Fischer, K.; Mueller, S.; Frey, H.; Schmidt, M.; Mohr, K. *Mol. Pharm.* **2016**, *13*, 3636-3647.