Supporting Information

Encapsulation of Pt(IV) Prodrugs within a Pt(II) Cage for Drug Delivery

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Experimental Section

General Information. *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)$(succinate)] was prepared as previously reported.[1] All reagents were purchased from Strem, Aldrich, or Alfa Aesar and used without further purification. All reactions were carried out under normal atmospheric conditions. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). Multinuclear 1D (^{1}H, ^{13}C and ^{195}Pt) NMR and 2D (DOSY and NOESY) NMR spectra were recorded on a Varian Unity 300 NMR spectrometer or a Bruker AVANCE-400 NMR spectrometer with a Spectro Spin superconducting magnet in the Massachusetts Institute of Technology Department of Chemistry Instrumentation Facility (MIT DCIF). Chemical shifts in ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were internally referenced to solvent signals (^{1}H NMR: acetone at δ = 2.05 ppm and DMSO at δ = 2.50 ppm; ^{13}C NMR: DMSO at δ = 40.45 ppm), and those in ^{195}Pt NMR spectra were externally referenced to K_2PtCl_4 in D_2O having δ = –1628 ppm. Electrospray ionization mass spectrometry (ESI-MS) was performed on an Agilent Technologies 1100 series liquid chromatography/MS instrument. Graphite furnace atomic absorption spectroscopic (GFAAS) measurements were taken on a Perkin Elmer AAnalyst 600 spectrometer. Fluorescence images of A2780 cells were acquired using a Zeiss Axiovert 200M inverted epifluorescence microscope equipped with an EM-CCD digital camera (Hamamatsu) and a MS200 XY Piezo Z stage (Applied Scientific Instruments). The light source was an X-Cite 120 metal-halide lamp (EXFO). The microscope was operated with Volocity software (PerkinElmer). Images were processed and intensities were quantified with ImageJ software (NIH). Distilled water was purified by passage through a Millipore Milli-Q Biocel water purification system (18.2 MΩ) equipped with a 0.22 μm filter.

*Synthesis and Characterization of **1***. To a mixture of *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)$(succinate)] (55 mg, 0.124 mmol) and 1-adamantyl isocyanate (50 mg, 0.282 mmol) was added 2.5 mL of anhydrous DMF, and the suspension was stirred at R.T. overnight. The suspension gradually turned clear, and the DMF was removed under reduced pressure at 65 ºC. To the oily residue was added Et_2O

to precipitate the product, and the suspension was ultrasonicated for 20 min. The solid product was isolated by centrifugation, washed with DCM and Et_2O, and dried in vacuum. Yield: 52 mg, 67%. ^{1}H NMR (400 MHz, acetone-d_6/DMSO-d_6: 10/1) δ 6.35 (m, 6H, NH_3), 5.53 (s, 1H, NH), 2.57 (m, 2H, succinate); 2.47 (m, 2H, succinate); 2.00 (s, 3H, adamantyl); 1.95 (s, 6H, adamantyl); 1.65 (s, 6H, adamantyl). ^{13}C {^{1}H} NMR (acetone-d_6, 75 MHz): 182.2, 175.5, 51.7, 43.1, 37.6, 32.0, 31.3, 30.1; ^{195}Pt NMR (acetone-d_6/DMSO-d_6, 86 MHz): 1250 ppm. DOSY NMR (DMSO-d_6, 400 MHz): D = 1.6 x 10^{-10} m^2/s. ESI-MS (Negative mode): cal.: 610.1, found: 609.9. Anal. Calcd. for: C, 29.47; H, 4.45; N, 6.87; Found: C, 29.79; H, 4.30; N, 6.78.

Synthesis and Characterization of ***2***. The synthesis of **2** was modified from the reported method.[2] To a mixture of $Pt(en)Cl_2$ (150 mg, 0.46 mmol) and silver nitrate (150 mg, 0.88 mmol) was added 4 mL of water, and the suspension was heated at 80 °C for 3 h with protection from light. The resulting suspension was filtered, and the filtrate was added to 2,4,6-tri-4-pyridyl-1,3,5-triazine (86 mg, 0.28 mmol). The suspension was heated at 100 °C for 40 min, and 20 µL 70 % HNO_3 and 1-adamantane carboxylic acid (80 mg, 0.44 mmol) was added. The mixture was further heated at 90 °C overnight, and then was cooled down to R.T. 1-adamantane carboxylic acid was removed by extraction with $CHCl_3$ (3 x 20 ml), and the aqueous solution was evaporated to dryness at 55 °C under reduced pressure. The solid residue was redissolved in 0.7 mL of water, and the mixture was filtered via a 0.2 µm syringe filter. To the clear solution was added 4 µL 70% HNO_3 slowly under stirring, and upon addition of HNO_3, a white precipitate formed immediately. The product was collected by centrifugation, washed with acetone, and dried in vacuum. Yield: 52 mg, 20 %. ^{1}H NMR (400 MHz, D_2O) δ 8.97 (d, J = 5.6 Hz, 24H, α-Pyridine), 8.44 (d, J = 5.6 Hz, 24H, β-pyridine), 2.72 (s, 24H, $NH_2CH_2CH_2NH_2$); DOSY NMR (400 MHz, D_2O): D = 1.8 x 10^{-10} m^2/s.

Formation of Host-guest Complex **3**. The cage **2** (6.7 mg, 2 µmol) was mixed with the Pt(IV) species (**1**) (4.8 mg, 8 µmol) in a 1:4 ratio in 1.0 mL of D_2O. The suspension was ultrasonicated for 5 min and then heated at 80 ºC for 15 min. Finally it became a clear colorless solution. The sample was lyophilized for further experiments. 1D and 2D ^{1}H NMR spectroscopy were used to characterize the supramolecular system. For **3**, ^{1}H NMR (400 MHz, D_2O) δ 9.26 (d, J = 5.1 Hz, 24H, α-Pyridine), 8.69 (d, J = 5.1 Hz, 24H, β-Pyridine), 2.75 (s, 24H, en), 2.64 (m, 8H, succinate); 2.49 (m, 8H, succinate); 1.25 (s, 24H, adamantyl); 0.07 (m, 24H, adamantyl); -0.71 (m, 12H, adamantyl). DOSY NMR (400 MHz, D_2O): D = 1.3 x 10^{-10} m^2/s. Anal. Calcd. for [**3**·$(H_2O)_6$] $C_{144}H_{216}Cl_8N_{60}O_{66}Pt_{10}$: C, 28.46; H, 3.58; N, 13.83; Found: C, 28.17; H, 3.37; N, 13.46.

Cyclic Voltammetry. Electrochemical experiments were performed at room temperature using a VersaSTAT3 potentiostat from Princeton Applied Research operated with the V3 Studio software. Cyclic voltammetry was performed using a three-electrode system comprising a glassy carbon working electrode, a platinum wire auxiliary electrode, and a Ag/AgCl reference electrode. Solutions of **2** and **3**, at concentrations of approximately 2 mM, were prepared in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO4, and 1.76 mM KH_2PO4, pH 7.4. The solutes of the saline acted as the supporting electrolyte and all solutions were sparged with nitrogen for 5 min prior to measurement. Voltammograms were obtained on quiescent solutions at a scan rate of 200 mV/s.

Cell Lines and Cell Culture. The human ovarian carcinoma A2780 and cisplatin resistant A2780CP70 cell lines were obtained from ATCC. The human lung carcinoma cell line A549 was kindly provided by David E. Root (Whitehead Institute for Biomedical Research). Unless otherwise specified, cells were incubated at 37 °C in 5% CO_2 and grown in RPMI (A2780 and A2780CP70) or DMEM (A549) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were passed every 3 to 4 days and restarted from a frozen stock upon reaching passage number 20.

MTT Assays. Cytotoxicity profiles of **1**–**3** and cisplatin against different cell lines (A549, A2780, and A2780CP70) were evaluated by the MTT assay. Cells were seeded on a 96 well plate (2000 cells per well) in 200 μL RPMI or DMEM and incubated for 24 h at 37 °C. The following day, solutions of the platinum compounds were freshly prepared in PBS and quantitated by GFAAS. The cells were then treated with the platinum compounds, separately at varying concentrations, and incubated for 72 h at 37 °C. The cells were then treated with 200 μL fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.8 mg/mL) and incubated for 3 h at 37 °C. The medium was removed, 200 μL of DMSO was added to the cells, and the absorbance of the purple formazan was recorded at 570 nm using a BioTek Synergy HT multi-detection microplate plate reader. Each experiment was performed in triplicate for each cell line.

LIVE/DEAD Cell Viability Assay and Nuclear Staining. Characteristic change in apoptotic cells treated with **3** was studied using the LIVE/DEAD cell viability assay (Molecular Probes) and nuclear staining in A2780 ovarian cancer cells. A2780 cells were cultured on 35 mm sterile glass bottom culture dishes (MATTEK corporation) for 24 h at 37 °C. The cells were then treated with 3 μM **3** for 72 h at 37 °C and 5% CO_2. Before the assay, cells were washed with 1 mL of PBS and 1 mL of dye-free RPMI to remove serum esterase activity generally present in serum-supplemented growth media. A 5 μL aliquot of calcein AM (4 mM in anhydrous DMSO) and 10 μL ethidium homodimer-1 (2 mM in DMSO/water, 1:4 vol/vol) were added to 10 mL of dye-free RPMI to produce a LIVE/DEAD working solution and an additional 5 μL Hoechst 33342 (1 mM) was added for nuclear staining. A 2 mL aliquot of LIVE/DEAD working solution was carefully added to the petri dishes, which were then incubated at R.T. for 30 min. Subsequently, the medium of the samples was replaced with 1 mL of dye-free RPMI before examination by fluorescence microscopy.

Cellular Uptake Experiment. One million A2780CP70 cells were seeded on 60 mm x 10 mm petri dishes and incubated for 24 h at 37 ^{0}C. These cells were then treated with cisplatin and **1**–**3** ([Pt] = 30 μM) and subsequently incubated for 4 h at 37 °C. Media was then removed and cells were washed with PBS (2 x 1 mL), harvested by trypsinization (1 mL), and washed with 0.5 mL of PBS. Solutions containing cells were centrifuged at 1500 rpm for 5 min at 4 °C and the platinum content in the whole cell was analyzed by GFAAS upon digestion by 70% HNO_3. All experiments were performed in triplicate.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay. DNA fragmentation in apoptotic A2780 cells caused by the treatment with 3 was studied using TUNEL assay (In Situ Cell Death Detection Kit, TMR red, Roche, 12156792910) A2780 cells were cultured on 25 mm petri dishes for 24 h at 37 °C and grown in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were then treated with 30 μM **3** for 24 h at 37 °C. TUNEL assay was further carried out following the manufacture protocol.

Flow Cytometric Analysis. A2780 cells were incubated with or without the test compounds for 24–72 h at 37 °C. Cells were harvested from adherent cultures by trypsinization. Following centrifugation at 1000 rpm for 5 min, cells were washed with PBS and then fixed with 70% ethanol in PBS. Fixed cells were collected by centrifugation at 2500 rpm for 3 min, washed with PBS, and centrifuged as before. Cellular pellets were resuspended in 50 μg/mL of propidium iodide (Sigma) in PBS for nucleic acids staining and treated with 100 μg/mL of RNaseA (Sigma). DNA content was measured on a FACSCalibur-HTS flow cytometer (BD Biosciences) using laser excitation at 488 nm and 20,000 events per sample were acquired. Cell cycle profiles were analyzed using the ModFit software. For the apoptosis experiments, an annexin V-FITC early apoptosis detection kit was used. The manufacture's protocol was followed to perform this experiment. Briefly, untreated and treated cells (1×10^5) were suspended in 1× annexin binding

buffer (96 µL) (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), then 1 µL FITC annexin V and 12.5 µL PI (10 µg/ mL) were added to each sample and incubated on ice for 15 min, after which, more binding buffer (150 µL) was added while gently mixing. The samples were kept on ice prior to analysis with the FACSCalibur-HTS flow cytometer and 20,000 events per sample were acquired. Cell populations were analyzed using the FlowJo software (Tree Star).

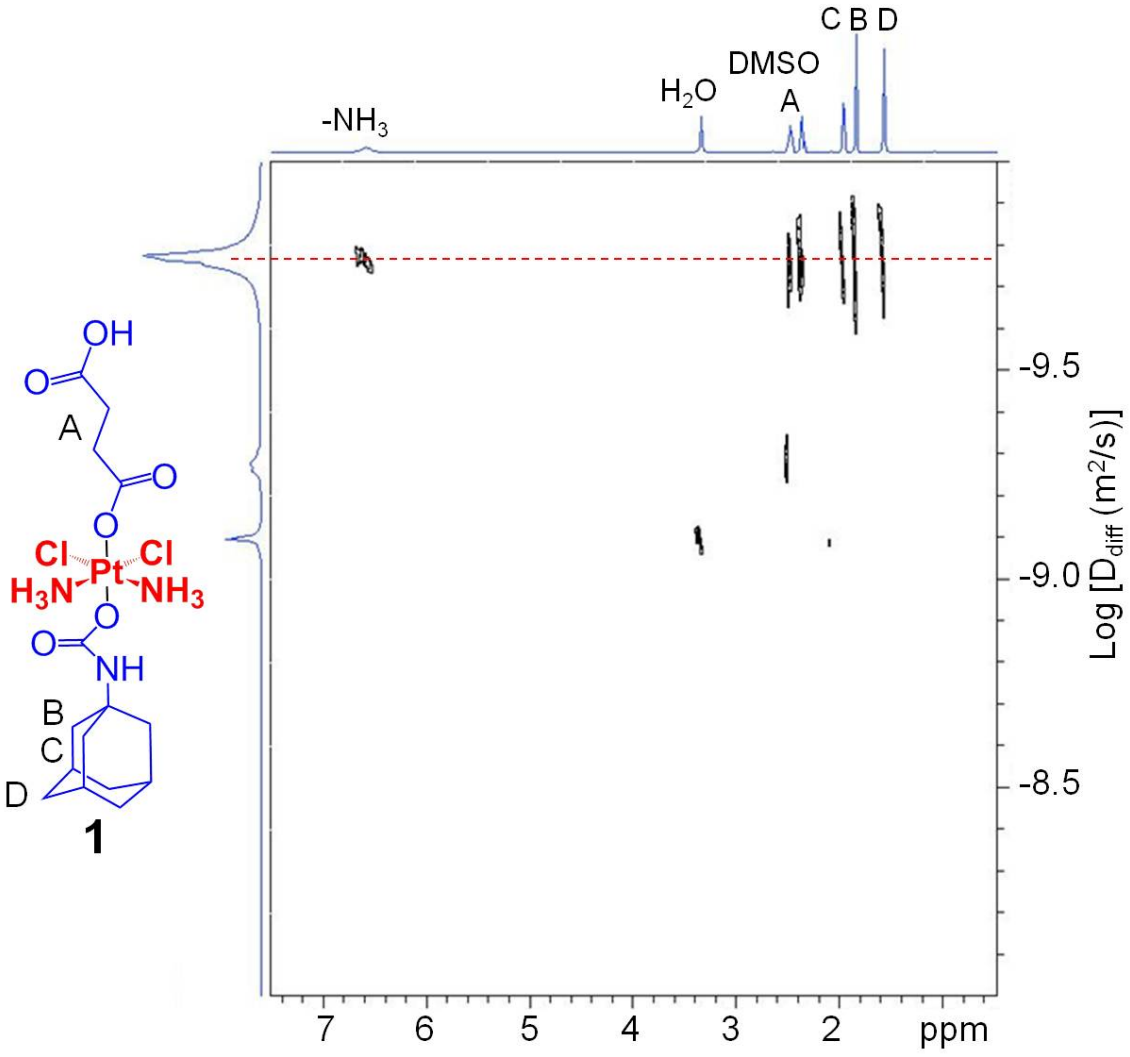

Figure S1. DOSY NMR (400 MHz, R.T.) spectrum of **1** (19 mM) in DMSO-d_6 (D = 1.6 x 10^{-10} m^2/s).

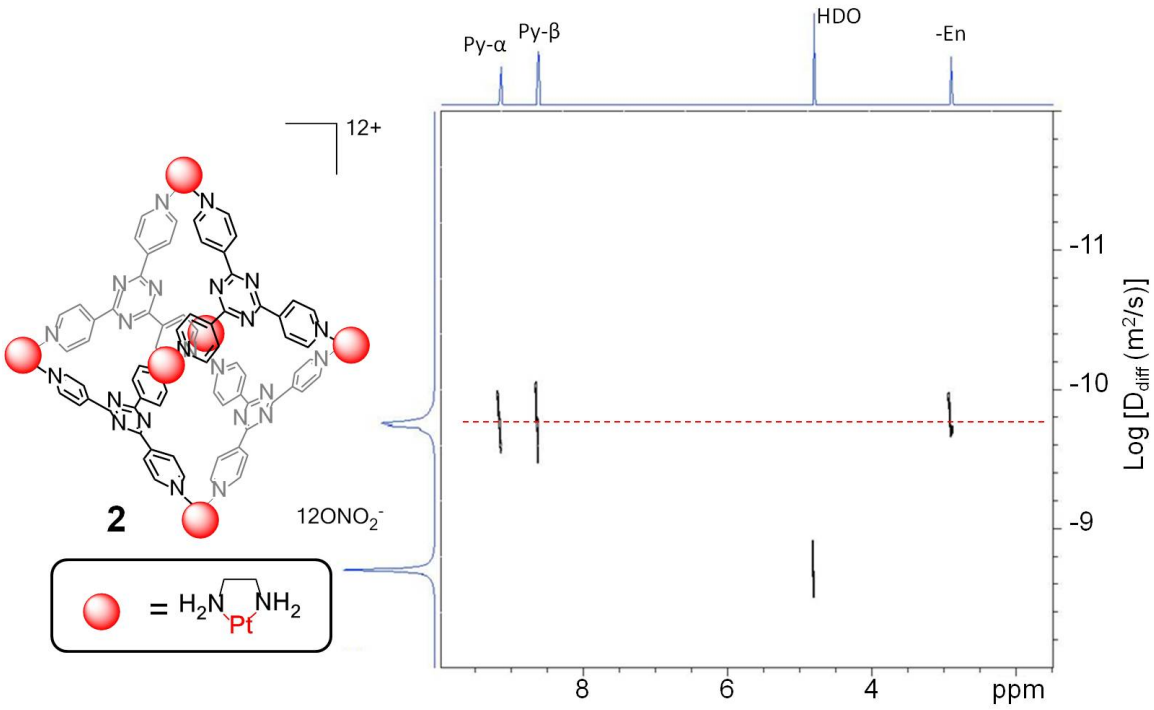

Figure S2. DOSY NMR (400 MHz, R.T.) spectrum of **2** (2.8 mM) in D_2O (D = 1.8 x 10^{-10} m^2/s).

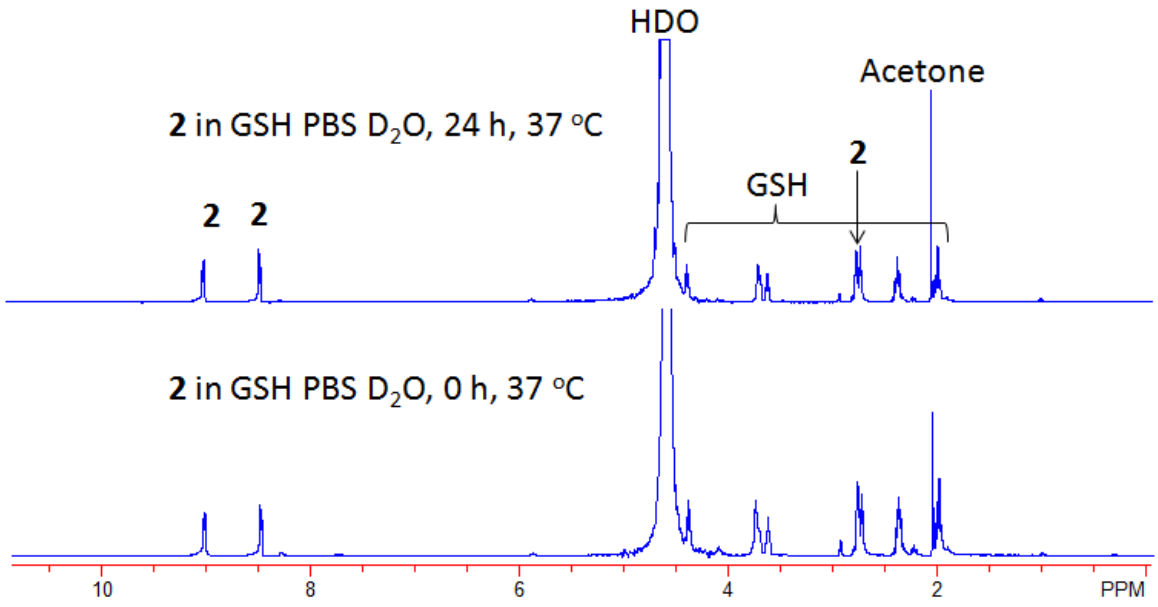

Figure S3. ^{1}H NMR spectra of the cage (**2**) in PBS D_2O with GSH.

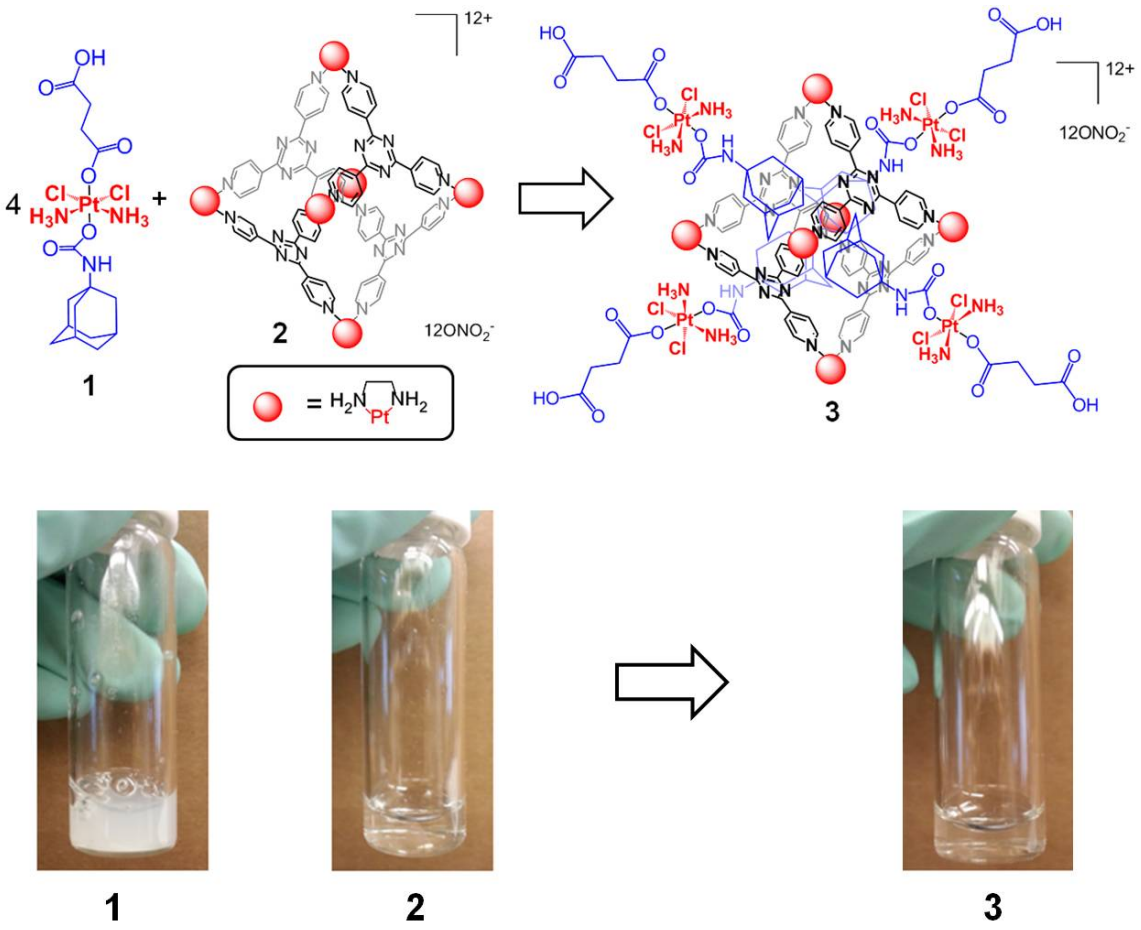

Figure S4. Change of solubility of **1** upon assembly into **3** with **2**.

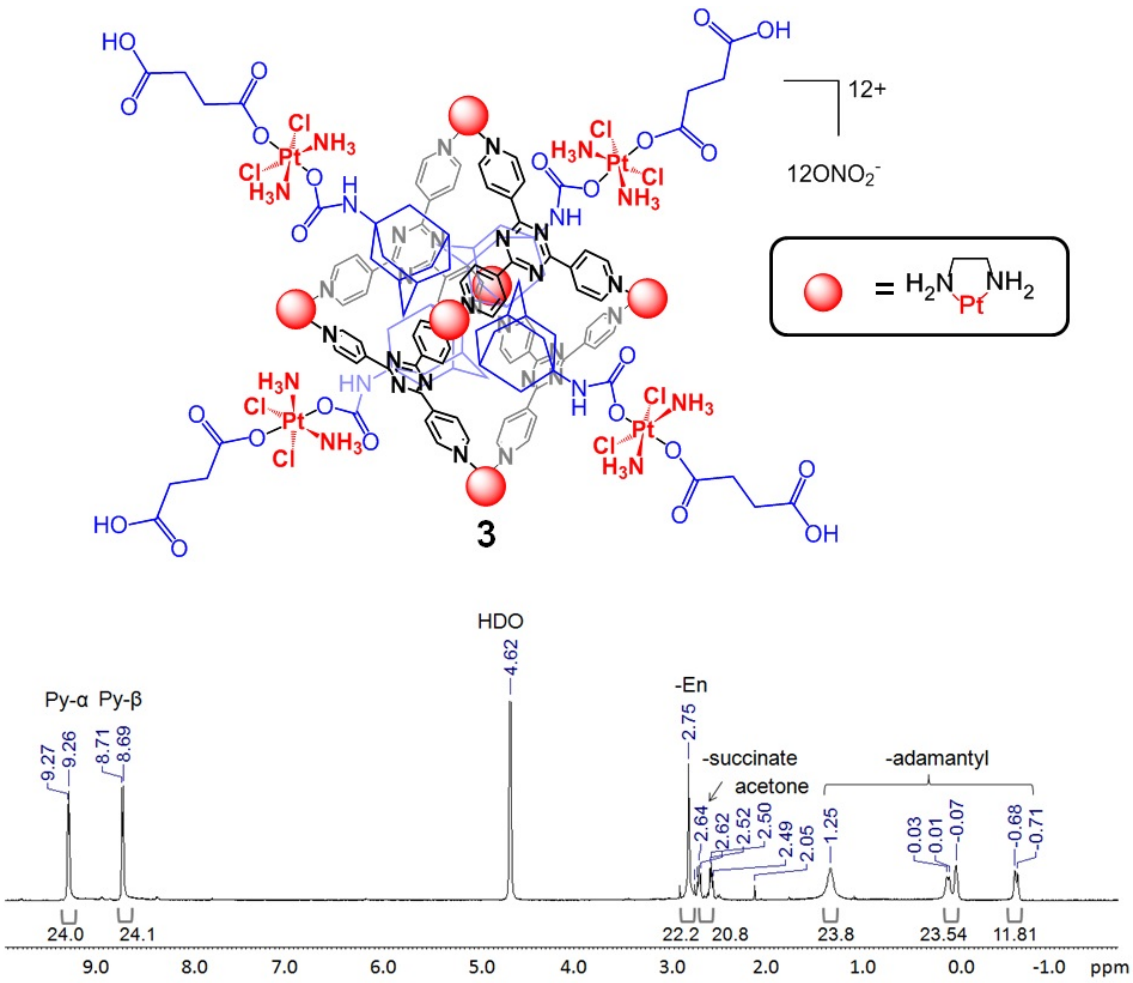

Figure S5. ^{1}H NMR (400 MHz, R.T.) spectrum of **3** (2.8 mM) in D_2O.

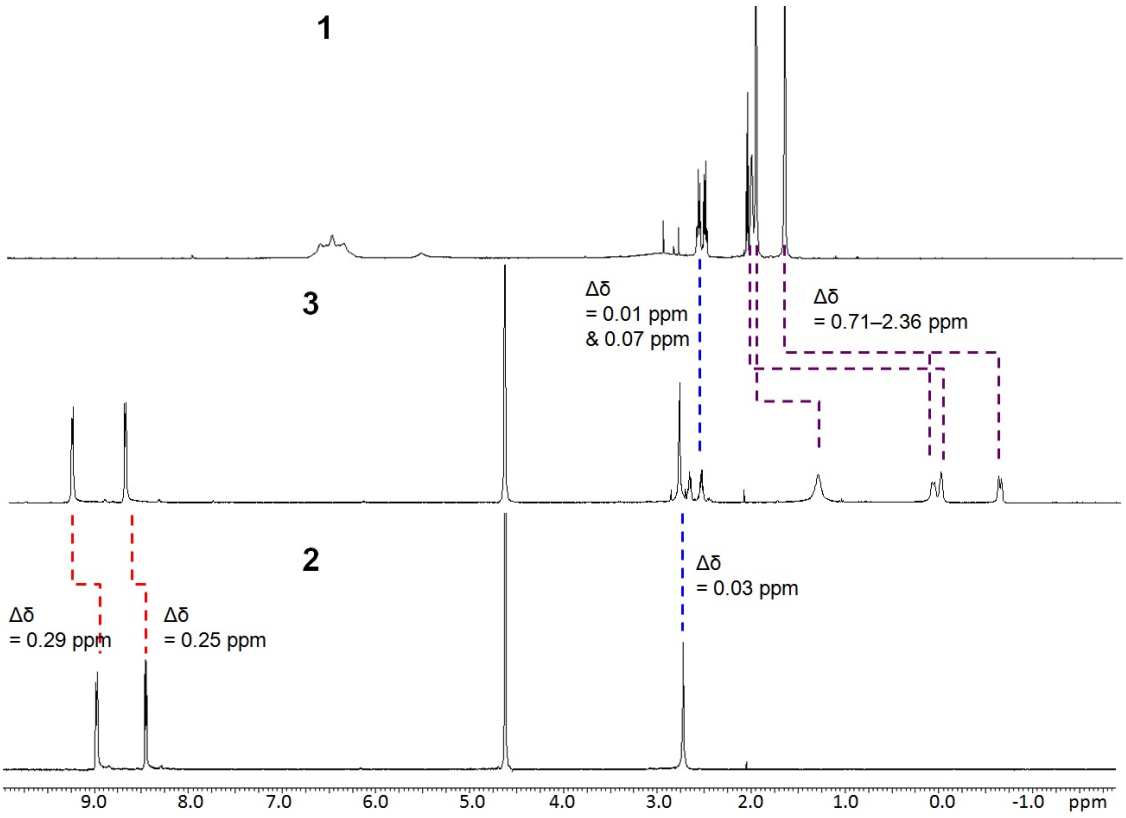

Figure S6. Change of chemical shifts of ^{1}H NMR spectra (400 MHz, R.T.) of **1** (top, 19 mM in acetone-d_6/DMSO-d_6 10:1) and **2** (bottom, 2.8 mM in D_2O) upon assembly into **3** (middle, 2.8 mM in D_2O).

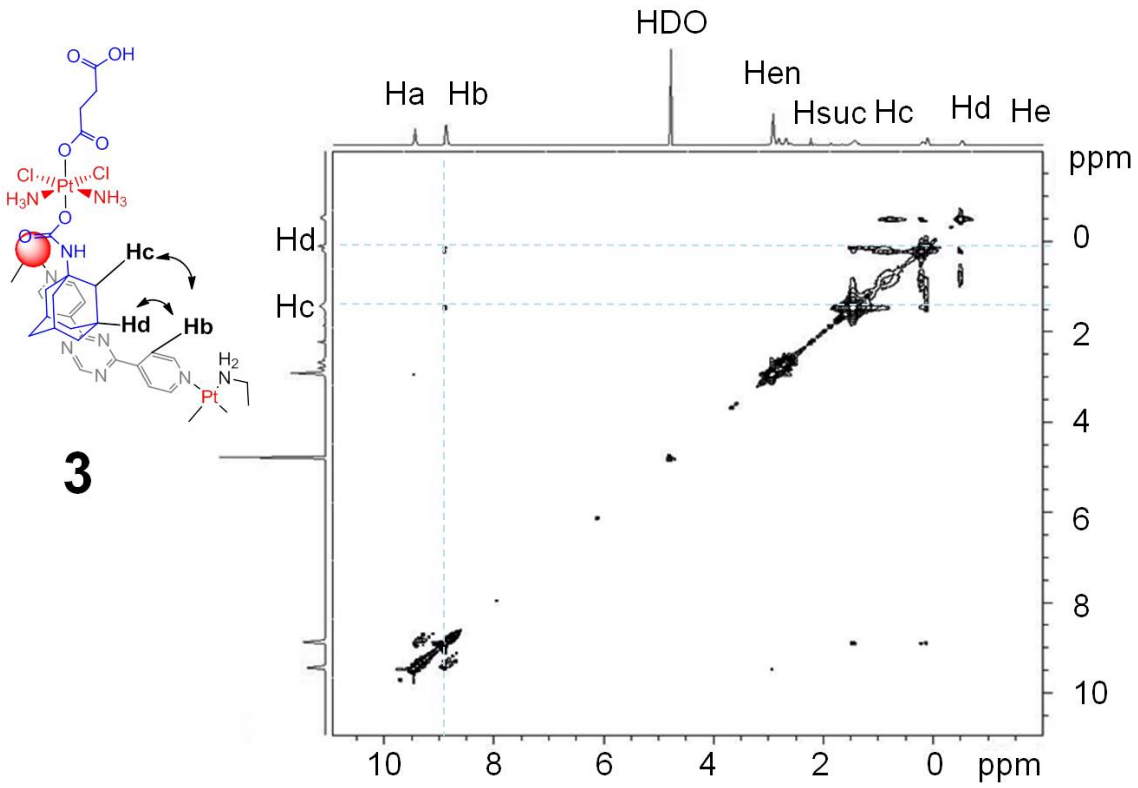

Figure S7. 2D NOESY (400 MHz, R.T.) NMR spectrum of **3** (2.8 mM) in D_2O.

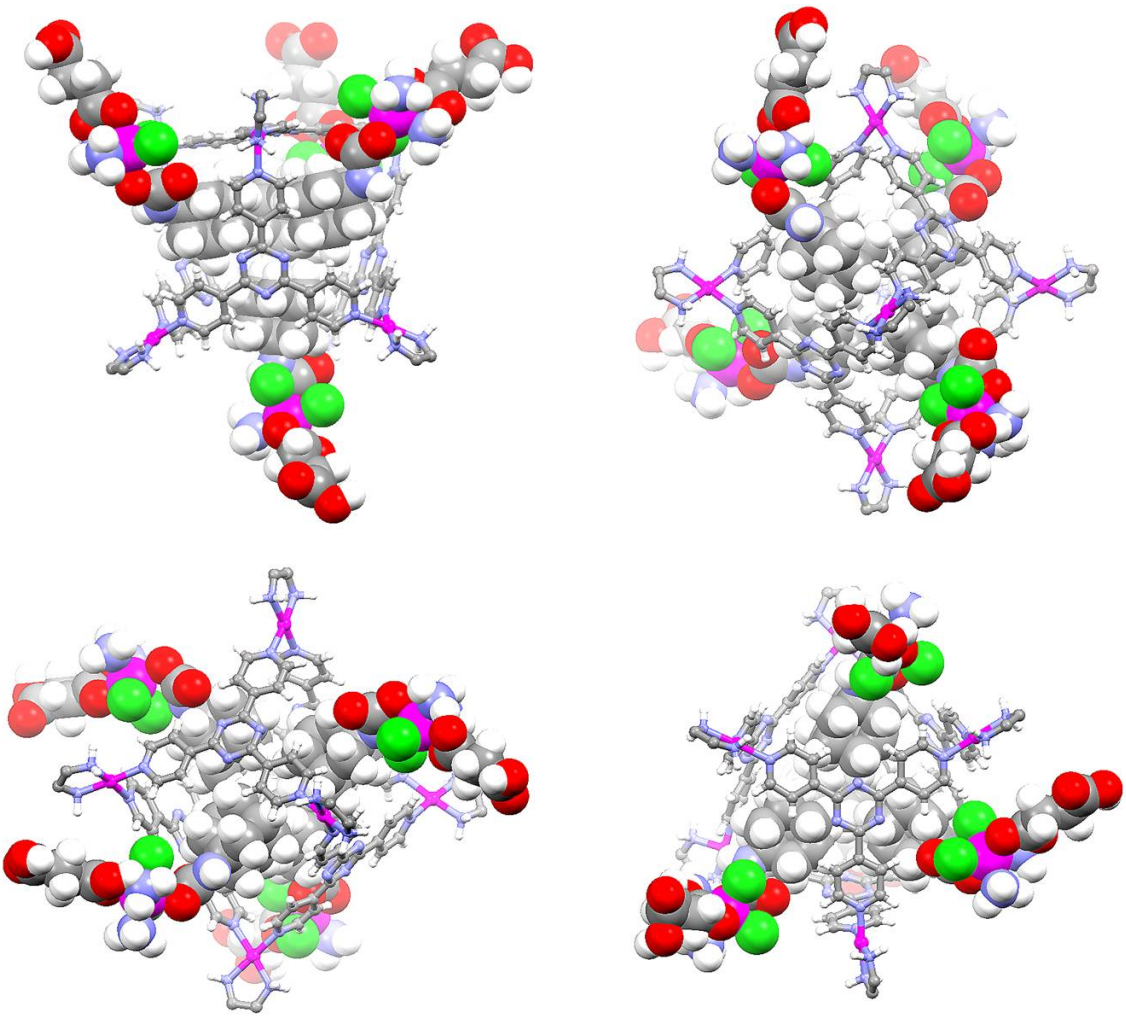

Figure S8. Different views of the 3D model of **3**, built in Maestro® based on the crystal structure of a Pd analogue[3] and minimized in Spartan® with MMFF force field.

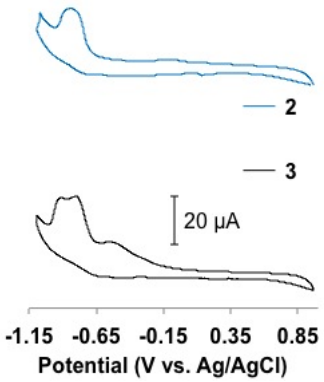

Figure S9. Cyclic voltammogram of **2** (top) and **3** (bottom) (2 mM) in PBS.

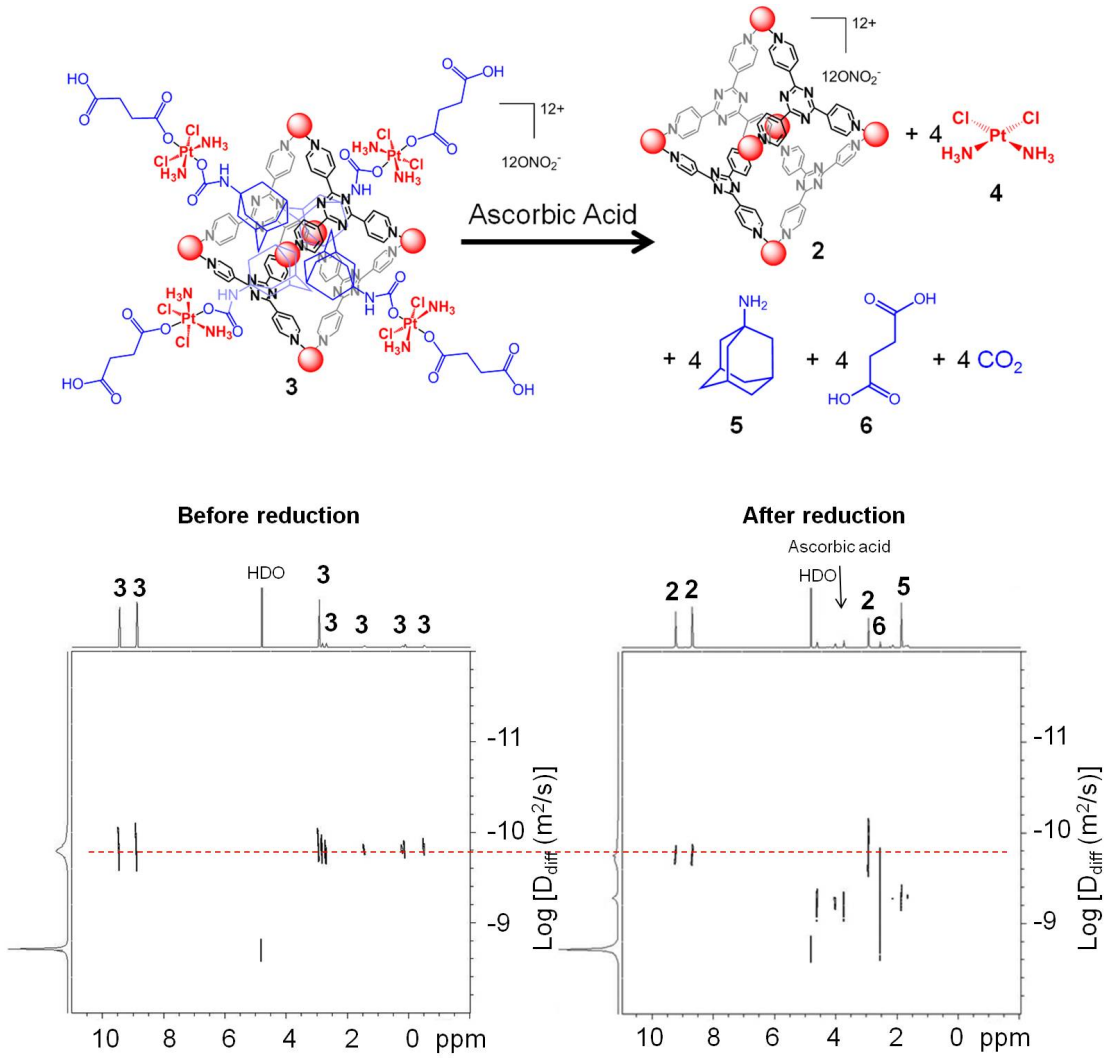

Figure S10. DOSY NMR (400 MHz, R.T.) spectra of **3** (2.9 mM in D_2O) before and after reduction by ascorbic acid (29 mM).

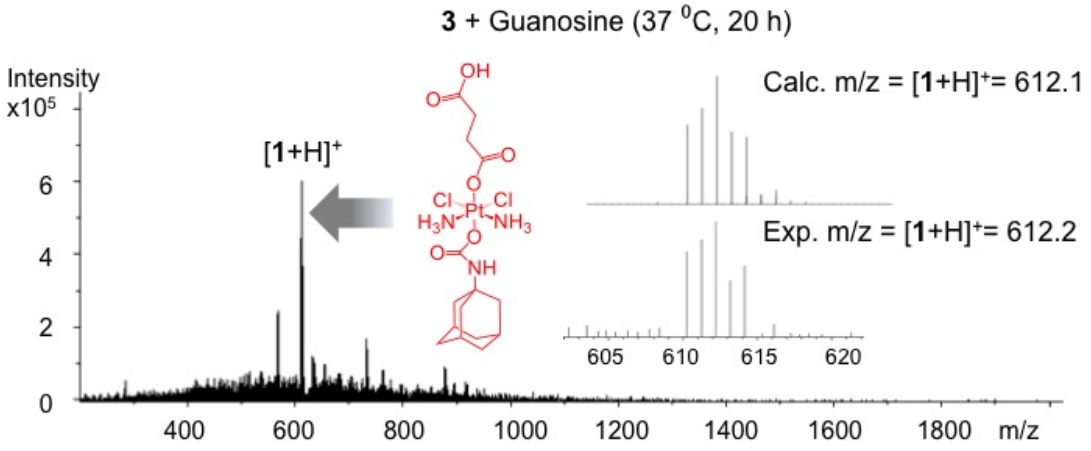

Figure S11. ESI-MS of **3** (0.16 mM) treated with guanosine (0.62 mM) after 20 h of incubation at 37 °C in water (1 mL).

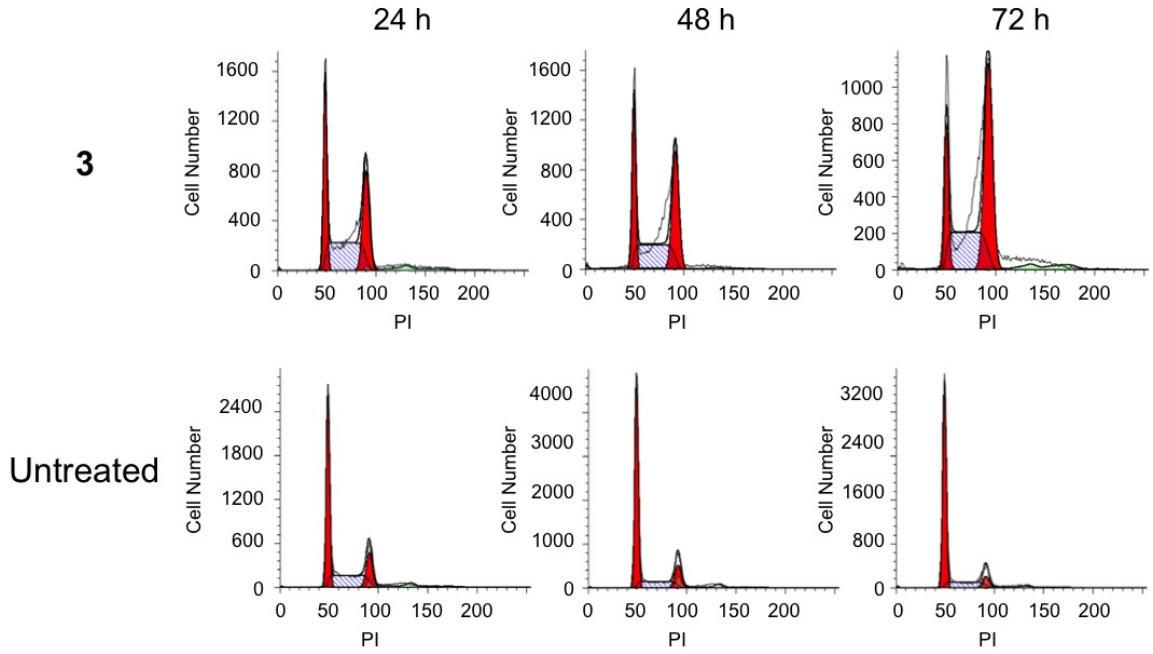

Figure S12. Flow cytometric cell cycle analysis of A2780 cells treated with and without **3**.

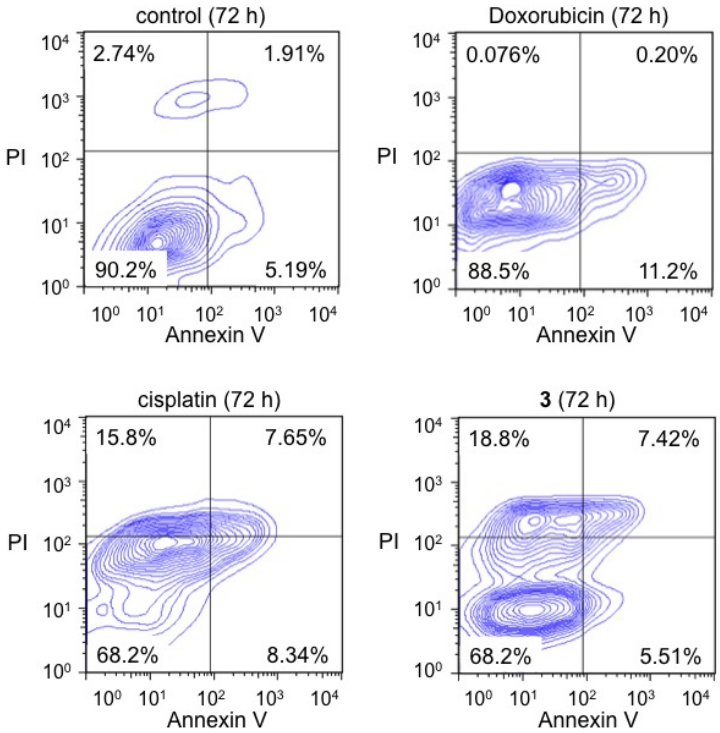

Figure S13. Annexin V/PI flow cytometric analysis of cisplatin, doxorubicin, and **3** in A2780 cell line after 72 h of incubation at 37 ^{0}C 5% CO_2 (FL1-H: Annexin V; FL2-H: PI).

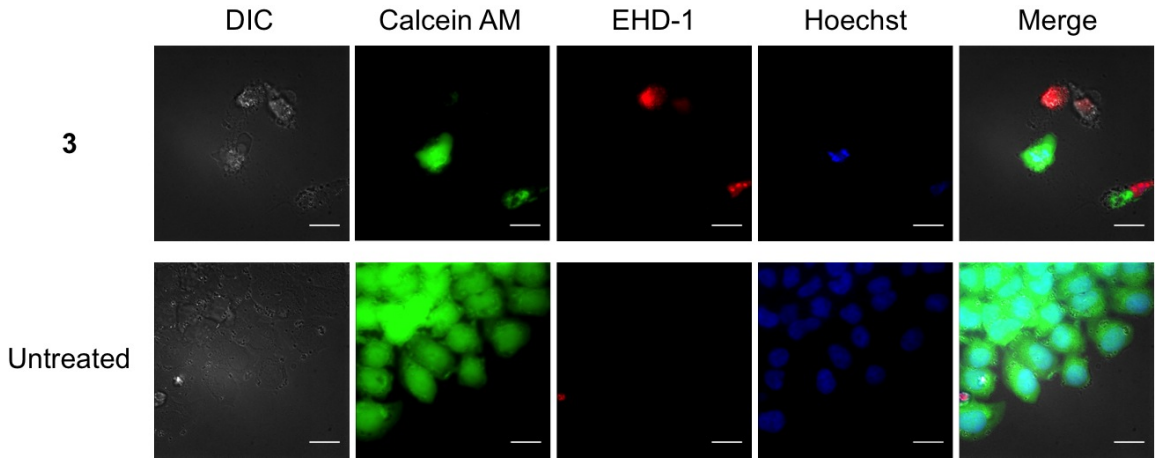

Figure S14. LIVE/DEAD cell assays and nuclear staining for A2780 cells treated with and without **3** (Scale bar: 20 μm).

References

1. S. Dhar, W. L. Daniel, D. A. Giljohann, C. A. Mirkin and S. J. Lippard, *J. Am. Chem. Soc.*, 2009, **131**, 14652-14653.
2. F. Ibukuro, T. Kusukawa and M. Fujita, *J. Am. Chem. Soc.*, 1998, **120**, 8561-8562.
3. T. Kusukawa and M. Fujita, *J. Am. Chem. Soc.*, 2002, **124**, 13576-13582.