Supplemental Information

Conjugation of Vitamin E Analog α-TOS to Pt(IV) Complexes for Dual-Targeting Anticancer Therapy

Kogularamanan Suntharalingam†‡, Ying Song†‡, Stephen J. Lippard†*
†Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

Table of Contents

Materials and methods

Cisplatin was purchased from Strem Chemicals (Newburyport, MA. USA). Unless otherwise noted, all chemicals were obtained from commercial sources and used as received. *c,c,t*-[$PtCl_2(NH_3)_2$ $(OH)_2$] was prepared according to a literature procedure. α-TOS anhydride was synthesized with a literature reported procedure. (G. Bartoli, M. Bosco, A. Carlone, R. Dalpozzo, E. Marcantoni, P. Melchiorre, L. Sambri, *Synthesis*, **2007**, 3489-3496.)

Synthesis of Pt(IV)(α-TOS)$_2$. α-TOS anhydride 2 g (1.94 mmol) and 162 mg (0.4850 mmol) of *c,c,t*-[$PtCl_2(NH_3)_2$ $(OH)_2$] were mixed in 5 mL of DMF and stirred at 50 ^oC overnight. The resulting clear solution was filtered through Celite. A 200 mL portion of water was added to the filtrate, which was subsequently extracted with 100 mL of ethyl acetate. The aqueous phase was further extracted with 100 mL of diethyl ether. The diethyl ether and ethyl acetate portions were combined and dried over Na_2SO_4. After removal of the solvent under vacuum, 50 mL of methanol was added and the solution was cooled to -20 ^oC. A yellowish-white precipitate formed and was collected by centrifugation. The solid was dried under vacuum to yield 390 mg, 60%. ^1H NMR (500 MHz, $CDCl_3$), δ (ppm) 5.61, (b, 3H), 2.92 (t, 2H), 2.69 (t, 2H), 2.57 (t, 2H), 2.08 (s, 3H), 1.99 (s, 3H), 1.95 (s, 3H), 1.76 (m, 2H), 1.53-1.09 (m, 30H), 0.86 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm)183.2, 173.9, 150.3, 141.0, 127.3, 125.7, 123.8, 118.2, 75.9, 40.1, 38.2, 38.0, 33.5, 33.4, 32.0, 30.8, 28.7, 25.5, 25.2, 23.4, 23.3, 21.7, 21.3, 20.5, 20.3, 13.9, 13.0, 12.6. $^{195}Pt\{^1H\}$c NMR (86 MHz, $CDCl_3$) δ (ppm) 1048.5. ESI-MS: *m/z* Calcd $[M-H]^-$ 1357.7, Found 1357.8. Anal. Calcd for $C_{66}H_{112}Cl_2N_2O_{10}Pt$: C, 58.30; H, 8.30; N, 2.06. Found: C, 57.98; H, 7.91; N, 1.92.

Synthesis of Pt(IV)(α-TOS)(OEt). α-TOS anhydride 200 mg (0.194 mmol) and 30 mg (0.082 mmol) of *c,c,t*-[$PtCl_2(NH_3)_2$ (OH)(OEt)] were mixed in 5 mL of DMF/EA (1:4 v/v) and stirred

at 50 ^{o}C overnight. The resulting clear solution was filtered through Celite. The solvent was removed under vacuum and the crude product was purified by column chromatography using EA/MeOH (10 %) as an eluent. The final product is a yellowish-white solid, yield 81 mg, 42%. ^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm) 5.2, (br, 5H), 2.3 (t, 2H), 2.9 (t, 2H), 2.7(t, 2H), 2.6 (t, 2H), 2.08 (s, 3H), 2.0-2.1 (2 s, 12H), 1.9 (m, 3H), 1.53-1.09 (m, 34H), 0.86 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm)181.8, 173.6, 149.7, 141.1, 127.2, 125.7, 123.0, 118.0, 75.4, 67.6, 37.7, 29.0, 28.5, 25.2, 24.8, 22.5, 22.4, 20.9, 19.6, 16.7, 12.7, 11.8, 11.4. ^{195}Pt{^{1}H} NMR (86 MHz, CDCl3) δ (ppm) 879.9. ESI-MS: m/z Calcd $[M-H]^-$ 873.8, Found 873.4. Anal. Calcd for ($M+H_2O$) $C_{35}H_{66}Cl_2N_2O_7Pt$: Calcd. C 47.08, H 7.45, N 3.14; Found C 47.11, H 6.97, N 3.03.

Instrumentation

NMR data were recorded on a Bruker DPX-400 or VARIAN Inova-500 spectrometer in the MIT Department of Chemistry Instrumentation Facility (DCIF). ^{1}H and ^{13}C NMR spectra were referenced internally to residual solvent peaks, and chemical shifts are expressed relative to tetramethylsilane (δ=0 ppm). ^{195}Pt NMR spectra were referenced externally using a standard of K_2PtCl_4 in D_2O (δ = -1628 ppm). Electrospray ionization mass spectrometry (ESI-MS) measurements were acquired on an Agilent Technologies 1100 series LC-MSD trap. Graphite furnace atomic absorption spectrometry was carried out using a Perkin-Elmer AAnalyst600 GF-AAS.

Cell Lines and Culture Conditions (a) Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS) and 0.25% trypsin/EDTA solutions were purchased from Cellgro (Manassas, VA). Penicillin-streptomycin solutions were purchased from Mediatech (Manassas, VA). (b) HeLa (CCL-2TM) cells, A549,

A2870, A2870/CP70, MCF-7, PC-3, and MRC-5 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). (c) HCT116 cells were kindly donated by Laura Trudel (MIT). HeLa, A549, MCF-7, and MRC-5 cells were cultured using DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. A2870, A2870/CP70, and PC-3 cells were cultured in RPMI supplemented with 10% FBS and 1% penicillin-streptomycin. HCT116 cells were cultured in McCoy's supplemented with 10% FBS and 1% penicillin-streptomycin. All cell-culture experiments were performed in the aforementioned media in an incubator operating at 37 °C and in the presence of a humidified atmosphere containing 5.0% CO_2. Cells were harvested using a 0.25% trypsin/EDTA solution.

Cytotoxicity Tests in Cancer Cell Lines The cytotoxicity of the compounds was assessed by means of the MTT assay (MTT= 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide). Typically, on day one, ~1×10^3 cells were seeded into 96-well plates in 100 μL media per well. The last row of the plate was left empty as a control for background absorption. The cells were allowed to grow for 24 h in the incubator at 37 °C. On day two, a series of drug dilutions in growth media were prepared immediately prior to cell treatment. The media in the wells of the 96 plates were replaced with 100 μL of growth media containing appropriate drug concentrations. The cells were treated for 48 h at 37 °C in the incubator. On day four, a stock solution of MTT (5 mg/mL in PBS buffer) was diluted with media so that, when 100 μL of the media was added to each well, 10 μL of MTT stock was applied. The cells were incubated for 2 h and then all the media was replaced with 100 μL of DMSO to dissolve the purple MTT-formazan crystals. The optical density for each well at 550 nm was measured with a microplate reader.

Cellular Uptake. To measure the cellular uptake of the platinum complexes, ca. 1 million A549 cells were treated with 10 μM of the complexes at 37 ºC for 3 h. Then the media was removed, the cells were washed with PBS solution (1 mL × 3), harvested, and centrifuged. The cellular pellet was suspended in an appropriate volume of PBS to obtain a homogeneous cell suspension (eg. 100 μL). The suspension was divided in two. One part was used to analyze the metal content in the whole cell and the other was used for analysis of the cytoplasmic and nucleus. The Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit was used to extract the separate cytoplasmic, nuclear, and membrane fractions. The remaining cell suspension was mineralized with 65% HNO_3 and then completely dried at 120 ºC. The solid extracts were re-dissolved in 2% HNO_3 and analysed using GF-AAS. Cellular platinum levels were expressed as pmol Pt per million cells. Results are presented as the mean of 3 determinations for each data point.

Intracellular DNA Platinum Content. To measure the amount platinum on genomic DNA, ca. 1 million A549 cells were treated with 10 μM of the test compounds at 37 ºC for 3 h. The nucleus was extracted using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit. The nuclear pellet was suspended in DNAzol (1 mL, genomic DNA isolation reagent, MRC). The genomic DNA was precipitated with ethanol (0.5 mL), washed with 75% ethanol (0.75 mL × 3), and re-dissolved in 200 μL of 8 mM NaOH. The DNA concentration was determined by UV−visible spectroscopy, and platinum, was quantified by GFAAS. The reported values are the average of at least three independent experiments with the error reported as the standard deviation.

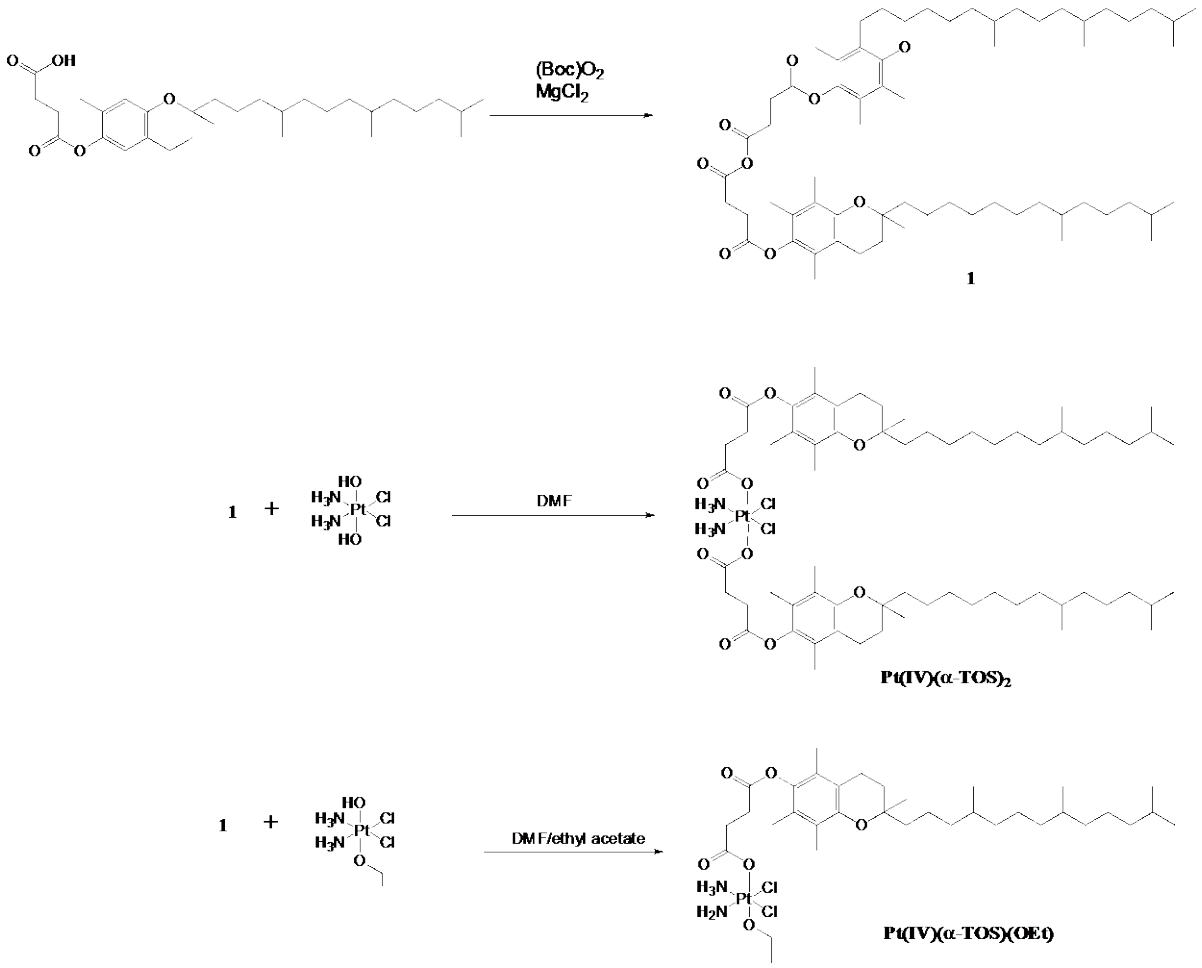
Flow Cytometry In order to monitor the cell cycle, flow cytometry studies were carried out. A549 cells were incubated with and without the test compounds for 24 and 48 h at 37 °C. Cells were harvested from adherent cultures by trypsinization and combined with all detached cells from the incubation medium to assess total cell viability. 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 re-suspended in 50 µg/mL propidium iodide (Sigma) in PBS for nucleic acids staining and treated with 100 µg/mL 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 analysed using the ModFit software. For the apoptosis experiments, the Annexin V-FITC Early Apoptosis Detection Kit (Cell Signaling Technology) was used. The manufacture's protocol was followed to carry out 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 propidium iodine (10 µg/ mL) were added to each sample and incubated on ice for 15 min. Subsequently, more binding buffer (150 µL) was added while gently mixing. The samples were kept on ice prior to being read on the FACSCalibur-HTS flow cytometer (BD Biosciences) (20,000 events per sample were acquired). Cell populations were analysed using the FlowJo software (Tree Star). For the JC-1 assay, the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman) was used. The manufacture's protocol was followed to carry out this experiment. Briefly, to untreated and treated cells grown in 6-well plates (at a density of 5×10^5 cells/ mL) was added the JC-1 staining solution (100 µL/ mL of cell media). The cells were incubated for 30 min, harvested, and analysed by using the FACSCalibur-HTS flow cytometer

(BD Biosciences) (20,000 events per sample were acquired). The FL1 channel was used to detect unhealthy or apoptotic cells with collapsed mitochondria. Cell populations were analysed using the FlowJo software (Tree Star).

Immunoblotting Analysis A549 cells (5×10^5 cells) were incubated with test compound for 48 h at 37 ºC. Cells were washed with PBS, scraped into SDS-PAGE loading buffer (64 mM Tris-HCl (pH6.8)/ 9.6% glycerol/ 2% SDS/ 5% β-mercaptoethanol/ 0.01% Bromophenol Blue) and incubated at 95 ºC for 10 min. Whole cell lysates were resolved by 4-20 % sodium dodecylsulphate polyacylamide gel electrophoresis (SDS-PAGE; 200 V for 1 h) followed by electro transfer to polyvinylidene difluoride membrane, PVDF (350 mA for 1 h). Membranes were blocked in 5% (w/v) non-fat milk in PBST (PBS/0.1% Tween 20) and incubated with the appropriate primary antibodies (Cell Signalling Technology and Santa Cruz). After incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signalling Technology), immune complexes were detected with the ECL detection reagent (BioRad) and analysed using an Alpha Innotech ChemiImager™ 5500 fitted with a chemiluminescence filter.

Co-immunoprecipitation. A549 cells (5×10^5 cells) were incubated with test compound for 48 h at 37 ºC. Cells were washed with PBS, scraped into RIPA buffer (250 µL, 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and 1% protease inhibitor mixture) and gently mixed for 15 min. The resultant cell lysate was treated with protein A-agarose bead slurry (50 µL) and centrifuged to remove proteins that bind non-specifically. Anti Bcl-XL antibody (Cell Signalling Technology) was added to the lysate and incubated for 2 h at room temperature. Protein A-agarose bead slurry (50 µL) was added and the

mixture was incubated at 4 °C overnight. Centrifugation allowed separation of the immunocomplex, which was washed with cold RIPA buffer (3 × 500 μL). The immunocomplex was then suspended in SDS-PAGE loading buffer (50 μL) and incubated at 95 ºC for 10 min. The solution containing the immunocomplex was isolated by centrifugation and then analysed for Bax content using the immunoblotting analysis protocol described above.



Scheme S1. Synthesis of the vitamin E analogue α-TOS conjugated Pt(IV) complexes: Pt(IV)(α-TOS)$_2$ and Pt(IV)(α-TOS)(OEt).

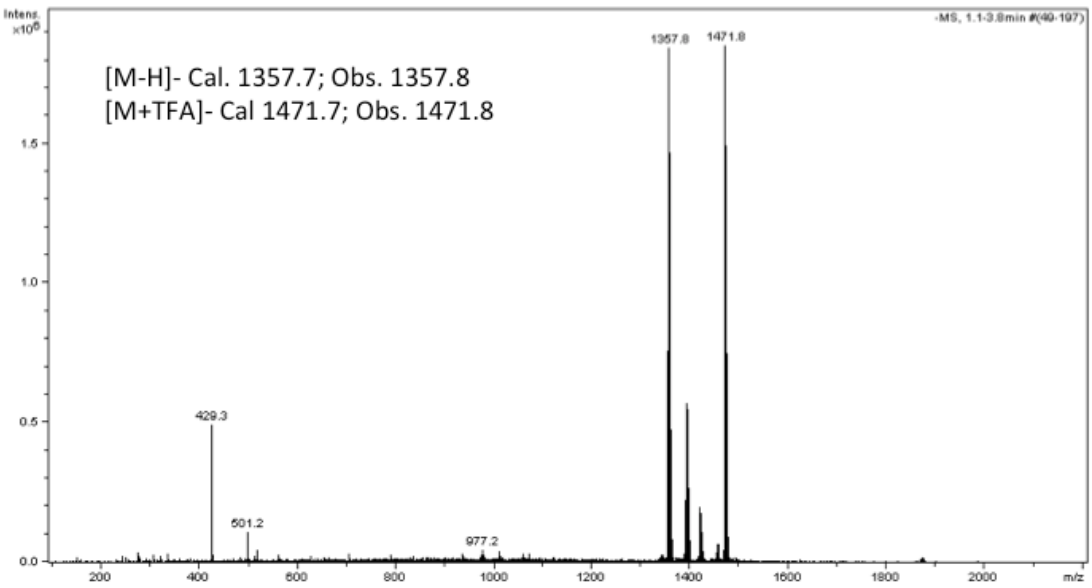


Fig. S1 ESI-MS(-) spectrum of Pt(IV)(α-TOS)$_2$, *m/z* calcd [M-H]$^-$ 1357.7, obsd 1357.8.

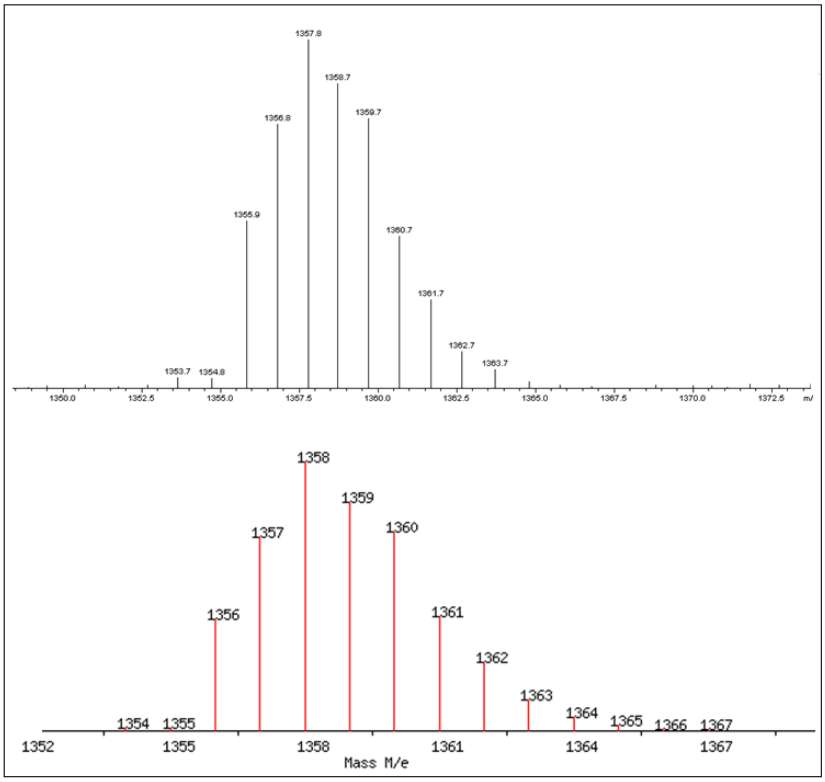


Fig. S2 ESI-MS(-) spectra of Pt(IV)(α-TOS)$_2$ showing the observed pattern (top) matching the corresponding calculated isotope pattern (bottom).

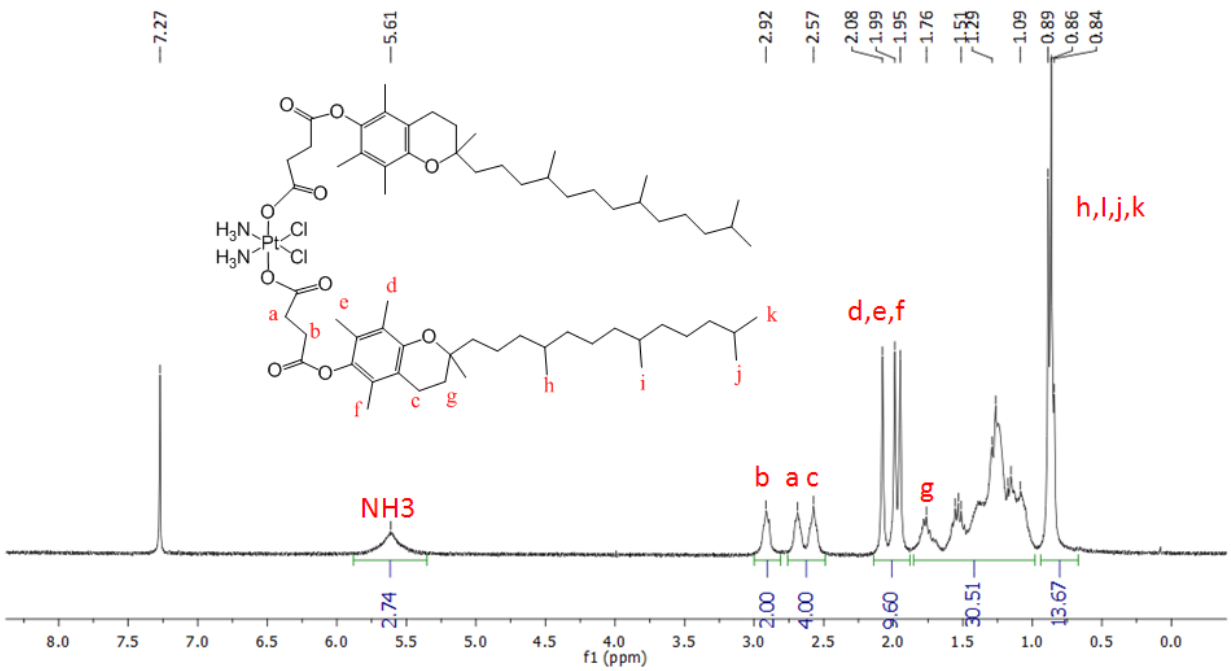


Fig. S3 ^{1}H NMR spectrum of in Pt(IV)(α-TOS)$_2$ in $CDCl_3$, 500 MHz.

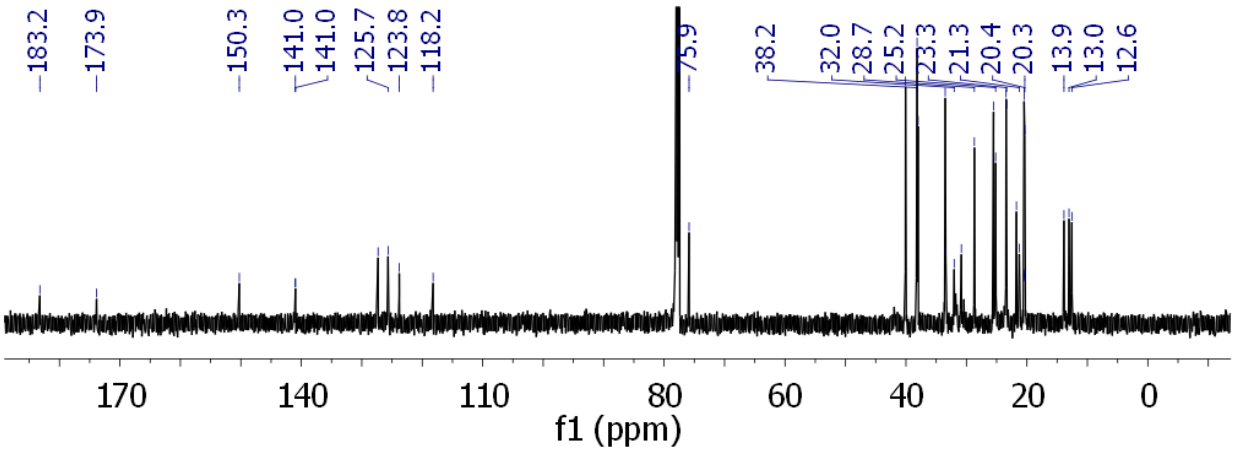


Fig. S4 $^{13}C\{^{1}H\}$ NMR spectrum of in Pt(IV)(α-TOS)$_2$ in $CDCl_3$, 125 MHz.

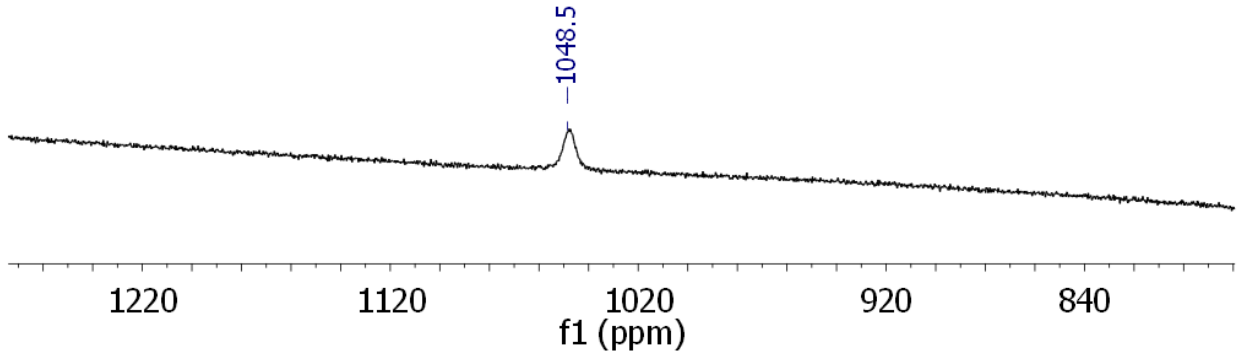


Fig. S5 $^{195}Pt\{^{1}H\}$ NMR spectrum of in Pt(IV)(α-TOS)$_2$ in $CDCl_3$, 86 MHz.

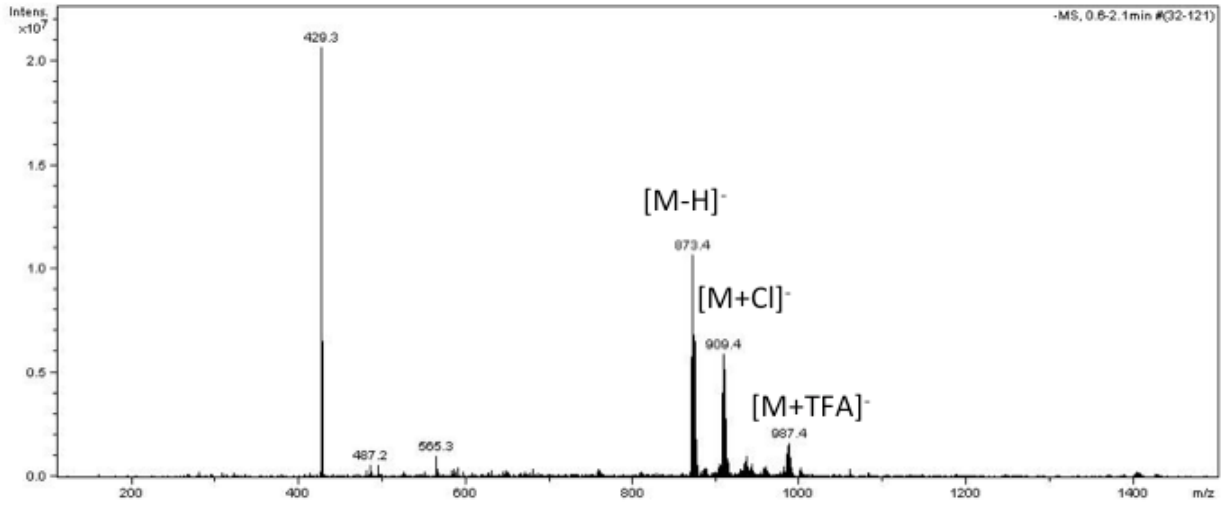


Fig. S6 ESI-MS(-) spectrum of Pt(IV)(α-TOS)(OEt), *m/z* calcd $[M-H]^-$ 873.8, obsd 873.4.

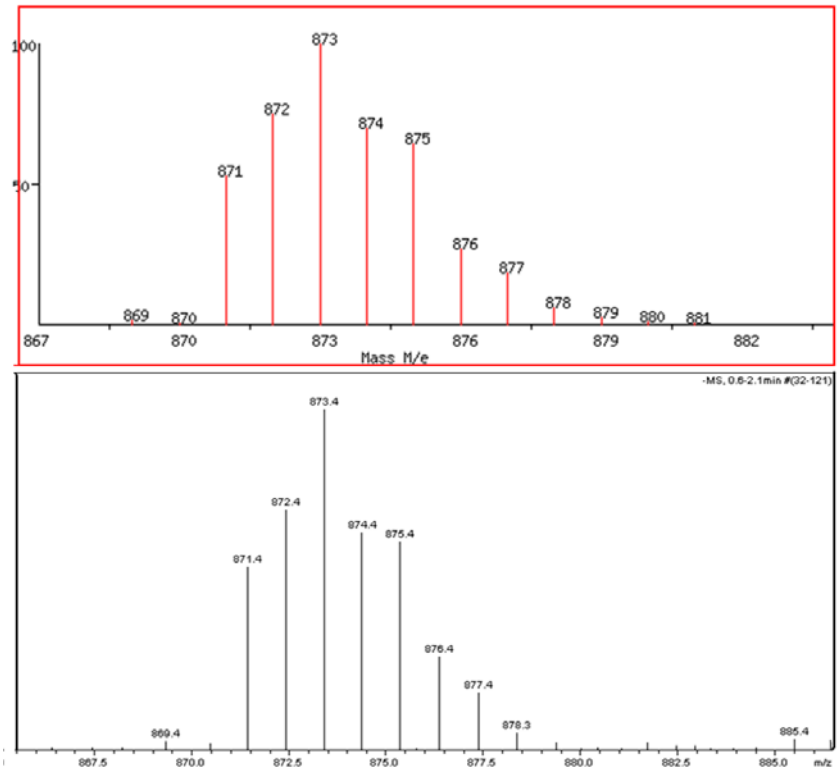


Fig. S7 ESI-MS(-) spectra of Pt(IV)(α-TOS)(OEt) showing the observed pattern (bottom) matching the corresponding calculated isotope pattern (top).

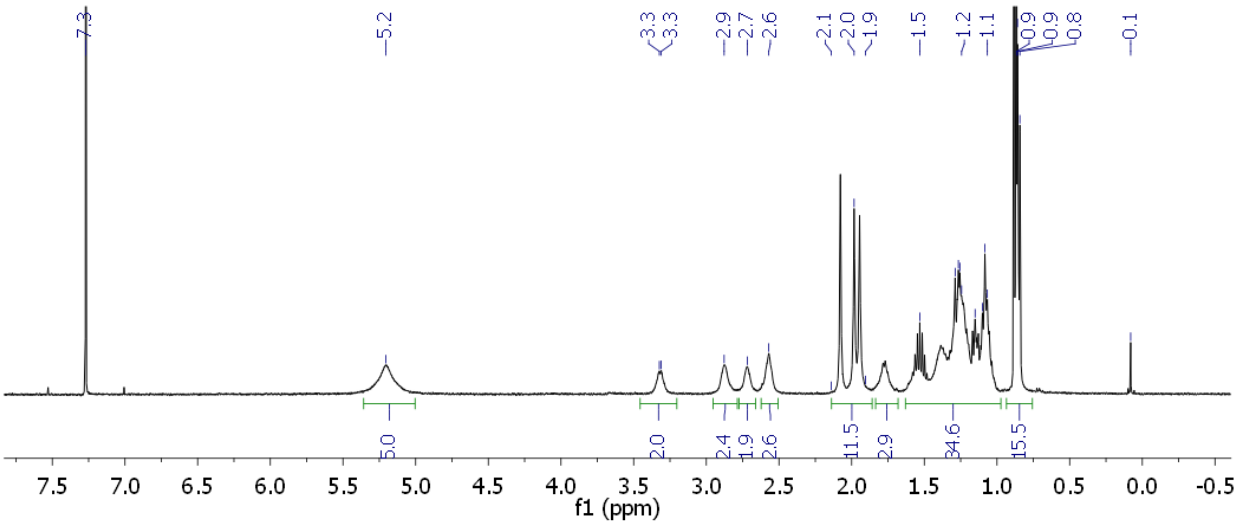


Fig. S8 ^{1}H NMR spectrum of in Pt(IV)(α-TOS)(OEt) in $CDCl_3$, 500 MHz.

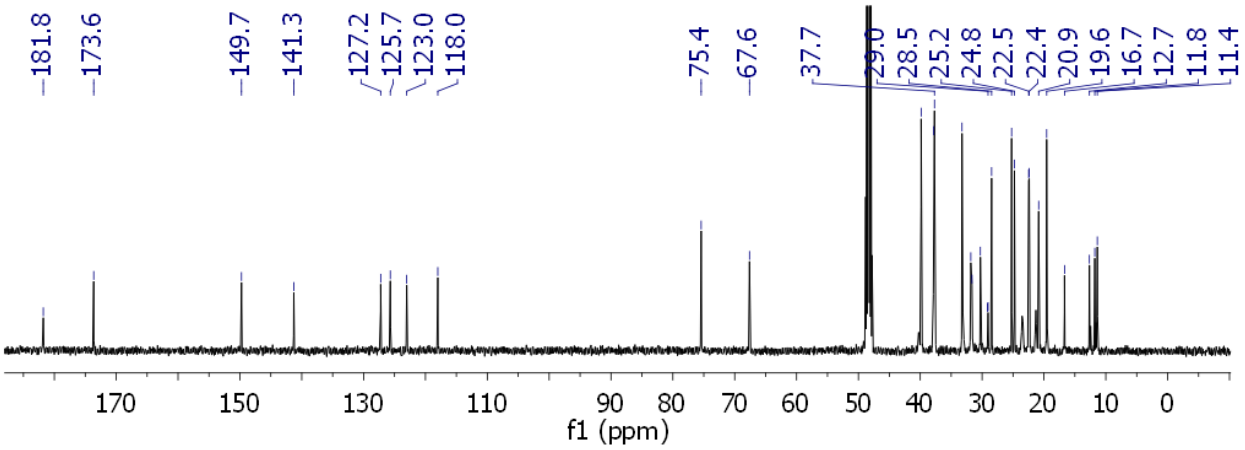


Fig. S9 $^{13}C\{^{1}H\}$ NMR spectrum of in Pt(IV)(α-TOS)(OEt) in $CDCl_3$, 125 MHz.

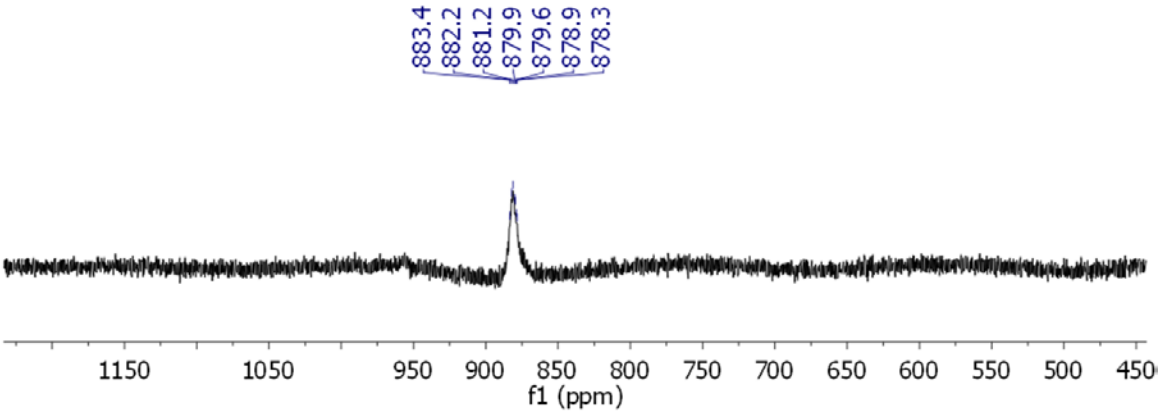


Fig. S10 $^{195}Pt\{^{1}H\}$ NMR spectrum of in Pt(IV)(α-TOS)(OEt) in $CDCl_3$, 86 MHz.

Cell name	Type	$Pt(IV)(OAc)_2$
PC-3	Prostate	> 100
HCT116	Colon	> 100
MCF-7	Breast	> 100
MRC-5	Lung (Normal)	> 100

Fig. S11 IC_{50} values (in μM) for $Pt(IV)(OAc)_2$ against cancer and healthy cell lines.

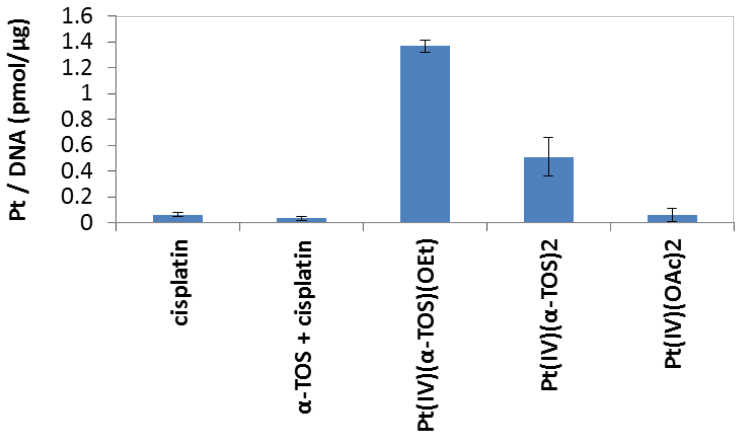


Fig. S12 Platinum content in genomic DNA extracted from A549 cells treated with cisplatin, mixtures of α-TOS and cisplatin, Pt(IV)(α-TOS)$_2$, Pt(IV)(α-TOS)(OEt), and Pt(IV)(OAc)$_2$ (10 µM for 3 h)

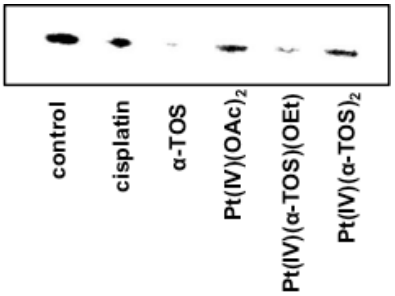


Fig. S13 The effect of cisplatin, mixtures of α-TOS and cisplatin, Pt(IV)(α-TOS)$_2$, Pt(IV)(α-TOS)(OEt), and Pt(IV)(OAc)$_2$ treatment (at the respective IC_{50} values for 48 h) on the Bcl-xL-Bak interaction dynamics. Immunoblotting analysis of immunoprecipitates for Bax levels.

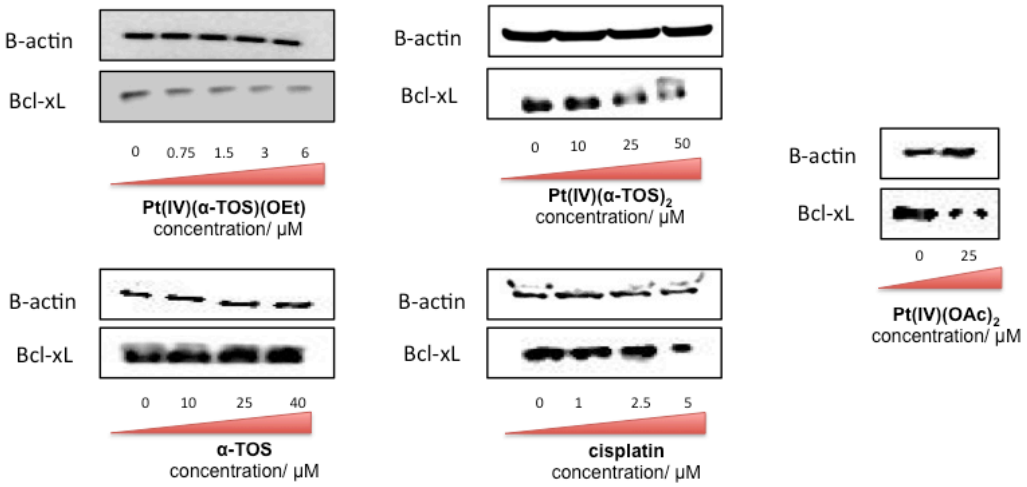


Fig. S14 Immunoblotting analysis of Bcl-xL expression upon cisplatin, mixtures of α-TOS and cisplatin, Pt(IV)(α-TOS)$_2$, Pt(IV)(α-TOS)(OEt), and Pt(IV)(OAc)$_2$ treatment

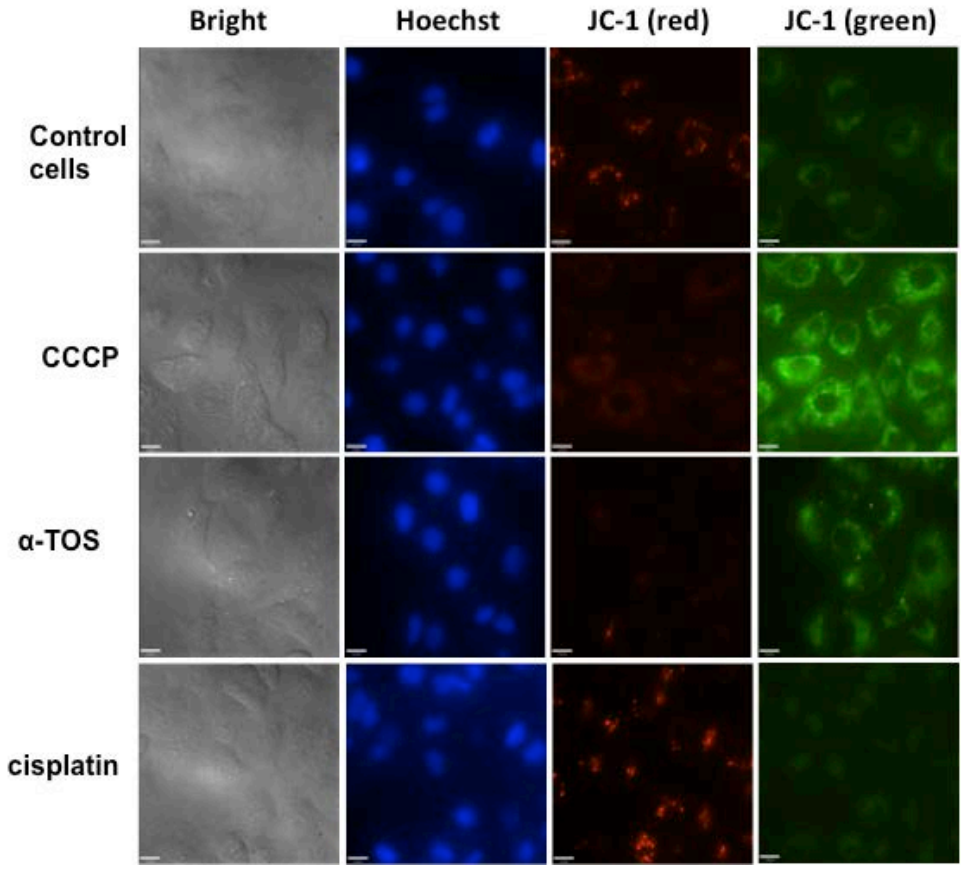


Fig. S15 Fluorescence images of A549 cells untreated and treated with CCCP, α-TOS at 100 μM, or cisplatin at 10 μM for 4 hours. JC-1 forms mainly red-emitting aggregates in mitochondria of in untreated control cells and green-emitting monomers in cytoplasm of CCCP or α-TOS treated cells. Scale bar = 13 μm.

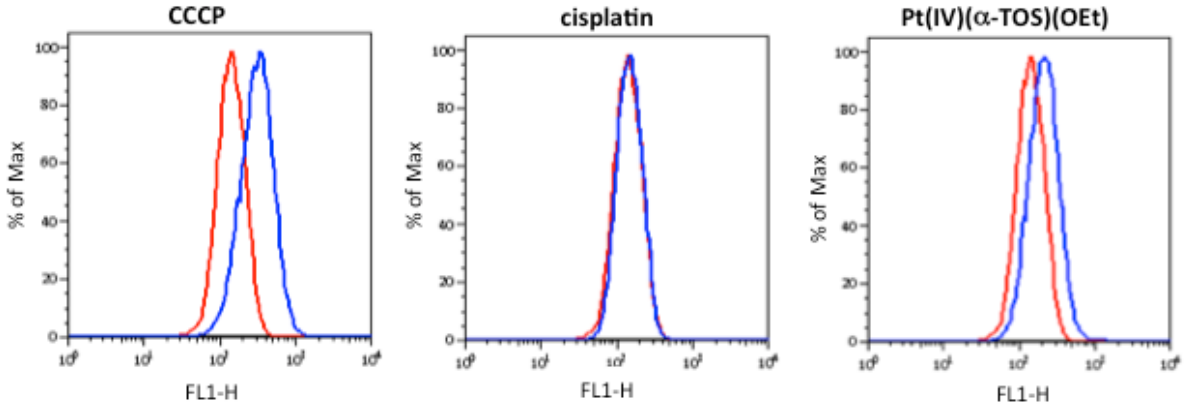


Fig. S16 Flow cytometry analysis of A549 cells treated with CCCP at 25 μM, cisplatin at 25 μM, and Pt(IV)(α-TOS)(OEt) at 25 μM, for 4 h. Histograms representing the change in FL1-H for cells stained with JC-1 in absence (red line) and presence (blue line) of CCCP, cisplatin, and Pt(IV)(α-TOS)(OEt).

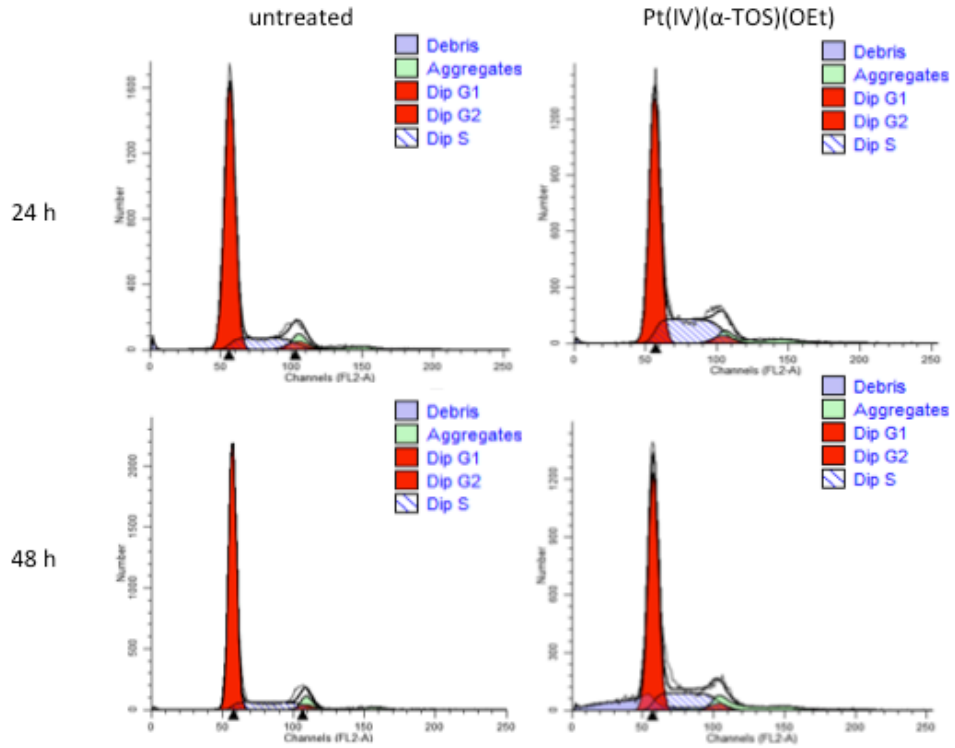


Fig. S17. Histograms representing the different phases of the cell cycle for A549 cells in absence and presence of Pt(IV)(α-TOS)(OEt) (3 μM) over the course of 48 h. 24 h untreated: G1: 78.1%, S: 17.7%, G2/M: 4.5%. 48 h untreated: G1: 81.0%, S: 16.0%, G2/M: 3.0%. 24 h treated with Pt(IV)(α-TOS)(OEt) : G1: 64.7%, S: 31.7%, G2/M: 3.6%. 48 h treated with Pt(IV)(α-TOS)(OEt): G1: 70.4%, S: 26.2%, G2/M: 3.4%

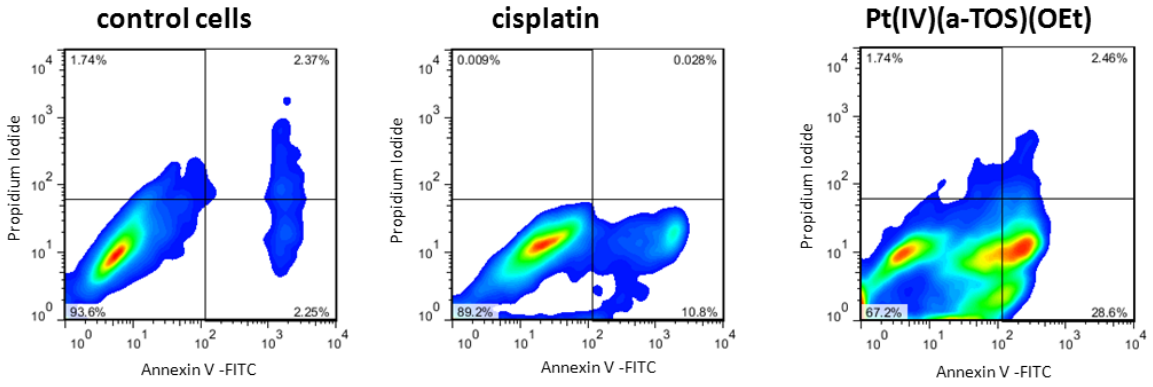


Fig. S18 FITC Annexin V/PI binding assay plots of untreated A2780 ovarian cancer cells (control), cells treated with Pt(IV)(α-TOS)(OEt) (4 μM for 48 h) and cisplatin (10 μM for 48 h).